Neurosteroids Induce Allosteric Effects on the NMDA Receptor

Nanomolar Concentrations of Neurosteroids Exert Non-Genomic Effects on the NMDA Receptor Complex

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Abstract

The neurosteroids constitute a group of powerful hormones synthesized and acting in the central nervous system. They participate in a number of important central processes, such as memory and learning, mood and neuroprotection. Their effects emerge from rapid interactions with membrane bound receptors, such as the N-methyl-D-aspartate (NMDA) receptor, the gamma-aminobutyric acid receptor and the sigma 1 receptor. The mechanisms of action are separate from classical genomic interactions.

The aims of this thesis were to identify and characterize the molecular mechanisms underlying the effects of nanomolar concentrations of neurosteroids at the NMDA receptor.

The results show that the neurosteroids pregnenolone sulfate (PS) and pregnanolone sulfate 3α5βS differently modulate the NMDA receptor, changing the kinetics for the NMDA receptor antagonist ifenprodil, through unique and separate targets at the NR2B subunit. The effects that appear to be temperature independent were further confirmed in a calcium imagining functional assay. A second functional study demonstrated that PS and 3α5βS affect glutamate-stimulated neurite outgrowth in NG108-15 cells.

Misuse of anabolic androgenic steroids (AAS) has powerful effects on emotional states. Since neurosteroids regulate processes involved in mood it can be hypothesised that AAS can interact with the action of neurosteroids in the brain. However, chronic administration of the AAS nandrolone decanoate did not alter the allosteric effects of PS or 3α5βS at the NMDA receptor, but changed the affinity for PS, 3α5βS and dehydroepiandrosterone sulfate to the sigma 1 receptor. The results also showed that the neurosteroids displace 3H-ifenprodil from the sigma 1 and 2 receptors without directly sharing the binding site for 3H-ifenprodil at the sigma 1 receptor. The decreased affinity for the neurosteroids at the sigma 1 receptor may be involved in the depressive symptoms associated with AAS misuse.

The NMDA receptor system is deeply involved in neurodegeneration and the NMDA receptor antagonist ifenprodil exert neuroprotective actions. The findings that neurosteroids interact with ifenprodil at the NMDA receptor may be an opportunity to obtain synergistic effects in neuroprotective treatment.

Keywords: Pharmacology, neurosteroids, NMDA receptor, NR2B subunit, ifenprodil, sigma receptor, anabolic androgenic steroids, allosteric modulation, non-genomic

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Johansson, T, Zhou, Q, Nyberg, F and Le Grevès, P. Alteration in the neurosteroid action at the sigma 1 receptor but not at the NMDA receptor after chronic AAS administration. *In manuscript*.

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## Contents

Introduction ................................................................................................... 11  
The definition of neurosteroids and their biosynthesis ......................... 11  
Functional significance of neurosteroids .............................................. 14  
  Memory and learning ............................................................................ 14  
  Mood ..................................................................................................... 14  
  Neurodegeneration and neuroprotection .............................................. 15  
The rapid effects of neurosteroids in the central nervous system .......... 17  
Anabolic androgenic steroids .................................................................. 17  
The NMDA receptor ................................................................................ 18  
  Composition, function and distribution .............................................. 18  
  Central processes involving the NMDA receptor ............................... 20  
  The pharmacology of the NMDA receptor ....................................... 20  
The Sigma receptor .............................................................................. 21  
  Classification and distribution ......................................................... 21  
  Central processes involving the sigma receptor ................................ 22  
  The pharmacology of the sigma receptor ........................................ 22  
Ligands as tools for the study of NMDA and sigma receptors ............... 23  
  Ifenprodil ............................................................................................. 23  
  Haloperidol ........................................................................................ 24  
  Blocking agents .................................................................................. 24  

Aims of this thesis ....................................................................................... 26  
  Specific aims ........................................................................................ 26  

Methods ........................................................................................................ 27  
  General procedures ............................................................................. 27  
  Animals and treatment ...................................................................... 27  
    Dissection ......................................................................................... 27  
  Cells ..................................................................................................... 28  
    Cell culturing ................................................................................... 28  
    CHO-E2 cells .................................................................................... 28  
    NG108-15 cells .................................................................................. 29  
Membrane preparation ............................................................................. 30  
  Rat tissue membrane preparation for receptor binding (paper I & V) .... 30  
  Cell membrane preparation for receptor binding (paper II & III) ....... 30  
  Ligand binding .................................................................................... 30
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>3α5βS</td>
<td>pregnanolone sulfate</td>
</tr>
<tr>
<td>3β-HSD</td>
<td>3β-hydroxysteroid dehydrogenase-isomerase</td>
</tr>
<tr>
<td>$^3$H-ifenprodil</td>
<td>tritium labeled ifenprodil</td>
</tr>
<tr>
<td>AAS</td>
<td>anabolic androgenic steroids</td>
</tr>
<tr>
<td>ALLOPREG</td>
<td>allopregnanolone, $3\alpha5\alpha$, $3\alpha,5\alpha$--TH PROG</td>
</tr>
<tr>
<td>ALLOPREGS</td>
<td>allopregnanolone sulfate, $3\alpha5\alpha$S</td>
</tr>
<tr>
<td>AMPA</td>
<td>$\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>APV</td>
<td>DL-2-amino-5-phosphonopentaoic acid</td>
</tr>
<tr>
<td>$B_{\text{max}}$</td>
<td>Maximum ligand binding</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>D-MEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DHEA</td>
<td>Dehydroepiandrosterone</td>
</tr>
<tr>
<td>DHEAS</td>
<td>Dehydroepiandrosterone sulfate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EPSC</td>
<td>Excitatory post-synaptic current</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>Fura-2 AM</td>
<td>Fura-2 acetomethoxy ester</td>
</tr>
<tr>
<td>GABA_A</td>
<td>Gamma-aminobutyric acid A</td>
</tr>
<tr>
<td>GBR 12,909</td>
<td>1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-[3-phenylpropyl]piperazine</td>
</tr>
<tr>
<td>GBR 12,935</td>
<td>1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl)-piperazine</td>
</tr>
<tr>
<td>HAT</td>
<td>Hypoxanthine, aminopterin, thymidine</td>
</tr>
<tr>
<td>IC$_{50}$</td>
<td>Inhibitory concentration 50%</td>
</tr>
<tr>
<td>icv</td>
<td>Intracerebroventricular</td>
</tr>
<tr>
<td>$K_d$</td>
<td>Apparent dissociation constant</td>
</tr>
<tr>
<td>$K_i$</td>
<td>Inhibition constant</td>
</tr>
<tr>
<td>$K_{off}$</td>
<td>Dissociation rate constant</td>
</tr>
<tr>
<td>$K_{on}$</td>
<td>Association rate constant</td>
</tr>
<tr>
<td>MK-801</td>
<td>$(\pm)$-5-methyl-10,11-dihydroxy-5h-dibenzo(a,d)cyclohepten-5,10-imine) a.k.a. dizocilpine</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-aspartate</td>
</tr>
</tbody>
</table>
$n_H$ the Hill coefficient
NPC Niemann-Pick C type disease
NR1 NMDA receptor subunit 1
NR2 NMDA receptor subunit 2
NR2A NMDA receptor subunit 2A
NR2B NMDA receptor subunit 2B
NR3 NMDA receptor subunit 3
PREG pregnenolone
PROG progesterone
PS pregnenolone sulfate
PTX pertussis toxin
(+)-SKF 10,047 (+)-N-Allylnormetazocine
SMD steroid modulatory binding site
SNRI serotonin and noradrenalin re-uptake inhibitor
SSRI selective serotonin re-uptake inhibitor
VTA Ventral tegmental area
Introduction

Steroid hormones are powerful mediators in the body. The findings that steroids not only act via nuclear receptors affecting the gene transcriptions, but also directly at membrane bound receptors, could both explain the rapid effects of steroids reported and present possible targets for treatment of diseases and pathological conditions.

The definition of neurosteroids and their biosynthesis

Neurosteroids are defined as steroid compounds, synthesized in and inducing rapid effects on the central nervous system (CNS). Neuro-active steroids are referring to all steroids with effects on the CNS. Neurosteroids are derived from cholesterol or as metabolites from other steroids. They exert both rapid and slow effects. Rapid effects are thought to be mediated via membrane bound receptors, a mechanism apart from the classical, slower genomic mechanism\(^\text{16}\).

In the CNS, six dominating neurosteroids have been identified: PROG and its metabolites allopregnanolone (ALLOPREG or 3α5α) and pregnanolone sulfate (3α5βS) (Figure 1) are considered as inhibitory neurosteroids. Pregnenolone (PREG), Dehydroepiandrosterone (DHEA) (Figure 2) and their respective sulfate (PS (Figure 2) and DHEAS) are stimulatory. PS and DHEAS are more active than the non-sulfated parent steroids\(^\text{205}\).

![Figure 1. The structures of three inhibitory neurosteroids, PROG (left), ALLOPREG (middle) and 3α5βS (right).](image-url)
Figure 2. The structures of two stimulatory neurosteroids, PS (left) and DHEA (right).

Enzymes that form PREG from cholesterol and other neurosteroids from PREG include two cytochrome P450s, 3β-hydroxysteroid dehydrogenase-isomerase (3βHSD), 3α-hydroxyoxidoreductase and 5α-reductase. Sulfotransferase and sulphatase add respectively remove the sulfate in 3-position (Figure 3). These enzymes are expressed in brain regions such as the hippocampus, the cortex and the hypothalamus.

Figure 3. (See next page) The synthesis of neurosteroids and the reversible metabolism. * indicates a pathway shown in vitro but still not in vivo.

Neurosteroids, and sulfated neurosteroids in particular, have been reported to be present at low amounts in the rat brain. Nevertheless, femtomoles of PS administered by intracerebroventricular (icv) injections have in different studies on rodents been demonstrated to affect processes involving the NMDA receptor. Examinations of whole brain have found around 6 ng/g PS in rat and in mice, but also 20 times lower amounts has been reported. In vitro assays have demonstrated that certain neurosteroids exert anti-apoptotic actions in concentrations around 1 nM. The effects of DHEA and DHEAS persist in pM concentration on neuritogenesis.
Functional significance of neurosteroids

Memory and learning

Neurosteroids are involved in cognitive performance and memory consolidation \textsuperscript{16}. Neurosteroids administrated exogenously show anti-amnesic and promnesic properties on memory and learning. The ability of learning and remembering things and tasks and their decline over the age is correlated to endogenous levels of neurosteroids and their decline \textsuperscript{188,205}. Memory and cognitive performances in rodents can be enhanced by PS \textsuperscript{79,80,201} and DHEAS \textsuperscript{81,82}. PS is also shown to reverse NMDA antagonist-induced memory deficits \textsuperscript{180,181}.

Mood

Neurosteroids are shown to be involved in mood and temper. The female sexual behaviour in rats is believed to be initiated by PROG, or more potently, by the neurosteroid ALLOPREG. It is regulated by neurosteroid interactions with NMDA receptors in the ventral tegmental area (VTA) and in the hypothalamus and can be banished by the NMDA receptor antagonist MK-801 \textsuperscript{237}. Lowered levels of ALLOPREG are correlated to aggression \textsuperscript{241} and women diagnosed with premenstrual dysphoric disorder have reduced serum levels of ALLOPREG compared to controls \textsuperscript{93}.

PROG is also targeting the sigma receptor, acting as an antagonist while its metabolites ALLOPREG and pregnanolone are acting as positive modulators of the GABA receptor \textsuperscript{205}. ALLOPREG is described to possess anxiolytic properties and its GABA\textsubscript{A} stimulation effects are believed to be responsible for this \textsuperscript{30}. Social isolation decreases the 5\alpha-reductase, an enzyme crucial in facilitating the conversion of PROG to ALLOPREG, in the hippocampus and in glutaminergic neurons in the frontal cortex, and the amygdala, resulting in symptoms in mice as aggression and anxiety \textsuperscript{3}.

ALLOPREG levels are lowered as a consequence of ethanol intake \textsuperscript{223} and as well during diseases as depression and panic disorders. The levels can be restored by selective serotonin re-uptake inhibitors (SSRI) as fluoxetine \textsuperscript{243,260,310} and fluvoxamine \textsuperscript{310}. Fluoxetine has also been recorded to re-establish levels of the neuroactive steroids 3\alpha\beta and 3\beta5\alpha \textsuperscript{260}. Norfluoxetine, another SSRI, has been shown to re-establish lowered ALLOPREG levels in doses too low for efficiently inhibit the serotonin reuptake \textsuperscript{242}, an effect that is proposed to be mediated by sigma 1 receptors \textsuperscript{263}.

Depressive behaviour in aged mice is believed to be the result of the decline of neurosteroids. It can be reversed by igmesine, a selective sigma 1
receptor agonist, in a dose-response manner. Treatment with PS or DHEAS reversed the depressive symptoms to levels of control, in a depression model, using adrenalectomized and castrated male mice.

Neurodegeneration and neuroprotection

Neurodegeneration occurs as a result of apoptosis or necrosis. It is induced by neurochemical modulators that cause injuries to the nervous system. One common injury is the NMDA receptor mediated excitotoxicity. Neurodegeneration is characterized by neuronal cell death and a progressive dysfunction. It is a core problem in diseases as Alzheimer’s disease, non-Alzheimer’s dementia, Parkinson’s disease, epilepsy and stroke. Neuroprotection is defined as the effect that prevents or recovers neural systems from neurodegeneration.

Key proteins involved in Alzheimer’s disease, such as tau and β-amyloid, regulate the synthesis of neurosteroids in vitro synthesis. A mutation in the gene for tau changes the involvement of the modified tau in neurosteroid synthesis. The data suggests that the neurodegeneration seen in Alzheimer’s disease are due to lowered production of certain neurosteroids. PREG has in vitro been shown to protect cells from β-amyloid toxicity and in vivo to compensate for the modification (hyperphosphorylation) of tau. In patients diagnosed with Alzheimer’s disease, PS and DHEAS were shown to be significantly lowered compared to non-dement controls, in certain brain regions and ALLOPREG was lowered in the frontal cortex as well as in plasma. These findings were supported by a negative correlation between cortical β-amyloid and the lowered PS levels. A recorded consequence of lowered levels of neurosteroids in patients suffering from Alzheimer’s disease is aggression, a symptom seen in other diagnoses as well, see sections below.

The NMDA receptor could very well be involved in these mechanisms, since glycine site agonists are shown to counteract memory impairment induced by β-amyloid and numerous reports are available considering the diseases influence on the NMDA receptor.

Parkinson’s disease is characterized by the loss of dopaminergic neurons in substantia nigra. This state is mimicked in vivo experiments by the administration of MPTP, a substance that bring damage to those neurons selectively. DHEA as well as 17β-estradiol have the ability to prevent the development of MPTP induced Parkinson’s disease, in a neuroprotective manner. Also PROG has shown neuroprotective effects against MPTP assault on neurons in the striatal region.

PROG and its metabolite 19-norprogesterone can protect primary hippocampal neuron cultures against glutamate induced toxicity. ALLOPREG
show neuroprotective effects in a Niemann-Pick C type disease (NPC) model 99. The NPC is an inherited neurodegenerative disease with parallels to Alzheimer’s disease in the cellular pathology 220.

Hypoxia is a certain way to induce neuronal damage. It can be found in the brain as well as in the spinal cord, e.g. after stroke or physical injury. Neurosteroids as DHEAS, the DHEAS metabolites 7α-OH-epiandrosterone or 7β-OH-epiandrosterone 134,209 are all shown to exert neuroprotection in hypoxia 138,154,247. PS and 3α,5βS are suggested to be useful in stroke therapy 281.

In the fetal brain the levels of ALLOPREG is much higher than in the newborn brain. It is believed that ALLOPREG induces a sleep-like state in the fetus. But ALLOPREG and other neurosteroids also protects the fetal brain from injury, from e.g. hypoxia 120. The fetus also responds to hypoxia with an up-regulation of ALLOPREG 26. PS is crucial in the neuronal plasticity in newborns, acting via α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), receptors and NMDA receptors. NMDA receptors in newborns are constituent of NR1/NR2D (see the part below about the NMDA receptor) presynaptically and NR1/NR2B postsynaptically 175. Cortical neurons from fetus, growth *in vitro*, change morphology as a direct response to DHEA or DHEAS; DHEA induce the axons to grow and DHEAS the dendrites to grow 197.

On the other hand, age tends to display a decline in neurosteroid levels, in particular PROG 238, the precursor to ALLOPREG. DHEA and its sulfate ester DHEAS are involved in the processes of aging 102,161 where levels of DHEA and DHEAS, both with antiglucocortical effects, decrease and levels of cortisol remain unaltered compared to young adults 2,102,160. The decline of DHEA and DHEAS levels results in levels for a 70 years old man at only about 25% the levels of a 25 year old. The two DHEA derivates 7α-OH-DHEA and 7β-OH-DHEA both show neuroprotective properties, which are believed to be mediated by their counteraction of glucocorticoids 209. This has been speculated to be subject for a diagnose as DHEA and DHEAS deficient and therefore be subject to treatment with supplements 2. DHEA and DHEAS are currently being sold on the US market as an anti-age drugs and clinical experiments have confirmed some positive results for the two neurosteroids as “youth drugs” 15,208. *In vitro* experiments have shown that neurosteroids as DHEAS, DHEA and ALLOPREG, are important mediators in aging, counteracting apoptosis 43 a process they can not keep inhibiting when levels are decreased with age 44.
The rapid effects of neurosteroids in the central nervous system

Neurosteroids interact with and modulate membrane bound receptors, such as the \( \gamma \)-amino butyric acid A (GABA\(_A\)) \(^{19,153,171}\), the sigma 1 \(^{191}\) and the \( N \)-methyl-D-aspartate (NMDA) receptor \(^{123,132,231,281}\), mechanisms which presumably mediate their non-genomic effects. There data presented in this field is still limited and most of the data originates from electrophysiological experiments. Electrophysiological experiments using high concentrations (micromolar) of the neurosteroids, detect modulatory effects on recombinant NMDA receptors. In those studies, pregnenolone sulfate (PS) (Figure 2) acts as a positive allosteric modulator, stimulating the agonist induced response of NMDA receptor complexes composed by NR1/NR2A or NR1/NR2B subunits \(^{174,333}\). \( 3\alpha 5\beta S \) is reported to exert inhibitory action in those studies \(^{174}\). Similar effects of PS and \( 3\alpha 5\beta S \) at micromolar concentrations were also found when \( \text{Ca}^{2+} \)-influx was measured in CHO (Chinese hamster ovary) cells stably expressing the NR1/NR2B subtype of the NMDA receptor \(^{211}\).

Anabolic androgenic steroids

Anabolic androgenic steroids (AAS) are the male sex hormone testosterone and synthetic derivates of testosterone (Figure 4). They were originally developed for therapeutic purposes, but nowadays also commonly used by athletes \(^{164,336}\) or by criminals \(^{149,292}\). Still, testosterone and its ester derivates with prolonged duration are clinically used to treat male hypogonadism as replacement therapy \(^{280}\). The testosterone derivate nandrolone (Figure 4) is being used in terminal diseases to promote well-being and increase appetite as well as to counteract the itching of chronic biliary obstruction. Nandrolone is also used to assist the recovery after severe burns or postoperative wounds. It is usually administrated as its ester nandrolone decanoate (Figure 4), which creates a slow-release depot in vivo, from where nandrolone is released \(^{13,252}\).

**Figure 4.** The structures of the three commonly used AAS’s testosterone (left), nandrolone (middle) and nandrolone decanoate (right).
The illegal use of AAS include the risk of side-effects from supratherapeutic doses, including influence on the genital system and impotence \(^{25,78}\), depression \(^{246,293}\), anxiety \(^{257,258}\), cardiac failure \(^{67,236,290}\), even though there could be other reasons for cardiac diseases in this group, such as being target for hostility \(^{250}\) or anger attacks \(^{75,76}\) shown to increase the risk for cardiac disease. Neither is the risks of being infected with lethal virus infections, as HIV or hepatitis C, negligible in the illegal use of AAS \(^4\). The desired effects of AAS in supraphysiological doses are increased muscle mass and strength \(^{24}\), aggression \(^{35,235,239,291}\) and self-confidence \(^{72}\). The aggressive behavior following AAS treatment is accompanied by lowered levels of ALLOPREG in the mouse brain \(^{241}\). This, both ALLOPREG levels and aggression could be reversed by administration of fluoxetine \(^{77,121}\) or its metabolite norfluoxetine \(^{77,121,244}\) or by the administration of estrogen and PROG \(^{242}\).

AAS are believed to interfere with several receptor systems within the CNS, e.g. the NMDA receptor. Chronically administered AAS changes receptor expression, while acutely they can act as allosteric modulators (for review see \(^{50}\)). Side effects of AAS are suggested to be mediated by interactions between AAS or its metabolites and other steroid hormones \(^{12}\). Through the rapid, allosteric modulation, AAS or metabolites of AAS, act as neuroactive steroids \(^{83}\), or interactions between AAS and neurosteroids could exist.

The NMDA receptor

Composition, function and distribution

Glutamate is the endogenous ligand of two major groups of receptors, the G-protein coupled metabotropic receptors and the ligand-gated ion channels. The latter ones are named after ligands, which selectively activate the receptor. These are the AMPA, the kainate and the NMDA receptors \(^{322}\). The NMDA receptor is blocked by Mg\(^{2+}\) in a voltage dependent manner. When activated by glutamate and the co-agonist glycine the blockade is removed and the ion-channel is permeable for Ca\(^{2+}\), Na\(^+\) and K\(^+\) \(^{192}\).

The NMDA receptor is composed out of two pairs of subunits, two NR1 and two NR2 subunits. The NR1s are either NR1a or NR1b, while the NR2Bs are a homologue or heterologue combination of the four available (NR2A-D) subunits \(^{206,207}\) (Figure 5). Each subunit consists of four transmembrane units connecting the extracellular N-terminal with the intracellular C-terminal (Figure 6). The combination of subunits determine the specific pharmacological and functional properties of the receptor \(^{322}\).
Figure 5. The NMDA receptor is constructed out of two pairs of subunits, two NR1s and two NR2s, forming the ion pore. The four proteins forming the receptor are situated in the cell membrane.

Figure 6. Each one of the four subunits forming the NMDA receptor is anchored in the cell membrane with four transmembrane units, M1-M4, connecting the extracellular N-terminal with the intracellular C-terminal. M1-M3 form the ion pore.

Different brain regions are hosting differently composed NMDA receptors. The NR1 subunits are distributed throughout the whole CNS, and the NR2 subunits are differently distributed in the CNS, NR2A and NR2B dominating.
in cortex and the hippocampus and NR2B is also present in the hypothalamus, while NR2A is found in all brain regions.

The distribution of differently composed NMDA receptors is also changed during development. The affinity for certain ligands is also altered during development. The NR2B subunit is to be found all over the embryonic brain, but in the postnatal brain NR2B subunits is restricted to the forebrain, the hippocampus and the hypothalamus. The NR1 and NR2D subunits are expressed unaltered during the brain development, NR1 all over the brain and NR2D in diencephalon and the brainstem. The NR2A and NR2C subunits both appear postpartum, the NR2A in all brain regions and NR2C in the cerebellum. The changes during development are believed to be crucial for the synaptic plasticity.

Central processes involving the NMDA receptor

Uncontrolled or excessive activation of the NMDA receptor leads to neurodegeneration, cell death and epilepsy, whereas impaired control of its activation is thought to be involved in schizophrenia and neurodegenerative diseases and also in drug addiction. It is also involved in memory and memory consolidation. An impairment or down-regulation of the NMDA receptor decreases spatial learning and other memory functions and pharmacological blockade has been shown to be neuroprotective, analgesic, amnesic and anticonvulsivative. The NMDA receptor antagonist memantine improves mood and cognitive functions in patients suffering from Alzheimer’s disease and memantine, ifenprodil and haloperidol have been proposed to display neuroprotection.

The pharmacology of the NMDA receptor

The NMDA receptor has several well-defined binding sites for a various number of ligands, endogenous as well as exogenous substances. This includes sites for the agonist glutamate and the co-agonist glycine and for polyamines, ifenprodil, protons, neurosteroids, zinc ions, magnesium ions, MK-801 (for review, see) and site for reduction/oxidation. The glycine site is located exclusively on the NR1 subunit and the others on different NR2 subunits or in the ion channel. However, it is described as many as three different sites for polyamines, and one of those is proposed to be situated on the NR1 subunit and increase the glycine binding. The other two are described to be located on the NR2B subunit, one of them is located near the ifenprodil site, which decreases the glutamate binding. The other one is reckoned as a glycine independent stimulation. The polyamine site on the NR1 subunit has been suggested to be accompanied by a separate ifenprodil binding site as well, both regulating the glycine binding.
It is also known that several of those ligands interact with each other when binding to the receptor: the main agonist glutamate increases the binding of ifenprodil and vice versa. Ifenprodil and polyamines decrease each other’s interaction with the receptor, to such an extent it once was believed they shared the same site \(^{41,142,256,268}\). Ifenprodil and polyamines are shown to induce their effects via increasing respectively decreasing the tonic proton inhibition of the receptor \(^{210,298}\).

Side effects from NMDA receptor antagonists, e.g. the channel blocker MK-801, limit their clinical usefulness. This is quite understandable due to the receptor distribution throughout the central nervous system (CNS) and the non-selectivity of the actual drugs. Subtype selectivity was asked for and has lead to the development of subunit selective antagonists. However, these compounds also proved to carry too many side effects. The all-or-nothing effect of channel opening, as occurs with competitive antagonists or high-affinity channel blockers, seems to be too powerful for clinical use. The existence of several modulatory sites, where allosteric modulators regulate the effect of endogenous agonists, shows promises of being therapeutic compounds \(^{322}\). Such group of substances includes the neurosteroids. An allosteric ligand enjoys several therapeutic advantages over conventional ligands. These include that allosteric ligands not being active other then when the receptor also bind the endogenous ligand. The allosteric ligand then attenuates or potentiates its signal. The effects of the allosteric ligand will follow the synaptic release of endogenous ligands, the release pulses and therefore better mimic a natural state. Treatment with allosteric ligands does not cause desensitisation or internalisation of the receptor, which can be a problem with conventional ligands. Thus will the effects of allosteric ligands persist over the treatment. The binding site for conventional ligands often share similarities with other receptors, as a result of the evolution. The conventional ligands will therefore bind to other targets as well, causing side-effects. The allosteric site seems to be much more specific for the allosteric ligand, why side-effects are far less expressed. The allosteric effect is also highly saturable, reducing the risk for overdosing and side-effects \(^{135}\).

The Sigma receptor

Classification and distribution

The sigma receptor is an atypical receptor \(^{271}\) associated with the NMDA receptor and also an important target for neurosteroids \(^{205}\). Progesterone is believed to be the endogenous ligand \(^{91,191}\). It was first defined as an opioid receptor \(^{176}\), but has then proven to be a completely different type of receptor family \(^{284,287}\). The sigma receptor family is divided in two subtypes \(^{248}\), sigma
1 and sigma 2, where the sigma 1 receptor is far more studied and better characterized than the sigma 2 receptor. The sigma 1 receptor is classified as a G\textsubscript{i} receptor, since it is sensitive to pertussis toxin (PTX) \cite{130, 295}, but shown little similarity with other G-protein receptors \cite{91}. The sigma receptors are present all over the rat CNS including the cortex \cite{6, 34, 56}. The distribution of the two sigma receptor subtypes are quite equal in the rat brain, apart from the motor cortex, which is only inhabited by sigma 2 receptors, a region responsible for locomotion \cite{34}. Also the nucleus accumbens and the substantia nigra predominantly host sigma 2 receptors, whereas the density of sigma 1 receptors was higher in the dentate gyrus and some nuclei of thalamus and hypothalamus \cite{34}.

Central processes involving the sigma receptor

The sigma receptor mediates dysphoric and hallucinogenic effects. The dysphoric properties can be used in antidepressive treatment \cite{182, 191, 277, 278, 309}. The sigma receptor is also believed to mediate the change in mood in pregnant women \cite{21}. The sigma receptor is also involved in memory function as sigma ligands promote anti-amnesic or pro-mnesic effects \cite{183, 185, 188, 190}.

The pharmacology of the sigma receptor

The effects of sigma receptors are mediated by controlling the mobilization of intracellular calcium \cite{177}. They cause dose dependent increase in intracellular calcium via the sigma 2 receptor, probably by releasing Ca\textsuperscript{2+} from intracellular storages. The raise in intracellular calcium is assumed to be the second messenger signal to the sigma 2 receptor activation \cite{328}. Sigma receptors also control intracellular calcium by modulation of NMDA receptor responses \cite{20, 22, 177, 204}. The modulation of the NMDA receptor can be shown as counteracting of effects, e.g. glutamate or NMDA induced postsynaptic response \cite{51, 294} or excitotoxicity \cite{275}, as changes in calcium influx \cite{112} or in changes in binding of NMDA receptor ligands, e.g. \textsuperscript{3}H-MK-801, using selective sigma ligands without affinity to the NMDA receptor, as MS-377 \cite{139}, (+)SKF 10,047 and (-)PAPP \cite{275}. This can be translated into functional studies, where selective sigma ligands are also shown to attenuate MK-801 induced learning impairment \cite{184, 190}. 

22
Ligands as tools for the study of NMDA and sigma receptors

Ifenprodil

Ifenprodil (Figure 7) is a phenylethanolamine compound, with affinity to many receptors, among them the NMDA receptor and the sigma receptor. It was originally developed as a vasodilating agent, exploiting its affinity for $\alpha_1$-adrenergic receptors $^{40,104,170}$ and used as a cerebral vasodilator $^{57}$ and tested as anti-ischemic agent in models for cerebral ischemia $^{95}$. Its affinity for the NMDA receptor was revealed in the 1980s. Ifenprodil acts neither as a channel blocker, nor as an antagonist to glutamate or glycine sites, why it is classified as a non-competitive NMDA receptor antagonist $^{159,256,321}$. Ifenprodil has its binding site on the NMDA receptor solely on the NR2B subunit and remains the best characterized of the subunit specific NMDA receptor ligands. It was discovered that ifenprodil possesses neuroprotective properties, while it lacks several of the side effects shown by classic NMDA receptor antagonists. Ifenprodil was first addressed as a polyamine antagonist $^{18,41,268,288}$, but further studies showed that the interaction between polyamines and ifenprodil is allosteric. That allosteric effect results in highly decreased affinity for the other ligand $^{86,87,142}$. However, due to its affinity to the sigma receptor $^{54,108}$, the piperazine acceptor site $^{59}$, the voltage-dependent Ca$^{2+}$ channels $^{14,49}$ and the 5-HT$_3$-receptor $^{11,194}$, ifenprodil was doomed to never enter the clinical area, instead forever being a good pharmacological tool.

![Figure 7. The structure of the NR2B selective agent ifenprodil.](image)

In the beginning ifenprodil was defined to have a 100-fold selectivity for NR1a/NR2B over NR1a/NR2A $^{321}$, but later studies claim that NR1a/NR2A, NR1a/NR2C and NR1a/NR2D all are almost insensitive to ifenprodil $^{63,100,324}$. Transgenic animal techniques using knockout mice further show that NMDA receptors on hippocampal neurons lacking the NR2B subunit do not respond to ifenprodil $^{296}$.

$^3$H-ifenprodil, a commonly used radioligand for in vitro experiments on NR2B, shows affinity for several other binding sites in rat brain membranes. To ascertain that the NR2B binding really is being studied, the other high affinity targets must be blocked. The high affinity sites include, apart from
NR2B, the sigma receptor \textsuperscript{109,111,140}, which can be blocked by GBR 12,909 \textsuperscript{55,59,101}, the piperazine acceptor site, which is blocked by trifluoperazine \textsuperscript{59}. Trifluoperazine also blocks the sigma receptor \textsuperscript{59,331} as well as the last high affinity target, the $\alpha_1$-adrenergic receptors \textsuperscript{304}. The pure binding to the NMDA receptor is then dose-dependently biphasic \textsuperscript{217}. The high affinity interaction is the one between the ifenprodil binding site on the NR2B subunit. The explanation for the low affinity interaction could be found in the putative interaction between ifenprodil and the glycine site on the NR1 subunit \textsuperscript{159,253,321}.

Haloperidol

Haloperidol (\textit{Figure 8}) (paper II-III & V) is a widely used anti-psychotic agent \textsuperscript{252} with positive effects on depression and social disabilities in chronic schizophrenia \textsuperscript{195,261}. The anti-psychotic effects are believed to reside from blocking dopaminergic receptor. Apart from the dopamine receptor affinity, haloperidol display binding characteristics reminding of that for ifenprodil. Haloperidol binds to NR2B \textsuperscript{60,86,122,126}, serotonin and $\alpha$-adrenergic receptors \textsuperscript{252} and sigma receptors \textsuperscript{56,111,287,313}. Haloperidol actually once was considered as the prototypic drug for the sigma receptor \textsuperscript{130,203,284}. It was used to discriminate the sigma site from the PCP-binding site on the NMDA receptor \textsuperscript{129,130,287}. Haloperidol was used in order to assure the $^3$H-ifenprodil binding to be to the NR2B target (in combination with trifluoperazine) and in the sigma receptor assay.

\textit{Figure 8}. The structure of haloperidol, which shares several binding sites with ifenprodil.

Blocking agents

The GBR 12,900-family is a series of compounds originally designed to block dopamine transporters, inhibiting the dopamine uptake, but was found to also label other targets. GBR 12,935 (\textit{Figure 9a}) and GBR 12,909 (\textit{Figure 9b}) are commonly used to isolate the NMDA receptor site in various binding assays. They share structure similarities with both ifenprodil (\textit{Figure 9c}) and haloperidol (\textit{Figure 9d}). GBR 12,935 is used preferably to label the piperazine acceptor site \textsuperscript{8,94} and GBR 12,909 labels the sigma receptor \textsuperscript{37,55}.
Their selectivity is however not utterly, why too high concentrations of GBR 12,935 would interfere with the sigma receptor binding, elucidated in the optimization process of the \textsuperscript{3}H-ifenprodil binding to the sigma receptor. Another ligand used to isolate the NMDA receptor site is trifluoroperazine \textsuperscript{59} (Figure 9e).

![Figure 9. The structures of (a) ifenprodil, (b) haloperidol, (c) GBR 12,935, (d) GBR 12,909 and trifluoroperazine show similarities, as do some of their receptor affinities.](image)
Aims of this thesis

The rapid physiological effects of neurosteroids are to quite an extent examined, but the molecular mechanisms underlying their actions are still not fully elucidated. The aim of this thesis was to increase the understanding of the mechanisms behind neurosteroid mediated modulation of NMDA and sigma receptors, to bring clarity and knowledge to the field.

Specific aims

Characterisation of the influence of PS and $3\alpha 5\beta S$ on ifenprodil binding to rat cortical NMDA receptors (paper I).
Characterisation of the influence of PS and $3\alpha 5\beta S$ on ifenprodil inhibitory action on glutamate stimulated calcium influx and ifenprodil binding recombinant NMDA receptors (paper II & III).
Investigate the impact of temperature on ifenprodil binding and the action of neurosteroids on such binding to the recombinant NMDA receptors (paper II)
Examine the effects of nanomolar concentrations of PS, $3\alpha 5\beta S$ and ifenprodil on glutamate stimulated neurite outgrowth in vitro (paper IV).
Investigate the consequences of in vivo AAS (nandrolone decanoate) treatment on the effects of nanomolar concentrations of PS and $3\alpha 5\beta S$ on NMDA and sigma receptors in rat cortical membranes (paper V).
Methods

General procedures
All experiments were carried out at the division of research on biological drug dependence, Biomedical center, Uppsala, Sweden. Animal experiments were conducted in the animal facilities. In vitro experiments were repeated at least three times on different days.

Animals and treatment
Adult male Sprague–Dawley rats (Alab AB, Sollentuna, Sweden) weighing 200–220 grams (approximately 11-week-old) at the start of the studies, were housed in groups of four in air-conditioned rooms under an artificial 12 hours light-dark cycle at a temperature of 22-23°C and a humidity of 50-60%, food (R36 standard pellets) and water ad libitum. The cages used elm chips as surface. Prior to tissue sampling or treatment, the rats were allowed to adapt to the laboratory environment for one week. The animal experimental procedure was approved by the local experimental animal committee in Uppsala, Sweden.

In paper I the animals received no treatment before tissue sampling, in paper V the animals were randomly divided into two groups, one receiving nandrolone decanoate (15 mg/kg) every third day and the other group received vehicle (arachidonis oleum). The treatment lasted for three weeks. The injections were administered subcutaneously in volumes of 100 μl. The sites of injection were shifted, never injecting the same location twice, in order to avoid local skin reactions.

Dissection
The animals were sacrificed by decapitation. Brain tissues were rapidly removed from the skulls and placed on ice. The frontal cortex, the cortex, the hippocampus and the hypothalamus were dissected, immediately frozen and stored at -80°C until further processed.
Cells

Cell culturing
Cells were grown in a controlled environment with a humidified atmosphere containing 5% CO₂ at 37°C (Sanyo MCO-15AC CO₂ incubator, Sanyo E&E Europe BV, Holland).

CHO-E2 cells
The CHO-E2 cell line (Figure 10) (paper II & III) is a cell line genetically enhanced by Uchino and co-workers (2001). It was a kind gift of Dr. Shigeo Uchino. It is carrying the genes for functional NMDA receptor [NR1/NR2B]. The CHO cell line is not capable to cope with such massive expression of NMDA receptor, why the genes are under the control of the Drosophila heat-shock protein promoter 70. Activation of the promoter is carried out by heat induction for 2 hours. The receptors are then present after 6 h for another 6 h. After the heat induction, the cells need efficient NMDA receptor blockade to survive until used in assay, provided by APV.

Figure 10. The CHO-E2 cells as seen in the microscope, look like any ordinary CHO cell, but possess the ability to express fully functional NMDA receptors, formed from two NR1s and two NR2Bs.

The CHO-E2 cells were grown on 100 mm culture dishes (Sarstedts, Nürnberg-Rommelsdorf, Germany). The culture medium (Dulbecco’s modified Eagle’s medium (D-MEM) supplemented with 10% foetal bovine serum, 1.2 mg/ml geneticin, 2 μg/ml blasticidin S HCl and 10 μg/ml puromycin) was changed every sixth day. The cells were grown to approx. 80% confluence and split 1:3, generally every sixth day, using trypsin/EDTA (0.05%/0.02%), before transferring to fresh 100 mm culture. Cells at passages 5 – 30 were used.

Heat induction of CHO-E2
To induce NMDA receptor expression in the CHO-E2 cells, culture dishes containing 150000 cells cm⁻² were incubated for 2 h at 43°C in a controlled humidified environment containing 5% CO₂ (Micro Galaxy, Zander Medical
Supplies, Vero Beach, FL, USA) and were then maintained at 37°C for 6 h, in order to express the proteins, in culture medium supplemented with 1 mM APV, before used in calcium assays or harvested for membrane preparation.

NG108-15 cells

The neuroblastoma x glioma NG108-15 hybrid cell line (Figure 13) (paper IV) was employed due to its ability to produce axon-like neurites as a result from elevated intracellular calcium and expresses functional NMDA receptors. In immature neurons, the NR2B subunit of the NMDA receptor is shown to be accumulated in growth cones of axonal processes suggesting a role for this subunit in neurite outgrowth. Neurite outgrowth from isolated cells also referred to as neuritogenesis, plays an important role for cell-cell interactions. It is dependent on cytosolic Ca²⁺ and accordingly, activation of the NMDA receptor stimulates neuritogenesis. The NMDA receptor is also known to regulate neuritogenesis in primary cultures and is believed to play an important role in neuritogenesis in the adult as well as in developing brain. However, it is noteworthy that the sigma 1 receptor, also in control of calcium homeostasis, is previously described to induce neuritogenesis.

NG108-15 (neuroblastoma x glioma) hybrid cells (obtained from ATCC, USA) were grown in 260 ml plastic culture flasks (75 cm² bottom area; Nunc A/S, Roskilde, Denmark). Culture medium [D-MEM, supplemented with 10% foetal bovine serum (FBS) 100 U/ml penicillin G, 100 mg/ml streptomycin and HAT-supplement (hypoxanthine 5 mM, aminopterin 20 μM, thymidine 0.8 mM) (diluted 1:50)] was changed every second day. Confluent grown cells were split 1:3 to fresh culture flasks. Cells at passages 5 – 25 were used.

Differentiation of NG108-15

The NG108-15 cells were allowed to grown to approximate 50% confluence, thereafter the cells were transferred to fresh 100 mm culture dishes (Sarstedts). The culture medium was changed to differentiating medium [D-MEM supplemented with 0.5% FBS, 1.5% DMSO, 100 U/ml penicillin G, 100 mg/ml streptomycin and HAT-supplement (diluted1:50)]. The cells were allowed to differentiate for 24 h, defined by growth characteristics according to Seidman and co-workers. Dishes were checked before treatment was started, using only dishes containing > 95% differentiated cells.
Membrane preparation

Rat tissue membrane preparation for receptor binding (paper I & V)

Membranes were prepared from the frontal cortex, a tissue rich in NMDA receptors and consists of circa 50% NR1/NR2A and 50% NR1/NR2B in adult animals (paper I and V), cortex (paper V), the hippocampus (paper V) and the hypothalamus (paper V) tissues. The tissue was homogenized by means of a Teflon pestle homogenizer in ten volumes of ice-cold 50 mM Tris/acetate buffer supplemented with 0.32 M sucrose at pH 7.4. The homogenate was centrifuged twice at 1,000 x g for ten minutes at 4°C. The supernatant was further centrifuged, at 17,000 x g, the resulting pellet was homogenized in 20 volumes of ice-cold 50 mM Tris/acetate buffer pH 7.0, and finally centrifuged three times at 50,000 x g for 30 minutes. The final pellet was re-suspended in ice-cold rat storage buffer [10 mM Tris/acetate (pH 7.0), 10% sucrose] and stored as 500 μl aliquots at -80°C. The protein content was determined by the method described by Lowry and co-workers.

Cell membrane preparation for receptor binding (paper II & III)

The CHO-E2 cells were harvested after brief incubation in trypsin/EDTA at 37°C and centrifuged at 1,000 x g for 5 minutes. The supernatant was decanted and the pellet was dissolved in cell buffer (50 mM Tris/HCl, pH 7.4). Centrifugation was performed three times and the final pellet was dissolved in a small amount of binding buffer, and homogenized in a polytron homogenizer. More cell buffer was added to the homogenate, which was then centrifuged at 35,000 x g for 15 minutes. The pellet was dissolved in ice-cold cell storage buffer [10 mM Tris/HCl (pH 7.4), 10% sucrose], and the well-washed membranes were stored at -80°C. The protein content was measured by the method described by Lowry and co-workers.

Ligand binding

³H-ifenprodil receptor binding was conducted as described in paper I-III & V. In short, the rat frontal cortex homogenate was incubated with ³H-ifenprodil, unlabeled ifenprodil and neurosteroids for two hours at room temperature (paper I-II & V), 24 h at 4°C (paper II) or 30 min (paper II-III & V) in order to reach equilibrium. All mixing and dilution steps were performed in an automated robotic work station (Biomek® 2000). Incubation was terminated by rapid filtration under vacuum, using a Tomtec® 96 well
harvester and GF/A (paper I) or GF/B (paper II-III & V) filters. Filters were scintillated and measured for beta radiation in a TriLux® beta counter.

Total specific binding, competition binding, and dissociation assays were carried out, using 0.6 nM (6.0 nM at 37°C; 1.5 nM in sigma receptor experiments) \(^{3}\)H-ifenprodil and in saturation analysis, concentrations were ranging between 0.01 and 32 nM (0.01 – 38 nM at 37°C). Non-specific bound \(^{3}\)H-ifenprodil was determined in the presence of 10 μM (100 μM at 37°C) unlabeled ifenprodil (NMDA receptor experiments) or 100 μM haloperidol (sigma receptor experiments). Dissociation assays started after equilibrium was reached and induced by the addition of 100 μM (1 mM at 37°C) unlabeled ifenprodil. In paper I-III & V, trifluoroperazine was used to block all other sites for \(^{3}\)H-ifenprodil apart from the NR2B site. In paper V, 1.0 mM spermidine, 1.0 μM prazosin \(^{272}\) and 50 nM GBR 12,935 (1-[2-(diphenylmethoxy) ethyl]-4-(3-phenylpropyl)-piperazine) \(^{7,8,94}\) were used to block other sites (NR2B subunits, \(\alpha_1\)-receptors and piperazine acceptor sites respectively) for \(^{3}\)H-ifenprodil, when studying the sigma receptor binding of \(^{3}\)H-ifenprodil. In paper III, glutamate and glycine were added to the buffer in order to set the NMDA receptor in an activated state, this for the comparison to the data from intracellular calcium measurements.

Ligand binding calculations

Saturation studies.

The results from saturation experiments reveals the number of binding sites available (\(B_{\text{max}}\)) for the radioligand, expressed as pmol bound radioligand per mg membrane proteins, and its affinity for the receptor, expressed in the apparent dissociation constant (\(K_d\)) in nanomolar. Saturation isotherms were created from three independent series of \(^{3}\)H-ifenprodil saturation binding experiments. The results from specific binding were analyzed using non-linear curve fitting and compared for the best fit (one or two binding sites), using the equations:

\[ B_{eq} = B_{\text{max}} \times \frac{[^{3}\text{H-ifenprodil}]}{K_d + [^{3}\text{H-ifenprodil}]} \quad \text{for 1 binding site and} \]

\[ B_{eq} = B_{\text{max 1st site}} \times \frac{[^{3}\text{H-ifenprodil}]}{K_{d 1st site} + [^{3}\text{H-ifenprodil}]} + B_{\text{max 2nd site}} \times \frac{[^{3}\text{H-ifenprodil}]}{K_{d 2nd site} + [^{3}\text{H-ifenprodil}]} \]

for 2 binding sites, where \(B_{\text{max}}\) is the maximum binding of \(^{3}\)H-ifenprodil and \(K_d\) is the apparent dissociation constant of \(^{3}\)H-ifenprodil.
The saturation experiments were conducted by adding subsequently higher concentrations of \(^3\)H-ifenprodil (paper I & III) or by using the same concentrations of \(^3\)H-ifenprodil and adding subsequently higher concentrations of ifenprodil (paper II & V), adding the concentrations of labeled and non-labeled ifenprodil, a method possible since the receptor has equal affinity for labeled and non-labeled ifenprodil.

**Competition studies**

The displacement studies of \(^3\)H-ifenprodil were performed with different concentrations of unlabeled ifenprodil (10 pM – 1 \(\mu\)M) (paper I-III & V) or haloperidol (100 pM – 100 \(\mu\)M) (paper II & V) or other tested substances (paper V). The results were analysed using non-linear curve fitting and compared for the best fit (one or two binding sites), using the equations:

**Equation 3**

\[
B_{eq} = B_{nsb} + \frac{(B_{sb} - B_{nsb})}{1 + 10^{([\text{competitor}] - \log IC_{50})}},
\]

for 1 binding site and

**Equation 4**

\[
B_{eq} = B_{nsb} + f_{1st\,site} \times \frac{(B_{sb} - B_{nsb})}{1 + 10^{([\text{competitor}] - \log IC_{50})}_{1st\,site}} + f_{2nd\,site} \times \frac{(B_{sb} - B_{nsb})}{1 + 10^{([\text{competitor}] - \log IC_{50})}_{2nd\,site}}
\]

for 2 binding sites, where \(B_{nsb}\) is non-specifically bound \(^3\)H-ifenprodil, \(B_{sb}\) is specifically bound \(^3\)H-ifenprodil, \(IC_{50}\) is the concentration of unlabeled ifenprodil that causes 50% inhibition at the binding site and \(f\) is the fraction of binding sites (1\(^{st}\) and 2\(^{nd}\) sites refer to binding sites with high and low affinity for \(^3\)H-ifenprodil). \(K_i\) values were calculated using the Cheng–Prusoff equation \(^{46}\):

**Equation 5**

\[
K_i = \frac{IC_{50}}{[\text{radioligand}]} K_d
\]

The Hill coefficient, \(n_H\), was introduced by Hill in the 1910\(^{th}\) in an attempt to describe the oxygen binding to hemoglobin, which occurs with a positively cooperative binding. When \(n_H = 1\), the interaction between ligand and receptor obey the law of mass action, for a bimolecular reaction \(^{118,163}\). A displacement curve diverged from the sigmoid shape display a \(n_H \neq 1\) \(^{163}\). A Hill coefficient exceeding 1 indicates a positive cooperativity and when the Hill
The Hill coefficient is below 1, the mechanism involved can be negative cooperativity, more than one binding site independently from each other or one binding site displaying different affinity states. The Hill coefficient was calculated using the equation:

\[
B_{eq} = B_{mb} + \frac{(B_{mb} - B_{mh})}{1 + 10^{(\log IC_{50} - [\text{competitor}]) \times n_H}}
\]

The results from competition experiments reveal the specificity of ligands to the binding sites and present non-labeled ligands affinity as IC\textsubscript{50} values recalculated to K\textsubscript{i} values (nanomolar). They also display the effects of allosteric modulation more visually than the saturation experiment.

**Dissociation studies**

The experiment is primly determining the dissociation rate constant (K\textsubscript{off}) (min\textsuperscript{-1}), which is depending on the affinity for the substance to the receptor. K\textsubscript{off} expresses the rate of the dissociation of the ligand-receptor complex. Its opposite is the association rate constant, K\textsubscript{on}, which is the rate of forming the ligand-receptor complex. Together, they calculate the apparent dissociation constant, K\textsubscript{d}:

\[
K_d = \frac{K_{off}}{K_{on}}
\]

The dissociation of ³H-ifenprodil from the NMDA receptor or the sigma receptor was studied at a fixed concentration of ³H-ifenprodil. The experiment began at equilibrium, reached after two hours at room temperature (paper I-II & V), 24 h at 4°C (paper II) or 30 min (paper II & III) and dissociation was initiated by the addition of excess unlabeled ifenprodil (paper I-III & V) or what is to be considered a large volume of buffer, 3000 μl (paper II). The results were analysed using non-linear curve fitting and compared for the best fit (one or two binding sites), using the equations:

\[
B_t = B_{eq} \times e^{-K_{off} \times t}
\] for 1 binding site and

\[
B_t = B_{eq_{1st\ site}} \times e^{-K_{off_{1st\ site}} \times t} + B_{eq_{2nd\ site}} \times e^{-K_{off_{2nd\ site}} \times t}
\] for 2 binding sites,
where $B_t$ is the specific binding at time point $t$ and $B_{eq}$ is the specific binding at equilibrium, $B_{1\text{nd site}}$ is the plateau between the 1\text{st} and 2\text{nd} binding sites and $K_{off,1}$ and $K_{off,2}$ are the dissociation rate constants.

Fluorescence measurements (paper III)

Cell preparation for $[Ca^{2+}]_i$ measurements

The cells were harvested after brief incubation in trypsin/EDTA at 37°C and centrifuged at 1000 x g for 5 minutes. The supernatant was decanted and the pellet was dissolved in a balanced salt solution (BSS). The BSS was used as a buffer with the purpose of keeping the cells viable and functional. The BSS contains 130 mM NaCl, 5.4 mM KCl, 2.0 mM CaCl$_2$, 5.5 mM glucose and 10 mM HEPES, pH 7.3. Centrifugation was performed three times and the pellet was then dissolved in a small amount of BSS.

Loading of fluorescence

Heat-activated cells were gently centrifuged (300 x g, 3 min), resuspended in 100 μL BSS supplemented with Fura-2 acetoxymethoxy ester (Fura-2 AM) (5.0 μM), 0.001% cremophore EL and probenecid (1.0 mM), and incubated for 45 minutes at 37°C. Extracellular Fura-2 AM was removed by dilution, gentle centrifugation (1000 x g for 5 minutes) and decantation, and the cells were resuspended in fresh BSS (50000 cells in 100 μL), now supplemented with 100 μM trifluoperazine and distributed in 96-well black, round-bottom plates (NUNC, Copenhagen, Denmark) for immediate $[Ca^{2+}]_i$ measurement.

$[Ca^{2+}]_i$ measurement

Intracellular calcium measurements were run in a fluorescence image plate reader (BMG Polarstar, Labvision, Stockholm, Sweden) at 37°C. The cells were illuminated alternately at 340 and 380 nm. The emitted signals were recorded at 510 nm. The quotient of the emission generated from 380 nm divided by the emission generated from 340 nm was calculated. Baseline measurements were established and a mixture of glutamate and glycine (final concentrations of 100 μM and 10 μM, respectively, dissolved in BSS) was added to the well by a pump (100 mL min$^{-1}$). This addition was performed in quadruplicate for every run and was used as an internal standard. The cells were pre-incubated, firstly with the neurosteroids and then with other substances, before measurement. The baseline was established and the glutamate and glycine solution was added. The peak signal was monitored
for 45 seconds per well. The peak counts were normalized to the internal standard and blank and were then translated into calcium ion concentrations.

Neurite measurement (paper IV)

The neurite outgrowth was morphometrically analyzed as described by Wu et al. The cells and the neurites were photographed (Minolta Z1 digital camera) through microscope (Olympus CK2) and 20x magnification. Each dish was photographed on three randomly picked spots at time zero (start of treatment), 24 h and 48 h after treatment. Photographs were transferred to, magnified and enhanced in Adobe Photoshop 3.0 (Adobe Systems Inc. 2005). The data recorded from each file include the number of neurites per cell, length of primary neurite, number of branch points per neurite and the size (thickness) of the neurites.

Data analyses

Data is presented as mean ± S.E.M. The binding (paper I-III & V) and fluorescence (paper III) data were assembled, and analyzed using nonlinear and linear regression in Microsoft Excel (Microsoft Corp.1995-2003) and Prism 4.0 (GraphPad, 2003). The biphasic curves were compared with the monophasic curves using the sum of squares (partial F-test) and the biphasic curve was chosen when considered to be a statistically significant improvement. One-way ANOVA was followed by Dunnett's multiple-comparisons post-hoc test (binding and fluorescence data) or Fischer’s Protected Least Significant Difference (PLSD) post-hoc test (morphometry data). $P < 0.05$ was considered as the significant level.
Results and discussion

Optimizing the processes

Evaluation of the membrane preparation
Stability of the test substances is vital to the process and the results. In order to assure that no sulfated neurosteroids were converted to non-sulfated neurosteroids, the membrane preparations were also conducted in the presence of estradiol (100 μM). Estradiol is an estrone sulfatase inhibitor which prevents the transformation of sulfated neurosteroids to non-sulfated neurosteroids. Since this membrane preparation yielded the same results as the membrane preparation without estradiol, estradiol was subsequently excluded.

The membrane preparations were also conducted in the presence of an enzyme inhibitor cocktail (Complete, Mini®), in order to evaluate receptor degradation during the homogenization procedure. Since there were no significant differences in total 3H-ifenprodil specific binding, enzyme inhibitors were subsequently excluded.

The discrimination of receptors (paper I-III & V)
The binding of 3H-ifenprodil to the NMDA receptor (paper I-III & V) was discriminated to the NMDA by the co-incubation of trifluoroperazine blocking all other sites. The low affinity NMDA receptor site was discriminated by the use of nanomolar levels of ifenprodil. The combination of GBR 12,909 and GBR 12,935, blocking the sigma 1 receptor and the piperazine acceptor site was a theoretical option for the study. The combination was discarded in favour for the use of trifluoroperazine, which provides the advantage to block α1-receptors as well, which is a target not only for ifenprodil but also for PS. The difference, in terms of receptor affinity, between 3H-ifenprodil and ifenprodil could be assumed to be neglected, why data from 3H-ifenprodil could be translated to non-labeled ifenprodil.

The binding was best described using one-site binding equations (paper I-III & V) and the specific binding was kept above 91%. $K_r$ and $K_{off}$
determinations are shown in Table 1. The $n_H$ showing the slope of the sigmoid displacement curve is close to -1 (perfect sigmoid shape), the evidence of one isolated binding site.

Table 1. Affinities of ifenprodil to the NMDA receptor. $K_i$-values are determined in competition experiments, displacing $^3$H-ifenprodil with unlabeled ifenprodil, $K_{off}$-values are determined in kinetic experiments, using an excess of unlabeled ifenprodil to induce the dissociation. Trifluoperazine was used to block all but the NR2B site for ifenprodil. n.d. = not determined. * using glutamate activated receptors. † Unpublished data obtained during experiments for paper III. See Material & Methods for full description of experimental procedures.

<table>
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<th>Tissue</th>
<th>Temp. (°C)</th>
<th>$K_i$ (nM)</th>
<th>$n_H$</th>
<th>$K_{off}$ (min$^{-1}$)</th>
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<td>Rat the frontal cortex</td>
<td>25</td>
<td>4.8 ± 0.7</td>
<td>-0.92</td>
<td>0.20 ± 0.022</td>
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<td>n.d.</td>
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<td>V</td>
</tr>
<tr>
<td>Rat the hypothalamus</td>
<td>25</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.16 ± 0.023</td>
<td>V</td>
</tr>
<tr>
<td>Recombinant CHO</td>
<td>4</td>
<td>60.7 ± 7.5</td>
<td>-0.88</td>
<td>4.9 ± 0.52</td>
<td>II</td>
</tr>
<tr>
<td>Recombinant CHO</td>
<td>25</td>
<td>57.2 ± 6.7</td>
<td>-0.91</td>
<td>5.1 ± 0.28</td>
<td>II</td>
</tr>
<tr>
<td>Recombinant CHO</td>
<td>37</td>
<td>60.9 ± 5.8</td>
<td>-0.90</td>
<td>4.7 ± 0.34</td>
<td>II</td>
</tr>
<tr>
<td>Recombinant CHO*</td>
<td>37</td>
<td>37.5 ± 5.5</td>
<td>-0.89</td>
<td>5.2 ± 0.013</td>
<td>III</td>
</tr>
</tbody>
</table>

The $^3$H-ifenprodil binding was limited to the sigma receptor by the presence of 1 mM spermidine, blocking the NMDA receptor$^{268}$, 50 nM GBR 12,935, blocking the piperazine acceptor site$^8$ and 1 μM prazosin, blocking the $\alpha_1$ receptor$^{272}$ (paper V). It was best described using one-site binding equations and the specific binding was kept above 93%.

The effects of neurosteroids involving the NMDA receptor

Allosteric modulation of the $^3$H-ifenprodil binding by neurosteroids (paper I-III & V)

The effects of PS and 3α5βS on $^3$H-ifenprodil binding to the NMDA receptor (paper I-III & V) and ifenprodil (paper III) were studied at low neurosteroid concentrations (0.1 – 100 nM), levels that possibly are more close to what could be considered as physiological$^{58,106,172,320}$ than experiments conducted with electrophysiological techniques using 100 μM of neurosteroids. The neurosteroids exerted no direct effects on neither the $^3$H-ifenprodil binding, such as competitive displacement, nor on the calcium influx through the NMDA receptor on their own. Instead, the distinct effects appear to be
modulatory, shifting the binding properties for $^3$H-ifenprodil and the blocking abilities for ifenprodil on the NMDA receptor in an allosteric manner.

An allosteric modulating agent by binding to its site, changes the affinity for another ligand to its site. The changes in affinity is then best measured as changes in dissociation rate $^{135}$ of the receptor-radioligand complex, measured as $K_{off}$, why the dissociation experiments is the best way of studying an allosteric modulation. $K_{on}$ is depending only on concentration and keeping the concentration constant means that changes in the dissociation curve is due to changes in the affinity / $K_{off}$ $^{163}$.

The possibility of the existence of two unique binding sites independent from each other or one binding site displaying different affinity states must be distinguished from negative cooperation. This is solved by performing to similar kinetic experiments: using “infinite dilution” only and compare this to “infinite dilution” plus excess of unlabeled ligand. The negative cooperative system will display an accelerating dissociation in the first case but not in the second one. When both experiments display the same dissociation profile, the system is composed of two binding sites or one binding sites with two different affinity states.

Different targets for PS and 3α5βS (paper I-III)

Many neurosteroids are suggested to have several different binding sites, not shared by all neurosteroids. PS and 3α5βS are shown not share binding sites in the high micromolar concentrations, this in studies on apoptosis $^{231,315,333}$.

In order to examine the possibility for the NMDA receptor of hosting two different high affinity sites for PS and 3α5βS in the nanomolar concentrations, we investigated their pharmacology (paper I). The first indication on the existence of two sites was the observation that PS and 3α5βS did not interact competitively. This conclusion is drawn from the observation that they did not significantly affect one another’s modulatory effect curves. It is indicating that PS positive and 3α5βS negative effects on $^3$H-ifenprodil specific binding are mediated through distinct sites. The existence of two sites is further supported by the findings that glutamate decreased the PS induced stimulatory effect by approximately 35% while glutamate had no effect on 3α5βS mediated inhibition. These results show that the binding site for PS is sensitive to the state of the NMDA receptor (glutamate activated or not), an effect that may be mediated through an allosteric interaction between the glutamate binding site located on the NR2B subunit $^{156}$ and the putative high affinity PS site. It is consistent with the findings in electrophysiological studies where the positive modulatory effect of PS is decreased during the activated state of the receptor $^{125}$. When present alone, glutamate increased $^3$H-ifenprodil binding, an effect that has been reported by others $^{101}$. Though in contrast, no effect of the competitive glutamate antagonist APV on $^3$H-ifenprodil binding was observed in this study, as was reported in the study of
Grimwood and co-workers (2000). The differences in the results could be explained by the absence (our cell homogenate) respectively the presence (tissue homogenate) of endogenous glutamate, so the described effect of APV in fact is the result of absence of the stimulating glutamate effect. No effects of APV were to observe on the modulatory effects of neurosteroids on $^3$H-ifenprodil binding. Thus, the present results further underline earlier findings that ifenprodil binds with higher affinity to activated NMDA receptors (state-dependent binding) \(^{144}\). The fact that glutamate had no effect on $3\alpha 5\beta$S induced changes of $^3$H-ifenprodil binding further support that its site is distinct from that of PS and suggests that the action of $3\alpha 5\beta$S is not dependent on the state of the NMDA receptor. Since neurosteroids appear to only have small structural differences (Figure 11) it may be difficult to believe that they possess such great differences in action. But when the structures are displayed in 3D (Figure 12) it can be more easily understood, where the double bond in steroid skeleton of PS forces a rather planar appearance of the molecule and $3\alpha 5\beta$S is heavily bent between the A- and B-ring.

![Figure 11. The 2D structures of PS (left) and $3\alpha 5\beta$S (right) show great similarities.](image)

![Figure 12. The 3D structures of PS (left) and $3\alpha 5\beta$S (right) reveal the big difference between the two neurosteroids. Illustration adopted from Weaver et al., 2000 \(^{315}\).](image)

The effects on $^3$H-ifenprodil binding to the NMDA receptor, of ligands acting at other modulatory sites of the NMDA receptor, were also studied (paper I). Reduction of two cysteine residues in the NR1 subunit is shown to potentiate the NMDA receptor response \(^{285}\). Such modification of the receptor complex, induced by the reducing agent dithiothreitol (DTT), had no
effect on the modulation of $^3$H-ifenprodil binding by PS or $3\alpha5\beta$S. The effects of the two neurosteroids were found to be unaffected in the presence of the co-agonist glycine which binding site resides exclusively on the NR1 subunit$^{19,151}$. Summing up the characteristics for the sites mediating the effects of PS and $3\alpha5\beta$S on $^3$H-ifenprodil binding, it is clear that the pharmacology display several similarities with those observed in electrophysiological experiments. In such studies on chick spinal cord neurons and on NMDA receptors expressed in *Xenopus laevis* oocytes, PS and $3\alpha5\beta$S were demonstrated also to act through different sites$^{232}$. Furthermore, the neurosteroids are shown not to interact with the redox modulatory or the glycine site$^{231,232,333}$. The interaction between PS (at the low-affinity site) and glutamate on one hand and ifenprodil and glutamate on the other, have been suggested to be mediated through conformational changes of their binding sites located on the NR2B subunit$^{101,125}$. Since we here observe interactions comparable to those found by these authors and no effect of the NR1 specific ligand glycine, it is tempting to speculate that the high-affinity site for PS, also is located on the NR2B subunit.

As none of the allosteric ligands tested affected $3\alpha5\beta$S mediated modulation of $^3$H-ifenprodil binding, it is likely that this neurosteroid act on a site not allosterically linked to any of those recognized by these ligands.

The neurosteroid effect curves are bell- or U-shaped (paper I-III)

The high affinity sites for the neurosteroids on the NMDA receptor are only available in small concentrations interval, where the action is present. PS and $3\alpha5\beta$S binding to the putative high affinity sites show opposite actions on the $^3$H-ifenprodil binding (paper I-III) and the ifenprodil calcium influx inhibition (paper III). Where PS increases the affinity for $^3$H-ifenprodil in a bell-shaped curve of action, $3\alpha5\beta$S decreases it in a U-shaped curve of action. In the narrow window of high affinity action for neurosteroids, binding data indicates that PS or $3\alpha5\beta$S both increase the number of accessible binding sites for $^3$H-ifenprodil and that the primary difference lays in the way they modulate the affinity for $^3$H-ifenprodil (paper I-III).

Two other neurosteroids were investigated: we found that neither allo-pregnanolone sulfate (ALLOPREGS; $3\alpha5\alpha$S) nor DHEAS changed the number of binding sites for $^3$H-ifenprodil. Nor did they affect the affinity for the radioligands in saturation and dissociation experiments. Further experiments could however bring more light to their modulatory activities, since competition data indicates that certain concentrations are able to change the curve fit into a two-site fit, 10 nM DHEAS slightly lowered the IC$_{50}$ for ifenprodil$^{136}$. Interestingly, where low doses of PS induced improvement of cognitive functions there were also a description of a biphasic dose-response curve$^{80}$ and the anti-depressant effect of DHEAS was limited to a bell-shaped dose
interval. Furthermore, biphasic effects have also been demonstrated to occur for other modulating factors acting on the NMDA receptor. For example, low doses of cations or polyamines stimulate binding of the non-competitive channel blocker $^3$H-MK-801 and the NMDA site selective antagonist $^3$H-CGP 39,653 to rat brain membranes while high doses reduce such binding. A similar effect has also been described for polyamines in studies on $^3$H-MK-801 binding to porcine hippocampal membranes. The inorganic divalent cation Zn$^{2+}$ has also been demonstrated to display bell-shaped dose-response curves in desensitization experiments measuring calcium current in HEK-293 cells expressing NR1/NR2A receptors. This phenomenon may reflect high and low affinity states of the same binding site or distinct sites with different affinities for the modulators.

**Temperature involvement (paper II)**

Ifenprodil binding is sensitive to temperature. In assays run at 4°C, ifenprodil binds approximately equally to the ifenprodil site on the NR2B subunit and to the sigma receptor. At 37°C, more than 90% of the ifenprodil binding is to the sigma receptor. With the temperature impact on ifenprodil selectivity in mind, the issue had to be investigated thoroughly: since the discovery of the neurosteroid modulatory effects on $^3$H-ifenprodil binding on rat frontal cortex membranes was made at 25°C and since the functional experiments had to be run at 37°C on recombinant NMDA receptors, the data would have been hard to translate. The recombinant NR1/NR2B receptors on CHO-E2 membranes were subsequently run at 4°C, room temperature (25°C) and body temperature (37°C) (paper II), evaluating the binding characteristics for $^3$H-ifenprodil. In agreement with the findings of Hashimoto and London (1993) and Hashimoto and co-workers (1994), the total available NR2B sites ($B_{max}$) for $^3$H-ifenprodil was significantly higher at 4°C compared to 37°C. Compared to 4°C, there was a tendency towards less number available at 25°C, but not statistically significant.

More important was the finding that the dissociation constant rate $K_{off}$ for $^3$H-ifenprodil to the NR2B subunit was independent of the temperature. The apparent affinity increased at 4°C compared to 37°C displaying a lowered $K_d$. Since $K_{off}$ was unaltered, the change in $K_d$ has to depend on the association rate (see Equation 7). This fact is supported by thermodynamic laws, when in the assay, the concentration of $^3$H-ifenprodil was far below the $K_d$, the association is directed by the concentration of receptors and ligands and the temperature. The more heat, the more energy, the faster association and $K_{on}$ are increased, resulting in a lowered $K_d$. $K_{on}$ and $K_{off}$ are generally targets for changes in temperature. But temperature does not always dictate the kinetics parameters. Haloperidol binding at dopaminergic receptors is non-sensitive to alterations in the temperature and ion channel kinetics often shown temperature independence. Since haloperidol and
ifenprodil shown similarities in kinetics and binding properties to the NMDA receptor, it seems reasonable that the dissociation could be insensitive to temperature.

Allosteric & direct effects of neurosteroids (paper I-III & V)

The NMDA receptor is modulated and fine-tuned, not only by single interactions with ligands, but by complex interactions between protons, polyamines, neurosteroids and ifenprodil. The protons are believed to play a central role in the regulation. Protons induce a tonic inhibition of the receptor at physiological pH. The inhibition is about 50% in the normal state \(^{289,297}\). Polyamines are relieving this inhibition, leading to a potentiation of the receptor activity, and ifenprodil is somewhat counteracting the polyamine action, increasing the proton inhibition \(^{167,179,210,229,298}\).

The present findings are examples of those complex interactions. Neurosteroids in nanomolar concentrations do not have any direct influence on the intracellular calcium. Neither did pre-incubation with the neurosteroids alter the response of the NMDA receptor when stimulated with glutamate and glycine. Instead they appeared able to modulate the calcium influx via interactions with ifenprodil at low concentrations. When glutamate and glycine stimulated and ifenprodil inhibited the receptors, the inhibiting action from ifenprodil was changed when pre-incubated with PS or 3α5βS. Pre-incubation with PS caused increased inhibition and pre-incubation with 3α5βS gave less inhibition. This suggests that PS increase the ifenprodil affinity and thereby its inhibiting effect, whereas 3α5βS produces an opposite action (paper III).

The neurite outgrowth and morphology in NG108-15 cells, is a process dependent on intracellular calcium. Experiments presented in this thesis confirmed that nanomolar concentrations of 3α5βS induce effects only via ifenprodil modulation, whereas nanomolar concentrations of PS exerted a somewhat more complex pattern of effects (paper IV). Neurosteroids are previously shown to regulate the morphology of astrocytes \(^{64}\). The changes in morphologic characteristics in the neurite outgrowth, due to NMDA receptor stimulation and modulation of this were studied in differentiated NG108-15 cells (Figure 13a). Stimulation with glutamate and glycine induced the neurites to grow thicker, longer and produce more branch point (Figure 13b). Branch points are nodes where neurites are connected or the possibility for a new neurite to launch. Stimulation with glutamate and glycine and inhibition with ifenprodil reduced the stimulatory outgrowth effect (Figure 13c). The combined addition of ifenprodil and PS to the glutamate and glycine stimulation was not different from ifenprodil blockade to glutamate and glycine stimulation after 24 h (no figure) but after 48 h the neurites were fragmented and the cells were detached from the surface (Figure 13d). The combined addition of ifenprodil and 3α5βS to the glutamate and glycine
reduced the ifenprodil blockade after 24 h (Figure 13e) but that effect was not to be seen after 48 h (Figure 13f).

Figure 13. The differentiated NG108-15 cells after 48 h of absence or presence of treatment [except from (e) which is after 24 h]. (a) non-treated, (b) glutamate and glycine treatment, (c) glutamate and glycine treatment combined with ifenprodil, (d) glutamate and glycine treatment combined with ifenprodil and PS, (e & f) glutamate and glycine treatment combined with ifenprodil and 3α5βS.

Since the outgrowth itself is a slow process, the neurites were examined after 24 h and 48 h. The experiment is troubled by the time span and cannot for certain exclude genomic effects from the neurosteroids. Indeed, the effects of PS alone, is as likely to be genomic as non-genomic, whereas the effects seen in the presence of 3α5βS at least partly may be due to interaction with ifenprodil since 3α5βS attenuate the effects of ifenprodil after 24 h, but not after 48 h (paper IV).

There are recordings that there are direct interactions between the NMDA receptor and neurosteroids, not involving other NMDA receptor agents. This is only documented for neurosteroids at high concentrations. The specific target for neurosteroids on the NMDA receptor is called steroid modulatory domain (SMD). Activation via SMD requires high concentrations of the neurosteroids (approx. 100 μM)\(^{132}\), why SMD could be considered as a low affinity site. Electrophysiological experiments using high micromolar concentrations of PS and 3α5βS define PS as a positive modulator and 3α5βS as a negative modulator of the NMDA receptor\(^{42,125,174}\), effects which possible originate from the interaction between the neurosteroids and SMD. These
effects are described to take place in the presence as well as in the absence
of glutamate activation.

The target or targets for lower concentrations of neurosteroids on the
NMDA receptor remain(s) undefined, but may well be high affinity site(s),
completely different from SMD, as the effects seen at much higher concen-
trations in electrophysiological experiments are independent of other ligands.
This is illustrated in a model (Figure 14) where one NR1 and one NR2B
with these and other known binding sites are shown, together with their in-
teractions. The existence of both high and low affinity binding sites for neu-
rosteroids on the NMDA receptor complex, could explain the difference
between the direct effects seen in electrophysiological experiments and the
modulatory action on cognitive functions\textsuperscript{79,80,180,181,193} as well as the modulation
of \(^3\)H-ifenprodil binding and ifenprodil inhibition, as seen in paper I-III & V.

\textbf{Figure 14.} Schematic view of the two subunit types forming the NMDA receptor
with binding sites and the results of binding to those sites. The ion pore is blocked
by Mg\textsuperscript{2+} in the non-activated state. Glutamate and glycine are the agonist and co-
agonist inducing the gate opening. Their binding also promotes the binding of the
channel blocker MK-801. Glutamate and ifenprodil increases each others binding.
Protons inhibit the gate opening and micromolar concentrations of neurosteroids at
SMD can either inhibit or promote the gate opening. Reduction or oxidation of the
sulfur bonds inhibit or amplify the amplifying effect of Zn\textsuperscript{2+} on the inhibiting effect
of protons. Polyamines and ifenprodil bind to separate targets which interfere with
each other causing decreased binding. Ifenprodil, as well as haloperidol, increase the
proton inhibition, whereas polyamines relieve the proton inhibition. Neurosteroids in
nanomolar concentrations seems to have different targets, one acting positively on
the ifenprodil binding and one negatively.
The effects of neurosteroids involving the sigma receptor (paper IV-V)

The sigma receptor is shown to bind neurosteroids. This raised an interest to investigate a possible interaction between ifenprodil and the neurosteroids at the sigma receptor. PS, 3α5βS and DHEAS at micromolar concentrations all displaced \(^{3}\text{H}-\text{ifenprodil}\) from the sigma 1 and the sigma 2 receptor (paper V). Previously, it is shown that PS, DHEAS and PROG have affinity to the sigma 1 receptor and to the sigma 2 receptor \(^{205}\). All these three neurosteroids display a planar structure due to the unsaturation in the A-rings. PS and DHEAS act as agonists and PROG acts as antagonist at the sigma 1 receptor \(^{20,204}\). An interesting difference is that the agonists are sulfated neurosteroids and the antagonist is not.

Those sigma 1 receptor agonistic properties of DHEAS have been demonstrated to counteract the memory deficit effect of MK-801 \(^{186}\) and inhibit persistent Na\(^+\) currents \(^{47}\). Concerning PS, its binding to and activation of a pre-synaptic sigma 1-like receptor in male Sprague-Dawley rat the hippocampus \(^{70,267}\) and cortex \(^{70}\), induce the NMDA receptor facilitated excitatory post-synaptic current (EPSC). PREG is suggested to induce glutamate release via the sigma 1 receptor \(^{200}\).

The effects seen on neurite outgrowth and morphology in NG108-15 cells were addressed to be mediated by interactions with the NMDA receptor (paper IV). However, it could as well be mediated by the sigma receptor, which is present in the cell line \(^{90,331}\). Talking against it, is the fact that it is believed that sigma receptors mainly exists in the endoplasmatic reticulum and not in the membrane \(^{113}\). But on the other hand, the use of the sigma receptor blocker trifluoroperazine, as in receptor binding and fluorescence experiments, was not possible due to unwanted reactions as ceased cell division, cell processes, as neurite growth, terminated and the cells assumed to spherical shape and ceased cell attachment to the surface. The assumption to spherical shape symptom is reported to be a sigma mediated effect in C6 glioma cells \(^{329,330}\). It is possible to speculate that the massive concentration of trifluoroperazine caused the cell detachment by interactions with sigma receptors. Then, it could be the same effect produced by the neurosteroids, mainly PS. It is strictly hypothetic and further studies are needed in order to shed light on the issue.

Influences of AAS on neurosteroid effects (paper V)

The AAS nandrolone decanoate treatment has been reported to demonstrate central side effects. The effects are rapid, occurring within minutes after administration. This is considered to be mediated by non-classical mecha-
nism. Possible explanations could be interference with neurosteroids, with the metabolism or formation of neurosteroids. In fact, the AASs danazol and stanozolol have been shown to have affinity for non-androgenic receptors. Since AAS act via classical (genomic) mechanisms as well, it is not unlikely that AAS could have influence on neurosteroids targets after chronic administration. Previous studies have shown alterations of the dopaminergic, the serotonergic, the GABAergic and the glutaminergic systems after chronic or sub-chronic treatment with nandrolone decanoate.

The theory of the interference between AAS and neurosteroids was studied in paper V. The glutaminergic system was previously studied on the mRNA level. This investigation of the NMDA receptor was conducted at protein level. The ifenprodil binding site on the NR2B subunit after chronic nandrolone decanoate treatment was studied, not only looking at the frontal cortex, but also at the hippocampus and the hypothalamus. There was no up- or down-regulation of the receptor, measured as $^3$H-ifenprodil binding to the NR2B subunit, as result of nandrolone decanoate administration. No difference was recorded in $^3$H-ifenprodil binding kinetics to the NR2B subunit between the AAS group and the control group. This was the case in all of the three regions. It is in agreement with previous studies of mRNA levels, except from the hypothalamus where an up-regulation of the mRNA for NR2B was seen. A change in mRNA levels does however not necessarily correspond to altered protein expression.

The kinetics of $^3$H-ifenprodil at the NMDA receptor was also unaltered of AAS administration, as was the modulatory effects of PS and 3α5βS at nanomolar concentrations. The conclusions drawn from those findings therefore became that chronic nandrolone decanoate treatment neither affect the neurosteroid and ifenprodil binding sites nor the NR2B subunits in the studied regions.

Turning the attention to another ifenprodil and neurosteroid target, the sigma receptor, the outcome was different. The chronic treatment with nandrolone decanoate changed the target for the neurosteroids on the sigma 1 receptor, decreasing their ability to displace $^3$H-ifenprodil from the sigma 1 receptor. The sigma 2 receptor was unaffected. Interestingly, the results also proved evidence that $^3$H-ifenprodil and neurosteroids do not share binding site on the sigma 1 receptor, but that the neurosteroid binding interferes with and blocks the $^3$H-ifenprodil binding, since $^3$H-ifenprodil, ifenprodil, haloperidol, trifluoperazine and (+)-SKF 10,047 binding to the sigma 1 receptor was unaltered by the AAS treatment. Another steroid overload that affects the sigma receptor function in the brain is pregnancy, why it seems reasonable that AAS could target the sigma receptor.

This is probably a result of genomic interaction with the complex of the androgen receptor and nandrolone, since the change is still there after decapitation, dissection and membrane preparation. It could also be the consequence of high concentrations around the receptor, which has changed its
nature due to input from the immediate environment. Whether such effect can persist to the *in vitro* assay has to be further investigated.

These experiments leave a gap to be filled, since it does not include direct and rapid effect of nandrolone or nandrolone decanoate similar to the allosteric effects produced by the neurosteroids. It does neither tell about interactions nor competitions between the AAS and the neurosteroids.

**Imagining the use for neurosteroids**

**Neurosteroids in combating drug abuse**

The NMDA receptor, and in particular the NR2B subunit, is believed to be involved in the development as well as the maintenance of drug and alcohol dependence. Antagonists to the NMDA receptor are proposed to be effective in drug addiction treatment, diminishing and attenuate both the responses from drug stimuli and the withdrawal symptoms. They also seem to counteract the physical dependence, sensitization and tolerance to opiates. Concerning the reported side effects from NMDA antagonists at therapeutic concentrations, the use of fine-tuning allosteric modulators as neurosteroids may replace or at least decrease the required doses of the antagonists, making the doses required tolerable for human therapeutic use.

Neurosteroids possessing antagonistic properties at the sigma 1 receptor could be useful in the treatment of addiction to methamphetamine or cocaine. The sigma 1 receptor is a target for methamphetamine and the sigma 1 receptor is up-regulated in the whole brain by cocaine. This is suggesting a possible therapeutic role for sigma ligands in cocaine and methamphetamine dependence treatment, for instance neurosteroids.

**Antidepressive and anxiolytic use of neurosteroids**

Several studies have provided evidence for the involvement of the sigma 1 receptor in depression and the anti-depressive treatment. There is upcoming evidence for that the anti-depressive effects of SSRI, like fluoxetine (Fontex®, Prozac®) and SNRI, like venlafaxin (Efexor®) are mediated by sigma 1 receptor interaction and that neurosteroids can be used in treatment of depression due to sigma 1 receptor interactions.

Sigma 2 receptor ligands produce potent anxiolytic effect, which probably but not certainly is sigma 2 receptor mediated, since the selectivity between the two sigma subtypes not is complete. They have been shown to induce apoptosis in cancer cells, why those ligands could be of use in anti-cancer therapy. Cancer might be a disease followed by anxiety, why anxiolytic properties of the anti-cancer drug could be of use.
The abuse of AAS is often accompanied by depression, anxiety and aggressive behavior. Chronic AAS treatment has been shown to induce anxiety by altering the GABA_A receptor in the cortex 29,30, but as mentioned above, these symptoms are also induced by the sigma receptors. Thus, those symptoms could be the result of altered sigma 1 receptors or altered levels of neurosteroids, induced by the AAS. Therefore, the hypothesis could be formed, that chronic AAS administration is followed by losses in sigma receptor function. This is expressed as above mentioned symptoms. Therefore, before it has been fully examined, the influences of AAS on the sigma receptor cannot be ruled out.

Thus, neurosteroids seems to be potential agents in the treatment of depression and anxiety, two often accompanied syndromes that causes big economical losses to the society and personal suffering.

Neuroprotective use of neurosteroids

The need for neuroprotection is present in diseases as Alzheimer’s disease, Parkinson’s disease, stroke, spinal cord injury and partum hypoxia injuries. Neurosteroids and NMDA receptor antagonists are both groups of substances with neuroprotective properties. The affinity for several other receptors has limited the clinical use of competitive and non-competitive drugs, e.g. ifenprodil. Ifenprodil was once first defined as an anti-hypertensive drug 104,170, then thought of as a neuroprotective drug 96,265,272 assigned to its NMDA antagonistic effects. The non-tolerable side-effects thwarted the plans.

The family of allosteric modulators is attractive as therapeutic agents with unique advantages to conventional agonists and antagonists due to their state-dependent action. In this context the effect of PS to stimulate the inhibitory effect of ifenprodil effect could be of clinical interest, since a maintained effect at decreased doses of the NR2B antagonist may help to reduce its side effects. Still, ifenprodil could face limited clinical usefulness, since NMDA receptor antagonists display side effects connected to the NMDA effect. The side effects include nausea, vomiting, psychosis and memory impairment. However, the weak or partial NMDA receptor antagonist memantine seems to be well-tolerable and still efficient in neuroprotection 88. And there have been/are derivates of ifenprodil in clinical trials for neuroprotection, lacking some of ifenprodil’s side-effects 45. Whether it is the lack of other targets for memantine or the weak effect on the NMDA receptor that makes it well-tolerable is not known, but if the tolerance is due to weak NMDA effect, it could mean that ifenprodil would be tolerated clinically if combined with 3α5βS, attenuating its NMDA antagonistic effect.

The sulfated neurosteroids themselves are metabolites of respective non-sulfated neurosteroid. Desulfatation is the reversed reaction, where non-sulfated neurosteroids are formed from sulfated neurosteroids. It does take
place and if it is not saturable, treatment with sulfated neurosteroids will result in elevation of non-sulfated neurosteroids and the risk for non-desired effects increases. Especially in the case of PS, which is converted to PREG, from which several, almost every neurosteroid can be formed (Figure 3). Another question mark using highly water soluble sulfated neurosteroids as drugs has to be raised, namely whether they pass the blood brain barrier and are able to reach their targets.
Conclusions

This thesis reports the findings that neurosteroids in physiological concentrations have distinct effects on the NMDA. Neurosteroids in therapeutic concentrations have affinity to sigma receptors. It also reports changes of the sigma receptor, but not the NMDA receptor, from chronic treatment with the AAS nandrolone decanoate.

- The neurosteroids PS and 3α5βS in nanomolar concentration mediate their influences on the NMDA receptor via allosteric interactions, not via competitive binding. The interactions are robust to changes in the temperature.

- The modulatory effects were seen within narrow windows of nanomolar concentrations (0.1 – 10 respectively 1 – 100 nM) of the neurosteroids suggesting that they act through binding sites with higher affinity for the neurosteroids compared to those mediating the action on NMDA receptors observed in electrophysiological studies. At least two different sites for neurosteroids are involved, which could be classified as high affinity sites.

- The neurosteroids in those nanomolar concentrations possess the ability to change the kinetics for 3H-ifenprodil binding to the NR2B subunit of the NMDA receptor, as well as the calcium influx inhibitory effect of ifenprodil. Whether this is the result of conformal changes of the known ifenprodil binding site or if the neurosteroids unveil other sites for ifenprodil remains to be further elucidated.

- The chronic treatment with the AAS nandrolone decanoate genetically altered the binding for the neurosteroids, but not for 3H-ifenprodil, to the sigma 1 receptor. The conditions at the NMDA receptor were unchanged for both the neurosteroids and 3H-ifenprodil.

- The neurosteroids did not displace all 3H-ifenprodil from the sigma 1 receptor. Together with the above mentioned, this indicates that 3H-ifenprodil and neurosteroids do not share the same binding site on the sigma 1 receptor. The partial ability to displace 3H-ifenprodil from the sigma 1 receptor could possibly be explained by the local-
ization of the two binding sites, perhaps in a similar way like ifen-prodil and polyamines do not share binding site on the NR2B sub-unit, but interfere in each others binding, probably due to very closely located binding sites.

This thesis concludes that there are two high affinity targets for neurosteroids on the NMDA receptor. They are not previously described and they are not shared by other common NMDA receptor ligands or neurosteroids in high concentrations. We speculate that the structure, especially the possibility or impossibility to bend the A-ring, is important for the effect of the neurosteroid. Together, this could be a valuable pathway for explaining effects of neurosteroids in vivo. These targets on the NMDA receptor are robust to chronic and supratherapeutic doses of the AAS nandrolone decanoate, doses that are comparable to those used by AAS abusers. The treatment altered the sigma target for the neurosteroids, why we can speculate that the depressions associated with AAS abuse could be the result of sigma receptor changes.
Future perspective

The interactions between ifenprodil, haloperidol and neurosteroids at the sigma receptor need further investigation, and their binding characteristics need to be elucidated.

Neurosteroids and ifenprodil both interact on both the NMDA receptor and the sigma 1 receptor, which receptors are linked together and both control intracellular calcium. The effects of neurosteroids on ifenprodil actions could be tested in an in vitro model, both receptors non-blocked, providing data on how the complete system controls the intracellular calcium.

The NMDA receptors are at tonic proton inhibition at physiological conditions. It is believed that ifenprodil increases the proton inhibition and that polyamines relieve the same. It has also been proposed that neurosteroids also are involved in regulating the proton inhibition. Taken together, the effects of neurosteroids at different pHs could not only add knowledge to the nature of the neurosteroids and the NMDA receptor, but also about diseases believed to be proton mediated, e.g. epilepsy \(^{216,224,318}\). Neurosteroids, and especially the balance between neurosteroids, are shown to be involved in epilepsy \(^{83,84,116}\). Phosphorylation of the NR2 subunits seems to be of great importance for its function \(^{155}\) and the NR2B subunit is the most prominent phosphorylated NMDA receptor protein \(^{198}\). NMDA receptors being phosphorylated on the NR2B subunit, by ERK dependent reactions, cause epilepsy when activated \(^{216}\).

There has recently been described that there is a third NMDA receptor subunit, the NR3 \(^{73,74,219}\) as well as non-classically composed NMDA receptors. Non-classically composed NMDA receptors are composed of other combinations of subunits than two similar NR1s and two similar NR2s, e.g. two NR1s, one NR2A and one NR2B \(^{274}\). The incorporation of NR3 subunits would also create a non-classically composed NMDA receptor. It would be of interest to investigate whether the NR3 subunit has binding sites for neurosteroids, and it would also be interesting to study and characterize the non-classically composed NMDA receptor, in terms of responses to and behaviour in the presence of neurosteroids. The lack of information of the NR3 subunit, binding sites and expression in the rat brain, makes it impossible to rule out that some of the data presented in this thesis actually derives from NR3 binding. Indeed, recombinant expressed non-classically composed NMDA receptors could be a useful tool in this question.
The allosteric effects on the ifenprodil binding site on the NMDA receptor could very well be unique, but imagining a conformal change in the binding site, this change could perhaps be present in a nearby situated site, namely the polyamine site. The ifenprodil binding site on the NMDA receptor is situated nearby one of the polyamine sites, so close that binding to one of the sites interfere with binding to the other. This once lead to the misunderstanding that ifenprodil was a polyamine antagonist, since polyamines apparently displaced radiolabeled ifenprodil. This could be elucidated using a radiolabeled polyamine, e.g. $^3$H-spermidine.

Lots of experiments that have been undertaken, considering neurosteroid *in vitro* assays, have been performed using solvents like ethanol or DMSO. In the initial phase of our experiments, we planned to run both sulfated and non-sulfated neurosteroids. However, the solvents necessary to dissolve the non-sulfated neurosteroids severely changed the binding results for the sulfated neurosteroids compared to running the assay in just water-based buffer, why we focused on the sulfated neurosteroids from the beginning. It would be a convenient continuation to optimize the present assay for solvents enough to keep non-sulfated neurosteroids in solution, to test those neurosteroids. It would be particularly interesting to compare PREG and pregnanolone with PS and 3α5βS in this new environment and also to compare those results to the results presented in this thesis.

Another issue, concerning the binding of neurosteroids, is the problem of measuring the direct binding, which seems impossible to membrane structures. This has been explained with the model (Figure 15) where the neurosteroid bind to its target, the target then alters configuration as a result of the interaction. The configuration alteration not only induces an allosteric modulation of other ligands, e.g. ifenprodil, but also ejects the neurosteroid itself. That means that the time of neurosteroid binding is too short to be measured with conventional binding assays. One way of dealing with and detecting this kind of binding could be to monitor the binding in real time.

![Figure 15](image)

*Figure 15. A hypothetical and schematic model of the binding of neurosteroids to membrane targets, their allosteric effect on ifenprodil and the ejection of the neurosteroids.*
Populärvetenskaplig sammanfattning

Neurosteroider kallas de hormoner av steroidtyp som kan bildas i och ger snabba effekter i hjärnan. De är viktiga för processer som minne och inlärning, neuroprotektion, sinnesstämning samt neuronal plasticitet.

Neurosteroider bildas från kolesterol eller från andra steroidhormoner, som t.ex. könshormonerna. Effekterna medieras via interaktioner med membranbundna receptorer, såsom NMDA-, sigma- och GABA-receptorer. Mechanismen som ger snabba effekter skiljer sig från den mer långsamma klassiska där steroider utövar sin effekt genom att verka på genomet.

Syftet med denna avhandling var att identifiera och karakterisera de molekylärer mekanismerna för nanomolära koncentrationer av neurosteroider på NMDA receptorn, en koncentration som kan antas vara fysiologisk.

Resultaten visar att neurosteroidererna pregnenolonsulfat (PS) och pregnanolonsulfat (3α5βS) förändrar bindningen av ifenprodil indirekt, allosteriskt, till NMDA-receptorn, genom att binda till separate, unika ställen på NR2B-subenheten. Effekterna verkar vara temperaturoberoende och bekräftades vidare in funktionell kalciummätningsstudie. En andra funktionell studie visade att PS och 3α5βS påverkar glutamatstimulerad neurittillväxt I NG108-15-celler.


NMDA-receptorsystemet är djupt inblandat i neurodegeneration, celldöd och neurotoxicitet. Genom att blockera NMDA-receptorer kan neuronala
skador, t.ex. efter stroke eller vid Alzheimers sjukdom, begränsas eller hävas. Blockad av NMDA-receptorn är dock förknippat med mycket biverkningar, som i fallet med ifenprodil. Dessa fynd att neurosteroider och ifenprodil interagerar med varandra på NMDA-receptorn kan öppna för möjligheten att utnyttja en neuroprotektiv synergism, och därigenom komma ned på tolererbara doser för människa.
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