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MARKUS STORVIK

# Molecular mechanisms of the effects of uncompetitive NMDA-antagonist MK-801 on CREB related transcription factors

Doctoral dissertation

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Department of Neurobiology
A.I. Virtanen Institute for Molecular Sciences
Department of Pharmacology and Toxicology
Faculty of Pharmacy
University of Kuopio

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**Series Editors:**
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A.I. Virtanen Institute

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A.I. Virtanen Institute

**Author's address:**
Department of Neurobiology
A.I. Virtanen Institute
University of Kuopio
P.O. Box 1627
FIN-70211 KUOPIO
FINLAND
Tel. +358 17 163 665
E-mail: markus.storvik@uku.fi

**Supervisors:**
Doc. Garry Wong, Ph.D.
Department of Neurobiology
A.I. Virtanen Institute
University of Kuopio

Professor Eero Castrén, M.D., Ph.D.
Neuroscience Center
University of Helsinki

Doc. James Callaway, Ph.D.
Department of Pharmaceutical Chemistry
University of Kuopio

**Reviewers:**
Professor Raimo K. Tuominen M. D.
Department of Pharmacy
University of Helsinki

Docent Petri Hyytiä, Ph.D.
National Public Health Institute
Helsinki

**Opponent:**
Professor Astrid Lægreid, Ph.D.
Department of Physiology and Biomedical Engineering
Norwegian University of Science and Technology
Trondheim, Norway

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## ABSTRACT

Uncompetitive N-methyl-d-aspartate (NMDA) receptor antagonists, such as dizocilpine (MK-801) and memantine, bind to the NMDA receptor channel, and block $Ca^{2+}$ influx. The increase in intracellular concentration of second messengers, such as $Ca^{2+}$ and cyclic adenosine monophosphate (cAMP), activate the transcription of certain genes. This process is controlled by cAMP response element binding protein (CREB), a transcription factor that can be activated by synaptic activity. The aim of the present study was to characterize alterations in the expression of transcription factors produced by NMDA receptor antagonists. Regulation of gene expression of CREB and its modulators, and glutamate receptor subunits were studied after MK-801 administration. In addition, the gene expression profiles in rat brains after MK-801, with or without cocaine, were studied with DNA micro- and macro arrays.

High, but transient induction of genes related to CREB, such as inducible cAMP early repressor (ICER) and other CREM-family transcripts, were found following uncompetitive NMDA antagonist treatment. These induced transcripts, alone or in heterodimers with other transcription factors, were found to bind CRE-elements in DNA. The uncompetitive NMDA antagonists are therefore capable of altering the transcription of genes expressed in neurons. The effect of the acute NMDA-antagonist MK-801 on the expression of glutamate receptor subunits was also determined and mGluR3, GluR3 and GluR4 were affected. Finally, two DNA microarray experiments were performed after treatment with acute MK-801, acute cocaine, or after a combination of MK-801 and cocaine. The expression of altered genes on parietal cortex, frontal cortex, and in nucleus accumbens was profiled. Although MK-801 did not prevent the alterations in gene expression caused by cocaine, a high number of transcription factors and signalling transduction genes were among the 850 candidate genes.

In conclusion, these findings provide new information about the acute effects of uncompetitive NMDA on the regulation of gene transcription, which in turn leads to long-term changes in the brain. These findings should help to determine the baseline of how alterations in gene expression in brain develop during NMDA antagonist treatment.

National Library of Medicine Classification: QU 26.5, QU 60, QV 76.5, QV 113, QZ 52
Medical Subject Headings: gene expression regulation; gene expression profiling; receptors, N-methyl-D-aspartate / antagonists & inhibitors; dizocilpine maleate; cocaine; DNA-binding protein, cyclic AMP-responsive; transcription factors; repressor proteins; oligonucleotide array sequence analysis; in situ hybridization; rats; brain; computational biology

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Markus Storvik,
Jyväskylä, March 2004

**ABBREVIATIONS**

| | |
|---|---|
| AMPA | α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid |
| ANOVA | analysis of variance |
| AP-1 | activation protein 1 |
| BDNF | brain-derived neurotrophic factor |
| CA1-3 | cornus ammonis fields 1-3 of hippocampus |
| CaMK | calmodulin-activated protein kinase |
| cAMP | cyclic adenosine monophosphate |
| CaMK | $Ca^{2+}$/calmodulin-dependent protein kinase |
| cDNA | complementary deoxyribonucleic acid |
| Cpu | caudate putamen |
| CBP | CREB binding protein |
| CREB | cAMP element binding protein |
| CREM | cAMP response element modulator |
| DA | dopamine |
| DG | dentate gyrus of hippocampus |
| EMSA | electromobility shift assay |
| EST | expressed sequence tag |
| Fr ctx | frontal cortex |
| GABA | gamma-amino-butyric acid |
| GluR1-4 | glutamate receptor subunits 1-4 |
| GO | gene ontology |
| ICER | inducible cAMP early repressor |
| IEG | immediate early gene |
| *i.p.* | intraperitoneal |
| LTP | long-term potentiation |
| MAPK | mitogen-activated protein kinase |
| mGluR | metabotropic glutamate receptor |
| mRNA | messenger ribonucleic acid |
| MK-801 | (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-iminehydrogen maleate; dizocipline maleate |
| NF-kB | uclear factor kappa-B |
| NMDA | N-methyl-D-aspartate |
| NR1 | N-methyl-D-aspartate receptor subtype 1 |
| NR2 | N-methyl-D-aspartate receptor subtype 2 |
| NAc | nucleus accumbens |
| Par ctx | parietal cortex |
| PBS | phosphate buffered saline |
| PKA | cyclic AMP dependent protein kinase, protein kinase A |
| PKC | protein kinase C |
| PCP | phencyclidine |
| RNA | ribodeoxynucleic acid |
| SOM | self-organising map |
| VGCC | voltage-gated $Ca^{2+}$ channel |
| VTA | ventral tegmental area |

## LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications referred to by their corresponding Roman numerals (I-IV):

**I** Storvik M, Lindén AM, Kontkanen O, Lakso M, Castrén E, Wong G. (2000) Induction of cAMP response element modulator (CREM) and inducible cAMP early repressor (ICER) expression in rat brain by uncompetitive N-methyl-D-aspartate receptor antagonists. J Pharmacol Exp Ther. 294(1):52-60.

**II** *Törönen P, *Storvik M, Lindén AM, Kontkanen O, Marvanová M, Lakso M, Castrén E, Wong G. (2002) Expression profiling to understand actions of NMDA/glutamate receptor antagonists in rat brain. Neurochem Res. 27(10):1209-20. *Shared first authorship.

**III** Storvik M, Lindén AM, Lakso M, Wong G. (2003) Subtype selective decrease of AMPA and metabotropic glutamate receptor mRNA expression in rat brain by the the systemic NMDA receptor blocker MK-801 J Mol Neurosci. 21(1):29-34.

**IV** Storvik M, Tiikkainen P, van Iersel M, Wong G. (2004) Gene expression profiles in rat brain after MK-801 and cocaine treatments.(submitted)

In addition, some unpublished data is presented.

**TABLE OF CONTENTS**

# 1 INTRODUCTION

Glutamate is the major excitatory amino acid (EAA) neurotransmitter in the mammalian brain (Headley and Grillner, 1990). Glutamate is synthesised in neurons from glutamine, and stored into vesicles. An action potential leads to the release of glutamate into the synaptic cleft, and from there it is taken mainly into glial cells and there converted into glutamine. Glutamate is released from pre-synaptic neurons when the action potential reaches the synapse. The released glutamate increases the likelihood that a cell will fire by binding post-synaptic excitatory ligand gated ion channel receptors. The glutamate receptors also modulate cell state by second messengers, $Ca^{2+}$ or cAMP. The effects of the receptor activation eventually alters the function of transcription factors and transcription of genes (Kornhuber and Weller, 1997). This is the point where the electrical, chemical, and macromolecular systems of the brain meet, and in which glutamate plays an important role in learning, memory, and neuronal plasticity (Sheng and Kim, 2002).

Most of the long-term effects of glutamate are transmitted through NMDA-receptors. NMDA receptor antagonists have antidepressant, analgesic, anxiolytic, anaesthetic, and according to some studies also antiaddictive properties (Kemp and McKernan, 2002). Many potent NMDA receptor antagonists such as ketamine, and phencyclidine (PCP) produce psychotic symptoms, which are very similar to those seen in schizophrenia. On the other hand, moderately potent uncompetitive NMDA antagonists such as memantine and amantadine are in clinical use for neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease (Reisberg et al., 2003), and they are well tolerated (Parsons et al., 1999).

Addiction disorders bring enormous costs to societies. Despite intensive research, there is no effective cure for addiction. Drug use can be decreased or prevented by medication causing adverse effects when the drug of abuse is taken. Another strategy for treating drug addiction is to ease the withdrawal state, by making the cessation of use more comfortable. These strategies do not alter the subjective elements of drug use, mainly the drug craving. Several lines of research suggest that uncompetitive NMDA antagonists could decrease drug self-administration by inhibiting the molecular mechanisms leading to addiction (Glick and Maisonneuve, 2000). It has been shown that in rats, MK-801 prevents the sensitisation to cocaine. MK-801 also decreases the self-administration of cocaine in rats (Pierce et al., 1997), although the findings are controversial (Hyytia et al., 1999). One of the most important of these molecular mechanisms is the cyclic AMP response element binding protein (CREB), which is a transcription factor activated by intracellular calcium, cAMP, and other intracellular

signalling pathways. CREB takes part in memory consolidation and it's role in addiction has been pointed out in several studies (Carlezon et al., 1998; Nestler, 1993).

In this dissertation, the effects of uncompetitive NMDA ion channel blockers on the CRE-mediated gene expression have been studied at several levels. The alterations in the expression of CREB and related proteins have been assayed. The changes of expression of glutamate receptors are assayed to see the alterations at the receptor level. Finally, the gene expression profiles in brains of NMDA antagonist MK-801 and cocaine treated rats are determined by DNA-microarray techniques.

# 2 REVIEW OF THE LITERATURE

## 2.1 Glutamate

Glutamate is the main excitatory amino acid (EAA) neurotransmitter in the mammalian brain, and is important for passing electrical signalling over the synaptic clefts (Headley and Grillner, 1990). Glutamate is synthesised in neurons from glutamine and stored into vesicles. Glutamate is released from the pre-synaptic neuron when the action potential reaches the synapse and the intracellular $Ca^{2+}$ concentration rises. From the synaptic cleft, glutamate is mainly taken into glial cells and converted into glutamine. The released glutamate binds to excitatory ionotropic glutamate receptors, or to metabotropic glutamate receptors, which modulate cell state by second messengers and eventually by altering transcription factor function and gene expression (Kornhuber and Weller, 1997).

Excitatory receptors open ion channels or modulate the cell state by second messengers. These changes increase the likelihood that the neuron will fire (Krystal et al., 1999). At these levels the electrical, chemical and macromolecular systems of the brain meet and form the system known as brain activity.

Glutamate plays an important role in learning and memory, in neuronal survival, genetic expression of axon guidance cues, associative synaptic connectivity, formation of networks, and in other forms of synaptic plasticity (Thomas, 1995). Nearly 70% of all synapses in the brain have glutamate receptors. Glutamate is the central neurotransmitter in cortico-cortical, cortico-limbic, and cortico-subcortical neuroconnections, and essentially in all systems important for higher brain functions.

### 2.1.1 Molecular biology of the glutamate receptors

The effects of glutamate are mediated via ion channel-forming receptors and via metabotropic G-protein-coupled glutamate receptors. The ionotropic glutamate receptors are named by their agonists. AMPA receptors are made of GluR1-4 subunits, and kainate receptors of GluR5-7 and KA1-2 subunits. NMDA-receptor subunits are NR1, NR2A-NR2D, and NR3. There are also orphan receptors δ1 and δ2 which are likely non-functional (Dingledine et al., 1999). The most distinct of all of the glutamate receptor subunits is NR1, which binds glycine instead of glutamate (Seeburg, 1993). The metabotropic glutamate receptors (mGluR) exist in 8 subtypes (mGluR1-mGluR8) (Conn, Pin, 1997).

#### 2.1.1.1 AMPA-receptors

AMPA receptors consist of 4 or 5 subunits (M1 – M5), forming an ion channel by their M2 loop. AMPA receptors are typically heteromers of all combinations from GluR1 to GluR4 (Dingledine et al., 1999; Nakanishi et al., 1998). The subunit composition affects the ion channel currents. The receptor function is further regulated by protein kinases that phosphorylate receptor subunits. Phosphorylation affects the ion channel kinetics and the properties of binding of receptor-associated proteins (Koles et al., 2001). Activated AMPA receptor ion channels pass $Na^+$ and $K^+$ ions, which cause short (10-20ms) excitatory postsynaptic potentials. AMPA receptors can also pass some calcium, depending on the subunit composition. The receptors with GluR2 subunits pass only a little calcium compared to the others (Dingledine et al., 1999).

#### 2.1.1.2 Kainate-receptors

Kainate receptors are composed of GluR5, GluR6, and GluR7 subunits, which are close relatives to genes encoding AMPA receptor subunits GluR1-GluR4. Kainate receptors contribute to excitatory postsynaptic currents in many regions of the central nervous system including hippocampus, cortex, spinal cord, and retina. Presynaptic kainate receptors occur at both excitatory and inhibitory synapses (Huettner, 2003). Once there, they cause short currents of $Na^+$ and $K^+$ ions, plus more modest amounts of $Ca^{2+}$ ions to pass (Dingledine et al., 1999; Huettner, 2003).

#### 2.1.1.3 NMDA-receptors

NMDA receptors consist of five or sometimes only four subunits coded by individual genes. NR1 unit exists in 8 variants, and NR2 in four variants (NR2A-NR2D) each with different properties. At least one of the subunits must be of the NR1 type to form a functioning channel (Laube et al., 1998). In addition, the receptor has NR2 units. Variants of NR3 has two variants, NR2A seen in developing brain and NR3B in motoneurons (Nishi et al., 2001). All the subunit proteins consist of 3 transmembrane loops and one loop that does not penetrate the cell membrane, but forms the edges for the ion channel (Dingledine et al., 1999; Platenik et al., 2000); see figure 1).

In the brain, NMDA receptors have typically NR1 subunits and either NR2A or NR2B subunits, or NR1, NR2A, and NR2B subunits (Dunah and Standaert, 2003). NMDA receptors containing NR1 and NR2A have fast deactivation kinetics. Receptors with NR1 and NR2B binary receptors exhibit slow deactivation kinetics. NMDA receptors containing NR1, NR2A, and NR2B subunits have slow deactivation kinetics and also low affinity to haloperidol which is also seen in receptors with NR1 and NR2A subunits (Dunah and Standaert, 2003).

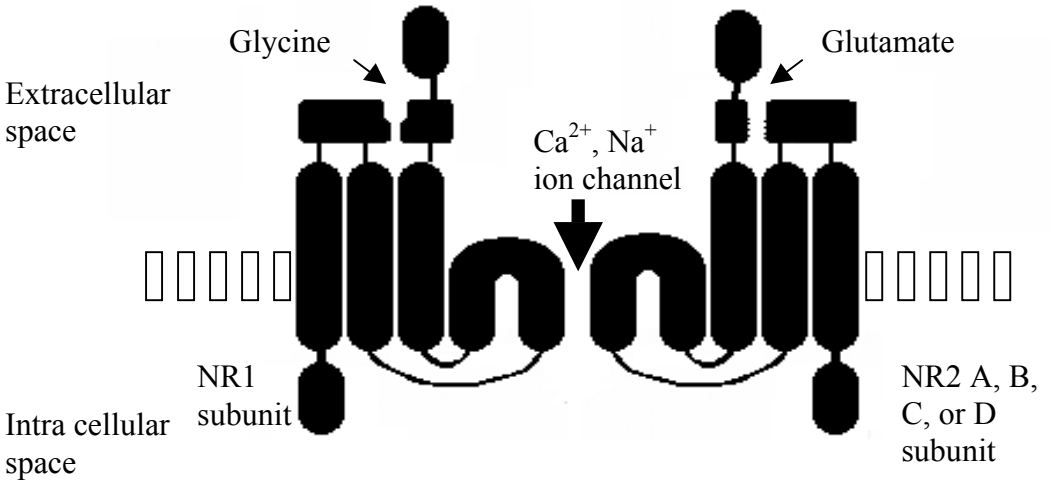


**Figure 1.** The organization of an NMDA-receptor complex. The NR1 and NR2 proteins have 3 transmembrane domains, and one intramembrane loop (adapted from Platenik et al., 2000).

NMDA receptors are located mainly in the postsynaptic side of the synapses, where they have a special role in synapse formation, and memory consolidation. These roles can be understood, as it's now known that their function is voltage dependent. They need two agonists to bind before activation, and they can pass significant amounts of calcium ions (Dingledine et al., 1999).

**The ligand binding sites in NMDA-receptors**

NMDA receptors have binding sites for two major neurotransmitters: glutamate and co-agonist glycine. Glutamate binds to the NR2 subunit, and glycine to the NR1 subunit, which is genetically far from other glutamate ion channel subunit genes (see figure 2) (Dingledine et al., 1999; Laube et al., 1998; Platenik et al., 2000). There is a polyamine binding site, which binds spermine, and spermidine. Polyamines decrease glutamate binding, and enhance the binding of competitive antagonists to the NMDA recognition site (Romano et al., 1991; Williams, 1997). This is likely to have a physiological role. In addition to these sites, a ligand can bind inside

the ion channel. Several uncompetitive NMDA antagonists, including ketamine, MK-801, and phencyclidine (PCP) bind here, but no endogenous ligand has been found (Porter and Greenamyre, 1995).

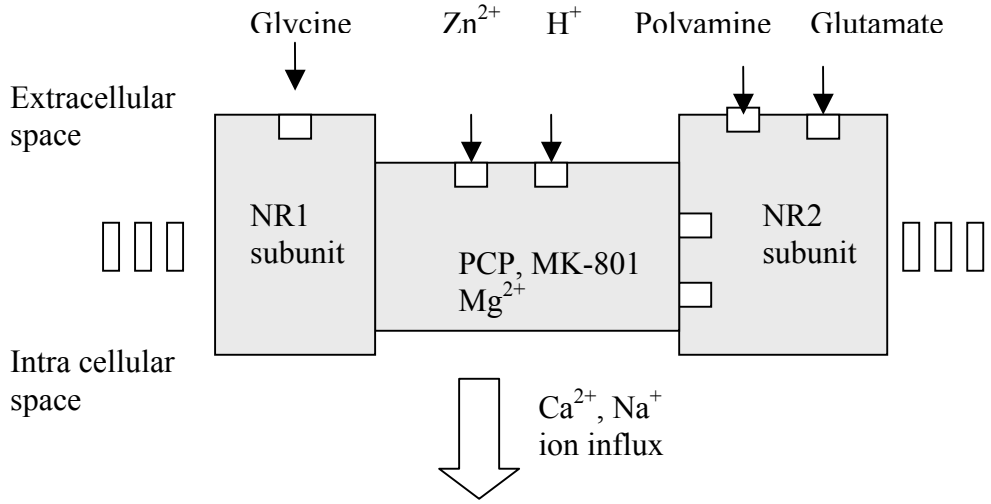


**Figure 2.** The ligand binding sites of an NMDA-receptor. Both glutamate and glycine need to bind the receptor in order to open the ion channel.

**NMDA-receptor ion channel passes $Ca^{2+}$ ions**

NMDA receptors differ from AMPA receptors in their function. AMPA-type glutamate receptor channels are relatively impermeable to $Ca^{2+}$, but NMDA-type receptor channels are highly permeable to $Ca^{2+}$. Compared to most ionotropic receptors, the ion channel of glutamatergic NMDA-receptors open and close slowly (Dingledine et al., 1999). The activation of NMDA-receptor requires depolarisation in the synaptic terminal, because it has voltage-dependent blockade by magnesium ions. This can be caused by prior activation of AMPA-receptors and their $Na^+/K^+$ ion currents. Because of the fast kinetics of AMPA-receptors, several bursts of activation are needed before the cell membrane reaches –50mV, when the $Mg^{2+}$ ion leaves the ion channel and glutamate binding can activate and open the ion channel for calcium currents (Bliss and Collingridge, 1993).

#### 2.1.1.4 Metabotropic glutamate receptors

Metabotropic glutamate receptors (mGluRs) belong to the large group of 7-transmembrane (7-TM) protein receptors, and do not form an ion channel, but mediate their effect via so-called G-proteins (Conn and Pin, 1997; Hermans and Challiss, 2001; Pin and Duvoisin, 1995). Metabotropic glutamate receptors are slowly acting and modulatory. The intracellular part of the metabotropic glutamate receptor binds G-protein, which activates or inhibits second messenger systems. Activated G-protein binds GTP and hydrolyses it into GDP. The activated G-protein then binds to adenylate cyclase or another mediator. The type of G-protein that a specific mGluR binds decides the type of effect.

Group I mGluRs, which are mGluR1 and mGluR5, activate phosphoinositide hydrolysis, which in turn leads to an increase of intracellular calcium, and cAMP concentration, and finally enhance NMDA-receptor function (Hermans and Challiss, 2001).

Group II mGluRs, mGluR2 and mGluR3 inhibit adenylate cyclase, which in turn decreases the amount of cAMP to be synthesised. Activation of the group II mGluRs inhibits glutamate release and decreases the effects of ionotropic receptors. They also inhibit the function of N-type voltage gated calcium channels.

Group III mGluRs, which are mGluR4, and mGluR6-8, also inhibit adenylyl cyclase, but are weaker than group II mGluRs. In some cases, their activation leads to an increased glutamate release, and they have a role in synaptic long-term depression.

Different mGluRs are located differently, depending on the brain areas and the group they belong to. Excitatory group I mGluRs, and inhibitory group III mGluRs are usually post-synaptic. Group II mGluRs are presynaptic and inhibitory (Conn and Pin, 1997; Schoepp, 2001). Metabotropic glutamate receptors modulate activities of ion channels and ion channel receptors. Interestingly, group I and II mGluRs both inhibit N-type voltage gated calcium channels (Conn and Pin, 1997).

#### 2.1.1.5 Phosphorylation and regulation of ionotropic receptors

The glutamate binding activity and ion channel properties are regulated by the phosphorylation of receptor subunits by protein kinases, and by the binding and transport of several proteins. The phosphorylation typically makes the receptor more active, or increases the ion currents. The protein kinases can be activated by second messenger systems activated by metabotropic or ionotropic receptors of several transmitters. The phosphorylation is transient, because phosphatase enzymes dephosphorylate proteins. The expression levels of the protein kinases and receptor-associated molecules are regulated by transcription factors, which are partially controlled by second messenger systems. The signalling activity between neurons mediated by transcription factors therefore has effects on the expression and phosphorylation, and the function of glutamate receptors in the area (Koles et al., 2001; Nakanishi et al., 1998).

Phosphorylation of a GluR1 subunit alters the functional properties of the AMPA receptor ion channel. $Ser^{845}$ phosphorylation of GluR1 increases $Ca^{2+}$ influx, but on the other hand, $Ca^{2+}$ influx leads to dephosphorylation of GluR1 subunit. $Ca^{2+}$ influx is responsible for AMPA-induced dephosphorylation of GluR1 subunit which occurs through L-type $Ca^{2+}$ channels and not by NMDA-receptors (Snyder et al., 2003). By this mechanism, dephosphorylation of GluR1 creates a negative feedback mechanism for the regulation of AMPA receptor activity in neurons (Snyder et al., 2003). The effects of the protein kinases on AMPA and NMDA receptors are presented in table 1.

The $Ca^{2+}$ influx stimulates the accumulation of AMPA receptors at the neuronal membrane of hippocampal neurons (Borgdorff and Choquet, 2002). Also, there are so-called silent synapses with only NMDA-receptors. With enough activity, AMPA receptors are also transported to the region to form a normal active synapse with both fast and slow glutamate receptors (Borgdorff and Choquet, 2002). These phenomena use several types of glutamate receptor associated proteins, such as glutamate receptor-interacting proteins GRIP-1 and GRIP-2.

The proteins associating with NMDA-receptors include synaptic organisers, which are PDZ-domain-containing proteins (e.g., proteins of the PSD-95 family, GRIP, AMPA receptor-binding protein (ABP)). They bind the C-terminus of NMDA-subunits, and function by clustering the receptor in the synaptic terminus. Also nitric oxide synthase can be bound to NMDA-receptors directly or by PDZ-proteins, where it is activated by $Ca^{2+}$/calmodulin activity (Watanabe et al., 2003).

AMPA receptors are targeted to excitatory synapses by PDZ domain-containing proteins (Hayashi et al., 2000). In synaptic long-term depression (LTD), AMPA receptors are rapidly internalised. Binding of GRIP1, ABP and PICK1 proteins are regulated by AMPA GluR2 which is phosphorylated on $Ser^{880}$ in LTD. GRIP1 and PICK1 may regulate AMPA receptor internalisation during LTD (Kim et al., 2001).

**Table 1.** Phosphorylation regulates AMPA and NMDA receptor kinetics and the properties of fast glutamatergic signalling. (CaMK, Ca2+/calmodulin-dependent protein kinase; PKA, protein kinase A; PKC, protein kinase C, $Ser^{831}$, $Ser^{845}$, and $Ser^{880}$, serine residues of glutamate receptor subunits; Src, Fyn, protein kinases; GRIP, glutamate receptor interacting protein; LTP, long term potentiation; PDZ, a domain in several proteins interacting with glutamate receptors) (Maj and Zhang, 2003; (Banke et al., 2000; Grosshans and Browning, 2001; Snyder et al., 2003)

| **Subunit** | **Kinase** | **Effect of phosphorylation** |
|---|---|---|
| GluR1 | CaMKII, $Ser^{831}$ | Phosphorylation increases the current flow |
| | PKA, $Ser^{845}$ | Peak response open probability increased. Dephosphorylation seen in LTD |
| | PKC, $Ser^{831}$ | Increase of current flow |
| GluR2 | PKC, $Ser^{880}$ | PDZ domain binding, dephosphorylation decreases GRIP1 binding |
| GluR3 | Not known, PKC suspected | Phosphorylation by PKC regulates the binding of the PDZ domain-containing proteins |
| GluR4 | PKA, PKC, CaMKII | Control of synaptic organization |
| NR1 | PKA | Potentiation of NMDA receptor function |
| | PKC | Increased opening probability |
| NR2A | PKA | Potentiation of NMDA receptor function |
| | PKC | Potentiation of NMDA receptor function |
| | Src, Fyn | Phosphorylation seen in LTP. Dephosphorylation in lithium treatment |
| NR2B | PKC | Potentiation of NMDA receptor function |
| | Fyn | Fyn-knockout impairs LTP |

### 2.1.2 Physiological significance of the ionotropic glutamate receptors

Glutamate receptors participate in synaptic plasticity, learning, and memory. A model for these phenomena is long-term potentiation (LTP) (Bliss and Collingridge, 1993). A key player in this mechanism is NMDA receptor activation and calcium influx. The short-term effects are formed by modulation of existing proteins, but long-lasting effects require synthesis of new proteins

LTP is an activity-dependent, long-lasting enhancement in the strength of synaptic connections between neurons. LTP is most easily demonstrated in the hippocampus, and was first reported in the rabbit by Bliss and Lømo (Bliss and Lomo, 1973). The phenomenon lasted for up to hours or even several days (Bliss and Lomo, 1973). LTP was then proposed as a candidate memory mechanism, since it exhibits associatively, co-operatively, and input specificity (Malenka, 2003). Input specificity means that LTP selectively enhances only the active pathways. Converging inputs that are not active at the time of stimulation are not potentiated. Hebb (1949) proposed, on theoretical grounds, that synaptic modification that only occurs as a consequence of coincidence between pre- and post-synaptic activity could support "learning". In this way, the co-occurrence of neuronal events might be recorded in networks of neurons and used as the building blocks for memory representations (Bliss and Collingridge, 1993).

In addition to memory, LTP and related modulations of synaptic functions are likely to be important in neuropsychiatric disorders where any kind of memory dysfunction is present, but is not necessarily the primary feature (Reid and Stewart, 1997). Rison and Stanton (1995) have reviewed its involvement in mechanisms of neuronal injury and in disorders such as epilepsy, Alzheimer's disease, and schizophrenia. There are significant alterations in the mechanisms of memory in other neuropsychiatric disorders. In patients suffering from depression, there are decreased neuronal activities on certain brain areas, which can be seen as alterations in LTP. Also antidepressant treatments can modify the same molecules needed in LTP, as discussed in chapter 2.2.3. Also in addiction, the memory mechanisms include pathways in common with LTP. Even a single cocaine exposure *in vivo* induces long-term potentiation in dopamine neurons, which can persist for days (Ungless et al., 2001).

Synapse activity can be enhanced for long after action potential burst. As calcium enters the neuron through NMDA-receptors, intracellular signalling pathways are activated (Nakanishi et al., 1998). This makes it possible for a neuronal network to sum up signals, either spatially, when several nerve termini activate simultaneously, or temporally when a

synapse is activated several times over a short period of time. Both pre-synaptic and post-synaptic neurons are required to be fired, or potentiation fails to become a long term potentiation (Bliss and Collingridge, 1993). In a classical model of LTP, the mechanism for this selectivity is explained by the $Mg^{2+}$ blockade in slowly activated NMDA-receptors, which is removed by depolarisation caused by high activity. NMDA receptor antagonists can prevent the induction of LTP and NMDA antagonists impair spatial learning tasks. The effect is dose-dependent (Davis et al., 1992).

Calcium entering neurons through active NMDA-receptors activates the kinases CaMKII and PKC controlling ion channels. NMDA-receptors phosphorylated by fyn, PKA, PKC, are activated by PKC; AMPA receptors recruited to "silent" synapses, conductance increased by phosphorylation, other kinases (PKA is activated by cAMP produced by CaMK activity), and transcription factors. In several brain areas, voltage-gated calcium channels and AMPA subtypes passing some calcium are required for LTP. It is also modulated by metabotropic glutamate receptors (Bashir et al., 1993), by regulating the function of voltage-gated calcium channels, and NMDA ion channel activities. The fast developing long-term potentiation of synapse and neural network is essential for short-term memory, and if the network activity is repeated, the synapses consolidate and form the base for long term memory, which can last for the rest of an individuals' life (Bliss and Collingridge, 1993).

## 2.2 Pharmacology of NMDA-receptors

A number of neurological and psychiatric conditions are associated with alterations in glutamatergic signalling. These include schizophrenia, depression, drug addiction, epilepsy, and several neurodegenerative conditions such as Parkinson's disease, Alzheimer's disease, and acute stroke. NMDA-receptors have been a focus of interest for drug development because of their unique properties (see chapter 2.1.1.3).

NMDA-receptors can be activated by ligand binding to glutamate or glycine sites, causing excitation. Ligands binding polyamine sites are modulators and could increase receptor functions (Bowe and Nadler, 1995; Romano et al., 1991; Williams, 1997).

There are competitive, non-competitive, and uncompetitive antagonists for NMDA receptors. The competitive antagonist competes with glutamate for it's binding site, whereas non-competitive antagonists do not, but bind somewhere else in the NMDA receptor complex, for example to the glycine site, polyamine site, or some unknown site. Uncompetitive antagonists are agents that bind inside the open NMDA-receptor ion channel, as depolarisation

and glutamate binding remove the $Mg^{2+}$ blockade (Dingledine et al., 1999). To avoid confusion, uncompetitive antagonists can be referred as NMDA-ion channel blockers.

### 2.2.1 NMDA-ion channel blockers

The agents acting by blocking NMDA receptor ion channels include many well-known drugs such as amantadine, memantine, ketamine, PCP, and the ethnopharmacological substance ibogaine (table 2). NMDA-channel blockers were first introduced as dissosiative anaesthetic agents that do not disrupt the control of breathing (Krystal et al., 1994). NMDA-channel blockers have also neuroprotective, and antidepressive effects (Palmer, 2001; Skolnick, 1999). NMDA channel blockers, especially memantine, have been also suggested to have antiaddictive properties, discussed in detail in chapter 2.4.7. In addition, high doses of high affinity NMDA-ion channel blockers produce psychotomimetic effects. The most potent NMDA-blockers are used in anaesthesia and they can induce psychotic symptoms (Krystal et al., 1999; Krystal et al., 1994). One exception is ketamine, which does not have higher affinity than memantine, but which belongs to the anaesthesia group because of its clinical profile. Amantadine, memantine, and the antitussive agent dextromethorphane, are well tolerated in their clinical uses. NMDA antagonists such as memantine protect against cocaine induced convulsions in non-sedating doses, whereas antiepileptic agents are required to be given in sedative doses (Witkin et al., 1999).

Very high affinity NMDA-ion channel blockers have a tendency to get stuck inside the ion channel (Krystal et al., 1999; Porter and Greenamyre, 1995). As an alternative explanation, some uncharacterised conformation change in the receptor protein complex 3D-structure could keep ligands attached. The prototype drug of the group is MK-801 (dizocipline) that is used for research purposes only. The affinity (Ki) of MK-801 is 0.003 μM which is hundreds of times higher than the Ki of ketamine (0.2 μM) or memantine (0.2 μM) (Krystal et al., 1999). MK-801 is a prototypical NMDA-receptor ion channel blocker. The drugs act by blocking the calcium ion current through NMDA-type glutamate receptors (Wong et al., 1986). MK-801 is a potent and selective antagonist of the NMDA receptor/channel complex and acts by binding to the so called PCP site located within the channel (Reynolds and Miller, 1988), thus blocking $Ca^{2+}$ influx.

**Table 2.** Uncompetitive NMDA-antagonists (Krystal et al., 1999; Rogawski, 2000). The binding properties and the suggested or clinical use of the agents vary greatly.

| **Common name** | **Affinity** | **Use** |
|---|---|---|
| Dextromethorphan | Low affinity | Antitussive |
| Amantatine | Low affinity | Neuroprotective, antidepressant |
| Memantine | Low affinity, 0.2 μM | Neuroprotective, antidepressant |
| Ibogaine | Low affinity | Traditional medicine |
| Ketamine | Dissociative Anaesthetic, 0.2 μM | Anesthetic |
| Phencyclidine | Dissociative Anaesthetic | Street drug |
| Dizocipline (MK-801) | Dissociative Anaesthetic 0.003 μM | Research |
| Dextrorphan | Dissociative Anaesthetic | Research |

### 2.2.2 Short-term effects

NMDA antagonists alter neurochemistry acutely, partially by direct inhibition, and partially by indirect circuits. Indirect mechanisms affect monoamines, catecholamines, GABA, and finally glutamate transmission.

In cortical regions, namely in the pyramidal cell layer, NMDA antagonists block the activation of GABAergic neurons with a greater potency than they inhibit the activation of glutamatergic neurons (Maccaferri and Dingledine, 2002). The decreased activation then reduces GABAergic transmission that inhibits glutamate neurons, resulting in increased glutamate release. This disinhibition of glutamate neurons then increases stimulation of non-NMDA glutamate receptors and leads to neuronal activation (Krystal et al., 2003). This will lead to an increase in action of monoaminergic neurons in cortex, limbic system, midbrain and brainstem. Also, the release of dopamine will increase. The dopaminergic activity on frontal cortex can be blocked by AMPA antagonists (Moghaddam et al., 1997).

Output of dopamine neurons projecting to the frontal cortex and prefrontal cortex is under tonic excitatory control of NMDA and AMPA receptors in the VTA, and AMPA receptors in the prefrontal cortex. NMDA receptors in the prefrontal cortex exert a tonic inhibitory control on dopamine release (Takahata and Moghaddam, 1998).

**Adverse effects**

The effects of the blockade of NMDA receptor mediated glutamatergic stimulation are seen as psychotomimetic symptoms, hallucinations, and thought impairment. In extreme cases of NMDA-antagonism, this mechanism leads to neurotoxicity (Olney et al., 1991). These are most often seen with high affinity NMDA-ion channel blockers, which have street value for drug abuse (Krystal et al., 1999). PCP and MK-801 are not used clinically because of these properties. Also ketamine, which is still in use, can produce nightmarish experiences for anaesthetised patients during the recovery/wake-up period.

In healthy people, ketamine and PCP induce euphoria in small doses. High doses induce also dissociation symptoms, disturbed thinking, hallucinations, and in even higher doses unconsciousness. These effects are similar to both the positive (e.g. perceptual disturbances) and negative symptoms (e.g. diminished emotional response) of schizophrenia, and PCP-induced symptoms in humans or in animals are the best schizophrenia model available (Krystal et al., 1994).

NMDA-antagonists can inhibit LTP, and they impair learning tasks in animals (Morris, 1989). Small doses of NMDA-antagonists seem to not only impair the calcium influx to nerve terminals, but also cause improper action of local circuits, as inhibition of glutamatergic neurons by GABA decreases because of NMDA-block on GABAergic neurons (Jevtovic-Todorovic et al., 2001).

Ketamine disturbs also working memory and information encoding short-term memory, but for a yet to be explained reason, small doses of NMDA-antagonists increase memory function in Alzheimer-patients, perhaps also through increased glutamate release (Krystal et al., 1999; Moghaddam et al., 1997). Ketamine has positive effect on moods, which helps in learning. In addition, the NMDA-antagonists have neuroprotective properties, which could be therapeutically valuable in neurodegenerative disorders. For memantine, the effective but tolerated doses have varied from 2 to 10 mg in pre-clinical trials, and 20 mg has been a typical dose in clinical trials (Kornhuber et al., 1994; Palmer, 2001). There is some evidence that the behavioural effects of NMDA-channel blockers can be disrupted by glycine-B receptor antagonists (Karcz-Kubicha et al., 1999).

**Toxicity**

Although NMDA antagonists protect neurons from death after ischemia and hypoxia (Schurr et al., 1995) by decreasing the excitotoxic damage in the brain through inhibition of calcium influx to neurons, they are neurotoxic themselves in high doses.

The neurotoxicity of low doses is reversible, higher doses leads to death by apoptosis and even higher to necrotic cell deaths. In rats, MK-801 has induced intracellular vacuolisation, and caused neuron deaths in a dose-dependant manner. The most sensitive brain region for this toxicity is the retrosplenial cortex (Olney et al., 1991).

In young rats, the toxicity is less intensive than in adult rats. It has been hypothesised that this could be related to the known phenomenon that ketamine does not induce psychotic effects in children, but does induce them in an adult human. The damage of uncompetitive NMDA-antagonists can be to some extent inhibited by antipsychotic agents (Nakki et al., 1996), or GABA-receptor binding anxiolytic agents (Jevtovic-Todorovic et al., 2001). This is interesting, as diazepam attenuates the psychotic symptoms induced by ketamine (Krystal et al., 1999; Olney et al., 1991).

### 2.2.3 Long-term effects of uncompetitive NMDA-antagonists

The mechanisms of the long-term effects of these drugs, like antidepressive and anti-addictive properties, are not completely understood. The development of these effects needs alterations in the expression of several types of neuronal proteins that participate in the regulation of neuronal transmission.

In monkeys, chronic blockade of NMDA receptors is relatively well tolerated, but in rats, MK-801 has significant and long-term adverse consequences on learning tasks (Paule et al., 2003). The NMDA antagonist MK-801 does not seem to damage working memory permanently, but in toxic doses the treatment does permanently damage some learning abilities, the effect can be seen even long after such treatments. The less potent NMDA antagonist memantine has a larger therapeutic range before such changes (Zajaczkowski et al., 2000), and clinically used doses do not induce neurotoxicity.

**The antidepressive effect of NMDA antagonists**

Clinical response to antidepressant drugs requires chronic administration, and there is usually a delayed onset in relief of symptoms (Schwaninger et al., 1995). NMDA antagonists have been shown to both block and enhance the action of antidepressants. In animals studies, low doses of NMDA-ion channel blockers have antidepressive actions, but in high doses, the effect of antidepressants is decreased by the coadministration of NMDA-blockers.

The effects of MK-801 in these tests may be due to an indirect activation of dopamine neurotransmission, and, therefore, it should be acknowledged that peripheral administration of MK-801 potentially could affect the release of other neurotransmitters (Callado et al., 2000). Although it is unlikely that NMDA receptor antagonists themselves could be clinically tolerated, the fact that the NMDA receptor-channel complex has multiple modulatory sites (and appears to be regulated by both adrenal steroids and neurotrophic factors) suggests that there is further scope for the development of new drug therapies with enhanced efficacy.

Uncompetitive NMDA-antagonists have some tendency to induce euphoria in high doses. In chronic treatment with non-euphoric doses, there has been seen some antidepressive effect (Petrie et al., 2000; Skolnick, 1999), which suggests that the antidepressive effect of NMDA antagonists is not an artifact caused by increased euphoria-related locomotor activity. Both preclinical and recent clinical studies support the finding. Also chronic administration of antidepressants to mice alters the expression of NMDA receptor subunits. It is hypothesized that traditional antidepressants and these agents converge to produce an identical functional

endpoint: a region-specific dampening of NMDA receptor function (Skolnick, 1999), but it is not necessary that both affect the same targets downstream.

Several studies in animals demonstrated that the activity of transcription factor CREB is upregulated by chronic antidepressant treatment. Therefore, it has been hypothesised that antidepressant treatment exerts its therapeutic effect by this mechanism (Koch et al., 2003a; Koch et al., 2002). CREB could be the mechanism that explains the antidepressive effect of NMDA antagonists. Since it is phosphorylated CREB (pCREB) that determines its transcriptional activity, it is pertinent that some antidepressants have been shown to reduce pCREB in the brain *in vivo* and in tissue culture *in vitro*. Moreover, pCREB is downregulated in human fibroblasts from patients with major depression and in the post-mortem brain of suicide victims with a history of depression (Manier et al., 2002; Sulser, 2002).

**Neuroprotection in neurodegenerative disorders**

Due to the efficacy of NMDA-ion channel blockers to reduce acute excitotoxity, it was suggested that these agents might have potential in treatment of neurodegenerative disorders. There were also case reports of cognitive improvement of amantadine treated senior influenza patients. Amantadine is a weak uncompetitive NMDA-antagonist, a close relative to memantine that usually does not produce severe adverse effects (Kornhuber et al., 1994). Also, there have not been street-markets for memantine, which could be used as evidence of the lack of abuse potential, which high-affinity blockers and to some extent even the unspecific low-affinity agent dextromethorphan have.

The clinical studies have demonstrated efficiency of memantine in moderate-to-severe Alzheimer's disease (Reisberg et al., 2003). NMDA-blocker treatment is expected to increase the life expectancy in patients with Alzheimer's disease (Danysz and Parsons, 2003). In Parkinson's disease, memantine can balance the dopaminergic and glutaminergic imbalance in basal ganglia, but NMDA-antagonists also increase the expression of genes needed in dopamine synthesis, neuronal signalling, and neuronal survival (Danysz and Parsons, 2003; Parsons et al., 1999).

## 2.3 Gene transcription and NMDA-antagonists

The response to external stimuli can lead to permanent alterations on neuronal structure and function by novel protein synthesis. The genes are transcribed into RNA, which are then translated as proteins with peptide-structure. This process is initiated by activation of transcription factors, which bind DNA and enable the RNA synthesis to begin. Some of the transcription factors are constitutive, which are activated by phosphorylation. Some other transcription factors are expressed only when needed. They are products of genes transcribed after activation of inducible transcription factors (Herdegen and Leah, 1998).

The transcription factors are classified into several large groups based on the structure, and the groups are further divided into families based on which DNA consensus sequences the factors recognize (Herdegen and Leah, 1998). Many neuronal functions are regulated through cAMP or $Ca^{2+}$ dependent signaling pathways. Neuronal stimulation mediates expression of genes containing $Ca^{2+}$/cAMP response elements. $Ca^{2+}$ and cAMP were found to induce transcription of genes containing cAMP response elements (CRE) in the promoter region. Transcriptional factors binding to CRE's in the promoters belong to the basic leucine zipper superfamily and are composed of three genes in mammals, CREB, CREM, and activating transcription factor 1 (ATF-1). The most notable transcription factor binding CRE is CREB. The family of transcription factors binding CRE contains both activators as CREB, and repressors, such as ICER proteins (De Cesare and Sassone-Corsi, 2000).

The CREB-CRE transcription factor pathway is necessary for long-term memory encoding, and neuronal cell survival. The stimuli requiring learning and memory activate CRE-mediated gene expression (Impey et al., 1998; Platenik et al., 2000).

### 2.3.1 CREB

CREB is a basic leucine zipper (bZIP) transcription factor. The bZIP's class of transcription factors include gene families similar to AP-1 proteins, CREB, C/EBP, and some minor families. CREB belongs to the same family as the CREM, and ATF-1 transcription factors. These transcription factors have a DNA binding domain (basic domain), and dimerization domain (leucine zipper). When PKA or CaMK(IV) phosphorylates CREB-proteins, they form dimers. The dimer can bind DNA specifically at CRE. This way $Ca^{2+}$/cAMP can increase expression of certain genes, which alters the cell function in a long-term way.

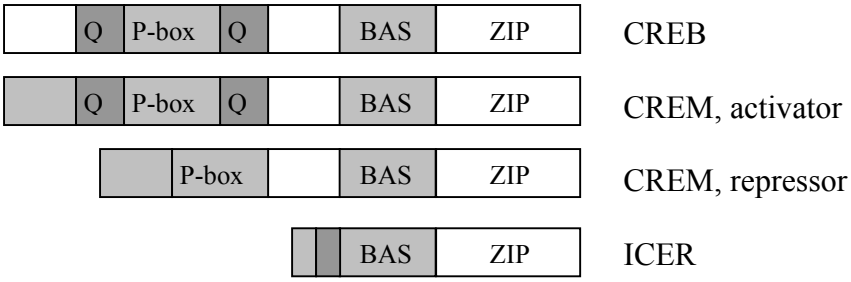


**Figure 3.** The CREB and CREM family bZIP transcription factors. Near the carboxy end on the left there are Q-domains required for basal activity, the protein kinase inducible domain, and near the amino end there is proline-rich dimerization domain and DNA-binding domain. Legend: Q, Q-domain; P-box, phosphorylation site; BAS, basic domain; ZIP DNA-binding domain (Adapted from Servillo et al., 2002)

The name for transcription factor CREB stands for cAMP response element binding protein, for it can bind to CRE sequences in DNA ( 5'TGACGTCA3'), (Gonzalez et al., 1989). CRE-mediated transcription begins after the increase of intracellular calcium concentration. A more correct name would be cAMP/$Ca^{2+}$ response-element binding protein, as CRE has been found to be in several genes, in which transcription is activated by both cAMP or $Ca^{2+}$ concentration increases in the cell. CREB is not the only protein that can bind CRE-element, but it is the most notable (Quinn, 2002), since other transcription factors can bind to CRE. Therefore CREB activity is not always the same as transcription of genes containing CREs. (Hu et al., 1999). CREB protein has three isoforms: CREBalfa, CREBbeta, and CREBdelta. CREBdelta is the prominent form consisting of 90% percent of all the forms (Herdegen and Leah, 1998). The CREB transcription factor is the connection point where the electrical stimulation induced second messenger signals in the cell can lead to transcription of the DNA (see figure 4).

**Glutamate stimulation can activate CREB**

Glutamate can activate CREB by all types of glutamate receptors. NMDA-receptors are permeable to calcium ions. Calcium influx activates protein kinases, for example PKA, which phosphorylates CREB. AMPA-dependent phosphorylation of the nuclear transcription factor, CREB, requires L-type $Ca^{2+}$ channels (Rajadhyaksha et al., 1999). In contrast, AMPA-dependent CREB phosphorylation is also blocked by NMDA receptor antagonists (Rajadhyaksha et al., 1999). Metabotropic glutamate receptors regulate CREB activity via cAMP or IP3 second messenger systems in a type specific manner.

**Regulation of CREB activity**

CREB is constitutively expressed, although some upregulation is reported by chronic antidepressant treatment. Regulation of CREB activity is mainly dependent on the phosphorylation state. CREB can bind to DNA without being phosphorylated, but the activity is low. The phosphorylation alters the 3D-structure and allows additional factors to interact with CREB.

Basal activity requires Q-boxes near the carboxyl end of the protein. This carboxy-terminal constitutive activation domain (CAD) recruits the promoter recognition factor TFIID, and RNA polymerases (Quinn, 2002). Next to that domain, there is kinase-inducible domain (KID) with phosphorylation sites for PKA, PKC, casein kinase, and CaMK IV (Quinn, 2002)**.** Intracellular signalling cascades leading to CREB-activity include Ras/MAPK, $Ca^{2+}$/CaMK, PI3K/Akt, and cAMP/PKA pathways (Dawson and Ginty, 2002; Deisseroth et al., 1998; Kim et al., 2000). CREB is dephosphorylated by serine/threonine phosphorylases (Koch et al., 2003b).

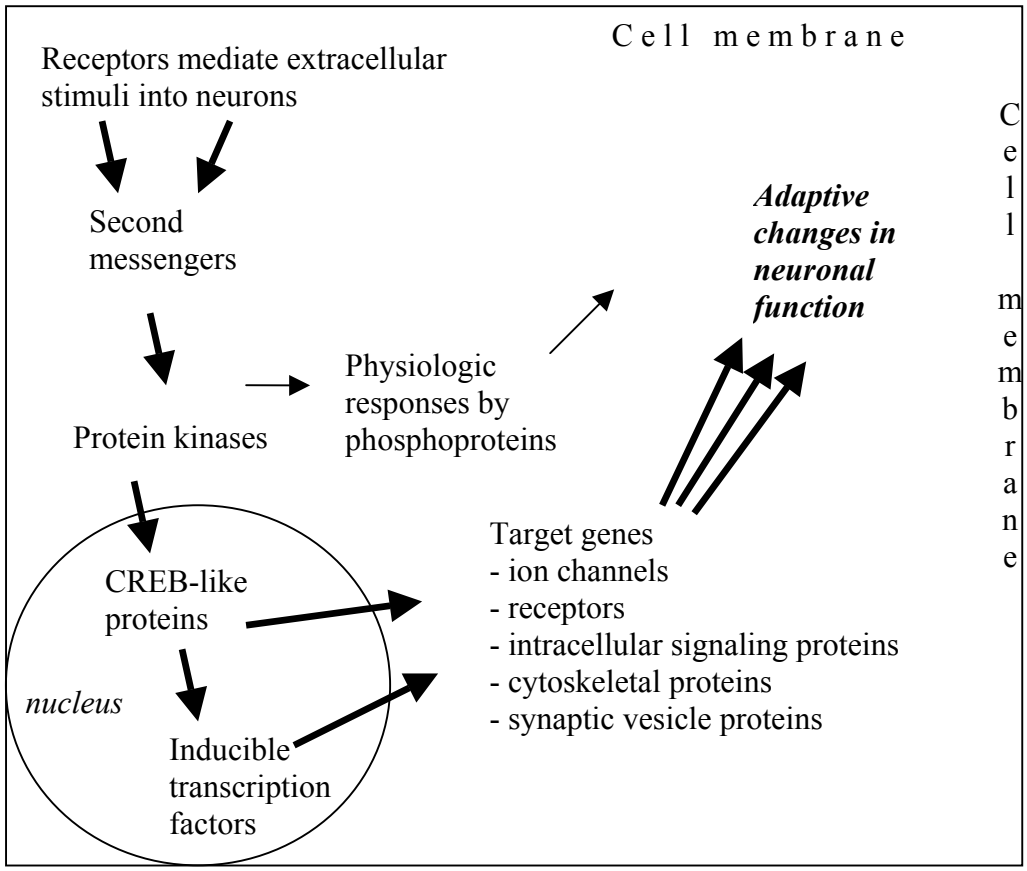


**Figure 4.** A schematic presentation of the function of CREB-like proteins in the cell. CREB-like proteins mediate extracellular stimuli into gene expression. The target genes mediate long lasting alterations in neuronal function.

**CREB binding proteins, CBP and TORC**

Phosphorylation at Ser-133 is critical for the induction of CREB activity, but this site does not fully explain the activation of CREB (Gonzalez et al., 1991). The other domain of CREB was found to specifically bind another nuclear protein and coactivator called CREB binding protein (CBP), which links CREB to the basal transcription factor machinery, including RNA polymerase II complexes (Chrivia et al., 1993; Kwok et al., 1994). CBP is recruited when CREB is phosphorylated. CBP does not bind to unphosphorylated CREB, although CBP does bind to unphosphorylated steroid-responsive element binding protein (Cardinaux et al., 2000).

These coactivators of CREB play the role of regulating transcription activated by calcium currents through NMDA and voltage-sensitive calcium channels. Activation of CaMKII, CaMKIV, PKA, but not Ras-MAP pathways can induce CBP mediated transcription. This induction is strength-dependent, and may be a regulatory switch for glutamate-induced CREB-mediated transcription (Hu et al., 1999).The function of CBP with CREB makes it possible for cells to react differently to calcium and non-calcium signals, cAMP and non-cAMP mediated signals, stress, and mitogens. There is also a nuclear inhibitor that binds to CREB and inhibits CBP recruitment in response to stress or mitogenic stimuli (Mayr et al., 2001).

Phosphorylated CREB would have to interact with the RNA polymerase complex to have an effect on transcription, whether at the recruitment step or at later steps (e.g. isomerization, promoter clearance, or reinitiation) (Kim et al., 2000). As seen in figure 5, phosphorylated CREB activates transcription through interaction with CBP and recruitment of RNA polymerase II complexes. CREB binds CBP in a strictly phosphorylation-dependent manner. CBP binds TFIIB and enhances the activation through recruitment of essential components of the polymerase complex. CBP has interactions with numerous other transcription factors, including c-Fos, c-Jun, Jun-B, c-Myb, Sap-1a,p62, (TCF), Stat1a, Stat2, SREBP, Myo-D, pp90(rks), GATA-1, p53, NNcoA, and p160 (Janknecht and Hunter, 1996).

CREB is a constitutive transcription factor with some activity even when it is not phosphorylated. There are transducers of regulated CREB activity (TORC), which are coactivators that enhance CRE-dependent transcription via a phosphorylation-independent interaction with the bZIP DNA binding/dimerization domain of CREB. TORCs enhance the interaction of CREB with the TAF(II)130 component of TFIID (Conkright et al., 2003a).

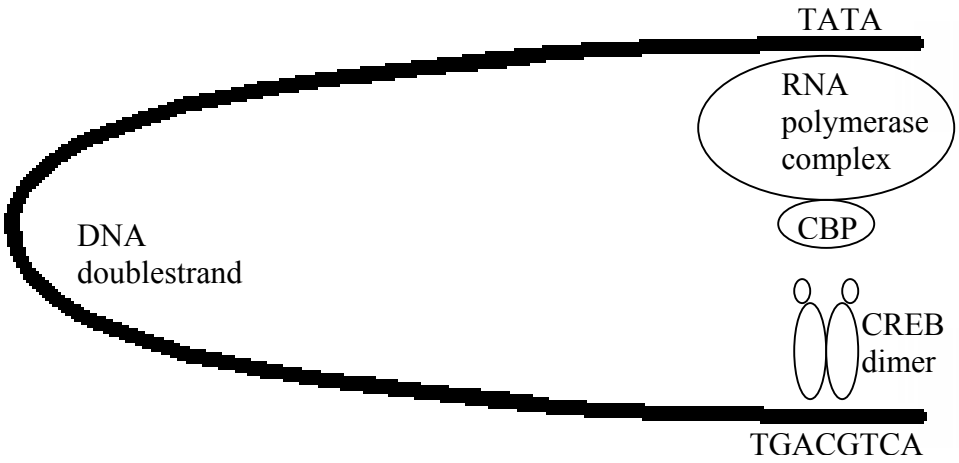


**Figure 5. CREB transcription factor**. CREB binds CRE sequence, and interacts with RNA polymerase II complex through associated proteins (CBP and others). TATA and CRE-elements are shown.

**Interplay by dimerization**

CREB can bind CREs either as a homo- or heterodimer. In neurons, CREB and CREM are much more expressed than ATF-1 **(**Mayr and Montminy, 2001; Dawson and Ginty 2002). The family has both activators like CREB and repressors like some CREM isoforms. Common for all of these is the phosphorylation site for PKA Arg-Arg-Pro-Ser-Tyr. This family binds mostly to CRE (5' TGACGTCA 3').

The leucine-zipper transcription factors share similarities. The closest relatives for the group of CREB and CREM are the C/EBP proteins (CAAT-box/enhancer binding proteins) and activation protein 1 (AP-1) proteins. AP-1 includes fos, jun, and krox proteins. AP-1 genes code for inducible transcription factors, which are induced after CREB-activity. CREB does not form dimers with most AP-1 proteins, in contrast to CREM/ICER proteins, which do form dimers with AP-1 (Herdegen and Leah, 1998). CREB can form dimers with ATF-1 and CREM/ICER proteins. ICER is an inducible repressor of CREB, and is transcribed from an alternative promoter within the CREM gene. The heterodimers of CREB-family transcription factors can bind a variety of sites.

CRE-sequence is similar to TPA responsive element (5' TGAGTCAG 3'), which is recognized by AP-1 family transcription factors. The heterodimers consisting of CREB, CREM, and AP-1 proteins recognize both sequences in variable degrees (Herdegen and Leah, 1998). An ICER form of CREM-factor can inhibit both systems.

### 2.3.1 Modulators of CREB, the CREM and ICER genes

The CREM gene is a close homolog to CREB. The products of the CREM gene are leucine zipper transcription factors or modulators, which can dimerize to a variety of other leucine zipper proteins (Foulkes et al., 1991).

Variable splicing produces the different CREM-proteins. The only activator, which activates gene transcription, is CREMtau. The other CREM isoforms (alfa, beta, and gamma) inhibit gene transcription. The properties of CREM isoforms are determined by the combination of exons, which lead to different functional domains in different proteins. CREMtau is the only isoform with two glutamine-rich regions, Q1, and Q2. Phosphorylation of sites in these regions is needed for transcription activation (Herdegen and Leah, 1998).

CREM-proteins that are not phosphorylated can still form heterodimers with leucine zipper transcription factors of CREB/ATF-1 and AP-1 families. Most of these dimers are less active than homodimers. In theory, the most minimal of all CREM-repressors would be a single leucine zipper domain, which could dimerize with CREB and other transcription factors, and repress CRE-mediated transcription.

**Inducible cAMP early repressor, ICER**

Expression of CREB/ATF-1 family transcription factors is steady and their activity is regulated by phosphorylation. Phosphorylation is then slowly removed by phosphorylases. To counterbalance the different activity states in neurons, new protein synthesis is required (Molina et al., 1993).

The small inducible cAMP early repressor ICER is a product of the CREM gene beginning from an alternative promoter in an otherwise intronic area. In the promoter of ICER, there are four CRE-sequences, which leads ICER to be transcribed efficently together with other CRE-containing genes (Della Fazia et al., 1997). It has been suggested that ICER functions as a $Ca^{2+}$ -activated repressor of CREB/CRE-mediated transcription, because the properties of CREs can differ. CREs of individual genes can be regulated separately by $Ca^{2+}$ and cAMP (Krueger et al., 1999). ICER expression peaks 2 hours after activation of CREB, and the production ends 4-6 hours after its beginning (Molina et al., 1993).

The structure of ICER is simple. It has only 120 amino acids and little more than the leucine zipper domain is left from CREM-structure. The four isoforms of ICER have only minor differences. ICER can bind CRE-elements either as homodimer, or as dimerized with some other leucine zipper transcription factor. As ICER cannot activate transcription machinery, ICER expression represses CRE-mediated gene transcription. This way ICER inhibits the activity of it's own CRE-containing promoter and forms a negative autoregulatory loop for balancing CRE-transcription (Foulkes et al., 1996). ICER dimerizes also with AP-1 family transcription factors, which are induced by CRE-transcription. This way ICER expression represses also the production of AP-1 transcripts.

### 2.3.3 Targets of CRE mediated transcription

The genes containing CRE and transcribed after CREB activation include enzymes needed in neurotransmitter synthesis, such as tyrosine hydroxylase, receptors like neuron-derived orphan receptor-1 (NOR-1), and somatostatin. The products of these genes have neuromodulatory activity and hormone-like effects, cell cycle regulators and antiapoptosis genes, such as B-cell leukaemia/lymphoma 2 protein (Bcl-2), and cyclin A, and neurotrophic factors and their receptors, as brain-derived neurotrophic factor, and its receptor trk-B (tyrosine kinase B). Perhaps the most dramatic effect of CREB-transcription is the induction of transcription factors, such as c-fos, or YY-1. The inducible transcription factors mediate further the long-term effects (Conkright et al., 2003b; Desdouets et al., 1995; Montminy, 1997).

### 2.3.4 CRE mediated transcription in neuronal processes

In the nervous system, the signalling pathways controlled by neurotransmitters, neuropeptides and other modulators, growth factors, and hormones can alter gene expression by activating protein kinases that activate CREB-mediated transcription (Kim et al., 2000).

Synaptic activity leading to cAMP/$Ca^{2+}$ signalling and phosphorylation of CREB by CaMKIV and other protein kinases can alter the expression of CRE-containing genes. The products of these genes participate in synaptic transmission (Lim et al., 2000). These genes include receptors and enzymes producing or degrading neurotransmitters. A more sustained and widespread effect is caused by soluble peptides such as neurotrophins, which carry proliferation messages to neurons. CREB is therefore important in synaptic plasticity. Ras/ERK/RSK pathways also drive these effects.

### 2.3.5 CREB in diseases and medication

The symptoms of depression include low mood, anhedonia, pessimism, and some physiological changes. Depression has also a negative effect on neuronal survival in brains (Garcia, 2002). CREB might play a role in these alterations. There is evidence that antidepressants stimulate components of the cAMP pathway in patients with depression while mood stabilisers blunt the same pathway in patients with bipolar disorder. All this suggests the involvement of CREB, and there is some evidence of alterations of cAMP signalling pathways in post-mortem brains (Stewart et al., 2001). It is still difficult to determine which alterations are primary and which secondary. An interesting finding is the involvement of the brain nerve growth factor (BDNF) in antidepressant drug action (Nibuya et al., 1996). BDNF is a well known target for CRE-mediated transcription, and it is secreted out of activated neurons, strengthening signals to other neurons nearby (Sulser, 2002).

There are many parallels between the molecular changes underlying addiction and those related to other forms of brain plasticity such as learning and memory. From a behavioral perspective, certain features of addiction, such as the ability of drug-associated cues to induce relapse, have been described as forms of memory. Also, activation of the cAMP pathway and of CREB-mediated transcription has been observed in forms of synaptic plasticity believed to constitute cellular correlates of memory (Nestler, 2001). The role of CREB in drug addiction is discussed in chapter 2.4.5.

### 2.3.6 CREM/ICER in development and diseases

As an inducible transcription repressor, ICER is important for many hormonal oscillations controlling metabolism. CREM plays a key physiological and developmental role within the hypothalamic-pituitary-gonad axis, and in other areas with requirement for transient transcription activation (Foulkes et al., 1996).

**Circadian rhythm**

ICER is expressed rhythmically in the suprachiasmatic nucleus. The rhythm is controlled by the light cycle, and driven by melatonin. ICER is cAMP inducible and undergoes a characteristic day-night oscillation in expression with a peak towards the end of the night. CREB is phosphorylated constitutively with a transient fall occurring at the beginning of the night. These transcription factors modulate the oscillatory levels of melatonin. The amplitude of oscillations of serotonin N-acetyl transferase, the rate-limiting enzyme of melatonin synthesis, is then reequilibrated by CREB and oscillations of ICER. Thus, a transcription factor modulates the oscillatory levels of a melatonin hormone (De Cesare and Sassone-Corsi, 2000; Foulkes et al., 1996).

**Spermatogenesis**

Spermatogenesis is under the control of hormonal signals, which are converted to protein expression by transcription factor activity. Spermatogenesis stops at the first step of spermatogenesis in the CREB or CREM knock-out mutants and there is a significant increase in apoptotic germ cells. In addition to CREB, CREM is highly expressed in testis and important for the control of spermatogenesis (De Cesare and Sassone-Corsi, 2000; Nantel and Sassone-Corsi, 1996).

**Gastrointestinal system**

The secretions of parachrinic and enzyme products in gastrointestinal system are also regulated in a rhythmical manner. Gastrin is a peptide hormone, which controls gastric acid secretion. CREB plays an important role in control of gastrin mediated signalling (Thommesen et al., 2000). Gastrin has CRE-element in its promoter, and gastrin activates CREB-transcription in its target cells, as the receptors activate PKC, MAPK, and other signaling systems. Gastrin can also activate CRE by $Ca^{2+}$-activated pathways.

Gastrin-induced expression of ICER is not inhibited by PKA and PKC, or calmodulin inhibitors, although CRE-mediated transcription is affected (Thommesen et al., 2001). It has been suggested that ICER promoter is activated by different mechanism that activates the minimal CRE promoter. A set of transcription factors that can bind ICER promoter does not belong to CREB/CREM/AFT-1 family (Thommesen et al., 2001).

**CREM/ICER in cancer**

ICER is an important mediator of cAMP antiproliferative activity that acts as a putative tumor suppressor gene product. The second messenger cAMP inhibits the proliferation of most cell types. The nuclear response of cAMP is mediated by CREB and CREM/ICER. ICER is a transcriptional repressor that negatively regulates cAMP-mediated gene expression. CREM/ICER is important for male reproduction and proliferative signaling. ICER specifically affects the tumorigenicity of the prostate cancer cell without affecting their growth. In leukemia cells ICER has anti-apoptotic effects diminishing the effect of prostaglandins that cause apoptosis (Krueger et al., 1999).

ICER-IIgamma is induced by cAMP. ICER-IIgamma blocks cells at the G2/M boundary of the cell cycle. (De Cesare and Sassone-Corsi, 2000). ICER-IIgamma inhibits the growth and DNA synthesis of mouse pituitary tumour cells and human choriocarcinoma cells. This alteration in cell growth is coupled with a reduced ability of these cells to grow in an anchorage-independent manner and to form tumours in mice. ICER-IIgamma is a tumour suppresser gene product mediating the antiproliferative activity of cAMP. Therefore, the manipulation of ICER expression could be used for the treatment of androgen-insensitive prostate tumours without causing undesirable toxicity to the cells (Memin et al., 2002).

## 2.4 Addiction

The defining feature of drug addiction is compulsive, out-of-control drug use, despite negative consequences. Addiction is a chronic brain disease, not a synonym to tolerance or dependence. Physical dependence is neither necessary nor sufficient to cause addiction, although these two phenomena often coexist. Drug taking leaves a permanent mark on the brain. These changes make the brain more sensitive to drugs and cause a person to crave drugs. In addiction, drugs lead to wanting, not necessary liking of the drug. There is medication to antagonize the effects of the drugs of abuse, and to relieve the physical withdrawal symptoms, but there is no efficient medication to cure the drug addiction and drug craving (Kreek et al., 2002). The loss of control that addicts show with respect to drug seeking and taking may relate to the ability of drugs of abuse to commandeer these natural-reward circuits and disrupt a person's motivation and drive for normal reinforcers (Franken, 2003; Koob and Le Moal, 2001; Self, 1998).

### 2.4.1 Neuropsychology of addiction

Drugs of abuse are both rewarding and reinforcing, although there are some exceptions, like nicotine, which is only reinforcing, not rewarding. Rewards are stimuli that the brain interprets as intrinsically positive, and reinforcing stimuli are those that increase the probability that behaviors paired with them will be repeated (Koob, 1992).

Total abstinence is difficult to achieve, and this is largely because of episodic craving, that may persist for years. This leads to high rates of relapse (Cami and Farre, 2003). A drug user loses the voluntary ability to control the drug use.

Recent theories of addiction describe a concept of incentive motivational processes, In most current conceptualisations of drug dependence, subjective craving is regarded as a central phenomenon, leading to relapse and continuation of drug use (Everitt et al., 1999; Robinson and Berridge, 2000). Drugs with high potential for addiction, such as cocaine or amphetamine, change the neurochemistry even after a single dose, although permanent changes require repeated administration (Nestler et al., 2001). Because of these alterations, addiction has begun to be seen as a neuropsychiatric disorder (Robbins and Everitt, 1999).

### 2.4.2 Molecular mechanisms of addiction

In neurochemical terms, there are four stages of development of an addiction (Nestler, 2000); (Hyman, 1996a; Hyman, 1996b):

1) When the drug is administrated, an acute drug state where the reward processes of the brain are active. The dopamine release in the mesolimbic regions of brain increases.

2) If drug use is continued, a chronic drug state develops, where the changes in the neurochemical systems show as tolerance, desensitisation, and addiction. Adaptations in mesoaccumbens brain reward circuitry itself alter emotional and motivational aspects.

3) As the drug concentration decreases in the body after continuous use, there is a short term withdraw state, and withdrawal symptoms occur. In the brain, glutamatergic and noradrenergic transmission increases, as the balance of homeostasis has shifted.

Long-term withdrawal. There has not been drug in the body for long times, but the craving for the drug remains. The systems for memory and learning in the brain are probably important for this. This stage can remain for the rest of the life.

### 6.4.1 The initial targets of the drugs of addiction

All drugs of abuse induce release of dopamine in the mesolimbic dopamine reward system, especially in nucleus accumbens (NAc) (Wise, 1998). Even though not all drugs of addiction are dopamine agonists, they have secondary effects involving dopamine in mesolimbic pathways (Robbins and Everitt, 1999). The drugs with high addiction potential include opiates and psychostimulants. The use of sedatives (benzodiazepines), NMDA-antagonists (PCP, ketamine), cannabinoids and alcohol can also lead to addiction. Cocaine is a psychostimulant, which acts through potentiating monoaminergic transmission. Cocaine inhibits the functioning of dopamine reuptake transporters, thereby increasing the duration of action of dopamine that is released in the nucleus accumbens (Hyman 1996). Serotonin and noradrenalin transporters are also affected. Amphetamine prevents reuptake of dopamine as cocaine does, but amphetamine also increases dopamine release without changing firing rate, and causes reverse transportation of dopamine by dopamine transporters (Giros 1996). Sites of action for the drugs of addiction are presented in figure 6.

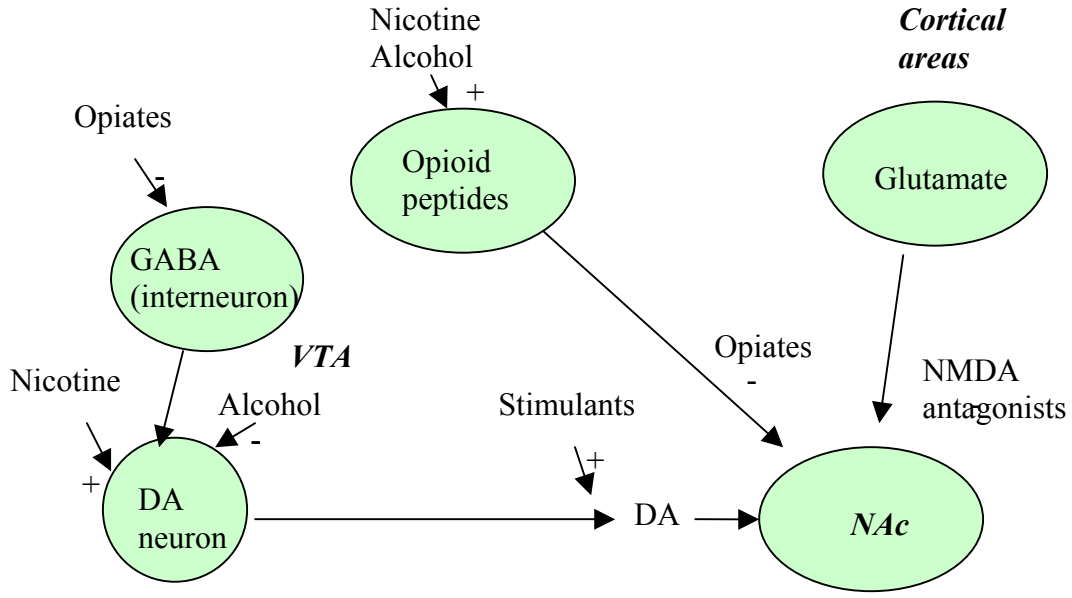


**Figure 6.** Sites for the action of drugs of abuse. Opiates increase firing of dopaminergic neurons by inhibiting GABAergic neurons in VTA, and directly by the receptors in nucleus accumbens. Cocaine and amphetamine potentiate dopaminergic transmission from VTA to NAc. Nicotine and ethanol activate dopaminergic neurons in VTA, and also have effect on opioid release. PCP and also to some extent ethanol block glutaminergic input to NAc (adapted from Nestler etal., 2001b).

### 2.4.4 Glutamate and addiction

The nucleus accumbens receives glutamatergic inputs from prefrontal cortex, amygdala and hippocampus, as seen in figures 7 ((Hummel and Unterwald, 2002; Ikeda et al., 2003)). Dopamine can control the release of glutamate, and glutamate can control the release of dopamine in nucleus accumbens, as seen in figure 10. Acute administration of NMDA antagonist MK-801 increases dopamine-mediated behaviour (Smith et al., 1997).

The effect of dopamine in nucleus accumbens can be studied by unilateral stimulation of dopamine receptors (Ikeda et al., 2003). Glutamatergic AMPA and NMDA receptor antagonists can inhibit the effects of dopamine, AMPA being more important and NMDA more modulatory (Ikeda et al., 2003). Paradoxically, NMDA antagonist MK-801 can induce activity in nucleus accumbens measured by locomotoric activity by some mechanism that is different than dopamine agonist-induced locomotor activity (De Leonibus et al., 2001).

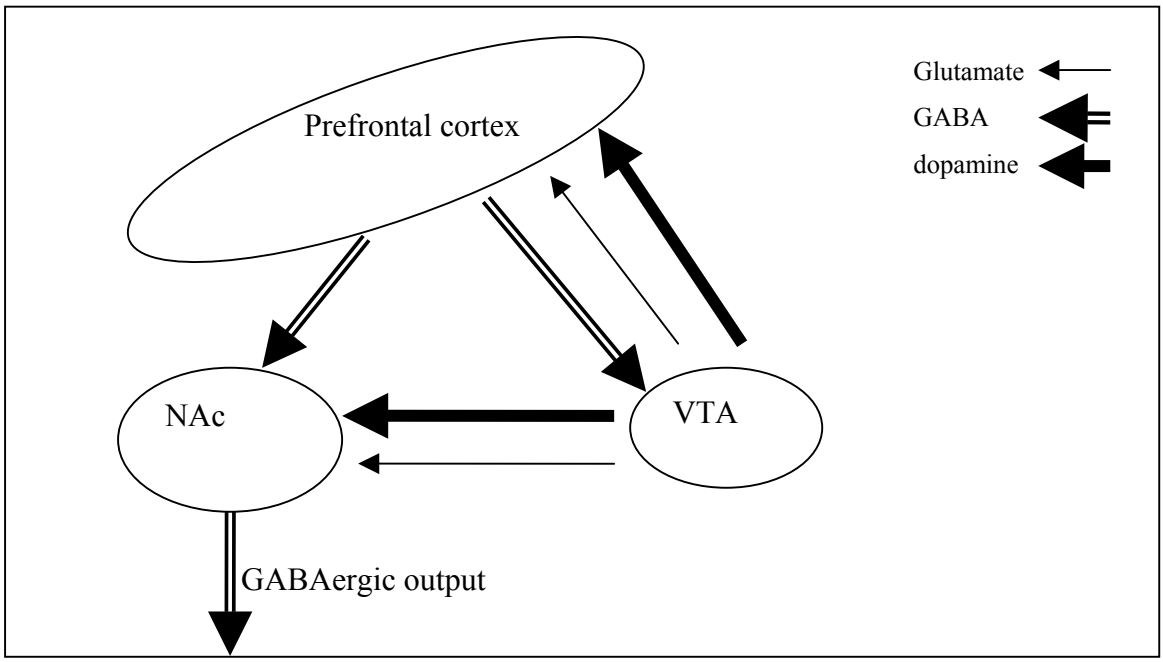


**Figure 7**. The role of glutamate in neuronal circuits in brain and in neuronal circuits associated with addiction. Nucleus accumbens receives dopaminergic input from ventral tegmental area, and glutamatergic input from cortical regions (Adapted from Hummel et al. 2002).

## Integration of psychology, behavior, and molecules

In order to be able to develop medication for drug addiction, the psychological mechanism must not be by-passed, as they contain the key to the problem – why are drug related stimuli able to elicit classically conditioned physiological (dependence) and subjective (craving) responses in persons with drug addiction? These psychological mechanisms include acute subjective reward, memory formation, and attention processes (Franken, 2003). The long-lasting effects require memory mechanisms, and activation of gene transcription and protein synthesis. Memory consolidation by synaptic plasticity in addition is also moderately well understood, although some mysteries remain, and one of these is the ultimate role delta-fosB plays in addiction (Nestler et al., 2001). Another mystery is the role of CREB in learning and unlearning of addiction, and whether can it be affected by medication?

Second messenger pathways play a critical role in learning (Sheng and Kim, 2002), and cAMP cascade leading to the activation of PKA is involved, for example in late long term potentiation in the rat hippocampus (Kandel, 2001) and working memory in the frontal cortex (Taylor et al., 1999). It is also needed in cocaine self-administration in rats (Self, 1998).

The increase in nucleus accumbens dopamine response to acute amphetamine is independent from calcium, but the sensitisation-related enhancement of this response appears to be calcium dependent (Warburton et al., 1996).

Chronic exposure to several drugs of abuse upregulates cAMP formation and PKA activity specifically in NAc (Shaw-Lutchman et al., 2003; Widnell et al., 1996). Does drug-induced upregulation of PKA activity in the NAc contribute to motivational disturbances in drug addiction, or is it also a part of a negative feedback mechanism that might even protect from those changes?

Infusion of PKA inhibitor into NAc induces a relapse in cocaine-seeking behavior (Self, 1998). Similar effects have been reported after systemic injections of D2-like dopamine agonists (Self, 1998). Also, PKA activator induces an impaired ability to discriminate the drug-effects. This is suggested by Self et al. (1998) to be more presynaptic, than post-synaptic. Based on these observations, it that has been suggested that sustained upregulation of PKA activity in chronic drug-abuse could explain the tolerance to cocaine reinforcement, and acute inhibition of this system could contribute to drug craving and relapse (Self, 1998).

### 2.4.5 CREB and addiction

The use of addictive drugs leads to long-term alterations in brain neurochemistry (Nestler, 1993; Nestler, 2001). These alterations in reward, motivation, mood, and arousal systems change beyond the ability of the system to return to its original set point (Koob and Le Moal, 1997; Koob and Le Moal, 2001). To produce these adaptations in the brain, the gene expression and protein synthesis must be altered some way in addiction, as these alterations are stable or permanent. Changes through novel protein synthesis reguire gene transcription, a process that is regulated by the transcription factors. One of the transcription factors associated with the mechanisms of addiction is CREB (cAMP response element binding protein), which is important in synaptic plasticity. CREB activity in the nucleus accumbens shell controls gating of behavioural responses to emotional stimuli (Barrot et al., 2002).

Increased transcription of the CREB gene accompanies the upregulation of the cAMP pathway known to occur in certain brain regions after repeated drug intake. Upregulation of the cAMP pathway and CREB might mediate a homeostatic adaptation that diminishes further drug responsiveness. However, as the increased levels of CREB have a relatively short life, additional mechanisms are thought to underlie the longer-lasting manifestations of addiction.

The genes regulated by CREB include transcription factors such as immediate early genes, and delta fosB, which then cause more changes in gene expression (Nestler et al., 2001).

It is suggested that CREB is needed in NAc for gating and controlling the effects of emotional stimuli. For acute control of information, CREB is too slow in response, but it could act as modulation for the overall response level. If drug addiction is thought of as a process of homeostasis, then CREB could be the key to how the static state is achieved at the neuronal level (Shaw-Lutchman et al., 2003). Over action of CREB in NAc leads to the diminishing of the rewarding effects of sugar and morphine. This has been proved by transgenic experiments. Underexpression of CREB in NAc increases the rewarding effects of sugar and morphine, and natural rewards. Also the response for aversive stimuli was diminished in a CREB knock-down experiment (Barrot et al., 2002).

The NAc is a critical neural substrate for the rewarding properties of opiates and most other drugs of abuse (Koob, 1999). Chronic morphine or cocaine treatment upregulates the cAMP pathway in this brain region (Terwilliger et al., 1991; Unterwald et al., 1993). Chronic exposure to amphetamine increases the state of phosphorylation of CREB in striatal regions (Cole et al., 1995; Turgeon et al., 1997). Using viral vectors to overexpress CREB, increased CREB activity in the NAc reduces the rewarding properties of morphine and of cocaine (Carlezon et al., 1998; Barrot et al., 2000). Increased CREB function in this region also produces a negative emotional state as inferred from an animal model of depression (Pliakas et al., 2001). Together, these data support the scheme that observed induction of CRE-mediated transcription in the NAc may mediate tolerance to the rewarding effects of morphine and contribute to aversive aspects of the withdrawal syndrome (Nestler, 2001). It is hard to know if the expression level of CREB stands for reduced activity, as it could be a compensatory alteration for prolonged activity.

Chronic cocaine administration decreases the basal activity of adenylyl cyclase in some parts of striatum outside nucleus accumbens. Chronic cocaine administration eventually increases the CREB activation in nucleus accumbens. This is seen as accumulation of fos-related proteins with long half-life.

Chronic treatment of enkephalin acting on mu and delta opioid receptors do decrease the basal activity of adenylyl cyclase in both of the regions, but cocaine attenuates the decrease created by a delta-agonist, but not the one created by a mu-agonist (Unterwald et al., 1993). Opiates acutely inhibit CREB by inhibition of adenylyl cyclase, but in chronic treatment, cAMP-mediated signalling is returned to basal level, because its regulators are upregulated (adenylyl cyclases I, and VIII, also PKA) and the phosphorylation state of CREB gradually

recovers toward normal levels during the course of chronic opiate administration. If opiate treatment is removed, the over activity of CREB system can be seen. This upregulation of molecules is seen in NAc, locus coeruleus and other limbic areas (Shaw-Lutchman et al., 2003). In withdrawal, CREB activity is increased in NAc but in VTA the CREB activity is decreased.

It appears that acute exposure to morphine reduces levels of CREB, but chronic exposure activates CREB in the nucleus accumbens, possibly through cAMP (Self et al., 1998; Self and Nestler, 1998; Widnell et al., 1996). The levels of FosB, another transcriptional regulator, are also increased after administration of drugs of abuse. As the half-life of this molecule is very long, it provides a molecular mechanism of addiction based in the stability of the protein by which drug-induced changes in gene expression can persist long after drug intake stops.

### 2.4.6 Antiaddictive drugs.

The main goal for treating of substance addiction is to prevent the uncontrolled use of drug and prevent subsequent relapses. The strategies for treating drug addiction includes the following five main time points, and several targets (Kreek et al., 2002).

1. Prevent the psychological and molecular mechanism
2. Substitute the drug of addiction with a less harmful drug
3. Ease physiological withdrawal symptoms
4. Balance the function of the reward system
5. Reverse the long term alterations in brain

These make it possible for the individual to unlearn (in a wider sense) out of addiction.

The first time point for treatment is the active drug state. The subjective effects of the drug of abuse could be prevented by drug-antagonists, or immunopharmaceutical means, such as cocaine or nicotine vaccinations. In the early stage of drug addiction where the drug user has been using the substance only for a short period of time, these might be a useful way of intervention, if only it happens before robust changes in the brain. Direct antagonising of the drug effect on the other hand might be dangerous, if there is strong physical dependence. A partial detoxification can be performed for opiates by partial-agonists such buprenorphine,

which blocks the effect of opiates by competing for mu receptors. Antagonising the effect of one group of substances does not prevent the use of any other drugs of abuse.

The second point to cure addiction is to relieve the withdrawal symptoms that keep up the drug abuse. This is the base of the opiate substitution by slowly decreasing the maintenance dose of methadone or buphrenorphine. For psychostimulant users, sedatives and antipsychotics are used to relieve the symptoms.

The third point is relapse prevention, in which the drug craving is the main target. It is typical for all addictions that relapses occur even after long stages of abstinence.

### 2.4.7 NMDA-channels, transcription, and addiction

NMDA antagonists are suggested to have significant potential in the treatments of drug dependence and addiction, including withdrawal effects, normalization of the neurochemical alterations, and attenuation of conditioned responses of drug related stimuli, diminishing the drug use (Bisaga and Popik, 2000).

The hypothesis for NMDA antagonists as potential anti-addictive drugs arise from several findings (Glick and Maisonneuve, 2000; Popik et al., 1995):

- Ibogaine, an alkaloid of an African plant, which has a reputation of being antiaddictive
- Acamprosate, a low affinity NMDA-antagonist that binds to the spermidine site of NMDA-receptor, in use to treat alcoholism.
- MK-801 and other uncompetitive NMDA-receptor blockers, evidence of diminished cocaine self-administration in rats.
- Lower affinity NMDA-blockers reduce drug self-administration in rats also.
- In clinical trials, low-affinity NMDA-blockers have not shown efficiency for diminishing drug craving, but they reduce the euphoria.
- Treatment of withdrawal symptoms with NMDA-antagonists.
    - Morphine withdrawal can be treated as a state of glutamate hyperactivity
- Treatment of drug craving with NMDA antagonists
    - Independent from withdrawal symptoms

The capacity of NMDA antagonists to reverse the effects of drugs of addiction is most well documented in opioid withdrawal, as memantine and ibogaine are reported to significantly ease the withdrawal symptoms (Leal et al., 2003; Maisonneuve and Glick, 2003; Popik et al., 1995). Even during gradual detoxification from opioids, there are dramatic symptoms. The

other way to ease the symptoms is to perform very fast detoxification with opioid antagonists in narcosis, which would then normalize the opioid system. Theoretically, use of NMDA-antagonists in anesthesia may be found to be useful.

**NMDA antagonists and drug craving**

The phenomenon often described as a reason for relapse after abstinence is craving for the drug. NMDA-antagonists seem to inhibit also the craving for the drug, which is demonstrated both in animal studies, and with human subjects. Ibogaine, a plant alkaloid with NMDA-antagonistic properties was shown to reduce mice morphine consumption (Popik and Skolnick, 1996). A specific NMDA-antagonist has been shown to reduce morphine use and this might be because of the glutamatergic blockade in ventral tegmental area (Xi and Stein, 2002). The strongest proof comes from treatment of alcoholism where there are very promising recent results on the use of acamprosate as additional medication.

Uncompetitive NMDA-antagonists interact in the reward and reinforcement systems with the dopaminergic transmission, as described in chapter 2.4.4. Although addictive psyhostimulants amphetamine and cocaine increase dopaminergic transmission, glutamate is involved in the induction and expression of the behavioural changes. Activating NMDA receptors in dopamine midbrain cell bodies is required for stimulant sensitisation.

Co-administration of dextromethorphan with morphine attenuates the morphine rewarding effect and related dopamine releases at the nucleus accumbens (Huang et al., 2003). In addition to dopaminergic transmission, NMDA-antagonists affect also noradrenergic and serotonergic transmission in several brain areas. Release of noradrenaline in locus coeruleus, serotonin in dorsal raphe nucleus, and also dopamine in nucleus accumbens increase (Callado et al., 2000). In the nucleus accumbens shell, but not in the core, NMDA-block increases dopamine release (Marcus et al., 2001). If serotonergic transmission is blocked, NMDA-antagonists seem to lose their effect on dopaminergic transmission. (Dall'Olio et al., 1999; Dall'Olio et al., 2000).

## 3. AIMS OF THE STUDY

Although neurotransmitter receptor binding by NMDA-antagonist drugs is well documented, the proposed clinical efficacy of NMDA-antagonist drug treatment is less well understood. The aim of the present studies was to examine the molecular biological mechanisms of the effects of NMDA-antagonists and CREB. This study was undertaken to:

1) Study the effect of uncompetitive NMDA receptor antagonists on the expression of the members of CREB family transcription and closely related leucine zipper transcription factors in rat brain.
2) Examine whether glutamate receptors and signal transduction proteins are affected by CREB or independent of it after NMDA-antagonist treatment.
3) Study how the alterations in gene expression by NMDA-antagonists correlate with the altered gene expression after cocaine treatment.
4) Identify the genes with an altered expression in response to acute MK-801 and cocaine treatments.

# 4. EXPERIMENTAL PROCEDURES

Experiments were carried out with the rats *in vivo*. All animal studies were performed in accordance with the guidelines of the Society for Neuroscience and were accepted by the Experimental Animal Ethics Committee of the University of Kuopio.

## 4.1 Experimental animals

Male Wistar rats (weight 180-260 g, National Laboratory Animal Centre, University of Kuopio and Harlan, Netherlands) were housed 3-6 per cage and kept under standardised temperature (22±1), and humidity. A circadian light cycle (12 hours light/12 hours dark) was maintained in the housing facilities with free access to food and water.

All drugs were administered with intraperitoneal (*i.p.*) injections. After drug treatments, the animals were anaesthetised with $CO_2$ and sacrificed by decapitation. The brains used for *in situ* hybridisations were frozen intact and stored at -70 C. The brains used for immmunoblotting, or RNA isolation, were dissected and brain area samples were stored at -70 C. The samples for DNA-microarrays in **publication IV** were treated with RNAlater (Ambion, UK) overnight.

## 4.2 Pharmacologic agents and treatments

In **publication I**, rats were given a single injection of saline, (+)-MK-801 (dizocilpine maleate; Research Biochemicals Inc., Natick, MA, USA) in doses ranging from 0.1 mg/kg to 10 mg/kg. The animals were sacrificed 1 hour, 4 hours, 8 hours, 1 day, 2 days, 3 days, or 6 days later. The moderate affinity NMDA-ion channel blockers, memantine (Akatinol-Memantine; gift from Dr.G.Quack, Merz + Co., Frankfurt/Main, Germany) and ketamine (Ketalar; Parke-Davis Scandinavia, Solna, Sweden) were given as a single injection, and the animals were sacrificed 6 hours later. The atypical antipsychotic drug clozapine (Leponex; Wander pharma GmpH, Nürnberg, Germany) was given as a single injection once a day for 17 days. Tricyclic antidepressant desipramine (Sigma Chemical Co.), typical antipsychotic haloperidol (Serenase; Orion, Espoo, Finland), kainate-type glutamate receptor agonist kainic acid and high-affinity uncompetitive NMDA-antagonists phencyclidine (Sigma chemical co., St. Louis, MO, USA), CPP [(+/-)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid)];

Research Biochemicals International) were given as a single injection and the animals were sacrificed 4 hours later.

In **publication II**, rats were given MK-801 (dizocipline maleate; Research Biochemicals Inc., Natick, MA, USA) (1mg/kg) dissolved in water and sacrificed 4 hours after the treatment.

In **publication III**, rats were given a high dose of MK-801 (dizocipline maleate; Research Biochemicals Inc., Natick, MA, USA) (5mg/kg; 4h) dissolved in water.

In **publication IV**, rats were given MK-801 (dizocipline maleate; Research Biochemicals Inc., Natick, MA, USA) (1mg/kg), cocaine (20mg/kg), dissolved in water. The drugs were given alone or in combined treatment, in which MK-801 injection was given 15 minutes prior to cocaine, the animals were sacrificed 4 hours after the last injection.

MK-801 induced stereotypical behaviour in rats dose dependently. Cocaine (20mg/kg) induced modest hyperactivity. The pre-treatment with MK-801 (1mg/kg) before cocaine (20mg/kg) induced stereotypical behaviour with high motoric activity.

### 4.3 *In situ* hybridisation experiments

Radioactive *in situ* hybridisation with antisense oligonucleotide probe on coronal brain sections was used to determine mRNA levels of CREB, CREM, and ICER (publications **I** and **IV**), and candidate (publications **II** and **IV**), and glutamate receptor subunits (publication **III**). Gene-specific anti-sense oligonucleotide probes (35-mer) were designed after sequence homology comparison to public databases to minimize non-specific hybridisation (table 4).

After the drug treatments, the rats were narcotized with $CO_2$ and decapitated. Brains were rapidly removed, frozen in dry ice, and stored at -70°C. Horizontal or coronal brain sections (14 µm) were cut on a Leica CM 3000 cryostat and thaw-mounted onto SuperFrost/Plus (Menzel-Gläser, Braunschweig, Germany) slides. Sections from saline (control) and treated rats were comounted onto the same slide. *In situ* hybridisation with oligonucleotide probes were performed as described by Wisden and Morris (1994).
Oligonucleotide probes were 3' end-labeled to a specific activity of 1-3 × $10^7$ cpm/pmol using terminal deoxynucleotidyl transferase (MBI Fermentas, Vilnius, Lithuania) and a 20:1 M ratio of [alpha-$^{33}$P]dATP (2000 Ci/mmol, New England Nuclear) to probe. Hybridisation was performed on 4% paraformaldehyde postfixed sections with 1-3 × $10^6$ cpm/ml labeled probe in buffer containing 50% formamide, 4× standard saline citrate (SSC) (1× SSC: 150 mM NaCl, 15 mM sodium citrate), 10% dextran sulfate, and 10 mM dithiothreitol (DTT). After overnight

hybridisation at 42°C, sections were dipped into 1× SSC at room temperature, washed for 30 min at 55°C in 1× SSC, washed sequentially for 3 min each at room temperature in 1× SSC, 0.1× SSC, 70% ethanol, and 94% ethanol. After a 1- to 5-week exposure, Hyperfilm-max films (Amersham, Buckinghamshire, England) were developed in D-19 (Kodak, France).

**Table 4**. Oligonucleotide sequences of probes for *in situ* hybridisation experiments.

| Gene | GenBank # | Sequence (5' - 3') |
|---|---|---|
| CREM/ICER | U04835 | CAGACTCCCTGGTGAGGCAGCCATCACCACACCTT |
| ICER | S66024 | CAGTTTCATCTCCAGTTACAGCCATGTTGGGCT |
| CREB | X14788 | TGGCTGGGCCGCCTGGATGACCCCATGGACCTGGA |
| RTN-1 | | AATAAGTTGTCCTTGATGAGCTTCCCCTCCACAGG |
| Erp29 | AY004254 | TAGTCTGAGATCCCCACCTCTGCCACCAAGAGATC |
| ABC2/ABCA2 transporter | | TCTTGATGAGGTCCAGAATGAGGTTCCACAGGAAG |
| GluR1 | M36418 | AGTTTGAAGAGGGACGAGACCAGACAACCAGTGAC |
| GluR2 | M36419 | AATTTGAAGATGGAAGAGAAACACAAAGTAGTGAA |
| GluR3 | M36420 | ACAACAATGAAGAAC CTCGTGACCC ACAAAGCCCT |
| GluR4 | M36421 | AGTAGGTTTAGCCCATATGAGTGGCACACAGAAGA |
| mGluR1 | M61099 | GTACACAGAAGCTAATCGCTATGACTATGTCCACG |
| mGluR2 | M92075 | ATCAGCCGGACCTCTTCCTGCCTCTCGCTGTAGCG |
| mGluR3 | M92076 | AACTTGCAGCAGACAGGTGGGAAGTATTCTTACTT |
| mGluR4 | M90518 | GTACCAACTGCGCAATGGCTCGGCCGAGTACAAGG |
| mGluR5 | D10891 | GACAATGGGGAATTAAAAATGGATGATGACGAAGT |
| mGluR6 | D13963 | GCAGA GGCCCTTAGACTGGATATGGAAGTTCTACG |
| mGluR7 | D16817 | TCTCATTGGGCAGTGGACAGATGAACTTCAGCTCA |
| mGluR8 | U63288 | CTGGACAAATCAACTTCATCTAAAGGTGGAAGATA |
| GPC19 | | GTTTGGTGGGTTTGAGTTGATGGGCCAGGCGAGCT |
| SNTA | | GAACAGAAAGGCTCCTCTTCCTCCTCTTGCCACTG |
| IP-bg | | GAAGCGCCGGACACAGTATGTGTTCCCATTTCATG |
| RAMP | | ATGGTGGTGATGAAGTATACCAGAGACCTGGCGCT |
| VAMP2 | | ATTGGGGAGGAAAGTTTTCAGTCCAACCTCTAGCA |
| RGS2 | | ACACTGGTTCTACAGCACGGCACAGCATTCACTCT |
| GRIP2 | | GTCGCCCGGCTCGAGGGTGCCAGTCCTGTGCGCCA |
| CBP | | AGCTCTGACAGTTGTTTATGTTTGGACGCAGCATC |
| c-fos | X06769 | GCAGCGGGAGGATGACGCCTCGTAGTCCGCGTTGAAACCCGAGAA |
| Sst (87) | | CGCCCAAAGCCAGGACGATGCAGAGCGCGGCCAGC |
| Sst (353) | | GAAGAAGTTCTTGCAGCCAGCTTTGCGTTCCCGGG |

*In situ* autoradiograms were quantified with video-based MCID™ image analysis software (Imaging Research Inc., St. Catherines, Canada). The appropriate brain areas were determined according to rat brain atlas (Paxinos, 1986).

## 4.4 Immunoblotting

In **publication I**, immunoblotting was performed in order to compare protein expression by MK-801 and saline treatments. Brains were removed and rapidly dissected and immediately homogenized by sonication for 5 s in 1% SDS and 1 mM sodium orthovanadate (SDS lysis buffer) and then heated for 5 min in a boiling water bath. After measuring protein concentrations (Dc Protein Assay; Bio-Rad, Richmond, CA), samples consisting of equal amounts of protein (20 μg) were diluted into 2× Laemmli's buffer and subjected to 15% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes (Protran; Schleicher & Schuell, Keene, NH) and blocked in PBS (pH 7.5) with 3% nonfat dried milk (blocking buffer), and immunoblotting was carried out with anti-CREM antibodies (X-12:sc-440, rabbit polyclonal IgG; Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200 dilutions at 4°C overnight. Filters were rinsed in water twice and in PBS containing 0.05% Tween 20 twice. Bound immunoglobulins were detected with anti-rabbit horseradish peroxidase-conjugated secondary antibody (Amersham) at 1:1000 dilution for 1 h at room temperature, rinsed, and followed by chemiluminescence detection using Luminol (Sigma Chemical Co.) as the substrate. Films were exposed for 1 to 5 min.

## 4.5 Electromobility shift assay

DNA-binding activity of proteins recognizing CRE-sequence was studied in **publication I**. The tissues from parietal cortex were homogenized in hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM NaCl, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM DTT) and homogenized manually for 12 strokes. Sample tubes were left on ice for 15 min, spun, and washed once in hypotonic buffer. Pellets were resuspended in an equal volume of low-salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 1.2 mM KCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM DTT), and then high-salt buffer (same as low-salt buffer except 1.2 M KCl) was added to obtain a final KCl concentration of 0.4 M. Samples were incubated on ice for 30 min and centrifuged at top speed (25,000*g*) for 30 min at 4°C. The supernatant was drawn off, and the nuclear protein concentration was then measured. Consensus doubled-stranded CRE oligonucleotide was labeled in a reaction consisting of 3.5 fmol of oligonucleotide, 1× kinase buffer, 3 μl of [-$^{32}$P]ATP (~3000 Ci/mmol at 10 mCi/ml), and 15 U of T4 polynucleotide kinase (Promega). Nuclear extracts (5 μg) were incubated for 20 min at room temperature with labeled oligonucleotide (~30,000

cpm/reaction), 2 µg of poly(dI/dC), 10 mM Tris-HCl, pH 7.5, 4% glycerol, 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, and 1 mM DTT. Samples were resolved by electrophoresis on 4% polyacrylamide gel. Gels were dried and exposed to X-films or Phosphor Screens (Molecular Dynamics Inc., Sunnyvale, USA). Some films were overexposed (3-7 days) to reveal low-expressing complexes.

For competition assays, 100-fold of double-stranded competitor oligonucleotide (activator protein-1, nuclear factor-B, CREB; Promega) was incubated with the nuclear protein before the addition of radiolabeled oligoprobe.

For supershift assays, 2 µg of anti-CREB or anti-CREM antibodies (sc-271 X, mouse monoclonal IgG, or sc-440 X, rabbit polyclonal IgG, respectively; Santa Cruz Biotechnology Inc.) was incubated for 2 h with the nuclear extract before the addition of radiolabeled CRE oligoprobe.

DNA-binding activity was quantified from X-ray films using MCID™ image analysis software (Imaging Research Inc., St Catherines, Canada). The results were converted to percent of the complex intensity in drug treated samples comparison to complex intensities of protein extracts from saline-treated animals.

### 4.6 DNA-microarray experiments

In **publication II**, differentially expressed genes in the rat parietal cortex were searched using a microarray with 300 aminolinked 65-mer oligonucleotides printed on 3D-Link slides printed at KIChip (Stockholm, Sweden). In **publication IV**, differentially expressed genes in the parietal cortex, frontal cortex, and nucleus accumbens, after MK-801 and/or cocaine treatments were determined using DNA-microarrays from the Norwegian University of Science and Technology with 14500 sequence verified rat cDNA clones printed in duplicate on Corning CMT Gaps II slides. Samples of frozen tissue from rat parietal cortex, frontal cortex, or nucleus accumbens were placed into TRIzol reagent (GIBCO, Langley, OK, USA), homogenized, and total RNA extracted. DNA was removed by DNase I treatment (Ambion, Stockholm, Sweden). Samples from two animals were pooled to create 3 samples of 6 animals in the treatment group (figure 8).

Hybridisation was performed using fluorescent cDNA probes synthesised from total RNA (20 µg) using Cy Script labelling (Amersham Biosciences, Buckinghamshire, England) according to the manufacturer's protocols with minor modifications. Hybridisation was in a total volume of 28 µl containing Cy3 and Cy5 labelled cDNAs, human Cot-1 DNA (10 µg)

and yeast tRNA (20 µg) (Invitrogen, Carlsbad, CA) in 3x SSC (1x SSC; 150 mM NaCl, 15 mM sodium citrate)/0.3% SDS at 55°C for 18 h. After hybridisation, microarrays were washed in 1x SSC/0.03% SDS, 0.2x SSC, and 0.1x SSC for 3 min each at room temperature. Slides were quantitated using ScanArray 5000 (Packard Bioscience, Meriden, CT) and converted to colour images using Array Vision (Imaging Research, St. Catherine's, Ontario, Canada) and TIGR Spotfinder software (Saeed et al., 2003). Spots were quantitated and background subtracted.

### 4.7 Expression array data analysis

In **publication II**, data was normalized for each channel using b-actin as a control and quantitated results are the average of two experiments, as the number of genes was too low for any other normalization. The fold-change of gene expression was determined dividing Cy3 channel expression by Cy5 channel expression, then corrected by the ratio of control genes.

In **publication IV**, microarray datasets were normalized by LOWESS method (Chen et al., 2003). The number of replicate spots with expression level over Arabidopsis spike controls, and deviation between replicates were used as quality controls and the most trusted data was taken into further analysis.

Z-ratios were created with a method modified from Cheadle et al. (2003). For each treatment and brain area, Z-ratios were used determine the level of significant alteration in the gene expression.

First, the Z-scores were calculated for the data from each channel of each of the replicate chips. A $\log_{10}$ transformation of the raw intensity data was applied to reduce the variance due to extreme values. The standard deviation of all genes in the replicate chips was calculated. The log fold expression of the gene was divided by the standard deviation of the logarithmic values of all genes. This yielded Z-scores for the each gene. Next, for each gene, the average Z-score of the 3 replicate chip controls (Cy3 channel) was subtracted from the average Z-score of the treatment (Cy5 channels). Finally, this difference was then divided with the standard deviation from the all genes in this group.

The method standardizes the cut-off limit for significant gene expression alteration. The genes with Z-ratios <-2 or >2 were considered to have expression with significant alteration.

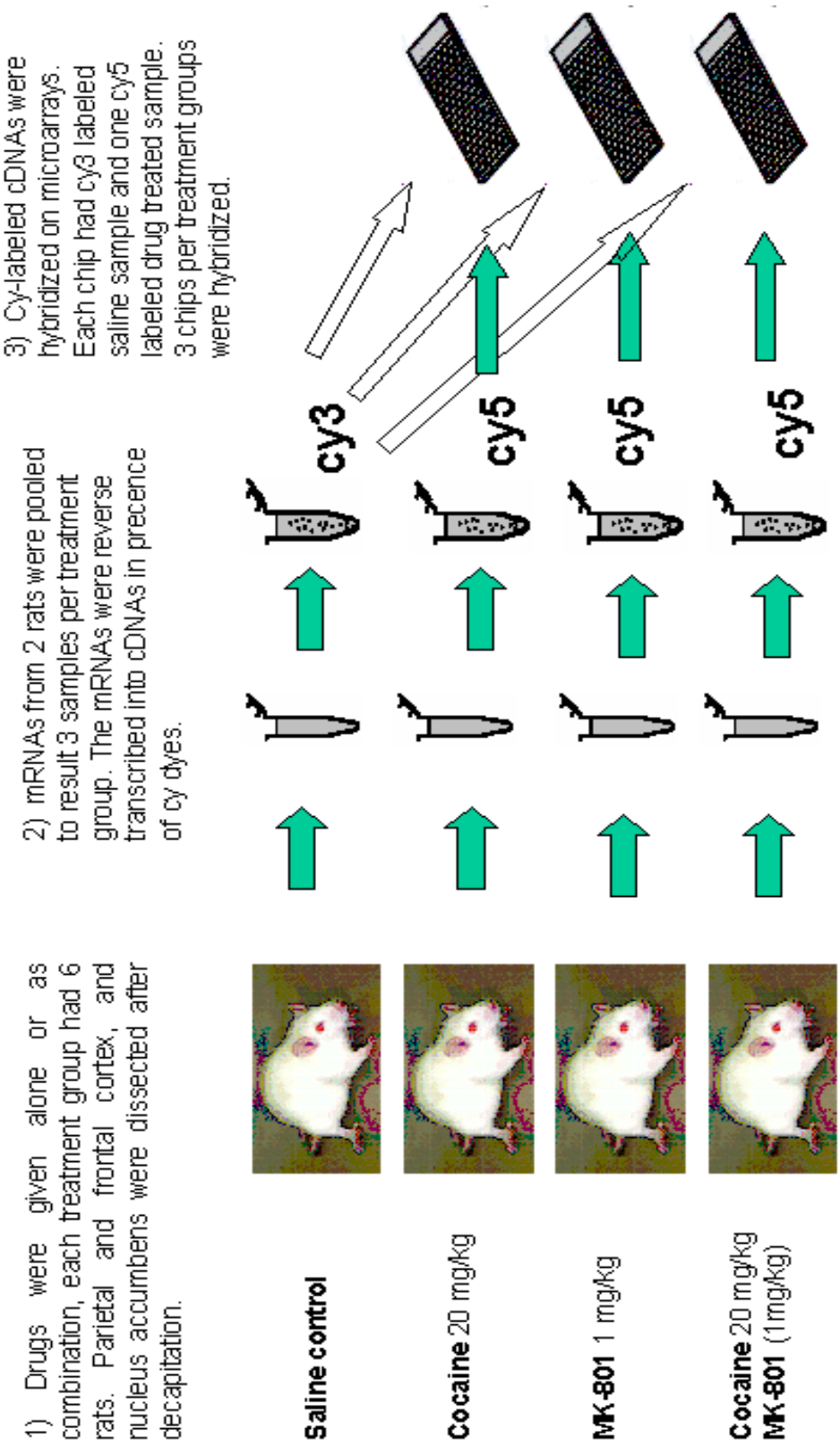


**Figure 8.** Workflow of a microarray experiment. In publication **II**, animals were treated with saline or MK-801. In publication **IV**, animals were treated with saline, cocaine, MK-801, or MK-801 + cocaine

## 4.8 Bioinformatic analysis

The information of genes in the datasets of **publication IV** was subjected to further bioinformatics analysis. The NTNU DNA-microarray had a total of 14500 cDNA clones printed on the microarray. In the beginning of the project, almost 7600 (52%) of these were unknown ESTs. Because of the high number of unidentified sequences and errors in the annotations, there was a need for reannotation according to the homology comparison in order to increase the information of the genes and functional groups that are affected by the treatment. The ESTs with altered expression were reannotated with results from sequence comparisons against all entries in the last available release of the GenBank (#137) and EMBL (#75) sequences using the BLAST (Basic Local Alignment Search Tool) program with human judgement. In order to facilitate the process, we wrote a minimalistic BLAST-parser for filtering and parsing the results (figure 9).

In order to detect strong patterns in the gene expression data, the difference of gene expression between treatments and brain was studied by hierarchical (tree) clustering with Pearson correlation to compute a dendrogram that assembles all elements into a single tree where the degree of similarity among gene profiles is represented by branch lengths and distances. The results were confirmed by determining the correlation between fold-changes from two datasets at a time. The genes with altered expression were clustered by 6 x 6 neuron Self-Organising Map (SOM) algorithm (Toronen et al., 1999) to produce synexpression groups, which are represented by genes sharing the same expression pattern.

The genes with altered expression were finally classified by the gene ontology (GO) terms. Gene ontology's (GO) is a common hierarchical terminology for genes and their products. GO-terms for the annotated genes or their human/mouse orthologs were obtained through DAVID Internet service, and completed with locuslink. Not all transcript ESTs represent genes with a function, and 42% of genes have no annotation even after reannotation, and less than half of the regulated ESTs were used in the grouping process.

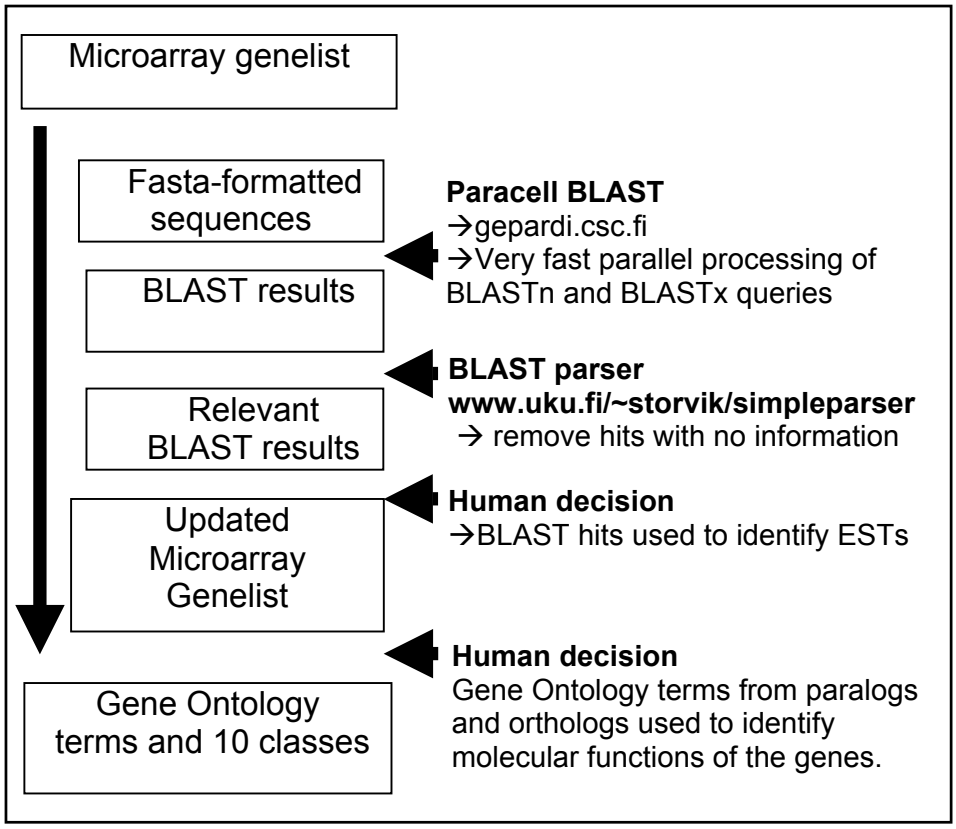


**Figure 9.** Reannotation process of 14K and 15K NTNU rat microarrays

### 4.9 Statistical analysis (I-IV)

Data analysis for *in situ* hybridisation's and immunoblotting and was performed using analysis of variance (ANOVA), or Student's t-test. For the electromobility assay, the data was analysed using ANOVA with Dunnett's post hoc test. Data of DNA array expression analyses **(II, IV)** were analysed as described in section 4.8.

# 5 RESULTS

## 5.1 Effects of uncompetitive NMDA receptor blockers on CREB/CREM family leucine-zipper transcription factor mRNA levels

NMDA receptor blockade by MK-801 greatly induced expression of CREM and ICER in the rat brain. (**I**, Fig 1). This induction was dose dependent and was seen already at a non-toxic dose of 0.2mg/kg, and even higher at toxic doses up to 10mg/kg (**I**, Fig. 2). Time course assay demonstrated the highest increase of CREM/ICER at the 4 hours time point, and return to the basal level between 8 to 24 hours. The effect was seen with all uncompetitive NMDA antagonists, and also by kainate activation, but not by competitive NMDA antagonist or by antipsychotic agents (**I**, table 1). The ICER was the predominant form of the CREM family to be induced by uncompetitive NMDA-treatment. (**I**, fig 5). CREB expression was not altered (**I**).

Expression level changes of several transcription factor transcripts were studied by *in situ* hybridisations. MK-801 alone and the pre-treatment of MK-801 combined with cocaine were similar. The inducible repressor ICER, CREM, and CBP were upregulated on parietal cortex. Expression of CREB was unaltered. Cocaine differed from other treatments, and c-fos was downregulated in the hippocampus (71.8±21.7) and parietal cortex (**IV**, table 6 and figure 5).

**Table 5.** Summary of expression of CREM and ICER mRNA in parietal cortex after MK-801 treatment, percents compared to saline treatments. In publication **I** and **IV**, the *in situ* hybridisation results, in publication **II**, DNA-microarray experiment result. Values are % of control ± SEM.

| **mRNA** | **treatment** | **publication and experiment** | | |
|---|---|---|---|---|
| | | **I** (*in situ*) | **II** (microarray) | **IV** (*in situ*) |
| CREM | MK-801 (5mg/kg; 4h) | 178 ± 22 | - | - |
| CREM | MK-801 (1mg/kg; 4h) | - | 141 | 166±9.9 |
| CREM | Phencyclidine (20 mg/kg; 6 h) | 136 ± 11 | - | - |
| CREM | Memantine (50 mg/kg; 6 h) | 150 ± 13 | - | - |
| CREM | Ketamine (250 mg/kg; 6 h) | 168 ± 18 | - | - |
| ICER | MK-801 (5mg/kg; 4h) | 238 ± 15 | - | - |
| ICER | MK-801 (1mg/kg; 4h) | - | 122 | 168±6.0 |
| ICER | Phencyclidine (20 mg/kg; 6 h) | 163 ± 13 | - | - |
| ICER | Memantine (50 mg/kg; 6 h) | 192 ± 19 | - | - |
| ICER | Ketamine (250 mg/kg; 6 h) | 131 ± 7 | - | - |

## 5.2 Effects of uncompetitive NMDA receptor blockers on CREM/ICER transcription factor protein levels

Immunoblotting revealed that not only mRNA levels, but also protein levels of CREM and ICER were increased after MK-801 treatment time and dose dependently (**I**, fig 2). The DNA-binding activity of the protein complexes recognising CRE-consensus sequence in DNA was studied in the rat parietal cortex preparation 1 hour to 6 days after administration of MK-801 to determine the functional activity of CREB and CREM/ICER proteins. Four complexes with different sizes were observed (**I**, Figure 6). The complexes named in the size order from I to IV were identified by electro mobility super shift with CREB and CREM antibodies. CREB antibody removed the complex II and some of the intensity of complexes I and III. CREM antibody affected all of the complexes, and most notably to the light complex III and very light complex IV (**I**, Figure 7A). As complex I was not removed by either CREB or CREM antibodies, electro mobility shift assay with unlabelled DNA sequences containing AP-1, CREB, or NF-kB binding sites were carried out. CREB competitor removed all of the complexes, NF-kB competitor was without an effect, and AP-1 competitor removed complex I almost totally (I, Figure 7B).

The time course of the complex forming (**I**, Figure 6) revealed acute induction of complex I containing CREB and AP-1 proteins, and complex II containing CREB-CREB-dimers. The induction of these complexes was most notable at the 1-day time point. This was followed by a statistically insignificant decrease of complexes I and II below baseline. At the 6 days time point complexes I and II reached baseline.

The time course of complexes III and IV, which contain CREM and ICER proteins, was faster and significant already at the 8 hours time point. The expression peaked significantly at 1 day time point, but returned to basal level at the 2 days time point (**I**, Figure 6).

## 5.3 Effect of NMDA-antagonists MK-801 on OCT-1, C/EBP and NF-kB

In addition to electromobility shift assay with CRE-oligo (publication **I**), also OCT-1, C/EBP, and NF-kB oligos were used to further characterise the binding activity of several transcription factor pathways. As seen in figure 10, the effects of uncompetitive NMDA-antagonists MK-801 (5mg/kg) can alter the binding of nuclear proteins in different brain areas significantly even days after the treatment (data unpublished).

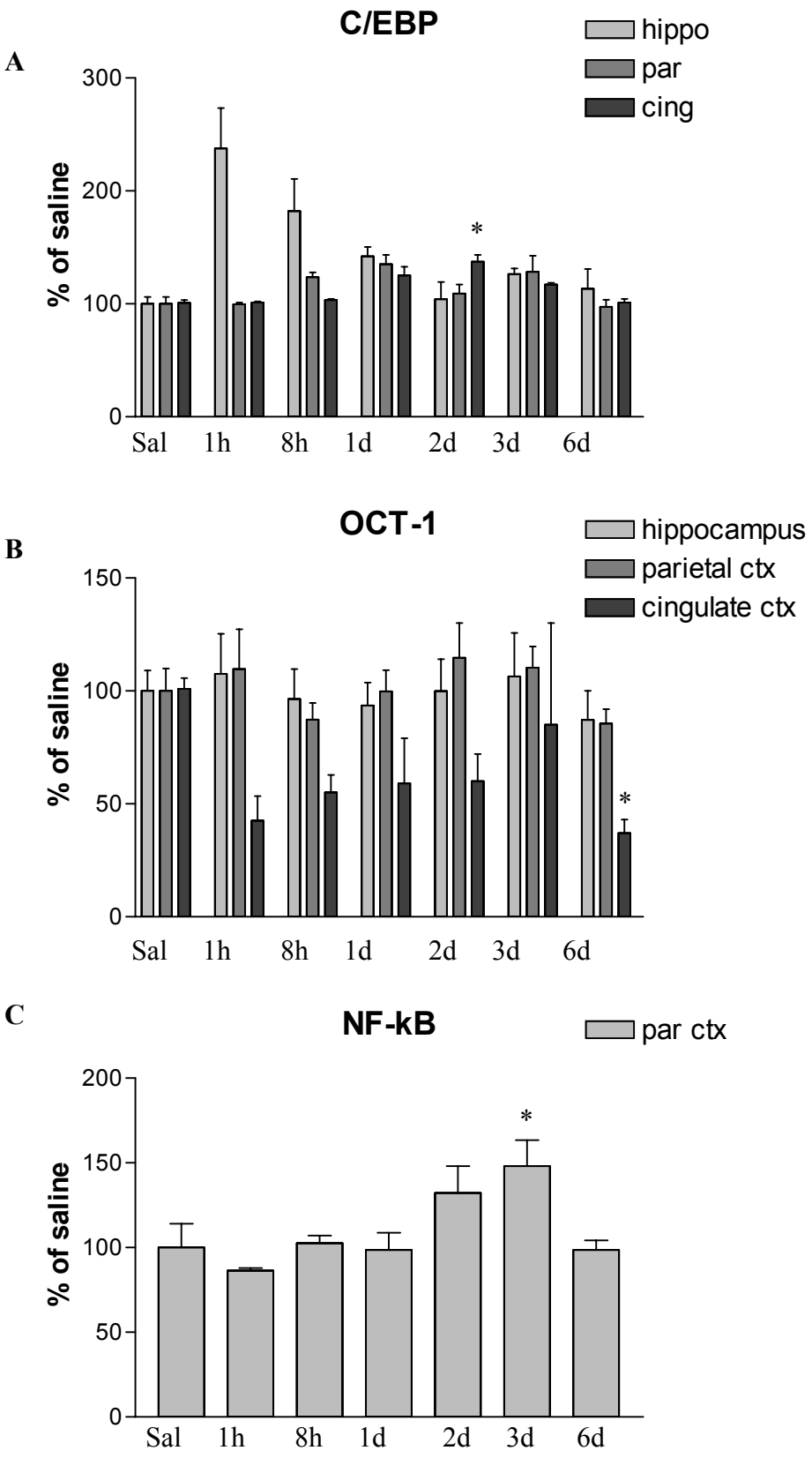


**Figure 10** The intensity on complexes between the C/EBP, OCT-1, and NF-kB oligonucleotides, and the rat brain nuclear proteins after MK-801 (5mg/kg) treatment. The significant alterations are delayed, although some trends do occur only hours after the treatment.

### 5.4 Alteration of glutamate receptor subunits

The expression of AMPA receptor subunit and metabotropic glutamate receptor mRNAs after systemic administration of MK-801 (5mg/kg i.p., 8 h) was determined by *in situ* hybridisation. Significant decreases of AMPA glutamate receptor subunits GluR3 and GluR4, and metabotropic glutamate receptor mGluR3 were observed (**III**, Table 1). GluR3 was most significantly altered in hippocampus, and GluR4 in cortical regions. In limbic regions no alteration was observed (**III**). Metabotropic glutamate receptor 3 was downregulated throughout the brain.

### 5.5 Gene expression ratios in DNA-microarrays after MK-801 treatment

Expression levels and profiles of genes following saline or MK-801 treatment (1 mg/kg; 4 h) in rat brain parietal cortices were obtained using microarrays. Average ratio values (MK-801 treated/saline treated) from 2 independent experiments were determined. Six genes were found to be up-regulated by at least 40%: EST-885, CBP, CREMact1,2, pan CREM/ICER, c-*fos*, and PKCγ. (table I, **II**). Higher expression was observed for ICER than CREM forms of CREM/ICER transcripts (figure I, **II).** Also a slight increase of nurr-1 and c-jun transcription factors were observed (Table I., **II**).

## 5.6 Expression of CREB-related transcription factors after MK-801 and cocaine treatments

The acute effect of cocaine (20 mg/kg), MK-801 (1mg/kg) and the combination of MK-801 pretreatment 15 minutes before cocaine on the expression of CREB and regulators of CREB were determined by *in situ* hybridisation (**IV** and unpublished results). The expression induced by MK-801 and cocaine differed greatly (table 6).

**Table 6**. Effect of cocaine and MK-801 (alone, or as combination) on CREB and ICER expression in rat brain. The brain areas are retro, retrospenial cortex; par ctx, parietal cortex; and DG, dental gyrus. (Mean% ± standard error of mean; 4 hours time point)

| **CREB** | **treatment** | **retro** | **Par ctx layer 4** | **striatum** | **DG** |
|---|---|---|---|---|---|
| | Cocaine | 88.0±18.6 | 91.8±17.7 | 136.1±21.8 | 88.9±12.1 |
| | MK-801 | 107.0±23.5 | 103.5±9.8 | 113.4±13.0 | 131.9±37.8 |
| | Cocaine+ MK-801 | 95.6±41.9 | 85.4±19.2 | 106.1±20.4 | 106.7±26.8 |
| **ICER** | | **retro** | **Par ctx layer 4** | **striatum** | **DG** |
| | Cocaine | 96.8±13.2 | 96.3±14.2 | 109.1±4.3 | 101.6±13.4 |
| | MK-801 | 144.5±14.5 | 158.8±11.1 | 103.2±6.4 | 115.8±19.6 |
| | Cocaine+ MK-801 | 143.1±20.6 | 182.1±30.5 | 103.4±6.2 | 134.6±28.9 |

A DNA-microarray experiment was carried out to determine the effects of MK-801 (5mg/kg) and cocaine (20mg/kg) on the gene expression in the rat parietal cortex, nucleus accumbens, and frontal cortex. In order to produce reliable data, the datasets originated from the chips with 14500 EST were filtered heavily. Only on average, 10% of the genes passed the filtering and was treated as trusted data. The strictest limiter was the number of replicate spots, which was required to be 4 or above. (**IV** table I).

The 850 genes with significantly altered expression in at least one of the sample sets, determined by z-transformation and z-ratios from all 3 replicate chips of the group, were taken to further analysis (**IV** figure I; table 10)

A hierarchical clustering with Pearson correlation was performed to find brain areas and treatments grouping together. Cocaine and MK-801 treated NAc data were grouped together (**IV** figure 2). Combined MK-801 + cocaine treatment in Nucleus accumbens was clustered together with parietal cortex MK-801 treatment. Parietal cortex data of cocaine and MK-801 + cocaine was on the other hand grouped together. The frontal cortex was different from all other data.

Synexpression groups of the genes with altered expression were created by Self Organizing Map algorithm. The main factor regulating gene expression in parietal cortex was MK-801, and in NAc cocaine and MK-801 had similar and as large effect of gene expression (**IV** figure 4).

The functional groups and biological significance of the genes with altered expression were constructed based on the Gene Ontology terms obtained from David www-based database and locuslink. A large group (30 genes) of transcription regulation associated genes was seen to be regulated by MK-801 or cocaine after MK-801 treatment (**IV** Figure 3). Also intracellular signalling pathways and enzyme-activity associated genes were highly altered. The percentage of the genes falling to the categories was similar in all studied groups (**IV** figure 3).

# 6 DISCUSSION

Uncompetitive antagonists of the NMDA receptors have neuroprotective, anesthetic, antiepileptic, and antidepressive effects (Krystal et al., 1999; Skolnick, 1999). According to some studies, these agents have also antiaddictive effects (Popik et al., 1995), although the reports are controversial. Despite therapeutic effects, potent uncompetitive antagonists produce also psychosis-like symptoms in rats and humans. Also, the antiaddictive potential requires special consideration, as PCP and ketamine have abuse value and are used as street drugs.

The uncompetitive NMDA-antagonists produce a number of molecular and cellular changes in the brain (Marvanová et al. 2003). The effects of NMDA-ion channel blockers on immediate early genes has been also reported (Kontkanen et al., 2000). We further studied the underlying transcriptional mechanisms, which could be involved in these alterations. The main focus of the research was the transcription factor CREB, which is one of the first transcription factors to be activated after neuronal stimuli, as well as modulators CREM and ICER. CREB is also known to be needed in neuronal survival, and likely a mediator for the development of antiaddictive and antidepressive effects of uncompetitive NMDA-antagonists (Bito and Takemoto-Kimura, 2003).

## 6.1 Analysis of microarray experiment results

The alterations in mRNA levels do not always match the protein levels, because of post-transcriptional steps. The verification techniques used in study include protein assays by immunoblotting, in which the protein studied is bound with a specific monoclonal antibody. By those methods we demonstrated that alterations in the studied CREB or CREM/ICER family transcription factors do occur similarly in mRNA and protein levels.

The reliability, sensitivity, and reproducibility of the DNA-microarray technique has been severely questioned, as in many studies known predicted alterations have not been seen, and at the same time alterations seen have not been verified by independent techniques. The most reliable method was the protein assay, but it does not give information of the accurate localization of the changes. The best information was given by *in situ* hybridisation, in which the cell groups with altered expression could be detected. None of the techniques gave information of the changes in individual neurons. This could be done either in cell cultures, which gives limited information because of the lack of circuits needed for the full actions of

NMDA-channel blockers. Slightly better could be the floating slice technique, in which the neuronal connections are mostly intact.

**Problems and solution with DNA-microarray data.**

Microarray data analysis has no canonical guidelines. The removal of background binding was done both in publications **II** and **IV** by diminishing the background intensity from the spot intensities. In publication **IV** there were spike control spots printed on chip. Because no mRNA was supposed to specifically bind to these spots, their average intensities could be used as measure of the unspecific hybridisation. The filtering of all raw values was performed on the level of spike control averages, which should markedly decrease false positives. The intensities of data spots vary greatly, and approximately 20 % of the spots with lowest expression were filtered out. This could create artifacts, if spots on one of the replicate chip have been filtered, leaving the spots from other two chips with higher expression to the datasets. This was taken into consideration by accepting to the datasets only the genes that had data from at least 4 spots.

The DNA-microarray technique can be based on synthesised well-designed oligonucleotide probes or on cDNA libraries. The Kuopiochip v 1.0 used in publication **II** had custom-designed oligomers, whereas NTNU Rat chips used in publication **IV** had cDNA-oligos. The genes on microarray in publication **II** were annotated reliably, because all of the probes were custom designed against one known gene. On the other hand, in publication **IV** the probes on the chip were ESTs, varying from 200 to 600 base pairs. Unfortunately, half of the ESTs do not have significant similarity with previously annotated genes in databases. The rest of the RNA-sequences are likely to have homologies with mRNAs, but in varying degree. Also cloning and sequencing mistakes are common. The basic annotation of the ESTs on microarray was found to be insufficient, and also in some cases incorrect. The reannotation produced more correct information.

In the reannotation process, we had a bioinformatics challenge to identify the ESTs as genes. The library, to which the NTNU microarray was based, was created in 1999, and the sequence databases have grown hugely since that time. For the annotation process, several options were considered. First, if the annotation is done for only the regulated genes instead of all genes, this reduces the work that is to be done by many hundred folds. Unfortunately this was not enough, as we needed to know the level of expression of CREB/CREM and fos/jun related transcription factors, even if they would not be regulated by the treatments. This required all of the EST sequences to be reannotated using the latest GenBank database.

For the annotation process, homology comparison with BLAST was chosen as the main tool. The strength for BLAST is that cDNA sequences can be compared not only to nucleotide, but also to protein sequences. Comparison of mRNAs to proteins via mRNA to protein sequence translation can on the other hand create false results, as the amino acid sequences of conserved regions of related proteins with different function might be sometimes close and masked by the mistakes of cDNA sequencing.

The BLAST parser created for the task represents the closest for the best found homologs. This parser had the advantage of simplicity, speed, ease of use, and the inbuilt filtering of homology finding pointing to cDNAs, libraries, and draft sequences. Even after diminishing false homology hits with the BLAST parser, the scientist is still required to make the final decision of whether the EST is likely matching an mRNA of coded protein. In many cases no good annotation can be found, maybe because of sequencing error of the cDNA. This information was used, although after careful consideration, in publication **IV** to decide functional classes of the ESTs with altered expression after drug treatments. As Locuslink www-service binds together information of orthologs from different species and homologous genes in same species, and information of functional domains, gene ontology terms, and other additional information, both manual inspection and semiautomatic scripts were successful in the data mining for following analysis, and the basic annotation of unknown sequences.

**Doses and treatments**

In the **publication I**, the doses used were relatively high in order to see the full range of effects. As the high response with lower doses of MK-801 was seen, the doses were lowered and a dose-response study was performed.

MK-801 used in **publications II to IV** has very high affinity for NMDA-ion channels, and it can cause toxicity in high doses. The dosage of 1mg/kg does not cause permanent damage in male rats (Olney et al., 1991), but the cell stress can possibly be seen in the microarray data as expression of stress-related genes. The 1mg/kg dose of MK-801 induced stereotypic behaviour in the male rats. Lower affinity NMDA-ion channel blockers such as dextromethorphan and memantine are well tolerated in clinical use. This may raise the question that the research should be focused on them, as the results could be more relevant for clinical questions. Dissociative anaesthetics have also addiction potential, and for lower affinity NMDA-blockers the risk for abuse is not as high, although some abuse does occur, when they are used in very high doses. Good clinical data for that is unfortunately missing. It

could be hypothesised that dosing and pharmacokinetics matter most, not the affinity on receptor or receptor binding kinetics.

The anecdotal tales of NMDA-antagonists abusers describe that the subjective effects of NMDA-antagonists can be described as separate plateaus, depending on dose and the potency of the agent. This would suggest that dose-dependent activation of the different neuronal circuits, but this requires still more research.

## 6.2 The effect of NMDA-blockers on glutamate system

It has been demonstrated that uncompetitive NMDA-antagonists increase glutamatergic activity via non-NMDA-receptors mediated mechanisms. This study does not answer whether the alterations in gene expression occur because of increased glutamate release, or by some other transmitter systems. There are reports of alterations in monoamine transmission after NMDA-blocker treatments (Callado et al., 2000), and dysregulation of GABAergic transmission (Olney et al., 1991). All these alterations will create a complex network of altered neurochemistry and neurotransmission.

The effect of NMDA-blockers on glutamate transmission itself can be seen in three areas at the gene-expression level. First, the regulators of synaptic and receptor functions may be altered. This happens mainly by existing proteins, but novel protein synthesis will soon follow, including protein kinases. Second, the receptor expression may alter as adaptation to the changes. Third, larger scale alterations will follow after altered activation and expression of transcription factors in glutamatergic neurons and their target neurons. In the series of studies, we observed alterations belonging to all of these categories, although pin-pointing them into specific neuronal populations is impossible with only gene-expression data. In the DNA-microarray results, uncompetitive NMDA-antagonists MK-801 altered greatly the expression of enzyme, signaling mediators, and transcription factors.

### The effects of NMDA-antagonists on gene expression

We have demonstrated that CREM/ICER expression can be induced by uncompetitive NMDA-antagonists. This was seen in mRNA level, protein level, and also in level of DNA-protein complex formation. The induction of CREM/ICER was seen after kainate-induced seizures, but not after other treatments, not even by competitive NMDA-antagonists. This demonstrates that strong CREM/ICER induction is a typical feature for NMDA-antagonists.

**The effect of NMDA-blockers on glutamate receptors**

Glutamate receptor expression and function undergo adaptive changes during chronic treatment of NMDA-antagonist (Toyooka et al., 2002). These alterations could begin early at mRNA levels. Linden et al. (1997, 2001) have studied the acute effects of uncompetitive NMDA-antagonists on glutamate receptor expression (Linden et al., 1997; Linden et al., 2001b).

Taken together, the results of that and this study suggest that the expressions of metabotropic glutamate receptors do not seem to alter greatly. The metabotropic glutamate receptors of group I, which potentiate NMDA-effects, are not affected. This could suggest lack of adaptive changes. The group II mGluRs are putative targets for antipsychotic drugs (Cartmell et al., 2000). The effects of mGluR2 and mGluR3 are not yet completely distinguished from each other, because of the lack of selective compounds.

Alterations in the expression of ionotropic glutamate receptors can be more often observed. The NMDA-receptor subunit NR2B is decreased by MK-801 treatment, which could then decrease the NMDA-receptor activity (Linden et al., 1997; Linden et al., 2001b). We saw a decrease in AMPA-receptor subunit GluR3 and Glu4 expression, which again could be adaptive changes. But is there really a system that increased/decreased activity which then leads to alterations in mRNA levels pointing to returning to basal level? The data does not tell. The altered expression of NMDA-receptors is controversial. Linden et al (2001) demonstrated increased phosphorylation of NR2A subunit of NMDA-receptors, which then could lead to increase of NMDA-receptor mediated $Ca^{2+}$ influx that is acutely blocked by MK-801 (Linden et al., 2001a). The electrophysiological data of NMDA-receptor antagonist treatment induced alterations on AMPA and NMDA-receptor functions is minimal. The microarray experiment demonstrated alteration of numerous kinases and receptor-binding protein coding genes (PKA, PKC, GRIPs) that could affect to glutamate receptor function. GRIP-2 which is involved in the targeting of AMPA receptors to synapses (Dong et al., 1999) was upregulated on parietal cortex by MK-801 treatment, which could been an adaptive effect, resulting from the acute decrease of glutamatergic transmission on cortical regions. If this is the general trend, it would suggest that the main effects of MK-801 are seen on cortical regions, and then the secondary effects would be seen as the cortical neurons project to lower areas such as VTA, and nucleus accumbens, where MK-801 would again block NMDA-mediated transmission.

## 6. 3 Uncompetitive NMDA-antagonists treatment alters function of CREB

In the series of studies, we clearly demonstrated that uncompetitive NMDA-antagonists induce of CREB-activity in rat brain – although not determined directly, it was seen as upregulation of genes containing CRE-elements (somatostatin, ICER, and others). Among these genes were CREB/CREM/ATF-1 family transcription factors. The most highly inducible was ICER, which is an early response repressor for CREB-mediated transcription.

As seen by dimerization between ICER, and AP-1, CREB, and CREM family transcription factors in the electromobility shift assay, the induction of ICER can alter the profile of these transcription factors. This could lead to decrease of activity of transcription factors other than CREB. In the electromobility shift assay, the binding of ICER to CRE-sequences was acutely increased, and then returned to basal level, but binding of AP-1 proteins to CRE-sequences was decreased for days.

There is interplay with different signalling pathways even at the transcription factor level. AP-1 family transcription factors are produced after cAMP-activity. The activation of AP-1 factors is then induced by MAP kinases, c-fos by ERK-type kinases, and Jun by JNK/SAPK type kinases (Servillo et al., 2002). AP-1 proteins can dimerize with CREB/ATF-1 family transcription, although not to CREB itself. One of the highly dimerizing proteins is ICER, inducible cAMP early repressor. When induced after CRE-activation, ICER can dimerize with AP-1 proteins. This way cAMP and MAPK pathways can interact even after transcription of the target genes has occurred. The binding properties of AP-1 containing heteromers to CRE-sequences were altered by MK-801 treatment. AP-1 binding to CRE rises slowly, and then is below basal state for several days after MK-801 treatment. This could be because of induction of ICER, which declines slowly.

C-fos is an immediate early gene that is known to influence transcription of other genes in many neural systems. C-fos is also a target gene for CREB and induced after its' activation Both psychostimulants and the uncompetitive NMDA antagonist MK-801 induce c-fos expression after systemic administration, but in different brain areas. MK-801 induces c-fos in primary motor cortex, infralimbic cortex, and in several thalamic nuclei, but does not affect the expression in nucleus accumbens. Amphetamine induces c-fos in nucleus accumbens shell. Both amphetamine and MK-801 induce c-fos in thalamus, but in partially different thalamic nuclei (De Leonibus et al., 2001). This makes it further unlikely that the motor effect exerted by NMDA antagonists depends on increased DA activity (Imperato et al., 1990), otherwise a

somewhat similar pattern of c-fos induction should have been observed (De Leonibus et al., 2001).

The decrease of mGluR3 to 80% from the basal level on parietal cortex pyramidal cell layer 8 hours after 1mg/kg MK-801 injection was interesting. The promoter of mGluR3 has several transcription factor binding sites (2 x AP-1, 2 x AP-2, 3 x C/EBP, 2 x HSF, 3 x NF-1, 2 x Oct-1, and 1 x Sp-1) (Minoshima and Nakanishi, 1999). In the time course assay of electromobility shift of DNA-protein complexes with Oct-1, NF-kB, CREB, AP-1, and C/EBP probes we saw some alterations in the time point of 1h, which could be soon enough to have impact on mGluR3 expression at 8h time point.

We saw an increase in the intensity of C/EBP binding complexes at 8-hour time point, but not at the 1 h time point. If MK-801 decreases the $Ca^{2+}$ currents, it could lead to dephosphorylation of C/EBP proteins. Moreover, transcriptional activity of C/EBP proteins might depend on CBP binding properties and phosphorylation (Kovacs et al., 2003). If there is impairment of CBP-function, it should be seen in the expression of CRE-dependent genes, but on the contrary we saw massive induction of such genes. If the promoter of gene coding C/EBP has CRE sites, it would suggest increase of C/EBP mRNA expression. There are also repressing forms of C/EBP, which makes it difficult to estimate the net effect.

NF-kB complexes were modestly decreased at the 1h time point, but it should not affect mGluR3 transcription. Also, intensity of AP-1 and CREB containing complexes was increased from 1 to 8 hours. The intensity of ICER-containing complexes was not affected at 1 hour, but in 8 hours indicating increased CREB-activity. The ICER protein itself did not increase until several hours later. There are no CRE-elements in mGluR3 promoter, but activity of genes induced by CREB-activity may play some role. It could be hypothesised that interaction of ICER with AP-1 proteins could lead to binding of transcription factor dimers without activity to the AP-1 binding sites on mGluR3 promoter. In this case, induction of AP-1 family immediate early genes by CREB could not be strong enough to balance the situation.

Oct-1 has not only activation function, but also transcription repressor function. Oct-1 activity has been reported to downregulate prolactin gene expression, which is known to be activated by dopaminergic transmission. We observed a rapid decrease of Oct-1 binding activity in the cingulate cortex, but not in other brain areas. MK-801 has been reported to decrease prolactin expression in the hippocampus (O'Donnell et al., 2003), but our EMSA assay results does not elucidate the role of Oct-1 in this issue.

**Co-regulators**

In both microarray experiments and *in situ* hybridisation, we observed upregulation of CREB binding protein (CBP). CBP serves in the integration of additional signal transduction pathways (Chawla et al., 1998; Hardingham et al., 1999). CBP mediates both positive and negative transcriptional responses to the JAK/STAT and Ras/Map signal pathways, and regulates also activation of AP-1 proteins and NF-kB (Horvai et al., 1997). CBP has also a signal-regulated transcriptional activation domain that is controlled by nuclear calcium via CaMKIV and by cAMP (Chawla et al., 1998). More research however is needed before the significance of the alteration of CBP can be determined, and if this is the mechanism of how alterations of CREB-mediated transcription can be modulated by uncompetitive NMDA-antagonists to alter AP-1 related transcription. These and other findings raise the question of CBP as a target of signalling pathways altered by NMDA-antagonists and cocaine.

## 6.4 Main findings and future studies

In *in situ* hybridisation and DNA-microarray studies, we found alteration in the expression of genes associated with all levels of glutamate signalling, starting from glutamate receptors and enzymes, continuing to signal transducers, and ending at transcription factors. These molecules play a role in neuronal activity, neuronal plasticity, in adaptive responses, and in addiction, therefore, they appeared to be putative targets for the actions of MK-801 and to the suggested antiaddictive properties of NMDA-channel blockers.

The most important finding of this series of studies was that MK-801 administration increased ICER expression, and that a low dose of MK-801 affected intracellular signalling pathways. This leads the way for future studies of uncompetitive NMDA-antagonists. Another rising question is the role of CBP, which could be differentially regulated after drug treatments. For the future studies, it remains to be seen what the effects of clinically well tolerated uncompetitive NMDA-antagonists such as memantine, and dextromethorphan have on gene expression in nucleus accumbens, striatum, and frontal cortex.

# 7 SUMMARY AND CONCLUSION

This study aimed to examine the molecular effects of the uncompetitive NMDA receptor antagonists to the expression of transcription factor CREB and to related gene products in the rat brain. The main findings and their relevance can be summarised as follows:

1. CREM/ICER expression is induced in the rat brain by uncompetitive (MK-801, PCP, memantine, ketamine), but not by competitive NMDA-antagonist drugs. The induction is transient and most of the induced forms are of the ICER form. The induced ICER form interacts with the transcription factors binding CRE-elements, as shown by electromobility shift assays. There is also interaction between AP-1 and CREM/ICER transcription factors.

2. The treatments with uncompetitive NMDA antagonist MK-801 alter the expression of AMPA and metabotropic glutamate receptors, sub-units GluR3, Glu4, and mGluR3. These alterations may lead the way to further characterise drug targets to modulate glutamate-signalling pathways.

3. CREM/ICER gene products repress CREB mediated transcription. The alteration of CREB function could be a possible mechanism for the anti-addictive properties of the uncompetitive NMDA antagonists, although CREM/ICER induction might be merely secondary effects for initial induction of CREB activity. However, no alteration of the expression of genes induced by acute cocaine treatment was observed to be blocked by the acute treatment of the uncompetitive NMDA-antagonist MK-801. Potential or reputed anti-addictive properties of NMDA antagonists are likely to occur through complex transcriptional events that indirectly require b-ZIP transcription factors and later, hundreds of their target genes.

4. Several genes with an altered expression in response to acute MK-801 and cocaine treatments were identified. These molecules play a role in neuronal activity, neuronal plasticity, in adaptive responses, and in addiction. They appeared to be putative targets for the actions of MK-801 and to the suggested antiaddictive properties of NMDA channel blockers. The gene expression profiles after acute treatments with MK-801 or cocaine were found to be similar in nucleus accumbens, but differed greatly in the cortical regions in rat brain. Characterisation of these genes may lead to a better understanding of the molecular pharmacology of uncompetitive NMDA receptor antagonists and some of these genes may constitute novel targets for new therapeutic use.

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# I

## Induction of cAMP response element modulator (CREM) and inducible cAMP early repressor (ICER) expression in rat brain by uncompetitive N-methyl-D-aspartate receptor antagonists

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Markus Storvik, Anni-Maija Lindén, Outi Kontkanen, Merja Lakso, Eero Castrén, and Garry Wong

# II

## Expression profiling to understand actions of NMDA/glutamate receptor antagonists in rat brain

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Petri Törönen*, Markus Storvik*, Anni-Maija Lindén, Outi Kontkanen, Markéta Marvanova, Merja Lakso, Eero Castrén, and Garry Wong. *Shared first authorship

# III

## Subtype selective decrease of AMPA and metabotropic glutamate receptor mRNA expression in rat brain by the the systemic NMDA receptor blocker MK-801

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Markus Storvik, Anni-Maija Lindén,
Merja Lakso, and Garry Wong

# IV

## Gene expression profiles in rat brain after MK-801 and cocaine treatments

*Submitted*

by

Markus Storvik, Pekka Tiikkainen,
Martijn van Iersel, and Garry Wong