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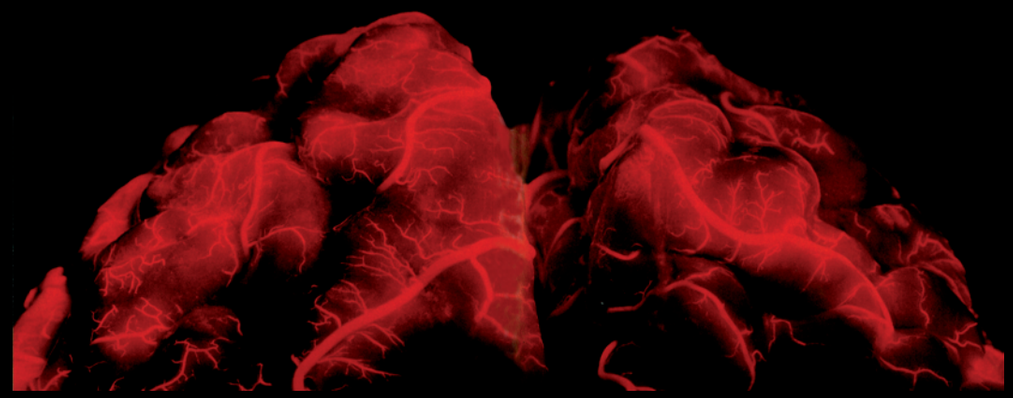

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To My Grandparents

ABSTRACT

Drug addiction is a chronic disorder characterized by craving and compulsive drug use despite adverse consequences and high rates of relapse during periods of abstinence. Therapeutic interventions for most addiction disorders are limited today, partly because the underlying neurobiology is still unknown. A growing body of evidence indicates that synaptic plasticity contributes to the development and persistence of addiction, however, most research have focused on rodent animal models and very limited knowledge exists about the effects of drugs of abuse on the glutamatergic system in the human brain. The aim of this thesis was therefore to gain deeper insight into the neurobiology of drugs of abuse, including alcohol, heroin and cocaine directly in the human brain relevant to synaptic plasticity in key neuronal circuits relevant for the development and persistence of addiction.

In the first study we examined the gene expression profile of sixteen endogenous control genes in the prefrontal and motor cortex of alcoholics. The results demonstrated differences in gene expression stability between the prefrontal and motor cortex as well as region-specific-alterations in several genes normally used as reference genes between alcoholic and controls. These observations implicate the importance of selecting proper genes for normalization when performing gene expression studies.

Next we investigated whether the NF-κB system was altered in the prefrontal and motor cortex of alcoholics. The results revealed a reduced DNA-binding activity of the NF-κB and p50 homodimer in the prefrontal cortex of alcoholics that was coupled to a reduction in *RELA* mRNA levels. NF-κB has been implicated in synaptic plasticity and memory consolidation, thus it is tempting to speculate that decreased NF-κB function could lead to a disruption of learning and memory formation, or effect alcohol-induced associative memory reconsolidation often linked to relapse.

Third, we examined the effect of alcohol consumption on modulators of synaptic strength (synaptophysin) and executors of glutamate release in the prefrontal and motor cortex. We observed increased synaptophysin I levels in the prefrontal cortex of alcoholics compared to controls, while levels of predominant members of the synaptic vesicular machinery important for glutamate release were unaltered. These results suggest a role for synaptophysin in the alcohol dependence–associated enduring neuroplasticity in the prefrontal cortical glutamate circuitry.

Finally, we evaluated glutamatergic receptors and their associated scaffolding proteins in the amygdala and striatum of heroin, cocaine and polysubstance (heroin/cocaine) abusers. The findings revealed region-specific disturbances in glutamatergic systems tightly coupled to PSD-95 and Homer in human drug abusers indicting an abberant regulation of glutamatergic signaling and function.

In conclusion, we have demonstrated disturbances in several key mechanisms underlying synaptic plasticity/function in the human brain of drug abusers that are in line with research findings from animal models. Altogether these findings emphasize pathology of neuroplasticity as a common feature in addiction disorders.

LIST OF PUBLICATIONS

I. Johansson S., Fuchs A., **Ökvist A**., Karimi M., Harper C., Garrick T., Sheedy D., Hurd Y., Bakalkin G., Ekström T.J., Validation of endogenous controls for quantitative gene expression analysis: Application on brain cortices of human chronic alcoholics. Brain Research, 2007, 1132(1): 20-28.

II. **Ökvist A**., Johansson S., Kuzmin A., Bazov I., Merino-Martinez R., Ponomarev I., Mayfield D., Harris R.A., Sheedy D., Garrick T., Harper C., Hurd Y.L., Terenius L., Ekström T.J., Bakalkin G., Yakovleva T. Neuroadaptations in Human Chronic Alcoholics: Dysregulation of the NFκB – system. PLoS ONE, 2007, 9: e930.

III. Henriksson R., Kuzmin A., **Ökvist A**., Harper C., Sheedy D., Garrick T., Yakovleva T., Bakalkin G., Elevated Synaptophysin I in the Prefrontal Cortex of Human Chronic Alcoholics. Synapse, 2008, 62: 829–833

IV. **Ökvist A**., Fagergren P., Garcia A., Drakenberg K., Bannon M., Horvath M., Keller E., Hurd Y.L. Dysregulation of the synaptic machinery in the striatum and amygdala of human drug abusers. Manuscript

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ACTB	Beta-actin
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
B2M	Beta-2-microglobulin
BA	Brodmann area
BDNF	Brain derived neurotrophic factor
CREB	cyclic AMP response element binding
CRF	Corticotrophin releasing factor
DSM IV	Diagnostic and statistic manual of mental disorders, fourth edition
EMSA	Electrophoretic mobility shift assay
GABA	γ-aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GluR	Glutamate receptor
GPCR	G-protein coupled receptor
IκB	Inhibitor of κB
IKK	IκB kinase
IPO8	importin 8
LTD	Long-term depression
LTP	Long-term potentiation
MC	Motor cortex
mGluR	Metabotropic glutamate receptor
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA	N-methyl-D-aspartate
PI3K	Phosphoinosititde 3-kinase
PFC	Prefrontal cortex
PGK1	Phosphoglycerate kinase 1
PPIA	Peptidylprolyl isomerase A
PKA	Protein kinase A
PKC	Protein kinas C
PMI	Post-mortem interval
POLR2A	RNA polymerase II
PSD-95	Post-synaptic density protein 95
ROS	Reactive oxygen species
RPLP0	Ribosomal large P0
RT-PCR	Real-time PCR
SNAP-25	Synaptosome-associated protein 25
SNARE	Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor
SYP1	Synaptophysin I
SYX1A	Syntaxin 1A
TFRC	Transferrin receptor
VAMP	Vesicle-associated membrane protein
VGLUT	Vesicular glutamate transporter

1 INTRODUCTION

Drug abuse is an enormous worldwide problem that impacts on the individual, family and society at multiple levels. Nonetheless, in Sweden 2009 many view addiction as a problem connected to lack of character or discipline i.e. if you are strong enough you can resist, you do not get stuck, you are not weak and give in, you do not become dependent! And if you have, you can still get yourself together. Unfortunately, this stigma associated with substance use and dependence prevents individuals accepting their disease, to seek treatment and to get adequate support from society. A study preformed by The World health organization (WHO) reported that out of 18 disabilities in 14 countries 'substance addiction' ranked at the top or near, in terms of social disapproval or stigma (Room, 2001). As we shall see by reading this thesis, addiction is clearly a disease caused by genetic, environmental and drug-induced changes that ultimately affect molecular and cellular systems as well as behavior. Importantly, these factors interact. Sadly, therapeutic interventions for most addiction disorders are limited today, partly because the underlying neurobiology is still unknown.

In writing this thesis I hope that I can provide you readers with some concept of the current theory regarding synaptic plasticity in relation to addiction disorders and to discuss the results in relation to these findings and models. Importantly, I have chose to consider myself an author who writes a story, with one particular perspective in mind, aware that the story could be written in many, many different ways, but that the angle chosen by its writer still provides an interesting story worth telling since it widens the horizon, at least a tiny bit. More specifically, this story is about how drugs of abuse affect common neurobiological mechanisms and neuronal circuits in the brain and how these plastic events effect the development and persistence of addiction. Even though I have chosen only one angle, one perspective, there will be many points that I have forgotten to make and many facts that could have been interpreted in another way. Therefore, I urge all of you reading this thesis who share my love and interest for the field, to use it to agree and disagree. Use it as a starting point for discussion and inspiration, for formulating new questions that needs answers. With this, I leave the thesis in your hands, to enjoy, to dislike, to explore, but most importantly of all, to intellectually challenge its content.

1.1 ADDICTION DISORDERS

Alcohol and other drugs of abuse are an increasing problem in the world today, with major impact on the individual as well as on society in general. Social and psychiatric problems as a consequence of addiction are very common and treatment interventions are limited. The World health organization estimated in their Global Status Report for Alcohol that 76.3 million people worldwide had a diagnosable alcohol use disorder (W.H.O, 2004) and that alcohol consumption is the fifth leading cause of death worldwide. It is also apparent that the prevalence and the proportion frequently using illicit drugs are increasing. According to ODC approximately 185 million people used illicit drugs during the period 1998-2001 (UNODCCP, (2002)). These numbers correspond to 4.3% of the world's population over 15 years old and above. In Europe, 8% of the population reported that they used at least one type of illicit drug other then cannabis. In the USA the number was 20% (WHO, 2004). Alcohol- and drug use are also a problem in Sweden. The alcohol consumption has increased during the last ten years. The average consumption per person was estimated to 9.8 liters in 2007. That is an increase with 2.5 liters since the 1990's (CAN, 2008). The number of heroin addicts has also increased during past years in Sweden and now represent the largest group of heavy users in the age group less than 35 years old (CAN, 2002). Furthermore, Fugelstad *et al* reported that heroin accounted for approximately 62% of the drug-related deaths in Sweden (Fugelstad et al., 1997). In the following sections (1.1.1-1.1.3) my aim is to give some background to addiction disorders, their clinical aspects, theoretical models and mechanisms of action.

1.1.1 Clinical features of addiction

From a clinical perspective there is a need to compartmentalize the drug-taking behaviors into different stages e.g. *use, abuse* and *dependence,* in order to intervene and guide, help and treat patients. Substance use is defined as recreational use for non-medical purposes (also referred to as social use) while substance abuse is characterized by continued drug use despite the harmful/negative consequences it has at the social and personal levels (failure to fulfill major roles at work, school, home, physical problems arising as a consequence of drug use etc.). According to the Diagnostic and Statistic Manual of Mental disorders fourth edition (DSM IV) formulated by the American Psychiatric Association in 1994, substance dependence is defined as a maladaptive pattern of substance use leading to clinical impairment or distress (APA., 1994) (Table 1). Three out of the seven criteria have to be fulfilled during a twelve-month period in order to be diagnosed with substance dependence. Being in a social setting, I often get questions regarding the psychological versus the physical nature of addiction. Important to note is that physical dependence *per se* is neither necessary nor sufficient to cause addiction. Not all drugs cause 'physical' dependence.

Table 1. DSM-IV criteria for substance dependence

The DSM-IV criteria define substance dependence as a maladaptive pattern of substance use, leading to clinically significant impairment or distress, as manifested by three (or more) of the following, occurring at anytime in the same 12-month period:

1. Tolerance, as defined by either of the following:
   (a) The need for markedly increased amounts of the substance to achieve intoxication or desired effect.
   (b) Markedly diminished effect with continued use of the same amount of the substance.
2. Withdrawal, as manifested by either of the following:
   (a) The characteristic withdrawal syndrome for the substance.
   (b) Use of the same (or a closely related) substance is taken to relieve or avoid withdrawal symptoms.
3. The substance is often taken in larger amounts or over a longer period than was intended.
4. A persistent desire or unsuccessful efforts to cut down or control substance use.
5. A great deal of time is spent in activities necessary to obtain the substance, use the substance, or recover from its effects.
6. Important social, occupational, or recreational activities are given up or reduced because of substance use.
7. Continued substance use despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by the substance.

1.1.2 Theoretical models of addiction

Addiction disorders are highly complex diseases and result from genetic, developmental and sociological vulnerabilities combined with drug-induced changes within the brain. Theoretical models have therefore emerged trying to explain the key features of addiction, including motivation, craving, relapse and control. Positive reinforcement, hedonic allostasis and dependence as pathology of staged neuroplasticity are three of the dominating theories in the addiction field today. Because my thesis relates to synaptic plasticity in addiction, I will elaborate most on the third model of addiction as a 'pathology of staged neuroplasticity' that was proposed by Kalivas and O´Brien in 2008 (Kalivas and O'Brien, 2008). However, it is my belief that the different theories contribute to the understanding of the disease even though dependence to a

particular drug may be better explained by one or the other theory, depending on the pharmacological effects of the drug, in combination with genetic vulnerability and, environmental factors.

The positive reinforcement theory of addiction postulates that repeated drug use causes neuroadaptations within the mesocorticolimbic dopamine system. These changes then result in a behavioral state where the motivation for drug seeking is enhanced (incentive motivation), which is manifested as increased drug apetite or 'wanting'. The specific increase in motivation that is separate from the 'liking' effect of the drug is referred to as incentive sensitization by Robinson and Berridge (Robinson and Berridge, 1993, 2001).

The theory of hedonic allostasis proposed by Koob and LeMoal assigns negative reinforcement (alleviating withdrawal symptoms or other negative effects) as an important regulator of the transition from controlled drug use to a compulsive relapsing disorder (Koob and Le Moal, 1997, 2001). In this model the positive reinforcing effects are important for the initial phases of dependence, but with time the negative reinforcing effects become more important and drive the progression from controlled drug use into a compulsive state as a result of an 'allostatic shift'. In contrast to homeostasis, which is a self-regulating process maintaining vital parameters around a normal operating level, allostasis, refers to maintaining stability at a setpoint outside the normal homeostatic range at times when physiological systems challanged. Koob and Moal suggest that repeated drug intake creates an 'allostatic shift' of reward circuits and hormonal stress responses affecting moods states, which results in counter-adaptive neuronal responses, in turn leading to a continuation of drug use and ultimately to compulsive intake.

In a recent review Kalivas and O´Brien propose the third theory where the 'core' addiction syndrome is hypothesized to be caused by a pathology in the mechanisms of brain neuroplasticity underlying motivated behaviors that also affects the ability to value natural rewards (Kalivas and O'Brien, 2008). The motivational circuitry allows us to learn about and behaviorally adapt to important environmental stimuli during normal physiological conditions. For example, they help us to choose among natural rewards or how to avoid dangerous situations (Everitt and Robbins, 2005; Kelley, 2004). However, drugs change these brain circuits and hence impair the ability to create a proper hierarchy among behaviors, instead favoring drug-related behaviors (Kalivas and Volkow, 2005). This can be exemplified as the inability of a substance abuser to appreciate natural rewards such as friendship, love etc. and being able to prioritize them over drug-seeking and -taking. Kalivas and O´Brien propose that transient as well as stable plasticity in the motivational circuit contribute to this pathology. Impairment of transient plasticity is largely due to the molecular pharmacological actions of the drug and probably contributes to the development of addiction by promoting acquisition of new memories coupled to the drug that favors drug-induced behaviors. Transient plasticity typically occurs during social use and critically involves dopamine cells in the ventral tegmental area that release dopamine into the prefrontal cortex, amygdala and nucleus accumbens (Berridge and Robinson, 1998; Jones and Bonci, 2005; Kauer, 2004; Kelley, 2004; Schultz, 2004; Wise, 2004). Conversely, stable plasticity is caused by repeated drug insults that cause changes in synaptic physiology, and lasts from weeks to being relatively permanent. These alterations are related to changes in the cognitive and emotional responses to important environmental stimuli and accounts for the high vulnerability to relapse after cessation of drug intake. Kalivas and O´Brien divide the relapse stage into regulated and compulsive relapses. Regulated relapse refers to the ability of the individual to consciously make a decision of whether to relapse or not. In compulsive relapse, on

the other hand, the addict is not making a conscious choice and automatically relapses. Progression from regulated relapse to compulsive relapse probably has a molecular basis. Regulated relapse relies on the retrieval of drug-associated memories and the integration of these memories, leading to execution of behaviour, through activation of glutamatergic projections from the PFC to nucleus accumbens (Cardinal and Everitt, 2004; Pierce and Kalivas, 1997b; Wolf et al., 2004). Conversely, in compulsive relapse, the glutamatergic circuitry has transitioned from a conscious executive PFC circuitry (PFC-nucleus accumbens) to a more habitual circuitry involving the dorsal striatum and motor pattern generators that are known to drive unconscious, well-learned behaviors (Barnes et al., 2005; Everitt and Robbins, 2005).

1.1.3 Drugs of abuse: clinical features and mechanisms of action

1.1.3.1 Alcohol

The acute behavioral effects of ethanol vary between individuals and are dependent on many factors including dose, rate of drinking, gender, body weight and the time since the previous dose. The blood alcohol level is also important for the behavioral consequences. At low doses the first effects that are observed are heightened activity and disinhibition (e.g. of normal social functioning and emotional restraint). At higher doses cognitive, perceptual and motor functions become impaired. Effects on mood and emotions vary greatly from person-to-person in. These effects are dependent on the effects ethanol has on the central nervous system, which are described below.

Ethanol is often referred to as the 'dirty drug' due to its quite complex effects on multiple neuronal systems. Intriguingly, its complexity is dependent on its simplistic chemical structure, and because of it, alcohol affects almost all systems within the brain directly or indirectly. For example, it directly interacts with multiple receptors and ion channels such as: γ-aminobutyric acid A ($GABA_A$), N-methyl-D-aspartate (NMDA) receptors, 5-hydroxytryptamine-$_3$ receptors, acetylcholine receptors, L-type Ca^{2+} channels, and inward rectifying K^+ channels. Furthermore, various neurotransmitters/neuromodulators mediate some of the acute and long-term effects of ethanol including dopamine, opioid neuropeptides, endocannabinoids, neuropeptide Y and corticotrophin releasing factor (CRF) that are all important for various aspects of ethanol dependence (Fadda and Rossetti, 1998; Manzanares et al., 2005; Vengeliene et al., 2008).

The effect of ethanol on receptor systems is concentration-dependent and state-dependent. Acute ethanol increases the inhibitory activity mediated by $GABA_A$ receptors (Mihic, 1999) but decreases the excitatory activity mediated by glutamate receptors, especially NMDA receptors. These two mechanisms of action may be related to the sedative and cognitive impairments associated with intoxication (Vengeliene et al., 2008). The reinforcing effects produced by ethanol are probably related to an increased firing rate of ventral tegmental area dopamine neurons (Gessa et al., 1985), and dopamine release in the nucleus accumbens (Di Chiara and Imperato, 1988), probably as a secondary consequence of activation of the GABA system or direct stimulation of endogenous opioids (Manzanares et al., 2005).

Chronic ethanol consumption alters the balance between the excitatory and inhibitory neurotransmitter and neuropeptide systems; the two most studied being the glutamate and GABA systems. Hence while acute alcohol increases $GABA_A$ receptor function, prolonged consumption

has the opposite affect Accordingly, acute NMDA receptor function is inhibited, while its excitatory activity is increased during chronic ethanol consumption (Vengeliene et al., 2008).

Alcohol can cause withdrawal symptoms severe enough to be fatal. Early signs of withdrawal are severe shaking, sweating, weakness, agitation, headache, nausea and vomiting, rapid heart rate and seizures. In severe cases the alcohol withdrawal can be complicated by the state of autonomic hyperactivity, hallucinations and delusions. It is believed that the disturbances in glutamate and GABA systems are responsible for the intense withdrawal symptoms.

1.1.3.2 Opiates

Opiate drugs such as morphine are compounds that initially were extracted from the poppy seed. Heroin is a synthetic opiate drug processed from morphine. Intravenous injection of opioids produces a warm flushing of the skin and sensations described by users as a "rush"; however, the first experience with opiates can also be unpleasant, and involve nausea and vomiting. In addition to inducing euphorogenic effects, opioids also produce analgesic, sedative, and respiratory depressant effects on the central and peripheral nervous systems (Jaffe, 1990). These effects of opiates could be explained by their effect on the endogenous opioid system, which has a central role in regulating pain as well as mood and well-being (Contet et al., 2004).

Three main families of opioid receptors (μ, δ and κ-receptors) mediate activities of both exogenous opioids (drugs) and endogenous opioid peptides (endorphins, enkephalins and dynorphins), and therefore represent the key players in the understanding of opioid-related behaviors. The μ-opioid receptor subtype is predominantly responsible for the rewarding and analgesic effects of heroin and morphine (De Vries and Shippenberg, 2002). It is strongly expressed in the central nervous system (Mansour et al., 1995), including structures involved in addiction-related behaviors such as cerebral cortex, ventral tegmental area, striatum and amygdala (Akil et al., 1998; Mansour et al., 1995). Opioid receptors belong to the superfamily of G protein-coupled receptors (GPCRs) and their activation causes hyperpolarization and inhibition of neuronal activity. Downstream signaling is also initiated through receptor activation and leads to activation of transcription factors and gene expression. Chronic opiate exposure leads to tolerance, which could be explained by modulation of a number factor including G-proteins involved, e.g., in receptor activation and signaling, ion channels, regulatory proteins, and transcription factors (Taylor and Fleming, 2001)

Withdrawal from chronic opioid use is associated with an intensely dysphoric withdrawal syndrome, which may be a negative drive to reinstate substance use. In addition, it is characterized by physical symptoms that vary in severity e.g. watering eyes, runny nose, yawning, sweating, restlessness, irritability, insomnia, panic, tremor, nausea, vomiting, diarrhoea, increased blood pressure and heart rate, chills, cramps and muscle aches (Jaffe, 1990).

1.1.3.3 Cocaine

Cocaine is derived from the coca plant (*Erythroxylum Coca*) and is one of the most addictive drugs used by Man. It increases alertness, feelings of well-being and euphoria, energy and motor activity, feelings of competence and sexuality. Anxiety, paranoia and restlessness are also frequent. In the brain, cocaine acts as a monoamine transporter blocker, with similar affinities for dopamine, serotonin, and norepinephrine transporters (Ritz et al., 1990). It is widely accepted

that the ability of cocaine to act as a reinforcer is due largely to its ability to block dopamine reuptake resulting in elevated dopamine levels (Sora et al., 2001; Wise and Bozarth, 1987; Woolverton and Johnson, 1992) in the mesocorticolimbic system. In general, there appears to be little physical tolerance to the effects of cocaine, although there may be acute tolerance within a single session of repeated substance use. Cocaine withdrawal does not result in severe physical reactions that characterize opioid withdrawal, but it does induce 'post-high down' that can be manifested as irritability, anxiety and depression.

1.2 SYNAPTIC PLASTICITY

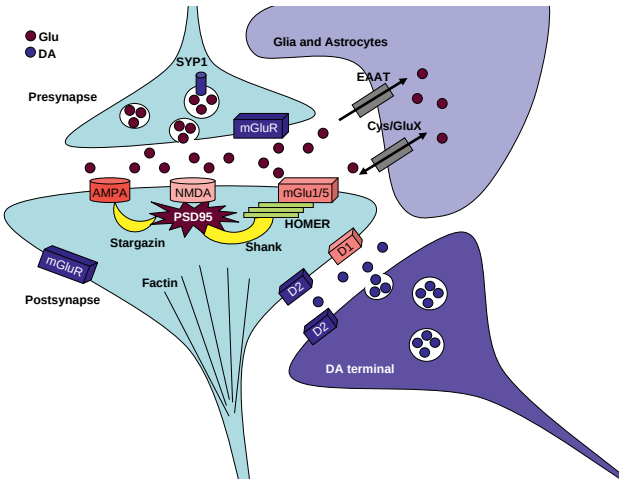
Synaptic plasticity: activity-dependent strengthening (long-term potentiation; LTP) or weakening (long-term depression; LTD) of synapses was suggested early on to represent a cellular building block for learning and memory. More recently it has been demonstrated that LTP and its counterpart long-term depression is used for brain functions other then learning and memory, such as stabilization and elimination of of synapses during early development of neuronal circuitry (Citri and Malenka, 2008; Foeller and Feldman, 2004; Kauer and Malenka, 2007). Moreover, accumulating evidence also suggests that impairments in synaptic plasticity contribute to the development of neuropsychiatric disorders e.g. addiction disorders (Kalivas et al., 2008).

Tom Bliss first discovered LTP in glutamatergic synapses of the hippocampus in 1973 (Bliss and Lomo, 1973). Thereafter, intense research was conducted in order to understand the molecular basis and behavior correlates of synaptic plasticity in the hippocampus because of the central role of the hippocampus in memory formation. However, it has since become clear that LTP and LTD are fundamental processes of most excitatory synapses of the central nervous system (Citri and Malenka, 2008) . Furthermore, it was recently discovered that these processes also exist in inhibitory GABAergic synapses (Nugent and Kauer, 2008). The molecular basis for synaptic plasticity has been widely studied using electrophysiological methods and brain slices. It is apparent that LTP and LTD is brain region- and cell type-specific. That implies that their induction and maintenance are regulated by specific receptors, signaling cascades and gene expression within a given neuron. In addition, LTP and LTD can be elicited both pre- and post-synaptically and its persistence is protein synthesis- dependent. Many forms of LTP and LTD have been reported to date, but the most common forms are NMDR-dependent LTP (Malenka and Bear, 2004), pre-synaptic LTP (Malenka and Bear, 2004; Nicoll and Schmitz, 2005), NMDR-dependent LTD (Malenka and Bear, 2004; Morishita et al., 2005), metabotrophic glutamate receptor-dependent LTD (Gubellini et al., 2004; Pfeiffer and Huber, 2006)) and endocannabinoid-dependent LTD (Wilson and Nicoll, 2002). One focus of this thesis was on molecular events associated with the glutamatergic system given its known contribution to compulsive, goal-directed behavior relevant for addiction disorders.

1.2.1 The glutamatergic synapse

The glutamatergic synapse, its components and regulators, are essential for synaptic plasticity to occur. Pre-synaptic as well as post-synaptic glutamate receptors, scaffolding proteins, signaling enzymes, transcription factors and glutamate transporters all contribute to the ability of the synapse to be plastic, to be strengthened and to be weakened. At the molecular level synaptic plasticity is dependent on the release of glutamate from synaptic vesicles located in the pre-

synaptic terminal. The released glutamate then activates either pre- or post-synaptic glutamate receptors which trigger a whole symphony of events including interaction with scaffolding proteins, induction of signaling cascades, receptor trafficking/redistribution and protein synthesis regulated by transcription factors, which are essential for the initial expression and maintenance phases of LTP and LTD. In this section I will briefly describe the endocytosis synaptic vesicular release machinery and a related protein (synaptophysin) that is important for glutamate release and hence also for the occurrence of pre-synaptic plasticity. In addition, I will describe different glutamate receptors and their interacting scaffolding proteins that are essential for further downstream signaling, gene expression and synaptic plasticity. An overview of a glutamatergic synapse and regulatory elements is depicted in *Figure 1*.



Source, Pernilla Fagergren

Figure 1. The glutamatergic synapse

A simplified schematic of the investigated markers and their interactions. Synaptophysin I is a abundant synaptic vesicle protein in the pre-synaptic terminal. GluR1 is a subunit of the AMPA receptor that via Stargazin is linked to PSD-95, a scaffolding protein. PSD-95 is also via Shank linked to the NMDA receptor and long Homer scaffolding proteins of the group I Metabotrophic Glutamate Receptors (mGluR1/).

1.2.1.1 The synaptic vesicle release machinery

Glutamate is stored in synaptic vesicles in the pre-synaptic terminal and waits to be released into the synaptic cleft upon stimulation. The regulation of neuroexocytosis and vesicle fusion (release of molecules contained in synaptic vesicles) is mainly regulated by the SNARE complex (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor), which is composed of the three synaptic proteins: syntaxin 1A (SYX1A), synaptosome-associated protein 25 (SNAP-25) and vesicle-associated membrane protein (VAMP) (Jahn and Scheller, 2006).

1.2.1.2 Synaptophysin

Syanaptophysin was one of the first synaptic vesicle proteins to be isolated and cloned. It accounts for 8% of the total protein content in synaptic vesicles but even so its role in the lifecycle of synaptic vesicles is relatively unknown. However, recent evidence suggests a role for synaptophysin (SYP1) in synaptic vesicle recycling or endocytosis. Interestingly, SYP1 seems (together with other isoforms of SYP1: synaptogyrin) to be important for synaptic plasticity without directly affecting neurotransmitter release (Evans and Cousin, 2005; Valtorta et al., 2004). A more detailed discussion of the role of SYP1 for plasticity and addiction is included in paragraph ***4.4.1*** in relation to results obtained from our research laboratory.

1.2.1.3 Glutamate receptors, scaffolding proteins and their relevance for plasticity

Glutamatergic effects are mediated by glutamate receptors (GluRs) such as ionotropic α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), NMDA, kinate and metabatropic glutamate receptors (mGluR) (Boeckers, 2006) located either pre- or post-synaptically. According to the simplistic view ionotrophic GluRs meditate direct and fast excitatory transmission by post-synaptically located receptors, while mGluRs tune neuronal transmission at the post-synaptic level or control glutamate release from pre-synaptic terminals. However, it is now clear that ionotropic GluRs also can act pre-synaptically but instead of tuning neuronal transmission as mGluRs they probably control the strength of synaptic transmission by altering the probability of transmitter release (Pinheiro and Mulle, 2008). This section will describe these glutamate receptor families, the role of their interactions with scaffolding proteins and their contribution to pre- and post-synaptic plasticity. Focus will be on their post-synaptic activity and interaction with specific scaffolding proteins since that has been our main area of research interest (paper IV).

1.2.1.3.1 Ionotrophic glutamate receptors

The NMDA receptors are tetrameric complexes composed of multiple NR1 subunits together with at least one NR2 subtype (NR2A-NR2D) (Monyer et al., 1992; Seeburg, 1993)). The obligatory NR1 subunit is necessary for channel function (Kennedy and Manzerra, 2001; Monyer et al., 1992) while the different NR2 subunits have a C- terminal domain that anchor downstream signaling molecules (Kennedy and Manzerra, 2001). The function of the NMDA receptor strongly depends on the combination of subunits, for example switching NR2B for the NR2A subunit results in synaptic currents with shorter duration (Quinlan et al., 1999). This change in channel property has been suggested to underlie experience-dependent plasticity. The scaffolding protein post-synaptic density protein 95 (PSD-95) interact with the NMDA receptor and plays a crucial role for the functional expression after receptor activation (Kornau et al., 1995). The NMDA receptor-PSD-95 complex is required for a number of events following NMDA receptor activation, including stabilization of receptors at the plasma membrane, downstream signaling, trafficking of receptor complexes and increased activity (Kim and Sheng, 2004; Lau and Zukin, 2007; Lin et al., 2004), events that control plasticity at post-synaptic sites. Importantly, NMDA receptors are the main triggers for the induction of LTP and LTD at excitatory synapses (pre- and post synaptic). The increased Ca^{2+} influx after NMDA receptor activation triggers the active insertion or removal (during LTP or LTD respectively) of AMPA receptors (post-synaptically)

(Collingridge et al., 2004; Lau and Zukin, 2007), which has been considered as a expression mechanism for synaptic plasticity (Malinow and Malenka, 2002) .

Most AMPA receptors are tetramers composed of a combination of GluR1, 2, 3 and 4 subunits (GluR1/GluR2, or GluR2/GluR3 heterodimers) (Kornau et al., 1997). However, it has recently become apparent that GluR1 homodimers also are present and functional at synaptic terminals, specifically during synaptic plasticity (Cull-Candy et al., 2006; Liu and Zukin, 2007), in which they are thought to play an important role. The AMPA receptor can bind several scaffolding and structural proteins within the post-synaptic terminal including the Pro/SAP/Shank complex, GluR interacting protein/AMPAR binding protein (GRIP/ABP), protein interacting with C kinase 1 (PICK-1) and PSD-95 via Stargazin (Boeckers, 2006). These interactions are dependent on different AMPA receptor subunits and their relevance for AMPA receptor functions seems to differ. The interaction between the AMPA receptor subunit GluR1 and Pro/SAP/Shank structurally attach the AMPA receptor complex to the other GluR complexes (Uchino et al., 2006). GRIP/ABP interacts with the GluR2 subunit and by forming complexes with, for example, the Ephrin receptor they help to stabilize, target and transport AMPA receptors (Bruckner et al., 1999; Kim and Sheng, 2004). GluR2 and GluR3 subunits can also bind PICK-1, which has been suggested to be involved in AMPA receptor internalization (Kim et al., 2001; Perez et al., 2001). In addition PICK-1 has been proposed to release AMPA receptors from intracellular membranes in order to facilitate the reversal of LTD (Daw et al., 2000), also referred to *as de-depression*. However, one of the most interesting interactions (in my opinion) with regard to the AMPA receptor involvement in LTP is the binding of GluR1 to PSD-95 via stargazin (TARP) (Chen et al., 2000). This interaction is one way that the AMPA receptor complex can interact with the NMDA receptor complex, thereby regulating NMDA-dependent LTP (Boeckers, 2006; Collingridge et al., 2004). More specifically, Bredt and Nicoll proposed a model in 2003 where the direct interaction between AMPA and stargazing is responsible for the trafficking of AMPA receptor to the plasma membrane while the PSD-95-Stargazin complex recruits AMPA receptors from the extra-synaptic sites to synapses during synaptic plasticity (Bredt and Nicoll, 2003). In 2004, Ehrilch *et al* reported that PSD-95 is required for the control of GluR1 incorporation during experience-driven synaptic plasticity (Ehrlich and Malinow, 2004), supporting the essential role for the PSD-95-AMPA receptor interaction for synaptic plasticity.

1.2.1.3.2 Metabotrophic glutamate receptors

mGluRs are G-protein coupled receptors that are divided into three groups (group I-III) depending on their sequence homology, transduction mechanisms and pharmacology (Anwyl, 1999; Conn and Pin, 1997). Group I mGluR comprises mGluR1 and mGluR5. They are linked to G-proteins of the Gq type and are primarily expressed post-synaptically (Conn and Pin, 1997). Conversely, group II mGluR (mGluR2 and mGluR3) are coupled to inhibitory Gi/o proteins and are located pre- and post-synaptically (Pin and Acher, 2002), whereas group III mGluRs (mGluR4, mGluR6, mGluR7 and mGluR8) interact with Gi/o proteins in the pre-synaptic terminal and thus play an important role for neurotransmitter release (Schoepp, 2001). However, the localization and composition of mGluR differs between brain regions. In the rat striatum for example, group I mGluRs have been identified post-synaptically, whereas both group II and III have only been recorded at a pre-synaptic level on excitatory terminals (Gubellini et al., 2004). This observation will be important for the results presented in this thesis.

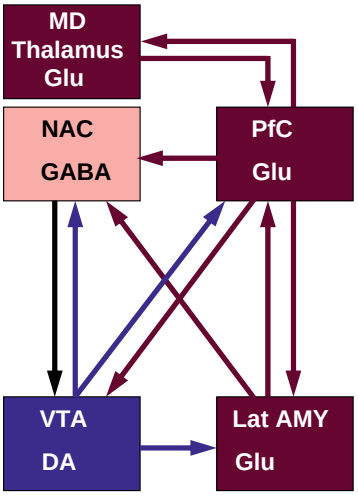
At post-synaptic sites mGluRs form complexes with Homer proteins (Homer 1-3) (Brakeman et al., 1997) and regulate downstream signaling, synaptic activity and surface clustering of mGluR [see (Boeckers, 2006; Duncan et al., 2005; Kammermeier, 2006). Homer is also tightly linked to the NMDA receptor complex via interactions with a trimeric Shank–GKAP–PSD-95 complex (Naisbitt et al., 1999; Thomas, 2002; Tu et al., 1999), thereby providing a possibility to regulate NMDA receptor activity (Pisani et al., 2001). Supporting this hypothesis, it has been reported that NMDA-dependent corticostriatal LTP can be blocked by inhibiting group 1 mGluRs (mGluR1 and mGluR5 dependent.

1.3 GLUTAMATE-DEPENDENT PLASTICITY IN DRUG ABUSE

Emerging evidence supports the notion that addictive drugs elicit or modify synaptic plasticity in key brain circuits (see detailed description below and *Figure 2*) involved in addiction and that these synaptic alterations have important behavioral consequences. Alterations at multiple levels have been reported, including behavior, LTP/LTD as well as molecular changes in pre- and post-synaptic terminals and gene expression corresponding to changes in plasticity (Hyman et al., 2006; Kalivas et al., 2008; Kalivas and O'Brien, 2008; Kalivas et al., 2005; Kauer and Malenka, 2007). Results presented within this thesis will describe alterations in these systems in relation to alcohol, as well as heroin and cocaine use. The next sections will therefore describe molecular correlates of pre- and post-synaptic plasticity in relation to drug abuse with emphasis on the glutamatergic system. But first a very brief description of the circuitry relevant for drug-induced synaptic plasticity.

1.3.1 Glutamatergic circuitry relevant for the development and persistence of addiction.

Glutamate is the most common excitatory neurotransmitter in the human brain, but only certain glutamatergic projections seem to be specifically targeted by drugs of abuse and contribute to the development and persistence of addiction (*Figure 2*). These include glutamatergic afferents from the prefrontal cortex, amygdala and hippocampus to the nucleus accumbens (Pierce and Kalivas, 1997a; Rogers and See, 2007; See et al., 2003). Moreover, recent reports also suggest that glutamatergic projections to the dorsal striatum (caudate nucleus and putamen) play an important role for maintaining a compulsive drug intake (For details see paragraph *1.1.2* in which the theoretic model 'pathology of staged neuroplasticity' by Kalivas and O´Brien is described). Furthermore, glutmatergic projections to the ventral tegmental area have been proposed to play an important role for the development of addiction (Pierce and Kalivas, 1997a; Vezina, 2004), although it is still unclear which glutamatergic afferents that are critical. Projections from the prefrontal cortex, bed nucleus of stria terminalis and penunculopontine region are important candidates (Carr and Sesack, 2000; Charara et al., 1996; Georges and Aston-Jones, 2002). Importantly, bidirectional glutamate projections are present between the amygdala and the prefrontal cortex (Ghashghaei et al., 2007).



Source, Pernilla Fagergren

Figure 2. Glutamatergic interactions of the reward pathway

MD, mediodorsal; NAc, Nucleus Accumbens; PFC, Prefrontal Cortex; VTA, Ventral Tegmental Area; Lat Amy, Lateral Amygdala; Glu, glutamate; GABA, γ-aminobutyric acid; DA, dopamine

1.3.2 Pre-synaptic plasticiy: relevance for drug abuse

Glutamate release is altered by alcohol as well as by cocaine and opiates (LaLumiere and Kalivas, 2008; McFarland et al., 2003; Ohi et al., 2007; Roberto et al., 2006; Siggins et al., 2005; Xiao et al., 2008). The effects on release vary with drug and region analyzed, but it is clear that pre-synaptic events play a significant role in drug-induced plasticity and its behavioral consequences, even though post-synaptic events are probably as essential. The current literature concerning pre-synaptic alterations is minimal compared to the information regarding post-synaptic plasticity and molecular events underlying the disturbance of synaptic strength in relation to addiction. Moreover, the specific mechanisms through which glutamate release is altered following administration of drugs in a particular brain region are not fully understood. However, pre-synaptic receptors, including dopamine and glutamate receptors have been implicated as well as the synaptic vesicular machinery and related factors (Deng et al., 2008; Xiao et al., 2008; Xie and Steketee, 2008a). In particular, dopamine-glutamate interactions have received increasing attention during recent years, and hence, it should be emphasized that plasticity arising from these interactions is probably crucial for addiction-related plasticity and alterations in neuronal circuitry, although I will not elaborate on dopamine-induced plasticity within this thesis.

A few studies have been implicated pre-synaptic glutamate receptors as being important regulators of pre-synaptic plasticity and alterations in glutamate release. For example, altering pre-synaptic mGluR2/3 receptor function in the medial PFC attenuates cocaine-induced sensitization in rats (Xie and Steketee, 2008a) and is correlated with modulation of glutamate transmission via reduced mGluR2/3 function (Xie and Steketee, 2008b). In addition, alterations in mGluR 2/3 induced LTD have also been observed in the nucleus accumbens following one-week withdrawal from mice chronically treated with morphine (Robbe et al., 2002). Several

studies have also reported alterations in the glutamate release machinery, e.g members of the SNARE complex and related factors, after morphine (SNAP-25) (Xu et al., 2004), cocaine (synaptotagmin4) (Courtin et al., 2006) and amphetamine (SYP1) treatment (Ujike et al., 2002). Last but not least, a few studies have also linked SYP1 to drug-induced behaviors (Rademacher et al., 2006; Rademacher et al., 2007). SYP1 appears to be involved in determination of synaptic strength without directly affecting basal glutamate release (Janz et al., 1999; McMahon et al., 1996). Such a factor could be partly responsible for the increase in synaptic strength in the PFC that results in increased glutamate release during craving and relapse following drug administration/intake. We therefore set out to evaluate whether SYP1 levels were altered in the human PFC of alcoholics (paper III). Detailed descriptions of our findings and a discussion are provided in paragraph *4.4.1*.

1.3.3 Post-synaptic plasticity: relevance for drug abuse.

A large body of evidence supports the involvement of post-synaptically elicited drug-induced plasticity in key brain circuits (see paragraph 1.3.1). For example, post-synaptically induced excitatory enhancement has been observed in the ventral tegmental area following alcohol, morphine, cocaine and amphetamine treatment, respectively (Argilli et al., 2008; Borgland et al., 2004; Saal et al., 2003; Stuber et al., 2008; Ungless et al., 2001). Moreover, both LTP-like and LTD-like effects (Kauer and Malenka, 2007) as well as abolished LTD have been observed in the nucleus accumbens (Martin et al., 2006) following drug administration. The contradicting results in the nucleus accumbens are probably related to differences in drug-administration paradigms, time analyzed (e.g. acute, chronic, withdrawal) or other methodological factors. However, it is clear that drugs affect post-synaptically-induced plasticity in both ventral tegmental area and the nucleus accumbens. Less is known about drug-induced plasticity in the PFC and amygdala, although a few studies have provided initial insights. Huang *et al* reported increased LTP in the medial PFC of rats repeatedly treated with cocaine (Huang and Kandel, 2007). Aberrant synaptic strength has also been reported in the amygdala following cocaine and alcohol withdrawal in rodents (Fu et al., 2007; Stephens et al., 2005).

Alterations in synaptic strength have been coupled to drug-induced behavioral effects and morphological alterations in spine density (Kauer and Malenka, 2007; Robinson and Kolb, 2004). Spine density is generally considered to increase during LTP and to be highly dependent on the dynamic organization of and scaffolding properties of its post-synaptic density (Segal, 2005). Most classes of addictive drugs effect spine density in reward-related brain circuits, yet the effect differs between drug and region analyzed. Cocaine increases spine density in the nucleus accumbens, whereas heroin and alcohol decrease spine density in the same brain region (Robinson and Kolb, 1999; Zhou et al., 2007). However, ethanol treatment of hippocampal cultures increases spine density (Carpenter-Hyland and Chandler, 2006).

Several studies using gene-deleted or transgenic mouse models have contributed to our current understanding of addiction disorders and have identified AMPA-, NMDA receptors and the scaffolding proteins PSD-95 and Homer has as being important contributors to glutamate plasticity and the development and persistence of addiction. A recent study by Engblom *et al* demonstrated that the NMDA NR1 subunit and the GluR1 are essential for cocaine drug-seeking behavior (Engblom et al., 2008). Furthermore, targeted deletion of PSD-95 augments the acute locomotor effects produced by cocaine (Yao et al., 2004). Many studies have also been

conducted evaluating the role of Homer proteins in addiction disorders (Szumlinski et al., 2008). For example, Homer1- and Homer2-gene deleted mice exhibit enhanced cocaine-induced place conditioning and cocaine-induced locomotor activity (Szumlinski et al., 2004). Furthermore, over-expression of long Homer isoforms in the nucleus accumbens abolishes cocaine-induced sensitization of locomotor hyperacitvity and prevents development of glutamate abnormalities normally elicited by cocaine (Szumlinski et al., 2006). Furthermore, mice lacking the gene encoding protein Homer2 exhibit a reduced preference for ethanol, an absence of conditional place preference and an absence of sensitization to the locomotor stimulant effects of ethanol (Szumlinski et al., 2005).

The majority of studies described above have been performed using various animal models following cocaine, morphine or alcohol administration, respectively. Many features of animal models do not mimic the complexity of human drug abuse, including the chronicity of drug use. Thus many critical questions remain to be answered as to the glutamatergic pathophysiology in human addiction disorders. Initial insights were obtained by Hemby *et al*, who demonstrated alterations in glutamatergic receptors (e.g. NMDA receptor subunits NR1 and AMPA receptor subunit GluR 2/3) in the nucleus accumbens of human cocaine overdose victims. A question thus asked in this thesis was whether dysregulation of markers of synaptic plasticity is also evident in opiate users, and thus may be a common feature of drugs of abuse, as suggested by some animal models (paper IV, paragraph 4.4.2)

1.4 THE NF-KB SYSTEM AND ITS ROLE IN GENE EXPRESSION AND PLASTICITY

The previous section was devoted to the description of synaptic plasticity and its main regulators. This section will instead focus on the end result of the downstream signaling events following glutamate activation, namely the activation of transcription factors and the induction of gene transcription. However, this section will not focus on the best characterized transcription factors in regard to plasticity, the cAMP response element binding (CREB) and the Fos family of transcription factors, but instead on a potentially unique transcription factor family, at least in so far as to its involvement in plasticity, the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) family of transcription factors.

1.4.1 NF-κB system

The transcription factor NF-κB was identified 23 years ago (Sen and Baltimore, 1986) as a nuclear factor that binds the κ light chain enhancer in B-cells (and hence, the name NF-κB). It has thereafter received tremendous attention for its central role in the immune system, and cancer (Packham, 2008). However, more recently it has become apparent that the NF-κB system not only is important for immune responses but also for cellular processes in other systems such as the nervous system, in which, it participates in the regulation of synaptic plasticity, myelination, cell survival/death and inflammation (Mattson, 2005; Memet, 2006). The potential relevance to synaptic plasticity made it of interest for our human post-mortem studies.

In mammals, the NF-κB/Rel family of transcription factors comprises five members, p65 (RelA), Rel-B, c-Rel, p50 and p52, which share a Rel homology domain allowing DNA-binding, dimerization and nuclear localization (Baeuerle and Henkel, 1994). The Rel-B, p65 (Rel-A) and c-Rel also contain a transactivation domain which is absent in the p52 and p50 subunits [Figure 1, (Tergaonkar, 2006). Of the various dimeric combinations, p65-p50 (NF-κB) is most common.

Binding of most NF-κB complexes to motifs in target promoters assists transcription, but homodimeric complexes of p50 or p52 can repress it (Guan et al., 2005; Li et al., 1994; Moynagh, 2005). NF-κB and Rel dimers are retained inactive in the cytoplasm by interacting with inhibitory molecules- called IκBs. The IκB family is composed of IκBα, IκBβ, IκBε, IκBγ, IκBζ, Bcl-3 and the precursors of p50 and p52, p105 and p100, respectively. NF-κB is induced by multiple extracellular stimuli (described in detail in the next paragraph) that trigger activation of an IκB kinase (IKK) complex, which phosphorylates the IκBs leading to their ubiquitination and proteasomal degradation. The released NF-κB migrates to the nucleus, binds to -κB binding sites with consensus sequence GGGRNNYYCC (N = any base, R = purine, Y = pyrimidine) in a target gene and activates transcription. The IKK complex contains the two kinases IKKα and IKKβ and the regulatory subunit NEMO/IKKγ and functions as integrator of signals thereby regulating NF-κB activity [Reviwed in (Ghosh and Karin, 2002; Moynagh, 2005; Whiteside and Israel, 1997).

The NF-κB transactivation capacity (capacity to increase gene expression) can be regulated at multiple levels by several factors. Most studies have focused on post-translational modifications such as phosphorylation or acetylation of the p65 subunit [Reviwed in (Schmitz et al., 2004), although a few studies have also demonstrated that phosphorylation of the p50 subunit is important for its DNA-binding activity (Guan et al., 2005; Li et al., 1994). p65 is phosphorylated by several kinases including protein kinase A (PKA), protein kinase C (PKC) and the IKK complex. Acetylation or deacetylation of p65 by CBP/p300 and histone deacetylases, respectively, have also been observed (Schmitz et al., 2004). The phosphorylation of many of these sites is associated with an increase in the transcriptional activity of p65, as is acetylation by CBP/p300. Conversely, deacetylation by histone deacetylases leads to repression of transactivation and also termination of NF-κB activation by increasing the affinity of NF-κB for IκBα (Moynagh, 2005). Interestingly, the p50 homodimer recruits co-repressor complexes containing histone deacetylaces that are removed by IKKα, (Hoberg et al., 2006; Hoberg et al., 2004) and thus this has been one of the proposed hypotheses underlying p50 homodimer repression. Another hypothesis states that p50 homodimers compete for binding to NF-κB sites. However, since the p50 homodimer has a weaker affinity for –κB sites then does NF-κB this scenario seems less plausible.

1.4.2 NF-κB in the nervous system

NF-κB participates in a number of cellular processes within the central nervous system. For example, it regulates synaptic plasticity, myelination, cell survival/death and inflammation. I order to do so NF-κB is present and functional in neurons as well as in glia and oligodendrocytes. Most research has been conducted on NF-κB within neurons and glial cells, but a few studies have demonstrated that NF-κB is important for myelination regulated by oligodendrocytes. In the brain the p65/p50 heterodimers and p50/p50 homodimer are the most common, together with the inhibitor IκBα. However, c-Rel-containing complexes are also present (Mattson, 2005; Memet,

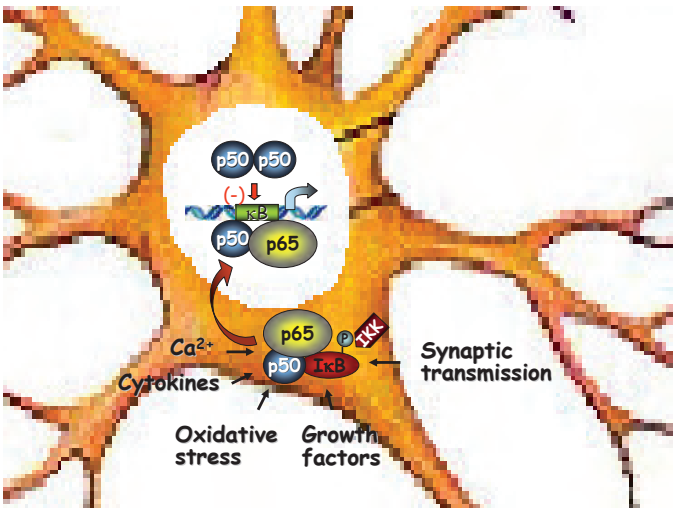
2006). These members of the NF-κB family occur in both neuronal and glial cells, yet NF-κB ligands as well as its activity patterns differ between the two cell types. For example, while glutamate, cell depolarization and Ca^{2+} exclusively induce neuronal NF-κB, NF-κB is activated by inflammatory mediators (e.g IL-1 and TNF), growth factors and oxidative stress in both neurons and glia (Kaltschmidt et al., 2005). Furthermore, NF-κB is constitutively active in neurons (i.e. in the nucleus free of IκB and capable of binding DNA) (O'Neill and Kaltschmidt, 1997), but present in a latent form in glial cells (Moynagh et al., 1993). These differences concord with the current literature, indicating specific roles for NF-κB in glial and neuronal cells, where NF-κB mainly is involved in inflammatory processes in glial cells while neuronal NF-κB seems to be important for a wider range of cellular processes. The neuronal-specific NF-κB functions related to its constitutive activity are probably linked to its regulation by synaptic transmission, as they can be repressed by inhibitors of action potential generation, glutamate receptors (e.g. mGluR5, NMDA) and L-type calcium channels, respectively.

As described previously, NF-κB is constitutively active in the nuclear compartment in neurons, but surprisingly the p65-p50 heterodimer is also present in its inactive form in synaptic terminals (Meffert et al., 2003). These complexes can be activated through synaptic transmission, Ca^{2+}, growth factors, oxidative stress and cytokines or retrograde transported to the nucleus (Meffert et al., 2003; Wellmann et al., 2001) (*Figure 3*). Hence it has been proposed that NF-κB serves dual functions within neurons: it acts as a signal transducer transmitting information from the active synapse to the nucleus, and it acts as a transcriptional regulator when it reaches the nucleus. As with other transcription factors, NF-κB regulates gene expression. In the immune system an enormous number of NF-κB target genes have been discovered (Pahl, 1999), although the knowledge about which genes are regulated by NF-κB in the nervous system is scanty and mainly relies on extrapolation of genes identified in the immune system. Nonetheless, some genes have been identified including: μ-opiod receptor, protein kinase A catalytic α subunit N-CAM, inducible nitric oxide synthase (NOS-II) amygdaloid precursor protein (APP), β secretase, brain derived neurotrophic factor (BDNF), inducible cycloxygenase-2 (COX-2), and calcium/calmodulin-dependent protein kinase II δ (Memet, 2006). During recent years the NF-κB family of transcription factors has also received increasing attention for its role in learning and memory processes. The next paragraph will briefly summarize some relevant findings that have emerged.

1.4.3 NF-κB in synaptic plasticity

Evidence has accumulated for a role of NF-κB in synaptic signaling and transcriptional regulation required for synaptic plasticity. For example, -κB decoy blocks LTP induction in the hippocampus and amygdala (Albensi and Mattson, 2000; Yeh et al., 2006) and transgenic animal models reveal alterations in learning as well as deficits in synaptic plasticity (discussed below). Both pre- and post-synaptic mechanisms have been proposed to play roles for NF-κB- induced plasticity, yet very limited information exists and detailed studies are needed in order to clarify the importance and specificities of these events (Kaltschmidt et al., 2005) (Kaltschmidt et al., 1993; Sulejczak and Skup, 2000). Even though the mechanisms underlying its role in synaptic plasticity are unclear, NF-κB is clearly important for learning and memory processes. Significant work has been performed evaluating transgenic mouse models of p65, p50, c-Rel. Several of these models exhibit profound changes in cell survival/apoptosis and partly in myelin-related processes. In addition, they show alterations in learning and memory. $p65^{-/-}$ on a $TNRF^{-/-}$

background yielded deficits in spatial memory formation in the radial arm maze (Meffert et al., 2003). p50$^{-/-}$ mice have disturbances in short-term spatial memory (Denis-Donini et al., 2008) and in the manifestation of emotional behavior (Kassed and Herkenham, 2004), while impairments in contextual fear and passive avoidance memory have been reported in c-Rel$^{-/-}$ deficient mice (Levenson et al., 2004; O'Riordan et al., 2006). In addition, when NF-κB was inhibited by inducing expression of transdominant negative IκB, animals also showed modulation of learning and memory (Fridmacher et al., 2003). A follow-up study by Kaltschmit *et al* investigating these animals revealed a specific alteration in spatial memory formation coupled to impairments in protein synthesis-dependent late phase LTP and LTD. Furthermore, NF-κB controlled spatial memory formation through activation of PKA and CREB activation in these animals (Kaltschmidt et al., 2006).



Source. Georgy Bakalkin

Figure 3. Regulation of synaptic NF-κB in neurons

The NF-κB (p65/p50) complex is present at synaptic terminals in its inactive form bound to the inhibitory protein IκB. Upon stimulation by, for example, through synaptic transmission, Ca2+, growth factors, oxidative stress or cytokines, IKK phosphorylates IκB that is targeted for degradation. The active NF-κB is retrogradely transported back to the nucleus where it can act as a transcription factor. p50 homodimers present in the nucleus act as repressors of NF-κB transcriptional activity.

1.4.4 NF-κB: relevance for drug abuse

Although increasing evidence indicates an important role for NF-κB in learning and memory processes (Kaltschmidt et al., 2005), and addiction disorders it is related to disturbances in plasticity (Kalivas and O'Brien, 2008; Kauer and Malenka, 2007), only a few studies have evaluated the role of NF-κB in relation to addiction. For example, cocaine administration in rats has been reported to increase the levels of the NF-κB family members, p105, p65 and IκB in the

nucleus accumbens (Ang et al., 2001). Furthermore, a recent study by Russo *et al* determined that chronic cocaine administration in mice up-regulates NF-κB activity in the nucleus accumbens (Russo et al., 2008). Several studies have also evaluated NF-κB activity in relation to ethanol treatment, and acute as well as chronic ethanol administration has been reported to alter NF-κB activity. However, it is still not known whether chronic alcohol consumption in humans affects the NF-κB system in the brain. We therefore investigated the NF-κB system in the brains of alcoholics (section *4.3.1*).

2 AIMS OF THE STUDY

A growing body of evidence indicates that synaptic plasticity is essential for the development and persistence of addiction, however, most research have focused on animal models and very limited knowledge exists to the effects of drugs of abuse on the these systems in the human brain. The aim of this thesis was therefore to gain deeper insight to how drugs of abuse, including alcohol, heroin and cocaine affect common neurobiological mechanisms connected to synaptic plasticity in key neuronal circuits relevant for the development and persistence of addiction.

Our main questions were:

First, is the transcription factor NF-kB, which recently has been suggested to play a role for synaptic plasticity, affected by alcohol consumption? Second, is there any evidence of pre-synaptic alterations in alcoholics that may explain changes in synaptic efficacy but not alter basal glutamate release? Third, is there evidence of post-synaptic rearrangements/alterations in heroin, cocaine or polysubstance (heroin-cocaine) users that could underlie synaptic plasticity?

3 MATERIALS

In this section I will describe the human post-mortem tissues that have been used in order to study the molecular and biochemical alterations in the brains of alcohol, heroin and polysubstance users. Additional material or the methods will not be discussed in this section since they are conventional techniques that are fully provided in the individual articles. However, a paragraph within the section entitled "Present investigations in perspective to the current literature" will describe the limitations as well as the process of working with molecular and biochemical techniques when studying the human post- mortem brain.

3.1 HUMAN SUBJECTS

3.1.1 Human alcoholics (papers I – III)

Human post-mortem brain tissues, from alcoholics and controls (paper I-III) were obtained from the New South Wales Tissue resource center (TRC), University of Sidney, Australia (Harper et al., 2003; Harper and Matsumoto, 2005). The TRC is an established brain bank that can provide researchers worldwide with post-mortem brain material from uncomplicated or complicated (e.g. liver cirrosis, Wernicke- Korsakoff syndrom) alcohol-dependent subjects. The tissue can be obtained both as fresh-frozen punctures or paraffin-embedded brain sections from brain regions of interest. The alcohol-dependent individuals used for biochemical evaluation in this thesis met the DSM–VI criteria and also the National Health and Medical Research Council (NHMRC/ World health organization criteria as individuals who consumed greater than 80g of ethanol per day for the majority of their adult lives, with one exception who consumed on average 60g alcohol per day. Controls had either abstained from alcohol completely or were social drinkers who consumed less then 20g of ethanol per day with one exception that consumed 35g ethanol per day. In addition to the DSM IV and NHMRC/WHO criteria the subjects also met stringent selection criteria defined by the TRC, presented below.

- No history of abuse to other drugs
- Not diagnosed with Wernicke-Korsakoff syndrome
- No history of development disorders
- No history of psychiatric or neurological disorders
- No history of cerebral infarction, head injury or neurodegenerative disease (e.g. Alzheimer´s disease)
- Above 18 years of age
- Negative screen for HIV and Hepatitis B/C
- No prolonged agonal life support
- No obvious brain abnormalities on gross examination

All individuals, alcoholics and controls, were male Caucasians who were matched group-wise with regard to sex, age, race and post-mortem interval (PMI). The general characteristics for the control (n = 15) and alcohol (n = 15) specimens are summarized in Table 2. For detailed demographic information see Table 1 in paper II. The studies were preformed on brain tissues from the prefrontal cortex (superior frontal gyrus, brodmann area 9) and motor cortex (brodmann area 4). Brain specimens were secured by a qualified pathologist under full ethical clearance

from the Sidney South West Area Health Service Human Ethics Committee (X03-01117) and informed written consent from the next of kin. The study was approved by the Ethics Committee at Karolinska Institutet (permit #04-849/4).

Table 2. Demographic data of alcohol population used for analyses.

Group	Controls	Alcoholics
Number	15	15
Age, year	58.80 ± 14.5 (34-82)	58.87 ± 14.0 (34-81)
Gender	Male	Male
PMI, hours	26.9 ± 16.4 (6.5-50)	31.8 ± 15.1 (8.5-60)
pH	6.46 ± 0.29 (5.88 – 6.9)	6.47 ± 0.27 (5.66 – 6.78)
Ethanol (blood)	n = 0	n = 2

Values presented as mean ± SD and range are shown in parentheses. PMI, post-mortem

3.1.2 Human heroin abusers (paper IV)

Human post-mortem brain tissues from heroin overdose and normal control Caucasian subjects (paper IV) were collected at the Department of Forensic Medicine at Semmelweis University, Hungary, as well as from the National Institute of Forensic Medicine, Karolinska Institutet, Stockholm, Sweden. Subjects included in the heroin group died from heroin overdose, which was verified by toxicology and they had physical signs of heroin use such as needle track marks. Most of the included subjects had a history of heroin abuse, which was determined after collecting information from police reports as well as from family interviews and medical records when available. The heroin group represented a unique drug abuse population, as they were predominant heroin users receiving no methadone treatment. Toxicological evaluation also revealed that the morphine/codeine concentration ratio was above 1, indicating heroin usage rather than use of codeine medication (Ceder and Jones, 2001). The control group had negative toxicology for opiates and other drugs of abuse except alcohol. All cases were assessed for common drugs of abuse (including alcohol) and also for therapeutic agents. The general characteristics and inclusion criteria for control (n = 19) and heroin (n = 35) subjects are described in Table 3. For detailed demographic information see Table 2 in paper IV. The studies were preformed on brain tissues from the amygdala and striatum (middle-rostral level of the striatum, where the nucleus accumbens is dissociated into shell and core subdivisions). Brain specimens were collected at autopsy within 24 hrs after death under the guidelines approved by the Semmelweis University Human Ethical Committee and the Ethics Committee at Karolinska Institutet and the Swedish Board of Social Welfare (permit #02-370), respectively.

3.1.3 Human polysubstance users (paper IV)

Post-mortem brain specimens from heroin, cocaine and heroin-cocaine polysubstance users as

well as normal control subjects (paper IV) were collected as part of the routine autopsy process according to a protocol approved by Wayne State University's Human Investigation Committee, Detroit, MI, USA. Heroin, cocaine and cocaine-heroin users exhibited a positive toxicology for heroin, cocaine, and combination of cocaine and heroin and/or their metabolites. Heroin abuse subjects tested negative for cocaine and cocaine abusers tested negative for opiates. Control subjects tested negative for opiates and cocaine. All subjects tested negative for other common drugs of abuse including barbiturates, benzodiazepines, and phencyclidine

Table 3. Demographic data of the heroin abuse population.

Group	**Control**	**Heroin**
Number	N = 19	N = 35
Age (yr)	36 ± 12 (15-58)	27 ± 5 (20-46)
Gender	M = 16 F = 3	M = 30 F = 5
PMI (h)	21.37 ± 6.40	23.46 ± 1.93
pH	6.73 ± 0.21 (6.15-6.98)	6.54 ± 0.20 (6.07– 6.85)
Ethanol (blood)	n = 2	n = 5
Drug (ug/ml blood)		0.39 ± 0.46
Inclusion criteria	Negative toxicology of opiate or other drug No history of opiate abuse No history of abuse to other drugs No physical body needle tracks No history of psychiatric disorder	Positive opiate toxicology History of opiate abuse No history of abuse to other drugs Physical body needle tracks

Values presented as mean ± SD, range in parenthesis. yr, year; M, male; F, female; PMI, postmortem interval; h, hours, OD, overdose

A few subjects tested positive for alcohol but did not exhibit common signs of chronic alcohol abuse such as liver pathology. The drug abuse populations were mixed with regard to race and sex. The general characteristics for control (n = 6), heroin (n = 5), cocaine (n = 6) and heroin-cocaine (n = 7) subjects are described in Table 4. Analyses were preformed on fresh-frozen brain sections from the amygdala and striatum. Brain specimen storage and experiments have been conducted in the United States under local ethical approvals. No Swedish ethical permit is therefore required.

Table 4. Demographic data of the heroin, cocaine and heroin-cocaine polysubstance abuse population

Group	**N**	**Age** year	**PMI hours**	**Race** B/W	**Gender** M/F	**Alcohol** tox	**Drug** μg/ml blood	**Brain pH**
Control	6	46 ± 3.3		3/3	6/0	3	-	6.82 ± 0.06
Heroin	5	40 ± 4.5		3/2	4/1	3	0.48 ± 0.28	6.59 ± 0.04
Cocaine	6	45 ± 4.1		4/2	6	0	2.16 ± 0.83	6.60 ± 0.12
Heroin-Cocaine	7	45 ± 2.7		3/4	5/2	2	0.28 ± 0.11 0.31 ± 0.15	6.64 ± 0.10

Values presented as mean ± SD, range in parenthesis. PMI, post-mortem interval; M, male; F, female; B, Black; W, White: tox, toxicology.

4 PRESENT INVESTIGATIONS IN PERSPECTIVE OF CURRENT LITERATURE

4.1 TECHNICAL CONSIDERATIONS FOR POST-MORTEM HUMAN BRAIN STUDIES.

The aim of my thesis work was to gain deeper insight into how the human brain is affected by drugs of abuse. In order to answer this question we chose to study human post-mortem brain specimens, specifically brain regions with relevance for addiction disorders. Brain tissues were collected from several sources, including: The Tissue Resource Center, University of Sidney, Australia (alcoholics); Department of Forensic Medicine at Semmelweis University, Hungary and National Institute of Forensic Medicine, Karolinska Institutet, Stockholm, Sweden (heroin abuse population); and Wayne State University, Detroit, MI, USA (heroin and cocaine polysubstance users). For detailed descriptions see *Materials* (paragraph three). Using human post-mortem tissues to analyze a large number of samples requires careful planning and execution of experiments as well as knowledge about the limitations of techniques and the material itself. In this section, I will address limitations and obstacles as well as ways in which we tried to minimize experimental errors when studying post-mortem human brain materials. The techniques most frequently used in our lab that will be discussed include real-time poly chain reaction (RT-PCR), *in situ* hybridization histochemistry, western blot analysis and electrophoretic mobility shift assay.

The quality of the starting material is essential for producing reliable results. Collection of human post-mortem material is an involved process with often long post-mortem interval, thus partial degradation of the tissue may render difficulties with accurate detection of RNA, protein or post-translational modifications. We are therefore extremely careful handling the tissue subsequent to initial collection in order to minimize additional degradation. In addition, we assessed the RNA quality before conducting RT-PCR and used preventive measures such as phosphatase and protease inhibitors when extracting proteins in order to minimize additional protein degradation. Evaluation of RNA revealed sharp 28S and 18S bands with only a few samples that exhibited some degree of smearing, indicating partial RNA degradation. These samples were not used for RT-PCR. Western blot analysis did not reveal additional bands or smears following antibody detection, suggesting that protein degradation was not a major issue for the markers studied.

Compared to using animal models for biochemical evaluation, human post-mortem studies require larger sample size in order to achieve the statistical power necessary to detect relevant changes in markers analyzed. This can be explained by the increased variability due to, for example, life style and genetic factors. The results presented in this thesis have generally been conducted using around 30-50 samples (excluding paper I). In using this number of samples, we have evaluated what methods that would be most suitable for analysis (mRNA: Low density array) or used ways to overcome technical issues when such has arisen (Protein: western blot).

We were early interested in evaluating a number of genes with RT-PCR (not all results form those studies are included in this thesis). However, evaluating 60 samples (2 brain regions 30 saples/region) and 47 genes would have been both time-consuming and expensive. Therefore we decided to use custom-made Taq-man low-density arrays from Applied Biosystems, which can analyze many genes at the same time using a small amount of starting material.

The majority of our results are based on work using semi-quantitative western blot analysis. There are gel systems for western blot that can analyze 30-50 samples/run, however, the quality of such big-scaled immunoblots suffers. We therefore run several gels, but with fewer samples per gel. This requires that, gel-gel differences have to be accounted for in order to quantify and compare samples on different gels. Reference samples that constitute a mixture of control samples (paper II and III) or one representative control sample (paper IV) are loaded into each gel to serve as an internal control. Following antibody detection the immunoreactivity for a given protein and sample is compared with the reference sample and a ratio is calculated. The calculated ratios can then be used for further analyses.

4.2 ENDOGENOUS CONTROLS OF THE HUMAN BRAIN AND DRUG ABUSE

We determined the most suitable endogenous controls for studying PFC and MC tissue from alcoholic and control subjects

The study of mRNA is a central theme of my thesis work. Few studies have evaluated which endogenous control genes can be utilized for optimal normalization of mRNA levels. Most studies to date have selected endogenous control genes based on previously published data. This approach may create unreliable results since a number of factors including brain region-specific functions and drug use could alter the activity of so called "housekeeping" genes. For example, transferrin receptor (*TFRC*), a frequently used "housekeeping gene", has been reported to be differentially expressed in alcoholics (Liu et al., 2004). In paper I, we therefore evaluated 16 genes frequently used as endogenous controls in gene expression studies to determine which genes were most stably expressed and could be used as reference genes in our subsequent mRNA expression studies. We were especially interested in evaluating whether there were differences between brain regions in terms of stability of markers and if any of the endogenous controls were differentially expressed between the drug abuse and control groups. These were important issues to address since our subsequent study encompassed post-mortem brain specimens from both alcoholics and controls and the specimens were derived specifically from the prefrontal cortex (PFC; brodmann area 9) and motor cortex (MC; brodmann area 4).

4.2.1 Using geNORM to evaluate stability of endogenous controls

To perform the analysis we used a TaqMan® Low Density Endogenous Control Panel, and a mathematical tool called geNORM (Vandesompele et al., 2002), which calculates the geometrical mean, a normalization factor based on the most stably expressed genes. One advantage using geNORM for selection of endogenous control genes is that it is not sensitive to the amount of cDNA used in the real-time amplification. Other analysis methods are usually dependent on equal cDNA input for optimal selection of genes. However, equal cDNA input is difficult to achieve due to inaccurate or insensitive technical procedures prior to the real time amplification. geNORM avoids the problem with equal cDNA input by calculating the expression ratio across samples. The genes with the lowest expression ratio across samples are the most stable and are represented with a low M-rank value (See Figure I A/B in paper I). In other words, the M-rank represents the relationship in stability between genes analyzed: the lower the M-rank the more stable are the genes. In addition to selecting the most stable "housekeeping genes" geNORM also determines the minimum number of endogenous control

genes required for an accurate normalization. Adding genes generally increases the accuracy of normalization since the expression ratio comes closer to the true mean value. This is represented as decreased V-values in the pair-wise variation plot (Figure 1 C/D, paper I). However, if the expression of genes is stable (low M values), two genes may be enough to perform a reliable normalization. Moreover, if many genes show stable expression it is likely that several genes and/or combinations that do not necessarily have the lowest M-rank value are suitable for normalization.

4.2.2 Brain region specificity

Our results demonstrate that beta-actin (*ACTB*) and ribosomal large P0 (*RPLP0*) were the most stably expressed genes in the PFC, while importin 8 (*IPO8*) and RNA polymerase II (*POLR2A*) were most stable in MC. The 'stability' of these genes was sufficient to ensure accurate and stable quantitation and they could therefore serve as good candidate reference genes (See Vandesompele, cut off: M = 0.15). However, the analyses indicated that different genes should be used for PFC and MC specimens, respectively, in order to obtain the most optimal results. There are no clear explanations for these observations, but it is likely that brain-specific expression patterns or environmental factors alone or in combination may influence the gene expression stability, possibly in a brain region-specific way. For example, Olsen *et al* reported distinct gene expression profiles in the nucleus accumbens and dorsal striatum, demonstrating the occurrence of regional gene expression differences in even closely related brain structures (Olsen et al., 2008). In addition, a microarray study performed by Flatsher-Balden evaluated the expression profile between alcoholics and controls in three brain regions and demonstrated that none of the differentially expressed genes in the ventral tegmental, nucleus accumbens and PFC, overlapped when compared (Flatscher-Bader et al., 2005). These results suggest that environmental factors such as alcohol intake exert specific effects in different brain regions. However, the functional expression profile for ventral tegmental area and nucleus accumbens were similar while the PFC exhibited a unique expression pattern. Thus brain structures that share functional properties may be affected in a more similar fashion by environmental factors then are functionally distant structures. Taken together, brain region-specific functions and environmental factors alone or in combination can affect the expression pattern of genes, thereby providing a possible explanation for the regional specific differences observed between the PFC and MC.

4.2.3 Drug-induced changes

In order to examine if any alcohol-related differences in gene expression were evident we used the normalization factor calculated from the selected endogenous control genes. We identified three genes that had increased expression in the alcoholic samples in the MC: peptidylprolyl isomerase A (*PPIA*, $p = 0.007$), phosphoglycerate kinase (*PGK1*, $p = 0.01$) and hypoxanthine phosphoribosyl transferase (*HPRT1*, $p = 0.04$), and one gene that had decreased expression in the PFC of alcoholics: beta-2-microglobulin (*B2M*, $p = 0.03$). Alcohol consumption may alter gene transcription via several mechanisms, thereby affecting genes involved in a wide range of cellular functions. Genes used as endogenous controls are often highly abundant proteins concerned with maintainence of basic cellular functions. For example, *PPIA*, one of the differentially expressed control genes in our study, is a member of the peptidyl-prolyl cis-trans isomerase (PPIAse)

family that are important regulators of protein folding, a basic cellular function. However, the expression of *PPIA* as well as many other "housekeeping genes" is affected by pathological conditions such as inflammatory diseases. The involvement of PPIA in inflammation is coupled to its induction by oxidative stress (Jin et al., 2004; Jin et al., 2000; Liao et al., 2000). Ethanol metabolism leads to generation of reactive oxygen species (ROS)(Zakhari, 2006). It is therefore likely that the alteration in *PPIA* expression is linked to alcohol-induced oxidative stress. Expression of the *PGK1* gene was also increased in the MC of alcoholics. It has been demonstrated that *PGK1* expression is increased by hypoxia (Kress et al., 1998). Alcohol attenuates the response to hypoxia in fetal tissue by altering the cerebral blood flow (Mayock et al., 2007), hence the increased *PGK1* mRNA levels in the brains of alcoholics could represent an adaptive response coupled to an hypoxic state. Furthermore, alterations in both *HPRT1* and *B2M* mRNA expression have been associated with pathological conditions (Jordanova et al., 2003; Yamada et al., 2008). Taken together, four genes often used as endogenous control genes were differentially expressed in the cerebral cortex of alcoholics. These genes are therefore not suitable reference genes in real-time quantification analyses when alcoholic samples are evaluated.

The results described in this section demonstrate that alcohol consumption alters the transcription of genes involved in even basic 'stable' cellular functions. The next section will discuss transcription factors and their roles in regulating gene expression related to synaptic plasticity and drug abuse, which was an important factor during the initial phase of this thesis.

4.3 TRANSCRIPTION FACTORS IN SYNAPTIC PLASTICITY AND DRUG ABUSE

Transcription factors (TF) such as CREB and deltaFosB are activated or repressed by drugs of abuse (Chao and Nestler, 2004; Pandey, 2004) and were originally suggested to play a major role in the neuroadaptations associated with the persistence of addiction. In concordance with these observations many gene expression studies provide strong evidence for altered transcriptional regulation in drug abusers (Flatscher-Bader et al., 2006; Lull et al., 2008; McClung, 2006). It is now widely accepted that addiction disorders are, at least partly, related to long-lasting memories of drug experience (Kauer and Malenka, 2007). As with other forms of memory it is hypothesized that gene expression or protein translation is required for memory storage (Berke and Hyman, 2000; Nestler, 2001). CREB has been extensively studied in the context of learning, memory and addiction (Carlezon et al., 2005; Chao and Nestler, 2004; Lonze and Ginty, 2002), but less is known about the transcription factor NF-κB in relation to these processes. Although increased attention has been drawn to the role of NF-κB in learning and memory (Kaltschmidt et al., 2005; Mattson, 2005), limited information still exists with regard to its role in plastic events relevant to addiction disorders. We were therefore interested in evaluating whether the NF-κB system was altered in the cortex of alcoholics. The next section will briefly describe the detection of NF-κB DNA-binding activity using the electrophoretic mobility shift assay (EMSA) in the human cortex (paper II). Thereafter a more extensive evaluation describes the effects of chronic alcohol consumption on the NF-κB system in the human prefrontal and motor cortices (paper II). The discussion will focus on the effects that an altered NF-κB system in alcoholics could have on memory, plasticity and addiction. However, I am aware that the results could imply disturbances in a number of other systems, including inflammation, myelination and cell survival/death, in which NF-κB also plays an essential role. The last paragraph in section 4.3 will summarize that

major findings from paper II and touch upon the importance of multiple transcription factors in controlling cellular events following chronic ethanol administration.

4.3.1 The NF-κB system

We demonstrated a reduced DNA-binding activity of the NF-κB and p50 homodimer in the PFC of alcoholics that was coupled to a reduction in *RELA* mRNA levels. In addition, the bioinformatic analysis in combination with the RT-PCR suggested that the alteration in NF-κB DNA-binding activity observed in alcoholics might affect transcription from NF-κB dependent genes.

4.3.1.1 NF-κB DNA binding activity in the human prefrontal and motor cortex (paper II)

Before studying the effects on chronic alcohol consumption on the NF-κB system, we first examined NF-κB DNA-binding activity in the human cortex. We used an electrophoretic mobility shift assay (EMSA) to identify the DNA-binding activity of NF-κB. The assay identified two specific complexes that had previously been identified by our group as the NF-κB (p65 /p50 heterodimer) and p50 homodimer complex (Bakalkin et al., 1993; Bakalkin et al., 1994). Consistent with these findings, inducible NF-κB is most frequently composed of the p65 and p50 DNA binding subunits (Reviewed in (Kaltschmidt et al., 2005). The p50 homodimer DNA-binding activity was 2.4 - 2.7 fold higher then the NF-κB DNA binding activity, suggesting that the p50 homodimer was the dominant NF-κB binding factor in the human cortex. Interestingly, inducing the NF-κB binding activity using deoxycholate (DOC), which dissociates the inhibitory IκB from the NF-κB complex (Baeuerle and Baltimore, 1988; Bakalkin et al., 1993; Bakalkin et al., 1994), did not increase the binding activity more than about 15% in the human cortical samples, which can be compared to 92% induction in the murine pre B-cell line 70Z/3 (Baeuerle and Baltimore, 1988). These findings concord with previous studies which demonstrated that NF-κB has a relatively high constitutive activity in neurons compared to non-neuronal cells (Mattson, 2005). We also observed that NF-κB and p50 homodimer DNA-binding activity was much lower in six of the 30 analyzed samples. These six subjects had the lowest brain tissue pH. Thus the low tissue pH probably inactivated the NF-κB and p50 DNA-binding activity. We therefore excluded these samples from our analyses when comparing the levels of NF-κB and p50 homodimer DNA-binding activity in samples from controls and alcoholics.

4.3.1.2 Decreased NF-κB and p50 homodimer DNA-binding activity and p65 mRNA levels in the prefrontal cortex of alcoholics (paper II)

In order to evaluate whether the NF-κB system was altered in the PFC (BA9) or MC (BA4) in alcoholics, we used EMSA, semi-quantitative western blot analysis and TaqMan® Low Density arrays to study the DNA binding activities, protein and mRNA levels of the key players within the system. We determined a reduction in the NF-κB (p65/p50) DNA-binding activity ($p = 0.010$). NF-κB activity is important for many aspects of learning and memory processes including fear conditioning, inhibitory avoidance and spatial memory (Dash et al., 2005; Freudenthal et al., 2005; Yeh et al., 2006). Moreover, Meffert *et al* demonstrated that p65 gene-deleted animal models exhibited a selective deficit in spatial learning that was coupled to a lack

of synaptic p65/p50 (Meffert et al., 2003). Downregulated NF-κB DNA-binding activity and decreased *RELA* mRNA levels could thus imply a disruption of learning and memory formation in alcoholics. Indeed, alcoholics show impairment in episodic and working memory performance (Pitel et al., 2008). Working memory performance has been linked to dorsolateral PFC function [Rewiewed in (Funahashi, 2006), in which brodmann area 9, used for our analysis, is situated.

It is well known that substance abusers in general, including alcoholics, have a lower basal prefrontal cortical activity [Rewieved in (Goldstein and Volkow, 2002) and that NF-κB is activated by glutamate. It is therefore tempting to speculate that the decreased glutamatergic tone in the PFC during basal conditions are responsible for the downregulated NF-κB DNA-binding activity apparent in this study. However, could NF-κB play a different role during alcohol intoxication or relapse, when the PFC activity is increased and drug-related memories are created or reconsolidated? There are a few reports indicating that NF-κB is activated during acute and chronic drug administration and may affect drug-related behaviors. First, experimental animal studies have revealed that acute administration of ethanol results in an activation of NF-κB and increased p65 protein levels (Rulten et al., 2006; Ward et al., 1996), while chronic ethanol intake did not induce NF-κB activity until after a challenge dose of ethanol (Ward et al., 1996), suggesting that NF-κB may be involved in drug-primed relapse. Second, inhibiting NF-κB activity in the nucleus accumbens blunts the sensitized response to chronic cocaine administration (Russo et al., 2008). Third, a more recent study reported by Lubin and Sweatt demonstrated that NF-κB activity is necessary for the reconsolidation of associative fear memories (Lubin and Sweatt, 2007). Fear conditioning is often used as a model to examine associative memory formation and reconsolidation, which is also thought to play a major role in the etiology of addiction. From this viewpoint it could be hypothesized that NF-κB is involved in drug-induced associative memory reconsolidation. However, further research is necessary in order to answer these and other questions related to NF-κB role in drug-induced plasticity. The reduction in DNA-binding activity observed in the PFC was accompanied by a trend of decreased NF-κB DNA-binding activity ($p = 0.06$) in the MC as well, indicating that the effect of chronic alcohol consumption on NF-κB activity may not be exclusive to the PFC.

In addition to a reduction in the NF-κB DNA binding activity we also recorded a downregulated PFC DNA-binding activity of the p50 homodimer complex ($p = 0.029$), which generally represses transcription from NF-κB sites (Flatscher-Bader et al., 2006; Guan et al., 2005; Li et al., 1994). The p50 homodimer is less well studied, specifically in relation to neuronal plasticity, although p50 homodimer gene deleted mice exibit disturbances in short-term spatial memory (Denis-Donini et al., 2008) and in the manifestation of emotional behavior (Kassed and Herkenham, 2004).

There are several ways in which the NF-κB and p50 DNA binding activity could be altered e.g. through altered mRNA/protein levels or by post-translational modifications of the p65 or p50 subunits (Chen and Greene, 2004; Guan et al., 2005; Li et al., 1994; Zhong et al., 2002). Protein phosphorylation by protein kinase A and other kinases is critical for the binding of the p50 homodimer to DNA (Guan et al., 2005; Li et al., 1994). We observed that the downregulation of NF-κB DNA-binding activity was accompanied by a decrease in *RELA* (p65) mRNA levels ($p = 0.003$), although the p65 protein levels were not altered. The lack of significantly altered p65 protein levels in alcoholics could be due to the limitations of protein quantification, since semi-

quantitative western blot analysis is far less sensitive then is RT-PCR. The downregulated NF-κB DNA-binding activity may thus result from decreased p65 levels. Conversely, *NFKB1* (p50) mRNA and p50 protein levels were not changed in alcoholics, suggesting that the downregulated p50 homodimer DNA-binding activity are related to post-translational modifications of the p50 subunit.

The overall reduction in NF-κB and p50 DNA binding activity suggests an altered transcription of both NF-κB- and p50 homodimer-regulated genes. In the next section I will describe the results from a bioinformatic approach we used in order to evaluate whether NF-κB regulated genes are differentially expressed in alcoholics.

4.3.1.3 Chronic alcohol consumption alters transcription of genes that are regulated by NF-κB or have -κB binding sites (paperII)

The altered DNA- binding activity in the PFC of alcoholics could possibly result in a decreased transcription of NF-κB- dependent genes and also to an increased transcription through genes normally repressed by the p50 homodimer. Many studies have been conducted investigating gene expression changes within the PFC of alcoholics. One of our collaborating groups had performed a genome-wide gene expression study using brain material from control and alcoholics that partly overlapped with our study population (Liu et al., 2004). They identified 479 differentially expressed genes, 209 upregulated and 270 downregulate in alcoholics. We used their set of differentially expressed genes in an attempt to investigate whether NF-κB binding sites were accumulated or under-represented in the set of upregulated and downregulated genes compared to a control set of genes. Answering this question would give us some insight as to whether the NF-κB or p50 homodimer changes that we observed could have any potential functional relevance. In addition, we performed RT-PCR for a few genes that are known to be regulated by NF-κB. The bioinformatic analysis revealed that κB- binding sites (NF-κB and p50 like) were under-represented in the downregulated set of genes compared to the control and upregulated genes.

These results can be interpreted in multiple ways. First, the accumulation of NF-κB and p50 binding sites within the upregulated genes could represent an induction of genes that is normally repressed by the p50 homodimer. Given the fact that we identified the p50 homodimer as the dominant NF-κB binding factor in the human brain, this would be a reasonable explanation. A downregulated DNA-binding activity would thus favor the induction of NF-κB genes. However, the frequency of upregulated genes containing NF-κB or p50 binding sites should optimally have been higher then the frequency of NF-κB or p50 binding sites in the control set. Our analysis indicate that the upregulated and control set of genes more-or-less contain the same frequency of genes containing -κB binding sites. The bioinformatic results presented in this thesis are therefore not clear-cut in respect to the potential effect of NF-κB in controlling differential gene expression in the PFC of alcoholics. Despite this, the RT-PCR performed to examine known targets of NF-κB revealed that expression of four of the evaluated 11 NF-κB- dependent gene transcripts was decreased in the alcohol group (our unpublished observations). These results suggest that at least a few known NF-κB target genes are differentially expressed, possibly through the downregulation of NF-κB DNA-binding activity that we observed.

In the following section I will conclude the results from paper I. In addition, I would like to make a few general comments with regard to the role of NF-κB and other transcription factors in addiction disorders.

4.3.2 Drug-induced transcription factor alterations

Extensive research has demonstrated that transcription factor activity and protein synthesis is crucial for the development of stable drug-induced plasticity. Recent findings also suggest that the NF-κB family of transcription factors plays a role in stable plasticity underlying learning and memory formation. The results from paper II demonstrate that the NF-κB system is altered in the cortex of human alcoholics. It is thus possible that NF-κB plays a role in drug-induced long-term plasticity underlying the pathophysiology of addiction. However, biological systems are rarely simple and often redundant and so it is likely that multiple transcription factors may act separately or together in different combinations in order to regulate the complex changes in gene transcription and plasticity that have been observed in alcoholics and other drug abusers. For example, NF-κB has been demonstrated to control CREB activation through activation of protein kinase A, thereby regulating, for example, spatial memory formation (Kaltschmidt et al., 2006). In addition, Rulten *et al* specifically identified both the NF-κB and SP1 signaling pathways in response to acute and chronic ethanol treatment in mice (Rulten et al., 2006). Moreover, both CREB and SP1 DNA-binding activity are reduced in the rat cortex following ethanol withdrawal (Mittal et al., 1999; Pandey et al., 1999). Together these studies indicate the complexity of transcription factor regulation by drugs of abuse.

The drug-induced alterations in transcription factor activity observed and discussed in this section are dependent on upstream signaling events regulated by, for example, neuronal activity. As discussed earlier, transcription factors regulate transcription of gene products e.g., Homer, Dynorphin, Fos and FosB (CREB). Several of these gene products are known to be crucial for long-term stable plasticity. The next section (*4.4*) will focus on alterations in pre-and post-synaptic proteins (e.g. vesicular proteins, glutamate receptors and scaffolding proteins), many of which are regulated by the above discussed transcription factors. Moreover, they are essential for synaptic plasticity to occur.

4.4 GLUTAMATE-DEPENDENT PLASTICITY: RELEVANCE FOR DRUG ABUSE

As the discussed in the introduction, pathology of staged neuroplasticity is an important theory of addiction disorders. Related neurobiological mechanisms are linked to glutamate-dependent plasticity in corticolimbic and corticostriatal brain circuits that mediate reward, emotional expression, cognitive control and motor function. Most research evaluating synaptic plasticity has focused on ventral tegmental area, striatum and amygdala. For example, glutamatergic plasticity of dopamine neurons in the ventral tegmental area is important for establishing addiction-related behavior (Borgland et al., 2004; Churchill et al., 1999; Fitzgerald et al., 1996; Ungless et al., 2001; White et al., 1995; Zhang et al., 1997) whereas glutamatergic plasticity in the striatum and amygdala is critical for the expression of these behaviors (Kalivas et al., 2005; Kalivas and Volkow, 2005). Less is known about glutamate plasticity within the PFC in relation to addiction. However, imaging studies together with rodent animal models have suggested that aberrant

glutamate transmission in the PFC contributes to the loss of control and compulsive relapse that is one of the hallmarks of addiction (Kalivas and Volkow, 2005; LaLumiere and Kalivas, 2008; Lyvers, 2000; McFarland et al., 2003).

At a molecular level, glutamate-plasticity can in part be explained by rearrangement of the pre-or post-synapse. This can potentially be explained by a pre-synaptic alteration in transmitter release (Stevens, 1993) or how the post-synaptic cell responds to the released transmitter upon stimulation (Nicoll and Malenka, 1995). The next section (paragraph *4.4.1*) will discuss pre-synaptic events important for transmitter release and plasticity in relation to drug abuse with emphasis on alcohol dependence. Specific focus will be directed towards synaptic vesicular proteins controlling transmitter release and SYPI, a modulator of synaptic strength. I will also discuss their relevance for PFC plasticity in relation to alcohol dependence (paper III). In paragraph *4.4.2* I will elaborate on the importance of post-synaptic alterations in drug-induced glutamatergic plasticity, focusing on amygdala and striatum. More specifically, I will present results investigating glutamatergic receptors and scaffolding proteins in heroin abusers but also in a small population of cocaine and polysubstance users (paper IV).

4.4.1 Pre-synaptic alterations: relevance for drug abuse

Increased glutamate release has been reported following chronic drug intake and is thought to be important for addiction-related behaviors (Kalivas et al., 2008). However, not much is known about how alcohol or other drugs of abuse alter glutamate release. Pre-synaptic plasticity leads to increased glutamate release following neuronal activation (Malenka and Bear, 2004; Nicoll and Schmitz, 2005) and can be regulated by rearrangement of pre-synaptic receptors or other components directly involved in the transmitter release machinery. Today it is known that both ionotrophic glutamate receptors as well as metabotrophic glutamate receptors are situated at the pre-synapse and can control short-term as well as long-term neurotransmitter release, respectively. Moreover, synaptic vesicles and associated proteins are important regulators of transmitter release by controlling the amount and frequency of transmitter release as well as recycling of vesicles, which is crucial to maintain a neurotransmitter supply for exocytosis and thus execution of synaptic strength.

Drugs of abuse can affect pre-synaptic transmitter release in several ways, either through direct interactions with pre-synaptic receptors or indirectly through affecting other mechanisms involved in plasticity. In addition, drugs could affect transmitter release in other communicating brain regions, thus regulating pre-synaptic activity in another regions. Altered glutamatergic transmitter release from pre-synaptic terminals has been observed following ethanol treatment although the effect seems to vary with conditions under which transmission is observed (Roberto et al., 2006; Siggins et al., 2005). For example, acute ethanol treatment reduces glutamate release in the nucleus accumbens, whereas a challenge dose with ethanol after a washout period from chronic ethanol treatment increases glutamate release in the central amygdala and hippocampus (Roberto et al., 2004; Roberto et al., 2006). However, basal release is often unaffected in ethanol treated animals (Roberto et al., 2006; Siggins et al., 2005). These data indicate that the conditions in which ethanol modulates synaptic transmission via pre-synaptic actions are still unclear and unresolved. However, considering that basal evoked release is unaffected, whereas ethanol induces glutamate release under certain circumstances, suggests that modulators of synaptic strength rather then executors of glutamate release would play an important role. We were

therefore interested in evaluating whether alcohol dependence in humans affects modulators of synaptic strength or executors of glutamate release. The following section will describe and discuss the findings from this study.

4.4.1.1 Pre-synaptic alterations in the prefrontal cortex of alcoholics (Paper III)

We observed increased synaptophysin I levels in the prefrontal cortex of alcoholics compared to controls, while levels of syntaxin 1A, synaptosome-associated protein 25, vesicle-associated membrane protein were unaltered. No alterations were observed in the motor cortex.

To address the hypothesis that repeated alcohol consumption affects modulators of synaptic strength but not executors of glutamate release, we used semi-quantititative western blot analysis to compare the immunoreactivities of SYPI and members of the SNAREs e.g SYX1A, SNAP-25 and VAMP that regulate transmitter release through controlling membrane fusion and exocytosis of synaptic vesicles (Montecucco et al., 2005). The levels were assessed in the PFC (BA9) and MC (BA4) of alcoholics and controls, as previously described. We determined that the protein levels of SYPI are increased in the PFC of alcoholics ($p < 0.01$) whereas no difference was detected in the SNARE proteins. No significant differences were observed in the MC.

SYPI is a major synaptic vesicle protein that has been implicated to regulate both short- and long-term synaptic plasticity without affecting basal glutamate release function (Janz et al., 1999; McMahon et al., 1996). However, SYP activity has been linked to increased glutamate release following LTP induction (Mullany and Lynch, 1998). Alcoholics exhibit a reduced basal activity in the PFC even though activity is induced following cue-induced craving (Goldstein and Volkow, 2002). It is therefore tempting to speculate that the increased SYPI protein levels observed within the PFC in alcoholics compared to control subjects could reflect an increased synaptic strength that may not affect basal glutamate release, but may be released during activity-dependent stimulation such as during drug craving or relapse. Interestingly, there are some findings that can be used to argue in favor of such a hypothesis. A recent paper by Bragina *et al* demonstrate that SYP1 co-localizes to a higher extent with the vesicular glutamate transporter 1 (VGLUT1) (95%) containing terminals then with VGLUT2 (30%) in the rat cerebral cortex (Bragina et al., 2007). According to a more simplistic view, VGLUT1-containing synapses are considered low release probability whereas VGLUT2 are considered high release probability. In addition, VGLUT1-containing synapses have a higher potential for synaptic plasticity (Fremeau et al., 2004; Liguz-Lecznar and Skangiel-Kramska, 2007). Accordingly, could the increased SYP1 in the PFC of alcoholics potentiate VGLUT1 containing synapses with low release probability, thus affecting transmitter release under specific circumstances when low probability release synapses are recruited?

At a molecular level it is still unclear how SYP contributes to increasing synaptic strength and glutamate release. However, it has been suggested to play a role in the availability of synaptic vesicles endocytosis or subsequent recycling steps (Evans and Cousin, 2005). For example, synaptophysin-deficient mice have defects in synaptic vesicle recycling and formation (Spiwoks-Becker et al., 2001). Furthermore, its interacting partner synaptobrevin-2 (VAMP-2) has been implicated in endocytosis that rapidly reuses synaptic vesicles that have just undergone

exocytosis (Deak et al., 2004; Valtorta et al., 2004). A role for SYP in these processes is compatible with a role in synaptic plasticity since synaptic vesicles have to recycle in order to maintain their supply for exocytosis.

The absence of significant differences in SYPI protein levels in the MC between alcoholics and controls suggests that the effect of chronic alcohol consumption on SYPI in PFC is region- and possibly also circuitry-specific. As discussed previously, SYP co-localizes to a higher extent with VGLUT1 then VGLUT 2 in the cortex (Bragina et al., 2007). VGLUT1 and 2 have regional as well as circuitry-specific distributions (Fremeau et al., 2004). Thus their distribution as well as co-localization of SYP with VGLUT and other factors regulating synaptic vesicle recycling may affect the effect of chronic alcohol consumption on SYP levels and furthermore glutamate-dependent plasticity in specific neuronal circuits.

In conclusion, the increase in SYPI immunoreactivity in the PFC of chronic alcoholics as compared to control subjects suggest a role for SYP in the alcohol dependence–associated enduring neuroplasticity in the prefrontal cortical glutamate circuitry. The absence of significant differences in the immunoreactivities of SYX1A, SNAP-25 and VAMP between alcoholics and control subjects are in agreement with no apparent effect of chronic ethanol exposure on basic glutamatergic release. In the next section I will briefly discuss a few studies that have evaluated the role of SYP in relation to drug abuse.

4.4.1.2 Drug-induced alterations in synaptophysin and its implications

There are only a few studies that have investigated SYP in relation to drug abuse. The results vary with drug, context and brain region evaluated. This is not surprising since SYP appears to have a regional-specific distribution (as discussed in the previous section), which together with generalized as well as drug-specific effects may alter the regulation of SYP levels. We determined increased levels of SYPI in the PFC (BA9) of alcoholics. Is this alcohol-specific or a common drug alteration? Only one additional study has been conducted evaluating the effects of drug abuse on SYP levels in the human cortex. Ferrer-Alcón and colleagues examined the protein levels of SYP in post-mortem PFC (BA9) specimens from opiate addicts and controls but did not report any significant differences between the two groups (Ferrer-Alcon et al., 2000). The lack of additional studies evaluating SYP in the human cortex from drug abusers prevents a proper answer to the question of drug-specific versus common effects. However, a few studies provide us with some clues. We examined the SYPI protein levels in the putamen of heroin and control subjects but did not observe any statistical difference between the groups (our unpublished observations, $p = 0.617$). Furthermore, no changes in SYP mRNA levels were observed in the rat caudate nucleus, putamen or midbrain following 7 days treatment with morphine (Spangler et al., 2003). It therefore appears that opiates do not alter SYP levels following repeated opiate treatment/use regardless of region examined. Conversely, a few studies have implicated SYP in amphetamine-induced molecular as well as behavioral events. For example, acute treatment with amphetamines increase SYP levels in the PFC and striatum, whereas no induction was observed following chronic treatment (Ujike et al., 2002). Moreover, the degree of amphetamine-induced conditioned place preference is positively correlated with increased SYP immunoreactivity in the nucleus accumbens core, basolateral amygdala and hippocampus (Rademacher et al., 2006). Furthermore Rademacher *et al* demonstrated that amphetamine-increased SYP

immunoreactivities in the balsolateral amygdala, dorsolateralstriatum and hippocampus are associated with conditioned motor sensitization in rats (Rademacher et al., 2007).

It appears that drug-induced alterations in SYP levels to some extent exhibit drug-specific regulation. However, it is far from clear how this difference in regulation occurs. It is possible that the discrepancies observed are dependent on the acute or protracted pharmacological actions of the specific drug. Specifically in the human studies evaluating SYP in the human cortex: only two alcoholics showed positive ethanol toxicology whereas the opiate addicts died from overdoses. It is therefore likely that drug-on-board versus no-drug-on-board may affect the observed results. So the question still remains: what importance does SYP have for the molecular and drug-induced behavioral effects of addiction disorders?

4.4.2 Post-synaptic alterations: relevance for drug abuse

The post-synaptic site is dense in glutamate receptors such as AMPA, NMDA and mGluRs, that are specifically targeted and clustered at the post-synaptic membrane by various scaffolding and adaptor proteins. (*Figure 1*) (Boeckers, 2006), all of which are essential for overall synaptic function and plasticity to occur. The majority of studies evaluating the role of post-synaptic glutamate plasticity in addiction disorders have been performed using various animal models following cocaine or morphine administration. Alterations in LTP/LTD and various of the above-mentioned receptors as well as scaffolding and structural proteins present in the post-synaptic density have been implicated in drug-induced plasticity and addiction-related behaviors (Gass and Olive, 2008; Kalivas et al., 2008; Kalivas and O'Brien, 2008; Kauer and Malenka, 2007). However, many features such as the complexity of human drug abuse e.g chronicity are not mimicked by the animal models. So many critical questions remain as to understanding the glutamatergic pathophysiology in human addiction disorders. The following paragraphs will discuss post-synaptic alterations in the human brain following heroin and polysubstance use (paper VI).

4.4.2.1 Post-synaptic alterations in drug abusers (paper IV)

We determined dysregulation of glutamate receptors and scaffolding proteins and a disturbed network coupling between these markers within the human brain of heroin abusers. There is a clear regional difference in the altered connectivity with amygdala more linked to strengthening of GluR1-PSD95 coupling, whereas diminished mGluR5-Homer occurs in the striatum. The striatum is also characterized by an overall decrease in glutamatergic markers.

We evaluated the mRNA and protein levels of glutamate receptors (GluR1, NMDA NR1 and mGluR5) and their scaffolding proteins (Homer, and PSD-95) in human heroin (Table 3) as well polysubstance users (Table 4; paper IV) using *in situ* hybridization histochemistry and semi-quantitative western blot analyses. In addition we wanted to evaluate whether drug use altered the biological organization/connectivity of the glutamtergic markers in the PSD. We focused our investigation on the amygdala and striatum given their essential roles in the emotional, rewarding, habitual and compulsive features of addiction disorders. The next paragraphs (*4.4.2.2& 4.4.2.3*) will describe and discuss findings from these studies. Focus will be directed

towards explaining the results from a post-synaptic perspective; however, as several of these receptors are also expressed pre-synaptically the results could also have potential impact on pre-synaptic plasticity (Pinheiro and Mulle, 2008). I have divided the results and discussion into two parts, one focused on the amygdala and the other on results obtained from the striatum. The reason for this is that the mechanisms underlying glutamate-dependent plasticity to some extent is regional- and circuitry-specific and is therefore easier to discuss separately in order to avoid confusion.

4.4.2.2 Alterations in the amygdala of human heroin, cocaine and polysubstance abusers (Paper IV).

The amygdala, which has bi-directional connectivity with the PFC, plays a major role in the emotional significance of sensory stimuli and enables emotional memory formation, a phenomenon with major relevance for the development and persistence of addiction. However, surprisingly little is known about the lateral amygdala with regard to drug abuse and synaptic plasticity although it is the major receptive amygdala nucleus of this structure integrating sensory information from most cortical modalities (Groenewegen and Uylings, 2000; Ongur and Price, 2000; Rolls et al., 1996). We examined the mRNA levels of GluR1, PSD-95 and Homer 1 in the amygdaloid complex (focused on the lateral, accessory basal and basal nuclei) of heroin, cocaine and heroin-cocaine users (See Table 4). Protein levels (GluR1, PSD-95, mGluR5 and NR1) were determined in the lateral amygdala of heroin abusers (Table 3) from which abundant tissue was available for western blot analyses. The results demonstrated a significant correlation between GluR1 and PSD-95 mRNA levels in the lateral amygdala in all substance abuse groups (heroin, $r = 0.95$ $p = 0.01$; cocaine $r = 0.94$ $p = 0.005$; heroin- cocaine- $r = 0.94$ $p = 0.002$) that was absent in control subjects ($r = 0.60$ $p = 0.20$). There was also evidence of disturbed glutamatergic network coupling in the lateral amygdala between heroin abusers and controls ($p = 0.006$). GluR1 and PSD-95 protein levels correlated positively in heroin abusers, but not in controls (control, $r = 0.029$ $p= 0.925$, heroin, $r = 0.534$ $p =0.003$). The fact that the correlation between GluR1 and PSD-95 was strong in all substance abuse groups (mRNA) and that it was evident at both mRNA level and protein levels in different heroin populations (Table 3 and 4) strongly suggests that it is not an artifact but a phenomenon in heroin as well as cocaine and polysubstance users that may be important for the common mechanisms underlying some aspects of addiction related to amygdala function.

PSD-95 induces GluR1 delivery into synapses that is coupled to the strengthening of excitatory synapses during experience-driven learning (Ehrlich and Malinow, 2004). GluR1 incorporation into the active synaptic site following drug administration has been demonstrated in the striatum (Anderson et al., 2008; Boudreau et al., 2007; Boudreau and Wolf, 2005), but little is known about these events in the lateral amygdala. Fear conditioning is dependent on activity in the basolateral amygdala (LeDoux, 2000; McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997; Rumpel et al., 2005) and is often used as a model to examine associative memory formation, which also is thought to play a major role in the etiology of addiction. Similar neurobiological mechanisms are therefore likely to play a significant role in both fear conditioning and the development of addiction disorders. Fear conditioning induces strengthening of excitatory synapses within the lateral amygdala and requires trafficking of GluR1 into synapses (LeDoux, 2000; McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997; Rumpel et al., 2005). Increased GluR1 plasma membrane levels following fear

conditioning have been reported, although the total amounts of GluR1 mRNA and protein levels are unchanged (Yeh et al., 2006). A correlation between GluR1 and PSD-95 in heroin abusers could therefore suggest an active trafficking of GluR1 into synaptic sites, since PSD-95-GluR1 interaction is required for the insertion of receptor into the plasma membrane. It would also be tempting to speculate that the strong coupling observed between GluR1 and PSD-95 in heroin abusers represents an induction of synaptic GluR1 that leads to strengthening of synaptic connectivity and furthermore increased responsiveness of the amygdala during e.g. craving or cue-induced relapse.

Apart from apparent disturbances in the network coupling, Homer 1b/c protein levels were increased ($p = 0.002$, covariate pH) in the lateral amygdala. A recent study by Befort *et al* reported increased Homer 1 mRNA levels following chronic morphine treatment in mice, but in the central amygdala (Befort et al., 2008). The Homer family consists of long constitutively expressed (e.g. Homer 1b/c/d) and short inducible Homer (Homer 1a) isoforms (Brakeman et al., 1997; Saito et al., 2002; Xiao et al., 1998). Decreased levels of short Homer 1a mRNA and protein levels has been observed following acute drug treatment (Zhang et al., 2007), whereas chronic treatment does not appear to alter Homer 1a levels (Ghasemzadeh et al., 2009). Thus increased Homer 1 levels following chronic morphine treatment in mice likely represents an induction of long Homer 1 isoforms and supports our finding that chronic heroin abuse increases long Homer isoforms within the amygdala. We did not observe additional differences between drug abusers (heroin, cocaine and heroin-cocaine) and control subjects within the amygdala. However, it is possible that alterations in protein levels are masked through measurement of total protein levels. In support of this notion a recent study by Glass *et al* reported increased GluR1 levels at the plasma membrane of dendrites, but no change was detected in total GluR1 content following chronic morphine administration (Glass et al., 2005). It is also possible that protein activity is altered although total levels are not changed.

4.4.2.3 Alterations in the Striatum of human heroin abusers (Paper IV)

We examined the mRNA levels of the glutamtergic markers and scaffolding proteins in the striatum (caudate nucleus, putamen, nucleus accumbens core and shell). Protein levels (GluR1, PSD-95, mGluR5 and NR1) were determined in the putamen of heroin abusers (Table 3) for which abundant tissue was available for western blot analyses. Compared to the amygdala the striatum (putamen) also exhibited disturbed glutamatergic coupling ($p = 0.032$), but was related to the relationship between mGluR5 and Homer; mGluR5 mRNA levels correlated with Homer 1 in control subjects ($r = 0.638$, $p = 0.0079$), but not in heroin abusers ($r = 0.256$, $p = 0.216$). There was no difference in correlation structure between heroin abusers and controls when analyzing the mRNA levels of the other striatal subregions or protein data in the putamen. However, when only considering correlations between mGluR5 and Homer a similar pattern was evident in the caudate nucleus (control $r = 0.778$, $p = 0.001$, heroin, $r = 0.142$, $p = 0.498$) and to some degree in the NA core (control $r = 0.568$, $p = 0.054$, heroin, $r = 0.279$, $p = 0.314$). Given that the protein levels in the putamen showed similar correlations as the mRNA (control $r = 0.8166$ $p = 0.001$, heroin $r = 0.4131$, $p = 0.029$), this phenomenon is probably related to striatal function and heroin-induced disruption of connectivity in the more motor-related striatal subregions. Indeed, experimental animal models have demonstrated that chronic cocaine administration disrupts long-term depression (LTD) in the nucleus accumbens core (Martin et al., 2006), thus losing the ability to undergo activity dependent weakening of synaptic strength.

The activation of mGluR5 is important for the induction of LTD and LTP in principle striatal medium spiny neurons (Shen et al., 2008; Sung et al., 2001) and Homer proteins appear to be a viable means to regulate mGluR5 localization and function in order to fine tune this process (Kammermeier, 2006, 2008; Kammermeier and Worley, 2007; Kammermeier et al., 2000; Thomas, 2002). The lack of correlation observed in our study could thus be important for proper LTD or LTP induction. The activation of mGluR5 induces the extracellular signal-regulated kinase (ERK) and phosphoinosititde 3-kinase (PI3K) signaling pathways through interaction with long Homer isoforms (Mao et al., 2005; Rong et al., 2003) that in turn induce protein synthesis required for mGluR5-LTD (Huber et al., 2000; Snyder et al., 2001). Acute interruption of the mGluR5-Homer interactions with a mGluR5 C-terminal tail containing the Homer ligand domain in hippocampal neurons severely reduced LTD and inhibited the PI3K pathway and translational activation (Ronesi and Huber, 2008). A disrupted network coupling in the heroin abusers could therefore lead to an aberrant mGluR5 signaling and disturbed protein synthesis occluding normal striatal-LTD. It has been suggested that failure to induce LTD within the striatum could reflect ongoing memory consolidation in relation to drug administration, failure to induce further learning or maybe even to facilitate reactivity to relapse-inducing stimuli (Kelley, 2004; Martin et al., 2006; Udo et al., 2004). However, the disturbed coupling between mGluR5 could potentially also reflect disturbed LTP since mGluR5 activity is coupled to NMDA receptor activity in striatal medium spiny neurons.

In addition to a disturbed network coupling we observed an overall decrease of glutamatergic markers in the putamen of heroin abusers. Specifically, we observed decreased PSD-95 ($p = 0.028$, covariate pH), Homer 1 ($p = 0.006$, covariate age and pH) and mGluR5 ($p = 0.013$) mRNA levels. The protein levels of GluR1 ($p = 0.011$, covariate age and GAPDH), PSD-95 ($p = 0.004$, covariate pH) and NR1 ($p= 0.039$, covariate age and GAPDH) were reduced in heroin abusers compared to controls. Decreased mRNA levels were also detected in the caudate nucleus (Homer 1, $p = 0.030$) and nucleus accumbens core (Homer 1, $p = 0.001$) and shell (mGluR5, $p = p = 0.040$). Moreover, several markers did not reach statistical significance but had trends for decreased mRNA levels (see Table 5, paper IV). There are not many studies that have examined the effect on glutamate receptors and scaffolding proteins following chronic morphine or heroin administration. Most studies have evaluated these factors after withdrawal from chronic intake or during relapse. However, recent data from our group demonstrate that chronic heroin self-administration in rats significantly reduces the mGluR5 mRNA levels within the striatum ($p <0.001$), supporting the findings from our human study. There are also morphological studies that have reported reduced spine density in the striatum following heroin administration (Robinson and Kolb, 2004). Spine density has been correlated with activity of glutamate receptors and accumulation of scaffolding proteins (El-Husseini et al., 2000; McKinney et al., 1999; Shi et al., 1999; Vanderklish and Edelman, 2002). The overall decrease of glutamate markers and associated scaffolding proteins within the striatum is therefore in agreement with the previously observed morphological alterations in striatum of heroin self-administering animals.

Evaluation of the acute pharmacological effects of heroin in our study demonstrate that several markers, including Homer 1 and PSD-95, have positive correlations with opiate toxicological measures in all striatal regions (**Homer 1**: putamen, $r = 0.4757$, $p = 0.0140$, caudate, $r = 0.4594$, $p = 0.0182$, nucleus accumbens core, $r = 0.4366$, $p = 0.0798$, nucleus accumbens shell, $r = 0.4291$, $p = 0.0755$; **PSD-95**: putamen, $r = 0.3971$, $p = 0.0547$; caudate, $r = 0.3969$, $p = 0.0548$; nucleus

accumbens core, r = 0.5376, p = 0.0176; nucleus accumbens shell, r = 0.6959, p = 0.0013). However, the fact that the levels of these markers are reduced in heroin abusers compared to in controls suggests that these levels may be even lower during non-acute drug conditions and that the overall decrease in glutamatergic markers potentially reflects the chronic state of drug use.

5 CONCLUDING REMARKS

The aim of this thesis was to describe how drugs of abuse affect common neurobiological mechanisms related to synaptic function and plasticity within the brain and to discuss how these plastic events may relate to the development and persistence of addiction. The results presented herein support a role of drug-induced effects on the glutamatergic system and regulators of gene transcription in human drug abusers that may be important for the underlying disturbances in synaptic plasticity.

At the molecular level, synaptic plasticity or activity-dependent alteration in synaptic strength is dependent upon pre-synaptic release of transmitter, post-synaptic response to the released transmitter and alterations in gene transcription. Even though synaptic plasticity can be mediated by pre- or post-synaptic mechanisms the interplay between the three events is still crucial. Animal models have provided a foundation of knowledge supporting altered synaptic plasticity following drug intake that is coupled to dysfunctional glutamatergic signaling, post-synaptic responses and alterations in gene expression. The results of this thesis thus provide evidence that alterations do exist at pre-synaptic, post-synaptic and transcription factor levels in the brain of human drug abusers:

First, we report alterations in SYP1, a synaptic vesicle protein and modulator of synaptic strength that has the potential to affect glutamate release. As previously suggested, increased SYP1 protein levels in the PFC of alcoholics could reflect an increased synaptic strength that may not affect basal glutamate release, but may be released during activity-dependent stimulation such as during drug craving or relapse. Emerging evidence has assigned the dorsal striatum as a critical regulator of habit-induced drug-seeking behavior and increased glutamate release from the PFC to the dorsal striatum is essential for inducing this behavior. The dorsolateral PFC and, specifically brodmann area 9, which was studied in our investigation, project to dorsal caudate and putamen, suggesting that increased SYP1 may contribute to the compulsive relapse connected to neuronal habit circuitry.

Second, we found evidence of disturbed coupling between post-synaptic glutamate receptors and scaffolding proteins and alterations in several glutamatergic receptors (GluR1, NR1, mGluR5) and scaffolding proteins (PSD-95, Homer). Region specific effects were observed. Amygdala showed a profound disturbance in the coupling between GluR1 and PSD-95. Reduced levels of both glutamatrgic receptors and scaffolding proteins and a disturbed correlation between mGluR5 and Homer characterized the striatum. The reduction in glutamatergic markers within the striatum is in line with evidence demonstrating a reduction in spine density following heroin administration. A decreased spine density is often accompanied by decreased LTP or increased LTD. We also found a lack of correlation between mGluR5 and Homer in the striatum of heroin abusers that was strongly evident in controls. mGluR5 activity induce NMDA receptor activation and LTP in the striatum (Gubellini et al., 2004). Furthermore, Homer is necessary for the interaction between the NMDA receptor and mGluR5, hence these results strongly suggest disturbance of glutamatergic signaling in the striatum of heroin abusers that may be linked to the pathology of neuroplasticity hypothesized in human drug abusers.

Finally, we report decreased NF-κB DNA-binding activity in the PFC of alcoholics that potentially could effect gene transcription as a consequence of glutamate receptor activation. In

fact, four out of eleven NF-κB dependent genes examined with RT-PCR was downregulated in alcoholics. A decreased NF-κB activity and reduced transcriptional activity from NF-κB dependent genes may be due to a reduced basal cortical activity and could, among other things, contribute to imparments in working memory seen in alcoholics. In addition to the already discussed aspects of NF-κB function, NF-κB has also been suggested to regulate synaptic spine density through activation of BDNF (Russo et al., 2008) and, the drosophila homolog was shown to be important for GluR1 trafficking and insertion into the plasma membrane (Heckscher et al., 2007). Active trafficking of GluR1to synaptic sites is associated with an increased spine density and LTP. Indeed, it has been proposed that NF-κB may be responsible for the increased spine density in the nucleus accumbens following chronic cocaine administration (Russo et al., 2008).

Many limitations are evident and must be considered in evaluating the thesis. There were of course noted challenges with working with the post-mortem human brain. Moreover, the studies comprised a wide range of differences including neuronal systems: pre- and post-synaptic markers and transcription factors; brain specimens: prefrontal cortex, motor cortex, striatum and amygdala; class of drug: alcohol, heroin, cocaine and polysubstance (heroin/cocaine) user. Thus, some observed changes could reflect drug-specific effects on neuronal systems as well as common mechanisms underlying addiction disorders. Future studies will help to answer these important questions.

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