Chapter 6

General discussion

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Drug addiction is a major cause of personal suffering and poses a great financial burden on society. Despite decades of addiction research, available treatments have limited efficacy and relapse rates remain high. A more detailed understanding of the neurobiological mechanisms underlying addiction is believed to contribute to the development of more effective cessation therapies in the future[21-23]. The aim of this thesis was to utilize animal models of addiction to identify and characterize novel molecular mechanisms that underlie relapse.

Chapter 2 described the identification of several extracellular matrix (ECM) proteins that are regulated by cue-induced reinstatement of heroin seeking and presented evidence that this behavior is controlled by ECM plasticity. In chapter 3, I investigated the involvement of the ECM protein brevican in more detail using the cocaine conditioned place preference (CPP) model. Reduced expression of this protein in the dorsal hippocampus (dHC) of heterozygous *brevican* knockout mice (*Bcan*$^{+/-}$) led to enhanced expression of a cocaine memory. The first part of the discussion will briefly summarize the properties and functions of the neural ECM and explore the current evidence for a role of ECM plasticity in addiction. Chapters 4 and 5 presented the search for molecular mechanisms underlying reinstatement of nicotine seeking and the identification of cue-induced regulation of $GABA_A$ receptors and Src homology 2 domain-containing protein tyrosine phosphatase substrate-1 (SHPS-1). The role of $GABA_A$ receptors in this relatively unexplored form of addiction was investigated in more detail in chapter 4. The findings described in this chapter indicate a role for GABAergic plasticity in the regulation of reinstatement of nicotine seeking. The second part of the discussion will focus on the current knowledge of the neurobiological mechanisms underlying reinstatement of nicotine seeking and compare these mechanisms with the neural processes that parallel reinstatement of drug seeking after self-administration of heroin and cocaine.

The neural extracellular matrix and drug addiction

The extracellular space between neurons and glial cells is comprised of ECM, containing the polysaccharide hyaluronan, and a large diversity of glycoproteins and proteoglycans[160]. During early development, the neural ECM forms a loose structure that can be extracted from tissue under relatively mild conditions[286]. At this stage, the ECM supports proliferation, migration, axonal outgrowth and synaptogenesis[159,287]. The adult brain ECM has a different protein composition and is less growth-permissive than the ECM in the juvenile brain[286]. In addition to the loose diffuse ECM, a distinctive highly condensed form of ECM is initially formed during the final stage of maturation of neuronal circuits. These so-called perineuronal nets (PNNs) are extracellular macromolecular aggregates, characterized by a much stronger association with cellular membranes[286], and form tight net-like structures around subpopulations of neurons[137]. The assumed function of PNNs in the adult brain is to reduce neural plasticity and stabilize established neural circuits[158,175,288].

Apart from *de novo* synthesis by glial cells and neurons, which both contribute to the secretion of ECM components into the extracellular space[147], ECM levels are locally regulated by proteolytic cleavage mediated by proteases. These include metalloproteases and serine proteases, which target virtually all components of the ECM[79,289,290]. The composition and turnover

Box 1. Paradigms, molecular techniques and interventions used in this thesis

To identify novel molecular mechanisms that underlie relapse, I combined a number of experimental strategies, in terms of paradigms, molecular techniques and interventions. The drug self-administration and reinstatement paradigm was used to model drug taking and relapse in rats[209,346]. Comparison of groups of animals in different stages of this paradigm (e.g., after cessation of drug taking versus after reinstatement) enables the identification of neural plasticity mechanisms that parallel specific addiction phases (e.g., reinstatement). In addition to the self-administration paradigm I used a heterozygous knock-out mouse model in combination with the conditioned place preference (CPP) paradigm[41]. An important site of neural plasticity is the synapse, where changes in protein composition determine adaptations in neurotransmission efficiency, network activity and ultimately behavior. I used a biochemical fractionation method to isolate synaptic membranes[123] from brain regions implicated in addiction and analyzed synaptic proteins by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Labeling of peptides with different isobaric tags (i.e., iTRAQ-based proteomics) allowed for the determination of relative protein levels in different samples[264,265]. In addition, immunoblotting was used to identify and quantify specific proteins of interest. Finally, to investigate the causative role in drug seeking of proteins found regulated after cessation of drug self-administration or reinstatement, I designed interventions aimed at manipulating the level or activity of these proteins prior to a reinstatement session.

of ECM are major determinants of neurotransmission rates and neural plasticity in the local environment[159]. They control the diffusion rate of membrane receptors, neurotransmitters and ions through the extracellular space, which has consequences for the dynamics of for example synaptic neurotransmitter receptors, volume transmission and calcium signaling[159]. As such, the ECM contributes to functional compartmentalization in the brain[161]. Furthermore, it controls signal transduction by regulating the activation of membrane proteins, such as ion channels, G protein-coupled receptors, integrins and kinases. For example, alterations in the ratio of growth-permissive and growth-restricting factors affect signal transduction, as well as protease-mediated release of growth factors and exposure of functional domains that are sterically hindered in intact proteins[291]. Via regulating cytoskeleton dynamics and other downstream signaling pathways, activation of membrane receptors ultimately affects structural (e.g., regulation of the number and shape of dendritic spines) and functional synaptic plasticity (i.e., LTP and LTD).

The growing evidence that the ECM is an important regulator of neuronal adaptation and plasticity[158,159] indicates that abnormal ECM composition and turnover might underlie the development and progression of psychiatric disorders, such as drug addiction. In support of this, a strong link between drug addiction and ECM remodeling has been established in the last decade.

Opioids and ECM remodeling

Direct manipulation of ECM levels has been shown to facilitate extinction of drug memories. Infusions of chondroitinase ABC, an enzyme that degrades PNNs by digesting glycosaminoglycan side chains of chondroitin sulfate proteoglycans (CSPGs), into the amygdala prior to extinction training attenuated subsequent reinstatement of morphine CPP, as well as reinstatement of heroin seeking after heroin self-administration[292].

In chapter 2, a causal link was established between changes in ECM constituents and

cue-induced reinstatement of heroin seeking. After three weeks of abstinence from heroin self-administration, rats expressed reduced synaptic levels of brevican, tenascin-R and hyaluronan and proteoglycan link protein 1 in the medial prefrontal cortex (mPFC). Furthermore, also after extinction of heroin self-administration, synaptically localized brevican and tenascin-R levels were reduced in the mPFC and a similar effect was observed in the nucleus accumbens (NAc).

Importantly, different ECM proteases have been implicated in opiate addiction. After a single or repeated injection of morphine, tissue plasminogen activator (tPA) was upregulated in a number of brain areas including the frontal cortex, NAc and hippocampus[164]. Tissue plasminogen activator is a serine protease that converts plasminogen into plasmin[79,293], which in turn cleaves a wide range of ECM components including proteoglycans and laminin[290,293]. Interestingly, tissue plasminogen activator$^{-/-}$ (*tPA*$^{-/-}$) mice exhibited an attenuated morphine-induced increase in locomotor activity and reduced morphine CPP[164], whereas tPA overexpression in the NAc augmented these behaviors[168]. Furthermore, self-administration rates were altered in *tPA*$^{-/-}$ mice[294]. These *tPA*$^{-/-}$ mice showed impaired release of dopamine in the NAc in response to morphine, which was rescued by local infusion of tPA[164,295], indicating that downstream effects include adaptations in dopamine neurotransmission.

In addition to tPA, increased protein levels of matrix metalloproteinase (MMP)-9 protein levels were observed in the midbrain after a single injection of morphine[296]. Tissue plasminogen activator and plasmin can upregulate MMP-9[297] and convert a number of MMPs into their active forms[290], suggesting that tPA might directly or indirectly mediate the reduced ECM levels observed after heroin self-administration (chapter 2). However, this would need to be established.

Restoration of ECM levels in the mPFC and NAc by systemic administration of the MMP inhibitor FN-439[136] prior to a reinstatement session attenuated cue-induced reinstatement of heroin seeking (chapter 2). In line with the study of Xue et al.[292], this indicates that opiate memories can be manipulated via the ECM, however the effect depends on the timing of the intervention, as well as the brain region that is targeted.

Psychostimulants and ECM remodeling

Similar to opiates, tPA levels in several brain areas are induced by administration of psychostimulants. For example, a single exposure to cocaine increased tPA expression in the mPFC, NAc and insula[168,298], whereas an increase in the hippocampus was only observed after repeated administration of cocaine[168]. In contrast, methamphetamine administration did not affect tPA in the NAc after a single, but only after repeated exposure[165]. Compared with wild-type mice, *tPA*$^{-/-}$ mice showed an attenuated potentiation of dopamine release in the NAc upon repeated methamphetamine administration[299] and decreased behavioral sensitization and CPP[165]. Injection of tPA into the NAc could partly restore methamphetamine-induced behavioral sensitization[165]. In line with this, lentiviral vector-mediated overexpression of tPA in the NAc led to an increase of cocaine- or amphetamine induced locomotor activity and CPP[166,168]. With respect to tobacco use, nicotine administration increased tPA activity levels in the NAc[188].

Nicotine-induced CPP was found to be diminished in *tPA*$^{-/-}$ compared with wild-type mice, possibly as a result of the impaired nicotine-induced dopamine release in the NAc also observed in these animals.

The role of MMPs in psychostimulant-induced locomotor sensitization and contextual learning has also been explored. Repeated methamphetamine administration leading to locomotor sensitization was associated with increased MMP-2 and MMP-9 levels in the frontal cortex and NAc[84]. *MMP-2*$^{-/-}$ and *MMP-9*$^{-/-}$ mice, as well as wild-type mice infused with a non-specific MMP inhibitor into the frontal cortex, showed impaired locomotor sensitization and CPP in response to methamphetamine[84]. Furthermore, repeated methamphetamine administration was associated with increased dopamine release and reduced dopamine reuptake in the NAc of wild-type mice, whereas these processes were attenuated in *MMP-2*$^{-/-}$ and *MMP-9*$^{-/-}$ mice[84].

Concerning cocaine, intracerebral ventricular infusion of MMP inhibitor FN-439 prior to conditioning sessions hindered the acquisition of a preference for the cocaine-paired context and led to a reduced cocaine-primed reinstatement of CPP after extinction training[119]. Although infusion of FN-439 did not affect extinction or reinstatement responding when given prior to extinction sessions, infusion prior to two consecutive drug-primed reinstatement sessions resulted in a reduced CPP during these sessions, and completely abolished preference in a subsequent third reinstatement session. Furthermore, when FN-439 was infused immediately after two reinstatement sessions, this resulted in a reduced preference for the drug-paired compartment in the second test, as well as a subsequent test[119]. Whereas MMP-2 and MMP-3 levels were unchanged in two areas involved in contextual learning and extinction, the dorsal hippocampus and the mPFC, MMP-9 activity was increased specifically in the mPFC after drug-primed reinstatement of cocaine CPP in animals that underwent extinction of cocaine CPP[300]. These results suggest that MMP-9 activity in the mPFC might contribute to the retrieval and/or reconsolidation of a cocaine-associated contextual memory by facilitating synaptic remodeling[300]. Alternatively, the observation that MMP-9 activity is unaltered in the mPFC of animals that did not receive extinction training before reinstatement supports the idea that mPFC MMP-9 activity might underlie a modification of the extinction memory[300].

In continuation of chapter 2, the role of brevican in addictive behavior was further explored in chapter 3. As a first step to determine whether reduced levels of ECM components could affect drug reward learning, we showed that genetic reduction of brevican levels using *Bcan*$^{+/-}$ mice augmented sensitivity to cocaine reward learning in the CPP paradigm. AAV-mediated rescue of brevican expression levels in the dHC of *Bcan*$^{-/-}$ mice was sufficient to reduce cocaine CPP during a remote test (chapter 3). In line with our observations that reduced ECM levels increase expression of addictive behavior, it was previously found that inhibition of ECM breakdown using a broad-spectrum MMP inhibitor interfered with acquisition and reinstatement of cocaine CPP[119]. However, the finding that MMP-9 was regulated in the mPFC after reinstatement of cocaine CPP, but not in the dHC[300], indicates that the nature of the involvement of ECM remodeling is likely to depend on the brain area, addiction phase and behavioral paradigm. Brevican cleavage might be a downstream effect of psychostimulant-induced activa-

tion of ECM degrading enzymes, as brevican is a substrate of a vast number of metalloproteinases[301]. Importantly, although brevican is not a substrate of MMP-9, this MMP might indirectly affect Bcan levels by processing other MMPs, such as MMP-2 and MMP-13, into their active forms[290].

Finally, two other ECM components, the glycoprotein reelin and the heparan sulfate proteoglycan syndecan-3, have been implicated in psychostimulant-induced addictive behavior. A single methamphetamine treatment resulted in the transient downregulation of reelin mRNA in the frontal cortex of rats[302], whereas reelin expression was upregulated in the NAc after cocaine self-administration[303]. Interestingly, histone deacetylase inhibitor trichostatin A, which decreased cocaine self-administration rates, also normalized the upregulation of reelin in the NAc and upregulated reelin levels in the mPFC[303]. In addition, overexpression of reelin in excitatory forebrain neurons was shown to attenuate cocaine-induced locomotor sensitization in mice[304]. Syndecan-3 mRNA was upregulated in the lateral hypothalamus of rats after cocaine

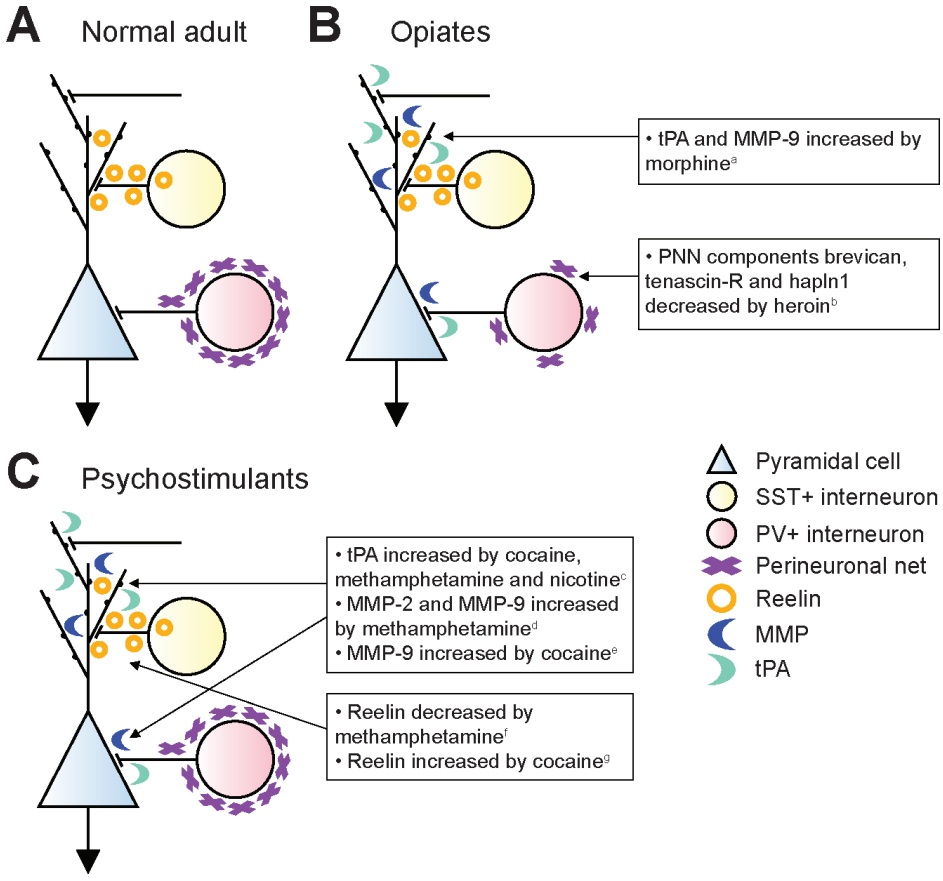

Figure 1. Schematic representation of the consequences of drug administration for ECM levels and proteases in animal models. Compared with their respective controls (**A**), levels of PNN components, reelin, tPA and MMPs are changed after exposure to opiates (**B**) and psychostimulants (**C**). a[164,296]; b[85]; c[165,168,298]; d[84]; e[300]; f[302]; g[303].

self-administration[305]. *Syndecan-3*$^{-/-}$ mice showed higher rates of cocaine self-administration, an effect that could be rescued by AAV-mediated re-expression of syndecan-3 in the lateral hypothalamus.

Conclusions: ECM and addiction

Exposure to opiates and psychostimulants has been associated with altered activity of ECM degrading proteases, in particular MMP-2, MMP-9 or tPA, and altered levels of structural ECM proteins in the mPFC, NAc and hippocampus (Figure 1). This supports the hypothesis that, by facilitating ECM remodeling, addictive drugs open up a plasticity window that supports drug-induced neuroadaptations and thereby underlie the persistent nature of addiction. Interestingly, an increasing number of studies show that experimental manipulations that reduce ECM breakdown or elevate ECM levels intervene with addictive behavior in different animal models of addiction, including morphine, cocaine and methamphetamine behavioral sensitization[84,164,165,306], morphine, cocaine, amphetamine, methamphetamine and nicotine CPP (chapter 3 and [84,119,164-166,188,306]), cocaine self-administration[305] and reinstatement of heroine seeking (chapter 2).

It becomes clear that different drugs of abuse have overlapping effects on ECM remodeling, in particular on the regulation of tPA. This ECM protease is released from neurons in an activity-dependent manner[307] and has been implicated in synaptic plasticity[308,309]. This suggests an important role for tPA in drug-induced neural alterations that underlie the development of addiction. Available evidence suggests that an important downstream effect is the adaptation of dopaminergic neurotransmission in the NAc[306], as this process is impaired in *tPA*$^{-/-}$ mice after exposure to morphine, nicotine and methamphetamine[164,188,295,299]. However, the drugs of abuse covered in this discussion do not exhibit identical effects on ECM remodeling. For example, morphine, cocaine and methamphetamine induce overlapping but distinct spatial patterns of tPA regulation (see above), whereas altered MMP2 levels in the frontal cortex appear to be involved in the response to methamphetamine but not cocaine[84,300]. The exact nature of drug-induced ECM changes likely depends on the type of drug, brain area, behavioral paradigm and addiction phase. Importantly, increased availability of literature comparing the same parameters (e.g., ECM protein expression levels and protease activity) with similar methods (e.g., immunoblot and zymography analysis of specific brain regions after different addiction phases) will enable a better evaluation of similarities and differences between different drugs of abuse.

Furthermore, available literature on neural ECM and drug addiction is currently largely focused on the drug-induced regulation of ECM proteases and their genetic or pharmacological manipulation. Little, however, is known about the relevant downstream targets that are affected. A small number of studies suggests the downstream involvement of MMP substrates and/or signal transduction proteins, such as integrins, intercellular adhesion molecules, epidermal growth factor receptor (EGFR) and extracellular signal-regulated kinase (ERK)[310-314]. Importantly, chapter 2 and 3 add to this knowledge by describing the identification of a number of ECM proteins, including brevican and tenascin-R, that were regulated after heroin expo-

sure, and the characterization of their functional involvement in reinstatement of heroin seeking after self-administration and in the expression of cocaine CPP. Future studies that analyze changes in protease activity, identify ECM components and quantify their expression levels in parallel, and evaluate causality between specific ECM components and addictive behavior will add to our understanding of the role of neural ECM in addiction.

Finally, it is important to explore to what extent it is possible to translate such findings from animal models to the human situation. Only two sets of studies currently allow the comparison between animal models and human subjects. MMP-9 activity was reduced in the hippocampus of human cocaine abusers, whereas it was elevated in the mPFC and unchanged in the dorsal hippocampus of mice after reinstatement of CPP[167,300]. Concerning alcohol, an addictive drug not discussed in this thesis, elevated levels of MMP-9 were found in the blood of alcoholics, whereas repeated exposure to alcohol resulted in reduced MMP-9 activity in the hippocampus and PFC of rats[87,187]. These are seemingly opposite results, however, the mismatch between the experimental designs and procedures could well explain the apparent lack of consistency between these studies. This underlines the importance of resolving the dynamics and the location of ECM modulation that specifically parallels different phases of the disease progression. In addition, it will be crucial to determine the translational value of altered ECM remodeling observed in animal models in the future.

Molecular mechanisms of relapse to nicotine seeking

Three decades ago, self-administration of nicotine by rats was reported for the first time[315]. Later studies showed the potential of nicotine-associated cue exposure, drug priming and stress to induce reinstatement of nicotine seeking[210,316-318]. Since then, a wide range of pharmacological compounds, including antagonists of nicotinic cholinergic, dopamine, $GABA_B$, metabotropic glutamate and cannabinoid receptors, have been reported to modulate reinstatement of nicotine seeking[210,228,229,231,319-321]. However, our knowledge of the involvement of specific brain areas and the molecular mechanisms that drive this behavior is limited.

Pharmacological inactivation of the insular cortex, in which hypocretin (Hcrt) neurotransmission via Hcrt-1 receptors regulates nicotine self-administration[262], decreased cue- and drug-induced reinstatement of nicotine seeking[263]. In apparent contrast to these and other reports implying the insular cortex in nicotine addiction[322], I did not observe any cue-induced regulation of synaptic proteins in the insular cortex (chapter 5). As discussed in this chapter, this might be explained by the heterogeneity of the insula, the absence of clear landmarks to ensure accurate dissection of this area or localization of plasticity mechanisms outside of the synapse. Alternatively, it is possible that the insula is subject to acute changes that we could not pick up with the employed technique (e.g., posttranslational modifications, such as phosphorylation).

Evidence for the involvement of such mechanisms in nicotine seeking comes from a study that analyzed the phosphorylation state of dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32), a protein implicated in the actions of several drugs of abuse[323]. A short incubation period after nicotine self-administration, which led to higher rates of

cue-induced reinstatement, was associated with altered phosphorylation of DARPP-32 in the insular cortex and the NAc core, but not in the mPFC and NAc shell[322]. However, the consequences of this modification for reinstatement of nicotine seeking were left uninvestigated.

A number of other neural mechanisms have been implied in nicotine relapse. Cue-induced reinstatement after nicotine self-administration has been associated with activation of Hcrt neurons in the lateral hypothalamus and phosphorylation levels of p38 mitogen-activated protein kinase (MAPK) and glutamate receptors GluA2 and GluN1 in total lysates of the NAc[324]. Systemic administration of an Hcrt-1, but not Hcrt-2, receptor antagonist normalized phosphorylation of p38 MAPK and GluN1 and attenuated reinstatement of nicotine seeking. Intra-accumbens infusion of an inhibitor of protein kinase C, a kinase that phosphorylates GluA2 and GluN1, decreased cue-induced reinstatement rates[324]. The phosphorylation state has consequences for the conductance and trafficking of glutamate receptors[325]. This underlines the involvement of glutamate plasticity in the NAc in regulating reinstatement of nicotine seeking[201].

In agreement with the common distinction between the two subregions of the NAc, the core and the shell[326], these regions have been differentially implicated in reinstatement of nicotine seeking. Whereas injection of LY235959, a competitive NMDA receptor antagonist, into the NAc core led to increased rates of cue-induced reinstatement of nicotine seeking, injection into the shell subregion was without effect[327]. After extinction of nicotine self-administration, dendritic spine head diameter and the AMPA / NMDA ratio were increased in the NAc core compared with saline-treated animals[248]. At the molecular level, increases in GluA1, GluN2A and GluN2B and a decrease in glutamate transporter-1 (GLT-1) protein levels were observed. After cue-induced reinstatement, the effect on spine diameter and AMPA / NMDA ratio was augmented and extracellular glutamate levels in the NAc core were increased. In contrast to injection of LY235959[327], injection of a GluN2A antagonist into the NAc core attenuated cue-induced reinstatement of nicotine seeking[248]. These contrasting findings can be due to different experimental parameters or pharmacological properties of the compounds, but are consistent with the idea that glutamate neurotransmission in the NAc core regulates reinstatement.

In chapters 4 and 5, acute neuroplasticity associated with cue-induced reinstatement of nicotine seeking was investigated. In the mPFC, reinstatement of nicotine seeking resulted in an altered composition of synaptic protein levels. SHPS-1, a transmembrane protein involved in intercellular communication[277,280], was downregulated after cue exposure (chapter 5), whereas levels of synaptic $GABA_A$ receptor subunits α1 and γ2 were elevated (chapter 4). Interestingly, reinstatement of nicotine seeking was increased by blocking membrane insertion of $GABA_A$ receptors in the dorsal mPFC, but not the ventral mPFC, with a peptide that interferes with the interaction between the γ2 subunit and trafficking protein GABARAP[230,243]. Furthermore, stimulation of GABAergic neurotransmission with the $GABA_A$ receptor agonist muscimol attenuated reinstatement after microinjection into the dorsal or ventral mPFC. These results indicate that both mPFC subregions are part of the neural circuitry mediating reinstatement of nicotine seeking, but emphasize the role of GABAergic plasticity in the dorsal mPFC in

controlling this behavior. Together with the data on glutamatergic plasticity in the NAc core[248], this points to a central role for the dorsal mPFC-to-NAc core projection in controlling cue-evoked nicotine seeking.

Cortical GABAergic interneurons exert local inhibitory control over pyramidal cells and interneurons[328]. In turn, pyramidal cells in the dorsal mPFC send a major glutamatergic projection to inhibitory medium spiny neurons in the NAc core[244,329]. I propose a model for cue-induced reinstatement of nicotine seeking (Figure 2) in which, upon cue exposure, neural circuitry that includes the insula and the mPFC-to-NAc projection is activated. The insula, which is involved in processing of interoceptive states, conscious feelings and decision-making, provides excitatory input to the mPFC[33,330]. Activation of the dorsal mPFC-to-NAc core projection results in glutamate release in the NAc core[248], paralleled by potentiation of glutamatergic synapses in this area[248,324] and reinstatement of nicotine seeking. Simultaneous upregulation of synaptic $GABA_A$ receptors in the dorsal mPFC acts to counterbalance activation of the glutamatergic projection from this region to the NAc core, thereby constraining response rates ("compensatory neuroplasticity", chapter 4). It follows that reinstatement can be inhibited by antagonizing glutamatergic neurotransmission in the NAc[248]. In contrast, reduced GABAergic neurotransmission in the dorsal mPFC, after blocking $GABA_A$ receptor membrane insertion, increases glutamatergic neurotransmission and augments reinstatement of nicotine seeking (chapter 4). It is likely that a more extended neural circuitry underlies reinstatement of nicotine seeking, however the other brain areas remain to be determined.

Parallels between reinstatement mechanisms of nicotine, heroin and cocaine seeking

Similar to nicotine, the glutamatergic dorsal mPFC-to-NAc core projection has a key role in reinstatement of heroin and cocaine seeking[73,76,201,218], the two other drugs of abuse investigated in this thesis. Here, I will compare the limited available data on the neurobiological mechanisms associated with cue-induced reinstatement of nicotine seeking with those of cue-evoked heroin and cocaine seeking to evaluate similarities and differences between these drugs. Neurobiological aspects of heroin and cocaine addiction have been compared elsewhere; for excellent reviews see[189,331,332].

In line with nicotine, pharmacological inactivation of the insular cortex decreased cue-induced reinstatement of cocaine seeking[333]. The effect of pharmacological manipulation of this area on heroin reinstatement has not been studied. In addition, whereas data on the regu_lation of DARPP-32 in the insula is lacking, protein levels and phosphorylation of this protein in the NAc were altered after cocaine administration[334]. Exposing DARPP-32 phosphomutant mice and controls to cocaine indicated that DARPP-32 phosphorylation in the NAc mediates molecular and behavioral effects of cocaine[335]. However, data on the role of DARPP-32 in reinstatement of heroin or cocaine seeking are currently lacking.

Systemic pharmacological blockade of the Hcrt-1 receptor attenuated cue-induced reinstatement of nicotine, but also of heroin and cocaine seeking[324,336,337]. Cue-induced food seeking was not affected by this compound[324], indicating that this effect does not extend to operant behavior for non-drug rewards. The absence of effect on reinstatement of an Hcrt-2

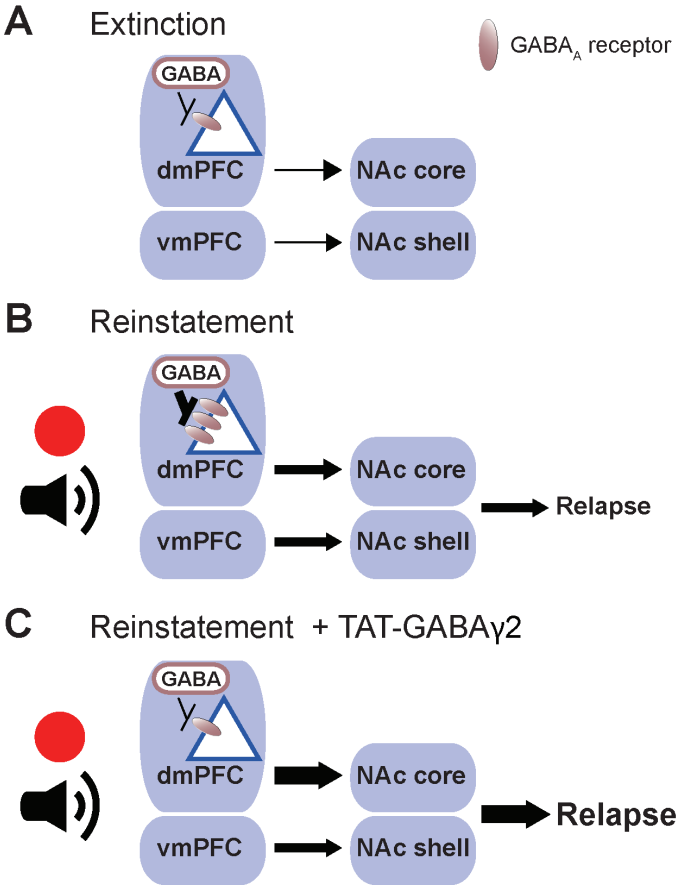

Figure 2. Working model for cue-induced reinstatement of nicotine seeking. After extinction of nicotine self-administration (**A**), cue exposure precipitates reinstatement (**B**). This behavior is mediated by activation of the mPFC and NAc (potentially receiving input from the insular cortex). The glutamatergic projection from the dorsal mPFC to the NAc core has a key role in regulating this process. GABAergic plasticity in the dorsal subregion of the mPFC limits reinstatement by increasing inhibitory input to pyramidal cells in the dorsal mPFC, whereas glutamatergic plasticity in the NAc core induces reinstatement by increasing glutamatergic neurotransmission. (**C**) Blocking membrane insertion of GABA$_A$ receptors with the GABAγ2 peptide in the dorsal mPFC prevents the reinstatement-associated increase of GABAergic inhibition in this area, resulting in a higher excitatory output to the NAc core and augmented reinstatement levels.

receptor antagonist has been shown for nicotine and cocaine[324,337].

Enhanced glutamate transmission in the nucleus accumbens is thought to have a central role in relapse to addictive drugs, including nicotine, heroin and cocaine[99,125,201]. For these three drugs, increased levels of extracellular glutamate in the NAc core have been observed that were associated with reinstatement[127,152,248]. Similar to nicotine[248,324,327], glutamatergic plasticity in the NAc has been associated with cue-induced reinstatement of heroin and cocaine seeking. Whereas dendritic spine size and AMPA/NMDA ratio in the NAc core were found reduced after extinction of heroin self-administration, these parameters were increased after cue-induced reinstatement[221]. Protein levels of GLT-1 and GluN2B, but not GluN2A, in this area were in-

creased after extinction[221,338]. Injection of a GluN2B antagonist into the NAc core abolished the changes in dendritic spine size and AMPA/NMDA ratio and attenuated cue-induced reinstatement. Furthermore, siRNA-mediated knockdown of GluN2B decreased reinstatement compared with knockdown of GluN2A[221]. Finally, injection of an AMPA/kainate receptor antagonist into the NAc core also decreased cue-induced reinstatement of heroin seeking[127].

With respect to cocaine, a decrease of GLT-1 and an increase of GluA1 and mGluR5 protein levels has been observed in the NAc after cocaine withdrawal[339-341]. Similar to nicotine and heroin[221], cue-induced reinstatement was paralleled by an increase in dendritic spine size and AMPA/NMDA ratio in the NAc core[266]. Local injection of antagonists of AMPA/kainate and NMDA receptors into the NAc core has been shown to attenuate cue-induced reinstatement of cocaine seeking[342]. Furthermore, data on nicotine reinstatement suggest a role for PKC-dependent regulation of glutamatergic plasticity[324]. Whereas phosphorylation of glutamate receptors has not been observed after cue-induced reinstatement of cocaine seeking, drug-induced reinstatement was associated with increased phosphorylation of GluA2 in the NAc shell[343]. Local disruption of GluA2 trafficking in the NAc core (which featured a non-significant increase of phosphorylated GluA2) or shell attenuated reinstatement. In addition, cocaine-induced reinstatement increased PKCγ phosphorylation in the NAc core and shell, whereas inhibition of PKC attenuated this behavior in both subregions of the NAc[344,345]. This suggests that PKC-mediated phosphorylation of glutamate receptors might also have a role in cue-induced reinstatement of cocaine seeking. Together, these studies indicate an important role of glutamatergic plasticity in the NAc in controlling reinstatement of heroin and cocaine seeking.

Pharmacological inactivation of the dorsal mPFC abolished the rise in accumbal extracellular glutamate and drug-induced reinstatement of heroin seeking[221], as well as the increase in dendritic spine size and AMPA/NMDA ratio in the NAc core and cue-induced reinstatement of cocaine seeking[266], which underscores the importance of the mPFC-NAc projection in heroin and cocaine seeking. In line with this, inactivation of the dorsal or ventral mPFC attenuated cue-induced reinstatement of heroin seeking[115]. In contrast, whereas inactivation of the dmPFC also decreased cue-induced reinstatement after self-administration of cocaine, inactivation of the ventral mPFC was without effect[252].

The increase of prefrontal synaptic levels of brevican and tenascin-R isoforms after reinstatement of heroin seeking (chapter 2) was not observed after reinstatement of nicotine seeking using quantitative proteomics (chapter 5). Furthermore, in contrast to the GABAergic plasticity in the mPFC found associated with nicotine reinstatement (chapter 4), the acute downregulation of GluN2B, GluA2 and GluA3 in mPFC synaptic membranes was identified as a key mechanism underlying cue-induced reinstatement of heroin seeking[109]. No regulation of $GABA_A$ receptor subunits α1 or γ2 was found associated with reinstatement of heroin seeking (unpublished results). Blocking GluA2 endocytosis, which resulted in decreased rates of heroin seeking after injection into the ventral mPFC[109], did not affect reinstatement of nicotine seeking (chapter 4). In line with this, no regulation of glutamate receptors was found after cue-induced reinstatement of nicotine seeking using either a proteomics approach (chapter 5) or immuno-

blotting (chapter 4). However, pharmacologically targeting GABAergic neurotransmission or plasticity (i.e., membrane insertion of $GABA_A$ receptors) in the dorsal mPFC was sufficient to bidirectionally regulate reinstatement of nicotine seeking (chapter 4).

Conclusions: Molecular mechanisms controlling relapse

Evidently, there are similarities and differences in the neurobiological mechanisms associated with cue-induced reinstatement of nicotine, heroin and cocaine seeking, despite the fact that not all mechanisms have consistently been investigated for all three drugs of abuse. A common finding is that activation of the glutamatergic mPFC-NAc projection has a key role in reinstatement, a process paralleled by increased extracellular glutamate concentrations and glutamatergic plasticity, such as an increase in dendritic spine head size and AMPA/NMDA ratio in the NAc core[221,248,266]. However, other glutamatergic plasticity mechanisms in this area, such as regulation and phosphorylation of glutamate receptors, only partially overlap (chapter 4 and [109,221,248,324,339,343]). In addition, after extinction of self-administration of nicotine and cocaine, synapses in the NAc core appear to be in a more potentiated state (e.g., increase in GluA1 levels, dendritic spine head size and AMPA/NMDA ratio) than after self-administration of heroin (decrease dendritic spine head size and AMPA/NMDA ratio)[221,248,339].

Strikingly, using identical experimental approaches, different neural plasticity mechanisms in the mPFC have been identified to be associated with cue-induced reinstatement of nicotine and heroin seeking. Nicotine and heroin reinstatement were paralleled by respectively GABAergic plasticity in the dorsal mPFC (chapter 4) and glutamatergic plasticity in the ventral mPFC[109]. Importantly, these mechanisms were shown to be drug-specific and causally related to reinstatement. Similarly, reinstatement of heroin seeking was associated with ECM remodeling (chapter 2), whereas the proteomics screen described in chapter 5 did not identify regulation of any ECM components after reinstatement of nicotine seeking. Manipulation of ECM levels altered heroin and cocaine seeking behavior in rodents (chapter 2 and 3). This suggests a drug-dependent involvement of ECM plasticity in reinstatement, although the possibility that manipulation of ECM levels also affects nicotine seeking cannot be ruled out at the moment.

To conclude, reinstatement of nicotine seeking entails similar but distinct neural circuitry and molecular mechanisms compared with heroin and cocaine. This provides a rational base for the development of interventions aimed at common targets, such as the glutamate transporter GLT-1, which is downregulated in the NAc after withdrawal from all three drugs of abuse[248,338,341]. However, the identification of drug-specific molecular mechanisms that drive reinstatement might permit the development of interventions that intervene with relapse to a specific drug of abuse but minimally affect other neural processes and behaviors.