Chapter 1

General introduction
Historical aspects of drug use

Religious, medicinal and recreational use of mind-altering substances by humans has a history of thousands of years. For example, smoking of tobacco to exploit the stimulating effects of nicotine and tobacco use during ceremonies date back at least 3000 years ago in Central America. Different hallucinogenic substances have been used by cultures in Asia and in parts of the New World to induce revelations and transcendental experiences. In addition, several psychoactive substances, including cocaine from coca leaves, opium from poppy seeds, cannabis and hallucinogens, have been widely exploited as anesthetic and to relieve pain and sorrow in the last millennia. Interestingly, these substances are still applied in a clinical setting today and continue to be the subject of investigation, for example to explore their potential for the treatment of neurological and psychiatric disorders.

Repeated exposure to a subset of psychoactive substances – or ‘drugs of abuse’ – can lead to drug addiction. This is a chronically relapsing disorder characterized by loss of control over drug intake despite negative consequences. Although addictive drugs have diverse molecular targets, they share the property that they activate the brain's mesocorticolimbic reward system. This evolutionary conserved system, with at its core the ventral tegmental area (VTA), nucleus accumbens (NAc) and prefrontal cortex (PFC; Figure 1), evolved to regulate motivation by reinforcing behavior that activates this circuitry. Upon extended use, addictive substances increase motivation to consume more frequently and with increasing amounts. Nonetheless, vulnerability to become addicted varies per individual and depends on both genetic (e.g., single nucleotide polymorphisms) and environmental factors (e.g., development, personal circumstances, drug availability).

Addiction has a major impact on society while effective pharmacotherapies are lacking

Due to our innate vulnerability to become addicted, along with developments that led to increased potency and availability of addictive drugs, drug addiction is presently a major societal problem. Besides being a significant source of social problems, such as relationship disturbances and unemployment, it is a primary cause of morbidity and mortality worldwide. For example, there is a very strong relation between smoking and cancer, lung disease and cardiovascular disease. According to the World Health Organization, the global annual death rate associated with tobacco use is nearly 6 million, or 1 in every 10 adult deaths, and this number is increasing. In the Netherlands, the number of smokers has decreased over the last decades, but is now stabilizing at approximately 3.6 million, with an estimated 18.900 deaths directly caused by smoking annually. In addition to personal suffering, huge societal costs are associated with drug addiction. Health care costs, crime and loss of productivity are associated with 600 billion dollars in the United States alone. In the Netherlands, merely the costs of health care related to drug addiction sum up to 1.2 billion euros.

Hence, a lot is to be gained from preventing drug use and supporting drug addicts to refrain from drug use, for example by anti-drug campaigns, psychological care and help with...
reintegration into society. Drug addiction is nowadays regarded as a neuropsychiatric disorder and is identified based on criteria that are described in the Diagnostic and Statistical Manual of Mental Disorders 5 (DSM-5). The view that drug addiction is a brain disease has attracted attention to the development of pharmacotherapies as tools to facilitate prolonged drug abstinence. By targeting the neural mechanisms underlying drug addiction and relapse, such therapies should reduce the rewarding effects of addictive drugs or craving for (i.e., desire to use) drugs. For a range of addictive drugs (i.e., opiates, nicotine and alcohol), a limited number of therapies is currently approved by the FDA. However, the effectiveness of pharmacotherapies to prevent relapse remains low, and for some drugs (e.g., cocaine) not even a single therapy has yet been approved. At the same time, the availability of techniques that allow a more detailed analysis of the neurobiological mechanisms underlying addiction (e.g., proteomics analysis) might offer a basis for developing more effective anti-addiction therapies.

**Human imaging studies revealed the brain circuitry of addiction**

Imaging studies have provided insight in the brain areas that are involved in initiation and persistence of addictive behavior. The principal circuit is the mesocorticolimbic system (Figure 1), in which the VTA provides dopaminergic input in the NAc and PFC. Human positron emission tomography (PET) studies, using radioligands that compete for binding to dopamine receptors, have shown for several drugs of abuse that their administration leads to increased release of the neurotransmitter dopamine in the striatum, including the NAc. During initial drug taking, especially the VTA-to-NAc dopaminergic projection is central to the rewarding effect of addictive drugs.

Upon repeated exposure, a more extensive circuitry gets involved, as has been shown by studies using functional magnetic resonance imaging (fMRI). This imaging technique monitors regional changes in neural activity, measured as altered blood flow. A key brain region

**Box 1. Molecular targets of nicotine, heroin and cocaine**

*Tobacco* is manufactured from dried leaves of plants of the genus *Nicotiana*. These plants produce the alkaloid nicotine, the major addictive component of tobacco, as a chemical defense to prevent being eaten. Nicotine functionally mimics the neurotransmitter acetylcholine by activating or desensitizing endogenous receptors in our brain, the so-called nicotinic acetylcholine receptors. *Heroin*, a synthetic drug, is a morphine-based diacetyl ester that crosses the blood-brain barrier more rapidly than its precursor. Morphine is an opiate produced by the opium poppy *Papaver somniferum*. In the brain, heroin is converted back to its active metabolite morphine and activates opioid receptors, such as the μ-opioid receptor. *Cocaine* is extracted from the coca plant (family *Erythroxylaceae*). Its central effect includes inhibition of dopamine reuptake by blocking monoamine transporters.

*Psychoactive substances such as nicotine, heroin and cocaine affect nervous system function, including cognitive processes, through modulating synaptic transmission between neurons. The psychostimulants nicotine and cocaine induce a feeling of relaxation and alertness, whereas administration of heroin and cocaine is associated with euphoria. However, repeated exposure to these substances can lead to addiction. Above a certain threshold, intake results in reduced performance and at high levels in death.*
implicated in drug addiction using fMRI is the PFC, and more specifically several of its sub-regions, such as the anterior cingulate cortex, the dorsolateral PFC and the orbitofrontal cortex. The PFC exerts executive control over limbic regions and is regarded important for cognitive functions including decision making and inhibitory control. For several drugs of abuse, fMRI studies show that drug administration leads to activation of the PFC, whereas a drug-free state is associated with decreased activation of this brain region in addicted individuals. Most importantly, craving for drugs induced by presentation of drug-associated cues results in increased activation of prefrontal circuitry in drug addicts but not in non-addicted controls. As craving is an important factor that often evokes relapse, the PFC is regarded as a promising target in addiction intervention strategies.

Evidently, other brain regions are involved in addiction. In addition to the NAc and PFC, dopaminergic projections from the VTA target the amygdala and hippocampus. These areas have important roles in associative learning and have been found to be activated by environmental stimuli that are associated with drug use. Upon extended access to drugs, the dorsal part of the striatum is thought to acquire an increasingly important role in habit-like, compulsive drug taking. Furthermore, craving is associated with activation of the insula, a cortical brain region involved in monitoring the body’s interoceptive state and maintaining internal homeostasis.

The involvement of the areas mentioned above is relatively independent of the type of drug, patient population and imaging method used in the study. Ethical considerations greatly restrict the use of invasive techniques in human subjects, limiting direct exploration of the neurobiological mechanisms that underlie addiction. To learn more about these mechanisms, animal models that mimic aspects of addictive behavior, predominantly employing rodents, have been developed and investigated over the last decades. Whereas subcortical regions of the addiction circuitry, such as the striatum and the hippocampus, are anatomically and functionally conserved in humans and rodents, comparison between cortical brain regions is less straightforward. Particularly, prefrontal regions do not allow one-to-one comparisons of
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subareas. Taking into account specific characteristics, such as the cytoarchitecture, pattern of neural connections and functional properties, the rodent medial prefrontal cortex (mPFC) is considered to resemble areas of the primate PFC involved in addiction in humans (i.e., anterior cingulate cortex and dorsolateral PFC)\(^3\).\

Paradigms to study addictive behavior in animals

Drug use is paralleled by the formation of an association between the rewarding effects of the drug and neutral environmental stimuli\(^3\). Through this associative memory, these stimuli (or cues) acquire rewarding and motivational properties\(^3\). As a consequence, drug-associated cues can precipitate craving and relapse in human addicts. Addiction paradigms combine aspects of classical and operant conditioning to provoke an association between addictive drugs and cues and exploit this association to investigate the neurobiological mechanisms underlying different aspects of drug addiction, including reward, reinforcement and drug seeking behavior.

Conditioned place preference: an animal model to study drug reward and associative learning

Reward-induced learning or conditioning can be modeled using the conditioned place preference (CPP) paradigm (Box 2). Temporal pairing of a rewarding drug and a neutral context transfers rewarding properties of the drug to the context, eliciting approach behaviors\(^3\). As such, higher rewarding properties are thought to contribute to an increase in time spent in the drug-paired compartment when animals are allowed to freely explore a drug-paired and neutral context\(^3-4\). CPP studies after physical or pharmacological manipulations of specific brain areas show involvement of brain areas that are also activated by drugs or drug-associated cues in humans\(^4\). A reduced expression of CPP has been observed after inactivation of brain areas including the VTA, NAc, mPFC and insula\(^4,5\). However, the involvement of specific areas can depend on experimental details of the CPP protocol used. For example, lesions of the prelimbic cortex were without consequences for the acquisition of cocaine CPP in one study\(^6\), whereas another study reported a reduced CPP\(^7\). This discrepancy was suggested to be due to procedural differences, such as differences in food availability\(^6\).

Box 2. Conditioned place preference

The CPP model is based on the principle that conditioned environmental stimuli are paired with an unconditioned stimulus, the drug, after which the association between the two, reflecting the strength of the drug memory, is evaluated. A CPP setup typically consists of two compartments that differ in terms of tactile and visual properties. An initial preference for one of the two compartments is determined in a pre-conditioning test, during which the animal can freely move between the compartments. Next, one of the two compartments is paired with a drug reward (a drug injection before restricting the animal to that compartment), while the other compartment is paired with vehicle. After a specific number of conditioning sessions, preference for any compartment is tested again and expressed relative to the initial preference. Formation of a preference for the drug-paired compartment is interpreted as being mediated by the drug’s rewarding effect\(^3\).
**Drug self-administration: an animal model to study drug reinforcement and relapse**

A second behavioral paradigm is the operant self-administration model of addictive drugs (Box 3). Due to its face and predictive validity, this is arguably the most sophisticated animal model to disentangle the different behavioral phases, and hence molecular mechanisms, of the addiction process. Typically, drug self-administration elicits other molecular responses in the brain than forced exposure, dissociating the cognitive aspects of self-administration from the pharmacological effect of the drug. As the model resembles different phases of the human addiction cycle, including relapse, face validity is generally considered to be high. Self-administration also has good predictive validity, as it can be used to assess drug abuse liability and has been recognized as reliable method to identify compounds affecting cocaine and heroin use in humans. Similar to humans, self-administration of addictive drugs is associated with activation of the mesocorticolimbic system and dopamine release in target areas of the VTA. Moreover, relapse to drug seeking entails brain regions identified in human imaging studies noted above, including the NAc, mPFC, insula and hippocampus.

**Box 3. Drug self-administration**

To allow active control over intake of drugs of abuse, animals are implanted with an intravenous catheter. During daily sessions in an operant cage, the catheter is connected to a syringe containing the drug, after which operant responding (i.e., lever pressing or nose poking) is reinforced by an infusion of a small dose of drugs. An exception is alcohol self-administration, during which the drug is supplied in a receptacle in the operant cage. Generally, each infusion is paired with audio-visual cues (i.e., a tone and a light cue) to provoke an association between the drug and specific environmental stimuli. These drug-paired cues can be used at a later stage to evoke recall of memory related to the previously self-administered drug. In addition to an active nose poke hole (or lever), the operant cage contains an inactive hole; an inactive response is without consequences and allows the experimenter to control for non-specific responding and locomotor effects.

Under fixed ratio (FR) responding, in which a fixed number of responses is required to obtain a single drug infusion, animals commonly acquire a preference for the active operandum within several days, independent of the type of drug used. Drug reinforcement is measured during this self-administration phase. A progressive ratio (PR) test can be used to measure motivation to obtain a drug reward. This reinforcement schedule requires the animal to work more for every subsequent reward, by increasing the number of responses necessary for each additional drug infusion.

Two variants of the self-administration procedure to model loss of control over drug use, or compulsive drug use, were introduced a decade ago. The first makes use of extended access to the drug of abuse (i.e., more and longer self-administration sessions). Extended access, but not limited access to cocaine, causes rats to continue cocaine seeking behavior despite negative consequences in the form of a foot shock. In the second model, three major DSM-IV criteria for drug addiction are modeled to distinguish between resilient non-addict and vulnerable addict drug-taking rats. Drug use despite negative consequences, a high motivation to take drugs and difficulty suppressing drug seeking are modeled by respectively coupling drug delivery with a foot shock, self-administration under a PR ratio and evaluation of drug seeking when no drugs are available. These models aim to better reflect an advanced addiction stage in humans, characterized by compulsive drug use despite negative consequences.
Drug-induced neuroplasticity and neuroadaptations support persistent drug taking and seeking

The neural effects of addictive drugs and drug-associated stimuli can be characterized as acute changes (neuroplasticity; minutes to hours) and long-term changes (neuroadaptations; days to months). In the weeks after discontinuation of drug use, an increase of cue-induced craving and relapse to drug seeking can be observed in humans and animals, respectively\(^{52,53}\). This phenomenon is known as incubation of drug craving\(^{53}\) and indicates that neuroadaptations parallel drug taking as well as abstinence. These changes are thought to embody a persistent drug memory that is the core of the chronically relapsing nature of addiction. Knowledge of the neural substrates of this memory yields potential targets for developing improved therapies for addiction\(^{54}\). Moreover, this knowledge might give new insights in factors contributing to the individual vulnerability to become addicted. This highlights the importance of increasing our knowledge of the neurobiological mechanisms underlying drug addiction.

As described above, drugs of abuse share the ability to stimulate dopamine signaling in the mesocorticolimbic system. Nicotine directly activates these dopamine neurons, whereas other psychostimulants, such as cocaine and amphetamine, interfere with the reuptake of dopamine by inhibiting reuptake or reversing its transport via binding to dopamine transporters. Opioids exert their effect by inhibiting VTA GABAergic cells, resulting in disinhibition of dopaminergic neurons in the VTA and increased dopamine release\(^{8,54}\). Neuroplasticity in dopaminergic neurons in the VTA, an “early drug trace”, can already be observed after a single injection of drugs\(^{54}\). All drugs of abuse share the property that they strengthen excitatory input to these neurons, measured as increased AMPA/NMDA ratio in VTA slices\(^{55}\). The molecular basis of changes in the AMPA/NMDA ratio are alterations in the number as well as the composition of AMPA and NMDA glutamate receptors.

Although evidence suggests that a single drug exposure does not lead to neuroadaptations in higher brain areas, repeated drug use does affect this circuitry\(^{56}\). In addition to structural changes\(^{57}\), drugs of abuse affect synapse physiology in the NAc and mPFC. Repeated injections of cocaine, for example, lead to a decreased AMPA/NMDA ratio in the NAc, whereas after a period of withdrawal the AMPA/NMDA ratio was increased in this area\(^{56}\). These neuroadaptations in the NAc might be hierarchically dependent on prior plastic changes in the VTA\(^{58}\). The involvement of drug-evoked plasticity in the transition from drug use to drug addiction was suggested by a set of experiments using an adapted self-administration protocol.

After drug self-administration, animals may undergo a drug-free period in the form of abstinence or extinction. During abstinence, animals are restricted to their home cage, resembling withdrawal in the absence of drug cues. Extinction is an active learning process during which animals are (repeatedly) exposed to the operant cage, but with the drug and drug-associated audio-visual cues made unavailable. Finally, the self-administration model is extensively used to investigate the neurobiological underpinnings of relapse to drug seeking. After a drug-free period (abstinence or extinction), relapse to drug seeking can be precipitated by a drug infusion, presentation of drug-associated cues or stress.
that differentiates between non-addict and addict rats (see Box 3). Cocaine self-administration leads to abolished NMDA-dependent long-term depression (LTD) in the NAc of both groups, whereas only the addict rats do not recover this form of LTD after prolonged self-administration. Interestingly, only these rats exhibit abolished mGluR2/3-dependent LTD in the mPFC. These observations are consistent with the suggestion that drug addiction can be viewed as a staged pathology. In addition, they emphasize the relevance of glutamatergic plasticity in the mesocorticolimbic system in addiction, in particular related to prefrontal glutamatergic projections that control NAc output.

These and other studies contributed to the concept that learning and memory formation and drug-evoked plasticity comprise the same fundamental synaptic plasticity mechanisms. Structural changes in dendritic branching as well as modification of the strength of neural connectivity, such as long-term potentiation (LTP) and LTD and their molecular correlates (alterations in the composition of neurotransmitter receptors and other synaptic proteins) have a central role in the storage of memories. This is underlined by studies using model systems for synaptic plasticity and animal models for learning and memory, for example auditory and contextual fear conditioning. Learning of an association between a tone and an aversive stimulus during auditory fear conditioning is paralleled by LTP in the lateral amygdala. In turn, LTP in this area depends on AMPA receptor exocytosis. In addition to the amygdala, the dorsal hippocampus (dHC) and the mPFC are important for the encoding and expression of memories. Whereas the amygdala and dHC have been implicated in respectively the emotional and contextual components of memory formation, the mPFC might have a role in the integration of these components. Molecular and physiological studies using the fear conditioning paradigm point out that glutamatergic plasticity in the hippocampus and the amygdala parallels the formation and modification of fear memories. Drug self-administration studies point towards an important role of the mPFC, hippocampus and the amygdala in addiction. In line with this, similar neuroplasticity mechanisms in these brain areas parallel addictive behavior.

The extracellular matrix and addiction
The appearance of studies linking addiction to alterations in the composition of the extracellular matrix (ECM) is consistent with the notion that fundamental plasticity mechanisms involved in learning and memory also underlie initiation and persistence of addictive behavior. The ECM in the brain principally consists of the polysaccharide hyaluronic acid and glycoproteins and proteoglycans, including members of the lectican family, tenascins and link proteins. In addition to their role in structural support, these proteins have roles in neural signaling and plasticity. A role for the ECM in the regulation of neuroplasticity can be concluded from studies investigating the visual cortex during early development, when formation of the ECM parallels maturation of neural circuits towards the end of the critical period. In turn, chondroitinase-mediated breakdown of the ECM is sufficient to reopen this critical period. LTP-inducing stimuli result in protease-mediated regulation of ECM components, whereas experimental manipulations of ECM levels generally impair LTP. Finally, at the molecular level,
the ECM may act as a scaffold for proteins that mediate plasticity and as a lateral diffusion barrier for neurotransmitter receptors, as has been shown for AMPA receptors. Several recent human and animal studies suggest a relation between addictive behavior and altered ECM levels, indicating the brain's ECM as an interesting subject of future addiction studies.

Summary
In summary, drug addiction is a great burden to society. Efforts to increase our understanding of the neurobiological mechanisms underlying drug addiction and relapse are critical in order to develop more effective pharmacotherapies. Examining the nature of drug-induced neuroplasticity and neuroadaptations will contribute to our knowledge of factors that increase vulnerability to become addicted and will reveal neural substrates that are potential targets for pharmacotherapy. Importantly, the neural mechanisms central to addiction might not be unique to this field of research, as evidence suggests they resemble the fundamental neural mechanisms that mediate learning and memory.

Overview of this thesis
This thesis describes the search for novel molecular mechanisms that underlie cue-induced relapse to heroin (chapter 2) and nicotine seeking (chapter 4 and 5) in rats. The mechanism identified in chapter 2 is further explored in a cocaine CPP model using a genetically modified mouse strain (chapter 3).

To identify novel proteins contributing to relapse to heroin seeking, a proteomics screen comparing animals that underwent heroin self-administration and a period of abstinence with drug-naïve animals was performed (chapter 2). Heroin-exposed animals demonstrated reduced levels of ECM proteins, including tenascin-R and the lectican brevican, in biochemical preparations of mPFC synaptic membranes. Furthermore, an identical analysis after extinction of heroin self-administration showed that ECM levels in both the NAc and mPFC were reduced. Cue-induced relapse resulted in an acute (partial) normalization of these levels and was accompanied by an increase in inhibitory input to prefrontal pyramidal cells. Considering that the identified ECM proteins condense around GABAergic interneurons of the mPFC, ECM turnover might be related to the changes observed in GABAergic neurotransmission. Recovery of ECM levels prior to relapse by pharmacological inhibition of ECM breakdown was sufficient to attenuate cue-induced relapse to heroin seeking, underlining the functional relevance of this neural mechanism.

To gain a better insight in the contribution of brevican, one of the ECM components regulated after heroin self-administration, to addictive behavior, I studied the brevican+/− mouse using the CPP paradigm (chapter 3). This mouse showed reduced protein expression levels of brevican similar to relapse-prone rats. Compared with wild-type littermates, brevican+/− mice showed enhanced expression of a cocaine-associated memory without an effect on fear learning, suggesting that reduced brevican levels specifically enhance cocaine reward learning. Adenoviral vector-mediated expression of brevican in the dHC of brevican+/− mice, but not in the mPFC and NAc, resulted in an attenuation of expression of the cocaine memory three
weeks after conditioning. This points towards a role for ECM plasticity in the dHC in the formation of a cocaine memory.

Knowledge of neurobiological mechanisms underlying nicotine relapse lags behind compared with knowledge about relapse to the illicit drugs heroin and cocaine, despite the fact that nicotine addiction is much more prevalent. To elucidate the molecular mechanisms driving relapse to nicotine seeking, I measured the regulation of neurotransmitter receptors most commonly implicated in neuroplasticity in synaptic membrane preparations of the mPFC (chapter 4). Although I observed no regulation of glutamate receptors, I found the α1 and γ2 subunits of the GABA_A receptor upregulated after cue-induced reinstatement of nicotine seeking. Local inhibition of GABA_A receptor membrane insertion in the dorsal mPFC resulted in augmented reinstatement rates, suggesting that GABAergic neurotransmission in this area acts to suppress relapse. Accordingly, the GABA_A receptor agonist muscimol attenuated reinstatement of nicotine seeking, suggesting that GABA_A receptors are a potential novel target for development of smoking cessation therapies.

To further investigate the role of the mPFC, and the insula – a brain region repeatedly implicated in nicotine addiction in the last decade – I performed a proteomic screen to identify novel proteins involved in cue-induced reinstatement of nicotine seeking (chapter 5). After both abstinence and extinction, no reinstatement-associated protein regulation was observed in the insula, suggesting a limited role for neuroplasticity in nicotine relapse in this brain area. However, in the mPFC, Src homology 2 domain-containing protein tyrosine phosphatase substrate-1 (SHPS-1) was found to be downregulated independent of the protocol used. This regulation was confirmed in an independent set of animals after extinction of nicotine self-administration. These results suggest that SHPS-1, a transmembrane protein involved in intercellular communication, is an interesting candidate for further research into the neurobiology of relapse to nicotine seeking.

Finally, the findings described in this thesis will be discussed in a broader context in chapter 6. Whereas the circuitry involved in addictive behavior appears to be similar for different drugs of abuse, recent evidence suggests that the molecular mechanisms regulating drug taking and seeking are at least in part unique for specific drugs. The first part of my general discussion will evaluate the current knowledge of the involvement of ECM remodeling in addictive behavior. The focus of the second part is on the molecular mechanisms underlying cue-induced reinstatement of nicotine seeking and the comparison with the other addictive drugs used in the research described in this thesis: heroin and cocaine.