Roles of forced nicotine exposure and *Comt* gene disruption in the development of addiction-related behavioural and neurochemical changes in mice.

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ACADEMIC DISSERTATION

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To my parents

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ABSTRACT

Activation of midbrain dopamine systems is thought to be critically involved in the addictive properties of abused substances. Drugs of abuse increase dopamine release in the nucleus accumbens and dorsal striatum, which are the target areas of mesolimbic and nigrostriatal dopamine pathways, respectively. Dopamine release in the nucleus accumbens is thought to mediate the attribution of incentive salience to rewards, and dorsal striatal dopamine release is involved in habit formation. In addition, changes in the function of prefrontal cortex (PFC), the target area of mesocortical dopamine pathway, may skew information processing and memory formation such that the addict pays an abnormal amount of attention to drug-related cues. In this study, we wanted to explore how long-term forced oral nicotine exposure or the lack of catechol-*O*-methyltransferase (COMT), one of the dopamine metabolizing enzymes, would affect the functioning of these pathways. We also wanted to find out how the forced nicotine exposure or the lack of COMT would affect the consumption of nicotine, alcohol, or cocaine.

First, we studied the effect of forced chronic nicotine exposure on the sensitivity of dopamine D_2-like autoreceptors in microdialysis and locomotor activity experiments. We found that the sensitivity of these receptors was unchanged after forced oral nicotine exposure, although an increase in the sensitivity was observed in mice treated with intermittent nicotine injections twice daily for 10 days. Thus, the effect of nicotine treatment on dopamine autoreceptor sensitivity depends on the route, frequency, and time course of drug administration.

Second, we investigated whether the forced oral nicotine exposure would affect the reinforcing properties of nicotine injections. The chronic nicotine exposure did not significantly affect the development of conditioned place preference to nicotine. In the intravenous self-administration paradigm, however, the nicotine-exposed animals self-administered nicotine at a lower unit dose than the control animals, indicating that their sensitivity to the reinforcing effects of nicotine was enhanced.

Next, we wanted to study whether the *Comt* gene knock-out animals would be a suitable model to study alcohol and cocaine consumption or addiction. Although previous work had shown male *Comt* knock-out mice to be less sensitive to the locomotor-activating effects of cocaine, the present study found that the lack of COMT did not affect the consumption of cocaine solutions or the development of cocaine-induced place preference. However, the present work did find that male *Comt* knock-out mice, but not female knock-out mice, consumed ethanol more avidly than their wild-type littermates. This finding suggests that COMT may be one of the factors, albeit not a primary one, contributing to the risk of alcoholism.

Last, we explored the effect of COMT deficiency on dorsal striatal, accumbal, and prefrontal cortical dopamine metabolism under no-net-flux conditions and under levodopa load in freely-moving mice. The lack of COMT did not affect the extracellular dopamine concentrations under baseline conditions in any of the brain areas studied. In the prefrontal cortex, the dopamine levels remained high for a prolonged time after levodopa treatment in male, but not female, *Comt* knock-out mice. COMT deficiency induced accumulation of 3,4-dihydroxyphenylacetic acid, which increased further under levodopa load. Homovanillic acid was not detectable in *Comt* knock-out animals either under baseline conditions or after levodopa treatment.

Taken together, the present results show that although forced chronic oral nicotine exposure affects the reinforcing properties of self-administered nicotine, it is not an addiction model itself. COMT seems to play a minor role in dopamine metabolism and in the development of addiction under baseline conditions, indicating that dopamine function in the brain is well-protected from perturbation. However, the role of COMT becomes more important when the dopaminergic system is challenged, such as by pharmacological manipulation.

TIIVISTELMÄ

Tammimäki, Anne 2008. *Pakotetun nikotiinialtistuksen ja* Comt*-geenipuutoksen vaikutus riippuvuuteen liittyvien neurokemiallisten ja käyttäytymismuutosten kehittymisessä hiirillä.*

Huumeiden ja muiden riippuvuutta aiheuttavien aineiden yhteinen nimittäjä on kyky aktivoida keskiaivojen dopamiinijärjestelmiä. Ne lisäävät dopamiinin vapautumista akkumbens-tumakkeessa ja dorsaalisessa striatumissa, jotka ovat mesolimbisen ja nigrostriataalisen dopamiiniradan päätealueet. Akkumbens-tumakkeessa dopamiinin ajatellaan välittävän palkitsevien tapahtumien muuttumista halutuiksi, ja dorsaalisessa striatumissa sen on todettu osallistuvan tapojen muodostukseen. Lisäksi muutokset mesokortikaalisen radan päätealueen, etuaivokuoren, toiminnassa voivat vääristää tiedonkäsittelyä ja muistin toimintaa siten, että addiktoitunut yksilö kiinnittää suhteettoman paljon huomiota huumeisiin liittyviin ympäristön tekijöihin. Tässä tutkimuksessa haluttiin selvittää, kuinka pitkäkestoinen pakotettu juomaveden kautta tapahtuva nikotiinialtistus tai katekoli-*O*-metyylitransferaasin (COMT) puutos vaikuttaa näiden dopamiiniratojen toimintaan. Lisäksi halusimme tutkia, miten pakotettu nikotiinialtistus tai COMT-puutos vaikuttaa nikotiinin, alkoholin tai kokaiinin kulutukseen.

Ensin tutkimme mikrodialyysi- ja liikeaktiivisuuskokein, miten pakotettu krooninen nikotiinialtistus vaikuttaa dopamiinin D_2-tyypin autoreseptorien herkkyyteen. Havaitsimme, että herkkyys ei muutu pakotetun nikotiinijuoton seurauksena, mutta se lisääntyy hiirillä, joille on annettu toistetusti nikotiinipistoksia kahdesti päivässä 10 päivän ajan. Näin ollen nikotiinikäsittelyn vaikutus autoreseptoriherkkyyteen riippuu antotavasta ja mahdollisesti myös käsittelyn kestosta.

Toiseksi selvitimme, vaikuttaako pakotettu nikotiinijuotto nikotiini-injektioiden palkitseviin vaikutuksiin. Krooninen nikotiinialtistus ei merkitsevästi muuta nikotiinin aiheuttamaa paikkahakuisuutta. Nikotiinialtistetut eläimet kuitenkin itseannostelevat nikotiinia matalammalla annostasolla kuin verrokkihiiret. Tämä viittaa siihen, että nikotiinialtistetut hiiret olivat verrokkeja herkempiä nikotiinin palkitseville vaikutuksille.

Kolmanneksi halusimme tutkia, olisivatko geenimuunnellut hiiret, joilta puuttuu COMT-entsyymi, käyttökelpoinen eläinmalli alkoholi- ja kokaiiniriippuvuuksien tutkimiseen. Vaikka aiemmin on todettu, että kokaiini ei kiihdytä liikeaktiivisuutta näissä hiirissä yhtä paljon kuin vastaavissa villityypin hiirissä, COMT-puutos ei vaikuta kokaiiniliuosten kulutukseen tai kokaiinin aiheuttamaan paikkahakuisuuteen. Alkoholiliuoksia *Comt*-poistogeeniset hiiriurokset kuitenkin juovat runsaammin kuin verrokkihiiret, mutta tätä vaikutusta ei havaita naarashiirissä. Tulosten perusteella näyttäisi siltä, että COMT saattaa olla yksi, vaikkakaan ei keskeisin, osatekijä alkoholismin kehittymisessä.

Lopuksi selvitimme miten COMT-puutos vaikuttaa dorsaalistriatumin, akkumbens-tumakkeen ja etuaivokuoren dopamiinimetaboliaan perustilassa sekä levodoparasituksen aikana hereillä olevilla hiirillä. Menetelminä käytettiin tavallista ja no-net-flux-mikrodialyysitekniikkaa. COMT-puutos ei vaikuta solunulkoiseen dopamiinipitoisuuteen millään tutkituista aivoalueista. Levodopa-annoksen jälkeen etuaivokuoren dopamiinitasot säilyvät kuitenkin pidempään korkeina *Comt*-poistogeenisillä hiiriuroksilla kuin verrokkihiirillä, mutta samanlaista vaikutusta ei ole nähtävissä naarashiirillä. COMT-puutos aiheuttaa 3,4-dihydroksifenyylietikkahapon kumuloitumisen, joka korostuu edelleen levodopan annon vaikutuksesta. Homovanilliinihappoa ei ole mitattavia pitoisuuksia *Comt*-poistogeenisien hiirien näytteissä perustilassa eikä levodoparasituksen aikana.

Yhteenvetona voidaan todeta, että vaikka krooninen nikotiinialtistus juomavedessä vaikuttaa itseannostellun nikotiinin palkitseviin vaikutuksiin, se ei itsessään ole riippuvuuden malli. COMT:n rooli addiktion riskitekijänä näyttää olevan pieni. Sen merkitys dopamiinimetaboliassa kuitenkin korostuu, kun dopaminerginen järjestelmää rasitetaan esim. farmakologisin keinoin.

ORIGINAL PUBLICATIONS

I **Tammimäki, A.**, Pietilä, K., Raattamaa, H., Ahtee, L. Effect of quinpirole on striatal dopamine release and locomotor activity in nicotine-treated mice. European Journal of Pharmacology 531:118-125, 2006.

II **Tammimäki, A.**, Chistyakov, V., Patkina, N., Skippari, J., Ahtee, L., Zvartau, E., Männistö, P.T. Effect of forced chronic oral nicotine exposure on intravenous self-administration and rewarding properties of acute nicotine. European Journal of Pharmacology, 591:164-170, 2008.

III **Tammimäki, A.**, Forsberg, M. M., Karayiorgou, M., Gogos, J. A., Männistö, P. T. Increase in free choice oral ethanol self-administration in male mice with *Comt* gene disruption. Basic & Clinical Pharmacology & Toxicology, 103:297-304, 2008.

IV **Tammimäki, A.**, Käenmäki, M., Pakarinen, K., Keisala, T., Karayiorgou, M., Gogos, J. A., Männistö, P. T. Minor effect of *Comt* gene disruption on striatal, accumbal and frontal cortical extracellular dopamine concentrations in no-net-flux conditions or under levodopa load in freely moving mice. Manuscript.

LIST OF ABBREVIATIONS

3-MT	3-Methoxytyramine
5-HIAA	5-Hydroxyindole acetic acid
5-HT	5-Hydroxytryptamine
ANOVA	Analysis of variance
AUC	Area under the curve
COMT	Catechol-*O*-methyl transferase
Comt	Catechol-*O*-methyl transferase gene
CPP	Conditioned place preference
CREB	Cyclic adenosine monophosphate responsive element binding protein
DAT	Dopamine transporter
Dat1	Dopamine transporter gene
DAT-CI	Mouse strain with cocaine-insensitive dopamine transporter
DOPA	3,4-Dihydroxyphenylalanine
DOPAC	3,4-Dihydroxyphenyl acetic acid
Drd1-5	Genes for dopamine receptors D_1-D_5
E_d	Extraction fraction
GABA	γ-Aminobutyric acid
GC-MS	Gas chromatograph with mass spectrometric detection
HET	Heterozygous
HOM	Homozygous
HPLC	High performance liquid chromatograph
HVA	Homovanillic acid
ICSS	Intracranial self-stimulation
i.p.	Intraperitoneally
i.v.	Intravenously
IVSA	Intravenous self-administration
L-DOPA	L-3,4-dihydroxyphenylalanine, levodopa
MAO	Monoamine oxidase
Maoa	Monoamine oxidase A gene
Maob	Monoamine oxidase B gene
MDMA	3,4-Methylenedioxymethamphetamine
MOPEG	3-Methoxy-4-hydroxy-phenylglycol
m/z	Mass-to-charge ratio
NA	Noradrenaline
nAChR	Nicotinic acetylcholine receptor
NET	Noradrenaline (norepinephrine) transporter
NMDA	N-methyl-D-aspartic acid
PCR	Polymerase chain reaction
R	Ratio criterion
s.c.	Subcutaneously
SEM	Standard error of mean
SIM	Single ion monitoring
siRNA	Small interfering ribonucleic acid
SNP	Single nucleotide polymorphism
TAAR1	Trace amine associated receptor 1
Th	Tyrosine hydroxylase gene
VMAT	Vesicular monoamine transporter
Vmat2	Vesicular monoamine transporter 2 gene
VNTR	Variable number tandem repeat (polymorphism)
WT	Wild-type littermates

1 INTRODUCTION

Addiction to drugs of abuse is a major cause of disability and health-care costs in Western countries. The overall cost of substance abuse (including alcohol and tobacco) has been estimated to be up to 3.5% of the gross domestic product in North American and European countries (Pouletty, 2002). Substance abuse is also predicted to be the leading preventable cause of premature deaths in the world by the year 2020 (Murray and Lopez, 1997). Recently, new drugs for the treatment of alcohol and tobacco dependence have been launched, but their efficacy is modest (Bouza et al., 2004; Wu et al., 2006). In addition, no drugs exist to support cessation of psychostimulant use, and we still do not have effective tools to fight relapses that can occur even after several years of abstinence. The development of drugs for addictions is hampered by incomplete knowledge of the mechanisms of addiction. Nevertheless, it is widely accepted that the mesocorticolimbic dopaminergic pathway is involved in the development of addiction (Hyman et al., 2006). One of the aims of this thesis was to elucidate some of those alterations that chronic nicotine exposure induces in this pathway. Studies in humans have shown that polymorphisms of the catechol-*O*-methyltransferase gene (*Comt*) may be linked to addiction (e.g. Beuten et al., 2006; Tiihonen et al., 1999), and quantitative trait loci studies in mice have suggested that COMT activity may contribute to the severity of drug or alcohol use (Grice et al., 2007). Therefore, another aim of this work was to find out how *Comt* disruption affects self-administration of oral drugs and the function of the mesocorticolimbic dopamine pathway in mice.

Dependence refers to a drug-induced state in which the cessation of drug use produces a physiological withdrawal syndrome (Koob and Le Moal, 2006). *Addiction* is a chronic disease characterized by relapses, compulsive, and uncontrollable drug use, as well as emergence of a negative emotional state when the drug is not available. Dependence is included in the diagnostic criteria for addiction and it can also occur without physical signs of withdrawal. On the other hand, dependence can develop towards a variety of drugs, most of which are not addictive. For example, a laxative-dependent individual is typically not addicted to laxatives; his or her bowels simply do not function properly any longer without the daily dose of the drug.

2 REVIEW OF LITERATURE: GENETICALLY MODIFIED MICE IN ADDICTION RESEARCH

2.1 Addiction theories

Early addiction theories suggested that addicts use drugs to alleviate withdrawal symptoms; in other words, substances of abuse were seen as negative reinforcers (Nestler, 1992; Wise and Bozarth, 1987). This hypothesis has several drawbacks. For example, both people and animals self-administer opioids in the absence of withdrawal symptoms or physical dependence, and several drugs produce withdrawal symptoms even though they are not readily self-administered (Robinson and Berridge, 1993). In addition, clinical evidence shows that relieving the withdrawal symptoms is marginally effective in the treatment of addiction (Wise and Bozarth, 1987).

The positive reinforcement theory of addiction is the basis of modern addiction theories. This view postulates that drugs are used because of the pleasant state that they induce, not because of the alleviation of an unpleasant state (Robinson and Berridge, 1993; Wise and Bozarth, 1987). However, there are several problems with the hypothesis that the pleasurable effects of the drugs alone are sufficient to maintain drug use. First, in order to maintain drug use, the rewarding properties of the drug should be enormous in proportion to the negative consequences of using the drug (Robinson and Berridge, 1993). For example, nicotine can produce very severe addiction although its psychotropic effects are mild (Anthony et al., 1994; Koob and Le Moal, 2006). In addition, drug addicts often experience diminished or disturbed reward effects from drug use (Lamb et al., 1991; Robinson and Berridge, 1993). Moreover, mere positive reinforcement does not explain craving or relapse elicited by conditioned stimuli (Robinson and Berridge, 1993).

Definitions of certain terms linked to the addiction theories are represented in Table 1.

Table 1 *A short glossary of terms used in the addiction theories.*

Term	Definition
Incentive salience	Attractiveness of external stimuli, events, places and their mental representations as well as their ability to capture attention. Incentive salience always applies to the perception of external events and their internal representation. Brain actively attributes incentive salience to particular perceptions based on their association with past activation of the mesocorticolimbic dopamine systems. The attribution is an unconscious process and only the product of it, "wanting", can be consciously experienced. (Robinson and Berridge, 1993) Incentive salience can also be described as a subcomponent of reward. According to this description it is nearly synonymous to drug wanting. (Koob and Le Moal, 2006)
Incentive stimulus	Stimulus that has been attributed with incentive salience. Consequently, it has become salient and "wanted". (Robinson and Berridge, 1993; Koob and Le Moal, 2006)
Natural incentive	Unconditioned stimulus, such as food, water, or sexual partner. These stimuli have evolved during the evolution and their meaning is to promote the survival of the individual or the species. Some of them are state dependent (hunger, thirst). (Robinson and Berridge, 1993; Koob and Le Moal, 2006)
Artificial incentive	Unconditioned stimulus, such as substances of abuse or electrical brain stimulation. These stimuli activate the process of incentive stimulus formation more directly than the natural incentives. Therefore, these stimuli can become wanted even in the absence of pleasure. (Robinson and Berridge, 1993; Koob and Le Moal, 2006)
Liking	Synonymous to pleasure or hedonia. Liking is usually the trigger that activates the components of associative learning and incentive salience. (Robinson and Berridge, 1993; Koob and Le Moal, 2006)
Wanting	The subjective feeling of needing or desiring something, or motivation to take drugs (Robinson and Berridge, 1993; Koob and Le Moal, 2006)
Craving	Pathologically intense wanting, only magnitude discriminates wanting from craving. (Robinson and Berridge, 1993; Koob and Le Moal, 2006) OR "Memory of the rewarding aspects of drug use superimposed on a negative affective state". (Koob and Le Moal, 2008)
Negative reinforcer	Negative reinforcers promote substance use by terminating an aversive state, e.g. stress or anxiety. (Wise and Bozarth, 1987; Koob and Le Moal, 2006)
Positive reinforcer	Positive reinforcers promote substance use, possibly by inducing a pleasurable state, e.g. pleasure, relief, or gratification. Positive reinforcement probably has a role in the induction phase of addiction that is characterized by impulsivity. Negative reinforcement, on the other hand, is linked with the expression phase of addiction, which is characterized by compulsivity. (Wise and Bozarth, 1987; Koob and Le Moal, 2006)
Antireward systems	The brain stress or emotional systems that are activated in response to excessive utilization of the brain reward system. (Koob and Le Moal, 2008)
Allostasis, allostatic state	Allostasis means stability through alteration, and allostatic systems tend to undergo constant change (Koob and Le Moal, 2008). Allostasis is a physiological term describing deviation from homeostasis. In the allostatic state referred to in the allostatic theory of addiction, the set-point of reward gradually shifts below the homeostatic range.

2.1.1 The incentive sensitisation theory of addiction

One widely accepted addiction theory is the incentive sensitisation model by Robinson and Berridge (1993, 2001). The basis of this theory is that repeated substance use causes progressive and persistent neuroadaptations and, as a consequence, addictive behaviour. The theory rests on the assumption that drug "liking" produced by unconditioned stimuli and drug "wanting" caused by conditioned stimuli are two different processes. During repeated drug use, drug "wanting" sensitises, whereas drug "liking" does not. Sensitisation of the neural substrate for "wanting" results in an enhancement of incentive salience attribution. As the associative learning processes are concomitantly activated, the focus of incentive salience is increasingly directed towards drug-related stimuli. These stimuli control behaviour with increasing efficiency, and "wanting" develops into obsessive craving. Since the sensitisation is virtually permanent, addicts are prone to relapse even after prolonged abstinence.

2.1.2 The allostatic theory of addiction

More recently, a theory focussing on the motivational aspects of addiction has evolved ("allostatic view of addiction"; Koob and Le Moal, 1997, 2008). The opponent-process theory suggests that during the development of addiction, the initial drug effect is opposed or counteracted by homeostatic changes (Figure 1; Koob and Le Moal, 1997; Solomon and Corbit, 1974). The opponent-process is divided into two phases. The a-process consists of hedonic responses to the drug. It occurs instantly after drug stimulus and correlates well with the stimulus intensity, quality, and duration. Furthermore, the a-process exhibits tolerance with repeated exposure to the drug. The b-process appears after the termination of the a-process. It begins and decays slowly, but it grows in magnitude with repeated exposure. More recently, the opponent-process theory has been expanded into an allostatic model of motivational systems in the brain. In this model, addiction is depicted as a cyclical process (Koob and Le Moal, 1997, 2008). The cycle consists of three stages: drug consumption and intoxication, withdrawal and negative mood after drug consumption, as well as drug preoccupation and anticipation between bouts of drug use. Repeated frequent drug use results in decreased function of brain reward systems, leading to progressive enhancement of antireward systems. Together, these changes produce an allostatic state of the reward systems and compulsive drug use.

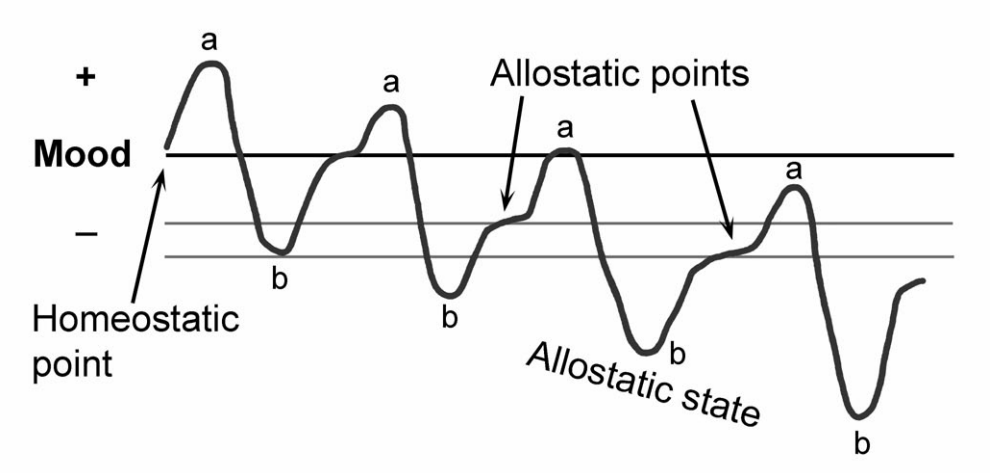

Figure 1 *The allostasis model of addiction. According to the opponent-process and allostasis model of addiction, the response to drugs can be divided into two successive processes. The first is an* a-process *(peaks of the curve), which presents a positive mood state, and the second is a counteradaptive* b-process *(valleys of the curve). If the b-process is appropriate, it only balances the activation state of a-process and retains the homeostatic mood state. However, when drugs are frequently readministered, b-process never returns to the homeostatic point. This results in development of allostatic points of mood and allostatic affective state in the addicted individual. In this state, the brain reward system is underactivated, whereas the brain stress system is overactivated. The allostatic state is sustained even during protracted abstinence. Modified from Koob and Le Moal (2001).*

2.1.3 The role of dopamine in addiction

All addictive drugs increase the synaptic levels of dopamine in the nucleus accumbens either directly or indirectly (Di Chiara and Imperato, 1988). Consequently, dopamine is generally considered to be the key neurotransmitter in the development of addiction (for a review, see Hyman et al., 2006). The mesolimbic dopamine pathway is the most important of the brain dopamine circuits in the development of addiction (Figure 2). It mediates reward prediction error (Schultz, 2006), cue learning (Ito et al., 2004), and motivation to obtain reward (Berridge and Robinson, 1998). It is involved in the induction and expression of behavioural sensitisation (Cador et al., 1995; Robinson and Berridge, 1993), and in the reinstatement of cocaine use (Shaham et al., 2003). However, there is evidence that drug-induced reward may not be directly correlated with extracellular dopamine concentration (Berridge and Robinson, 1998; Cannon and Palmiter, 2003; Robinson et al., 2005). Instead, the burst activity of mesolimbic dopamine neurons occurs during reward prediction but the activity stops when the reward actually occurs (Schultz, 1998, 2006). Therefore, dopamine seems to be involved in the acute effects of drug use and the initiation of addiction, but during the transition process from recreational drug use to end-stage

addiction, the importance of dopaminergic mechanisms gradually decays. The changes in the function of the glutamatergic projections from the prefrontal cortex to the nucleus accumbens probably become more prominent in end-stage addiction (Kalivas and Volkow, 2005).

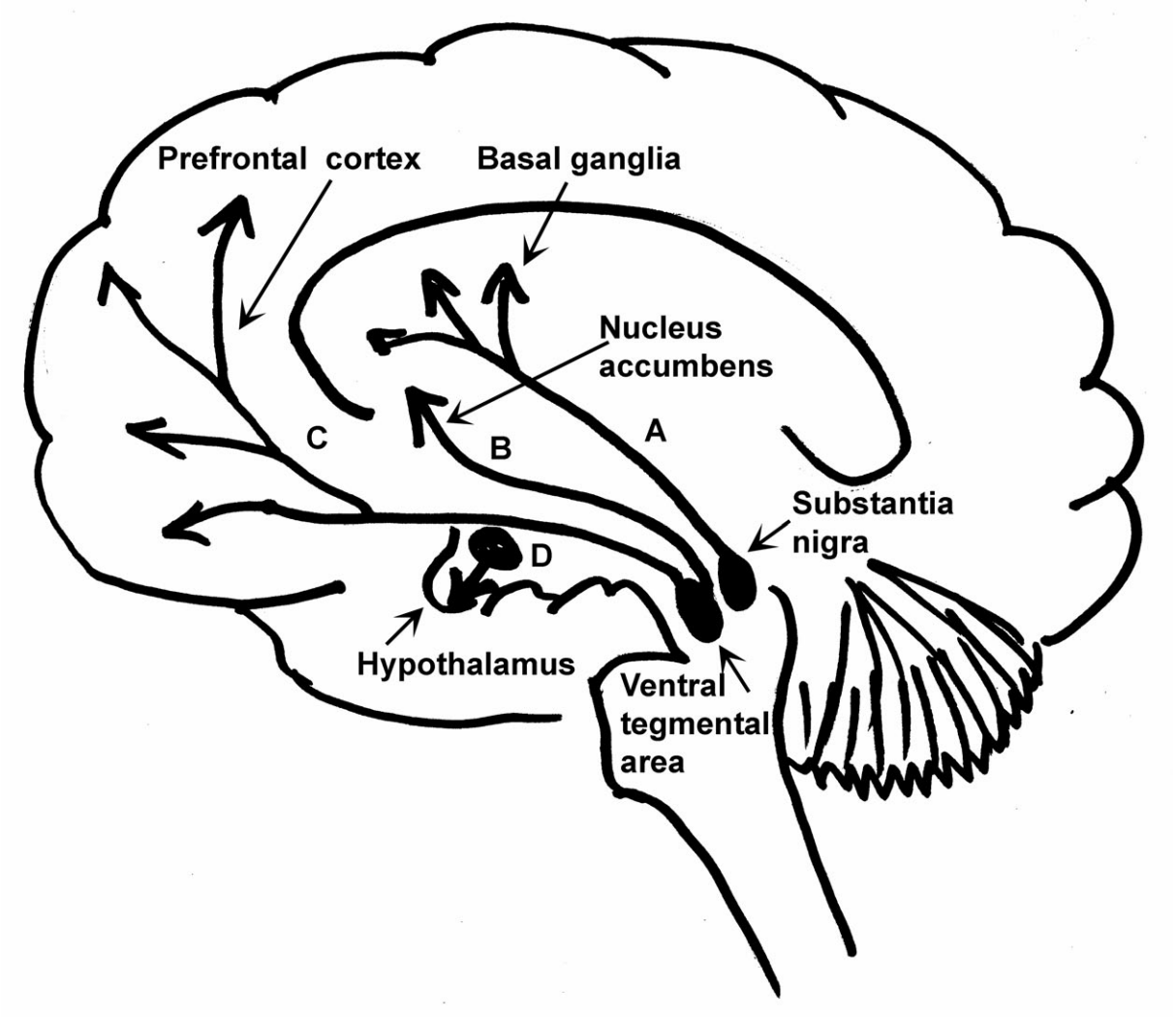

Figure 2 *Main dopaminergic pathways in the brain. A = nigrostriatal pathway, B = mesolimbic pathway, C = mesocortical pathway, D = tuberoinfundibular pathway.*

In addition, the nigrostriatal dopamine pathway and the mesocortical dopamine pathway play particular roles in addiction-related processes. The former is involved in habit formation (Yin and Knowlton, 2006), and the latter in reinstatement of drug-seeking (Kalivas et al., 2005; Shaham et al., 2003), modulation of goal-directed behaviour (Grace et al., 2007; Montague et al., 2004), and impulsivity (Hyman et al., 2006).

2.2 Mouse models to study addiction

None of the existing animal models of addiction completely emulates the human condition, but they do permit investigation of certain elements of the addiction process. In rats, several sophisticated methods have been created to study different aspects of addiction (see Koob and Le Moal, 2006 and Sanchis-Segura and Spanagel, 2006 for comprehensive reviews). However, many of them are difficult to implement in mice, and often only modified models can be used. The following sections present those addiction models that have been used in genetically modified mice bearing mutations in the dopaminergic system.

2.2.1 Behavioural sensitisation

Behavioural sensitisation is an extensively exploited model of behavioural plasticity. It refers to the progressive and long-lasting enhancement of certain behaviours, e.g. horizontal locomotor activity and stereotypic movements, in response to repeated, intermittent treatment with psychostimulants, μ-opioids, nicotine, alcohol, dopamine D_2 receptor agonists and NMDA antagonists (Babbini and Davis, 1972; Badiani et al., 1995; Difranza and Wellman, 2007; Phillips et al., 1997; Segal and Mandell, 1974; Stolerman et al., 1973; Vezina, 2004). In addition, sensitised behaviours may occur at lower doses, have shorter latencies, and be more intense than before sensitisation (Babbini and Davis, 1972; Segal et al., 1980). For most substances except nicotine, this enhanced behavioural response can also be detected in mice (Itzhak and Martin, 1999; Pietilä et al., 1998).

Behavioural sensitisation can be separated into two components. The first phase is induction, which involves activation of dopamine D_1 and glutamate receptors in the ventral tegmental area and substantia nigra, as well as regulation of the firing of ventral tegmental area dopamine neurons by glutamatergic projections from the prefrontal cortex and amygdala (Bjijou et al., 1996; Kalivas and Alesdatter, 1993; Wolf et al., 1995; Wolf, 1998). The neurochemical changes during the induction phase, such as the desensitisation of dopamine D_2 autoreceptors, are transient (Ackerman and White, 1990; Henry et al., 1989). The second phase is the expression of sensitisation, which is assumed to result from persistent neurochemical alterations in the nucleus accumbens (Cador et al., 1995; Wolf, 1998). These changes include increased responsiveness of the dopamine D_1 receptors (Henry and White, 1991; Higashi et al., 1989), upregulation of cAMP signal transduction (Nestler et al., 1990; Terwilliger et al., 1991), and enhanced dopamine release induced by stimulant drugs (Kalivas and Duffy, 1990).

Behavioural sensitisation has an equivalent in human behaviour. Repetitive psychostimulant use first results in intense curiosity and exploration of the environment that may be stereotypic in nature (Ellinwood et al., 1973; Rang et al.,

1999). Later, this suspiciousness of the environment turns into paranoia and even psychosis. Although behavioural sensitisation in rodents does not correspond directly to human psychosis or drug abuse, it is possible that all of these disorders involve plastic changes in the limbic neurochemical systems (Richtand, 2006).

2.2.2 Conditioned place preference and conditioned place aversion paradigms

Conditioned place preference (CPP) or conditioned place aversion paradigms assess the positive or negative reinforcing efficacy of drugs by Pavlovian conditioning (Koob and Le Moal, 2006; Sanchis-Segura and Spanagel, 2006; Tzschentke, 1998). In these paradigms, animals experience two or more distinct environments that are paired with drug or vehicle. After conditioning, the animals are allowed to freely explore all the environments. The time spent in the drug-paired environment is considered an index of the reinforcing value of the drug. With some modifications to the basic model, it is also possible to measure extinction and reinstatement of drug use in the conditioned place preference paradigm (Sanchis-Segura and Spanagel, 2006; Shaham et al., 2003).

One of the assets, but also one of the handicaps, in the conditioned place preference paradigm is the assessment of place conditioning in a drug-free state (Bardo and Bevins, 2000; Sanchis-Segura and Spanagel, 2006). On one hand, the pharmacological effects of drugs do not interfere with the measurement of preference. On the other hand, state-dependent learning may influence place conditioning, and a lack of drug cue in the test phase may confound the results. In addition, the drug effect during the conditioning phase may impede familiarization with the drug-paired context, which may render it more novel than the saline-paired context. Another drawback in the conditioned place preference paradigm is that it does not provide robust dose-response curves.

2.2.3 Drug discrimination

Although more commonly used as a tool to investigate the mechanisms of action of various drugs, drug discrimination can also be used to explore the abuse potential of substances in genetically modified rodent strains. In drug discrimination experiments, the animal is trained to produce a particular response in a given drug state for a food reinforcer, and a different response in a vehicle or drug-free state (Koob and Le Moal, 2006). The drug effect acts as a discriminative stimulus or cue, which guides the animal to respond correctly to gain reinforcement. In the beginning of the drug discrimination test, the animals are taught to respond to food reinforcement that is

paired with a training drug, e.g. morphine. If desired, generalization of the drug discrimination to a different drug can be measured subsequently.

2.2.4 Intracranial self-stimulation

Intracranial self-stimulation (ICSS), also called brain stimulation reward, can be used to evaluate changes in the reward threshold (Borisenko et al., 1996; Koob and Le Moal, 2006; Sanchis-Segura and Spanagel, 2006). It is based on self-administration of short electrical trains of stimulation to, for example, the medial forebrain bundle, the nucleus accumbens, or the lateral hypothalamus. Drugs of abuse decrease the ICSS threshold, whereas withdrawal symptoms increase it, and there is a good correlation between abuse potential and the ability to lower ICSS thresholds (Markou and Koob, 1992). Different procedures yield either a rate-frequency curve or the current intensity threshold at which the animal makes at least two positive responses out of three stimulus presentations (Koob and Le Moal, 2006). Drugs of abuse shift the rate-frequency curve to the left and decrease the current intensity threshold. Alterations in the shape of the curve or response latency are signs of motor or performance deficits.

2.2.5 Drug self-administration models

2.2.5.1 Intravenous self-administration

The intravenous drug self-administration paradigm models the binge or intoxication phase of the addiction cycle (Koob and Le Moal, 2006). Drugs that have a high abuse potential in humans are readily self-administered by laboratory animals and, therefore, intravenous drug self-administration is considered to have good construct validity. It measures the primary rewarding or positive reinforcing properties of the substance. The i.v. self-administration tests can be carried out either in a fixed-ratio or progressive ratio fashion (Sanchis-Segura and Spanagel, 2006). In the fixed-ratio approach, the drug infusions are delivered in a constant ratio throughout the session (e.g. one drug infusion after five operandum responses in a fixed ratio of 1:5, or drug infusion after every operandum response in a fixed ratio of 1:1). In the progressive ratio approach, the number of responses required for a drug infusion increases arithmetically during the test session. The fixed ratio setup measures the potency of the reinforcer; the progressive ratio setup, its efficacy (Koob and Le Moal, 2006).

In addition to the amount of drug consumed, time needed to exhibit self-administration can be determined in the intravenous self-administration paradigm (Koob and Le Moal, 2006). Furthermore, responding for non-drug reward can be

evaluated during the training period. Extinction and drug reinstatement tests can be added to the basic i.v. self-administration setup, yielding an animal model of relapse (Epstein et al., 2006). It is also possible to exploit second-order schedules that include additional conditioned stimuli contingent on drug delivery (Koob and Le Moal, 2006). These schedules test the motivational effects of the drugs.

The intravenous self-administration method can also be used in mice. Rats are usually taught initially to lever-press for food reward. However, in mice the feasibility of training lever pressing depends on the specific mouse strain, and more often nose-poking holes are used as operandi in mouse experiments. Mice have high basal nose-poking activity and, thus, they learn quickly to respond to reward in this setup.

2.2.5.2 Oral self-administration

Oral self-administration setup is a natural choice for the animal model of ethanol consumption, but it has also been used for cocaine, opioids, and nicotine. However, the validity of the method for substances other than ethanol has been questioned (Sanchis-Segura and Spanagel, 2006).

Oral self-administration setups can be either operant or non-operant (Koob and Le Moal, 2006). In the former, the liquid is delivered after the animal completes the task with the operandum (see section 2.2.3.1). In the non-operant paradigm, two or more burettes ('Richter tubes') containing drug solution or water are presented to the animal either continuously or on a limited-access schedule (Koob and Le Moal, 2006; Sanchis-Segura and Spanagel, 2006). The animal can choose freely between different solutions, but access to the solution may be limited. For instance, ethanol consumption in the continuous access schedule often fails to produce alcohol intoxication, whereas appropriate blood alcohol levels can readily be achieved using limited-access schedules (Rhodes et al., 2007). Tastants can be added to the drug solutions to improve the discrimination between drug solution and water, or to ameliorate the otherwise aversive taste of the solution (Sanchis-Segura and Spanagel, 2006). Since genetically modified mice may have deficits in their taste sensations, testing their basal preferences for sweet and bitter tastes is recommended (see section 2.4.2).

2.3 Chronic oral nicotine treatment as a model of chronic nicotine exposure

Nicotine is the psychoactive and addictive compound in tobacco, and it is an agonist on nicotinic acetylcholine receptors (nAChRs) (Stolerman and Jarvis, 1995). It binds with high affinity to nAChRs $\alpha4\alpha6\beta2\beta3$, $\alpha4\alpha5\beta2$, and to $\alpha4\beta2$ subunits, but it also

activates other nAChR subtypes with lower affinity, such as α7 containing receptors (Grady et al., 2007). Although the initial effect of nicotine is to activate nAChRs, continuous chronic exposure to nicotine causes loss of receptor function, which is called desensitisation (Pidoplichko et al., 1997). Therefore, chronic nicotine treatment typically results in upregulation in the number and/or function of nAChRs (Fenster et al., 1999; Buisson and Bertrand, 2001).

Physiologically, nAChRs act pre- and postsynaptically to modulate neurotransmitter release (Grady et al., 2007). The activation of somatodendritic nAChRs in the ventral tegmental area or presynaptic nAChRs in the nucleus accumbens induces dopamine release in the nucleus accumbens, which probably plays a role in the reinforcing properties of nicotine (Di Chiara, 2000; Grady et al., 2007). In addition, dopamine release is modulated indirectly by the nicotinic receptors situated presynaptically on glutamatergic neurons projecting from cortical areas to the ventral tegmental area and the nucleus accumbens (Schilström et al., 1998, 2000; Reid et al. 2000).

Traditionally, nicotine has been administered to rodents by repeated injections or osmotic minipumps (Pietilä and Ahtee, 2000). However, repeated handling causes stress to the animals, and frequent needle pricks induce formation of scar tissue. The implantation and removal of minipumps requires some surgery, but their use reduces handling-related stress. Nevertheless, because they involve constant delivery of nicotine, studies using minipumps are poor models for human intermittent nicotine intake. To overcome these drawbacks, nicotine has been administered to mice in their drinking water (Pietilä and Ahtee, 2000; Sparks and Pauly, 2000).

The plasma nicotine and cotinine concentrations of mice exposed orally to nicotine are similar to or higher than those of heavy smokers (Pietilä and Ahtee, 2000). The 7-week chronic nicotine exposure enhances striatal and accumbal dopamine metabolism. It also induces tolerance to the hypothermia-inducing and locomotion-inhibitory effects of acute nicotine injections. After seven weeks of oral nicotine exposure, locomotor activity is enhanced and circadian locomotor activity rhythm is altered (Gäddnäs et al., 2000, 2001).

The mechanisms of tolerance induced by chronic oral nicotine exposure are poorly understood. It may be mediated by alterations either in the nAChRs or in the dopamine receptors. During withdrawal from 4- or 7-week chronic nicotine exposure, the nAChRs are upregulated (Pietilä and Ahtee, 2000; Nuutinen et al., 2005), and *c-fos* and *fosB* are activated (Marttila et al. 2006). However, the number of dopamine D_1 and D_2 receptors does not change (Pietilä et al. 1996). It is unknown whether there are changes in the function of dopamine receptors. In addition, the consequences of forced chronic nicotine exposure on voluntary nicotine self-administration or sensitivity to nicotine's psychoactive properties remain a mystery.

2.4 Genetically modified mice

2.4.1 Techniques to generate genetically modified mice

Two basic techniques can be used to generate genetically modified mice (see Crawley, 2006 and Stephens et al., 2002 for a review). One is to introduce a mutation at a targeted site in the genome using homologous recombination (Figure 3, panel A). The mutation can be a selective deletion of a portion of the gene of interest, yielding knock-out mice (also known as “null mutant mice”) that completely lack the gene product. The homologous recombination technique can also be used to insert multiple copies of the target gene, or point mutations of the target gene, into the genome. The former yields knock-in mice overexpressing the gene product, and the latter results in mutant mice with either altered function, lower expression level, or complete deficiency of the target gene product. With this technique, the site of the mutation can be carefully selected and the process of gene manipulation can be effectively controlled.

The other technique to create genetically modified mice is to insert multiple copies of the gene of interest or foreign DNA into the host genome by non-homologous recombination, which results in the production of a transgenic mouse line either expressing the foreign gene or overexpressing the native gene (Figure 3, panel B). With this technique, it is not possible to control the exact site of the gene insertion.

The mutation can also be designed to be restricted to a certain anatomical area (conditional knock-outs), and also to be inducible (inducible knock-outs; Crawley, 2006; Jaisser, 2000; Mishina and Sakimura, 2007). In the former, the targeted gene is under the control of a promoter that is specific to the tissue that expresses the promoter gene. In the latter, the mutation includes a drug-sensitive element that permits its activation or inactivation by drug treatment.

Several triggering techniques have been applied in inducible and conditional transgenic mice, but the tetracycline-inducible system and the *Cre*/*loxP* system are used most often (Jaisser, 2000; Mishina and Sakimura, 2007). To generate a conditional knock-out mouse line, two sets of transgenic mice are needed. One expresses the activator (tetracycline transactivator or *Cre* recombinase) under the control of a selected tissue-specific promoter. The other expresses the so-called acceptor construct, where the expression of the target gene or transgene is under the control of a tetracycline transactivator or is flanked by *loxP* sequences. When these sets of mice are mated, the expression of the mutation can be temporally controlled. In the case of the tetracycline-inducible system, gene expression is controlled by administering tetracycline or its derivatives to the animals. In the *Cre*/*loxP* system, temporal control is reached by using inducible *Cre* recombinase, which is activated

by an exogenous ligand. The *Cre*/*loxP* system also allows the spatial control of gene expression if tissue-specific promoters are used.

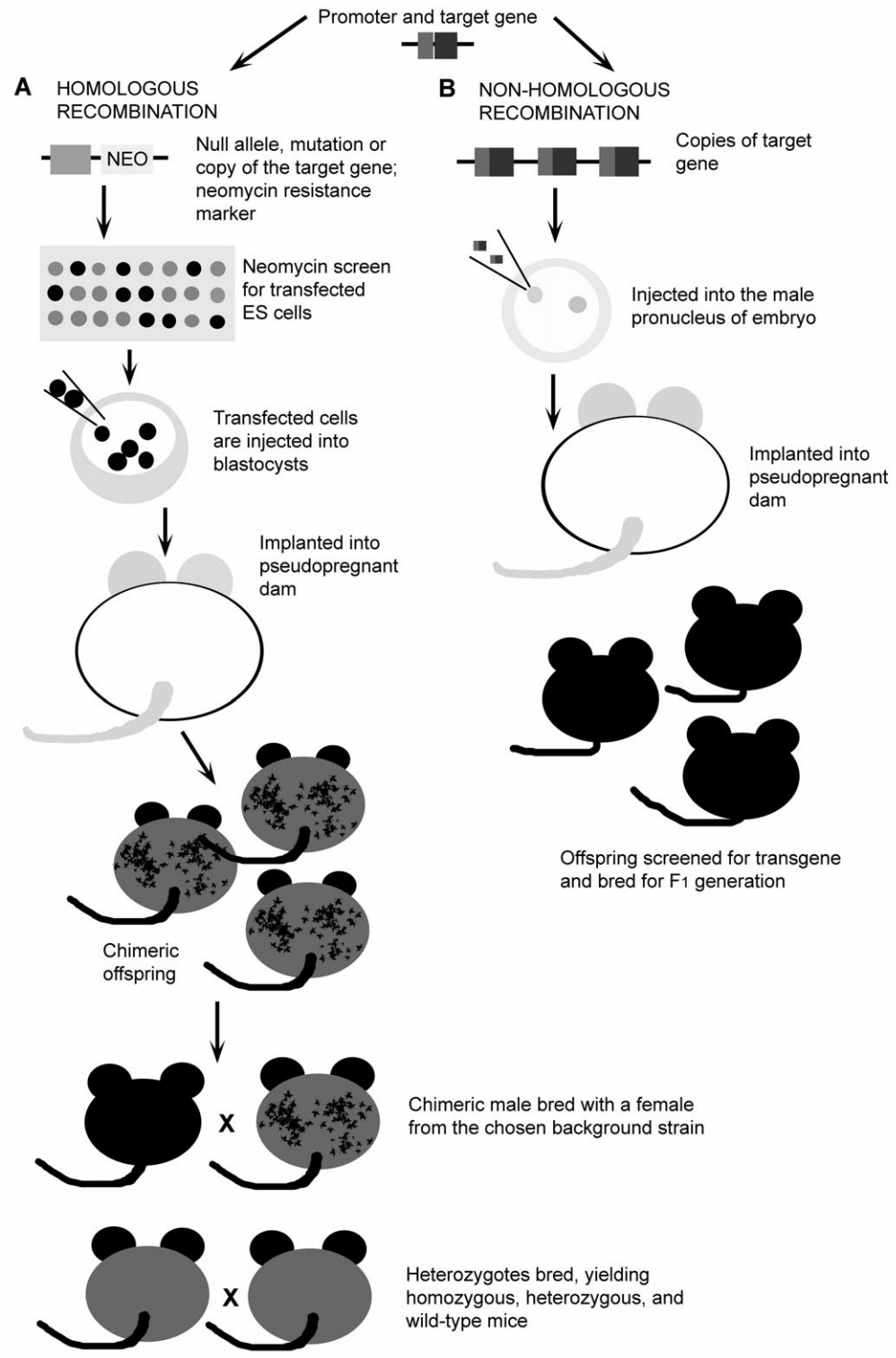

Figure 3 *The two basic techniques to generate genetically modified mice. Panel A: Homologous recombination technique. Panel B: Non-homologous recombination. Modified from Stephens et al. (2002).*

2.4.2 Genetically modified mice as research tools

Genetically modified mice are a valuable research tool (Stephens et al., 2002). For instance, ligands that reliably distinguish the D_1 and D_5 dopamine receptor subtypes, or that distinguish among the D_2, D_3, and D_4 subtypes remain unavailable, but knock-out animals have provided important information about their respective roles. With genetically modified animals, it is also possible to study those targets for which no ligands are available. In addition, even when appropriate pharmacological agents exist, animal studies avoid the need for stressful, long-term drug administration. These animals are also useful for testing the putative mechanisms of action of novel ligands.

There are important caveats for both non-homologous and homologous gene manipulation techniques (Stephens et al., 2002). In non-homologous recombination, the foreign DNA is inserted into a random location in the genome, which may disrupt other genes. With this technique, it is also not possible to control how many copies of the gene have been inserted. In homologous recombination, introduction of the neomycin resistance gene (*neo*) into the genome may alter genetic function. Furthermore, if the *neo* gene is not subsequently removed from the genome, it may phosphorylate normal proteins in the offspring of the knock-out mice. It seems that the very process of gene manipulation may induce additional changes in the genome, e.g. in the expression of a heat shock protein, mortalin, or mitochondrial antioxidant protein 2, indicative of increased oxidative stress (Skynner et al., 2002). In addition, the gene knock-out and *neo* insertion may affect the function of other, functionally related genes that often appear clustered in the genome and are transcribed together (Stephens et al., 2002), and the targeting vector and disrupted reading frame can introduce “hitchhiker” genes, which may also affect gene functioning (Crawley, 2006). Therefore, the deletion of one gene may disrupt the control of a whole group of genes with related functions.

The presence of the gene deletion during the entire ontogeny may induce compensatory adaptations in the physiology and behaviour of the grown-up mouse (Stephens et al., 2002). The deleted gene may also play a role in development, which interferes with the interpretation of the physiological or behavioural changes observed in the adult mice. Furthermore, another protein with a similar function may take over the role of the target protein. This is the case with the noradrenaline transporter (NET) in the dopamine transporter gene knock-out mice (Carboni et al., 2001). With the traditional knock-out, knock-down, or knock-in animals, it is also not possible to achieve ideal temporal and spatial resolution. These problems can be overcome by the use of conditional knock-outs that can be activated even in adult animals.

Mutations have been introduced into several different inbred mouse strains and hybrids made of two or more strains. However, the functional consequences of gene deletion may be different depending on the background strain (Gerlai, 1996).

Most often this is exhibited as behavioural or neurochemical differences between mouse lines (see below), but it can even be associated with the survival rate of pups in knock-out strains whose health is fragile (Morice et al., 2004). Some variability may be due to different expression levels of modifier genes between the mouse strains (Kido et al., 2000; Nadeau, 2001). The development of knock-out or transgenic strains may also be compromised by the fact that the mutation is on a more or less hybrid background if the background strain does not match with the one providing the embryonic stem cells (Gerlai, 1996). Embryonic stem cells are usually from the 129Sv mouse strain, which is seldom the strain of choice for maintaining a knock-out line. The first generation of mice derived from breeding with the background strain mice, e.g. C57BL/6J, are essentially C57BL/6J x 129Sv hybrids. The behavioural profiles of these strains are different, and therefore the influence of 129Sv genes may be even greater than that of the target gene manipulation. The same is true for strains intentionally maintained with hybrid backgrounds. This problem can be solved by generating a congenic strain by backcrossing the mice into the C57BL/6J background. However, even after 12 generations of backcrossing into the background strain (ca. two years), 1% of the genes will be from the 129Sv strain. Regardless, it is not always clear whether the differences are due to genetic or methodological deviations. Even when similar, validated methods are used to measure animal behaviour, experiments carried out in different laboratories may yield divergent results (Crabbe et al., 1999).

2.5 Genetically modified mouse lines targeting the dopaminergic system

The following sections present genetically modified mouse lines (referred to as “mutants”) that have a targeted mutation in the dopaminergic system (Figure 4). The focus is on lines that have been used in addiction studies. A summary of the main findings related to addiction-like behaviour is given in Table 3.

2.5.1 Tyrosine hydroxylase mutant mouse lines

Tyrosine hydroxylase converts the amino acid L-tyrosine into L-3,4-dihydroxyphenylalanine (L-DOPA), the precursor of dopamine (Figure 4, Cooper et al., 2003). Since this conversion is the rate-limiting step in dopamine biosynthesis, it is subject to complex physiological regulation. In addition to dopaminergic neurons, tyrosine hydroxylase is expressed in noradrenergic neurons, since dopamine is the precursor of noradrenaline. In humans, the 7-repeat K4 allele of an intronic tetranucleotide repeat polymorphism of tyrosine hydroxylase has been postulated to protect against nicotine dependence (Anney et al., 2004). Unfortunately, it is not

known how this polymorphism affects the expression or activity of tyrosine hydroxylase, and therefore dopamine synthesis. Such an association has not been found between smoking and the 10-repeat K1 allele, which leads to decreased dopamine synthesis (Anney et al., 2004; Ton et al., 2007).

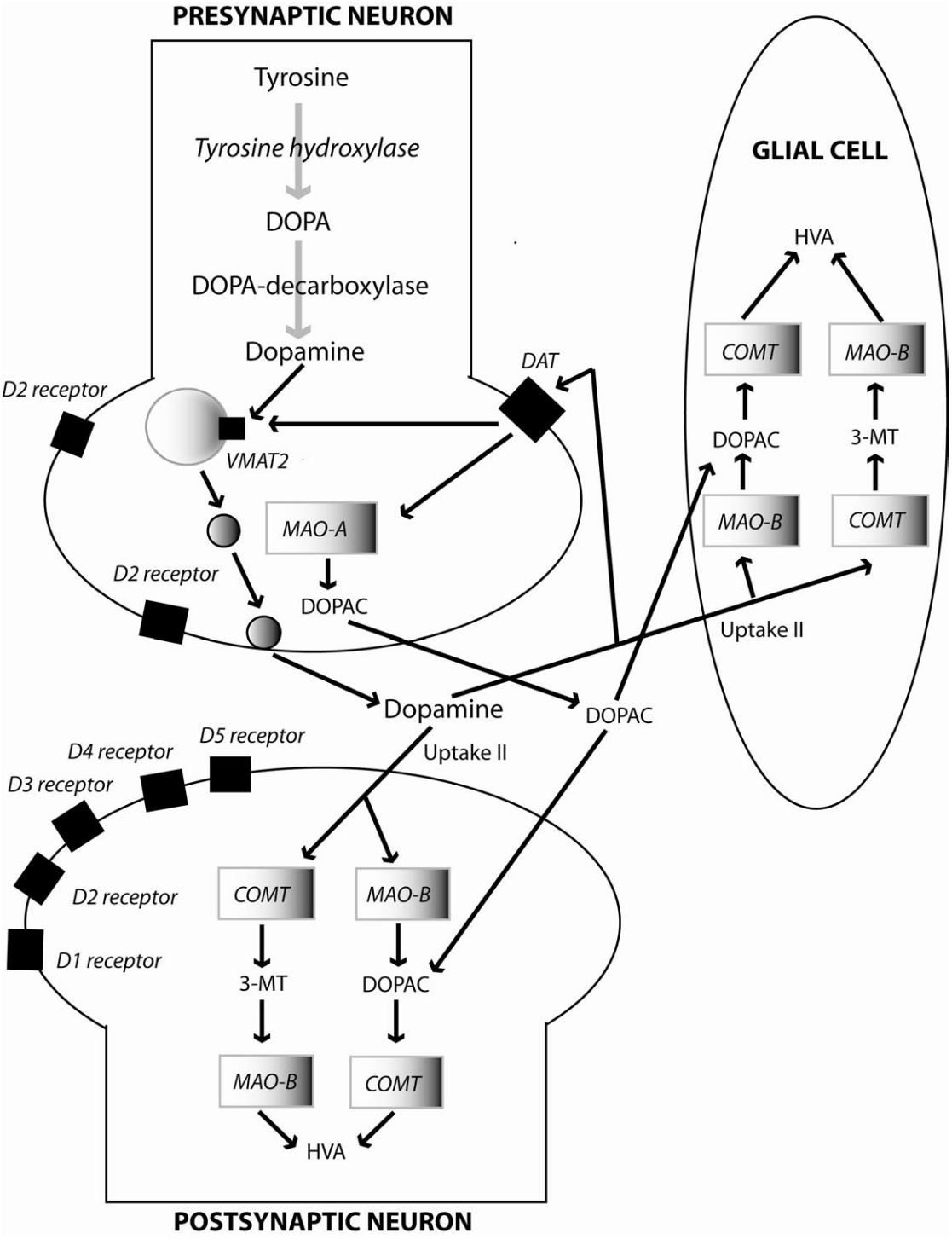

Figure 4 *Dopamine synthesis and metabolic pathways. DOPA = 3,4-dihydroxyphenylalanine, VMAT2 = vesicular monoamine transporter 2, DAT = dopamine transporter, MAO-A and MAO-B = monoamine oxidases A and B, COMT = catechol-O-methyltransferase, DOPAC = 3,4-dihydroxyphenylacetic acid, HVA = homovanillic acid, 3-MT = 3-methoxytyramine.* Italics *indicate the sites for mutations that are covered by this literature review.*

Dopamine-deficient mice are a mouse strain in which the gene for tyrosine hydroxylase (*Th*) is knocked out and dopamine is not formed in dopaminergic neurons (Zhou and Palmiter, 1995). To restore the tyrosine hydroxylase activity in noradrenergic cells, the coding region of the *Th* gene was introduced near the dopamine β-hydroxylase gene locus. Homozygous dopamine-deficient mice survive early postnatal period, but at 3-4 weeks of age they become hypoactive and hypophagic and die unless a daily levodopa treatment is initiated. Interestingly, locomotor activity and eating can also be restored with caffeine treatment (Kim and Palmiter, 2003). Failure to eat is not a consequence of motor disturbances, since the animals are able to grasp, chew, and swallow food (Szczypka et al., 1999; Zhou and Palmiter, 1995). They also show normal liking, wanting, and learning about rewards (Robinson et al., 2005), as well as normal preference for sucrose (Cannon and Palmiter, 2003). Instead, they seem to lack the motivation that would drive them to eat (Robinson et al., 2007; Szczypka et al., 1999). As compensatory changes to the severe hypodopaminergia, the striatal dopamine D_1 and D_2 receptors are sensitised, and the proportion of D_2 receptors in the high activity state (D_2^{High}) is increased 2.2-fold, but the number of dopamine receptors is unchanged (Kim et al., 2000; Seeman et al., 2005).

In addition to the dopamine-deficient mice, a knock-in mouse strain overexpressing the human *Th* gene has been designed (Kaneda et al., 1991). These mice have 50-fold higher tyrosine hydroxylase mRNA expression and three-fold higher tyrosine hydroxylase activity than their wild-type littermates. Despite these alterations in the rate-limiting enzyme, the level of DOPA in the striatum is normal, as are the levels of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) (Kiuchi et al., 1993). In addition, spontaneous locomotor activity and habituation to novel environments are intact (Nabeshima et al., 1994).

The dopamine-deficient mice exhibit reduced locomotor response to an acute dose of amphetamine, and in the course of repeated administration, the effect of amphetamine is completely abolished (Heusner et al., 2003; Szczypka et al., 1999). The fact that a single dose of amphetamine can induce an increase in locomotion may be due to the earlier levodopa supplementation as suggested by Heusner et al. (2003), or formation of 3,4-dihydrophenylalanine (L-DOPA) through the tyrosinase-mediated “rescue pathway” (Sanchez-Ferrer et al., 1995). When *Th* expression is virally restored specifically in the nucleus accumbens of dopamine-deficient animals, amphetamine causes locomotor hyperactivity (Heusner et al., 2003). These mice also show a reduced locomotor response to morphine, but the response normalises when levodopa is administered one hour before the morphine treatment (Hnasko et al., 2005). Furthermore, they develop morphine- and cocaine-conditioned place preference over a range of doses when treated with stimulatory caffeine or levodopa (Hnasko et al., 2005, 2007). As in dopamine transporter gene knock-out animals (see section 2.3.2), a serotonin-dependent mechanism has been suggested for morphine and cocaine reward in dopamine-deficient animals (Hnasko et al., 2007).

Th knock-in mice are less sensitive to the locomotion-enhancing effect of methamphetamine and the initial hypolocomotor effect of acute nicotine (Nabeshima et al., 1994). This unexpected finding may be due to compensatory mechanisms that suppress excess dopaminergic activity in the knock-in animals. However, the nature of such mechanisms remains unclear.

In conclusion, studies in dopamine-deficient mice have shown that they are capable of exhibiting addiction-related behaviour, which seems to challenge the notion that dopamine plays a pivotal role in the development of addiction. However, complete lack of a neurotransmitter is likely to be a dramatic alteration that induces compensatory changes in brain neurochemistry. Indeed, there is some evidence that serotonergic mechanisms may account for drug reward in the dopamine-deficient animals.

2.5.2 Dopamine transporter mutant mouse lines

Dopamine transporter (DAT) takes up dopamine that has diffused out of the synaptic cleft (Figure 4; Cooper et al., 2003; Hersch et al., 1997), and this reuptake is the main mechanism for terminating the dopamine signal in the caudate-putamen (Giros et al., 1996; Mazei et al., 2002; Yavich et al., 2007). However, in prefrontal cortex the density of DAT is lower than in the caudate-putamen or nucleus accumbens, and uptake into glial cells through an 'uptake II' mechanism or uptake by noradrenaline transporter are more important (Mazei et al., 2002; Morón et al., 2002; Sesack et al., 1998). DAT is the target for psychomotor stimulants, such as cocaine and amphetamine (O'Brien, 1996). In humans, the A9 allele of variable number tandem repeat (VNTR) polymorphism and A allele of rs27072G/A single nucleotide polymorphism (SNP) of the dopamine transporter gene, which decrease the expression of DAT, may be associated with alcoholism (Köhnke et al., 2005; Samochowiec et al., 2006; Ueno et al., 1999), cocaine use (Guindalini et al., 2006), and smoking behaviour (Erblich et al., 2004, 2005; Ling et al., 2004; Stapleton et al., 2007; Timberlake et al., 2006). However, studies on this topic do not always agree (e.g. Choi et al., 2006; Franke et al., 1999; Parsian and Zhang, 1997; Ton et al., 2007).

Homozygous dopamine transporter gene (*Dat1*) knock-out mice have an extremely high extracellular dopamine concentration in the striatum and nuclear accumbens (Jones et al., 1998; Rocha et al., 1998; Shen et al., 2004; Spielewoy et al., 2000a), and the elimination time of extracellular dopamine is at least 100-300 times longer than in wild-type mice (Giros et al., 1996; Jones et al., 1998). As compensatory changes to the profoundly altered dopamine clearance, these mice show a 90% decrease below wild-type in the sensitivity of dopamine D_2 autoreceptor to dopamine D_2 receptor agonist treatment (Jones et al., 1999), and a 50% decrease in the striatal dopamine D_1 and D_2 receptor expression (Giros et al., 1996; Sora et al.,

2001). Consequently, stimulus-evoked dopamine release from striatal brain slices is decreased due to depleted intracellular dopamine stores (Jones et al., 1998). However, the number of dorsal and ventral striatal D_2 receptors that are in the functional high-affinity state (D_2^{High}) is considerably higher than in wild-type mice (Seeman et al., 2007).

The homozygous *Dat1* knock-out animals demonstrate marked random locomotor hyperactivity, with reduced rearings and increased thigmotaxis, as well as deficits in performing more demanding motor tasks (Barr et al., 2004; Fernagut et al., 2003; Gainetdinov et al., 1999; Giros et al., 1996; Mead et al., 2002; Powell et al., 2004; Ralph et al., 2001; Sora et al., 1998, 2001; Spielewoy et al., 2000b, 2001). Accordingly, they show impaired habituation to novel environment, decreased exploratory behaviour in the open field test, as well as disturbed maternal behaviour (Spielewoy et al., 2000b). Interestingly, the heterozygous *Dat1* knock-out animals show neurochemistry and behaviours that differ little from those of wild-type mice (Giros et al., 1996; Ralph et al., 2001; Salahpour et al., 2007; Shen et al., 2004; Sora et al., 1998, 2001; Spielewoy et al., 2000a, 2000b). The *Dat1* knock-out animals also have altered preference to sweet and bitter solutions: both sexes exhibit increased preference for bitter quinine solutions and decreased preference for sweet saccharin solutions (Savelieva et al., 2002), which may be due to the deficit in olfactory discrimination observed in these mice (Tillerson et al., 2006). When responding for food, the *Dat1* knock-outs do not differ from their wild-type littermates in response activity, time required for acquisition of stable response, or response under fixed ratio or progressive ratio conditions; however, the time needed for extinction of the food self-administration behaviour is longer in the knock-out mice (Hironaka et al., 2004).

In addition to a *Dat1* knock-out mouse line, a *Dat1* knock-down line and a mouse line featuring local knock-down of *Dat1* using small interfering ribonucleic acid (siRNA) have been developed. The *Dat1* knock-down mice show a 90% reduction in the expression of DAT, increased extracellular dopamine levels (Zhuang et al., 2001), locomotor hyperactivity in novel environments (Tilley et al., 2007; Zhuang et al., 2001), compromised response habituation (Zhuang et al., 2001), and changes in dopamine D_2 receptor function (Wu et al., 2007). The local knock-down of *Dat1* with siRNA was targeted to the substantia nigra and ventral tegmental area, and it caused a 35% loss in striatal DAT levels without affecting novelty-induced locomotion (Salahpour et al., 2007). The latter finding is consistent with findings in the heterozygous *Dat1* knock-out animals that show a 50% decrease in DAT protein but only slightly increased locomotor activity (Giros et al., 1996; Ralph et al., 2001; Salahpour et al., 2007; Sora et al., 1998; Sora et al., 2001; Spielewoy et al., 2000b).

In contrast to what has been found in the *Dat1* knock-outs, *Dat1* knock-down animals demonstrate enhanced response to food reward in progressive ratio conditions (Cagniard et al., 2006), and they exhibit enhanced incentive motivation for sweet reward in a runway task (Peciña et al., 2003). The conflicting results concerning sweet reward in the *Dat1* knock-out vs. *Dat1* knock-down mice may be

due to methodological differences between the free-choice oral self-administration test and the runway task, since the former measures only preference between two solutions, whereas the latter demands completion of a more complicated task and it measures reward learning.

Donovan et al. (1999) and Salahpour et al. (2008) have engineered knock-in mouse strains overexpressing dopamine transporter. The *Dat1* knock-in mice show a 20-30% increase in striatal DAT expression, an increased rate of dopamine reuptake, decreased striatal tissue dopamine and DOPAC levels under baseline conditions, and accelerated habituation to novel environments. In addition, the *Dat1* knock-in mice show a reduced response rate to a reward of sweetened milk (Salahpour et al., 2008). Chen and co-workers (2005) have developed a knock-in mouse strain with a functional but cocaine-insensitive DAT (DAT-CI). In these animals, DAT uptake activity is around 50% of the activity in wild-type mice. The DAT-CI mice display increased accumbal basal extracellular dopamine levels and enhanced baseline locomotor activity (Chen et al., 2006).

Sotnikova et al. (2005) have introduced the *Dat1* knock-out into the dopamine-deficient mice. The resulting triple mutant mice exhibit severe akinesia, rigidity, tremor, and ptosis, and they are postulated to be a useful model of Parkinson's disease.

To elucidate the mechanisms of action of psychostimulants, researchers have studied them extensively in mouse strains carrying a modified *Dat1* gene. Although psychostimulants do not affect striatal extracellular dopamine levels in the *Dat1* knock-out mice (Fumagalli et al., 1998; Giros et al., 1996; Rocha et al., 1998; but see Shen et al., 2004), they increase accumbal (Budygin et al., 2004; Carboni et al., 2001; Mateo et al., 2004; but see Shen et al., 2004) and prefrontal cortical (Shen et al., 2004) extracellular dopamine levels in these animals. The explanation for this is probably that the psychostimulant blocks noradrenaline transporter-mediated dopamine uptake in these brain areas, since the selective noradrenaline transporter blocker reboxetine also increases accumbal dopamine levels in wild-type but not in knock-out mice (Carboni et al., 2001). However, another study has provided evidence of a non-serotonergic and non-noradrenergic mechanism in the cell body level for the cocaine-induced increase in extracellular dopamine (Budygin et al., 2002). The DAT-CI animals do not exhibit increased extracellular dopamine levels in response to cocaine (Chen et al., 2006). This difference in cocaine effects observed with the full *Dat1* knock-out and the DAT-CI knock-in is probably due to the stronger compensatory mechanisms in the former.

DAT deficiency results in a lack of behavioural sensitivity to psychostimulants (Giros et al., 1996; Mead et al., 2002; Rocha et al., 1998; Sora et al., 1998). As a matter of fact, psychostimulants can even suppress hyperactivity in the *Dat1* knock-out mice (Gainetdinov et al., 1999; Powell et al., 2004; Spielewoy et al., 2001) and also in the *Dat1* knock-down (Zhuang et al., 2001; but see Tilley et al., 2007), *Dat1* local knock-down (Salahpour et al., 2007), and DAT-CI mice (Chen et al., 2006).

Despite the paradoxical effect of psychostimulants, *Dat1* knock-out mice have been found to develop place preference induced by cocaine (Mateo et al., 2004; Medvedev et al., 2005; Sora et al., 1998), amphetamine (Budygin et al., 2004), and methylphenidate (Sora et al., 1998), and to self-administer cocaine (Rocha et al., 1998). Cocaine-induced place conditioning can also be detected in the *Dat1* knock-down animals (Tilley et al., 2007). These findings challenge the classical psychomotor stimulant theory of addiction (Wise and Bozarth, 1987) and suggest that, at least under some conditions, the psychostimulatory and reinforcing effects of drugs do not share the same mechanisms. Nevertheless, the DAT-CI mice fail to exhibit cocaine reward, as expected (Chen et al., 2006). Consistent with the idea that DAT is critical for psychostimulant reinforcement, cocaine and amphetamine reward is enhanced in the *Dat1* knock-in mice (Donovan et al., 1999; Salahpour et al. 2008). Thus, DAT still seems to be the major target for cocaine's action, and the non-dopaminergic reinforcing effects of cocaine are apparently due to the compensatory mechanisms present in the *Dat1* knock-out mice born with the mutation.

The unexpected finding that *Dat1* mutants show no psychostimulant-induced locomotor activity but retain place conditioning has been attributed to the cocaine-induced stimulation of the serotonergic system and the concomitant strong dopaminergic activation (Barr et al., 2004; Budygin et al., 2004; Rocha et al., 1998; Sora et al., 2001; Trinh et al., 2003). This activation leads to inhibition of ERK signalling cascade in the striatum (Beaulieu et al., 2006). However, the dopamine-deficient *Dat1* knock-outs also respond to amphetamines, but they do not show altered behaviour in response to manipulation of noradrenaline or serotonin activity (Sotnikova et al., 2005). Amphetamine is an agonist of trace amine-associated TAAR1 receptors, which are found in brain regions containing somata and projections of dopaminergic neurons, e.g. in the limbic system and basal ganglia (Borowsky et al., 2001; Bunzow et al., 2001). Therefore, trace amines can modify locomotor and motivated behaviours usually associated with dopamine function. This suggests that TAAR1 receptors may at least partially mediate the paradoxical locomotor activation seen in the DAT-deficient mice.

In addition to the effects of psychostimulants, the effects of morphine, nicotine, and ethanol have also been studied in *Dat1* knock-out animals. The effect of the gene knock-out on locomotor response or drug reward differs considerably between substances. The *Dat1* knock-out mice show enhanced reward but, paradoxically, decreased locomotor activity in response to morphine (Spielewoy et al., 2000a). They are hypersensitive to the initial hypolocomotor effect of nicotine, and they fail to develop tolerance to it during chronic oral nicotine exposure (Weiss et al., 2007a, 2007b). They are also resistant to the locomotion-increasing effect of chronic oral nicotine exposure (Weiss et al., 2007a). On the other hand, the anxiogenic effect usually elicited by nicotine is missing in the *Dat1* knock-outs (Weiss et al., 2007a, 2007b). Furthermore, chronic nicotine exposure markedly improves the impaired cue and spatial learning performance of the *Dat1* knock-out animals (Weiss

et al., 2007a). DAT deficiency does not modify the effects of an acute ethanol injection on the extracellular levels of dopamine (Mathews et al., 2006). However, the *Dat1* knock-out mice take less time to to lose the righting reflex after an i.p. injection of ethanol; on the other hand, they regain the reflex more rapidly than their wild-type littermates (Savelieva et al., 2002). Studies of oral ethanol self-administration have revealed a sexually dimorphic, albeit not very robust, association between *Dat1* gene disruption and ethanol consumption. In female mice, DAT deficiency causes a decline (Savelieva et al., 2002) or no change (Hall et al., 2003) in ethanol consumption and preference. In male mice, however, the lack of DAT causes an increase (Hall et al., 2003) or no change (Savelieva et al., 2002) in ethanol consumption and preference.

Collectively, these data show that DAT is important for psychostimulant reinforcement. In DAT knock-out animals, serotonergic mechanisms compensate for the lack of DAT. However, the loss of cocaine reinforcement in DAT-CI animals, together with the enhanced cocaine reinforcement in DAT knock-in mice, suggests that DAT is indeed the most critical binding site in addiction to psychostimulants. However, the role of DAT in the development of addiction-like behaviour to nicotine, ethanol, or opioids is less clear.

2.5.3 Vesicular monoamine transporter 2 mutant mouse lines

Vesicular monoamine transporters (VMAT) are responsible for sequestering monoamines into intracellular vesicles for storage, to protect them from cytoplasmic oxidation and to regulate stimulated quantal monoamine release (Figure 4; Cooper et al., 2003). VMAT1 is expressed mainly in neuroendocrine and paracrine cells of peripheral organs, whereas VMAT2 is expressed in most parts of the nervous system and in the histaminergic cells of the gastrointestinal tract (Erickson et al., 1996; Weihe et al., 1994). In addition to DAT, VMAT2 is one of the targets of psychostimulants (Zheng et al., 2006). In humans, SNP polymorphisms in the VMAT2 gene (*Vmat2*), the effects of which are not yet fully known, may be associated with alcoholism (Lin et al., 2005; Schwab et al., 2005) and nicotine dependence (Sullivan et al., 2004). However, *Vmat2* polymorphism may not be associated with polysubstance abuse (Uhl et al., 2000).

Homozygous *Vmat2* knock-out animals show very low brain monoamine levels in spite of the increased synthesis rates, and most of them die within the first week after birth (Fon et al., 1997; Takahashi et al., 1997; Wang et al., 1997). There are reports of both decreased (Wang et al., 1997) and increased (Takahashi et al., 1997) striatal tissue dopamine levels in the adult heterozygous *Vmat2* knock-out mice, although the DOPAC levels are increased in this brain area (Takahashi et al., 1997; Wang et al., 1997). Furthermore, striatal extracellular dopamine levels are reduced despite the decreased number of DAT, probably because of a smaller

releasable pool of dopamine (Takahashi et al., 1997; Wang et al., 1997). These changes are similar to the ones observed in the striatum of reserpine-treated rats (Parker and Cubeddu, 1986). Although the changes in the dopamine levels are moderate, the heterozygous *Vmat2* knock-out mice show a nearly 90% increase in the proportion of accumbal D_2 receptors in the high-affinity state (Seeman et al., 2007). Despite these neurochemical alterations, these mice retain wild-type motor coordination, locomotor activity, and passive avoidance and stress responses (Takahashi et al., 1997). In taste preference experiments, both male and female *Vmat2* knock-out animals show normal aversion to quinine solution, but reduced preference for saccharin solution (Savelieva et al., 2006).

There is also a mouse strain deficient in both DAT and VMAT2. *Dat1*/*Vmat2* heterozygous mice show normal locomotor activity and habituation to novel environments (Fukushima et al., 2007); this behaviour resembles more that of *Vmat2* than of *Dat1* knock-out mice.

Mooslehner and co-workers (2001) have developed a knock-down mouse strain with considerably reduced expression of the *Vmat2* gene (5% of the wild-type level). Even the homozygous mice of this mutant strain survive into adulthood, but they exhibit considerably reduced dopamine levels in the striatum, hippocampus, cortex, and midbrain; in addition, their dopamine receptors are hypersensitive and their motor coordination is impaired.

The amphetamine-induced increase in the extracellular dopamine is attenuated in the heterozygous *Vmat2* knock-out animals, but the acute behavioural responses to amphetamine, methamphetamine, and cocaine are enhanced (Fukushima et al., 2007; Takahashi et al., 1997; Wang et al., 1997). As might be expected, homozygous *Vmat2* knock-down mice show a similar response to amphetamine (Mooslehner et al., 2001; Patel et al., 2003). The *Vmat2*/*Dat1* double-mutated mice exhibit weaker methamphetamine response, which resembles that of *Dat1* knock-out mice (Fukushima et al., 2007). However, the *Vmat2* knock-outs do not develop behavioural sensitisation to amphetamine, although they do develop it to cocaine and methamphetamine (Fukushima et al., 2007; Uhl et al., 2000). The *Vmat2* knock-outs show decreased amphetamine and ethanol reward (Savelieva et al., 2006; Takahashi et al., 1997), but cocaine induced place preference is intact (Takahashi et al., 1997). The fact that VMAT2 deficiency alters the reward or sensitisation effects of amphetamine, but not of cocaine, may be due to the slightly different mechanisms of action of these psychostimulants. Cocaine blocks the plasma membrane monoamine transporters, whereas amphetamine also reverses the function of dopamine transporter, inhibits MAO, and interacts with VMAT2 to release vesicular monoamines into the cytoplasm (Gainetdinov et al., 2002).

Studies on voluntary alcohol consumption in the heterozygous *Vmat2* knock-out males have yielded conflicting results. In one study, the male heterozygous *Vmat2* knock-outs showed decreased ethanol preference and consumption in a two-bottle free-choice oral self-administration setup (Savelieva et al., 2006), whereas in

another study, they consumed more ethanol than their wild-type littermates (Hall et al., 2003). However, Savelieva et al. (2006) were using a narrower range of ethanol concentrations (3-15%) than Hall et al. (2003; 1-32 %); moreover, the latter study found differences between knock-outs and wild-type animals only at the highest ethanol concentrations. It should also be pointed out that the two groups were using knock-out mouse lines generated in different laboratories, which may help to account for the different results. Nevertheless, both groups found that ethanol consumption was not altered in female mice.

Along with DAT, VMAT2 is essential in addiction-like behaviour induced by psychostimulants. However, VMAT2 is more important in amphetamine reinforcement than in cocaine reinforcement, obviously due to their slightly different mechanisms of action. As in the case of DAT, VMAT2 may not be linked in this way to ethanol reinforcement.

2.5.4 Dopamine D_1 receptor mutant mouse lines

Dopamine D_1 receptors (formerly known as D_{1A} receptors) are abundantly expressed in caudate putamen, nucleus accumbens, olfactory tubercle, and amygdala (Missale et al., 1998; Sealfon and Olanow, 2000). In striatum, D_1 receptors are expressed mainly in the medium spiny neurons, which project to the internal segment of globus pallidus or substantia nigra pars reticulata and which express substance P (Gerfen et al., 1990; Le Moine et al., 1991). Dopamine D_1 receptors have been shown to localize both pre- and postsynaptically, although the latter is considerably more common (Missale et al., 1998). Dopamine D_1 receptors are important, e.g. in reward-related learning (Sutton and Beninger, 1999) and in reinstatement of drug-seeking (Bossert et al., 2007; Hamlin et al., 2007). Dopamine D_1 receptors are also involved in the regulation of locomotor activity in concert with other dopamine receptor subtypes (Missale et al., 1998). Although stimulation of D_1 receptors alone has little or no effect on locomotor activity, the simultaneous stimulation of D_1 and D_2 receptors is crucial for maximal locomotor activation. In humans, a synonymous SNP Dde I A/G polymorphism and a -800 T/C polymorphism of dopamine D_1 receptor gene have been associated with smoking and other addictive behaviours, such as gambling and compulsive eating (Comings et al., 1997; da Silva Lobo et al., 2007).

Unless offered soft food mash, homozygous dopamine D_1 receptor gene (*Drd1*) knock-out mice show retarded growth, and they die at the age of 3-4 weeks (Drago et al., 1994). Their brain, especially the striatum, is reduced in size (Xu et al., 1994). Cortical lamination as well as cell number and density are normal in the cerebral cortex of adult *Drd1* knock-out mice (Drago et al., 1994; Stanwood et al., 2005). However, the lack of dopamine D_1 receptors affects the organization of the

dendrites of pyramidal cells in the anterior cingulate cortex and medial prefrontal cortex, which are brain areas involved in attention, cognition, and emotion (Stanwood et al., 2005). In these regions, dendrites are less bundled than in the brains of wild-type animals, and their patterning is irregular and intricate. Nevertheless, the neuronal processes of neostriatal cells are intact (Levine et al., 1996). The *Drd1* knock-out mice show increased levels of dopamine and DOPAC in the medulla pons, olfactory tubercle, and dorsal striatum (El-Ghundi et al., 1998; Parish et al., 2001), and increased expression of dopamine D_2 receptor in the striatum (Wong et al., 2003a). However, dorsal striatal DOPAC levels and dopamine activity are reduced (Parish et al., 2001). When the activity of mesolimbic dopaminergic neurons in *Drd1* knock-out animals was recorded during the reward-seeking phase, the mesolimbic neurons were found to give no pre-reward excitatory response, whereas the pre-reward inhibitory response was intact (Tran et al., 2005).

The first reports from the two laboratories where the *Drd1* knock-out mice were independently generated showed conflicting locomotor activity profiles (Drago et al., 1994, 1996; Smith et al., 1998; Xu et al., 1994a, 1994b). One mouse line showed intact horizontal locomotor activity but fewer rearings (Drago et al., 1994, 1996; Smith et al., 1998), whereas the other line showed locomotor hyperactivity but unchanged rearings as compared with wild-type animals (Xu et al., 1994a, 1994b). More recently, increased locomotor activity has also been detected in the *Drd1* knock-out strain created by Drago and co-workers (Centonze et al., 2003; Clifford et al., 1998; Crawford et al., 1997; Karasinska et al., 2005; McNamara et al., 2003). The *Drd1* knock-out mice exhibit retarded habituation for sniffing, locomotion, rearing to the wall or rearing from a seated position (see Table 2 for descriptions; McNamara et al., 2003), as well as decreased levels of free rearing, sifting, and chewing (Centonze et al., 2003; Clifford et al., 1998; Drago et al., 1994; McNamara et al., 2003). In addition, the mice showed increased grooming behaviour (Clifford et al., 1998) and impairments in sequencing motor acts (Cromwell et al., 1998). In the initial report, these mice were reported to have intact motor coordination in the beam-walking test (Drago et al., 1994), but a subsequent study that exploited the rotarod test suggested impaired motor control (Karasinska et al., 2000). However, the authors of the latter report propose that the failure of *Drd1* knock-out animals to stay on the rotating rod may instead be related to their decreased ability to initiate movements. Another motor disturbance expressed by the *Drd1* knock-out mice is an altered orofacial movement pattern (Tomiyama et al., 2002), which may contribute to their eating difficulties.

The *Drd1* knock-outs seem to have a deficit in initiating spontaneous behaviour, as well as in engaging in cue or spatial learning (El-Ghundi et al., 1999; Karasinska et al., 2000; Smith et al., 1998; Tran et al., 2005), but the aversive learning and goal-directed behaviours are intact (El-Ghundi et al., 2001; Tran et al., 2005). Furthermore, they show impaired reinforcement learning if the interval between task and reward is prolonged (Nitz et al., 2007). Long-term potentiation is

impaired in hippocampal and corticostriatal neurons (Centonze et al., 2003; Matthies et al., 1997), which may explain the compromised spatial learning skills. Nevertheless, the conditioned locomotor activity response, which represents one form of associative learning, is enhanced in these mice (McDougall et al., 2005). Under conditions of operant self-administration, the *Drd1* knock-out mice respond to food reward, although their response frequency is lower than that of their wild-type littermates (Caine et al., 2007). They also show diminished response rate to sucrose reward (El-Ghundi et al., 2003; Short et al., 2006), suggesting that either the reward mechanisms or the animals' motivation to work for a reward may be impaired, which may also contribute to their feeding problems. Another indicator of the compromised reward mechanisms is an increased intracranial self-stimulation (ICSS) threshold (Tran et al., 2005).

Table 2 *Descriptions of the behaviours observed in genetically modified mice in the ethogram analysis according to McNamara et al. (2002).*

Behaviour	**Description**
Locomotion	Coordinated movement of all four limbs that results in a change of location
Rearing seated	Front paws reach upwards, while hind limbs are on the floor in a sitting position.
Rearing free	Front paws reach upwards away from walls, while the animal stands on hind limbs.
Rearing to wall	Front paws reach upwards towards or onto a cage wall, while the animal stands on hind limbs.
Climbing	Jumping onto cage lid with climbing along the grill in an inverted or hanging position
Sifting	Characteristic sifting movements of the forepaws through the bedding material on the floor.
Grooming	Serial front paw movements to clean and condition the fur
Intense grooming	Characteristic pattern of grooming of the snout and then the face with the forepaws, followed by vigorous grooming of the hind flank or anogenital region with the snout
Sniffing	Flaring of nostrils with movement of whiskers
Chewing	Chewing movements directed onto physical material
Vacuous chewing	Chewing movements not directed onto physical material
Eating	Chewing with consumption
Stillness	Asleep or motionless, no behaviour observed

In wild-type animals, administration of cocaine into the nucleus accumbens inhibits the generation of action potentials, but this effect is reduced in *Drd1* gene

knock-out mice (Xu et al., 1994b). Cocaine and amphetamine also fail to induce the immediate-early genes *c-fos*, *fosB*, *junB*, and *zif268* in the striatum, nucleus accumbens, and cerebral cortex of these mice (Drago et al., 1996; Moratalla et al., 1996; Zhang et al., 2002). In microarray studies, the gene knock-out has been shown to affect the cocaine-induced changes in the expression of more than 100 genes, including those encoding gene expression modulators and intracellular signalling molecules (Zhang et al., 2005). The *Drd1* knock-out mice exhibit decreased phosphorylation levels of cyclic adenosine monophosphate responsive element binding protein (CREB) in the striatum in response to an acute dose of cocaine (Karasinska et al., 2005). Psychostimulants do not alter the protein kinase A activity in these animals (Crawford et al., 1997).

In response to an acute dose of cocaine or amphetamine, homozygous *Drd1* knock-out mice exhibit a diminished locomotor response, while *Drd1* knock-in mice show the same response as wild-type littermates, (Crawford et al., 1997; Dracheva et al., 1999; Drago et al., 1996; Karasinska et al., 2005; Xu et al., 1994b, 2000). On the other hand, one study has shown cocaine to increase sniffing and head bobbing in the *Drd1* knock-out mice (Drago et al., 1996), but another study found different results (Xu et al., 1994b). The *Drd1* knock-out animals show attenuated behavioural sensitisation when amphetamine or cocaine is given repeatedly at low doses (Crawford et al., 1997; Xu et al., 1994b, 2000), while at higher doses they show a pronounced sensitised locomotor response (Karper et al., 2002; McDougall et al., 2005). However, the knock-outs exhibit increased locomotor response to 3,4-methylenedioxymethamphetamine (MDMA; Risbrough et al., 2006), which is probably due to the dominance of non-dopaminergic mechanisms in MDMA-induced locomotor activation (Bengel et al., 1998; Callaway et al., 1990; Crespi et al., 1997). Despite the attenuated locomotor responses of *Drd1* knock-out mice to psychostimulants, cocaine induces conditioned place preference in these animals over a wide range of doses (Karasinska et al., 2005; Miner et al., 1995). Paradoxically, the knock-outs fail to achieve cocaine self-administration behaviour (Caine et al., 2007). In principle, the discordant results from the CPP and i.v. self-administration studies may be due to the contribution of 129Sv strain genes in the animals used by Caine et al. (2007). However, both C57BL/6J and 129Sv mice show cocaine self-administration behaviour, although the reinforcing effects of cocaine and food are diminished in the 129 substrains (Thomsen and Caine, 2006).

Drd1 knock-out mice show blunted locomotor response to an acute dose of morphine, and they fail to develop behavioural sensitisation to morphine (Becker et al., 2001). In spite of this and in spite of their failure to self-administer cocaine, they self-administer the opioid agonist remifentanil (Caine et al., 2007). Furthermore, the ethanol consumption of homozygous *Drd1* knock-out mice is markedly diminished in the two-bottle free-choice setup, as well as when force-fed a 12% ethanol solution (El-Ghundi et al., 1998; Short et al., 2006).

The reduced sucrose reward, the elevated ICSS threshold, and the attenuated drug effects in the *Drd1* knock-out mice all point to the likelihood that an absence of D_1 signalling results in a generalized impairment of either reward or motivation.

2.5.5 Dopamine D_2 receptor mutant mouse lines

Dopamine D_2 receptors are abundantly expressed in the substantia nigra, ventral tegmental area, caudatus-putamen, nucleus accumbens, and olfactory tubercle (Missale et al., 1998; Sealfon and Olanow, 2000). In striatum, the D_2 receptors are expressed mainly in GABAergic medium spiny neurons that coexpress enkephalins and project to the external segment of the globus pallidus (Gerfen et al., 1990; Le Moine et al., 1990). The dopamine D_2 receptor gene codes for two different receptor isoforms (Dal Toso et al., 1989): the short D_{2S} receptor, which has been suggested to act as an autoreceptor; and the long D_{2L} receptor, which is postulated to be a postsynaptic receptor (Usiello et al., 2000). Dopamine D_2 receptors are important in processes that initiate drug seeking (Anderson et al., 2006, but see Graham et al., 2007), and they also regulate forward locomotion in conjunction with D_1 and D_3 receptors (Missale et al., 1998). Gene association studies in human populations suggest that the A1 and probably also B1 allele of the Taq I polymorphism in the *Drd2* gene are associated with smoking (see Ho and Tyndale, 2007, for a recent review), alcoholism (Blum et al., 1990; Hallikainen et al., 2003; Hill et al., 2008; Noble, 2003), polysubstance abuse (Comings et al., 1994; Persico et al., 1996), and heroin dependence (Li et al., 2006; Xu et al., 2004). However, some studies disagree with these associations (e.g. Berrettini and Persico, 1996; Timberlake et al., 2006).

The dopamine D_2 receptor gene (*Drd2*) knock-out mice lack the autoreceptor function (L'Hirondel et al., 1998; Mercuri et al., 1997). D_2 receptor-deficient dopamine neurons have intact basic electrophysiological properties, but they fail to exhibit hyperpolarization or inhibition of spontaneous firing in response to dopamine or to the dopamine D_2-type receptor agonist quinpirole. Some studies have shown levels of dopamine and dopamine metabolites in the striatal tissue and extracellular fluid of *Drd2* knock-out mice to be normal (Dickinson et al., 1999; Kelly et al., 1998; Schmitz et al., 2001; Zapata and Shippenberg, 2005), whereas others have measured wild-type dopamine levels with increases in the level of metabolites and in dopamine activity (Jung et al., 1999b; Parish et al., 2001) or a decrease in dopamine levels (Job et al., 2006). On the other hand, *Drd2* knock-out mice have decreased accumbal extracellular dopamine levels (Job et al., 2006; Zapata and Shippenberg, 2005). They also show decreased DAT function (Dickinson et al., 1999) in spite of increased DAT expression (Parish et al., 2001), and they show decreased D_1 receptor expression (Baik et al., 1995; Jung et al., 1999b; Kelly et al., 1998) and increased

dopamine D_3 receptor expression during the late stages of postnatal development (Jung et al., 1999b).

Some studies have found that D_2 receptor deficiency leads to motor impairment resembling Parkinson's disease (Baik et al., 1995; Fowler et al., 2002). However, others have suggested that the absence of dopamine D_2 receptors does not cause parkinsonian behaviour (Cunningham et al., 2000; Kelly et al., 1998; Phillips et al., 1998). Although *Drd2* knock-out mice exhibit reduced distance travelled, time in motion, and number of movements, the movement speed and length as well as motor coordination are comparable to those of wild-type animals. Ethological analysis has shown that, in addition to moderately reduced horizontal locomotor activity during habituation to novel environments, *Drd2* knock-out animals exhibit reduced grooming, free rearing, and rearing towards the wall (see Table 2 for descriptions; Clifford et al., 2000). These inconsistent results have been associated with the different techniques used to generate the strains. The mice of Baik and co-workers (1995), as well as those of Jung and co-workers (1999b), are null mutants whose entire dopamine D_2 receptor gene is deleted; in contrast, only the C-terminal fragment of the gene is deleted in the mice of Kelly et al. (1998). Another explanation for the divergent results is that they are due to the different background strains on which the knock-out mouse lines are maintained. The 129 substrains show poorer motor coordination and lower locomotor activity than the C57BL/6 strain (Holmes et al., 2002; Tarantino et al., 2000; Võikar et al., 2004). Thus, the mice with a hybrid C57BL/6J x 129Sv background seem to exhibit the locomotor profile of the 129Sv strain, exacerbated by dopamine D_2 receptor deficiency. In fact, several generations of backcrossing onto the C57BL/6J background improves the motor coordination of the *Drd2* knock-outs (Kelly et al., 1998). Besides the motor disturbances, the knock-outs have been shown to have deficits in spatial, reverse, and avoidance learning (Glickstein et al., 2002; Kruzich et al., 2006; Smith et al., 2002; Tran et al., 2002).

In addition to the *Drd2* knock-out animals completely deficient in the dopamine D_2 receptor, D_{2L} receptor knock-out mouse strains have been developed (Usiello et al., 2000; Wang et al., 2000). The D_{2L} knock-out strains generated in different laboratories have shown different behaviour, with one group reporting intact locomotor activity under normal conditions (Usiello et al., 2000), and the other group reporting reduced locomotion and rearing, impaired motor coordination, and a decrease in avoidance learning (Fetsko et al., 2005; Wang et al., 2000).

Recently, Kellendonk and co-workers (2006) designed a mouse strain with a transient knock-in of striatal dopamine D_2 receptors. These mice possess increased dopamine levels but decreased dopamine turnover, as well as decreased activation of D_1 receptors in the medial prefrontal cortex. However, their locomotor activity and anxiety levels are normal. The transient dopamine D_2 receptor knock-in animals also show impairments in working memory tasks, whereas their general cognitive skills are intact. Interestingly, the disturbances in D_1 receptor activation and working

memory function persist even after the D_2 receptor overexpression has returned to normal.

When measured in conditioned place preference setup, response of the *Drd2* knock-outs to food reward is intact (Maldonado et al., 1997), but in operant self-administration conditions these mice respond less to food, milk, or saccharin (Caine et al., 2002; Fowler et al., 2002; Kruzich et al., 2006; Risinger et al., 2000). The decreased rate of response is especially evident in the progressive ratio setup, which measures motivation to work for the reward (Rowlett, 2000; Stafford et al., 1998). One reason for this may be lower basal locomotor activity, since the latency from operandum press to milk consumption is longer in the *Drd2* knock-outs (Fowler et al., 2002). In addition, these mice show delayed acquisition of the operant response. Furthermore, electrophysiological recordings of nucleus accumbens neurons have shown that the pre-reward inhibitory response to the predictable reward is lacking in these animals, which may influence the incentive salience of drug-related stimuli (Tran et al., 2002). However, the *Drd2* knock-outs also show impaired olfactory discrimination, which may interfere with the food or sweet reward experience (Tillerson et al., 2006). The ICSS threshold of the *Drd2* knock-outs has been reported to be intact in one study (Tran et al., 2002), whereas another study has suggested an elevation of the threshold (Elmer et al., 2005). Interestingly, the transient dopamine D_2 receptor knock-in animals also show reduced motivation to work for a food reward in an operant task, but their sucrose preference is not altered (Drew et al., 2007).

Due to the absence of dopamine autoreceptor function in *Drd2* knock-out mice, the increase in extracellular dopamine induced by cocaine and morphine is potentiated in these animals, but not in the dopamine D_{2L} receptor knock-outs (Rougé-Pont et al., 2002). On the other hand, *Drd2* knock-out animals show attenuated ethanol-induced increase in striatal extracellular dopamine levels (Job et al., 2006). Cyclic voltammetry studies have shown that the *Drd2* knock-out potentiates the amphetamine-induced increase of the stimulus-evoked dopamine overflow (Schmitz et al., 2001). However, the amphetamine-induced efflux of dopamine from striatal synaptosomes is not altered, whereas that induced by cocaine is decreased (L'Hirondel et al., 1998).

Amphetamine-induced locomotor activation is intact in homozygous *Drd2* knock-out mice (Chen et al., 2001), but they show reduced MDMA, phencyclidine, and cocaine-induced locomotor activation (Chausmer et al., 2002; Risbrough et al., 2006; Welter et al., 2007). However, the dopamine D_{2L} receptor deficiency does not influence the stimulatory effect of cocaine on locomotor activity (Welter et al., 2007). The same is true in the drug discrimination test for cocaine (Chausmer et al., 2002). Amphetamine potentiates the rewarding effect of ICSS to a similar extent in knock-out and wild-type animals (Elmer et al., 2005). In addition, the *Drd2* knock-out mice show reduced sensitivity to cocaine in the conditioned place preference paradigm (Welter et al., 2007), but they show enhanced cocaine self-administration behaviour at high doses of cocaine (Caine et al., 2002). The dopamine D_{2L} receptor knock-outs

show intact cocaine-induced place preference (Smith et al., 2002; Welter et al., 2007), indicating that only the dopamine D_{2S} receptor subtype is involved in cocaine place conditioning. The discordant results from CPP and i.v. self-administration studies may reflect the different background strains, since Welter et al. (2007) used a hybrid strain with 25% 129Sv genes and 75% C57BL/6J genes, whereas the *Drd2* knock-out line used by Caine et al. (2002) was maintained on a congenic C57BL/6J background. Another explanation for these discrepant findings may be the higher sensitivity of the self-administration paradigm for uncovering small changes in the reinforcing value of the drugs (Blokhina et al., 2004).

In some studies, neither the *Drd2* knock-out mice nor the D_{2L} receptor knock-out mice develop place preference to morphine (Maldonado et al., 1997; Smith et al., 2002). However, Dockstader and co-workers (2001) found that morphine does induce place conditioning in drug-naïve, but not opiate-dependent, *Drd2* knock-out mice. Again, the failure of Maldonado and co-workers (1997) and of Smith and co-workers (2002) to observe morphine place conditioning may be due to the incompletely congenic background strain. In both the opiate-dependent *Drd2* knock-outs and dopamine D_{2L} receptor knock-outs, naloxone-induced place aversion is abolished, but the somatic signs of morphine withdrawal are not altered (Dockstader et al., 2001; Smith et al., 2002). *Drd2* knock-out mice also fail to self-administer morphine intravenously both under fixed-ratio and progressive ratio schedules (Elmer et al., 2002). Furthermore, morphine antagonizes the rewarding effect of ICSS in these animals, although potentiation of the rewarding effect is seen in the wild-type animals (Elmer et al., 2005). *Drd2* knock-outs also show decreased oral ethanol self-administration (Palmer et al., 2003; Phillips et al., 1998; Risinger et al., 2000; Thanos et al., 2005), reduced ethanol place preference (Cunningham et al., 2000), diminished sensitivity to ethanol-induced motor impairment (Phillips et al., 1998), and enhanced susceptibility to locomotor sensitisation with repeated ethanol administration (Palmer et al., 2003). In addition, decreased ethanol consumption is not observed in the knock-outs if they have previously been sensitised to ethanol (Palmer et al., 2003).

In summary, the D_2 receptors, especially the D_{2S} form, seem to be important in the actions of abused drugs. There is also evidence that intact function of the dopamine D_2 receptors is needed for drug, food, and sugar reward or for the motivation to work to gain these rewards. However, these studies are weakened by the fact that the transient dopamine D_2 receptor knock-in mice show reduced motivation in an operant task.

2.5.6 Dopamine D_3 receptor mutant mouse lines

Dopamine D_3 receptors belong to the dopamine D_2 type receptor family and they are largely expressed in the limbic areas of the brain: the nucleus accumbens, islands of

Calleja, olfactory tubercle, ventral pallidum, and amygdala (Heidbreder et al., 2005; Sokoloff et al., 1990; Xu et al., 1997). The dopamine D_3 receptors have been suggested to act as autoreceptors, although they seem to be subordinate to the dopamine D_2 receptors in this function (Diaz et al., 2000; Joseph et al., 2002; Nissbrandt et al., 1995; Tepper et al., 1997). Stimulation of dopamine D_3 receptors reduces locomotor activity, and they regulate locomotor activity in concert with dopamine D_1 and D_2 receptors (Missale et al., 1998). The persistent inactivation of locomotor inhibition mediated by dopamine D_3 receptors seems to play a pivotal role in behavioural sensitisation (Richtand et al., 2003). Dopamine D_3 receptors are also involved in several aspects of drug dependence and abuse, e.g. in brain stimulation reward, reinforcement, drug seeking as well as cue, drug, and stress-induced drug reinstatement (Heidbreder et al., 2005).

Dopamine D_3 receptor gene (*Drd3*) knock-out mice are healthy, they breed normally, and they do not show any major physical abnormalities (Accili et al., 1996). This deficiency has been linked to elevated striatal extracellular dopamine levels (Joseph et al., 2002; Koeltzow et al., 1998), but in the absence of any change in the ratio of dopamine/DOPAC in the limbic forebrain (Chen et al., 2007; Joseph et al., 2002). However, in other *Drd3* knock-out animals coming from different laboratories, no alterations are found in the extracellular dopamine levels or dopamine clearance in the ventral striatum, or in the dopamine and dopamine metabolite levels in the striatum (Narita et al., 2003; Zapata et al., 2001). Dopamine D_1 receptor expression is reduced in the striatum but enhanced in the limbic forebrain of the *Drd3* knock-outs (Chen et al., 2007; Wong et al., 2003a). Furthermore, these mice have been shown to demonstrate decreased levels of tyrosine hydroxylase mRNA but increased levels of DAT mRNA, as well as enhanced DAT function (Le Foll et al., 2005).

Drd3 knock-out mice show increased horizontal locomotor activity, rearing, sniffing, and stereotypic behaviour, but their grooming is reduced (Accili et al., 1996; Boyce-Rustay and Risinger, 2003; Joseph et al., 2002; Steiner et al., 1997; Wong et al., 2003b; Xu et al., 1997). Some studies, however, have reported different observations (Boulay et al., 1999; Carta et al., 2000; Jung et al., 1999b), and an ethologically-based behavioural analysis found only increased rearing behaviour in females during prolonged assessment (McNamara et al., 2002). Nevertheless, *Drd3* knock-outs show reduced anxiety-related behaviour (Steiner et al., 1997) and impaired spatial working memory function (Glickstein et al., 2002), but intact taste reactivity for sweet and bitter solutions (McQuade et al., 2003).

The *Drd1* knock-out has been combined with the *Drd3* knock-out, yielding mice that show normal or slightly increased horizontal locomotor activity, reduced rearing, poor motor coordination and spatial learning performance, but wild-type anxiety-like behaviour (Karasinska et al., 2000, 2005; Wong et al., 2003b). They also exhibit increased sniffing, but decreased free rearing, rearing from a seated position, grooming, chewing, and stillness (Wong et al., 2003b). Furthermore, a *Drd2*/*Drd3* double mutant mouse line has been created (Jung et al., 1999b). Similar to the *Drd2*

knock-out animals, the double knock-outs show increased striatal dopamine turnover (Jung et al., 1999b). They also show similar behaviour to *Drd2* knock-outs, with reduced horizontal and vertical locomotor activity (Jung et al., 1999b; Vallone et al., 2002), although the alterations in the behavioural and neurochemical tests are apparently more severe in the *Drd2*/*Drd3* double-mutant than single-mutant knock-out mice.

Dopamine D_3 receptor deficiency does not affect the morphine-induced increase in dopamine turnover in the limbic forebrain (Narita et al., 2003). However, after chronic methamphetamine treatment, the dopamine/DOPAC ratio decreases in the limbic forebrain of wild-type animals, but increases in the *Drd3* knock-out animals (Chen et al., 2007). Cocaine-induced *c-Fos* expression is enhanced in the dorsal and ventral striatum of *Drd3* knock-out mice (Carta et al., 2000).

The *Drd3* knock-out animals show enhanced motor responsiveness to acute doses of cocaine (Betancur et al., 2001; Carta et al., 2000; Karasinska et al., 2005; Xu et al., 1997), but they develop behavioural sensitisation to cocaine similar to wild-type mice (Betancur et al., 2001). In addition, they may be more sensitive to cocaine-paired cues than their wild-type littermates (Le Foll et al., 2002). On the other hand, the *Drd1* and *Drd3* double knock-out mice fail to show locomotor activation following an acute dose of cocaine (Karasinska et al., 2005). At low amphetamine doses, the *Drd3* knock-out mice show enhanced increase in horizontal locomotor activity but no stereotypic behaviour (McNamara et al., 2006). However, at higher doses the amphetamine response is similar between the knock-out and wild-type animals. Acute methamphetamine-induced horizontal locomotor activity and stereotypic behaviour are, on the other hand, enhanced in the *Drd3* knock-outs, and they also develop behavioural sensitisation to methamphetamine faster than the wild-type animals (Chen et al., 2007). Furthermore, they exhibit slightly enhanced methamphetamine-conditioned place preference. Female, but not male, *Drd3* knock-out animals show reduced MDMA-induced locomotor activation (Risbrough et al., 2006). As was noted for the *Drd1* knock-out mice (see 2.3.4), the fact that the effect of MDMA is different from that of cocaine or amphetamine may be due to the contribution of 5-HT-mediated mechanisms in the action of MDMA (Bengel et al., 1998; Callaway et al., 1990; Crespi et al., 1997). The *Drd3* knock-out mice are more sensitive than their wild-type littermates to the positive reinforcing effects of amphetamine in the conditioned place preference setup (Xu et al., 1997). However, the *Drd3* knock-outs, as well as the *Drd1* and *Drd3* double knock-outs, show intact cocaine-induced conditioned place preference (Karasinska et al., 2005).

Morphine-induced behavioural sensitisation and morphine-conditioned place preference are remarkably enhanced in the *Drd3* knock-out animals that are congenic with C57BL/6J background (Narita et al., 2003), but not in those knock-outs that are maintained on a C57BL/6J x 129Sv hybrid background (Francès et al., 2004). Ethanol self-administration, development of ethanol place preference, or conditioned taste aversion to ethanol are not affected by dopamine D_3 receptor

deficiency (Boyce-Rustay and Risinger, 2003; McQuade et al., 2003). Although ethanol reward is intact, *Drd3* knock-out animals exhibit more severe withdrawal symptoms after a 4-day forced oral ethanol exposure, and they are more sensitive to the hypnotic effect of ethanol (Narita et al., 2002). After an intraperitoneal ethanol injection, the *Drd3* knock-out mice develop higher blood alcohol levels than their wild-type littermates, suggesting that they exhibit slower ethanol metabolism (McQuade et al., 2003).

In conclusion, dopamine D_3 receptors seem to be involved in the effects of psychostimulants and morphine. In addition, some effects of ethanol are affected by *Drd3* gene disruption. However, since these receptors are assumed to be involved in drug reinstatement, further studies examining drug extinction and reinstatement in conditioned place preference or intravenous self-administration setups are warranted. Furthermore, studies using *Drd3* knock-in mice may provide valuable information about the role of dopamine D_3 receptors.

2.5.7 Dopamine D_4 receptor mutant mouse line

Dopamine D_4 receptors also belong to the dopamine D_2 type receptor family and they are abundantly expressed in the frontal cortex, amygdala, olfactory bulb, hippocampus, and hypothalamus (Missale et al., 1998; Sealfon and Olanow, 2000). The role of these receptors is not yet very clear, but they may function as synthesis-regulating autoreceptors and as regulators of locomotor activity with other dopamine receptor subtypes (Rubinstein et al., 1997). In humans, the long allele (~7 repeats) of exon III VNTR polymorphism of the dopamine D_4 receptor gene has been linked to opioid addiction (Kotler et al., 1997; Li et al., 1997; Shao et al., 2006, but see Franke et al., 2000; Li et al., 2000), smoking (see Ho and Tyndale, 2007, for a review), and methamphetamine abuse (Li et al., 2004).

Dopamine D_4 receptor gene (*Drd4*) knock-out mice appear physically normal (Rubinstein et al., 1997). They exhibit reduced levels of striatal extracellular dopamine, DOPAC, and HVA as well as wild-type dopamine concentration and decreased DOPAC levels in striatal and accumbal tissue (Thomas et al., 2007). Furthermore, the nucleus accumbens shows reduced dopamine turnover, potassium chloride-evoked dopamine release, and rate of dopamine uptake. However, other studies have suggested enhanced dopamine synthesis and turnover in the striatum, but not in the nucleus accumbens or frontal cortex of the knock-outs (Rubinstein et al., 1997, 2001). In addition, the expression of striatal and accumbal dopamine D_1 receptors, together with that of striatal, accumbal, and hippocampal NMDA receptors is up-regulated, and the proportion of striatal dopamine D_2 receptors in a highly active state increases at least two-fold (Gan et al., 2004; Seeman et al., 2005).

One study found *Drd4* knock-out mice to exhibit reduced horizontal and vertical locomotor activity but improved motor coordination (Rubinstein et al., 1997),

whereas other studies have not found this to be the case (Dulawa et al., 1999; O'Sullivan et al., 2006). An ethologically based analysis of the behaviour of these mice revealed only a small decrease in sniffing and delayed habituation of sifting (see Table 2 for descriptions; O'Sullivan et al., 2006). They are also less behaviourally responsive to novelty than their wild-type littermates (Dulawa et al., 1999). In addition, they show increased anxiety in the elevated plus maze and the light-dark exploration test, whereas the conditioned fear responses and emotionality are intact (Falzone et al., 2002).

Drd4 knock-out animals are more sensitive than their wild-type littermates to the stimulatory effects of alcohol, cocaine, and metamphetamine on locomotor activity (Katz et al., 2003; Rubinstein et al., 1997). The cocaine discriminative stimulus effects are also enhanced in the knock-out animals (Katz et al., 2003). Furthermore, when amphetamine is given repeatedly, the mice show increased behavioural sensitisation, although the acute amphetamine response is normal (Kruzich et al., 2004). However, ethanol preference and consumption are not altered (Falzone et al., 2002).

All in all, surprisingly few studies explore addiction-like behaviour in *Drd4* knock-out mice. There is some evidence that dopamine D_4 receptor may play a role in psychostimulant addiction. However, these receptors may not be involved in ethanol consumption.

2.5.8 Dopamine D_5 receptor mutant mouse line

Dopamine D_5 receptors (formerly known as D_{1B} receptors) belong to the dopamine D_1 type receptor family. They are expressed at quite low concentrations in the brain, particularly in the cortex, hippocampus, and striatum, as well as the lateral and medial thalamus (Choi et al., 1995; Ciliax et al., 2000; Meador-Woodruff et al., 1992). The dopamine D_1 and D_5 receptors appear to have a similar pharmacological profile, which makes distinguishing the two subtypes of the receptor family practically impossible. Thus, the role of these receptors in the brain is poorly understood, although they have been suggested to be involved in regulating cell migration during brain development (Wang et al., 1997). There is some evidence that a dinucleotide repeat polymorphism of the dopamine D_5 receptor gene may be involved in drug or alcohol abuse in humans (Vanyukov et al., 2000), but other studies have failed to support this hypothesis (Li et al., 2006; Sullivan et al., 2001).

Dopamine D_5 receptor gene (*Drd5*) knock-out mice are healthy and viable without the growth retardation seen in D_1-knock-out mice, but 30% of the homozygous animals lack whiskers (Holmes et al., 2001). Their horizontal locomotor activity seems to be slightly reduced and they show increased sifting during the exploration phase (see Table 2 for descriptions; O'Sullivan et al., 2005). In addition, grooming is decreased and habituation of rearing behaviour is delayed. The anxiety

level, motor coordination, spatial learning, memory, and fear conditioning are intact (Holmes et al., 2001). However, in the Porsolt's forced swim test, male *Drd5* knock-out mice show reduced immobility levels, indicating an "antidepressant-like" phenotype. There is apparently only one study reporting the effects of drugs of abuse in *Drd5* knock-out mice (Elliot et al., 2003). The knock-out mice show slightly reduced locomotor response to cocaine, but their cocaine discrimination behaviour is similar to that of their wild-type littermates.

Studies on the role of dopamine D_5 receptors in the brain are limited, and studies using abused substances are even scarcer. However, the limited distribution of these receptors in the brain suggests that they are not critically involved in addiction-like behaviour.

2.5.9 Monoamine oxidase A and B mutant mouse lines

After its reuptake into nerve terminals or alternatively into glial cells, dopamine is converted to DOPAC by monoamine oxidase (Figure 4; Cooper et al., 2003). There are two subtypes of monoamine oxidase (MAO), MAO-A and MAO-B, and these subtypes differ in their substrate specificity. In rodents, MAO-A preferentially metabolizes 5-HT (Cooper et al., 2003; Strolin Benedetti et al., 1992), and in mouse brain it is expressed in all the dopaminergic and noradrenergic neurons that also express tyrosine hydroxylase (Vitalis et al., 2002). MAO-B metabolizes primarily trace amines, such as β-phenylethylamine and benzylamine (Cooper et al., 2003; Strolin Benedetti et al., 1992), and it is expressed mostly in serotonergic neurons and non-neuronal cells (Vitalis et al., 2002). Dopamine is metabolized by both enzyme forms (Cooper et al., 2003; Strolin Benedetti et al., 1992). In humans, smoking has been associated with the T allele of the SNP T1460C polymorphism in the MAO-A gene, which leads to lower enzyme activity (McKinney et al., 2000), or the 4-repeat allele of the VNTR polymorphism in the promoter region, which enhances the transcription of the gene (Ito et al., 2003). Furthermore, both the A allele of the polymorphism in intron 13 of the MAO-B gene and allele B12 of the Taq IB polymorphism in the *Drd2* gene has been linked to chronic smoker and former smoker status in men, but not in women (Costa-Mallen et al., 2005).

In mice, the knock-out of the MAO-A gene (*Maoa*) results in an increase in levels of 5-HT, noradrenaline, and dopamine in brain tissue, as well as a decline in levels of DOPAC and 5-hydroxyindole acetic acid (5-HIAA; Cases et al., 1995; Popova et al., 2004). The levels of MAO-B are normal in brain and peripheral tissues (Cases et al., 1995; Holschneider et al., 2001). As pups, *Maoa* knock-out animals exhibit severely altered behaviour, including trembling, difficulty in righting, and fearfulness (Cases et al., 1995).

In adulthood, they show enhanced locomotor activity (Agatsuma et al., 2006), reduced beam-walking ability (Cases et al., 1995; Salichon et al., 2001), decreased

exploratory activity (Popova et al., 2000; Vishnivetskaya et al., 2007), delayed habituation to novel environments (Agatsuma et al., 2006), and increased fear conditioning avoidance learning (Kim et al., 1997). In the Porsolt swim test, their swimming time is prolonged, suggesting an “antidepressant” phenotype (Cases et al., 1995). They also exhibit increased aggression, and reduced time spent in social interaction (Cases et al., 1995; Vishnivetskaya et al., 2007). On the other hand, the *Maoa* knock-out animals respond normally to sucrose reward (Agatsuma et al., 2006).

In the MAO-B gene (*Maob*) knock-out animals, the tissue levels of dopamine, 5-HT, noradrenaline, and their metabolites are normal in striatum, cortex, hippocampus, raphe nucleus, substantia nigra, and thalamus (Grimsby et al., 1997). In addition, the striatal extracellular dopamine levels are normal (Chen et al., 1999), and the levels of MAO-A are unchanged in brain and peripheral tissues (Grimsby et al., 1997; Holschneider et al., 2001). However, the sensitivity, but not the number, of accumbal dopamine D_1 receptors and the density of striatal and accumbens shell dopamine D_2 receptors are increased (Chen et al., 1999). The knock-out animals also show delayed habituation to an inescapable open field (Lee et al., 2004), but they do not exhibit alterations in general locomotor activity (Grimsby et al., 1997; Lee et al., 2004). Moreover, they are not more aggressive than their wild-type littermates, and their working memory and visuo-spatial learning abilities are intact (Grimsby et al., 1997; Holschneider et al., 1999). Like *Maoa* knock-outs, *Maob* knock-outs show prolonged swimming time in the Porsolt swim test (Grimsby et al., 1997).

The development of nicotine place preference is abolished in *Maoa* knock-out mice, and they exhibit diminished preference for oral nicotine in two-bottle free-choice oral self-administration conditions (Agatsuma et al., 2006). However, nicotine preference is not altered in *Maob* knock-out mice (Lee et al., 2004). *Maoa* knock-out animals also show enhanced resistance to the hypnotic and hypothermic effects of ethanol (Ivanova and Popova, 2002; Popova et al., 2000), but ethanol consumption and ethanol preference remain normal (Popova et al., 2000). Furthermore, *Maob* knock-outs show reduced locomotor activity in response to acute or repeated doses of amphetamine (Yin et al., 2006).

Again, disappointingly few studies have examined addiction-like behaviour in *Maoa* and *Maob* knockout animals. There is, however, some evidence for an effect of MAO-A and MAO-B levels on the development of addiction. It should be noted that although MAO-A oxidizes dopamine, it is also critically involved in serotonin metabolism. Therefore, it is likely that at least part of the observed effects of MAO deficiency, e.g. increased aggression, are due to decreased serotonin elimination. Furthermore, the differences in the effects of MAO deficiency may be due to the different extents to which different substances activate serotonergic and dopaminergic pathways.

2.5.10 Catechol-*O*-methyltransferase mutant mouse line

Catechol-*O*-methyl transferase (COMT) catalyzes the metabolism of catecholamines and other catechols in the brain and peripheral tissues (Männistö and Kaakkola, 1999). There are two types of COMT enzyme, soluble S-COMT and membrane-bound MB-COMT, which are both products of the same gene (Lundström et al., 1991; Salminen et al., 1990). In most human and rodent tissues, S-COMT is the dominant enzyme form, but in the human brain MB-COMT is 2.5-fold more abundant than S-COMT (Tenhunen and Ulmanen, 1993; Tenhunen et al., 1994). In the brain, COMT has been localized to glial cells and postsynaptic neurons, but not to presynaptic dopaminergic neurons (Figure 4; Kaakkola et al., 1987; Karhunen et al., 1995a, 1995b). In striatum, reuptake by DAT and subsequent oxidation by MAO are the primary means of removing dopamine from the synaptic cleft (Cass et al., 1993; Eisenhofer et al., 2004; Giros et al., 1996). Nevertheless, in brain areas with low DAT density, e.g. in the prefrontal cortex, the role of COMT in the control of dopaminergic transmission is greater (Mazei et al., 2002; Morón et al., 2002; Sesack et al., 1998; Yavich et al., 2007).

In humans, a common functional Val108/158Met polymorphism affects COMT activity. The Met-allele results in a heat-labile enzyme with considerably lower activity (Boudíková et al., 1990; Chen et al., 2004; Lotta et al., 1995). COMT Val108/158Met polymorphism has been linked to drug abuse, although the influence of the human COMT Val108/158Met polymorphism on addiction remains unclear. Some studies have detected a correlation between high COMT activity (Val/Val genotype, resulting in low dopamine levels), and polysubstance abuse (Vandenbergh et al., 1997), heroin addiction (Horowitz et al., 2000), metamphetamine use (Li et al., 2004), alcoholism in males (Sery et al., 2006), and co-existence of alcoholism and smoking in females (Enoch et al., 2006). Others have pointed to an association between low enzyme activity (Met/Met genotype, resulting in high dopamine levels) and nicotine dependence in females (Beuten et al., 2006), alcohol consumption in non-alcoholic males (Kauhanen et al., 2000), and type 1 and type 2 alcoholism in males (Tiihonen et al., 1999; Wang et al., 2001). In addition, adolescent Val allele carriers who use cannabis seem to be at an increased risk to exhibit psychotomimetic side effects and to develop psychosis in adulthood (Caspi et al., 2005). However, several reports indicate that COMT polymorphism may not be linked to nicotine dependence (Colilla et al., 2005; David et al., 2002; Foroud et al., 2007; McKinney et al., 2000; Redden et al., 2005) or alcoholism (Foroud et al., 2007; Hallikainen et al., 2000; Kweon et al., 2005; Köhnke et al., 2003; Samochowiec et al., 2006).

COMT knock-out mice show normal locomotor behaviour (Haasio et al., 2003), but heterozygous animals exhibit decreased rearing and increased sifting and chewing during the exploration phase (see Table 2 for descriptions; Babovic et al., 2007). Male heterozygous mice also appear more aggressive than other genotypes

(Gogos et al., 1998). On the other hand, female homozygous mice, and lately also male homozygotes, have been shown to exhibit increased anxiety levels (Gogos et al., 1998; Papaleo et al., 2008). Furthermore, male COMT knock-out mice perform better in a T-maze test measuring working memory performance, but they show exaggerated acoustic startle response (Papaleo et al., 2008). Under basal conditions, DOPAC levels in brain tissue and striatal extracellular fluid are 3- to 4-fold higher in COMT deficient mice than wild-type littermates (Gogos et al., 1998; Huotari et al., 2002a), but striatal dopamine levels are normal (Gogos et al., 1998; Huotari et al., 2004). One study showed a 2.5-fold increase in prefrontal cortex dopamine levels in homozygous males but not females (Gogos et al., 1998), but another study failed to replicate this finding (Huotari et al., 2002a). However, homozygous animals show a 20-25% increase in stimulus-evoked dopamine release and a 50% lengthening of the dopamine elimination time (Yavich et al., 2007). In these animals, striatal dopamine D_1 and D_2 receptor binding is intact, but the proportion of D_2 receptors in the highly active state is increased 1.9-fold (Huotari et al., 2004; Seeman et al., 2005). In spite of these changes, the activities or protein levels of dopamine transporter, dopamine synthesizing enzymes, and other metabolic enzymes are normal (Haasio et al., 2003; Huotari et al., 2002a; Huotari et al., 2002b; Odlind et al., 2002). Interestingly, the liver cytochrome P450 enzyme profile shows that these mice still show some influence of the 129Sv strain in their genome (Forsberg et al., 2004).

COMT knock-out male mice show reduced sensitivity to the motor activation caused by cocaine and GBR 12909 (Huotari et al., 2002b), as well as to the initial motor depression caused by a large dose of amphetamine (Huotari et al., 2004). Amphetamine or GBR 12909 induce similar increases in the levels of dopamine in the striatal extracellular fluid in mutant and wild-type mice (Huotari et al., 2002b; Huotari et al., 2004). Even in prefrontal cortex, the effect of cocaine on dopamine neurotransmission is normal (Yavich et al., 2007).

The sexually dimorphic effects of *Comt* polymorphism or disruption may reflect different roles of this enzyme in males and females. The *Comt* promoters are down-regulated by estrogens (Jiang et al., 2003; Xie et al., 1999) and, therefore, females show less COMT activity than males despite having similar levels of COMT protein and mRNA (Boudíková et al., 1990; Chen et al., 2004; Tunbridge et al., 2004a). This may create a different background for the changes in the function of the dopamine system.

Although human studies have suggested a possible link between COMT polymorphism and substance use, animal studies exploring the neurochemical and behavioural responses to drugs of abuse in *Comt* knock-out animals are scarce. Published biochemical and locomotor activity studies with psychostimulants warrant further exploration of brain neurochemistry and addiction-like behaviour in these animals.

Table 3 *Summary of the addiction-related behavioural effects of mutations targeted to the dopaminergic system.*

Target and mutation		Sensitisation	CPP	IVSA/OSA[a]
TH	Dopamine deficient mice	↓↓ (AMPH)	0[b] (COC, MO)	NT
DAT	DAT KO	↓↓ (COC, AMPH)	0 (COC, AMPH, MPH) ↑ (MO)	0 (COC) ↔ (ETOH)
	DAT KD	NT	0 (COC)	NT
	DAT CI	NT	↓↓ (COC)	NT
	DAT KI	NT	↑ (COC, AMPH)	NT
VMAT2	VMAT2 KO[c]	↓↓ (AMPH) 0 (COC, METH)	↓ (AMPH, ETOH)	↔ (ETOH)
DRD1	DRD1 KO	↓↓ (MO) ↓ (AMPH, COC low) ↑ (AMPH, COC high)	0 (COC)	↓↓ (COC, ETOH) 0 (REM)
DRD2	DRD2 KO	↑ (ETOH)	↓ (COC, ETOH) ↔ (MO)	↑ (COC high) ↓ (ETOH) ↓↓ (MO)
	$DRD2_L$ KO	NT	0 (COC) ↓↓ (MO)	NT
DRD3	DRD3 KO	↑↑ (MO) ↑ (METH) 0 (COC)	↔ (MO) ↑ (AMPH, METH) 0 (COC, ETOH)	0 (ETOH)
	DRD1/DRD3 KO	NT	0 (COC)	NT
DRD4	DRD4 KO	↑ (AMPH)	0 (ETOH)	0 (ETOH)
DRD5	DRD5 KO	NT	NT	NT
MAO	MAOA KO	NT	↓↓ (NIC)	0 (ETOH)
	MAOB KO	↓ (AMPH,)	NT	NT
COMT	COMT KO	NT	NT	NT

[a] Oral self-administration: ethanol, intravenous self-administration: all other substances.
[b] Under levodopa or caffeine treatment.
[c] Only heterozygous individuals survive to adulthood.

CPP = conditioned place preference
OSA = oral self-administration
DAT = dopamine transporter
DRD1-5 = dopamine receptor 1-5
COMT = catechol-*O*-methyltransferase
COC = cocaine
METH = methamphetamine
MPH = methylphenidate
REM = remifentanil
KD = knock-down
CI = cocaine insensitive
IVSA = intravenous self-administration
TH = tyrosine hydroxylase
VMAT2 = vesicular monoamine transporter 2
MAO = monoamine oxidase
AMPH = amphetamine
ETOH = ethanol
MO = morphine
NIC = nicotine
KO = knock-out
KI = knock-in

↓	= diminished	↓↓	= considerably diminished
↑	= enhanced	↑↑	= considerably enhanced
↔	= controversial results	0	= no change
NT	= not tested		

2.6 Concluding remarks of the literature review

This literature review has pointed out several important issues that needed to be studied in the following experimental part of the study. First, the effects of chronic oral nicotine exposure on brain monoamines, the number of nAChRs, and forward locomotion have been thoroughly studied. However, the mechanisms of changes observed during chronic nicotine exposure are incompletely understood. Second, the effects of forced chronic nicotine exposure on reinforcing properties have not been studied. The effect of involuntary nicotine exposure on the development of addiction-like behaviour is relevant when the risks of passive tobacco smoke exposure are evaluated. Third, population studies have produced some evidence of a link between COMT activity levels and risk of addiction. However, *Comt* gene knock-out mice have not been used to clarify the association between addiction-like behaviour and *Comt* genotype. Fourth, the effect of *Comt* gene disruption on the extracellular levels of dopamine and dopamine metabolites in the nucleus accumbens or prefrontal cortex has not been studied.

3 AIMS OF THE STUDY

In general terms, this study aimed to further clarify the role of dopamine in addiction-related neurochemical and behavioural changes, and to examine whether *Comt* gene knock-out mice are a useful tool in addiction research.

The specific aims were:

1. To explore the effect of chronic oral nicotine treatment on D_2-like dopamine receptor sensitivity in the dorsal striatum and nucleus accumbens in mice after a 50-day forced oral nicotine exposure (I).

2. To study whether the 50-day forced oral nicotine exposure would affect the reinforcing effects of nicotine (II).

3. To investigate the effect of *Comt* gene disruption on the reinforcing effects of ethanol and cocaine solutions in male and female mice (III).

4. To clarify the effect of *Comt* gene disruption on extracellular levels of dopamine and on dopamine kinetics in striatum, nucleus accumbens, and prefrontal cortex under normal conditions and after levodopa-carbidopa treatment in freely-moving mice carrying a disruption of the *Comt* gene (IV).

4 MATERIALS AND METHODS

4.1 Animals

Male NMRI mice were bred at the Helsinki University Laboratory Animal Centre. At the beginning of forced chronic oral nicotine exposure, the mice were 4-5 weeks old and weighed 20-30 g (I, II). In locomotor activity tests after repeated nicotine injections (I) and in dose-response experiments for intravenous self-administration (II), 10-week old mice weighing 35-45 g were used.

The *Comt* disrupted mouse strain was originally generated by Gogos et al. (1998) on a mixed 129Sv x C57BL/6J background and later backcrossed for more than 20 generations on a pure C57BL/6J background. Mice were bred in the National Laboratory Animal Center, Kuopio, Finland (III), or in Helsinki University Laboratory Animal Centre, Helsinki, Finland (IV). Heterozygous males and females were used as breeding couples. To keep the strain viable, it was enriched regularly by mating C57BL/6J females (Harlan, The Netherlands) with heterozygous males, and their heterozygous offspring were used for further breeding. Mouse pups were weaned and ear-marked at three weeks of age, and a 3-5 mm tail clipping was taken for genotyping. Studies used both male and female *Comt* gene disrupted homozygous [COMT(-/-)] and heterozygous [COMT(+/-)] mice and their wild-type littermates, with all mice aged two to eight months at the beginning of the experiments.

Animals were housed in groups of 2-10 mice at an ambient temperature of 21-23 °C and relative humidity of 50 ± 10% under a 12:12 light cycle (lights on at 6:00 am). The mice had *ad libitum* access to mouse chow and drinking fluid. The oestrus phase of female mice was not determined. All procedures with animals were performed according to European Community Guidelines for the use of experimental animals (European Communities Council Directive 86/609/EEC) and reviewed and approved by the Animal Ethics Committees at the University of Helsinki in conformity with current legislation.

4.1.1 Genotyping of *Comt* disrupted mice (III, IV)

Genomic DNA was isolated from tail clippings as described by Laird et al. (1991). For genotyping, a polymerase chain reaction (PCR) method was used. Two primer sets were used: 5'-ACCATGGAGATTAACCCTGACTACG-3' (sense) and 5'-GTGTGTCTGGAA GGTAGCGGTC-3' (antisense) for the detection of COMT gene (comt) alleles, and 5'-GTGTTCCGGCTGTCAGCGCA-3' (sense) and 5'-GTCCTGATAGCGGTCCGCCA-3' (antisense) for the detection of mutant alleles containing the neomycin gene (*neo*) cassette that replaces exons 2-4 of the *Comt* gene. Genomic PCR was carried out with the Fail Safe PCR system (Epicentre

Technologies, Madison, WI, United States) using Fail Safe buffer B and the following thermal cycles: an initial denaturing at 98°C for 1 min and 35 cycles consisting of a denaturing temperature of 94°C for 30 s, annealing temperature of 65°C for 1 min, and extension at 72°C for 3 min, with a final extension of 70°C for 10 min. The amplified fragments were visualized by ethidium bromide staining under ultraviolet light after electrophoresis in a 1.7% agarose gel (Study III, Figure 1).

4.2 Drugs and drug treatments

4.2.1 Drugs

(-)-Nicotine base for nicotine solutions and nicotine injections in conditioned place preference and locomotor activity experiments was from Fluka BioChemika (Buchs, Switzerland). (-)-Nicotine tartrate for intravenous self-administration studies was purchased from Sigma (St. Louis, MO, USA). For the oral self-administration studies, ethanol was from Altia (Rajamäki, Finland), cocaine hydrochloride from University Pharmacy (Helsinki, Finland), and etonitazene hydrochloride from Ciba-Geigy Limited (Basel, Switzerland). (-)-Quinpirole for the microdialysis and locomotor activity studies was purchased from RBI (Natick, MA, USA). For the microdialysis studies, carbidopa was from Orion Pharma (Espoo, Finland), and levodopa from Sigma (St. Louis, MO, USA). Carbidopa and levodopa were suspended in 0.25% methylcellulose gel. All other drugs were dissolved in saline. For nicotine injections, the pH of nicotine solutions was adjusted to 7 with hydrochloric acid. Injection volume was 10 ml/kg in subcutaneous (s.c.) and intraperitoneal (i.p.) injections. All the drug doses refer to the free base.

4.2.2 Chronic nicotine treatments (I, II)

To study the effect of forced chronic oral nicotine exposure on sensitivity of dopamine D_2 receptors (I) and the rewarding and reinforcing properties of acute nicotine administration (II), (-)-nicotine base was administered to NMRI mice for seven weeks in the drinking water as described by Pekonen et al. (1993). In brief, the concentration of nicotine in the drinking solution was increased gradually at intervals of 3-4 days from 50 to 350 μg/ml, and subsequently at 7-day intervals from 350 to 500 μg/ml to make the mice drink as steadily as possible. The pH of the solutions was adjusted to 6.8 with hydrochloric acid. The nicotine solution was the sole source of fluid for the nicotine-exposed animals, and the control mice drank tap water during the entire exposure period. Body weights and fluid intake were measured once a week. For locomotor activity, conditioned place preference, intravenous self-

administration, and tissue monoamine experiments, some or all of the nicotine-exposed mice were withdrawn from nicotine by replacing the nicotine solution with tap water.

To compare the effect of two different ways of administering nicotine in locomotor activity measurements, a group of mice was treated with repeated nicotine injections. In this experiment, drug-naïve mice were given (-)-nicotine (0.4 mg/kg s.c.) or saline injections twice daily for 10 days.

4.2.3 Other drug treatments

(-)-Quinpirole (dopamine D_2-type receptor agonist) was given only acutely for the locomotor activity, extracellular dopamine, body temperature, and tissue dopamine measurements in chronically nicotine-exposed animals to test the sensitivity of presynaptic D_2-type dopamine autoreceptors. Low quinpirole doses were chosen because they are thought to activate mainly presynaptic dopamine autoreceptors. For locomotor activity and microdialysis experiments, the doses were 0.01 and 0.03 mg/kg (s.c.). For body temperature and tissue dopamine measurements, doses of 0.03 and 0.1 mg/kg (s.c.) were used.

Carbidopa (peripheral dopadecarboxylase inhibitor; 30 mg/kg i.p.) and levodopa (dopamine precursor; 10 mg/kg i.p.) were given only acutely for the striatal, accumbal, and prefrontal cortical microdialysis studies. The doses were chosen based on earlier studies by Huotari and coworkers (2002a, 2002b).

The dosing regimens for nicotine and cocaine are indicated below for the conditioned place preference experiments (4.3.2), experiments testing intravenous nicotine self-administration (4.3.3), and free choice oral self-administration studies (4.3.4).

4.3 Behavioural testing methods

4.3.1 Locomotor activity (I)

Locomotor experiments were performed during the light time of the day in a room reserved exclusively for behavioural experiments and fitted with ordinary artificial lighting and ventilation. On the test day the mice were weighed and carried in their home cages to the experimental room 30 min before the first test. In the test situation the animals were placed one to a cage in Macrolon III cages (18 x 33 x 15 cm) that were identical to their home cages except that no sawdust, food, or water was available. The locomotor activity of chronically nicotine-exposed mice was measured for 60 min on the 50^{th} day of forced oral nicotine exposure or after a withdrawal

period of 23-25 h. Immediately before the test the mice were given an injection of quinpirole (0.01 or 0.03 mg/kg, s.c.) or saline. The test cages were placed in locomotor activity chambers and covered with perforated plastic lids. Interruptions of infrared photo beams were registered and analysed by the software of the computer-controlled locomotor activity apparatus (Activity Monitor, MED Associates Inc., Georgia, VT, USA). For the locomotor activity measurements in mice treated repeatedly with nicotine, the basal locomotor activity was measured for 30 min at 19-24 h after the last injection. After the basal activity was measured, the animals were given quinpirole (0.03 mg/kg s.c.), and the locomotor activity was measured for an additional 60 min. Locomotor activation ratio was calculated for each 5-min segment by dividing the distance travelled in the postinjection period with the average distance travelled during the 5-min segments in the preinjection period. This design allowed comparison to the earlier study by Sershen et al. (1991).

4.3.2 Conditioned place preference (II, unpublished)

Mice were taken to the experiment room 1 h before the testing began. All tests were performed during the light phase of the day. The experiment room was fitted with ordinary artificial lighting and ventilation and continuous white noise to mask disturbing sounds from the environment. The tests were conducted in a computer-controlled apparatus consisting of eight rectangular boxes (42 x 42 x 41 cm), each of which was divided into two equal-sized compartments by a separating wall equipped with a guillotine door and covered by a perforated plastic lid (Activity Monitor, MED Associate Inc., Georgia, VT, USA). One compartment was black with a wire mesh floor, and the other one white with a metal bar floor. In cocaine experiments, the metal bar floor was covered with a transparent Plexiglas plate.

The CPP design consisted of three parts: preconditioning, conditioning, and postconditioning (Table 4). In the preconditioning phase, the mice were allowed to freely explore both compartments for 30 min and the time spent in each compartment was recorded for 15 min. This showed the animal's initial preference for either side. Mice that had a strong initial preference for either compartment (> 650 s) were excluded from further testing. For the conditioning phase, animals were randomly assigned to receive either saline or drug. Since the conditioning regimens were different for nicotine and cocaine, these regimens are described below in detail. Throughout the conditioning phase, the guillotine door was closed. In the postconditioning phase, the guillotine door was opened again and the time spent in each compartment was recorded for 15 min.

Table 4 *Experimental design of conditioned place preference experiments. On conditioning days, saline was given in the forenoon session and conditioning drug was given in the afternoon session.*

Day	Chronic nicotine exposure + nicotine 0.3 mg/kg (II)	Chronic nicotine exposure + nicotine 0.5 mg/kg (II)	*Comt* gene disruption + cocaine 10 mg/kg
1	Preconditioning 1x 30 min	Preconditioning 1x30 min	Preconditioning 1x30 min
2	Conditioning 2x15 min	Conditioning 2x15 min	Preconditioning 2x30 min
3	Conditioning 2x15 min	Conditioning 2x15 min	Conditioning 2x45 min
4	Conditioning 2x15 min	Conditioning 2x15 min	Conditioning 2x45 min
5	Conditioning 2x15 min	Conditioning 2x15 min	Conditioning 2x45 min
6	Postconditioning 1x15 min	Postconditioning 1x15 min	Conditioning 2x45 min
7			Postconditioning 1x15 min
8		Postconditioning 1x15 min	
15		Postconditioning 1x15 min	
22		Postconditioning 1x15 min	

In nicotine experiments (II), a biased design was used to determine the development of place preference to nicotine, i.e. the animals were assigned to the less preferred side, which was designated the drug-paired compartment. In the morning of the conditioning days, each mouse received a saline injection and was placed immediately into the more preferred compartment for 15 min. On the afternoon of the same day, the animals received either a nicotine (0.3 or 0.5 mg/kg s.c.) or saline injection and were placed in the less preferred compartment again for 15 min.

In cocaine experiments, a counterbalanced design was used. Animals were counterbalanced into the groups according to their initial preference and time spent in the boxes during the third preliminary trial. Care was taken that the initial average time spent in the drug-paired compartment was between 435 and 465 s in each group. Before noon on the conditioning days, each mouse received a saline injection and was placed immediately into the non-drug-paired compartment for 45 min. On the afternoon of the same day, the animals received either a cocaine (10 mg/kg i.p.) or saline injection and were placed into the drug-paired compartment again for 45 min.

4.3.3 Intravenous nicotine self-administration (II)

Initially, dose-response experiments were done in drug-naïve NMRI mice. Nicotine tartrate was dissolved in sterile saline and infused intravenously at unit doses of 0.00-0.24 μg/infusion. Unit dose refers to a single dose of nicotine that is delivered in

response to a nose-poke. Based on these studies, nicotine tartrate unit doses of 0, 0.04, and 0.08 μg/infusion were chosen for the experiments in chronically nicotine-exposed mice. For the intravenous self-administration (IVSA) experiments in nicotine-exposed animals, the mice were withdrawn for seven days by replacing the nicotine solution with tap water.

Apparatus and procedures of IVSA into the tail vein were similar to those described by Semenova et al. (1995). A schematic view of the arrangement is shown in Figure 5. Initially, the mice were placed in test chambers for 10 min and basal nose-poking activity was recorded. Based on these pre-test values, the mice were assigned into activity-matched pairs. Within 1 h after the pre-test, the IVSA session began. A pair of mice was placed into adjacent test chambers and needles were inserted into the lateral tail veins. Mice were allowed to habituate to the test chambers for 10 min. During the next 30 min, each nose poke of one animal in a pair ("master" mouse) resulted in an injection of a unit dose of nicotine or saline to both animals in the pair. The other mice in the pair were "yoked" control mice, and they were used to control for changes in locomotor activity that were unrelated to the reinforcing properties of nicotine.

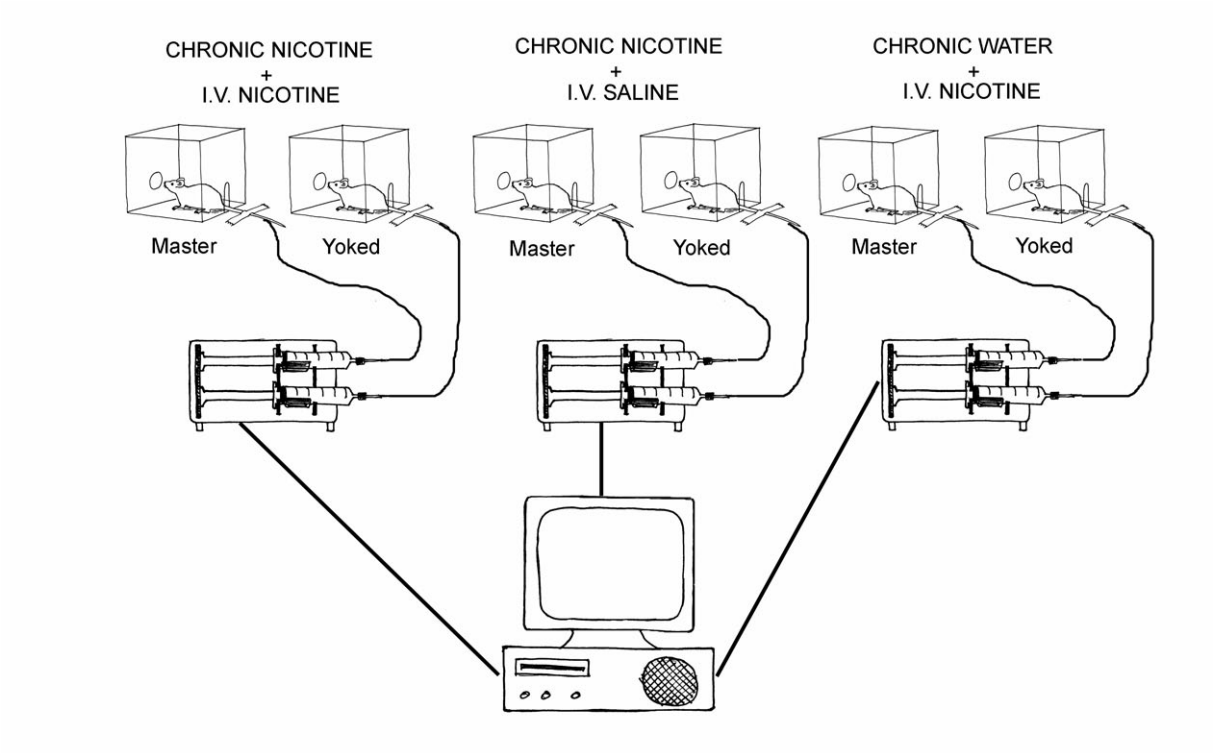

Figure 5 *The intravenous self-administration set-up. Nicotine or saline is delivered according to the nose pokes of the "master" mouse. The nose pokes of the "yoked" mouse are counted, but they do not affect the rate of nicotine administration. I.V. = intravenous.*

4.3.4 Free-choice oral self-administration of nicotine, ethanol, cocaine, and etonitazene (II, III)

4.3.4.1 Free-choice oral self-administration of nicotine (II)

A two-bottle free choice test was carried out after the 7-week forced nicotine exposure in order to investigate whether the nicotine-exposed mice would voluntarily maintain consumption of nicotine solution. The animals were divided into four groups: nicotine-exposed mice offered only nicotine solution (nicotine-nicotine treatment), nicotine-exposed mice offered nicotine solution and water (nicotine-nicotine/water treatment), controls offered only water (water-water treatment), and controls offered nicotine solution and water (water-water/nicotine treatment). The concentration of the nicotine solution was lowered to 250 μg/ml to increase its palatability. Because the animals in the free-choice nicotine self-administration experiment were group-housed, intake of nicotine by individual mice was estimated by measuring their plasma nicotine and cotinine concentrations (see 4.5.1).

4.3.4.2 Cage arrangements for ethanol, cocaine, and etonitazene study (III)

It is practically impossible to measure from ordinary water bottles exactly the small volumes that mice drink per day. Therefore, to simplify the measurements and to improve reliability in Study III, we chose drinking tubes, which have been used in alcohol drinking studies (Nurmi et al., 1999). The burettes were composed of custom-made glass tips (Laborexin, Helsinki, Finland) and 25-ml electronic pipette tips connected to each other with plastic tubing (Figures 6 and 7). Upper ends of the burettes were strengthened with laboratory tape to prevent cracking and capped with rubber plugs. A burette mount was made of stainless steel wire and a partition was cut from birch plywood. The partition was fitted with two holes in the middle for the burette tips, two round doors on the sides, and two small holes for the attaching the tubes to the cage. The mount and partition were fixed to the metal grid cage top with cable ties. The burettes were attached to the mount with 5-cm office bindings that anchored them to an optimal position. The floor of the drinking compartment was fitted with a metal grid consisting of a folded piece of stainless steel wire mesh. The floor of the other compartment was covered with aspen chip bedding. This system was found to be practical: Even though the animals managed to carry bedding to the burette tip compartment despite the partition, the grid floor let it fall on the bottom of the cage. Therefore, it was unusual to find crumbs of bedding in the burettes and they were never blocked by the bedding material. Since the partition was made of plywood, it also enriched the environment by providing something for the mice to gnaw.

4.3.4.3 Experimental setup for the free choice oral self-administration

Free choice between tap water and ethanol, cocaine (DAT, noradrenaline transporter and serotonin transporter blocker), or etonitazene solution (μ opioid receptor agonist) was given to male and female mice of all genotypes. Before the presentation of drug solutions, the mice were given tap water in both burettes for two days. Evaporation was assessed by measuring the water loss from burettes kept in empty boxes. Evaporated volumes were found to be less than 0.1 ml/day and, therefore, they were not subtracted from the measured consumption values. The fluid consumption was registered daily for four weeks and, to avoid the development of place preference, the positions of control and drug solution burettes were interchanged every two or four days.

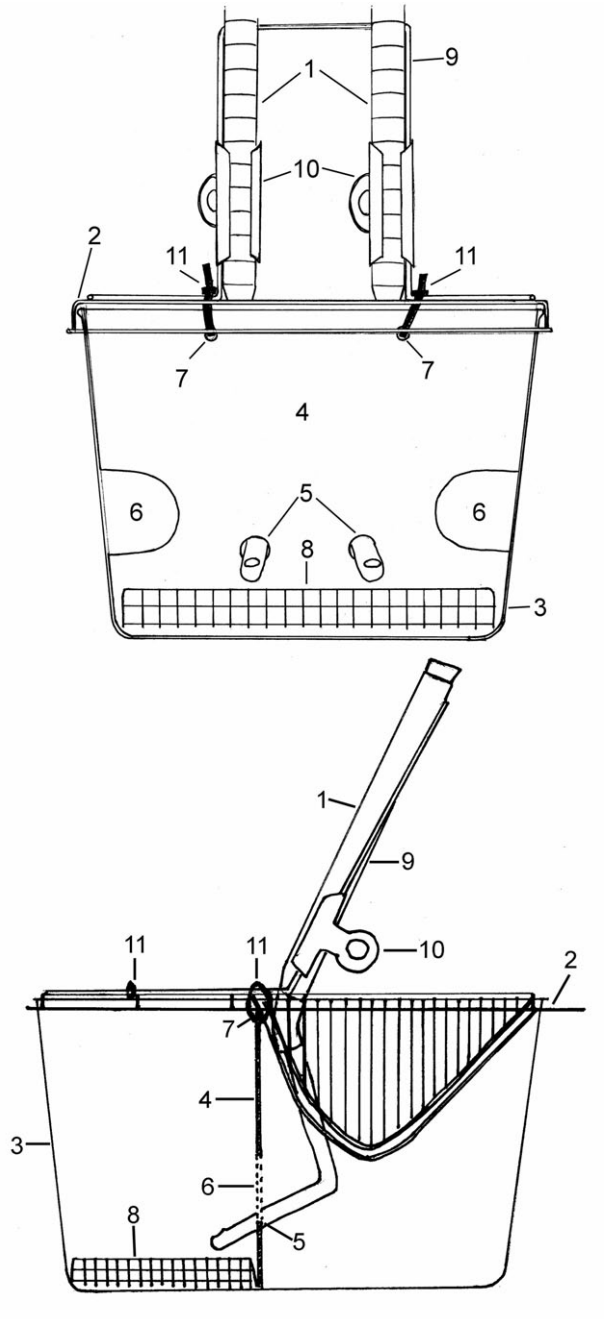

Figure 6 *The cage system used in the free-choice oral self-administration experiments in* Comt *disrupted mice. 1 = drinking burette, 2 = cage lid, 3 = Macrolon II cage, 4 = partition, 5 = holes for burette tips, 6 = mouse holes, 7 = holes for cable bands, 8 = metal grid floor, 9 = burette mount, 10 = office clips, 11 = cable bands. Food pellets, bedding, or nesting material are not shown in the pictures*

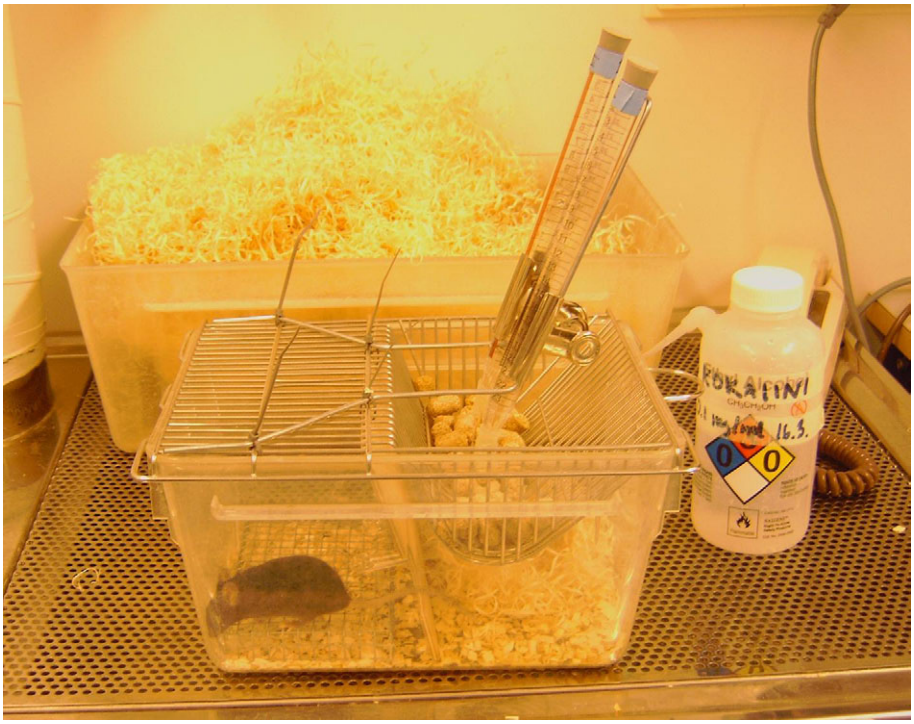

Figure 7 *Mouse in the free-choice oral self-administration cage.*

Etonitazene is a potent μ opioid receptor agonist; the optimal concentration in drinking solutions seems to be as low as 1-2 μg/ml in rats (Carlson, 1989; Carlson et al., 1996; Hyytiä and Sinclair, 1993). Etonitazene was chosen for the oral self-administration experiment instead of morphine because its high potency allows the preparation of low concentration solutions with nearly neutral taste.

4.3.4.4 Drinking solutions

Ethanol was given in the following concentrations: 2.5% (v/v; 20 mg/ml; days 1-7), 5% (40 mg/ml; days 8-14), 10% (80 mg/ml; days 15-21), and 20% (160 mg/ml; days 22-28). In a separate set of experiments, ethanol was given only as a 10% solution throughout the 4-week test. Dilutions were made from 96% ethanol without any additives. Cocaine was given in the following concentrations: 0.1 mg/ml (days 1-4), 0.2 mg/ml (days 5-12), 0.4 mg/ml (days 13-20), and 0.8 mg/ml (days 21-28). Cocaine hydrochloride was dissolved in tap water and the pH was adjusted to 3.2 with hydrochloric acid. Etonitazene was given in concentrations of 1 μg/ml (days 1-5) and 2 μg/ml (days 6-30). Etonitazene hydrochloride was dissolved in tap water and the pH of the solution was adjusted to 4.8 (1 μg/ml solution) or 4.5 (2 μg/ml solution) using acetic acid (Heyne, 1996). The pH of cocaine and etonitazene solutions was adjusted in order to improve their stability. Also the pH of water was adjusted

similarly in order to ensure that the mice would make the decision based on the drug's effects and not on any possible aversive taste or smell of the acid. Fresh ethanol, cocaine, or etonitazene solutions were prepared every four days and stored in a refrigerator. Burettes were refilled either every day (cocaine), every second day (etonitazene), or every four days (ethanol), depending on the stability of the solution.

4.4 Microdialysis in freely moving mice (I, IV)

Conventional microdialysis was used to determine extracellular dopamine and dopamine metabolite concentrations in chronically nicotine-exposed NMRI mice (I) and in *Comt* disrupted mice (IV). A quantitative no-net-flux microdialysis technique was used to examine the absolute extracellular dopamine concentrations and extraction fraction in *Comt* disrupted mice (IV).

4.4.1 Surgery

The mice were implanted with guide cannulae (MAB-4, Agn Tho's AB, Lidingö, Sweden) under isoflurane anaesthesia (induction, 4.5%; maintenance 1.5-2.5%). The mice were also given a buprenorphine injection (0.05-0.1 mg/kg s.c.) for pain relief before the operation. The coordinates for guide cannulas were calculated relative to bregma. They were aimed at the nucleus accumbens (A/P = +1.4, L/M = +0.9. D/V = -3.8), the dorsal striatum (A/P = +0.6, L/M = +1.8, D/V = -2.7), or the medial prefrontal cortex (A/P = +2.0, L/M = +0.5, D/V = -1.0) according to the mouse brain atlas by Franklin and Paxinos (1997). After the surgery the animals were placed one per cage into test cages (30 x 30 x 40 cm) and allowed to recover for 5-7 days before the experiment.

4.4.2 Conventional microdialysis (I, IV)

Approximately 16 h before the experiment, a microdialysis probe was inserted into the guide cannula. The probe (MAB-4, Agn Tho's AB, Lidingö, Sweden) had a membrane length of 1 mm for nucleus accumbens and dorsal striatum and 2 mm for prefrontal cortex; its outer diameter was 0.2 mm. The probe was infused with a modified Ringer solution (147 mM NaCl, 1.2 mM $CaCl_2$, 2.7 mM KCl, 1.0 mM $MgCl_2$, and 0.04 mM ascorbic acid) at a flow rate of 0.5 μl/min. On the morning of the experiment day, the flow rate of the infusion was increased to 2 μl/min. Collection of microdialysis samples for HPLC analysis was performed every 20 min (40 μl/sample) starting after a 120-min stabilization period. Detailed description of the HPLC methods can be found in studies I and IV. Baseline samples were collected for 80

min. In study I, the mice were injected with (-)-quinpirole (0.01 or 0.03 mg/kg, s.c.; dissolved in saline). In study IV, the animals received first carbidopa (30 mg/kg, i.p.) and 40 minutes later levodopa (10 mg/kg, i.p.). At the end of the experiment, the animals were decapitated and the brains were removed from the skull and frozen rapidly on dry ice. The positions of the microdialysis probes were verified histologically from brain slices prepared *post mortem*.

4.4.3 No-net-flux microdialysis (IV)

On the morning of the experiment day, a microdialysis probe (MAB-4; membrane length, 1 mm; outer diameter, 0.2 mm) was inserted into the guide cannula, and the probe was infused with Ringer solution at a flow rate of 0.6 μl/min. After a 3- hour stabilization period, four different concentrations of dopamine in Ringer solution (C_{in}; 0, 2, 10, and 20 nM) were perfused through the probes in a random order. Following a 30-min equilibration period, two 30-min samples (18 μl each) were collected at each C_{in} for HPLC analysis. The animals were sacrificed and the probe placements were verified as described in section 4.6.2.

In the no-net-flux studies, a linear equation was constructed for each animal by plotting the net flux of dopamine through the probe (DA_{in}-DA_{out}) against DA_{in}, where DA_{out} is the dialysate dopamine concentration acquired during the perfusion and DA_{in} the dopamine concentration of the perfusion fluid. Based on this equation, the extracellular dopamine level (DA_{ext}) and the *in vivo* extraction fraction (E_d) were calculated as described by Parsons and Justice (1992). The DA_{ext} value stands for the perfusion fluid dopamine concentration at which there is no net flux of dopamine through the probe ($DA_{in} – DA_{out} = 0$). E_d, on the other hand, has been shown to indicate differences in the function of DAT-mediated dopamine uptake (Chefer et al., 2006; Justice, 1993).

4.5 Biochemical analyses

4.5.1 Nicotine assay (II)

4.5.1.1 Collection of samples

The mice were decapitated at 6:00 am at 10 days after the beginning of the free-choice phase; nicotine solution was available until the moment of blood collection. Trunk blood was collected into tubes containing 0.5% sodium citrate solution, and

plasma was separated from blood by centrifugation at 800 *g* for 20 min. Samples were stored in plastic tubes at -80°C until assayed.

4.5.1.2 Nicotine and cotinine assay

Concentrations of nicotine and its main metabolite, cotinine, were determined by a gas chromatographic-mass spectrometric (GC-MS) method that was slightly modified from previously published methods (Leikola-Pelho et al., 1990; Pekonen et al., 1993). The modifications served to decrease sample loss during sample preparation. First, glassware was silylated to prevent nicotine from adhering to the surfaces. Second, after dichloromethane extraction, the samples were evaporated to a volume of 35 μl under a nitrogen stream instead of evaporation in a water bath. Third, in addition to quinoline (Sigma, St. Louis, MO, USA), the tobacco-derived alkaloid myosmine (Sigma, St. Louis, MO, USA), which is structurally close to nicotine, was used as an internal standard. GC-MS analyses were performed on a Hewlett-Packard 5970 quadrupole MS connected to a Hewlett-Packard 5890 GC using an NB-54 fused silica column (15 m; internal diameter, 0.20 mm). In single ion monitoring (SIM) analyses, fragment ions of m/z 84 (nicotine), m/z 98 (cotinine), and 129 (quinoline, internal standard) were used. The sensitivity of the assay was 5 ng/ml for both nicotine and cotinine.

Since airborne nicotine contamination is known to be a severe problem in nicotine analysis (Curvall et al., 1982; Feyerabend and Russell, 1980), all laboratory glassware was carefully rinsed with denatured ethanol (96%) and kept in an oven at 100°C overnight. In addition, hoods were wiped with alkaline detergent and ethanol, and smokers were not allowed to handle the samples. Despite the efforts, nicotine contamination could not be completely prevented. The background level of nicotine obtained from blank plasma samples was subtracted from the results.

4.5.2 Determination of monoamines from brain tissue (unpublished)

Chronically nicotine-exposed mice were killed on the 50th day of nicotine treatment or 23-25 h after the nicotine solution was replaced with tap water. (-)-Quinpirole (0.03 or 0.1 mg/kg, s.c.) was administered to mice 60 min before dissection. Rectal temperature was measured immediately before and 30 and 60 min after quinpirole. Striatums were dissected and frozen rapidly on dry ice. Tissue samples were weighed (mean weights: hypothalamus, 16 mg; striatum, 24 mg; cortex, 160 mg) and stored at -80°C until assayed. The tissues were homogenized in 0.2 N perchloric acid and centrifuged at 27 800 *g* (4ºC, 30 min). The supernatant was removed and subsequently purified and fractionated in Sephadex G-10 gel chromatographic columns as described by Haikala (1987). Monoamines and their metabolites were

analyzed by high performance liquid chromatography (HPLC) equipped with electrochemical detection. The dopamine, noradrenaline, and metabolite concentrations were calculated as micrograms per gram (μg/g) wet weight of tissue.

4.6 Data analysis and statistics

Data concerning brain tissue monoamines, locomotor activity (I), or conditioned place preference (II) were tested with two-way analysis of variance (ANOVA; chronic treatment x acute treatment/conditioning drug). The unpublished cocaine place preference data were analyzed with three-way ANOVA (sex x genotype x conditioning drug).

Data from the free-choice oral self-administration experiments were analyzed with two-way ANOVA for repeated measures (sex x genotype x days/weeks, III). Nicotine and cotinine concentration data were tested with one-way ANOVA (II).

For nicotine self-administration studies, a ratio (R) criterion was calculated for each pair of experimental animals according to the formula $R = \log(M_T/Y_T) - \log(M_{BL}/Y_{BL})$, where M_T and Y_T are the total number of nose-poke responses of the "master" and "yoked" control mouse, respectively, during the 30 min test. M_{BL} and Y_{BL} are the total number of nose-poke responses in the "master" and the "yoked" control mouse, respectively, during the 10 min pre-test (baseline). The pre-test data were tested with one-way ANOVA and the data from chronically nicotine-treated animals were tested with two-way ANOVA (chronic treatment x acute treatment, II).

The effect of quinpirole on extracellular concentration of dopamine and dopamine metabolites was calculated as a percentage change from the baseline value, and these data were tested with two-way ANOVA for repeated measures (100-180 min; I). Locomotor activity data from mice given nicotine repeatedly subcutaneously were analysed using a one-way ANOVA for repeated measures (25-45 min; I). Results of the rectal temperature measurements were analysed using a two-way ANOVA for repeated measures (0-60 min).

For the microdialysis studies, Student's t-test (I) or one-way ANOVA (IV) was used to test the baseline dialysate data of accumbal and striatal dopamine and metabolites. Area under the curve (AUC) was calculated for dopamine, DOPAC, and HVA after carbidopa and levodopa treatments (100-420 min). AUC values were analyzed with two-way ANOVA (IV; sex x genotype). A linear regression model was used to create the no-net-flux curves for dopamine. The no-net-flux data were further analyzed with two-way ANOVA (sex x genotype).

5 RESULTS

5.1 The effects of forced chronic oral nicotine exposure on the sensitivity of dopamine D_2 receptors (I, unpublished)

5.1.1 The effects of chronic oral nicotine exposure and subsequent withdrawal on catecholamine concentrations in brain tissue and extracellular fluid, as well as on motor activity and body temperature

Table 5 summarizes the effects of chronic oral nicotine exposure and a subsequent withdrawal lasting 23-25 h on catecholamine concentrations in the extracellular fluid and brain tissue, as well as on motor activity and body temperature. As shown in previous studies, the concentrations of dopamine metabolites in striatal tissue were elevated during the chronic nicotine exposure, but they returned to control levels during the withdrawal period. Furthermore, metabolite/dopamine ratios were calculated to examine the effect of the chronic nicotine exposure on dopamine metabolism inside and outside the dopamine neuron. Both DOPAC/DA and HVA/DA ratios were higher in nicotine-exposed than in nicotine-withdrawn or control animals, indicating that both intracellular dopamine levels and dopamine release increase during chronic nicotine exposure.

Table 5 *The effects of chronic oral nicotine exposure and a subsequent withdrawal lasting 23-25 h on tissue and extracellular fluid catecholamine concentrations, motor activity and body temperature.*

		Nicotine exposed	Nicotine withdrawn
Striatum tissue	DA	±	±
	DOPAC	↑	↓
	HVA	↑	↓
	DOPAC/DA	↑	↓
	HVA/DA	↑	↓
Hypothalamus tissue	NA	±	↑
	MOPEG	±	±
Cortex tissue	NA	↑	↓
	MOPEG	±	↓
Nucleus accumbens extracellular	DA	±	ND
	DOPAC	±	ND
	HVA	±	ND
Dorsal striatum extracellular	DA	↑	ND
	DOPAC	(↑)	ND
	HVA	(↑)	ND
Motor activity		↑	±

↑ = significantly increased as compared to water exposed controls
(↑) = non-significantly increased as compared to water exposed controls
↓ = significantly decreased as compared to water exposed controls
± = no change
ND = not determined.

Elevated dopamine concentrations were also found in the dorsal striatal extracellular fluid during chronic oral nicotine exposure, confirming the result derived from measurements of tissue dopamine levels. In addition, cortical tissue noradrenaline concentration was found to increase during the exposure, but after the withdrawal the levels of both noradrenaline and 3-methoxy-4-hydroxy-phenylglycol (MOPEG) were lower than in water-exposed control animals.

During chronic nicotine treatment, the nicotine-exposed animals were more active than nicotine-withdrawn or control animals. This indicates that the mice had developed tolerance to the suppressive effect of nicotine on motor activity, and this tolerance had unmasked the drug's stimulatory effect. The mice also developed tolerance to the hypothermic effect of nicotine.

5.1.2 Effect of quinpirole on levels of dopamine and dopamine metabolites and on locomotor activity in chronically nicotine-exposed, nicotine-withdrawn, and naïve mice

Quinpirole (0.03 or 0.1 mg/kg) decreased striatal tissue DOPAC and HVA concentrations, as well as DOPAC/DA and HVA/DA ratios (Table 6). The 0.03 mg/kg dose of quinpirole also decreased the accumbal and striatal extracellular levels of dopamine and dopamine metabolites (Study I, Figure 2). The 0.01 mg/kg dose was tested only in nucleus accumbens, and it was found to have no effect on extracellular levels of dopamine or dopamine metabolites. Quinpirole also decreased motor activity and rectal temperature in a dose-dependent manner (Figure 8, Table 6). The impact of quinpirole was similar on all measured parameters in nicotine-exposed, nicotine-withdrawn, and control mice. On the other hand, quinpirole was less effective at reducing motor activity in mice treated with repeated nicotine injections than in control animals (Study I, Figure 4).

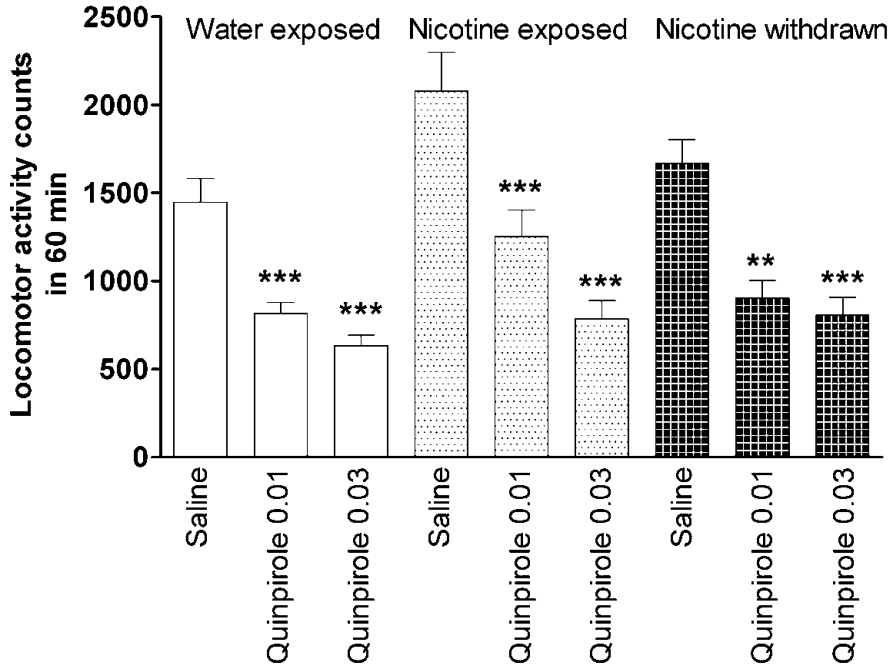

Figure 8 *Effect of quinpirole (0.01 or 0.03 mg/kg s.c.) and saline (10 ml/kg s.c.) on locomotor activity in chronically water exposed, nicotine exposed and withdrawn (23-25 h) mice.* **p < *0.01,* ***p < *0.001, quinpirole vs. saline within each chronic treatment group. n = 14-31.*

Table 6 *The effect of quinpirole on striatal tissue dopamine and dopamine metabolites in nicotine exposed, nicotine withdrawn (23-25 h) and control animals. DOPAC/DA and HVA/DA ratios are also shown.*

Chronic treatment	Acute treatment	DA	DOPAC	HVA	DOPAC/DA	HVA/DA
Water control	Saline	13266 ± 821	924 ± 42	1250 ± 80	0.072 ± 0.004	0.095 ± 0.006
	Quinpirole 0.03 mg/kg	13818 ± 529	704 ± 44 ***	1026 ± 83 ***	0.052 ± 0.004 ***	0.075 ± 0.006 ***
	Quinpirole 0.1 mg/kg	13245 ± 846	574 ± 26 *** #	844 ± 52 ***	0.044 ± 0.002 *** #	0.065 ± 0.004 ***
Nicotine exposed	Saline	13280 ± 749	932 ± 45	1460 ± 83	0.072 ± 0.005	0.112 ± 0.006
	Quinpirole 0.03 mg/kg	13750 ± 900	864 ± 57	1221 ± 37 **	0.064 ± 0.004	0.092 ± 0.006
	Quinpirole 0.1 mg/kg	14731 ± 1065	735 ± 27 *	1081 ± 41 **	0.052 ± 0.002 **	0.077 ± 0.007 **
Nicotine withdrawn	Saline	12578 ± 599	768 ± 41	1085 ± 69	0.062 ± 0.003	0.087 ± 0.005
	Quinpirole 0.03 mg/kg	14392 ± 622	762 ± 55	931 ± 55	0.053 ± 0.003	0.065 ± 0.003 **
	Quinpirole 0.1 mg/kg	14756 ± 644 *	587 ± 46 * #	750 ± 65 *	0.040 ± 0.003 *** ##	0.051 ± 0.004 ***

Two-way ANOVA:

Chronic treatment effect:
DA F (2, 119) = 0.051, not significant;
DOPAC F (2, 119) =7.838, $p < 0.01$;
HVA F (2, 119) = 17.329 $p < 0.001$;
DOPAC/DA ratio F (2, 119) = 7.180, $p < 0.01$;
HVA/DA ratio F (2, 119) = 15.930, $p < 0.001$;

Acute treatment effect:
DA F (2, 119) = 4.314, $p < 0.05$;
DOPAC F (2, 119) = 23.447, $p < 0.001$;
HVA F (2, 119) = 27.278, $p < 0.001$;
DOPAC/DA ratio F (2, 119) = 36.692, $p < 0.001$;
HVA/DA ratio F (2, 119) = 37.509, $p < 0.001$;

Chronic x acute treatment interaction: all not significant.

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ as compared to the saline treated control animals within the same chronic treatment group;

#$p < 0.05$, ##$p < 0.01$ as compared to the 0.03 mg/kg quinpirole treated animals within the same chronic treatment group. n = 9-23

Table 7 *The effect of quinpirole (0.03 or 0.1 mg/kg s.c.) on body temperature in nicotine exposed, nicotine withdrawn (23-25 h) and control animals.*

Treatment	ΔTºC	
	30 min	**60 min**
Water + Saline	0.2 ± 0.1	0.2 ± 0.1
Water + Quinpirole 0.03 mg/kg	-0.7 ± 0.1 **	-0.1 ± 0.1
Water + Quinpirole 0.1 mg/kg	-1.9 ± 0.3 ***	-0.8 ± 0.2 ***
Nicotine exposed + Saline	0.1 ± 0.2	0.4 ± 0.2
Nicotine exposed + Quinpirole 0.03 mg/kg	-0.6 ± 0.1	0.0 ± 0.1
Nicotine exposed + Quinpirole 0.1 mg/kg	-2.2 ± 0.4 ** ##	-1.3 ± 0.5 ** #
Nicotine withdrawn + Saline	0.3 ± 0.1	0.3 ± 0.1
Nicotine withdrawn + Quinpirole 0.03 mg/kg	-0.6 ± 0.2 *	-0.3 ± 0.2
Nicotine withdrawn + Quinpirole 0.1 mg/kg	-1.0 ± 0.3 ***	-0.4 ± 0.2 *

ΔT˚C = body temperature after quinpirole – baseline body temperature
**p* < 0.05, ***p* < 0.01, ****p* < 0.001 as compared to the saline treated control animals within the same chronic treatment group
#*p* < 0.05, ##*p* < 0.01 as compared to the 0.03 mg/kg quinpirole treated animals within the same chronic treatment group. n = 12-24

5.2 Effect of chronic forced nicotine exposure on the reinforcing properties of nicotine (II)

5.2.1 Two-bottle free choice self-administration test after forced chronic oral nicotine or water exposure

Weight development and nicotine and cotinine concentrations in mouse plasma immediately following the two-bottle free choice self-administration test are summarized in Table 8. Unfortunately, data on liquid consumption by individual mice are unavailable because the animals were housed in groups. Relatively large concentrations of nicotine were found in the plasma of nicotine-nicotine treated mice and small amounts in the plasma of mice in the nicotine-nicotine/water and water-water/nicotine groups. The concentration of cotinine was also high in the nicotine-nicotine treated mice, and some cotinine was found in the plasma of animals treated with nicotine-nicotine/water and water-water/nicotine.

Table 8 *Weight gain as well as nicotine and cotinine concentrations in mouse plasma immediately after the two-bottle free-choice oral nicotine administration experiment.*

	Weight at the end of forced nicotine exposure phase	Weight at the end of two-bottle free-choice phase	Nicotine concentration ng/ml	Cotinine concentration ng/ml
Nicotine - nicotine	38.1 ± 0.6	40.7 ± 0.8	65.4 ± 22.2	735.4 ± 67.4
Nicotine - water/nicotine	38.7 ± 0.9	43.0 ± 1.3	2.9 ± 1.5 *	40.2 ± 14.8 ***
Water - water/nicotine	39.4 ± 1.2	39.8 ± 1.3	9.8 ± 3.5 *	33.2 ± 14.1 ***
Water - water	39.0 ± 1.2	39.5 ± 1.4	0	0

*$p < 0.05$, ***$p < 0.001$ as compared to nicotine-nicotine group. n = 9-10

5.2.2 Development of conditioned place preference to nicotine after forced chronic oral nicotine or water exposure

The smaller dose of nicotine (0.3 mg/kg) did not induce significant place preference in either of the two chronic exposure groups, whereas the larger dose of nicotine (0.5 mg/kg) induced equally strong place preference in nicotine-exposed as well as control animals (Study II, Figure 3). The preference was not observed anymore in the repeated postconditioning measurements on days 8, 15, and 22.

5.2.3 Acquisition of intravenous self-administration of nicotine after forced chronic oral nicotine or water exposure

The optimal nicotine tartrate unit dose in NMRI mice was 0.08 μg/infusion (0.028 μg/infusion based on nicotine free base; Study II, Figure 1). At the unit dose 0.04 μg/infusion (0.014 μg/infusion based on free base), self-administration activity of the nicotine group did not differ from saline control group. The nicotine pre-exposed mice self-administered nicotine at a lower unit dose than the water-exposed control animals, indicating that the chronically nicotine-treated mice were more sensitive to the reinforcing effects of intravenous nicotine (Study II, Figure 2). Nicotine pre-exposure did not affect the dose of nicotine consumed during the self-administration session.

5.3 Free-choice oral self-administration of abused substances in *Comt* gene knock-out mice (III, unpublished)

5.3.1 Ethanol

Consumption of ethanol solution (ml/kg) and ethanol preference ratios (%) per day in male and female mice are shown in Study III, Figure 2. Again, female mice consumed more ethanol solutions than male mice. In addition, the effect of genotype on ethanol consumption was different between sexes. Male heterozygous and homozygous mice drank more ethanol solution than their wild-type littermates. The male mice liked 5 and 10% ethanol solutions the most, whereas ethanol consumption decreased at the highest ethanol concentration. Female homozygous mice showed decreased ethanol preference and consumption at the two lowest ethanol concentrations. However, genotype did not affect ethanol drinking at the higher concentrations and the genotype effect failed to reach statistical significance. Overall, the concentration of ethanol solution did not affect ethanol consumption in female mice.

5.3.2 Cocaine

Consumption of cocaine solution (ml/kg) and cocaine preference ratios (%) per day in male and female mice are shown in Study III, Figure 3. Again in this experiment, female mice consumed more fluid than males. After the first two changes of the burette position, the male mice of all genotypes developed either a side preference or a side aversion to the cocaine solution. This resulted in a peculiar drinking pattern where cocaine preference and cocaine aversion alternated in four-day cycles (Study III, Figure 3). The pattern persisted during the remainder of the experiment and obscured any possible preference for the drug. The overall consumption of cocaine solution was higher in females because of their more stable drinking pattern, but genotype did not affect cocaine intake in either sex. The mice reduced cocaine solution consumption when the cocaine concentration increased, and this effect was most evident in female animals. Cocaine treatment resulted in weight loss in both sexes, indicating that the dose of cocaine was high enough to cause anorexia. This effect was greater in females, but it was not affected by genotype.

5.3.3 Etonitazene

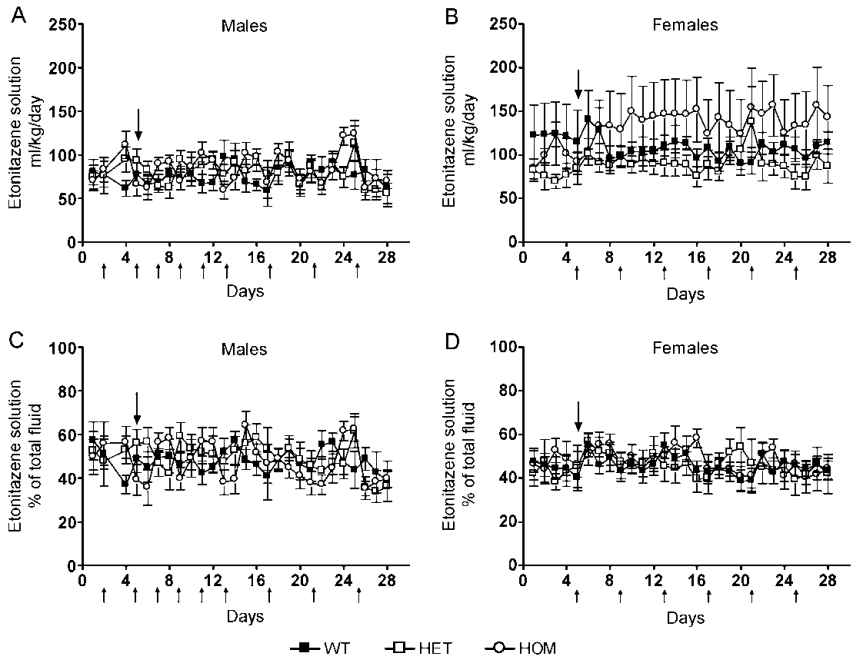

Figure 9 *Consumption of etonitazene solution (1 or 2 µg/ml, panels A and B) and etonitazene preference (panels C and D) in male and female* Comt *knock-out mice. WT = wild-type, HET = heterozygous, HOM = homozygous. Large arrows indicate the days when the concentration of the drug solution was increased; small arrows, the days when burette places were interchanged.* n = *7-11 in each group. Data for day 3 are missing for the males due to burette leakage. Two-way ANOVA for repeated measures showed that female mice drank more etonitazene solution than male mice [sex effect:* F *(1, 46) = 6.466,* p < *0.05; genotype effect:* F *(2, 45) = 1.498, not significant; sex x genotype interaction:* F *(2, 45) = 1.181, not significant]. Etonitazene preference was similar in both sexes and all genotypes.*

The consumption of etonitazene solution (ml/kg) as well as total liquid consumption (ml/kg) and etonitazene preference ratios (%) per day in male and female mice are presented in Figure 9. The COMT genotype did not affect etonitazene consumption. However, female mice drank more etonitazene solution than male mice. This was due to the higher total liquid consumption in females than males, since both sexes showed a similar preference for etonitazene. The voluntary consumption of etonitazene solution did not affect the weight of the animals.

5.4 Development of conditioned place preference to cocaine in *Comt* gene disrupted mice (unpublished)

Because the free-choice oral self-administration experiment yielded unexpected results, the effect of *Comt* gene disruption on the rewarding properties of cocaine was tested in the conditioned place preference set-up. The CPP experiments showed that the rewarding properties of cocaine (10 mg/kg, i.p.) were similar in *Comt* disrupted and wild-type animals in both sexes (Figure 10).

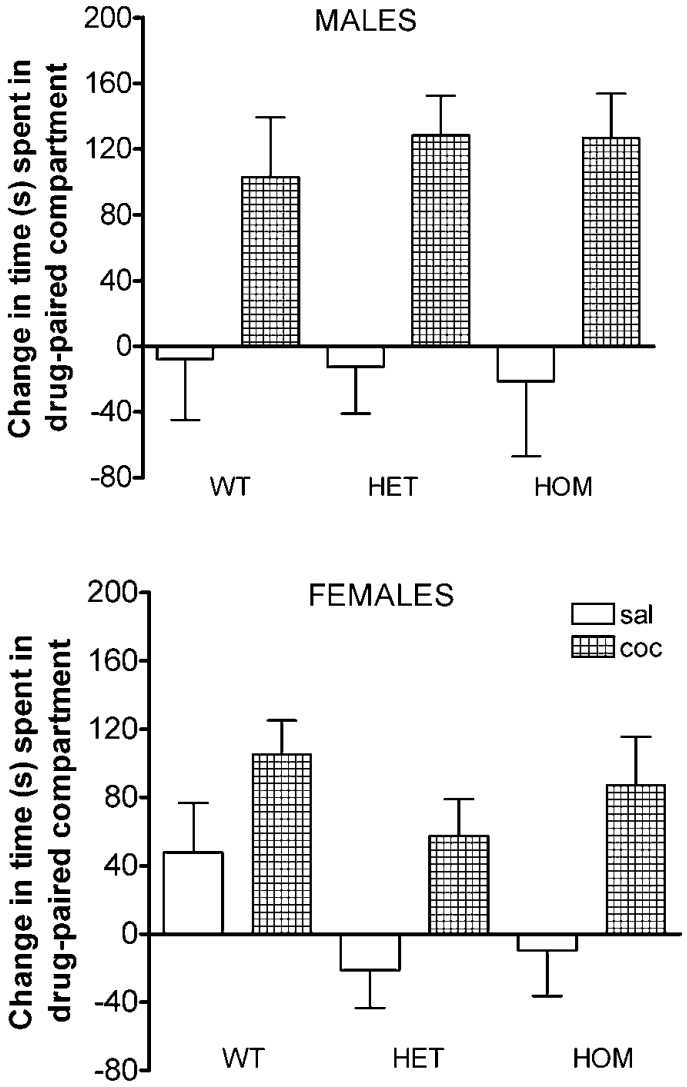

Figure 10 *Cocaine (10 mg/kg i.p.) place conditioning in COMT knock-out mice. The results (means ± SEM.) are expressed as change in seconds spent in the drug-paired compartment. Control mice received saline (sal) injections. Animals conditioned with cocaine (coc) increased the time spent in the drug-paired compartment significantly more than the saline-conditioned control animals. However, the three-way ANOVA showed that sex or genotype did not significantly affect cocaine place conditioning [sex effect* F *(1, 160) = 0.252, not significant; genotype effect* F *(2, 160) = 0.551, not significant; drug effect* F *(1, 160) = 42.160,* p *< 0.001; sex x drug interaction* F *(1, 160) = 2.760, not significant; genotype x drug interaction* F *(2, 160) = 0.522, not significant]. n = 11-18 in each group. WT = wild-type, HET = COMT(+/-), HOM = COMT(-/-), sal = saline (0.9% sodium chloride solution), coc = cocaine.*

5.5 The effect of levodopa loading on striatal, accumbal and cortical levels of extracellular dopamine and dopamine metabolites in *Comt* knock-out mice (IV)

The baseline values of dopamine, DOPAC, and HVA in male and female *Comt* knock-out mice and their wild-type littermates are shown in Study IV, Table 1. Extracellular dopamine levels were similar in both sexes and genotypes in dorsal striatum, nucleus accumbens, and prefrontal cortex. DOPAC levels were higher in homozygous animals, although in dorsal striatum the only significant difference was between female wild-type and homozygous mice. HVA was not detected in homozygous animals.

Dopamine and DOPAC levels in the dorsal striatal, accumbal, and prefrontal cortical extracellular fluid of *Comt* knock-out and their wild-type littermates after the administration of carbidopa (30 mg/kg i.p.) and levodopa (10 mg/kg i.p.) are shown in Study IV, Figures 1-3. The corresponding AUC values are given in Study IV, Table 2. Sex or the lack of COMT did not affect the levodopa-induced extracellular dopamine levels in dorsal striatum. In the nucleus accumbens, the mice lacking COMT seemed to have higher extracellular dopamine concentration than the wild-type animals, but sex did not play a role in this difference. In the prefrontal cortex, the *Comt* genotype seemed to affect the levodopa-induced dopamine levels in a sex-dependent manner; the elevated extracellular dopamine levels persisted in the male mice for an extended period. DOPAC levels were higher in *Comt* knock-out animals than in wild-type mice in all three brain areas, but sex did not affect the accumulation of DOPAC. HVA was not detected in *Comt* knock-out mice after levodopa treatment in any of the brain areas studied. In wild-type animals HVA levels were similar in both sexes.

5.6 Absolute striatal and accumbal extracellular dopamine concentrations in *Comt* knock-out mice (IV)

The absolute extracellular dopamine concentrations and extraction fractions in the dorsal striatum and nucleus accumbens are shown in Figure 11. *Comt* gene disruption did not alter extracellular dopamine levels in these regions, since the points of no net flux were similar in both genotypes. In addition, the gene disruption did not affect the function of DAT, based on the unaltered extraction fractions. Sex did not have an effect on these parameters.

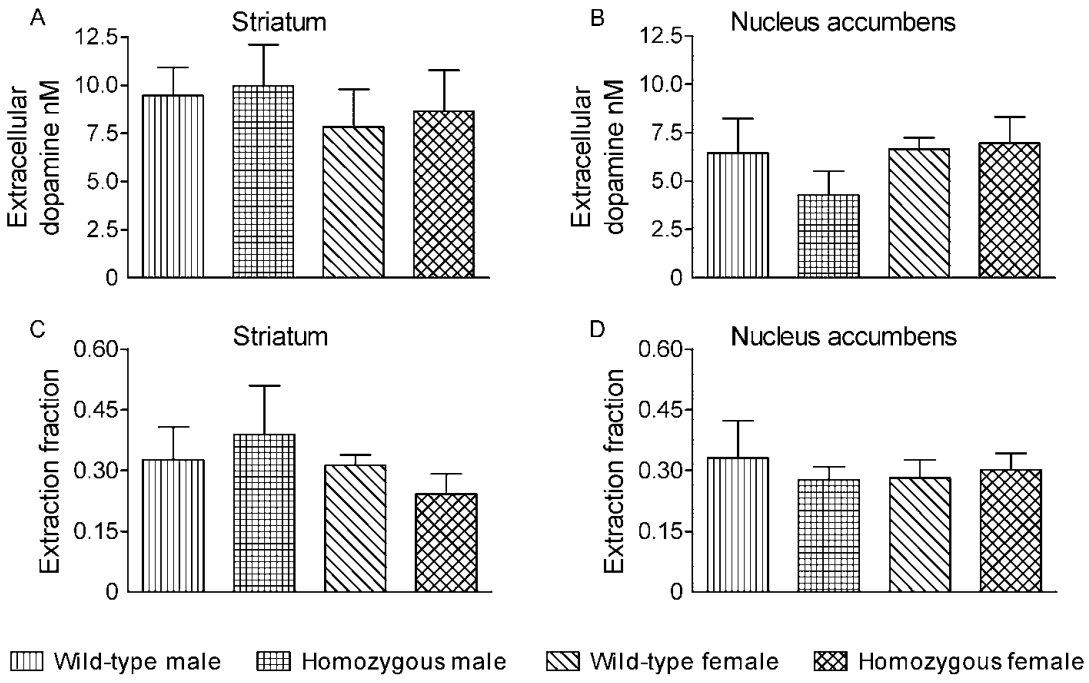

Figure 11 *The effect of* Comt *gene disruption on extracellular dopamine and the function of dopamine transporter. Panels A and B: Absolute extracellular dopamine levels in the dorsal striatum and nucleus accumbens of* Comt *knock-out mice and their wild-type littermates. Panels C and D: The extraction fractions of dopamine in striatum and nucleus accumbens of* Comt *knock-out mice and their wild-type littermates. The extraction fraction reflects changes in the function of dopamine transporter. Data are expressed as means ± SEM of 6-7 animals.*

6 DISCUSSION

6.1 Some methodological considerations

6.1.1 Microdialysis

In vivo microdialysis is a valuable tool for studying changes in the extracellular levels of neurotransmitters and their metabolites in specific brain areas (Chaurasia et al., 2007; Ungerstedt, 1991; Westerink, 1995). It is based on diffusion of molecules through the semipermeable membrane of a microdialysis probe into a pool of circulating artificial cerebrospinal fluid, which can be used both to collect endogenous compounds from the brain extracellular fluid and to administer drugs into the brain. Microdialysis assesses the extrasynaptic dopamine that is secreted due to tonic release (Grace et al., 2007). The concentration of extrasynaptic dopamine changes much more slowly than that of intrasynaptic dopamine, with the changes occurring over seconds to minutes in the extracellular space, and over milliseconds within the synapse. Phasic burst-firing dopamine mediates the behaviourally relevant dopamine signal to post-synaptic nerve cells. The tonically released dopamine activates the extrasynaptic presynaptic autoreceptors and has several effects, such as on the activity of medial prefrontal cortex afferents to the nucleus accumbens.

The advantage of microdialysis is that it allows repeated sampling of neurotransmitter release over hours in awake and freely moving animals (Ungerstedt, 1991; Westerink, 1995). The drawbacks are the relatively large outer diameter of the microdialysis probe (200 μm), tissue damage caused by the insertion of the guide cannula and probe, and poor time resolution, which is several minutes at best.

Baseline dialysate samples can provide an estimate of alterations in the extracellular dopamine concentrations caused by drug treatments or genetic modifications. However, with conventional microdialysis, it is not possible to measure the true extracellular concentrations of neurotransmitters. In addition, the estimates derived from the baseline samples may be poorly reproducible. Therefore, we used quantitative no-net-flux microdialysis to measure the extracellular dopamine concentrations in the striatum and nucleus accumbens of *Comt* disrupted mice. The no-net-flux method also allows the calculation of the *in vivo* extraction fraction that reflects differences in the function of DAT-mediated dopamine uptake (Chefer et al., 2006; Justice, 1993).

6.1.2 Conditioned place preference

The CPP paradigm is intended to measure the rewarding properties of drug stimuli that have been associated with distinct environmental cues (Bardo and Bevins, 2000; Tzschentke, 1998). If the drug stimulus is rewarding, the animal will show a preference for the drug-conditioned environment over the placebo-conditioned environment. The CPP paradigm is readily adaptable to mice and does not require extensive training or surgery. On the other hand, the effects of the compounds under study on animals' state-dependent learning, novelty-seeking behaviour, anxiety, and stress may complicate interpretation of the results.

The CPP design is called biased if the animals show a clear preference for either of the compartments during the preconditioning phase, and they are conditioned to the less preferred side of the apparatus (James et al., 2000; Tzschentke, 1998). If there is no such initial preference, an equal number of animals within each group is assigned to receive the conditioning drug in the light and dark compartments of the apparatus; this design is called unbiased. The unbiased model is recommended, since interpretation of the data is more straightforward. This study used an unbiased design in the cocaine experiments in *Comt* disrupted mice, but a biased design in the nicotine experiments in chronically nicotine-exposed animals. Several studies in rats have suggested that the biased setup is preferable in nicotine-conditioned place preference studies (Le Foll and Goldberg, 2005). Nevertheless, some authors have successfully used the unbiased, counterbalanced setup in nicotine studies in rats and mice (e.g. Castañe et al., 2006; Grabus et al., 2006; Philibin et al., 2005; Walters et al., 2006; Wilkinson and Bevins, 2007).

The biased design may yield false positive results if the compound of interest has anxiolytic properties (Bardo and Bevins, 2000). Therefore, precautions were taken in the present study to minimize the disadvantages of this design. Animals showing a strong initial preference for either of the compartments during the preconditioning phase were excluded from the experiment. In addition, the effect of nicotine was not compared with the performance during the preconditioning session but instead with the performance of a saline-conditioned group. Although smokers report anxiolysis after having smoked a cigarette (Kassel and Unrod, 2000), it is not clear whether nicotine is anxiolytic in rodents. Anxiety tests with low doses of nicotine (0.1-0.45 mg/kg) have yielded conflicting results (Brioni et al., 1993; File et al., 2002; Irvine et al., 2001), and in our laboratory a 0.3-mg/kg dose of nicotine has been found to be anxiogenic in NMRI mice (Raattamaa, Soininen & Ahtee, 2001, unpublished observations). Therefore, it is unlikely that the nicotine place conditioning observed with the 0.5 mg/kg dose is due to nicotine's anxiolytic effects.

Another concern with the biased design is that the animals may show a preference shift because of a reduction of aversion. A recent study compared nicotine place conditioning in biased and counterbalanced setups (Brielmaier et al., 2007). The results suggested that the shift in preference in the biased group was

probably not due to nicotine-induced unconditioned reduction of aversion, but rather to true place conditioning. Nevertheless, we cannot exclude the possibility that unconditioned effects of nicotine may have contributed to the results in the present study.

The third problem linked to the biased design is the possibility that the difference is due to different “conditionability” of the drug-paired compartment (Le Foll and Goldberg, 2005). Higher salience cues in one of the compartments may produce stronger conditioning, which would skew the results. In the present study this is unlikely, since 40% of the mice were conditioned to the white compartment and 60% to the black compartment of the apparatus. Moreover, cocaine induced place preference in the same apparatus in an unbiased setup.

6.1.3 Oral drug self-administration

Oral self-administration is a widely used model in alcohol research, and it has also been used for several other substances, including nicotine, opioids, and psychostimulants. Recently, however, the validity of this method has been questioned in the case of substances other than ethanol (Sanchis-Segura and Spanagel, 2006). In addition, it is difficult to reach relevant blood drug or ethanol concentrations in oral self-administration setups that offer unlimited access to the solutions. This can be avoided using limited access paradigms, e.g. the method of drinking in the dark for ethanol (Rhodes et al., 2007). Another problem is the relatively slow absorption of the substances from the gastrointestinal tract, which may prevent the animal from associating the drug effects with the drinking solution (Jung et al., 1999a).

Drugs can also be delivered in the drinking solution in a forced manner; in other words, the animals cannot choose between fresh water and the drug solution. However, in this case the drug-induced alterations in behaviour or brain neurochemistry must be interpreted with caution. In forced oral administration, the animals drink the solution to maintain their water balance, not to obtain the drug. Thus, they also lack expectations about the drug effects (Jacobs et al., 2003). Therefore, the neurochemical or behavioural changes caused by passive drug exposure should not be considered addiction-related processes.

This and earlier studies have used a very high nicotine dose in the chronic oral nicotine exposure experiments (Gäddnäs et al., 2000; Gäddnäs et al., 2001; Pekonen et al., 1993; Pietilä et al., 1995; Pietilä et al., 1996; Pietilä et al., 1998). Other laboratories have used nicotine concentrations ranging from 10 to 200 μg/ml (Adriani et al., 2002; Butt et al., 2005; Grabus et al., 2005; Klein et al., 2004; Li et al., 2005; Sparks and Pauly, 1999). Strong nicotine solutions are bitter, and we did not use tastants to mask the taste. Although we do not know how mice experience the taste of nicotine, we assume that they find it unpleasant since the fluid intake of the

mice decreased, and their weight gain slowed, over the course of the experiments. On the other hand, the strong solution may also have induced other aversive effects unrelated to the bad taste. This argues for the importance of using less concentrated nicotine solutions in future studies.

6.2 The sensitivity of presynaptic dopamine D_2 receptors in response to forced chronic oral nicotine exposure

This study intended to clarify the mechanisms of tolerance to the effects of acute nicotine after chronic oral nicotine exposure. Earlier results from our laboratory did not find alterations in the number of dopamine D_1- or D_2-like receptors (Pietilä et al., 1996). In the present study, in both nicotine-exposed and control animals, a small autoreceptor-preferring dose of dopamine D_2/D_3 receptor agonist quinpirole reduced the extracellular and tissue concentrations of dopamine and its metabolites in the dorsal striatum and the nucleus accumbens in a similar manner. They also exhibited a similar decrease in locomotor activity and body temperature after quinpirole administration. These results suggest that although nicotine still enhances striatal dopamine release and metabolism, as well as locomotor activity, after the 7-week exposure (present results; Gäddnäs et al., 2001; Pietilä et al., 1995), the sensitivity of striatal dopamine D_2-like receptors remains unchanged (Table 9).

Altered sensitivity of the dopamine D_2-like autoreceptors has been associated with the development of behavioural sensitisation in response to intermittent administration of amphetamine or cocaine (Ackerman and White, 1990; Henry et al., 1989; Pierce et al., 1995). In rats, nicotine has been shown to induce behavioural sensitisation (Benwell and Balfour, 1992), which reduces the sensitivity of dopamine D_2 autoreceptors (Balfour et al., 1998). Nevertheless, there is only vague evidence that nicotine produces behavioural sensitisation in mice (Kuribara, 1999; Sahraei et al., 2007), although they do develop tolerance to the locomotion-reducing effects of nicotine (Pietilä et al., 1998; Sershen et al., 1991). In this study and previously, a 10-day repeated subcutaneous nicotine treatment has been found to attenuate dopamine autoreceptor sensitivity, although signs of behavioural sensitisation are absent (Sershen et al., 1991). Cocaine has been shown to induce sensitisation of the dopamine D_2 autoreceptors after repeated injections, but no changes when it is given as a continuous infusion (Davidson et al., 2000). Thus, it seems that, like cocaine's effects, nicotine's effects on dopamine D_2-like receptors depend on how it is administered.

6.3 The effect of forced chronic oral nicotine exposure on the rewarding or reinforcing properties of nicotine

Since chronic oral nicotine exposure induces changes in brain neurochemistry, and especially because it causes tolerance to the effects of acute nicotine, we wanted to explore whether chronic exposure would affect the primary or secondary reinforcing effects of nicotine. An earlier study had shown that 14-day continuous nicotine infusion did not alter nicotine self-administration behaviour in DBA/2J mice (Semenova et al., 2003). Another report showed that after 7-day repeated nicotine injections, the acquisition of nicotine self-administration was accelerated in Sprague-Dawley rats, but impaired in Long-Evans rats (Shoaib et al., 1997). Furthermore, after 10-day repeated nicotine injections or 6-day forced oral nicotine exposure during adolescence, Sprague-Dawley rats demonstrated enhanced sensitivity to self-administer nicotine (Adriani et al., 2003; Maehler et al., 2000). The NMRI mice chronically pre-exposed to nicotine were found to self-administer nicotine at lower unit doses than the control mice, suggesting that their sensitivity to the reinforcing effects of nicotine was enhanced. Since the genetic strain affects the acquisition of nicotine self-administration in rats, it is possible that the difference between the present results and those of Semenova et al. (2003) is due to the mouse strain. Another possibility is that the discordant results again reflect the different mode of nicotine delivery and the length of treatment.

The optimal nicotine unit dose differs considerably for different strains. In conditions similar to those used in the present study, the optimal dose ranges from 0.0168 μg/infusion in DBA/2J mice (Paterson et al., 2003; Semenova et al., 2003) to 0.056 μg/infusion in Swiss mice (Blokhina et al., 2005). In drug-naïve NMRI male mice, the optimal nicotine unit dose (0.028 μg/infusion) fell between these two extremes. Thus, their nicotine sensitivity is average.

A 7-day pretreatment with nicotine injections has been shown to enhance the development of nicotine-induced place preference in Lister rats, although the magnitude of the effect was small (Shoaib et al., 1994). However, in Sprague-Dawley rats, a 10-day repeated nicotine treatment did not affect the place conditioning if given in periadolescence, but it decreased sensitivity to develop nicotine place conditioning if given in postadolescence (Adriani et al., 2006). In the present study, nicotine pre-exposure did not affect the development of place preference to nicotine. Since we also started the forced oral nicotine exposure when the mice were early adolescents (4 weeks old), our results are consistent with those of Adriani and co-workers.

Other studies using both biased and unbiased, counterbalanced setups have shown the development of place preference with 0.5-0.8 mg/kg doses of nicotine in mice (Castañe et al., 2002; Grabus et al., 2006; Risinger and Oakes, 1995; Schechter et al., 1995; Walters et al., 2006). In addition, the present study has shown that a nicotine dose of 0.5 mg/kg induced significant conditioned place preference in

male NMRI mice. However, it can be argued that the observed shift in preference (~80 seconds) is not large enough to be reliable. Nevertheless, others have reported relatively small place preferences with nicotine, probably due to the limited reinforcing properties of nicotine (Castañe et al., 2002; Grabus et al., 2006; Kota et al., 2008; Schechter et al., 1995). The weak nicotine-induced place preference may also be due to insufficient handling prior to the experiments, since more robust nicotine-induced changes in place preference have been observed with pre-handled animals (Grabus et al., 2006). Our protocol did not include a pre-handling procedure.

We also studied whether nicotine-exposed mice would prefer nicotine solution when they were given the possibility to choose between nicotine solution and plain tap water. Only small amounts of nicotine and cotinine were found in the plasma of mice that were assigned to the free-choice groups. These concentrations were much lower than those observed in continuously nicotine-drinking mice, indicating that the mice had switched to drink mainly water without any evidence of nicotine preference. In this study, nicotine consumption was estimated by determining the nicotine and cotinine levels in an end-point plasma sample instead of housing the animals singly and measuring the nicotine consumption daily. This approach has certain drawbacks. First, it provides an estimate of the nicotine consumption at only one time point instead of creating a time-consumption curve. Second, differences in nicotine metabolism affect both nicotine and cotinine levels in plasma (Tyndale and Sellers, 2002). However, it is unlikely that different metabolic activity could have affected the present results. It is also unlikely that the mice would have preferred the nicotine solution more in the beginning of the free-choice phase.

Collectively, these results suggest that nicotine pre-exposure enhances the reinforcing effects of the acutely administered nicotine (Table 9). In our experiments, however, this effect was significant only in the intravenous self-administration model, which has proved to be very sensitive in detecting the reinforcing properties of a substance (Blokhina et al., 2004). Our conditioned place preference experiments also showed a trend in this direction.

Table 9 *The influence of chronic oral nicotine exposure on the effects of quinpirole and the reinforcing properties of nicotine.*

	During nicotine treatment	After withdrawal from nicotine
Effect of quinpirole on locomotor activity	0	0
Effect of quinpirole on tissue or extracellular dopamine metabolites	0	0
Effect of quinpirole on body temperature	0	0
Free-choice oral self-administration of nicotine	0	NT
Nicotine conditioned place preference	NT	(↑)
Intravenous nicotine self-administration	NT	↑

0 = no change, (↑) = non-significantly enhanced, ↑ = significantly enhanced, NT = not tested

6.4 The effect of *Comt* gene disruption on consumption of oral ethanol, cocaine, and etonitazene, as well as on the rewarding properties of cocaine

This study aimed to explore the effect of *Comt* gene disruption on preference or aversion for increasing concentrations of ethanol (2.5-20%, v/v), cocaine (0.1-0.8 mg/ml), and etonitazene (1 or 2 μg/ml) solutions in male and female mice. We also studied whether the mutation would affect the development of cocaine-induced place preference.

First, the animals were tested with a range of ethanol or drug solutions in order to screen for genotype differences at different concentrations. This approach has been used in investigations of the preference of different rat or mouse strains for ethanol or drugs (e.g. Bachmanov et al., 1996; Blednov et al., 2005; Hall et al., 2003; Hyytiä and Sinclair, 1993; Meliska et al., 1995; Savelieva et al., 2002). Administering ethanol in increasing concentrations habituated the animals to its taste. Male *Comt*(-/-) and *Comt*(+/-) mice consumed significantly more ethanol than their male wild-type littermates at all concentrations tested, which may indicate enhanced reinforcing effects of ethanol in the knock-out animals. Ethanol preference was sex-dependent, since ethanol consumption of female mice was not linked to genotype. A similar sex-dependent genotype effect on alcohol consumption has also been reported in *Dat1* knock-out animals (Hall et al., 2003; Savelieva et al., 2002).

Cocaine doses chosen for the present study have previously been used in oral self-administration studies in rodents (Carlson and Perez, 1997; Davidson et al., 2004; Hyytiä and Sinclair, 1993; Marquardt et al., 2004). Oral cocaine has been shown to be behaviourally active in rats (Falk et al., 1991; Lau et al., 1991, 1992), and in the present study the cocaine dose proved to be high and effective enough to cause anorexia in mice. However, male mice exhibited a strange cocaine drinking pattern, which impaired the interpretation of the results. In females, neither cocaine intake nor preference was associated with *Comt* genotype.

Etonitazene has been used in oral opioid consumption studies in rats and mice in concentrations ranging from 0.3 to 17.5 μg/ml (Forgie et al., 1988; Hyytiä and Sinclair, 1993; Sala et al., 1993). It is preferable to morphine because its high potency allows the preparation of low-concentration solutions with neutral taste. In rats, the optimal concentration seems to be as low as 1-2 μg/ml (Carlson, 1989; Carlson et al., 1996; Hyytiä and Sinclair, 1993). Despite the fact that C57BL/6 mice have been found to consume opiate-containing drinking solutions more eagerly than several other mouse strains (Belknap et al., 1993; Elmer et al., 1995; Eriksson and Kiianmaa, 1971), researchers have suggested that they are relatively insensitive to the reinforcing effects of etonitazene, at least when relatively dilute (0.3 μg/ml) drinking solutions are used (Forgie et al., 1988). This may explain why the mice were indifferent to the etonitazene solution in the present study.

Conditioned place preference studies were carried out to clarify the effect of *Comt* disruption on the reinforcing properties of cocaine. These experiments were inspired by the earlier observation that homozygous *Comt* knock-out males are less sensitive to cocaine-induced motor activation (Huotari et al., 2002b), and to the initial motor depression caused by large doses of amphetamine (Huotari et al., 2004). Cocaine increased the time spent in the drug-paired environment similarly in both sexes and in all genotypes. Therefore, the *Comt* gene disruption does not seem to affect the reinforcing properties of cocaine. However, this experiment would have been stronger if smaller cocaine doses had also been tested and if the extinction of the place conditioning had been assessed.

Quantitative trait locus studies have suggested an association between high COMT activity and high alcohol consumption (Grice et al., 2007). The “drug-seeking” C57Bl/6 mice had higher COMT activity in the nucleus accumbens and prefrontal cortex than the “drug-avoiding” DBA mice. On the other hand, one study found no differences in the *Comt* mRNA expression between these strains (Kerns et al., 2005). The present results add complexity to the situation by suggesting that low COMT activity or *Comt* gene dose levels may be associated with increased ethanol consumption. In conclusion, considering the inconsistent results of animal studies and human population studies (see section 2.4.10 for discussion about the Val108/158Met polymorphism and addiction), it appears that *Comt* gene is not the primary factor in the development of human drug addiction or alcoholism.

6.5 The effect of *Comt* gene disruption on extracellular dopamine levels in the striatum, nucleus accumbens, and cortex

In dorsal and ventral striatum, uptake by DAT is the primary way to terminate the dopamine signal (Cass et al., 1993; Eisenhofer et al., 2004; Giros et al., 1996). DAT rapidly clears up most of the released dopamine back into the nerve ending, where it is either packed into storage vesicles by VMAT2 or oxidized by MAO-A. Therefore, only a fraction of dopamine is taken up into glial cells or postsynaptic neurons, where it is methylated. However, in brain areas with low dopamine transporter density, e.g. in the prefrontal cortex and hypothalamus, COMT is the primary enzyme in dopamine metabolism (Mazei et al., 2002; Morón et al., 2002; Sesack et al., 1998).

In the *Comt* knock-out mice, extracellular dopamine concentrations in the basal dorsal striatum or the nucleus accumbens were not altered in either sex under normal conditions. This is consistent with previous findings in these mice (Huotari et al., 2004). Earlier studies have also shown that the genotypes have similar dopamine concentrations in the striatal tissue under normal conditions (Gogos et al., 1998; Huotari et al., 2002a). Furthermore, treatment with selective COMT inhibitors tolcapone or entacapone does not affect dopamine levels or dopamine outflow in the extracellular fluid or tissues of the dorsal striatum or nucleus accumbens (Acquas et al., 1992; Budygin et al., 1999; Huotari et al., 1999; Kaakkola and Wurtman, 1992, 1993; Katajamäki et al., 1998; Li et al., 1998; Napolitano et al., 2003).

The basal dopamine concentration in the prefrontal cortex is not associated with *Comt* genotype. Similarly, treating rats with tolcapone alone did not affect extracellular dopamine levels in prefrontal cortex, although it potentiated clozapine-induced dopamine efflux (Tunbridge et al., 2004b). There are conflicting reports about the effect of COMT deficiency on dopamine levels in prefrontal cortex tissue. One study found a 2.5-fold increase in prefrontal cortex dopamine levels in homozygous *Comt* knock-out males (Gogos et al., 1998), whereas another study failed to replicate this finding (Huotari et al., 2002a). An *in vivo* voltammetry study showed that, in male *Comt* knock-out mice, stimulus-evoked dopamine release is higher by 20-25%, and the dopamine elimination time is two-fold longer, than in wild-type animals (Yavich et al., 2007). However, since the sampling time in microdialysis is longer than in voltammetry, it is possible that short-lived phenomena associated with dopamine release were missed in the present study.

The baseline levels of DOPAC in dorsal striatum, nucleus accumbens, and prefrontal cortex were elevated 1.6- to 2.6-fold in the *Comt* knock-out mice, and HVA was not detectable. Other studies have found similarly elevated DOPAC levels in striatal tissue and extracellular fluid of these mice (Huotari et al., 2002a). Oral administration of tolcapone or entacapone (15 mg/kg) cause a decrease of at most 90% in duodenal and liver COMT activity (Napolitano et al., 2003). These inhibitors have been shown to induce accumulation of DOPAC in striatal tissue as well as in striatal and accumbal extracellular fluid (Budygin et al., 1999; Kaakkola and

Wurtman, 1992, 1993; Li et al., 1998; Napolitano et al., 2003). The inhibitors also cause a transient decrease in striatal and accumbal HVA levels.

In conclusion, these results emphasize that DAT-mediated uptake and oxidation by MAO is the preferred metabolic route for dopamine (Fornai et al., 1999; Giros et al., 1996). Brain dopamine systems are apparently very well-protected from perturbation, and a 50% decrease in COMT enzyme levels in heterozygous *Comt* knock-outs (Huotari et al., 2002a; 2002b, 2004) or in S-COMT mutant mice (Study IV) only marginally affects dopamine metabolism. MAO apparently has a considerable enzymatic reserve, since the lack of COMT does not affect MAO protein levels or MAO activity in brain or kidneys (Huotari et al., 2002a; Odlind et al., 2002). We did not find evidence of altered DAT function in *Comt* knock-out mice. This finding agrees with previous results suggesting that these animals have wild-type levels of DAT protein (Huotari et al., 2002b). In addition, the effects of cocaine or the selective DAT inhibitor GBR 12909 have been shown to be unaltered in the striatum and prefrontal cortex (Huotari et al., 2002b; Yavich et al., 2007), which further suggests that DAT function is unchanged.

Figure 12 shows how levodopa and dopamine are metabolized in wild-type and *Comt* knock-out mice following treatment with carbidopa and levodopa. Levodopa can increase dopamine levels in the extracellular fluid of striatum and COMT inhibition potentiates this effect mainly by blocking the peripheral metabolism of levodopa to 3-OMD (Männistö and Kaakkola, 1999). In this study, treatment with carbidopa (30 mg/kg, i.p.) and levodopa (10 mg/kg, i.p.) modestly increased striatal, but not accumbal, extracellular dopamine levels. The lack of COMT did not significantly increase striatal or accumbal extracellular dopamine levels following carbidopa and levodopa treatment. In homozygous males the area under the striatal dopamine concentration curve is smaller than in the male wild-type animals or female wild-type and homozygous mice, which reflects the overall reduced dopamine levels in these mice. Other microdialysis studies have shown that COMT inhibitor treatment enhances the increase in extracellular dopamine concentration induced by levodopa and carbidopa (Kaakkola and Wurtman, 1992; Napolitano et al., 2003; Törnwall et al., 1994). The levodopa dose used in the present study was relatively low, which may explain why no effect of the *Comt* genotype on extracellular dopamine was seen. An earlier study found a similar lack of effect when striatal tissue dopamine levels were measured after the same carbidopa and levodopa dose (Huotari et al., 2002a), as did a third study in which the effects of a very high dose of levodopa and entacapone were studied in the absence of peripheral dopadecarboxylase inhibitor (Katajamäki et al., 1998).

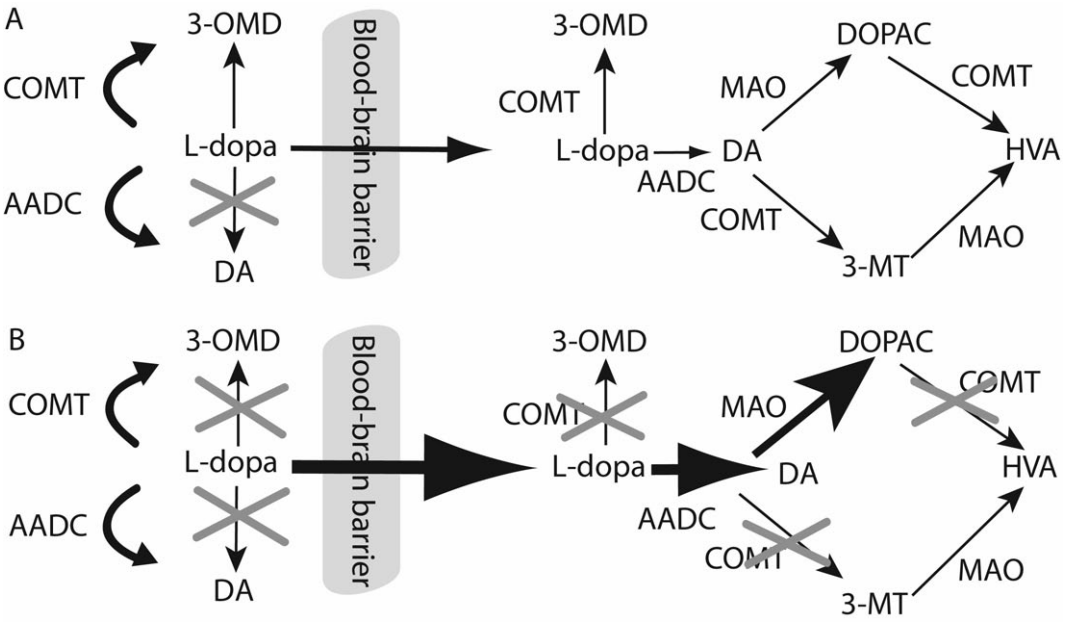

Figure 12 *Levodopa and dopamine metabolism after carbidopa and levodopa treatment in wild-type (A) and* Comt *knock-out mice (B). 3-MT = 3-methoxytyramine, 3-OMD = 3-O-methyldopa, AADC = L-aromatic acid decarboxylase, COMT = catechol-O-methyltransferase, DOPAC = 3,4-dihydroxyphenylacetic acid, HVA = homovanillic acid, L-dopa = L-3,4-dihydroxyphenylalanine, MAO = monoamine oxidase*

In the prefrontal cortex, carbidopa and levodopa treatment increased the extracellular levels of dopamine to similar extents in *Comt* knock-out and wild-type mice. The dopamine levels remained elevated in male mice longer than in the control animals. An earlier study found that the lack of COMT potentiates the enhanced dopamine concentration induced by carbidopa and levodopa treatment in the hypothalamic and prefrontal cortical tissue (Huotari et al., 2002a). The findings about males in the present study are consistent with this result. Under levodopa load the increased quantities of freshly synthesized dopamine are probably either packaged into storage vesicles or degraded in the cytoplasm by MAO (Eisenhofer et al., 2004). This may explain why the increase in dopamine concentration is not as high in the extracellular fluid as it is in the tissue.

Levodopa load further enhanced the accumulation of DOPAC in the striatal and accumbal extracellular fluid of *Comt* knock-out animals. Such enhancement of DOPAC accumulation in striatal extracellular fluid and tissue has been reported in *Comt* knock-out mice and in animals treated with COMT inhibitors under anaesthesia (Huotari et al., 2002a; Kaakkola and Wurtman, 1992; Napolitano et al., 2003; Törnwall et al., 1994). The present study now replicates these findings in awake and freely behaving *Comt* knock-out animals. The accumulated evidence therefore

indicates that the importance of the methylation pathway in dopamine metabolism is accentuated when the dopaminergic system is challenged.

6.6 General discussion

The prefrontal cortex is involved in the modulation of working memory, processing of information, specific aspects of cognition, and development of addiction (Brodal, 1992; D'Esposito et al., 1995; Koob and Le Moal, 2006). Studies on the Val108/158Met polymorphism in the human *Comt* gene have shown that individuals homozygous for the low-activity Met form of the enzyme have lower levels of tyrosine hydroxylase mRNA in the midbrain than do individuals homozygous for the high-activity Val protein. This difference probably reflects the elevated dopamine levels in the prefrontal cortex in the Met homozygotes (Akil et al., 2003). Cortical dopamine levels, on the other hand, have been shown to influence the effect of amphetamine on cognitive abilities (Mattay et al., 2003). Following administration of amphetamine, the cognitive performance of Met homozygous individuals deteriorates, whereas it improves in Val homozygotes (Figure 13). Since females apparently have lower COMT activity than males (Boudíková et al., 1990; Chen et al., 2004), females may be in a different position than males along the prefrontal cortex function/dopamine signalling curve under baseline conditions (Figure 13). Therefore, sex may affect the influence of pharmacological manipulations on cortical dopamine function. The sex differences observed in this study were in several cases even greater than the genotype differences, and they seem to be large enough to mask the relatively small changes induced by the lack of COMT.

We observed some sex-dependent differences in dopamine function in the prefrontal cortex in this study. Under levodopa load, the dopamine levels were higher in the male than female mice. This may indicate a different prefrontal cortical dopaminergic tone between the sexes, which may explain the sex-dependent effect of COMT deficiency on alcohol consumption. Although our microdialysis study found similar dopamine levels in prefrontal cortical in wild-type and *Comt* homozygous mice, a previous *in vivo* voltammetry study found these animals to differ in their dopamine release and elimination (Yavich et al., 2007). Therefore, the male homozygous mice may experience a greater increase in dopamine release in response to ethanol, which may explain their higher ethanol consumption. In humans, the prefrontal cortical dopamine levels are probably more tightly linked to the levels of COMT activity, since *O*-methylation is a more frequent pathway of dopamine metabolism in humans and primates than in rodents (Kopin, 1985).

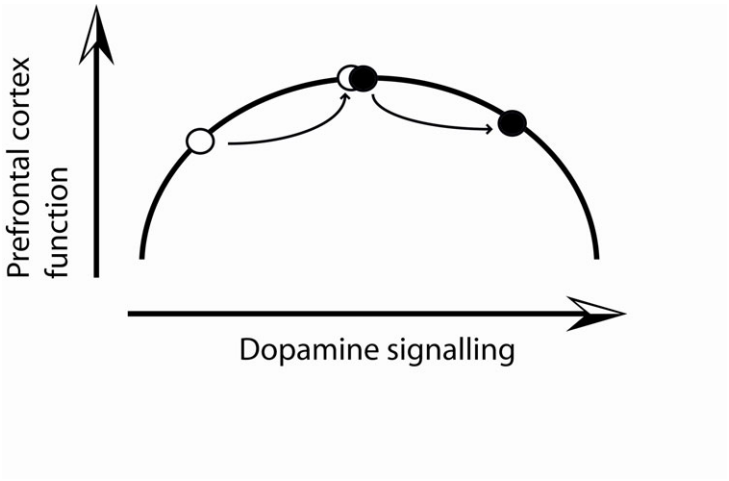

Figure 13 *The inverted U-shape model of the association between dopamine signalling and function of the prefrontal cortex. White circles denote Val homozygous individuals; black circles, Met homozygous individuals. The small arrows show the change in the dopamine signalling and prefrontal cortex function in response to amphetamine. Modified from Mattay et al. (2003).*

It is evident that dopamine's role in the actions of different drugs varies, even though to some extent it seems to be universally involved in the development of addiction. For psychostimulants the dopaminergic system is obviously the main target, but these agents also involve serotonergic and noradrenergic pathways (Koob and Le Moal, 2006). In addition to the dopaminergic system, opioids naturally affect the function of the endogenous opioid system; cannabinoids, the endocannabinoid system; nicotine, the cholinergic system; and ethanol, the GABAergic system. In this way, the lack of COMT or *Comt* Val108/158Met polymorphism seems to be differently associated with the consumption of, or addiction to, different drugs. Although the previous studies showed that locomotor response to cocaine and amphetamine is altered in *Comt* knock-out mice, neither the consumption of cocaine solutions nor cocaine-conditioned place preference was associated with COMT deficiency. Instead, we found that alcohol consumption was associated with the lack of COMT in a sex-dependent manner. There is also some evidence for a similar association between the low-activity COMT Met genotype and alcohol consumption or alcoholism in the humans.

Lack of association between cocaine use and *Comt* genotype may be due to cocaine's effect on the serotonergic system. Cocaine is able to induce place preference and self-administration in the animals lacking the DAT protein, apparently via serotonergic mechanisms (Mateo et al., 2004; Rocha et al., 1998; Sora et al., 1998). Therefore, it is understandable that the absence of the secondary dopamine metabolic enzyme, COMT, which does not participate in the metabolism of serotonin, does not appreciably influence the consumption of cocaine solutions.

Overall, this study shows that although forced chronic oral nicotine exposure affects the reinforcing properties of self-administered nicotine, it produces different neurochemical changes than other methods of administration, such as intermittent nicotine injections. Forced chronic oral nicotine exposure does not model addiction, but instead passive, involuntary exposure to nicotine. Studies evaluating the effect of nicotine pre-exposure on nicotine IVSA are scarce. Therefore, it would be interesting to study how different types of nicotine administration, such as via osmotic minipumps or intermittent injections, affects the acquisition of nicotine IVSA in mice. It would also be useful to compare the treatments in an IVSA model that allows repeated testing.

COMT is likely to be one factor in the development of addiction, but its role seems to be minor. The IVSA method would be more sensitive than CPP or oral self-administration methods for revealing differences in the reinforcing properties of the drugs. Thus, IVSA studies in the *Comt* knock-out mice over a wide dose range of cocaine and/or morphine may help to define the significance of COMT in addiction-like behaviour. Moreover, COMT involvement in dopamine in the brain is minimal under basal conditions, which indicates that dopamine function in the brain is well-protected from perturbation. Nevertheless, the significance of this metabolic enzyme is greater when the dopaminergic system is challenged for example by pharmacological manipulation. The *Comt* knockout mice show changes in the dopaminergic function of prefrontal cortex, but these changes are smaller than expected. Therefore, it would be important to study whether these animals show changes in monoamine uptake systems in prefrontal cortex.

7 CONCLUSIONS

Generally, this study showed that neurochemical changes in the dopaminergic system induced by nicotine treatment depend on the type of nicotine administration. Furthermore, the role of COMT in the development of addiction-like behaviour proved to be small. Thus, *Comt* knock-out mice have limited importance for addiction studies.

The following specific conclusions were drawn from the results of this study:

1. Chronic oral nicotine exposure does not affect the sensitivity of dopamine D_2-like autoreceptors in male NMRI mice, whereas intermittent nicotine injections reduce their sensitivity slightly but significantly. Thus, the effect of nicotine treatment on dopamine autoreceptor sensitivity depends on the route, frequency, and possibly also time course of drug administration.

2. Chronic oral nicotine exposure enhances the animal's sensitivity to the reinforcing properties of nicotine. However, only the intravenous self-administration paradigm is sensitive enough to detect this enhancement.

3. Deletion of COMT has different effects on the consumption of different drugs of abuse. *Comt* gene disruption enhances ethanol consumption in a sex-dependent manner. However, despite its influence on the motor effects of psychostimulants, it does not affect oral consumption of cocaine solutions or cocaine-induced place preference. Therefore, although COMT may contribute to the development of addiction, its role appears to be relatively minor.

4. In dorsal striatum, nucleus accumbens, and prefrontal cortex *Comt* gene disruption does not affect the extracellular levels of dopamine under drug-free conditions, although it does increase DOPAC concentration in these brain regions. This DOPAC accumulation is greater in all three brain areas when the genetically manipulated mice are given levodopa, but even under levodopa load the effect of COMT deficiency on dopamine efflux is small. Thus, the role of COMT in dopamine metabolism becomes important only when the dopaminergic system is acutely challenged.

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