THE ROLE OF DOPAMINE D3 RECEPTORS IN THE CONTROL OF ALCOHOL INTAKE

Giovanni Camillieri

UNIVERSITY OF CATANIA

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Giovanni Camillieri

Coordinator: Prof. Filippo Drago

Tutor: Prof. Salvatore Salomone

UNIVERSITY OF CATANIA

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List of abbreviations

5-HT	serotonin
BDNF	brain development neurotrophic factor
CAAs	competing amino-acids
CNS	central nervous system
$D_3^{-/-}$	dopamine D_3 receptor deficient mice
D_3R	dopamine D_3 receptor
DA	dopamine
DMSO	dimethyl sulfoxid
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, 4th edition
EPM	elevated plus maze test
FST	forced swimming test
GABA	γ-aminobutyric acid
$GABA_A$	γ-aminobutyric acid receptor A
GAD	generalized anxiety disorder
HF	Familiar conditions
HU	Unfamiliar conditions
i.p.	intraperitoneal injection
ICD-11	Classification of Diseases, 11th edition
NGT	novelty induced grooming test
PD	Panic disorders
SIT	Social Interaction Test
SSRI	selective serotonin reuptake inhibitor
TCA	tricyclic antidepressant drug
TRP	tryptophan
W.T.	wild type mice

Preface

Mesolimbic dopamine controls drug and alcohol seeking behavior. Stimulation of dopamine D_3 autoreceptor reduces extracellular levels of dopamine. We tested the hypothesis that dopamine D_3 receptor (D_3R) gene deletion or its pharmacological blockade counteracts alcohol preference and intake in a long-term voluntary ethanol intake paradigm. Mice $D_3R^{-/-}$ and their wild type (WT) littermates, treated or not with the D_3R antagonists U99194A and SB277011A, were tested. The selectivity of the D_3R antagonists was further assessed by molecular modeling. Activation of dopamine (DA) transmission and D_3R expression was assessed at the end of the experiment. After 8 days, daily ethanol intake was negligible in $D_3R^{-/-}$ and robust in WT; this behavior was stably maintained for 44 days. Treatment with D_3R antagonists counteracted ethanol intake in WT and was associated to increased DA transmission (assessed as phosphorylation of DARPP-32 and GSK3β) in striatum and prefrontal cortex. Forced ethanol intake increased the expression of RACK1 and BDNF in both WT and $D_3R^{-/-}$; in WT there was also a robust overexpression of D_3R. Thus, increased expression of D_3R associated with activation of RACK1/BDNF seems to operate as a reinforcing mechanism in voluntary ethanol intake. Taking into account that ethanol intake increases mesolimbic DA, low levels of extracellular DA resulting from D_3R

overexpression would facilitate ethanol intake, and high levels of extracellular DA, from either gene deletion of D_3R blockade, would inhibit ethanol intake. Thus, modulation of DA mesolimbic pathway by selective targeting of D_3 receptor might provide a basis for novel weaning treatments in alcoholism.

Introduction

1. Alcholism

Although alcohol has been recognized as an agent of abuse since the 17th century, the disease model was accepted only in the last century. Alcoholism is defined as a chronic and progressive disease characterized by loss of control over the use of alcohol with subsequent social, legal, psychological and ethical consequences. Recently, monoamines were shown to have a predominant role in the etiology of alcoholism. Dopamine has been implicated by virtue of its actions on the reward center. Endogenous opioids which lessen stress and produce euphoria, are released upon alcohol intake, whereas increased serotonin facilitates tolerance and thereby fosters increased alcohol consumption. Alcohol has facilitative effects on inhibitory action of GABA, while the stimulatory effects of glutamate are decreased. Drugs used for treatment of alcohol dependence can be broadly classified into 4 groups: sensitizing drugs, opioid antagonists, drugs acting on serotonergic systems and acamprosate. Disulfiram has been shown to be most effective for patients who believe in its efficacy and remain compliant with the treatment. The opioid antagonist naltrexone lowers relapse rate, reduces drinking days and prolongs periods of abstinence, while serotonin reuptake inhibitors and serotonin antagonists at best have equivocal

efficacy. The treatment of alcoholism should not be restricted to pharmacotherapy alone but should also be supplemented with interventions addressing the psychological, medical and social needs of patients.

In industrialized countries, alcohol use alone causes about 10% of total disability-adjusted life years lost (Rehm, Mathers et al. 2009), and a recent evaluation in the United Kingdom concluded that in aggregate, the harm to self and others inflicted by alcohol exceeds that caused by heroin or cocaine (Nutt, King et al. 2010). Alcohol consumption in the population is markedly skewed, and a large proportion of alcohol-related disability is due to alcohol addiction, hereafter equated with alcoholism. This is a condition that in the United States affects more than 12% of the population at some point in their life (Hasin, Stinson et al. 2007). Alcoholism is a chronic, relapsing disorder that shares many characteristics with other complex chronic conditions, such as diabetes or hypertension: it has a considerable component of genetic susceptibility, is under marked influence of environmental factors, and its onset and course are fundamentally shaped by behavioral choices (McLellan, Lewis et al. 2000; Goldman, Oroszi et al. 2005). This prompts the question of whether alcoholism can be tackled with medical treatments. Some efficacy of medications for alcoholism (Bouza, Angeles et al. 2004) as well as opiate (Amato, Davoli et al. 2005) and nicotine (Wu, Wilson et al. 2006) addiction has been documented and supports the feasibility of addiction pharmacotherapy. However, with the

exception of methadone or buprenorphine maintenance therapy for opioid addictions, the effect sizes of these treatments are small. Despite evidence-based guidelines that pharmacotherapy be considered in all patients with alcoholism, and in particular in those who are not successfully treated with behavioral interventions alone (Willenbring, Massey et al. 2009), only a small minority of patients receive medication for their alcoholism (Mark, Kranzler et al. 2003).

Clearly, extensive unmet medical needs remain in this therapeutic area.

A clinical diagnosis of alcoholism is currently made on the basis of diagnostic criteria that are standardized across addictive disorders by the Diagnostic and Statistical Manual of Mental Disorders, which is currently in its fourth edition (DSM IV). In the absence of reliable biomarkers, this approach eliminates some of the subjective judgment involved in making diagnoses, and has clinical utility. However, there is reason to believe that patients diagnosed using this approach are markedly heterogeneous. In fact, such heterogeneity was already proposed in the 1980s on the basis of clinical characteristics such as age of onset, but also on family history, which is a marker of genetic susceptibility (Cloninger 1987). Numerous other attempts at clinical subtyping of people with alcoholism have since followed. The use of genetic markers offers the possibility of more reliably and consistently capturing the heterogeneity of people with alcoholism, in ways that are closer to its biological underpinnings.

Among individuals in the general population who fulfill diagnostic criteria for alcoholism, the majority — about three-quarters — never receive treatment (Hasin, Stinson et al. 2007). Available data indicate that those people who go on to enter treatment and those who do not are fundamentally different with regard to personality traits, alcohol use patterns and long-term outcomes (Vaillant 1983; Vaillant 1996; Fein and Landman 2005). Furthermore, classic longitudinal studies show that long-term outcomes and alcohol-related harm vary markedly between individuals in ways that do not seem to have a simple correlation with participation in treatment or the level of alcohol use (Vaillant 1983; Vaillant 1996). A clinical diagnosis of alcoholism is probably best viewed as an 'end-stage disease', similar to congestive heart failure. In this view, the diagnostic category of alcoholism consists of conditions that are phenotypically similar (or constitute 'phenocopies'), but patients arrive at the disease state through fundamentally different trajectories. This is captured by a conceptualization that was first put forward for major depression (Kendler, Thornton et al. 2001), but is also likely to apply to addiction. In a kindling-like process, brain exposure to cycles of intoxication and withdrawal induces progressive neuro-adaptations that ultimately result in escalation of alcohol intake (Ballenger and Post 1978; Heilig and Koob 2007). In the absence of significant genetic susceptibility, escalation will only result following prolonged exposure to alcohol and the environmental factors with which it

interacts, such as stress. By contrast, when genetic risk factors are present, progression can be fast. These individuals can be viewed, in terminology borrowed from the depression literature (Kendler, Thornton et al. 2001), as 'pre-kindled', or 'already there'.

Emerging evidence indicates that individuals with alcohol addiction who are on trajectories that are driven by different biological mechanisms or who are in different stages of addiction can be expected to respond to different treatments. Fundamentally, treatments for alcohol addiction must intervene with biological mechanisms that provide motivation for alcohol seeking and consumption (Heilig and Egli 2006). These mechanisms largely fall into two main categories. First, in a similar way to other drugs of abuse, alcohol can activate brain reward pathways, leading to positively reinforced alcohol seeking and use. Secondly, alcohol can acutely suppress negative emotions that result from stress or withdrawal from alcohol itself, such as anxiety and dysphoria, thus setting the scene for negatively reinforced alcohol use (Heilig and Koob 2007; Koob and Volkow 2010). To highlight the distinction between these two incentives for alcohol use, the terms 'reward drinking' and 'relief drinking' have been introduced (Heinz, Lober et al. 2003). It is reasonable to expect that these different types of excessive alcohol use will require different treatments.

Alcoholism has a moderate to high heritability, and in part shares genetic susceptibility factors with other addictions (Goldman, Oroszi et al. 2005).

Genetic and environmental factors in alcoholism can result in very different types of vulnerability, ranging from heightened impulsivity and reward from alcohol to enhanced stress responses and anxious personality traits (Cloninger 1987). Genetic variants that alter alcohol reward- or stress-related emotional processing are therefore probable modifiers of disease trajectories and of responses to treatments that target reward and stress systems.

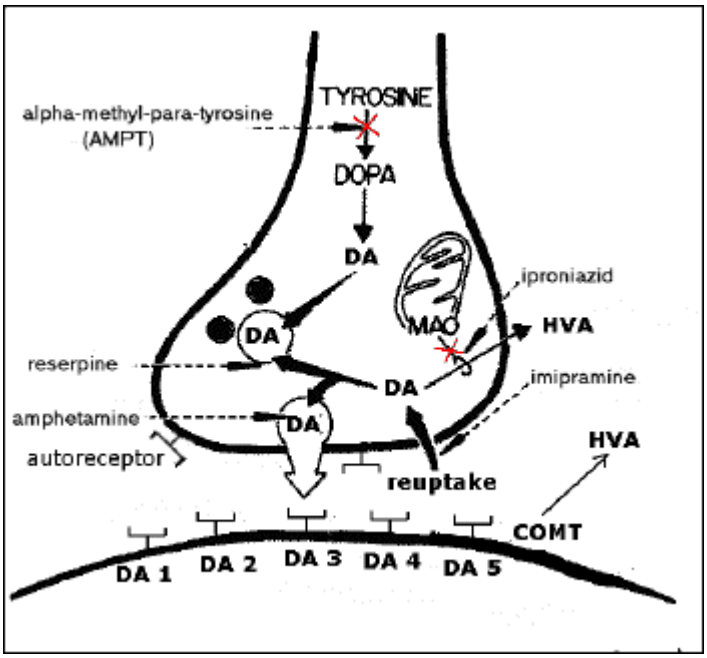
2. Dopamine pathways and receptors in the central nervous system

Four main dopaminergic pathways were identified within the CNS. The ventral tegmental area is the place of origin of two projection pathways towards the cortex (the mesocortical pathway) and the limbic area (the mesolimbic pathway); the hypothalamus is the place of origin of a projection towards the pituitary gland which controls prolactin secretion (the tubero-infundibular pathway) and a dopaminergic projection extends from the substantia nigra to the striatum (the nigrostriatal pathway) the degeneration of which is implicated in Parkinson's disease. Using these pathways, dopamine receptors are located. Five genes encoding dopamine receptors were identified.

These receptors are divided in two subfamilies: the D1-like receptor subtypes (D1 and D5) coupled with the Gs protein activate adenylyl cyclase and the D2-like subfamily (D2, D3, and D4) coupled with G proteins inhibit adenylate cyclase (Missale, Nash et al. 1998). D1 and D2 dopamine receptors are the most abundant subtypes in the central nervous system, but D1 dopamine receptor is the most widespread. D1 mRNA was found in the striatum, nucleus accumbens, olfactory tubercule, hypothalamus and thalamus. In other areas such as substantia nigra pars reticula with numerous binding sites for the D1 dopamine receptor, no mRNA was detected, suggesting that in these areas the D1

dopamine receptor is present in projections only (Jaber, Robinson et al. 1996). The D5 dopamine receptor is expressed at much lower level than the D1 dopamine receptor and its distribution is limited to the hippocampus and thalamus (the lateral mamillary nucleus and the parafascicular nucleus of the thalamus). The D2 dopamine receptors are located mainly in the striatum, olfactory tubercule, nucleus accumbens, the substantia nigra pars compacta, the ventral tegmental area and the pituitary gland. D2 dopamine receptors are pre- and post-synaptic receptors contrary to D1-like receptors which are mainly post-synaptic receptors (Jaber, Robinson et al. 1996). D4 dopamine receptors were found with a low expression in the basal ganglia and a higher expression in the frontal cortex, medulla, amygdala, hypothalamus and mesencephalon. However, this high expression is weak in comparison with other dopamine receptors (Jaber, Robinson et al. 1996). D3 dopamine receptors are expressed in the limbic area (nucleus accumbens, olfactory tubercule and islands of Calleja) and at a lower level in the striatum(Jaber, Robinson et al. 1996). The D3 dopamine receptors exist as autoreceptors that inhibit neuronal dopamine synthesis and post-synaptic receptors. These receptors by negatively regulating dopamine neuronal activity and/or by post-synaptic action exhibit an inhibitory influence on locomotor activity (Sibley 1999). The genetic techniques for negatively modulating dopamine receptor expression such as knockout animals and antisense technology showed that the disruption of D3, D4, D5 dopamine

receptor functions involved an increase or an improvement in the behavioral activity of animals contrary to the results observed with the disruption of D1, D2 dopamine receptor functions. Although these results have to be interpreted with caution as a compensatory mechanism could develop, these observations suggest that the most abundant dopamine receptors D1 and D2 are involved in positive regulation of behavioral activity whereas the D3, D4, D5 receptors are inhibitory by likely negative modulation of D1 and/or D2 receptor function in some cases (Sibley 1999).



Dopaminergic synapse

3. Alcoholism and dopamine

Credit for discovering that striatal DA neurotransmission is fundamental to drug self-administration is due to two independent groups using different means: James Olds and colleagues in the 1950s and 60s, and the pharmacologists associated with Arvid Carlsson, including Nils Hillarp, Annica Dahlstrom, Kjell Fuxe, and Urban Ungerstedt, who were establishing DA as a neurotransmitter.

In 1954, Olds and Peter Milner introduced intracranial self- stimulation by implanting electrodes in the brains of rats and providing them with a lever that they could press to apply current(Olds and Milner 1954). Soon after, Olds introduced ‘‘intracranial self-administration,’’ a lever-operated device that would allow rats to inject drugs via a pipette directly into defined areas of the brain (Olds and Olds 1958). The self-stimulation paradigm provided the initial evidence of how activity in specific brain regions correlated with the fraction of the time the animals pressed the lever, while self-administration provided insights into drug effects at specific brain regions.

Initial experiments demonstrated that rats with electrodes in the septal area used as much as 92% of the time to bar press, in contrast to regions where ‘‘animals do everything possible to avoid stimulation.’’ By 1956, Olds and collaborators found that stimulation of the hypothalamus was even more

rewarding, eliciting as many as 5000 bar presses per hour. They soon suspected that the efficacy of stimulation of the lateral hypothalamus was due to activation of the medial forebrain bundle, through which DA neurons course from cell bodies in the midbrain to striatal and cortical targets.

They then attempted to interfere with bar presses by administering drugs(Olds, Killam et al. 1956). Successful inhibitors of self- administration included reserpine, which blocks uptake of catecholamines into synaptic vesicles(Carlsson, Hillarp et al. 1962; Kirshner 1962), and chlorpromazine, the antipsychotic, which Carlsson later showed blocks DA receptors.

By 1958, Olds concluded: ‘‘The cells which mediate primary rewarding effects are located in a midline system running from the midbrain through and into the subcortical and cortical groups of the rhinencephalon. The cell groups which mediate primary rewarding effects are different from those which mediate primary punishing effects’’(Olds and Olds 1958). They validated the role of medial forebrain projection from the midbrain to the cortical and subcortical areas in these rewarding effects in later lesion studies (Olds and Olds 1969).

Also in 1969, Olds and Phillips introduced the concept that salient stimuli are responsible for the firing of DA neurons by showing that ventral midbrain neurons fired at a higher frequency following a tone paired with food presentation, and lower rates following other tones not paired with a reinforce

(Phillips and Olds 1969). Remarkably, they conclude with essentially the contemporary understanding of the rules of DA neuronal firing: ‘‘Thus it was surmised that expectancy of reward, rather than response to the tones per se, accounted for the differing rates of firing in midbrain unit activity. These responses reflect an integration of sensory input with the internal state, where the response to tones which signified a reward appropriate to the submotivational state of the organism was amplified by the degree of that motivation.’’ These insights, along with a posthumous study that outlines a specific role for VTA neurons(Brauth and Olds 1977), led to subsequent explorations to decipher the rules by which reinforcement control the activity of DA neurons(Schultz 2011).

By 1976, the year that he died in an accident, Olds wrote a review on the state of self-stimulation and drive, concluding that ‘‘noradrenaline neurons might be the reward neurons addressed to negative drives and DA neurons to positive drives’’(Olds 1976). By introducing self-stimulation paradigms using electrical current and direct drug application, exploiting this to map the brain regions involved, and characterizing firing modes by these neurons to reward and stimuli associated with reward, Olds perhaps provided the strongest contribution to our under- standing of addiction.

Evidence that Addictive Drugs Enhance Striatal DA Neurotransmission During this same period, Carlsson’s colleagues developed

histochemical fluorescent techniques showing that DA neurons originated in the ventral mesencephalon and projected to the cortex and striatum (Hillarp, Fuxe et al. 1966), in the pathway Olds had identified. Similarities between the behavioral response to AMPH and electric self-stimulation of this pathway were observed, and led Crow and colleagues to suggest that ‘‘the dopamine-containing system arising from the ventral mesencephalon may function as an activating system involved in the effects of positive reward on operant behaviour’’ (Anlezark, Arbuthnott et al. 1971). A variety of neurochemical experiments to measure catecholamine release during self-stimulation were conducted, as well as additional lesioning studies, as reviewed(German and Bowden 1974).

Experiments by Wise, Fibiger, Phillips, and others were influential in convincing the field that DA release was particularly important for reward, for example by showing that partial DA receptor blockade increased self-administration of AMPH by rats, while rats would self-administer direct DA receptor agonists(Yokel and Wise 1978). The memorably named De Wit and Wise (1977) showed that a DA D2 receptor antagonist, but not norepinephrine antagonists, blocked cocaine reinforcement.

An approach that convinced the field at large that DA was responsible for the actions of addictive drugs was in vivo micro-dialysis using electrochemical detection. DA release in vivo had been studied using ‘‘push-pull cannula’’, but

this caused significant tissue damage, while voltammetry(Kissinger, Hart et al. 1973) measured catecholamine release and reuptake with extraordinary time resolution(Millar, Stamford et al. 1985; Rice, Oke et al. 1985) and was effective for studying cocaine and AMPH(Caviness and Wightman 1982; Ewing, Alloway et al. 1983), but could not differentiate between electroactive compounds (Gonon, Buda et al. 1980) including norepinephrine and DA. Microdialysis with HPLC electrochemical detection was pioneered by Ralph Adams and collaborators(Adams 1976; Plotsky, Wightman et al. 1977), who showed that AMPH released DA, and Ungerstedt's lab(Ungerstedt and Pycock 1974), who confirmed this response in the striatum.

Microdialysis studies were extended by Imperato and Di Chiara and colleagues, who demonstrated that ethanol (Di Chiara and Imperato 1985), opiates and barbituates (Di Chiara and Imperato 1986), and nicotine (Imperato, Mulas et al. 1986) increased DA concentrations in striatum, particularly in the ventral striatum / nAc. Drugs with aversive properties decreased DA release, and non-abused drugs did not modify synaptic DA (Di Chiara and Imperato 1988).

Most recently, studies in human psychostimulant-naive individuals have shown that AMPH-mediated DA release as measured by D2 receptor availability is significantly associated with effects of the drug reported by the subject as ''happiness'' and ''energy''(Abi-Dargham, Kegeles et al. 2003).

In summary, a role for enhanced striatal DA neurotransmission in the addictive properties of drugs is supported by an increase in extracellular DA levels in the striatum measured following all of the classic addictive drugs; an increased level of drug self-administration when DA receptors are partially antagonized, with cessation at more complete blockade; inhibition of drug self-administration when catecholamines release is decreased by VMAT inhibition or when DA synthesis is blocked; cessation of self-administration when DA neurons or their axons are ablated; reports by AMPH naïve individuals providing a correlation between DA release and self-reported euphoria. Each mechanism of action essentially decouples DA transmitter levels from normal physiological control. From Olds's studies, this would be expected to assign the stimuli that were associated with acquiring these drugs as reinforcements, providing a conceptual synaptic framework for addiction.

Voluntary drinking releases DA in humans (Boileau, Assaad et al. 2003), with recent human PET imaging showing that the equivalent of three drinks increased extrasynaptic DA in the striatum by 138% in men and 69% in women(Urban, Kegeles et al. 2010).

It is thus startling that we do not know how ethanol enhances DA transmission. One reason is because there are many possible receptor and channel targets for ethanol, as recently reviewed(Melis, Diana et al. 2009; Morikawa and Morrisett 2010), but none that clearly show sufficient effect on

DA release at levels achieved by individuals who consume alcohol for its reinforcing properties. How Many Are Enough? Ethanol is typically measured as blood fraction (blood alcohol content [BAC], in the U.S. in units of g/100 ml written as a percentage). As there appears to be no blood/brain barrier to ethanol penetration, the extracellular levels in brain are close to those in blood (Robinson, Brunner et al. 2002). A glass of wine (150 ml, 2.6 M ethanol) yields 0.02% BAC (4.8 mM) in blood of non-alcoholic 68 kg men, with women achieving 34% higher levels(Frezza, di Padova et al. 1990). A typical level for legal intoxication is 0.08% BAC (17 mM), stupor occurs at 0.25% (54 mM), blackout at 0.35% (76 mM), and lethality at 0.4% (87 mM). Effects on DA transmission related to self-administration for reinforcing properties should therefore be present at 5–20 mM ethanol, lower than that often studied experimentally. Note however that alcoholics develop tolerance and can achieve extraordinary levels, as high as 1.20% BAC (260 mM)(Brick and Erickson 2009). How Does Ethanol Cause DA Release? It has been suggested that alcohol activates VTA neurons directly to release DA into nAc. However, some but not all dialysis experiments show that alcohol application into the nAc alone locally increases DA overflow while application of alcohol into the VTA does not(Yim, Schallert et al. 1998; Ericson, Molander et al. 2003), suggesting that effects at the axons may be required. In contrast, studies of ethanol using cyclic voltammetry in the striatal slice (Budygin, Phillips et al. 2001)

demonstrated effects of ethanol on evoked DA release only at very high (100–200 mM) alcohol levels, which depressed release. Thus, the relevant brain sites in striatum are still unclear.

Ethanol might increase DA release by direct excitation of DA neurons. Some studies show an effect of alcohol to excite DA neurons in the VTA at concentrations of 20–320 mM(Brodie and Dunwiddie 1990; Okamoto, Harnett et al. 2006). This might involve an inhibition of potassium channels, including those that regulate after-hyperpolarizations and the rate of burst firing, as well as sustained K+ currents(Koyama, Brodie et al. 2007).

Alternatively, ethanol may act via disinhibition of DA neurons, most likely at GABA receptors. Ethanol effects on GABA are suspected to play a part in its effects, notably the motor-impairing and anxiolytic responses, in a manner related to the benzodiazepines and barbiturates. Recent evidence suggests a possible role for extrasynaptic GABAa receptors, as a population of extra-synaptic receptors containing a-4 subunits were found that provide a steady inhibition of thalamic neurons with sedative levels (50 mM) ethanol(Jia, Chandra et al. 2008). A recent paper showed that viral knockdown of the a-4 subunit in the nAc shell but not the core, decreased alcohol drinking and preference in the rat(Rewal, Jurd et al. 2009); perhaps the receptors in that area are more sensitive than those in the thalamus.

Several classes of striatal interneurons exert ‘‘veto’’ power on the ability of MSN neurons to fire(Tepper, Koos et al. 2004), and if either these or medium spiny neuron collaterals were inhibited, the net result of ethanol inhibition of GABAergic activity (or cholinergic) activity via GABAergic disinhibition could underlie a presynaptic component of enhanced DA release.

A similar pathway mediated by an ethanol-mediated GABA receptor disinhibition of DA neurons by ethanol could also occur in the ventral midbrain(Mereu and Gessa 1985), as found with opioids and sedatives. It should be noted that, in contrast, ethanol appears to enhance GABAb currents on midbrain DA neurons by activating GIRK currents(Federici, Nistico et al. 2009), which may reinstate some inhibition. Alternatively, opiate receptors on GABAergic VTA projection neurons have been implicated in the action of ethanol(Xiao and Ye 2008), perhaps via ethanol-mediated release of b-endorphin, which would similarly disinhibit DA neurons.

Other hypotheses have been offered for network effects via cannabinoids, serotonin, glycine, NMDA channels, nAChR, and NMDA receptors, but to date none have been shown to clearly cause enhanced firing of DA neurons at levels of 5– 20 mM ethanol, although the enhancement of burst firing seems to involve endocannabioids(Cheer, Wassum et al. 2007). In summary, while there is good evidence that levels of ethanol achieved during moderate drinking release DA, and while there are a multitude of potential targets, the means by

which ethanol releases DA during drinking at nominally rewarding levels remain unclear.

4. Experimental animal models of alcoholism

Researchers have known since 1940 that some rodents voluntarily consume alcohol in a laboratory setting. One can also assume that voluntary alcohol consumption by rodents and other mammals occurs in the wild, because some mammals, including rodents, occasionally consume large amounts of rotten fruits and exhibit abnormal behavioral patterns that may result from intoxication. Consequently, voluntary alcohol consumption, which is often observed in combination with palatable food or fluid intake, can be considered a part of the normal behavioral repertoire of rodents. These observations position rats and mice as ideal subjects for studying various aspects of human alcohol use, including alcohol reinforcement.

One commonly used approach to modeling human alcohol consumption in rodents are alcohol preference studies, in which the animals are given a choice between water and alcohol solutions and the investigators measure the amount consumed of each fluid. In comparison to other behavioral studies (e.g., anxiety tests), data on alcohol consumption levels obtained by such alcohol preference experiments show little variation, even when conducted in different laboratories(Crabbe, Wahlsten et al. 1999) and different settings. Moreover, because alcohol reinforcement is mediated by brain structures that have been strongly conserved during evolution (i.e., subcortical structures), rodent studies

have an enormous potential for further elucidating the neurobiological basis of alcohol consumption and alcohol reinforcement processes in humans.

This article presents several rodent models that have been used in recent years to study various aspects of alcohol addiction. The article first reviews traditional alcohol preference models and their limitations. It then describes newer models aimed at helping researchers investigate the rodent equivalent of complex human behaviors, such as craving, relapse, and loss of control over drinking. These models have been validated in pharmacological studies and have provided some insight into the neurochemical and cellular changes underlying addictive behaviors.

Alcohol Preference Models

As mentioned previously, researchers have conducted numerous alcohol preference studies in which the animals were offered a free choice between water and alcohol solutions of various concentrations. These studies found that when offered low alcohol concentrations (i.e., up to 6 percent weight/volume), which have a "sweet" taste, rats and mice generally drink more alcohol than water. At higher alcohol concentrations, however, at which the taste of the solution usually is aversive to rodents, large differences exist among individuals and among strains in alcohol preference. These observations suggest that animals primarily prefer alcohol because of such factors as taste, rather than

because of its stimulatory effect on the central nervous system. Only a few animals exhibit an alcohol preference that results from alcohol's pharmacological (e.g., reinforcing) effects.

The large variability in alcohol preference among individual animals and strains has allowed researchers to selectively breed rats for differential alcohol preference, generating pairs of animal strains that are characterized by particularly low or high alcohol consumption levels. The best studied pairs of lines were generated in Finland, the United States, and Sardinia. The Finnish model—called Alko Alcohol (AA) and Alko Non-alcohol (ANA) rats—comprises two strains of albino rats that based on their selection or rejection of a 10-percent alcohol solution and water, were selectively bred starting in 1963(Eriksson 1968). The alcohol-preferring (P) rats, originally bred in Indiana, voluntarily consume 5–8 grams of alcohol per kilogram of body weight per day (g/kg/day), attaining blood alcohol concentrations of 50–200 mg/100 mL, whereas the non-alcohol-preferring rats (NP) consume less than 0.5 g/kg/day alcohol(McBride and Li 1998). The Sardinian alcohol-preferring (sP) rats also have been selectively bred for high alcohol preference and consumption for more than 20 years (Colombo 1997). These models have been used as a tool for characterizing the behavioral, neurochemical, and molecular correlates of differential voluntary alcohol consumption and preference.

A major limitation of these models is that alcohol preference alone does not necessarily indicate addictive behavior but often reflects controlled alcohol consumption. For example, animals from an alcohol-preferring strain of inbred mice called C57BL/6 have a high alcohol preference but do not meet important criteria of addictive behavior, such as loss of control over drinking. Thus, the animals' alcohol intake decreases dramatically when they are offered diets augmented with sugar. Furthermore, the close correlation of food and alcohol consumption and the occurrence of alcohol consumption at normal times in the circadian cycle demonstrate that alcohol intake in C57BL/6 mice is controlled by normal behaviors(Dole, Ho et al. 1985). Consequently, the usefulness of alcohol-preferring inbred mouse strains as valid animal models of alcoholism is questionable.

Nevertheless, the aforementioned alcohol-preferring rat lines have allowed researchers to study numerous aspects of alcohol's effects and their role in alcohol use. For example, studies have demonstrated clearly that these animals maintain voluntary alcohol consumption even in the presence of other palatable solutions(Lankford, Roscoe et al. 1991). Moreover, the alcohol-preferring rats find alcohol reinforcing, because they will orally self-administer alcohol even if they have to perform a task to obtain the alcohol (i.e., under operant conditions). Finally, an elegant set of experiments has shown that P- rats consume alcohol for its reinforcing actions on the central nervous system. In

those studies, the animals self-administered small amounts of alcohol via a special infusion device directly into a brain region called the ventral tegmental area(Gatto, McBride et al. 1994; Rodd-Henricks, McKinzie et al. 2000). This brain site is critically involved in initiating the reinforcing effects of drugs of abuse .

The Reinstatement Model

The main criterion of alcohol dependence is loss of control over drinking. Compulsive, uncontrolled alcohol-seeking and alcohol-taking behavior can occur even after long periods of abstinence and is usually associated with craving and relapse. Accordingly, animal models that measure relapse behaviors may allow researchers to investigate aspects of human alcohol dependence that are not easily addressed by preference models. One approach for measuring craving and relapse behaviors in animals is the reinstatement model (Stewart and de Wit 1987).

In a typical reinstatement experiment a rat is initially trained to press a lever for receiving alcohol or another drug. After the rat has learned this specific task, the drug is withheld, even if the animal presses the lever. After a while the rat stops pressing the lever, indicating that the lever-pressing behaviour has become extinguished. Following extinction, investigators present various stimuli and assess whether these stimuli reinstate the drug-seeking behavior—that is, if they cause renewed lever responding even if the animal does not

receive the drug. At least three types of stimuli can reinstate responding: (1) injection of a small dose of the drug (i.e., drug priming), (2) stress, and (3) conditioned stimuli that were previously paired during the initial training session with the delivery of the drug.

Although reinstatement models of intravenous self-administration of psycho-stimulants and opioids have existed for many years, few attempts have been made to transfer this paradigm to the alcohol field. Chiamulera and colleagues (1995) reported the first alcohol reinstatement study in rats. In that study, rats were trained over several months to press a lever in order to receive alcohol. After stable lever pressing was obtained, the rats were tested in extinction, receiving water instead of alcohol following lever pressing. After 8 to 10 extinction sessions, administration of a small quantity of alcohol reinstated previously extinguished alcohol-seeking behavior. These results are consistent with the widely reported

description of the "first - drink" phenomenon—that is, ingestion of a small alcohol amount may induce a strong subjective state of craving and, subsequently, relapse to drug-taking behavior in abstinent alcoholics(Ludwig, Wikler et al. 1974). This priming effect can occur even after years of abstinence (Besancon 1993)

Recently, other research groups also have used the alcohol reinstatement paradigm. In those studies, stress caused by intermittent mild electric shocks

to the animals' feet (Le, Quan et al. 1998) as well as alcohol-associated olfactory cues (Katner, Magalong et al. 1999) could reinstate previously extinguished responding for alcohol. In conclusion, the characteristics of reinstatement of alcohol-seeking and -taking behavior are similar to those for other drugs of abuse. Furthermore, the reinstatement approach can be used to study the effects of putative anti- craving and anti-relapse medications.

Nevertheless, the usefulness of the reinstatement model in representing human alcohol dependence has two important limitations. First, researchers to date have not conclusively demonstrated that rats which go through a reinstatement procedure are truly alcohol dependent in the sense that they exhibit alcohol responding that is no longer controlled by normal behavioral mechanisms (i.e., is uncontrolled). Second, it appears that extinction of alcohol-seeking behavior usually plays only a minor role in alcoholic patients trying to achieve and maintain abstinence. With the exception of patients undergoing focused extinction therapy, alcoholics generally try to avoid exposure to external alcohol cues during abstinence. In most cases, alcoholics stay abstinent for a while but may experience craving and subsequent relapse if they are re-exposed to external cues (e.g., the sight of a bar or smell of alcohol), particularly if they are in a vulnerable internal state. Consequently, the animal reinstatement procedure may not accurately reflect the situation of abstinent alcoholics experiencing craving and relapse. This situation may be better mimicked by the

so-called alcohol deprivation effect (ADE), which is represented in an animal model in which long-term alcohol self-administration alternates with repeated alcohol deprivation phases.

Long-Term Alcohol Self-Administration With Repeated Alcohol Deprivation Phases: An Animal Model of Alcoholism

To model the compulsive, uncontrolled alcohol-seeking and alcohol-taking behavior characteristic of human alcoholics, Spanagel and Hölter (1999) developed a long-term model of alcohol self-administration with repeated alcohol deprivation phases. In this model, male Wistar rats have free access to food, water, and three alcohol solutions of 5, 10, and 20 percent (volume/volume) in their cage. After two months of continuous alcohol access, the rats are deprived of alcohol for several days before again being offered all alcohol solutions. This procedure is repeated monthly for the following year. The renewed availability of the alcohol solutions following a deprivation phase leads to a pronounced but temporary rise in alcohol intake and preference, the ADE. This pattern of relapse-like drinking is observed across several species, including rats, mice, monkeys and human social drinkers (Burish, Maisto et al. 1981).

In addition to the ADE, alcohol consumption behavior after long-term consumption followed by deprivation also is characterized by changes in the animals' alcohol intake patterns. Thus, the animals not only consume more

alcohol but also consume large amounts of highly concentrated alcohol solutions at inappropriate times during their daily cycle (e.g., during the light phase when the animals are normally inactive and drinking activity is low).

Interestingly, the ADE in chronically drinking rats can persist over long abstinence periods (i.e., several months), demonstrating that a specific memory for the drug exists. This persistence is similar to the behavior of human alcoholics, who can easily relapse even after years of abstinence.

An ADE can also be observed under operant conditions—that is, if the animals have to perform a task to receive the alcohol. In these experiments, the animals' alcohol intake and preference increase significantly following an alcohol deprivation phase of 2 weeks (Holter, Landgraf et al. 1997), implying that a strong motivation exists for the drug. This strong motivation to drink a highly concentrated alcohol solution following deprivation is further demonstrated by the introduction of various progressive-ratio tasks, in which the animals have to work more and more (e.g., press a lever more often) in order to receive a rein-forcer (e.g., alcohol). In such studies the maximum number of consecutive lever responses the animals will per- form in order to receive one alcohol dose (i.e., the breaking point) is significantly higher following deprivation compared with baseline responding(Spanagel and Holter 2000). These findings suggest that at least in chronically drinking rats, the ADE represents a situation of increased

motivation to work for alcohol, which is compatible with the operational definition of craving(Markou, Weiss et al. 1993).

Such increased motivation to work for alcohol, however, is not the primary criterion for defining addiction in animals—the loss of control over drinking also must be demonstrated. In an attempt to assess uncontrolled drinking behavior, researchers have sought to influence the ADE by either adulterating the taste of the alcohol solution with quinine or by offering a highly palatable sugar solution instead of water. In the first experiment, the investigators added quinine hydrochloride to the alcohol solution, but not to the water(Spanagel, Holter et al. 1996). Quinine is a very bitter tasting substance that usually produces a strong taste aversion in rats. Despite the aversive taste, however, the long-term alcohol-drinking rats consumed large amounts of the quinine-containing alcohol solution following a deprivation phase. In fact, alcohol intake and preference, as well as the time course of the ADE in the quinine-exposed animals, were similar to those of control animals that had the same experimental history and which received unadulterated alcohol. It is important to note, however, that increasing quinine concentrations did affect the expression of the ADE. Thus, when the alcohol was adulterated with high quinine concentrations, alcohol consumption and preference after deprivation dropped even below baseline drinking and preference. These results show that in long-term alcohol-drinking rats, alcohol intake following a deprivation phase

is relatively resistant to modification by taste adulteration; in other words, drinking behavior to a certain point becomes inflexible and uncontrolled.

These conclusions were further sup-ported by an experiment during which rats had a free choice between a sugar solution and alcohol after a period of alcohol deprivation (Spanagel and Weiss 1999). In general, rats have a high preference for the sugar solution over alcohol. Nevertheless, in this study, chronically drinking rats still consumed more alcohol following deprivation than before the deprivation period, indicating that the ADE was still present despite the availability of the sugar solution.

Thus, the two studies demonstrated that alcohol intake during the ADE remained unchanged after presentation of either an adulterated alcohol solution or a highly palatable sugar solution. These findings suggest that alcohol consumption in animals serves not only nutritional purposes but also is at least partly motivated by alcohol's pharmacological effects. In other words, alcohol consumption during the ADE seems to involve compulsive, uncontrolled drug-seeking and drug-taking behavior and can clearly be dissociated from normal eating and drinking behaviors.

This conclusion is further supported by pronounced changes in the diurnal rhythm of drinking activity following alcohol deprivation in chronically drinking rats. For these experiments, the animals were tested in a fully automated electronic drinkometer device(Holter, Engelmann et al. 1998) that

allows researchers to monitor drinking patterns constantly on a computer. In the experiment, age matched control animals exhibited normal drinking activity—that is, high drinking activity during the active night phase and low, and for some hours no, drinking activity during the inactive light phase. In contrast, the pattern of drinking activity changed in the chronically drinking rats during the ADE.

In particular, most of the animals still showed high drinking activity during the inactive phase, and some animals even showed no differences in drinking activity during the dark and light phases of the daily cycle. Such a level of drinking activity is far beyond normal con trolled behavior seen in the appropriate control animals.

In summary, the results of the alcohol deprivation studies indicate a non-nutritional component of alcohol consumption and pharmacologically motivated drinking behavior in long-term alcohol self-administering rats. Moreover, because the animals' drinking behavior was difficult to modify, alcohol drinking during the ADE appears to represent compulsive, uncontrolled drug-seeking and drug-taking behavior. Additional studies demonstrated that chronically drinking rats that underwent repeated alcohol deprivation phases exhibited tolerance, physical and psychological signs of withdrawal, and stress-induced drinking (Holter, Engelmann et al. 1998) (Spanagel and Holter 2000). In particular, the animals showed augmented anxiety when experiencing

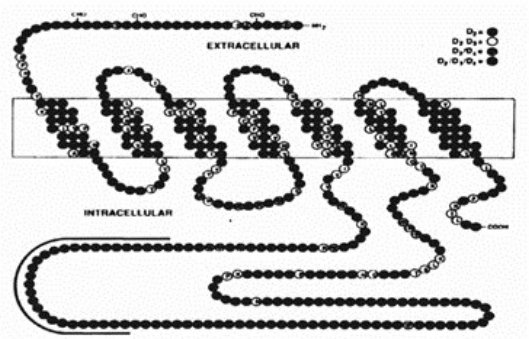
alcohol deprivation, similar to the anxiety attacks observed in human alcoholics undergoing withdrawal. This experience of anxiety after alcohol deprivation also might contribute to the relapse-like drinking behavior observed in the rats. Taken together these observations reflect some of the diagnostic criteria for alcoholism listed in the fourth edition of the *Diagnostic and Statistical Manual of Mental Disorders* (DSM–IV) of the American Psychiatric Association (1994). Thus, the alcohol deprivation model can serve as an animal model of alcoholism that covers most of the DSM–IV criteria. Any valid animal model of alcoholism, however, should have predictive value for the human situation. Therefore, anti-craving drugs that can effectively prevent relapse in human alcoholics also should be effective in the animal model.

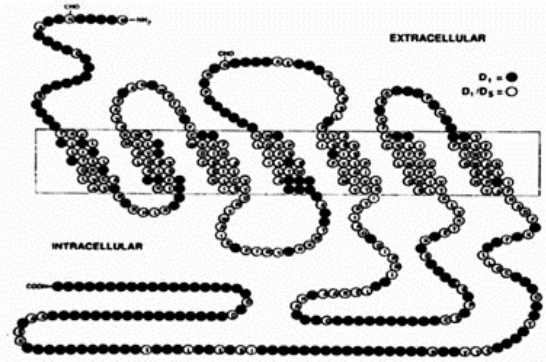
5. Dopamine D3 receptor

The D3 receptor belongs to the family of G protein-coupled receptors, whose topography is characterized by the occurrence of seven transmembrane domains. Its primary sequence is close to that of the D2 receptor, and to a lesser extent, to the D4 receptor. Visualization of D3 receptor mRNA in rat brain by in situ hybridization indicated a predominant expression of the message in the ventral striatum and other limbic areas. In substantia nigra and ventral tegmental area, lesion studies showed that both D2 and D3 receptors are expressed by dopaminergic neurons and constitute autoreceptors. This lesion, downregulates postsynaptic D3 receptor in nucleus accumbens (Levesque, Martres et al. 1995), by deprivation of brain-derived neurotrophic factor (BDNF), an anterograde factor of dopamine neurons. The lesion also induced decrease in areas of dopamine cell bodies could reflect a similar processes occurring in non dopaminergic neurons. Dopamine release (Tang, Todd et al. 1994) and synthesis (O'Hara, Uhland-Smith et al. 1996) are inhibited by stimulation of the D3 receptor expressed in a transfected mesencephalic cell line and various agonists, with limited preference for the D3 receptor (Sautel, Griffon et al. 1995), inhibit dopamine release, synthesis and neuron electrical activity, giving support to the existence of D3 autoreceptors. Direct confirmation of its autoreceptor distribution has been a demonstrated by

immunocytochemical methods showing that D3R is expressed in all dopaminergic neurons (Diaz, Pilon et al. 2000). However, the selectively of agonists towards the D3 receptor in vivo has been strongly questioned, because they elicit similar inhibition of dopamine neuron activities in wild-type and D3 receptor-deficient mice (Koeltzow, Xu et al. 1998). In addiction, dopamine autoreceptor functions are suppressed in D2-receptor deficient mice (Mercuri, Saiardi et al. 1997; L'Hirondel, Cheramy et al. 1998). Nevertheless, dopamine extracellular levels in the nucleus accumbens (Koeltzow, Xu et al. 1998) and striatum (Joseph, Wang et al. 2002) are twice as high in D3 receptor-deficient as in wild-type mice, suggesting a control of dopamine neurons activity by the D3 receptor. A possible explanation for the increased dopaminergic tone in D3R-deficient mice might an increase in DA synthesis, release and /or decrease in DA uptake. The hypothesis of a control exerted by D3 autoreceptors is also supported by the observations that D3 receptor-deficient mice display signs reminiscent of hyperdopaminergia, presumably resulting from the lack of autoreceptors controlling DA neuron activity. These alterations may represent adaptative changes to increased DA tone (Le Foll, Diaz et al. 2005). Finally, D3 autoreceptors may mediate yet unrecognized control by DA of other activities of DA neurons, such as synthesis or release of neuropeptides co-expressed with DA in these neurons (neurotensin, cholecystokinin or neurotrophins. These convergent results suggested that D3 deficient mice seem to be more responsive

in several physiological situations compared to their W. T. littermates (Le Foll, Diaz et al. 2005).





Dopamine receptors structure

6. Design of the present research

Based on the reviewed data of the literature, the aim of the present thesis was to asses the role of DA receptors, in particular D_3 receptors in ethanol addiction. The following aspects were investigated:

1. To determine the basal behavior of $D_3^{-/-}$ and their WT littermates in an experimental model of ethanol intake (Two Bottle Free Choice Paradigm)
2. To determinate the effect of different D3 selective antagonists in $D_3^{-/-}$ and WT mice in the long-term ethanol voluntary intake paradigm
3. To investigate the effect of buspirone in $D_3^{-/-}$ and WT mice exposed to the long-term ethanol voluntary intake paradigm.

Chapter I

Dopamine D_3 receptor mediates alcohol intake

Gian Marco Leggio[1*], Giovanni Camillieri[1*], Alessandro Castorina[2], Chiara Bianca Maria Platania[1], Giuseppina Marrazzo[1], Velia D'Agata[2], Filippo Drago[1], Claudio Bucolo[1], Salvatore Salomone[1]

[1]Department of Clinical and Molecular Biomedicine, Section of Pharmacology and Biochemistry, [2] Department of Bio-Medical Sciences; Catania University, Catania, Italy

*Equally contributing authors

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Abstract

Mesolimbic dopamine controls drug and alcohol seeking behavior. Stimulation of dopamine D_3 autoreceptor reduces extracellular levels of dopamine. We tested the hypothesis that dopamine D_3 receptor (D_3R) gene deletion or its pharmacological blockade counteracts alcohol preference and intake in a long-term voluntary ethanol intake paradigm. Mice $D_3R^{-/-}$ and their wild type (WT) littermates, treated or not with the D_3R antagonists U99194A and SB277011A, were tested. The selectivity of the D_3R antagonists was further assessed by molecular modeling. Activation of dopamine (DA) transmission and D_3R expression was assessed at the end of the experiment. After 8 days, daily ethanol intake was negligible in $D_3R^{-/-}$ and robust in WT; this behavior was stably maintained for 44 days. Treatment with D_3R antagonists counteracted ethanol intake in WT and was associated to increased DA transmission (assessed as phosphorylation of DARPP-32 and GSK3β) in striatum and prefrontal cortex. Forced ethanol intake increased the expression of RACK1 and BDNF in both WT and $D_3R^{-/-}$; in WT there was also a robust overexpression of D_3R. Thus, increased expression of D_3R associated with activation of RACK1/BDNF seems to operate as a reinforcing mechanism in voluntary ethanol intake. Taking into account that ethanol intake increases mesolimbic DA, low levels of extracellular DA resulting from D_3R overexpression would facilitate ethanol intake, and high levels of extracellular DA, from either gene deletion of D_3R blockade, would inhibit ethanol intake. Thus, modulation of DA mesolimbic pathway by selective targeting of D_3 receptor might provide a basis for novel weaning treatments in alcoholism.

Introduction

Alcohol is the most widely used addictive drug. Alcohol consumption in the population is underestimated, and a large portion of alcohol-related disability is due to alcohol addiction. The reinforcing properties of ethanol are mediated by the mesolimbic dopamine (DA) pathway[1]; both oral self-administration[2] and systemic administration of ethanol increase the firing rate of mesolimbic dopaminergic neurons[3, 4] and stimulate extracellular DA release in the striatum and in the nucleus accumbens[5, 6]. Moreover, reduced extracellular levels of DA have been observed in ethanol preferring rats and mice, suggesting that alterations in the mesolimbic DA pathway is an important factor predisposing to ethanol preference and intake[7, 8].

DA exerts its action through five receptor subtypes (D_{1-5}); the D_3 receptor (D_3R) subtype plays an important role in the modulation of mesolimbic DA pathway and in the control of drug-seeking behavior[9, 10]. D_3R is located both at pre- and post-synaptic level, primarily in the nucleus accumbens and granulate cells of the island of Calleja[11-14]; in these structures, stimulation of presynaptic D_3R inhibits DA synthesis and release[15]. A number of different experimental paradigms have explored the involvement of D_3R in the mechanism of ethanol addiction[16-19]. However, few studies have focused on voluntary ethanol intake[16, 20], and only two[21, 22] on voluntary ethanol intake in D_3R null mice ($D_3R^{-/-}$). Pharmacological studies generally report that the D_3R blockade decreases

ethanol consumption[16, 20]; in contrast, genetic studies did not find a change in ethanol intake following D_3R gene deletion[21]. These controversial results might be, at least in part, accounted for by species differences (rat *vs.* mouse), and by the short duration of the experimental paradigm, limited to few days, a time not sufficient to establishing an ethanol preferring behavior.

The present study was therefore, designed to test the hypothesis that D_3R gene deletion or D_3R pharmacological blockade counteracts the alcohol preference and intake in mice tested in a long-term ethanol-drinking paradigm. Mice $D_3R^{-/-}$ and their wild type (WT) littermates, treated or not with D_3R antagonists were tested for 44 days in a voluntary ethanol intake paradigm[21, 23]. Activation of DA transmission and D_3R expression was assessed at the end of the experiment.

Materials and Methods

Animals

Mice D_3R null ($D_3R^{-/-}$) and WT littermates (males, 8–12 weeks old) were individually housed, with free access to chow and water (except in the ethanol drinking procedure), in an air-conditioned room, with a 12-h light–dark cycle. Mice $D_3R^{-/-}$ were 5th–8th generation of congenic C57BL/6J mice, generated by a backcrossing strategy[24]. All experiments were carried out according to the Directive 2010/63/EU and to the Institutional Animal Care and Use Committee of the Catania University.

Alcohol-drinking procedures

Mice $D_3R^{-/-}$ (n=30) and WT (n=30) received 24 h free access to tap water and 10% ethanol solution (v/v), contained in 100 ml graduated tubes with stainless steel drinking spouts; the position of tubes was interchanged (left/right) every 24 h, to prevent the acquisition of position. Ethanol and water intake was measured as daily consumption in grams. The experiment lasted 59 days. For the first 15 days (habituation period) animals received 24 h free access to two tubes containing only tap water (time 0 in Figure 1a). After the habituation period (from 15 to 59 day) 10% ethanol solution was available in one of the bottles. In the forced alcohol-drinking procedure, $D_3R^{-/-}$ (N = 12) and WT (N =

18) received for the first 15 days (habituation period) tap water only (time 0), followed (from 15 to 59 day) by 10% ethanol only.

Drugs and treatments

Alcohol drinking solution (10% ethanol v/v) was prepared from 99 % ethanol (Sigma, St Louis, MO) and tap water. The D_3R antagonists U99194A maleate and SB277011A hydrochloride, both from Sigma, were dissolved in saline. Drugs solutions were freshly prepared and 10 mg/kg doses were intraperitoneally (i.p.) injected (in a volume of 10 ml/kg). Control animals received saline (vehicle) injections.

After 30 days of voluntary alcohol drinking procedure, both $D_3R^{-/-}$ mice and WT showed stable ethanol and water intake; at this time, they were randomly allocated to the 6 experimental groups (n=10 per group): WT/vehicle, WT/U99194A, WT/SB277011A, $D_3R^{-/-}$/vehicle, $D_3R^{-/-}$/U99194A, $D_3R^{-/-}$ /SB277011A. Animals were i.p. injected once a day, for 14 consecutive days, with a 23-gauge stainless steel needle of 31 mm length.

Analysis of mRNA expression by real-time quantitative RT-PCR

Total RNA was isolated by TRIzol (Invitrogen, Carlsbad, CA). Single-stranded cDNA was synthesized with SuperScript III (Invitrogen), by priming with oligo-$(dT)_{20}$. Aliquots of cDNA were amplified in parallel reactions with

external standards at known amounts, using specific primer pairs for D_3R, RACK1, BDNF and S18 ribosomal RNA (reference gene). Each PCR reaction (20 µl final volume) contained 0.5 µM primers, 1.6 mM Mg^{2+}, and 1× Light Cycler-Fast Start DNA Master SYBR Green I (Roche Diagnostics, Indianapolis, IN). Amplifications were carried out in a Light Cycler 1.5 instrument (Roche Diagnostics). Quantification was obtained by the ΔCt comparative method[25].

Western Blot Analysis

Protein extracts from prefrontal cortex, striatum and cerebellum were run in SDS-PAGE, blotted and probed for non-phosphorylated and phosphorylated forms of DARPP-32 and GSK-3β, with primary antibodies (Cell Signalling Technology, Beverly, MA), diluted at 1:1,000, and secondary antibody (goat anti-rabbit IRDye; Li-Cor Biosciences, Lincoln, NE). Blots were scanned with an Odyssey Infrared Imaging System (Li-Cor Biosciences). And analyzed with ImageJ software (NIH, Bethesda, MD; http://rsb.info.nih.gov/ij/index.html).

Molecular modeling

Molecular docking studies were carried out to model the interactions of SB277011A and U99194A with D_3R and D_2R. Structures of ligands were obtained by the ProDrg web server (http://davapc1.bioch.dundee.ac.uk/prodrg/),

as .mol2 files[26]. When necessary, Open Babel was used to convert file formats. Protonation state of ligands was assigned at pH=7.4. Structure models of human D_3R and $D_{2L}R$, optimized by explicit molecular dynamics simulation in water-lipid environment, were obtained as previously described[27]; human receptors were used instead of the murine ones because their crystallographic and/or theoretical structures are available; furthermore, their protein sequences are conserved between the two species, particularly in the transmembrane domain and in the binding pocket. For more details see Supplementary information.

Statistical analysis

Data were analyzed using one- or two-way analysis of variance (ANOVA). The post hoc Newman-Keuls test was used for multiple comparisons, taking P value less than 0.05 as significant.

Results

Dopamine $D_3R^{-/-}$ mice exhibit lower voluntary ethanol intake as compared to control

As shown in Figure 1a, WT preferred the ethanol-containing solution since the beginning. In contrast, during the first 8 days of observation, $D_3R^{-/-}$ mice did not show any preference for either ethanol or water (Figure 1a). During the entire period of observation (44 days) WT mice maintained their preferential intake of ethanol ($p<0.01$), whereas $D_3R^{-/-}$ mice developed and maintained a preference for water ($p<0.01$). Furthermore, there was no difference between WT and $D_3R^{-/-}$ in terms of total amount of fluid intake (ethanol + water) (Figure 1a).

In silico analysis of U99194A and SB277011A interaction with D_2R and D_3R shows their high selectivity for the D_3R subtype.

To examine pharmacological antagonism at D_3R, we docked U99194A and SB277011A into models of D_3R and $D_{2L}R$ receptors[27]. As shown in Table 1, consensus-scoring of poses confirmed the higher affinity of docked compounds for the D_3R in comparison to the $D_{2L}R$ subtype. The binding energy of complexes was strictly related to ligand-protein interactions, each ligand bound in a different manner to D_3R and $D_{2L}R$ receptors (Figure S3, Supplementary information) and different hydrogen bonds (H-bonds) were formed (Table S1,

Supplementary information). Ligands docked into D_3R formed less polar contacts than ligands docked into $D_{2L}R$. Moreover, contacts of SB277011A with hydrophobic residues were more numerous into D_3R receptor than into $D_{2L}R$ binding pocket; in contrast, U99194A interacted with the same number of hydrophobic residues, though these residues were different in the two receptors.

Blockade of D_3R counteracts voluntary ethanol intake

After 30 days of stable ethanol/water intake, mice received daily i.p. injections of either vehicle or D_3R antagonists (U99194A or SB277011A). As shown in Figure 1b, treatment of WT with each D_3R antagonist significantly decreased voluntary ethanol intake as compared to the vehicle control group ($p<0.01$, for both U99194A and SB277011A). Treatment of $D_3R^{-/-}$ with U99194A and SB277011A did not change ethanol intake. Neither in WT nor in $D_3R^{-/-}$ total fluid intake was affected by treatment with D_3R antagonists (Figure 1b).

Dopamine D_3R is up-regulated in striatum and prefrontal cortex (PFC) of WT mice following chronic ethanol intake

Figure 2 (a, b) shows the D_3R gene expression profile in striatum and PFC of control WT (not exposed to ethanol) or WT that had free access to water and ethanol, treated with vehicle or the D_3R antagonist SB277011A. Chronic ethanol intake increased D_3R mRNA expression in both striatum ($P<0.05$) and

PFC ($P<0.01$). Treatment with SB277011A induced a further increase of D_3R mRNA expression ($P<0.05$, $P<0.01$).

Expression of BDNF and RACK1 was increased following chronic ethanol intake in both WT and D3$^{-/-}$ mice

BDNF induces D_3 receptor expression in nucleus accumbens, both during development and in adulthood[28]. RACK1, a mediator of chromatin remodeling, regulates in an exon-specific manner the expression of BDNF gene[29] and the RACK1/BDNF pathway is activated upon exposure to ethanol[30]. We therefore assessed BDNF and RACK1 mRNA expression in striatum and PFC of WT and $D_3R^{-/-}$ that had free access to either water only or to both water and ethanol. As shown in Figure 2, long-term free access to ethanol increased BDNF (panels c and d) and RACK1 mRNA (panels g and h) in striatum and PFC of WT mice ($p<0.05$, $p<0,01$; RACK1 increase in PFC did not reach statistical significance). The ethanol-dependent increase of BDNF and RACK1 expression was unaffected by chronic treatment with SB277011A. Mice $D_3R^{-/-}$ showed an increased basal expression of BDNF in the PFC as compared with their WT littermates ($p<0.05$, Figure 2f), that was not affected by long-term free access to ethanol or by the treatment with SB277011A. Surprisingly, long-term free access to ethanol increased BDNF and RACK1 expression also in the striatum of $D_3R^{-/-}$ mice ($p<0.01$, Figure 2, panels e and i), while no change in RACK1

expression was found in the PFC of the of $D_3R^{-/-}$ (Figure 2j).

Forced chronic ethanol intake results in increased BDNF and RACK1 expression both in WT and $D_3R^{-/-}$ mice

Long-term free access to ethanol appeared to be associated with BDNF/RACK1 overexpression, but the interpretation of these data was made difficult by the different ethanol intake in the two genetic groups, being very high in WT and very low in $D_3R^{-/-}$. Furthermore, the finding that free access to ethanol was associated with similar BDNF/RACK1 overexpression in striatum, regardless of genotype, suggested that this effect might be very sensitive to low levels of ethanol, while on the other hand, BDNF/RACK1 activation in PFC seemed less affected by ethanol exposure. To address these issues, some WT and $D_3R^{-/-}$ mice were subjected to forced ethanol intake, i.e. they had access to ethanol 10% solution only. As shown in Figure 3, forced ethanol intake induced a significant overexpression of BDNF and RACK1 mRNAs in striatum and PFC of both WT and $D_3R^{-/-}$ mice ($p<0.05$, $p<0.01$). Of note, BDNF and RACK1 mRNA levels induced in $D_3R^{-/-}$ mice were even higher than those induced in WT; in these latter, treatment with SB277011A induced a further increase in BDNF and RACK1 ($p<0.05$ vs. ethanol alone), up to the level of ethanol-treated $D_3R^{-/-}$ mice.

Dopamine receptor signaling is enhanced in PFC and striatum of $D_3R^{-/-}$ mice and of SB277011A-treated WT mice

Activation of D_1 receptor results in activation of adenylyl cyclase/cAMP/protein kinase A (PKA) signaling; a major substrate for PKA in the striatum is DA and cAMP-regulated phosphoprotein (DARPP-32). D_2-like receptors regulate the activity of the protein kinases Akt and GSK3β; stimulation of either D_2 or D_3 receptors results in phosphorylation of Akt and GSK3β[31]. In order to assess activation of dopaminergic transmission in different CNS areas, we determined, by immunoblot, the abundance of phosphorylated DARPP-32 (Thr 34) and of phosphorylated GSK3β (Ser 9). As shown in Figure 4, both phosphoDARPP-32 and posphoGSK3β were more abundant in PFC and striatum of $D_3R^{-/-}$ than in WT mice. Treatment of WT mice with SB277011A induced phosphorylation of DARPP-32 and GSK3β in both brain areas, up to the level of $D_3R^{-/-}$ mice. In contrast, in cerebellum there was no difference in the level phosphoDARPP-32 and posphoGSK3β between WT e $D_3R^{-/-}$, nor it was influenced by SB277011A-treatment in WT.

Discussion

This study was intended to elucidate the role of D_3R in voluntary ethanol intake. Importantly, these data have been obtained in a chronic model (44 days), whereas most published data have been generated from short term (< 7 days) models. The results show that either D_3R gene deletion or D_3R pharmacological blockade counteracted chronic alcohol preference and intake in mice. Analysis of DARPP-32 and GSK3β phosphorylation indicated that both D_3R gene deletion and D_3R pharmacological blockade were associated with enhanced DA transmission in PFC and striatum. Furthermore, chronic ethanol intake induced D_3R receptor expression associated with activation of RACK1/BDNF, which might represent the basis for a reinforcing mechanism in voluntary ethanol intake.

An important interpretative issue is the genetic background where the D_3R null mutation was placed. Specific behavioral phenotypes are differently expressed in different strains of mice[32]. The $D_3R^{-/-}$ mice we used are on the C57BL/6J background[24], a strain where ethanol preference and sensitivity is well documented[33]. Interestingly, ethanol-preferring C57BL/6J mice show reduced DA content and turnover in the terminals of mesolimbic and mesostriatal DA neurons compared with ethanol-avoiding DBA/2J and BALBc mice[8]; furthermore, increase in extracellular DA levels decreases ethanol preference

and consumption in C57BL/6J mice[8], suggesting that a reduction of limbic DA increases ethanol preference and intake. On the other hand, $D_3R^{-/-}$ mice have extracellular DA levels twice as high as their WT littermates[34, 35]; this enhanced DA tone and the resulting adaptations may reflect the removal of the inhibitory role for D_3R in the control of basal extracellular DA levels[36], giving support to an autoreceptor role for D_3R. The increased DA activity in $D_3R^{-/-}$ mice is consistent with their phenotype, including higher basal levels of grooming behavior, hyper-locomotion and reactivity to drug-paired environmental cues[24, 36, 37].

Here we found that $D_3R^{-/-}$ mice chronically (44 days) exposed to the voluntary ethanol intake paradigm, drank very low quantities of ethanol in comparison with their WT littermates (Figure 1). This observation is apparently in contrast with the only two previous studies testing $D_3R^{-/-}$ mice in the ethanol voluntary intake paradigm[21, 22]; both studies, in fact, reported no differences between $D_3R^{-/-}$ and C57BL/6J control mice. It is likely that the different conclusion between these studies and our data is due to the different period of observation. Their observation was, in fact, limited to 8 days[21, 22] and when looking at the same time frame (Figure 1a), we too did not observe significant differences between $D_3R^{-/-}$ and WT littermates, mainly because $D_3R^{-/-}$ mice did not yet show a preference for either ethanol or water. However, after 8 days, $D_3R^{-/-}$ mice started to show a marked preference for water, such that the difference with WT

became obvious; this behavior was stably maintained for the following 5-6 weeks of observation.

To obtain pharmacological evidence for a functional role of D_3R in the control of voluntary ethanol intake we tested two D_3R antagonists, U99194A and SB277011A (Figure 1) at doses reported to target D_3R[38, 39]. Before administering these drugs, we performed a molecular modeling study to gain information on the interaction of U99194A and SB277011A with D_3R. *In silico* analysis showed that the two D_3R antagonists were i) highly selective for the D_3R subtype and ii) displayed a distinct interaction (different binding energy, different interaction patterns) with D_3R, consistent with their distinct chemical structure. Both U99194A and SB277011A induced a significant decrease of voluntary ethanol intake in WT but not in $D_3R^{-/-}$ (Figure 1b). This pharmacological evidence reinforces the view that activation of D_3R is required for voluntary ethanol intake and is consistent with rat data, showing that D_3R antagonism reduces relapse-like drinking and cue-induced ethanol-seeking behavior[20]. Furthermore, it opens perspectives for a novel, D_3R–based, drug treatment for alcoholism.

Enhanced D_3R expression in striatum following long-term alcohol consumption has been previously reported in both mice and rats[20, 40]. Our data confirm that chronic voluntary ethanol intake up-regulated D_3R mRNA expression in both striatum and PFC of WT mice (Figure 2). Interestingly, D_3R expression is

increased by exposure to other addictive drugs, such as nicotine and cocaine, in caudate-putamen[41] and in nucleus accumbens[42, 43] of rats, as well as in human cocaine overdose victims[44]. Expression of D_3R therefore appears as a potential basis for a reinforcing mechanism in reward-related behavior associated with voluntary intake of addictive drugs and ethanol. A number of studies have linked D_3R expression in accumbens to BDNF derived from cortical sources[28, 43]; furthermore, ethanol exposure increases both BDNF and D_3R within the dorsal striatum itself[30, 40]. The scaffolding protein RACK1 is a key regulator of BDNF expression; RACK1 translocates to the nucleus after exposure of neurons to ethanol and increases expression of BDNF[30]. Our current hypothesis is therefore that activation of RACK1/BDNF by ethanol may induce expression of D_3R, which in turn maintains ethanol consumption. This hypothesis is at odds with Jeanblanc[40] who proposed that RACK1/BDNF-induced expression of D_3R may, instead, attenuate ethanol consumption, based on the observation that systemic administration of RACK1, expressed as a Tat-fusion protein (Tat-RACK1), reduces ethanol consumption. Their study, however, cannot account for the following facts: i) ethanol intake is negligible in $D_3R^{-/-}$ and robust in WT; ii) increase in RACK1/BDNF/D_3R is maintained during chronic (44 days) ethanol intake in WT; iii) forced ethanol intake increases RACK1/BDNF even in $D_3R^{-/-}$, where this effect is not accompanied by D_3R overepression, D_3R gene being inactivated. If RACK1/BDNF/D_3R overexpression attenuated ethanol

consumption, as claimed by Jeanblanc[40], this would have induced a progressive decline of ethanol preference in WT, which was not observed in our setting. The different effect reported following Tat-RACK1-treatment might be at least in part related to the fact that ethanol intake was measured acutely, after overnight treatment[40], whereas we were using a chronic model.

Here we show that chronic voluntary ethanol intake increased D_3R expression in striatum and PFC concomitant with increased expression of BDNF (Figure 2). It is noteworthy that, in basal condition, $D_3R^{-/-}$ mice exhibited higher BDNF than WT in PFC, consistent with a tendency reported in a recent study[45]. When subjected to forced ethanol intake, $D_3R^{-/-}$ mice showed a robust increase in BDNF expression in PFC. Therefore, chronic ethanol intake increases BDNF independently of D_3R receptor stimulation. The finding that chronic ethanol intake increased RACK1 in striatum and PFC of both WT and $D_3R^{-/-}$ (Figure 3) provides additional evidence for the role of RACK1/BDNF/D_3R pathway in alcohol addiction; chronic ethanol intake stimulates RACK1/BDNF pathway leading to D_3R overexpression and addictive behavior in WT, but not in $D_3R^{-/-}$, because this latter lacks D_3R .

The effect of the D_3R antagonist SB277011A deserves a specific discussion. We observed that 14-day treatment with SB277011A induced a robust overexpression of D_3R in both striatum and PFC (Figure 2). This was not surprising, since chronic treatment with an antagonist generally induces the up

regulation of the targeted receptor, a phenomenon well known for DA receptors[46]. However, an additional interesting observation in our study is that the effect of SB277011A on D_3R expression was, again, associated with the activation of RACK1/BDNF, as seen in the forced ethanol paradigm (Figure 3). Therefore, RACK1/BDNF pathway seems to be a key regulator of D_3R dynamical expression, not only related to reward mechanisms and addiction, but also in other conditions, such as exposure to specific ligands.

As $D_3R^{-/-}$ has been shown to exhibit extracellular DA levels substantially higher than WT, as assessed by microdialysis[35], a phenomenon related to the lack of autoreceptor function[34], we hypothesized that ethanol intake effectively stimulates DA release and transmission in WT, but not in $D_3R^{-/-}$, presumably because this latter already displays high extracellular DA levels. To test the hypothesis that treatment with D_3R antagonists mimicked the high DA phenotype documented in $D_3R^{-/-}$[35] we assessed phosphorylation of DARPP32 (Thr 34), that is increased by different addictive drugs, including ethanol[47, 48], and of GSK3β (Ser 9), that is linked to D_2-like receptors signaling cascade[49, 50], particularly under hyper-DAergic conditions[50]. Treatment with SB277011A increased phosphorylation of DARPP32 and of GSK3β to a level similar to that of $D_3R^{-/-}$. Thus, chronic blockade of the D_3R or its genetic deletion increased DA transmission in both striatum and PFC, consistent with increased extracellular DA[34, 35].

In conclusion, chronic ethanol intake stimulates RACK1/BDNF pathway, leading to D_3R overexpression and subsequent low levels of extracellular DA. This low DA may be responsible for further ethanol intake. On the contrary, high levels of extracellular DA, from either gene deletion of D_3R blockade, would inhibit ethanol intake. Thus, pharmacological antagonism selectively targeting D_3R might provide a basis for novel weaning treatments in alcoholism.

Conflict of interest

None of the authors have completing financial interests in relation to the work described.

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References

1. Samson HH, Tolliver GA, Haraguchi M, Hodge CW. Alcohol self-administration: role of mesolimbic dopamine. *Ann N Y Acad Sci* 1992; **654:** 242-253.

2. Weiss F, Hurd YL, Ungerstedt U, Markou A, Plotsky PM, Koob GF. Neurochemical correlates of cocaine and ethanol self-administration. *Ann N Y Acad Sci* 1992; **654:** 220-241.

3. Mereu G, Fadda F, Gessa GL. Ethanol stimulates the firing rate of nigral dopaminergic neurons in unanesthetized rats. *Brain Res* 1984; **292**(1)**:** 63-69.

4. Gessa GL, Muntoni F, Collu M, Vargiu L, Mereu G. Low doses of ethanol activate dopaminergic neurons in the ventral tegmental area. *Brain Res* 1985; **348**(1)**:** 201-203.

5. Imperato A, Di Chiara G. Preferential stimulation of dopamine release in the nucleus accumbens of freely moving rats by ethanol. *J Pharmacol Exp Ther* 1986; **239**(1)**:** 219-228.

6. Yoshimoto K, McBride WJ, Lumeng L, Li TK. Alcohol stimulates the release of dopamine and serotonin in the nucleus accumbens. *Alcohol* 1992; **9**(1)**:** 17-22.

7. Murphy JM, McBride WJ, Lumeng L, Li TK. Contents of monoamines in forebrain regions of alcohol-preferring (P) and -nonpreferring (NP) lines of rats. *Pharmacol Biochem Behav* 1987; **26**(2)**:** 389-392.

8. George SR, Fan T, Ng GY, Jung SY, O'Dowd BF, Naranjo CA. Low endogenous dopamine function in brain predisposes to high alcohol preference and consumption: reversal by increasing synaptic dopamine. *J Pharmacol Exp Ther* 1995; **273**(1)**:** 373-379.

9. Heidbreder CA, Gardner EL, Xi ZX, Thanos PK, Mugnaini M, Hagan JJ *et al.* The role of central dopamine D3 receptors in drug addiction: a review of pharmacological evidence. *Brain Res Brain Res Rev* 2005; **49**(1)**:** 77-105.

10. Joyce JN, Millan MJ. Dopamine D3 receptor antagonists as therapeutic agents. *Drug Discov Today* 2005; **10**(13)**:** 917-925.

11. Bouthenet ML, Souil E, Martres MP, Sokoloff P, Giros B, Schwartz JC. Localization of dopamine D3 receptor mRNA in the rat brain using in situ hybridization histochemistry: comparison with dopamine D2 receptor mRNA. *Brain Res* 1991; **564**(2)**:** 203-219.

12. Murray AM, Ryoo HL, Gurevich E, Joyce JN. Localization of dopamine D3 receptors to mesolimbic and D2 receptors to mesostriatal regions of human forebrain. *Proc Natl Acad Sci U S A* 1994; **91**(23)**:** 11271-11275.

13. Diaz J, Levesque D, Lammers CH, Griffon N, Martres MP, Schwartz JC *et al.* Phenotypical characterization of neurons expressing the dopamine D3 receptor in the rat brain. *Neuroscience* 1995; **65**(3)**:** 731-745.

14. Diaz J, Pilon C, Le Foll B, Gros C, Triller A, Schwartz JC *et al.* Dopamine D3 receptors expressed by all mesencephalic dopamine neurons. *J Neurosci* 2000; **20**(23)**:** 8677-8684.

15. Levant B. The D3 dopamine receptor: neurobiology and potential clinical relevance. *Pharmacol Rev* 1997; **49**(3)**:** 231-252.

16. Silvestre JS, O'Neill MF, Fernandez AG, Palacios JM. Effects of a range of dopamine receptor agonists and antagonists on ethanol intake in the rat. *Eur J Pharmacol* 1996; **318**(2-3)**:** 257-265.

17. Cohen C, Perrault G, Sanger DJ. Preferential involvement of D3 versus D2 dopamine receptors in the effects of dopamine receptor ligands on oral ethanol self-administration in rats. *Psychopharmacology (Berl)* 1998; **140**(4)**:** 478-485.

18. Thanos PK, Katana JM, Ashby CR, Jr., Michaelides M, Gardner EL, Heidbreder CA *et al.* The selective dopamine D3 receptor antagonist SB-277011-A attenuates ethanol consumption in ethanol preferring (P) and non-preferring (NP) rats. *Pharmacol Biochem Behav* 2005; **81**(1)**:** 190-197.

19. Harrison SJ, Nobrega JN. A functional role for the dopamine D3 receptor in the induction and expression of behavioural sensitization to ethanol in mice. *Psychopharmacology (Berl)* 2009; **207**(1)**:** 47-56.

20. Vengeliene V, Leonardi-Essmann F, Perreau-Lenz S, Gebicke-Haerter P, Drescher K, Gross G *et al.* The dopamine D3 receptor plays an essential role in alcohol-seeking and relapse. *Faseb J* 2006; **20**(13)**:** 2223-2233.

21. McQuade JA, Xu M, Woods SC, Seeley RJ, Benoit SC. Ethanol consumption in mice with a targeted disruption of the dopamine-3 receptor gene. *Addict Biol* 2003; **8**(3)**:** 295-303.

22. Boyce-Rustay JM, Risinger FO. Dopamine D3 receptor knockout mice and the motivational effects of ethanol. *Pharmacol Biochem Behav* 2003; **75**(2)**:** 373-379.

23. Wise RA. Voluntary ethanol intake in rats following exposure to ethanol on various schedules. *Psychopharmacologia* 1973; **29**(3)**:** 203-210.

24. Accili D, Fishburn CS, Drago J, Steiner H, Lachowicz JE, Park BH *et al.* A targeted mutation of the D3 dopamine receptor gene is associated with hyperactivity in mice. *Proc Natl Acad Sci U S A* 1996; **93**(5)**:** 1945-1949.

25. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 2008; **3**(6)**:** 1101-1108.

26. Schuttelkopf AW, van Aalten DM. PRODRG: a tool for high-throughput crystallography of protein-ligand complexes. *Acta Crystallogr D Biol Crystallogr* 2004; **60**(Pt 8)**:** 1355-1363.

27. Platania CB, Salomone S, Leggio GM, Drago F, Bucolo C. Homology modeling of dopamine d(2) and d(3) receptors: molecular dynamics refinement and docking evaluation. *PLoS One* 2012; **7**(9)**:** e44316.

28. Guillin O, Diaz J, Carroll P, Griffon N, Schwartz JC, Sokoloff P. BDNF controls dopamine D3 receptor expression and triggers behavioural sensitization. *Nature* 2001; **411**(6833)**:** 86-89.

29. He DY, Neasta J, Ron D. Epigenetic regulation of BDNF expression via the scaffolding protein RACK1. *J Biol Chem* 2010; **285**(25)**:** 19043-19050.

30. McGough NN, He DY, Logrip ML, Jeanblanc J, Phamluong K, Luong K *et al.* RACK1 and brain-derived neurotrophic factor: a homeostatic

pathway that regulates alcohol addiction. *J Neurosci* 2004; **24**(46)**:** 10542-10552.

31. Mannoury la Cour C, Salles MJ, Pasteau V, Millan MJ. Signaling pathways leading to phosphorylation of Akt and GSK-3beta by activation of cloned human and rat cerebral D(2)and D(3) receptors. *Mol Pharmacol* 2011; **79**(1)**:** 91-105.

32. Nelson RJ, Young KA. Behavior in mice with targeted disruption of single genes. *Neurosci Biobehav Rev* 1998; **22**(3)**:** 453-462.

33. Crabbe JC, Kosobud A, Young ER, Janowsky JS. Polygenic and single-gene determination of responses to ethanol in BXD/Ty recombinant inbred mouse strains. *Neurobehav Toxicol Teratol* 1983; **5**(2)**:** 181-187.

34. Joseph JD, Wang YM, Miles PR, Budygin EA, Picetti R, Gainetdinov RR *et al.* Dopamine autoreceptor regulation of release and uptake in mouse brain slices in the absence of D(3) receptors. *Neuroscience* 2002; **112**(1)**:** 39-49.

35. Koeltzow TE, Xu M, Cooper DC, Hu XT, Tonegawa S, Wolf ME *et al.* Alterations in dopamine release but not dopamine autoreceptor function in dopamine D3 receptor mutant mice. *J Neurosci* 1998; **18**(6)**:** 2231-2238.

36. Le Foll B, Diaz J, Sokoloff P. Neuroadaptations to hyperdopaminergia in dopamine D3 receptor-deficient mice. *Life Sci* 2005; **76**(11)**:** 1281-1296.

37. Le Foll B, Frances H, Diaz J, Schwartz JC, Sokoloff P. Role of the dopamine D3 receptor in reactivity to cocaine-associated cues in mice. *Eur J Neurosci* 2002; **15**(12)**:** 2016-2026.

38. Reavill C, Taylor SG, Wood MD, Ashmeade T, Austin NE, Avenell KY *et al.* Pharmacological actions of a novel, high-affinity, and selective human dopamine D(3) receptor antagonist, SB-277011-A. *J Pharmacol Exp Ther* 2000; **294**(3)**:** 1154-1165.

39. Carr KD, Yamamoto N, Omura M, Cabeza de Vaca S, Krahne L. Effects of the D(3) dopamine receptor antagonist, U99194A, on brain stimulation and d-amphetamine reward, motor activity, and c-fos

expression in ad libitum fed and food-restricted rats. *Psychopharmacology (Berl)* 2002; **163**(1)**:** 76-84.

40. Jeanblanc J, He DY, McGough NN, Logrip ML, Phamluong K, Janak PH *et al.* The dopamine D3 receptor is part of a homeostatic pathway regulating ethanol consumption. *J Neurosci* 2006; **26**(5)**:** 1457-1464.

41. Neisewander JL, Fuchs RA, Tran-Nguyen LT, Weber SM, Coffey GP, Joyce JN. Increases in dopamine D3 receptor binding in rats receiving a cocaine challenge at various time points after cocaine self-administration: implications for cocaine-seeking behavior. *Neuropsychopharmacology* 2004; **29**(8)**:** 1479-1487.

42. Le Foll B, Diaz J, Sokoloff P. Increased dopamine D3 receptor expression accompanying behavioral sensitization to nicotine in rats. *Synapse* 2003; **47**(3)**:** 176-183.

43. Le Foll B, Diaz J, Sokoloff P. A single cocaine exposure increases BDNF and D3 receptor expression: implications for drug-conditioning. *Neuroreport* 2005; **16**(2)**:** 175-178.

44. Staley JK, Mash DC. Adaptive increase in D3 dopamine receptors in the brain reward circuits of human cocaine fatalities. *J Neurosci* 1996; **16**(19)**:** 6100-6106.

45. Xing B, Guo J, Meng X, Wei SG, Li SB. The dopamine D1 but not D3 receptor plays a fundamental role in spatial working memory and BDNF expression in prefrontal cortex of mice. *Behav Brain Res* 2012; **235**(1)**:** 36-41.

46. Creese I, Snyder SH. Chronic neuroleptic treatment and dopamine receptor regulation. *Adv Biochem Psychopharmacol* 1980; **24:** 89-94.

47. Svenningsson P, Nairn AC, Greengard P. DARPP-32 mediates the actions of multiple drugs of abuse. *Aaps J* 2005; **7**(2)**:** E353-360.

48. Nuutinen S, Kiianmaa K, Panula P. DARPP-32 and Akt regulation in ethanol-preferring AA and ethanol-avoiding ANA rats. *Neurosci Lett* 2011; **503**(1)**:** 31-36.

49. Beaulieu JM, Gainetdinov RR, Caron MG. The Akt-GSK-3 signaling cascade in the actions of dopamine. *Trends Pharmacol Sci* 2007; **28**(4)**:** 166-172.

50. Li YC, Xi D, Roman J, Huang YQ, Gao WJ. Activation of glycogen synthase kinase-3 beta is required for hyperdopamine and D2 receptor-mediated inhibition of synaptic NMDA receptor function in the rat prefrontal cortex. *J Neurosci* 2009; **29**(49)**:** 15551-15563.

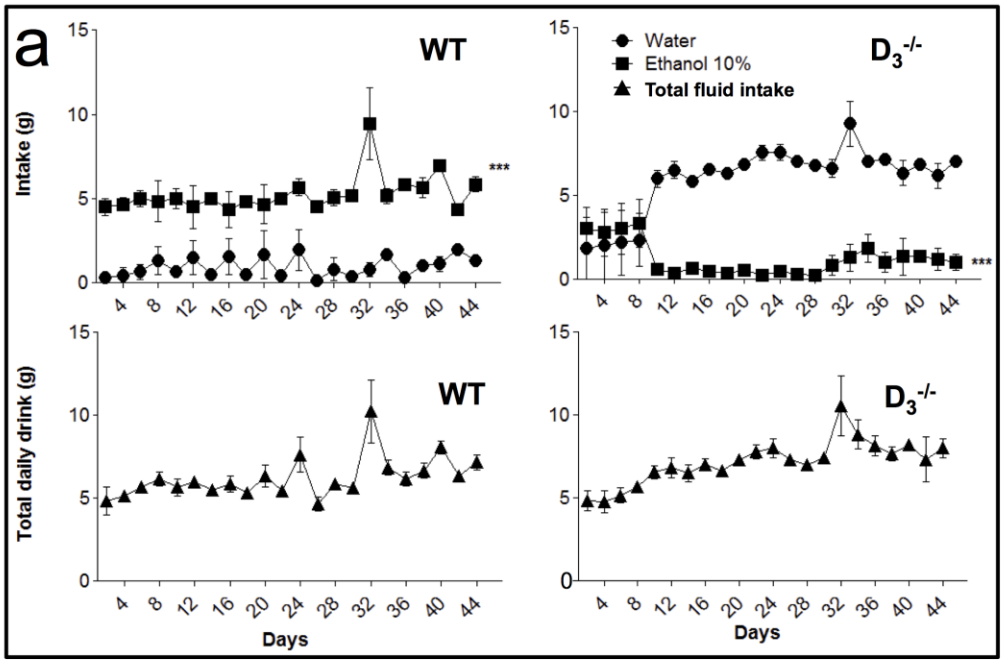
51. Audinot V, Newman-Tancredi A, Gobert A, Rivet JM, Brocco M, Lejeune F *et al.* A comparative in vitro and in vivo pharmacological characterization of the novel dopamine D3 receptor antagonists (+)-S 14297, nafadotride, GR 103,691 and U 99194. *J Pharmacol Exp Ther* 1998; **287**(1)**:** 187-197.

Table 1. Autodock 4.2 (AD4.2) binding energies and DSX scores of ligand-receptor complexes.

	hD_3 (binding Energy Kcal/mol)		hD_{2L} (binding energy Kcal/mol)		Experimental K_i (nM) [pK_i]	
Ligand	AD4.2	DSX-Score	AD4.2	DSX-Score	hD_3	hD_2
SB277011A	-9.9	-127	-8.2	-66	11[38] [7.9]	1032[38] [6.0]
U99194A	-5.0	-104	-5.4	-87	160[51] [6.8]	2281[51] [5.6]

Experimental Ki are from literature[38, 51]; pKi (in square brackets) is the negative logarithm of Ki.

Figures


a
WT
D3-/-
Water
Ethanol 10%
Total fluid intake
Intake (g)
Total daily drink (g)
Days
***

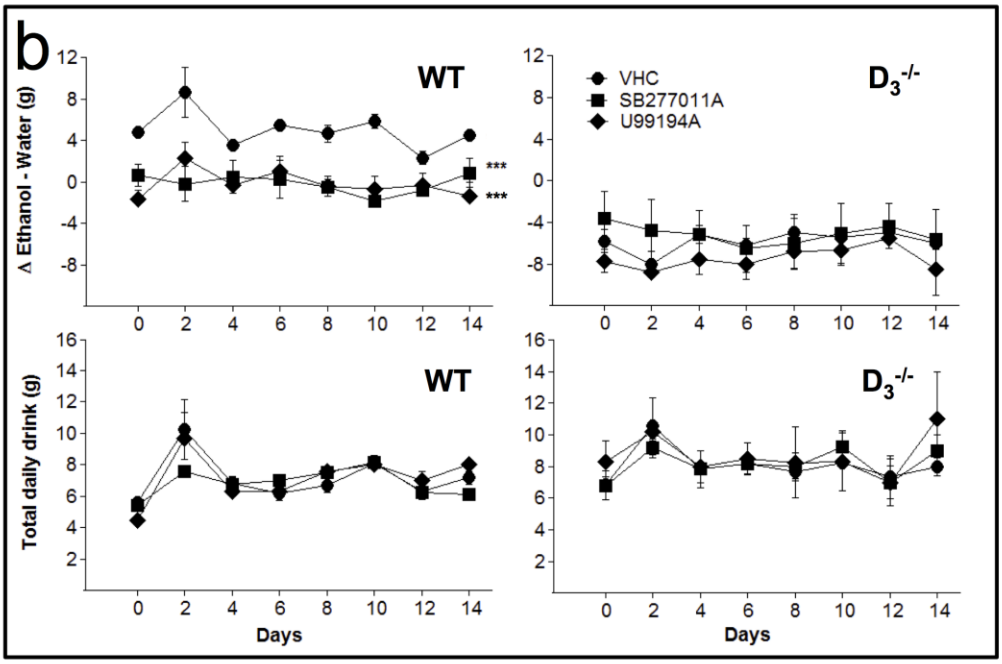

b
WT
D3-/-
VHC
SB277011A
U99194A
Δ Ethanol - Water (g)
Total daily drink (g)
Days
***

Figure 1

Wild type (WT) mice show a stable ethanol preference ($D_3^{-/-}$ mice do not) that is counteracted by D_3 pharmacological antagonist. In **a**, voluntary ethanol intake was measured every 48 h, for 44 days, in WT (n=30) and $D_3^{-/-}$ (n=30) mice that had free access to water and ethanol solution (10%). Daily intake of either water or ethanol solution is expressed in g. Lower panels show total fluid intake that was not different in the two groups. In **b**, voluntary ethanol intake was measured as in **a**, but in mice that had received the day before and kept receiving daily i.p. injection of either saline (vehicle, VHC, n=10), U99194A (n=10) or SB277011A (n=10), either drug at 10 mg/kg. To outline the effect of drug-treatment on ethanol preference, in the ordinates of upper graphs the difference in g between daily water and ethanol intake is shown. Lower panels show total fluid intake in either group that was not affected by drug-treatment.

*** $P<0.001$ vs water or vehicle (VHC). One-way ANOVA and Newman-Keuls post hoc test.

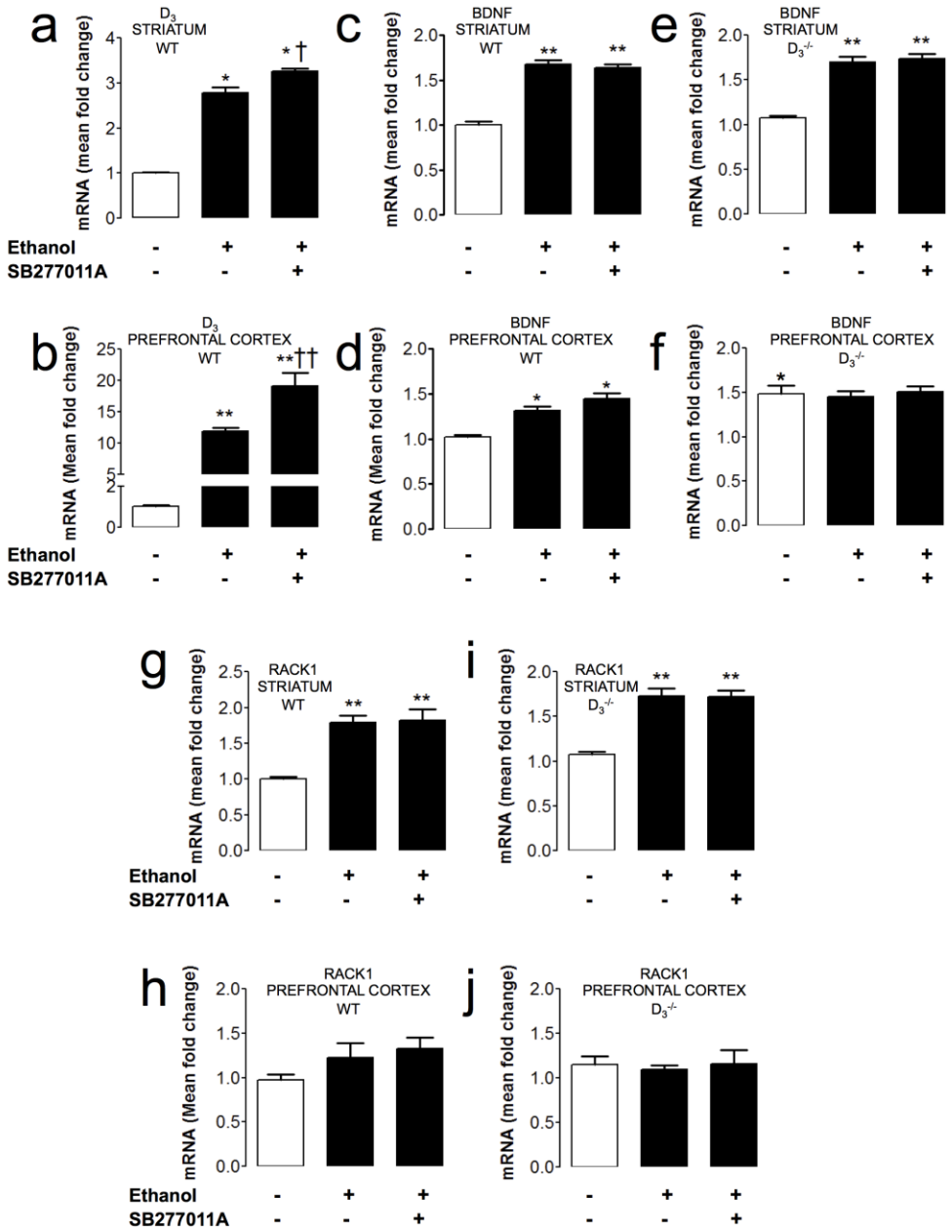

a
D3 STRIATUM WT
mRNA (mean fold change)
Ethanol - + +
SB277011A - - +
b
D3 PREFRONTAL CORTEX WT
mRNA (Mean fold change)
Ethanol - + +
SB277011A - - +
c
BDNF STRIATUM WT
mRNA (mean fold change)
d
BDNF PREFRONTAL CORTEX WT
mRNA (Mean fold change)
e
BDNF STRIATUM D3-/-
mRNA (mean fold change)
f
BDNF PREFRONTAL CORTEX D3-/-
mRNA (mean fold change)
g
RACK1 STRIATUM WT
mRNA (mean fold change)
h
RACK1 PREFRONTAL CORTEX WT
mRNA (Mean fold change)
i
RACK1 STRIATUM D3-/-
mRNA (mean fold change)
j
RACK1 PREFRONTAL CORTEX D3-/-
mRNA (mean fold change)

Figure 2

Chronic ethanol intake induces D_3 up-regulation, associated with activation of BDNF/RACK1 pathway. Abundance of transcripts in prefrontal cortex and striatum was assessed by quantitative RT-PCR after 44 days of free access to water only (white columns), or to both water and ethanol (black columns), in mice treated or not with SB277011A (see also methods). In **a**-**b**, D_3 expression profile in WT; in **c**-**f**, brain-derived neurotrophic factor (BDNF) expression profile in WT and $D_3^{-/-}$; in **g**-**j** RACK1 expression profile in WT and $D_3^{-/-}$. Mean fold changes are expressed relative to transcript levels in controls (WT having access to water only). Each column is the mean (± S.E.M.) from 5 different samples. $^{*}P<0.05$, $^{**}P<0.01$ vs water only; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ vs water/ethanol, vehicle-injected. One-way ANOVA and Newman-Keuls post hoc test.

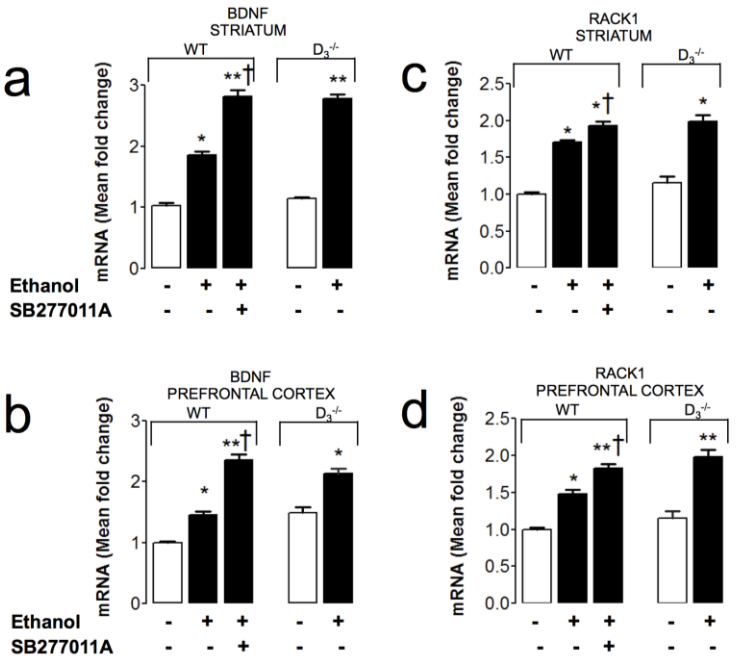


Figure 3

Forced chronic ethanol intake induces the same activation of BDNF/RACK1 pathway in WT and $D_3^{-/-}$. Abundance of transcripts in prefrontal cortex and striatum was assessed by quantitative RT-PCR after 44 days of free access to water only (white columns), or to ethanol 10% only (black columns), in mice treated or not with SB277011A (see also methods). Mean fold changes are expressed relative to transcript levels in controls (WT having access to water only). In **a**-**b**, brain-derived neurotrophic factor (BDNF) expression profile in WT and $D_3^{-/-}$; in **c**-**d** RACK1 expression profile in WT and $D_3^{-/-}$. Each column is the mean (± S.E.M.) from 5 different samples. $^{*}P<0.05$, $^{**}P<0.01$ vs water

only; †P<0.05, vs. water/ethanol, vehicle-injected. One-way ANOVA and Newman-Keuls post hoc test.

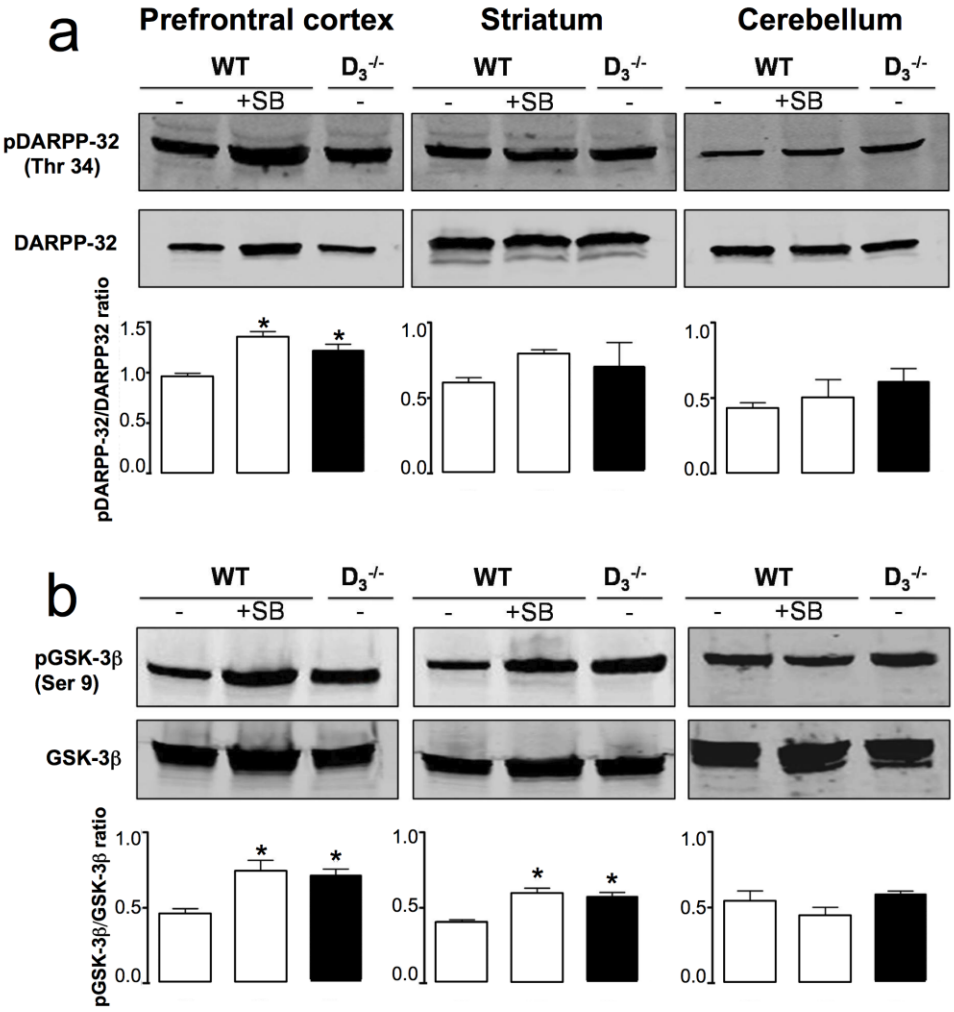


Figure 4

Activation of dopaminergic transmission following SB277011A-treatment. The abundance of phosphorylated DARPP-32 (Thr 34, **a**) and phosphorylated GSK3β (Ser 9, **b**) was assessed by immunoblot, in different CNS areas of WT mice (white columns), injected i.p. for 14 days with either vehicle or 10 mg/kg SB277011A and in $D_3^{-/-}$ (black columns). Bar graphs show mean (± S.E.M.) of intensities normalized against the respective non-phosphorylated protein. Notice that both phosphoDARPP-32 and posphoGSK3β were increased by treatment of WT mice with SB277011A, up to the level of $D_3^{-/-}$ mice, in prefrontal cortex and striatum, but not in cerebellum. Each column is the mean (± S.E.M.) from 5 different samples.* $P<0.05$, vs contro (vehicle-injected WT). Two-way ANOVA and Newman-Keuls post hoc test.

SUPPLEMENTARY INFORMATION

Molecular modeling

Autodock 4.2 (AD4.2)[1] was chosen as docking software, because it gave the best prediction for pose of eticlopride docked in the human D_3R homology model[2]. Search space included the extracellular site (binding pocked and extracellular loops) of the receptors. Input grid maps of search space were created applying Amber parameters running the AD4.2 executable Autogrid. In docking calculations, the Lamarkian genetic algorithm was used; hundred iterations with 2,500,000 energy evaluations per run were carried out. Population size was set to 150 and a maximum of 27,000 generations per run was carried out, followed by automatic clusterization of poses. Top scored (lowest energy and more populated) poses with orthosteric binding, such as eticlopride in human D_3-lysozime chimera[3], were selected for analysis of ligand-protein interactions using the graphical user interface AutoDock Tools. The free energy function was calibrated using a set of 188 structurally known ligand-complexes with experimentally determined binding constant[4]. The binding energy of ligand poses (kcal/mol) represents the sum of intermolecular energy, internal energy and torsional free energy of the ligand minus the unbound-system energy (see also Supplemental Information).

Ligand-protein complexes were rescored with DSX-score online (http://pc1664.pharmazie.uni-marburg.de/drugscore/), that uses a knowledge-

based scoring function based on comprehensive crystallographic information on ligand-protein complexes[5]; furthermore, solvent accessible surface potential is introduced in DSX-score to account for the desolvation effects. Binding mode of antagonists was represented by two dimensional diagrams generated by LigPlot+[6] (see also Supplemental Information, Figure S3).

Table S1. Residues of hD_3 and hD_{2L} receptors, involved in ligand binding.

Ligand	hD_3 residues		hD_{2L} residues	
	H-bond and polar contacts	Hydrophobic contacts	**H-bond** and polar contacts	Hydrophobic contacts
SB277011A	**Asp 110**, **(-C=O) Cys 181**, Ser 182.	Gly 94, Val 86, Leu 89, Val 111, Trp 342, Phe 345.	**(-NH) Ile 183**, Asp 114, Ser 193, Tyr 408.	Phe 110, Val 115, Phe 390, His 393.
U99194A	**Asp 110**, Ser 192.	Val 107, Val 111, Ile 183, Phe 345, Phe 346, His 349.	**Asp 114, His 393**, Ser 194.	Phe 110, Val 111, Val 115, Val 190, Phe 389, Phe 390.

Residues involved in H-Bond are reported in bold.

Comparison of protein sequences of human and murine D_3R and $D_{2L}R$ isoforms

The translational approach was applied because protein sequences are higly conserved between the human and mouse (Table S2 and Figure S1). A significant difference between mouse D_3R and human D_3R is found in the 3th citoplasmatic loop, where human D_3R has a deletion of 46 aminoacids (Figure S2); this deletion is found also between human $D_{2L}R$ and mouse $D_{2L}R$ receptors. The transmembrane domains and consequently the binding pocket is highly conserved (Figure S2), between human and mouse.

Table S2. Results of DA receptor sequence alignment. Sequences were retrieved from Protein-NCBI. pBLAST (NCBI web server) was used for sequence alignments.

Alignment	Identities	Identities+conserved mutations	Gaps
$hD_{2L}R$-$mD_{2L}R$	95 %	98 %	0
hD_3R-mD_3R	81 %	85 %	**11 %

** Gaps are related to the 3th citoplasmic loop (G-protein binding domain).

Figure S1. Phylogenetic distance of human (h) and murine (m) DA receptors. The tree was generated by accessing to http://www.cbrg.ethz.ch/services/PhylogeneticTree, after protein sequences were retrieved from Protein-NCBI database.

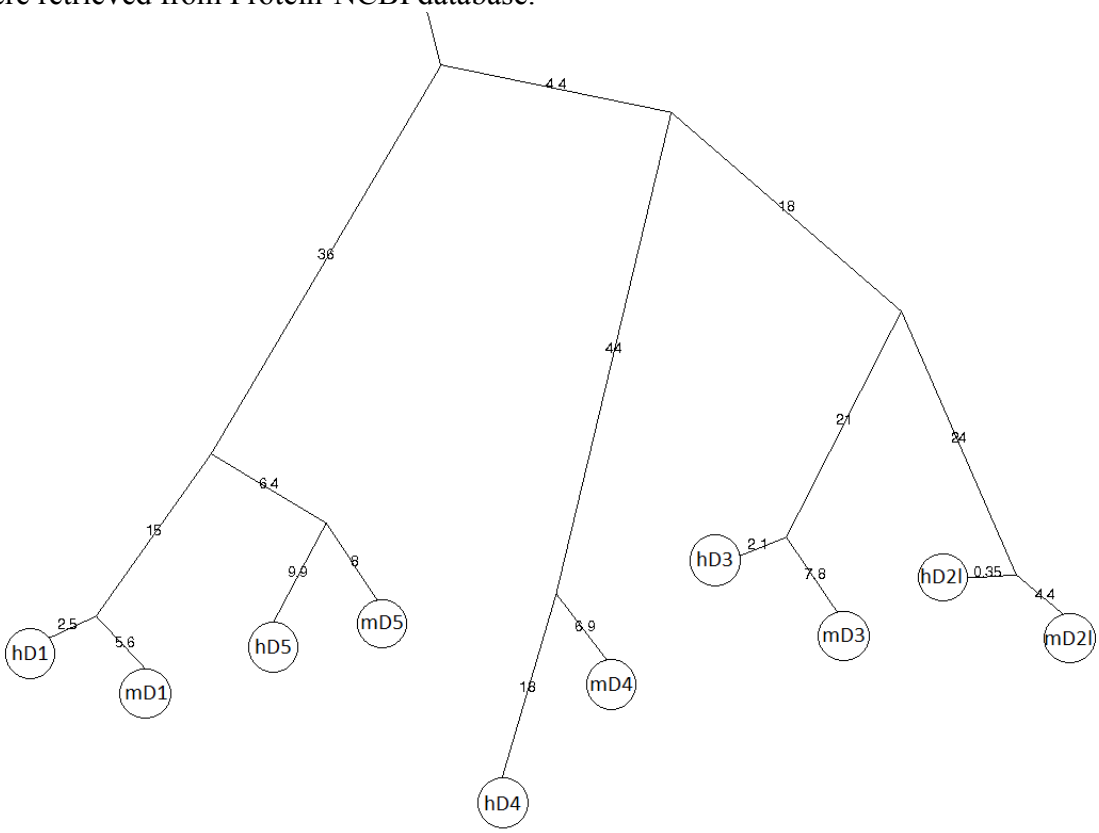


Figure S2. Multiple sequence alignment, performed with CLUSTAL W and visualized with JMOL, between murine and human sequences of D_3R and $D_{2L}R$.

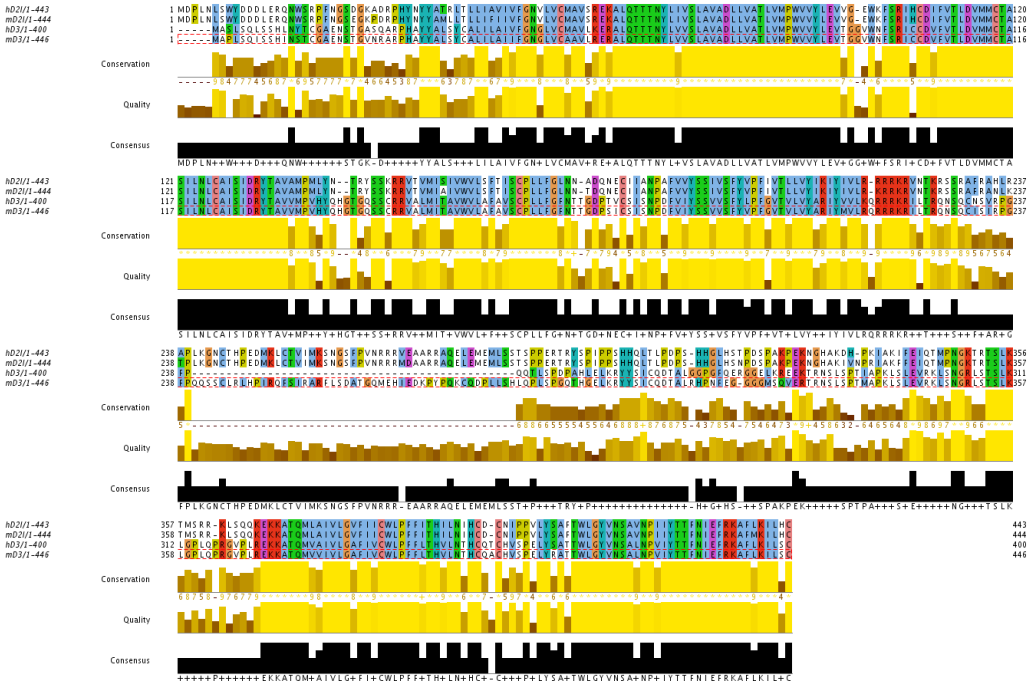
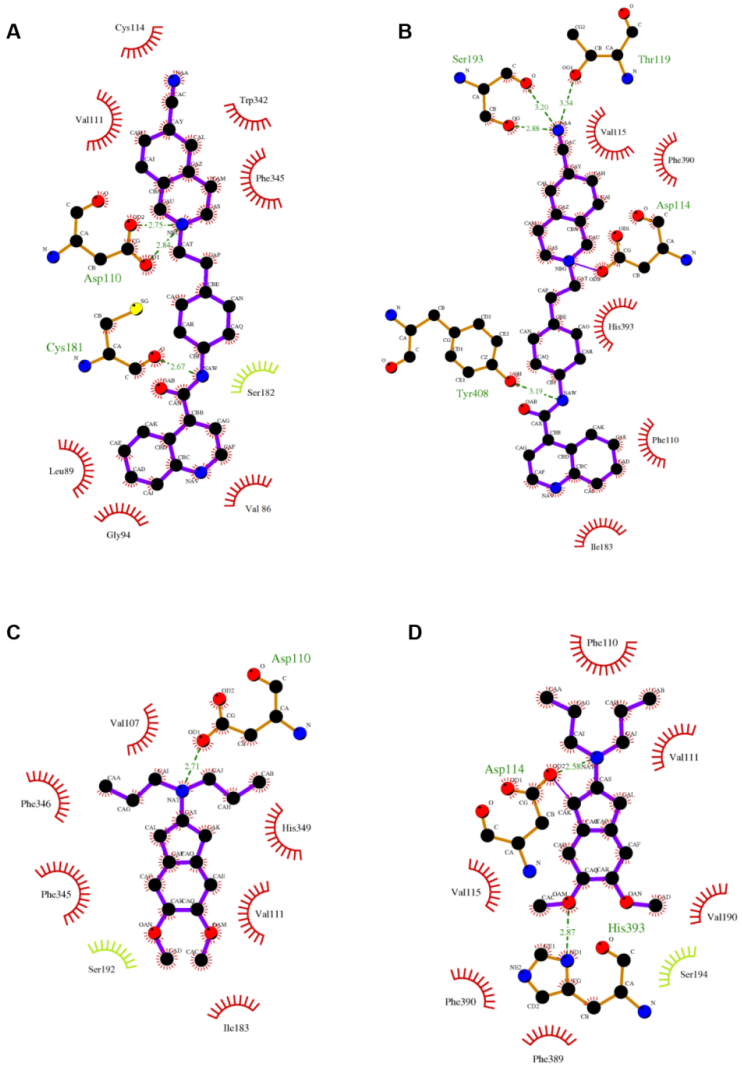


Figure S3. Two dimensional diagrams of binding mode of ligands docked into hD_3R and $hD_{2L}R$. Ligand binding mode of SB277011A (panels A and B) and U99194A (panels C and D) docked into hD_3R (panels A and C) and $hD_{2L}R$ (panels B and D) refined structures.



References

1. Morris GM, Goodsell DS, Huey R, Olson AJ. Distributed automated docking of flexible ligands to proteins: parallel applications of AutoDock 2.4. *J Comput Aided Mol Des* 1996; **10**(4)**:** 293-304.

2. Platania CB, Salomone S, Leggio GM, Drago F, Bucolo C. Homology modeling of dopamine d(2) and d(3) receptors: molecular dynamics refinement and docking evaluation. *PLoS One* 2012; **7**(9)**:** e44316.

3. Chien EY, Liu W, Zhao Q, Katritch V, Han GW, Hanson MA *et al.* Structure of the human dopamine D3 receptor in complex with a D2/D3 selective antagonist. *Science* 2010; **330**(6007)**:** 1091-1095.

4. Huey R, Morris GM, Olson AJ, Goodsell DS. A semiempirical free energy force field with charge-based desolvation. *J Comput Chem* 2007; **28**(6)**:** 1145-1152.

5. Neudert G, Klebe G. DSX: a knowledge-based scoring function for the assessment of protein-ligand complexes. *J Chem Inf Model* 2011; **51**(10)**:** 2731-2745.

6. Laskowski RA, Swindells MB. LigPlot+: multiple ligand-protein interaction diagrams for drug discovery. *J Chem Inf Model* 2011; **51**(10)**:** 2778-2786.

General Discussion

7.1 D3 KO mice show an aversion for the ethanol intake

Here we found that $D_3R^{-/-}$ mice chronically (44 days) exposed to the voluntary ethanol intake paradigm, drank very low quantities of ethanol in comparison with their WT littermates (Figure 1). This observation is apparently in contrast with the only two previous studies testing $D_3R^{-/-}$ mice in the ethanol voluntary intake paradigm(Boyce-Rustay and Risinger 2003; McQuade, Xu et al. 2003); both studies, in fact, reported no differences between $D_3R^{-/-}$ and C57BL/6J control mice. It is likely that the different conclusion between these studies and our data is due to the different period of observation. Their observation was, in fact, limited to 8 days(Boyce-Rustay and Risinger 2003; McQuade, Xu et al. 2003) and when looking at the same time frame (Figure 1a), we too did not observe significant differences between $D_3R^{-/-}$ and WT littermates, mainly because $D_3R^{-/-}$ mice did not yet show a preference for either ethanol or water. However, after 8 days, $D_3R^{-/-}$ mice started to show a marked preference for water, such that the difference with WT became obvious; this behavior was stably maintained for the following 5-6 weeks of observation.

7.2 Dopamine D3 selective antagonists counteract ethanol intake

To obtain pharmacological evidence for a functional role of D_3R in the control of voluntary ethanol intake we tested two D_3R antagonists, U99194A and SB277011A (Figure 1) at doses reported to target D_3R (Reavill, Taylor et al. 2000; Carr, Yamamoto et al. 2002). Before administering these drugs, we performed a molecular modeling study to gain information on the interaction of U99194A and SB277011A with D_3R. *In silico* analysis showed that the two D_3R antagonists were i) highly selective for the D_3R subtype and ii) displayed a distinct interaction (different binding energy, different interaction patterns) with D_3R, consistent with their distinct chemical structure. Both U99194A and SB277011A induced a significant decrease of voluntary ethanol intake in WT but not in $D_3R^{-/-}$ (Figure 1b). This pharmacological evidence reinforces the view that activation of D_3R is required for voluntary ethanol intake and is consistent with rat data, showing that D_3R antagonism reduces relapse-like drinking and cue-induced ethanol-seeking behavior (Vengeliene, Leonardi-Essmann et al. 2006). Furthermore, it opens perspectives for a novel, D_3R–based, drug treatment for alcoholism.

Concluding Remarks

In conclusion, I would like to underline the following remarks:

1. $D_3^{-/-}$ mice exhibit a lower basal "anxiety-like behavior" in EPM in comparison by their WT littermates.
2. $D_3^{-/-}$ mice show a higher basal level of grooming behavior in NGT, as compared to WT controls. If the higher grooming score may be considered as an index of anxiety (as it decrease after anxiolytic treatment), on the other and it is a phenotypical peculiarity of the hyperdopaminergic status of $D_3^{-/-}$ mice
3. Different sensitivity to the acute treatment with various doses of diazepam in $D_3^{-/-}$ and WT mice, tested in EPM and NGT
4. WT mice after repeated treatment for 7 days with diazepam (1 mg/kg/day) show in EPM "anxiolytic-like" effect in comparison with $D_3^{-/-}$ mice
5. The down-regulation of $GABA_A$ rteceptor in CA1 area of hippocampus of $D_3^{-/-}$ mice, but not of WT animals, found in these studies, might be considered as a compensatory mechanism of the GABAergic hyperactivity linked to the lack of inhibitory action of D_3Rs on GABAergic neurotransmitter system
6. $D_3^{-/-}$ intact mice, tested in FST procedure, exhibited a decrease (not significant) of immobility time in comparison to their WT. The different

between the two groups (decrease of immobility time of $D_3^{-/-}$ mice) is significant after intraperitoneal vehicle administration, suggesting that WT mice are more sensitive to the stress induced by injection per se

7. $D_3^{-/-}$ mice exhibit different sensitivity to several antidepressant drugs in comparison to WT mice in FST, suggesting that both a deficiency of mesolimbic DA is involved in depression and that D_3Rs, acting also as autoreceptors, could play a potential role in mood disorders

Taken together, our result reveal a role of D_3Rs in "anxiety-like behavior" and in the effect of anxiolytic treatment and antidepressant drugs, demonstrating an important aspect of the physiophatology of anxiety and depression.

References

Abi-Dargham, A., L. S. Kegeles, et al. (2003). "Dopamine mediation of positive reinforcing effects of amphetamine in stimulant naive healthy volunteers: results from a large cohort." Eur Neuropsychopharmacol **13**(6): 459-468.

Adams, R. N. (1976). "Probing brain chemistry with electroanalytical techniques." Anal Chem **48**(14): 1126A-1138A.

Amato, L., M. Davoli, et al. (2005). "An overview of systematic reviews of the effectiveness of opiate maintenance therapies: available evidence to inform clinical practice and research." J Subst Abuse Treat **28**(4): 321-329.

Anlezark, G. M., G. W. Arbuthnott, et al. (1971). "Role of cerebral dopamine in the action of psychotropic drugs." Br J Pharmacol **41**(2): 406P-407P.

Ballenger, J. C. and R. M. Post (1978). "Kindling as a model for alcohol withdrawal syndromes." Br J Psychiatry **133**: 1-14.

Besancon, F. (1993). "Time to alcohol dependence after abstinence and first drink." Addiction **88**(12): 1647-1650.

Boileau, I., J. M. Assaad, et al. (2003). "Alcohol promotes dopamine release in the human nucleus accumbens." Synapse **49**(4): 226-231.

Bouza, C., M. Angeles, et al. (2004). "Efficacy and safety of naltrexone and acamprosate in the treatment of alcohol dependence: a systematic review." Addiction **99**(7): 811-828.

Boyce-Rustay, J. M. and F. O. Risinger (2003). "Dopamine D3 receptor knockout mice and the motivational effects of ethanol." Pharmacology, biochemistry, and behavior **75**(2): 373-379.

Brauth, S. E. and J. Olds (1977). "Midbrain unit activity during classical conditioning." Brain Res **134**(1): 73-82.

Brick, J. and C. K. Erickson (2009). "Intoxication is not always visible: an unrecognized prevention challenge." Alcohol Clin Exp Res **33**(9): 1489-1507.

Brodie, M. S. and T. V. Dunwiddie (1990). "Cocaine effects in the ventral tegmental area: evidence for an indirect dopaminergic mechanism of action." Naunyn Schmiedebergs Arch Pharmacol **342**(6): 660-665.

Budygin, E. A., P. E. Phillips, et al. (2001). "Effect of acute ethanol on striatal dopamine neurotransmission in ambulatory rats." J Pharmacol Exp Ther **297**(1): 27-34.

Burish, T. G., S. A. Maisto, et al. (1981). "Effects of voluntary short-term abstinence from alcohol on subsequent drinking patterns of college students." J Stud Alcohol **42**(11): 1013-1020.

Carlsson, A., N. A. Hillarp, et al. (1962). "A Mg-ATP dependent storage mechanism in the amine granules of the adrenal medulla." Med Exp Int J Exp Med **6**: 47-53.

Carr, K. D., N. Yamamoto, et al. (2002). "Effects of the D(3) dopamine receptor antagonist, U99194A, on brain stimulation and d-amphetamine reward, motor activity, and c-fos expression in ad libitum fed and food-restricted rats." Psychopharmacology **163**(1): 76-84.

Caviness, J. N. and R. M. Wightman (1982). "Use of rapid superfusion to differentiate the release of dopamine from striatal tissue induced by sympathomimetic amines from release induced by potassium." J Pharmacol Exp Ther **223**(1): 90-96.

Cheer, J. F., K. M. Wassum, et al. (2007). "Phasic dopamine release evoked by abused substances requires cannabinoid receptor activation." J Neurosci **27**(4): 791-795.

Cloninger, C. R. (1987). "Neurogenetic adaptive mechanisms in alcoholism." Science **236**(4800): 410-416.

Crabbe, J. C., D. Wahlsten, et al. (1999). "Genetics of mouse behavior: interactions with laboratory environment." Science **284**(5420): 1670-1672.

Di Chiara, G. and A. Imperato (1985). "Ethanol preferentially stimulates dopamine release in the nucleus accumbens of freely moving rats." Eur J Pharmacol **115**(1): 131-132.

Di Chiara, G. and A. Imperato (1986). "Preferential stimulation of dopamine release in the nucleus accumbens by opiates, alcohol, and barbiturates: studies with transcerebral dialysis in freely moving rats." Ann N Y Acad Sci **473**: 367-381.

Di Chiara, G. and A. Imperato (1988). "Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats." Proc Natl Acad Sci U S A **85**(14): 5274-5278.

Diaz, J., C. Pilon, et al. (2000). "Dopamine D3 receptors expressed by all mesencephalic dopamine neurons." J Neurosci **20**(23): 8677-8684.

Dole, V. P., A. Ho, et al. (1985). "Toward an analogue of alcoholism in mice: criteria for recognition of pharmacologically motivated drinking." Proc Natl Acad Sci U S A **82**(10): 3469-3471.

Ericson, M., A. Molander, et al. (2003). "Ethanol elevates accumbal dopamine levels via indirect activation of ventral tegmental nicotinic acetylcholine receptors." Eur J Pharmacol **467**(1-3): 85-93.

Eriksson, K. (1968). "Genetic selection for voluntary alcohol consumption in the albino rat." Science **159**(3816): 739-741.

Ewing, A. G., K. D. Alloway, et al. (1983). "Simultaneous electrochemical and unit recording measurements: characterization of the effects of D-

amphetamine and ascorbic acid on neostriatal neurons." Brain Res **261**(1): 101-108.

Federici, M., R. Nistico, et al. (2009). "Ethanol enhances GABAB-mediated inhibitory postsynaptic transmission on rat midbrain dopaminergic neurons by facilitating GIRK currents." Eur J Neurosci **29**(7): 1369-1377.

Fein, G. and B. Landman (2005). "Treated and treatment-naive alcoholics come from different populations." Alcohol **36**(2): 19-26.

Fitzgerald, S. D., J. M. Sullivan, et al. (1990). "Suspected ethanol toxicosis in two wild cedar waxwings." Avian Dis **34**(2): 488-490.

Frezza, M., C. di Padova, et al. (1990). "High blood alcohol levels in women. The role of decreased gastric alcohol dehydrogenase activity and first-pass metabolism." N Engl J Med **322**(2): 95-99.

Gatto, G. J., W. J. McBride, et al. (1994). "Ethanol self-infusion into the ventral tegmental area by alcohol-preferring rats." Alcohol **11**(6): 557-564.

German, D. C. and D. M. Bowden (1974). "Catecholamine systems as the neural substrate for intracranial self-stimulation: a hypothesis." Brain Res **73**(3): 381-419.

Goldman, D., G. Oroszi, et al. (2005). "The genetics of addictions: uncovering the genes." Nat Rev Genet **6**(7): 521-532.

Gonon, F., M. Buda, et al. (1980). "In vivo electrochemical detection of catechols in the neostriatum of anaesthetized rats: dopamine or DOPAC?" Nature **286**(5776): 902-904.

Griffin, W. C., 3rd, L. D. Middaugh, et al. (2007). "Voluntary ethanol drinking in mice and ethanol concentrations in the nucleus accumbens." Brain Res **1138**: 208-213.

Hasin, D. S., F. S. Stinson, et al. (2007). "Prevalence, correlates, disability, and comorbidity of DSM-IV alcohol abuse and dependence in the United States: results from the National Epidemiologic Survey on Alcohol and Related Conditions." Arch Gen Psychiatry **64**(7): 830-842.

Heilig, M. and M. Egli (2006). "Pharmacological treatment of alcohol dependence: target symptoms and target mechanisms." Pharmacol Ther **111**(3): 855-876.

Heilig, M. and G. F. Koob (2007). "A key role for corticotropin-releasing factor in alcohol dependence." Trends Neurosci **30**(8): 399-406.

Heinz, A., S. Lober, et al. (2003). "Reward craving and withdrawal relief craving: assessment of different motivational pathways to alcohol intake." Alcohol Alcohol **38**(1): 35-39.

Hillarp, N. A., K. Fuxe, et al. (1966). "Demonstration and mapping of central neurons containing dopamine, noradrenaline, and 5-hydroxytryptamine and their reactions to psychopharmaca." Pharmacol Rev **18**(1): 727-741.

Holter, S. M., M. Engelmann, et al. (1998). "Long-term ethanol self-administration with repeated ethanol deprivation episodes changes ethanol drinking pattern and increases anxiety-related behaviour during ethanol deprivation in rats." Behav Pharmacol **9**(1): 41-48.

Holter, S. M., R. Landgraf, et al. (1997). "Time course of acamprosate action on operant ethanol self-administration after ethanol deprivation." Alcohol Clin Exp Res **21**(5): 862-868.

Imperato, A., A. Mulas, et al. (1986). "Nicotine preferentially stimulates dopamine release in the limbic system of freely moving rats." Eur J Pharmacol **132**(2-3): 337-338.

Jaber, M., S. W. Robinson, et al. (1996). "Dopamine receptors and brain function." Neuropharmacology **35**(11): 1503-1519.

Jia, F., D. Chandra, et al. (2008). "Ethanol modulates synaptic and extrasynaptic GABAA receptors in the thalamus." J Pharmacol Exp Ther **326**(2): 475-482.

Joseph, J. D., Y. M. Wang, et al. (2002). "Dopamine autoreceptor regulation of release and uptake in mouse brain slices in the absence of D(3) receptors." Neuroscience **112**(1): 39-49.

Katner, S. N., J. G. Magalong, et al. (1999). "Reinstatement of alcohol-seeking behavior by drug-associated discriminative stimuli after prolonged extinction in the rat." Neuropsychopharmacology **20**(5): 471-479.

Kendler, K. S., L. M. Thornton, et al. (2001). "Genetic risk, number of previous depressive episodes, and stressful life events in predicting onset of major depression." Am J Psychiatry **158**(4): 582-586.

Kirshner, N. (1962). "Uptake of catecholamines by a particulate fraction of the adrenal medulla." J Biol Chem **237**: 2311-2317.

Kissinger, P. T., J. B. Hart, et al. (1973). "Voltammetry in brain tissue--a new neurophysiological measurement." Brain Res **55**(1): 209-213.

Koeltzow, T. E., M. Xu, et al. (1998). "Alterations in dopamine release but not dopamine autoreceptor function in dopamine D3 receptor mutant mice." J Neurosci **18**(6): 2231-2238.

Koob, G. F. and N. D. Volkow (2010). "Neurocircuitry of addiction." Neuropsychopharmacology **35**(1): 217-238.

Koyama, S., M. S. Brodie, et al. (2007). "Ethanol inhibition of m-current and ethanol-induced direct excitation of ventral tegmental area dopamine neurons." J Neurophysiol **97**(3): 1977-1985.

L'Hirondel, M., A. Cheramy, et al. (1998). "Lack of autoreceptor-mediated inhibitory control of dopamine release in striatal synaptosomes of D2 receptor-deficient mice." Brain Res **792**(2): 253-262.

Lankford, M. F., A. K. Roscoe, et al. (1991). "Drinking of high concentrations of ethanol versus palatable fluids in alcohol-preferring (P) rats: valid animal model of alcoholism." Alcohol **8**(4): 293-299.

Le, A. D., B. Quan, et al. (1998). "Reinstatement of alcohol-seeking by priming injections of alcohol and exposure to stress in rats." Psychopharmacology (Berl) **135**(2): 169-174.

Le Foll, B., J. Diaz, et al. (2005). "Neuroadaptations to hyperdopaminergia in dopamine D3 receptor-deficient mice." Life Sci **76**(11): 1281-1296.

Levesque, D., M. P. Martres, et al. (1995). "A paradoxical regulation of the dopamine D3 receptor expression suggests the involvement of an anterograde factor from dopamine neurons." Proc Natl Acad Sci U S A **92**(5): 1719-1723.

Ludwig, A. M., A. Wikler, et al. (1974). "The first drink: psychobiological aspects of craving." Arch Gen Psychiatry **30**(4): 539-547.

Mark, T. L., H. R. Kranzler, et al. (2003). "Understanding US addiction physicians' low rate of naltrexone prescription." Drug Alcohol Depend **71**(3): 219-228.

Markou, A., F. Weiss, et al. (1993). "Animal models of drug craving." Psychopharmacology (Berl) **112**(2-3): 163-182.

McBride, W. J. and T. K. Li (1998). "Animal models of alcoholism: neurobiology of high alcohol-drinking behavior in rodents." Crit Rev Neurobiol **12**(4): 339-369.

McLellan, A. T., D. C. Lewis, et al. (2000). "Drug dependence, a chronic medical illness: implications for treatment, insurance, and outcomes evaluation." JAMA **284**(13): 1689-1695.

McQuade, J. A., M. Xu, et al. (2003). "Ethanol consumption in mice with a targeted disruption of the dopamine-3 receptor gene." Addiction biology **8**(3): 295-303.

Melis, M., M. Diana, et al. (2009). "Ethanol and acetaldehyde action on central dopamine systems: mechanisms, modulation, and relationship to stress." Alcohol **43**(7): 531-539.

Mercuri, N. B., A. Saiardi, et al. (1997). "Loss of autoreceptor function in dopaminergic neurons from dopamine D2 receptor deficient mice." Neuroscience **79**(2): 323-327.

Mereu, G. and G. L. Gessa (1985). "Low doses of ethanol inhibit the firing of neurons in the substantia nigra, pars reticulata: a GABAergic effect?" Brain Res **360**(1-2): 325-330.

Millar, J., J. A. Stamford, et al. (1985). "Electrochemical, pharmacological and electrophysiological evidence of rapid dopamine release and removal in the rat caudate nucleus following electrical stimulation of the median forebrain bundle." Eur J Pharmacol **109**(3): 341-348.

Missale, C., S. R. Nash, et al. (1998). "Dopamine receptors: from structure to function." Physiol Rev **78**(1): 189-225.

Morikawa, H. and R. A. Morrisett (2010). "Ethanol action on dopaminergic neurons in the ventral tegmental area: interaction with intrinsic ion channels and neurotransmitter inputs." Int Rev Neurobiol **91**: 235-288.

Nutt, D. J., L. A. King, et al. (2010). "Drug harms in the UK: a multicriteria decision analysis." Lancet **376**(9752): 1558-1565.

O'Hara, C. M., A. Uhland-Smith, et al. (1996). "Inhibition of dopamine synthesis by dopamine D2 and D3 but not D4 receptors." J Pharmacol Exp Ther **277**(1): 186-192.

Okamoto, T., M. T. Harnett, et al. (2006). "Hyperpolarization-activated cation current (Ih) is an ethanol target in midbrain dopamine neurons of mice." J Neurophysiol **95**(2): 619-626.

Olds, J. (1976). "Brain stimulation and the motivation of behavior." Prog Brain Res **45**: 401-426.

Olds, J., K. F. Killam, et al. (1956). "Self-stimulation of the brain used as a screening method for tranquilizing drugs." Science **124**(3215): 265-266.

Olds, J. and P. Milner (1954). "Positive reinforcement produced by electrical stimulation of septal area and other regions of rat brain." J Comp Physiol Psychol **47**(6): 419-427.

Olds, J. and M. E. Olds (1958). "Positive reinforcement produced by stimulating hypothalamus with iproniazid and other compounds." Science **127**(3307): 1175-1176.

Olds, M. E. and J. Olds (1969). "Effects of lesions in medial forebrain bundle on self-stimulation behavior." Am J Physiol **217**(5): 1253-1264.

Phillips, M. I. and J. Olds (1969). "Unit activity: motiviation-dependent responses from midbrain neurons." Science **165**(3899): 1269-1271.

Plotsky, P. M., R. M. Wightman, et al. (1977). "Liquid chromatographic analysis of endogenous catecholamine release from brain slices." Science **197**(4306): 904-906.

Reavill, C., S. G. Taylor, et al. (2000). "Pharmacological actions of a novel, high-affinity, and selective human dopamine D(3) receptor antagonist, SB-277011-A." The Journal of pharmacology and experimental therapeutics **294**(3): 1154-1165.

Rehm, J., C. Mathers, et al. (2009). "Global burden of disease and injury and economic cost attributable to alcohol use and alcohol-use disorders." Lancet **373**(9682): 2223-2233.

Rewal, M., R. Jurd, et al. (2009). "Alpha4-containing GABAA receptors in the nucleus accumbens mediate moderate intake of alcohol." J Neurosci **29**(2): 543-549.

Rice, M. E., A. F. Oke, et al. (1985). "Simultaneous voltammetric and chemical monitoring of dopamine release in situ." Brain Res **340**(1): 151-155.

Robinson, D. L., L. J. Brunner, et al. (2002). "Effect of gender and estrous cycle on the pharmacokinetics of ethanol in the rat brain." Alcohol Clin Exp Res **26**(2): 165-172.

Rodd-Henricks, Z. A., D. L. McKinzie, et al. (2000). "Alcohol deprivation effect is prolonged in the alcohol preferring (P) rat after repeated deprivations." Alcohol Clin Exp Res **24**(1): 8-16.

Sautel, F., N. Griffon, et al. (1995). "Nafadotride, a potent preferential dopamine D3 receptor antagonist, activates locomotion in rodents." J Pharmacol Exp Ther **275**(3): 1239-1246.

Schultz, W. (2011). "Potential vulnerabilities of neuronal reward, risk, and decision mechanisms to addictive drugs." Neuron **69**(4): 603-617.

Schwandt, M. L., S. G. Lindell, et al. (2010). "Alcohol response and consumption in adolescent rhesus macaques: life history and genetic influences." Alcohol **44**(1): 67-80.

Sibley, D. R. (1999). "New insights into dopaminergic receptor function using antisense and genetically altered animals." Annu Rev Pharmacol Toxicol **39**: 313-341.

Spanagel, R. and S. M. Holter (2000). "Pharmacological validation of a new animal model of alcoholism." J Neural Transm **107**(6): 669-680.

Spanagel, R., S. M. Holter, et al. (1996). "Acamprosate and alcohol: I. Effects on alcohol intake following alcohol deprivation in the rat." Eur J Pharmacol **305**(1-3): 39-44.

Spanagel, R. and F. Weiss (1999). "The dopamine hypothesis of reward: past and current status." Trends Neurosci **22**(11): 521-527.

Tang, L., R. D. Todd, et al. (1994). "Dopamine D2 and D3 receptors inhibit dopamine release." J Pharmacol Exp Ther **270**(2): 475-479.

Tepper, J. M., T. Koos, et al. (2004). "GABAergic microcircuits in the neostriatum." Trends Neurosci **27**(11): 662-669.

Ungerstedt, U. and C. Pycock (1974). "Functional correlates of dopamine neurotransmission." Bull Schweiz Akad Med Wiss **30**(1-3): 44-55.

Urban, N. B., L. S. Kegeles, et al. (2010). "Sex differences in striatal dopamine release in young adults after oral alcohol challenge: a positron emission tomography imaging study with [(1)(1)C]raclopride." Biol Psychiatry **68**(8): 689-696.

Vaillant, G. E. (1983). "Natural history of male alcoholism V: is alcoholism the cart or the horse to sociopathy?" Br J Addict **78**(3): 317-326.

Vaillant, G. E. (1996). "A long-term follow-up of male alcohol abuse." Arch Gen Psychiatry **53**(3): 243-249.

Vengeliene, V., F. Leonardi-Essmann, et al. (2006). "The dopamine D3 receptor plays an essential role in alcohol-seeking and relapse." FASEB journal : official publication of the Federation of American Societies for Experimental Biology **20**(13): 2223-2233.

Willenbring, M. L., S. H. Massey, et al. (2009). "Helping patients who drink too much: an evidence-based guide for primary care clinicians." Am Fam Physician **80**(1): 44-50.

Wise, R. A. (2009). "Roles for nigrostriatal--not just mesocorticolimbic--dopamine in reward and addiction." Trends Neurosci **32**(10): 517-524.

Wu, P., K. Wilson, et al. (2006). "Effectiveness of smoking cessation therapies: a systematic review and meta-analysis." BMC Public Health **6**: 300.

Xiao, C. and J. H. Ye (2008). "Ethanol dually modulates GABAergic synaptic transmission onto dopaminergic neurons in ventral tegmental area: role of mu-opioid receptors." Neuroscience **153**(1): 240-248.

Yim, H. J., T. Schallert, et al. (1998). "Comparison of local and systemic ethanol effects on extracellular dopamine concentration in rat nucleus accumbens by microdialysis." Alcohol Clin Exp Res **22**(2): 367-374.

Yokel, R. A. and R. A. Wise (1978). "Amphetamine- type reinforcement by dopaminergic agonists in the rat." Psychopharmacology (Berl) **58**(3): 289-296.