UNIVERSITY OF CATANIA
International Ph.D. Program in Neuropharmacology
XXVIII Cycle

TARGETING DOPAMINE D3 RECEPTOR: NEW INSIGHTS INTO THE PATHOPHYSIOLOGY OF ALCOHOL ADDICTION AND ANXIETY

PhD Thesis

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During the 4th year of my PhD program, I spent six months working as visiting PhD student at University of Cambridge. I worked on an original project entitled: *Noradrenergic mechanisms in the nucleus accumbens shell: regulation of impulse control and the transition to compulsive behaviour*.

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List of abbreviations

7-OH-DPAT ((+/-)-7-hydroxy-N,N-(di-n-propyl-2-aminotetralin))

AD aldehyde dehydrogenase

AD4.2 Autodock 4.2

ADE alcohol deprivation effect

ADT AutoDock Tools

ANOVA analysis of variance

BDNF brain development neurotrophic factor

BLA basolateral amygdala

CNS central nervous system

COMT cathecol-O-methyl transferase

CSD Cambridge Structural Database

D_3R dopamine D_3 receptor

$D_3R^{-/-}$ dopamine D_3 receptor deficient mice

DA dopamine

DID drinking in the dark

DMSO dimethyl sulfoxid

DSM-IV Diagnostic and Statistical Manual of Mental Disorders, 4^{th} edition

EPM elevated plus maze test

GABA γ-aminobutyric acid

$GABA_A$ γ-aminobutyric acid receptor A

GAD generalized anxiety disorder

i.p. intraperitoneal injection

ICD-11 International Classification of Diseases, 11th edition

MAO monoamine oxidase

NAc nucleus accumbens

NP alcohol Non-Preferring rats

P alcohol Preferring rats

PDB Protein Data Bank

PKA protein kinase A

SAS-potential Solvent Accessible Surface potential

SI single injection

VEH vehicle

VMAT-2 the vesicular monoamine transporter 2

VTA ventral tegmental area

WT wild type mice

Preface

The dopaminergic neurotransmission in the central nervous system (CNS) is mediated by two different classes of G protein-coupled receptors, the "D_1R-like" receptors (D_1R and D_5R) and the "D_2R-like" receptors (D_2R, D_3R and D_4R) (Seeman et al., 1994). Since its discovery in the early 90's, the dopamine D3 receptor (D_3R) has aroused great interest in the scientific community. Indeed, its limited distribution in the limbic brain areas involved in the control of cognitive and emotional functions has made this receptor a promising target for the treatment of several neuropsychiatric disorders such as drug addiction, depression and schizophrenia (Leggio et al., 2016).

Several data suggest that D_3R, likely acting as autoreceptor, modulates the activity of dopaminergic neurons throughout the mesolimbic, mesocortical and nigrostriatal dopaminergic pathways (Gobert et al., 1995; Tepper et al., 1997; Diaz et al., 2000). Yet, D_3R-deficient mice ($D_3R^{-/-}$) ehibit extracellular levels of dopamine (DA) twice as high as their wild-type (WT) littermates suggesting that D_3R could play a inhibitory role in the control of basal extracellular DA levels (Koeltzow et al., 1998; Joseph et al., 2002).

The central hypothesis of my PhD research project has been that D_3R exerting a pivotal role in the control of the mesolimbic dopamine pathway, is involved in the pathophysiological mechanisms subserving neuropsychiatric

disorders linked to dysfunctionality of this dopaminergic pathway. In particular, the present thesis aimed to: 1) investigate the role of D_3R in the mesolimbic DA control of ethanol reward; 2) assess the involvement of a γ-aminobutyric acid receptor A ($GABA_A$)/D_3R interaction in the mesolimbic DA modulation of anxiety-like behavior by using both genetic and pharmacological approaches.

Chapter I

General Introduction

1. Dopamine system in the central nervous system

In 1957, Arvid Carlsson, a Swedish neuropharmacologist, discovered that DA was a neurotransmitter and not only the precursore of noradrenaline. Since this discovery, DA has attracted a great amount of attention leading to numerous breakthroughs in neuroscience. Four main dopaminergic pathways have been mapped in the brain: the nigrostriatal pathway originating in the substantia nigra and projecting to the dorsal striatum; the mesolimbic pathway that arises from the ventral tegmental area (VTA) and sends dopaminergic fibers to the nucleus accumbens (NAc); the mesocortical pathway that also arises from the VTA and projects to the cerebral cortex and the tuberoinfundibular pathway that connects the hypothalamus to the pituitary gland (Anden et al., 1964; Dahlstroem and Fuxe, 1964). DA activity (figure 1) is mediated by five dopaminergic receptors. These receptors are divided in two subfamilies: the D_1-like receptor subtypes (D_1R and D_5R) coupled to Gαs proteins and the D_2-like subfamily (D_2R, D_3R, and D_4R) coupled to Gαi proteins (Missale et al., 1998). D_1Rs and D_2Rs are the most abundant subtypes in the central nervous system, but D_1R is the most widespread (Jaber et al., 1996). D_1R mRNA has been detected in striatum, nucleus accumbens, olfactory tubercule, hypothalamus and thalamus (Jaber et al., 1996). D_5R is expressed at much lower level than the D_1R dopamine receptor and its distribution is limited to the hippocampus and thalamus (the lateral mamillary

nucleus and the parafascicular nucleus of the thalamus). D_2Rs are localized mainly in striatum, olfactory tubercule, nucleus accumbens, substantia nigra pars compacta, VTA and the pituitary gland. D_2Rs are both pre- and post-synaptic receptors contrary to D_1-like receptors which are mainly post-synaptic receptors (Jaber et al., 1996). D_4Rs have been found with a low expression in basal ganglia and a higher expression in frontal cortex, medulla, amygdala, hypothalamus and mesencephalon. However, this high expression is weak in comparison with other dopamine receptors (Jaber et al., 1996). D_3Rs are primarily confined in the limbic system (nucleus accumbens, olfactory tubercule and islands of Calleja). However, D_3R mRNA has been found in the medial prefrontal cortex (mPFC)–NAc–ventral pallidum loop (Koob and Le Moal, 1997) as well as in the ventral striatal, ventral pallidal, thalamic, and orbitofrontal loops (Everitt and Robbins, 2005).

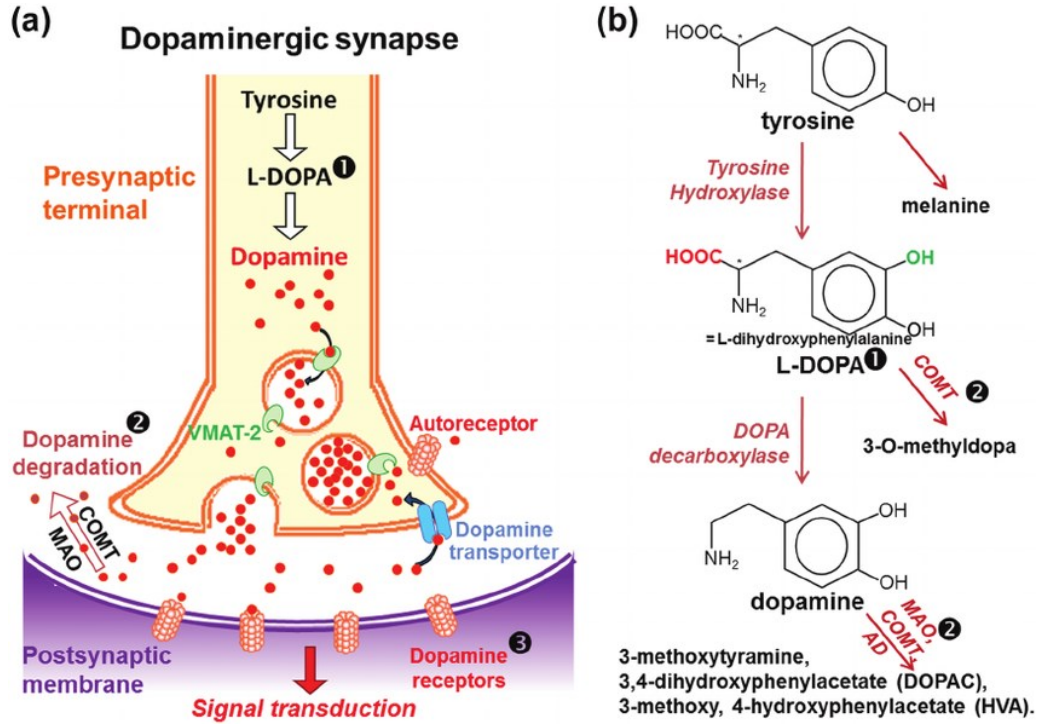

Figure 1: Dopaminergic synapse and dopamine metabolism. (a,b) In the presynaptic terminal of dopaminergic neurons, tyrosine is converted in L-DOPA by the activity of tyrosine hydroxylase. L-DOPA is subsequently transformed to the neurotransmitter DA by action of DOPA decarboxylase. (b) DA is then transferred in vesicles by the vesicular monoamine transporter 2 (VMAT-2). After exocytosis of the DA vesicles, DA binds to DA receptors on the postsynaptic membrane, leading to the transduction of the signal in the postsynaptic neuron. (a,b) DA is then recycled by reuptake via the DA transporter, or catabolized by the action of monoamine oxidase (MAO), cathecol-O-methyl transferase (COMT) and aldehyde dehydrogenase (AD) enzymes. (modified from Jones et at., 2012)

2. Dopamine D3 receptor

In 1990, for the first time, the rat D_3R was cloned and characterized (Sokoloff et al., 1990). Among DA receptors, D_3R exhibits the highest affinity for DA (70-fold higher than D_2R receptors), suggesting that DA may occupy D_3R in vivo for extended periods of time, leading to high spontaneous activation of D_3R (Richtand et al., 2001; Vanhauwe et al., 2000).

In rat, the largest D_3R densities have been found in granule cells of the islands of Calleja and in medium-sized spiny neurons of the rostral and ventromedial shell of nucleus accumbens (Diaz et al., 1994, 1995; Le Moine and Bloch, 1996). PET studies, carried out on baboons and mice by using the D2/3 PET agonist (with preferential selectivity and affinity for D_3R) [11C]-(+)-PHNO, have revealed a high expression of D_3R in ventral pallidum, substantia nigra, thalamus, and habenula (Rabiner et al., 2009). Its primary sequence is similar to that of D_2R, and to a lesser extent, to the D_4R. Activation of D_3R expressed in a transfected mesencephalic cell line inhibits dopamine release (Tang et al., 1994) and synthesis (O'Hara et al., 1996). Moreover, agonists with limited preference for the D_3R (Sautel et al., 1995) likewise inhibit dopamine release, synthesis and neuron electrical activity giving support to the hypothesis that D_3R could operate as autoreceptor. However, since DA agonists produce analogous inhibition of dopamine neuron activities in both WT and $D_3R^{-/-}$ mice, their selectivity towards D_3R *in vivo* has been put into question (Koeltzow et al., 1998). Clearer evidence

of its autoreceptor function arise from immunocytochemical experiments showing that D_3R is expressed in all dopaminergic neurons (Diaz et al., 2000). Yet, dopamine extracellular levels in NAc (Koeltzow et al., 1998) and striatum (Joseph et al., 2002) are higher in $D_3R^{-/-}$ compared to their WT littermates, suggesting a D_3R-mediated control of dopamine neurons activity. These convergent results supported the fact that $D_3R^{-/-}$ mice seem to be more responsive in several physiological situations compared to their WT littermates (Le Foll et al., 2005). By contrast, the study by Simpson et al. (2014) has been demonstrated that mice with a striatal overexpression of D_3Rs have less marked, but still noteworthy phenotype. Indeed, these mice exhibit a disrupted motivation, suggesting that targeting D_3R might have effect on motivational symptoms.

3. Dopamine D3 receptor and alcohol addiction

Alcohol addiction is a chronic relapsing disorder and shares many features of other chronic diseases such as hypertension and diabetes. Indeed, it is charachterized by a strong component of genetic susceptibility and is under influence of environmental factors (Heilig et al., 2011). Alcoholism produces about 10% of total disability-adjusted life years lost (measure of disease burden) in industrialized countries. Genetic and environmental factors in alcohol addiction may flow in very dissimilar sorts of vulnerability, ranging from amplified impulsivity and reward from alcohol to heightened stress responses and anxious personality traits (Goldman et al., 2005). Alcoholics are very heterogeneous in terms clinical features such as age of onset and family history (McLellan et al., 2000). The pathophysiology and etiology of alcohol addiction is still poorly understood and there are no effective pharmacological treatments.

It is well established in literature tha DA neurotransmission is involved in the pathophysiology of drug addiction. The mesolimbic DA pathway (figure 2) modulates the rewarding properties of drugs of abuse (Ikemoto and Bonci, 2013), such as ethanol and opiates (Pierce and Kumaresan, 2006). Alcohol induces an increase of DA release in the shell, but not in the core of NAc. (Bassareo et al., 2003; Cadoni et al., 2000). Moreover, in rats, intravenous administration of alcohol produces an increase in the firing rate of dopamine mesolimbic neurons in

a dose-dependent manner (Gessa et al., 1985). In line with these preclinical evidence, it has been reported that intoxicating doses of alcohol trigger dopamine release in the ventral striatum of humans (Boileau et al., 2003) and an activation of this brain area by alcohol-associated cues in abstinent high-risk drinkers and alcohol-dependent individuals has been found as well (Braus et al., 2001; Kareken et al., 2004). It is well demonstrated that D_3R, which is highly expressed in the shell of NAc, regulates the mesolimbic DA pathway and is involved in the neural mechanisms underlying drug seeking behavior (Heidbreder et al., 2005). Numerous studies have investigated the involvement of D_3R in ethanol-drinking paradigms (Cohen et al., 1998; Harrison and Nobrega, 2009; Heidbreder et al., 2007; Rice et al., 2012; Silvestre et al., 1996; Thanos et al., 2005). In this regard, the pharmacological manipulation of D_3R seems to produce different behavioural effects compared to the genetic manipulation of this dopaminergic receptor. Heidbreder and colleagues (2007) demonstrated that the selective D_3R antagonist SB277011A reduces alcohol intake and prevents relapse to alcohol-seeking behavior of male C57BL/6N mice exposed to oral operant self-administration. It has also been reported that the preferential DA D_3R antagonist S33138 decreases the binge drinking of ethanol without significantly affect the consumption of water (Rice et al., 2012). In agreement with these preclinical evidence, the dopamine receptor agonist with reasonable selectivity for the D_3R 7-OH-DPAT ((+/-)-7-hydroxy-N,N-(di-n-propyl-2-aminotetralin)) enhance both ethanol intake and

preference at the dose of 0.01 mg/kg (Silvestre et al., 1996). The study by Vengeliene et al. (2006) reported that the selective D_3R antagonist SB277011A induces a dose-dependent decrease of relapse-like drinking in the alcohol deprivation effect (ADE) model as well as a reduction in cue-induced ethanol-seeking behavior. Moreover, SB277011A significantly diminishes ethanol preference, intake and lick responses both in alcohol Preferring (P) and Non-Preferring (NP) rats tested in the two bottle choice paradigm (Thanos et al., 2005). Regarding the genetic manipulation of D_3R, $D_3R^{-/-}$ mice are resistant to ethanol sensitization (Harrison and Nobrega, 2009) and seem to have similar levels of ethanol intake (McQuade et al., 2003) compared to their WT littermates. Despite several studies have investigated the involvement of D_3R in ethanol reward, its precise role is largely unknown.

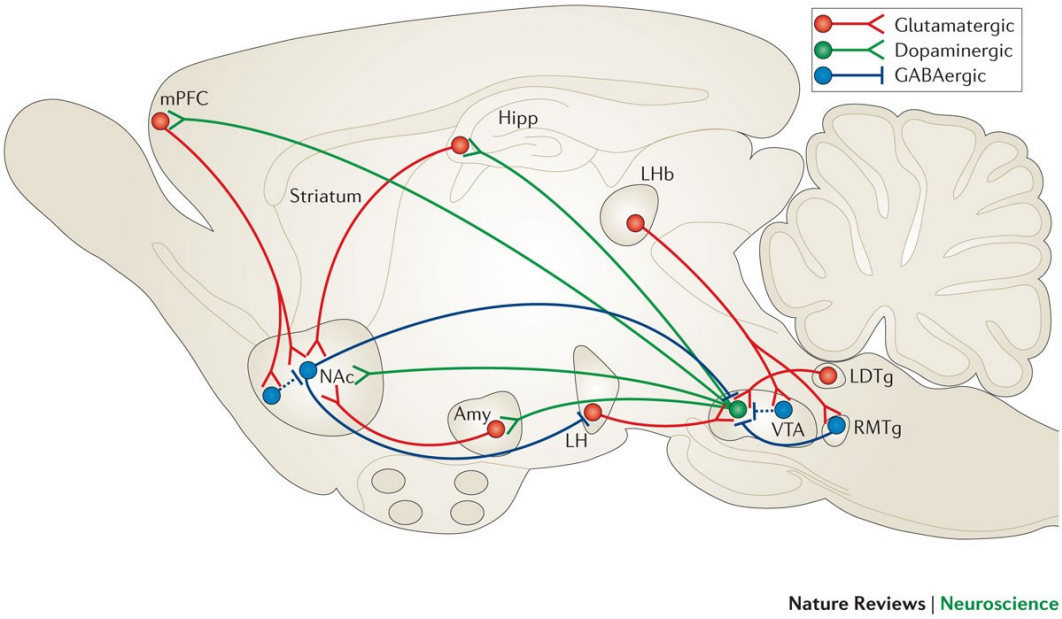

Figure 2: VTA– NAc reward circuit. The major reward circuit consists of dopaminergic fibers originating from the VTA and projecting to the NAc (in green), which release dopamine in response to reward-related stimuli. (from Russo and Nestler, 2013)

4. Dopamine D3 receptor and anxiety

Anxiety is a physiologic emotion under stressful and dangerous situations and is believed to be part of the evolutionary "fight or flight" reaction of survival. In many circumstances, the presence of anxiety may become maladaptive and constitutes a psychiatric disorder. By using significant prognostic tools, it is possible to classify anxiety disorders by their diagnostic subtype (obsessive-compulsive disorders, panic disorder, social phobia, generalized anxiety disorder [GAD] etc.). Diagnostic criteria for these several subtypes are given in the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV, text revision) and the International Classification of Diseases, 11th edition (ICD-11). The contribution of GABA system and $GABA_A$ receptor complex in the modulation of emotional processes is well known (Clement et al., 2002; Mehta and Ticku, 1999). However, the involvement of GABA-ergic innervation of particular brain structures in the regulation of anxiety is not satisfactorily documented. The mechanism of action of the most used anxiolytic drugs in clinic and preclinic research, benzodiazepines, relies on an enhancement in the affinity of the recognition site of $GABA_A$ receptors for GABA, ultimately potentiating its inhibitory action in the limbic system (Mehta and Ticku, 1999; Mohler et al., 2002).

A large body of behavioral and biochemical data indicate the involvement of DA neurotransmission in the pathophysiology of anxiety (Kienast et al., 2008). It is well established that stress triggers the mesocorticolimbic DA system activation and induces an increase of DA extracellular levels in the nucleus accumbens and medial prefrontal cortex, generating anxiolytic-like behavioral effects (Cabib et al., 1994; Dunn, 1988; Salamone, 1994; Simon et al., 1993). It is also demonstrated that anxiolytic drugs, such as diazepam and ICS 205930 (Finlay et al., 1995; Imperato et al., 1990), can attenuate stress-induced increase in DA concentration. However, behavioral evidences of the involvement of D_1R in anxiety are week and inconsistent (Bartoszyk, 1998; Rodgers et al., 1994). By contrast, animal studies described the anxiolytic-like effects of D_2R antagonists such as haloperidol or sulpiride (Costall et al., 1987; Pich and Samanin, 1986). Biochemical studies have indicated that haloperidol, sulpiride and quinpirole show affinity not only for D_2R but also for D_3R (Sokoloff et al., 1990). It has been demonstrated that $D_3R^{-/-}$ mice display low levels of anxiety tested in the open field arena and plus-maze tests (Steiner et al., 1997). Putative D_3R antagonists such as PNU-99194A and nafadotride showed anti-anxiety effects in the conflict drinking test in rats and exploration models in rats or mice (Gendreau et al., 1997; Rogoz et al., 2000). Furthermore, D_3R agonists, used at low doses, have been suggested to be involved in modulation of anxiety levels (Bartoszyk, 1998).

A dopaminergic-GABAergic interaction in the mesolimbic DA pathway is a well-documented phenomenon (figure 3). Indeed, binding of benzodiazepines to the α1-containing $GABA_A$ receptors on GABAergic VTA neurons leads to a reduction of the activity of these cells and a consequent decrease of GABA release, which results in a disinhibition of the dopaminergic VTA neurons and a resulting increase in DA release in the ventral striatum (Rudolph and Knoflach, 2011). Growing data suggest a D_3R mesolimbic modulation of GABA system. Indeed, dopamine via D_3R, may control the expression of innate anxiety-like behaviors through a down-regulation of GABAergic control over lateral/basolateral amygdala neurons (Diaz et al., 2011). A dynamic-dependent inhibition of $GABA_A$ modulated by D_3R receptor has also been found in NAc (Chen et al., 2006) and hippocampus (Hammad and Wagner, 2006; Swant et al., 2008). $D_3R^{-/-}$ mice exhibit low baseline anxiety levels and acute administration of diazepam is more effective in $D_3R^{-/-}$ than in WT littermates when tested in the elevated plus maze test (EPM) (Leggio et al., 2011). However, the precise role of the $D_3R/GABA_A$ systems interaction in both the modulation of anxiety-like behaviors and the effect of anxiolytic drugs remains poorly understood.

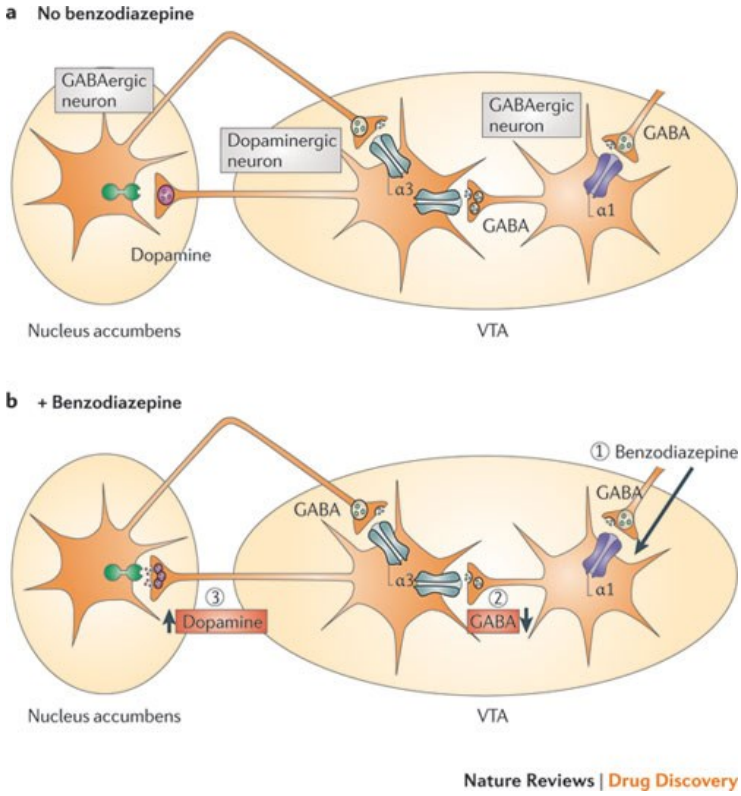

Figure 3: $GABA_A$ receptor subtypes in the mesolimbic dopaminergic systems. VTA GABAergic neurons express the α1 subunit, whereas dopaminergic neurons predominantly express the α3 subunit. Binding of benzodiazepines to the α1-containing $GABA_A$ receptors on GABAergic VTA neurons leads to a reduction of the activity of these cells and consequently to a decrease of GABA release, which in turn disinhibits the dopaminergic VTA neurons leading to an increase of DA release in the ventral striatum. In principle, benzodiazepines likely have functionally opposing actions via the α1-containing $GABA_A$ receptors on GABAergic neurons and on α3- containing $GABA_A$ receptors on the dopaminergic neurons of the VTA. However, the effect on the α1-containing $GABA_A$ receptors on the dopaminergic neuron is functionally predominant. (modified from Rudolph and Knoflach, 2011)

5. Design of the present research

Based on the reviewed data present in literature, the aim of the present thesis has been to assess the role of D_3R in the mesolimbic DA control of ethanol reward and to evaluate the recruitment of $GABA_A/D_3R$ interaction in the mesolimbic DA modulation of anxiety-like behavior. The following aspects were investigated:

1. Evaluating the basal behavior of $D_3R^{-/-}$ mice and their WT littermates in experimental models of anxiety and ethanol reward [two bottle choice, drinking in the dark (DID) and EPM]
2. Assessing the behavioural response of $D_3R^{-/-}$ mice and their WT littermates to selective D_3R antagonists, at different doses and testing time in different models of ethanol reward
3. Investigating the possible involvement of RACK1/BDNF/D_3R pathway in ethanol seeking behavior and the activation of dopaminergic neurotransmission in striatum of our mice.
4. Assessing the sensitivity of $D_3R^{-/-}$ mice and their WT littermates tested in the EPM test to repeated administration of diazepam.
5. Testing the hypothesis that genetic deletion or pharmacological blockade of D_3R affect $GABA_A$ subunit expression.

Chapter II

Dopamine D3 Receptor Is Necessary for Ethanol Consumption: An Approach with Buspirone

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Abstract

Mesolimbic dopamine (DA) controls drug- and alcohol-seeking behavior, but the role of specific DA receptor subtypes is unclear. We tested the hypothesis that D_3R gene deletion or the D_3R pharmacological blockade inhibits ethanol preference in mice. D_3R-deficient mice ($D_3R^{-/-}$) and their wild-type (WT)

littermates, treated or not with the D_3R antagonists SB277011A and U99194A, were tested in a long-term free choice ethanol-drinking (two-bottle choice) and in a binge-like ethanol-drinking paradigm (drinking in the dark, DID).

The selectivity of the D_3R antagonists was further assessed by molecular modeling. Ethanol intake was negligible in $D_3R^{-/-}$ and robust in WT both in the two-bottle choice and DID paradigms. Treatment with D_3R antagonists inhibited ethanol intake in WT but was ineffective in $D_3R^{-/-}$ mice. Ethanol intake increased the expression of RACK1 and brain-derived neurotrophic factor (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. Indeed, blockade of the BDNF pathway by the TrkB selective antagonist ANA-12 reversed chronic stable ethanol intake and strongly decreased the striatal expression of D_3R. Finally, we evaluated buspirone, an approved drug for anxiety disorders endowed with D_3R antagonist activity (confirmed by molecular modeling analysis), that resulted effective in inhibiting ethanol intake. Thus, DA signaling via D_3R is essential for ethanol-related reward and consumption and may represent a novel therapeutic target for weaning.

Keywords: Dopamine D_3 receptor; Knockout mice; Animal models of ethanol reward; Buspirone; BDNF

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1. Introduction

The mesolimbic dopamine (DA) pathway mediates the rewarding effects of drugs of abuse (Bowers et al., 2010; Ikemoto and Bonci, 2013; Koob, 1992; Robbins and Everitt, 1996; Wise and Bozarth, 1987), including ethanol and opiates (Pierce and Kumaresan, 2006; Wise and Bozarth, 1987). Both oral self-administration (Weiss et al., 1992) and systemic administration of ethanol increase the firing rate of mesolimbic dopaminergic neurons (Gessa et al., 1985; Mereu et al., 1984) and stimulate extracellular DA release in the striatum and in the nucleus accumbens (Imperato and Di Chiara, 1986; Yoshimoto et al., 1992). In a recent metaanalysis on published data sets of in vivo microdialysis in rat brain, the acute administrations of ethanol appear to increase the level of monoamines, including DA, globally and independent of the brain sites up to 270% of the basal concentrations (Brand et al., 2013). DA exerts its action through five receptor subtypes (D1–5R); the D3 receptor (D_3R) subtype has an important role in the modulation of the mesolimbic DA pathway and in the control of drug-seeking behavior (Heidbreder et al., 2005; Joyce and Millan, 2005). The D_3R is located both at pre- and post-synapses, in the ventral striatum (nucleus accumbens and island of Calleja) (Bouthenet et al., 1991; Murray et al., 1994); in these structures, stimulation of presynaptic D_3R may modulate DA synthesis and release (Levant, 1997). Several studies have explored the involvement of D_3R in ethanol-drinking

paradigms (Cohen et al., 1998; Harrison and Nobrega, 2009; Heidbreder et al., 2007; Rice et al., 2012; Silvestre et al., 1996; Thanos et al., 2005), but their precise role remains unclear. Indeed, pharmacological studies generally report that D_3R blockade decreases ethanol consumption (Heidbreder et al., 2007; Rice et al., 2012; Silvestre et al., 1996; Vengeliene et al., 2006); in contrast, genetic manipulation studies did not find a change in ethanol intake following D_3R gene deletion (McQuade et al., 2003). In the present study, we tested the hypothesis that D_3R gene deletion or the D_3R pharmacological blockade inhibits the ethanol preference and the voluntary intake in mice. Mice $D_3R^{-/-}$ and their wild-type (WT) littermates, treated or not with D_3R selective antagonists, were tested in a long-term free choice ethanol-drinking paradigm (two-bottle choice) (McQuade et al., 2003; Wise, 1973) and in a binge-like ethanol-drinking paradigm (drinking in the dark, DID). Activation of the RACK1/BDNF (brain-derived neurotrophic factor)/D_3R pathway (Jeanblanc et al., 2006) and activation of DA transmission were assessed at the end of behavioral experiments. The RACK1/BDNF/D_3R pathway was here considered because D_3R expression is related to BDNF (Guillin et al., 2001; Le Foll et al., 2005b) and ethanol exposure is able to increase RACK1 translocation into the nucleus of neurons, which increases expression of BDNF (Jeanblanc et al., 2006; McGough et al., 2004). Finally, the effect of buspirone was evaluated in the drinking paradigms. Because buspirone is an already

approved drug for anxiety disorders, endowed with D_3R antagonist activity, it may be easier to translate to the clinic practice.

2. Materials and methods

2.1. Animals

D_3R null ($D_3R^{-/-}$) mice and their WT littermates (males, 8–12 weeks old) were individually housed, with free access to chow and water (except in the ethanol-drinking procedures), in an air-conditioned room, with a 12-h light–dark cycle. $D_3R^{-/-}$ mice were 10th–12th generation of congenic C57BL/6J mice, generated by a back-crossing strategy (Accili et al., 1996). All experiments were carried out according to the Directive 2010/63/EU and to the Institutional Animal Care and Use Committee of Catania University.

2.2. Drugs and treatment

Ethanol, U99194A maleate, SB277011A hydrochloride, buspirone hydrochloride, 8-OH-DPAT and ANA-12 were from Sigma (St Louis, MO). All drugs were dissolved in saline and intraperitoneally (i.p.) injected (in a volume of 10 ml/kg), except ANA-12 that was dissolved in 10% dimethyl sulfoxide. U99194A was used at 10 mg/kg (Harrison and Nobrega, 2009), SB277011A was used at 10 mg/kg (Song et al., 2012), buspirone was used in the range 0.1–10 mg/kg (Martin et al., 1992), 8-OH-DPAT was used at 1 mg/kg (Martin et al., 1992), and ANA-12 was used at 0.5 mg/kg (Cazorla et al., 2011).

In the two-bottle choice paradigm, after 30 days of voluntary alcohol-drinking procedure, $D_3R^{-/-}$ and WT were randomly allocated to the eight

experimental groups (n= 6/10 per group): WT/vehicle, WT/U99194A, WT/SB277011A, WT/buspirone, $D_3R^{-/-}$/vehicle, $D_3R^{-/-}$/U99194A, $D_3R^{-/-}$/SB277011A, and $D_3R^{-/-}$/buspirone. Animals were i.p. injected once a day, for 14 consecutive days. On day 14, animals were sacrificed 1 h after the last administration and brain tissues were taken. In another set of experiments, after 30 days of voluntary alcohol-drinking procedure, mice were randomly allocated to five experimental groups (n= 5/7 per group): WT naive, WT/vehicle, WT/ANA-12, $D_3R^{-/-}$/ vehicle, and $D_3R^{-/-}$/ANA-12. Animals were i.p. injected once a day, for 4 consecutive days with the selective Trkb antagonist ANA-12 at 0.5 mg/kg (Cazorla et al., 2011; Vassoler et al., 2013). On day 4, animals were sacrificed 1 h after the last administration and brain tissues were taken.

In the DID paradigm, mice were allocated to 10 experimental groups (n= 5/6 per group): WT naive, $D_3R^{-/-}$ naive, WT/vehicle, $D_3R^{-/-}$/vehicle, WT/SB277011A, $D_3R^{-/-}$/ SB277011A, WT/buspirone 0.1 mg/kg, WT/buspirone 1 mg/ kg, WT/buspirone 3 mg/kg, and WT/buspirone 10 mg/kg. In another set of experiments, mice were allocated to four experimental groups (n= 5/6 per group): WT/vehicle, WT/8-OH-DPAT, $D_3R^{-/-}$/vehicle, and $D_3R^{-/-}$/8-OH-DPAT, and they were tested in the DID paradigm. Animals were i.p. injected 1 h before the behavioral procedure.

2.3 8-OH-DPAT-Induced Hypothermia

Body temperature was measured intrarectally using a lubricated probe inserted B2 cm and a digital thermometer (CEM advanced thermometer; DT-610B). Mice were moved to the behavioral room and two baseline temperature measurements were taken. After 10 min, animals received an i.p. injection of vehicle or 1 mg/kg 8-OH-DPAT or 3 mg/kg buspirone. The body temperature was recorded every 15 min for a total of 45 min.

2.4. Behavioral tests

2.4.1. Two-Bottle Choice Paradigm

$D_3R^{-/-}$ (n=30) and WT (n=30) mice 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 acquisition of position bias. Ethanol and water intake was measured as daily consumption in grams. The experiments 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 days), 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 days) by 10% ethanol only.

2.4.2. Drinking in the dark (DID)

The 4th version of the behavioral paradigm was used, as described by Rhodes et al. (2005). The procedure started 3 h after lights off in the animal room. Water bottles were replaced with graduated tubes with stainless steel drinking spouts containing 20% (v/v) ethanol in tap water. This was done in home cages where animals were singly housed (Rhodes et al., 2005). The ethanol tubes remained in place for 2 h. After the 2-h period, intakes were recorded, and the ethanol tubes were replaced with water tubes. This procedure was repeated on days 2 and 3. On day 4, the procedure was again repeated except that the ethanol tubes were left in place for 4 h, and intakes were recorded after 4 h.

2.5 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 ml final volume) contained 0.5 mM primers, 1.6 mM Mg^{2+} , and 1 x 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 DCt comparative method.

2.6 Western Blot Analysis

Protein extracts from striatum and cerebellum were run in SDS-PAGE, blotted, and probed for non-phosphorylated and phosphorylated forms of DARPP-32, GSK-3b, and Trkb, with primary antibodies (Cell Signalling Technology, Beverly, MA), diluted at 1:1000, 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).

2.7. Statistical analysis of data

Data were analyzed using one- or two-way analysis of variance (ANOVA). The post hoc Newman–Keuls test was used for multiple comparisons; p-values <0.05 were considered as significant.

3. Results

3.1 $D_3R^{-/-}$ mice exhibited lower ethanol intake

As shown in Figure 1a and b, WT mice exhibited a high intake of ethanol-containing solution. In contrast, $D_3R^{-/-}$ mice showed a low ethanol intake (Figure 1a and b). During the entire period of observation (44 days), WT mice maintained their preferential intake of ethanol, whereas $D_3R^{-/-}$ mice maintained a preferential intake of water ($F_{(1,307)}$=1170.08, $p<0.001$). There was no difference between WT and $D_3R^{-/-}$ mice in terms of total amount of fluid intake (ethanol + water) (Figure 1c). In the DID paradigm, $D_3R^{-/-}$ mice also showed a lower ethanol intake compared with their WT counterparts ($F_{(3,97)}$= 13.90, $p<0.01$, 2nd day; $F_{(3,97)}$=21.04, $p<0.001$, 3rd day; Figure 2a).

3.2 Blockade of D_3R Inhibited Ethanol Intake

In the two-bottle choice paradigm, after 30 days of stable ethanol/water intake, mice were treated with D_3R antagonists (U99194A or SB277011A). As shown in Figure 1d and e, treatment of WT with each D_3R antagonist decreased voluntary ethanol intake ($F_{(2,56)}$= 55.23 $p<0.01$, for both U99194A and SB277011A). Treatment of $D_3R^{-/-}$ mice with U99194A and SB277011A did not change ethanol intake (data not shown). Neither in WT nor in $D_3R^{-/-}$ mice total fluid intake was affected by treatments (Figure 1f and data not shown).

SB277011A also significantly decreased ethanol intake in WT mice tested in the DID ($F_{(3,48)}$= 8.67, $p<0.01$, 1st day; $p<0.05$ 2nd day; Figure 2b), while it did not change ethanol intake of $D_3R^{-/-}$ in the DID paradigm (Figure 2c).

3.3 RACK1, BDNF, and DA D_3R expression were increased in the striatum of WT mice following chronic ethanol intake

BDNF induces D_3R expression in the ventral striatum, both during development and in adulthood (Guillin et al., 2001). RACK1, a mediator of chromatin remodeling, regulates in an exon-specific manner the expression of the BDNF gene (He et al., 2010) and the RACK1/BDNF pathway is activated upon exposure to ethanol (McGough et al., 2004). We therefore assessed D_3R, BDNF, and RACK1 mRNA expression in striatum of WT that had free access to either water only or to both water and ethanol. Figure 3a shows that chronic ethanol intake increased D_3R mRNA expression in striatum ($F_{(3,23)}$= 170.4, $p<0.05$). Long-term access to ethanol also increased BDNF (Figure 3b, $F_{(7,47)}$= 48.05, $p<0.01$) and RACK1 (Figure 3c, $F_{(7,47)}$= 21.14, $p<0.01$) mRNA in striatum of WT mice. Long-term ethanol exposure appeared to be associated with BDNF/RACK1 overexpression, but interpretation of these data was made difficult by the different ethanol intake in the two genetic groups, as it was very high in WT and very low in $D_3R^{-/-}$ mice. To address this issue, some WT and $D_3R^{-/-}$ mice were subjected to forced ethanol intake, that is, they had access to ethanol 10% solution only. As shown in Figure 3d and e, forced ethanol intake induced a significant

overexpression of BDNF ($F_{(7,47)}$=48.05, p<0.05, p<0.01) and RACK1 ($F_{(7,47)}$= 21.14, p<0.05, p<0.05) mRNAs in striatum of both WT and $D_3R^{-/-}$ mice. We also tested the effects of the D_3R antagonists SB277011A and buspirone (see also below) on mRNA expression of D_3R, BDNF, and RACK1. None of these values were changed by a 14-day treatment with SB277011A or buspirone (Figure 3f–h).

3.4 Blockade of the BDNF receptor TrkB inhibited ethanol intake and decreased D_3R expression

TrkB is the high affinity receptor for BDNF, belonging to the family of tyrosine kinase receptors, which undergo autophosphorylation upon agonist binding (Soppet et al., 1991). In order to assess the role of BDNF pathway in ethanol intake, we used the recently available TrkB selective antagonist ANA-12 (Cazorla et al., 2011). After 30 days of stable ethanol/water intake, mice received daily i.p. injections of either vehicle or ANA-12 (Figure 4a and b). ANA-12 reversed the stable ethanol intake of WT mice ($F_{(7,42)}$=30.53, p<0.001) but did not change the voluntary and the forced ethanol intake of $D_3R^{-/-}$ mice (data not shown). Neither in WT nor in $D_3R^{-/-}$ mice total fluid intake was affected by treatment with ANA-12 (Figure 4c and data not shown). Also in the DID paradigm ANA-12 was effective in reducing ethanol intake in WT mice ($F_{(3,55)}$=6.64, P<0.05, Figure 4d), whereas it did not change ethanol intake in $D_3R^{-/-}$ mice (Figure 4e). To assess the selective blockade of the BDNF receptor in

striatum by ANA-12, we determined, by immunoblot, the abundance of phosphorylated TrkB. As shown in Figure 4f, treatment of WT with ANA-12 significantly decreased phosphorylation of TrkB ($F_{(3,35)}$=184.5, p<0.01). Finally and more interestingly, ANA-12 strongly decreased D_3R mRNA expression in the striatum of WT mice exposed to voluntary ethanol intake (Figure 4f, $F_{(3,35)}$=184.5, P<0.001).

3.5 Buspirone Inhibited Ethanol Intake

In the two-bottle choice paradigm, after 30 days of stable ethanol/water intake, mice were treated with buspirone (1 mg/kg/day). As shown in Figure 5a and b, treatment of WT with buspirone significantly decreased voluntary ethanol intake ($F_{(1,28)}$=20.88, p<0.05). Treatment of $D_3R^{-/-}$ mice with buspirone did not change ethanol intake (data not shown). Neither in WT nor in $D_3R^{-/-}$ mice total fluid intake was affected by treatment (Figure 5c and data not shown). The treatment with buspirone also significantly decreased ethanol intake in WT mice when tested in the DID. Dose ranging of buspirone (0.1, 1, 3, and 10 mg/kg) showed that treatment of WT with buspirone at the doses of 3 and 10 mg/kg significantly decreased ethanol intake both in the 1st day ($F_{(4,75)}$=31.24, p<0.05) and in the 2nd day ($F_{(4,75)}$= 31.24, p<0.01 3 mg/kg; p<0.05 10 mg/kg) of the behavioral paradigm (Figure 5d). Buspirone did not change ethanol intake of WT in the 3rd and 4th days of DID (Figure 5d). Furthermore, in the DID paradigm, 3

mg/kg buspirone did not change ethanol intake in $D_3R^{-/-}$ mice (data not shown). Because buspirone is also known as a 5-HT1A agonist, the D_3R specific effect of buspirone in decreasing ethanol intake was confirmed by using the selective 5-HT1A agonist, 8-OH-DPAT. As shown in Figure 5e, treatment with 8-OH-DPAT (1 mg/kg, i.p.) in WT and $D_3R^{-/-}$ mice mice did not affect ethanol intake (Figure 5e and data not shown). As expected, the 5-HT1A selective agonist 8-OH-DPAT decreased the body temperature of WT mice ($F_{(2,39)}$=14.99, $p<0.001$) (Figure 5f). Buspirone (3 mg/kg) decreased the body temperature of WT mice only transiently (Figure 5f).

3.6 DA receptor signaling in striatum of WT and $D_3R^{-/-}$ mice exposed to ethanol

Activation of D1 receptor results in activation of adenylyl cyclase/cAMP/protein kinase A (PKA) signaling; a major substrate for PKA in the striatum is DARPP-32. D2-like receptors regulate the activity of the protein kinases Akt and GSK3b; stimulation of either D_2R or D_3R results in phosphorylation of Akt and GSK3b (Mannoury la Cour et al., 2011). In order to assess activation of dopaminergic transmission in striatum, we determined, by immunoblot, the abundance of phosphorylated DARPP-32 (Thr 34) and of phosphorylated GSK3b (Ser 9). As shown in Figure 6, posphoGSK3b was more abundant in striatum of $D_3R^{-/-}$ mice than in WT mice, whereas phosphoDARPP-32 showed the same tendency, though it did not reach statistical significance.

Treatment of WT mice with SB277011A induced phosphorylation of DARPP-32 and GSK3b, up to the level seen in $D_3R^{-/-}$ mice. In contrast, in cerebellum, there was no difference in the level phosphoDARPP-32 and posphoGSK3b between WT e $D_3R^{-/-}$ mice, nor it was influenced by SB277011A treatment in WT.

4. Discussion

This study demonstrates that D_3R is necessary for ethanol consumption in mice, because either D_3R gene deletion or D_3R pharmacological blockade by selective D_3R experimental antagonists or the approved drug buspirone, inhibits alcohol intake. The D_3R overexpression induced by ethanol intake associated with the activation of RACK1/BDNF may represent the basis for a reinforcing mechanism of ethanol intake. Indeed, although selective blockade of the TrkB reversed stable intake of ethanol in WT mice and decreased D_3R expression levels in their striatum, it was ineffective in $D_3R^{-/-}$ mice. It seems that D_3R, among D2-like receptors, is the key player in addiction, particularly in reward mechanisms. Indeed, although the D_2R is associated with mesocortical and mesohippocampal DA pathway, the D_3R is associated with the ventral mesolimbic DA system (Sokoloff et al, 1990). Previous studies reported low levels of D_2R both in animal models and in patients addicted to cocaine, alcohol, metamphetamine, and nicotine (Volkow et al., 2009). Conversely, upregulation of D_3R expression has been reported following exposure to DA elevating drugs (Boileau et al., 2012; Heidbreder and Newman, 2010; Le Foll et al., 2005b; Mash, 1997; Segal et al., 1997; Staley and Mash, 1996). An important interpretative issue is the genetic background on which the D_3R null mutation was placed. Specific behavioral phenotypes are differently expressed in different strains of mice (Nelson and

Young, 1998). The $D_3R^{-/-}$ mice we used are on the C57BL/6J background (Accili et al., 1996), a strain where ethanol preference and sensitivity is well documented (Crabbe et al., 1983). Interestingly, $D_3R^{-/-}$ mice have extracellular DA levels twice as high as their WT littermates (Joseph et al., 2002; Koeltzow et al., 1998); this enhanced DA tone and the resulting adaptations may reflect removal of the inhibitory influence of D_3R in the control of basal extracellular DA levels (Le Foll et al., 2005a), giving support to an autoreceptor role for D_3R in the mesolimbic areas of the brain (Diaz et al., 2000). 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 (Accili et al., 1996; Le Foll et al., 2005a; Le Foll et al., 2002). Here we found that $D_3R^{-/-}$ mice chronically exposed to the voluntary ethanol intake paradigm, drink very low quantities of ethanol in comparison with their WT littermates. This observation cannot be attributed to differences in metabolism (McQuade et al., 2003), locomotor activity (Harrison and Nobrega, 2009), or taste reactivity (McQuade et al., 2003) between WT and $D_3R^{-/-}$ mice. The lower ethanol intake of $D_3R^{-/-}$ mice in comparison with their WT control mice seems 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 et al., 2003). This may be due, at least in part, to some important differences in experimental procedures used. Indeed, McQuade et al. (2003), that have shown no

difference between $D_3R^{-/-}$ mice and WT in the 24-h access paradigm, used a different experimental procedure in the two-bottle choice paradigm. First, they used just 4 days of adaptation period before ethanol exposure. Second, they tested both $D_3R^{-/-}$ mice and WT animals with increasing concentrations of ethanol in subsequent 7-day steps. In the first step, 3% ethanol, in the second step 6%, in the third step 10%, in the 4th 15%, and finally, in the 5th 20% ethanol. Thus, the behavioral paradigm used by McQuade and co-workers is quite different from our paradigm. From our experience, for these mice it is to have a long period of habituation in the two-bottle paradigm (15 days) before to start with the ethanol access procedure. It is likely that the progressive increase of the ethanol concentration every 7 days, may induce an adaptation to the ethanol that damps the difference between $D_3R^{-/-}$ and WT mice. Furthermore, in the McQuade's study, the relative positions of the ethanol and water bottle were determined randomly each day, whereas in our experiments the position of tubes was interchanged (left/right) every 24 h, to prevent acquisition of position bias. The random change of bottles may expose a given animal to access the same solution (either ethanol or water) in the same position for two/three days consecutively, which may interfere with the results of the experiment during a short period of observation (7 days). In the study by Boyce-Rustay and Risinger (2003), C57 animals were used as control of $D_3R^{-/-}$ mice. These experiments are not comparable to our experiments using WT littermates as controls. Moreover, again,

in this study increasing concentrations of ethanol were used in 8-day steps (3 and 10%). Thus, (i) the behavioral procedure is different; (ii) an adaptation to ethanol may occur and damp the difference between genotypes. 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 at doses reported to selectively target the D_3R (Carr et al., 2002; Reavill et al., 2000). Before administering these drugs, we performed a molecular modeling study to gain information on the interaction of U99194A and SB277011A with D_3R. As illustrated in Supplementary Information, 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. We found that both U99194A and SB277011A induced a significant decrease in voluntary ethanol intake in WT but not in $D_3R^{-/-}$ mice. This pharmacological evidence reinforces the view that the D_3R is necessary for ethanol consumption in mice and is consistent with rat data showing that D_3R antagonism reduces relapse-like drinking and cue-induced ethanol-seeking behavior (Vengeliene et al., 2006).

We confirmed the primary role of D_3R in the control of ethanol-drinking behavior in a binge-like ethanol-drinking paradigm (Crabbe et al., 2011; Rhodes et al., 2005; Rhodes et al., 2007). Here, again, $D_3R^{-/-}$ mice exposed to DID drank lower quantities of ethanol in comparison with their WT littermates, and D_3R

blockade by SB277011A decreased ethanol intake in WT but not in $D_3R^{-/-}$ mice. No differences were recorded in the DID at day 4. Indeed, there was neither a genotype effect between WT and $D_3R^{-/-}$ mice nor a treatment effect with the SB277011A in WT mice. In general, the binge-like behavior is captured by the 2 h time window that detects differences between treatments/genotypes better than the 4 h window, because the cumulative intake over 4 h makes smaller the proportion of differences (Rhodes et al., 2005). Thus, it is likely that, the lack of differences on day 4 is due to the longer lasting access to ethanol that produced overall a higher consumption, potentially masking the genotype/treatment effect on binge-like drinking behavior occurring in the first 2 h. Enhanced D_3R expression in striatum following long-term alcohol consumption has been previously reported in both mice and rats (Jeanblanc et al., 2006; Vengeliene et al., 2006). Our data show and confirm that chronic voluntary ethanol intake upregulated D_3R mRNA expression in the striatum of WT mice. Interestingly, D_3R expression is increased by exposure to other addictive drugs, such as nicotine and cocaine, in caudate–putamen (Neisewander et al., 2004) and in nucleus accumbens of rats (Le Foll et al., 2003, 2005b) and humans (Staley and Mash, 1996). Expression of D_3R therefore appears to be 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 the nucleus accumbens to BDNF derived from cortical sources (Guillin et al., 2001; Le Foll et al., 2005b);

furthermore, ethanol exposure increases both BDNF and D_3R within the striatum itself (Jeanblanc et al., 2006; McGough et al., 2004). 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 (McGough et al., 2004). Jeanblanc et al. (2006) proposed that the RACK1/BDNF/D_3R pathway is involved in the control of ethanol consumption in mice. Our hypothesis is that activation of RACK1/BDNF by ethanol may induce expression of D_3R, which in turn controls and maintains ethanol consumption. This hypothesis is supported by the data we generated showing that: (i) ethanol intake is negligible in $D_3R^{-/-}$ mice and robust in WT; (ii) increase in RACK1/BDNF/D_3R is maintained during chronic ethanol intake in WT; (iii) forced ethanol intake increases RACK1/BDNF even in $D_3R^{-/-}$ mice. Furthermore, chronic voluntary ethanol intake increased D_3R expression in striatum concomitant with increased expression of BDNF. It is noteworthy that, in the basal condition, $D_3R^{-/-}$ mice exhibited higher BDNF than WT, consistent with a tendency reported in a recent study (Xing et al., 2012). When subjected to forced ethanol intake, $D_3R^{-/-}$ mice showed a robust increase in BDNF expression in the striatum. Therefore, chronic ethanol intake increases BDNF independently of D_3R receptor stimulation. The finding that chronic ethanol intake increased RACK1 in striatum of both WT and $D_3R^{-/-}$ mice provides additional evidence for the role of RACK1/BDNF/D_3R pathway in ethanol intake; chronic ethanol intake stimulates

RACK1/BDNF pathway leading to D_3R overexpression and addictive behavior in WT, but not in $D_3R^{-/-}$ mice, because this latter lacks D_3R. To provide additional evidence, we blocked the BDNF pathway by using the TrkB specific antagonist, ANA-12. We found that ANA-12 reversed ethanol intake both in the twobottle choice and DID paradigms and strongly decreased the expression of D_3R in the striatum of WT-treated mice. Recently, D_3R on VTA-SN dopaminergic neurons were found to mediate neuroplasticity effects of several addictive drugs (Collo et al., 2012; Collo et al., 2013). Therefore, our conclusion about the engagement of striatal RACK1, BDNF, and D_3R in mediating ethanol consumption may be only a part of a more complex mechanism, whose elucidation may require an assessment of the effects of ethanol intake in the VTA-SN dopaminergic neurons.

Finally, in a translational perspective, we tested buspirone, a drug marketed for anxiety disorders, endowed with D_3R antagonist (Bergman et al., 2013; Le Foll and Boileau, 2013; Newman et al., 2012) and 5-HT1A partial agonist activity (Wong et al., 2007). Notably, buspirone shows also high affinity for other D2-like receptors (Bergman et al., 2013; Kula et al., 1994; Tallman et al., 1997). D_3R antagonists may be effective for treating substance use disorders and buspirone has proven effective in several preclinical model of drug abuse (Heidbreder and Newman, 2010; Higley et al., 2011; Song et al., 2012), but no studies have, so far, investigated its D_3R antagonist action in ethanol consumption. By both radioligand binding and molecular modelling studies (see Supplementary

Information), we found that buspirone: (i) shows slight higher affinity at D_3R than at D_2R (Ki, 29 vs 62 nM, respectively) and may form interactions comparable with those of SB277011A in D_3R, having the antagonist binding mode at D_3R, (ii) displays a distinct interaction from the other two antagonists SB277011A and U99194A (different binding energy, different interaction patterns) with D_3R, consistent with their distinct chemical structure. Thereafter, we found that buspirone induced a significant decrease in ethanol intake in both two-bottle choice and DID paradigms. The dose of 1 mg/kg inhibited ethanol intake in both paradigms, though its effect did not reach statistical significance in DID; 3 and 10 mg/kg, however produced a significant effect in DID. We confirmed the specificity of D_3R effect by using a selective 5-HT1A agonist, 8-OH-DPAT, in the DID. Treatment with 8-OH-DPAT did not impact ethanol intake, whereas, as expected, decreased the body temperature in a stable manner. In a translational perspective, an important issue is the actual availability of buspirone to bind D_3R in human CNS. Reported buspirone's affinity toward human recombinant D_3R ranges from 3.5 to 98 nM (Bergman et al., 2013; Newman et al., 2012), which partially overlaps its affinity for 5-HT1A receptors; because buspirone binding to 5-HT1A is considered the basis of its anxiolytic activity in humans, it is likely that anxiolytic doses are sufficient to occupy also D_3R in human CNS. However, the D_3R-related therapeutic potential of buspirone requires more detailed information,

including measurements of D_3R receptor occupancy in human PET studies, as an essential prerequisite to clinical application.

Finally, as $D_3R^{-/-}$ mice have been shown to exhibit extracellular DA levels substantially higher than WT, as assessed by microdialysis (Koeltzow et al., 1998), a phenomenon related to the lack of autoreceptor function (Joseph et al., 2002), we hypothesized that ethanol intake effectively stimulates DA release and transmission in WT, but not in $D_3R^{-/-}$ mice, presumably because this latter already displays high extracellular DA levels. To test the hypothesis that treatment with D3R antagonists mimicked the high DA phenotype documented in $D_3R^{-/-}$ mice (Koeltzow et al., 1998), we assessed phosphorylation of DARPP32, that is increased by different addictive drugs, including ethanol (Nuutinen et al., 2011; Svenningsson et al., 2005), and of GSK3b, that is linked to D2-like receptors signaling cascade (Beaulieu et al., 2007; Li et al., 2009), particularly under hyper-DAergic conditions (Li et al., 2009). Treatment with SB277011A increased phosphorylation of DARPP32 and of GSK3b to a level similar to that of $D_3R^{-/-}$ mice. Thus, chronic blockade of the D_3R or its genetic deletion increased DA transmission in striatum, consistent with increased extracellular DA (Joseph et al., 2002; Koeltzow et al., 1998).

In conclusion, either D_3R gene deletion or D_3R pharmacological blockade inhibit ethanol intake. Thus, pharmacological antagonism selectively targeting D_3R may provide a basis for novel weaning treatments to inhibit ethanol

consumption. In this context, buspirone, a drug marketed as anxiolytic since more than 25 years and endowed with D_3R antagonist activity, exhibits, translational potential for treating alcohol addiction.

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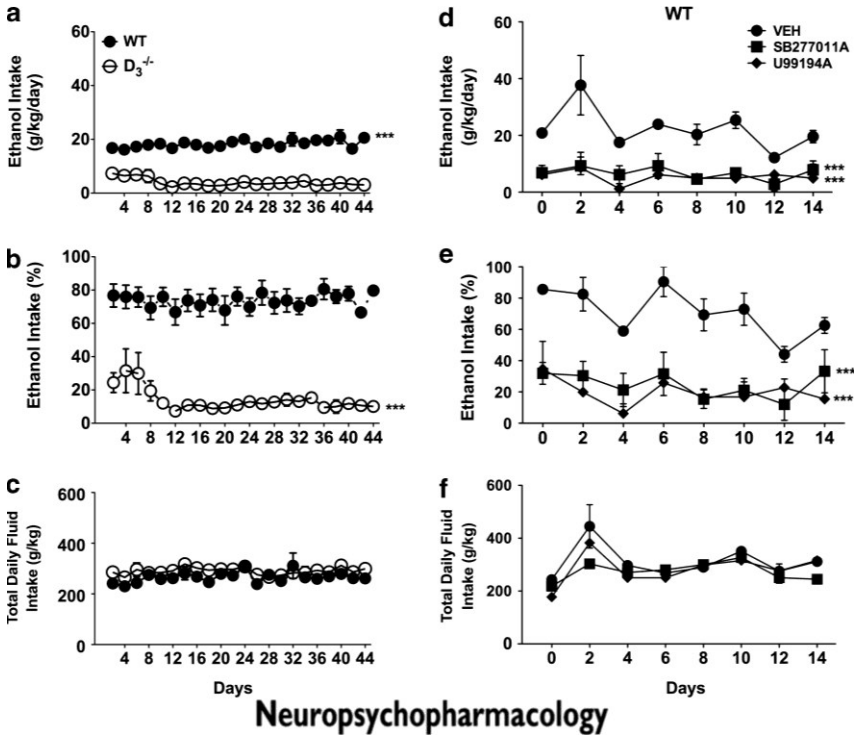

Figure 1 In the two-bottle choice paradigm, $D_3R^{-/-}$ mice show a lower voluntary ethanol intake as compared with wild-type (WT). D3 pharmacological antagonism inhibits ethanol intake in WT mice. (a, b) Voluntary ethanol intake was measured every 24 h, for 44 days, in WT (n=30) and $D_3R^{-/-}$ (n=30) mice that had free access to water and ethanol solution (10%). (c) Shows total fluid intake that was not different in the two groups. (d, e), 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, VEH, n=10), U99194A (n=10) or SB277011A (n=10), either drug at 10 mg/kg. (f) Total fluid intake in either group that was not affected by drug treatment. ***$p<0.001$ vs water or vehicle (VEH). One-way ANOVA and Newman–Keuls post hoc test.

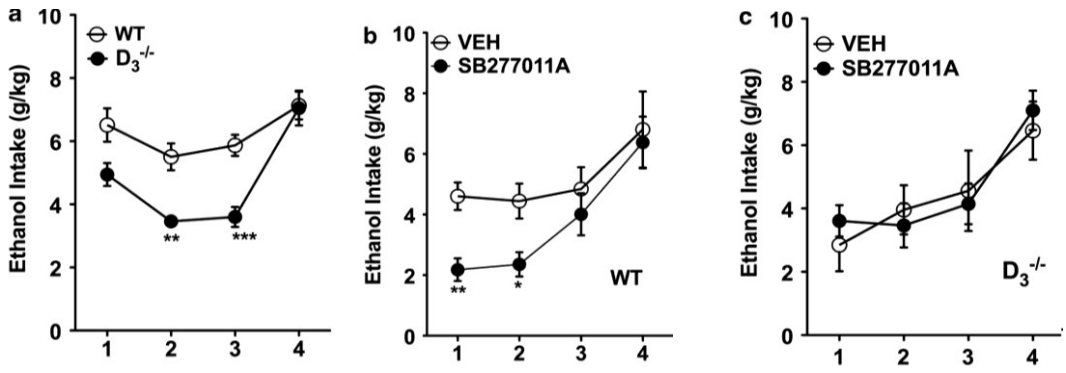

Figure 2 In the drinking in the dark (DID) paradigm, $D_3R^{-/-}$ mice show a lower ethanol intake as compared with their wild-type (WT) littermates. The D3 antagonist SB277011A inhibits ethanol intake of WT but not in $D_3R^{-/-}$ mice. (a) DID was measured, for 4 days, in WT (n=12) and $D_3R^{-/-}$ (n=12) mice that had limited access (2 h/day for 3 days and 4 h the 4th day) to ethanol solution (20%). (b, c) 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, VEH, n=10), or SB277011A (n=10), at 10 mg/kg. *p<0.05, **p<0.01, ***p<0.001 vs wild-type (WT) or vehicle (VEH). One-way ANOVA and Newman–Keuls post hoc test.

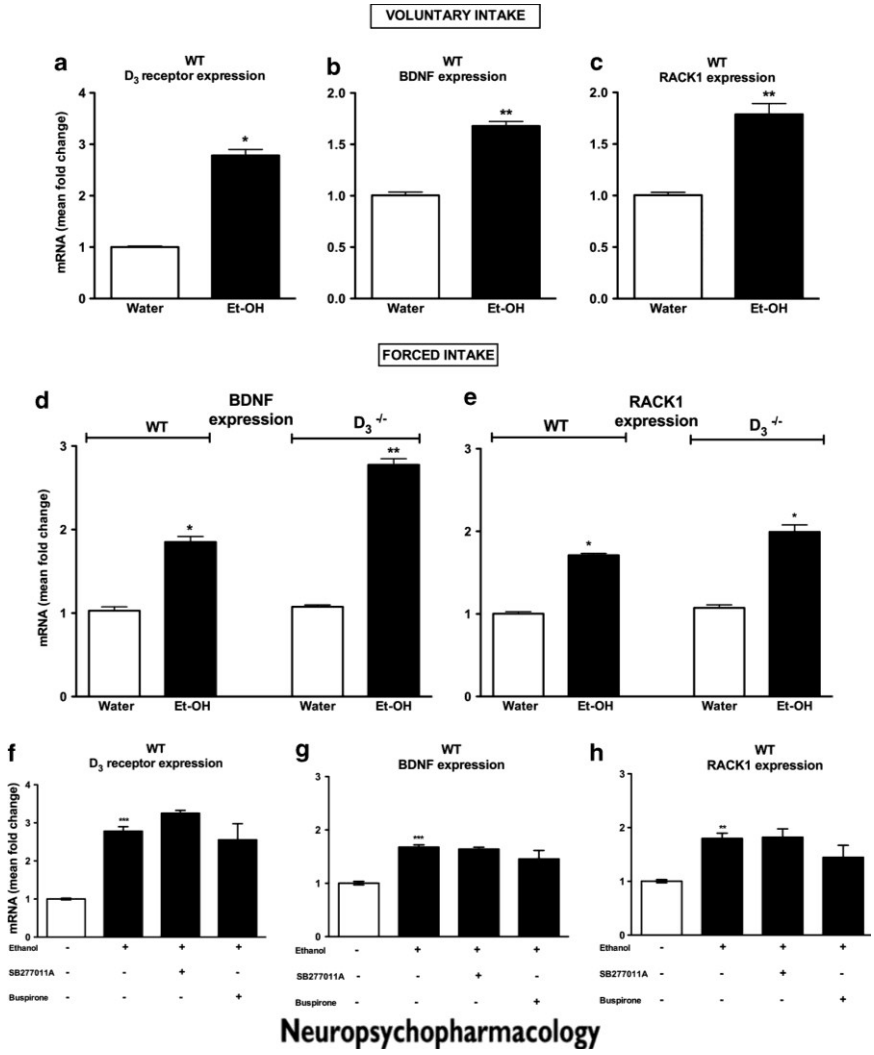

Figure 3 Chronic ethanol intake induces D_3R upregulation, associated with the activation of BDNF/RACK1 pathway. Abundance of transcripts in striatum was assessed by quantitative RT-PCR after 44 days of free access to water only (white columns), or to both water and ethanol (black columns, upper panels) or forced ethanol intake (black columns lower panels). In the forced alcohol-drinking procedure (d–h), $D_3R^{-/-}$ and WT received 10% ethanol only, with or without SB277011A or buspirone for 14 days. (a, f) D3 Expression profile in WT; (b, d, g) brain-derived neurotrophic factor (BDNF) expression profile in WT and $D_3R^{-/-}$ mice; (c, e, h) RACK1 expression profile in WT and $D_3R^{-/-}$ mice. Mean fold changes are expressed relative to transcript levels in controls (WT having access to water only). Each column is the mean (± SEM) from five different samples. *p<0.05, **p<0.01 vs water. One-way ANOVA and Newman–Keuls post hoc test.

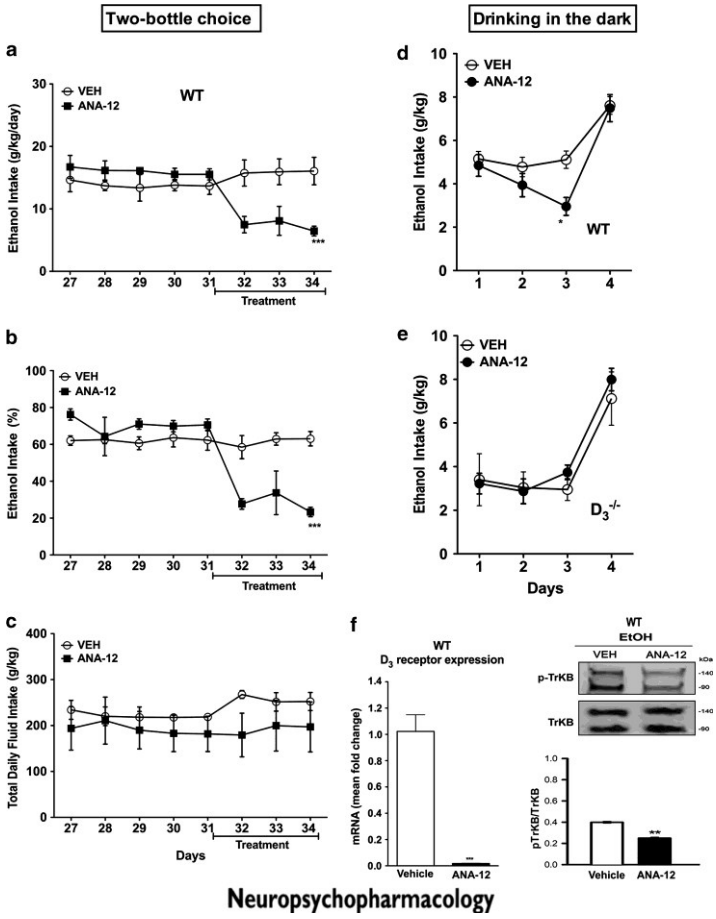

Figure 4 The selective TrKB antagonist, ANA-12 reverses ethanol intake of WT mice and induces D3 receptor downregulation but does not change ethanol intake of $D_3R^{-/-}$ mice. (a, b and c) Voluntary ethanol intake was measured every 24 h, for 34 days, in WT (n=30) and $D_3R^{-/-}$ (n=20) mice that had free access to water and ethanol solution (10%). At day 31, mice received daily i.p. injection of either vehicle (VEH), or ANA-12 at 0.5 mg/kg. (d, e) Drinking in the dark (DID) was measured, for 4 days, in WT (n=9) and D3 $D_3R^{-/-}$ (n=9) mice that had limited access (2 h/day for 3 days and 4 h the 4th day) to ethanol solution (20%), daily injected with vehicle or ANA-12 1 h before the test. *p<0.05, **p<0.01, ***p<0.001 vs VEH, one-way ANOVA and Newman–Keuls post hoc test. (f) The abundance of transcripts of D3 receptor in striatum was assessed by quantitative RT-PCR in WT mice exposed to chronic voluntary ethanol intake. Mean fold changes are expressed relative to transcript levels in controls. The abundance of phosphorylated TrkB was assessed by immunoblot, in the striatum WT treated with ANA-12 and exposed to the voluntary ethanol intake. Bars show mean (± SEM). **p<0.01, ***p<0.001 vs vehicle. One-way ANOVA and Newman–Keuls post hoc test.

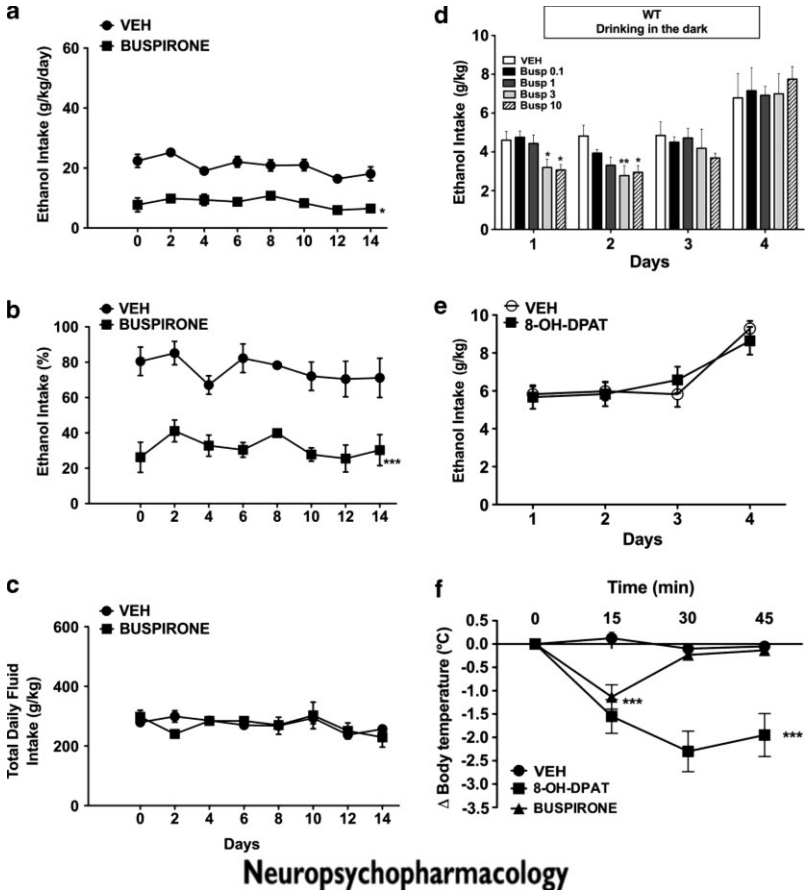

Figure 5 Buspirone inhibits ethanol intake in WT mice both in the two bottle choice and DID paradigm. (a, b) Voluntary ethanol intake was measured every 24 h, for 44 days, in WT (n=20) and $D_3R^{-/-}$ (n=20) mice that had free access to water and ethanol solution (10%). Mice received for 14 days, from day 31, daily i.p. injection of either vehicle (VEH) or buspirone at 1 mg/kg. (c) Total fluid intake that was not changed by buspirone. *p<0.05, ***p<0.01 vs VEH. One-way ANOVA and Newman–Keuls post hoc test. (d) The dose ranging of buspirone (0.1, 1, 3, and 10 mg/kg) in WT mice exposed to the drinking in the dark (DID) paradigm. DID was measured, for 4 days, in WT (n=33) that had limited access (2 h/day for 3 days and 4 h the 4th day) to ethanol solution (20%). *p<0.05, **p<0.01 vs VEH. One-way ANOVA and Newman–Keuls post hoc test. (e) The effect of the selective 5-HT1A agonist, 8-OHDPAT in DID paradigm. 8-OH-DPAT at 1 mg/kg did not change ethanol intake. (f) The action on 5-HT1A of 3 mg/kg buspirone was compared with 1 mg/kg 8-OH-DPAT by assessing the pharmacologically induced hypothermia. ***p<0.001 vs VEH. One-way ANOVA and Newman–Keuls post hoc test.

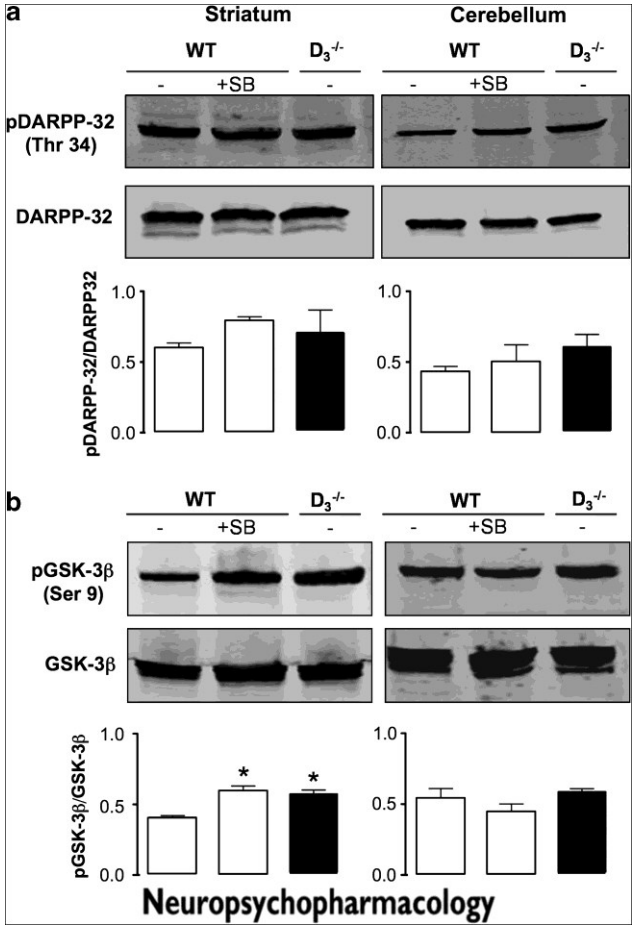

Figure 6 DA receptor signaling is enhanced in striatum of $D_3R^{-/-}$ mice and of SB277011A-treated WT mice. The abundance of phosphorylated DARPP-32 (Thr 34) (a) and phosphorylated GSK3b (Ser 9) (b) was assessed by immunoblot, in the striatum of WT mice exposed to the long term voluntary ethanol intake (white columns) and injected i.p. for 14 days with either vehicle or 10 mg/kg SB277011A and in $D_3R^{-/-}$ (black columns). Brain tissues were taken 1 h after the last administration of either vehicle or SB277011A. Bar graphs show mean (± SEM) of intensities normalized against the respective non-phosphorylated protein. Each column is the mean (± SEM) from five different samples. *$p<0.05$ vs control (vehicle-injected WT). Two-way ANOVA and Newman–Keuls post hoc test.

Supplementary Information – Leggio G.M. et al.

Dopamine receptor radioligand binding studies

All radioligands were obtained from Perkin Elmer Live and Analytical Sciences (Rodgau, Germany). Reference substances were purchased from Sigma Aldrich (Steinheim,Germany).

CHO-K1 cells expressing human dopamine D_3 or D_2 receptors were used for membrane preparation. Membrane protein concentration was determined by the method of Bradford (Bradford, 1976) . Radioligand competition binding assay was performed as described before (Sasse et al., 2007). Crude membrane preparations were incubated with 0.2 nM of [^{3}H]Spiperone and test ligand in a concentration range of 0.1 nM to 100 µM. For determination of non-specific binding 10 µM of haloperidol was used. All values are means of at least two independent measurements, each in triplicates and seven different concentrations. Binding data were analyzed by the software GraphPad Prism^{TM} (2000, version 3.02, San Diego, CA, USA).

Table 1S. Recombinant hD_3 versus hD_2 receptor binding.

Compound	hD_2 Ki [nM] $\bar{x}$ ± SD	hD_3 Ki [nM] $\bar{x}$ ± SD
U2991941	2639 ± 410	102.3 ± 37.3
SB277011 A	1914 ± 329	10.04 ± 2.65
Buspirone	62.26 ± 17.6	29.98 ± 11.5
Haloperidol	1.91 ± 0.15	11.03 ± 1.86

Methods molecular modeling

Structures of ligands were obtained by the ProDrg web server (http://davapc1.bioch.dundee.ac.uk/prodrg/) as .mol2 files (Schuttelkopf and van Aalten 2004) or retrieved from the PubChem database [http://pubchem.ncbi.nlm.nih.gov/]. When necessary, Open Babel (O'Boyle et al., 2011) was used to convert file formats. Protonation state of ligands was assigned at pH=7.4. Structure models of human D3R and D2LR, optimized by explicit molecular dynamics simulation in water-lipid environment, were obtained as previously described (Platania et al., 2012). 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. The optimized structure model of human 5HT1A receptor was obtained as described in

Platania et al. (Platania et al., 2013). Autodock 4.2 (AD4.2) (Morris et al., 1996) was chosen as docking software, because it provided the best prediction of pose of eticlopride docked in the hD3 homology modeling (Platania et al., 2012). Search space included the extracellular site (binding pocket 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 we chose, as search algorithm, the time-consuming Lamarkian genetic algorithm (GA). Hundred iterations of GA 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 hD3-lysozime chimera (Chien et al., 2010), were selected for analysis of ligand-protein interactions using the graphical user interface AutoDock Tools (ADT). AD 4.2 uses a semi-empirical free energy function and a charge-based method for desolvation contributes; the free energy function was calibrated using a set of 188 structurally known ligand-complexes with experimentally determined binding constant (Huey et al., 2007). The binding energy of ligand poses (Kcal/mol) is the sum of intermolecular energy, internal energy and torsional free energy of the ligand minus the unbound-system energy. Ligand-protein complexes were rescored with DSX-score online http://pc1664.pharmazie.uni-marburg.de/drugscore/. DSX-score uses a

knowledge-based scoring function (Neudert and Klebe, 2011). In particular DSX-score uses statistical pair potentials derived from Cambridge Structural Database (CSD) and from Protein Data Bank (PDB). Moreover, associated to PDB potential, Solvent Accessible Surface potential (SAS-potential) is introduced in DSX-score in order to account for the desolvation effects. PDB and SAS potentials were used in this work. Binding mode of antagonist was represented by two dimensional diagrams generated by LigPlot+ (Laskowski and Swindells 2011). LigPlot diagrams were further edited according to interactions revealed by ADT.

Results molecular modeling

In silico analysis of U99194A and SB277011A interactions with D2R and D3R shows their high selectivity for the D3R subtype.

To examine pharmacological antagonism at D_3R, we docked U99194A and SB277011A into models of D_3R and $D_{2L}R$ receptors (Platania et al., 2013). As shown in Table S2, consensus-scoring of poses confirmed the higher affinity of docked compounds for the D_3R in comparison to the $D_{2L}R$ subtype. As expected the binding energy of complexes is strictly related to ligand-protein interactions, each ligand bound in a different manner to D_3R and $D_{2L}R$ receptors (Figure 1S) and different hydrogen bonds (H-bonds) were formed (Table 3S). 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. In particularly the interaction of SB277011A and U99194A at the D_2R with the conserved aspartate residue in III helix is not optimal.

In silico analysis of buspirone showed its preferential interaction with D3R subtype

To examine pharmacological antagonism at D_3R, we docked buspirone into models of D_3R and $D_{2L}R$ receptors (Platania et al., 2012). As shown in Table 2S, consensus-scoring of poses confirmed the higher affinity of buspirone for the D_3R in comparison to the $D_{2L}R$ subtype.

Buspirone is an antagonist both at D_3R and D_2R, and interaction of its terminal pyrimidine and piperazine moiety involves the same residues of 6-ciano-3,4-dihydroisoquinolin moiety of SB277011A docked into D3 (Figure 2S, A,B). When docking buspirone into 5-HT1A receptor binding pocket we obtained two most populated poses of the compound, with differently oriented orthosteric binding, (Fig 3S). The orientation in the first pose was similar to the one of buspirone into the D_3R binding pocket. The orientation in the second pose was opposed to the first one and involved an H-bond between Ser 199 (helix V) and a

carbonyl oxygen of the azaspiro moiety. The involvement of helix V seemed fundamental for the activation of 5-HT1A receptor, together with the conserved Asp 116 residue, as reported by Ho et al. (Ho et al., 1992). Thus, the first orientation could lead to partial activation of the 5-HT1A receptor. The selective 5-HT1A agonist r-8-OH-DPAT was also docked into 5-HT1A and D_3Rs; although the poses looked very similar, the computational results confirmed the higher affinity of 8-OH-DPAT for 5-HT1A receptor (Fig 3S). Moreover, we have found that 8-OH-DPAT is not able to form the same interaction that 7-OH-DPAT, a recognized D_3R agonist, forms into the D_3R binding pocket (Platania et al., 2012). In conclusion, in silico analysis (DSX-score) shows that i) SB277011A, a D_3R antagonist, is highly selective for the D_3R subtype; ii) Buspirone may form interactions comparable to SB277011A in D_3R, having the antagonist binding mode at D_3R.

Table 1S. 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**
Buspirone	-9.0	-122	-9.7	-97	8.04 [8.1] (Tadori, Forbes et al. 2011)	35.6 [7.5] (Tadori, Forbes et al. 2011)
SB277011A	-9.9	-127	-8.2	-66	11 [7.9] (Reavill, Taylor et al. 2000)	1032 [6.0] (Reavill, Taylor et al. 2000)
U99194A	-5.0	-104	-5.4	-87	160 [6.8] (Audinot, Newman-Tancredi et al. 1998)	2281 [5.6] (Audinot, Newman-Tancredi et al. 1998)

Experimental K_i are from literature. pK_i (in square brackets) is the negative logarithm of K_i.

Table 2S. Residues interacting with tested ligands.

Ligand	hD_3 residues		hD_{2L} residues	
	H-bond, polar contacts	**Hydrophobic contacts**	**H-bond, polar contacts**	**Hydrophobic contacts**
Buspirone	Asp110, Ser 192	Val 86, Val 107, Val 111, Ile 183, Trp 342, Phe 345, Phe 346, Thr 369, Tyr 373	Asp 114, His 393, Cys 118	Phe 110, Val 115, Cys 182, Val 190, Ser 193, Phe 389, Phe 390
SB277011A	Asp 110, (-C=O)Cys 181, Ser 182	Gly 94, Val 86, Leu 89, Val 111, Trp 342, Phe 345	(-NH)Ile183, Asp 114, Thr 119, 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

Underlined residues represent H-bonds

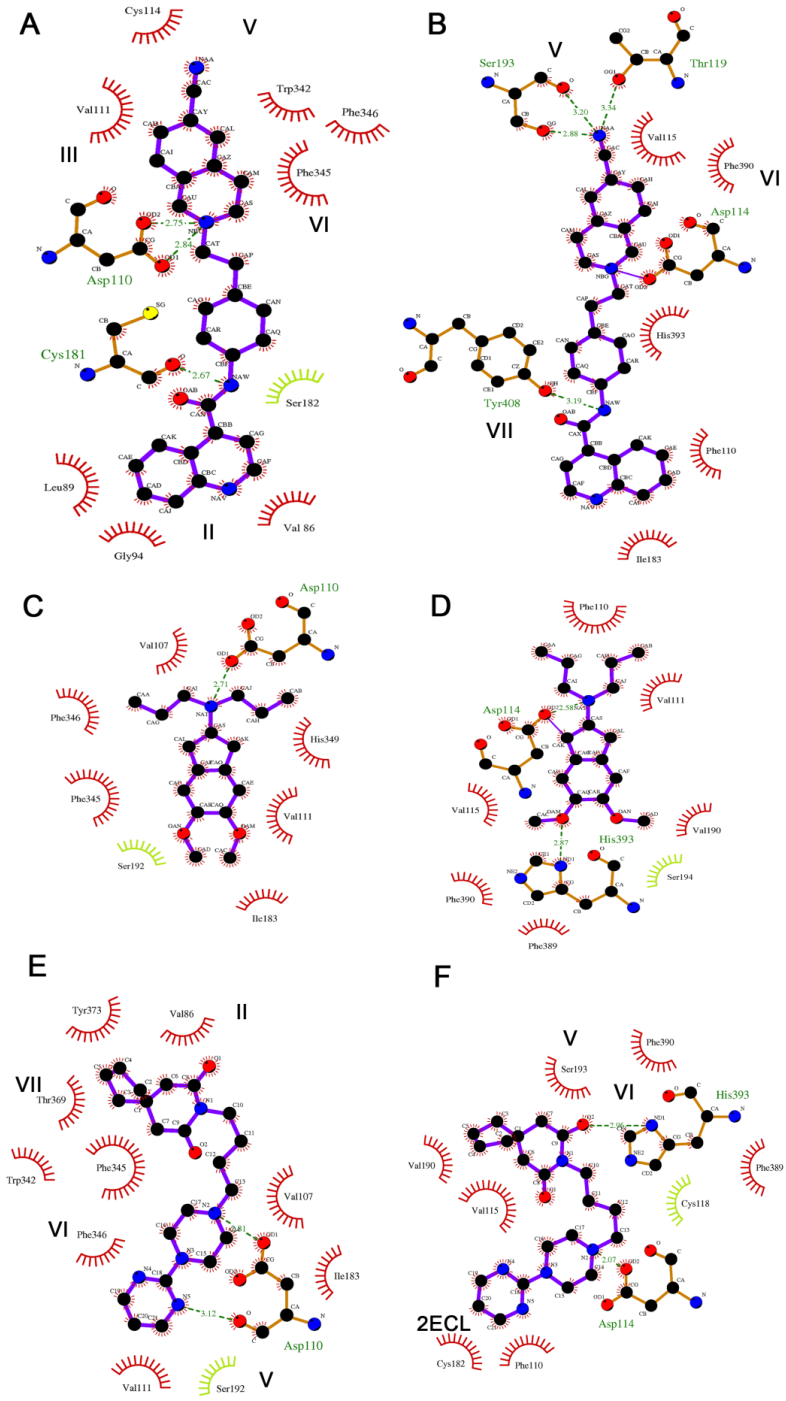

Figure 1S. 2D representation of ligands docked into dopaminergic receptors. SB277011A docked into D_3R (A) and D_2R (B); U99011A docked into D_3R (C) and D_2R (D) binding pockets; buspirone docked into D_3R (E) and D_2R (F).

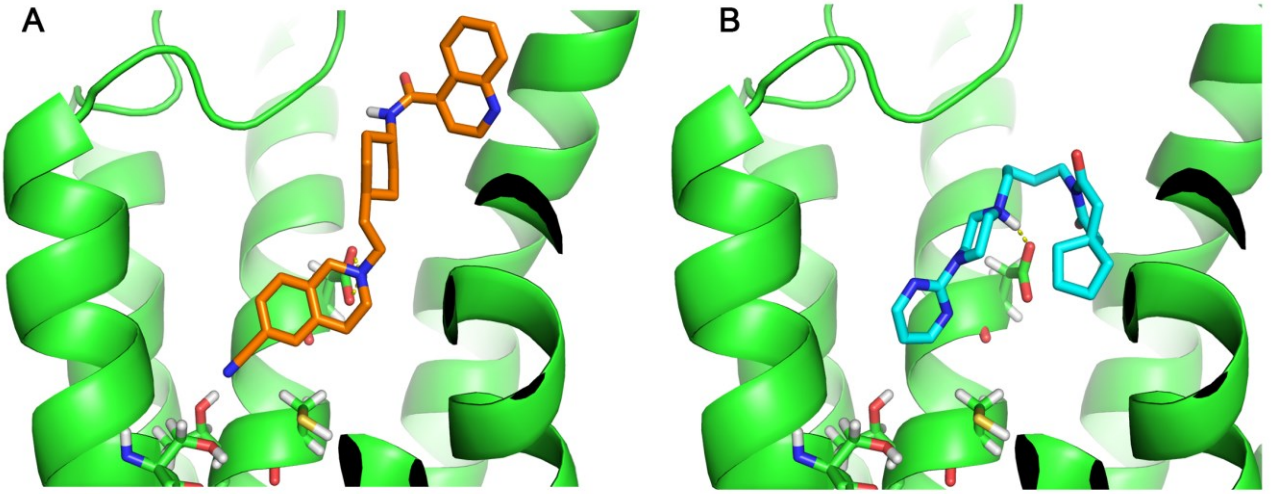

Figure 2S. In silico analysis of SB277011A and buspirone interactions with D_3R. 3D representation of SB277011A (**A**, orange stick) and buspirone (**B**, cyan stick) docked into D_3R**.** Buspirone is an antagonist at D_3R and interaction of its terminal pyrimidine and piperazine moiety involves the same residues of 6-ciano-3,4-dihydroisoquinolin moiety of SB277011A docked into D_3**.**

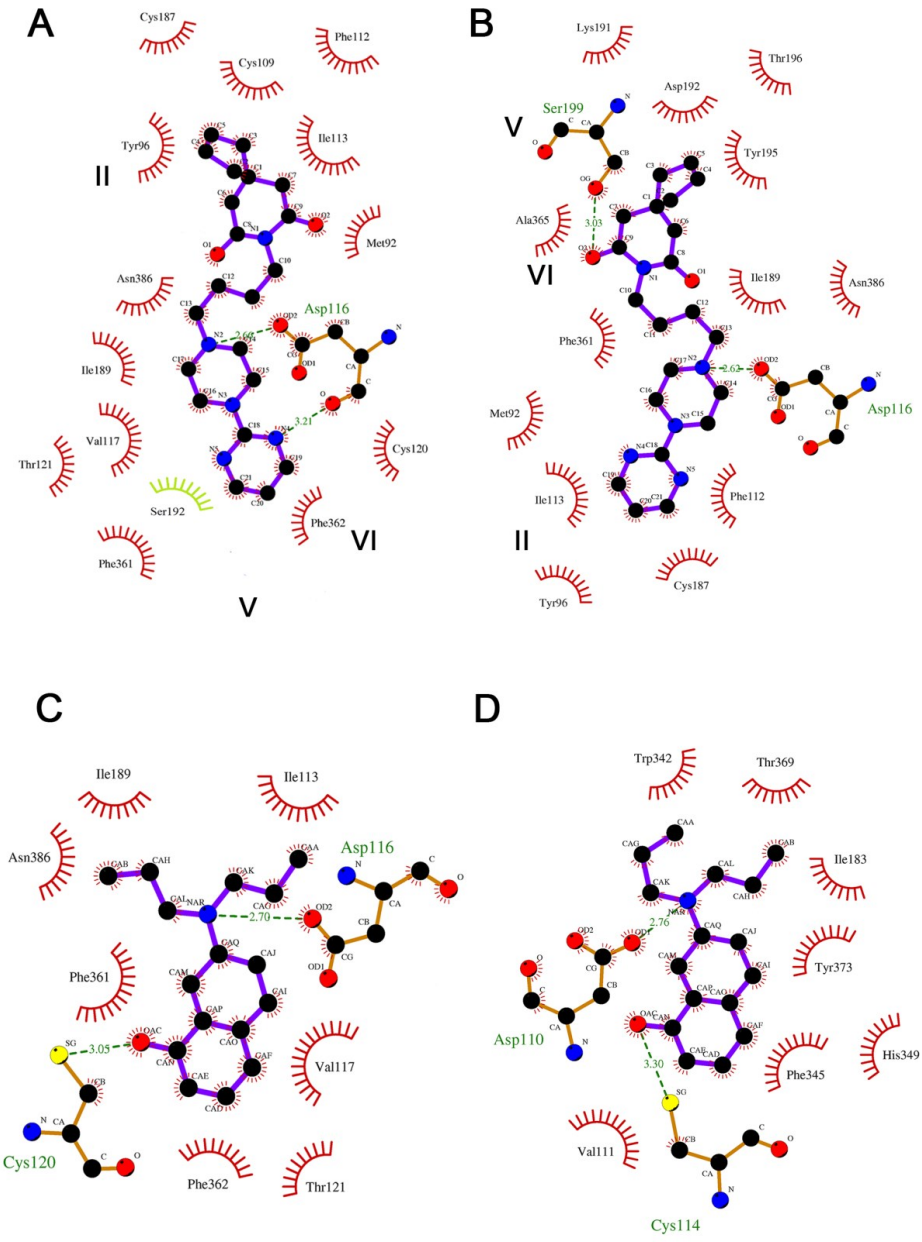

Figure 3S. 2D representation of ligands docked into $5HT_{1A}$ and D_3 receptors. Buspirone interacting with $5HT_{1A}$ receptor with two orientations 1(A) and 2 (B); r-8-OH-DPAT into the binding pocket of $5HT_{1A}$ (C) and D_3R

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Chapter III

Dopamine D3 receptor-dependent changes in alpha6 GABAA subunit expression in striatum modulate anxiety-like behaviour: Responsiveness and tolerance to diazepam

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Abstract

Increasing evidence indicates that central dopamine (DA) neurotransmission is involved in pathophysiology of anxiety, in particular the DA receptor subtype 3 (D_3R). We previously reported that D_3R null mice ($D_3R^{-/-}$) exhibit low baseline anxiety levels and that acutely administrated diazepam is more effective in $D_3R^{-/-}$ than in wild type (WT) when tested in the elevated plus maze test (EPM). Here we tested the hypothesis that genetic deletion or pharmacological blockade of D_3R

affect $GABA_A$ subunit expression, which in turn modulates anxiety-like behaviour as well as responsiveness and tolerance to diazepam. $D_3R^{-/-}$ mice exhibited tolerance to diazepam (0.5 mg/kg, i.p.), assessed by EPM, as fast as after 3 daytreatment, performing similarly to untreated $D_3R^{-/-}$ mice; conversely, WT exhibited tolerance to diazepam after a 14–21 day-treatment. Analysis of GABAA α6 subunit mRNA expression by qPCR in striatum showed that it was about 15-fold higher in $D_3R^{-/-}$ than in WT. Diazepam treatment did not modify α6 expression in $D_3R^{-/-}$, but progressively increased α6 expression in WT, to the level of untreated $D_3R^{-/-}$ after 14–21 day-treatment. BDNF mRNA expression in striatum was remarkably (410-fold) increased after 3 days of diazepam-treatment in both WT and $D_3R^{-/-}$; such expression level, however, slowly declined below control levels, by 14–21 days. Following a 7 day-treatment with the selective D_3R antagonist SB277011A, WT exhibited a fast tolerance to diazepam accompanied by a robust increase in α6 subunit expression. In conclusion, genetic deletion or pharmacological blockade of D_3R accelerate the development of tolerance to repeated administrations of diazepam and increase α6 subunit expression, a $GABA_A$ subunit that has been linked to diazepam insensitivity. Modulation of $GABA_A$ receptor by DA transmission may be involved in the mechanisms of anxiety and, if occurring in humans, may have therapeutic relevance following repeated use of drugs targeting D_3R.

Keywords: Anxiety; Dopamine D3 receptor; GABAA; Alpha6 subunit; Diazepam; BDNF

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1. Introduction

Increasing evidence indicates that dopamine (DA) neurotransmission is involved in the pathophysiology of anxiety, in particular, a large literature points to a correlation between anxiety-like behavior and the mesolimbic DA pathway (Cabib and Puglisi-Allegra, 1994; Kienast et al., 2008; Talalaenko et al., 1994). DA exerts its action through five G protein-coupled receptor subtypes (D1–5R); D_3R has an important role in the modulation of the mesolimbic DA pathway and in the control of several DA-related disorders such as addiction, depression and anxiety (Joyce and Millan, 2005; Leggio et al., 2014). The D_3R is, in fact, highly represented both at pre- and post-synapses, in the ventral striatum (nucleus accumbens and island of Calleja) (Bouthenet et al., 1991; Murray et al., 1994). In fact, D_3R is expressed also by tyrosine hydroxylase positive neurons, corresponding to its role as autoreceptor (Diaz et al., 2000). This is consistent with reports that mutant mice lacking D_3R receptors are hyperactive (Xu et al., 1997), presumably due to increases in DA resulting from a lack of negative feedback normally mediated through D_3R autoreceptors (Levant, 1997; Song et al., 2012b). Dopamine acting through the D_3R, may modulate the expression of innate anxiety-like behaviors involving a long-lasting, dynamic-dependent down-regulation of GABAergic control over lateral/basolateral amygdala neurons (Diaz et al., 2011). Such a D_3R mediated, dynamic-dependent inhibition of $GABA_A$ receptor has also been found in the nucleus accumbens (NAc) (Chen et al., 2006)

and hippocampus (Hammad and Wagner, 2006; Swant et al., 2008). However, the precise role of the D_3R/$GABA_A$ systems interaction both in the modulation of anxiety-like behaviors and the effect of anxiolytic drugs have not been completely explored. In a previous study, we found that $D_3R^{-/-}$ mice are more sensitive to the anxiolytic effect of diazepam than their WT littermates, which suggested potential alterations in the $GABA_A$ transmission. In the present study, we tested the hypothesis that genetic deletion or pharmacological blockade of D_3R affect $GABA_A$ subunit expression, which in turn may modulate anxiety-like behaviour as well as responsiveness and tolerance to diazepam. In this respect, we assessed the behavioral response of $D_3R^{-/-}$ mice and their WT littermates, tested in the elevated plus maze (EPM), an experimental model of anxiety, and their sensitivity to repeated administration of diazepam. At the end of behavioural experiments, we analyzed the expression of $GABA_A$ receptor subunit α6 mRNA in the ventral striatum, a brain area where D_3R is predominantly expressed. Similarly, we assessed the behavioral response and the sensitivity to diazepam in WT mice following repeated treatment with SB277011A, a selective D_3R antagonist. Finally, since D_3R expression is related to brain-derived neurotrophic factor (BDNF) (Guillin et al., 2001; Le Foll et al., 2005; Leggio et al., 2014), and BDNF is involved in the control of $GABA_A$ receptor response in NAc (Koo et al., 2014), we also analyzed the expression of BDNF and D_3R.

2. Materials and methods

2.1. Animals

Mice D_3R null ($D_3R^{-/-}$) and their WT littermates (males, 8–12 weeks old) were group-housed (3–5 mice per cage), with free access to chow and water, in an air-conditioned room, with a 12 h light–dark cycle. Mice $D_3R^{-/-}$ were 10th–12th generation of congenic C57BL/6 J mice, generated by a back crossing strategy (Accili et al., 1996). The genotypes of $D_3R^{-/-}$ and WT mice were assessed by a PCR method with two pairs of primers flanking either exon 3 of the wild-type D3R or the phosphoglycerate kinase 1 gene promoter cassette of the mutated gene (Accili et al., 1996).

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.

2.2. Drugs and treatment

All drugs were purchased from Sigma (St Louis, MO). Diazepam was dissolved in physiological saline containing Tween 80 (0.1%), SB277011A hydrochloride was dissolved in physiological saline containing dimethyl sulfoxide (10%). All drugs were intraperitoneally (i.p.) injected (in a volume of 10 ml/kg). SB277011A was used at 10 mg/kg (Song et al., 2012a), diazepam was used at 0.5 mg/kg (Leggio et al., 2011). All animals were gently manipulated by experienced

facility keepers to avoid any environmental or physical stresses. In a first set of experiments $D_3R^{-/-}$and WT were randomly allocated to the 10 experimental groups (n=6/8 per group): WT/naïve, $D_3R^{-/-}$/naïve, WT/vehicle single injection (SI), WT/diazepam SI, $D_3R^{-/-}$/vehicle SI, $D_3R^{-/-}$/diazepam SI, WT/vehicle (3 days), WT/ diazepam (3 days), $D_3R^{-/-}$/vehicle (3 days), $D_3R^{-/-}$/diazepam (3 days). For the 3-day treatment, the animals were i.p. injected once a day, for 3 consecutive days. On day 3 animals were i.p. injected 1 h before the EPM test. Animals were sacrificed 1 h after the EPM and brains tissues were taken. In a second set of experiments $D_3R^{-/-}$and WT mice were randomly allocated to the 10 experimental groups (n=6/8 per group): WT/vehicle (7 days), WT/ diazepam (7 days), $D_3R^{-/-}$/vehicle (7 days), $D_3R^{-/-}$/diazepam (7 days), WT/vehicle (14 days), WT/diazepam (14 days), $D_3R^{-/-}$/vehicle (14 days), $D_3R^{-/-}$/diazepam (14 days), WT/vehicle (21 days), WT/diazepam (21 days). Animals were i.p. injected once a day, for 7, 14 and 21 consecutive days. On day 7, 14 and 21 animals were i.p. injected 1 h before the EPM test. Animals were sacrificed 1 h after the EPM and brain tissues were taken.

2.3. Elevated plus maze (EPM) test

The apparatus consisted of two opposite open arms, (30 x 5 cm) and two arms with walls (30 x 5 x 14 cm) that were attached to a central platform (5 x 5 cm) to form a cross. The maze was elevated 50 cm from the floor (Pellow et al.,

1985). Illumination measured at the center of the maze was 40 lx. After treatment, each animal was placed at the center of the maze with its nose in the direction of one of the closed arms, and observed for 5 min, to measure the following parameters: number of entries in the open and closed arms, and time of permanence in each arm. The time of permanence measures the time spent by the animal in the open and closed arms. An arm entry was defined as two paws having crossed the dividing line between an arm and the central area. The anxiolytic effect of a drug treatment is indicated by a significant increase in parameters in open arms (time and/or number of entries). Total entries reflect the motor component of the exploratory activity. After removal of each mouse, the maze floor was carefully wiped with a wet towel. The behavior of animals was recorded using a video camera (Sony Videocam PJ330E) and then scored from the monitor display by an independent observer.

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

Total RNA was isolated from striatum 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, GABAA α6 subunit, BDNF and S18 ribosomal protein (reference gene). Each PCR reaction (20 µl final volume) contained 0.5 µM primers, 1.6 mM Mg^{2+}, and 1

x 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.

2.5. 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; p values less than 0.05 were considered as significant.

3. Results

3.1. $D_3R^{-/-}$ but not WT mice exhibited a fast tolerance to the anxiolytic effects of diazepam after 3 and 7 days of treatment

As previously reported (Leggio et al., 2011), naïve untreated $D_3R^{-/-}$ mice spent more time in open arms than WT and seemed more sensitive to the acute injection of diazepam (Fig. 1). In WT mice, diazepam (0.5 mg/kg, daily) maintained its anxiolytic effect for up to 7 days, as indicated by the time that diazepam-treated WT spent in the open arms of the EPM and the score number of entries, significantly higher than in the vehicle control groups (Figs. 1 and 2). In contrast, $D_3R^{-/-}$ mice became tolerant to diazepam (0.5 mg/kg, daily) already after a 3-day treatment, as shown by the lack of difference both in the time spent and in the score number of entries in open arms of the EPM as compared with the vehicle control group (Fig. 1). Similar results were obtained after a 7- and 14-day treatments (Figs. 2 and 3). In both WT and $D_3R^{-/-}$ the total amount of entries (open arms+close arms) was not changed by diazepamtreatment, indicating that locomotor activity was not affected (Figs. 1 and 2). In order to achieve tolerance to the anxiolytic effect of diazepam in WT mice, we carried out daily treatments for up to 21 days. As shown in Fig. 3, 14-day treatment with diazepam (0.5 mg/kg) induced tolerance in WT, as indicated by the lack of difference both in the time spent and in the score number of entries in open arms of the EPM as

compared with the vehicle control group (Fig. 3). Similar results were obtained in WT following a 21-day treatment (not shown).

3.2. Expression of α6 $GABA_A$ subunit in striatum

The expression of mRNA of α6 $GABA_A$ subunit was assessed in the striatum of mice by qPCR, after the EPM test. As shown in Fig. 4, $D_3R^{-/-}$ mice exhibited about 15-fold higher basal expression of α6 ($p<0.001$) than WT. Diazepam-treatment (for 3 or 7 days), did not modify α6 subunit expression in $D_3R^{-/-}$ (Fig. 4). In contrast, diazepam treatment (3–21 days) increased α6 subunit expression in WT. The increase in α6 was already significant after a 3-day treatment ($p<0.001$) and continued to rise in a time-dependent manner for up to 21 days, reaching a level similar to that of $D_3R^{-/-}$ mice(Fig. 4).

3.3. BDNF and D_3R expression in striatum

Expression of different $GABA_A$ subunits may be influenced by BDNF (Bulleit and Hsieh, 2000); furthermore, D_3R is critically controlled by BDNF (Guillin et al., 2001). We therefore wondered whether or not changes in α6 $GABA_A$ subunit expression were related to mRNA abundance of BDNF and/or D_3R in striatum. As shown in Fig. 5, BDNF mRNA expression was increased by a 3-day diazepam treatment in the striatum of both WT and $D_3R^{-/-}$ ($p<0.001$). The increase in BDNF expression, however, was not maintained when the diazepam

treatment was carried out for longer period of time, progressively declining below control levels, after 14–21 days (Fig. 5b). In contrast, D_3R expression progressively increased in striatum of WT following diazepam treatment, for up to 21 days ($p<0.001$, Fig. 5c).

3.4. Effect of pharmacological blockade of D_3R on responsiveness and tolerance to diazepam and on α6 $GABA_A$ subunit expression

Mice were treated with the D_3R antagonist SB277011A (10 mg/kg) for 7 days in combination or not with diazepam (0.5 mg/kg). As shown in Fig. 6, after SB277011A-treatment, WT mice were sensitive to the anxiolytic effect of diazepam, as indicated by the EPM paradigm where they increase both the time spent in open arms and the number of entries ($p<0.05$). However, when WT mice had received for 7 days administration of diazepam in combination with SB277011A, they became tolerant to diazepam, as indicated by the EPM paradigm where there was no change in time and entries in open arms, at variance with SB277011A-untreated WT, that needed at least 14 day diazepam treatment to become tolerant (Figs. 2 and 3).

Analysis of mRNA of α6 $GABA_A$ subunit in striatum of WT treated with SB277011A showed a marked increase in expression that was further enhanced by concurrent diazepam-treatment (Fig. 6d); a similar pattern of increased expression by treatments was seen for D_3R (Fig. 6e), whereas a slight decrease in BDNF

expression was seen following SB277011A-treatment alone or in combination with diazepam (Fig. 6f, $p<0.05$ for both conditions).

4. Discussion

The present data show that genetic deletion or pharmacological blockade of D_3R induce a fast development of tolerance to the anxiolytic effect of diazepam and produce a remarkable increase of $GABA_A$ α6 subunit expression in striatum. Because $GABA_A$ bearing α6 shows higher affinity for GABA but is poorly sensitive to diazepam (Minier and Sigel, 2004), we propose that D_3R mediated signalling participates to modulation of $GABA_A$ receptors, potentially affecting anxiety-like behavior and sensitivity to $GABA_A$ targeting anxiolytic drugs.

It has been reported that $D_3R^{-/-}$ mice exhibit reduced anxiety-like behavior (Accili et al., 1996; Leggio et al., 2011; Steiner et al., 1997), even though this finding was not noticed by others (Chourbaji et al., 2008; Xu et al., 1997). However, consistent with a role of D_3R in anxiety, putative D_3R antagonists have shown anxiolytic-like effects in rodents (Diaz et al., 2011; Gendreau et al., 1997; Leggio et al., 2011; Rogoz et al., 2000), while $D_3R^{-/-}$ mice exhibit an increased sensitivity to a single administration of diazepam (Leggio et al., 2011), the prototype of anxiolytic drugs. DA, acting through D_3R, modulates the expression of innate anxiety-like behaviors involving a long-lasting, dynamic-dependent down-regulation of GABAergic control over lateral/basolateral amygdala neurons (Diaz et al., 2011). A D_3R-mediated, dynamic-dependent inhibition of $GABA_A$ receptor has also been found in NAc (Chen et al., 2006) and hippocampus (Hammad and Wagner, 2006; Swant et al., 2008). GABAergic medium spiny

neurons (MSN) represent a predominant cell type regulating neuronal activity and function in NAc, through $GABA_A$ receptors (Brog et al., 1993; Koo et al., 2014; Pennartz et al., 1994). Thus, there is evidence that the interaction of $GABA_A/D_3R$ systems in the mesolimbic DA pathway contributes to modulate anxiety-like behaviors. In a previous study, we reported an higher sensitivity to a single administration of diazepam in $D_3R^{-/-}$ mice, suggesting potential alterations in the $GABA_A$ transmission in these mice, where, however, there was no difference in [3H]flunitrazepam binding, indicating that the number of $GABA_A$ receptors was not different (Leggio et al., 2011). $GABA_A$ receptors are heteropentameric chloride channels that may include subunits from the 19 known up to now, α1-6, β1-3, γ1-3, δ, ε, θ, π, and ρ1-3 (Rudolph and Knoflach, 2011). They exhibit an orthosteric site for GABA and a number of allosteric sites for endogenous and exogenous compounds. Electrophysiological and pharmacological analysis of $GABA_A$ in Xenopus Oocytes, expressing predefined subunits, has elucidated how the composition in subunits differently affect the affinity of benzodiazepine binding to $GABA_A$ and their efficacy as allosteric modulator; in particular, co-expression of α6 and α1 blunts the effect of diazepam, while $GABA_A$ expressing α6/β2/γ2 lacks diazepam binding site (Minier and Sigel, 2004). Interestingly, polymorphisms of α6 subunit have been found to be associated both to anxiety-related traits (Arias et al., 2012) and to benzodiazepine sensitivity in humans (Iwata et al., 1999). As illustrated in Supplementary Information, based on

sequence analysis and molecular modelling and because most of mutations between α1 and α6 lie in regions outside of the benzodiazepine binding site, we believe that the insensitiveness to diazepam of $GABA_A$ bearing α6 could be largely related to residues involved in contacts with other $GABA_A$ subunits, which may influence the conformational transition of the pore upon binding of GABA and/or other endogenous or exogenous modulators (Barnard et al., 1998; Williams and Akabas, 2000). Furthermore, the Arg119 in α6 might influence the conformational movements of the pore, forming an H-bond with Tyr58 of γ2 (Supplementary information). The analysis of the conformation of $GABA_A$ by molecular dynamics simulation reinforces the view that benzodiazepine binding is weakly affected by the presence of a single α6, because the predicted interaction energy of diazepam bound to α6 is comparable to that of diazepam bound to α1. This prediction is consistent with reported data (Minier and Sigel, 2004) as well as with our previous finding, that [3H]flunitrazepam binding is not different between WT and $D_3R^{-/-}$, in spite of the much larger α6 expression in $D_3R^{-/-}$ (Leggio et al., 2011). We do recognize that our data provide correlations between deletion or pharmacological blockade of D_3R and expression of behavior (as readout of diazepam effect) and/or expression of α6 (as readout of potential diazepam insensitivity of $GABA_A$), without providing a causal link between D_3R function and $GABA_A$ expression and function. However, the significance of the correlation we report here is strengthen by the fact that D_3R blockade by SB277011A

produced changes in the expression of α6 and tolerance to diazepam symmetrical to those observed in $D_3R^{-/-}$ mice. The link we propose here is consistent with the dynamic regulation of $GABA_A$ receptors by D_3R mentioned above (Chen et al., 2006; Diaz et al., 2011), but may find further support by studies showing that dopaminergic transmission modulates $GABA_A$ subunit expression in other brain areas (Katz et al., 2005) and by the evidence we present here that D_3R deletion or pharmacological blockade increase $GABA_A$ α6 subunit expression. However, we also considered the potential role of other players in $D_3R/GABA_A$ interaction such as BDNF. This latter is known to modulate, through its receptor TrkB, $GABA_A$ receptor function in several areas of CNS, such as hippocampus, cerebellum and striatum (Huang et al., 1999; Jovanovic et al., 2004; Rico et al., 2002). Furthermore, BDNF plays a relevant role in normal adaptive responses to stress as well as in the response to antidepressant drug treatments (Duman, 2002; Duman and Monteggia, 2006). When assessing BDNF mRNA expression in striatum, we found a robust (410-fold) increase after 3 days of diazepam-treatment in both WT and $D_3R^{-/-}$ mice; such an high BDNF expression level, however, was not maintained, but slowly declined below control levels, by 14–21 days. Of note, after 7 days, BDNF levels were still 2–3-fold higher than control (Fig. 5, $p<0.05$ vs VHE). Hence, increase in BDNF levels seem here to be a close consequence of $GABA_A$ receptor activation by diazepam, particularly because BDNF levels declined, when the diazepam-treatment had been prolonged, in parallel with the

increase in α6 expression and with the appearance of tolerance to the anxiolytic effect of diazepam. This conclusion is consistent with reports showing that other $GABA_A$-activating agents, such as ethanol, increase BDNF expression (Jeanblanc et al., 2006; Leggio et al., 2014), perhaps, we speculate, by a cellular mechanism similar to that elicited by diazepam, whose elucidation, however, is beyond the scope of the present study. Of note, a number of studies (Huopaniemi et al., 2004; Licata et al., 2013) report opposite effects of diazepam on BDNF levels in other brain areas (hippocampus, cortex), but these studies have been carried out with higher doses (10–30 mg/kg) than in the present study (0.5 mg/kg). Caution should therefore been taken when comparing BDNF levels from different brain areas and from animals treated with different doses of diazepam. Finally, we looked at D3R expression because it is known to be related to BDNF (Guillin et al., 2001; Le Foll et al., 2005; Leggio et al., 2014). We found that diazepam induced an increase in striatum D_3R mRNA expression (up to 5-fold) in WT mice; this might be related to the earlier increase in BDNF, that peaked at 3 days and remained elevated at 7 days. The interpretation of this finding, however, is made difficult by the fact that we measured the transcripts of BDNF, while protein levels might conceivably have a delayed profile. In mice treated with SB277011A, D_3R transcripts were also increased and were further augmented by a 7-day co-administration of diazepam. The increase in D_3R expression here is likely due to the chronic block by the antagonist, which induces receptor up-regulation (D'Souza et al., 1997). Of

note, in these latter groups of mice, we did not detect any increase in BDNF mRNA, that appeared, instead, reduced. Again, changes in $GABA_A$ expression and function induced by prolonged D_3R blockade, as revealed by the robust increase in α6 expression and the fast development of tolerance to diazepam, seemed associated with decreased BDNF, similarly to what we observed after prolonged (14–21 days) diazepam treatment. The fact that most $GABA_A$ receptors are, in this condition, poorly responsive to diazepam, may account for the lack of stimulatory effect of diazepam-treatment on BDNF expression; the slight reduction in BDNF, however, remains to be explained.

In conclusion, D_3R modulates $GABA_A$ receptors function in striatum. Chronic blockade of D_3R increases α6 subunit expression and induces insensitivity to the anxiolytic effect of diazepam. This mechanism may have therapeutic relevance following repeated use of drugs targeting D_3R.

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6. Contributors

Gian Marco Leggio, Filippo Drago and Salvatore Salomone planned the experiments, analyzed the data and wrote the report. Gian Marco Leggio, Sebastiano Alfio Torrisi, Alessandro Castorina, Agata Antonia Rita Impellizzeri, Annamaria Fidilio and Filippo Caraci treated the animals, carried out the beavioral experiments and the mRNA analysis. Chiara Bianca Maria Platania and Claudio Bucolo carried out the in silico study.

7. Conflict of interest

The authors do not have any conflict of interest to disclose related to this work.

8.Acknowledgements

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University of Catania Medical School, Catania, Italy. The authors do not have any potential conflict of interest to disclose.

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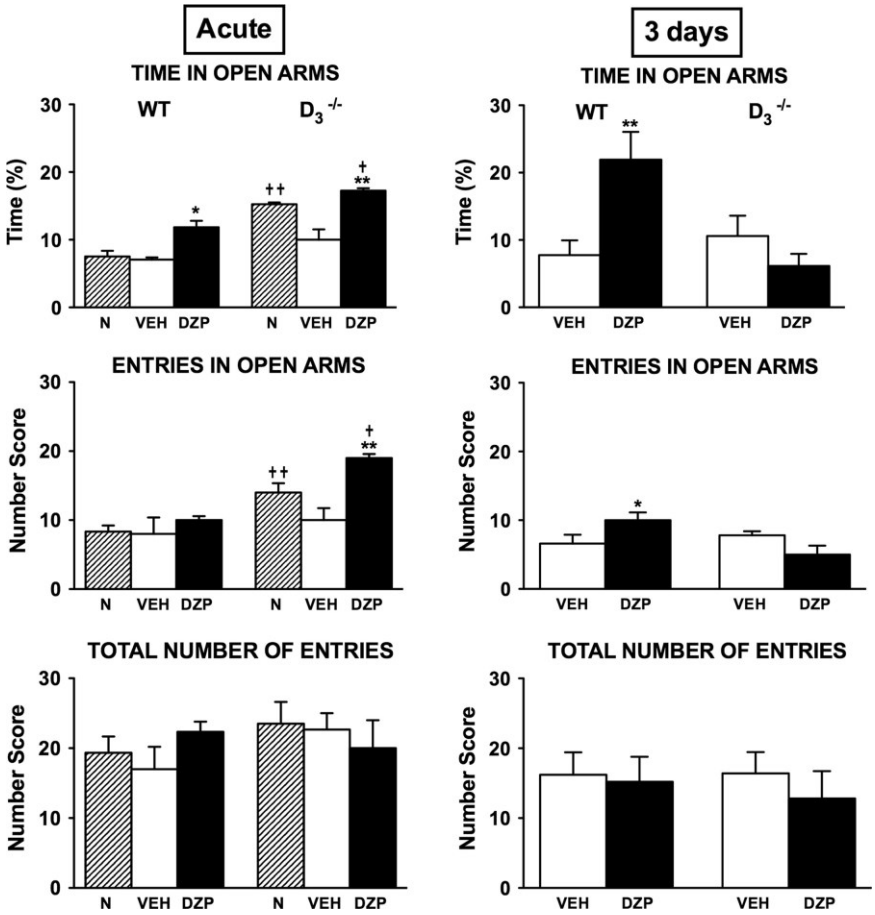

Fig. 1 Tolerance to the anxiolytic effects of diazepam after 3 days of treatment in $D_3R^{-/-}$ mice, but not in WT, as assessed in the Elevated Plus Maze (EPM) test. The time spent in open arms and the relative number of entries in open arms was measured during the 5-min test in WT (n=36) and $D_3R^{-/-}$ (n=36) that were untreated (naïve, N) or had received daily i.p. injection of either vehicle (VEH), or 0.5 mg/kg diazepam (DZP) for 3 days. The total number of entries (entries in open+entries in close arms) is an index of the exploratory activity, notice that it was not affected by drug-treatment in either group. Data are presented as means ± S.E.M. *P<0.05 and **P<0.01 vs. VEH. † P<0.05 and ††P<0.01 vs. the corresponding WT group, N or DZP. One-way ANOVA and Newman– Keuls post hoc test.

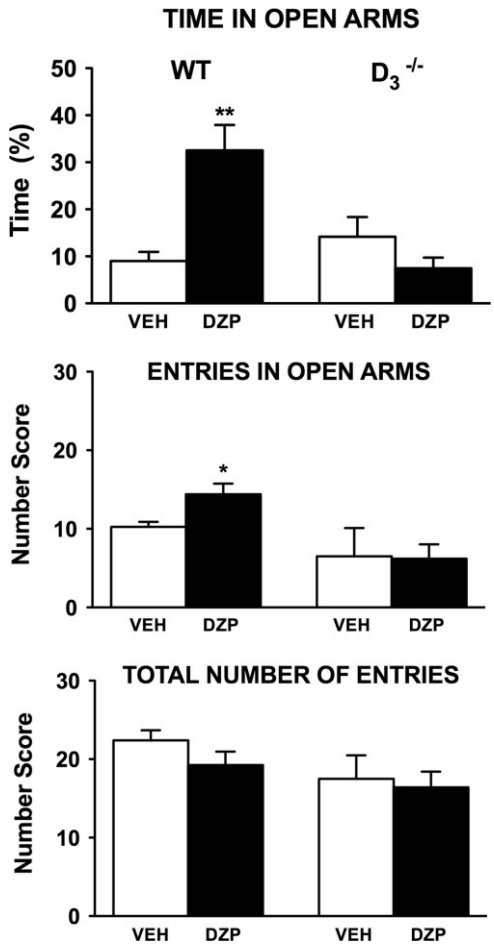

Fig. 2 Tolerance to the anxiolytic effects of diazepam after 7 days of treatment in $D_3R^{-/-}$ mice, but not in WT, as assessed in the Elevated Plus Maze (EPM) test. The time spent in open arms and the relative number of entries in open arms was measured during the 5-min test in WT (n=20) and $D_3R^{-/-}$ (n=20) that had received daily i.p. injection of either vehicle (VEH), or 0.5 mg/kg diazepam (DZP) for 3 days. The total number of entries (entries in open+entries in close arms) is an index of the exploratory activity, notice that it was not affected by drugtreatment in either group. Data are presented as means ± S.E.M. *P<0.05 and **P<0.01 vs. VEH. One-way ANOVA and Newman–Keuls post hoc test.

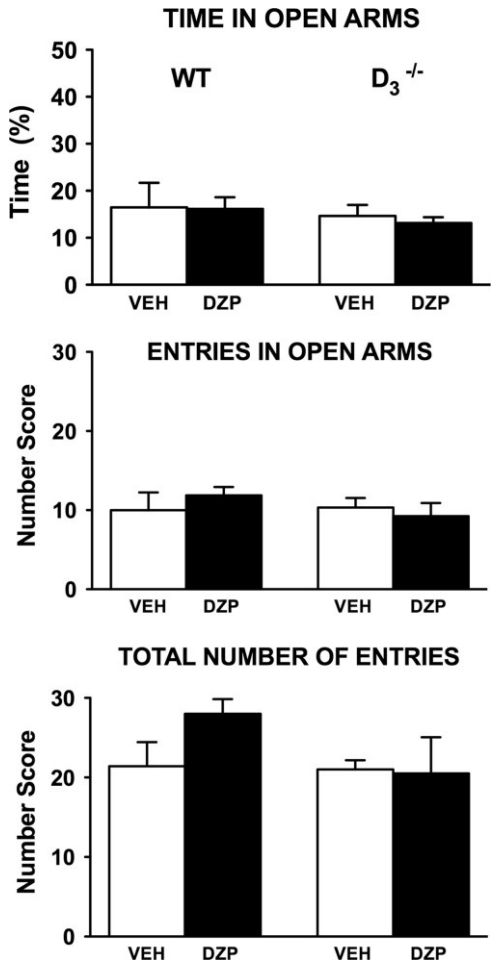

Fig. 3 Tolerance to the anxiolytic effects of diazepam, as assessed in the Elevated Plus Maze (EPM) test, appeared after 14-day treatment. The time spent in open arms and the relative number of entries in open arms was measured during the 5-min test in WT (n=20) and $D_3R^{-/-}$ (n=20) that had received daily i. p. injection of either vehicle (VEH), or 0.5 mg/kg diazepam (DZP) for 14 days. Data are presented as means ± S.E.M. One-way ANOVA and Newman–Keuls post hoc test did not show statistically significant differences.

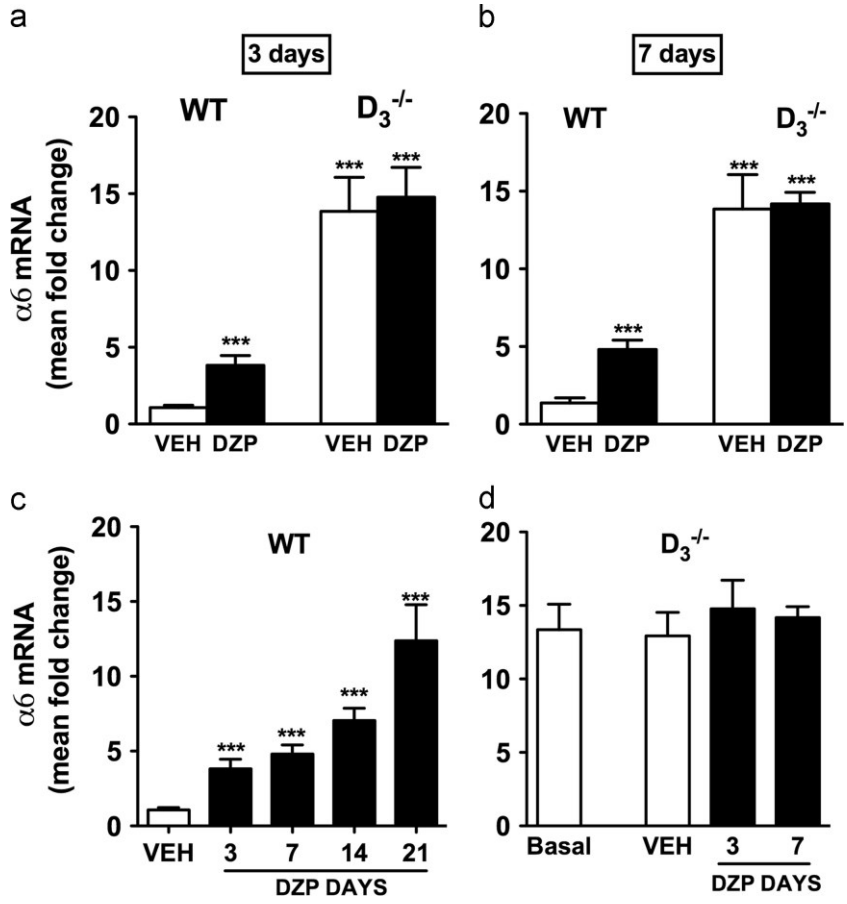

Fig. 4 $GABA_A$ α6 subunit expression was much higher in $D_3R^{-/-}$ than in WT; chronic treatment with diazepam induced α6 subunit overexpression in WT, up to the level seen in $D_3R^{-/-}$. Abundance of transcripts in striatum was assessed by quantitative RT-PCR after 3, 7, 14 and 21 days of treatment with either vehicle (VEH, white columns) or 0.5 mg/kg diazepam (DZP, black columns). In (a) and (b), α6 expression profile after treatment for 3 or 7 days; in (c) time course of α6 expression in WT (treatment 3–21 days); in (d) time course of α6 expression in $D_3R^{-/-}$ (treatment 3–7 days). Mean fold changes are expressed relative to transcript levels in controls (WT VEH). Each column is the mean (± S.E.M.) from 5 different samples. ***P<0.001 vs. VEH. One way ANOVA and Newman–Keuls post hoc test.

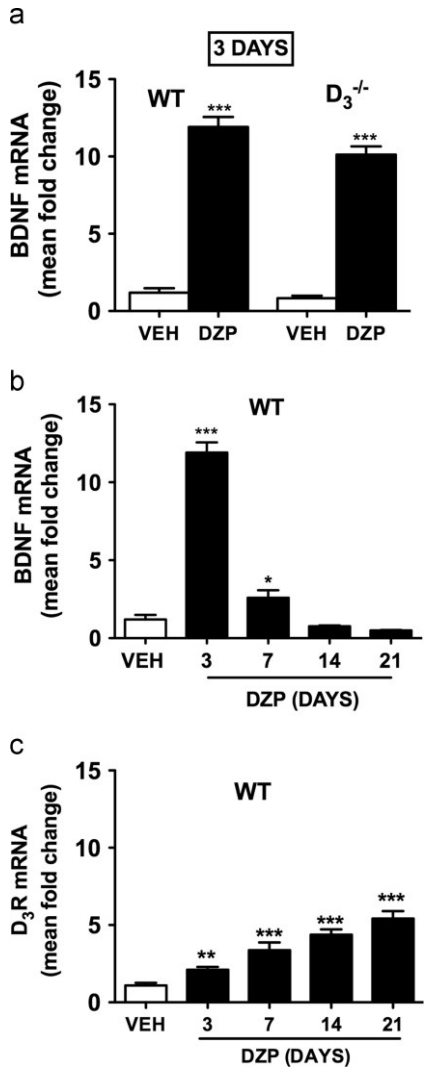

Fig. 5 Changes in abundance of BDNF and D_3R mRNA induced by chronic diazepam treatment. Abundance of transcripts in striatum was assessed by quantitative RT-PCR after 3,7,14 and 21 days of either vehicle (VEH, white columns), or 0.5 mg/kg diazepam (DZP, black columns). In (a), BDNF expression profile in WT and $D_3R^{-/-}$ mice treated for 3 days; in (b) time course of BDNF expression in WT (treatment 3–21 days); in (c) time course of D_3R expression in WT (treatment 3–21 days). Mean fold changes are expressed relative to transcript levels in controls (WT VEH). Each column is the mean (± S.E.M.) from 5 different samples. *P<0.05, **P<0.01 and ***P<0.001 vs. VEH. One-way ANOVA and Newman–Keuls post hoc test.

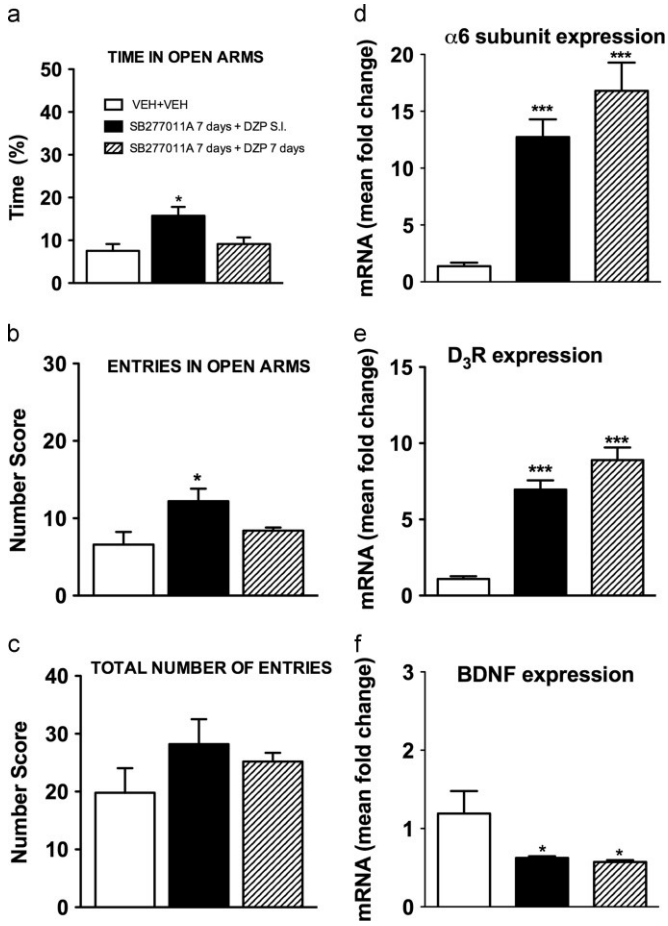

Fig. 6 Prolonged pharmacological blockade of D_3R induces rapid tolerance to the anxiolytic effect of diazepam and overexpression of $GABA_A$ α6 subunit. In (a)–(c), the anxiolytic effect of diazepam was assessed in the Elevated Plus Maze (EPM) test. The time spent in open arms and the relative number of entries in open arms was measured during the 5-min test in WT (n=30) that had received daily i.p. injection of either vehicle (VEH) or 10 mg/kg SB277011A with/without 0.5 mg/kg diazepam (DZP) for 7 days. Notice the tolerance to diazepam in SB277011A 7 days+DZP 7 days. In (d) $GABA_A$ α6 subunit expression profile; in (e) D3R expression profile; in (f) BDNF expression profile. Abundance of transcripts in striatum was assessed by quantitative RT-PCR. Mean fold changes are expressed relative to transcript levels in controls (WT VEH). Each column is the mean (± S.E.M.) from 5 different samples. *P<0.05 and ***P<0.001 vs. VEH. One-way ANOVA and Newman–Keuls post hoc test.

Supplementary Information

Molecular modeling of $GABA_A$ receptor with α6 subunit and docking of diazepam

Pairwise alignment of primary sequences of human α1 (BAD97167.1) and α6 (EAW61542.1) was carried out with protein-protein BLAST (BLASTp) (Altschul et al., 1990). The alignment was analyzed with Jalview 2.0 (Clamp et al., 2004). The homology model of $GABA_A$ receptor with α6 subunit was built by accessing to the Swiss-Model web service (http://swissmodel.expasy.org/) (Biasini et al., 2014). The template used for the homology model was the model of $GABA_A$ with α1 subunit obtained by Bergmann (Bergmann et al., 2013).

Molecular docking of diazepam was carried out in the benzodiazepine orthosteric binding site of $GABA_A$, containing either α1 or α6 subunit. The benzodiazepine binding site is characterized by residues at interface of αx and γ2 subunits. We used as reference the reported pose of diazepam (Bergmann et al., 2013) and repeated the docking of diazepam in α1γ2 and α6γ2 interface with AutoDock 4.2 (Huey et al., 2007) and rescoring with DSX-score (Neudert and Klebe, 2011), as previously described (Platania et al., 2013). Poses of diazepam into α1γ2 and α6γ2 were chosen within those most populated and with higher energy; furthermore, we considered the binding of the phenyl group of diazepam into the hydrophobic pocked as that conserved in the benzodiazepine site αxγ2.

Sequence alignment of α1 and α6 showed 62% identity, 73% homology and 4% gaps (Figure S1a). The unique difference at the benzodiazepine binding site

was His to Arg in α6. Arg119 in α6γ2 could form an H-bond with Tyr58 of γ2, whereas His101 could not. Furthermore, there were other poorly conserved regions, which may influence the conformational transition of the pore, particularly near the benzodiazepine binding site and at the interface with β subunit (Figure S1b). Predicted poses of diazepam in α1γ2 and α6γ2 were superimposable (Figure S1c), while both predicted interaction energies (-7.90 and -7.94 kj/mol, respectively) and DSX scores (-89 and -88 arbitrary units, respectively) were comparable. Diazepam appeared to interact with His101 in α1γ2 by means of a π-π stacking interaction, whereas it appeared to interact with Arg119 in α6γ2 by means of an halogen bond. An alternative pose of diazepam bared an H-bond with Arg 119 of α6γ2, but it excluded the interaction of phenyl of diazepam with the conserved hydrophobic pocket (data not shown).

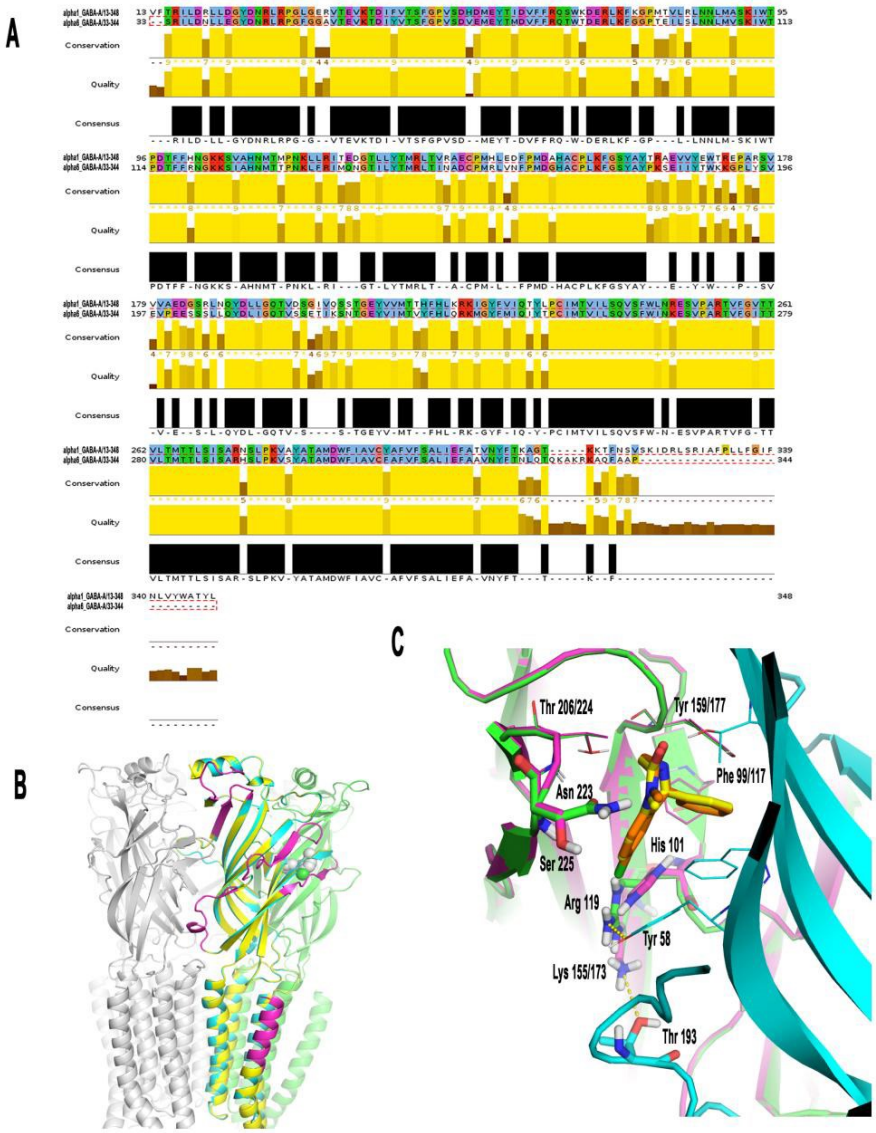

Figure S1

Docking of Diazepam in molecular models of $GABA_A$. A. Sequence alignment of α1 and α6 subunits. Blosum color code for residues. Yellow box corresponds to conservation and quality scores. Black box corresponds to consensus sequence. B. Modeling of $GABA_A$ receptor. Grey corresponds to the published model of β subunit (Bergmann et al., 2013), cyan corresponds to α6 subunit, yellow corresponds to α1 subunit, green corresponds to γ2 subunit, spheres represent diazepam. Notice the poorly conserved regions within α1 and α6, represented in magenta. C. Diazepam docked into α1γ2 (yellow stick) and into α6γ2 (orange stick). γ2 is represented in cyan, α1 is represented in magenta and α6 in green. For residues indicated as "Res x/y", x represents the position in α1 and y represents the position in α6.

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Chapter IV

General Discussion

6.1 Both genetic deletion and pharmacological blockade of D_3R inhibit ethanol intake

The data obtained during the first part of this thesis demonstrate that the genetic deletion, as well as the pharmacological blockade of D_3R inhibit ethanol intake in mice. Particularly, we discovered that $D_3R^{-/-}$ mice chronically exposed to the two bottle choice paradigm, exhibit a very low ethanol consumption compared to their WT littermates. This behaviour cannot be due to differences in metabolism (McQuade et al., 2003), locomotion (Harrison and Nobrega, 2009), or taste reactivity (McQuade et al., 2003) between WT and $D_3R^{-/-}$ mice. These results seems apparently in contrast with two earlier studies testing $D_3R^{-/-}$ mice in the ethanol voluntary intake paradigm (Boyce-Rustay and Risinger, 2003; McQuade et al., 2003). Different experimental procedures adopted in these works could explain these contrasting results. Turning to the pharmacological approach, in this work we used two previously reported selective D_3R antagonists, U99194A and SB277011A. We further assessed the selectivity of these pharmacological tools carrying out a molecular modeling study and we found that they were highly selective for the D_3R subtype and exhibited a distinct interaction with D_3R, in according to their dissimilar chemical structure. We discovered that both U99194A and SB277011A significantly decrease voluntary ethanol intake in WT but not in $D_3R^{-/-}$ mice. This pharmacological proof strengthens the view that the

D_3R is necessary for ethanol consumption in mice and is in agreement with previous rat data demonstrating that D_3R antagonism diminishes relapse-like drinking and cue-induced ethanol-seeking behavior (Vengeliene et al., 2006). Moreover, our results further confirmed the pivotal role of D_3R in the control of ethanol-drinking behavior in a binge-like ethanol-drinking paradigm (Crabbe et al., 2011; Rhodes et al., 2005; Rhodes et al., 2007). Indeed, $D_3R^{-/-}$ mice exposed to DID showed a very low ethanol consumption in comparison with their WT littermates, and D_3R blockade by SB277011A reduced ethanol intake in WT but not in $D_3R^{-/-}$ mice.

At the neurobiological level, our data demonstrate and confirm that chronic voluntary ethanol intake upregulated D_3R mRNA expression in the striatum of WT mice. Interestingly, an increase of D_3R expression after exposure to several drugs of abuse, such as nicotine and cocaine, in caudate–putamen (Neisewander et al, 2004) and in nucleus accumbens of rats (Le Foll et al., 2003, 2005b) has been reported. Similar results have been found in humans (Staley and Mash, 1996). Thus, D_3R expression seems to be a potential basis for a reinforcing mechanism in reward-related behavior associated with voluntary intake of addictive drugs and ethanol. Together with an increased D_3R mRNA expression, the activation of RACK1/BDNF pathway play an important role in this reinforcing mechanism.

Finally, I would like to underline that it is well-known that an excessive ethanol use causes a striatal hypodopaminergia in human and preclinical models

(Wolkov et al., 2007). It is thought that this hypodopaminergic state is a crucial pathophysiological feature of alcohol abuse, underlying the alcohol seeking and taking behaviour in an attempt to restore dopamine levels (Koob, 2013). The basal hyperdopaminergia of $D_3R^{-/-}$ mice (Koeltzow et al., 1998; Joseph et al., 2002) and the possible hyperdopaminergia induced by selective dopamine D_3R antagonists (Congestri et al., 2008) might be responsible for the low ethanol intake displayed by our mice.

6.2 Both genetic deletion and pharmacological blockade of D_3R accelerate the development of tolerance to diazepam

In the second part of this PhD thesis, we demonstrated that both genetic deletion and the pharmacological blockade of D_3R hasten the development of tolerance to repeated administrations of diazepam and increase α6 mRNA expression, a $GABA_A$ subunit that has been associated with diazepam insensitivity. Several studies have been reported that $D_3R^{-/-}$ mice exhibit low basal levels of anxiety-like behavior (Accili et al., 1996; Leggio et al., 2011), even if these data are in contrast with others (Chourbaji et al., 2008; Xu et al., 1997). Yet, D_3R antagonists have displayed anxiolytic-like effects in rodents suggesting a pivotal role of this receptor in anxiety, (Diaz et al., 2011; Gendreau et al., 1997; Rogoz et al., 2000). It has been discovered that dopaminergic system, via D_3R, down-regulate the GABAergic control of lateral/basolateral amygdala neurons and modulates the expression of innate anxiety-like behaviors (Diaz et al., 2011). Furthermore, a D_3R-mediated, dopaminergic-GABAergic interaction has also been discovered in NAc (Chen et al., 2006) and hippocampus (Hammad and Wagner, 2006; Swant et al., 2008). Thus, it is clear that the interaction of $GABA_A/D_3R$ systems in the mesolimbic DA pathway is fundamental to the expression of anxiety-like behaviors. The study by Leggio et al. (2011) previously demonstrated a higher sensitivity to the anxiolytic effect of a single injection of

diazepam in $D_3R^{-/-}$ mice, suggesting a potential dysfunctionality of the $GABA_A$ system in these mice. Our data demonstrate that the $GABA_A/D_3R$ mesolimbic interaction is associated with a remarkable increase of α6 mRNA expression induced by both genetic deletion and pharmacological blockade of D_3R. In this regard, α6 subunit has been linked to diazepam insensitivity together with other $GABA_A$ subunits such as α4 (Mizokami et al., 2010) and extrasynaptic $GABA_A$ receptors containing α4 or α6 subunits are known to mediate the tonic or extrasynaptic inhibition (Santhakumar et al., 2006). Recently, it has been reported that an extrasynaptic inhibition mediated by α5 subunit-containing $GABA_A$ receptors is responsible for the modulation of anxiety and fear generalization (Botta et al., 2015). Noteworthy, extrasynaptic $GABA_A$ receptors in the dorsomedial shell of the NAc, brain area rich of D_3R, are critical for ethanol intake (Olsen, 2011). In according to this evidence, it might be interesting to analyze the possible D_3R-mediated control of the mesolimbic extrasynaptic GABAergic inhibition.

Altogether, since a strong relationship between anxiety disorders and alcohol addiction is a well-documented phenomenon (Marquenie et al., 2007; Merikangas et al., 1998), we can state that a mesolimbic $GABA_A/D_3R$ interaction could represent a common neural substrate subserving the pathophysiology of these related neuropsychiatric disorders.

Concluding Remarks

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

1. $D_3R^{-/-}$ mice exhibit a very low ethanol intake compared to their WT littermates both in the two bottle choice and in the DID paradigm
2. Chronic blockade of D_3R reduces ethanol intake of WT mice without affecting total amount of fluid intake (ethanol + water)
3. Chonic ethanol intake induces an overexpression of D_3R and an activation of RACK1/BDNF pathway in WT mice. These neural processes seems to operate as reinforcing mechanism
4. The selective TrkB antagonist ANA-12, chronically injected, decreases ethanol intake and reduces D_3R expression. It has no effect on $D_3R^{-/-}$ mice
5. Buspirone, a commercially available anxiolytic drug endowed with D_3R antagonist activity, inhibits ethanol intake in WT mice both in the two bottle choice and in DID paradigm
6. Chronic blockade of D_3R or its genetic deletion increases DA transmission in striatum
7. $D_3R^{-/-}$ but not WT mice tested in the EPM exhibit a fast tolerance to the anxiolytic effect of diazepam

8. $D_3R^{-/-}$ mice have high basal level of $GABA_A$ α6 mRNA. After diazepam treatment, $GABA_A$ α6 mRNA expression does not change and increases in a time-dependent manner in $D_3R^{-/-}$ and WT mice, respectively
9. A 3-day diazepam treatment increases BDNF mRNA expression in the striatum of both WT and $D_3R^{-/-}$ mice. This increase gradually drops below control levels after 14–21 days
10. A progressively increase of D_3R expression was seen following chronic diazepam treatment in WT mice
11. Co-administration of the selective D_3R antagonist SB277011A and diazepam induces a fast development of tolerance as well as an increased mRNA expression of α6 subunit and D_3R in striatum of WT mice.

Taken together, the results present in this PhD thesis reveal a prominent role of D_3R in the pathophysiology of two related neuropsychiatric diseases. This may open new avenues for the design of new therapeutic strategies.

Chapter V

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Chapter VI

Annexes

List of Publications and Scientific Contributions

List of Publications

- Leggio GM, Catania MV, Puzzo D, Spatuzza M, Pellitteri R, Gulisano W, **Torrisi SA**, Giurdanella G, Piazza C, Impellizzeri AR, Gozzo L, Navarria A, Bucolo C, Nicoletti F, Palmeri A, Salomone S, Copani A, Caraci F, Drago F (2016). The antineoplastic drug flavopiridol reverses memory impairment induced by Amyloid-ß1-42 oligomers in mice. *Pharmacol Res*. 106:10-20.

- Leggio GM*, **Torrisi SA***, Castorina A, Platania CB, Impellizzeri AA, Fidilio A, Caraci F, Bucolo C, Drago F, Salomone S (2015). Dopamine D3 receptor-dependent changes in alpha6 GABAA subunit expression in striatum modulate anxiety-like behaviour: Responsiveness and tolerance to diazepam. *Eur Neuropsychopharmacol*. 25:1427-36. *co-first authorship.

- Leggio GM, Camillieri G, Platania CB, Castorina A, Marrazzo G, **Torrisi SA**, Nona CN, D'Agata V, Nobrega J, Stark H, Bucolo C, Le Foll B, Drago F, Salomone S (2014). Dopamine D3 Receptor is Necessary for Ethanol Consumption: An Approach with Buspirone. *Neuropsychopharmacology*. 39:2017-28.

Oral communications

- Genetic-driven reduction of D3R ameliorates dysbindin-dependent schizophrenia-relevant abnormalities. *Joint Annual PhD Students Retreat* 2015, Bordeaux.

- Role of central dopamine D3 and serotonin 2C receptors in the control of the mesoaccumbens dopaminergic pathway: implications for the treatment of depression. *37° Congresso Nazionale della Società Italiana di Farmacologia*. I NUOVI ORIZZONTI DELLA RICERCA FARMACOLOGICA: TRA ETICA E SCIENZA. NAPOLI 27 - 30 ottobre 2015.

Award

- 26 May 2016: IBRO InEurope Short Stay Grants Program award

Poster communications

- **Torrisi SA***, Leggio GM*, Giurdanella G, Caraci F, Platania CBM, Bucolo C, Salomone S and Drago F. Role of central dopamine D3 and serotonin 2C receptors in the control of the mesoaccumbens dopaminergic pathway: implications for the treatment of depression. *Convegno monotematico SIF. The stressed brain: psychopathologic implications and pharmacological intervention*. March 3rd - 4th, 2016. Università degli Studi di Milano *co-first authorship

- **Torrisi SA***, Leggio GM*, Castorina A, Bucolo C, Caraci F, Salomone S and Drago F. Genetic deletion of dopamine D3 receptor accelerate the development of tolerance to the anxiolytic effect of diazepam. *27th ECNP Congress.* October 18-21, 2014, Berlin, Germany *co-first authorship

- Platania CB, Leggio GM, Camillieri G, Castorina A, **Torrisi SA**, Nona CN, D'Agata V, Nobrega J, Le Foll B, Drago F. Molecular basis of alcohol intake: role of D3 dopaminergic receptor. *ECNP Workshop.* March 6-9, 2014 in Nice, France.

- **Torrisi SA**, Platania CBM, Drago F, Salomone S, Bucolo C. Effects of topical NSAIDs on LPS-induced inflammation. *36° congresso nazionale della società italiana di farmacologia.* October, 23-26 2013 Torino, Italy.