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THE ROLE OF DOPAMINE D_3 RECEPTOR IN ALCOHOL ADDICTION AND SCHIZOPHRENIA

PhD Thesis

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During the 2nd year of my PhD program, I spent two months working as visiting PhD student at University of Helsinki. During this period, I worked on the project entitled: *Dopamine D_3 receptor-dependent ectopic expression of α6 $GABA_A$ subunit counteracts alcohol intake by increasing GABA inhibition in the nucleus accumbens*

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List of abbreviations

5-HT 5-hydroxytryptamine

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

AC adenilate cyclase

ADE alcohol deprivation effect

ANOVA analysis of variance

BDNF brain development neurotrophic factor

cAMP cyclic adenosine monophosphate

CB1 cannabinoid receptor 1

CBD cannabidiol

CNS central nervous system

DAG diacylglycerol

D2L dopamine D2 long

D2S dopamine D2 short

D3R dopamine D3 receptor

D3R-/- dopamine D3 receptor deficient mice

DA dopamine

DID drinking in the dark

DMSO dimethyl sulfoxide

DYS dysbindin

EC endocannabinoid

GABA γ-aminobutyric acid

GABAA γ-aminobutyric acid receptor A

GD gestional day

GIRK G-protein gated inwardly rectifying K^+ channel

GPCR G protein-coupled receptors

GRK GPCR kinases

HIP hippocampus

IP3 inositol 1,4,5 trisphosphate

i.p. intraperitoneal injection

MAM methylazoxymethanol acetate

MSN Medium Spiny Neurons

NAc nucleus accumbens

NMDA N-methyl-D-aspartate

NP alcohol Non-Preferring rats

P alcohol Preferring rats

PFC prefrontal cortex

PKA protein kinase A

PLC phospholipase C

PV parvalbumin

RGS regulators of G protein signalling

SCZ schizophrenia

SI single injection

STR striatum

VEH vehicle

VTA ventral tegmental area

WT wild type mice

Preface

Dopamine (DA) modulates several essential functions of the central nervous system (CNS), including reward and cognition. The dopaminergic neurotransmission in the CNS is mediated by two different classes of G protein-coupled receptors (GPCR), the "D_1R-like" receptors (D_1R and D_5R) and "D_2R-like" receptors (D_2R, D_3R and D_4R, Seeman et al., 1994). Among dopamine receptors, the dopamine D_3 receptor (D_3R) has captured the scientific interest because of its restricted distribution in the brain, seemingly related to functions of dopamine associated with the limbic brain. So, the dopamine D_3R shows a limited distribution in the limbic brain areas involved in the control of cognitive and emotional functions, and it seems to represent a target for the treatment of several neuropsychiatric disorders such as drug addiction and schizophrenia (Leggio et al., 2016). D_3R, acting as autoreceptor, regulates the activity of DAergic neurons throughout the mesolimbic, mesocortical and nigrostriatal DAergic pathways (Gobert et al., 1995; Tepper et al., 1997; Diaz et al., 2000). D_3 deficient mice ($D_3R^{-/-}$) exhibit extracellular levels of dopamine twice as high as their wild-type (WT) littermates suggesting that D_3R could play an inhibitory role in the control of basal extracellular DA levels (Koeltzow et al., 1998; Joseph et al., 2002). Moreover, Leggio and colleagues (2014) demonstrated that $D_3R^{-/-}$ mice display significant lower levels of alcohol intake compared to their

WT littermates, in several ethanol-drinking paradigms. Furthermore, $D_3R^{-/-}$ mice show a 15-fold higher expression of γ-aminobutyric acid receptor A ($GABA_A$) α6 subunit in striatum compared to their WT littermates (Leggio et al., 2015). Based on previous data present in literature, the main hypothesis of my research project has been that the D_3R, showing a main role in the control of the mesolimbic DAergic pathway, is involved in neuropsychiatric disorders linked to alteration of DAergic pathways. So, the aims of this thesis were: 1) to investigate the role of the cross-talk between $GABA_A/D_3R$ in the mesolimbic DA control of ethanol consumption; 2) to assess the involvement of D_3R in the pathophysiology of schizophrenia.

Chapter I

General Introduction

1. Dopamine pathways in the central nervous system

DA regulates important physiological brain's functions, including reward and cognition, through four different DAergic pathways: the nigrostriatal pathway arising from the substantia nigra pars compacta (SNpc; Dahlström and Fuxe, 1964) and projecting to the dorsal striatum; the mesolimbic pathway originating in the ventral tegmantal area (VTA) and projecting to the nucleus accumbes (NAc); the mesocortical pathway that arises from the VTA and projects to the central cortex and the tuberoinfundibular pathway that connects the hypothalamus to the pituitary gland (Anden et al., 1964; Dahlstroem and Fuxe, 1964). The different DAergic pathways mediate several physiological functions, with the mesolimbic/mesocortical pathways implicated in reward and cognition. The evidence that neuropsychiatric disorders, like schizophrenia and addiction, involve a dysregulation of mesolimbic/mesocortical pathways, reinforces the hypothesis of a functional segregation of DAergic pathways. So, a key role of the mesocorticolimbic pathway has been recognized in reward, craving and aversion (Wise, 2009) and in schizophrenia (Perez-Costas et al., 2010; Yoon et al., 2013; Weinstein et al., 2017).

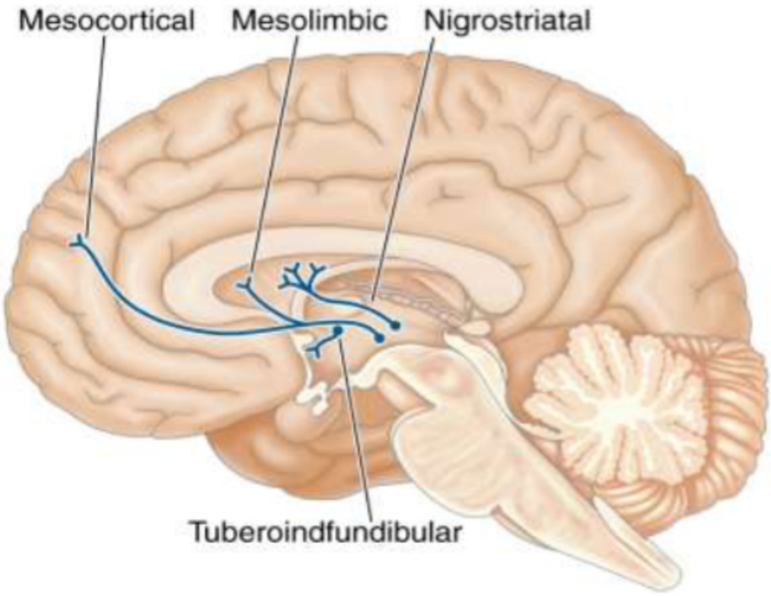

Figure 1: Representative image of DAergic pathways in the CNS. Nigrostriatal pathway, Mesolimbic pathway, Mesocortical pathway and Tuberoinfundibular pathway (Goodman and Gilman's Therapeutic Basis of Pharmacology).

2. DAergic receptors

DA mediates its effect through two families of GPCR, classified into "D_1R-like" and "D_2R-like" receptors. D_1R-like receptors (D_1R and D_5R) are coupled to Gs proteins and stimulate adenylate cyclase (AC), with production of cyclic adenosine monophosphate (cAMP) and activation of cAMP-dependent pathways, including protein kinase A (PKA) and other downstream signals. D_1R modulate different ionic channels, including voltage-activated Na^+-(Na_v), K^+-(K_v) and Ca^{2+} (Ca_v) channels, Ca^{2+}-activated K^+-(KCa) and G-protein gated inwardly rectifying K^+ (GIRK) channels (Maurice et al., 2001; Witkowski et al., 2008; Yang et al., 2013). D_2R-like receptors (D_2R, D_3R and D_4R), by coupling to G_i proteins, induce inhibition of AC and PKA-dependent pathways, as well as activation of GIRK and closure of Ca_V (Missale et al., 1998**,** Figure 2)**.** D_2R-like receptors genes generate variants. D_2R exists in two functional isoforms, D_2 long (D_2L) and D_2 short (D_2S; Giros et al., 1989), whereas several D_3R isoforms have been identified (Giros et al., 1991). Multiple D_4R variants are produced, mostly having a domain repeated 2 (2R), 4 (4R) and 7 (7R) times (Van Tol et al., 1992). In addition to act as monomers, DAergic receptors constitute dimeric and/or oligomeric complexes by association of different subtypes either with DA receptors or with other GPCRs and ligand-gated channels. Furthermore, D_1R-D_2R dimers are linked to G_q proteins, thus modulating phospholipase C (PLC), which produces inositol 1,4,5 trisphosphate (IP_3) and diacylglycerol

(DAG) to regulate intracellular Ca^{2+} (Lee et al., 2004, Figure 2). While the prevailing belief is that DAergic receptors act through G proteins, they can also activate G protein-independent mechanisms. A role in the G protein-independent signaling is played by arrestins, multifunctional adaptor proteins which bind DAergic receptors phosphorylated by GPCR kinases (GRKs; Gainetdinov et al., 2004). Binding of arrestins recruits several other proteins, including Akt, GSK-3, MAPK, c-Src, Mdm2 and N-ethylmaleimide-sensitive factor, thereby enhancing DA-activated pathways (Beaulieu and Gainetdinov, 2011; Figure 2). GRKs also regulate DAergic receptors by mediating their desensitization, since their phosphorylation elicits receptor endocytosis. Besides GRKs, the regulators of G protein signaling (RGS), a group of GTPase-activating proteins acting on G protein, negatively modulate DAergic receptors.

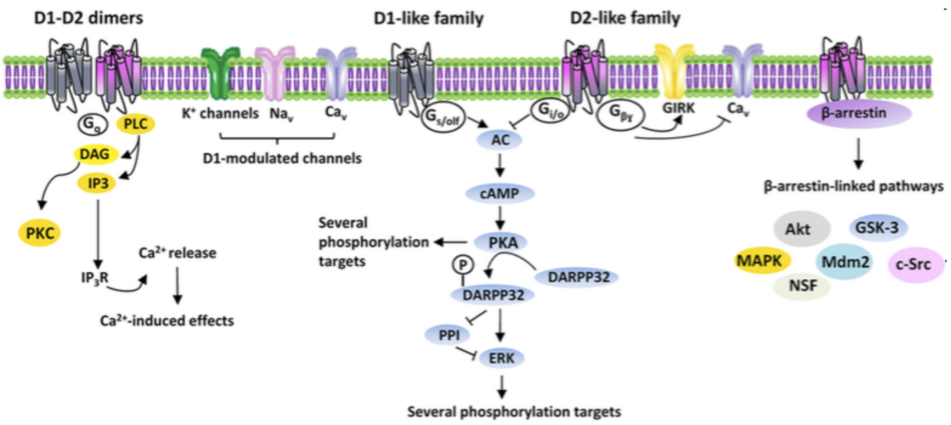

Figure 2: DAergic receptors signaling. Intracellular signaling pathways activated by D_1- and D_2-like receptors families. Gs, Gi, Gq, Gβγ, G proteins; AC, adenylyl cyclase; cAMP, 3'-5'-cyclic adenosine monophosphate; DARPP32, cyclic AMP-regulated phosphoprotein, 32 kDa; PPI, protein-phosphatase 1; ERK, extracellular signal-regulated kinase protein kinase; PLC, phospholipase C; DAG, diacylglycerol; PKC, protein kinase C; IP_3, inositol triphosphate; IP_3R, inositol triphosphate receptor; Ca^{2+},calcium; GIRK, G-protein gated inwardly rectifying K^+ channels; Cav, voltage-activated Ca^{2+} channels; Nav, voltage-activated Na^+ channels; Akt, thymoma viral proto-oncogene; GSK-3, Glycogen Synthase Kinase-3; MAPK, mitogen-activated protein kinase; Mdm2, mouse double minute 2 homolog; c-Src, proto-oncogene non-receptor tyrosine kinase; NSF, N-ethylmaleimide-sensitive factor. (Ledonne and Mercuri, 2017).

3. Dopamine D_3 Receptor

The D_3R was cloned in 1990 and characterized for its high sequence homology with the D_2R (Sokoloff et al., 1990). The D_3R subtype has an important role in the modulation of the mesolimbic DA pathway and in the control of drug-seeking behaviour (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). 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). 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 densieties have been found in granule cells of the island of Calleja and in medium spiny neurons (MSN) on the rostral and ventromedial shell of NAc (Diaz et al., 1994, 1995; Le Moine and Bloch, 1996). In addition, recent advances in technologies for the identification of specific cell types, including BAC transgenic mice expressing fluorescent reporter or the Cre recombinase, allow a more comprehensive understanding of the involvement of D_3R-expressing MSNs in various physiological and pathological conditions

(Gangarossa et al., 2013). Analysis of GFP expression in *Drd3- Cre* crossed with the *Rosa26:loxP* reporter mouse line (Genstat, Gene Expression Nervous System Atlas) makes possible the detailed characterization of the microanatomical distribution of D_3R-expressing MSNs in the mouse NAc. D_3R activates Gαi/o proteins to inhibit cAMP production and decrease PKA activity (Missale et al., 1998; Robinson and Caron, 1997), but D_3R also regulates other intracellular pathways, including the extracellular signal regulated kinase 1/2 and Akt cascades through G protein-dependent and/or independent mechanism, this latter involves β-arrestin (Collo et al., 2008, 2012; Cussac et al., 1999). The ability of ligands to differentially affect signaling through these pathways, referred to as biased agonism or functional selectivity, may be therapeutically exploitable. Recently, ligands that are devoid of D_2R-mediated Gαi/o protein signaling, but behave as partial agonists for D_2R/β-arrestin interactions, have been found to exert a number of effects in preclinical models of schizophrenia-like behavior while causing lower catalepsy (Park et al., 2016). As the majority of G protein-coupled receptors, D_3R forms both homo and heteromers (Maggio et al., 2015). Heteromers have been reported with D_2R (Scarselli et al., 2001), D_1R (Fiorentini et al., 2008; Marcellino et al., 2008), and also with the adenosine receptor A2AR (Torvinen et al., 2005). Immunocytochemical experiments, showing that D_3R is expressed in all dopaminergic neurons (Diaz et al., 2000), support the notion of D_3R functioning as autoreceptor. The higher

dopamine extracellular levels in NAc (Koeltzow et al., 1998) and striatum (Joseph et al., 2002) in $D_3R^{-/-}$ compared to their WT littermates suggest a D_3R-mediated control of dopamine release. 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, it has been demonstrated that mice with a striatal overexpression of D_3Rs have less marked, but still noteworthy phenotype (Simpson et al., 2014). Indeed, these mice exhibit a disrupted motivation, suggesting that targeting D_3R might have effect on motivational symptoms.

Yet, D_3R is important for prefrontal executive function, as pharmacological and genetic manipulations that affect prefrontal D_3Rs alter anxiety, social interaction and reversal learning. So, Clarkson and colleagues (2017) showed that D_3R expression defines a novel subclass of layer 5 glutamatergic pyramidal cell in mouse prefrontal cortex. D_3 receptor-expressing pyramidal neurons are electrophysiologically and anatomically separable from neighboring neurons expressing D_1 and D_2 receptors. Moreover, they discovered that D_3R activation, within these neurons, regulates low-voltage activated $Ca_v3.2$ calcium channels localized to the axon initial segment. Thus, Clarkson and colleagues' data indicate that D_3 receptors regulate the excitability of a unique, intratelencephalic prefrontal cell population, thereby defining novel circuit and cellular action for D_3Rs in PFC.

4. D_3R and alcohol addiction

Addiction is a neuropsychiatric disorder characterized by compulsive engagement in rewarding stimuli, despite adverse consequences. Modifications in the mesolimbic/mesocortical DAergic pathways represent core biological changes underlying addictive behaviors. Synthetic/natural rewards increase extracellular DA in limbic/cortical areas, besides producing other long-term modifications, including a potentiation of glutamatergic transmission in midbrain DAergic nuclei, NAc, striatum and cortex (Volkow and Morales, 2015). Moreover, long-term changes in DAergic receptor responsiveness possibly contribute to synaptic/neuronal adaptations leading to psychostimulant-induced sensitization and compulsion (Hyman et al., 2006). Actually, addictive drugs down-regulate D_2R-like receptors, with a reduced expression of striatal D_2R and D_3R in individuals addicted to cocaine, methamphetamine, alcohol or heroin (Volkow et al., 1993, 1996, 2001). Particularly, 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 this 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 widely expressed in the shell of NAc, regulates the mesolimbic DA pathway and is involved in the neural mechanism underlying drug seeking behaviour (Heidbreder et al., 2005). 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). As was demonstrated by Heidbreder and colleagues in 2007, the selective D_3R antagonist SB277011A reduces alcohol intake and prevents relapse to alcohol-seeking behaviour of male C57BL/6N mice exposed to oral operant self-administration. Moreover, the preferential 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) enhances both ethanol intake and preference at the dose of 0.01 mg/kg (Silvestre et al., 1996). Vengeliene and colleagues (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 behaviour. Yet, SB277011A significantly decreases 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). Furthermore, Leggio and colleagues (2014) demonstrated that $D_3R^{-/-}$ show significant lower levels of ethanol intake compared to their WT littermates. Despite several studies have investigated the involvement of D_3R in ethanol reward, its precise role is largely unknown.

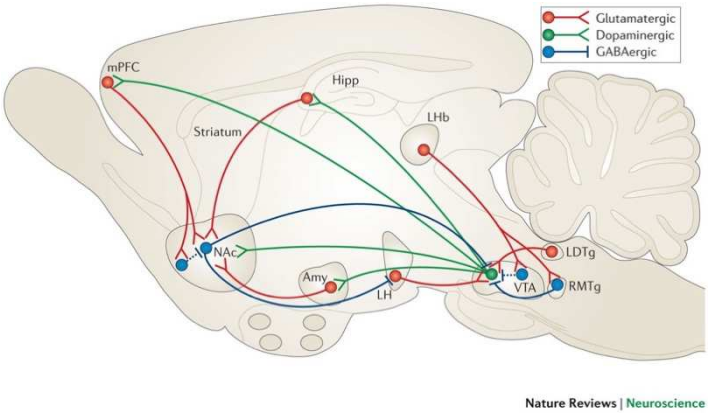

Figure 3: VTA– NAc reward circuit. The major reward circuit consists of dopaminergic fibers originating from the VTA and projecting to the NAc (in green), which release dopamine in response to reward-related stimuli. (Russo and Nestler, 2013).

5. D_3R and Schizophrenia

Schizophrenia is a disease affecting about 1% of population worldwide, characterized by abnormalities of behaviour and thinking with inability to understand reality. The ‘‘DA theory’’ for a dysfunction of DAergic transmission represents the first pathogenetic hypothesis of psychosis, being postulated following the fortuitous discovery of antipsychotics, acting as D_2R antagonists. Actually, schizophrenia is characterised by a hyperactivation of DAergic mesencephalic nuclei associated to a DAergic hypofunction in prefrontal cortex (PFC; Howes and Kapur, 2009; Perez-Costas et al., 2010; Yoon et al., 2013; Weinstein et al., 2017). Besides DAergic dysfunctions, alterations in glutamatergic transmission occur, with the ‘‘DA-Glutamate hypothesis’’; this hypothesis represents the current pathogenetic theory for schizophrenia, suggesting that this condition is associated with excessive stimulation of striatal DA D_2 receptors, deficient stimulation of prefrontal DA D_1 receptors and, alterations in prefrontal connectivity involving glutamate transmission at N-methyl -D-aspartate (NMDA) receptors (Laruelle et al., 2003). Supersensitivity to DA, due to modified DAergic receptors expression and/or functions, might contribute to schizophrenic symptomatology (Seeman et al., 2005). The first-line pharmacological treatment for schizophrenia is represented by antipsychotics. At first, antipsychotics were considered D_2R antagonists (Kapur & Mamo, 2003), and later on reconsidered as D_2R-like

antagonist, to indicate their low selective binding at D_2R, D_3R and D_4R. D_3R has been proposed as an available target for schizophrenia treatment, as a result of their restricted localization in limbic areas (Gurevich et al., 1997). D_3R modulation could improve cognitive/negative schizophrenic symptoms, without producing extrapyramidal/motor effects as D_2R antagonists (Joyce and Millan, 2005). Actually, novel antipsychotics acting as D_3R partial agonists/antagonists, ameliorate cognitive/negative schizophrenic symptoms (Leggio et al., 2016). None of the antipsychotic currently available act as selective ligand for D_3R (Schotte et al., 1996; McCormick et al., 2010); for example, in vivo human PET studies have shown that clozapine, olanzapine and risperidone poorly occupy D_3R in the brain of patients with schizophrenia (Graff-Guerrero et al., 2009; Mizrahi et al., 2011). In contrast with human studies, a number of D_3R selective ligands have recently become available for animal studies, where they have been tested, together with genetic deletion, to sort out the role of D_3R in schizophrenia. Available drug treatments are effective in improving positive symptoms (delusions, hallucinations), but show limited activity on negative symptoms (anhedonia, social withdrawal, lack of motivation) and on cognitive dysfunction. Although preclinical data are conflicting, it has been suggested that blockade of D_3R may impact cognitive impairment; indeed, $D_3R^{-/-}$ show a better performance than WT in a step-through passive-avoidance paradigm (Micale et al., 2010), while treatment with the D_3R selective antagonist

SB277011A does not improve the performance in the Morris water maze test (Tanyeri et al., 2015). On the other hand, while overexpression of D_3R in striatum does not induce cognitive deficits, it disrupts motivation, suggesting that changes in D_3R may be involved in the negative symptoms of schizophrenia (Simpson et al., 2014). Most antipsychotics, either first or second generation, do not display selectivity for D_3R over D_2R, but few compounds, including aripiprazole, blonanserin and cariprazine, show some D_3R selectivity. Asenapine has higher affinity at D_3R compared to D_2R, but displays higher affinity at some 5-hydroxytryptamine (5-HT) receptor subtypes (Shahid et al., 2009).

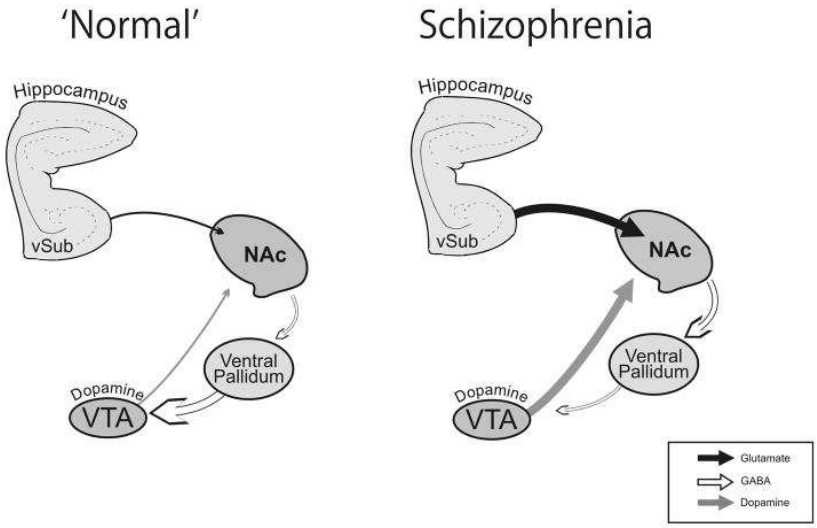

Figure 4: Cartoon depicting the aberrant regulation of the dopamine system in Schizophrenia. Hippocampal hyperactivity (suggested to be associated with a decrease in GABA transmission) results in an increased activation of the nucleus accumbens (NAc). The subsequent increase in NAc output inhibits the ventral pallidum (VP) resulting in the disinhibition of VTA dopamine neurons. (Logde and Grace, 2010).

6. Design of the present research

Based on the reviewed data present in literature, the aim of the present thesis has been to assess: (i) The $GABA_A/D_3R$ interaction in the mesolimbic DA modulation of alcohol reward; (ii) the involvement of D_3R in the pathophysiology of schizophrenia, using the methylazoxymethanol acetate **(**MAM) rats, an animal model of SCZ. The following aspects were investigated:

1. Testing the hypothesis that D_3R-dependent changes in $GABA_A$ α6 subunit expression, in NAc, affect the alcohol intake behavior, and, at the cell level, the electrical activity of MSN, thereby influencing the inhibitory synaptic transmission of NAc.
2. Assessing if the pharmacological blockade of D_3R affect α6 $GABA_A$ subunit expression in the NAc of WT littermates.
3. Investigating the involvement of D_3R in pathophysiology of MAM-induced SCZ-like phenotype, in rats
4. Evaluating D_3R expression in different brain areas of MAM rats, where alterations have been documented in SCZ**.**
5. Testing the hypothesis that the cannabinoid receptor 1 (CB1) pharmacological blockade controls dopaminergic alterations, via a modulation of the EC signalling.

Chapter II

Submitted

Dopamine D3 receptor-dependent expression of alpha6 GABAA subunit in the NAc counteracts alcohol intake by increasing GABA inhibition

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Abstract

We tested the hypothesis that dopamine D_3 receptor (D_3R)-dependent changes in $GABA_A$ α6 subunit expression in the nucleus accumbens (NAc) affect

voluntary alcohol intake, and, at the cell level, the electrical activity of medium spiny neurons (MSN), thereby influencing the inhibitory synaptic transmission of NAc. We revealed $GABA_A$ α6 activity by using Ro 15-4513. At baseline, α6 expression in NAc is negligible in wild type (WT) mice, whereas it is robust in $D_3R^{-/-}$. In the drinking-in-the-dark paradigm (DID), Ro 15-4513 inhibited alcohol intake in WT, but it increased it in $D_3R^{-/-}$. Treatment with SB 277011A, a D_3R antagonist, increased α6 expression and partially reversed the behavioral effect of Ro 15-4513 in the DID. In situ hybridization and qPCR confirmed α6 subunit mRNA expression especially in the NAc, being very low in other forebrain areas, while other relevant $GABA_A$ subunits were not changed in $D_3R^{-/-}$. Peak amplitudes of miniature inhibitory postsynaptic currents in NAc MSN showed a significant increase in $D_3R^{-/-}$ compared to WT. Furthermore, Ro 15-4513 reduced the peak amplitude in the NAc of $D_3R^{-/-}$, but not in that of WT. These data indicate that D_3R-dependent enhanced expression of α6 $GABA_A$ receptor subunit inhibits voluntary alcohol intake by increasing GABA inhibition in the NAc.

Key words: dopamine D3 receptor, $GABA_A$ receptor, alpha6 subunit, ethanol, Ro 15-4513

Significance

The dopamine D_3 receptor (D_3R), highly expressed in the nucleus accumbens (NAc), plays an important role in reinforcement and reward mechanisms of substance abuse disorders, including alcohol addiction. CNS effects of ethanol are partly mediated by stimulation of $GABA_A$ receptor, a ligand-gated anion channel composed of 5 subunits. Here, we show that D_3R-dependent changes in $GABA_A$ α6 subunit expression in the NAc control the alcohol intake behavior by increasing inhibitory $GABA_A$ currents in medium spiny neurons, the major cell population in the NAc. Thus, $D_3R/GABA_A$ cross talk is operative in the reinforcing mechanisms of alcohol, and represents a potential target for treatment of alcohol abuse.

1. Introduction

Alcohol is the most widely used and abused of all psychoactive drugs. Despite its mechanism of action being still elusive, a general consensus recognizes its major impact on the brain reward system. In fact, repeated intake of ethanol induces alterations in the nucleus accumbens (NAc), a main component of the mesolimbic reward circuit, as do several other drugs of abuse (1, 2). In this brain region more than 95% of the cells are GABAergic Medium Spiny Neurons (MSNs), whose activity is regulated by dopaminergic and glutamatergic inputs (3). MSNs comprise three distinct cell subpopulations; one expresses dopamine D1-like receptors (D_1R and D_5R), a second one expresses dopamine D_2-like receptors (D_2R, D_3R, D_4R), and a small third one expresses both D_1-like and D_2-like receptors (4, 5). $GABA_A$ receptors ($GABA_A$Rs) in the NAc have long been considered as a primary target for alcohol, and may be involved in voluntary alcohol consumption (6-9); moreover, chronic alcohol intake alters GABAergic function in the NAc, which sustains behavioral addictive patterns (2, 9). $GABA_A$Rs are pentamers assembled from a variety of subunits to form multiple isoforms that are likely to differ in their alcohol sensitivity (10). The $GABA_A$R is an heteromeric chloride channel comprising five subunits from the 19 known up to now, α1-6, β1-3, γ1-3, δ, ε, θ, π, ρ1-3 (11). This ionotropic receptor represents a major pharmacological target for many drugs, including benzodiazepines, barbiturates and ethanol. While GABA

binds to an orthosteric site, these exogenous compounds (and some endogenous modulators) bind to allosteric sites, which affect the gating of the channel and/or the response to GABA (11). In some experiments, the $GABA_A$Rs containing α6 subunit seem particularly sensitive to alcohol; indeed, rats expressing the naturally occurring R100Q allelic variation of α6 exhibit a higher sensitivity to motor incoordination induced by moderate doses of ethanol (12) and avoid alcohol consumption (13). This mutation was originally found enriched in a selectively bred, alcohol-sensitive rat line (14), which also shows reduced voluntary acceptance of alcohol solutions (15). Furthermore, the hypersensitivity to ethanol is also seen in tonic inhibitory currents mediated by the α6βδ-type $GABA_A$Rs in cerebellar slices (16). GABAergic MSNs receive dopaminergic inputs from the ventral tegmental area (VTA)(17); activation of this circuitry, the dopaminergic mesolimbic pathway, is classically considered as responsible for the reward response to physiological (e.g. food intake, sexual activity) or pathological (drug of abuse) stimuli. Activation of D_3R, highly expressed in the NAc, is involved in the control of alcohol consumption (18-20). Indeed, either D_3R gene deletion or D_3R pharmacological blockade inhibit alcohol intake (18). Because DRs and $GABA_A$Rs are co-localized in MSNs, both contributing to the control of NAc output (21), we hypothesized that some cross-talk may exist between D_3R and $GABA_A$Rs in the regulation of reward system. In this respect, we have already shown that genetic deletion or

pharmacological blockade of D_3R increases $GABA_A$ α6 subunit expression in the ventral striatum (22).

Here, we tested the hypothesis that D_3R-dependent changes in $GABA_A$ α6 subunit expression in the NAc affect the alcohol intake behavior, and, at the cell level, the electrical activity of MSNs, thereby influencing the inhibitory synaptic transmission in the NAc. To do so, we attempted to directly reveal $GABA_A$ α6 activity, by using Ro 15-4513, an imidazobenzodiazepine $GABA_A$ ligand exerting differential effects depending on the α subunit present in the $GABA_AR$ isoform, showing negative allosteric agonism with α1,2,3 and 5, but positive agonism with α4 and α6 (23, 24). Interestingly, based on molecular docking analysis and ligand binding interactions, Ro 15-4513 has been proposed to compete with ethanol within a binding pocket involving α6 (25-27). More importantly, Ro 15-4513 has shown efficacy in reducing alcohol drinking in rodents (28-30), but the detailed mechanisms of action have remained unknown. However, Ro 15-4513 may be considered α6-specific, since its binding is obvious in a α6 reach brain structure, such as the cerebellum, while it is hardly detectable in the very same structure in α6 null mice (31).

2. Materials and Methods

Male mice $D_3R^{-/-}$, $D3R^{+/-}$ and WT littermates (57) were used; experiments were carried out according to the Directive 2010/63/EU and to the Institutional Animal Care and Use Committee of the Catania University. The 4-hour version of the Drinking in the dark paradigm (DID) was used, according to Rhodes et al. (58). All drugs were intraperitoneally (i.p.) injected (for dose-regimens, solubility and commercial sources see *SI*). Analysis of mRNA Expression was carried out by Real-Time Quantitative RT-PCR and in situ hybridization (59); for more details see *SI*. [^{3}H]Ro 15-4513 autoradiography protocol is described in *SI*; Preparation of brain slices for electrophysiology followed the protocol by Scala et al. (60); for further details see *SI*. Data are expressed as means ± standard errors of the means (SEM). Statistical significance was assessed with the Student's t test (when used, paired-t test has been indicated in the text), one or two-way analysis of variance (ANOVA). The post hoc Newman-Keuls test was used for multiple comparisons. The level of significance was set at 0.05.

3. Results

3.1 Alcohol intake inversely correlated with $GABA_A$ α6 subunit expression.

We previously reported that $D_3R^{-/-}$ mice have low ethanol intake (18) and exhibit higher basal ectopic expression of $GABA_A$ α6 in the ventral striatum (22). Here, we assessed whether or not a correlation exists between alcohol consumption and $GABA_A$ α6 subunit expression in the NAc. Based on our previous data, we compared here WT ($D_3R^{+/+}$), heterozygous $D_3R^{+/-}$ and homozygous $D_3R^{-/-}$. As shown in Fig. 1, $D_3R^{+/-}$ exhibited low α6 expression, similar to WT. In contrast, $D_3R^{-/-}$ exhibited about 5-fold higher basal mRNA expression of α6 subunit as compared with WT and $D_3R^{+/-}$ [main effect of genotype $F(2, 14) = 9.447$, $P<0.01$; *post hoc:* $P<0.01$]. As expected, WT showed obvious ethanol preference in the drinking-in-the-dark (DID) paradigm, whereas $D_3R^{-/-}$ showed significantly lower ethanol intake [Fig. 1a, main effect of day: $F(3, 60) = 40.58$, $P<0.01$; main effect of genotype $F(2, 20) = 7.812$, $P<0.01$; *post hoc:* $P<0.01$ and $P<0.05$]. $D_3R^{+/-}$ showed alcohol intakes similar to WT, indicating that, the partial D_3R deletion in $D_3R^{+/-}$ did not modify the ethanol-preferring phenotype. Overall, the inverse correlation between α6 mRNA expression and alcohol intake was statistically significant ($R^2=0.44$, $P<0.01$, Fig. 1G). Thus, the high level of $GABA_A$ α6 subunit expression in the NAc mediates the reduction of alcohol consumption of $D_3R^{-/-}$.

3.2 Alcohol antagonist Ro 15-4513 increased ethanol consumption in mice expressing $GABA_A$ α6 in NAc.

The imidazobenzodiazepine Ro15-4513 was earlier named "alcohol antagonist" (32), because, in some studies, it inhibited alcohol intoxication, preference and self-administration in wildtype rodents (33-35). Furthermore, Ro 15-4513 has been proposed to bind to alcohol-sensitive competitive sites at α4/6β3δ-type $GABA_A$ receptors with high affinity ($K_D \approx 10$ nM)(36, 37), and to exert an effect on $GABA_AR$ currents which might depend on α6 and δ subunits (12), while in the cerebellum, the Ro 15-4513 binding seems to be almost entirely α6 subunit-dependent (31). Therefore, we tested here the hypothesis that Ro 15-4513 differently affects ethanol intake in mice expressing different levels of α6 in the NAc. As shown in Fig. 1C, Ro 15-4513 decreased voluntary ethanol intake in WT [main effect of day $F(3, 63) = 55.62$, $P<0.01$; main effect of treatment $F(1, 21) = 7.198$, $P < 0.05$; post hoc: $P<0.05$], but increased voluntary ethanol intake in $D_3R^{-/-}$ (Fig. 1D) [main effect of day $F(3, 39) = 34.87$, $P<0.01$; main effect of treatment $F(1, 13) = 9.384$, $P<0.01$; post hoc: $P<0.05$]. Worthy of note, $D_3R^{-/-}$, which normally show low preference for alcohol (18), following Ro 15-4513–treatment reached a level of ethanol consumption similar to that of WT. These data indicate that the paradoxical response to Ro 15-4513 seen in $D_3R^{-/-}$ is related to increased expression of

$GABA_AR$ containing α6 subunit in the NAc, and reinforce the negative association between α6 expression and ethanol consumption mentioned above.

Changes of $GABA_AR$ function induced by alterations in dopaminergic transmission may have clinical relevance, because a number of DR ligands, including D_3R selective ligands, are currently used to treat different neuropsychiatric disorders (38). In this respect, consistent with data obtained in $D_3R^{-/-}$ mice, we previously reported that chronic treatment with the selective D_3R antagonist SB 277011A increases α6 expression in the ventral striatum and accelerates the appearance of tolerance to the anxiolytic effect of diazepam (22). Here, to obtain further evidence of a functionally relevant cross-talk between D_3R and $GABA_AR$ containing α6 subunit in the control of voluntary alcohol consumption, we treated WT with SB 277011A for 7 days, before testing in the DID paradigm. As shown in Fig. 1E, SB 277011A-treatment reduced ethanol voluntary intake [main effect of day $F(3, 90) = 42.90$ $P<0.01$; main effect of treatment $F(2, 30) = 6.754$ $P<0.01$; post hoc: $P<0.05$, $P<0.01$]; the inhibition by SB 277011A was partially reverted by a treatment with Ro 15-4513, that increased ethanol intake on day 4, though not attaining statistical significance. Consistent with our previous finding (18), repeated treatment with SB 277011A also robustly increased the $GABA_A$ α6 subunit expression in the NAc of WT animals [Fig. 1F, main treatment effect $F(2, 9) = 17.07$ $P < 0.01$; post hoc: $P<0.01$]. Therefore, following D_3R blockade, increased expression of

$GABA_AR$ containing α6 subunit in the NAc was accompanied by a tendency toward a paradoxical effect of Ro 15-4513, similar to $D_3R^{-/-}$.

To precisely assess the spatial expression of α6 subunit in CNS, we carried out in situ hybridization (ISH) experiments. These experiments confirmed that, while heavily enriched in the cerebellar granule cell layer (not shown), significant α6 expression in the forebrain occurred specifically in the NAc, being very low in the other examined brain areas (Fig. 2A, B, Tab. 1, S1, S2). Furthermore, the expression of other relevant $GABA_A$ subunits was not changed in $D_3R^{-/-}$ (Tab. 1). Data obtained by ISH were confirmed by qPCR (Fig. S3). Autoradiography following incubation with a high 15-nM concentration of [^{3}H]Ro15-4513 showed a modest but statistically significant increase of [^{3}H]Ro15-4513 binding in the NAc (Fig. 2C, D), consistent with an increase in total number of $GABA_A$ receptors in the NAc, due to increase in α6 expression.

3.3 $D_3R^{-/-}$ mice exhibited Ro 15-4513-driven decrease of mIPSC amplitude in Medium Spiny Neurons.

To test the hypothesis that α6 subunit expression in the NAc shell, as seen in $D_3R^{-/-}$ mice, modifies inhibitory transmission, we performed whole-cell patch-clamp recordings on GABAergic MSNs, which represent >95% of the cell population in this brain region, and recorded miniature inhibitory postsynaptic

currents (mIPSCs). Analysis of the peak amplitudes of mIPSCs revealed a significant increase in $D_3R^{-/-}$ compared to WT (Fig. 3; A-D; 38.58 ± 3.35 pA, n = 19 versus 29.51 ± 2.96 pA, n = 16; $P<0.05$). In contrast, there was no significant difference in mIPSC frequency ($D_3R^{-/-}$: 1.98 ± 0.30 Hz, WT: 1.77 ± 0.26 ms) and mIPSC kinetics (Fig. 3 G, H; rise time, $D_3R^{-/-}$: 0.72 ± 0.06 ms; WT: 0.72 ± 0.06 ms; decay time, $D_3R^{-/-}$: 16.96 ± 1.10 ms; WT: 16.14 ± 1.31 ms). Next, we tested the effects of Ro 15-4513, which preferentially acts on α4/6 subunit-containing $GABA_A$Rs (36, 37) on mIPSCs in MSNs from WT and $D_3R^{-/-}$. Based on ISH and qPCR data indicating that $GABA_A$Rs containing α6 subunit in the NAc are almost absent in naïve WT mice and given the opposite effect of Ro 15-4513 treatment on ethanol intake observed in $D_3R^{-/-}$ mice, we expected that Ro 15-4513 would have differential effects on mIPSCs. For this in vitro experiment we selected the 0.3 μM Ro 15-4513 concentration, because it completely antagonizes ethanol enhancement of α4β3δ-type $GABA_A$R current (36). As shown in Fig. 3, bath application of 0.3 μM Ro 15-4513 did not significantly alter the frequency, rise time, decay time and amplitude of mIPSCs in WT (n = 16; paired t test). Surprisingly, 0.3 μM Ro 15-4513 induced a significant reduction of amplitude in the NAc of $D_3R^{-/-}$ (Figure 3; B-F; 38.58 ± 3.35 pA, versus 31.93± 3.03 pA, n = 19 $P<0.05$; paired t test) while frequency, rise time and decay time were not affected. These results suggest that the activity of α6-subunit-containing $GABA_A$R in $D_3R^{-/-}$ influences inhibitory

synaptic transmission of MSN within NAc shell. Since this effect of Ro 15-4513 resulted in a negative modulation, it is possible that the induced α6 subunit expression generates a novel population of $GABA_A$ receptor with heteromeric α subunits, such as α1 and α6 (39).

4. Discussion

We found that D_3R-dependent increased expression of α6 $GABA_A$ subunit counteracts alcohol intake by increasing GABA inhibition in the NAc. We revealed $GABA_A$ α6 activity by using Ro 15-4513, a $GABA_A$ ligand which appeared to exert α6-dependent effects, both in terms of behavior (ethanol intake) as well as of neuronal excitability (electrophysiology). The α6 subunit came to the attention of the alcohol addiction studies following the identification of the R100Q mutation in the Sardinian non-ethanol-preferring rat line, suggesting a possible involvement of the $GABA_A$R containing α6 subunit in the genetic predisposition to alcohol preference (13). This mutation is associated to hypersensitivity to motor-impairing effects of ethanol as well as to tonic inhibitory currents mediated by the α6βδ-type $GABA_A$R, measured by patch-clamp in cerebellar granule cells (10, 16). Worthy of note, this mutation strongly increases diazepam effect on GABA-evoked currents (14), consistent with a model where the amino acid residue at position 100 that affects ethanol sensitivity in the $GABA_A$Rs is part of the benzodiazepine ligand-binding pocket on the α6-subunit (22, 40, 41). Other studies have also described in humans α6 polymorphisms that correlate to alcohol dependence (42, 43). Our observation that genetic deletion or pharmacological blockade of D_3R increases $GABA_A$ α6 subunit expression in the ventral striatum (19, the present study), a brain structure involved in voluntary ethanol intake, provided a tool to study how

increased expression of α6 subunit-containing receptors may affect alcohol intake. Several studies, in the last two decades, have tried to elucidate how the subunit composition of different $GABA_A$Rs determines their electrophysiological and pharmacological features (inhibitory currents, ligand binding), or, at the organism level, the animal behavior (anxiety, addiction, response to anxiolytics). While most studies have dealt with recombinant systems, such as *Xenopus laevis* oocytes injected either with cRNA coding for the different subunits (12, 36) or with cRNA coding for concatenated subunits (44), no studies had the opportunity to examine native systems, i.e. animals spontaneously and stably expressing ectopic specific subunits in defined CNS structures. Polymorphisms of α6 subunit have been found to be associated both to anxiety-related traits (45) and to benzodiazepine sensitivity in humans (46). It is not yet known whether ectopic expression of α6 subunit containing $GABA_A$R isoforms in brain areas that normally express negligible amounts of α6 produces different responses to GABA (i.e. different inhibitory currents) and/or to exogenous modulators, including benzodiazepines and ethanol, probably because *in vivo* systems with significant ectopic α6 expression are not commonly available. The early studies with α6 subunit knockout mice (31, 47) remained inconclusive as it was later discovered that the knockout construct affected the expression of neighboring subunits in the $GABA_A$ gene cluster (48). We took advantage of Ro 15-4513, because it has been proposed to

compete with ethanol within a binding pocket involving α6 (25). We expected a different effect of Ro 15-4513 in WT, which barely express α6 in the NAc, versus $D_3R^{-/-}$, which robustly express α6. Indeed, we found an opposite effect of Ro 15-4513 in the two groups; in WT Ro 15-4513 reduced ethanol intake, presumably as a result of its action as a negative allosteric modulator in multiple $GABA_AR$s (36), where it would behave as an "ethanol antagonist" (25, 26); in contrast, in $D_3R^{-/-}$ Ro 15-4513 paradoxically increased ethanol intake, a surprising finding that might be explained in terms of differential modulation of the $GABA_AR$ containing α6 subunit by Ro 15-4513. Indeed, the antagonism between Ro 15-4513 and ethanol might be more at the functional level, rather than at the binding level; in fact, while the reported affinity of Ro 15-4513 for α4 and α6 containing $GABA_AR$ is quite close, in the nanomolar range (12, 25, 36, 49), the effect on the GABA-dependent currents in cells expressing either isoform is not clear, but might be quite different, as suggested by a paradoxical activation of neurons by gaboxadol in a transgenic Thy1α6 mouse line, ectopically expressing the $GABA_AR$ α6 subunit gene under the Thy-1.2 promoter (23). We directly address this issue by measuring MSN mIPSCs in the NAc and their sensitivity to Ro 15-4513. Based on the above premises, we hypothesized that a change in $GABA_A$ α6 subunit expression would increase spontaneous mIPSCs and that Ro 15-4513 would inhibit mIPSCs in MSN from $D_3R^{-/-}$, robustly expressing α6, whereas it would be ineffective in α6-deficient

MSNs from WT. The electrophysiological analysis of MSNs revealed a significant increase in mIPSC amplitude in $D_3R^{-/-}$, which expressed $GABA_AR$ containing α6 subunit in NAc, compared to WT; perfusion with Ro 15-4513 induced a significant reduction of amplitude in the NAc of $D_3R^{-/-}$, but was ineffective in WT. This latter observation clearly indicates that the modulation of the $GABA_AR$ channel by Ro 15-4513 depends on the presence of α6 subunit and is consistent with the observation of opposite effects of this drug on ethanol intake in WT and $D_3R^{-/-}$. Systematic assessment of α6 expression in the CNS by ISH, confirmed by qPCR, indicates that "ectopic" α6 expression in $D_3R^{-/-}$ was restricted to a limited brain area, corresponding to ventral striatum and including the NAc, a finding reinforced by autoradiography data obtained with [^{3}H]Ro 15-4513. The fact that genetic or pharmacological manipulation of D_3R specifically induced changes in the NAc, leaving relatively unchanged other brain areas is not so surprisingly, when considering that, at variance with D_2R, the expression of D_3R is mainly restricted to the very same structures where we observe increased α6 expression (50). To the best of our knowledge, it is not known in detail how D_3R control $GABA_AR$ subunit mRNA expression; however, other studies have shown dynamic D_3R-dependent down-regulation of GABAergic control over lateral/basolateral amygdala neurons (51), NAc (52) and hippocampus (53, 54). A direct dynamic interplay between metabotropic dopamine receptors and ionotropic (NMDA) receptors in plasma membrane has

been documented by single-molecule detection imaging and electrophysiology in live hippocampal neurons (55). Furthermore, cell signaling downstream of D_3R affects $GABA_ARs$ in the NAc (52), but numerous other complex mechanisms may impact $GABA_ARs$ trafficking (56) and deserve further studies to be elucidated. Finally, because these changes in $GABA_AR$ function can be related to dopaminergic transmission, they may assume further relevance in clinical situations, such as schizophrenia and Parkinson's disease, where D_3R are chronically blocked or stimulated by drug-treatments (38).

In conclusion, these data indicate that α6-containing $GABA_ARs$ in the NAc play an important role in controlling alcohol intake by increasing GABAergic-inhibition in the MSNs. Because changes in α6-containing $GABA_ARs$ are specifically induced in the NAc by D_3R-blockade, the interplay between DAergic and GABAergic transmission may present a novel relevant mechanisms in reinforcing properties of alcohol and other addictive drugs.

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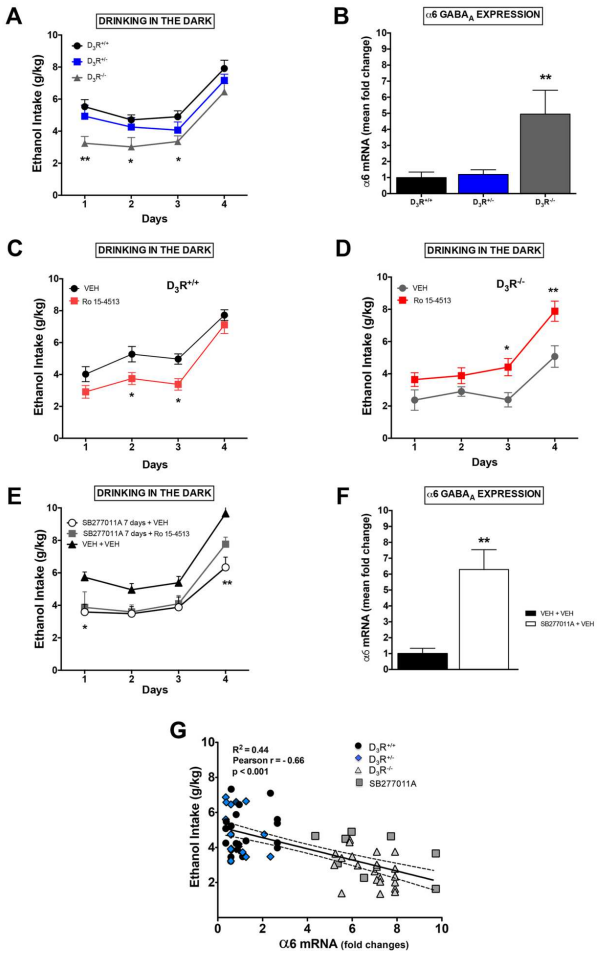

Figure 1. Alcohol intake inversely correlated with D_3R-dependent $GABA_A$ α6 subunit mRNA expression in the NAc; effect of Ro 15-4513 on ethanol consumption in mice expressing different levels of α6. A and B, ethanol intake (in the drinking in the dark paradigm, DID) and α6 expression in wild type ($D_3R^{+/+}$) heterozygous ($D_3R^{+/-}$) and null mice ($D_3R^{-/-}$). DID was measured for 4 days, in mice with limited access (2h/day for 3 days and 4h the 4th day) to ethanol solution (20%). Abundance of transcripts in the NAc was assessed by qPCR after DID; expression level is expressed as mean fold changes relative controls. C and D, ethanol intake (DID) in $D_3R^{+/+}$ and $D_3R^{-/-}$ treated with vehicle (VEH) or Ro 15-4513 (5 mg/kg, i.p.). E and F, ethanol intake (DID) and α6 expression in $D_3R^{+/+}$ treated with vehicle (VEH) or the selective D_3R antagonist, SB 277011A (10 mg/kg, i.p.) with or without Ro 15-4513. Each experimental group included 8-10 mice.

*P<0.05, **P<0.01 vs. the corresponding control ($D_3R^{+/+}$, VEH or VEH+VEH); one- or two-way ANOVA and Newman–Keuls post hoc test.

G, correlation between differences ethanol intake (DID) and α6 expression ($R^2 = 0.44$, Pearson r = -0.66, $p < 0.001$); different symbols represent different genotypes and/or treatments, as indicated.

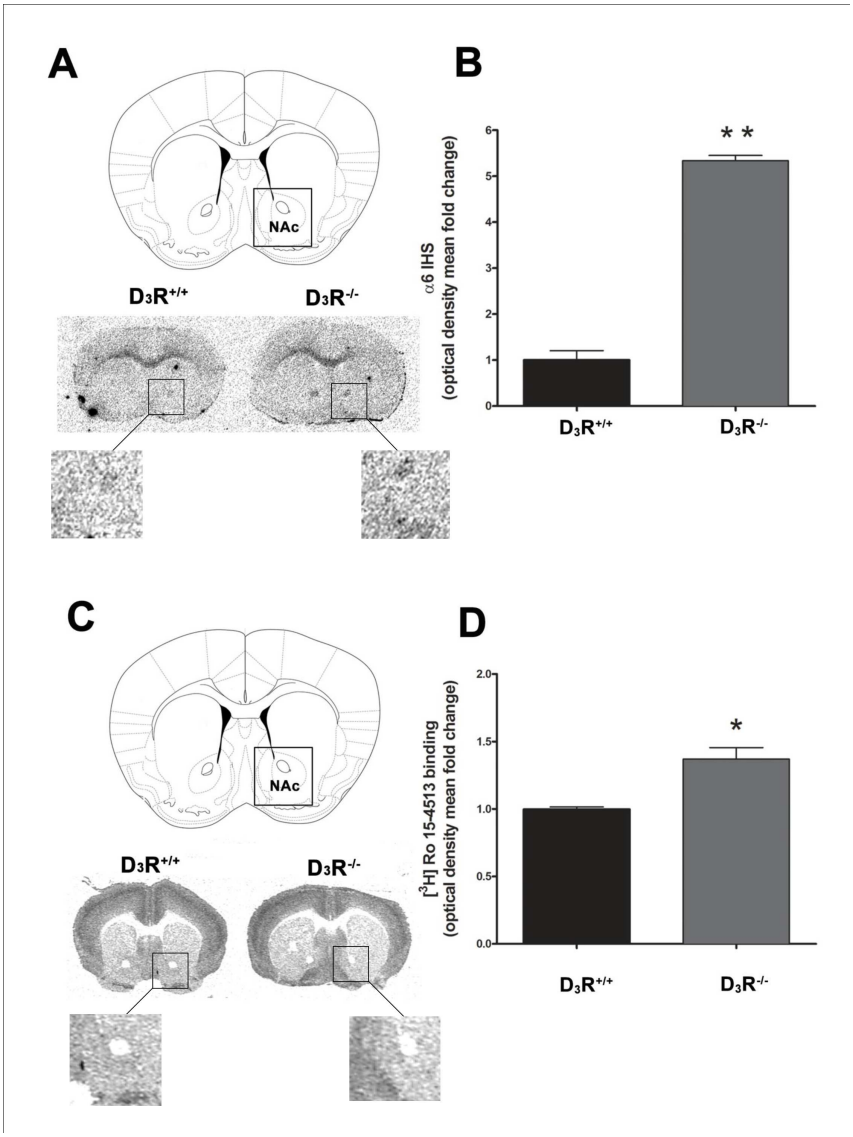

Figure 2. Ectopic expression of α6 $GABA_A$ subunit mRNA and [^{3}H]-Ro 15-4513 binding in the NAc of $D_3R^{+/+}$ and $D_3R^{-/-}$ mice. A and B, *In situ* hybridization (ISH) detection of α6; C and D, [^{3}H]-Ro 15-4513 autoradiography. Representative images and averaged optical density, mean fold changes relative to control (n=6-8 per group).
*P < 0.05, **P < 0.01 vs $D_3R^{+/+}$, unpaired *t* test).

Table 1. *In situ* hybridization (ISH) signals for of $GABA_A$ **α**1, **α**2, **α**4, **γ**2, δ mRNA subunits in the nucleus accumbens from $D_3R^{+/+}$ and $D_3R^{-/-}$ mice.

$GABA_A$ subunit	**ISH signal ($D_3R^{-/-}$ over $D_3R^{+/+}$ ratio)**
α1	1.19 ± 0.18
α2	1.12 ± 0.19
α4	1.03 ± 0.11
γ2	0.98 ± 0.18
δ	0.89 ± 0.19

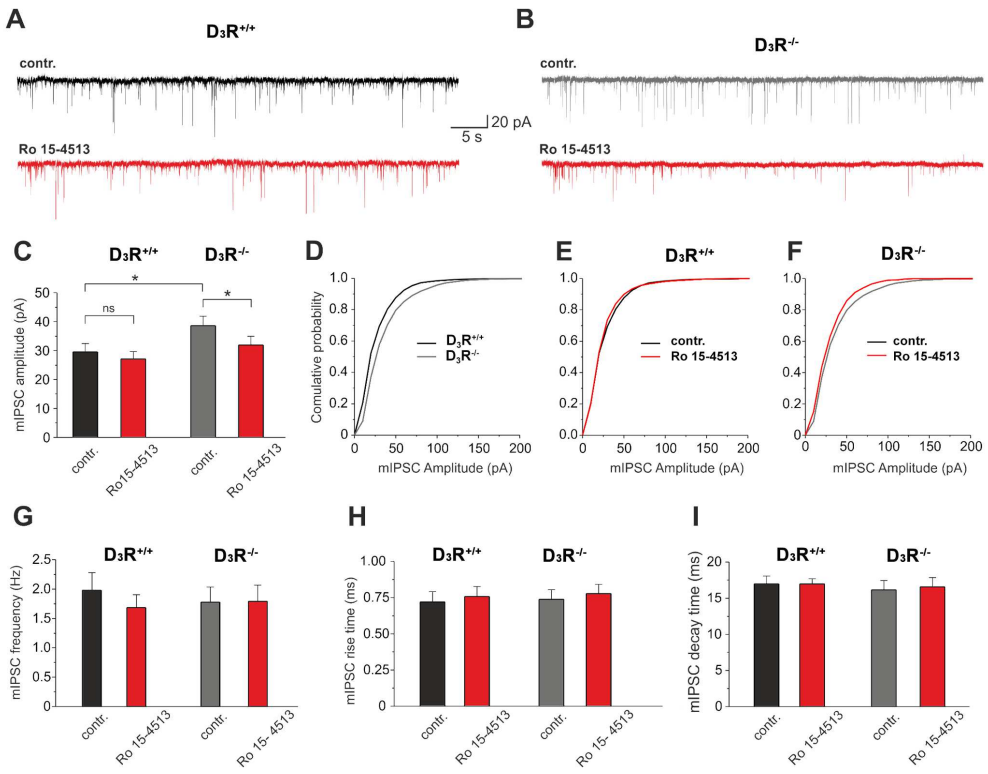

Figure 3. NAc medium spiny neurons from $D_3R^{-/-}$ mice exhibited increased $GABA_A$ inhibitory currents sensitive to Ro 15-4513. A and B, representative traces showing mIPSC recordings in slice from $D3R^{+/+}$ (WT) and $D3R^{-/-}$ mice before and after treatment with Ro-154513 (0.3 µM; in red). C, analysis of the peak amplitudes of mIPSCs; notice an increase in $D_3R^{-/-}$ compared to $D_3R^{+/+}$ and a decrease following Ro 15-4513 application in $D_3R^{-/-}$ only. *P<0.05, unpaired ($D_3R^{-/-}$ vs $D_3R^{+/+}$) or paired (pre- vs post- Ro 15-4513) *t* test ($D_3R^{-/-}$, n=19; $D_3R^{+/+}$, n=16). D-F, cumulative frequency distributions for mIPSC amplitude in the experimental conditions shown in A and B. G-I, analysis of mIPSC frequency, rise time and decay time.

Supporting Information - Leggio et al.

Materials and Methods

Animals.

Mice $D_3R^{-/-}$, $D_3R^{+/-}$ and WT ($D_3R^{+/+}$) 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. Mice $D_3R^{-/-}$ and $D_3R^{+/-}$ were congenic after 10th–12th generation of back crossing into C57BL/6J mouse line (1). 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.

Drinking in the dark paradigm (DID).

The 4-hour version of the behavioral paradigm was used, as described by Rhodes et al. (2). The procedure started 3h 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 (2); 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.

Drugs and Treatments.

Ethanol, Ro 15-4513 and SB 277011A hydrochloride were from Tocris (Ellisville, MO). All drugs were intraperitoneally (i.p.) injected. Ro 15-4513 (5 mg/kg) (3) was dissolved in 10% dimethyl sulfoxide, SB 277011A hydrochloride (10 mg/kg) (4, 5) was dissolved in saline. All drugs were injected in a volume of 10 ml/kg. In the DID paradigm, mice were allocated to seven experimental groups (n = 8/10 per group): WT naive, $D_3R^{+/-}$ naive , $D_3R^{-/-}$ naive, WT/vehicle, $D_3R^{-/-}$ /vehicle, WT/ Ro 15-4513, $D_3R^{-/-}$/ Ro 15-4513. In another set of experiments, mice were randomly allocated to three experimental groups (n= 8/10 per group): WT SB 277011A (pre-treatment for 7 days)/ Ro 15-4513, WT SB 277011A (pre-treatment for 7 days)/vehicle and WT Vehicle (pre-treatment for 7 days)/Vehicle and tested in the DID paradigm. Animals were i.p. injected 1 h before the behavioral procedure. On day 4, animals were sacrificed 1 h after ethanol-drinking procedure and the brain tissues were taken.

A*nalysis of mRNA Expression by Real-Time Quantitative RT-PCR.*

Total RNA was isolated by TRIzol (Invitrogen, Carlsbad, CA) from the NAc. Single-stranded cDNA was synthesized with Super-Script III (Invitrogen), by random priming. Aliquots of cDNA were amplified in parallel reactions with external standards at known amounts, using specific primer pairs for α6-$GABA_A$ subunit, D_3R and GAPDH (reference gene). Each PCR reaction (20 µl 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, IN). Amplifications were carried out in a Light Cycler 1.5 instrument (Roche Diagnostics). Quantification was obtained by the ΔCt comparative method.

In situ hybridization.

The in situ hybridization (ISH) was carried out as described earlier (6). Air-dried slides were fixed in ice-cold 4% paraformaldehyde for 5 min. The sections were washed in 1 PBS at room temperature for 5 min, dehydrated in 70% ethanol for 5 min and stored in 95% ethanol at 4 °C until used. The antisense DNA oligonucleotide probe (Oligomer Oy, Helsinki, Finland) sequences were as follows: α6, 5'-CAG TCT CTC ATC AGT CCA AGT CAT-3'; was complementary to the mouse $GABA_AR$ subunit mRNA sequence. Poly[^{35}S]dATP (PerkinElmer Life and Analytical Sciences, Boston, MA) tails were added to the 3'-ends of the probes by deoxynucleotidyl transferase (Promega Corporation, Madison, WI). Unincorporated nucleotides were removed by Illustra ProbeQuant G-50 Micro Columns (Amersham Biosciences, Buckinghamshire, UK). Labelling efficiency (360,000 cpm/µl) was determined by a scintillation counter. The labeled probe was diluted to 0.06 fmol/µl of hybridization buffer consisting of 50% formamide and 10% dextran sulfate in 4X Saline Sodium Citrate (SSC). Nonspecific controls for the antisense probes were produced by adding 100-fold excess of unlabeled probes. The hybridization occurred under glass Menzel-Gläser coverslips (Thermo Fisher

Scientific, Boston, MA) overnight at 42 °C. Finally, the slides were washed in 1X SSC at room temperature for 10 min, in 1X SSC at 55°C for 30 min, and 1X SSC, 0.1X SSC, 70% EtOH and 95% EtOH at room temperature for 1 min each. The slides were then air-dried and exposed with plastic [^{14}C]-radioactivity standards (GE Healthcare) to BioMax MR films (Eastman Kodak Company, Rochester, NY). Films were scanned (Epson expression 1680 Pro) and binding density was measured as optical density values. Images were imported into the *FIJI* version of the free image processing software *ImageJ*. The [^{14}C]-standards were exposed simultaneously with the brain sections as the reference. The hybridization values were converted to optical density values. Non-specific signal was subtracted to obtain the specific signal.

[^{3}H]Ro 15-4513 autoradiography.

Slides were pre-incubated in ice-cold 50 mM Tris–HCl buffer, pH 7.4, containing 120 mM NaCl for 15 min. The final incubation for basal [^{3}H]Ro 15-4513 binding was performed in the pre-incubation buffer containing 15 nM [^{3}H]Ro 15-4513 (PerkinElmer Life and Analytical Sciences) at 4 °C for 1 h. (7). This high ligand concentration was aimed at estimating the receptor number rather than affinity. The non-specific binding was determined in the presence of 10 μM flumazenil. The sections were then washed in ice-cold pre-incubation buffer twice for 1 min, dipped in ice-cold distilled water, air-dried at room temperature and exposed with [^{3}H]-plastic standards (GE Healthcare) to

Biomax MR films (Eastman Kodak). The films were scanned (Epson expression 1680 Pro) and binding density was measured as optical density values (*FIJI IMAGE-J*). The [^{3}H]-standards were exposed simultaneously with the sections as the reference. Non-specific binding was subtracted to obtain the specific binding values.

*Electrophysiology***.**

For the preparation of brain slices, we followed the protocol described by Scala et al. (8), with minor modifications. Animals were sacrificed by cervical dislocation. Brains were rapidly removed and placed in ice-cold cutting solution containing (in mM): TRIS-HCl 72, TRIZMA base 18, NaH_2PO_4 1.2, $NaHCO_3$ 30, KCl 2.5, glucose 25, HEPES 20, $MgSO_4$ 10, Na-pyruvate 3, ascorbic acid 5, $CaCl_2$ 0.5, sucrose 20. Slices (300 μm thick) were cut on a vibratome (VT1200S; Leica Microsystems, Germany) and immediately transferred to an incubation chamber held at 32°C and filled with a recovery solution containing (in mM): TRIS-HCl 72, TRIZMA base 18, NaH_2PO_4 1.2, $NaHCO_3$ 25, KCl 2.5, glucose 25, HEPES 20, $MgSO_4$ 10, Na-pyruvate 3, ascorbic acid 5, $CaCl_2$ 0.5, sucrose 20. After 30 min, slices were transferred to a second incubation chamber held at 32°C and filled with artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl 124, KCl 3.2, NaH_2PO_4 1.2, $MgCl_2$ 1, $CaCl_2$ 2, $NaHCO_3$ 26, and glucose 10, pH 7.4. During incubations, the chambers were continuously bubbled with 95% O_2/5% CO_2. Slices were equilibrated at room

temperature for at least 45 min. Slices were then transferred to a submerged recording chamber constantly perfused with heated aCSF (32°C) and bubbled with 95% O_2/5% CO_2. Medium spiny neurons (MSNs) within the NAc shell subregion were identified with a 40X water-immersion objective on an upright microscope equipped with differential interface contrast optics under infrared illumination (BX5IWI, Olympus, Center Valley, PA) and video observation. Electrodes were made from borosilicate glass micropipettes (Warner Instruments, Hamden, CT) prepared with a P-97 Flaming-Brown micropipette puller (Sutter Instruments, Novato, CA). Patch pipettes had a resistance of 4-6 MΩ when filled with an internal solution containing (in mM): CsCl 135, HEPES 10, EGTA 1.1, $CaCl_2$ 0.1; Mg-ATP 2.5, Na-GTP 0.25, phosphocreatine 5, pH 7.2. After establishing a gigaseal, the patch was broken by applying negative pressure to achieve a whole-cell configuration. A series resistance lower than 15 MΩ was considered acceptable, and monitored constantly throughout the entire recording. Neurons were held at -70 mV. Tetrodotoxin (TTX, 0.5 μM, Tocris), D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5, 50 μM, Tocris) and 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide (NBQX, 10 μM, Tocris) were applied to the bath to block action potential-mediated neurotransmitter release, NMDA and AMPA receptors, respectively. Ro 15-4513 (0.3 μM) was applied in the bath after 5-7 min of TTX, APV and NBQX perfusion. All recordings were carried out at least 10

min after application of any drug to the bath. Recordings were performed using a Multiclamp 700B/Digidata 1550A system (Molecular Devices, Sunnyvale, CA) and digitized at a 10000 Hz sampling frequency. The electrophysiological recordings were analyzed using the Clampfit 10.7 software (Molecular Devices). A template was constructed using the "Event detection/create template" function, as described in (9), then, mIPSCs were detected using the "Event detection/template search" function. All the waveforms detected during a single recording using template analysis were averaged and amplitude, rise time and decay time calculated.

Statistical analysis.

Data are expressed as means ± standard errors of the means (SEM). Statistical significance was assessed with the Student's t test (when used, paired-t test has been indicated in the text), one or two-way analysis of variance (ANOVA). The post hoc Newman-Keuls test was used for multiple comparisons. The level of significance was set at 0.05.

Table S1. *In situ* hybridization (ISH) signals for $GABA_A$ **α**1, **α**2, **α**4, **α**6, **γ**2 and δ subunit mRNA in the prefrontal cortex from $D_3R^{+/+}$ and $D_3R^{-/-}$ mice.

$GABA_A$ subunit	**ISH signal ($D_3R^{-/-}$ over $D_3R^{+/+}$ ratio)**
α1	0.92 ± 0.02
α2	1.10 ± 0.11
α4	0.80 ± 0.07
α6	1.15 ± 0.13
γ2	0.89 ± 0.30
δ	0.83 ± 0.17

Table S2. *In situ* hybridization (ISH) signals for $GABA_A$ **α**1, **α**2, **α**4, **α**6, **γ**2 and δ subunit mRNA in the hippocampus from $D_3R^{+/+}$ and $D_3R^{-/-}$ mice.

$GABA_A$ subunit	**ISH signal ($D_3R^{-/-}$ over $D_3R^{+/+}$ ratio)**
α1	0.91 ± 0.09
α2	1.19 ± 0.13
α4	1.07 ± 0.03
α6	1.20 ± 0.10
γ2	0.96 ± 0.26
δ	0.85 ± 0.16

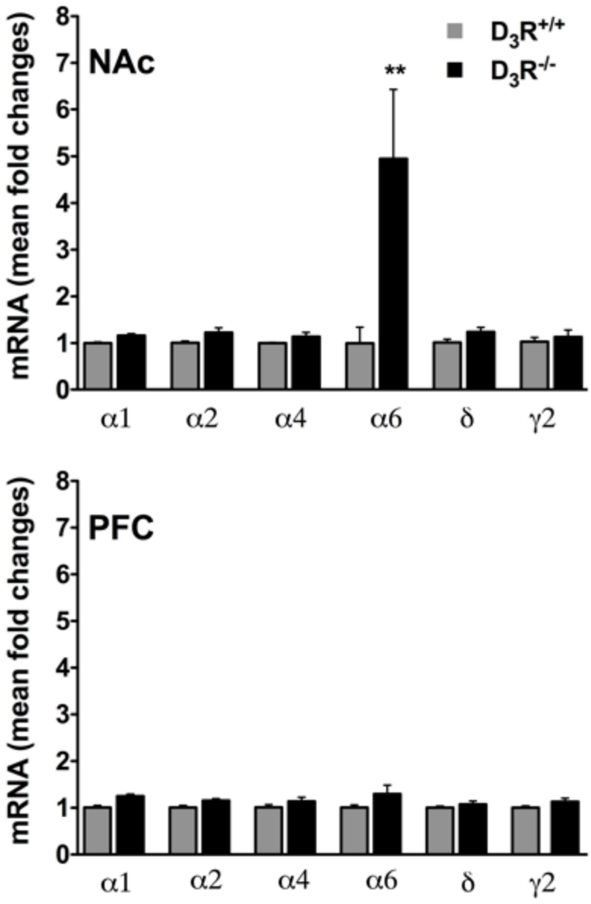

Figure S3. $GABA_AR$ α1, α2, α4, α6, γ2 and δ subunits mRNA expression in the nucleus accumbens (NAc) and in the prefrontal cortex (PFC) of wild type ($D_3R^{+/+}$) and D_3R null mice ($D_3R^{-/-}$). Abundance of transcripts was assessed by qPCR (primer sequences are reported in Tab. S4); expression level is expressed as mean fold changes (± standard error of the mean, vertical bars) relative to controls. Each experimental group included 6-8 mice.
**P<0.01 vs. the corresponding control ($D_3R^{+/+}$); one-way ANOVA and Newman–Keuls post hoc test.

Table S4. Primers for Real-Time PCR

Target gene	**Primer sequence**
Gabra1	5’-GACCAGGTTTGGGAGAGCGTGT-3’
	3’-GCCGGAGCACTGTCATGGGTC-5’
Gabra2	5’-CCCAGTCAGGTTGGTGCTGGC-3’
	3’-ACAGGGCCAAAACTGGTCACGT-5’
Gabra4	5’-CCTGTGCCTGGCGGCTTGTTTA-3’
	3’-CCCCAAATCCAGGACGCAGCC-5’
Gabra6	5’-GGCCAGGATTTGGGGGTGCTG-3’
	3’-TCAGTCCAAGTCTGGCGGAAGA-5’
Gabrg2	5’-ACCCAGAGGCGAGAGGCGAG-3’
	3’-GCTTGTGAAGCCTGGGTAGAGCG-5’
Gabrd	5’-CCGACCAGGCATTGGAGGTGC-3’
	3’-TGCTGTCCCGCCAGCTCTGA-5’
Gapdh	5’-CAACTCACTCAAGATTGTCAGCAA-3’
	3’-GGCATGGACTGTGGTCATGA-5’

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Chapter III

Submitted

Early modulation of endocannabinoid signaling prevents neurochemical and behavioral alterations in a neurodevelopmental model of schizophrenia

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Abstract

In accordance to the neurodevelopmental hypothesis of schizophrenia, prenatal exposure of rats to the antimitotic agent methylazoxymethanol acetate (MAM) at gestational day 17 produced long-lasting behavioral (i.e., social withdrawal and cognitive impairment) and molecular abnormalities at level of both prefrontal cortex (i.e., increased in cannabinoid CB1 receptor mRNA and protein expression; reduction in DNA methylation at specific CpG sites of CB1 gene promoter) and nucleus accumbens (i.e. increased mRNA expression of brain derived neurotrophic factor and dopamine D3 receptor; decreased mRNA expression of parvalbumin and dysbindin). These adult abnormalities were preceded at neonatal age by delayed appearance of neonatal reflexes, paralleled by higher 2-arachidonoylglycerol (2-AG) brain level. The schizophrenia-like deficits were reversed by treatment (from postnatal day 19 to 39) with cannabidiol (30 mg/kg/day), or, in part, by treatment with the cannabinoid CB1

receptor antagonist/inverse agonist AM251 (0.5 mg/kg/day), but not with haloperidol (0.6 mg/kg/day). These results suggest that prenatal MAM insult leads to premorbid anomalies at neonatal age, that may be considered as early marker preceding the development of schizophrenia-relevant manifestations at adulthood; early treatment with cannabidiol prevented the appearance of schizophrenia-like deficits in the adulthood, suggesting that early modulation of the endocannabinoid signaling might be protective against MAM-induced insult.

Keywords: Cannabidiol, Cannabinoid CB1 receptor, Antipsychotic drugs, MAM model

1. Introduction

It is well accepted that schizophrenia (SCZ) is primarily a developmental disease that becomes evident in adulthood[1]. Early neurodevelopmental anomalies may affect the typical brain maturation processes in the perinatal period causing a range of relatively benign changes that are markers of developmental disruption[2]. Emerging evidence indicates that preventive treatment with antipsychotics in the early phase of the disease could reduce the risk of progression to first-episode psychosis in patients[3] as well as the behavioral and structural abnormalities in experimental models[4-5]. Thus, identification of susceptible individuals based on early life stress events and the presence of premorbid anomalies could give the chance of early pharmacological intervention in the prodromal period to prevent the transition to SCZ later in life[6]. In this regard, prenatal methylazoxymethanol acetate (MAM) exposure, which induces SCZ-relevant functional and neuropathological deficits in adult but not in adolescent rats, result to be a very useful experimental tool to reproduce the human condition[7-8-9-10].

Recent evidences reported that the endocannabinoid system (ECS) plays an important role in SCZ. In particular, it is believed that ECS participates in the pathophysiology of SCZ[11-12-13-14]. However, very little is known about the potential therapeutic effects of the pharmacological modulation of ECS in the early stage of SCZ, and even less in periods prior to the onset of psychosis.

To this purpose, in this study we have investigated the behavioral phenotype and possible early presence of subtle neurodevelopmental impairment potentially associated with changes in ECS levels, in rats at neonatal age prenatally MAM exposed. Moreover, at adult age, we assessed behavioral phenotype and measured potential ECS changes in specific brain regions involved in SCZ symptoms (prefrontal cortex - PFC, hippocampus - HIP, nucleus accumbens - NAc and striatum - STR) which we attempted to revert by prepubertal treatment with the cannabinoid CB1 antagonist/inverse agonist AM251 and with cannabidiol, a non-psychotropic cannabinoid[15-16-17], compared to the typical antipsychotic haloperidol used here as positive control[9-18].

To further position our study towards clinical aspects we measured regional cerebral blood flow (rCBF) by arterial spin labeling (ASL) magnetic resonance imaging (MRI)[19-20]. In order to assess potential effects of ECS modulation on other (non-ECS) neural circuitries/mechanisms, we evaluated mRNA expression levels of brain derived neurotrophic factor (BDNF)[21], dopamine D3 receptors (D3R)[22], parvalbumin (PV)[23] and dysbindin (DYS)[24], whose changes as index of potential neurotransmitter alterations seem to be evident in the pathophysiology of SCZ.

2. Experimental procedures

2.1. Animals, MAM model and experimental design

The prenatal MAM administration was based on previous studies[7-8-9]. A detailed description of the experimental procedures can be found in Fig. 1 and supplementary data.

2.2. Drugs, experimental design and methods

Methods, along with any additional supplemental data are available in supplementary data.

3. Results

3.1. MAM rats at neonatal age

3.1.1. Behavioral phenotype

The appearance rate of neonatal reflexes and the nest seeking behavior in prenatally MAM-exposed rats and respective control (CNT) group are shown in Fig. 2. The percent appearance and the completion of neonatal reflexes had a significant delay in MAM rats. Fisher's exact t-test revealed several time points where the percentage of pups exhibiting righting (postnatal day (PND)1 $P<0.05$; Fig. 2A), cliff aversion (PND4-6, $P<0.05$; Fig. 2B), forelimb placing (PND2-4, $P<0.05$; Fig. 2C), forelimb grasping (PND2-3, $P<0.05$; Fig. 2D), bar holding (PND6-7, $P<0.05$; Fig. 2E) and negative geotaxis (PND3-4, $P<0.05$; Fig. 2F) was significantly lower in MAM group as compared to CNT. No difference was found either in the number of approaches (*t*-test=NS; Fig. 2G) or in the number of explorations (*t*-test=NS; Fig. 2H) of maternal nest between MAM and CNT group.

3.1.2. Biochemical analyses

In the whole brain of independent MAM rats (not subjected to behavioral testing) there was a significant increase of 2-arachidonoylglycerol (2-AG, *t*-test=2.448, $P<0.05$), but not of anandamide (AEA), palmitoylethanolamine (PEA) or oleoylethanolamine (OEA) (*t*-test=NS) concentration (Fig. 2I-J) at PND10. In order to extend these findings, we performed a wide transcriptomic

analysis for all the genes known to encode for the large class of enzymes involved in the metabolism of the two endocannabinoids (AEA and 2-AG). The two AEA-related compounds PEA and OEA, can be also produced and degraded via the action of the same class of enzymes (Iannotti et al. 2016). By means of this approach, we found that in the brain of MAM animals, with respect to their control, the expression levels of the two endocannabinoids responsive receptors CB1 and TRPV1 was not significantly different (Fig. 2K). By contrast, among the genes involved in the AEA metabolism, we found that α/β-hydrolase 4 (Abdh4; for the synthesis of AEA) and fatty acid amide hydrolase (FAAH; for the degradation of AEA) were slightly increased and reduced, respectively. On the other hand, among the class of genes regulating the metabolism of 2-AG, we found a slight increase in diacylglycerol-lipase (Dagl ß, involved in the synthesis of 2-AG) transcript levels (Fig. 2K). Western blotting analysis was also carried out to further corroborate the results of the transcriptomic analysis (Fig. 2 L and M). In conclusion, we demonstrate that, due to changes in the expression of genes involved in its metabolism, the endogenous 2-AG levels were significantly increased in the brain of MAM pups; whilst, AEA just showed a tendency toward upregulation.

3.2. MAM rats and the response to the treatment at adulthood

3.2.1. Behavioral phenotype

Figure 3A depicts that MAM/VHC group spent less time in social interaction (2-way ANOVA interaction $F_{4,79}$=12.23, P<0.001) compared to CNT/VHC rats (P<0.001), indicating impaired social behavior. Treatment with CBD 30 mg/kg (CBD30) and AM251, but neither CBD10 nor HAL, improved social performance in the MAM group as compared to the MAM/VHC group (P<0.001). However, in CNT groups both AM251 and HAL reduced the social activity (P<0.05, P<0.001), which was not affected by CBD treatment.

In the novel object recognition test (NOR) (Fig. 3B), prenatal MAM exposure caused an impairment of cognitive functions as demonstrated by a significant reduction (P<0.01) in the discrimination index (2-way ANOVA interaction $F_{4,116}$=2.757, *P*<0.05) during the test phase, which was reversed by CBD30 (P<0.01). The behavioral performance in all CNT groups as well as the exploration time in the familiarization phase were not affected by MAM exposure or treatments (data not shown). In addition, neither prenatal MAM exposure nor treatments affected the spontaneous horizontal (number of crossings) or vertical (number of rearing) locomotor activity in a novel environment, or the number of interactions assessed in social interaction (SI) at adulthood (data not shown).

3.2.2. Biochemical analyses

Among the brain regions (PFC, HIP, STR, NAc) where we assessed the ECS changes in the MAM model and interrogated how the treatment could revert them, the type-1 cannabinoid receptor (CB1) in the PFC was the most significant canonical target affected (Fig 4). Consistently with the significant increase of CB1 mRNA expression (2-way ANOVA interaction: $F_{3,30}$=4.658, P<0.01; Fig 4A), we observed a significant reduction of DNA methylation at level CB1 gene (CNR1) promoter in the average of the 5 CpGs of MAM/VHC group (2-way ANOVA interaction: $F_{3,31}$=3.585, P<0.01; Fig 4B) as well as the increase in CB1 receptor levels (2-way ANOVA interaction: $F_{3,15}$=3.010, P=0.06; *t*-test=6.685, P<0.01; Fig 4C), but not of CB1 receptor binding activity (CNT:23.00±4.398 fmol/mg; MAM:25.68±1.348 fmol/mg, *t*-test=NS).

Treatment with CBD30 reversed the MAM-induced changes in DNA methylation (P<0.05), mRNA (P<0.001) and protein (1-way ANOVA: $F_{3,10}$=5.703, P<0.05) expression. Treatment with AM251 reversed CB1 mRNA expression (P<0.001) in MAM rats and increased DNA methylation in the CNT rats (P<0.01). HAL did not induce modifications of CB1 receptors in MAM rats, except for an increased DNA methylation in CNT group (P<0.05). No difference in others ECS genes expression has been found (data not shown).

At the level of the NAc (Fig. 5A-D), prenatal MAM insult significantly increased the mRNA expression of BDNF (2-way ANOVA interaction:

$F_{4,24}$=4.352; *P*<0.001) and D3R (2-way ANOVA interaction: $F_{4,25}$=6.724; *P*<0.001) which were reversed by all treatments (P<0.01; P<0.001). However, the decreased PV expression (2-way ANOVA interaction: $F_{4,25}$=15.47, *P*<0.001; *t*-test=3.016, P<0.05) in the MAM group was reversed only by HAL (P<0.001), while the decreased DYS expression (2-way ANOVA interaction: $F_{4,24}$=6.410, *P*<0.001; *t*-test=2.501, P<0.05) was counteracted by all the drugs (P<0.05, P<0.001).

4. Discussion

4.1 Neonatal Age

For the first time, we showed that gestational MAM treatment delayed the onset of neonatal reflexes, which could represent a predictive factor for SCZ-like phenotype in adulthood[26-27-28], but it failed to affect the infant offspring`s learning ability based on olfactory cues in the nest-seeking behaviour. Thus, we could speculate that the prenatal MAM insult effects can be partially detected before puberty, in line with the clinical course of the SCZ that develops at stages with subtle deficits during early childhood recognized as premorbid phase[29].

At the molecular level the impaired neurobehavioral development of MAM pups was evident at PND 10 (when the reflexes are fully expressed) with elevated brain 2-AG level, partially due to an increased expression of the biosynthesis enzyme DAGL-β, in agreement with previous results on different SCZ animal models[30-31]. Given that 2-AG suppresses glutamate release by activating the cannabinoid CB1 receptors in presynaptic glutamatergic axon terminals[32-33-34], the prenatal MAM exposure transiently enhancing brain 2-AG signaling at neonatal age could reduce the glutamatergic neurotransmission – typical hallmark both in SCZ patients[35-36-37] and in the MAM model[38-39-40], in this latter already at neonatal age[41]. Interestingly, the lack of MAM-induced alterations in the brain neonatal expression of mRNA encoding for PV, D3R,

DYS and BDNF extends for the first time the previous observations to these two latter genes[42]. Thus, these data support the idea that altered EC signaling at neonatal age could negatively affect the maturation processes within the CNS leading to abnormal neurotransmission as well as to social and cognitive deficits at adulthood[7-8].

4.2 Adulthood

We further confirmed that MAM administration at GD 17 induces social deficit and recognition memory impairment [7-8-43], as suggested by the reduced time of interaction in the SI and by lower discrimination ratio in the NOR, respectively, whose validity is further supported by unaltered spontaneous locomotor activity.

For the first time, behavioral changes mimicking negative and cognitive-like symptoms in MAM rats coincides with alteration in CB1 expression (i.e. reduced DNA methylation, increased mRNA and protein expression), specifically restricted to the PFC, which expand our previous observations further supporting the translational value of this experimental model.[44] The gene-specific DNA methylation levels, which are involved in the pathophysiology of SCZ as developmental disorder[45] are inversely correlated with gene activation and protein[46-47], in agreement with our results.

Our results are consistent with previous findings showing altered CB1 mRNA expression at the level of PFC in a diverse variety of neurodevelopmental

models[48] as well as in clinical studies[11]. However, further investigations are deemed to explore the involvement of different epigenetic mechanisms at the level of the PFC transcriptional regulation of CB1 receptor gene in PFC. We also found a trend towards higher 2-AG content in the PFC of adult MAM rats, in line with previous preclinical[50] and human studies[51], again pointing to the role of EC signaling in the pathophysiology of SCZ. Collectively, these results suggest that the negative- and cognitive-like symptoms of MAM offspring could be in part due to a maladaptation of the EC tone (in terms of CB1 receptor expression and partially 2-AG content) in the PFC, a brain region involved in the modulation of emotionality and cognition, two behavioral dimensions which are disrupted in SCZ.

To further characterize the molecular underpinning of MAM model we found an increased mRNA expression of D3Rs in PFC, HIP, NAc and STR of MAM rats, as index of abnormal dopaminergic neurotransmission. These results are consistent with a previous observation at the level of NAc[52], and expand it suggesting that abnormal dopaminergic neurotransmission in SCZ symptoms could be also due to alteration of D3R in different brain regions, in agreement with clinical[53-54] and experimental observations[22]. We also supported the NAc as a key structure in the neurobiology of SCZ given the significant increase of BDNF and the significant decrease in the mRNA expression of PV and DYS. The deficit of PV expression in different brain regions as an index of altered

GABAergic neurotransmission is a consistent observation reported in SCZ patients[55] as well as in the MAM model[42-56], which we have further confirmed (see Fig 5C). Interestingly, for the first time here we observed that PV and D3R alterations in MAM model are paralleled with a reduced DYS expression. The disruption of this gene is typically associated with impaired glutamatergic[57], dopaminergic[24-58] and GABAergic signaling[59-60] in animal models and in schizophrenic population as well. Despite controversial evidence of BDNF and SCZ relation due to the chronicity of the illness and to the effects of antipsychotics[21-61], we cannot exclude that the SCZ-like phenotype of MAM rats could be also due to abnormal BDNF expression at the level of NAc and STR, which seems to be related to the D3R overexpression too[62]. However, additional evaluation at level both of epigenetic regulation of these gene expression and of their protein expression are necessary.

In addition, the evident dysregulation of dopaminergic system[7] (Fig. 5A; Supplementary figures S3A, S4A, and S5A) and the lower glutamatergic neurotransmission as well[38-39-40-41] could play a role in the altered rCBF in MAM rats (Fig. S6) as an index of impaired neural activity[63-64-65-66], in line with observations in SCZ patients[37-67-68]. This is the first report to show altered rCBF in MAM model, therefore more detailed studies are needed in order to better understand the neural mechanisms involved in the modification of cerebral blood perfusion in SCZ.

In agreement with the growing evidence suggesting that preventive antipsychotic treatment may reduce the risk of progression to the SCZ[3], here we showed for the first time that the prepubertal treatment with higher dose of CBD and partially AM251 rescued the decreased sociability and the recognition memory deficit as index of negative-and cognitive-like symptoms in MAM offspring, respectively. By contrast, HAL failed to reproduce the CBD effect in MAM rats and, much alike AM251, impaired the social behavior in control rats. Noteworthy, lack of efficacy of HAL in MAM rats is in line with the clinical evidence that antipsychotics are poorly effective towards negative and cognitive symptoms, thus stimulating the development of innovative pharmacological approaches[69]. Therefore, our results align with antipsychotic effects of CBD seen in SCZ patients[70-71] and in most of the preclinical models[72-73-74]. The locomotor activity paradigms served as an internal control for possible unspecific stimulant effects, which may confound the interpretation of other behavioral parameters. In this respect, it should be pointed out that none of the drugs tested in the present study affected rat motor behavior, indicating that the effects of pharmacological treatment on social and cognitive parameters of rats are not influenced by modification of locomotor activity.

The mechanisms underlying the beneficial effects of CBD on SCZ-like symptoms are still elusive. We have clearly demonstrated that early and repeated CBD treatment completely normalized MAM-induced alterations,

such as cannabinoid CB1 receptor and 2-AG content in the PFC, without having *per se* any effect in CNT rats. The decreased DNA methylation and paralleled increased mRNA and protein expression were attenuated by CBD which may at least in part contribute to its antipsychotic-like effects. Our results do not fully support the previous association of the antipsychotic effect of CBD with an increased AEA levels in serum[70], since there was higher AEA concentration in the PFC of CNT/CBD30 but not of MAM/CBD30 rats. Given that CBD interacts with several molecular targets that elevate AEA content, such as the hydrolysing enzyme FAAH[75] and the AEA transporter FABPs[76], at this stage we can only speculate that CBD reduced AEA inactivation through a not yet fully specified mechanism in CNT but not in MAM rats. Moreover, CBD modifies the function of several receptors in the CNS including CB1, CB2, TRPV1, GPR55, 5-HT_{1A}, PPARγ, μ and δ opioid receptors which could be involved at least in part in its antipsychotic activity. Besides its effect on CB1 receptor, we cannot rule out that the antipsychotic-like activity of CBD on MAM rats could be also due to its capacity to normalize the 2-AG content in PFC. Indeed, much alike negative allosteric modulation of AM251, CBD could also exert an antagonistic activity on CB1 receptor on orthosteric binding site[77]. This in turn could restore glutamatergic and/or dopaminergic neurotransmission. Therefore, the chronic inhibition of CB1 receptor might

limit the effect of excessive 2-AG, ultimately alleviating the behavioral impairment.

Another intriguing finding of the present study is that AM251 and HAL increased 2-AG levels in control rats, an effect that could be in part responsible for the social withdrawal similar to that observed in MAM rats. The mechanisms underlying the 2-AG increase in PFC of CNT rats remain undetermined and may be linked to changes in the dopaminergic and/or glutamatergic neurotransmission, as already suggested[50-78]. However, these results provide evidence that AM251 and HAL trigger different behavioral responses in the context of negative-like symptoms based on experimental groups (CNT vs. MAM), consistent with previous reports in healthy subjects[79] and in laboratory-based studies[78].

Altered dopaminergic transmission in several brain regions is a well-established neuropathological feature of SCZ, which is the main target of currently available and potentially innovative antipsychotic treatments. Consistent with previous observations showing that CBD can modulate the aberrant mesolimbic dopaminergic transmission and associated behavioral phenomena in humans and rodent models[80], the beneficial effects of early CBD treatment at higher dose on social and cognitive dysfunctions in MAM rats seem to be also due to its capacity to reverse D3R overexpression in several brain regions of MAM rats, in line with the effects of antipsychotic treatment in schizophrenic

patients[53-81]. The latter effect could also underlie the changes in the rCBF of somatosensory cortex, consistent with available evidence showing that the antipsychotic activity is associated with modulating rCBF abnormalities in schizophrenic patients[20-64-82].

Among the brain regions analyzed in this study, our data further support the role of the NAc as one of the main regions implicated in SCZ pathogenesis since each alteration we found in the mRNA expression of D3R, BDNF, PV and DYS was reversed by early pharmacological intervention. For the first time we showed that cannabinoids with potential antipsychotic activity and HAL reversed both aberrant BDNF and DYS expression. However, HAL but not the cannabinoids modified the reduced PV expression in the NAc suggesting a lack of effect of the EC signaling modulators on PV expression. Given that CBD showed better efficacy than AM251 and HAL to revert the SCZ-like phenotype, we could speculate that this difference could be due to its capacity to modulate the ECS elements better than D3R, BDNF, PV of DYS expression.

5. Conclusion

For the first time, we showed that prenatal MAM exposure induced neonatal behavioral and molecular alterations as predictive signs of SCZ-like deficits at adulthood. In agreement with the hypothesis of preventive antipsychotic treatment[3], repeated prepubertal CBD treatment prevented MAM-induced negative-and cognitive-like symptoms at adulthood, which are insensitive to currently used antipsychotics. Conveniently, CBD did not negatively affect control offspring, supporting its safety profile. We confirmed that a dysregulation of the ECS elements plays a role in the pathophysiology of the disease, which seems to be a promising target for effective treatment. As highlighted in recent reviews[74-80] several possible mechanisms of action have been suggested, but today to the best of our knowledge none of them has been conclusively identified as responsible for the antipsychotic effect of CBD. Based on our result, early intervention with CBD seems to revert dopaminergic alterations via a modulation of the EC signaling. Further studies are necessary to assess its effects on different neurotransmitter systems.

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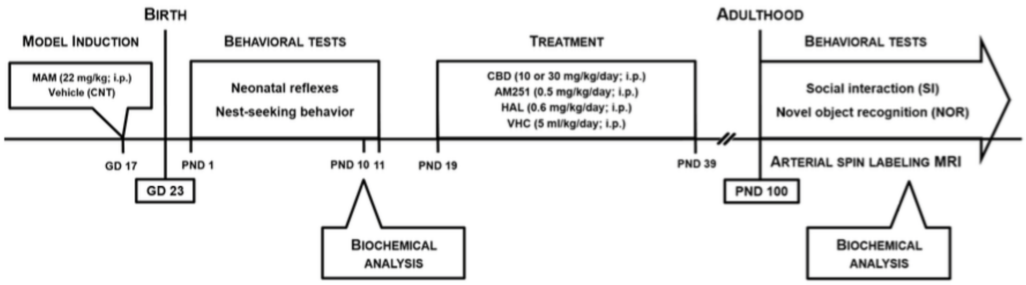

Figure 1. Experimental design used to investigate the effects of early pharmacological modulation of the endocannabinoid system (ECS) on offspring in MAM rat model of schizophrenia.

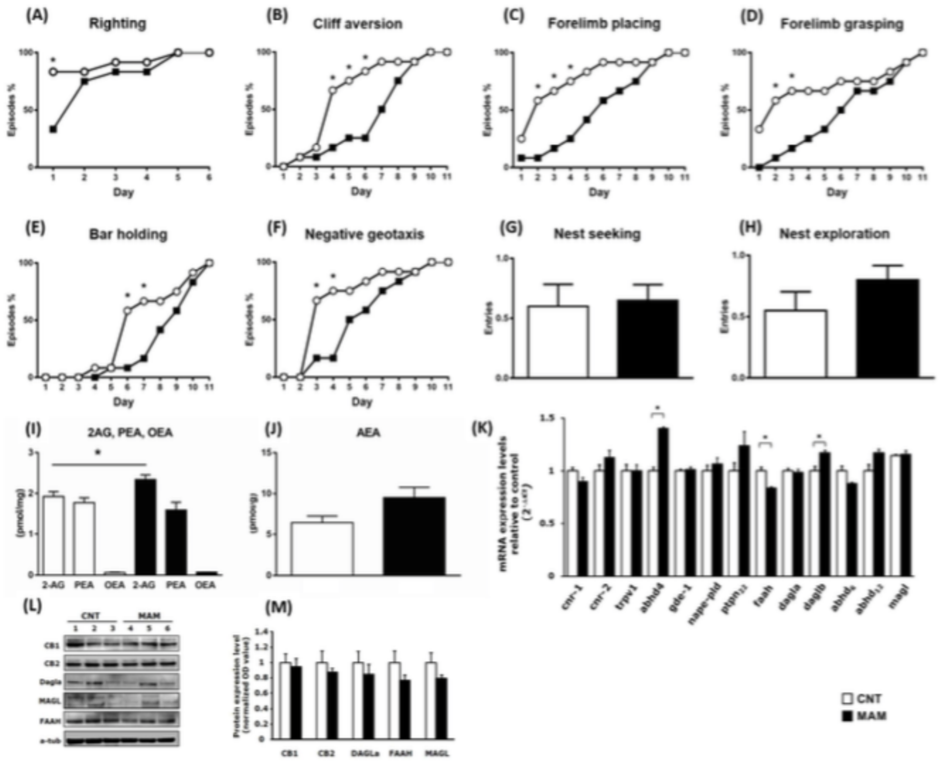

Figure 2. Effects of prenatal MAM exposure on neonatal behaviour and endocannabinoid system (ECS) elements in rat pups. Values are mean of percent cumulative appearance of each reflex (A-F) in each day, per group of animals (n=12), Fisher's exact *t*-test: *P<0.05 vs CNT. Data are presented as mean± S.E.M. (n=20) of (G) the number of approaches toward maternal nest (nest-seeking behaviour) and of (H) exploration number of the nest (nest exploration), of (n=3-5) (I) 2-AG, PEA, OEA and (J) AEA levels, of (K) gene and (L,M) protein expression of ECS elements (receptors and metabolic enzymes). *P<0.05 vs CNT, *t*-test.

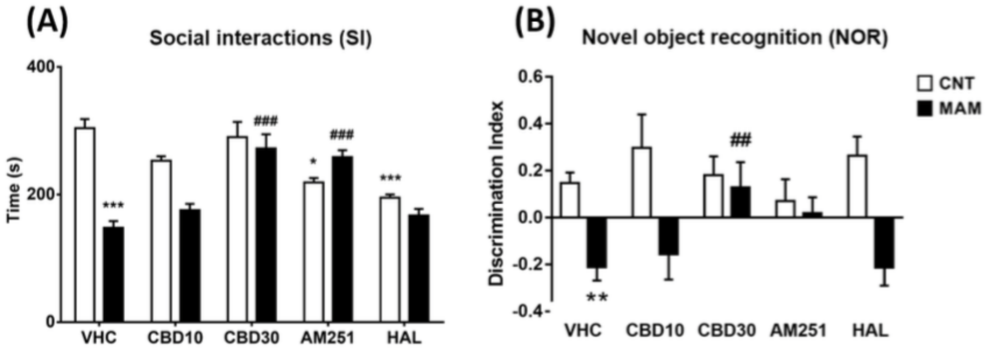

Figure 3. Effects of prepubertal modulation of the endocannabinoid tone on the behavioral phenotype of MAM rats in (A) the social interaction (SI) test and in (B) the novel object recognition (NOR) test at adulthood. Data are presented as mean±S.E.M. (n=12-15). *P<0.05, **P<0.01 and ***P<0.001 vs CNT/VHC; ##P<0.01 and ###P<0.001 vs MAM/VHC, Fisher`s LSD.

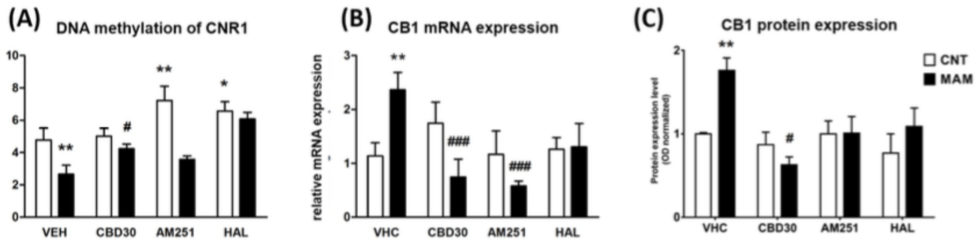

Figure 4. Effects of prepubertal modulation of the endocannabinoid tone on CB1 receptor in the prefrontal cortex (PFC) of MAM rats at adulthood. Data are presented as mean±S.E.M (n=3-7) of (A) DNA methylation of CB1 gene, (B) CB1 mRNA expression, (C) CB1 protein expression. *P<0.05 and **P<0.01vs CNT/VHC; #P<0.05 and ###P<0.001 vs MAM/VHC, Fisher`s LSD or *t*-test.

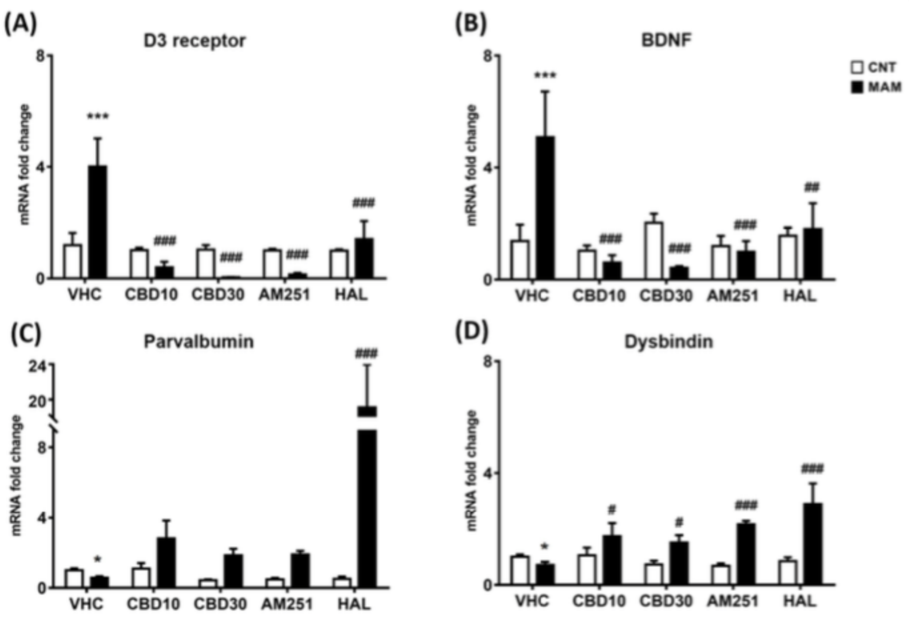

Figure 5. Effects of prepubertal modulation of the endocannabinoid tone on mRNA expression of (A) D3R, (B) BDNF, (C) PV and (D) DYS in the nucleus accumbens (NAc) of MAM rats at adulthood. Data are presented as mean±S.E.M (n=3-8). *P<0.05 and ***P<0.001vs CNT/VHC; #P<0.05, ##P<0.01 and ### P<0.001vs MAM/VHC, Fisher`s LSD or *t*-test.

Supplementary Information –Stark et al.

Materials and Methods

Animals and MAM model

Pregnant Sprague-Dawley rats were obtained from Charles River (Germany) at gestational day (GD) 13 and housed individually. They were randomly assigned to experimental groups and injected intraperitoneally (i.p.) with methylazoxymethanol acetate (MAM, 22 mg/kg) or vehicle (CNT, 0.9% NaCl) on GD 17, as previously described[1-2-3-4-5]. The mothers were regularly weighted and no differences were observed between the two experimental groups. No cross-fostering was used in this study, since in previous studies it did not impact the MAM phenotype[1]. Newborn litters, found up to 5 pm were considered to be born on that day (postnatal day 0 = PND 0). At birth, no difference was found in pregnancy length, total number of pups per litter, body weight, malformations, eye opening time. The offspring were weaned on PND 22 and housed in groups of 2-3 with littermates until adult, at which time they were used for behavioural and neurochemical experiments, with food and water available *ad libitum* and under a constant environmental conditions: relative humidity 50-60 %, temperature 23 ºC ± 1 ºC, 12-hour light-dark cycle (lights on at 6 a.m.). Only male offspring were used in the study. The rats showed a normal body weight gain (measured every day from PND19 to PND 39 and

every other day from PND 40 to PND85) that was independent both of prenatal treatment (MAM or CNT) and of preadolescent drug administration. As adults (from PND100) the animals were submitted to the behavioural tests. After completion of behavioural testing the rats were decapitated in short aether anaesthesia and their brain were removed. Prefrontal cortex (PFC), hippocampus (HIP), striatum (STR) and nucleus accumbens (NAc) were dissected, frozen on liquid nitrogen and stored at -80 until ready for analysis. For binding assay coronal sections (20-mm-thick) were cut on a cryostat and mounted on gelatin-coated slides. The sections were stored at –80°C until processing.

All experiments were conducted in accordance with all relevant laws and regulations of animal care and welfare. The experimental protocol was approved by the Animal Care Committee of the Masaryk University, Faculty of Medicine, Czech Republic and carried out under the European Community guidelines for the use of experimental animals.

Drugs

All compounds were administered i.p. in a volume of 5 ml/kg of body weight. The CB1 antagonist/inverse agonist AM251 (Sigma-Aldrich) was dissolved in dimethylsulfoxide (DMSO), Tween80 and saline (1:1:8). The non-psychotropic cannabinoid cannabidiol kindly provided by Prof. Raphael Mechoulam (Hebrew University, Jerusalem, Israel) was dissolved in Tween80 (2%) and

saline (98%). The typical antipsychotic haloperidol (Haloperidol-Richter®, Czech Republic) used here as positive control was dissolved in saline. Three groups of control animals were injected i.p. with AM251, cannabidiol or haloperidol vehicle, respectively. As similar results were obtained from these three control groups, vehicle data were pooled. As described in Fig 1, from postnatal day (PND) 19 to PND 39 [the period prior to puberty defined as PND 43.6+1 in Sprague-Dawley rats based on previous observations of balano-preputial separation and increases in circulating androgens[6-7]] different groups of rats (n=12-15) were treated i.p. with the cannabidiol (CBD: 10 or 30 mg/kg/day), AM251 (0.5 mg/kg/day), haloperidol (HAL: 0.6 mg/kg/day) or vehicles, based on previous[8-9-10-11-12]. To avoid litter effects each experimental group consisted of animals chosen randomly from different litters (at least four MAM-exposed litters and four vehicle-exposed litters). The experimental design resulted in 10 final groups: (1) offspring of control dams administered vehicle (CNT/VHC), (2) offspring of control dams administered CBD 10 (CNT/CBD 10), (3) offspring of control dams administered CBD 30 (CNT/CBD 30), (4) offspring of control dams administered AM251 (CNT/AM251), (5) offspring of control dams administered HAL (CNT/HAL), (6) offspring of MAM dams administered vehicle (MAM/VHC), (7) offspring of MAM dams administered CBD 10 (MAM/CBD10), (8) offspring of MAM dams administered CBD 30 (MAM/CBD30), (9) offspring of MAM dams

administered AM251 (MAM/AM251) and (10) offspring of MAM dams administered HAL (MAM/HAL).

Behavioral Phenotyping

Neonatal reflexes

Development of neonatal behaviour was studied by applying a battery of tests to assess neonatal reflexes, which are considered a reliable index of neurological and behavioral development, as previously described[13-14-15-16]. One or two males from each CNT and MAM litter were used for postnatal assessment of neurobehavioral development by a single examiner blind to the treatment conditions. Starting on PND 1, newborn pups were daily weighed and observed for neonatal reflexes, until "maximum appearance" was scored (i.e., 100 % of the brood was found to exhibit the full repertoire of reflexes). The following reflexes were scored: a) *righting:* the rat is capable of rapidly returning to its feet when placed on its back; b) *cliff aversion:* the rat withdraws from the edge of a flat surface when its snout and forepaws are placed over a cliff 60 cm high; c) *forelimb placing:* the rat places its forepaw up to a cardboard when it has been stroked against the dorsal surface of the paw; d) *forelimb grasping:* the rat grasps strongly the barrel of the 16-gauge needle, 1.0-mm diameter, when it is touched against the palm of each forepaw; e) *bar holding:* the rat holds itself on to a wooden stick, 2.0-mm diameter, for 5 s and

f) *negative geotactic reaction:* the pups were placed head down on an inclined surface (30 degrees). Each pup was observed for 60 s. to turn and move toward the upper end of the surface.

Nest-seeking behavior (Olfactory Discrimination)

On PND 11, twenty pups per treatment underwent determination of nest-seeking behavior, as previously described[16]. Briefly, the testing box consisted of a rectangular polycarbonate cage (40×20×18 cm) divided into three equal compartments by a permanent ink marker: a central arena and two side compartments, one side containing nest bedding from the test pup's home cage and the same quantity of fresh clean bedding on the opposite side. Each pup was placed in the central arena; for nest seeking, crossing of the line toward nest compartment with the forepaws and head was considered a positive entry. For nest exploration, crossing of the line plus sniffing and exploration of the nest were considered a positive score (cut-off time 60 s).

Social interaction (SI) test

The test was carried out in a moderately illuminated room, as previously described[10]. Each animal was allowed to freely explore an unfamiliar congener in a metal arena (60×60×60 cm) for 10 min. The arena was cleaned with 0.1% acetic acid and dried after each trial. Social behaviors were defined as sniffing, following, grooming, mounting, and nosing. The whole testing phase was

recorded and analyzed by two observers blind to the treatment groups. We recorded the time spent in social behaviors and the number of interactions.

Novel object recognition (NOR) test

The experimental apparatus used for the NOR test was an arena (43×43×32 cm) made of plexiglas, placed in a moderately illuminated room. As previously described[10], each animal was placed in the arena and allowed to explore two identical previously unseen objects for 5 min (familiarization phase). After an inter-trial interval of 3 min, one of the two familiar objects was replaced by a novel, previously unseen object and rats were returned to the arena for the 5-min test phase. During the test phase, the time spent exploring the familiar object (Ef) and the new object (En) was videotaped and analyzed separately by two observers blind to the treatment groups, and the discrimination index (DI) was calculated as follows: (En-Ef)/(En+Ef). The arena and all objects were cleaned with 0.1% acetic acid and dried after each trial.

Spontaneous locomotor activity

The exploratory activity was evaluated in moderately illuminated cubic metal arena (60×60×60 cm), as previously described[17]. Animals were placed gently in the center of the arena and allowed to explore. The horizontal (the number of squares crossed with all paws) and the vertical (number of rearing episodes) exploratory activity was scored recorded for 30 min and scored offline by two

observers blind to the treatment groups. The arena was cleaned with 0.1% acetic acid and dried after each trial.

Biochemical methods

Extraction, purification and quantification of endocannabinoids and endocannabinoid-related compounds

The endocannabinoids anandamide (AEA) and 2-Arachidonoylglycerol (2-AG), and endocannabinoid-related molecules *N*-palmitoylethanolamide (PEA) and *N*-oleoylethanolamide (OEA) were extracted from tissues and then purified and quantified as described elsewhere[18]. First, tissues were dounce-homogenized and extracted with chloroform/methanol/Tris-HCl 50 mM pH 7.5 (2:1:1, v/v) containing internal deuterated standards for AEA, 2-AG, PEA and OEA quantification by isotope dilution (5 pmol of $[^2H]_8$AEA, 50 pmol of $[^2H]_5$2AG, $[^2H]_4$ PEA, $[^2H]_2$ OEA (Cayman Chemicals, MI, USA). The lipid-containing organic phase was dried down, weighed and pre-purified by open bed chromatography on silica gel. Fractions were obtained by eluting the column with 99:1, 90:10 and 50:50 (v/v) chloroform/methanol. The 90:10 fraction was used for AEA, 2-AG, PEA and OEA quantification by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS), as previously described and using selected ion monitoring at M + 1 values for the four compounds and their deuterated homologues, as described in[19-20-21].

Real-Time qPCR (RT-qPCR)

Total RNA was isolated from native tissues by using TRI-Reagent (Sigma-Aldrich, Milan, Italy), according to the manufacturer's instructions, reacted with DNase-I (1 U/ml; Sigma-Aldrich) for 15 min at room temperature, and followed by spectrophotometric quantification. Final preparation of RNA was considered DNA- and protein-free if the ratio between readings at 260/280 nm was ≥1.7. 1 μg of isolated mRNA was reverse transcribed by use of iScript reverse transcriptase [Biorad (MI), Italy] in a 20 μl reaction volume with 1μl of iScript Reverse Transcriptase in 1X iScript Reaction Mix. The reaction mixes were incubated 5 min at 25°C, 20 min at 46°C and 1min at 95°C. Quantitative real-time PCR was carried out in CFX384 real-time PCR detection system [Bio-Rad, Segrate (MI), Italy] by using SYBR Green master mix kit [Bio-Rad, Segrate (MI)] with specific primers for target genes[22]. Samples were amplified simultaneously in quadruplicate in one- assay run with a non-template control blank for each primer pair to control for contamination or primer-dimers formation, and the ct (cycle threshold) value for each experimental group was determined. Each PCR reaction (20 ml final volume) was carried out with 100 ng of cDNA, 8μM of primers and 1X SYBR green master mix. The housekeeping genes (the hypoxanthine-guanine phosphoribosyltransferase, hprt and/or ribosomial protein S16) were used as an internal control to normalize the ct values, using the ΔΔCt method. Differences in mRNA level between groups

are reported as 2−ΔΔCt (fold change)[22]. The primers used for PCR amplification are reported in Table S1.

Western blotting analysis

Each animal, previously anesthetized, was decapitated and brains were collected immediately after sacrifice and frozen in liquid nitrogen for subsequent dissection. The whole brain was dissected and washed twice in cold PBS (without Ca2+ and Mg2+, pH 7.4) and homogenized as previously described[23]. Lysates were then centrifuged for 15 min at 13,000 × g at 4°C, and the supernatants transferred into clear tubes and quantified by DC Protein Assay (Bio-Rad, Segrate MI, Italy). Subsequently the samples (60-80 μg of total protein) were boiled for 5 min in Laemmli SDS loading buffer and loaded on 8–10%SDS-polyacrylamide gel electrophoresis and then transferred to a PVDF membrane. Membranes were incubated overnight at 4◦C with the following antibodies: (a) rabbit policlonal anti-CB1 Receptor Antibody (Y080037) Applied Biological Materials Inc. (CANADA); (b) rabbit polyclonal anti- DAGL α from Santa Cruz Biotechnology, Inc. CA USA; (c) rabbit policlonal anti-MAGL from Cayman Chemical (USA): d) mouse anti-BDNF (N-20): sc-546, diluted 1:500, Santa Cruz Biotechnology, CA, USA); e) mouse monoclonal anti- FAAH (WH0002166M7-100UG) from Sigma Aldrich MI Italy. The mouse monoclonal anti-tubulin clone B-5-1-2 (dilution 1:5000; Sigma–Aldrich, MI Italy) antibody was used to check for equal protein loading.

Reactive bands were detected by chemiluminescence (ECL or ECL-plus; Perkin-Elmer). Images were acquired and analyzed on a Chemi-Doc station with Quantity-one software (Bio-Rad, Segrate MI, Italy[24]).

Analysis of DNA methylation

Methylation status of CNR1 promoter was determined using pyrosequencing of bisulfite converted DNA. After DNA extraction, 0.5 µg of DNA from each sample was treated with bisulfite, using the EZ DNA Methylation-Gold™ Kit (Zymo Research, Orange, CA, USA). CNR1 was analyzed in clinical samples with PM00122031 (Qiagen, Hilden, Germany) and in rat brain tissues using the following primers: forward: 5′-GGAAGAGAGTAGGAAGATGATAG-3′; reverse: 5′-biotin-TTCTACCAA AACTAATATACCTAACACC-3′; and sequencing: 5′-AGAGAGTAGGAAG ATGATAGT-3′. Bisulfite treated DNA was amplified by PyroMark PCR Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's protocol. PCR conditions were as follows: 95 °C for 15 min, followed by 45 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s, and, finally, 72 °C for 10 min. PCR products were verified by agarose electrophoresis. Pyrose- quencing methylation analysis was conducted using the PyroMark Q24 (Qiagen). The level of methylation was analyzed using PyroMark Q24 Software (Qiagen, Hilden, Germany), which calculates the methylation percentage (mC/(mC + C)) for each CpG site, allowing quantitative comparisons (mC is methylated cytosine, C is unmethylated cytosine)[4].

Cannabinoid Receptor Binding Assays

Rat brain cortex was resuspended in 2 mM Tris–EDTA, 320 mM sucrose, 5 mM MgCl2 (pH 7.4), then was homogenized in a Potter homogenizer and centrifuged twice at 1000 x g (10 min), and the pellet was discharged. The supernatant was centrifuged at 15000 x g (20 min), and the pellet was resuspended in assay buffer (50 mM Tris–HCl, 3 mM MgCl2, pH 7.4), and the Bradford protein assay to measure the concentration of the sample protein was performed. These membrane fractions were used in rapid filtration assays with radiolabel agonist ([3H]CP-55,940; Perkin Elmer Life Sciences, Boston, Ma, U.S.A.), at 37 °C with incubation time of 60 minutes. At the beginning we performed binding assays with [3H]-CP-55,940 at different concentrations (0.1-4 nM) to create the nonlinear graph of specific binding, and to calculate the Kd, using the Prism 4 program (GraphPAD Software for Science, San Diego, CA), value necessary for subsequent evaluation of Ki. Then in all binding experiments, nonspecific binding was determined in the presence of 10 μM "cold" agonist (CP-55,940; Cayman Chemicals, Ann Arbor, Mi, U.S.A) that was tested by adding directly to the incubation medium during a preincubation time of 15 minutes at room temperature[25].

Magnetic resonance imaging (MRI)

All MRI was performed on a 9.4 T Bruker BioSpec 94/30USR scanner a with 2×2 surface array rat head RF receive coil and a volume transmit RF coil. The

measurement was conducted under anesthesia: 2% isoflurane and 1000 ml/min of oxygen. This anesthetic protocol was shown to be suitable for brain perfusion measurements[26-27]. The animals were laid on a thermal pad, their body temperature and respiratory curve were monitored during the measurement process. T2-weighted anatomical images were taken using the RARE sequence with TR = 3500 ms, TE = 36 ms, FOV 40.3 × 30.5 mm, image matrix 256 × 256. Fifteen axial slices with the thickness of 1.25 mm were acquired; these slices covered the brain from the root of the olfactory bulbs to the cerebellum. These anatomical pictures provided the background for the selection of axial slices suitable for the arterial spin labeling (ASL) sequence, for which the hippocampus, nucleus accumbens, somatosensory and prefrontal cortices were the regions of interest. ASL is a non-invasive perfusion imaging modality that uses magnetically spin-labelled blood water protons as an endogenous tracer of cerebral blood flow. By subtracting labelled and unlabeled images, a cerebral blood flow map is generated and all together could be used for quantification of cerebral blood flow[28]. In these measurements, two axial slices (bregma position: 0 and -3,14[29])with 1.25 mm thickness was obtained with FAIR-RARE sequence was applied with TR = 10000 ms, TE = 37.78 ms, TI stepped through 30, 50, 100, 200, 300, 500, 700, 900, 1000, 1100, 1500, 1800, 2200, 2800, 3200 ms, FOV 40.3×30.5 mm, and image matrix 128×96, with the adiabatic inversion pulse length of 16 ms, the bandwidth of 4866.2 Hz

and, the inversion slab thickness of 4.25 mm. By repeating measurement twice, with slice-selective and nonselective inversion, two images were obtained, from which the perfusion map could be calculated according to the equation[30].

$$CBF = \lambda . \frac{T_{1,\text{nonsel}}}{T_{1,\text{blood}}} \left(\frac{1}{T_{1,\text{sel}}} - \frac{1}{T_{1,\text{nonsel}}} \right)$$

where CBF is the cerebral blood flow (usually expressed in mL/min in 100g of tissue, ʎ is the blood-brain partition coefficient, expressing the ratio of the quantity of water per gram of tissue to the quantity of water per milliliter of blood, which is known to be 0.89±0.03 mL(blood)/g(tissue) in the rat brain[31], $T_{1,\text{nonsel}}$ and $T_{1,\text{sel}}$ are the apparent longitudinal relaxation times derived from the image series applying nonselective and slice-selective inversions, respectively, and $T_{1,\text{blood}}$ is the longitudinal relaxation of capillary blood.

Statistical analysis

Data were analyzed using 1- or 2-way ANOVA (factor 1: model; factor 2: treatment) followed by post-hoc Fisher`s LSD if appropriate or by unpaired *t*-test for independent data using specialized software (Graph-Pad Prism 6.0). The Fisher's exact *t*-test was used for frequencies (comparisons of percent data of reflex appearance). A P-value <0.05 was considered statistically significant.

Results

mRNA expression of dopamine D3 receptor (D3R), brain derived neurotrofic factor (BDNF), parvalbumin (PV) and dysbindin (DYS) in neonatal MAM rats

In our transcriptomic analysis at PND10, we found that mRNA brain expression levels of D3R (t=1.095, P=NS vs respective CNT group, unpaired *t*-test), BDNF (t=1.004, P=NS vs respective CNT group, unpaired *t*-test), PV (t=1.220, P=NS vs respective CNT group, unpaired *t*-test) and DYS (t=1.757, P=NS vs respective CNT group, unpaired *t*-test) were unaffected by prenatal MAM exposure (see Fig. S1A-D).

Endocannabinoid (EC) brain levels in adult MAM rats

We found a non-significant trend of increased 2-AG in the prefrontal cortex (PFC) of MAM/VHC animals (2-way ANOVA interaction, $F_{3,27}$= 8.242, P<0.01), which was significantly decreased by CBD30 and AM251 (P<0.05), but not by HAL (P=NS). However, the latter two compounds increased the 2-AG content in CNT rats (P<0.05, P<0.001) (Fig. S2A). The level of AEA was not altered in the PFC of MAM or CNT group by VHC treatment, but it was increased by CBD30 in the CNT group (Fig. S2B). No difference was found between the two experimental groups in the PFC levels of OEA and PEA (data not shown) as well as in the ECs levels in HIP, NAc and STR (data not shown).

mRNA expression of dopamine 3 receptor (D3R), brain derived neurotrofic factor (BDNF), parvalbumin (PV) and dysbindin (DYS) in prefrontal cortex (PFC), hippocampus (HIP) and striatum (STR) of MAM rats

In the PFC (Fig. S3A-D) 2-way ANOVA revealed a significant effect of treatment ($F_{4,23}$=3.499, *P*<0.05; $F_{4,23}$=7.219, *P*<0.001) and a model x treatment interaction (**(**$F_{4,23}$=5.984, *P*<0.001; $F_{4,23}$=4.601, *P*<0.01) for PV and D3R mRNA expression, respectively. However, a main effect of model was found for PV ($F_{1,23}$=7.810; *P*<0.01), but not for D3R ($F_{1,23}$=1.002; *P*=NS) mRNA expression. *Post-hoc* analysis revealed a significantly increased expression of dopamine D3R mRNA (P<0.001) in MAM rats, which was reversed by cannabinoids (P<0.001), but not by HAL (P= NS). Although the PV expression was slightly decreased (P=NS) in MAM rats, the highest dose of CBD (30 mg/kg) and AM251 increased significantly its expression (P<0.05, P<0.001). Neither MAM exposure nor pharmacological treatment affected BDNF (model x treatment interaction: $F_{4,25}$=1.615, P=NS) and DYS expression (model x treatment interaction: $F_{4,21}$=1.020, P=NS).

As depicted in Fig. S4A-D, in the HIP a main effect of model ($F_{1,24}$=25.60, *P*<0.001; $F_{1,24}$=29.39, *P*<0.001), treatment ($F_{4,24}$=9.888, *P*<0.001; $F_{4,24}$=6.087, *P*<0.01) and a model x treatment interaction ($F_{4,24}$=8.167, *P*<0.001; $F_{4,24}$=4.616; *P*<0.01) for D3R and DYS mRNA expression was found, respectively. *Post-*

hoc analyses revealed that the increased expression of D3R ($P<0.05$) in MAM rats was reversed by the pharmacological treatment ($P<0.001$). Although the prenatal MAM expression did not affect DYS expression as compared to the CNT group, both the CB1 antagonist AM251 and the antipsychotic HAL increased its expression in the MAM group ($P<0.001$). Neither MAM exposure nor pharmacological treatment affected BDNF (model x treatment interaction: $F_{4,24}=0.9167$; *P*=NS) and PV expression (model x treatment interaction: $F_{4,24}=0.5557$; *P*=NS).

In the STR (Fig. S5A-D) 2-way ANOVA revealed a main effect of model ($F_{1,44}=8.560$, $P<0.01$), treatment ($F_{4,44}=5.985$, $P<0.001$) and a model x treatment interaction ($F_{4,44}=4.169$, $P<0.01$) for BDNF mRNA expression. Its higher expression in MAM rats ($P<0.05$) was reversed both by CBD in a dose dependent manner ($P<0.01$, $P<0.001$) and by AM251 ($P<0.001$), but not by HAL (P=NS). Furthermore, MAM exposure was also able to increase the D3R expression (model effect: $F_{1,44}=8.560$, $P<0.01$; *t*-test $P<0.01$ vs CNT), which was unaffected by the pharmacological treatment (model x treatment interaction: $F_{4,44}=0.6835$, *P*=NS). Neither MAM exposure nor pharmacological treatment affected PV (model x treatment interaction: $F_{4,45}=1.9996$, *P*=NS) and DYS (model x treatment interaction: $F_{4,23}=0.7693$, *P*=NS) mRNA expression.

Regional cerebral blood flow (rCBF)

CBD30, but neither AM251 nor HAL, reversed the increased blood flow (2-way ANOVA interaction $F_{3,58}$=3.278, P<0.05 Fisher's LSD P=0.054; Fig S6A) in the somatosensory cortex of MAM rats (P<0.05). At hippocampal level (Fig. S6B) there was a significant decreased of rCBF in MAM rats (model effect: $F_{1,58}$=28.71, P<0.001; t-test P<0.01 vs CNT), which was unaffected by the pharmacological treatment (model x treatment interaction: $F_{3,58}$=0.2998, P=NS). Neither MAM exposure nor pharmacological treatment affected rCBF at level of NAc (model x treatment interaction: $F_{3,54}$=1.376, P=NS; Fig S6C) or PFC (model x treatment interaction: $F_{3,53}$=1.251, P=NS; Fig S6D).

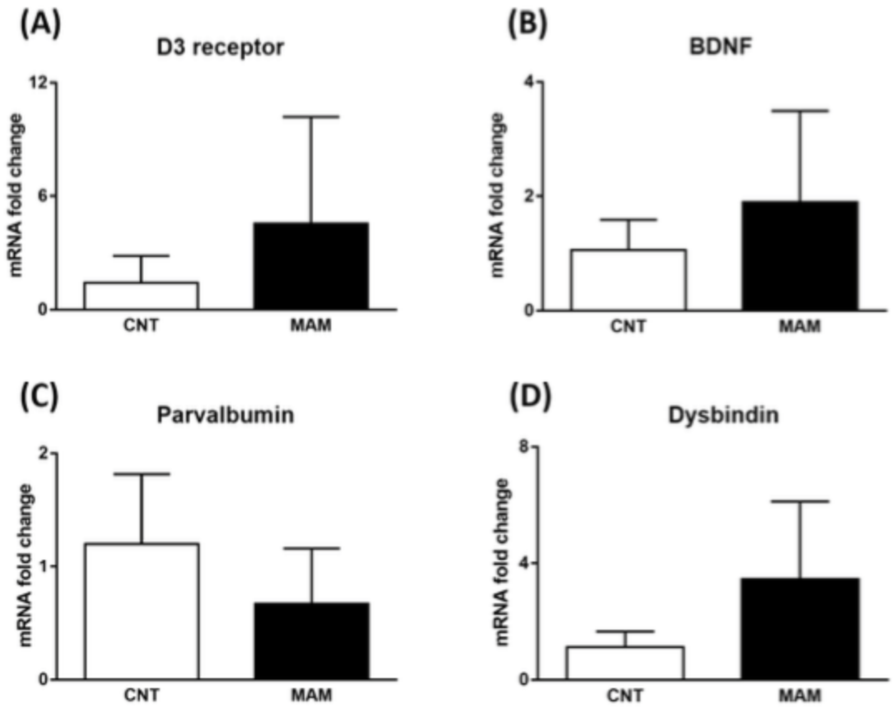

Figure S1. Effects of prenatal methylazoxymethanol (MAM) acetate exposure on mRNA expression of (A) dopamine D3 receptor (D3R), (B) brain derived neurotrophic factor (BDNF), (C) parvalbumin (PV) and (D) dysbindin (DYS) in the whole rat brain at neonatal age. Data are presented as mean±SEM (n=3-4).

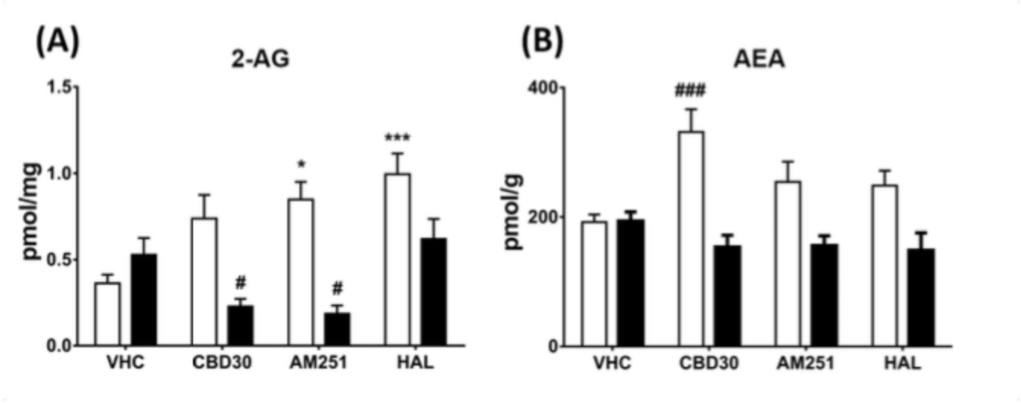

Figure S2. Effects of prepubertal modulation of the endocannabinoid tone on (A) 2-arachidonoylglycerol (2-AG) and (B) anandamide (AEA) in the prefrontal cortex (PFC) of rats at adulthood. Data are presented as mean±SEM (n=5-6). *P<0.05 and ***P<0.001 vs CNT/VHC; #P<0.05 and ###P<0.001 vs MAM/VHC, Fisher's LSD.

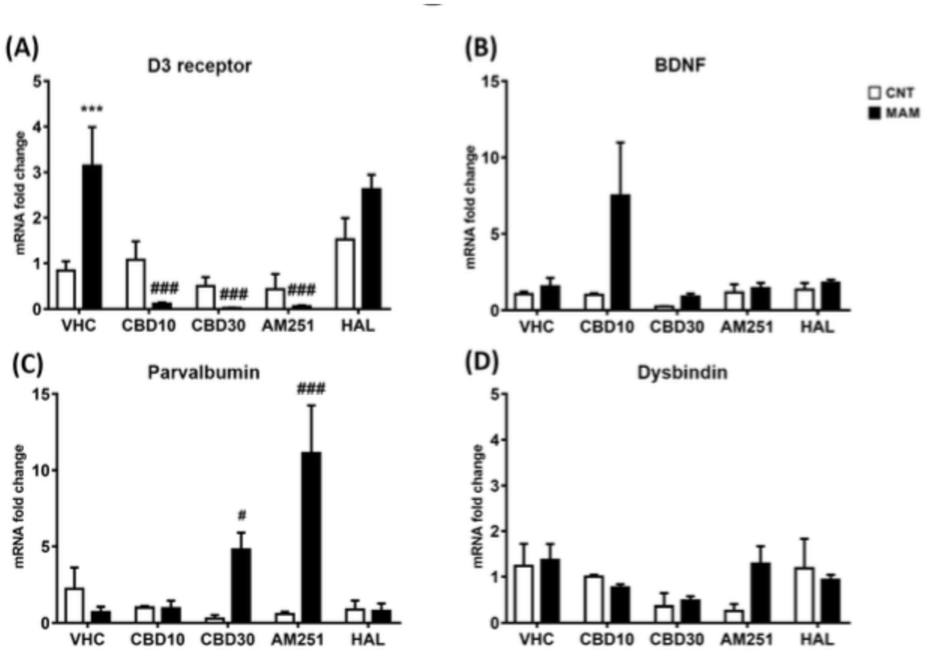

Figure S3. Effects of prepubertal modulation of the endocannabinoid tone on mRNA expression of (A) dopamine D3 receptor (D3R), (B) brain derived neurotrophic factor (BDNF), (C) parvalbumin (PV) and (D) dysbindin (DYS) in the prefrontal cortex (PFC) of rats at adulthood. Data are presented as mean±SEM (n=3-8). ***P<0.001 vs CNT/VHC, #P<0.05 and ###P<0.001 vs MAM/VHC, Fisher's LSD.

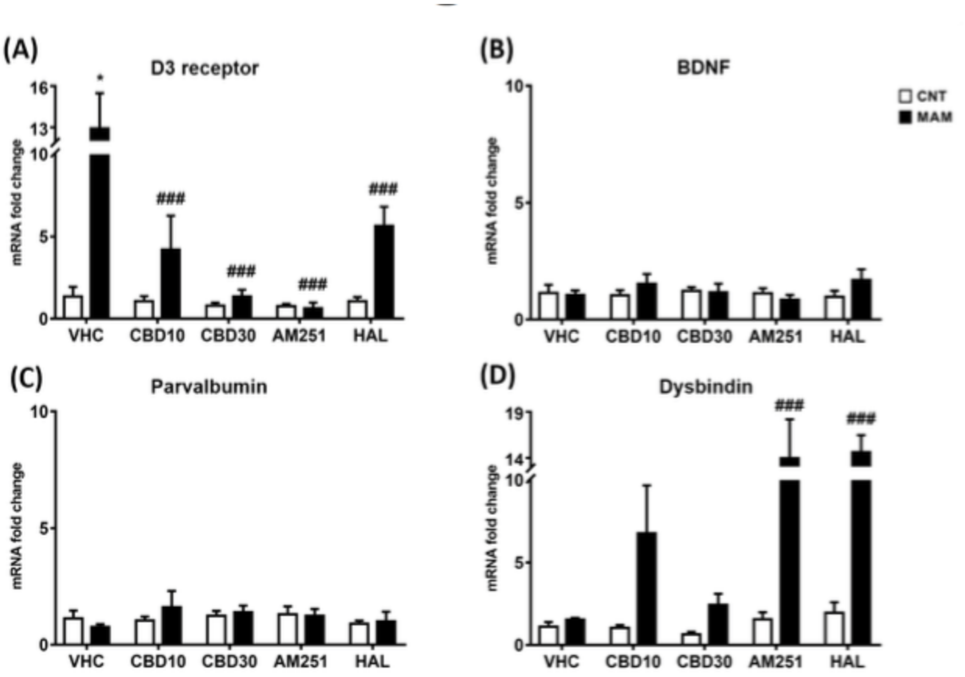

Figure S4. Effects of prepubertal modulation of the endocannabinoid tone on mRNA expression of (A) dopamine D3 receptor (D3R), (B) brain derived neurotrophic factor (BDNF), (C) parvalbumin (PV) and (D) dysbindin (DYS) in the hippocampus (HIP) of rats at adulthood. Data are presented as mean±SEM (n=3-8). *P<0.05vs CNT/VHC, ###P<0.001vs MAM/VHC, Fisher's LSD.

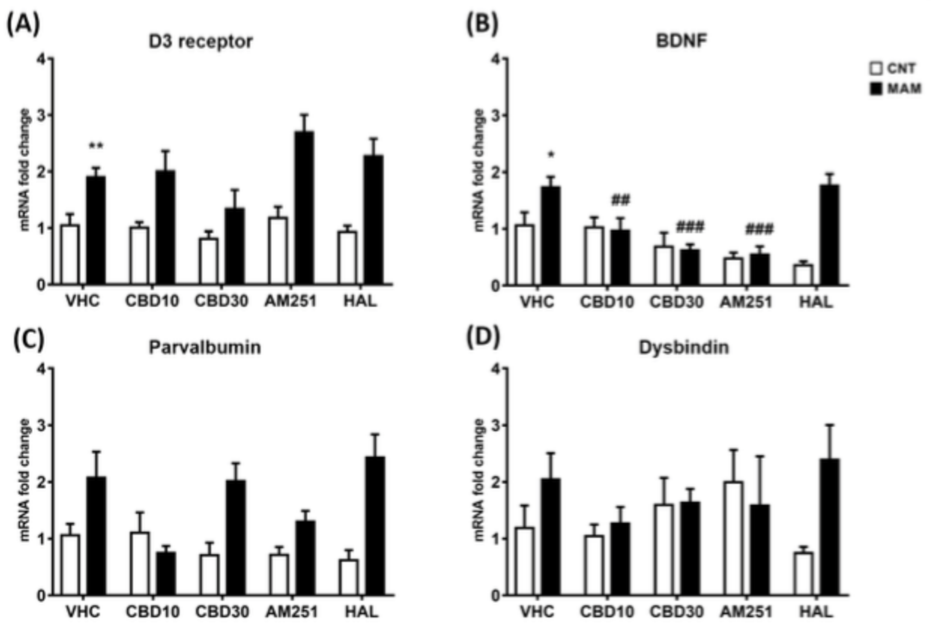

Figure S5. Effects of prepubertal modulation of the endocannabinoid tone on mRNA expression of (A) dopamine D3 receptor (D3R), (B) brain derived neurotrophic factor (BDNF), (C) parvalbumin (PV) and (D) dysbindin (DYS) in the striatum (STR) of rats. Data are presented as mean±SEM (n=3-8). *P<0.05vs CNT/VHC, ##P<0.01 and ###P<0.001vs MAM/VHC, Fisher's LSD or *t*-test.

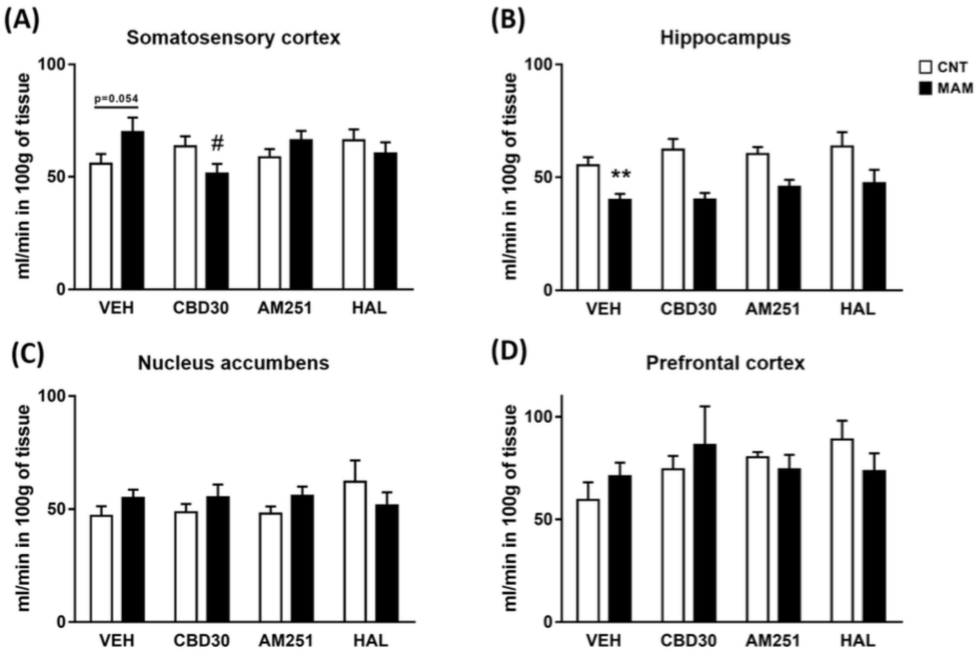

Figure S6. Effects of prepubertal modulation of the endocannabinoid tone on regional cerebral blood flow (rCBF) in (A) somatosensory cortex, (B) hippocampus (HIP), (C) nucleus accumbens (NAc) and (D) prefrontal cortex of rats. Data are presented as mean±SEM (n=8-10). **P<0.01vs CNT/VHC, #P<0.05vs MAM/VHC, Fisher's LSD or *t*-test.

Table S1. List of primer sequences used for quantitative real-time RT-PCR analysis

Gene name:	Forward sequence (5'- 3')	Reverse sequence (5'- 3')
Abhd4	TCTGGCGTCAAGCGGAGGGA	ACGCCACCCCCAAAGCCATG
Abdh6	AGCGTCTGCTCCCATCCCCA	TGGCTTGCCAGTGGCGTGAA
Abdh12	CAGGCGTGCGGTCGAAACCA	TCAAGCTGCAGTCGGCGTCC
Cnr1	CTGAGGGTTCCCTCCCGGCA	TGCTGGGACCAACGGGGAGT
DAGLα	GGCCGCACCTTCGTCAAGCT	ATCCAGCACCGCATTGCGCT
Daglβ	AGACCCGGGTGCAATGCTGC	GCCCTGGTGTGTGGGTCACG
Faah	GGCAGAGCCACAGGGGCTATCA	TGGGGCTACAGTGCACAGCG
Gde1	GCAGCCCCTTCAACGCCTGT	GATGGCCGCCAGCGTGTTCT
Napepld	AGGCTGGCCTACGAATCACGT	ATGGTACACGGGGGACGGCG
Ptpn22	TGGTCGTGGGAGAGCCGCTT	GGGCCACTTTTTGCGCCTGC
Trpv1	AGACATCAGCGCCCGGGACT	CCAGCTTCAGCGTGGGGTGG
Hprt	ACAGGCCAGACTTTGTTGGATTTGA	AGGCTGCCTACAGGCTCATAGTG
S16	TCCGCTGCAGTCCGTTCAAGTCTT	GCCAAACTTCTTGGATTCGCAGCG
D3	GGGGTGACTGTCCTGGTCTA	AAGCCAGGTCTGATACTGAT
BDNF	CGAGTGGGTCACAGCGGCAG	GCCCCTGCAGCCTTCCTTCG
Parvalbumin	TTCTGAAGGGCTTCTCCTCA	AAGCAGTCAGCGCCACTTAG
Dysbindin	TGAAGGAGCGGCAGAAGTT	GTCCACATTCACTTCCATG

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Chapter IV

General Discussion

6.1 Cross-talk between D_3R and $GABA_A$ receptor in alcohol addiction

The data obtained during the first part of this thesis demonstrate that D_3R-dependent ectopic expression of α6 $GABA_A$ subunit counteracts alcohol intake by increasing GABA inhibition in the NAc. In particular, we discovered that Ro 15-4513, an α6 $GABA_A$ ligand, modulates ethanol intake in $D_3R^{-/-}$ and WT mice, that express different levels of ectopic α6 $GABA_A$ in NAc. So, Ro 15-4513 decreased voluntary ethanol intake in WT mice and, very interesting, $D_3R^{-/-}$ mice, which normally do not prefer alcohol (Leggio et al., 2014), following Ro 15-4513 treatment consumed a level of ethanol similar to that of WT. These data indicate that in $D_3R^{-/-}$ mice the response to Ro 1545-13 is related to the α6 increased ectopic expression in the NAc, these results are supported by the previous observations of Leggio and collegues (2015), which demonstrated that $D_3R^{-/-}$ show a higher α6 $GABA_A$ subunit expression in striatum compared to their WT littermates. As showed by Saba et al in 2001, the Sardinian non-ethanol-preffering rat line report a R100Q mutation in α6 subunit, this suggest a possible involvement of $GABA_AR$ containing α6 in the predisposition to consume alcohol. This mutation is associated to tonic inhibitory currents mediated by the α6βδ-type $GABA_AR$, measured by patch-clamp in cerebellar granule cells (Olsen et al., 2009, Santhakumar et al., 2007). Moreover, our electrophysiology analysis of MSN in the NAc, robustly

expressing α6, revealed a significant increase in mIPSCs peak amplitudes in $D_3R^{-/-}$ mice compared to WT; perfusion with Ro 15-4513 induced a significant reduction of the peak amplitude in the NAc of $D_3R^{-/-}$, but no effects have been observed in WT. These results clearly indicate that the modulation of the $GABA_AR$ channel by Ro 15-4513 depends on the presence of α6 subunit and is consistent with the observation of opposite effects of this drug on ethanol intake in WT and $D_3R^{-/-}$. At neurobiological level, systematic assessment of α6 expression in CNS by ISH, confirmed by qPCR, indicates that ectopic α6 expression was restricted to NAc, these results have been confirmed by autoradiography data obtained with [^{3}H] Ro 15-4513. Turning to the pharmacological approach, in order to obtain another evidence of the relevant cross-talk between D_3R and $GABA_AR$ containing α6 in the modulation of alcohol consumption, we used the D_3R selective antagonist, SB 277011A.

Very interesting, the treatment with SB 277011A increased the $GABA_A$ α6 subunit expression in the NAc of WT mice. Moreover, following D_3R pharmacological blockade, ectopic expression of $GABA_AR$ containing α6 subunit in the NAc was accompanied by a tendency toward a paradoxical effect of Ro 15-4513, similar to $D_3R^{-/-}$ mice.

Finally, our data suggest that there is a cross-talk between $GABA_ARs$ containing α6 and D_3R in the modulation of alcohol consumption. Moreover, these data show a role played by α6 $GABA_ARS$ in the NAc in the control of

alcohol intake by increasing GABA-inhibition in MSN. Supported by the pharmacological treatment, the cross-talk between D_3R and $GABA_A$ may assume relevance in the reinforcing proprieties of alcohol and may represent a potential target for wean treatment.

6.2 D_3R plays a key role in the pathophysiology of schizophrenia: implications in MAM model

In the second part of this PhD thesis, we demonstrated that prenatal MAM exposure induced neonatal behavioural and molecular alterations as predictive signs of SCZ-like deficits at adulthood. Our results confirmed that MAM administration at GD 17 induces social and recognition memory impairment, as described by the reduced time of interaction in the SI and by lower discrimination ratio in the NOR, respectively, which validity is further supported by unaltered spontaneous locomotor activity. Particulary, at molecular level we focused our interest on the involvement of D_3R in MAM model, because we found an increased mRNA expression of D_3Rs in PFC, HIP, NAc and STR of adult MAM rats, as index of abnormal dopaminergic neurotransmission. These results suggest that abnormal dopaminergic neurotransmission in SCZ could be also due to alteration of D_3R in different brain regions, in agreement with clinical (Cui et al., 2015) and preclinical observation (Sokoloff and Le Foll, 2017). The significant increase of BDNF gene expression and a significant decrease in the mRNA expression of PV and DYS in the NAc reinforced the hypothesis that this area can represent a key structure in the neurobiology of SCZ. We showed a deficit in PV expression in different brain regions as index of altered GABAergic neurotransmission, these

results are consistent with the previous observation reported in SCZ patients (Brisch et al., 2015) and in MAM model (Gill and Grace, 2014; Chan et al., 2014) as well. One the main feature of SCZ is the altered dopaminergic transmission in several brain regions, this one represent the main target of the current and potential antipsychotic treatment. We demonstrate that the early cannabidiol (CBD) treatment at highest dose has beneficial effects on social and cognitive dysfunctions in MAM rats, these effects seem to be also due to its capacity to reverse D_3R overexpression in several brain regions of MAM rats, these results are consistent to previous Renard and collegues' observations (2017) showing that CBD can modulate the aberrant mesolimbic dopaminergic transmission. Finally, based on our results, early intervention with CBD seems to revert dopaminergic alterations via a modulation of the EC signalling, a key role seems to be played by the D_3R in the NAc, these data support the use of antipsychotics, acting on altered dopaminergic transmission, in SCZ treatment.

Concluding Remarks

Finally, I would like to conclude with these remarks:

1. Ethanol intake is inversely correlated with $GABA_A$ α6 subunit expression
2. The α6 $GABA_A$ ligand, Ro 15-4513, increases ethanol consumption in $D_3R^{-/-}$ mice, that express elevated levels of $GABA_A$ α6 in NAc, and decreases ethanol intake in WT littermates
3. Chronic blockade of D_3R increases the $GABA_A$ α6 subunit expression in the NAc of WT mice
4. $D_3R^{-/-}$ mice reveal a significant increase in mIPSCs peak amplitudes in MSN compared to WT
5. Perfusion with Ro 15-4513 induce a significant reduction of the peak amplitude in the NAc of $D_3R^{-/-}$ MSN, but it has no effects in WT
6. MAM rats show an increased mRNA expression of D_3Rs in PFC, HIP, NAc and STR, as index of abnormal dopaminergic neurotransmission
7. CBD treatment at highest dose reverse D_3R overexpression in several brain regions of MAM rats
8. MAM rats show a significant increase of BDNF gene expression and a decrease in PV and DYS expression in the NAc, a key structure in the neurobiology of SCZ.

9. CBD reverts dopaminergic alterations via a modulation of the EC signalling

Taken together, the data of this PhD Thesis reveal a prominent role of D_3R in the pathophysiology of alcohol addiction and schizophrenia, very common neuropsychiatric disorders. This may open avenues for the design of new therapeutic strategies.

Chapter V

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Chapter VI

Annexes

List of Publications and Scientific Contributons

List of Publications

- Gian Marco Leggio*, **Roberta Di Marco***, Marcello D'Ascenzo, Sebastiano Alfio Torrisi, Kristiina Dahl, Giovanni Giurdanella, Alessandro Castorina, Teemu Aitta-Aho, Giuseppe Aceto, Claudio Bucolo, Claudio Grassi, Esa R. Korpi, Filippo Drago and Salvatore Salomone. Dopamine D3 receptor-dependent ectopic expression of alpha6 GABAA subunit counteracts alcohol intake by increasing GABA inhibition in the nucleus accumbens. * co-first authorship.

- Tibor Stark, Jana Ruda-Kucerova, Fabio Arturo Iannotti, Claudio D'Addario, **Roberta Di Marco**, Giovanni Giurdanella, Vladimír Pekařík, Eva Drazanova, Fabiana Piscitelli, Monica Bari, Zuzana Babinska, Giovanni Giurdanella, Martina Di Bartolomeo, Salvatore Salomone, Alexandra Sulcova, Mauro Maccarrone, Carsten T Wotjak, Zenon Starkuc Jr, Filippo Drago, Raphael Mechoulam, Vincenzo Di Marzo, Vincenzo Micale**.** Early modulation of the endocannabinoid signaling prevents the brain neurochemical and behavioral alterations in a neurodevelopmental model of schizophrenia at adulthood.

Oral communications

- Dopamine D3 receptor-dependent changes in GABAA receptor alpha 6 subunit expression control voluntary ethanol intake. *Convegno monotematico SIF. Farmaci, Salute e Qualità della Vita. 25-28 Ottobre 2017, Rimini*

Award

- 12 March 2016: ECNP travel grant

Poster communications:

- **R. Di Marco***, G.M. Leggio*, S.A. Torrisi, G.Giurdanella, A.Fidilio, F.Caraci, C.Bucolo, S.Salomone, F.Drago. Cross-talk between GABAergic and dopaminergic system: role of GABAA alpha 6 subunit and D3 receptor in ethanol addiction in mice. European Nuropsychopharmacology Neuroscience Applied Volume 27 Supplementary 1 March 2017-07-05. * co-first authorship.

- T. Stark, **R. Di Marco**, J. Ruda-Kucerova, G.Giurdanella, V. Pekrik, Z. Babinska, S.Salomone, R.Mechoulam, F.Drago, A. Sulcoa, V.Micale. Early modulation of the endocannabinoid tone prevents molecular and behavioral alterations in MAM model of schizophrenia. European Nuropsychopharmacology Neuroscience Applied Volume 27 Supplementary 1 March 2017-07-05