UNIVERSITA' DEGLI STUDI DELL'INSUBRIA

DIPARTIMENTO DI SCIENZE TEORICHE E APPLICATE (DiSTA)

DOTTORATO DI RICERCA IN NEUROBIOLOGIA

Coordinatore: Prof. Daniela Parolaro

CICLO XXVI°

THE DUAL FACE OF THE ENDOCANNABINOID SYSTEM IN SCHIZOPHRENIA: EXPERIMENTAL EVIDENCE

Candidato:

ERICA ZAMBERLETTI

Matr. N. 616866

Tutor:

PROF. DANIELA PAROLARO

ANNO ACCADEMICO 2012/2013

This PhD Project was conducted at the University of Insubria in the Neuropsychopharmacology Laboratory under the supervision of Prof. Daniela Parolaro and Dr. Tiziana Rubino

TABLE OF CONTENTS

ABSTRACT

Investigating the association between Cannabis, the endogenous cannabinoid system and schizophrenia must take into account two aspects of major relevance.

On the one hand, Cannabis is the most widely used illegal drug and there is substantial evidence that its consumption has to be classified as an independent risk factor for psychosis that may lead to a worse outcome of the disease. On the other hand, there are several lines of evidence that clearly indicate the presence of a dysregulation in the endocannabinoid system in animal models of psychosis and at least in a subgroup of schizophrenic patients.

Concerning the first point, our recent studies in female rats demonstrated that chronic THC treatment during adolescence induced a complex phenotype in adulthood characterized by the presence of anhedonia, behavioral despair in the forced swim test, reduced sociability as well as significant deficits in spatial working and object recognition memory. Moreover, adolescent THC administration also sensitizes to the locomotor activating effect induced by acute psychostimulant administration in adulthood. This response is consistent with the presence of a psychotic-like phenotype. Thus, the simultaneous presence of pronounced depressive-like behaviors, cognitive deficits as well as psychotic-like signs suggests that adolescent THC exposure had led to a behavioral phenotype in adulthood that reflects the presence of complex schizoaffective-like disorder. Interestingly, when the same protocol of THC exposure was performed in adult animals, no behavioral alterations were observed, thus highlighting the specific vulnerability of the adolescent brain to the long-lasting adverse effects of THC.

The neurobiology of cannabis-induced schizophrenia is still unknown. However, we recently demonstrated that adolescent exposure to THC produces-long lasting alterations in the endocannabinoid system, that ultimately could result in imbalances of excitatory and inhibitory neurotransmission within specific brain regions. Accordingly, our data demonstrate that the schizoaffective-like disorder induced by adolescent THC exposure in female rats is strictly associated with alterations in the inhibitory neurotransmission which are particularly prominent within the prefrontal cortex, a brain region that has been found to be severely affected in schizophrenia.

With regard to the second aspect, there are several lines of evidence that clearly indicate the presence of a dysregulation in the endocannabinoid system in different animal models of psychosis and based on these observations, the pharmacological modulation of the endocannabinoid system has been taken into account as a new therapeutic possibility for

psychotic disorders. In line with this, we demonstrated that chronic treatment with the cannabinoid CB1 receptor antagonist, AM251, counteracts the psychotic-like phenotype in a neurodevelopmental model of schizophrenia in rats. This recovery at behavioral level was paralleled by the normalization of the endocannabinoid system functionality both in terms of endocannabinoid levels and CB1 receptor/G protein coupling in all the brain areas analyzed, possibly suggesting that the ability of AM251 to restore normal endocannabinoid system may account for its antipsychotic action.

As a whole, data reported in this thesis, strongly suggest an association between an altered endocannabinoid tone and the development of psychotic symptoms, thus supporting the exploitation of compounds acting on the endocannabinoid system as new therapeutic agents in the treatment of schizophrenia and related disorders.

INTRODUCTION

1. The endocannabinoid system

The term ‘endocannabinoid system’ refers to a neuromodulatory system present both in the brain and periphery comprising the cannabinoid CB1 and CB2 receptors, their intrinsic lipid ligands, endocannabinoids, such as the N-arachidonoylethanolamide (anandamide, AEA) and the 2-arachidonoylglycerol (2-AG), and the associated enzymatic machinery (transporters, biosynthetic and degradative enzymes).

Two types of cannabinoid receptors have been characterized, named CB1 and CB2 based on the order of their discovery (Matsuda et al., 1990; Munro et al., 1993), both belonging to the superfamily of G protein coupled receptors, whose activation mediates most of the effects of cannabinoid drugs. CB1 receptors are highly distributed in the CNS with low to moderate expression in periphery whereas CB2 receptors are predominately located in immune cells in tissues and recent papers demonstrate that they may also be expressed in neurons (Brusco et al., 2008; Gong et al., 2006; Van Sickle et al., 2005). The CB1 receptor activation through both Gi/o proteins inhibits adenylyl cyclase activity, activates potassium channels and inhibits voltage-gated calcium channels, while the CB2 receptor is known only to couple to Gi proteins (Howlett, 2002). Some indirect evidence suggests the presence of additional cannabinoid receptors (GPR55, GPR119, PPAR) (Ryberg et al., 2007; O’Sullivan, 2007) but to date only CB1 and CB2 receptors are recognized by The International Union of Basic and Clinical Pharmacology.

Endogenous ligands for the cannabinoid receptors were discovered soon after their characterization. The two major known endogenous ligands are AEA and 2-AG (Devane et al., 1992; Stella et al., 1997; Sugiura et al., 2006). Both are arachidonic acid derivatives produced from phospholipid precursors postsynaptically through activity-dependent activation of specific phospholipase enzymes (Piomelli, 2003). Later on, a number of other endocannabinoid ligands have been identified including N- arachidonoyldopamine (NADA), N-arachidonoylglycerolether and O-arachidonoylethanolamine (De Petrocellis and Di Marzo, 2009).

These ligands do not share the same biosynthetic or metabolic pathways, indicating distinct mechanisms of regulation. Different pathways can produce AEA from the phospholipid precursors N-arachidonoyl-phosphatidylethanolamine, the most relevant being a direct conversion catalysed by an N-acyl-phosphatidylethanolamine selective phosphodiesterase. 2-AG is mainly synthesized through activation of phospholipase C and the subsequent production of diacylglycerol, which is rapidly converted to 2-AG by

diacylglycerol lipase. After its re-uptake, AEA is hydrolysed by the enzyme fatty acid amide hydrolase (FAAH), producing arachidonic acid and ethanolamine, while 2-AG is primarily metabolized by monoacylglycerol lipase (MAG lipase), leading to the formation of arachidonic acid and glycerol (Di Marzo and Petrosino, 2007). Apart from their well known binding to CB1 and CB2 receptors, endocannabinoids may also bind to other receptors: for example AEA may activate intracellularly the potential vanilloid receptor type 1 (TRPV1) (Ross, 2003). Moreover, other putative cannabinoid receptors are the 'orphan' G protein coupled receptor, GPR55 (Ryberg et al., 2007), and the peroxisome proliferator activated receptor, PPAR (O'Sullivan, 2007). However, CB1 and CB2 receptors are certainly the most known targets for AEA and 2-AG, which activate them with different affinity: AEA has the highest affinity in both cases, whereas 2-AG has the highest efficacy in both cases (McPartland et al., 2007).

Importantly, endocannabinoids are synthetized and released "on demand" by post synaptic cells through the cleavage of membrane phospholipid precursors in response to different physiological and pathological stimuli. Released endocannabinoids act as retrograde transmitters and traverse back across the synapse where they bind pre synaptically located CB1 receptors and reduce synaptic transmitter release (Freund et al., 2003).

On this basis, the endocannabinoid system can be considered one of the major players in regulating the activity state of various neurotransmitters, and endocannabinoids are involved in several physiological functions and dysregulation of the endocannabinoid system has been associated with various pathological states, including psychiatric disorders. Recent data suggest that changes in endocannabinoid signaling and their consequences on neuronal activity might be important in the aetiology of schizophrenia and may explain the impact of cannabis abuse in psychotic disorders.

2. Cannabis and schizophrenia

The association between cannabis use and psychosis has long been recognized. Recent advances in knowledge about cannabinoid receptor function have renewed interest in this association. Converging lines of evidence suggest that cannabinoids can produce a full range of transient schizophrenia-like positive, negative, and cognitive symptoms in some healthy individuals. Also clear is that in individuals with an established psychotic disorder, cannabinoids can exacerbate symptoms, trigger relapse, and have negative consequences on the course of the illness. The mechanisms by which cannabinoids produce transient psychotic symptoms, while unclear may involve dopamine, GABA, and

glutamate neurotransmission. However, only a very small proportion of the general population exposed to cannabinoids develop a psychotic illness. It is likely that cannabis exposure is a “component cause” that interacts with other factors to “cause” schizophrenia or a psychotic disorder, but is still debated whether it could represent either a sufficient or necessary factor to do so alone. Nevertheless, in the absence of known causes of schizophrenia, the role of component causes remains important and warrants further study. Dose, duration of exposure, and the age of first exposure to cannabinoids may be important factors, and genetic factors that interact with cannabinoid exposure to moderate or amplify the risk of a psychotic disorder are beginning to be elucidated (D’Souza et al., 2009). In particular, the risk to develop psychotic symptoms associated with cannabis consumption appears to be highly dependent upon the frequency of cannabis use (Di Forti et al, 2009) as well as the age when drug use begins, adolescence representing a more vulnerable time window for the long-term adverse effect of cannabis consumption (Malone et al, 2010; Rubino et al, 2012).

2.1 The endocannabinoid system and schizophrenia

Besides the “exogenous cannabinoid hypothesis” of schizophrenia referring to the risk factor associated with cannabis abuse (see above), an “endogenous hypothesis” has been recently put forward referring to the presence of dysregulations of the endocannabinoid system contributing to the pathophysiology of schizophrenia (Müller-Vahl and Emrich, 2008). Indeed, the distribution of cannabinoid receptors in brain areas implicated with schizophrenia such as prefrontal cortex (PFC), basal ganglia, hippocampus and anterior cingulated cortex (ACC) is consistent with a crucial role of the endocannabinoid system in this pathology.

Based on these considerations, in the following sections the effect of chronic cannabis exposure in adolescence as a risk factor for developing psychotic-like behavior later in life (exogenous stimulation) as well as the alterations of the endocannabinoid system present in schizophrenia and their possible role as target for new therapeutic approaches (endogenous hypothesis) will be reviewed, taking into account both experimental data and human studies.

Before reviewing available data on the above-mentioned topics, a brief overview on animal models of schizophrenia will be provided.

2.2 Methodological considerations on rodent models of schizophrenia

Animal models are important for studying psychiatric diseases as they allow the use of methods that for ethical reasons cannot be used in humans and enable the researcher to examine hypotheses regarding the development of these diseases in a controlled environment that would not be possible in human studies. It is, however, critical to note that due to the complex human psychopathology of psychiatric diseases, it is not possible to model a disease like schizophrenia in its entirety. In fact, it is impossible to model in rodents most symptoms observed in schizophrenic patients, such as delusions, hallucinations and disorganized thinking. Current approaches to the development of relevant animal models rely on focusing on specific signs or symptoms associated with schizophrenia, rather than mimicking the entire syndrome. Cognitive functioning is moderately to severely impaired in patients with schizophrenia. The profile of deficits in schizophrenia involves several domains of learning and memory. For example, recognition memory in both nonverbal and verbal modalities is impaired in schizophrenia (McGuire et al. 2013) and impairments in this domain of memory can be easily assessed in rodents through the novel object recognition test. Behavioral models of negative symptoms that are currently used in the development of novel antipsychotic agents include the social withdrawal model and the forced swim test. For instance, social withdrawal is often one of the earliest symptoms to occur in schizophrenia. The social interaction test allows to measures explorative and social behaviors between pairs of animals in a either familiar or unfamiliar environment. Finally, the assessment of the immobility in the FST represents a useful model for the negative symptoms of the disease, particularly the depressive features (Chindo et al. 2012). However, the cognitive deficits and the negative symptoms of schizophrenia have a partial overlap with other neurological disorders, therefore one limitations of the above mentioned tasks is that they are not specific for schizophrenia. In contrast, PPI response as well as increased sensitivity to the locomotor-activating effects of psychostimulants such as PCP are considered more specific to schizophrenia and this model is currently used to validate animal models of schizophrenia and to assess the efficacy of novel antipsychotic treatments.

Despite the limitations, studies in experimental animals can provide potentially important new insight into a range of brain mechanisms with relevance to schizophrenia, such as detailed investigations on the role of certain brain areas in behavior, the mechanism of action of psychoactive and antipsychotic drugs, the interaction of classical and ‘novel’ neurotransmitters and genes in brain function, and neurodevelopmental mechanisms. Several of these studies would be very difficult to carry out in humans, both from a

technical and an ethical point of view. Clearly, complex psychiatric illnesses, such as schizophrenia, cannot be exactly reproduced in species such as rats and mice. Nevertheless, animal models are an important tool in studying the symptoms and development of such illnesses, alongside approaches such as post-mortem studies, psychophysiological studies, imaging and epidemiology.

2.3 Effect of Adolescent exposure to cannabinoids and schizophrenia-like behaviour: animal studies

Hyperlocomotion and sensorimotor gating

Experimental studies dealing with long-lasting effects of adolescent cannabinoid exposure on psychosis-related behaviors in adult rodents are very scarce.

Only two papers have addressed the effect of chronic pubertal cannabinoid exposure on sensorimotor gating at adulthood and report a long-lasting PPI deficit (Schneider and Koch, 2003; Wegener and Koch, 2009).

The concept of testing for locomotor hyperactivity in animal models as a symptom of psychosis is based upon the premises that enhanced dopaminergic activity in rodents leads to enhanced motor activity (Geyer, 2008) and changes in dopaminergic activity, and although they are unlikely to be the primary cause of positive symptoms of schizophrenia, may be involved in varying degrees of symptomatology (Van den Buuse, 2010). Therefore locomotor hyperactivity, either at baseline or after treatment with psychoactive drugs, such as amphetamine or phencyclidine, has become widely used as a behavioral tool to investigate psychosis-like behaviors.

As for PPI, few papers extensively investigated basal locomotor activity in adult animals pre-exposed to cannabinoids during adolescence and they reported confounding results: some of them showed no significant alterations in the open field recordings (Biscaia et al., 2003; Rubino et al., 2008), while others stated the presence of locomotor hyperactivity (Wegener and Koch, 2009).

So far, no published paper dealt with the consequence of cannabinoid adolescent exposure on psychoactive drug-induced locomotor activation.

Cognitive deficits

When recognition memory was tested through the novel object recognition test, most papers reported impairments after chronic cannabinoid treatment during adolescence that

were evident from 15 days to 30 days after discontinuing the treatment (O'Shea et al., 2004, 2006; Quinn et al., 2008; Realini et al., 2010; Schneider and Koch, 2003). These impairments were present in both males and females, were induced by both the natural and synthetic cannabinoid compounds, and did not occur when the treatment was performed at adulthood. The only discrepant finding was by Higuera-Matas et al. (2009), who reported no significant effect of chronic CP55,940 during adolescence on recognition memory in both male and female adult rats. However, it is worth noting that they tested animals after a longer withdrawal period, i.e. 59 days, and maybe at this interval of time the long-lasting behavioral changes were recovered. If this is true, the effect of adolescent cannabis exposure on cognition might be considered as long-lasting and not irreversible, a feature really relevant at epidemiological level. Further studies are needed to make this point clearer. Spatial learning assessed through the Morris water maze was not affected by adolescent exposure to natural or synthetic cannabinoids in both male or female rats (Cha et al., 2006, 2007; Higuera-Matas et al., 2009). However, spatial working memory in the radial maze was impaired in both genders (Rubino et al., 2009a, 2009b).

As a general conclusion, the cognitive deficit appears to be task-specific, thus suggesting impairments in specific components of learning and memory rather than widespread effects: in particular, it appears that whenever the working memory component is involved in the test performance, a deficit is observed.

Social behaviors

Characteristic features of negative symptoms of schizophrenia are social withdrawal and aggressive behaviors. These behaviors can be assessed in the social interaction test, that measures explorative and social behaviors between pairs of animals in a either familiar or unfamiliar environment.

When the social interaction test was performed, all the authors found an impairment in social behaviours after adolescent exposure to cannabinoids (Leweke and Schneider, 2010; O'Shea et al., 2004, 2006; Realini et al.,2010).

2.4 Adolescent Cannabis use and psychosis: human studies

Different longitudinal studies have confirmed that a large intake of cannabis during adolescence triggers acute psychosis and may worsen outcomes in established psychosis (D'Souza et al 2009, Henquet et al 2005a, Le Bec et al 2009). An increased incidence of psychotic symptoms has been demonstrated by Henquet et al. (2005b) in a 4 year follow

up study involving young german individuals aged 12-24 years as well as by Ferdinand et al (2005) in a 14-year follow-up study of 4–16 year old subjects from the Dutch population, indicating that cannabis use predicted future psychotic symptoms in individuals who did not have such symptoms before they began using cannabis. Accordingly, McGrath et al. (2010), using a sibling pair analysis nested within a prospective birth cohort, showed that early cannabis use is associated with psychosis-related outcomes in young adults. The use of sibling pair analysis provides a better control of several potential confounding variables, thus making the results more compelling.

Interestingly, the inverse relationship has also been demonstrated: that is, the presence of psychotic symptoms in those who had never used cannabis predicted future cannabis use (Dekker et al., 2009).

The age when cannabis use begins is one of the more important determinant for susceptibility to psychotic illness Cannabis users had an earlier age at onset of psychosis, and there was a strong linear relationship between age at first cannabis use and age at onset of both prodromal and psychotic symptoms (Dragtet al., 2010; Sugranyes et al., 2009, Leeson et al 2011). Initiation by the age of 18 years doubled, whereas initiation by 15 years quadrupled the odds of subsequent psychotic disorders at follow-up at the age of 26 years (Arseneault et al., 2002). Accordingly, later age at first cannabis use predicted earlier cessation of use and this in turn was linked to fewer positive psychotic symptoms and days in hospital during the first 2 years (Leeson et al 2011).

It has been suggested that cannabis abuse may affect brain circuits involved in reward, decision making, attention, learning and memory, and behavioural control, all of which are still maturing into early adulthood, thus leading to alterations in neurobiology that increase psychosis. In this view, the role of the endocannabinoid system in neural development and modulation of neurotransmitter systems during adolescence should be better investigated to understand the mechanisms contributing to the enhanced vulnerability to psychosis observed in adolescent cannabis users.

Not all young cannabis users develop psychosis, indicating that cannabis use interacts with other variables, such as genetic vulnerability (i.e. the COMT Val108Met polymorphism) and other environmental factors, finally leading to psychosis onset (Caspi et al., 2005; Dominguez et al., 2010; Fergusson et al., 2006; Henquet et al., 2008, 2009; Semple et al., 2005).

As a whole, data available so far suggest that exposure to cannabis is neither a necessary nor a sufficient cause of schizophrenia but, more likely, cannabis exposure is a component or contributing cause that interacts with other known (genetic, environmental) and unknown factors, culminating in schizophrenia.

2.5 Alterations of the endocannabinoid system in schizophrenia: animal studies

CB1 receptor

Vigano' et al. (2009) demonstrated that the cognitive impairment induced by repeated PCP injections in juvenile rats was associated with a significant enhancement in CB1 receptor binding in the amygdala and ventral tegmental area (VTA). Moreover, PCP treatment induced a widely distributed decrease in CB1 receptor functionality in the PFC, hippocampus substantia nigra and cerebellum and a slight increase in the globus pallidus. These results suggest that a maladaptation in CB1 receptor density and functionality could be related to the cognitive impairment associated with the glutamatergic hypothesis of schizophrenia. Differently, Seillier et al. (2009) using a subchronic PCP model in adult rats found no changes in CB1 receptor expression whereas increases in CB receptor/G protein coupling in the ACC and nucleus accumbens (NAc) and a reduction in the CA2/3 fields of the hippocampus were observed. Differences in drug regimen and the different age of the animals might explain the discrepant results.

Using a rat model based on social isolation, Malone et al. (2008) found a down-regulation in CB1 receptor density in the caudate putamen (CPu) and amygdala. In contrast, increases in CB1 receptor binding in the PFC, CPu, thalamic nuclei and posterior area of the hypothalamus were recently reported by Sciolino et al. (2010) and Robinson et al. (2010), using the same experimental model. The different techniques used, the different rat species and the duration of the isolation rearing protocol may account for the observed discrepancies.

CB1 receptor expression and functionality was also studied in another neurodevelopmental model of schizophrenia based on the maternal deprivation protocol. A long-lasting decrease in CB1 receptor expression was showed in both male and in female rats submitted to 24-hour maternal deprivation at PND 9. The down-regulation was evident either in the short term (at PND 13) as well as in the long term period (at adulthood) (Suarez et al., 2009; Llorente-Berzal et al., 2011), strongly supporting the idea that long-lasting alterations in the endocannabinoid system are involved in the development of schizophrenia-like symptoms.

Another important approach to clarify CB1 receptor's role in schizophrenia is the use of knockout (ko) mice. Acute PCP administration is known to induce locomotor activation, stereotyped behaviors as well as reduced social interactions in wild-type animals. Interestingly, PCP-induced behavioral alterations were different in CB1 ko mice (Haller et al., 2005), since reduced locomotion, greater enhancement in ataxia and stereotypies as well as no alterations in social behavior were observed. These findings indicate that CB1 gene disruption dramatically affects the behavioral effects of PCP, strongly supporting its involvement in schizophrenia. As social disruption and stereotypy are believed to model respectively negative and positive symptoms of schizophrenia, it can be tentatively suggested that cannabinoids may play different parts in these two symptoms, possibly inhibiting positive but facilitating negative ones.

CB2 receptor

In the last years, evidence has been accumulated pointing to a role for central CB2 receptors in schizophrenia. CB2 deletion in mice caused a decreased motor activity, enhanced response to acute cocaine, PPI deficit, and cognitive impairment (Ortega-Alvaro et al., 2011). This preliminary evidence raises the possibility that a pharmacological manipulation of CB2 receptor could be a potential therapeutic target for the treatment of schizophrenia-related disorders.

Endocannabinoid levels

Besides CB1 and CB2 receptors, there is experimental evidence for an involvement of alterations in endocannabinoid levels in schizophrenia.

Using an animal model based on PCP administration, Vigano' et al. (2009) found no changes in AEA levels in either the PFC and hippocampus, whereas 2-AG content rose significantly in the PFC of PCP-treated rats. In contrast, Seillier et al. (2009) measured AEA and 2-AG in PCP-treated rats not tested for behaviors and found increases in AEA levels in the NAc and VTA and a trend, although not significant, in the PFC whereas 2-AG levels were increased only in the VTA. One of the more relevant reason for the discrepancy of the above quoted data, both based on PCP injection, is the time of endocannabinoids level measurements: immediately after the behavioral test (as in Vigano's study) or in animals that had not been tested (as in Seillier's one). Thus, in the former case endocannabinoids levels reflect their production in response to neuronal activity, in the latter one they reflect basal levels.

More recently, a paper of Eisenstein et al. (2010) looked at the role of 2-AG and endocannabinoid signaling in rodents with bilateral olfactory bulbectomy (OBX), a procedure that induces increased locomotor activity in response to the open field, a behavior attributed to hyperdopaminergic activity resembling schizophrenia. OBX-induced hyperactivity was restricted to the first 3 minutes of the open field test, showing the presence of novelty (0-3min) and habituation (3-30) phases of the locomotor response. 2-AG and AEA levels were reduced in the ventral striatum in OBX animals. While in sham-operated the levels of 2-AG were negatively correlated with the distance travelled during the novelty phase, in OBX rats 2-AG levels correlated with the distance traveled during the habituation phase, strongly support the idea that the endocannabinoid system and, particularly, 2-AG are implicated in the hyperactive locomotor response induced by OBX and suggest that drugs able to increase 2-AG content can be suggested in human disorders modeled by OBX, such as schizophrenia.

Alterations of the endocannabinoid system triggered by adolescent THC exposure

As already mentioned above, the adolescent brain appears to be particularly vulnerable to the long-lasting adverse effects of cannabinoids, especially THC. In order to analyze the molecular underpinnings of this vulnerability, we recently investigated whether and how chronic THC exposure interferes with different maturational events occurring in the prefrontal cortex during adolescence. In the same experimental model used in the present thesis, we found that the endocannabinoid system undergoes maturational processes from mid adolescence into adulthood. Adolescent THC exposure disrupts these processes, leading to impaired endocannabinoid signaling and deficits in endocannabinoid-mediated LTD in the adult prefrontal cortex.

In this same developmental window, neuronal refinement occurs at prefrontal cortex glutamatergic synapses, and adolescent exposure to THC impairs it. THC altered the maturational fluctuations of NMDA subunits leading to larger amounts of gluN2B at adulthood. Moreover, adult animals pre-exposed to THC showed increased AMPA gluA1 with no changes in gluA2 subunits. This suggests the presence of more calcium permeable AMPA receptors at adulthood, also supported by reduced rectification index for AMPA EPSCs.

This severe effect induced by adolescent THC in the prefrontal cortex might be due to the disruption of the physiological role played by the endocannabinoid system during this developmental window. Indeed, the blockade of CB1 receptor specifically from early to late

adolescence seems to prevent the occurrence of pruning at glutamatergic synapses. Thus, vulnerability of adolescent animals to long-lasting THC adverse effects might partly reside in the disruption of the pivotal role played by the endocannabinoid system in the adolescent prefrontal cortex maturation (Rubino et al. *submitted*).

2.6 Alterations of the endocannabinoid system in schizophrenia: human studies

CB1receptor

Several lines of evidence support the presence of alterations in CB1 receptors in the brain of schizophrenic patients. Up-regulations of CB1 receptors have been demonstrated in the dorsolateral prefrontal cortex (DLPFC), ACC and posterior cingulated cortex (PCC) of schizophrenic patients (Dean et al., 2001; Zavitsanou et al., 2004; Newell et al., 2007). In contrast, Eggan et al. (2008) showed a down-regulation of CB1 mRNA levels in the DLPFC of subjects with schizophrenia that was significantly correlated with a reduction in glutammic acid decarboxilase and cholecystokinin mRNA levels in the same brain region. Finally, no changes in the superior temporal gyrus and ACC have been found by Deng et al. (Deng et al., 2007) and Koethe et al. (2007) respectively. Moreover, Uriguen et al. (2009) showed that the immunodensity of CB1 receptors was significantly decreased in antipsychotic-treated subjects but not in drug-free schizophrenic subjects. Both cannabis consumption and treatment with antipsychotic drugs as well as the different techniques used for determining CB1 receptor levels could explain the apparent inconsistent results obtained in these studies. More recently, Dalton et al. (2011), employing a more rigorous control for potentially confounding factors, reported a significant increase in CB1 receptor binding in the DLPFC in a subgroup of patients who suffered from paranoid schizophrenia compared to normal controls and patients with non-paranoid schizophrenia. This finding suggests a different contribution of the endocannabinoid system in different subtypes of schizophrenia, arguing for distinct neurochemical correlates of clinical subtypes and raising the possibility of instituting psychopharmacological treatment accordingly.

Finally, a recent Positron Emission Tomography (PET) study showed an elevated binding of a specific CB1 tracer in the pons of schizophrenic patients (Wong et al., 2010). More importantly, this study suggested that CB1 binding is positively associated with severity of positive symptoms and negatively with severity of negative symptoms.

As a whole, human studies highlight the presence of CB1 receptor dysregulations in specific brain areas mainly involved in cognition and memory, two functions highly

compromised in schizophrenia. Importantly, variation in the CB1 receptor gene may confer risk for disorganized schizophrenia (Chavarría-Siles et al., 2008).

CB2 receptors

To date, there is little clinical evidence supporting a role for CB2 receptors in schizophrenia. In 2003, De Marchi et al. (2003) showed that decreased AEA and CB2 receptor mRNA levels in peripheral blood mononuclear cells were associated with a clinical remission of schizophrenia. Moreover, a close correlation between a polymorphism of the CB2 receptor gene and increased susceptibility to schizophrenia has been demonstrated (Ishiguro et al., 2010). More recently, Minocci et al. (2011), in a case–control study, found a significant association between bipolar disorders and a polymorphism in CB2 receptor gene, supporting the hypothesis that CB2 is involved in the pathogenetic mechanism underlying this affective disorder and, presumably, other psychotic diseases.

Endocannabinoid levels

Besides alterations in the CB1 receptor, clinical studies show that also changes in endocannabinoid levels are implicated in schizophrenia. De Marchi et al. (2003), measuring AEA levels from blood of volunteers or patients with schizophrenia, found significantly more AEA in schizophrenic patients, and clinical remission was accompanied by a significant drop in the levels of AEA and the mRNA transcript for CB2 receptors and FAAH. Thus, endocannabinoid signaling might be altered during the acute phase of schizophrenia not only in the central nervous system but also in the blood. These researchers suggested that the observed changes might be related to the immunological alterations described in schizophrenia.

Leweke et al. (1999) and Giuffrida et al. (2004) reported a significant increase of cerebrospinal AEA levels in acutely schizophrenic antipsychotic naïve patients which was not observed in patients suffering from affective disorders or dementia. Differently from the work of De Marchi et al. (2003), no changes in AEA content were observed in serum (Leweke et al., 1999), suggesting that the changes in AEA levels were presumably of central origin. Other lipid molecules, such as oleoylethanolamide (OEA) and palmitoylethanolamide (PEA), were not altered in schizophrenics, thus excluding any generalized alteration in lipid signaling.

The negative correlation between cerebrospinal AEA levels with psychopathological symptoms in acute, non-medicated, schizophrenic patients suggests the existence of an

"anandamidergic" dysregulation in schizophrenia. In this context, AEA might play an adaptive role, counteracting the neurotransmitter abnormalities in acute schizophrenia. Moreover, patients treated with atypical antipsychotics, did not differ from antipsychotic-naive schizophrenic individuals with respect to cerebrospinal AEA, whereas patients receiving preferentially D2 receptor-blocking antipsychotic medication had AEA levels comparable to healthy volunteers. This is consistent with the theory that "typical" antipsychotics normalize AEA levels by blocking D2-like receptors that initiate AEA synthesis in limbic and motor areas, while "atypical" preferentially interact with serotonin 5-HT2A receptors. In a hypothetical model of the pathophysiological relevance of the endocannabinoid system, binding of endogenous agonists like AEA or 2-AG to CB1 receptors counteracts increased dopaminergic neurotransmission. This mechanism might be protective because as above quoted, levels of AEA in cerebrospinal fluid of schizophrenic patients were inversely correlated with the severity of psychotic symptoms (Giuffrida et al., 2004). Finally, Koethe et al. (2009) investigated the levels of AEA and OEA in cerebrospinal fluid and serum of patients with initial prodromal states of psychosis. AEA levels in the cerebrospinal fluid were significantly elevated in these patients, in a manner comparable to that described for first-onset, antipsychotic-naïve, schizophrenic subjects whereas OEA concentrations did not differ significantly between patients and controls, thus ruling out the possibility that changes in AEA levels were caused by a generalized alteration in ethanolamide signaling.

3. Pharmacological modulation of the endocannabinoid system as therapeutic tool for schizophrenia

Several preclinical studies have been published in the last ten years to clarify the effect of a pharmacological modulation of CB1 receptors (through agonists, antagonists and indirect agonists) on schizophrenia-like symptoms. In contrast, human data on the effect of cannabinoid drugs in schizophrenia are limited to cannabidiol (CBD, a non-psychoactive phytocannabinoid) and rimonabant, a CB1 receptor antagonist/inverse agonist.

3.1 Animal studies

Hyperlocomotion and stereotipy

Several papers reported the effects of both acute and chronic manipulation of the endocannabinoid system on hyperlocomotion and stereotypies induced by psychotomimetic agents. The majority of these studies highlights the ability of both natural and synthetic cannabinoid agonists to reduce locomotor activation induced by

amphetamine (Gorriti et al., 1999; Long et al., 2009), cocaine (Przegalinski et al., 2005) and quinpirole (Marcellino et al., 2008) in rats. In contrast, Gorriti et al. (2005) and Moreno et al. (2005) showed that THC and HU-210 exacerbated quinpirole-induced hyperlocomotion in rats whereas CP-55,940 administration had no effect on amphetamine-induced locomotor activation in monkeys (Madsen et al., 2006). Very different doses and regimens of both cannabinoid agonists and psychotomimetic agents were used, and this could explain the discrepant results obtained. However, the reported improvement of positive symptoms following cannabinoid agonists administration agrees with the hypothesis of a protective role of AEA in schizophrenia (Giuffrida et al., 2004). Interestingly, the AEA transport inhibitor, AM404, reversed apomorphine- and quinpirole-induced stimulation of motor behaviors (Beltramo et al., 2000). Accordingly, unpublished data from our laboratory demonstrated that indirect enhancement of AEA levels, through URB597 and AM404 injections, recovered PCP-induced hyperlocomotion and stereotyped behaviors in rats (Rubino et al., unpublished observations).

Also the potential anti-psychotic action of the non-psychoactive component of Cannabis sativa, CBD, has been investigated. Both acute and chronic administration of this cannabinoid compound have been proven to reduce hyperlocomotion induced by dopaminergic and glutamatergic agents in rodents (Gururajan et al., 2011; Long et al., 2009; Moreira and Guimaraes, 2005; Zuardi et al., 1991). As a general comment, cannabinoid agonists can differently modulate hyperlocomotion and stereotipy improving, exacerbating or having no effect on these behaviors. The protective effect strengthens Giuffrida's hypothesis of the protective role of AEA whereas an excessive stimulation of CB1 receptors could reduce the inhibitory control of the endocannabinoid system on dopaminergic functions (through receptor desensitization) thus worsening the symptoms.

Confounding data have been reported on the effect of cannabinoid antagonists administration on schizophrenia-like symptoms. In fact, administration of the CB1 antagonist/inverse agonist, rimonabant, either augmented (Ferrer et al., 2007; Masserano et al., 1999; Thiemann et al., 2008), attenuated (Cheer et al., 2007; Poncelet et al., 1999; Tzavara et al., 2003; 2009) or unaltered (Gerdeman et al., 2009; Lesscher et al., 2005; Madsen et al., 2006; Martin et al., 2003) locomotor responses or stereotyped behaviors induced by dopaminergic agents. In contrast, results obtained with the CB1 receptor antagonist, AM251, appear to be less ambiguous, since its administration was effective in reducing the psychomotor effect induced by amphetamine (Seillier et al., 2010; Thiemann

et al., 2008) or A2A receptor antagonists (Lerner et al., 2010) in rodents. Interestingly, the effect of cannabinoid antagonists on psychomotor activation was reported to be mediated by the selective blockade of CB1 receptors in the nucleus accumbens core (Morra et al., 2010), but not shell (Chiang and Chen, 2007). The protective effect of CB1 receptor blockade is supported by data demonstrating that genetic inactivation of CB1 receptors reduced hyperlocomotion induced by amphetamine, PCP and SCH442416, a A2A receptor antagonist (Corbillè et al., 2007; Haller et al., 2005; Lerner et al., 2010).

However, the protective effect of CB1 receptor antagonists are often limited to habituated animals and needs further investigation.

Social behaviors

Self-administration of WIN 55,212-2 has been reported to prevent the social deficit induced by chronic intermittent PCP in adult rats (Spano et al., 2010). PCP-induced social withdrawal was reversed also by enhancement of the endocannabinoid tone through URB597 or OMDM-2 (Seillier et al., 2010), suggesting that CB1 receptor activation may exert a protective action on PCP-induced reduction in sociability. Also CBD has been proven to prevent social withdrawal induced by glutamatergic agents (Long et al., 2006; Gururajan et al., 2011). Surprisingly, a potential benefit deriving from CB1 receptor blockade was also demonstrated by the study of Haller et al. (2005) showing that genetic CB1 receptor disruption prevented PCP-induced social deficit in mice.

Cognitive deficits

Cannabinoids have been reported to produce transient cognitive impairments including deficits in learning, short-term memory, working memory, executive function and attention in both human (Sewell et al., 2010) and animals (Zanettini et al., 2011). However, so far only few studies examined the effect of a pharmacological modulation of the endocannabinoid system on cognitive deficits in animal models of schizophrenia. Chronic THC treatment worsened PCP-induced cognitive impairment in the novel object recognition test (Vigano et al., 2009). In contrast, Spano et al. (2010) showed that WIN 55,212-2 self-administration in adult rats prevented PCP-induced deficits in recognition memory. These opposite results could be related to the different properties of THC and WIN 55,212-2 at CB1 receptors as well as the different ages at cannabinoid treatment, since younger animals (Vigano et al., 2010) display different vulnerability to aversive stimuli. In Vigano's work, the observed reduction of cognitive functions in THC-treated rats was accompanied by a marked decrease in AEA levels, suggesting that reduced AEA

content could represent one of the molecular mechanisms underlying the worsening of cognitive performances. This hypothesis is further confirmed by the work of Seillier et al. (2010) demonstrating that enhancement of AEA levels, through URB597 administration, reversed PCP-induced cognitive deficit.

Concerning CB1 receptor antagonists, AM251 was shown to ameliorate the working-memory deficit in PCP-treated rats (Seillier et al., 2010). In agreement, our recent work (Guidali et al., 2010) supported the beneficial effect of chronic AM251 treatment on cognitive performance in the novel object recognition test in a pharmacological model of schizophrenia. More recently, another CB1 receptor antagonist, AVE1625, was reported to improve both working and episodic memory impairments induced by MK-801 or neonatal nitric oxide synthase inhibition in rodents (Black et al., 2011), further suggesting that CB1 receptor antagonist may be useful to treat the cognitive deficits in schizophrenia.

Sensorimotor gating

PPI response can be disrupted in animals through various experimental conditions such as pharmacological manipulation of different transmitter systems as well as exposure to adverse environmental conditions such as social isolation and psychosocial stress. The effects of cannabinoid agonists on PPI disruption induced by either pharmacological and non-pharmacological conditions appear to be confounding and inconclusive. WIN55212-2 administration attenuated PCP-induced disruption of PPI (Spano et al., 2010) but led to a significant improvement of PPI in psychosocially stressed rats (Brzozka et al., 2011). In contrast, Malone and Taylor (2006) demonstrated that THC disrupted PPI response in socially isolated rats. Finally, THC improved PPI response in NRG1 mutant mice, a genetic model for schizophrenia (Boucher et al., 2007). Thus, depending on the doses of cannabinoid agonists, duration of administration, environmental conditions used or animals' genetic background, cannabinoid administration can evoke opposite effects in PPI response.

Controversial findings have been reported also with the non-psychoactive cannabinoid, CBD. Long et al. (2006) showed that in mice CBD significantly reversed PPI deficits induced by MK-801, suggesting an antipsychotic potential of this compound. However, this finding was not replicated by Gururajan et al. (2011) who demonstrated that CBD could not recover MK-801-induced disruption of PPI. It is worth to note that in these two studies different animal models (mice/rats) and experimental conditions (number and duration of

behavioral testing) were used, thus explaining at least in part the reported contradictory findings.

Finally, Mansbach et al. (1996) showed that the CB1 receptor antagonist SR141716 did not reverse the PPI disruption induced by both dopaminergic and glutamatergic agents. This finding was not confirmed by later studies demonstrating the ability of CB1 receptor antagonists to reverse the decrease in PPI induced by both pharmacological and environmental conditions. Ballmaier et al. (2007) reported that SR141716 and AM251 acted as antipsychotic compounds reversing PCP- and MK-801-induced disruption of PPI in rats. More recently, Black et al. (2011) showed the ability of another CB1 receptor antagonist AVE1625 to reverse LI induced by MK-801. Similarly, Malone et al. (2004) demonstrated that SR141716 antagonized the disruptive PPI effects of apomorphine also in mice. When SR141716 was tested in social isolated rats, Malone and Taylor (2006) found that its administration only reversed the decrease in PPI induced by THC but failed to recover the isolation-induced reductions in PPI, indicating that THC-induced disruption of PPI depend on activation of CB1 receptors but antagonism of CB1 receptors is probably not enough to normalize the PPI disruption induced by developmental manipulations. As a whole, CB1 receptor antagonists reversed the PPI disruption induced by pharmacological agents (cannabinoid drugs included) whereas their administration failed to recover the PPI deficit induced by adverse environmental conditions.

Recently, as above reported, PPI response has been reported to be disrupted in CB2 ko mice (Ortega-Alvaro et al., 2011), suggesting that CB2 receptor activation could represent a promising target for the rescue of PPI deficits.

3.2 Human studies

So far, only the potential therapeutic applications of CBD and rimonabant have been investigated.

CBD was effective in attenuating psychotic symptoms induced by nabilone and ketamine in healthy individuals (Leweke et al., 2010; Bosi et al., 2003). A recent fMRI trial observed that THC and CBD activated in opposite ways different brain areas in response to different tasks and, moreover, CBD treatment prevented the acute induction of psychotic symptoms by THC (Bhattacharyya et al., 2010). However, a recent study of Hallak et al. (2010) investigated the effects of CDB on selective attention and on the pattern of electrodermal responsiveness to auditory stimuli in patients and observed no significant beneficial effect of acute CBD administration, suggesting that a single CBD injection may not be sufficient

to induce an improvement of cognitive functioning. The absence of toxic effects associated with CBD, made its chronic administration acceptable in human trials. In line with this, a pilot study by Zuardi et al. (1995) demonstrated that a 19-year old female diagnosed with schizophrenia treated for four weeks with increasing doses of CBD showed a significant improvement of the Brief Psychiatric Rating Scale (BPRS) similar the one produced by haloperidol but avoiding the severe side effects after treatment with the conventional antipsychotic. In 2006, the study was expanded to three male subjects with 22 and 23 years of age, diagnosed with schizophrenia resistant to the treatment with conventional antipsychotics (Zuardi et al., 2006). Increasing doses of CBD failed to induce a significant improvement of BPRS in two of the subjects whereas the third did not respond to CBD at all. The lack of beneficial effect of CBD in these patients may be ascribed to their clinically resistance to antipsychotics. Later on, the potential antipsychotic properties of CBD were investigated by using a four-week exploratory, double-blind, controlled trial, with forty-two subjects (Leweke et al., 2007). In this study, CBD was tested in patients diagnosed with schizophrenia compared with the atypical antipsychotic amisulpride. Both CBD and amisulpride induced a significant reduction of psychotic symptoms but CBD induced significantly less side effects compared to the atypical antipsychotic used. Interestingly, CBD has been proven to be effective in alleviating psychotic symptoms associated with other neuropsychiatric conditions, such as Parkinson disease (Zuardi et al., 2009).

Although with the limitation due to the restricted number of subjects used in the studies, these preliminary findings point to a potential exploitation of CBD in the treatment of psychosis, particularly because of its safety and tolerability compared to traditional antipsychotics. However, long-term, double-blind, placebo-controlled trials in larger samples of subjects are strictly necessary to clearly address this issue.

Rimonabant has been used in several countries for treating obesity and, before being removed from the market because of its severe adverse effects (Ugur et al., 2008), it was tested in some psychiatric conditions with promising results. Based on the possibility that a hyperactivity of the endocannabinoid system in schizophrenia could be related to the development of clinical symptoms, rimonabant was tested in a six-week, double-blind controlled trial in schizophrenic patients (Meltzer et al., 2004). Contrary to expectations, rimonabant did not improve the Positive and Negative Syndrome Scale total score, CGI severity of illness score, BPRS scores compared to the group receiving placebo. More

recently, Kelly et al. (2011) conducted a sixteen-week, double-blind, placebo-controlled study with rimonabant in people with schizophrenia or schizoaffective disorder, based on the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition criteria, who were clinically stable on second-generation antipsychotics. They found a significant improvement in BPRS total score in the rimonabant group compared with the placebo group. A major limitation of this study is the small sample size, due to its premature termination (rimonabant marketing was suspended in 2008), and consequent limited power to detect efficacy or rare adverse events. Nonetheless, rimonabant was well tolerated and was not associated with worsening of psychiatric symptoms, thus CB1 inverse agonism/antagonism remains a promising approach for pharmacotherapy of schizophrenia.

In conclusion, it can be stated that the cannabinoid system is a promising target for novel therapeutic interventions in schizophrenia. However, additional controlled trials are still required to confirm the possible therapeutic exploitation and determine the safety of cannabinoid compounds. Moreover, besides CBD and rimonabant, other phytocannabinoids, such as cannabigerol (CBG), tetrahydrocannabivarin (THCV), cannabidivarin (CBDV), or the association of THC and CBD, which is already used in some neurological disorders, can represent promising approach for treating psychotic disorders.

AIMS

Objectives and hypotheses

The studies described in this thesis were designed with the intent to shed light on the complex relationship between cannabis, the endocannabinoid system and schizophrenia, taking into account both the exogenous and the endogenous cannabinoid hypothesis of schizophrenia.

The first approach is based on our recent demonstration that chronic THC treatment in adolescent rats induces permanent alteration in the endocannabinoid system and aims at verifying if dysregulations of the endocannabinoid system can affect the correct maturation of other neurotransmitter system, specifically the GABAergic system, thus favoring the appearance of a psychotic-like phenotype in adulthood .

The second part of this thesis highlights the presence of alterations of the endocannabinoid system in a validated neurodevelopmental model of schizophrenia and suggests that the pharmacological modulation of this system might represent a promising tool for treating schizophrenia.

With that said, the aims of the present PhD project were as follows.

Aim of study n. 1

“Adolescent delta-9-tetrahydrocannabinol (THC) exposure as a risk factor for schizophrenia”

The aim of this study was to assess whether adolescent exposure to delta-9-tertrahydrocannabinol (THC), the main psychoactive component of Cannabis sativa, is associated with an increased vulnerability to develop psychotic symptoms in adult female rats.

In order to answer this question, the following points were examined:

- The behavioral phenotype present in adult female Sprague-Dawley rats after adolescent THC exposure was characterized through a series of behavioral tests measuring specific endophenotypes of schizophrenia in animals. Specifically, cognitive deficits were assessed in terms of recognition memory, negative-like symptoms in terms of social behavior and behavioral despair, whereas sensitization to phencyclidine (PCP)-induced hyperlocomotion and stereotypy was determined as a measure of positive-like symptoms.
- The impact of adolescent THC exposure on the GABAergic transmission in the adult prefrontal cortex (PFC) and its possible involvement in the development of the observed behavioral phenotype were assessed..

Aim of study n. 2

“Modulation of the endocannabinoid system as potential antipsychotic strategy”

The aims of this study was to investigate the effect of a pharmacological manipulation of the endocannabinoid system by acute and chronic treatment with a CB1 receptor antagonist, AM251, on schizophrenia-like symptoms produced by a neurodevelopmental animal model of schizophrenia based on the social isolation procedure.

To this aim, the following points were examined:

- The goodness of the isolation rearing protocol was assessed in male Lister Hooded rats after 5 weeks of post weaning social isolation before performing other behavioral and neurochemical analyses. Therefore, behavioral analyses were performed in order to check for the presence of schizophrenia-like symptoms such as hyperlocomotion in a novel environment, cognitive impairment, aggressive behaviors in the social interaction test and PPI responses.

- The presence of changes in CB1 receptor density and functionality as well as endocannabinoid levels was monitored following the isolation rearing procedure in specific brain regions in order to highlight a possible contribution of imbalances in the endocannabinoid system to the development of schizophrenia-like traits in isolated animals. Moreover, the presence of alterations in dopamine D1 and D2 and glutamate NMDA receptor densities was analyzed in different brain areas following post-weaning social isolation.

- The ability of acute and chronic treatment with a selective CB1 receptor antagonist/inverse agonist, AM251, to reverse schizophrenia-like symptoms in isolation reared rats was investigated.

- The effect of AM251 treatment on the isolation-induced alterations of biochemical parameters (endocannabinoid system, dopamine and NMDA receptor densities) was examined to find a possible neural substrate of the improved phenotype.

MATERIALS AND METHODS

1) Methods in Study n.1

Animals

Female Sprague Dawley rats aged 28 days at the time of arrival were obtained from Charles River laboratories (Calco, Italy) and were housed in clear plastic cages on a 12 hour light-dark cycle (lights on 08:00h) and in a temperature (22 ± 2°C) and humidity controlled environment (50±10%). All animals were allowed free access to food and water. All experiments took place during the light phase and were performed in accordance with the guidelines released by the Italian Ministry of Health (D.L.116/92) and (D.L.111/94-B), and the European Community directives regulating animal research (86/609/EEC). All efforts were made to minimize the number of animals used and their suffering.

Adolescent THC Treatment

Delta-9-tetrahydrocannabinol (THC), a generous gift from GW Pharmaceutical (Salisbury, UK), was further purified to reach THC concentrations as high as 90% and dissolved in ethanol, cremophor and saline (1:1:18). Rats were injected with increasing doses of THC, or vehicle, twice a day from PND 35 to PND 45 (2.5mg/kg, PND 35-37; 5mg/kg, PND 38-41; 10mg/kg, PND 42-45), according to our previous published protocol (Rubino et al, 2008) (Fig. 1A). This protocol resembles a heavy use of marijuana, inasmuch as, according to the transformation in human equivalent dose proposed by FDA and the average content of THC in a joint, our first dose roughly corresponds to one joint, the second one to two joints, and the higher one to four joints. These moderate to high doses were chosen to be within the range known to produce behavioral effects in rats. Animals were then left undisturbed till adulthood. At PND 75 the behavioral effect triggered by adolescent THC were compared to those induced by sub-chronic administration of the non-competitive NMDA receptor antagonist, phencyclidine (PCP) (Neill et al, 2010).

Sub-chronic PCP treatment in adult animals

Sub-chronic PCP administration is known to mimic the cognitive impairment and some negative-like symptoms of schizophrenia, such as social withdrawal and behavioral despair, even after long-term withdrawal (Enomoto et al. 2007; Mouri et al., 2007). Phencyclidine hydrochloride (PCP, Sigma-Aldrich, UK) was dissolved in saline and administered at a dose of 5 mg/kg (volume of injection 1 ml/kg), i.p.

Animals were treated with either saline or PCP once a day for 7 days, according to a treatment schedule slightly modified from Seillier et al. (2010) and reported in Fig. 1B.

Acute PCP administration in THC pre-treated rats

Sensitization to hyperlocomotion and stereotyped behaviors induced by acute PCP administration is widely recognized as another endophenotype of schizophrenia in rodents. In order to determine whether adolescent THC exposure could sensitize to the locomotor-activating effects of acute PCP administration, at PND 75, THC- and vehicle-treated rats received an acute injection of PCP (or saline) at the dose of 2.5 mg/kg (Fig. 1C) and their behavior in terms of locomotor activation and stereotyped behaviors was monitored for 50 minutes after the injection.

Behavioral Tests

Classic and spatial versions of the novel object recognition (NOR) test

The experimental apparatus used for the object recognition test was an open-field box (43 x 43 x 32 cm) made of Plexiglas, placed in a dimly illuminated room. Animals performed each test individually. The experiment was performed and analyzed as previously described (Realini et al, 2011; Zamberletti et al, 2012). Briefly, each animal was placed in the arena and allowed to explore two identical previously unseen objects for 5 minutes (familiarization phase). After an inter-trial interval of 3 minutes one of the two familiar objects was replaced by a novel, previously unseen object and rats were returned to the arena for the 5-minute test phase. In the spatial variant of the test, both novel and familiar objects were placed in different positions compared to the familiarization phase, that is a spatial cue was added in the test. During the test phase the time spent exploring the familiar object (Ef) and the new object (En) was videotaped and recorded separately by two observers blind to the treatment groups and the discrimination index was calculated as follows : [(En-Ef)/(En+Ef)] x 100

Social Interaction Test

The test was carried out as previously reported (Realini et al, 2011; Zamberletti et al, 2012). On the day of testing, each animal was habituated for 10 minutes in the test arena (60 x 60 x 60 cm), an open-field box made of Plexiglas. During the test session, each animal was allowed to freely explore an unfamiliar congener in the arena for 10 minutes and we recorded the time spent in social behaviors and the number of aggressive behaviors. Social behaviors were defined as sniffing, following, grooming, mounting and nosing. Aggressive behaviors were defined as attacking, biting, tail rattling and aggressive grooming.

Forced Swim Test (FST)

Animals were tested in a modified version of the FST with only the first session of swimming as previously reported (Realini et al, 2011; Zamberletti et al, 2012), to measure a preexisting behavioral deficit induced by the behavioral manipulation. Briefly, rats were forced to swim for 15 minutes inside a clear 50 cm tall, 20 cm diameter glass cylinder filled to 30 cm with 25°C water. The session was videotaped for later analysis of the following parameters: immobility (time spent by the animal floating in the water making only those movements necessary to keep its head above the water), swimming (active swimming movements to the center of the cylinder), climbing (forceful thrashing movements with forelimbs against the walls of the cylinder). The time spent in each of these behaviors was measured by an experimenter blind to the treatment groups.

Locomotor activity and stereotyped behaviors

Rats were placed in a computer-controlled infra-red activity monitor arena. The arena consisted of a clear acrylic box, 43×43×32 cm (Ugo Basile, Varese, Italy) placed in a sound-attenuating room and fitted with two parallel infrared beams, located 2 and 6 cm from the floor to measure horizontal movement. Each animal was habituated in the arena for 10 minutes and then injected with acute PCP or vehicle. After the injection, horizontal movement and stereotyped behavior were recorded for 50 minutes (five 10-minute trials) and analyzed by two observers blind to the treatment groups. Stereotyped behavior was defined as episodes of repetitive licking, stereotyped searching, side-to-side waving or turning, dorso-ventral head movements, frequent repetitive rearing, forepaw flattering and repetitive gnawing.

Biochemical analyses

Biochemical investigations were performed in animals not tested for behavior. For histochemical analyses, rats were deeply anaesthetized with a 400 mg/kg dose of chloral hydrate and then perfused with 4% paraformaldehyde (PFA). For c-Fos detection brains were collected 20 minutes after acute PCP injection. Following extraction, brains were stored at 4°C in 4% PFA for 24 h and cryoprotected in 30% sucrose for a minimum of 24 h. Brains were included in OCT, cut in 40 µm-thick sections using a cryostat and stored at -20°C in anti-freezing solution (30% glycerol, 30% etylenglycol, 0.02% sodium azide in PBS).

Histochemical analyses

For GAD67 staining, slices were incubated in PBS 1x for 10 minutes at 95°C for antigen retrieval whereas no antigen retrieval was necessary for cFos staining. After blocking peroxidase activity with 3% H2O2 for 15 minutes, sections were incubated in blocking buffer (10% normal goat serum, 0.5% Triton X-100 in PBS) for 1 hour at room temperature and then incubated with rabbit polyclonal anti-cFos antibody (1:50, Abcam, UK) or mouse anti-GAD67 antibody (1:500, Millipore, Italy) in blocking solution overnight at 4°C. After washing, sections were incubated with the biotinylated goat anti-rabbit, or goat anti-mouse secondary antibody (1:200; Millipore, Italy) for 1 hour at room temperature. Labelled cells were visualized using the ABC system (Vectastain Elite; Vector Laboratories) with 3,3'-diaminobenzidine as chromogen. The sections were then mounted onto gelatine-coated slides, dehydrated and coverslipped with DPX. To control for specificity of immunostaining, some sections were processed without the primary antibody. Positive neurons were counted as previously described by Guidali et al. (2011), using Image Pro Plus 7.0 (MediaCybernetics, USA).

For double immunofluorescence, the sections were processed for antigen retrieval, pre-incubated in blocking solution for 2 hours at room temperature and then incubated with anti-GAD67 primary antibody together with parvalbumin (rabbit anti-parvalbumin, 1:1000, Abcam, UK) or cholecystokinin (rabbit anti-cholecystokinin, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. Double immunofluorescence was revealed by incubation in a mixture of Alexa Fluor 488 goat anti-mouse and Alexa Fluor 594 goat anti-rabbit (Invitrogen, Eugene, OR) diluted 1:2000 in blocking solution for 2 hours at room temperature. After several washes in PBS, sections were mounted onto Superfrost slides, dehydrated and coverslipped with ProLong Gold antifade reagent with DAPI (Invitrogen, Eugene, OR).

Digital Images were captured using a Olympus DP50 camera attached to an Olympus BX51-P polarizing/light microscope. Viewfinder Lite 1.0.135 software was used to import images from the camera. The quantification was carried out by first delineating the brain sections and the regions of interest (ROI) at low magnification (x4 objective). The ROI outlines were further refined under a x40 objective and labelled cells were then counted manually within the ROI using a 300x300 µm grid as reference and expressed as cells/mm2. For each animal, positive cells were counted in four 50µm-sections coursing the PFC (bregma +3.10 to 2.50 mm). Counts were determined for each hemisphere individually, and an average value for both hemispheres of each section was calculated.

To validate siRNA silencing, GAD67 labeled cells were counted within the ROI surrounding the site of injection in four 50µm-sections coursing the PFC. For double immunofluorescence analysis, four sections per rat were analysed and digital images were merged and adjusted only for contrast and brightness using Adobe Photoshop 5.0. Co-localization was defined by the experimenter as any overlapping staining. The investigator was blind to the grouping while taking the photomicrographs and performing the image analysis. Quantification was manually determined using Image-Pro Plus 7.0 software (Media Cybernetics, Bethesda, MD, USA) and expressed as the percentage of GAD67 and PV or CCK double positive cells among the total number of PV or CCK positive cells.

Western blot analyses

For western blot analyses, rats were decapitated and brains quickly removed. The cerebral areas (PFC and hippocampus) were obtained by regional dissection on ice, immediately frozen in liquid nitrogen and stored at -80°C until processing.

For total protein lysate, each brain region was homogenized in an appropriate volume of ice-cold buffer (10 mM Hepes pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 2 mM DTT, 1 mM PMSF, 1 mM EDTA, 1 mM EGTA, 2 mM sodium orthovanadate, 50 mM NaF, 10 mM sodium pyrophosphate, 0.5% Triton, 5 µg/ml aprotinin, and 5 µg/ml leupeptin) and centrifuged at 13000 rpm at 4°C for 3 minutes. The supernatant was used as total protein lysate and protein concentrations were determined according to the Micro-BCA assay kit (Pierce, Rockford, IL).

Protein lysates were prepared in boiling sodium dodecyl sulphate (SDS) sample buffer and equal amounts (30 µg) of total protein were run on a 10% SDS-polyacrylamide gel (15% for parvalbumin detection). The proteins were then transferred to polyvinylidene difluoride (PVDF) membranes, blocked for 2 h at room temperature in 5% dry skimmed milk in TBS1x 0.1% tween20 before incubation overnight at 4°C with the primary antibody. The following primary antibodies were used: mouse monoclonal anti-GAD67 (1:5000; Millipore, Italy), rabbit polyclonal anti-parvalbumin (1:7000; AbCam, UK), mouse monoclonal anti-VGAT (1:1000; Synaptic Sistems, Gottingen, Germany) and rabbit polyclonal anti-cholecystokinin (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). Bound antibodies were detected with horseradish peroxidase (HRP) conjugated secondary anti-rabbit or anti-mouse antibody (1:5000-15000; Chemicon International, Temecula, CA) for 1 hour at room temperature and and visualized using ECL Western Blotting Detection Reagents (GE Healthcare, UK). For detection of beta-actin, the blot was stripped with Restore Western

Blot Stripping Buffer (Thermo Scientific, Rockford, IL) and re-blotted with anti-beta-Actin antibody anti-beta-actin monoclonal antibody (1:15000-60000; Sigma Aldrich, Italy) overnight at 4°C and visualized as described. Bands were detected by autoradiography. For densitometry, images were digitally scanned and optical density of the bands was quantified using Image Pro Plus 7.0 software (MediaCybernetics, USA) and normalized to control. To allow comparison between different autoradiographic films, the density of the bands was expressed as arbitrary units.

Microdialysis experiments

Surgery

On the day of surgery, the animals, kept under isoflurane anaesthesia (1.5% mixture of isoflurane and air), were mounted in a David Kopf stereotaxic frame (Tujunga, CA, USA) with the upper incisor bar set at -2.5 mm below the interaural line. After exposing the skull and drilling a burr hole, a microdialysis probe of concentric design (CMA 12, MW cutoff 20,000 daltons; outer diameter 0.5 mm, length of dialyzing membrane 2 mm; Alfatech S.p.A., Genova, Italy) was implanted into the dorsal striatum (coordinates relative to the bregma were: AP: 0.7 mm anterior to bregma, L: 2.8 mm from the midline, V: -5.6 mm below the dura) or the PFC (coordinates relative to bregma were AP: 3.2 mm anterior to bregma, L: 0.6 mm from the midline, V: -2.2 mm below the dura) (Paxinos & Watson, 1986). After the implantation, the probe was permanently secured to the skull with methacrylic cement and 36 hours later microdialysis was performed in the awake freely moving rats.

Experimental protocol

On the day of the microdialysis experiment, the probe was continuously perfused with Ringer solution (in mM: NaCl, 144; KCl, 4.8; $MgSO_4$, 1.2; $CaCl_2$, 1.7) at a constant flow rate of 2 µl/min, using a CMA100 microinfusion pump (CMA 100, Carnegie Medicin, Stockholm, Sweden).

The collection of dialysate samples commenced 300 min after the onset of perfusion to achieve stable dialysis glutamate levels (Ferraro et al, 2012) and perfusates were collected every 20 minutes thereafter. Following the collection of three stable basal values, PCP (2.5 mg/kg) or saline were administered i.p. and other six perfusate samples were then collected (total sample collection time: 180 minutes).

Following each experiment, the brain was removed from the skull, and the position of the dialysis probe was verified using 30 µm-thick coronal cryostat sections. Only those animals in which the probe was correctly located were included in this study.

Glutamate and GABA analysis

Glutamate and GABA levels in the dialysate were measured by HPLC coupled to fluorimetric detection. Briefly, 20 µl samples were pipetted into glass microvials and placed in a temperature-controlled (4°C) Triathlon autosampler (Spark Holland, Emmen, The Netherlands). 30 µl of o-phthaldialdehyde/mercaptoethanol reagent were added to each sample, and 30 µl of the mixture were injected onto a Chromsep analytical column (3 mm inner diameter, 10 cm length; Chrompack, Middelburg, The Netherlands). The column was eluted at a flow rate of 0.48 ml/minute (Beckman125 pump; Beckman Instruments, Fullerton, CA, USA) with a mobile phase containing 0.1 M sodium acetate, 10% methanol and 2.2% tetrahydrofuran (pH 6.5). Glutamate and GABA were detected by means of a Jasco fluorescence spectrophotometer FP-2020 Plus (Jasco, Tokyo, Japan). The retention times of glutamate and GABA were ~3.5 minutes and ~ 15.0 minutes, respectively.

Data management and statistical analysis

Basal glutamate and GABA levels are expressed in µM and nM, respectively, and were not adjusted for the recovery from the microdialysis probe. Results from individual time points are reported as percentages of the mean ± SEM of the three baseline samples collected before treatment. Statistical analysis was carried out by analysis of variance (ANOVA), followed by the Newman-Keuls test for multiple comparisons.

In vivo delivery of GAD67 siRNA

Female Sprague Dawley rats aged 75 PND were anesthetized with chloral hydrate 400 mg/kg, i.p. (Sigma Aldrich, Italy) and placed in a stereotaxic instrument.

The target sequence used for GAD67 (Abnova, Walnut, CA) was GUAACUGCACACAtggtttcc (sense) and aaaccaUGUGUGCAGUUACCA (antisense). Two separate groups of animals were either subjected to sham-operation or injected with the Silencer Negative Control, scramble siRNA (Abnova, Walnut, CA), as a negative control.

GAD67 siRNA was suspended at a final concentration of 20 µg/µl in sterile RNAse free water containing 3% MaxSuppressor In vivo RNA-LANCEr II (Bioo Scientific Corporation, Austin, TX) and 10% sterile RNAse-free 10x PBS.

1.5 µl of the obtained solution were bilaterally injected into the PFC using the following coordinates: anterior, 2.70 mm; lateral, 1 mm; depth: -3 mm according to the atlas of Paxinos & Watson (1986). Injections were made with a Hamilton syringe at a rate of 0.5 µl/min using a motorized injector. Following surgery, rats were given an injection of amoxicilline (20 mg/kg, i.p., Sigma Aldrich, Italy) and then allowed to recover.

To investigate the efficacy of siRNA for silencing GAD67 expression, immunohistochemistry was performed 3 days after the surgery.

Data were expressed as mean ± SEM and analyzed by one-way or two-way ANOVA, followed by Bonferroni's post-hoc test. The level of statistical significance was set at $p<0.05$.

Statistical analysis

Behavioral and biochemical data were expressed as mean ± SEM and analyzed by unpaired Student's t test and by one-way or two-way ANOVA, followed by Bonferroni's post-hoc test, when appropriate. The level of statistical significance was set at $p<0.05$.

2) Methods in Study n.2

Animals and Isolation rearing procedure

At weaning (PND 21), male Lister Hooded rats (Harlan, Italy) were randomly housed in groups of 4 (grouped) or alone (isolated). All animals were housed in the same room and had visual, auditory and olfactory contact with animals caged nearby, on a 12 hour light-dark cycle (lights on 08:00h) and in a temperature (24 ± 2°C) and humidity controlled environment (50±10%). All animals had free access to food and water. The isolated animals were left undisturbed in their cages and received the minimal handling associated with husbandry (cage and bedding changed weekly).

All experiments were carried out during the light phase and performed in accordance with the guidelines released by the Italian Ministry of Health (D.L.116/92) and (D.L.111/94-B), and the European Community directives regulating animal research (86/609/EEC). All efforts were made to minimize the number of animals used and their suffering.

Drug administration

AM251 (Tocris, Italy) was dissolved in DMSO, Tween-80 and saline (1:1:8). The drug was acutely or chronically administered at 0.5mg/kg (with the injection volume of 0.5ml/kg), i.p.

For acute treatment each animal received a single injection 80 minutes before the test session, whereas for chronic administration AM251 was given daily for 3 weeks and animals underwent a series of behavioral tests 24 hours, 72 hours and 10 days after the last AM251 (or vehicle) administration. The different behavioral tests were performed on separate groups of animals, according to the timeline shown in Fig. 7.

Behavioral tests

Spontaneous Locomotor Activity

Rats were placed in a computer-controlled infra-red activity monitor arena. The arena consisted of a clear acrylic box, 43×43×32 cm (Ugo Basile, Varese, Italy) placed in a sound-attenuating room. The cage was fitted with two parallel infrared beams, located 2 and 6 cm from the floor and cumulative horizontal and vertical movement counts were recorded for 1 hour.

Novel object recognition (NOR) test

The experimental apparatus used for the object recognition test was an open-field box (cm60x60x60cm) made of plexiglass, placed in a dimly illuminated room. Animals performed each test individually. 10 minutes habituation session preceded the experimental trials. The experiment was performed and analyzed as previously described in Viganò et al., (2009). Briefly, after the habituation each animal was placed in the arena and allowed to explore two identical previously unseen objects for 10 minutes (familiarization phase). After an inter-trial interval of 1 hour one of the two familiar objects were replaced by a novel previously unseen object and rats were returned to the arena for the 3 minutes test phase. During the test phase the time spent exploring the familiar objects (Ef) and the new object (En) was videotaped and recorded separately by two observers blind to the treatment groups and .the discrimination index was calculated as follow: [(En-Ef)/(En+Ef)]*100.

Social interaction test

The test was carried out in a room illuminated with a dim overhead light. On the day of testing, each animal was habituated for 10 minutes in the test arena (cm60x60x60cm) made of plexiglass. During the test session, each animal was allowed to freely exploring an unfamiliar congener in the arena for 10 minutes. 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. Aggressive behaviors were defined as attacking, biting,

tail rattling and aggressive grooming. The whole testing phase was videotaped, analyzed by two observers blind to the treatment groups and we reported the time spent in social behaviors and the number of aggressive behaviors.

Prepulse inhibition of startle reflex apparatus

The startle reflex system is composed by 4 standard cages each placed into a sound-attenuated and ventilated chamber (Med Associated, USA). Plexiglas cylinders (diameter 9 cm),closed by two doors to restrict the animals, were mounted on a piezoelectric accelerometer platform connected to an analogue-digital converter. Background noise and acoustic bursts were conveyed through two speakers placed in proximity to the startle cage so as to produce a variation in sound intensity within 1 dB. On test day, each rat was placed in the experimental cage for a 5 min acclimatization period with a 70 dB white noise background; this was continued for the remainder of the session. Animals were then tested on 3 consecutive trial blocks. The first and the third blocks consisted of 5 pulse-alone trials of 40 ms at 115 dB, while the second block (test block) was a pseudorandom sequence of 50 trials including 12 pulse-alone trials, 30 pulse trials preceded by 74, 78 or 82 dB prepulses (10 for each level of prepulse loudness), and 8 no-stimulus trials (where the only background noise was delivered). The percent (%) PPI was calculated based only on the values relative to the second block, and using the following formula: 100-[(mean startle amplitude for prepulse+pulse trials/mean startle amplitude for pulse-alone trials)×100].

Biochemical assays

Autoradiographic-binding assays

For assessment of long-term effects of isolation rearing and AM251 treatment on CB1 receptor function, biochemical analysis were performed on separate groups of animals not tested for behavior. Rats were decapitated and brains were rapidly removed, frozen in liquid nitrogen and stored at -80°C until processing.

Coronal sections (20μm thick) were cut on a cryostat and mounted on gelatin-coated slides. The sections were stored at –80°C until processing.

[^{3}H]CP-55,940 receptor autoradiographic binding

The [^{3}H]CP-55,940 receptor autoradiographic binding was performed as previously described (Rubino et al., 2000; Viganò et al., 2009).

CP-55,940-stimulated [^{35}S]GTPγS binding in autoradiography

This was determined as previously described (Rubino et al., 2000; Viganò et al., 2009).

[^{3}H]SCH23390 and [^{3}H]Raclopride receptor autoradiographic binding

The experiments were performed as described previously (Léna et al., 2004). For the D1 receptor binding, sections were prewashed for 20 min in 50 mM Tris–HCl buffer, pH 7.4, containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$ at room temperature. The sections were then incubated in the same buffer at room temperature in the presence of 4 nM [^{3}H]SCH23390 (PerkinElmer Life Sciences, Milan, Italy) for 90 min to label the D1 receptors. For D2 receptors detection, slides were pre-incubated in the same Tris buffer as the D1 receptors. Incubation was carried out in the presence of 4 nM [^{3}H]raclopride for 60 min. Both incubations were terminated by rapid rinses in icecold 50 mM Tris–HCl buffer pH 7.4, followed by a dip into ice-cold distilled water, before they were rapidly dried in a stream of cold air. Sections were exposed to Kodak Biomax MR films (PerkinElmer Life Sciences, Milan, Italy) and developed after 6 (D1 receptor) or 10 (D2 receptor) weeks.

[^{3}H]MK-801 receptor autoradiographic binding

The experiment was performed as previously described by Newell et al. (2007). Sections were incubated at room temperature for 2.5 h in 30 mM HEPES buffer containing 20 nM [^{3}H]MK801 (Perkin Elmer Life Sciences, Milan, Italy) and exposed to Kodak Biomax MR films for 5 weeks.

Image analysis

The intensity of the autoradiographic films was assessed as previously reported in Rubino et al. (2000). Data were expressed as fmol/mg of tissue.

Endocannabinoid levels

Separate animals not subjected to PPI procedure were used for the dissection of PFC, CPu, NAc and Hippo after 5 weeks of isolation or social rearing and 24 hours after the last AM251 (or vehicle) injection. The collected areas were immediately frozen in liquid nitrogen and stored at -80°C.

Extraction and quantification of endocannabinoids

Frozen tissue samples were homogenized in chloroform/methanol/TRIS-HCl 50 mM pH 7.4 (2:1:1, v/v) containing 10 pmol of [^{2}H]8-AEA, [^{2}H]4-PEA and [^{2}H]4-OEA, and 50 pmol of [^{2}H]5-2-AG as internal deuterated standards (purchased from Cayman Chemicals, Ann Arbor, MI). The extract was purified by means of silica gel mini-columns as described in

Bisogno et al. (1997), and the eluted fraction containing AEA and 2-AG analysed by means of liquid chromatography-atmospheric pressure-mass spectrometry (LC-APCI-MS) conducted as described previously (Marsicano et al., 2002). Analyses were carried out in the selected ion-monitoring mode using m/z values of 356 and 348 (molecular ions +1 for deuterated and undeuterated AEA), 304 and 300 (molecular ions +1 for deuterated and undeuterated PEA), 330 and 326 (molecular ions +1 for deuterated and undeuterated OEA), and 384.35 and 379.35 (molecular ions +1 for deuterated and undeuterated 2-AG). AEA, OEA, PEA and 2-AG concentrations were calculated by isotope dilution and are expressed as pmol per g or mg of wet tissue weight. The concentrations of 2-AG were obtained by adding up to the amounts of the 2-isomer also those of the 1(3)-isomer, which mostly originates from the isomerization of the former during work-up.

Statistical analysis

Behavioral and biochemical results were analyzed by unpaired Student's t-test or a two-way ANOVA with housing conditions and drug treatment as independent variables, followed up by Bonferroni's post hoc test. PPI data were analyzed with unpaired Student's t-test or two-factor analysis of variance (ANOVA) with rearing condition or drug treatment as between-subjects factor and trial type (prepulse intensity) as repeated measure (within-subjects factor) followed by Bonferroni's post hoc test. All data were expressed as mean ± SEM. The level of statistical significance was set at $p<0.05$.

RESULTS

1) Results of Study n.1

Behavior

Adolescent THC treatment leads to the development of a psychotic-like phenotype in adult female rats

Negative-like symptoms and cognitive signs

Figure 2 shows the behavioral phenotype of adult rats treated with THC during adolescence in comparison with the behavioral effect observed in animals sub-chronically administered with PCP, a validated animal model of schizophrenia (Fig. 1A and B).

Panel A represents the effect of adolescent THC administration and sub-chronic PCP treatment in the NOR test. As expected, sub-chronic PCP administration induced a significant cognitive impairment in the classic version of the NOR test, as stated by the significant reduction in the discrimination index by about 100% compared to controls. A greater cognitive deficit was observed in the spatial version of the test, the discrimination index being reduced by about 190%. Interestingly, adolescent THC treatment triggered the development of a cognitive impairment in adulthood similar to that induced by sub-chronic PCP administration both in the classic and in the spatial variant of the NOR test, the discrimination index being significantly reduced by about 100% and 200% respectively. The time spent exploring the two identical objects during the familiarization phase and the locomotor activity was not altered in any of the groups analyzed (data not shown).

Panel B represents the effect of adolescent THC administration and sub-chronic PCP treatment in the social interaction test. As expected, sub-chronic PCP administration significantly reduced the time spent in active social behaviors in the 10-minute test session by about 50% when compared to vehicle-treated rats. Adolescent THC exposure induced a similar reduction in social behaviors (40%). Aggressive behaviors were not observed in any of the groups under investigation.

Panel C shows the effect of adolescent THC administration and sub-chronic PCP treatment in the FST. The sub-chronic PCP protocol induced a significant increase by about 80% in the time spent in immobility during the 15-minute test session. A significant increase in the immobility time by about 80% was also observed in adult animals that underwent adolescent THC treatment. In both experimental groups the increase in the immobility time was paralleled by a significant reduction of the time spent in swimming activity.

Sensitization to PCP-induced hyperlocomotion

Another endophenotype of schizophrenia is the presence of hypersensitivity to acute PCP administration (Lazar et al, 2011). To test whether adolescent THC treatment sensitizes the behavioral response to acute PCP on measures of locomotor activity and stereotyped behaviors, adult rats exposed to THC during adolescence were injected with PCP 2.5 mg/kg according to the treatment schedule reported in Figure 1C. Rat behavior was then monitored for 50 minutes in the activity cage.

Figure 2, panel D, represents locomotor activation and stereotyped behaviors in response to acute PCP administration in adult rats exposed to THC during adolescence. No differences were observed in the basal locomotor activity between vehicle- and THC-treated rats during the whole test session. The dose of PCP we used (2.5 mg/kg) did not induced locomotor activation in vehicle-treated animals but significantly increased locomotion in THC-treated rats. In particular, no locomotor activation was observed during the first and the last 10-minute trials, but two-way ANOVA revealed significant THC, PCP and THCxPCP main effects during the 20-, 30- and 40-minute trials (20-minutes: THC: $_{F1-20}$=34.62; p<0.0001; PCP: F_{1-20}=11.78; p=0.0050; THCxPCP interaction: F_{1-20}=26.39; p=0.0002; 30-minutes: THC: F_{1-20}=22.32; p=0.0005; PCP: F_{1-20}=9.424; p=0.0097; THCxPCP interaction: F1-20=17.25; p=0.0013; 40-minutes: THC: F_{1-20}=12.67; p=0.0039; PCP: F_{1-20}=0.6995; p=0.4193; THCxPCP interaction: F_{1-20}=7.584; p=0.0175). In fact, THC-treated rats showed an enhanced locomotor activation in response to acute PCP by about 320%, 370% and 200% respectively compared to the other experimental groups.

Statistical analysis also revealed significant THC, PCP and THCxPCP main effects on stereotyped behaviors during the whole 50-minute test session (10-minutes: THC: F_{1-20}=5.428; p=0.0381; PCP: F_{1-20}=5.428; p=0.0381; THCxPCP interaction: F_{1-20}=5.428; p=0.0381 20-minutes: THC: F_{1-20}=23.17; p=0.0004; PCP: F_{1-20}=210.2; p<0.0001; THCxPCP interaction: F_{1-20}=23.17; p=0.0004; 30-minutes: THC: F_{1-20}=19.70; p=0.0008; PCP: F_{1-20}=240.5; p<0.0001; THCxPCP interaction: F_{1-20}=19.70; p=0.0008; 40-minutes: THC: F_{1-20}=21.48; p=0.0006; PCP: F_{1-20}=322.1; p<0.0001; THCxPCP interaction: F_{1-20}=21.48; p=0.0006; 50-minutes: THC: F_{1-20}=8.040; p=0.0150; PCP: F_{1-20}=60.89; p<0.0001; THCxPCP interaction: F_{1-20}=8.040; p=0.0150). In fact, stereotyped behaviors were observed in vehicle-treated animals following acute PCP injection and this effect was significantly enhanced in THC-treated rats, PCP-induced stereotyped behaviors being

significantly higher in THC-treated rats than in vehicle-treated rats during the whole 50-minute test session.

Biochemical analyses

Role of PFC GABAergic signaling in the negative-like symptoms and cognitive signs induced by adolescent THC treatment

Research to date suggests that cortical GABAergic deficits contribute to both the pathophysiology and symptomatology of schizophrenia (Nakazawa et al, 2012). Since important interactions between the endocannabinoid and the GABAergic systems have been largely demonstrated (Lopez-Moreno et al, 2008), we checked whether adolescent THC administration could lead to long-term alterations in the GABAergic transmission in terms of GAD67, parvalbumin (PV), VGAT and cholecystokinin (CCK) protein levels as well as basal GABA levels in the PFC of adult rats.

Figure 3A represents the impact of adolescent THC exposure on GAD67, PV, VGAT and CCK protein levels in the PFC of adult rats.

No changes were observed in PV, VGAT and CCK levels in the PFC of adult animals administered with THC during adolescence. In contrast, adolescent THC administration significantly reduced the enzyme responsible for most GABA synthesis in adulthood, GAD67 protein levels being significantly reduced in the PFC by about 45% compared to vehicle-treated rats.

As shown in figure 3B, the reduction of GAD67 expression was not evident in the middle and late adolescence (PND 46 and 60), but only developed in adulthood (PND 75) (two way ANOVA for age: $F_{2\text{-}37}=59.23$; $p<0.0001$; agexTHC interaction: $F_{2\text{-}37}=13.31$; $p<0.0001$), suggesting a possible reduction in GABA synthesis.

Figure 3C shows that extracellular GABA levels in the PFC of THC-treated animals were significantly lower than those measured in vehicle-treated rats.

Since GAD67 is expressed either in PV- as well as in CCK-containing interneurons, double immunofluorescence for GAD67, PV and CCK proteins was performed to check in which neuronal subpopulation was present the reduction in GAD67 protein expression.

Immunocolocalization of GAD67 and PV and of GAD67 and CCK proteins in the PFC is represented in figure 4A and B, respectively. GAD67 was reduced by about 60% both in

PV- and CCK-containing interneurons in the PFC of THC-treated rats compared to vehicle-treated animals.

To assess whether the alterations in GABAergic transmission could be extended to other brain areas relevant to schizophrenia, the same biochemical analyses were performed in the hippocampus of adult animals that underwent adolescent THC administration. No changes were observed in GAD67, PV, VGAT and CCK levels in the hippocampus of adult animals chronically administered with THC during adolescence (data not shown).

Finally, to understand the functional meaning of the specific reduction of GAD67 levels in the PFC and the possible relationship between this molecular alteration and the behavioral phenotype induced by adolescent THC treatment, we knocked down GAD67 expression through a siRNA-mediated gene silencing approach. GAD67 siRNA was bilaterally injected in the PFC of adult naïve animals and their behavior was investigated in the NOR test, social interaction test, FST and on the locomotor response to acute PCP administration.

Silencing efficacy of GAD67 siRNA was analyzed by immunohistochemistry. As reported in figure 5A, 72 hours after an acute bilateral injection of GAD67 siRNA, GAD67 expression was significantly reduced by about 70% compared with controls.

Figure 5 (B-E) shows the effect of GAD67 siRNA delivery in the PFC on the rat behavioral responses as measured in the NOR test (fig. 5B), social interaction test (fig. 5C), FST (fig. 5D) and on PCP-induced locomotor activation (fig. 5E). Animals were tested 72 hours after the injection. Sham-operation and scramble siRNA injection did not alter the behavioral responses in all the test performed. Silencing GAD67 expression in the PFC by in vivo siRNA injection did not induce any behavioral alteration in the NOR (fig. 5B) and in the social interaction test (fig. 5C) and did not affect the locomotor response induced by acute PCP injection (fig. 5E). In fact, no changes in locomotor activity were observed following PCP administration in any of the groups analyzed. Statistical analysis only revealed a PCP effect on stereotyped behaviors during the whole test session (10-minutes: $F_{1\text{-}24}$=142.3; p<0.0001; 20-minutes: $F_{1\text{-}24}$=252.1; p<0.0001; 30-minutes: $F_{1\text{-}24}$=287.9; p<0.0001; 40-minutes: $F_{1\text{-}24}$=105.4; p<0.0001; 50-minutes: $F_{1\text{-}24}$=85.39; p<0.0001).

In contrast, lower GAD67 expression in the PFC resulted in a significant increase in the time spent in immobility (80%) in the FST compared with controls (fig. 5D). This increase in immobility was paralleled by a significant reduction in climbing and swimming activities.

Alterations in neural activation and glutamate release underlying THC-induced sensitization to acute PCP

To assess whether a different neuronal activation in response to acute PCP could underlie the increased behavioral response observed in THC-treated rats, c-Fos immunoreactivity was monitored in the PFC and CPu of THC- and vehicle-exposed animals, two brain regions involved in the development of behavioral sensitization (Vanderschuren and Kalivas, 2000)

Figure 6A represents c-Fos immunoreactivity in response to acute PCP in the PFC and CPu of THC-exposed rats and vehicle-treated animals.

In the PFC, statistical analysis revealed significant THC, PCP and THCxPCP main effects (THC: $F_{1\text{-}12}$=54.36; p<0.0001; PCP: $F_{1\text{-}12}$=55.00; p<0.0001; THCxPCP interaction: $F_{1\text{-}12}$=5.377; p=0.0484). Vehicle administration did not alter c-Fos expression in THC pre-treated rats. The low dose of PCP we used (2.5 mg/kg) did not induce by itself neuronal activation in this brain area but its administration in THC-treated animals caused a significant increase in c-Fos immunoreactivity by about 35% compared to vehicle-treated animals.

In the CPu, statistical analysis revealed significant THC, PCP and THCxPCP main effects (THC: $F_{1\text{-}12}$=54.36; p<0.0001; PCP: $F_{1\text{-}12}$=55.00; p<0.0001; THCxPCP interaction: $F_{1\text{-}12}$=4.377; p=0.0494). In fact, both THC and PCP by themselves significantly increased c-Fos expression by about 53%, and a further increase in c-Fos immunoreactivity was evident when PCP was administered in THC-treated rats (+60% compared to THC and PCP alone).

Figure 6B represents the effects of acute PCP injection (2.5 mg/kg) on PFC extracellular glutamate and GABA levels in vehicle- and THC-treated rats.

A slight increase in PFC extracellular glutamate levels was observed after PCP injection in control animals. In contrast, PCP significantly increased extracellular glutamate levels in the PFC of THC-treated rats. This effect reached statistical significance 40-minutes after the drug injection and was associated with a slight, not significant, reduction of extracellular GABA levels. No significant changes in PFC extracellular GABA levels were observed after PCP injection in control animals.

Similarly, PCP induced a significant increase of extracellular glutamate levels in the dorsal striatum of THC-treated rats, but not in control rats. No changes in striatal extracellular

GABA levels were found after PCP administration in both groups under investigation (Fig. 6C).

2) Results of Study n.2

Behavioral assessment of isolation-rearing protocol

Figure 8 shows the behavioral scene after 5 weeks of isolation rearing.

Isolation-reared rats were significantly more active in the novel environment than group-housed rats ($t=8.820$, $p<0.0001$) without any alteration in rearing activity between the two groups ($t=1.876$, $p=0.0832$) (Fig. 8a). Moreover, the isolation-rearing protocol caused an impairment of cognitive functions as demonstrated by a significant reduction in the discrimination index during the test phase of the NOR test ($t=10.25$, $p<0.0001$) compared to group-reared controls. In both groups there was no difference in the time spent exploring the two identical objects during the familiarization phase (data not shown), but isolated animals failed to discriminate between the new and familiar object in the test phase (Fig. 8b). The locomotor activity was not altered both in grouped and isolated rats (data not shown). In the social interaction test, no differences were found in the time spent in active behaviors between isolation- and group-reared animals ($t=0.1969$, $p=0.8504$) but isolation rearing caused a significant increase in aggressive behaviors compared to group-reared controls ($t=32.75$, $p<0.0001$) (Fig. 8c). Figure 8d represents the effect of rearing condition on % pre-pulse inhibition startle magnitude (% PPI). Both housing condition and AM251 treatment did not alter the magnitude of the startle response (data not shown). At a pre-pulse intensity of 74 dB, isolation-reared rats showed a significant lower %PPI than group-housed controls PPI (n=19) ($t=6.756$; $p<0.0001$). A significant disruption in the PPI response, although less intense, was still evident in isolated rats at a pre-pulse intensity of 78 dB ($t=3.466$; $p=0.0026$) and 82 dB ($t=2.491$; $p=0.0221$) compared with social controls.

Effect of the isolation-rearing protocol on CB1R functionality

After 5 weeks of isolation rearing, we investigated the effects of housing condition on CB1R density and functionality.

Figure 9a shows the effects of isolation rearing on CB1R density. Isolation rearing had no effect on CB1R density in all the cerebral regions analyzed.

We found significant changes in CB1R functionality as shown by the GTPγS binding assay (Fig. 9b). Particularly, the isolation-rearing protocol induced a significant reduction in CB1R functionality in the PFC ($t=2.093$, $p=0.0481$), NAc ($t=3.572$, $p=0.0017$), caudate putamen

(CPu) (t=3.507, p=0.0020), hippocampus (Hippo) (t=2.546, p=0.0216) and ventral tegmental area (VTA) (t=3.438, p=0.0044).

Effect of the isolation-rearing protocol on endocannabinoid levels

Figure 10 shows the effect of 5 weeks of social isolation on endocannabinoid levels in the PFC, NAc, CPu and Hippo.

In the PFC, social isolation induced a significant reduction in 2-AG levels by about 52% (t=2.525; p=0.0450) compared with group-reared controls. Moreover, a significant increase in PEA content (+59%) was evident in isolated rats (t=3.827; p=0.0187). No changes were found in AEA and OEA levels following 5 weeks of isolation rearing (AEA: t=0.3344; p=0.7494; OEA: t=0.9077; p=0.3990).

Similarly, in the NAc, a significant reduction in 2-AG content (-23%) was present following the isolation rearing procedure (t=2.744; p=0.0336). No changes in AEA, PEA and OEA levels were observed (AEA: t=0.4032; p=0.7035; PEA: t=0.1621; p=0.8765; OEA: t=1.183; p=0.2814).

Finally, isolated animals showed a significant increase in 2-AG levels in both the CPu (t=3.051; p=0.0284) and hippocampus (t=2.957; p=0.0316) by about 170% and 67% respectively. AEA, PEA and OEA levels did not differ from controls in these two brain areas (CPu: AEA: t=0.3681; p=0.7254; PEA: t=0.2863; p=0.7843; OEA: t=1.091; p=0.3250. Hippo: AEA: t=1.106; p=0.3112; PEA: t=0.2914; p=0.7805; OEA: t=1.166; p=0.2877).

Effect of the isolation-rearing protocol on dopamine D1 and D2 receptor densities

The effect of social isolation on dopamine D1 and D2 receptor densities in the PFC, CPu and NAc is illustrated in Figure 11A.

Isolated animals showed a significant reduction in D1 receptor density in the PFC by about 71% compared with group-reared controls (t= 6.680; p<0.0001). No significant changes in [^{3}H]-SCH23390 binding were observed in the CPu (t=1.651; p=0.1130) and NAc (t=1.480; p=0.1529) following the isolation procedure. In contrast, D2 receptor density was significantly increased in the PFC of isolated rats by about 82% (t=5.181; p=0.0001). Isolation rearing did not alter [^{3}H]-raclopride binding in the CPu (t=2.045; p=0.0601) and NAc (t=0.3168; p=0.7560).

Effect of the isolation-rearing protocol on glutamate NMDA receptor density

Figure 11B shows the effect of isolation rearing on glutamate NMDA receptor binding in the PFC, CPu, NAc, Hippo, amygdala (Amy) and thalamus (Thal).

A significant decrease in NMDA receptor density was present in the CPu following 5 weeks of isolation rearing (t=2.766; p=0.0113). Isolated animals also displayed a significant increase in [^{3}H]-MK-801 binding in the NAc compared with group-housed controls (t=2.566; p=0.0184). No changes were observed in the PFC (t=1.819; p=0.0825), Hippo (t=0.5691; p=0.5751), Amy (t=0.7774; p=0.4452) and Thal (t=0.4034; p=0.6905) following isolation rearing.

Effect of acute AM251 administration on cognitive impairment and aggressive behavior induced by isolation rearing protocol

Figure 12 shows the effects of acute AM251 administration on isolation-induced cognitive deficit and aggressiveness.

In the novel object recognition test, acute AM251 treatment did not alter the exploration time in the familiarization phase both in grouped and isolated rats (data not shown) and failed to improve the recognition memory disrupted by social isolation rearing (two-way ANOVA for housing: $F_{1,12}$=75.24, p<0.0001; two-way ANOVA for drug: $F_{1,12}$=0.0001214, *p*=0.9914; no interactions) (Fig.12a). Moreover, acute AM251 alone did not affect the discrimination index in socially reared rats and there were no differences in the locomotor activity between all the groups considered (data not shown).

In the social interaction test acute AM251 did not significantly reduce the number of aggressive events in isolation reared rats (two-way ANOVA for housing: $F_{1,12}$=148.6, *p*=0.9914; two-way ANOVA for drug: $F_{1,12}$=2.673, *p*=0.1280; no interactions). Neither housing conditions or AM251 treatment affected the time spent in social behaviors during the social interaction test (two-way ANOVA for housing: $F_{1,12}$=0.3768, *p*=0.5508; two-way ANOVA for drug: $F_{1,12}$=1.844, *p*=0.1995; no interactions) (Fig.12B).

Behavioral characterization after chronic AM251 (or vehicle) treatment in isolated and group-housed rats

Figure 13 shows the effects of chronic AM251 administration on isolation-induced cognitive impairment.

The discrimination index impaired in isolation-reared rats was restored after 3 weeks of chronic AM251 treatment (housing: $F_{1,12}$=6.834, p=0.0226; drug: $F_{1,12}$=4.755, p=0.0498; drug x housing interaction : $F_{1,12}$=19.43, p=0.0023) and the recovery of this parameter was still evident at 72 hours (housing: $F_{1,12}$=5.482, p=0.0373; drug: $F_{1,12}$=12.27, p=0.0044; no interaction) and 10 days after the last AM251 administration (housing: $F_{1,12}$=10.48, p=0.0071; drug: $F_{1,12}$=6.403, p=0.0264; drug x housing interaction : $F_{1,12}$=10.39, p=0.0073). Neither housing conditions nor AM251 treatment altered the time spent exploring the two identical objects during the familiarization phase (data not shown) and AM251 alone did not affect the recognition memory in socially reared rats. The locomotor activity was not altered in any of the groups analyzed (data not shown).

Figure 14 shows the effects of chronic AM251 treatment on the aggressive behaviors in the social interaction test.

We observed a reduction in the number of aggressive behaviors in isolation-reared rats 72 hours and 10 days after the last AM251 administration compared to what was observed in the social interaction test performed 24 h after the last injection. However, the aggressive behaviors in isolation-reared rats were still significantly increased compared to group-reared controls at both time-points. Isolation-reared rats chronically administered with AM251 showed a significant reduction in the number of aggressive events in the social interaction test performed 24 hours after discontinuing treatment (drug: $F_{1,12}$=13.71, p=0.0030; drug x housing interaction : $F_{1,12}$=13.71, p=0.0030) and this recovery was still evident at 72 hours (drug: $F_{1,12}$=11.37, p=0.0056; drug x housing interaction: $F_{1,12}$=11.37, p=0.0056) and 10 days (drug: $F_{1,12}$=6.716, p=0.0236; drug x housing interaction : $F_{1,12}$=6.716, p=0.0236) after the last AM251 administration. Chronic AM251 per se did not alter the time spent in social behaviors.

Figure 15 shows the effect of chronic AM251 (or vehicle) treatment on PPI response in isolation- and group-reared rats.

Two-way ANOVA revealed a significant effect of rearing conditions on PPI response at 74 and 78 dB intensities (74 dB: $F_{1\text{-}15}$=13.41; p=0.0023; 78 dB: $F_{1\text{-}15}$=16.15; p=0.0011), PPI disruption being still evident in isolation-reared animals compared to group-housed controls following vehicle treatment. The effect of rearing conditions on PPI response was not present at 82 dB intensities ($F_{1\text{-}15}$=2.471; p=0.1368).

AM251 treatment did not affect PPI response in control animals at any of the dB intensities tested (74 dB: F_{1-15}=2.263; p=0.1533; 78 dB: F_{1-15}=0.4114; p=0.5309; 82 dB: F_{1-15}=0.02212; p=0.8837).

In contrast, AM251 administration completely counteracted PPI disruption in isolated rats at 74 as well as 78 dB intensities (74 dB: F_{1-15}=5.750; p=0.0299; 78 dB: F_{1-15}=4.562; p=0.0496).

Neurochemical characterization after chronic AM251 (or vehicle) treatment in isolated and group-housed rats

Effect of AM251 chronic treatment on CB1 receptor functionality

After 3 weeks of chronic AM251 (or vehicle) treatment we found no alterations in the CB1 receptor density, both in group housed and isolation reared rats, in all the brain regions analyzed (Figure 16a).

Figure 16b represents the results of CP-55,940-stimulated GTPγS autoradiographic-binding assay performed 24 hours after the last chronic AM251 (or vehicle) administration.

AM251 had no effect on CB1 receptor functionality in group housed controls but counteracted the alterations observed in rats reared in isolation in the PFC, NAc, Hippo and VTA. After 3 weeks of chronic vehicle treatment, isolation reared rats still showed a significant reduction in the CB1 receptor functionality in the PFC, NAc, Hippo and VTA compared to group housed controls (PFC: two-way ANOVA for housing: $F_{1,28}$=6.610, p=0.0157; NAc: two-way ANOVA for housing: $F_{1,28}$=7.083, p=0.0127; Hippo: two-way ANOVA for housing: $F_{1,28}$=8.549, p=0.0068; VTA: two-way ANOVA for housing: $F_{1,28}$=8.549, p=0.0068), indicating that these alterations were not influenced by daily handling. The reduction reported in isolation reared rats were counteracted by AM251 chronic administration (PFC: two-way ANOVA for drug x housing interaction: $F_{1,28}$=8.881, p=0.0059; NAc: two-way ANOVA for drug x housing interaction: $F_{1,28}$=5.848, p=0.0223; Hippo: two-way ANOVA for drug x housing interaction: $F_{1,28}$=8.410, p=0.0072).

Endocannabinoid levels

Figure 17 represents endocannabinoid levels following chronic AM251 (or vehicle) administration in respect to housing conditions.

Effect of chronic handling on endocannabinoid content

Endocannabinoid levels appeared to be particularly influenced by chronic handling due to vehicle/drug administration in isolated animals. Thus we first checked whether handling procedure per se further modify the isolation rearing-induced alteration in endocannabinoid levels.

In the PFC, a significant effect of handling was evident on OEA content in isolated rats (F_{1-12}=9.588; p=0.0092), OEA levels being reduced in both vehicle- and AM251-treated animals. AM251 administration did not affect OEA content in group housed controls (F_{1-12}=0.08697; p=0.7731). Moreover, in the same brain regions, handling counteracted isolation-induced increase in PEA levels (F_{1-12}=4.83; p=0.036).

Similarly, in the NAc, a significant handling effect was found on OEA levels (F_{1-12}=12.38; p=0.0042), with reduction of OEA content both in vehicle and in AM251 treated rats.

Finally, in the CPu and hippocampus, two-way ANOVA revealed a significant main effect of handling on endocannabinoid content (F_{1-12}=6.57; p=0.017). Specifically, chronic handling completely counteracted the isolation-induced increase in 2-AG in the CPu, but caused an increase in AEA levels in .the hippocampus

In summary, 3 weeks of handling induced in the PFC and NAc a significant reduction in OEA levels, resistant to AM251 treatment, completely reversed in the CPu the 5 weeks isolation-induced increase in 2-AG and induced an increase in AEA levels in the hippocampus. In contrast, handling did not affect the significant alterations in 2-AG levels measured in the PFC, NAc and hippocampus after 5 weeks of isolation.

Effect of AM251 administration on endocannabinoid content

In the PFC, social isolation procedure significantly reduced 2-AG levels in vehicle treated rats (F_{1-12}=6.637; p=0.0258). Interestingly, AM251 administration did not alter 2-AG levels in control animals (F_{1-12}=1.472; p=0.2504) but completely restored 2-AG levels in isolated rats (F_{1-12}=10.72; p=0.0074).

Neither isolation rearing nor AM251 treatment altered AEA and PEA levels in this brain area (AEA: housing condition: F_{1-12}=1.732; p=0.2120; drug: F_{1-12}=0.00007932; p=0.9930. PEA: housing condition: F_{1-12}=0.2793; p=0.6068; drug: F_{1-12}=0.06082; p=0.8094).

Similarly, in the NAc, two-way ANOVA revealed a significant effect of housing conditions on 2-AG content (F_{1-12}=10.23; p=0.0095), its levels being significantly reduced in isolation reared rats chronically treated with vehicle. AM251 administration counteracted the

isolation-induced reduction of 2-AG (F_{1-12}=5.167; p=0.0463) without affecting 2-AG content in control animals (F_{1-12}=3.944; p=0.0751).

No effect of isolation rearing were observed on AEA and PEA content (AEA: F_{1-12}=0.2595; p=0.6205; PEA: F_{1-12}=3.298; p=0.0944) and AM251 treatment did not affect AEA, PEA and OEA levels in this brain region (AEA: F_{1-12}=0.6064; p=0.4526; PEA: F_{1-12}=0.2132; p=0.6526; OEA: F_{1-12}=1.103; p=0.3143).

In the Hippo, AEA (F_{1-12}=5.112; p=0.0450) and 2-AG (F_{1-12}=7.271; p=0.0224) levels were significantly increased in isolated rats compared to group housed controls, although, as mentioned above, the effect on AEA was probably due to handling. Interestingly, AM251 administration completely reversed the isolation-induced increase in 2-AG (F_{1-12}=7.061; p=0.0240) and the handling -induced increase in AEA (F_{1-12}=38.99; p<0.0001), without affecting per se their contents in group housed controls (AEA: F_{1-12}=2.438; p=0.1467; 2-AG: F_{1-12}=4.493; p=0.0601).

Neither social isolation nor AM251 administration altered PEA (housing condition: F_{1-12}=0.7320; p=0.4123; Drug: F_{1-12}=0.1241; p=0.7319) and OEA (housing condition: F_{1-12}=0.7937; p=0.3921; Drug: F_{1-12}=1.530; p=0.2419) levels in this brain area.

Finally, in the CPu, isolation rearing procedure did not alter AEA, 2-AG, PEA and OEA levels (AEA: F_{1-12}=0.5577; p=0.4696; 2-AG: F_{1-12}=0.07726; p=0.7862; PEA: F_{1-12}=0.1744; p=0.6836; OEA: F_{1-12}=1.262; p=0.2833). In contrast, two-way ANOVA revealed a significant effect for drug treatment on PEA levels (F_{1-12}=10.61; p=0.0069), with AM251 treatment slightly increasing PEA content in both isolated and group-housed rats. AM251 administration did not alter AEA (F_{1-12}=0.03262; p=0.8597), 2-AG (F_{1-12}=1.120; p=0.3127) and OEA (F_{1-12}=0.3095; p=0.5882) levels in this brain region.

Effect of AM251 administration on dopamine D1 and D2 receptor densities

Figure 18a shows the effect of AM251 (or vehicle) administration on dopamine D1 and D2 receptor densities in isolated and group-reared animals.

Isolation rearing significantly reduced D1 receptor density in the PFC (F_{1-12}=46.50; p<0.0001), the effect being evident both in AM251 and vehicle-treated rats. No effect of isolation rearing on [^{3}H]SCH23390 binding was observed in the CPu (F_{1-12}=0.1546; p=0.6970) and NAc (F_{1-12}=2.577; p=0.1193).

AM251 treatment did not affect [^{3}H]SCH23390 binding in the PFC (F_{1-12}=0.04747; p=0.8291) and CPu (F_{1-12}=0.7729; p=0.3866). In contrast, two-way ANOVA showed a

significant effect of drug treatment in the NAc (F_{1-12}=5.439; p=0.0268), D1 receptor density being significantly down-regulated in isolation reared rats (F_{1-12}=8.015; p=0.0083).

In the PFC, statistical analysis revealed a significant effect of housing conditions (F_{1-12}=5.360; p=0.0264), drug treatment (F_{1-12}=8.387; p=0.0064) and housing condition x drug treatment interaction (F_{1-12}=20.50; p<0.0001) on dopamine D2 receptor density. Isolation rearing significantly increased [^{3}H]Raclopride binding in this brain area and AM251 administration completely antagonized the isolation-induced alteration.

Neither isolation rearing nor AM251 treatment altered D2 receptor density in the CPu and NAc (CPu: housing conditions: F_{1-12}=0.01988; p=0.8887; drug treatment: F_{1-12}=1.858; p=0.1813; NAc: housing conditions: F_{1-12}=0.1370; p=0.7140; drug treatment: F_{1-12}=0.2175; p=0.6446).

Effect of AM251 administration on glutamate NMDA receptor density

Figure 18b represents the effect of AM251 (or vehicle) treatment on NMDA receptor density in respect to the different housing conditions.

A significant down-regulation of NMDA receptor density was evident in isolated rats in the CPu (F_{1-12}=9.408; p=0.0050). Moreover, isolation rearing induced a significant up-regulation of the receptor in the NAc (F_{1-12}=23.32; p<0.0001). No changes were instead observed in the PFC (F_{1-12}=0.8202; p=0.3734), Hippo (F_{1-12}=3.485; p=0.0728), Amy (F_{1-12}=0.8727; p=0.3599) and Thal (F_{1-12}=0.4155; p=0.5248) following isolation rearing.

AM251 treatment did not affect [^{3}H]MK-801 binding in group-reared controls in any of the brain areas analyzed but completely recovered isolation rearing-induced alterations in the CPu (drug: F_{1-12}=6.718; p=0.01550; housing conditions x drug interaction: F_{1-12}=4.404; p=0.0457) and NAc (drug: F_{1-12}=6.254; p=0.0190; housing conditions x drug interaction: F_{1-12}=14.01; p=0.0009). Moreover, in the Hippo two-way ANOVA revealed a significant housing conditions x drug treatment interaction (F_{1-12}=20.38; p=0.0001), NMDA receptor binding being significantly down-regulated in isolated rats following AM251 treatment.

GENERAL DISCUSSION

Overview

The overarching goal of this collective body of research was to examine whether alterations of the endocannabinoid signaling, either induced by exogenous administration of THC during adolescence or by a neurodevelopment procedure such as the social isolation rearing from weaning, could be associated with the development of psychotic-like symptoms in rats and to determine the efficacy of a modulation of the endocannabinoid system, through AM251, in reverting schizophrenia-like signs and in restoring endocannabinoid system functionality.

The research described in Study n. 1 argues that prolonged exposure to THC during adolescence represents a risk factor for the development of a complex schizoaffective-like phenotype later in life. This behavioral picture is associated with marked alterations in the GABAergic system within the PFC. The observed alterations of GABA transmission may be directly related to the altered behavior in the FST observed in adult THC-treated rats, as silencing GAD67 in the PFC results in increased time spent in immobility in the test. In contrast, they do not appear to be related to the other behavioral alterations triggered by adolescent THC treatment. Instead, the psychotic-like component of THC-induced behavioral phenotype (i.e. sensitization to acute PCP) may be ascribed to increased neuronal activation as well as enhanced glutamate levels within the PFC and CPu.

In Study n. 2, it was demonstrated that alterations of the endocannabinoid system, in terms of CB1 receptor functionality and endocannabinoid content, were present in a neurodevelopmental animal model of schizophrenia based on the post-weaning social isolation procedure. Moreover, in these animals we also found significant alterations in dopamine D1 and D2 as well as glutamate NMDA receptor densities. More interestingly, the pharmacological modulation of the endocannabinoid system through AM251 was effective in counteracting the schizophrenia-like phenotype observed in isolation-reared rats and this recovery at behavioral level was paralleled by the rescue of CB1 receptor functionality as well as the normalization of endocannabinoid contents within specific brain regions. Moreover, AM251 administration also partially counteracted the isolation-induced changes in dopamine and glutamate receptors, possibly contributing to the observed recovery at behavioral level.

A detailed discussion of the studies presented in this thesis is provided in the following sections.

Adolescent delta-9-tetrahydrocannabinol (THC) exposure as a risk factor for developing schizophrenia later in life

Behavioral results

The results here presented provide evidence that adolescent THC treatment leads to long-term behavioral alterations in adult female rats characterized by the presence of recognition memory deficits, social withdrawal and altered emotional reactivity, closely resembling the ones induced by sub-chronic PCP administration.

These findings confirm and expand our previous studies demonstrating the presence of a complex depressive-like phenotype in adult female rats that underwent adolescent THC exposure, characterized by the presence of anhedonia, behavioral despair in the FST and reduced sociability as well as significant deficits in spatial working memory and object recognition memory (Realini et al, 2011; Rubino et al, 2008, 2009).

Furthermore, here we demonstrated for the first time that, besides the cognitive impairments and altered emotional reactivity, adolescent THC administration also induces a sensitization to the locomotor activating effect of acute PCP when administered in adulthood. In fact, adult animals exposed to THC during adolescence showed an exacerbated behavioral response to acute PCP compared with vehicle-treated controls, in terms of both locomotor activation and stereotyped behaviors. This effect is consistent with the presence of a psychotic-like phenotype, since sensitization to PCP-induced hyperactivity is widely recognized as an index with translational relevance to the positive symptoms of schizophrenia. To our knowledge, this is the first time that the long-lasting consequences of adolescent cannabinoid exposure on psychoactive drug-induced locomotor activation were assessed.

Thus, the present data extend our earlier findings and indicate that the behavioral picture triggered by adolescent THC treatment is more complex than what previously thought. In fact, the simultaneous presence of pronounced depressive-like behaviors, cognitive deficits as well as psychotic-like signs suggests that adolescent THC exposure had led to a behavioral phenotype that cannot be defined as depressive-like behaviors, but rather be ascribed to a more complex schizoaffective-like disorder.

As a whole, our data support the hypothesis that adolescent exposure to THC might represent a risk factor for developing psychotic-like symptoms in adulthood.

Biochemical results

Alterations in several markers of the inhibitory neurotransmission have been demonstrated in multiple cortical regions of schizophrenic patients (Curley and Lewis, 2012) as well as in different animal models of the pathology (Powell et al, 2012).

Here, we demonstrated that the psychotic-like phenotype observed in adult rats that underwent adolescent THC treatment is associated with marked alterations in the GABAergic system within the PFC. Specifically, adolescent THC exposure resulted in lower levels of GAD67, the enzyme responsible for most GABA synthesis, and, accordingly, PFC basal GABA levels were reduced in adult THC-treated rats compared with vehicle-treated animals. In contrast, adolescent THC exposure did not affect PV, VGAT and CCK expression.

These data suggest that THC exposure during adolescence disturbs the physiological maturation of the GABA system within the PFC, possibly delaying the physiological increase in GAD67 that occurs during adolescence.

In line with this, in studies examining brain tissue from schizophrenic patients, concomitant reductions of GAD67 and CB1 receptor mRNA levels within cortical regions have been observed (Akbarian and Huang, 2006; Eggan et al, 2008). Accordingly, in the present study we found that adolescent THC exposure resulted in long-term alterations of GAD67 levels in the PFC, whereas no changes were observed in the hippocampus. Interestingly, a reduction in CB1 receptor was also observed within this brain region following adolescent THC exposure (Rubino et al., personal communication).

Cortical GAD67 expression has been found to be reduced in PV-positive interneurons in both schizophrenic brains (Hashimoto et al, 2003) and animal models of schizophrenia (Wang et al, 2008). Here, we demonstrated that GAD67 levels were reduced in cortical PV-containing interneurons following adolescent THC exposure. However, we also reported, for the first time, that adolescent THC treatment also resulted in reduced GAD67 immunoreactivity in CCK-containing interneurons, possibly suggesting that also alterations in this neuronal GABAergic subpopulation might play a role in THC-induced schizoaffective-like disorder.

Within the neocortex, CB1 receptor is expressed at high levels in a subpopulation of CCK-positive basket interneurons whereas it is not expressed in PV-positive interneurons (Marsicano and Lutz, 1999). It has been recently demonstrated that agonism at CB1

receptor affects GABAergic transmission in the PFC. Indeed, reduced gamma oscillations have been reported in the PFC following administration of the synthetic cannabinoid agonist, CP-55,940 (Kucewicz et al, 2011). Interestingly, at cellular level, the decrease in gamma oscillations has been linked to reduced function of PV-positive interneurons (Volman et al, 2011), which lack CB1 receptor. Since CCK-positive and PV-positive cells have been demonstrated to be anatomically and functionally coupled (Armstrong and Soltesz, 2012), we can suggest that, following adolescent THC administration, primary alterations in CCK-containing interneurons might have occurred triggering, in turn, the alterations observed in PV-positive cells.

As a whole, lower GAD67 expression appears to be a critical hallmark of schizophrenia, but it is still unknown if and to what extent deficits in GAD67 contribute to the pathogenesis of the disease. In this study, we demonstrated that the deficit in GAD67 was not present 24 hours (PND 46) nor 15 days (PND 60) after discontinuing THC administration but only developed in adulthood (PND 75), suggesting a strong positive correlation among the deficit in GAD67 and the behavioral impairments observed in adult THC-treated rats.

To investigate whether reduced GAD67 expression is sufficient in determining the behavioral alterations observed in adult rats that underwent adolescent THC exposure, we silenced GAD67 expression selectively in the PFC of adult naïve rats and tested them for behavior. Silencing GAD67 expression in the PFC was sufficient to impact rats' behavior in the FST: indeed, we observed an 80% increase in the time spent in immobility compared with controls, an effect that was paralleled by reductions in climbing and swimming activities. In contrast, reduced GAD67 expression did not affect animal behaviors in the NOR and SI tests and did not alter the response to acute PCP.

The present results suggest that lower GAD67 levels may be directly related to the behavioral despair observed in adult THC-treated rats whereas THC-induced cognitive deficits and social withdrawal may rely on other molecular mechanisms, different from the ones considered in the present study, or may be dependent upon concomitant alterations that involve multiple neurotransmitter systems.

Moreover, here we demonstrated that an altered neuronal activation may underlie the behavioral sensitization to acute PCP observed in adult THC-treated rats.

Using c-Fos as a functional anatomical marker of activated neurons within the central nervous system (Kovacs, 2008), we reported that c-Fos immunoreactivity was significantly enhanced in the PFC and CPu of adult THC-exposed animals in response to acute PCP.

This increase in neuronal activation may be dependent upon enhanced extracellular glutamate levels following acute PCP injection (Adams and Moghaddam, 1998). Accordingly, acute PCP administration resulted in enhanced PFC and CPu glutamate levels in THC-treated rats, but not in vehicle-treated animals.

As a whole, these findings support the hypothesis that prolonged exposure to THC during adolescence represents a risk factor for the development of a complex schizoaffective-like phenotype later in life. This behavioral picture is associated with marked alterations in the GABAergic system within the PFC. Indeed, adolescent THC exposure results in reduced GABA synthesis and levels in the PFC. Reduced cortical GAD67 expression may be directly related to the altered behavior in the FST observed in adult THC-treated rats, as silencing GAD67 in the PFC results in increased time spent in immobility in the test, but does not appear to be related to the psychotic-like component of the THC-induced behavioral phenotype, as delivery of GAD67 siRNA in the PFC did not affect the behavioral response induced by acute PCP administration. In contrast, the sensitization to acute PCP reported in adult THC-treated rats may be ascribed to increased neuronal activation as well as enhanced glutamate levels within the PFC and CPu.

Modulation of the endocannabinoid system in a neurodevelpmental model of schizophrenia

Behavioral and biochemical characterization of the isolation induced phenotype

Rearing rats in isolation from weaning results in long-term abnormalities on brain structure, neurotransmitter function and behavior compared to group housed controls (Lapiz et al., 2003). In our hands, after five weeks of isolation rearing, rats presented a marked increase in total horizontal locomotor activity, in isolation reared rats compared to group housed controls without any differences in total vertical activity between the two groups. Moreover, rats reared in isolation showed a cognitive impairment in the novel object recognition test and a significant increase in the number of aggressive behaviors. compared to socially-reared controls. Consistent with the presence of a schizophrenia-like phenotype, we demonstrated here the presence of a robust deficits in PPI response in isolated rats compared to socially reared littermates thus reinforcing the original findings that this environmental manipulation provides a viable, non-pharmacological model of impaired PPI.

Several lines of evidence suggest that dysregulation of endocannabinoid signaling might be specifically implicated in the behavioral phenotype exhibited by isolated animals

(Malone et al., 2008; Robinson et al., 2010). Thus, in these animals, using autoradiographic techniques, we explored CB1 receptor levels in isolation reared rats and group housed controls but found no changes in CB1 receptor binding sites in all the brain regions considered. These data appear in contrast with the results reported in recent papers that demonstrated a reduction in CB1 immunoreactivity restricted to caudate putamen and amygdala (Malone et al., 2008) or, more recently, an increase of the CB1 receptor mRNA expression (Robinson et al., 2010). The discrepancies between our study and the ones of Malone and Robinson can be due to the different techniques used to evaluate CB1 receptor density (autoradiographic binding assay or immunohistochemistry), the different rat species (Lister Hooded or Sprague-Dawley) and the duration of isolation rearing protocol (5 weeks or 8 weeks), in fact it can be possible that a downregulation in CB1 receptor could appear after longer periods of isolation.

Despite the results concerning the CB1 receptor density, our data show a widely diffused alterations in the CB1 receptor/G protein coupling in isolated reared rats. In particular, it was significantly reduced in the prefrontal cortex (pf ctx) (-35%), caudate putamen (CPu) (-45%), nucleus accumbens (NAc) (-52%), hippocampus (Hippo) (-39%) and in the ventral tegmental area (VTA) (-58%).

The presence of a widespread desensitization of CB1 receptors in specific brain regions of isolated rats suggests the possibility of an enhanced endocannabinoid tone in isolation reared rats. Accordingly, significant differences in the endocannabinoid content were evident in isolated rats compared to group housed controls in all the brain regions analyzed. The most intriguing finding was that these alterations mainly involved the endocannabinoid 2-AG, the levels of which were differentially regulated in isolated rats depending on the brain area considered. Particularly, 2-AG levels were significantly higher in the CPu and Hippo, whereas significant decreases were present in the PFC and NAc following 5 weeks of isolation rearing. No changes in the other endocannabinoid, AEA, or in the AEA-related mediators, OEA and PEA, occurred in any of the brain regions under investigation, except for a significant increase in PEA levels in the PFC.

Previous studies demonstrated that environmental stressors alter the expression of enzymes involved in 2-AG biosynthesis and degradation in brain regions controlling emotion and motor behavior (Suarez et al., 2010; Sutt et al., 2008). Moreover, the presence of alterations in 2-AG content following isolation rearing procedure is further supported by the recent findings of Robinson et al. (2010), demonstrating the presence of

widespread alterations in DAGLα and DAGLβ mRNA as well as MAGL mRNA in isolation reared rats. In line with these data we speculate that the alteration in 2-AG tissue concentrations reported here might be due to alteration in the enzymes involved in 2-AG synthesis or degradation. To date only the work of Sciolino et al. (2010) directly investigated the presence of alterations in the endocannabinoid levels in isolated rats. In this study, increases in 2-AG levels were present in the PFC while no changes were found in the NAc and Hippo. This discrepancy with our present findings could be ascribed to the different isolation rearing protocol as well as to the different rat strain used, since strain differences in the isolation-induced alterations have already been reported for other behavioral parameters (Weiss et al., 2000).

As a whole, these data clearly indicate that post-weaning social isolation procedures in rats induce marked alterations in specific component of the endocannabinoid system, suggesting that abnormal endocannabinoid signaling could represent one of the molecular underpinnings of isolation-induced behavioral deficits. Inasmuch as the endocannabinoid system plays a homeostatic role during brain development, it is tempting to speculate that disrupted endocannabinoid signaling could negatively affect the maturation of other neurotransmitter systems within the central nervous system, leading to abnormal neurotransmission. For this reason, we investigated here also the possible impact of altered endocannabinoid neuromodulatory function on the interface between glutamatergic and dopaminergic function by assessing dopamine D1 and D2 as well as NMDA receptor densities in isolated and group housed rats.

Interestingly, a significant reduction in D1 receptor and a significant increase in D2 receptor density were observed in the PFC of isolated rats compared to group reared controls. An imbalance between D1 and D2 receptors has been suggested to contribute to the symptoms of schizophrenia (Scott and Aperia, 2009). In agreement with our present findings, a PET study showed that schizophrenic patients have reduced dopamine D1 receptor binding in the PFC, which is related to the severity of the negative symptoms (Okubo et al., 1997; Sedvall and Farde, 1996), and, recently, a reduction of D1 receptor density was reported in the PFC of isolated rats (Toua et al., 2010 Moreover, the increase in D2 receptors we observed in the PFC of isolated animals agrees with first PET studies suggesting that dopamine D2 receptors are indeed up-regulated in schizophrenia patients (Wong et al., 1986). Based on the suggestion of a strong interaction between D1 and D2 second messenger systems (Seeman et al., 1989; Strange, 1991), it could be suggested

that the opposite effect of isolation rearing on D1 and D2 receptor densities might reflect a compensatory mechanism. Furthermore, both D1 and D2 receptors are involved in the regulation of PPI in rats (Geyer et al., 2001), with most of the evidence indicating a major contribution of D2 rather than D1 receptors to this behavior. However, a role of D1 receptors in the modulation of PPI has been also highlighted (Ralph-Williams et al., 2003), and thus it is more likely that D1 and D2 receptors work together to modulate PPI in rats. As a whole, these data suggest the presence of altered dopamine transmission in the PFC following isolation rearing procedure.

Intriguingly, changes in NMDA receptor density were also present in rats reared in isolation. Particularly, we found a significant reduction of NMDA receptor binding in the CPu paralleled by a significant increase in the NAc. Functional interactions involving dopamine and NMDA receptors have been documented in several forebrain regions and associated with the modulation of locomotor activity and memory processes (Adriani et al., 1998; Tseng and O'Donnell, 2003). Moreover, interactions at the dopamine-glutamate interface have been implicated in the regulation of sensorimotor gating (Wan and Swerdlow, 1996). Therefore, altered dopamine and glutamate transmission in the forebrain regions might underlie the disrupted behavior we observed in isolated rats.

Although a causal link cannot be established based uniquely on our present data, it is tempting to hypothesize that aberrant endocannabinoid signaling, such as that observed here in isolation reared rats, could negatively impact on normal behavior and neurotransmission in these animals and contribute, through this mechanism, to the behavioral alterations that accompany this condition. In this scenario, pharmacological interventions aimed at restoring normal endocannabinoid transmission could be effective in recovering isolation-induced schizophrenia-like phenotype as well as the related neurochemical alterations.

Effect of AM251 treatment on isolation-induced schizophrenia-like phenotype

Recent findings suggest that CB1 receptor antagonists possess a pharmacological profile reminiscent of atypical antipsychotics (Guidali et al., 2010). In the present work, acute AM251 administration did not reverse isolation-induced cognitive impairment and social withdrawal, suggesting that a single AM251 administration may not be sufficient to reach a beneficial effect on isolation rearing-induced behavioral deficits. The results obtained with chronic AM251 treatment appear more interesting. To the best of our knowledge, this is the first study evaluating the effect of a chronic manipulation of the endocannabinoid

system in isolation reared rats. Rats were chronically administered with AM251 (or vehicle) daily for 3 weeks as previously reported for the atypical antipsychotic clozapine (Li et al., 2007b). It is worth to note that the behavioral alterations observed in rats reared in isolation are particularly influenced by chronic handling (Sciolino et al., 2010) and this might be taken into consideration when performing a chronic pharmacological treatment in socially-isolated rats. In our model, chronic handling did not affect the cognitive impairment induced by isolation whereas the aggressive behaviors were reduced by handling but remained still significantly elevated when compared to group housed controls.

Intriguingly, chronic AM251 alone did not affect the cognitive functions in group housed controls. However, chronic treatment of AM251 to isolation reared rats, significantly improved the performance in the novel object recognition test and reduced the aggressive behaviors in the social interaction test. It is worth to note that this recovery due to chronic AM251 administration on isolation rearing-induced cognitive impairment and aggressive behaviors persisted till 10 days after discontinuing the treatment, indicating a long lasting effect of the cannabinoid antagonist on psychotic-like symptoms. This is an intriguing property of AM251 since, following treatment with antipsychotic drugs, it has been demonstrated a relapse of psychotic symptoms when taken off the drug (Li et al., 2007a) and, moreover, antipsychotics are have been associated with untoward effects upon withdrawal (Lee and Robertson, 1997).

Intriguingly, AM251 also completely counteracted PPI deficits in isolation reared rats. Since both typical or atypical antipsychotic treatments are effective in normalizing PPI responses in schizophrenic patients as well as in isolated rats (Kumari and Sharma, 2002; Powell and Geyer, 2002), the beneficial effect of AM251 is consistent with a potential antipsychotic-like profile of this compound. However, the mechanisms underlying this antipsychotic effect of AM251 are still unclear

Effect of AM251 treatment on isolation-induced alterations of the endocannabinoid system, and dopamine/glutamate receptor densities

Here we demonstrated that psychotic symptoms in isolation reared rats are accompanied by alterations in the endocannabinoid system, thus we first investigated a direct effect of AM251 treatment on CB1 receptor functionality and endocannabinoid content.

Chronic AM251 had no effects on CB1 receptor density both in grouped and isolated rats. The alterations in CB1 receptor/G protein coupling reported in isolated rats in the pf ctx, NAc, hHippo and VTA were still evident after 3 weeks of chronic vehicle chronic treatment,

except for the reduction in the CPu that seemed to have been counteracted by chronic handling. Interestingly, chronic AM251 completely restored CB1 receptor functionality in the pf ctx, NAc, hHippo and VTA in isolated rats without having per se any effect in all the brain areas analyzed.

As far as endocannabinoid levels is concerned, there are some isolation-induced changes in endocannabinoids that seem to be transient and can be therefore reversed by even a mild environmental manipulation, such as, as shown here, daily handling. In fact, a handling effect was clearly evident in the CPu of isolated rats, where chronic handling completely counteracted the isolation-induced increase in 2-AG content. Since in this same brain area, handling counteracted also the isolation-induced desensitization of CB1 receptors, this latter effect might be ascribed to the normalization of 2-AG levels in this brain area. Consistent with a role of the CPu in the development of aggressiveness, the above mentioned handling effects might also have contributed to the partial recovery of aggressive behaviors observed in isolated rats following vehicle administration.

On the other hand, some of the other isolation-induced changes observed here were less sensitive to chronic handling and might therefore play a more relevant role in the development of the psychotic-like phenotype. Indeed, the reductions in 2-AG levels in the PFC and NAc, as well as the increase of 2-AG in the Hippo, were still evident in isolated rats undergoing 3 weeks of vehicle treatment. More importantly, these persistent alterations in 2-AG contents were completely counteracted by chronic AM251 administration in all the corresponding brain regions. Therefore, given the observation that AM251 concomitantly reversed also the behavioral and schizophrenia-like consequences of isolation, it seems reasonable to suggest that these specific long-lasting changes in endocannabinoid signaling may account for the behavioral recovery observed in isolation reared rats, although further studies are needed to clarify the underlying mechanisms.

Intriguingly, AM251 administration also partially counteracted the isolation-induced changes in dopamine and glutamate receptors, possibly through the rescue of normal endocannabinoid system functionality. It is possible that only persistent alterations in endocannabinoid levels induced by isolation can affect to some extent dopamine and glutamate receptor expression. However, AM251 restored D2 receptor binding in the PFC of isolated rats but failed to normalize D1 receptor density in the same brain area. While much attention has been focused on striatal D2 receptors in schizophrenia, recent evidence has implicated cortical D2 receptors as the important sites of action of

antipsychotic drugs and changes in the dopamine receptors in the cerebral cortex need to be taken into account when evaluating the regulatory actions of neuroleptics (Lidow et al., 1998). Therefore, the observed recovery at D2 receptor in the PFC following AM251 treatment could be one of the molecular underpinnings of the antipsychotic-like profile of this compound. Furthermore, a role for the glutamatergic system in the behavioral recovery due to AM251 administration could not be ruled out, since AM251 treatment completely rescued NMDA receptor binding in the CPu and NAc of isolated animals. In conclusion, the present results demonstrate that chronic AM251 administration is effective in reversing schizophrenia-like signs in isolation reared rats, thus providing further evidence of an antipsychotic potential for antagonism at CB1 receptors. Imbalances in the endocannabinoid system, specifically CB1 receptor and 2-AG levels, could represent one of the molecular abnormalities related to the disrupted behavior observed in isolated rats and their normalization may account for AM251-induced recovery of psychotic-like symptoms in this animal model. Furthermore, the present findings indicate the occurrence, following isolation rearing procedure, of dopamine and glutamate disturbances in brain regions relevant to schizophrenia-like behaviors. The ability of AM251 to partially normalize these disturbances may also participate in its antipsychotic action.

CONCLUSIONS

A unifying theory of endocannabinoid system and schizophrenia

The body of research described in this thesis has highlighted that maladaptations of the endocannabinoid system either induced by a direct manipulation of the system (through adolescent THC exposure, or triggered by a neurodevelopmental insult (such as post-weaning social isolation) are associated with the development of psychotic-like symptoms in rats.

We speculate that exogenous cannabinoids or other insults during sensitive periods of brain development, such as adolescence, may disrupt the neuromodulatory role exerted by the endocannabinoid system on neurotransmission (particularly GABA) ultimately resulting in a major vulnerability to develop psychiatric disorders. Accordingly, in the present thesis the psychotic-like phenotype present in adult animals that underwent adolescent THC exposure was associated with marked changes in the GABAergic system within the PFC.

If cannabinoid dysregulation is considered as one of the potential causes of schizophrenia symptomology or a setting event for the disorder, then it may be possible to design therapeutic interventions to address this issue by using the wide range of ligands that directly or indirectly act on CB1 receptors and on the enzymes that synthesize or inactivate the endocannabinoids.

In line with this, we here demonstrated that CB1 receptor blockade through chronic AM251 treatment is effective in recovering the schizophrenia-like symptoms associated with post-weaning social isolation in rats.

Intriguingly, rimonabant, a CB1 receptor antagonist/inverse agonist, was tested with promising results in a sixteen-week, double-blind, placebo-controlled study in people with schizophrenia or schizoaffective disorder before being removed from the market because of its severe adverse effects (Kelly et al. 2011). However, a major limitation of this study is the small sample size, due to its premature termination (rimonabant marketing was suspended in 2008), and consequent limited power to detect efficacy or rare adverse events. Thus, additional controlled trials are required to confirm the possible therapeutic exploitation and determine the safety of cannabinoid compounds. More consistent findings have been obtained using the non-psychoactive cannabinoid, cannabidiol. In fact, its administration was effective in reverting the positive and negative symptoms of schizophrenia both in animal studies and clinical trials and its antipsychotic action may be

ascribed to its ability to increase serum levels of AEA by inhibiting the enzymatic degradation of this endocannabinoid (Leweke et al. 2012).

Of course, additional controlled trials are still required to confirm the possible therapeutic exploitation and determine the safety of cannabinoid compounds.

FIGURES

Figure 1

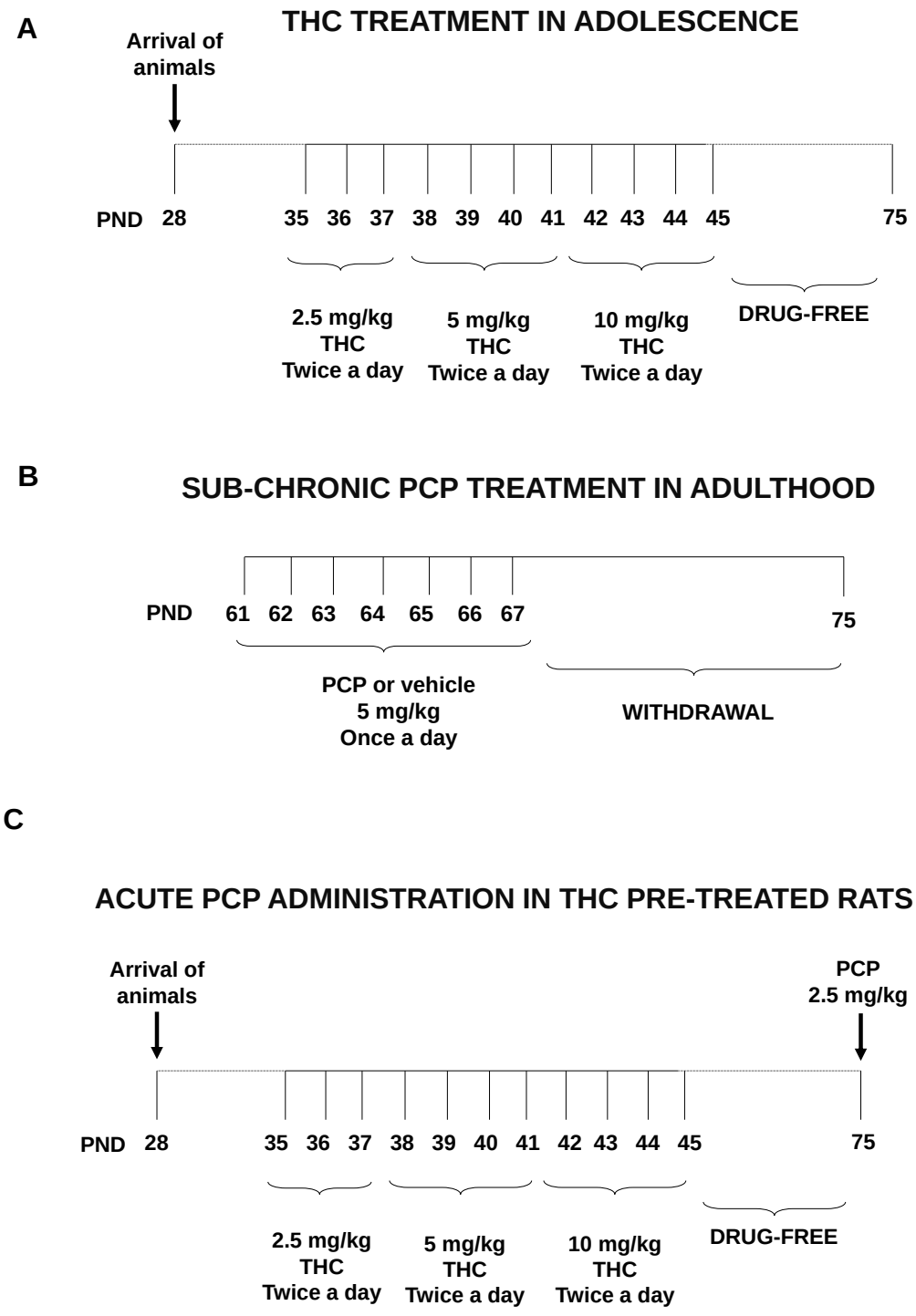

Figure 1: Adolescent THC treatment schedule (**A**) and sub-chronic PCP protocol (**B**). (**C**) Protocol of acute PCP injection in adult rats that underwent adolescent THC administration.

Figure 2

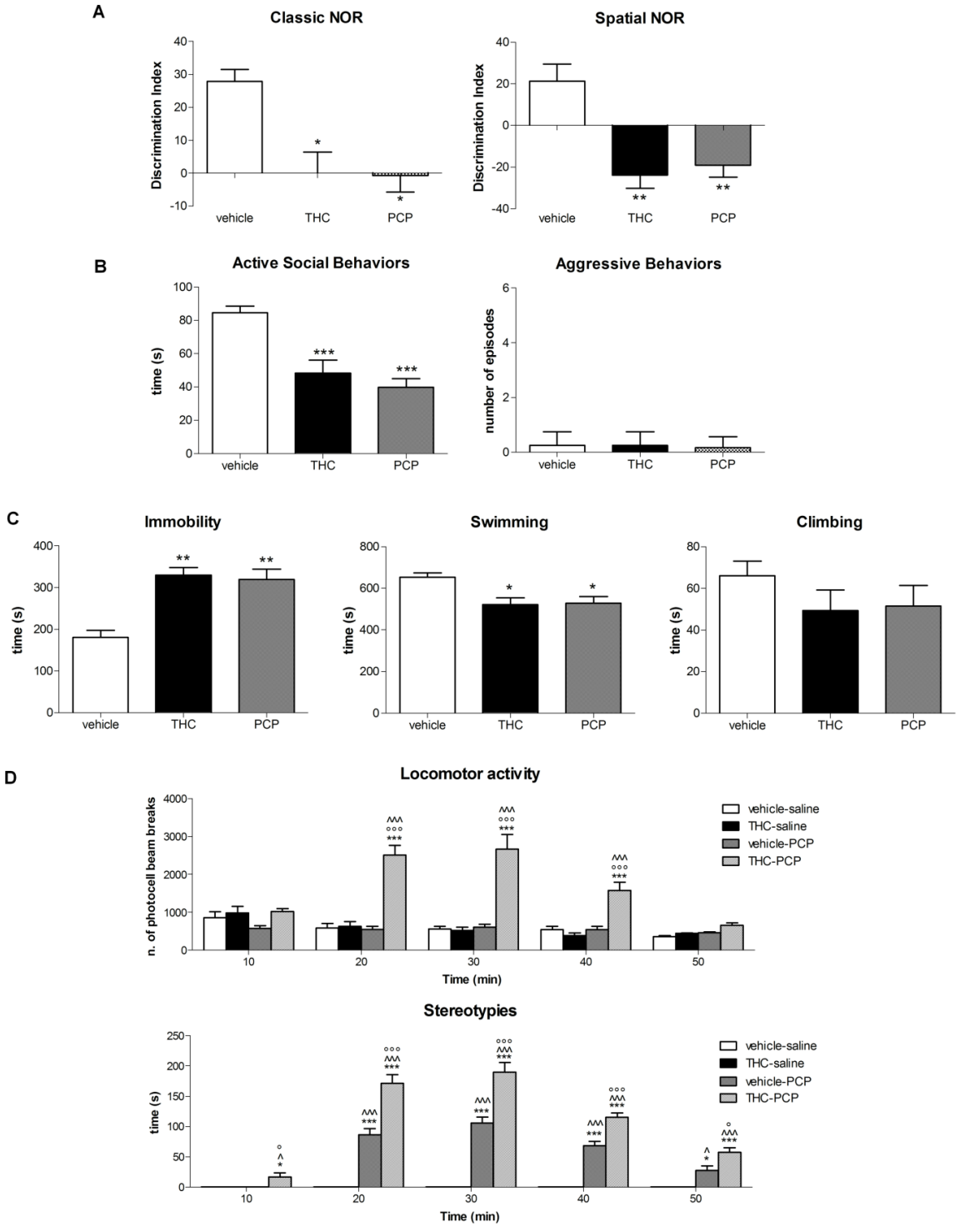

Figure 2: Comparison between the behavioral alterations induced by adolescent THC exposure and sub-chronic PCP treatment in adult rats in the NOR test (A), social interaction test (B) and FST (C). Data are expressed as mean ± S.E.M. of six animals per group. ***p<0.001; **p<0.01; *p<0.05 vs vehicle (Bonferroni's post hoc test). (D) Locomotor activity and stereotyped behaviors in response to acute PCP administration in THC-treated rats. Data are expressed as mean ± S.E.M. of six animals per group. ***p<0.001; *p<0.05 vs vehicle-saline; °°°p<0.001; °p<0.05 vs vehicle-PCP; ^^^p<0.001; ^p<0.05 vs THC-saline (Bonferroni's post hoc test).

Figure 3

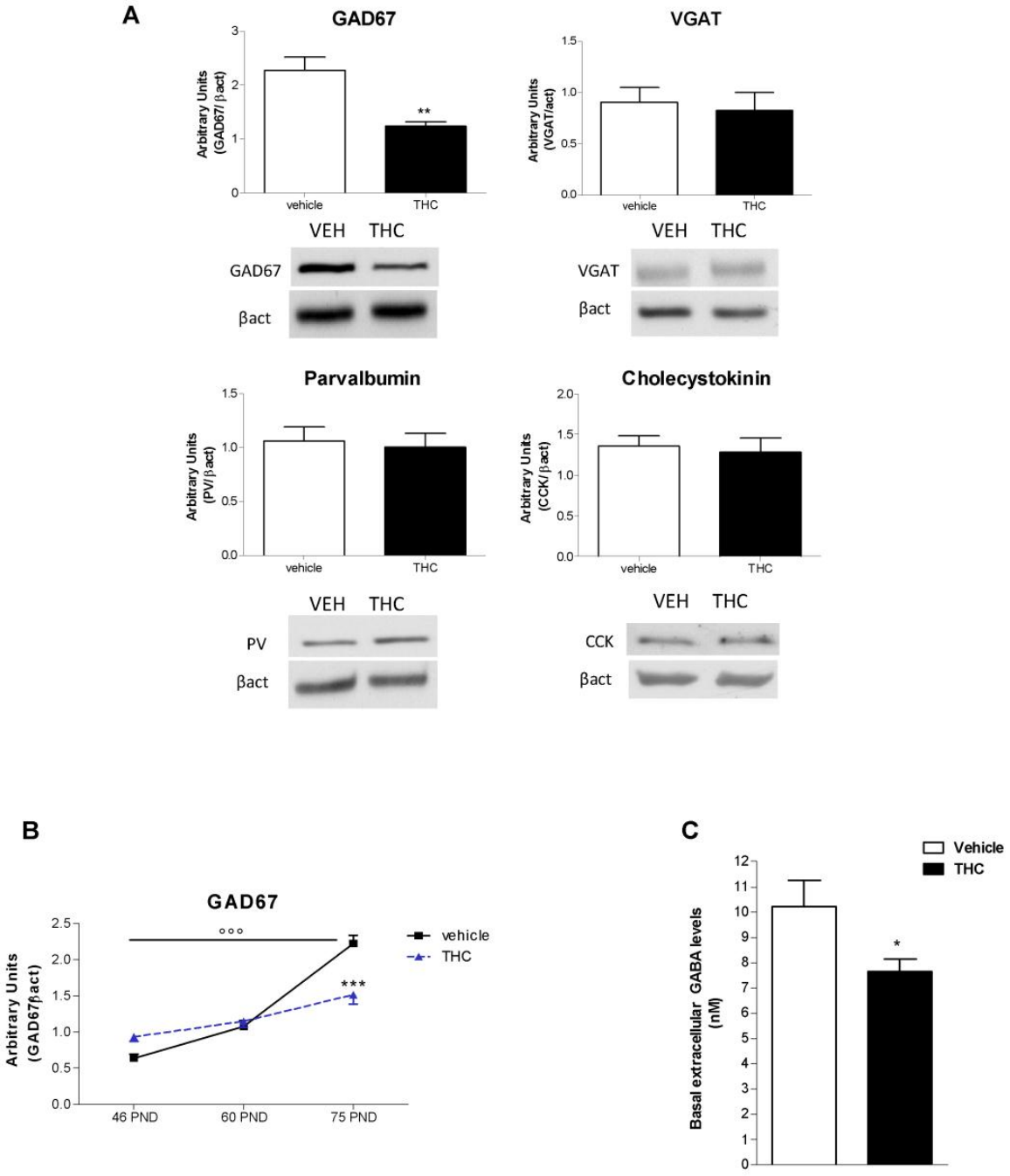

Figure 3: (**A**) GAD67, parvalbumin (PV), VGAT and cholecystokinin (CCK) levels in the PFC of adult animals exposed to THC during adolescence. Data are expressed as mean ± S.E.M. of four animals per group. **p<0.01 vs vehicle (unpaired Student's t test). (**B**) Characterization of the kinetics of GAD67 levels from mid adolescence to adulthood in the PFC of THC-exposed animals. Data are expressed as mean ± S.E.M. of four animals per group at each time point. ***p<0.001 vs vehicle at PND 75; °°°p<0.001 vs PND 46 (unpaired Student's t test). (**C**) Effects of adolescent THC exposure on basal extracellular GABA levels in the prefrontal cortex of the awake rat. Each value represents the mean ± S.E.M. (n=8). Statistical analysis was performed using Student's t-test; *P<0.05 significantly different from the vehicle-treated group.

Figure 4

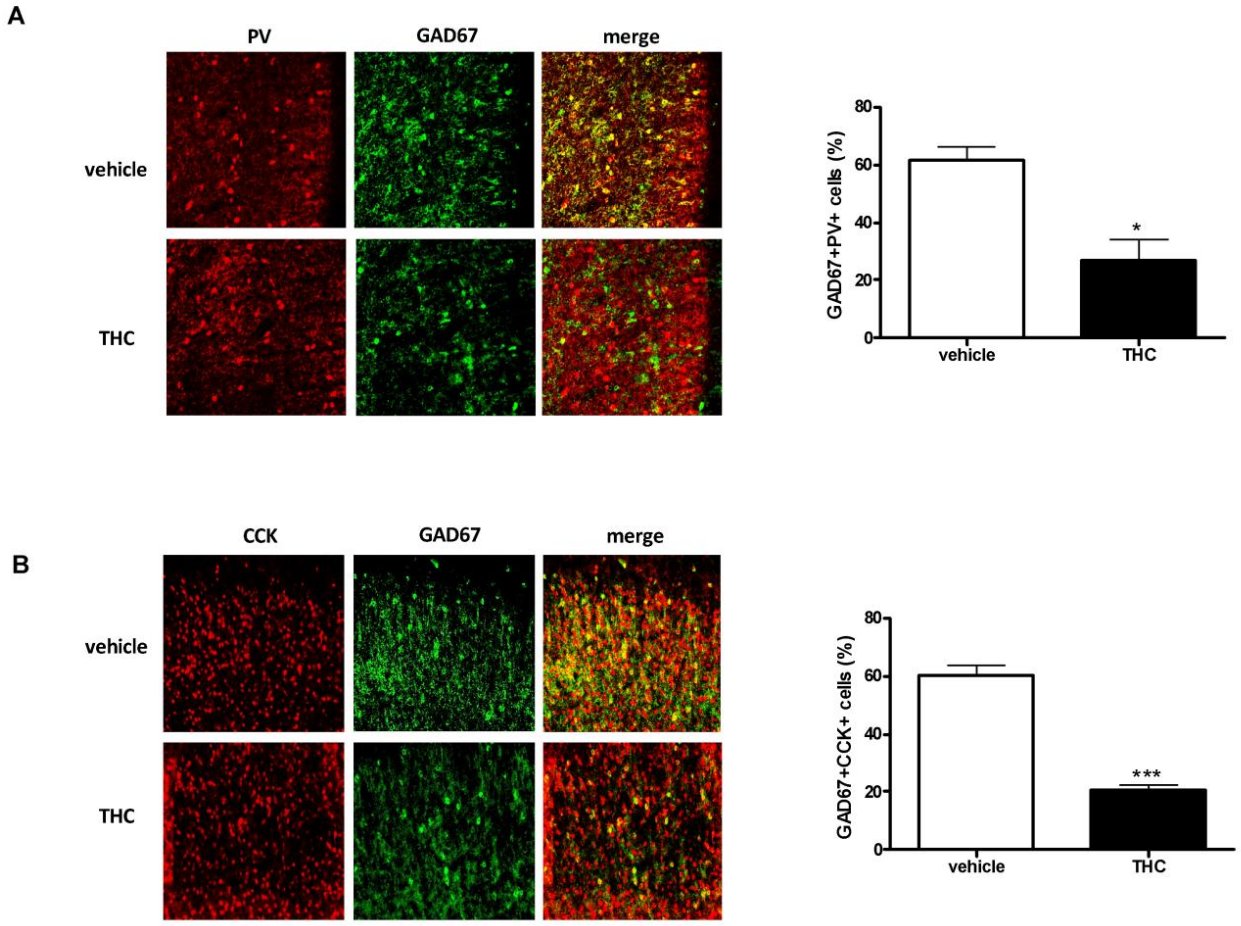

Figure 4: Representative dual-label immunofluorescence images for GAD67 and PV (**A**) and for GAD67 and CCK (**B**) in the PFC of THC-exposed animals. Quantification was determined as the percentage of GAD67 and PV or CCK double positive cells among the total number of PV or CCK positive cells. Data are expressed as mean ± S.E.M. of four animals per group. ***p<0.001; *p<0.05 vs vehicle (unpaired Student's t test).

Figure 5

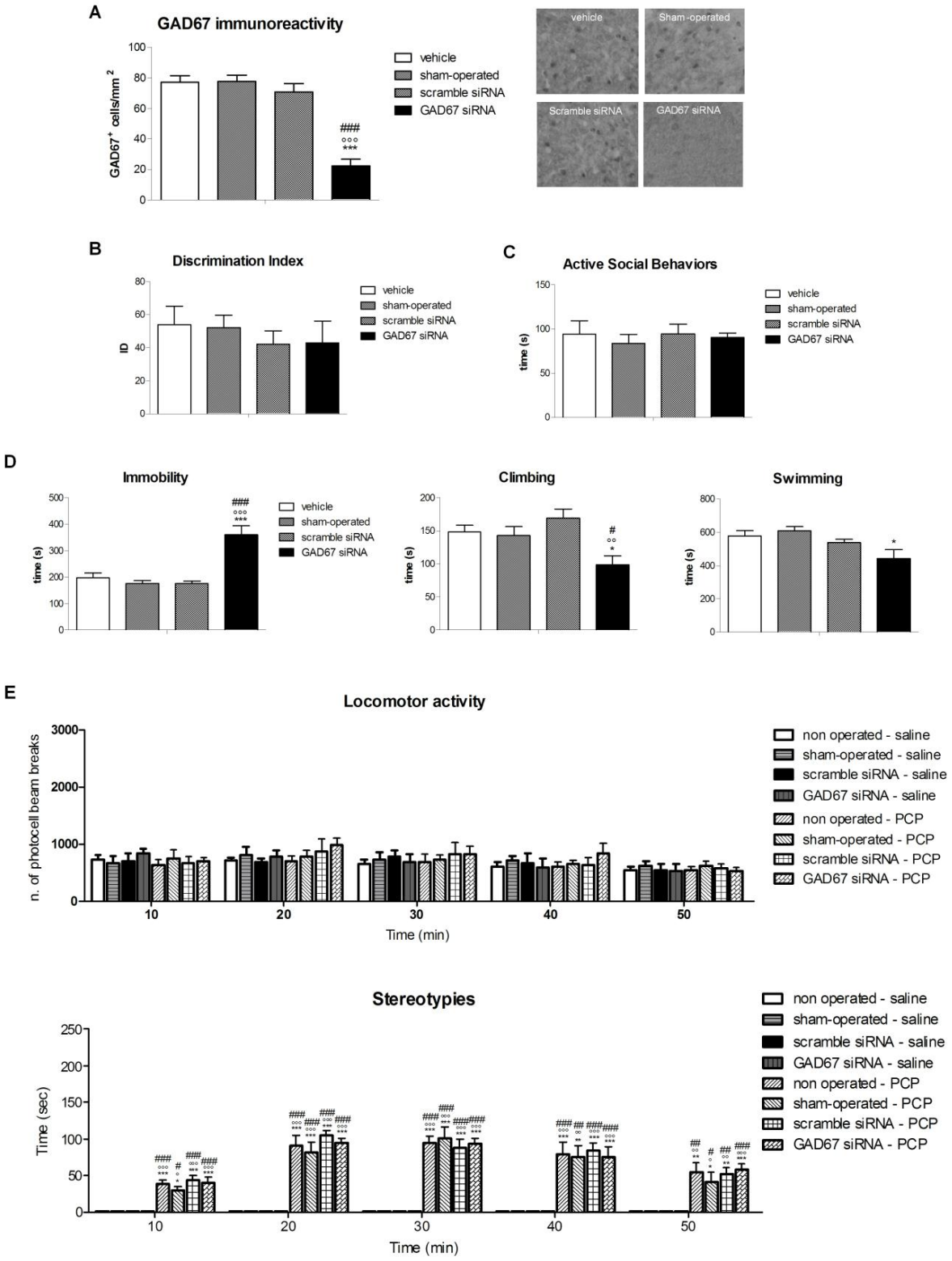

Figure 5: (**A**) GAD67 immunoreactivity in the PFC 72 hours after bilateral injection of GAD67 siRNA. Behavioral responses in the NOR test (**B**), social interaction test (**C**), FST (**D**) and effect of acute PCP administration (2.5 mg/kg) (**E**) 72 hours after bilateral injection of GAD67 siRNA. Data are expressed as mean ± S.E.M. of five animals per group. ***p<0.001; **p<0.01; *p<0.05 vs non-operated animals; °°°p<0.001; °°p<0.01; °p<0.05 vs sham-operated animals; ###p<0.001; ##p<0.01; #p<0.05 vs scramble siRNA (Bonferroni's post hoc test).

Figure 6

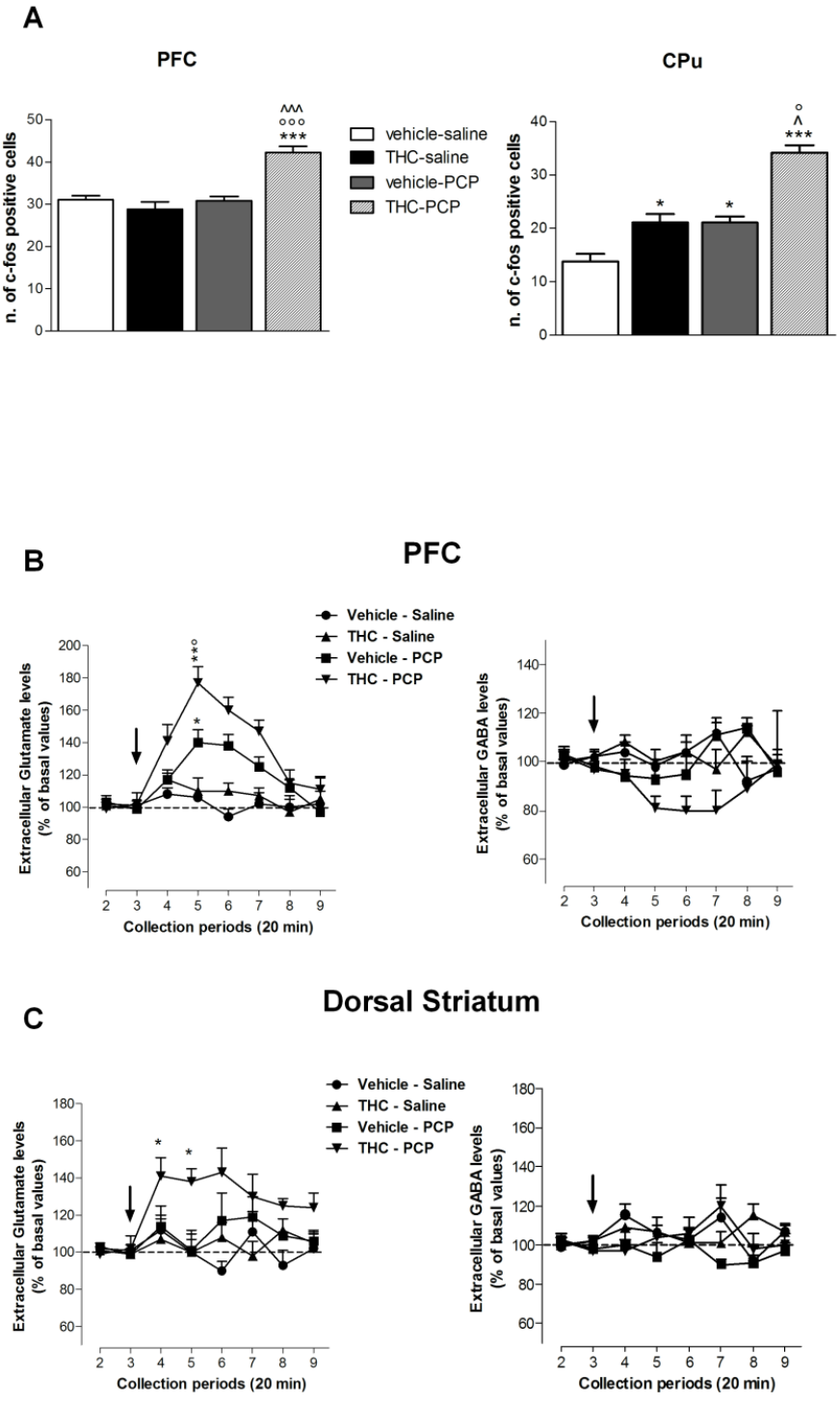

Figure 6: (**A**) c-Fos immunoreactivity in response to acute PCP administration in THC-treated rats in the Prefrontal Cortex (PFC) and Caudate Putamen (CPu). Data are expressed as mean ± S.E.M. of four animals per group. ***p<0.001; *p<0.05 vs vehicle-saline; °°°p<0.001; °p<0.05 vs vehicle-PCP; ^^^p<0.001; ^^p<0.01; ^p<0.05 vs THC-saline (Bonferroni's post hoc test). (B and C): Effects of an acute phencyclidine (PCP) injection (2.5 mg/kg, i.p.) on prefrontal cortex (**B**) and dorsal striatum (**C**) extracellular glutamate (left panel) and GABA (right panel) levels in rats treated during adolescence with vehicle or THC. The vertical arrows indicate the time of the injection of PCP. The results are expressed as percentage of the mean of the three basal values before PCP injection. Each point represents the mean ± S.E.M. of six animals. Left panel: *P<0.05, ** P<0.01 significantly different from vehicle groups; °P<0.05 significantly different from vehicle – PCP group according to ANOVA followed by the Newman-Keuls test for multiple comparisons.

Figure 7

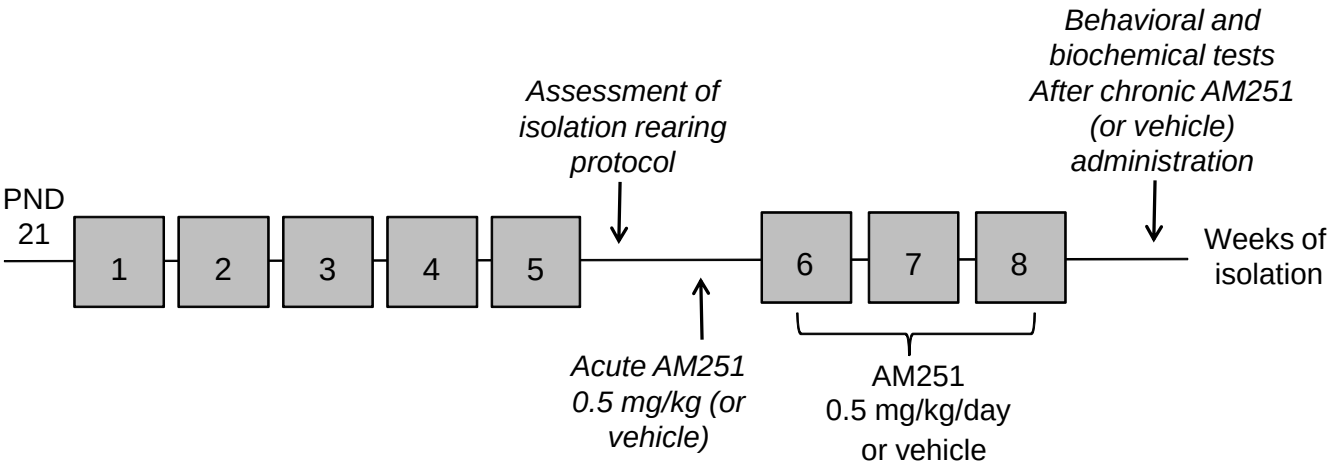

Figure 7: Treatment schedule

Figure 8

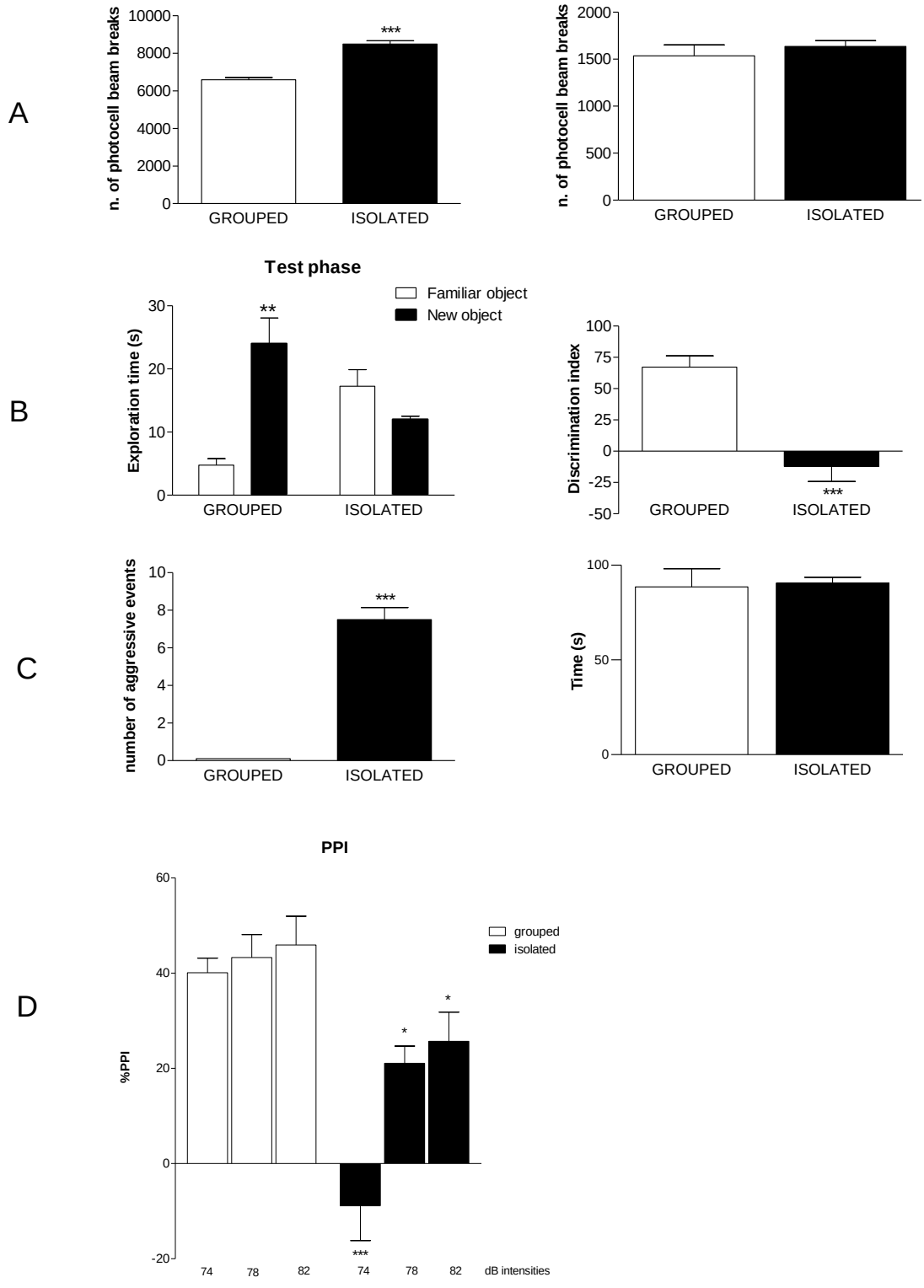

Figure 8: Behavioral phenotype after 5 weeks of isolation rearing. **A**: Horizontal (left) and vertical (right) activity assessed in the activity cage. **B**: Exploration time of the familiar versus novel object and the discrimination index during the test phase in the novel object recognition (NOR) test. **C**: number of aggressive behaviors (left) and time spent in social behaviors (right) during the social interaction test. Results are expressed as mean± S.E.M. of six animals per group. **D**: Percent prepulse inhibition (%PPI) after 5 weeks of isolation rearing. Data are expressed as the average PPI response of 10 animals per group over the 3 prepulse intensities. ***p<0.001; *p<0.05 vs grouped at the respective dB intensity (Unpaired Student's t-test)

Figure 9

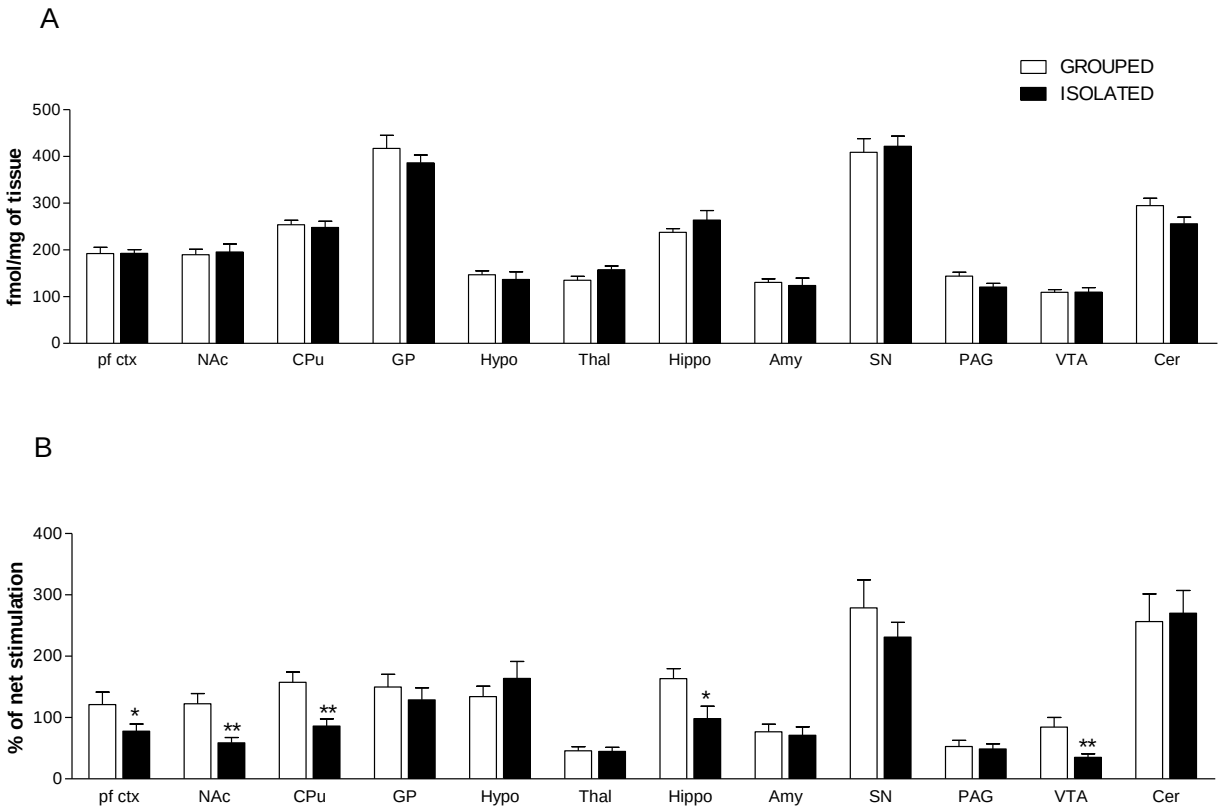

Figure 9: Effect of 5 weeks of isolation rearing on CB1 receptor density and functionality. A: [^{3}H]CP-55,940 receptor autoradiographic binding. B: CP-55,940-stimulated [^{35}S]GTPγS binding in autoradiography. Prefrontal cortex (pf ctx), nucleus accumbens (NAc), caudate putamen (CPu), globus pallidus (GP), hypothalamus (hypo), thalamus (thal), hippocampus (hippo), amygdale (amy), substantia nigra (SN), periaqueductal grey (PAG), ventral tegmental area (VTA), cerebellum (cer). Results are expressed as mean± S.E.M. of four animals per group. *p<0.05, **p<0.01 vs GROUPED (unpaired Student's t-test)

Figure 10

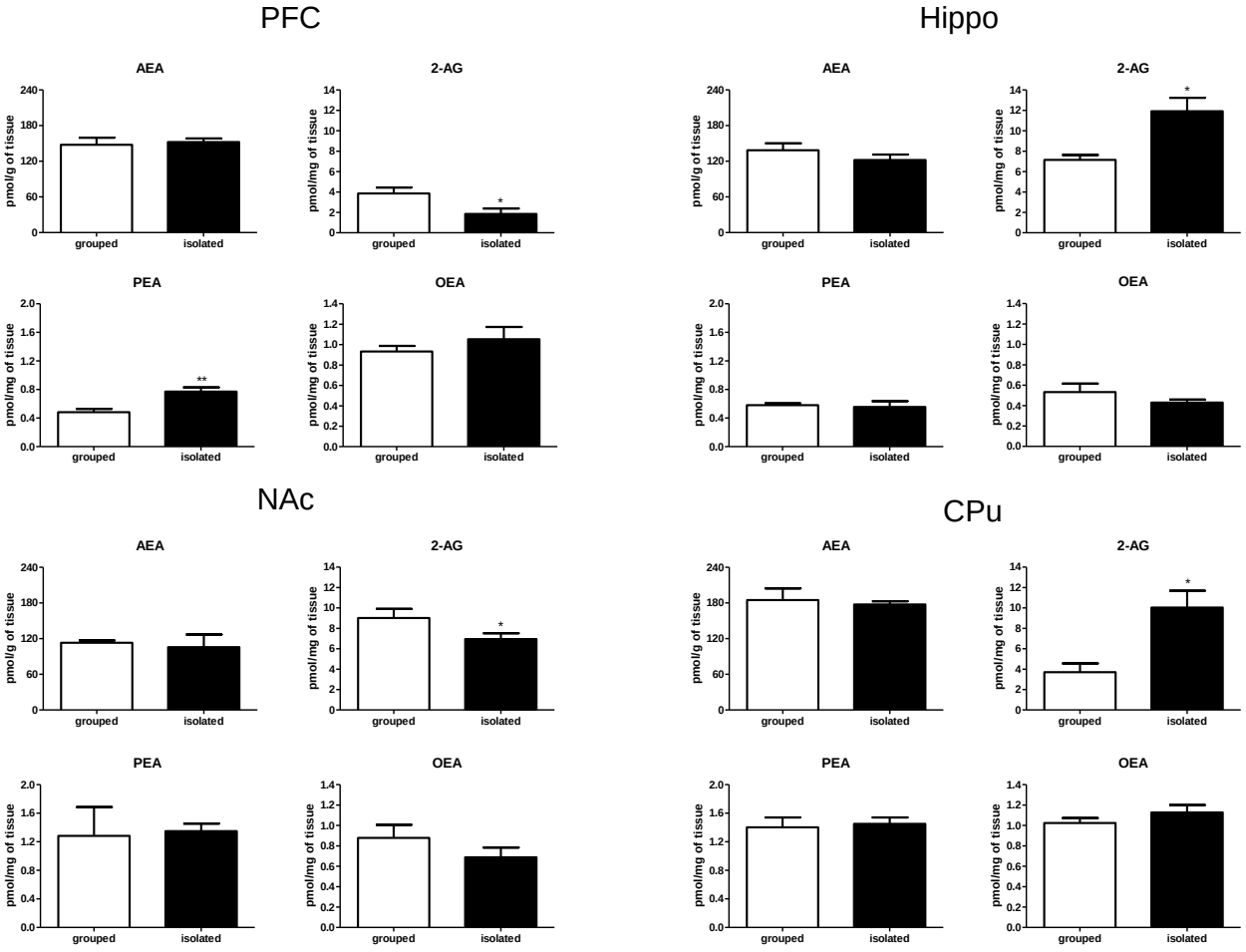

Figure 10: Brain tissue concentrations of endocannabinoids (AEA, 2-AG) and AEA-like mediators (PEA, OEA) after 5 weeks of isolation rearing. Brain areas: Prefrontal cortex (PFC); Caudate putamen (CPu); Nucleus accumbens (NAc); Hippocampus (Hippo). Data are expressed as the mean ± S.E.M. of four animals per group. *p<0.05 vs grouped (Unpaired Student's t-test).

Figure 11

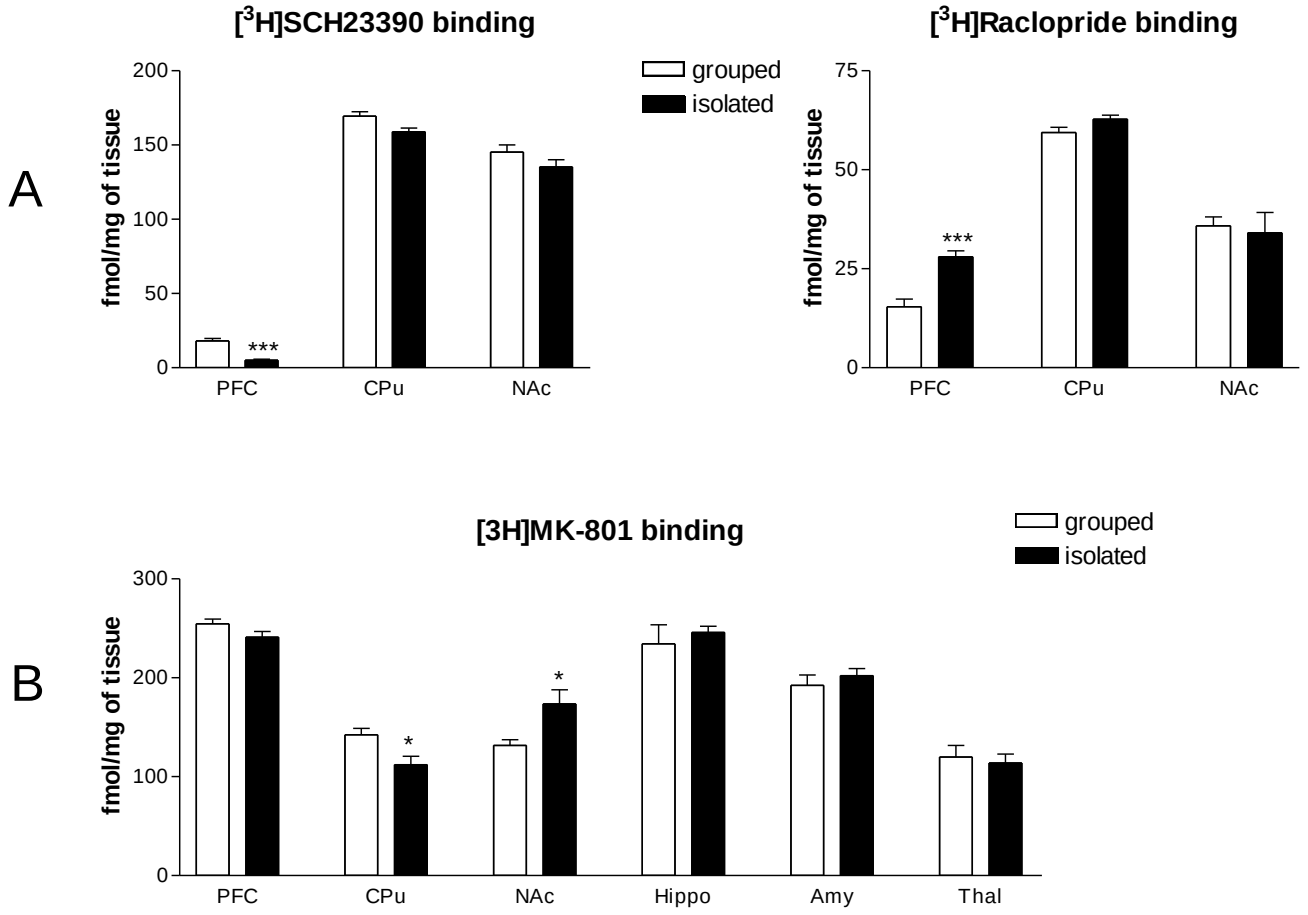

Figure 11: Effect of 5 weeks of isolation rearing on (**A**) D1 and D2 receptor density and (**B**) NMDA receptor density. D1 and D2 receptor densities were assessed through [^{3}H]SCH23390 and [^{3}H]Raclopride receptor binding, respectively. NMDA receptor density was assessed through [^{3}H]MK801 receptor binding. Results are expressed as fmol/mg of tissue. Brain areas: Prefrontal cortex (PFC); Caudate putamen (CPu); Nucleus accumbens (NAc); Hippocampus (Hippo); Amygdala (Amy); Thalamus (Thal). Data are expressed as mean ± SEM of four animals per group. ***p<0.001; *p<0.05 vs grouped (Unpaired Student's t-test).

Figure 12

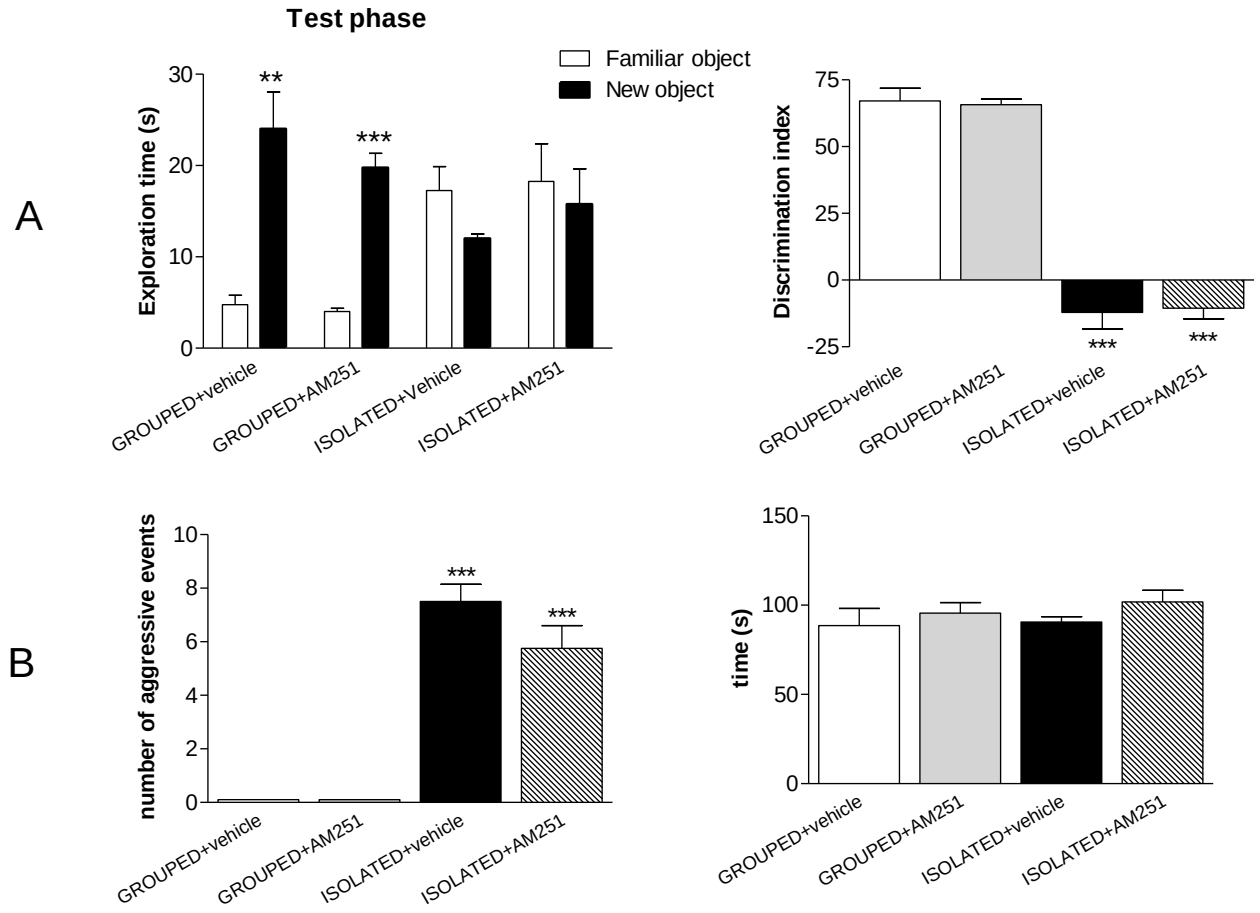

Figure 12: **A**: Effect of acute AM251 treatment (0.5 mg/kg) on isolation-induced cognitive deficit in the novel object recognition (NOR) test. The test was performed 80 minutes after AM251 administration. Represents the exploration time of the familiar versus novel object and the discrimination index during the test phase. **B**: Effect of acute AM251 treatment (0.5 mg/kg) in the social interaction test. The test was performed 80 minutes after AM251 administration. (Left) Represents the number of aggressive behaviors during the test session. (Right) Represents the time spent in active social behaviors. Data are expressed as mean± S.E.M. of five animals per group. **p<0.01, ***p<0.001 vs familiar object (unpaired Student's t-test); ***p<0.001 vs grouped+vehicle (Bonferroni's post-hoc test).

Figure 13

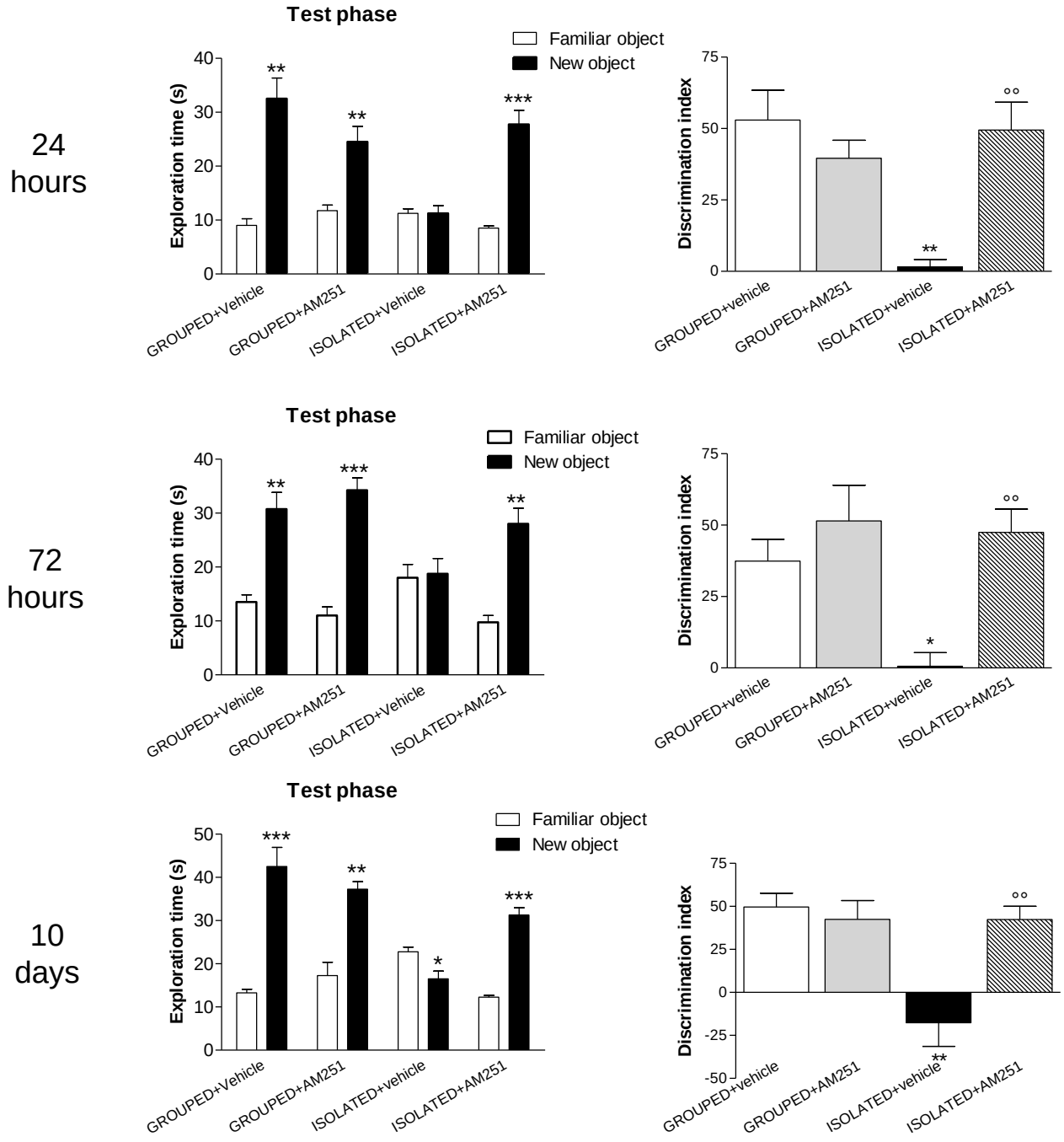

Figure 13: Effect of chronic AM251 treatment (0.5 mg/kg) on isolation-induced cognitive deficit in the novel object recognition (NOR) performed 24 hours, 72 hours and 10 days after the last AM251 administration. Represents the exploration time of the familiar versus novel object and the discrimination index during the test phase. Data are expressed as mean± S.E.M. of five animals per group. **p<0.01, ***p<0.001 vs familiar object (unpaired Student's t-test); **p<0.01,*p<0.05 vs grouped+vehicle; °°p<0.01 vs isolated+vehicle (Bonferroni's post-hoc test).

Figure 14

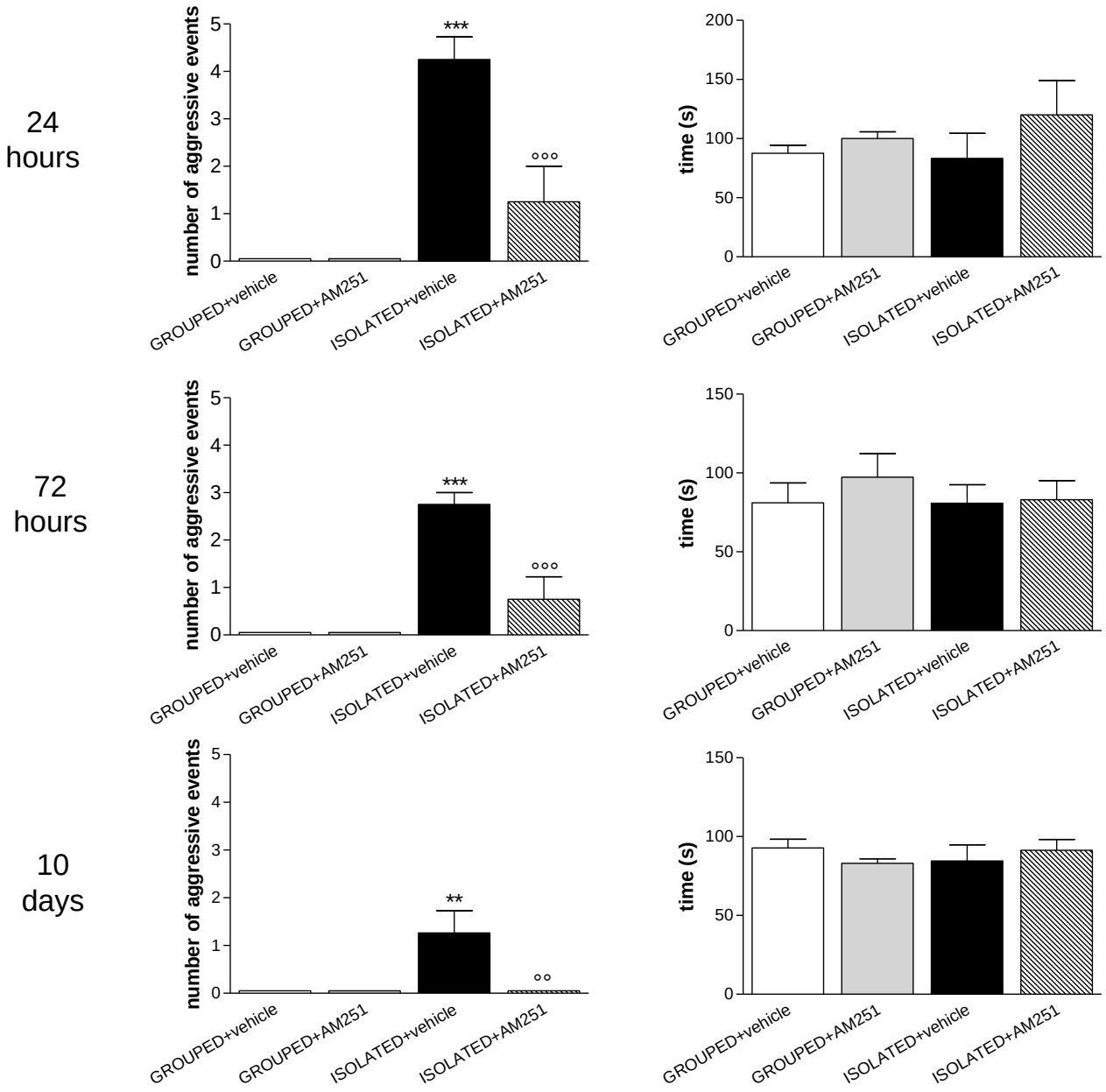

Figure 14: Effect of chronic AM251 treatment (0.5 mg/kg) during the social interaction test performed 24 hours, 72 hours and 10 days after the last AM251 administration. (Left): Number of aggressive events (Right) Time spent in active social behavior during the test session. Results are expressed as mean± S.E.M. of five animals per group. **p<0.01,***p<0.001 vs grouped+vehicle °°p<0.01,°°°p<0.01 vs isolated+vehicle (Bonferroni's post-hoc test).

Figure 15

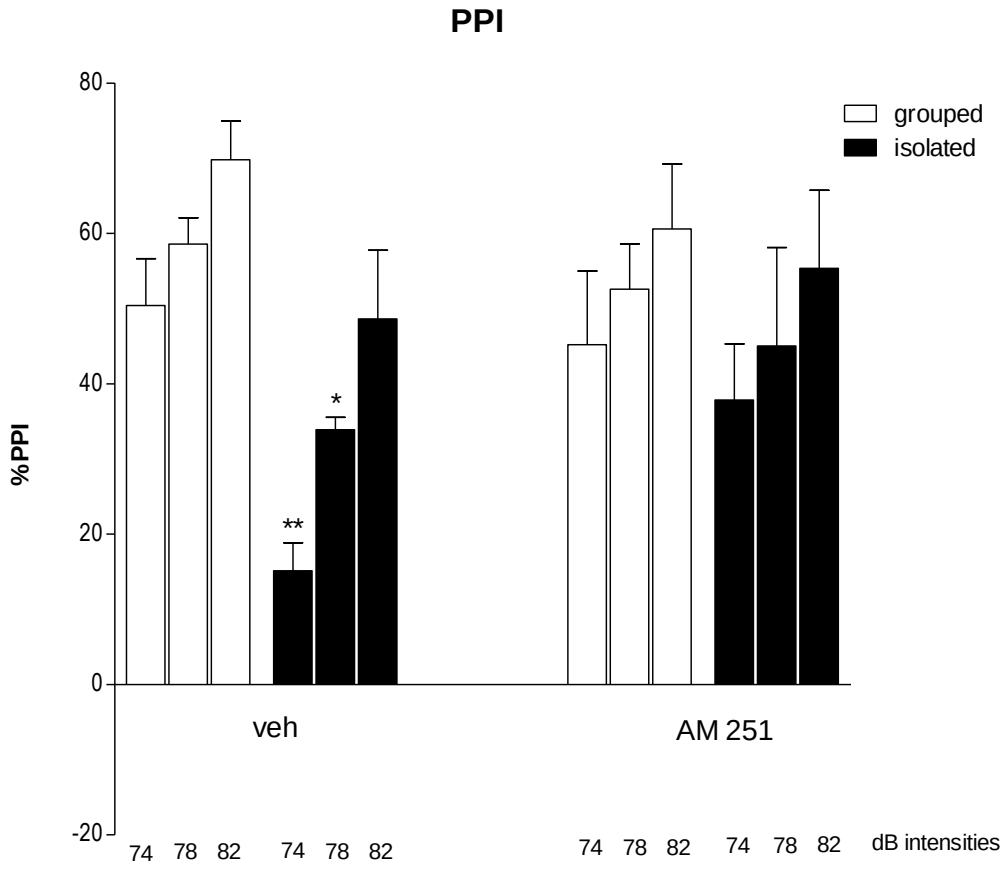

Figure 15: Percent prepulse inhibition (%PPI) after chronic AM251 (or vehicle) treatment in isolated and group housed rats. Data are expressed as the average PPI response of five animals per group over the 3 prepulse intensities. **p<0.01; *p<0.05 vs grouped at the respective dB intensity (Bonferroni's post-hoc test).

Figure 16

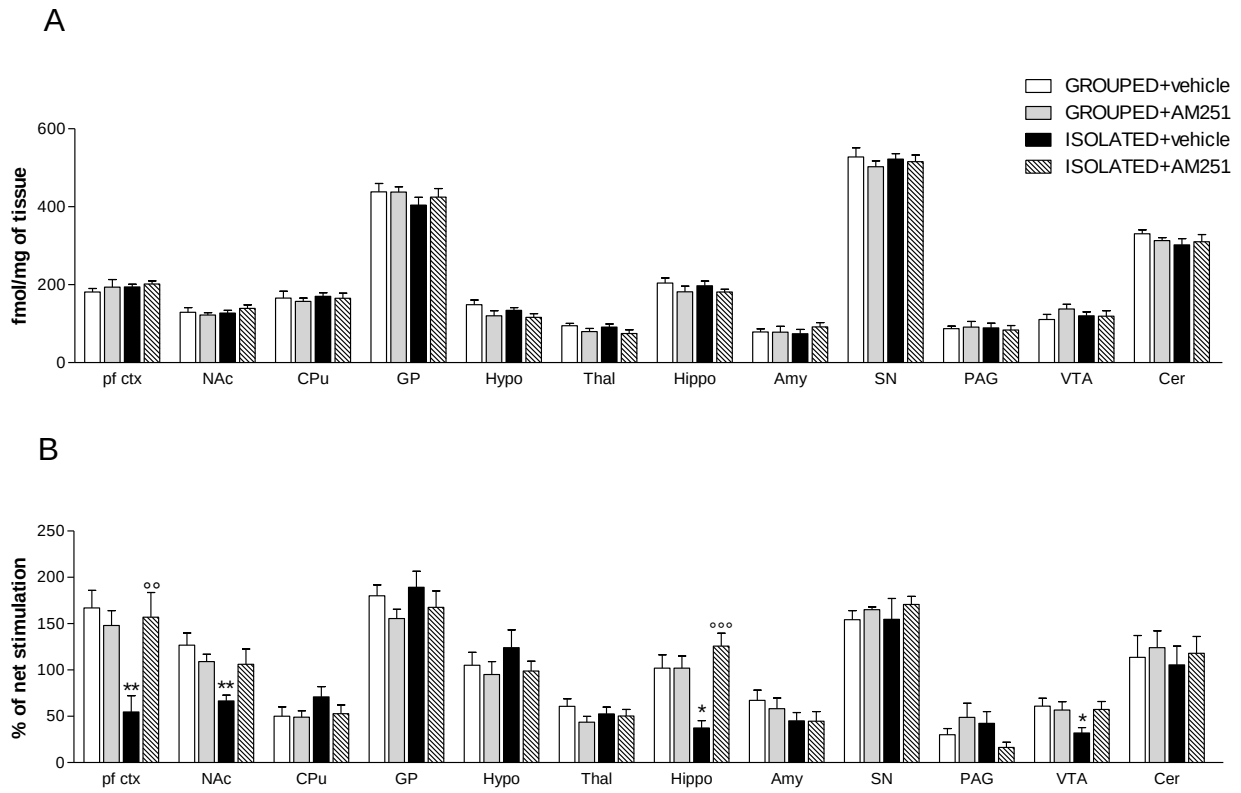

Figure 16: Effect of chronic AM251 on CB1 receptor density and functionality. **A**: [^{3}H]CP-55,940 receptor autoradiographic binding. **B**: CP-55,940-stimulated [^{35}S]GTPγS binding in autoradiography. Results are expressed as mean± S.E.M. of four animals per group. *p<0.05, **p<0.01 vs GROUPED+vehicle; °p<0.01, °°°p<0.001 vs ISOLATED+vehicle (Bonferroni's post-hoc test)

Figure 17

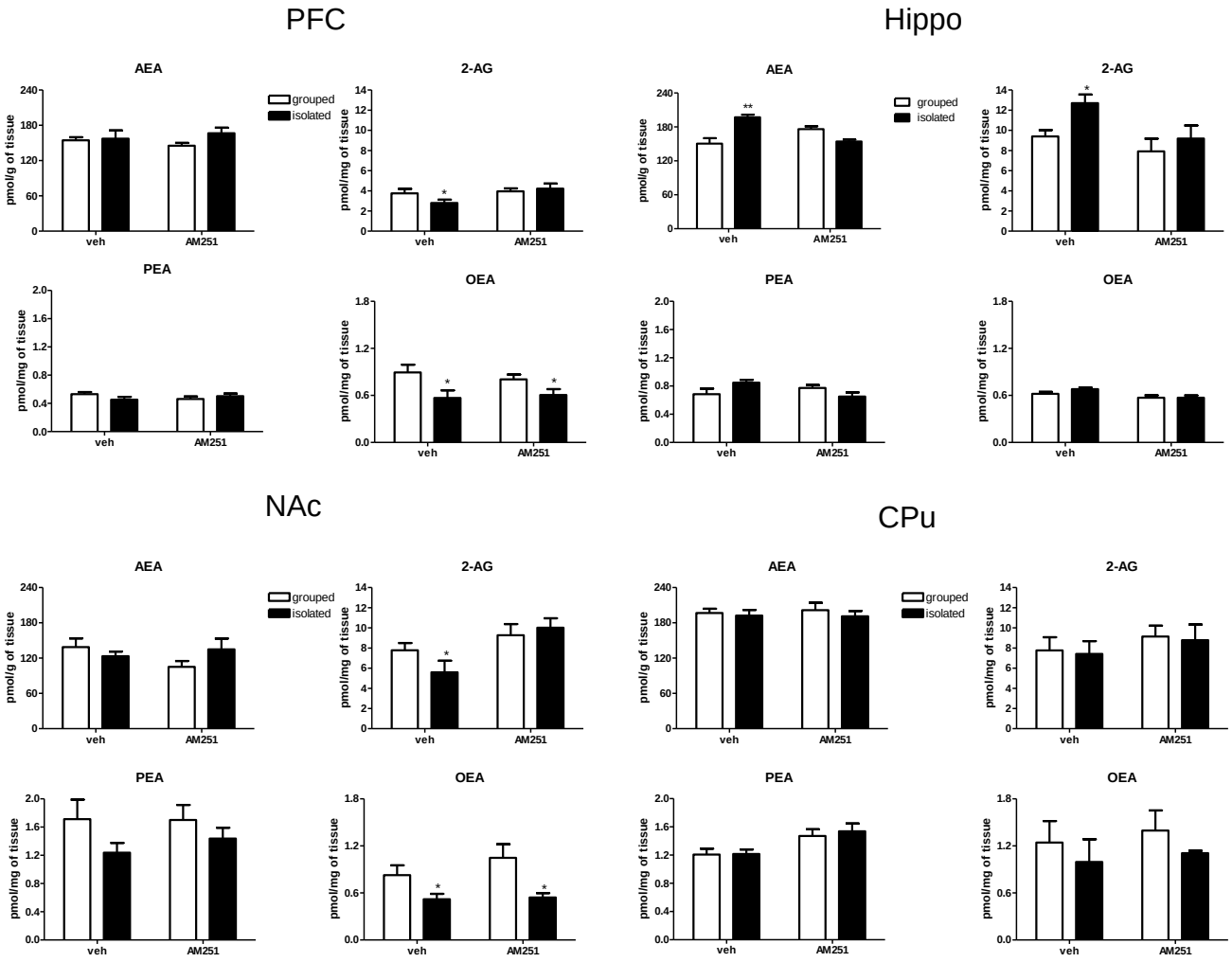

Figure 17: Brain tissue concentrations of endocannabinoids (AEA, 2-AG) and AEA-like mediators (PEA, OEA) after chronic AM251 or vehicle treatment in isolated and group housed rats. Brain areas: Prefrontal cortex (PFC); Nucleus accumbens (NAc); Caudate putamen (CPu); Hippocampus (Hippo). Data are expressed as the mean ± S.E.M. of four animals per group. *p<0.05 vs grouped (Bonferroni's post-hoc test).

Figure 18

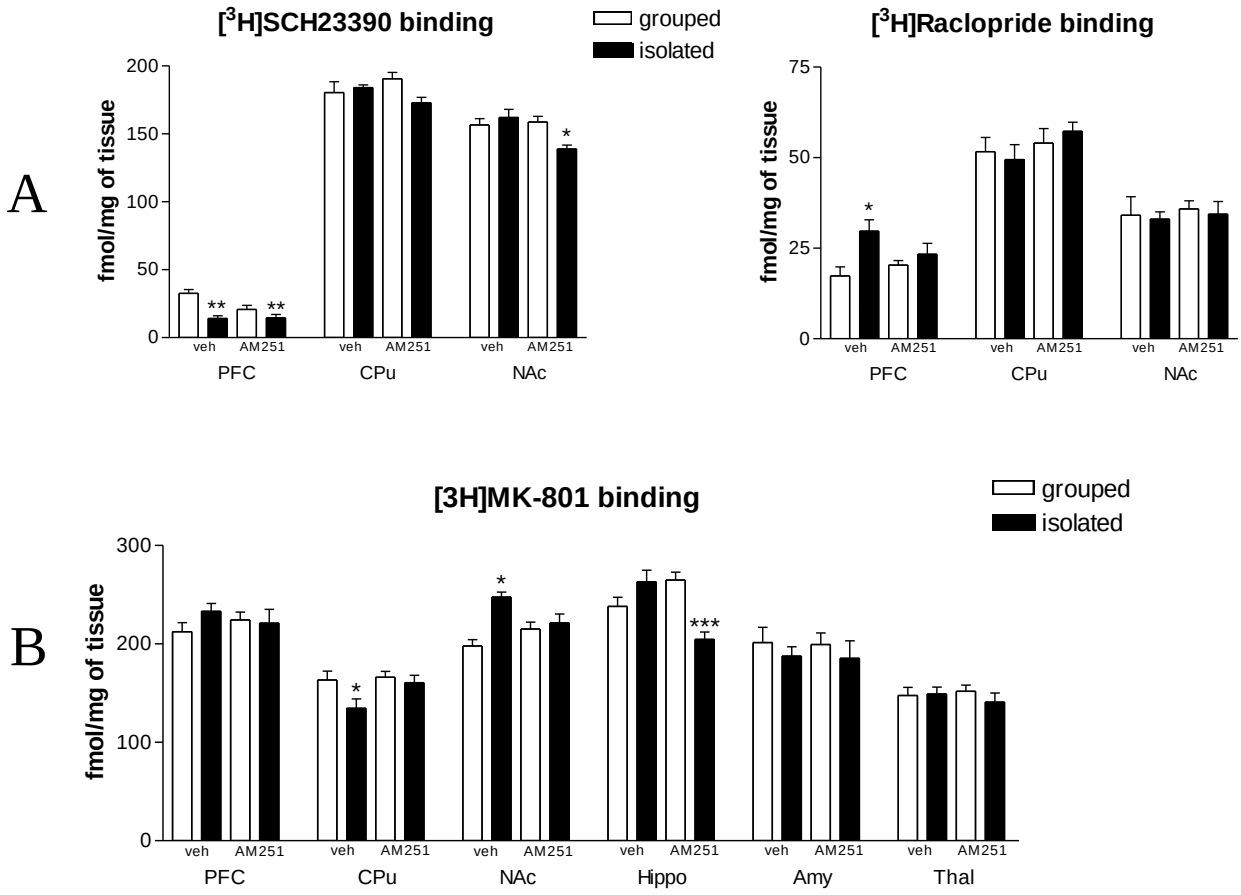

Figure 18: Effect of chronic AM251 (or vehicle) treatment on (**A**) D1 and D2 receptor density and (**B**) NMDA receptor density. D1 and D2 receptor densities were assessed through [^{3}H]SCH23390 and [^{3}H]Raclopride receptor binding, respectively. NMDA receptor density was assessed through [^{3}H]MK801 receptor binding. Results are expressed as fmol/mg of tissue. Brain areas: Prefrontal cortex (PFC); Caudate putamen (CPu); Nucleus accumbens (NAc); Hippocampus (Hippo); Amygdala (Amy); Thalamus (Thal). Data are expressed as mean ± SEM of four animals per group. ***p<0.001; **p<0.01; *p<0.05 vs grouped (Bonferroni's post-hoc test)

LIST OF PUBLICATIONS

Data presented in this PhD thesis have been published or are currently under publication in international peer-reviewed journals.

A list of publications is provided below.

Zamberletti E, Viganò D, Guidali C, Rubino T, Parolaro D. (2012). Long-lasting recovery of psychotic-like symptoms in isolation-reared rats after chronic but not acute treatment with the cannabinoid antagonist AM251. *Int J Neuropsychopharmacol* **15**:267-280.

Zamberletti E, Piscitelli F, Cadeddu F, Rubino T, Fratta W, Fadda P, Di Marzo V, Parolaro D. (2012). Chronic blockade of CB(1) receptors reverses startle gating deficits and associated neurochemical alterations in rats reared in isolation. *Br J Pharmacol* **167**:1652-1664.

Zamberletti E, Beggiato S, Steardo L. Jr, Prini P, Antonelli T, Ferraro F, Rubino T, Parolaro D. Alterations of prefrontal cortex GABAergic transmission in the complex psychotic-like phenotype induced by adolescent delta-9-tetrahydrocannabinol exposure in rats. Accepted for publication, *Neurobiology of Disease*

REFERENCES

Adams B, Moghaddam B. (1998). Corticolimbic dopamine neurotransmission is temporally dissociated from the cognitive and locomotor effects of phencyclidine. J Neurosci. 18: 5545-5554.

Adriani W, Felici A, Sargolini F, Roullet P, Usiello A, Oliverio A, et al. (1998). N-methyl-D-aspartate and dopamine receptor involvement in the modulation of locomotor activity and memory processes. Exp Brain Res 123: 52–59.

Akbarian S, Huang HS. (2006). Molecular and cellular mechanisms of altered GAD1/GAD67 expression in schizophrenia and related disorders. Brain Res Rev. 52: 293-304.

Armstrong C, Soltesz I. (2012). Basket cell dichotomy in microcircuit function. J Physiol. 590: 683-694.

Arseneault L, Cannon M, Poulton R, Murray R, Caspi A, Moffitt TE. Cannabis use in adolescence and risk for adult psychosis: longitudinal prospective study. BMJ 2002; 325: 1212–3.

Ballmaier M, Bortolato M, Rizzetti C, et al. Cannabinoid receptor antagonists counteract sensorimotor gating deficits in the phencyclidine model of psychosis. Neuropsychopharmacology 2007; 32: 2098-107.

Bhattacharyya S, Morrison PD, Fusar-Poli P, et al. Opposite effects of delta-9-tetrahydrocannabinol and cannabidiol on human brain function and psychopathology. Neuropsychopharmacology 2010; 35: 764-74.

Biscaia M, Marı´n S, Ferna´ ndez B, Marco EM, Rubio M, Guaza C, et al. (2003) Chronic treatment with CP 55,940 during the periadolescent period differentially affects the behavioural responses of male and female rats in adulthood. Psychopharmacology (Berl) 170: 301–308.

Bisogno T, Sepe N, Melck D, Maurelli S, De Petrocellis L, Di Marzo V. (1997). Biosynthesis, release and degradation of the novel endogenous cannabimimetic metabolite 2-arachidonoylglycerol in mouse neuroblastoma cells. Biochem J 322: 671-677.

Black MD, Stevens RJ, Rogacki N, et al. AVE1625, a cannabinoid CB1 receptor antagonist, as a co-treatment with antipsychotics for schizophrenia: improvement in cognitive function and reduction of antipsychotic-side effects in rodents. Psychopharmacology (Berl). 2011; 215: 149-63.

Boucher AA, Arnold JC, Duffy L, Schofield PR, Micheau J, Karl T. Heterozygous neuregulin 1 mice are more sensitive to the behavioural effects of Delta9-tetrahydrocannabinol. Psychopharmacology (Berl) 2007; 192: 325-36.

Brusco A, Tagliaferro PA, Saez T and Onaivi ES (2008) Ultrastructural localization of neuronal brain CB2 cannabinoid receptors. Ann NY Acad Sci 1139: 450–457.

Brzózka MM, Fischer A, Falkai P, Havemann-Reinecke U. Acute treatment with cannabinoid receptor agonist WIN55212.2 improves prepulse inhibition in psychosocially stressed mice. Behav Brain Res 2011; 218: 280-7.

Caspi A, Moffitt TE, Cannon M, et al. Moderation of the effect of adolescent onset cannabis use on adult psychosis by a functional polymorphism in the catechol-O-methyltransferase gene: longitudinal evidence of a gene x environment interaction. Biol Psychiatry 2005; 57: 1117–27.

Cha YM, Jones KH, Kuhn CM, Wilson WA and Swartzwelder HS (2007) Sex differences in the effects of delta9-tetrahydrocannabinol on spatial learning in adolescent and adult rats. Behav Pharmacol 18: 563–569.

Cha YM, White AM, Kuhn CM, Wilson WA and Swartzwelder HS (2006) Differential effects of delta9-THC on learning in adolescent and adult rats. Pharmacol Biochem Behav 83: 448–455.

Chavarría-Siles I, Contreras-Rojas J, Hare E, et al. Cannabinoid receptor 1 gene (CNR1) and susceptibility to a quantitative phenotype for hebephrenic schizophrenia. Am J Med Genet B Neuropsychiatr Genet 2008; 147: 279-84.

Cheer JF, Wassum KM, Sombers LA, et al. Phasic dopamine release evoked by abused substances requires cannabinoid receptor activation.J Neurosci 2007; 27: 791-5.

Chiang YC, Chen JC. The role of the cannabinoid type 1 receptor and down-stream cAMP/DARPP-32 signal in the nucleus accumbens of methamphetamine-sensitized rats. J Neurochem 2007; 103: 2505-17.

Chindo BA, Adzu B, Yahaya TA, Gamaniel KS. (2012). Ketamine-enhanced immobility in forced swim test: a possible animal model for the negative symptoms of schizophrenia. Prog Neuropsychopharmacol Biol Psychiatry 38:310-6.

Corbillé AG, Valjent E, Marsicano G, et al. Role of cannabinoid type 1 receptors in locomotor activity and striatal signaling in response to psychostimulants. J Neurosci 2007; 27: 6937-47.

Curley AA, Lewis DA. (2012). Cortical basket cell dysfunction in schizophrenia. J Physiol. 590: 715-724.

Dalton VS, Long LE, Weickert CS, Zavitsanou K. Paranoid schizophrenia is characterized by increased CB1 receptor binding in the dorsolateral prefrontal cortex. Neuropsychopharmacology 2011; 36:1620-30.

De Marchi N, De Petrocellis L, Orlando P, Daniele F, Fezza F, Di Marzo V. Endocannabinoid signalling in the blood of patients with schizophrenia. Lipids Health Dis 2003; 2: 5.

De Petrocellis L, Di Marzo V. An introduction to the endocannabinoid system: from the early to the latest concepts. Best Pract Res Clin Endocrinol Metab 2009; 23: 1-15.

Dean B, Sundram S, Bradbury R, Scarr E, Copolov D. Studies on [3H]CP-55940 binding in the human central nervous system: regional specific changes in density of cannabinoid-1 receptors associated with schizophrenia and cannabis use. Neuroscience 2001; 103: 9–15.

Dekker N, Linszen DH, De Haan L. Reasons for cannabis use and effects of cannabis use as reported by patients with psychotic disorders. Psychopathology 2009; 42: 350–60.

Deng C, Han M, Huang XF. No changes in densities of cannabinoid receptors in the superior temporal gyrus in schizophrenia. Neurosci Bull 2007; 23: 341-7.

Devane WA, Hanus L, Breuer A, et al. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 1992; 258: 1946-9.

Di Forti M, Morgan C, Dazzan P, Pariante C, Mondelli V, Marques TR, et al. (2009). High-potency cannabis and the risk of psychosis. Br J Psychiatry 195: 488-491.

Di Marzo V, Petrosino S. Endocannabinoids and the regulation of their levels in health and disease. Curr Opin Lipidol 2007; 18: 129-40.

Dominguez MD, Saka MC, Lieb R, Wittchen HU, van Os J. Early expression of negative/disorganized symptoms predicting psychotic experiences and subsequent clinical psychosis: a 10-year study. Am J Psychiatry 2010; 167: 1075–82.

Dragt S, Nieman DH, Becker HE, et al. Age of onset of cannabis use is associated with age of onset of high-risk symptoms for psychosis. Can J Psychiatry 2010; 55: 165–71.

D'Souza DC, Sewell RA, Ranganathan M. (2009). Cannabis and psychosis/schizophrenia: human studies. Eur Arch Psychiatry Clin Neurosci. 259: 413-431.

D'Souza DC, Sewell RA, Ranganathan M. (2009). Cannabis and psychosis/schizophrenia: human studies. Eur Arch Psychiatry Clin Neurosci. 259:413-31.

Eggan SM, Hashimoto T, Lewis DA. (2008). Reduced cortical cannabinoid 1 receptor messenger RNA and protein expression in schizophrenia. Arch Gen Psychiatry. 65: 772-784.

Eisenstein SA, Clapper JR, Holmes PV, Piomelli D, Hohmann AG. A role for 2-arachidonoylglycerol and endocannabinoid signaling in the locomotor response to novelty induced by olfactory bulbectomy. Pharmacol Res 2010; 61: 419-29.

Enomoto, T., Noda, Y., Nabeshima, T. (2007). Phencyclidine and genetic animal models of schizophrenia developed in relation to the glutamate hypothesis. Methods Find Exp Clin Pharmacol 29: 291.

Evins AE, Green AI, Kane JM, Murray RM. (2012). The effect of marijuana use on the risk for schizophrenia. J Clin Psychiatry 73:1463-8.

Ferdinand RF, van der Ende J, Bongers I, Selten JP, Huizink A, Verhulst FC. Cannabis–psychosis pathway independent of other types of psychopathology. Schizophr Res 2005; 79: 289–95.

Fergusson DM, Poulton R, Smith PF, Boden JM. Cannabis and psychosis. BMJ 2006; 332: 172–5.

Ferraro L, O'Connor WT, Beggiato S, Tomasini MC, Fuxe K, Tanganelli S, et al. (2012). Striatal NTS1 , dopamine D2 and NMDA receptor regulation of pallidal GABA and glutamate release--a dual-probe microdialysis study in the intranigral 6-hydroxydopamine unilaterally lesioned rat. Eur J Neurosci 35: 207-220.

Ferrer B, Gorriti MA, Palomino A, et al. Cannabinoid CB1 receptor antagonism markedly increases dopamine receptor-mediated stereotypies. Eur J Pharmacol 2007; 559: 180-3.

Freund TF, Katona I, Piomelli D. Role of endogenous cannabinoids in synaptic signaling. Physiol Rev 2003; 83: 1017-66.

Gerdeman GL, Schechter JB, French ED. Context-specific reversal of cocaine sensitization by the CB1 cannabinoid receptor antagonist rimonabant. Neuropsychopharmacology 2008; 33: 2747-59.

Geyer MA (2008) Developing translational animal models for symptoms of schizophrenia or bipolar mania. Neurotox Res 14: 71–78.

Giuffrida A, Leweke FM, Gerth CW, et al. Cerebrospinal anandamide levels are elevated in acute schizophrenia and are inversely correlated with psychotic symptoms. Neuropsychopharmacology 2004; 29: 2108-14.

Gong JP, Onaivi ES, Ishiguro H, Liu QR, Tagliaferro PA, Brusco A, et al. (2006) Cannabinoid CB2 receptors: immunohistochemical localization in rat brain. Brain Res 1071: 10–23.

Gorriti MA, Ferrer B, del Arco I, et al. Acute delta9-tetrahydrocannabinol exposure facilitates quinpirole-induced hyperlocomotion. Pharmacol Biochem Behav 2005; 81:71-7.

Gorriti MA, Rodríguez de Fonseca F, Navarro M, Palomo T. Chronic (-)-delta9-tetrahydrocannabinol treatment induces sensitization to the psychomotor effects of amphetamine in rats. Eur J Pharmacol 1999; 365:133-42.

Guidali C, Viganò D, Petrosino S, Zamberletti E, Realini N, Binelli G, et al. (2010). Cannabinoid CB1 receptor antagonism prevents neurochemical and behavioural deficits induced by chronic phencyclidine. Int J Neuropsychopharmacol 14: 17-28.

Gururajan A, Taylor DA, Malone DT. Effect of cannabidiol in a MK-801-rodent model of aspects of schizophrenia. Behav Brain Res 2011; 222: 299-308.

Hallak JE, Machado-de-Sousa JP, Crippa JA, et al. Performance of schizophrenic patients in the Stroop Color Word Test and electrodermal responsiveness after acute administration of cannabidiol (CBD). Rev Bras Psiquiatr 2010; 32: 56-61.

Haller J, Szirmai M, Varga B, Ledent C, Freund TF. Cannabinoid CB1 receptor dependent effects of the NMDA antagonist phencyclidine in the social withdrawal model of schizophrenia. Behav Pharmacol 2005; 16: 415-22.

Hashimoto T, Volk DW, Eggan SM, Mirnics K, Pierri JN, Sun Z, et al. (2003). Gene expression deficits in a subclass of GABA neurons in the prefrontal cortex of subjects with schizophrenia. J Neurosci. 23: 6315-6326.

Henquet C, Krabbendam L, Spauwen J, et al. Prospective cohort study of cannabis use, predisposition for psychosis, and psychotic symptoms in young people. BMJ 2005b; 330: 11.

Henquet C, Murray R, Linszen D, van Os J. The environment and schizophrenia: the role of cannabis use. Schizophr Bull 2005a; 31: 608–12.

Henquet C, Rosa A, Delespaul P, et al. COMT ValMet moderation of cannabis-induced psychosis: a momentary assessment study of 'switching on' hallucinations in the flow of daily life. Acta Psychiatr Scand 2009; 119: 156–60.

Higuera-Matas A, Botreau F, Migue´ns M, Del Olmo N, Borcel E, Pe´rez-Alvarez L, et al. (2009) Chronic periadolescent cannabinoid treatment enhances adult hippocampal PSA-NCAM expression in male Wistar rats but only has marginal effects on anxiety, learning and memory. Pharmacol Biochem Behav 93: 482–490.

Howlett AC. The cannabinoid receptors. Prostaglandins Other Lipid Mediat 2002; 68-69: 619-31.

Ishiguro H, Horiuchi Y, Ishikawa M, et al. Brain cannabinoid CB2 receptor in schizophrenia. Biol Psychiatry 2010; 67: 974-82.

Kelly DL, Gorelick DA, Conley RR, et al. Effects of the cannabinoid-1 receptor antagonist rimonabant on psychiatric symptoms in overweight people with schizophrenia: a randomized, double-blind, pilot study. J Clin Psychopharmacol 2011; 31: 86-91.

Koethe D, Giuffrida A, Schreiber D, et al. Anandamide elevation in cerebrospinal fluid in initial prodromal states of psychosis. Br J Psychiatry 2009; 194: 371-2.

Koethe D, Llenos IC, Dulay JR, Hoyer C, Torrey EF, Leweke FM, Weis S. Expression of CB1 cannabinoid receptor in the anterior cingulate cortex in schizophrenia, bipolar disorder, and major depression. J Neural Transm 2007;114: 1055-63.

Kovács KJ. (2008). Measurement of immediate-early gene activation- c-fos and beyond. J Neuroendocrinol. 20: 665-672.

Kucewicz MT, Tricklebank MD, Bogacz R, Jones MW. (2011). Dysfunctional prefrontal cortical network activity and interactions following cannabinoid receptor activation. J Neurosci. 31: 15560-15568.

Kumari V, Sharma T. (2002). Effects of typical and atypical antipsychotics on prepulse inhibition in schizophrenia: a critical evaluation of current evidence and directions for future research. Psychopharmacology 162: 97–101.

Lapiz MD, Fulford A, Muchimapura S, Mason R, et al. (2003). Influence of postweaning social isolation in the rat on brain development, conditioned behavior, and neurotransmission. Neuroscience and Behavioural Physiology 33, 13-29.

Lazar NL, Neufeld RWJ, Cain DP. (2011). Contribution of nonprimate animal models in understanding the etiology of schizophrenia. J Psychiatry Neurosci 36: E5–E29.

Le Bec PY, Fatse´as M, Denis C, Lavie E, Auriacombe M. Cannabis and psychosis: search of a causal link through a critical and systematic review. Encephale 2009; 35: 377–85.

Lee JWY, Robertson S (1997). Clozapine withdrawal catatonia and neuroleptic malignant syndrome: a case report. Annals of Clinical Psychiatry 9, 165-169.

Leeson VC, Harrison I, Ron MA, Barnes TR, Joyce EM. The Effect of Cannabis Use and Cognitive Reserve on Age at Onset and Psychosis Outcomes in First-Episode Schizophrenia. Schizophr Bull 2011; Epub Mar 9 doi: 10.1093/schbul/sbq153.

Léna I, Matthes H, Kieffer B, Kitchen I. (2004). Quantitative autoradiography of dopamine receptors in the brains of micro-opioid receptor knockout mice. Neurosci Lett 356: 220-224.

Lerner TN, Horne EA, Stella N, Kreitzer AC. Endocannabinoid signaling mediates psychomotor activation by adenosine A2A antagonists. J Neurosci 2010; 30: 2160-4.

Lesscher HM, Hoogveld E, Burbach JP, van Ree JM, Gerrits MA. Endogenous cannabinoids are not involved in cocaine reinforcement and development of cocaine-induced behavioural sensitization. Eur Neuropsychopharmacol 2005; 15:31-7.

Leweke FM and Schneider M (2010) Chronic pubertal cannabinoid treatment as a behavioural model for aspects of schizophrenia: effects of the atypical antipsychotic quetiapine. Int J Neuropsychopharmacol 3: 1–9.

Leweke FM, Giuffrida A, Wurster U, Emrich HM, Piomelli D. Elevated endogenous cannabinoids in schizophrenia. Neuroreport 1999; 10: 1665-9.

Leweke FM, Koethe D, Gerth CW, et al. Cannabidiol as an antipsychotic. A double-blind, controlled clinical trial on cannabidiol vs. amisulpride in acute schizophrenia. Eur Psychiatry 2007; 22: S14.02.

Leweke FM, Piomelli D, Pahlisch F, Muhl D, Gerth CW, Hoyer C, Klosterkötter J, Hellmich M, Koethe D. (2012). Cannabidiol enhances anandamide signaling and alleviates psychotic symptoms of schizophrenia. Transl Psychiatry 20;2:e94.

Leweke FM, Schneider U, Radwan M, Schmidt E, Emrich HM. Different effects of nabilone and cannabidiol on binocular depth inversion in Man. Pharmacol Biochem Behav 2000; 66: 175-81.

Li M, Fletcher PJ, Kapur S (2007a). Time course of the antipsychotic effect and the underlying behavioral mechanisms. Neuropsychopharmacology 32, 263–272.

Li N, Xihong W, Liang L (2007b). Chronic administration of clozapine alleviates reversal-learning impairment in isolation-reared rats. Behavioural Pharmacology 18, 135-145.

Lidow MS, Williams GV, Goldman-Rakic PS. (1998). The cerebral cortex: a case for a common site of action of antipsychotics. Trends in Pharmacological Sciences 19: 136-140.

Llorente-Berzal A, Fuentes S, Gagliano H, et al. Sex-dependent effects of maternal deprivation and adolescent cannabinoid treatment on adult rat behaviour. Addict Biol 2011; 16: 624-37.

Long LE, Chesworth R, Huang XF, McGregor IS, Arnold JC, Karl T. A behavioural comparison of acute and chronic Delta9-tetrahydrocannabinol and cannabidiol in C57BL/6JArc mice. Int J Neuropsychopharmacol 2010; 13: 861-76.

Long LE, Malone DT, Taylor DA. Cannabidiol reverses MK-801-induced disruption of prepulse inhibition in mice. Neuropsychopharmacology 2006; 31: 795-803.

Lopez-Moreno JA, Gonzalez-Cuevas G, Moreno G, Navarro M. (2008). The pharmacology of the endocannabinoid system: functional and structural interactions with other neurotransmitter systems and their repercussions in behavioral addiction. Addict Biol. 13:160-187.

Madsen MV, Peacock L, Werge T, Andersen MB. Effects of the cannabinoid CB1 receptor agonist CP55,940 and antagonist SR141716A on d-amphetamine-induced behaviours in Cebus monkeys. J Psychopharmacol 2006; 20: 622-8.

Malone DT, Hill MN, Rubino T. (2010). Adolescent cannabis use and psychosis: epidemiology and neurodevelopmental models. Br J Pharmacol 160: 511-522.

Malone DT, Kearn CS, Chongue L, Mackie K, et al. (2008). Effect of social isolation on CB1 and D2 receptor and fatty acid amide hydrolase expression in rats. Neuroscience 152, 265–272.

Malone DT, Long LE, Taylor DA. The effect of SR 141716 and apomorphine on sensorimotor gating in Swiss mice. Pharmacol Biochem Behav 2004; 77: 839-45.

Malone DT, Taylor DA. The effect of Delta9-tetrahydrocannabinol on sensorimotor gating in socially isolated rats. Behav Brain Res 2006; 166: 101-9.

Mansbach RS, Rovetti CC, Winston EN, Lowe JA 3rd. Effects of the cannabinoid CB1 receptor antagonist SR141716A on the behavior of pigeons and rats. Psychopharmacology (Berl) 1996; 124: 315-22.

Marcellino D, Carriba P, Filip M, et al. Antagonistic cannabinoid CB1/dopamine D2 receptor interactions in striatal CB1/D2 heteromers. A combined neurochemical and behavioral analysis. Neuropharmacology 2008; 54: 815-23.

Marsicano G, Wotjak CT, Azad SC, Bisogno T, Rammes G, Cascio MG, et al. (2002). The endogenous cannabinoid system controls extinction of aversive memories. Nature 418: 530-534.

Marsicano G., Lutz B. (1999). Expression of the cannabinoid receptor CB1 in distinct neuronal subpopulations in the adult mouse forebrain. Eur J Neurosci. 11: 4213–4225.

Martin RS, Secchi RL, Sung E, et al. Effects of cannabinoid receptor ligands on psychosis-relevant behavior models in the rat. Psychopharmacology (Berl) 2003; 165: 128-35.

Masserano JM, Karoum F, Wyatt RJ. SR141716A, a CB1 cannabinoid receptor antagonist, potentiates the locomotor stimulant effects of amphetamine and apomorphine. Behav Pharmacol. 1999l; 10: 429-32.

Matsuda LA, Lolait SJ, Brownstein MJ, Young AC and Bonner TI (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346: 561–564.

McGuire KA, Blahnik MM, Sponheim SR. (2013). Discrimination within Recognition Memory in Schizophrenia. Behav. Sci. 3, 273–297.

McPartland JM, Norris RW, Kilpatrick CW. Coevolution between cannabinoid receptors and endocannabinoid ligands. Gene 2007; 397: 126-35.

Meltzer HY, Arvanitis L, Bauer D, Rein W. Placebo-controlled evaluation of four novel compounds for the treatment of schizophrenia and schizoaffective disorder. Am J Psychiatry 2004; 161: 975-84.

Minocci D, Massei J, Martino A, et al. Genetic association between bipolar disorder and 524A>C (Leu133Ile) polymorphism of CNR2 gene, encoding for CB2 cannabinoid receptor. J Affect Disord 2011; 134: 427-30.

Moreira FA, Guimarães FS. Cannabidiol inhibits the hyperlocomotion induced by psychotomimetic drugs in mice. Eur J Pharmacol 2005; 512:199-205.

Morra JT, Glick SD, Cheer JF. Neural encoding of psychomotor activation in the nucleus accumbens core, but not the shell, requires cannabinoid receptor signaling. J Neurosci 2010; 30: 5102-7.

Mouri A, Noda Y, Enomoto T, Nabeshima T. (2007). Phencyclidine animal models of schizophrenia: approaches from abnormality of glutamatergic neurotransmission and neurodevelopment. Neurochem Int 51:173-84.

Müller-Vahl KR, Emrich HM. Cannabis and schizophrenia: towards a cannabinoid hypothesis of schizophrenia. Expert Rev Neurother 2008; 8: 1037-48.

Munro S, Thomas KL and Abu-Shaar M (1993) Molecular characterization of a peripheral receptor for cannabinoids. Nature 365: 61–65.

Nakazawa K, Zsiros V, Jiang Z, Nakao K, Kolata S, Zhang S. et al. (2012). GABAergic interneuron origin of schizophrenia pathophysiology. Neuropharmacology. 62: 1574-1583.

Neill JC, Barnes S, Cook S, Grayson B, Idris NF, McLean SL, et al. (2010). Animal models of cognitive dysfunction and negative symptoms of schizophrenia: focus on NMDA receptor antagonism. Pharmacol Ther 128: 419-432.

Newell KA, Deng C, Huang XF. Increased cannabinoid receptor density in the posterior cingulate cortex in schizophrenia. Exp Brain Res 2006; 172: 556-60.

Newell KA, Zavitsanou K, Huang XF. (2007). Short and long term changes in NMDA receptor binding in mouse brain following chronic phencyclidine treatment. J Neural Transm 114: 995-1001.

O'Shea M, McGregor IS and Mallet PE (2006) Repeated cannabinoid exposure during perinatal, adolescent or early adult ages produces similar longlasting deficits in object recognition and reduced social interaction in rats. J Psychopharmacol 20: 611–621.

O'Shea M, Singh ME, McGregor IS and Mallet PE (2004) Chronic cannabinoid exposure produces lasting memory impairment and increased anxiety in adolescent but not adult rats. J Psychopharmacol 18: 502–508.

Okubo Y, Suhara T, Suzuki K, Kobayashi K, Inoue O, Terasaki O, et al. (1997). Decreased prefrontal dopamine D1 receptors in schizophrenia revealed by PET. Nature 385: 634–636.

Ortega-Alvaro A, Aracil-Fernández A, García-Gutiérrez MS, Navarrete F, Manzanares J. Deletion of CB2 cannabinoid receptor induces schizophrenia-related behaviors in mice. Neuropsychopharmacology 2011; 36: 1489-504.

O'Sullivan SE. Cannabinoids go nuclear: evidence for activation of peroxisome proliferator-activated receptors. Br J Pharmacol 2007; 152: 576-82.

Paxinos G, Watson C. (1986). The rat brain in stereotaxic coordinates. Academic Press, New York.

Piomelli D. The molecular logic of endocannabinoid signalling. Nat Rev Neurosci 2003; 4: 873-84.

Poncelet M, Barnouin MC, Brelière JC, Le Fur G, Soubrié P. Blockade of cannabinoid (CB1) receptors by 141716 selectively antagonizes drug-induced reinstatement of exploratory behaviour in gerbils. Psychopharmacology (Berl) 1999; 144: 144-50.

Powell SB, Geyer MA. (2002). Developmental markers of psychiatric disorders as identified by sensorimotor gating. Neurotox Res 4: 489-502.

Powell SB, Sejnowski TJ, Behrens MM. (2012). Behavioral and neurochemical consequences of cortical oxidative stress on parvalbumin-interneuron maturation in rodent models of schizophrenia. Neuropharmacology. 62: 1322-1331.

Przegaliński E, Göthert M, Frankowska M, Filip M. WIN55,212-2-induced reduction of cocaine hyperlocomotion: possible inhibition of 5-HT(3) receptor function. Eur J Pharmacol 2005; 517: 68-73.

Quinn HR, Matsumoto I, Callaghan PD, Long LE, Arnold JC, Gunasekaran N, et al. (2008) Adolescent rats find repeated Delta(9)-THC less aversive than adult rats but display greater residual cognitive deficits and changes in hippocampal protein expression following exposure. Neuropsychopharmacology 33: 1113–1126.

Ralph-Williams RJ, Lehmann-Masten V, Geyer MA. (2003). Dopamine D1 rather than D2 receptor agonists disrupt prepulse inhibition of startle in mice. Neuropsychopharmacology 28: 108-118.

Realini N, Vigano' D, Guidali C, Zamberletti E, Rubino T, Parolaro D. (2011). Chronic URB597 treatment at adulthood reverted most depressive-like symptoms induced by adolescent exposure to THC in female rats. Neuropharmacology 60: 235-243.

Robinson SA, Loiacono RE, Christopoulos A, Sexton PM, et al. (2010). The effect of social isolation on rat brain expression of genes associated with endocannabinoid signaling. Brain Research 1343, 153-167.

Roser P, Vollenweider FX, Kawohl W. Potential antipsychotic properties of central cannabinoid (CB1) receptor antagonists. World J Biol Psychiatry 2010; 11: 208-19.

Ross RA. Anandamide and vanilloid TRPV1 receptors. Br J Pharmacol 2003; 140: 790-801.

Rubino T, Realini N, Braida D, Alberio T, Capurro V, Viganò D, et al. (2009). The depressive phenotype induced in adult female rats by adolescent exposure to THC is associated with cognitive impairment and altered neuroplasticity in the prefrontal cortex. Neurotox Res 15: 291-302.

Rubino T, Realini N, Braida D, Guidi S, Capurro V, Vigano` D, et al. (2009b) Changes in hippocampal morphology and neuroplasticity induced by adolescent THC treatment are associated with cognitive impairment in adulthood. Hippocampus 19: 763–772.

Rubino T, Viganò D, Massi P, Parolaro D. (2000). Changes in the cannabinoid receptor binding, G protein coupling, and cyclic AMP cascade in the CNS of rats tolerant to and dependent on the synthetic cannabinoid compound CP55,940. J Neurochem 75: 2080-2086.

Rubino T, Vigano D, Realini N, Guidali C, Braida D, Capurro V, et al. (2008). Chronic delta 9-tetrahydrocannabinol during adolescence provokes sex-dependent changes in the emotional profile in adult rats: behavioral and biochemical correlates. Neuropsychopharmacology 33: 2760-2771.

Rubino T, Zamberletti E, Parolaro D. (2012). Adolescent exposure to cannabis as a risk factor for psychiatric disorders. J Psychopharmacol. 26: 177-188.

Ryberg E, Larsson N, Sjögren S, et al. The orphan receptor GPR55 is a novel cannabinoid receptor. Br J Pharmacol 2007; 152:1092-101.

Schneider M and Koch M (2003) Chronic pubertal, but not adult chronic cannabinoid treatment impairs sensorimotor gating, recognition memory, and the performance in a progressive ratio task in adult rats. Neuropsychopharmacology 28: 1760–1769.

Sciolino NR, Bortolato M, Eisenstein SA, Fu J, et al. (2010). Social isolation and chronic handling alter endocannabinoid signaling and behavioral reactivity to context in adult rats. Neuroscience 168, 371-386.

Scott L, Aperia A. (2009). Interaction between N-methyl-D-aspartic acid receptors and D1 dopamine receptors: an important mechanism for brain plasticity. Neuroscience 158: 62–66.

Sedvall, G. and Farde, L. (1996) Lancet 347, 264

Seeman P, Niznik HB, Guan HC, Booth G, Ulpian C. (1989). Link between D1 and D2 dopamine receptors is reduced in schizophrenia and Huntington diseased brain. Proc Natl Acad Sci U S A 86: 10156-10160.

Seillier A, Advani T, Cassano T, Hensler JG, Giuffrida A. (2010). Inhibition of fatty-acid amide hydrolase and CB1 receptor antagonism differentially affect behavioural responses in normal and PCP-treated rats. Int J Neuropsychopharmacol 13:373-86.

Seillier A, Giuffrida A. Evaluation of NMDA receptor models of schizophrenia: divergences in the behavioral effects of sub-chronic PCP and MK-801. Behav Brain Res 2009; 204: 410-5.

Semple DM, McIntosh AM, Lawrie SM. Cannabis as a risk factor for psychosis: systematic review. J Psychopharmacol 2005; 19: 187–94.

Sewell RA, Perry EB Jr, Karper LP, et al. Clinical significance of neurological soft signs in schizophrenia: factor analysis of the Neurological Evaluation Scale. Schizophr Res 2010; 124: 1-12.

Spano MS, Fadda P, Frau R, Fattore L, Fratta W. Cannabinoid self-administration attenuates PCP-induced schizophrenia-like symptoms in adult rats. Eur Neuropsychopharmacol 2010; 20: 25-36.

Stella N, Schweitzer P, Piomelli D. A second endogenous cannabinoid that modulates long-term potentiation. Nature 1997; 388: 773-8.

Strange PG. (1991). D1/D2 dopamine receptor interaction at the biochemical level. Trends Pharmacol Sci 12: 48-49.

Suárez J, Llorente R, Romero-Zerbo SY, et al. Early maternal deprivation induces gender-dependent changes on the expression of hippocampal CB(1) and CB(2) cannabinoid receptors of neonatal rats. Hippocampus 2009; 19: 623-32.

Suarez J, Rivera P, Llorente R, Romero-Zerbo SY, Bermúdez-Silva FJ, de Fonseca FR, Viveros MP. (2010). Early maternal deprivation induces changes on the expression of 2-AG biosynthesis and degradation enzymes in neonatal rat hippocampus. Brain Res. 1349: 162-173.

Sugiura T, Kishimoto S, Oka S, Gokoh M. Biochemistry, pharmacology and physiology of 2-arachidonoylglycerol, an endogenous cannabinoid receptor ligand. Prog Lipid Res 2006; 45: 405-46.

Sugranyes G, Flamarique I, Parellada E, et al. Cannabis use and age of diagnosis of schizophrenia. Eur Psychiatry 2009; 24: 282–6.

Sutt S, Raud S, Areda T, Reimets A, Kõks S, Vasar E. (2008). Cat odour-induced anxiety--a study of the involvement of the endocannabinoid system. Psychopharmacology (Berl) 198: 509-520.

Thiemann G, van der Stelt M, Petrosino S, Molleman A, Di Marzo V, Hasenöhrl RU. The role of the CB1 cannabinoid receptor and its endogenous ligands, anandamide and 2-arachidonoylglycerol, in amphetamine-induced behavioural sensitization. Behav Brain Res 2008; 187: 289-96.

Toua C, Brand L, Möller M, Emsley RA, Harvey BH. (2010). The effects of sub-chronic clozapine and haloperidol administration on isolation rearing induced changes in frontal cortical N-methyl-D-aspartate and D1 receptor binding in rats. Neuroscience 165: 492-499.

Tseng KY, O'Donnell P. (2003). Dopamine-glutamate interactions in the control of cell excitability in medial prefrontal cortical pyramidal neurons from adult rats. Ann N Y Acad Sci 1003: 476–478.

Tzavara ET, Davis RJ, Perry KW, et al. The CB1 receptor antagonist SR141716A selectively increases monoaminergic neurotransmission in the medial prefrontal cortex: implications for therapeutic actions. Br J Pharmacol 2003; 138: 544-53.

Tzavara ET, Degroot A, Wade MR, Davis RJ, Nomikos GG. CB1 receptor knockout mice are hyporesponsive to the behavior-stimulating actions of d-amphetamine: role of mGlu5 receptors. Eur Neuropsychopharmacol 2009; 19: 196-204.

Ugur T, Bartels M, Kis B, Scherbaum N. Psychosis following anti-obesity treatment with rimonabant. Obes Facts 2008; 1: 103-5.

Urigüen L, García-Fuster MJ, Callado LF, et al. Immunodensity and mRNA expression of A2A adenosine, D2 dopamine, and CB1 cannabinoid receptors in postmortem frontal cortex of subjects with schizophrenia: effect of antipsychotic treatment. Psychopharmacology (Berl) 2009; 206: 313-24.

Van den Buuse M (2010) Modeling the positive symptoms of schizophrenia in genetically modified mice: pharmacology and methodology aspects. Schizophr Bull 36: 246–270.

Van Sickle MD, Duncan M, Kingsley PJ, et al. Identification and functional characterization of brainstem cannabinoid CB2 receptors. Science 2005; 310: 329-32.

Vanderschuren LJ, Kalivas PW. (2000) Alterations in dopaminergic and glutamatergic transmission in the induction and expression of behavioral sensitization: a critical review of preclinical studies. Psychopharmacology (Berl) 151: 99–120.

Vigano D, Guidali C, Petrosino S, Realini N, Rubino T, Di Marzo V, et al. (2009). Involvement of the endocannabinoid system in phencyclidine-induced cognitive deficits modelling schizophrenia. Int J Neuropsychopharmacol 12: 599-614.

Volman V, Behrens MM, Sejnowski TJ. (2011). Downregulation of parvalbumin at cortical GABA synapses reduces network gamma oscillatory activity. J Neurosci. 31: 18137-18148.

Wan FJ and Swerdlow NR. (1996). Sensorimotor gating in rats is regulated by different dopamine-glutamate interactions in the nucleus accumbens core and shell subregions. Brain Research 722: 168-176.

Wang CZ, Yang SF, Xia Y, Johnson KM. (2008). Postnatal phencyclidine administration selectively reduces adult cortical parvalbumin-containing interneurons. Neuropsychopharmacology. 33: 2442-2455.

Wegener N and Koch M (2009) Behavioural disturbances and altered Fos protein expression in adult rats after chronic pubertal cannabinoid treatment. Brain Res 1253: 81–91.

Weiss IC, Di Iorio L, Feldon J, Domeney AM. (2000). Strain differences in the isolation-induced effects on prepulse inhibition of the acoustic startle response and on locomotor activity. Behav Neurosci 114: 364-373.

Wong DF, Kuwabara H, Horti AG, et al. Quantification of cerebral cannabinoid receptors subtype 1 (CB1) in healthy subjects and schizophrenia by the novel PET radioligand [11C]OMAR. Neuroimage 2010; 52: 1505-13.

Wong DF, Wagner HN Jr, Tune LE, Dannals RF, Pearlson GD, Links JM et al. (1986). Positron emission tomography reveals elevated D2 dopamine receptors in drug-naive schizophrenics. Science 234: 1558-1563.

Zamberletti E, Prini P, Speziali S, Gabaglio M, Solinas M, Parolaro D, et al. (2012). Gender-dependent behavioral and biochemical effects of adolescent delta-9-tetrahydrocannabinol in adult maternally deprived rats. Neuroscience 204: 245-257.

Zamberletti E, Viganò D, Guidali C, Rubino T, Parolaro D. Long-lasting recovery of psychotic-like symptoms in isolation-reared rats after chronic but not acute treatment with the cannabinoid antagonist AM251. Int J Neuropsychopharmacol. 2010 Oct 6:1-14.

Zanettini C, Panlilio LV, Alicki M, Goldberg SR, Haller J, Yasar S. Effects of endocannabinoid system modulation on cognitive and emotional behavior. Front Behav Neurosci 2011; 5: 57.

Zavitsanou K, Garrick T, Huang XF. Selective antagonist [3H]SR141716A binding to cannabinoid CB1 receptors is increased in the anterior cingulate cortex in schizophrenia. Prog Neuropsychopharmacol Biol Psychiatry 2004; 28: 355-60.

Zuardi AW, Crippa JA, Hallak JE, et al. Cannabidiol for the treatment of psychosis in Parkinson's disease. J Psychopharmacol 2009; 23: 979-83.

Zuardi AW, Hallak JE, Dursun SM, et al. Cannabidiol monotherapy for treatment-resistant schizophrenia. J Psychopharmacol. 2006; 20: 683-6.

Zuardi AW, Morais SL, Guimarães FS, Mechoulam R. Antipsychotic effect of cannabidiol. J Clin Psychiatry 1995; 56: 485-6.

Zuardi AW, Rodrigues JA, Cunha JM. Effects of cannabidiol in animal models predictive of antipsychotic activity. Psychopharmacology (Berl) 1991; 104: 260-4.