Cannabinoid and neuregulin 1 gene interaction as an animal model of increased vulnerability to schizophrenia

---

Aurélie A. Boucher

A thesis submitted in fulfilment
of the requirements for the degree of
Doctor of Philosophy

Department of Pharmacology
The University of Sydney

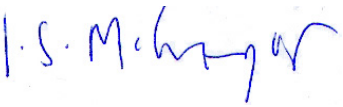
The publication of the two research papers from Chapter 2 and 3 relied on close collaboration with researchers from diverse laboratories in Sydney and in France. Most of the experiments were conducted in different Departments within the University of Sydney and at the Garvan Institute of Medical Research. I have been involved at all stages of the work. Specifically, I prepared the drug under the supervision of Dr. Arnold in the Department of Pharmacology before treating all the animals intraperitoneally prior to behavioural testing. I then tested them in different behavioural tasks with the help of Dr. Karl and Ms Liesl Duffy at the Garvan Institute of Medical Research. For the work published in the *Neuroscience* paper, the animals were then perfused by Dr. Arnold and I collected and sliced the perfused brains at 40 µm. I then performed c-Fos immunohistochemistry under the supervision of Dr. Hunt before mounting and coverslipping all the tissues. I counted some regions of the brain but Dr. Hunt counted the majority of the areas.

For the papers, I wrote the entire manuscript as a first draft. I then improved the Introduction, Methods and Results sections after comments from my supervisor Dr Arnold. We then worked on the Discussion together. I am also very grateful of Dr. Tim Karl, Dr. Glenn Hunt, Prof. Jacques Micheau, Prof. Iain McGregor and Prof. Peter Schofield for their valuable feedback on the manuscript and for the laboratory space they have offered to aid in the completion of my PhD research.

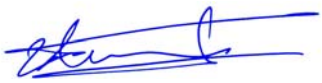
Ms Aurélie Boucher 

Dr. Jonathon Arnold 

Dr. Tim Karl 

Prof. Iain McGregor 

Dr. Glenn Hunt 

Prof. Jacques Micheau 

Ms Liesl Duffy 

Prof. Peter Schofield 

TABLE OF CONTENTS

ACKNOWLEDGMENTS

First and foremost I would like to thank my supervisor Dr. Jonathon Arnold for his constant support and precious help throughout this amazing journey. I am very grateful of his assistance in the lab, for always being considerate especially with my English at the beginning, for all the interesting discussions about my project and help in Pharmacology and for teaching me how to write and present scientific research, and also for his cheerfulness that made this PhD so much easier. Thanks for the fantastic mentor that he has been during all these years.

Also I want to sincerely thank Prof. Jacques Micheau for his support since the beginning of my undergraduate studies, for his precious encouragement in the cotutelle project and all his help for the completion of this thesis. I although want to thank Dr Jean-Louis Guillou for first introducing me to behavioural neurosciences almost 10 years ago. I am also thankful to Dr. Georges Di Scala and everyone from the Centre for Integrative and Cognitive Neuroscience at the University of Bordeaux 1 for always welcoming me in their laboratory; Merci!

Then I want to thank my associate supervisor and friend Dr. Tim Karl for all the help in the behavioural testing and discussions about this project as well as for his support during all these years. I also want to thank Dr. Glenn Hunt for his valuable expertise in all the immunohistochemistry experiments and his kindness; it has been a pleasure to work by his side. I also want to thank Prof. Iain McGregor for his feedback on the manuscripts and to allow me to use lab facilities whenever I needed. I also give a huge thanks to Ms Liesl Duffy for her help in the behavioural testing and for always being a friend even in difficult moments.

I now wish to say a special thanks to the Schizophrenia Research Institute for not only offering a scholarship for all these years, but also for their amazing kindness and compassion especially when arriving in Australia. Daren, Debby, thank you so much for taking care of me.

I also wish to thank the Clive and Vera Ramaciotti foundation for providing financial support to this project and the Garvan Institute of Medical Research for letting me use their behavioural facilities. I also want to thank the reviewers that will examine this thesis, thanks for your time spent and your feeback on this work.

I want to give a huge thank to Silvana Hourcade and all the department of Pharmacology at the University of Sydney for always being so helpful, kind and funny, I had an amazing time during all these years in such a pleasant atmosphere.

Finally I want to thank my family for supporting me in the project to work in Sydney even if I know the distance has not always been easy to deal with, all my new Aussie friends and family that taught me how to be “no worries mate” and all my friends from France who have always been there for me. Finally, the most important person I would like to thank is my amazing fiancé Jono, I would never thought I would have had to go so far away to find someone so wonderful.

PUBLICATIONS AND PRESENTATIONS

Publications and manuscripts

A. A. Boucher, T. Karl, L. Duffy, G. E. Hunt, P. R. Schofield, J. Micheau, J. C. Arnold. Differential neurobehavioural effects of chronic cannabinoid treatment on heterozygous Neuregulin 1 mice. *In preparation.*

A. A. Boucher, L. Vivier, M. Metna-Laurent, C. Darnet, L. Brayda-Bruno, N. Mons, J. C. Arnold, J. Micheau. Acute and chronic treatment of Δ9-tetrahydrocannabinol on learning and memory – correlates with Zif 268 expression in mouse forebrain. *accepted for publication in Behavioural Pharmacology on the 3rd of November 2008.*

A. A. Boucher, G. E. Hunt, T. Karl, J. Micheau, I. S. McGregor, J. C. Arnold. Heterozygous neuregulin 1 mice display greater baseline and Delta(9)-tetrahydrocannabinol-induced c-Fos expression. *Neuroscience*. 2007 Nov 23;149(4):861-870.

A. A. Boucher, J. C. Arnold, L. Duffy, P. R. Schofield, J. Micheau, T. Karl. Heterozygous neuregulin 1 mice are more sensitive to the behavioural effects of Delta9-tetrahydrocannabinol. *Psychopharmacology*. 2007 Jun;192(3):325-36.

Conference presentations

A. A. Boucher. Heterozygous Neuregulin 1 mice display altered sensitivity to the neurobehavioural effects of cannabinoids: an animal model of drug-induced psychosis. Bosch Institute Seminar, Sydney, Australia, May 2008.

A. A. Boucher. Heterozygous Neuregulin 1 mice display altered sensitivity to the neurobehavioural effects of cannabinoids**.** VII Annual Young Investigators Symposium, Bosch Institute, Sydney, Australia, December 2007.

A. A. Boucher, J. C. Arnold, T. Karl, J. Micheau. Acute behavioral effects of Δ9-tetrahydrocannabinol on the neuregulin 1 knockout mice: a new animal model of drug induced psychosis. Bosch Institute Monthly Seminar, Sydney, Australia, April 2006.

Conference poster presentations

A.A. Boucher, L. Vivier, M. Metna-Laurent, C. Darnet, L. Brayda-Bruno, N. Mons, J.C. Arnold, J. Micheau. Acute and chronic treatment of Δ9-tetrahydrocannabinol on learning and memory – correlates with Zif 268 expression in mouse forebrain. 12th European Behavioural Pharmacology Society Meeting, Tübingen, Germany, August-September 2007.

A. A. Boucher, J. C. Arnold, L. Duffy, B. Dean, G. Pavey, J. Micheau , P. R. Schofield, T. Karl. Behavioural investigation of a neuregulin 1 knockout model in schizophrenia research? International Brain Research Organization, World Congress of Neuroscience, Melbourne, Australia, July 2007.

A. A. Boucher, J. C. Arnold, T. Karl, J. Micheau. Acute behavioral effects of Δ9-tetrahydrocannabinol on the neuregulin 1 knockout mice: a new animal model of drug induced psychosis. VI Annual Young Investigators Symposium, Bosch Institute, Sydney, Australia, December 2006.

A. A. Boucher, L. Duffy, P. R. Schofield, J. Micheau, T. Karl, J. C. Arnold. Cannabis use in an animal model for schizophrenia: effects of acute Δ^9-tetrahydrocannabinol treatment in neuregulin1 knockout mice. Inaugural Addiction Neuroscience Network Australia Workshop, Hamilton Island, Australia, September 2006.

A. A. Boucher, T. Karl, L. Duffy, P. R. Schofield, J. Micheau, J. C. Arnold. Cannabis use in an animal model for schizophrenia: effects of acute Δ^9-tetrahydrocannabinol treatment in neuregulin1 knockout mice. 5th Forum of European Neurosciences, Vienna, Austria, July 2006.

LIST OF FIGURES AND TABLES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

ABBREVIATIONS

Ac	nucleus accumbens
Am	amygdala
ANOVA	analysis of variance
BNST	bed nucleus of the stria terminalis
BSA	bovine serum albumine
BT	body temperature
BW	body weight
C	cerebellum
CEA	central nucleus of the amygdala
CNS	central nervous system
COMT	catechol-O-methyltransferase
CPU	caudate putamen
CSF	cerebrospinal fluid
DA	dopamine
DISC-1	disrupted-in-schizophrenia 1
DSM	diagnostic and statistical manual of mental disorders
DTNBP1	dysbindin
EGF	epidermal growth factor
EPM	elevated plus maze
G72	D-amino-acid oxidase activator
GABA	gamma-aminobutyric acid
GIRK	G-protein-responsive potassium channel
HB	hole board
Hip	hippocampus
Hyp	hypothalamus
HT	hypothalamus
Ig	immunoglobuline
i.p.	intraperitoneally
LD	light-dark
LH	lateral hypothalamus
LI	latent inhibition
LSD	lysergic acid diethylamide
LSV	ventrolateral septum
MAPK	mitogen-activated protein kinase
NAC	nucleus accumbens / nucleus accumbens core
NAS	nucleus accumbens shell
NMDA	N-methyl-D-aspartate
NPT	non pre-treated
Nrg1	animal neuregulin 1 gene
NRG1	human neuregulin 1 gene
NRG1	neuregulin 1 protein
Nrg1 HET	heterozygous neuregulin 1 transmembrane-domain knockout
OF	open field
P	pituitary
PAG	periaqueductal gray

PB	phosphate buffer
PBH	phosphate-buffered horse serum
PCP	phencyclidine
PFA	paraformaldehyde
PFC	prefrontal cortex
PKA	protein kinase A
PPI	prepulse inhibition
PRODH	proline dehydrogenase
PT	pre-treated
PV or PVT	paraventricular nucleus of the thalamus
PVN	paraventricular nucleus of the hypothalamus
RGS4	regulator of G-protein signalling 4
SEM	standard error of the mean
sep	septum
SI	social interaction
SN	substantia nigra
Str	striatum
Th	thalamus
THC	Δ^9-tetrahydrocannabinol
TX	triton X100
VEH	vehicle
VMH	ventromedial hypothalamus
VOC	voltage-operated calcium channel
VTA	ventral tegmental area
WHO	world health organisation
WT	wild type-like

RÉSUMÉ FRANCAIS

La schizophrénie est un désordre mental chronique et sévère avec une prévalence mondiale d'environ 1 %. Les premiers symptômes psychotiques de cette maladie apparaissent généralement à la fin de l'adolescence ou au début de la vie adulte. Les symptômes de la schizophrénie sont classés en symptômes positifs, négatifs et cognitifs. Les symptômes positifs tels que les hallucinations et délires correspondent à la phase psychotique de la maladie, alors que les symptômes négatifs de retrait social et diminution de l'expression des émotions sont constamment présents. Les causes de la schizophrénie proviennent d'une interaction entre facteurs génétiques et facteurs environnementaux, parmi lesquels l'usage du cannabis.

Des études ont montré que l'utilisation du cannabis peut augmenter le risque de développer la schizophrénie en précipitant la maladie chez les individus qui présentent une vulnérabilité génétique aux désordres mentaux. Des recherches portant chez l'homme et l'animal indiquent que la neuréguline 1 (*Nrg1*) est un gène de susceptibilité à la schizophrénie. L'objectif de cette thèse a donc été d'examiner si un dérèglement du gène *Nrg1* module les effets comportementaux et neuronaux des cannabinoïdes.

Dans une première partie (Chapitre 2), les effets comportementaux de l'administration de Δ^9-tétrahydrocannabinol (THC), le principal composant psychotrope du cannabis, ont été évalués. Les souris mutantes hétérozygotes pour le domaine transmembranaire de *Nrg1* (*Nrg1* HET) montrent des différences de comportement d'activité locomotrice, d'exploration et d'anxiété. D'une manière primordiale, les souris *Nrg1* HET sont plus sensibles aux effets du THC sur la

suppression de l'activité locomotrice comparées aux souris sauvages. De plus, les souris *Nrg1* HET expriment une plus grande augmentation du % d'inhibition par pré-pulse (PPI) induite par le THC que les souris sauvages. Ces résultats indiquent pour la première fois que la perturbation d'un gène impliqué dans la susceptibilité à la schizophrénie module les effets des cannabinoïdes.

Apres avoir démontré que les souris *Nrg1* HET sont plus sensibles aux effets aigus du THC dans différents tests comportementaux y compris ceux qui modélisent des symptômes de la schizophrénie, nous avons mesuré les effets du THC sur l'activité neuronale chez les souris *Nrg1* HET et sauvages en utilisant l'expression de c-Fos mesurée par immunohistochimie (Chapitre 3). Dans le septum latéral, le THC accentue l'expression de c-Fos uniquement chez les souris *Nrg1* HET. De plus, le THC induit une augmentation de l'expression de c-Fos dans le noyau central de l'amygdale, le noyau du lit de la strie terminale et dans le noyau paraventriculaire de l'hypothalamus plus masquée chez les souris *Nrg1* HET que chez les souris sauvage. Conformément à l'expression d'un phénotype associé à la schizophrénie chez les souris *Nrg1* HET, ces animaux présentent une augmentation d'expression de c-Fos dans le septum latéral et la coquille du noyau accumbens. De plus, l'effet du génotype sur l'expression de c-Fos, sans drogue ou après exposition au THC, est observé seulement quand les animaux ont été soumis à des tests comportements avant d'être perfusés. Ceci suggère qu'un stress est nécessaire pour promouvoir ces effets. Ces données fournissent des corrélations neurobiologiques attestant l'augmentation de la sensibilité comportementale des souris *Nrg1* HET au THC. Ces travaux rapportés dans le Chapitre 2 renforcent l'hypothèse de l'existence d'une interaction entre les cannabinoïdes et la neuréguline 1.

Comme le risque de développer la schizophrénie chez les individus vulnérables augmente avec la fréquence d'utilisation du cannabis, le Chapitre 4 évalue les effets de l'administration répétée de CP 55,940, un analogue synthétique du THC. Les tests comportementaux ont montré que la tolérance aux effets hypothermiques et hypolocomoteurs du CP 55,940 apparait plus rapidement chez les souris *Nrg1* HET que chez les souris contrôles. Inversement, une tolérance à l'effet anxiogène du CP 55,940 s'est développée chez les souris témoins, mais pas chez les souris *Nrg1* HET. Dans la PPI, des effets opposés du CP 55,940 ont été observés lors du premier jour d'exposition, avec une facilitation de la PPI chez les souris *Nrg1* HET souris et une diminution chez les souris contrôles. Toutefois, cet effet différentiel entre les souris *Nrg1* HET et témoins ne se maintient pas avec la répétition des administrations de CP 55,940, les deux génotypes devenant tolérants aux effets du cannabinoïde sur la PPI. En outre, une augmentation de l'expression de Fos B / ΔFos B, un marqueur des changements neuronaux à long terme, a été observée dans le septum ventrolatéral des souris *Nrg1* HET après exposition chronique au CP 55,940 mais cet effet n'a pas été observé chez les souris contrôles. Ces résultats indiquent que le système de la neuréguline 1 est impliqué dans la réponse neuroadaptative à l'exposition répétée aux cannabinoïdes.

Les déficits cognitifs figurent parmi les principaux endophénotypes observés dans la schizophrénie. Ainsi, le chapitre 5 propose une procédure qui permettrait d'évaluer les effets des cannabinoïdes sur les déficits de mémoire de travail chez la souris wild-type. Peu d'études se sont intéressées aux effets de l'exposition chronique aux cannabinoïdes sur la mémoire et ont examiné si une tolérance à ces effets se produit. Ici, nous avons étudié les effets de l'exposition répétée au THC sur la mémoire spatiale et l'expression de zif268, un autre gène de mesure d'activité

neuronale. Un groupe d'animaux n'a pas été prétraité avec du THC alors qu'un autre groupe a reçu 13 injections quotidiennes de THC (1mg/kg/jour; i.p.) avant d'être soumis aux tests de mémoire dans le labyrinthe aquatique de Morris. Les deux groupes ont reçu le THC durant toute la phase comportementale. Le THC a diminué la mémoire spatiale et la mémoire de travail, en particulier la flexibilité comportementale, même chez les animaux prétraités par le THC et qui étaient déjà tolérants à ses effets hypolocomoteurs. L'expression de zif268 a été significativement réduite dans l'aire CA3 de l'hippocampe et dans le cortex préfrontal seulement chez les animaux n'ayant pas reçu de prétraitement. Ceci suggère que la tolérance aux effets des cannabinoïdes apparait plus rapidement sur l'activité neuronale que sur les effets comportementaux, ces derniers persistant après 24 jours d'exposition. Cette étude montre que l'administration répétée de THC perturbe la flexibilité comportementale et que ces effets sont résistants au phénomène de tolérance. Il serait intéressant d'examiner si ces déficits de mémoire induits par les cannabinoïdes sont modifiés chez les souris *Nrg1* HET par rapport aux souris contrôles.

En conclusion, les résultats présentés dans cette thèse fournissent la première preuve que la suppression partielle d'un gène de susceptibilité de la schizophrénie, le *Nrg1,* module l'action comportementale et neurobiologique des cannabinoïdes administrés en traitement aigu ou chronique. Cette recherche met en évidence que les systèmes de la neuréguline et des cannabinoïdes interagissent dans le system nerveux central, et accroît nos connaissances sur la façon dont les facteurs génétiques augmentent la vulnérabilité d'un individu à la schizophrénie et à la psychose induite par le cannabis.

ABSTRACT

Schizophrenia is a severe, chronic and disabling mental disorder with a worldwide prevalence of approximately 1 %. It is a lifelong illness characterized by psychotic symptoms which typically first appear in late adolescence/early adulthood. The symptoms of schizophrenia are usually categorized as positive (hallucinations and delusions), negative (blunted affect and poverty of speech) and cognitive (memory, attention and executive function impairments). Schizophrenia is thought to arise from an interaction between several susceptibility genes and environmental factors, one of them being the use of cannabis, the most widely used illicit drug in the world. Human population studies show that cannabis use is associated with schizophrenia, and it is now well recognised that cannabis use increases the risk of developing schizophrenia by approximately twofold.

The reasons for the association between cannabis and schizophrenia remain controversial and different theories have been proposed to explain the nature of this relationship. According to the self-medication hypothesis of schizophrenia, patients with psychotic disorders use cannabis to alleviate aversive symptoms of the disorder or the side effects associated with antipsychotic medications. Other theories posit that cannabis is a component cause contributing to the development of schizophrenia. Supporting this, an increasing body of evidence shows that cannabis use increases the incidence and severity of psychotic symptoms and that cannabis use most frequently precedes the onset of schizophrenia. As a large majority of cannabis users do not develop schizophrenia, a gene-environment interaction appears necessary for the

development of the disorder. That is, cannabis use may unmask latent schizophrenia in individuals that have a genetic predisposition to the disorder.

Family studies provide strong evidence of a genetic contribution to the aetiology of schizophrenia. Several candidate genes are likely involved in the disorder, but this thesis will specifically focus on the neuregulin 1 (*NRG1*) gene. *NRG1* was first proposed as a schizophrenia susceptibility gene in 2002 and linkage studies have since replicated this association in diverse populations around the world. In addition, changes in expression of Nrg1 isoforms and its receptor ErbB4 have been reported in the brain of schizophrenia patients. *NRG1* polymorphism has also been associated with cognitive and behavioural differences in schizophrenia patients compared to healthy individuals. Collectively, *NRG1* is now recognized as one of the most promising genes that confer an increased risk of developing schizophrenia

The creation of knockout mice lacking a specific gene offers an exciting new approach in the study of mental disorders. While several mutant mice for *Nrg1* and ErbB4 receptor have been developed, this thesis focussed on mice that are heterozygous for the transmembrane domain of the *Nrg1* gene (named *Nrg1* HET mice). These mice exhibit a schizophrenia-like phenotype including hyperactivity that can be used as a reflection of positive symptoms of schizophrenia. Furthermore, they display impairments in social recognition memory and prepulse inhibition (PPI), a model of attentional deficits observed in schizophrenia patients. In addition, the brains of *Nrg1* HET contain fewer functional NMDA receptors and more 5-HT_{2A} receptors than wild type-like (WT) animals which is consistent with the neurotransmitters imbalance observed in schizophrenic patients. The phenotype of *Nrg1* HET mice is age-dependent, another aspect that mirror the late adolescent/early adulthood onset of schizophrenia symptoms. The present thesis aimed at developing an animal model of

genetic vulnerability to cannabinoid-precipitated schizophrenia by utilising *Nrg1* HET mice to observe if these animals show an altered behavioural and neuronal response to cannabinoid exposure. We hypothesise that *Nrg1* deficiency will alter the neurobehavioural responses of animals to cannabinoids.

The experiments detailed within the first research chapter (Chapter 2) aimed at examining the behavioural effects of an acute exposure to the main psychoactive constituent of cannabis, Δ^9-tetrahydrocannabinol (THC), in *Nrg1* HET mice using a range of behavioural tests of locomotion, exploration, anxiety and sensorimotor gating. Relative to WT control mice, *Nrg1* HET mice were more sensitive to both the locomotor suppressant action of THC, as measured in the open field test, and to the anxiogenic effects of THC in the light-dark test, although the effects in this procedure may be confounded by the drug-free hyperactive phenotype of *Nrg1* HET mice. Importantly, *Nrg1* HET mice expressed a greater THC-induced enhancement in PPI than WT mice. Taken together, the data presented in Chapter 2 show that a deficiency in a schizophrenia susceptibility gene *Nrg1* enhanced the behavioural impact of THC.

After having established a link between *Nrg1* deficiency and increased sensitivity to the behavioural effects of cannabinoids in Chapter 2, Chapter 3 assessed the neuronal activity underlying the effects of an acute THC exposure on *Nrg1* HET mice by using c-Fos immunohistochemistry. In the ventral part of the lateral septum (LSV), THC selectively increased c-Fos expression in *Nrg1* HET mice with no corresponding effect being observed in WT mice. In addition, a non-significant trend for THC to promote a greater increase in c-Fos expression in *Nrg1* HET mice than WT mice was observed in the central nucleus of the amygdala, the bed nucleus of the stria terminalis and the paraventricular nucleus of the hypothalamus. Consistent with *Nrg1* HET mice exhibiting a schizophrenia-related phenotype, these mice expressed

greater drug-free levels of c-Fos in the shell of the nucleus accumbens and the LSV. Interestingly, the effects of genotype on c-Fos expression, drug-free or following THC exposure, were only observed when animals experienced behavioural testing prior to perfusion. This suggests that an interaction with stress was necessary for the promotion of these effects.

As the risk of developing psychosis in vulnerable individuals increases with the frequency of cannabis use, Chapter 4 assessed the effects of repeated exposure to cannabinoids on *Nrg1* HET mice. As THC was not available at the time, the synthetic analogue of THC, CP 55,940, was used in this experiment. Behavioural testing showed that tolerance to CP 55,940-induced hypothermia and locomotor suppression developed more rapidly in *Nrg1* HET mice compared to WT mice. On the contrary, tolerance to the anxiogenic-like effect of CP 55,940 in the light-dark test was observed in WT mice, however no such tolerance occurred to this effect in *Nrg1* HET mice. Similarly, no tolerance developed to CP 55,940-induced thigmotaxis in *Nrg1* HET mice as measured in the open field. For PPI, on the first day of exposure opposite effects were observed, with CP 55,940 treatment facilitating PPI in *Nrg1* HET mice and decreasing it in WT mice. However, the differential effect of CP 55,940 on PPI was not maintained with repeated testing as both genotypes became tolerant to the effects of the cannabinoid on sensorimotor gating. In addition, a selective increase in Fos B/ΔFos B expression, a marker of longer-term neuronal changes, was observed in the LSV of *Nrg1* HET mice following chronic CP 55,940 exposure, with no corresponding effect seen in WT mice. These results collectively demonstrate that the neuregulin system is involved in the neuroadaptive response to repeated cannabinoid exposure.

One of the main schizophrenia endophenotypes observed in human studies are cognitive impairments of higher executive functions. Thus Chapter 5 aimed to develop a procedure to allow evaluation of cannabis-induced working memory deficits in mice. Few studies have investigated the effects of chronic cannabinoid exposure on memory performance and whether tolerance occurs to cannabinoid-induced memory impairment. Here we studied the effects of repeated exposure to THC on spatial memory and the expression of the immediate early gene zif268 in mice. One group of animals were not pre-treated with THC while another group was given 13 daily injections of THC prior to memory training and testing in the Morris water maze. Both groups were administered THC throughout the memory training and testing phases of the experiment. THC decreased spatial memory and reversal learning, even in animals that received the THC pre-treatment and were tolerant to the locomotor suppressant effects of the drug. Zif268 immunoreactivity was reduced in the CA3 of the hippocampus and in the prefrontal cortex only in non pre-treated animals, indicating that while tolerance to the effects of cannabinoids on neuronal activity arose, cannabinoid-promoted memory impairment in these animals persisted even after 24 days of exposure. Taken together these data demonstrate that the spatial memory impairing effects of THC are resistant to tolerance following extended administration of the drug. Such a model could be applied to *Nrg1* HET mice in future studies to observe if cannabinoid-induced working memory impairments and the development of tolerance to this effect are altered relative to WT mice.

In conclusion, this thesis provides the first evidence that partial deletion of the schizophrenia susceptibility gene *Nrg1* modulates the neurobehavioural actions of acutely and chronically administered cannabinoids. *Nrg1* HET mice appear more sensitive to the acute neurobehavioural effects of cannabinoids. Notably, acutely

administered THC facilitated attentional function by increasing PPI in *Nrg1* HET mice. However, with repeated cannabinoid administration this acute benefit was lost. The *Nrg1* HET mice displayed a long-lasting anxiogenic profile that was resistant to tolerance. Conversely, *Nrg1* HET mice developed tolerance to the locomotor suppressant and hypothermic effects of cannabinoids more rapidly than WT mice, indicating a distorted neuroadaptive response in these animals. Another major finding of this thesis is that the lateral septum appears to be an important brain region dysregulated by cannabinoids in *Nrg1* HET mice. Cumulatively, this research highlights the fact that neuregulin 1 and cannabinoid systems appear to interact in the central nervous system. This may ultimately enhance our understanding of how gene-environment interactions are responsible for cannabis-induced development of schizophrenia.

Chapter 1

CANNABIS AND NEUREGULIN AS A MODEL OF SUSCEPTIBILTY TO SCHIZOPHRENIA

1. Schizophrenia

1.1. Epidemiology

Schizophrenia is a severe, chronic and disabling mental disorder. It was first described as specific brain pathology, "dementia praecox", in the early 20th century by Emil Kraepelin, and then renamed "schizophrenia" (from the Greek roots *schizein* "to spilt" and *phrēn* "the mind") by Eugen Bleuler (Black and Boffeli, 1989) (see Fig. 1). A recent epidemiological study indicates that about 7 individuals per 1000 will develop schizophrenia during their lifetime (McGrath et al., 2008), and data from the World Health Organization (WHO) report that approximately 25 million persons are suffering from this disorder in the world (WHO, 2008). Schizophrenia is a lifelong illness with symptoms typically beginning in late adolescence/early adulthood, and males usually having an earlier onset than females (Jablensky, 2000). This disorder causes significant distress to the patient and also to the patient's family and friends. Approximately 10 % of schizophrenia patients commit suicide (Meltzer, 2002). People with schizophrenia are often unemployed, poor and homeless with many spending long periods of time in mental health facilities. This incurs a major cost to society, where 2 % of Australia's health and community expenditure is accounted for by this disorder (Carr et al., 2004) and the annual cost of medical management of schizophrenia in France is estimated to be billions of dollars (Rouillon et al., 1997).

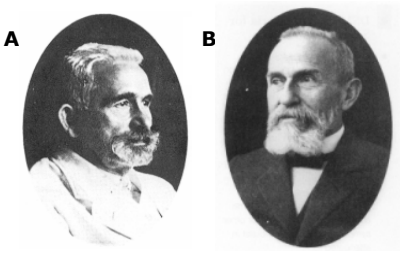


Figure 1. (A) Emil Kraepelin (1856-1926) and (B) Eugen Bleuler (1857-1939), were German and Swiss psychiatists.

1.2. Symptoms

Many different types of psychotic disorders have been characterized and they are categorized by the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) as schizoaffective, schizophreniform, delusional, shared psychotic and brief psychotic disorders (DSM-IV). They all have a slightly different symptomatology but this thesis will focus specifically on schizophrenia. This disorder must last for at least 6 months to be classified as schizophrenia and include the presence of at least 2 of the characteristic symptoms of impaired contact with reality and evidence of social or occupational dysfunction (each of 1 month duration minimum). These symptoms should not be attributable to mood, medical and substance abuse disorders (DSM-IV). More specifically, the psychiatric symptoms of schizophrenia are usually classified as positive and negative. The positive symptoms reflect an excess or distortion of normal functions, and include psychotic symptoms such as distortion in perception (hallucinations) and thought content (delusions), disorganized speech and abnormal behaviours (DSM-IV). These positive symptoms are usually present at discrete periods in time which are often classified as "psychotic episodes", while the negative symptoms of schizophrenia are present persistently during the course of the illness. Negative symptoms reflect a loss of normal functions and include decreased emotions (affective flattening/blunted affect) and pleasure (anhedonia), poverty of thought and speech (alogia) and lack of motivation (avolition/apathy) (DSM-IV). These symptoms are usually associated with a general intellectual decline with decreased performance in most cognitive functions like memory, attention and executive functions (Mitchell et al., 2001).

1.3. Treatments and early theories of schizophrenia

The neuronal mechanisms that underline the symptoms of schizophrenia have been investigated for more than half a century, and are thought to arise from an imbalance of many different neurotransmitter systems. Advances in this area came from the introduction in 1952 of the first antipsychotic drug, chlorpromazine. The therapeutic actions of this neuroleptic were first observed by a French surgeon, Henri Laborit, who used the drug as a surgical anaesthetic because of its highly sedative and calming effects (Shen, 1999). It was then realized that these so-called classical or typical antipsychotic drugs like chlorpromazine or haloperidol are effective in reducing symptoms of schizophrenia, particularly positive symptoms such as hallucinations and delusions (Levinson, 1991).

The observation that typical antipsychotic drugs are antagonists for dopamine D2 receptors, and that drugs such as amphetamine which enhance dopamine release may promote psychotic symptoms, led to the formulation of the "dopamine hypothesis" that positive symptoms of schizophrenia are attributable to a hyperactivity of dopamine function (Seeman, 1987). There are 4 different dopaminergic pathways in the brain (Fig. 2) and the activation of the mesolimbic dopaminergic pathway that extends from cell bodies in the ventral tegmental area (VTA) to the nucleus accumbens (ventral striatum) is most likely dysregulated in schizophrenia. This pathway is involved in addiction and studies

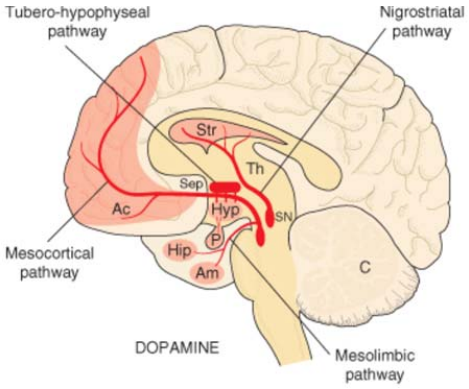


Figure 2. The different dopaminergic pathways in the brain. The location of the main groups of cell bodies and fibre tracts is shown in red. Pink areas show the location of dopaminergic terminals. (Ac, nucleus accumbens; Am, Amygdala; C, cerebellum; Hip, hippocampus; Hyp, hypothalamus; p, pituitary; sep, septum; SN, substantia nigra; Str, striatum; Th, thalamus). *Adapted from Rang and Dale's Pharmacology, 2007, Churchill Livingstone.*

suggest that the elevated levels of dopamine in response to drugs accentuate the attribution of salience to stimuli and conditioned learning, inducing addictive behaviours (Volkow et al., 2007). Within the pathophysiological framework of schizophrenia, increased activity of this pathway could be responsible for the association of abnormal importance to internal or external stimuli, thus producing hallucinations and delusions (Stone et al., 2007, Murray et al., 2008). The strong dopamine D2 receptor antagonism of typical antipsychotic drugs renders them markedly efficient in reducing positive symptoms of schizophrenia.

However typical antipsychotics are inefficient in reducing the negative and cognitive symptoms of schizophrenia and these drugs are associated with severe side effects. They are extremely sedative and induce weight gain, metabolic disorders or sexual dysfunction as well as the development of unwanted extrapyramidal motor symptoms (Levinson, 1991, Nikam and Awasthi, 2008). These Parkinson-like symptoms are thought to be caused by the dopamine antagonism of antipsychotic drugs that also block dopamine transmission in the nigrostriatal pathway (Fig. 2), a region known to regulate motor behaviour.

The absence of effect of antipsychotic drugs on the negative symptoms of schizophrenia suggests that other neurotransmitter systems are involved in the disorder. Evidence of decreased glutamatergic levels in the cerebrospinal fluid (CSF) of schizophrenic patients led to the hypothesis of a dysfunction in glutamatergic function in schizophrenia (Kim et al., 1980). In addition, glutamate NMDA receptor antagonists, such as phencyclidine or ketamine, induce most of the positive, negative and cognitive symptomatic features of schizophrenia in healthy individuals and exacerbate psychosis in schizophrenic patients (Allen and Young, 1978, Coyle, 1996, Javitt, 2007). Clinical studies also showed that drugs which enhance NMDA receptor

function reduce negative and cognitive symptoms in schizophrenia patients. From these observations developed the theory that hypoglutamatergic neurotransmission in limbic and cortical regions such as frontal cortex and hippocampal formation might subserve the negative and cognitive symptoms of schizophrenia (Coyle, 1996, Mitchell et al., 2001).

The diversity of the neurotransmitter systems involved in schizophrenia is also highlighted by the introduction in the 1970's of the first atypical antipsychotic, clozapine. This provided a turning point in schizophrenia treatment as these drugs induce fewer extrapyramidal side-effects as they are weaker antagonists for the dopamine D2 receptor than typical antipsychotics. In addition, besides decreasing positive symptoms, atypical antipsychotics are also effective in reducing the negative symptoms of schizophrenia (Meltzer et al., 1989, Lindenmayer, 1995, Shen, 1999, Nikam and Awasthi, 2008). Even if all antipsychotic drugs available are dopamine D2 antagonists, these atypical antipsychotic drugs have different selectivity for other dopamine receptor subtypes and they are known to interact with additional neurotransmitter receptors having particularly high affinity for serotonin 5-HT_2 receptors. This reinforces the notion that multiple neurotransmitters are involved in the pathophysiology of schizophrenia (Gerlach, 1991, Lindenmayer, 1995).

The most recent theories of schizophrenia acknowledge that both genetic (see section 3) and environmental factors are involved in the pathogenesis of the disorder. The current theories within the literature posit that schizophrenia cannot be attributed to a single cause, but rather is a multifactorial neurodevelopmental disorder, involving the interaction of several susceptibility genes with environmental factors. This thesis will focus on modelling the interaction between genes and the environment. Environmental factors play an important role in triggering the development of

schizophrenia (Henquet et al., 2008). Examples of environmental factors associated with the disorder include pregnancy and delivery complications, exposure to viral infections or to stress during gestation or childhood, nutritional deficiencies or psychosocial deficiencies like low intelligence quotient, urban upbringing, being an immigrant, having an older father, low socioeconomic status and winter birth (Jablensky, 2000, Tandon et al., 2008). While all these factors may indeed play a role in the emergence of schizophrenia, this thesis is specifically concerned with the relationship between cannabis use and genetic vulnerability in the development of the disorder.

2. The role of cannabis in the development of schizophrenia

2.1. Cannabinoid pharmacology

Extracts of the plant *Cannabis sativa* have been used for medicinal and recreational purposes for millennia, the first documented case dating back 5000 years ago in China (Mikuriya, 1969). It was introduced to the western world during the ninetieth century, and its recreational use has dramatically increased since the 1960's. Today, it is estimated that about 147 million people consume cannabis, making it the most widely used illicit drug in the world (WHO, 2008).

In the past 20 years extensive research has been undertaken to characterize cannabis pharmacology, as there is increasing interest in the use of this drug as a therapeutic agent. Cannabinoids promote analgesia and show potential in the treatment of neuropathic pain and inflammation associated with multiple sclerosis. Further, their antiemetic and appetite stimulant properties are potentially useful in treating patients with chemotherapy-induced nausea and vomiting (Hosking and Zajicek, 2008). In addition, blockade of cannabinoid CB1 receptors using rimonabant

(SR 141716) is effective in treating obesity, inducing weight loss via both central and peripheral mechanisms of action (Isoldi and Aronne, 2008). While rimonabant has recently been suspended for its use as an anti-obesity drug in Europe (Jones, 2008), it might also be developed for other applications, for example as an anti-craving compound against nicotine and alcohol dependence (Colombo et al., 2007, Le Foll et al., 2008). Interestingly, the USA's Food and Drug Administration has been reticent to introduce rimonabant as a therapeutic agent given evidence that it might increase the risk of psychiatric disturbances in patients. The association between cannabinoids and mental disorder remains one of the major stumbling blocks for the introduction of cannabinoid-based therapies.

Cannabinoid consumption produces a range of effects on the central nervous system (CNS) including euphoria, sensory magnification and impairment of short-term memory, attention and motor function. Cannabis use also induces some peripheral effects such as tachycardia, vasodilatation of the eyes, reduction of intraocular pressure, bronchodilatation and immunosuppression (Abood and Martin, 1992, Iversen, 2003). Cannabis is relatively safe in overdose, and does not induce death in itself. There are however a lot of adverse effects associated with the use of the drug. Even at low doses, cannabis produces drowsiness and confusion, sometimes accompanied by anxiety, panic attacks, paranoia, hallucinations and depersonalisation. Cannabis also decreases motivation promoting a so-called amotivational syndrome that resembles the negative symptoms of schizophrenia (Manzanares et al., 2004). Further, the long-term exposure to the drug is associated with the development of tolerance and dependence, and also induces long term cognitive deficits in attention, memory and executive functions (Hall and Solowij, 1998).

Cannabis extracts from the plant contain more than 60 cannabinoid compounds that are highly lipid soluble (Di Marzo and Petrocellis, 2006). These phytocannabinoids include the main psychoactive constituent of cannabis, Δ^9-tetrahydrocannabinol (THC), and cannabidiol, a major non-psychotropic constituent of cannabis that may possess antipsychotic actions (Zuardi et al., 2006). From these chemical structures, synthetic cannabinoids have been developed such as CP 55,940, WIN 55,212-2 and HU 210 (Di Marzo and Petrocellis, 2006, Pertwee, 2008). The existence of an endocannabinoid system was discovered by the isolation of the endogenous ligands anandamide (Devane et al., 1992) and 2-arachidonoylglycerol (Mechoulam et al., 1995), that are chemically distinct from the phytocannabinoids and synthetic cannabinoids (see Fig. 3).

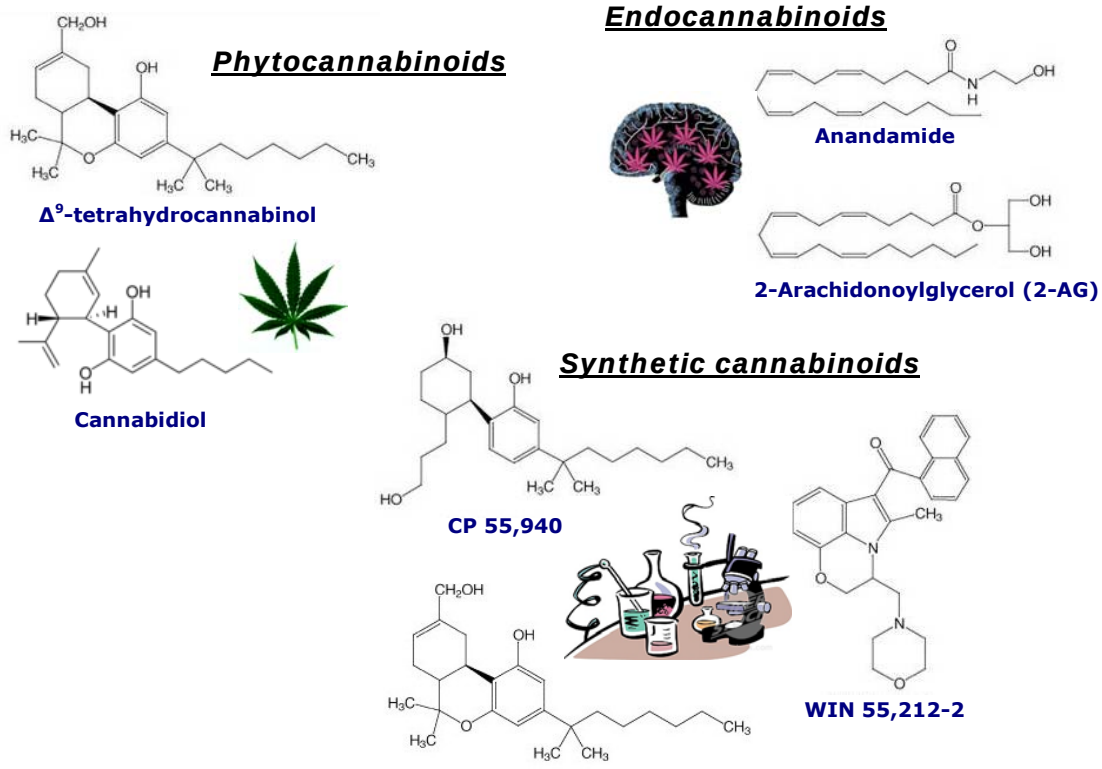


Figure 3. Cannabinoid ligands.

As their high lipophilicity allows passage across the blood brain barrier to the CNS, all cannabinoids affect brain functioning. They exert their effects by binding to the cannabinoid CB1 receptor, which was first isolated in 1988 (Devane et al., 1988). A cannabinoid CB2 receptor has also been isolated and is mainly present in the periphery where it is located predominantly on immune cells (Pertwee, 2008). However, CB2 receptors have recently been shown to be also present in the brain where they may be involved in depression and substance abuse (Onaivi et al., 2006). In addition, a growing body of evidence suggests the presence of an additional cannabinoid receptor (CB3) that is yet to be cloned (Di Marzo et al., 2000, Fride et al., 2003).

The CB1 receptor is one of the most abundant G-protein-coupled receptors in the CNS. CB1 is coupled to $G_{i/o}$ proteins that inhibit adenylate cyclase activity thus decreasing cAMP production and Ca^{2+} ion channel activity (see Fig 4). The activation of CB1 receptors also increases K^+ conductance and increases mitogen-activated protein kinase (MAPK) activity (Howlett et al., 2004). CB1 receptors are predominantly localized presynaptically, exerting inhibitory effects on synaptic transmission (Di Marzo and Petrocellis, 2006). Endocannabinoids are synthesized and released on demand and appear to act as retrograde messengers that modulate the release of various neurotransmitters implicated in the aetiology of schizophrenia,

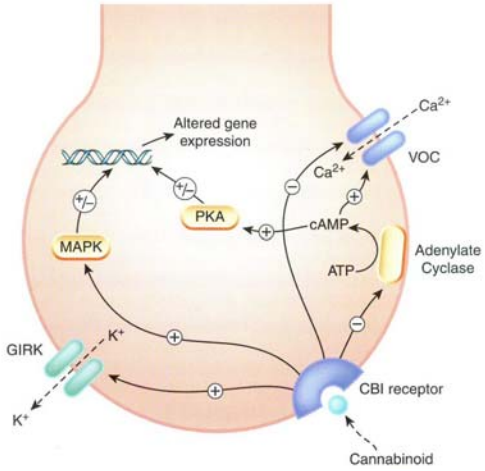


Figure 4. Intracellular pathway of CB1 receptor activation. CB1 receptor activation inhibits neurotransmitter release via inhibition of Ca^{2+} entry and hyperpolarisation due to activation of potassium channels. It also alters gene expression. (GIRK, G-protein-responsive potassium channel; MAPK, mitogen-activated protein kinase; PKA, protein kinase A; VOC, voltage-operated calcium channel). *Adapted from Rang and Dale's Pharmacology, 2007, Churchill Livingstone.*

including GABA, glutamate and dopamine (van der Stelt and Di Marzo, 2003). Some of the highest densities of CB1 receptors are found in brain regions implicated in schizophrenia that regulate diverse functions affected by cannabinoids. Specifically, CB1 receptors are localised in the hippocampus (memory), the amygdala (emotion), the cerebellum and the substantia nigra (motor function), the hypothalamus (hypothermia), the pituitary gland (hormone secretion), as well as in the cortex (alteration of higher cognitive function) (Herkenham et al., 1990, Abood and Martin, 1992).

Consistent with the localisation of the CB1 receptor in the brain, the effects of cannabinoids on humans are mirrored in animals. The administration of cannabinoids in rodents produces a characteristic combination of four symptoms: hypothermia, analgesia, hypoactivity, and catalepsy (Chaperon and Thiebot, 1999). The effects of cannabinoids on general reduction of spontaneous locomotor activity are widely reported and involve modulation of dopamine transmission in the basal ganglia where the levels of CB1 receptors are the highest in the brain (Giuffrida et al., 1999). When injected with high doses of cannabinoids, a state of hyper-reflexia can be observed where sedated mice jump in response to auditory or tactile stimuli producing a "popcorn effect" that is due to the depressant effects of the drug (Dewey, 1986). Catalepsy is another classic type of motor depression observed in rodents following high dose cannabinoid administration (Dewey, 1986).

Administration of exogenous cannabinoids in animals bidirectionally influences anxiety. While low doses have anxiolytic properties, high doses of cannabinoids induce anxiogenic responses in animal models of anxiety correlating with activation of the hypothalamo-pituitary axis (HPA) and the release of stress hormones such as ACTH and corticosterone (Valjent et al., 2002, Valverde, 2005).

Anxiety can also be measured as decreased social interaction in animals, a behaviour that is decreased by cannabinoids (Genn et al., 2004). However unlike in humans, cannabinoids exposure normally decreases food intake in laboratory animals (Valverde, 2005). In addition they modulate reward-related behaviours such as intracranial self stimulation or conditioned place aversion (Chaperon et al., 1998, Arnold et al., 2001a), and modulate cognitive processes such as memory (Riedel and Davies, 2005), attention (Solowij and Michie, 2007) and executive functions (Pattij et al., 2008).

2.2. The endogenous cannabinoid system and schizophrenia

The cannabinoid system and schizophrenia have neuronal, functional and psychological similarities, and there is increasing evidence that the endogenous cannabinoid system may in itself be dysfunctional in schizophrenia and other psychoses. In 1997, the cannabinoid hypothesis of schizophrenia was proposed (Emrich et al., 1997). The authors suggested that some schizophrenia symptoms may be related to a dysregulation of the endogenous cannabinoid system because normal healthy volunteers intoxicated with THC exhibited strong neuropsychological similarities to schizophrenia patients. Association between schizophrenia and a polymorphism for the gene that encodes the CB1 receptor, CNR1, has been delineated (Ujike et al., 2002). Interestingly, this association was only observed for the hebephrenic type of schizophrenia. This type of schizophrenia is characterized predominantly by negative symptoms that resemble the "amotivational syndrome" that can develop following chronic cannabis use (Campbell, 1976), genetically supporting that altered cannabinoid system may be involved in the pathogenesis of hebephrenic schizophrenia.

Neuroanatomical studies have analysed the levels of CB1 receptors and endocannabinoids in the brain of schizophrenia patients. Using autoradiography, a significant increase of CB1 receptor binding was found in the dorsolateral prefrontal cortex (PFC) (Dean et al., 2001), as well as in the posterior (Newell et al., 2006) and anterior (Zavitsanou et al., 2004) cingulate cortex in post mortem brains from patients. Decreased gray matter density in the right posterior cingulate cortex was also observed in cannabis-abusing first episode schizophrenia patients compared to non using schizophrenia patients (Bangalore et al., 2008). These data suggest that modulation of the endocannabinoid system in the cingulate cortex and the PFC may play a role in the impairments of higher cognitive functions observed in schizophrenia patients (Schlosser et al., 2008).

An imbalance of the endocannabinoid system has also been linked to schizophrenia, as the concentration of endocannabinoids were elevated in the blood (De Marchi et al., 2003) and the CSF of schizophrenic patients (Leweke et al., 1999). An 8-fold increased anandamide level in the CSF was observed specifically in non-medicated first episode paranoid type schizophrenia patients (Giuffrida et al., 2004). Interestingly, this alteration was absent in schizophrenia patients treated with typical antipsychotic drugs which predominately antagonise dopamine D2 receptors. Paranoid schizophrenia is characterized mainly by positive symptoms which are thought to arise from increased levels of dopamine (Oades et al., 2002). Thus acute psychotic episodes likely involve increased dopamine levels which trigger anandamide release as a homeostatic mechanism to counter excessive dopamine levels in the brain. Indeed, the increased anandamide level in schizophrenia patients was inversely correlated with psychotic symptoms (Giuffrida et al., 2004). Further, this fits with endocannabinoids acting as retrograde transmitters which inhibit the

release of dopamine, a phenomenon that has been demonstrated in the dorsal striatum (Giuffrida et al., 1999). In another study from the same group, increased CSF anandamide level was only observed in low-frequency cannabis users-schizophrenia patients, but was absent if these patients had high-frequency level of cannabis use or in healthy controls (Leweke et al., 2007). This suggests that the frequent use of cannabis may down-regulate anandamide signalling thereby removing the homeostatic role such signalling has in schizophrenia patients leading to uninhibited dopamine release and the precipitation of psychosis.

2.3. Association between cannabis and schizophrenia

Epidemiological studies show that there exists significant comorbidity between cannabis abuse disorders and schizophrenia with the rate of cannabis use among patients with schizophrenia being about twice as high than among the general population (Degenhardt et al., 2001). Although the association between cannabis and psychosis is well supported, the exact nature of this relationship is controversial with often polarized viewpoints being offered to interpret the association.

Some posit that *schizophrenia causes cannabis abuse/dependence*. For example, a polymorphism in the CB1 receptor gene might increase the risk of substance abuse in schizophrenia patients (Leroy et al., 2001). This is consistent with the view that the relationship between cannabis abuse and schizophrenia may be explained by a common brain circuitry subserving these disorders. Therefore, schizophrenia patients exhibit an enhanced vulnerability to addictive behaviour as a part of the disorder's symptomology (Chambers et al., 2001). At face value this appears compelling as the mesolimbic dopamine system is involved in both schizophrenia and drug addiction.

The self-medication hypothesis provides another explanation consistent with the notion that schizophrenia causes cannabis abuse. According to this, patients with psychotic disorders use cannabis to alleviate aversive symptoms of the disorder or the side effects associated with antipsychotic medications (Peralta and Cuesta, 1992, Khantzian, 1997, Chambers et al., 2001). However, there is now a growing body of evidence highlighting the limitations of this theory. One compelling argument against the self-medication hypothesis is that the initiation to substance use often precedes the first episode of psychosis (Hall, 1998). In addition, the effects of cannabis can often be unpleasant (e.g. increased anxiety and sedation), and may also exacerbate cognitive and attentional dysfunction in schizophrenia patients (D'Souza et al., 2004).

The opposing viewpoint is that *cannabis use causes schizophrenia*. This originates from reports of cannabis-induced psychotic episodes in individuals using high doses of cannabis. Indeed, the symptoms of cannabis intoxication resemble those of schizophrenia leading some to believe it provides a model state for the study of this disorder (D'Souza et al., 2004). However, most cases of cannabis psychosis are reversible with many patients remitting following the cessation of drug use. In this sense "cannabis psychosis" refers only to an acute, toxic reaction to heavy cannabis use that can be distinguished from persistent schizophrenia-like psychotic reactions that have a more prolonged time course (Hall et al., 2004b, Leweke et al., 2004).

Be that as it may, epidemiological studies strongly support that cannabis may play a component causal role in the development of schizophrenia with a meta-analysis showing that cannabis use increases the risk of developing schizophrenia by approximately twofold (Henquet et al., 2005). The argument that cannabis causes schizophrenia is compelling as it meets many of the criteria for causality in epidemiology. For example, the relationship is dose-dependent, with a greater risk of

schizophrenia in people who use cannabis more frequently than those that rarely use the drug (Andreasson et al., 1987, Henquet et al., 2005, Moore et al., 2007). Further, the relationship between cannabis and psychosis shows directionality as demonstrated by the association being maintained even when controlling for many of the potential confounding variables that could explain the association (D'Souza, 2007). In addition, studies have also shown that temporality exists in the association with cannabis use pre-dating or coinciding with the onset of schizophrenia symptoms (Allebeck et al., 1993, Linszen et al., 1994, Hambrecht and Hafner, 2000, Arseneault et al., 2002).

However, the view that cannabis use is necessary and sufficient to cause shizophrenia is undermined by the fact that the vast majority of people that use cannabis do not develop schizophrenia. This has led to the theory that *cannabis is a component cause in the development of schizophrenia* that might interact with vulnerability factors to promote schizophrenia. Supportive of this view is that cannabis use is associated with an earlier onset of symptoms (Barnes et al., 2006, Mauri et al., 2006) and an increased incidence and severity of psychotic symptoms in schizophrenia patients at high risk of developing the disorder (Johns, 2001, Miller et al., 2001, Degenhardt and Hall, 2002, D'Souza et al., 2005, Hides et al., 2006)

Here we specifically hypothesise that cannabis might unmask latent schizophrenia in individuals that have a genetic predisposition to the disorder (Caspi et al., 2005). This is consistent with the neurodevelopmental theory of schizophrenia which postulates that gene-environment interactions are required for the development of this debilitating mental disorder (Bayer et al., 1999).

3. Neuregulin 1 gene and schizophrenia

3.1. Genetic factors and susceptibility to schizophrenia

Family studies provide strong evidence that genetic predisposition is involved in the aetiology of schizophrenia. These studies report that the estimate risk for acquiring schizophrenia is 6-13 % for first-degree relatives like parents or siblings and 3-6 % for second-degree relatives (Faraone et al., 2002, Tandon et al., 2008). If both parents are affected, the risk of the offspring developing schizophrenia is more than 40 %. Twin studies showed that the risk is at least 50 % for monozygous twins that share 100 % of their genetic material, while it is around 15 % for dizygotic twins that only share 50 % of their genetic material like for siblings (Faraone et al., 2002). The heritability of schizophrenia is estimated at 80 % (Faraone et al., 2002, Tandon et al., 2008). However this hereditary factor is incomplete, which supports the neurodevelopmental theory of schizophrenia of the need of environmental factors like cannabis to trigger the development of the disorder. Expression of schizophrenia is likely to involve interaction of multiple susceptibility genes of small effects with non-genetic factors to modulate susceptibility to the disorder.

To identify the genes involved in the vulnerability to schizophrenia, genetic linkage and association studies have isolated several specific susceptibility genes including neuregulin 1 (*NRG1*) from chromosome 8p21 and *DTNBP1* (dysbindin) from chromosome 6p22 (Owen et al., 2005), that both influence glutamatergic transmission (Sawa and Snyder, 2003). Another strong candidate gene for schizophrenia is *DISC-1* (disrupted-in-schizophrenia 1) from chromosome 1q42, that interacts with cytoskeletal proteins which might affect neuronal migration (Owen et al., 2005). The chromosome 22q11 is also reported to be a particular susceptibility locus for schizophrenia risk (Owen et al., 2005) and two genes from this chromosome

have been identified as candidate genes for schizophrenia. *COMT* (catechol-O-methyltransferase) is one of the main enzymes that degrade catecholamines thus decreasing dopamine levels especially in the PFC (Sawa and Snyder, 2003). The second gene isolated is *PRODH* (proline dehydrogenase), a precursor of glutamate and a neuromodulator of glutamatergic transmission (Sawa and Snyder, 2003).

Other promising susceptibility genes have been implicated in schizophrenia, but the evidence of their role is not yet compelling (Owen et al., 2005). They include *G72* (D-amino-acid oxidase activator) from chromosome 13q34 that activates D-amino acid oxidase, the enzyme that catabolizes D-serine, the glial neuromodulator of glutamatergic NMDA receptors (Sawa and Snyder, 2003), or *RGS4* (regulator of G-protein signalling 4) from chromosome 1q22 that modulates G-protein signalling. Interestingly, most of these genes influence neurotransmission and especially the glutamatergic pathway, including NMDA receptors, that is deficient in schizophrenia. Although the association of these genes with schizophrenia is sometimes contentious (Sanders et al., 2008), their identification is essential in understanding the pathogenesis of schizophrenia.

3.2. Association between neuregulin and schizophrenia

Schizophrenia is a multifactorial polygenic disorder, and one of its most promising susceptibility genes is *NRG1*. Neuregulins are a family of growth and differentiation factors that are encoded by 4 genes (*NRG1-4*), with *NRG1* being the most characterized. It is a huge gene of around 1.4 megabases that generates 6 types of protein, and more than 31 isoforms (Fig. 5). Nrg1 contains an epidermal growth factor (EGF)-like domain that activates ErbB receptor tyrosine kinases. There are 4 ErbB

receptors (ErbB1-4) but Nrg1 binds predominantly to ErbB4 in the CNS (Falls, 2003, Mei and Xiong, 2008).

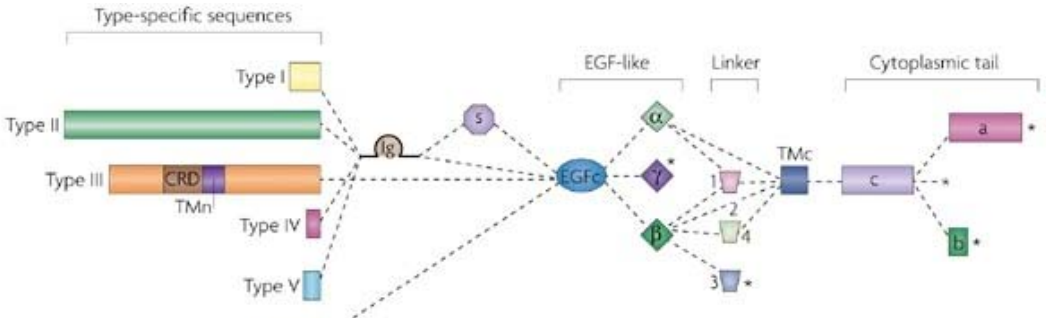


Figure 5. The different types of neuregulin 1. The 6 types of neuregulin 1 (NRG1) isoforms are classified according to their distinct amino-terminal sequences. In the type III isoforms, this sequence contains a cysteine-rich domain (CRD) that has a transmembrane domain (TMn). All six types of NRG1 isoforms have an epidermal growth factor (EGF)-like domain. Types I, II, IV and V have an immunoglobulin (Ig)-like domain between the N-terminal sequence and the EGF domain, with or without the spacer region (S), whereas the N-terminal-specific region of types III and VI is connected directly to the EGF domain.Variants are also generated by splicing in the linker regions and the C-terminal regions. Between the two regions is a C-terminal transmembrane domain (TMc). *Adapted from Mei, L. and Xiong, W. C., 2008. Neuregulin 1 in neural development, synaptic plasticity and schizophrenia. Nat Rev Neurosci. 9, 437-452.*

The possible linkage of schizophrenia to the chromosome 8p has been postulated since 1995 (Pulver et al., 1995, Kendler et al., 1996) and Stefansson and colleagues first associated *NRG1* with schizophrenia in 2002 (Stefansson et al., 2002). Since that time associations between *NRG1* and schizophrenia have been replicated in diverse Caucasian (Stefansson et al., 2002, Stefansson et al., 2003, Williams et al., 2003, Bakker et al., 2004, Corvin et al., 2004, Petryshen et al., 2005, Thomson et al., 2007, Georgieva et al., 2008), Asian (Yang et al., 2003, Li et al., 2004, Tang et al., 2004, Zhao et al., 2004, Fukui et al., 2006, Kim et al., 2006) and African American (Lachman et al., 2006) populations. However, some negative findings have also been reported (Hall et al., 2004a, Hong et al., 2004, Iwata et al., 2004, Kampman et al., 2004, Thiselton et al., 2004, Ingason et al., 2006). A meta-analysis of these data indicates that the problems associated with replication of findings lie in the heterogeneity of these studies in their methods, markers, sample ancestry and phenotype definition (Munafo et al., 2008). An association between *NRG1* with schizotypal personality has also been shown (Lin et al., 2005) further supporting the

role of this gene in schizophrenia. In addition, the Nrg1 receptor ErbB4 has also been associated with schizophrenia (Nicodemus et al., 2006, Norton et al., 2006, Silberberg et al., 2006, Law et al., 2007).

Together with these linkage studies, gene expression evidences also support a role of *NRG1* in schizophrenia. A first report showed an increase in Nrg1 isoform type I expression mRNA levels and a decrease in type II/type I and type II/type III ratios in the dorsolateral PFC of patients with schizophrenia (Hashimoto et al., 2004). However, more recent studies revealed a decreased Nrg1 isoform type I and an increased type II in the PFC (Parlapani et al., 2008) and a decreased Nrg1 isoform α in the PFC of schizophrenic patients (Bertram et al., 2007). At the protein level, elevated Nrg1 and ErbB4 have also been observed in the PFC of schizophrenia patients (Chong et al., 2008). In the hippocampus, increased levels of Nrg1 isoform type I has also been observed post mortem in patients (Law et al., 2006) and *NRG1* genetic variation is associated with hippocampal volume reduction in patients with schizophrenia and their relatives (Gruber et al., 2008). Taken together, these results show that abnormal expression of Nrg1 isoforms in the foremost brain regions implicated in schizophrenia might be one of the features that induce susceptibility to schizophrenia.

On a behavioural level, *NRG1* is also associated with psychoaffective and cognitive aspects of schizophrenia. *NRG1* is associated with personality difference in healthy individuals (Krug et al., 2008), and with increased reactivity to psychosocial stress in schizophrenic patients (Keri et al., 2008). Among the number of symptoms of schizophrenia, deficits in PPI and working memory are part of the main endophenotypes of the disorder. Interestingly, *NRG1* polymorphism has been associated with both deficits in prepulse inhibition (PPI) in schizophrenia and healthy

populations (Hong et al., 2008) and with cognitive deficits seen in schizophrenia and development of psychotic symptoms (Hall et al., 2006). These cognitive disruptions associated with *NRG1* polymorphism have been linked to impaired frontal and temporal lobe activation (Hall et al., 2006). *NRG1* is also associated with hyperactivation of the superior frontal gyrus during performance of a working memory task in healthy individuals (Krug et al., 2008) and with disruption in neural connectivity that, by leading to a slower speed of cognitive processing, may contribute to cognitive impairments observed in schizophrenia (Bramon et al., 2008). These associations of *NRG1* with schizophrenia shows that it is one of the main susceptibility genes for the disorder, however the mechanism by which *NRG1* signalling may influence the disorder remains largely unknown.

3.3. Functional role of neuregulin in schizophrenia

Nrg1-ErbB4 signalling plays essential roles in neurodevelopment, neurotransmission and synaptic plasticity that could influence the development of of psychiatric and neurological disorders such as schizophrenia (Mei and Xiong, 2008). Evidence now suggests that schizophrenia arises from disconnection between different brain regions, and the disruption of this circuitry could underlie the disturbed behaviours and perceptions observed in schizophrenia (Mitchell et al., 2001). Cognitive impairments of executive function subserved by the PFC can be considered as an endophenotype of schizophrenia (Wobrock et al., 2008), and it was recently shown that there is an abnormal activation in the dorsolateral PFC and in the anterior cingulate cortex of schizophrenia patients during working memory and executive processing (Schlosser et al., 2008). ErbB4 is associated with variation in fronto-temporal structural connectivity that is correlated with working memory (Konrad et al., 2008), and *NRG1*

gene variation is also associated with changes in medial frontal white matter structural integrity (Winterer et al., 2008). Further it has been reported that Nrg1-induced cell migration is impaired in schizophrenia (Sei et al., 2007). Thus, deficiency in Nrg1-ErbB4 function could subserve the deficits observed in schizophrenia.

Neuregulins are important regulators of glial cells including oligodendrocytes and myelination (Mei and Xiong, 2008). It is suggested that altered fronto-temporal connectivity could result from disruption in oligodendrocyte function in schizophrenia as altered expression of genes involved in myelination were observed in the postmortem dorsolateral PFC of schizophrenia patients (Hakak et al., 2001). Reduction of oligodendrocytes in the PFC (Vostrikov et al., 2008) and of key oligodendrocyte-related and myelin-related genes (Tkachev et al., 2003) as well as abnormalities of myelination (Flynn et al., 2003) were also observed in schizophrenia patients. Furthermore, it has recently been proposed that an increased risk of developing schizophrenia could result from inherited genetic variation that would affect oligodendrocyte development and myelination and that could also lead to a deficiency in dopaminergic functioning (Feng, 2008). In summary these studies suggest that deficits in Nrg1-ErbB4 signalling may contribute to the risk of schizophrenia by altering neuronal myelination and synaptic plasticity during brain development, resulting in defects in brain circuitry maturation. This would result in disruption of synaptic connectivity and subsequently lead to abnormal information processing in the brain that could contribute to the behavioural perturbation observed in schizophrenia.

Nrg1 is expressed in the adult human brain like the dorsolateral PFC, the hippocampal formation or the cerebellum (Law et al., 2004), regions of the brain that have been implicated in schizophrenia (Mitchell et al., 2001, Baldacara et al., 2008,

Segarra et al., 2008). Abnormalities in *NRG1* signalling might also influence schizophrenia susceptibility by altering the expression and function of neurotransmitter receptors, including glutamate receptors (Ozaki et al., 1997). Interestingly, *NRG1*-ErbB4 signalling is involved in glutamatergic NMDA receptor hypofunction in the PFC (Gu et al., 2005, Hahn et al., 2006) and the NMDA receptor is altered in the PFC of postmortem schizophrenia brain (Woo et al., 2008), consistent with the glutamatergic hypothesis of schizophrenia. *NRG1* also regulates other neurotransmitters systems, including dopamine (Yurek et al., 2004), GABA (Woo et al., 2007), acetylcholine (Mathew et al., 2007) and serotonin (Dean et al., 2008) which is of particular interest given that these neurotransmitters are involved in schizophrenia and modulated by atypical antipsychotics (Gray and Roth, 2007, Huang et al., 2008). Finally, ErbB4 signalling is also involved in the neuroendocrine control of puberty (Ma et al., 1999), which is important as the age of onset of the first episode of schizophrenia is usually associated with the post-pubertal period corresponding to sexual maturation (Stevens, 2002, Walker and Bollini, 2002). Thus, defects in Nrg1 signalling could disrupt hormonal changes associated with neurodevelopmental processes and neuroendocrine maturation underlying abnormal brain development (Walker and Bollini, 2002).

3.4. Animal models in schizophrenia research

A wide range of pharmacological, lesion and developmental manipulations have been developed to replicate some of the features of schizophrenia as modelled in animals. Pharmacology-based models have frequently been used since it was discovered that drugs which enhance dopamine function like amphetamine or antagonise NMDA receptor function like PCP promote psychotic symptoms in

humans (Gainetdinov et al., 2001). These drugs induce hyperlocomotion and stereotypy in animals, but glutamate hypofunction additionally induces negative symptoms like deficits in social interaction that can be reversed by atypical antipsychotic drugs (Corbett et al., 1995). In an attempt to model the neurodevelopmental theory of schizophrenia, aetiological models have been developed. For example, disrupted neurogenesis using X-ray or toxin exposure during gestation promote morphological changes in the hippocampus, frontal and entorhinal cortices as well as behavioural alterations such as hyperactivity, cognitive impairments and PPI and latent inhibition (LI) deficits (for a review, see Lipska and Weinberger, 2000). Similar changes are observed using other models including neonatal hippocampal lesions and early stress exposure like maternal separation or social isolation (Lipska and Weinberger, 2000).

The creation of knockout mice lacking a specific gene offers an exciting new approach in the study of mental disorders, especially schizophrenia which is partly genetically determined. Genetic animal models using mutations of dopamine transporter or NMDA receptor subunits have been reported (for a review, see Gainetdinov et al., 2001), but mutations of specific schizophrenia susceptibility genes provide particularly relevant animal models. Such genetic models of schizophrenia offer valuable tools in investigating the neurobiological basis and potential therapeutic targets of schizophrenia.

Given the complex nature of schizophrenia, animal research is limited to modelling specific schizophrenia-like symptoms and may not approximate all aspects of the disorder (Geyer, 2008). Modelling such aspects requires the need for appropriate endophenotypic measures. Endophenotypes are measurable components, unseen by the unaided eye, which are determined by an underlying genotype related

to a disease/disorder state. Such endophenotypes include behavioural, neuroanatomical, biochemical and cognitive measures (Gottesman and Gould, 2003). Some endophenotypes of schizophrenia can be directly reproduced in animal research. The most relevant example is deficits in PPI. This phenomenon is the decreased startle response produced by a prepulse stimulus, and reflects deficits in sensorimotor gating (Swerdlow et al., 1994). PPI is impaired in schizophrenia patients and can be reproduced in animals (Swerdlow et al., 1994). LI that is defined as the poorer conditioning to a previously irrelevant stimulus, is another model that measures the capacity of an organism to ignore irrelevant stimuli (Lubow and Moore, 1959). Importantly, LI is impaired in schizophrenia (Gray et al., 1995) providing support for its use in animal modelling of psychosis (Lubow, 2005). Schizophrenia is also characterized by social withdrawal in humans, a key component of negative symptoms (DSM-IV). This effect can be studied in animals as social interaction (SI), as developmental models such as neonatal hippocampal lesion and prenatal stress, and pharmacological models of schizophrenia using PCP induce deficits in SI in rat (Sams-Dodd et al., 1997, Sams-Dodd, 1999, Lee et al., 2007). Other negative symtoms of schizophrenia such as anhedonia can also be modelised in animals using reward sensitivity, a behaviour that is reduced in adult rats that received neonatal ventral hippocampal lesions (Le Pen et al., 2002).

While positive and negative symptoms have long been considered as hallmarks of schizophrenia, cognitive impairments are now considered as core features of the disorder (Elvevag and Goldberg, 2000). Working memory deficits are the most consistently observed cognitive symptoms in schizophrenia patients (Park et al., 1999) and antipsychotic drugs reverse working memory impairments (Weinberger and Gallhofer, 1997). The measurement of working memory provides an additional

endophenotype that can be discerned in animals. Interestingly, animal working memory deficits are subserved by dysfunctional dopamine and glutamate signalling in the prefrontal cortex (for a review, see Castner et al., 2004), areas thought to underlie cognitive impairments seen in schizophrenia patients. In addition to behavioural changes, endophenotypes might take the form of underlying anatomical or neurochemical changes in animals that parallel those observed in schizophrenia patients. For example, enlarged ventricles are consistently observed in schizophrenia patients and their relatives (McDonald et al., 2006) and thus provide a potential morphometric endophenotype of schizophrenia which might be reproduced in animal models of the disorder (Hikida et al., 2007, Pletnikov et al., 2008, Verma et al., 2008). Further, alteration in levels of neurotransmitters or receptors implicated in schizophrenia such as dopamine or NMDA receptors might also provide an endophenotypic measure in animal studies.

Of course reproducing the characteristic positive symptoms of schizophrenia such as hallucinations and delusion in animals is more difficult and subject to anthropomorphism. However, the positive symptoms might be modelled by increased locomotor activity as this is usually subserved by increased dopamine release, a proposed feature of schizophrenia neurochemistry. Indeed, drugs like amphetamine that increase psychotic symptoms in humans induce hyperactivity in rodents, and this effect is reversed by antipsychotic drugs (Ellison and Eison, 1983, Gleason and Shannon, 1997, Millan et al., 1999).

3.5. *Nrg1* HET mice as an animal model for schizophrenia

In the last decade mutant mice have been developed to disrupt *Nrg1* signalling in an attempt to model schizophrenia. The behavioural and neuronal manifestations of such

genetically modified mice are summarized in Table 1. Overall, the different manipulation of genes encoding for the different isoforms of *Nrg1* and ErbB4 receptors reproduce many of the important endophenotypes relevant to animal modelling of schizophrenia. The present thesis will concentrate on studying mutant mice heterozygous for the transmembrane domain of *Nrg1* gene, as this is the best characterised neuregulin 1-ErbB system genetic model. Further, a mutation of the transmembrane domain of the *Nrg1* gene has been specifically associated with schizophrenia in human populations (Walss-Bass et al., 2006). Interestingly, *Nrg1* knockout mice were originally developed to better understand cardiac development, and *Nrg1* homozygous knockout animals die embryonically due to heart defects (Meyer and Birchmeier, 1995). Mice heterozygous for *Nrg1* (*Nrg1* HET) are viable and fertile and have since been behaviourally characterised as an animal model of schizophrenia. These mice provide a very promising phenotype for the study of schizophrenia displaying hyperlocomotion that can be reversed by the antipsychotic drug clozapine, PPI deficits and a reduction in NMDA receptors (Stefansson et al., 2002). They also show a retarded exploratory habituation profile in a novel environment and impairments in social recognition memory (O'Tuathaigh et al., 2006, O'Tuathaigh et al., 2007). In addition, *Nrg1* HET mice show an age-dependent phenotype where the hyper-locomotor and hyper-exploratory behaviours of *Nrg1* HET mice is only seen in animals older than 4 months, which somewhat models the post-pubescent onset of schizophrenia (Karl et al., 2007). More recently these mice have been shown to have increased serotonin 5-HT_{2A} receptor levels in the brain (Dean et al., 2008) – this receptor is thought to be involved in hallucination as hallucinogenic drugs such as lysergic acid diethylamide (LSD) and psilocybin target 5-HT receptors (Gonzalez-Maeso et al., 2007).

Table 1. The different animal models of *Nrg1* deficiency

Gene dysfunction	Study	Behaviour modified
TM *Nrg1*	(Stefansson et al., 2002)	Hyperactivity, reversed by clozapine PPI impairments Reduction of functional NMDA receptors
	(O'Tuathaigh et al., 2006)	Hyperactivity and increased rearing Decreased habituation to exploration
	(Karl et al., 2007)	Hyperactivity and increased exploration Moderate anxiolytic-like effect Age-dependent phenotype Environment enrichment-dependent phenotype
	(O'Tuathaigh et al., 2007)	Hyperactivity Impairments in social novelty
	(O'Tuathaigh et al., 2008)	Hyperactivity Increased aggressive behaviour
	(Dean et al., 2008)	Increased serotonin 2A receptor and serotonin transporter
EGF-like *Nrg1*	(Gerlai et al., 2000)	Hyperactivity
	(Duffy et al., 2008)	Hyperactivity Increased habituation to new environment Moderate impairment in PPI following NMDA antagonism
immunoglobulin (Ig)-like *Nrg1*	(Rimer et al., 2005)	Clozapine-induced decreased exploration Latent inhibition deficit
type III *Nrg1*	(Chen et al., 2008)	Enlarged ventricles Hypofunction of the PFC, hippocampus and subiculum Reduced spine density Memory deficits PPI impairments
BACE1	(Savonenko et al., 2008)	Hyperactivity, reversed by clozapine Social impairment Cognitive deficits PPI impairments Hypersensitivity to NMDA receptor antagonist Reduced spine density
Aph1B/C	(Dejaegere et al., 2008)	PPI impairments, reversed by antipsychotics Memory deficits
ErbB4	(Stefansson et al., 2002)	hyperactivity
CNS-specific *ErbB4* null	(Golub et al., 2004)	Decreased locomotion Disrupted cue use in memory test
ErbB signalling in oligodendrocytes	(Roy et al., 2007)	Decreased locomotion Social impairment Anxiety-like behaviour increased levels of dopamine receptors and transporters

4. Aims of the current thesis

The work described in this thesis aims at examining if vulnerability to cannabinoid-induced effects could represent an additional phenotype of schizophrenia reproduced by *Nrg1* HET mice. While human population studies indicate that a gene-environment interaction is likely required for the development of schizophrenia they are restricted in the mechanistic detail they can provide. Indeed epidemiologists have made a call to basic scientists to assist in the quest to delineate the exact genetic and neuropharmacological bases for cannabis-induced schizophrenia (Henquet et al., 2005). Therefore, the present thesis attempts to answer such a call by examining the role of *Nrg1* in the neurobehavioural actions of cannabinoids.

Chapter 2 aimed to examine the behavioural effects of acute exposure to THC, the main psychoactive constituent of cannabis, in *Nrg1* HET mice and their control wild-type-like (WT) littermates. This study focussed on testing the animals in a wide range of behavioural domains such as locomotion, exploration, anxiety and sensorimotor gating using a multi-tiered behavioural phenotyping strategy. Here we hypothesised that deficiency in *Nrg1* would modulate the behavioural effects of acute THC exposure.

In Chapter 3 we aimed to assess the underlying neuronal changes that might subserve any distinct behavioural effects of acute THC administration on *Nrg1* HET mice observed in Chapter 2. To achieve this *Nrg1* HET mice and WT were exposed to THC before their brains were retained for c-Fos immunohistochemical analysis. C-Fos is a marker of neuronal activity as it is rapidly and transiently expressed to novel stimuli. Further, Chapter 3 also aimed to investigate whether the stress of behavioural testing contributed to any differential effect of THC observed on *Nrg1* HET mice.

As the relationship between cannabis and schizophrenia increases with the frequency of drug use, chronic cannabis consumption is more reliably associated with precipitating psychosis. Thus Chapter 4 aimed to assess the effect of chronic cannabinoid exposure on the neurobehavioural *responses* of *Nrg1* HET and WT mice. It was hypothesised that repeated cannabinoid exposure might induce altered neuroadaptive responses as measured by Fos B/ΔFos B expression in *Nrg1* HET compared to WT mice which might correlate with altered effects of the drug as measured on the behavioural level.

One of the main schizophrenia endophenotypes observed in human studies are cognitive impairments of higher executive functions. However, behavioural phenotyping in animal models of schizophrenia does not provide enough cognitive testing to evaluate higher functional deficits. Importantly, it is difficult to assess the cognitive effects of cannabinoids as these drugs are highly sedative and produce tolerance to their effects. Thus Chapter 5 aimed at setting up a procedure that would allow evaluating cannabis-induced behavioural flexibility deficits that could be extended to phenotyping cognitive deficits in *Nrg1* HET and WT animals in the future.

Chapter 2

HETEROZYGOUS NEUREGULIN 1 MICE ARE MORE SENSITIVE TO THE BEHAVIOURAL EFFECTS OF Δ^9-TETRAHYDROCANNABINOL

Psychopharmacology
DOI 10.1007/s00213-007-0721-3

ORIGINAL INVESTIGATION

Heterozygous neuregulin 1 mice are more sensitive to the behavioural effects of Δ^9-tetrahydrocannabinol

A. A. Boucher · J. C. Arnold · L. Duffy · P. R. Schofield · J. Micheau · T. Karl

Received: 13 July 2006 / Accepted: 22 January 2007

Abstract
Rationale Cannabis use may precipitate schizophrenia especially if the individual has a genetic vulnerability to this mental disorder. Human and animal research indicates that neuregulin 1 (Nrg1) is a susceptibility gene for schizophrenia.
Objectives The aim of this study was to investigate whether dysfunction in the *Nrg1* gene modulates the behavioural effects of Δ^9-tetrahydrocannabinol (THC), the major psychotropic component of cannabis.
Materials and methods Heterozygous *Nrg1* transmembrane-domain knockout mice (*Nrg1* HET) were treated with acute THC (0, 5 or 10 mg/kg i.p.) 30 min before being tested using open field (OF), hole board (HB), light-dark (LD), elevated plus maze (EPM), social interaction (SI) and prepulse inhibition (PPI) tests.
Results Nrg1 HET mice showed differences in baseline behaviour with regard to locomotor activity, exploration and anxiety. More importantly, they were more sensitive to the locomotor suppressant actions of THC compared to wild type-like (WT) mice. In addition, *Nrg1* HET mice expressed a greater THC-induced enhancement in % PPI than WT mice. The effects of THC on anxiety-related behaviour were task-dependent, with *Nrg1* HET mice being more susceptible than WT mice to the anxiogenic effects of THC in LD, but not in the EPM, SI and OF tests.
Conclusions Nrg1 HET mice were more sensitive to the acute effects of THC in an array of different behaviours including those that model symptoms of schizophrenia. It appears that variation in the schizophrenia-related neuregulin 1 gene alters the sensitivity to the behavioural effects of cannabinoids.

Keywords Cannabinoid · THC · Neuregulin 1 · Knockout mouse · Schizophrenia · Motor activity · Anxiety · Exploration · Prepulse inhibition · Sensorimotor gating

A. A. Boucher · J. C. Arnold · L. Duffy · P. R. Schofield · T. Karl
Neuroscience Institute of Schizophrenia and Allied Disorders (NISAD),
384 Victoria Street,
Darlinghurst, NSW 2010, Australia

A. A. Boucher · J. C. Arnold
Department of Pharmacology, University of Sydney,
Sydney, NSW 2006, Australia

A. A. Boucher · J. Micheau
Laboratoire de Neurosciences Cognitives, Université Bordeaux I,
33405 Talence, France

L. Duffy · T. Karl (✉)
Neuroscience Research Program,
Garvan Institute of Medical Research,
Sydney, NSW 2010, Australia
e-mail: t.karl@garvan.org.au

P. R. Schofield
Prince of Wales Medical Research Institute,
Sydney, NSW 2031, Australia

P. R. Schofield
University of New South Wales,
Sydney, NSW 2052, Australia

Introduction

Part of a clinical diagnosis of schizophrenia is the characterisation of this mental disorder by a range of psychiatric symptoms frequently classified into positive (e.g., hallucinations, delusions), negative (e.g., impaired emotion, social withdrawal) and cognitive symptoms (e.g., impairment of attention, language, memory) (Wong and Van Tol 2003). These symptoms are at least partially based on dopamine

(DA) hyperstimulation and a dysfunction of either glutamate or its *N*-methyl-D-aspartate (NMDA) receptor subtype (Coyle 1996; Wong and Van Tol 2003). According to the neurodevelopmental theory of schizophrenia, such altered neurochemistry is thought to arise due to an interaction between genetic and environmental factors promoting defects of brain circuitry maturation (Duncan et al. 1999; Farber et al. 1995). This results in abnormalities of synaptic connectivity, myelination or structural changes in the cortex of schizophrenia patients.

Genetic factors play an important role in the aetiology of schizophrenia (Cardno and Gottesman 2000; Sullivan et al. 2003). A meta-analysis of genome scans has reported a schizophrenia susceptibility locus on chromosomes 8p12–p23.1. Furthermore, significant associations with a candidate gene on 8p12, neuregulin 1 (*NRG1*), have been reported in Caucasian (Stefansson et al. 2002) and Asian populations (Tang et al. 2004; Yang et al. 2003), although some studies failed to replicate this association (Duan et al. 2005; Iwata et al. 2004). Nevertheless, Nrg1 with its more than 15 isoforms (Falls 2003), has an impact on schizophrenia-related brain processes such as the expression and function of central nervous system (CNS) neurotransmitter receptors for NMDA and gamma-aminobutyric acid (GABA), the activation of glial cells and the regulation of myelin and oligodendrocyte-related gene expression. Furthermore, altered *NRG1* mRNA expression can be found in postmortem dorsolateral prefrontal cortex and hippocampus of schizophrenia patients (Hashimoto et al. 2004; Law et al. 2006).

Several studies have proposed a schizophrenia-related phenotype for transgenic mice heterozygous for transmembrane Nrg1 (Falls 2003): the phenotype includes hyperactivity (reversible with clozapine), deficits in prepulse inhibition and habituation processes and fewer functional NMDA receptors (O'Tuathaigh et al. 2006; Stefansson et al. 2002). Importantly, transmembrane *NRG1* was recently found to associate with schizophrenia (Walss-Bass et al. 2006). Thus, the heterozygous *Nrg1* transmembrane-domain knockout mouse may provide a putative animal model of genetic vulnerability to schizophrenia.

The use of cannabis increases the risk of developing schizophrenia (Linszen et al. 1994), and people with psychotic disorders are more likely to use or have used psychoactive drugs than other psychiatric patients or the general population (Arseneault et al. 2004; Schneier and Siris 1987). It has been hypothesised that chronic (Degenhardt and Hall 2002; Leweke et al. 2004; Linszen et al. 1994) and less robustly acute (Favrat et al. 2005) cannabis consumption has an impact on the aetiology of schizophrenia in subjects with a predisposition for this mental disorder. Importantly, not only cannabis consumption during puberty/adolescence but also a variety of other external/environmental factors such as obstetric complications (Boksa 2004) or nutritional deficiency (Susser and Lin 1992) may unmask schizophrenia in individuals who have a prior vulnerability to the disorder (Degenhardt and Hall 2002; Leweke et al. 2004). This is consistent with the two-hit hypothesis of schizophrenia, which poses that psychotic patients harbour one or various susceptibility genes that are necessary but not sufficient to cause schizophrenia (hit 1). Environmental stressors, such as cannabis abuse, may then help to trigger the onset of schizophrenia by interacting with this genetic vulnerability to the mental disorder (hit 2) (Bayer et al. 1999).

The availability of transgenic mice offers a unique opportunity to systematically investigate such gene–environment interactions, while avoiding the many confounding factors that plague human studies on cannabis-induced psychosis (Henquet et al. 2005). In this study, we examine, whether dysfunction in the *Nrg1* gene modulates the behavioural effects of Δ^9-tetrahydrocannabinol (THC), the major psychotropic component of cannabis, using heterozygous *Nrg1* transmembrane domain knockout (*Nrg1* HET) mice. The effects of different dosages of acutely administered THC on wild type-like and *Nrg1* HET mice were assessed using a multitiered phenotyping strategy focusing on behavioural domains of locomotion, exploration, anxiety and sensorimotor gating.

Materials and methods

Animals

The heterozygous *Nrg1* transmembrane domain knockout mice were provided by Prof. Richard Harvey (Victor Chang Cardiac Research Institute, Sydney Australia) and have been described previously (Stefansson et al. 2002). Test animals were heterozygous $Nrg1^{+/-}$ (*Nrg1* HET) and wild type-like control $Nrg1^{+/+}$ (WT) littermates (backcrossed for 15 generations onto a C57BL6/J background). Genotypes were determined after weaning (postnatal day 21) by tail biopsy and polymerase chain reaction (primers for mutant *Nrg1* mice: Neo173F 5′-ATGAACTGCAGGACGAGG CA-3′ and Neo6301R 5′-GCCACAGTCGATGAATCCA G-3′; primers for wild-type-like control mice: 5′-AACAGC CTGACTGTTAACACC-3′ and 5′-TGCTGTCCATCTGCA CGAGACTA-3′). Male, adult, age-matched (±14 days) test animals of the same genotype were pair-housed in Macrolon cages under a 12:12 h light:dark schedule [light phase: white light (illumination: 80 lx) - dark phase: red light (illumination: <2 lx)] with food and water available ad libitum. Male, adult, age-matched (±7 days), group-housed A/J mice (Animal Resources Centre, Canning Vale, Australia) were used as standard opponents in the social interaction test. All research and animal care procedures were approved by the "Garvan Institute/St. Vincent's

Hospital Animal Experimentation Ethics Committee" and were in agreement with the "Australian Code of Practice for the Care and Use of Animals for Scientific Purposes".

Drug treatment

Δ^9-tetrahydrocannabinol (THC: Sigma-Aldrich, Sydney, Australia) was dissolved in 4% ethanol, 1% Tween 80 and 95% saline (Balerio et al. 2006; Varvel et al. 2006). The drug was administered intraperitoneally (i.p.) in a volume of 10 ml/kg of body weight (BW), 30 min before behavioural testing. Two doses of THC were used in this study, 5 and 10 mg/kg BW (latter one was shown to be CB1 receptor-specific: see e.g., Compton et al. 1996). Relatively high doses of THC were chosen, as cannabis psychosis has been associated with acute administration of high doses of cannabis (Hall and Degenhardt 2000; Johns 2001) and as the C57BL/6J background strain is known for its reduced sensitivity to some of the neurobehavioural effects of THC compared to other mouse strains (Onaivi et al. 1995). Mice were treated with similar volumes of either vehicle (4% ethanol, 1% Tween 80 and 95% saline) or one of the two dosages of THC in a counterbalanced, quasi-randomised design, allowing for within-animal comparison. A washout period of at least 7 days was guaranteed before the animals were retested.

Behavioural testing

All animals were tested at the age of 6–7 months to investigate whether acute THC treatment has a differential effect on the behavioural performance of *Nrg1* HET compared to WT mice. Nrg1 hypomorphic mice exhibit an age-dependent hyperactive phenotype, as mutant animals of 3–4 months of age show similar behaviours as wild type-like control littermates, whereas Nrg1 *HET* mice older than 4 months exhibit an increase in locomotion and exploration compared to WT mice (Karl et al. 2006). A first set of animals (n=10 per genotype) was tested 30 min postinjection in domains such as locomotion, exploration and anxiety using the open field (OF), the hole board (HB) and the elevated plus maze (EPM) tasks, respectively. A second set of mice (n=10–13) was tested 30 min postinjection for anxiety, social behaviours and sensorimotor gating using the light–dark (LD), the social interaction (SI) and the prepulse inhibition (PPI) tests (see Table 1). All behavioural testing was conducted during the light cycle under highly standardized test conditions (Crawley 1999; Karl et al. 2003). For habituation purposes, all test animals were transported to the testing room 1 h before behavioural testing (holding and test room were part of the same facility having therefore identical light, air pressure and temperature conditions). Environmental odours were removed from the different test apparatus by cleaning the equipment after each trial with a 30% ethanol solution.

Table 1 Test biography of both sets of wild type-like (WT) and heterozygous *Nrg1* transmembrane domain knockout (*Nrg1* HET) mice

Sets	Genotype (and animal numbers)	Battery of behavioural paradigms (30 min after i.p. injection of either vehicle or THC)
Set 1	WT: n=10; *Nrg1* HET: n=10	Behavioural test battery A OF HB EPM
Set 2	WT: n=10; *Nrg1* HET: n=13	Behavioural test battery B LD SI PPI

A washout period of at least 7 days was guaranteed before the animals were retested in the same battery of behavioural paradigms. Thus, all test animals were observed repeatedly 30 min postinjection in the behavioural test battery A [open field (OF), the hole board (HB) and the elevated plus maze (EPM); set 1] or test battery B [light-dark test (LD), the social interaction test (SI) and the prepulse inhibition paradigm (PPI); set 2]. Two sets of animals were tested over a period of 3 weeks. Mice were treated with either vehicle or one of the two dosages of THC (5 or 10 mg/kg bodyweight) in a counterbalanced, quasi-randomised design, allowing for within-animal comparison.

Open field (OF) Locomotor activity, explorative-like tendencies and anxiety-related behaviours were evaluated by placing the mouse into an infrared photobeam-controlled (x-, y-, and z-axis) open field activity test chamber (MED, Vermont, USA). This paradigm mimics the natural conflict in mice between the tendency to explore a novel environment and to avoid an exposed open area (Crawley 1985; DeFries et al. 1966). The arena (43.2 cm×43.2 cm) was divided into a central and a peripheral zone (MED software coordinates for central zone: 3/3, 3/13, 13/3, 13/13). Animals were tested for 30 min (illumination at floor level: 20 lx), and the animal's total distance travelled (horizontal activity: x- and y-axis) and vertical activity (z-axis) was recorded automatically (software settings: box size: 3; ambulatory trigger: 2; resting delay: 1,000 ms; resolution: 100 ms) in the central and peripheral areas. The ratio of central to total distance travelled and the time spent in the central zone were taken as measures of anxiety (Denenberg 1969).

Hole board (HB) The HB test provides independent measures of locomotor activity and directed exploration (Boissier et al. 1964; van Gaalen and Steckler 2000). Mice were placed into the open field activity test chamber, which was equipped with a hole board floor insert for mice (MED: 16 holes; diameter: 1.6 cm). The infrared photobeams

provided automated measures of the distance travelled, ambulatory frequency and *head-dipping* frequency.

Elevated plus maze (EPM) The EPM represents the natural conflict between the tendency of mice to explore a novel environment and the tendency to avoid a brightly lit, elevated, open area (Montgomery 1958). The grey plus maze had a central platform (6 cm×6 cm), two alternate enclosed arms (35 cm×6 cm; height of enclosing walls 28 cm; dimly illuminated: 10 lx) and two alternate open arms (35 cm×6 cm; without side walls; highly illuminated: 70 lx) with ledges (4 mm×6 mm). The arms' surface was raised 70 cm above the floor. The mouse was placed onto the centre field of the "+" (faced to an enclosed arm) and was allowed to explore the maze for 5 min. Closed arm entries were taken as measures of motor activity, whereas entries in and time spent on open arms and the percentage of open arm entries (open arm entry ratio) were recorded online as a measure of anxiety (Hogg 1996; Pellow et al. 1985). In addition to recording behaviours online by repeatedly trained research staff, the sessions were reanalysed for parameters such as time spent in the different compartments of the EPM. An individual entry was recorded when the animal entered the arm with at least half of its body length.

Light-dark (LD) In the LD test, the time spent in a brightly illuminated zone compared to a dark zone and the occurrence of associated exploratory behaviour (vertical activity) were used to assess anxiety in rodents (Costall et al. 1989; Crawley 1999). The test animals were placed into the open field chamber, which was equipped with a dark box insert for mice (covering half the area of the chamber: MED). An opening located in the centre of the partition connected light (illumination: 20 lx) and dark (illumination: <2 lx) compartments. At the start of the experiment, mice were placed into the lit compartment. The time spent in, entries into and horizontal activity in the differentially illuminated compartments, and vertical activity were recorded during a 10 min test session. The ratio of distance travelled in the light compartment to total distance travelled (distance ratio) and the time mice spent in the light compartment were taken as measures of anxiety.

Social interaction (SI) The SI model is widely used to measure anxiety-like behaviours and to detect anxiogenic- and anxiolytic-like effects of drugs (File 1988; Kask et al. 2001). Test animals were placed together with an A/J standard opponent into the open field activity test chamber (in opposite corners), where they were allowed to explore the arena and each other freely for 10 min. The behaviour of the test mouse was recorded online. Frequency and total duration (so-called active social interaction time) of the active socio-positive behaviours *general sniffing*, *anogenital sniffing*, *allogrooming*, *following* and *crawling over/under* were recorded.

Prepulse inhibition (PPI) Patients with schizophrenia show impaired sensorimotor gating. PPI is the operational measure of sensorimotor gating, in which a weak pre-stimulus (prepulse) attenuates the startle response (Wang et al. 2003). PPI was tested in two startle chambers (SR-Lab: San Diego Instruments, San Diego, USA). Animals were habituated to the test device for three consecutive days (day 1: 2×2 min; days 2–3: 1×10 min) before being tested 24 h later. The protocol used was adapted from methods developed by Geyer and Swerdlow (1998). Briefly, after a 5 min acclimation period with a 70 dB background noise, the test session began. Each session consisted of 76 trials in a pseudorandomised order: 10×90 dB startle response trials, 18×120 dB startle response trials, 2× prepulse alone trials (prepulse intensities of 74, 78, 82 or 86 dB), 8× PPI response trials (prepulse followed 80 ms later by a 120 dB startle stimulus), and 8× no pulse trials (background noise only). We chose a variable intertrial interval of averaged 15 s (range 10–20 s), prepulse duration of 20 ms and startle duration of 40 ms. Startle response was measured as the average mean amplitude. Percentage of PPI was calculated as [(startle response 120 dB - PPI response)×100/startle response 120 dB]. We also analysed the mean % PPI of all summed prepulse responses for each genotype.

Statistical analysis

One-way analysis of variance (ANOVA) was applied to investigate "baseline" differences in the behavioural performance of vehicle-treated WT and *Nrg1* HET mice. The behavioural response of WT and *Nrg1* HET mice to THC was analysed using two-way ANOVA to investigate the effect of the factors "genotype" and "THC dose" on several behavioural domains. This was followed by one-way ANOVA (factor: "genotype" or "THC dose" split by the corresponding factor) and the Student–Newman–Keuls post hoc test, in which vehicle data were compared to each individual dose of THC (5 and 10 mg/kg) within each genotype. Differences were regarded as statistically significant when $p<0.05$. Results present the *degrees of freedom*, *F*-values and *p*-values of one-way (for baseline comparison) or two-way ANOVA (for "THC dose" effects), while in the figures and tables, the *p*-values of the corresponding post hoc tests are provided. Significant post hoc effects of *Nrg1* HET animals vs WT are indicated by "#" ($^{\#}p<0.05$ and $^{\#\#}p<0.01$), whereas significant effects of acute THC treatment vs vehicle treatment are shown by asterisk ($^{*}p<0.05$ and $^{**}p<0.01$). All data are presented as means ± standard error of the mean (SEM).

Results

Locomotor activity and exploration

The results from the OF, HB and LD are shown in Table 2. One-way ANOVA revealed that vehicle-treated *Nrg1* HET mice exhibited a hyperactive phenotype at baseline as the total distance travelled was significantly increased in the OF [$F(1,18)=21.0$, $p<0.001$] and in the LD [$F(1,21)=8.8$, $p<0.01$] compared to vehicle-treated WT mice. Furthermore, *Nrg1* HET mice crossed significantly more often between the light and the dark compartment of the LD [$F(1,21)=9.6$, $p<0.01$] and entered more often the enclosed arms of the EPM [$F(1,18)=5.1$, $p<0.05$] than WT mice. This hyperactivity was not only evident with regard to locomotion but could be confirmed in explorative-like behaviours as measured by an increased frequency of head dipping in the HB [$F(1,17)=5.1$, $p<0.05$] and of exploration-related vertical activity in the LD test [$F(1,21)=7.1$, $p<0.05$] compared to vehicle-treated WT mice.

THC treatment significantly decreased the total distance travelled in the OF [$F(2,52)=34.6$, $p<0.001$] and the LD test [$F(2,59)=9.9$, $p<0.001$], and the frequency of total crossings [$F(2,59)=11.4$, $p<0.01$] and vertical activity [$F(2,59)=15.4$, $p<0.001$] in the LD test as measured by two-way ANOVA. Interestingly, a differential motor activity- and exploration-suppressing effect of THC was observed between WT and *Nrg1* HET mice as we detected significant genotype by drug interactions for all these parameters: total distance travelled in OF [$F(2,52)=10.3$, $p<0.001$] and LD [$F(2,59)=5.2$, $p<0.01$], frequency of total crossings in LD [$F(2,59)=5.2$, $p<0.01$], and vertical activity [$F(2,59)=4.2$, $p<0.05$] in LD. Thus, there exists a genotype-specific difference in the motor activity-suppressant actions of THC. This conclusion was further supported by post hoc analyses for motor activity/exploration measures conducted within each genotype. In the OF, 10 mg/kg THC significantly reduced the total distance travelled in both genotypes compared to vehicle. However, 5 mg/kg THC significantly inhibited the total distance travelled selectively in the *Nrg1* HET mice (see Table 2). In the LD test, both the 5 and 10 mg/kg doses of THC were effective in reducing the total number of crossings and the frequency of vertical activity of *Nrg1* HET mice. Nevertheless, both cannabinoid doses (5 and 10 mg/kg) were ineffective in reducing these measures in WT mice (see Table 2). Acute THC treatment also decreased the frequency of head dipping [$F(2,50)=44.1$, $p<0.001$] in the HB test as measured by two-way ANOVA. No significant genotype by

Table 2 Locomotor activity, exploration and anxiety-related behaviours

Parameters	WT mice			*Nrg1* HET mice		
	Vehicle	THC 5 mg/kg	THC 10 mg/kg	Vehicle	THC 5 mg/kg	THC 10 mg/kg
Open field						
Total distance [cm] "+"	2,017±121	1,607±131	1,190±194**	3,382±272#	1,358±241**	1,073±200**
Time spent in the centre [s]	294±59	68±21**	31±25**	370±50	134±50**	9±2**
Hole board						
Head dipping fq	15±2	3±1**	1±0.4**	23±3#	5±3**	1±0.5**
Elevated plus maze						
Enclosed arm entries	6±1	9±2	4±1	9±1#	12±3	12±3
Time spent in open arms [s]	16±4	10±5	7±3	30±6#	11±5*	14±6
Open arm entries ratio [%]	36±2	19±5	25±7	38±3	16±6*	24±5
Light–dark						
Total distance [cm] "+"	1,248±166	1,172±186	859±145	1,881±137##	981±156**	911±156**
Total crossings "+"	49±12	46±16	22±11	105±13##	29±12**	18±6**
Vertical activity "+"	35±5	25±10	11±7	63±8#	13±8**	6±4**
Time spent in light zone [s] "+"	72±15	86±32	40±25	147±18##	51±27**	25±11**
Distance travelled in light zone [cm] "+"	274±62	264±87	147±77	542±63##	182±78**	71±35**
Entries into light zone fq "+"	24±6	23±8	11±6	52±6##	14.5±6**	9±3**
Distance ratio [%] "+"	18±4	17±5	12±5	29±2#	13±4**	6±3**
Social interaction						
SI duration [s]	42±5	22±5**	12±2**	44±3	16±2**	9±2**
Sniffing fq	29±2	15±1**	9±1**	32±2	14±2**	7±1**
Anogenital sniffing fq	6±1	2±0.3**	1±0.3**	8±1	2±0.5**	0.4±0.2**

"fq" represent frequencies of any given behaviour; parameters with a significant genotype by THC dose interaction are presented with "+"; significant post hoc effects of *Nrg1* HET animals vs WT control animals at baseline are indicated by "#" or "##" (#$p<0.05$ and ##$p<0.01$), whereas significant effects of acute THC treatment vs vehicle treatment are shown by "*" or "**"(*$p<0.05$ and **$p<0.01$); all data are presented as means ± standard error of the mean (SEM)

dose interaction was observed for this parameter. Both doses of THC (5 and 10 mg/kg) significantly reduced head dipping compared to vehicle in either genotype (Table 2).

Anxiety

The results from the animal models of anxiety are shown in Table 2. Mice were repeatedly tested in the various animal models of anxiety (OF, EPM, LD and SI) according to a within-subjects, counterbalanced, quasi-randomised design with a 1 week interval between testing. To confirm that the effects of THC were consistent with repeated testing over weeks of administration, we analysed the data using two-way ANOVA (factor 1: week of testing and factor 2: THC dose). Our analyses found no significant week of testing by THC dose interaction effects for any parameter recorded in the different anxiety paradigms (data not shown). This highlights that the effects of THC were reproducible irrespective of whether the mice were naïve or pre-exposed to the test apparatus.

In the LD, one-way ANOVA for baseline behaviour revealed an anxiolytic-like phenotype for the vehicle-treated *Nrg1* HET mice. Accordingly, *Nrg1* HET mice spent significantly more time in the light compartment [$F(1,21)=9.0$, $p<0.01$], showed increased locomotion rates [$F(1,21)=8.8$, $p<0.01$] in the area and exhibited an elevated light compartment entry score [$F(1,21)=9.6$, $p<0.01$] compared to vehicle-treated WT mice. Importantly, this anxiolytic-like profile of *Nrg1* HET mice holds when the locomotion-related parameters were corrected for the total distance travelled in this paradigm as highlighted by a significant increase in distance ratio [$F(1,21)=6.8$, $p<0.05$] for *Nrg1* hypomorphs within the aversive light compartment. Furthermore, vehicle-treated *Nrg1* HET mice spent significantly more time on the more aversive open arms of the EPM [$F(1,18)=4.4$, $p=0.05$]. The reduced anxiety observed in these mutant mice appears to be task-dependent as SI performance was not different between vehicle-treated WT and *Nrg1* HET mice.

Two-way ANOVA revealed that THC had a significant anxiogenic-like effect as it reduced the time mice spent on the open arms of the EPM [$F(2,52)=4.7$, $p<0.05$] and the ratio of open arm entries [$F(2,52)=7.7$, $p<0.01$]. Furthermore, THC treatment resulted in a decrease of time spent [$F(2,59)=5.8$, $p<0.01$] and distance travelled [$F(2,59)=9.3$, $p<0.001$] in the light compartment of the LD, of the frequency of entries into the same compartment [$F(2,59)=11.4$, $p<0.001$] and of distance ratio in this area [$F(2,59)=7.7$, $p<0.001$]. We also found a genotype by THC dose interaction for time spent in the light compartment of the LD [$F(2,59)=3.4$, $p<0.05$], for the frequency of entries into [$F(2,59)=5.2$, $p<0.01$] and the distance travelled in [$F(2,59)=4.2$, $p<0.05$] the same area. In addition, there was a trend for a genotype by THC dose interaction for the distance ratio in the light compartment [$F(2,59)=2.9$, $p=0.06$]. Post hoc analysis revealed that THC, at 5 mg/kg, decreased the open arm entry ratio in the EPM for *Nrg1* HET but not WT mice. Furthermore, THC administration, at both doses of 5 and 10 mg/kg, reduced all behaviours measured in the LD in the *Nrg1* HET but not the WT mice (see Table 2).

In the social interaction test, two-way ANOVA revealed no differential effect of THC treatment on *Nrg1* HET and WT mice in any measures derived from this test. THC treatment dose-dependently decreased the total time spent in social interaction [$F(2,59)=51.8$, $p<0.001$], the frequency of sniffing [$F(2,59)=82.2$, $p<0.001$] and the frequency of anogenital sniffing [$F(2,59)=55.8$, $p<0.001$] in a similar fashion in WT and *Nrg1* HET mice. No aggressive interactions were observed between test animals and the opponent A/J mice.

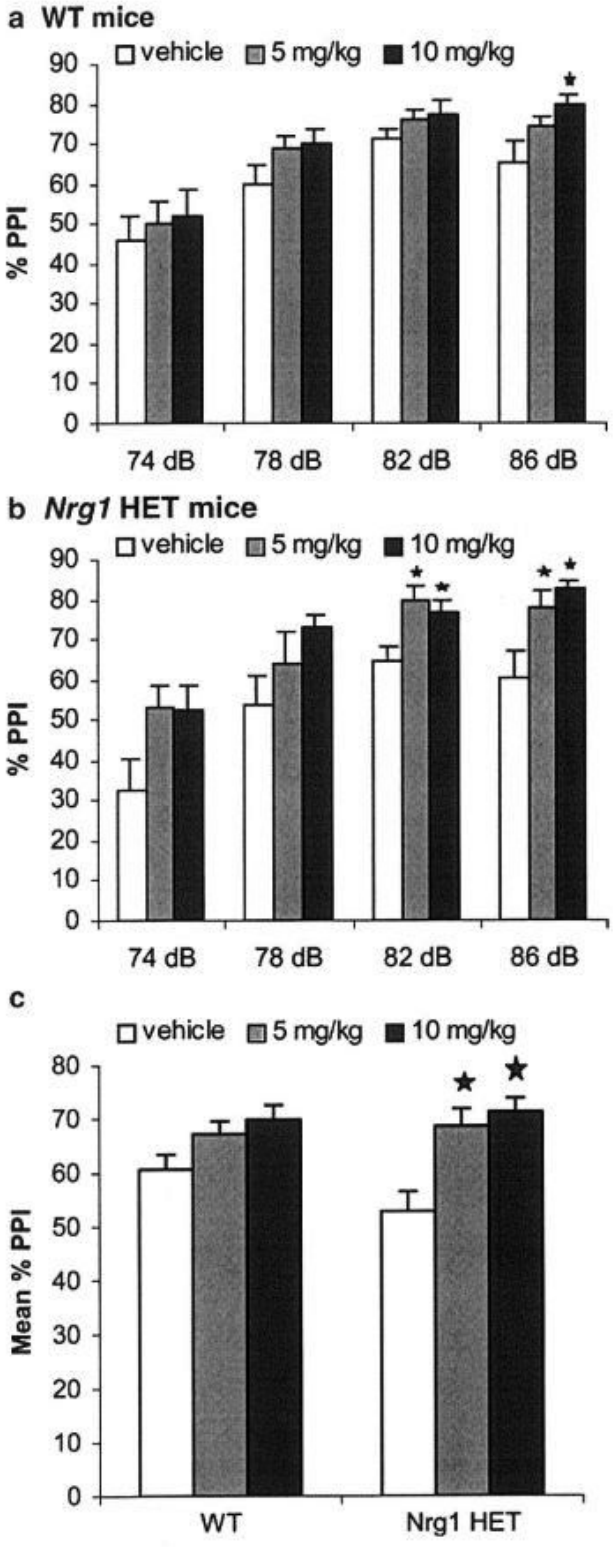
Vehicle-treated *Nrg1* HET mice showed no significant difference to vehicle-treated WT mice in the total time spent in the centre of the arena of the OF. However, two-way ANOVA showed that THC treatment significantly decreased the time spent in the centre of the OF [$F(2,52)=32.7$, $p<0.001$], which is a parameter for anxiety-related behaviour. Post hoc analysis revealed that in both genotypes, acute THC treatment (both 5 and 10 mg/kg) decreased the time spent in the centre of the OF compared to vehicle-treated animals.

Prepulse inhibition

Figure 1a–c presents the data from PPI testing. The prepulse inhibition (measured as % PPI) was elevated for both genotypes with increased prepulse intensities. At baseline, no differences were observed between vehicle-treated WT and *Nrg1* HET mice with regard to % PPI (Fig. 1a,b).

Two-way ANOVA revealed that acute THC administration significantly enhanced % PPI [$F(2,59)=6.4$, $p<0.01$; Fig. 1a,b]. Post hoc analyses indicated that in the *Nrg1* HET mice, the increased % PPI was observed for both THC doses at prepulses of 82 and 86 dB (Fig. 1b), whereas in WT mice, only treatment with 10 mg/kg THC significantly increased % PPI at the 86 dB prepulse (Fig. 1a). When % PPI was calculated as the % PPI of the mean PPI responses across all prepulse intensities, post hoc analysis revealed that both the 5 and 10 mg/kg THC doses increased % PPI in the *Nrg1* HET animals with no corresponding effects being observed in the WT mice (Fig. 1c).

No differences were observed between vehicle-treated *Nrg1* HET and WT mice when measuring their startle response to a 120 dB startle stimulus (Fig. 2). Two-way ANOVA revealed that THC treatment [$F(2,59)=6.1$, $p<0.01$] significantly reduced the startle response. Post hoc analysis revealed that the startle response-inhibiting effect of THC was only evident in *Nrg1* HET after treatment with 10 mg/kg THC and was not observed in the WT animals.



◀ **Fig. 1a–c** Prepulse Inhibition (PPI). The effect of acute THC treatment (30 min after i.p. injection of either vehicle or THC: 5 or 10 mg/kg BW) on % PPI [(startle response 120 dB–PPI response)×100/startle response 120 dB] was measured in WT and *Nrg1* HET mice (n=10–13). Different prepulse stimuli were used (4/8/12/16 dB above 70 dB background noise): **a** % PPI of WT mice, **b** % PPI of *Nrg1* HET mice, and **c** mean of % PPI of all summed prepulse responses for WT and *Nrg1* HET test animals. All data are presented as means ± standard error of the mean (SEM). Significant effects of acute THC treatment vs vehicle treatment are shown by "*" (p<0.05)

Discussion

Vehicle-treated *Nrg1* HET mice expressed a hyperactive, hyper-explorative and anxiolytic-like phenotype compared to WT animals. Importantly, mice hypomorphic for the *Nrg1* gene were more susceptible to THC's locomotor suppressant actions and its stimulating effect in an animal model for sensorimotor gating. In animal models of anxiety, less anxious *Nrg1* HET animals were no more affected by acute THC administration than their WT controls in an array of different models (e.g., social interaction and EPM). However, the *Nrg1* HET mice showed a greater anxiogenic response to THC than WT in the LD test, highlighting that the effects of THC on anxiety in these animals might be task-dependent.

The acute administration of THC decreased spontaneous locomotor activity and exploration in the open field test. Importantly, THC promoted a greater reduction in locomotor and exploratory activity in the *Nrg1* HET mice

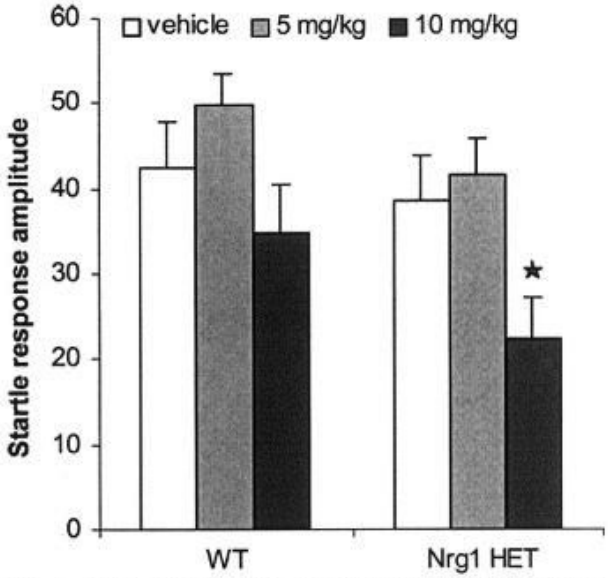


Fig. 2 Startle response. The startle response (averaged startle amplitude) in WT and *Nrg1* HET mice (n=10–13) was measured for a 120-dB startle stimulus 30 min after i.p. injection of vehicle or THC (5 or 10 mg/kg BW). All data are presented as means ± standard error of the mean (SEM). Significant effects of acute THC treatment vs vehicle treatment are shown by "*" (p<0.05)

compared to their WT controls, suggesting that the mutant mice are more susceptible to the well-established locomotor suppressant effects of cannabinoids (Arnold et al. 1998, 2001). It could be argued that this hypersensitivity may simply reflect the *Nrg1* HET mice being relatively hyperactive at baseline, thus, providing more room to delineate the locomotor suppressant effects of THC. However, a floor effect can be ruled out, as locomotor activity of both genotypes is not completely suppressed by the acute THC treatment (see, e.g., total distance in OF—Table 2). One likely explanation for the *Nrg1* HET animals' enhanced sensitivity to the locomotor inhibitory effects of cannabinoids may be based on developmental adaptations during ontogeny in the germline Nrg1 hypomorphic mice (e.g., increased expression of CB1 receptors or greater sensitivity of CB1 receptors to cannabinoid ligands in motor-related regions). The observation that *Nrg1* HET mice are hyperactive, combined with the finding that Nrg1 administration acts to inhibit locomotor behaviour in hamsters (Snodgrass-Belt et al. 2005), indicates that Nrg1 may function as an inhibitory counterbalance to excitatory neural processes mediating locomotor behaviour.

Vehicle-treated *Nrg1* hypomorphic mice exhibit an anxiolytic-like phenotype, which might be influenced by their hyperactivity. However, we detected an increase in the time hypomorphs spent in aversive areas. Thus, the decrease in anxiety is evident within a behavioural parameter, which is relatively unaffected by an unspecific increase in motor activity. These baseline differences exist for spatio-temporal (i.e., LD and EPM) rather than socio-temporal paradigms (i.e., SI). This task-dependent anxiolytic phenotype suggests an impact of Nrg1 on certain explorative/locomotive aspects of anxiety (Rodgers 1997; Rodgers and Johnson 1995). Importantly, anxiety symptoms (and high autonomic arousal) are very variable accompaniments to schizophrenia—often schizophrenia patients are diagnosed with more than one anxiety disorder (Braga et al. 2005; Tibbo et al. 2003; Townsend and Wilson 2005). Obviously, any animal model for candidate genes of schizophrenia models only some, but not all, aspects of the mental disorder. Future research has to clarify if the specific anxiolysis of Nrg1 hypomorphs is related to the comorbidity between anxiety disorders and psychosis reported in schizophrenia or if it is a direct effect of reductions in Nrg1 expression levels.

It has been widely reported that administration of cannabinoids stimulates anxiety-related behaviours in rodents (Onaivi et al. 1990, 1995; Rutkowska et al. 2006). In our study, we observed a task-specific increased susceptibility of *Nrg1* HET mice to the anxiogenic effects of THC compared to control animals. A confounding effect of the increased sensitivity of the *Nrg1* HET mice to the locomotor suppressant effects of THC can be ruled out, as spatiotemporal parameters of other anxiety paradigms (i.e., OF and EPM) were not differentially affected by THC treatment. It is possible that the enhanced sensitivity of the *Nrg1* HET animals to the anxiogenic effects of THC, as measured in the LD test, may reflect a specific anxiogenic-like action of THC on exploration/locomotion-related features of anxiety, which might be expressed to a greater extent in the mutant mice. These features are linked to how an organism adapts and responds to its spatial surrounding rather than to its social environment. Interestingly, both altered habituation to a novel environment in animal models (O'Tuathaigh et al. 2006) and social withdrawal in humans (Dixon et al. 1994) are described within the schizophrenia context. Further investigations should use a battery of different anxiety models screening for conditioned (conflict and other paradigms) and unconditioned responses (exploration, social, antipredator and other) after THC treatment to clarify this task-specificity (Rodgers 1997; Rodgers and Johnson 1995).

Within-subjects designs are commonly utilised in psychopharmacological mutant mice studies due to the difficulty in generating sufficient animal numbers. Importantly, altered anxiety-related behaviour (Espejo 1997; Lee and Rodgers 1990; Nyberg et al. 2003; Rodgers et al. 1996) and modulated sensitivity to the effects of psychotropic drugs upon repeated testing of mice in the same anxiety model (Holmes and Rodgers 1999; Rodgers et al. 1992; Rodgers and Shepherd 1993) are well-known phenomena. For instance, modulators of $GABA_A$ function (e.g., benzodiazepines) have diminished anxiolytic effects when administered to animals with prior test experience of the EPM. This phenomenon is known as "one trial tolerance" (Bertoglio and Carobrez 2002; File et al. 1990; Holmes and Rodgers 1999; Rodgers et al. 1992; Rodgers and Shepherd 1993). Our statistical analyses revealed no such altered effectiveness of THC with repeated testing, as mice displayed consistent anxiogenic responses to THC, whether they were naïve or had prior experience of the test apparatus. However, many studies demonstrating "one trial tolerance" to the effects of benzodiazepines use a 24 h test–retest interval (Albrechet-Souza et al. 2005; Cruz-Morales et al. 2002; File 1990; Holmes and Rodgers 1999). Therefore, it is possible that THC may have distorted effects with a shorter than 7 day intertrial interval, although the selective CB1 receptor antagonist AM 251 also showed consistent anxiety-modulating effects on EPM-naïve or -experienced mice using a 24 h test–retest interval (Rodgers et al. 2005). Interestingly, Bouwknecht et al. (2004) reported a relatively small impact of repeated testing on baseline anxiety behaviour of C57BL/6 mice using a 7 day intertrial interval. Furthermore, they showed consistent effects of the 5HT1A receptor agonist, flesinoxan, with repeated testing in anxiety models. Our results suggest that with a 1 week intertrial period, THC administered according to a within-subjects design provides

consistent behavioural results in various animal models of anxiety.

PPI is a phenomenon, whereby a pre-stimulus reduces the magnitude of the normal startle response to an intense startling stimulus (Graham 1975; Hoffman and Searle 1968). Importantly, motor activity differences (as shown for our animal model) do not to have an impact on the startle reflex to an acoustic startle stimulus (Leng et al. 2004). In the current study, 5 and 10 mg/kg THC exposure significantly increased PPI in the *Nrg1* HET mice, with the corresponding effect of THC not being as pronounced in WT animals. This increased effect of acute THC on PPI in the *Nrg1* HET mice appears to be valid, at least for the 5 mg/kg THC dose, as it was ineffective in reducing the startle response. The observation that 10 mg/kg THC significantly reduced startle in the *Nrg1* HET animals further supports that these mice are more sensitive to sedative actions of cannabinoids than WT animals.

Studies investigating the effects of cannabinoids on PPI in rodents are replete with inconsistencies. The anandamide reuptake and degradation inhibitor, AM404, and the synthetic cannabinoid receptor agonists, WIN 55,212-2 and CP 55,940, have been shown to promote deficits in PPI in some studies (Fernandez-Espejo and Galan-Rodriguez 2004; Mansbach et al. 1996; Martin et al. 2003; Schneider and Koch 2002) but not in others (Bortolato et al. 2005, 2006; Stanley-Cary et al. 2002). Two recent studies have directly examined the effects of THC on PPI (Malone and Taylor 2006; Nagai et al. 2006). Similar to our findings using wild type-like control mice, Malone and Taylor (2006) showed that THC (1 and 3 mg/kg) had no effect on PPI when administered to group-housed control rats. However, isolation-reared rats—suggested as another animal model for schizophrenia—exhibited PPI disruptions after THC treatment. Importantly, a genetic and environmental model should not be treated as identical models for schizophrenia as too little is known about biochemical and behavioural differences between these models. For example, socially isolated rats express increased anxiety-like behaviours (Wright et al. 1991) unlike *Nrg1* HET mice, which display reduced anxiety-related behaviour. Such differences and the fact that these studies were conducted on distinct species may account for the opposing effects of THC on PPI. Furthermore, it is widely accepted that any given animal model for schizophrenia can only represent certain aspects of this mental disorder not its entire complexity (Ellenbroek and Cools 2000; van den Buuse et al. 2005). Therefore, a direct comparison of a singular animal model (i.e., isolation rearing) with a two-hit model (i.e., Nrg1 depletion combined with drug abuse) would be overly simplistic.

Nagai et al. (2006), using almost identical doses of THC (6 and 10 mg/kg) to that employed here, demonstrated CB1 receptor-mediated PPI deficits in mice. The lack of correlation between the findings of Nagai et al. (2006) and our results in wild type-like mice are likely due to strain differences in the effects of cannabinoids as the C57BL6/J background strain used in our study has been shown to be less sensitive to the neurobehavioural effects of THC compared to other inbred strains (Arnold et al. 2001; Onaivi et al. 1995).

Given that chronic and - less robust - acute (Favrat et al. 2005; Linszen et al. 1994) THC exposure is thought to precipitate psychosis in vulnerable individuals (Degenhardt et al. 2003; Hall 1998) and that other known psychosis-promoting agents such as phencyclidine and amphetamine (Geyer et al. 2001; Mansbach and Geyer 1989; Mansbach et al. 1988; Martin et al. 2003) disrupt PPI, the observation that THC exposure increased PPI selectively in *Nrg1* HET animals was unexpected. The self-medication theory is an alternative theory for why people with schizophrenia are more likely to administer drugs of abuse than the general population (Arseneault et al. 2004; Degenhardt et al. 2001; Schneier and Siris 1987; Schofield et al. 2006). According to this theory, schizophrenia patients try to alleviate the symptoms associated with the disorder and/or to counter the side effects of antipsychotic medication. Interestingly, the most widely used drug in the schizophrenia population, nicotine, increases PPI in rats and reverses PPI deficits in schizophrenia patients (Acri et al. 1994; Kumari et al. 2001; Postma et al. 2006). Thus, schizophrenia patients may self-medicate with nicotine to reverse their cognitive deficits. Similarly, our observation of THC-induced enhancement of PPI supports the hypothesis that acute THC may have a partly beneficial action in schizophrenia patients by improving a negative symptom such as attentional dysfunction. Unfortunately, there is no satisfactory animal model for a variety of positive symptoms (i.e., hallucinations, delusions) of schizophrenia; hence, the effects of THC on these core aspects of the mental disorder cannot be concluded upon by the current investigation. However, it is interesting to note that THC, like clozapine, acts to reverse the hyperactive phenotype of *Nrg1* HET mice (Stefansson et al. 2002). On the contrary in human studies, it appears cannabis may acutely exacerbate positive schizophrenia symptoms (Grech et al. 2005; Linszen et al. 1994; van Os et al. 2002). Another study discusses the potential bidirectional interactions between cannabis use and psychosis (Hides et al. 2006).

Given that *Nrg1* HET mice provide a putative animal model of schizophrenia, the current results are consistent with the notion that *acutely* administered THC may reverse this schizophrenia phenotype, decreasing hyperactivity and enhancing attention as measured by PPI. As mice were tested repeatedly, behavioural alterations in *Nrg1* HET mice could be influenced by disrupted habituation and novelty processing-both have been considered central to the cognitive deficits observed in schizophrenia (O'Tuathaigh

et al. 2006). These results may provide an animal model of genetic vulnerability to self-medication using cannabinoids, where users may initiate cannabis use as a means to dampen some symptoms of schizophrenia. However, future investigations are needed to examine the effects of *chronic use* of cannabinoids on the *Nrg1* HET mice, as it is the extended use of cannabis that is more strongly associated with precipitating psychosis and exacerbating symptoms in schizophrenia patients.

Acknowledgements This research was supported by NISAD, utilizing infrastructure funding from NSW Health, by the Sylvia and Charles Viertel Charitable Foundation, by the Deutsche Forschungsgemeinschaft (Forschungsstipendium: Ka 1837/1-1 and Ka 1837/1-2 awarded to TK), by the National Alliance for Research on Schizophrenia and Depression (Young Investigator Award awarded to TK) and by a Clive and Vera Ramaciotti Foundation Young Investigator Grant awarded to JCA. AAB was supported by a NISAD Postgraduate Research Scholarship. We thank Prof. Richard Harvey for providing the *Nrg1* mice and the biological testing facility staff (especially J. Fisher, M. Pickering and K. Kerr) for their support. The critical comments by Jerry Tanda on the manuscript are gratefully acknowledged.

References

Acri JB, Morse DE, Popke EJ, Grunberg NE (1994) Nicotine increases sensory gating measured as inhibition of the acoustic startle reflex in rats. Psychopharmacology (Berl) 114:369–374

Albrechet-Souza L, Oliveira AR, De Luca MC, Tomazini FM, Santos NR, Brandao ML (2005) A comparative study with two types of elevated plus-maze (transparent vs. opaque walls) on the anxiolytic effects of midazolam, one-trial tolerance and fear-induced analgesia. Prog Neuropsychopharmacol Biol Psychiatry 29:571–579

Arnold JC, Topple AN, Hunt GE, McGregor IS (1998) Effects of pre-exposure and co-administration of the cannabinoid receptor agonist CP 55,940 on behavioral sensitization to cocaine. Eur J Pharmacol 354:9–16

Arnold JC, Topple AN, Mallet PE, Hunt GE, McGregor IS (2001) The distribution of cannabinoid-induced Fos expression in rat brain: differences between the Lewis and Wistar strain. Brain Res 921:240–255

Arseneault L, Cannon M, Witton J, Murray RM (2004) Causal association between cannabis and psychosis: examination of the evidence. Br J Psychiatry 184:110–117

Balerio GN, Aso E, Maldonado R (2006) Role of the cannabinoid system in the effects induced by nicotine on anxiety-like behaviour in mice. Psychopharmacology (Berl) 184:504–513

Bayer TA, Falkai P, Maier W (1999) Genetic and non-genetic vulnerability factors in schizophrenia: the basis of the "two hit hypothesis". J Psychiatr Res 33:543–548

Bertoglio LJ, Carobrez AP (2002) Anxiolytic effects of ethanol and phenobarbital are abolished in test-experienced rats submitted to the elevated plus maze. Pharmacol Biochem Behav 73:963–969

Boissier JR, Simon P, Lwoff JM (1964) Use of a particular mouse reaction (hole board method) for the study of psychotropic drugs. Therapie 19:571–583

Boksa P (2004) Animal models of obstetric complications in relation to schizophrenia. Brain Res Brain Res Rev 45:1–17

Bortolato M, Aru GN, Frau R, Orru M, Luckey GC, Boi G, Gessa GL (2005) The CB receptor agonist WIN 55,212-2 fails to elicit disruption of prepulse inhibition of the startle in Sprague Dawley rats. Psychopharmacology (Berl) 177:264–271

Bortolato M, Campolongo P, Mangieri RA, Scattoni ML, Frau R, Trezza V, La Rana G, Russo R, Calignano A, Gessa GL, Cuomo V, Piomelli D (2006) Anxiolytic-like properties of the anandamide transport inhibitor AM404. Neuropsychopharmacology 31 (12):2652–2659

Bouwknecht JA, van der Gugten J, Groenink L, Olivier B, Paylor RE (2004) Effects of repeated testing in two inbred strains on flesinoxan dose–response curves in three mouse models for anxiety. Eur J Pharmacol 494:35–44

Braga RJ, Mendlowicz MV, Marrocos RP, Figueira IL (2005) Anxiety disorders in outpatients with schizophrenia: prevalence and impact on the subjective quality of life. J Psychiatr Res 39:409–414

Cardno AG, Gottesman II (2000) Twin studies of schizophrenia: from bow-and-arrow concordances to star wars Mx and functional genomics. Am J Med Genet 97:12–17

Compton DR, Aceto MD, Lowe J, Martin BR (1996) In vivo characterization of a specific cannabinoid receptor antagonist (SR141716A): inhibition of delta 9-tetrahydrocannabinol-induced responses and apparent agonist activity. J Pharmacol Exp Ther 277:586–594

Costall B, Jones BJ, Kelly ME, Naylor RJ, Tomkins DM (1989) Exploration of mice in a black and white test box: validation as a model of anxiety. Pharmacol Biochem Behav 32:777–785

Coyle JT (1996) The glutamatergic dysfunction hypothesis for schizophrenia. Harv Rev Psychiatry 3:241–253

Crawley JN (1985) Exploratory behavior models of anxiety in mice. Neurosci Biobehav Rev 9:37–44

Crawley JN (1999) Behavioral phenotyping of transgenic and knockout mice: experimental design and evaluation of general health, sensory functions, motor abilities, and specific behavioral tests. Brain Res 835:18–26

Cruz-Morales SE, Santos NR, Brandao ML (2002) One-trial tolerance to midazolam is due to enhancement of fear and reduction of anxiolytic-sensitive behaviors in the elevated plus-maze retest in the rat. Pharmacol Biochem Behav 72:973–978

DeFries JC, Hegmann JP, Weir MW (1966) Open-field behavior in mice: evidence for a major gene effect mediated by the visual system. Science 154:1577–1579

Degenhardt L, Hall W (2002) Cannabis and psychosis. Curr Psychiatry Rep 4:191–196

Degenhardt L, Hall W, Lynskey M (2001) Alcohol, cannabis and tobacco use among Australians: a comparison of their associations with other drug use and use disorders, affective and anxiety disorders, and psychosis. Addiction 96:1603–1614

Degenhardt L, Hall W, Lynskey M (2003) Testing hypotheses about the relationship between cannabis use and psychosis. Drug Alcohol Depend 71:37–48

Denenberg VH (1969) Open-field behavior in the rat: what does it mean? Ann N Y Acad Sci 159:852–859

Dixon AK, Huber C, Lowe DA (1994) Clozapine promotes approach-oriented behavior in male mice. J Clin Psychiatry 55:4–7

Duan J, Martinez M, Sanders AR, Hou C, Krasner AJ, Schwartz DB, Gejman PV (2005) Neuregulin 1 (NRG1) and schizophrenia: analysis of a US family sample and the evidence in the balance. Psychol Med 35:1599–1610

Duncan GE, Sheitman BB, Lieberman JA (1999) An integrated view of pathophysiological models of schizophrenia. Brain Res Brain Res Rev 29:250–264

Ellenbroek BA, Cools AR (2000) Animal models for the negative symptoms of schizophrenia. Behav Pharmacol 11:223–233

Espejo EF (1997) Effects of weekly or daily exposure to the elevated plus-maze in male mice. Behav Brain Res 87:233–238

Falls DL (2003) Neuregulins: functions, forms, and signaling strategies. Exp Cell Res 284:14–30

Farber NB, Wozniak DF, Price MT, Labruyere J, Huss J, St Peter H, Olney JW (1995) Age-specific neurotoxicity in the rat associated with NMDA receptor blockade: potential relevance to schizophrenia? Biol Psychiatry 38:788–796

Favrat B, Menetrey A, Augsburger M, Rothuizen LE, Appenzeller M, Buclin T, Pin M, Mangin P, Giroud C (2005) Two cases of "cannabis acute psychosis" following the administration of oral cannabis. BMC Psychiatry 5:17

Fernandez-Espejo E, Galan-Rodriguez B (2004) Sensorimotor gating in mice is disrupted after AM404, an anandamide reuptake and degradation inhibitor. Psychopharmacology (Berl) 175:220–224

File SE (1988) How good is social interaction as a test of anxiety? In: Simon P, Soubrié P, Wildlocher D (eds) Animal models of psychiatric disorders. Selected models of anxiety, depression and psychosis. Karger, Basel, München, Paris, London, New York, New Delhi, Singapore, Tokyo, and Sydney, pp 151–166

File SE (1990) One-trial tolerance to the anxiolytic effects of chlordiazepoxide in the plus-maze. Psychopharmacology (Berl) 100:281–282

File SE, Mabbutt PS, Hitchcott PK (1990) Characterisation of the phenomenon of "one-trial tolerance" to the anxiolytic effect of chlordiazepoxide in the elevated plus-maze. Psychopharmacology (Berl) 102:98–101

Geyer MA, Swerdlow NR (1998) Measurement of startle response, prepulse inhibition, and habituation. In: Crawley JN, Skolnick P (eds) Current protocols in neuroscience, New York, pp 8.7.1–8.7.15

Geyer MA, Krebs-Thomson K, Braff DL, Swerdlow NR (2001) Pharmacological studies of prepulse inhibition models of sensorimotor gating deficits in schizophrenia: a decade in review. Psychopharmacology (Berl) 156:117–154

Graham FK (1975) Presidential Address, 1974. The more or less startling effects of weak prestimulation. Psychophysiology 12:238–248

Grech A, Van Os J, Jones PB, Lewis SW, Murray RM (2005) Cannabis use and outcome of recent onset psychosis. Eur Psychiatr 20:349–353

Hall W (1998) Cannabis use and psychosis. Drug Alcohol Rev 17:433–444

Hall W, Degenhardt L (2000) Cannabis use and psychosis: a review of clinical and epidemiological evidence. Aust N Z J Psychiatry 34:26–34

Hashimoto R, Straub RE, Weickert CS, Hyde TM, Kleinman JE, Weinberger DR (2004) Expression analysis of neuregulin-1 in the dorsolateral prefrontal cortex in schizophrenia. Mol Psychiatry 9:299–307

Henquet C, Murray R, Linszen D, van Os J (2005) The environment and schizophrenia: the role of cannabis use. Schizophr Bull 31:608–612

Hides L, Dawe S, Kavanagh DJ, Young RM (2006) Psychotic symptom and cannabis relapse in recent-onset psychosis. Prospective study. Br J Psychiatry 189:137–143

Hoffman HS, Searle JL (1968) Acoustic and temporal factors in the evocation of startle. J Acoust Soc Am 43:269–282

Hogg S (1996) A review of the validity and variability of the elevated plus-maze as an animal model of anxiety. Pharmacol Biochem Behav 54:21–30

Holmes A, Rodgers RJ (1999) Influence of spatial and temporal manipulations on the anxiolytic efficacy of chlordiazepoxide in mice previously exposed to the elevated plus-maze. Neurosci Biobehav Rev 23:971–980

Iwata N, Suzuki T, Ikeda M, Kitajima T, Yamanouchi Y, Inada T, Ozaki N (2004) No association with the neuregulin 1 haplotype to Japanese schizophrenia. Mol Psychiatry 9:126–127

Johns A (2001) Psychiatric effects of cannabis. Br J Psychiatry 178:116–122

Karl T, Pabst R, von Horsten S (2003) Behavioral phenotyping of mice in pharmacological and toxicological research. Exp Toxicol Pathol 55:69–83

Karl T, Duffy L, Scimone A, Harvey RP, Schofield P (2006) Altered motor activity, exploration, and anxiety in heterozygous neuregulin 1 mutant mice: implications for understanding schizophrenia. Genes Brain Behav (in press)

Kask A, Nguyen HP, Pabst R, von Horsten S (2001) Factors influencing behavior of group-housed male rats in the social interaction test: focus on cohort removal. Physiol Behav 74:277–282

Kumari V, Soni W, Sharma T (2001) Influence of cigarette smoking on prepulse inhibition of the acoustic startle response in schizophrenia. Hum Psychopharmacol 16:321–326

Law AJ, Lipska BK, Weickert CS, Hyde TM, Straub RE, Hashimoto R, Harrison PJ, Kleinman JE, Weinberger DR (2006) Neuregulin 1 transcripts are differentially expressed in schizophrenia and regulated by 5′ SNPs associated with the disease. Proc Natl Acad Sci USA 103:6747–6752

Lee C, Rodgers RJ (1990) Antinociceptive effects of elevated plus-maze exposure: influence of opiate receptor manipulations. Psychopharmacology (Berl) 102:507–513

Leng A, Yee BK, Feldon J, Ferger B (2004) Acoustic startle response, prepulse inhibition, and spontaneous locomotor activity in MPTP-treated mice. Behav Brain Res 154:449–456

Leweke FM, Gerth CW, Klosterkotter J (2004) Cannabis-associated psychosis: current status of research. CNS Drugs 18:895–910

Linszen DH, Dingemans PM, Lenior ME (1994) Cannabis abuse and the course of recent-onset schizophrenic disorders. Arch Gen Psychiatry 51:273–279

Malone DT, Taylor DA (2006) The effect of delta9-tetrahydrocannabinol on sensorimotor gating in socially isolated rats. Behav Brain Res 166:101–109

Mansbach RS, Geyer MA (1989) Effects of phencyclidine and phencyclidine biologs on sensorimotor gating in the rat. Neuropsychopharmacology 2:299–308

Mansbach RS, Geyer MA, Braff DL (1988) Dopaminergic stimulation disrupts sensorimotor gating in the rat. Psychopharmacology (Berl) 94:507–514

Mansbach RS, Rovetti CC, Winston EN, Lowe JA 3rd (1996) Effects of the cannabinoid CB1 receptor antagonist SR141716A on the behavior of pigeons and rats. Psychopharmacology (Berl) 124:315–322

Martin RS, Secchi RL, Sung E, Lemaire M, Bonhaus DW, Hedley LR, Lowe DA (2003) Effects of cannabinoid receptor ligands on psychosis-relevant behavior models in the rat. Psychopharmacology (Berl) 165:128–135

Montgomery KC (1958) The relation between fear induced by novel stimulation and exploratory behavior. J Comp Physiol Psychol 48:254–260

Nagai H, Egashira N, Sano K, Ogata A, Mizuki A, Mishima K, Iwasaki K, Shoyama Y, Nishimura R, Fujiwara M (2006) Antipsychotics improve delta9-tetrahydrocannabinol-induced impairment of the prepulse inhibition of the startle reflex in mice. Pharmacol Biochem Behav 84:330–336

Nyberg JM, Vekovischeva O, Sandnabba NK (2003) Anxiety profiles of mice selectively bred for intermale aggression. Behav Genet 33:503–511

Onaivi ES, Green MR, Martin BR (1990) Pharmacological characterization of cannabinoids in the elevated plus maze. J Pharmacol Exp Ther 253:1002–1009

Onaivi ES, Chakrabarti A, Gwebu ET, Chaudhuri G (1995) Neurobehavioral effects of delta 9-THC and cannabinoid (CB1) receptor gene expression in mice. Behav Brain Res 72:115–125

O'Tuathaigh CM, O'Sullivan GJ, Kinsella A, Harvey RP, Tighe O, Croke DT, Waddington JL (2006) Sexually dimorphic changes in

the exploratory and habituation profiles of heterozygous neuregulin-1 knockout mice. Neuroreport 17:79–83
Pellow S, Chopin P, File SE, Briley M (1985) Validation of open: closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods 14:149–167
Postma P, Gray JA, Sharma T, Geyer M, Mehrotra R, Das M, Zachariah E, Hines M, Williams SC, Kumari V (2006) A behavioural and functional neuroimaging investigation into the effects of nicotine on sensorimotor gating in healthy subjects and persons with schizophrenia. Psychopharmacology (Berl) 184:589–599
Rodgers RJ (1997) Animal models of 'anxiety': where next? Behav Pharmacol 8:477–496; discussion 497–504
Rodgers RJ, Johnson NJ (1995) Factor analysis of spatiotemporal and ethological measures in the murine elevated plus-maze test of anxiety. Pharmacol Biochem Behav 52:297–303
Rodgers RJ, Shepherd JK (1993) Influence of prior maze experience on behaviour and response to diazepam in the elevated plus-maze and light/dark tests of anxiety in mice. Psychopharmacology (Berl) 113:237–242
Rodgers RJ, Lee C, Shepherd JK (1992) Effects of diazepam on behavioural and antinociceptive responses to the elevated plus-maze in male mice depend upon treatment regimen and prior maze experience. Psychopharmacology (Berl) 106:102–110
Rodgers RJ, Johnson NJ, Cole JC, Dewar CV, Kidd GR, Kimpson PH (1996) Plus-maze retest profile in mice: importance of initial stages of trail 1 and response to post-trail cholinergic receptor blockade. Pharmacol Biochem Behav 54:41–50
Rodgers RJ, Evans PM, Murphy A (2005) Anxiogenic profile of AM-251, a selective cannabinoid CB1 receptor antagonist, in plus-maze-naive and plus-maze-experienced mice. Behav Pharmacol 16:405–413
Rutkowska M, Jamontt J, Gliniak H (2006) Effects of cannabinoids on the anxiety-like response in mice. Pharmacol Rep 58:200–206
Schneider M, Koch M (2002) The cannabinoid agonist WIN 55,212-2 reduces sensorimotor gating and recognition memory in rats. Behav Pharmacol 13:29–37
Schneier FR, Siris SG (1987) A review of psychoactive substance use and abuse in schizophrenia. Patterns of drug choice. J Nerv Ment Dis 175:641–652
Schofield D, Tennant C, Nash L, Degenhardt L, Cornish A, Hobbs C, Brennan G (2006) Reasons for cannabis use in psychosis. Aust N Z J Psychiatry 40:570–574
Snodgrass-Belt P, Gilbert JL, Davis FC (2005) Central administration of transforming growth factor-alpha and neuregulin-1 suppress active behaviors and cause weight loss in hamsters. Brain Res 1038:171–182
Stanley-Cary CC, Harris C, Martin-Iverson MT (2002) Differing effects of the cannabinoid agonist, CP 55,940, in an alcohol or Tween 80 solvent, on prepulse inhibition of the acoustic startle reflex in the rat. Behav Pharmacol 13:15–28
Stefansson H, Sigurdsson E, Steinthorsdottir V, Bjornsdottir S, Sigmundsson T, Ghosh S, Brynjolfsson J, Gunnarsdottir S, Ivarsson O, Chou TT, Hjaltason O, Birgisdottir B, Jonsson H, Gudnadottir VG, Gudmundsdottir E, Bjornsson A, Ingvarsson B, Ingason A, Sigfusson S, Hardardottir H, Harvey RP, Lai D, Zhou M, Brunner D, Mutel V, Gonzalo A, Lemke G, Sainz J, Johannesson G, Andresson T, Gudbjartsson D, Manolescu A, Frigge ML, Gurney ME, Kong A, Gulcher JR, Petursson H, Stefansson K (2002) Neuregulin 1 and susceptibility to schizophrenia. Am J Hum Genet 71:877–892
Sullivan PF, Kendler KS, Neale MC (2003) Schizophrenia as a complex trait: evidence from a meta-analysis of twin studies. Arch Gen Psychiatry 60:1187–1192
Susser ES, Lin SP (1992) Schizophrenia after prenatal exposure to the Dutch Hunger Winter of 1944–1945. Arch Gen Psychiatry 49:983–988
Tang JX, Chen WY, He G, Zhou J, Gu NF, Feng GY, He L (2004) Polymorphisms within 5′ end of the neuregulin 1 gene are genetically associated with schizophrenia in the Chinese population. Mol Psychiatry 9:11–12
Tibbo P, Swainson J, Chue P, LeMelledo JM (2003) Prevalence and relationship to delusions and hallucinations of anxiety disorders in schizophrenia. Depress Anxiety 17:65–72
Townsend MH, Wilson MS (2005) Comorbid anxiety disorders and divalproex sodium use among partial hospital patients with psychotic disorders. Compr Psychiatry 46:368–370
van den Buuse M, Garner B, Gogos A, Kusljic S (2005) Importance of animal models in schizophrenia research. Aust N Z J Psychiatry 39:550–557
van Gaalen MM, Steckler T (2000) Behavioural analysis of four mouse strains in an anxiety test battery. Behav Brain Res 115:95–106
van Os J, Bak M, Hanssen M, Bijl RV, de Graaf R, Verdoux H (2002) Cannabis use and psychosis: a longitudinal population-based study. Am J Epidemiol 156:319–327
Varvel SA, Wiley JL, Yang R, Bridgen DT, Long K, Lichtman AH, Martin BR (2006) Interactions between THC and cannabidiol in mouse models of cannabinoid activity. Psychopharmacology (Berl) 186:226–234
Walss-Bass C, Liu W, Lew DF, Villegas R, Montero P, Dassori A, Leach RJ, Almasy L, Escamilla M, Raventos H (2006) A novel missense mutation in the transmembrane domain of neuregulin 1 is associated with schizophrenia. Biol Psychiatry 60(6):548–553
Wang JH, Short J, Ledent C, Lawrence AJ, van den Buuse M (2003) Reduced startle habituation and prepulse inhibition in mice lacking the adenosine A2A receptor. Behav Brain Res 143:201–207
Wong AH, Van Tol HH (2003) Schizophrenia: from phenomenology to neurobiology. Neurosci Biobehav Rev 27:269–306
Wright IK, Upton N, Marsden CA (1991) Resocialisation of isolation-reared rats does not alter their anxiogenic profile on the elevated X-maze model of anxiety. Physiol Behav 50:1129–1132
Yang JZ, Si TM, Ruan Y, Ling YS, Han YH, Wang XL, Zhou M, Zhang HY, Kong QM, Liu C, Zhang DR, Yu YQ, Liu SZ, Ju GZ, Shu L, Ma DL, Zhang D (2003) Association study of neuregulin 1 gene with schizophrenia. Mol Psychiatry 8:706–709

Summary of the main results at the end of Chapter 2

Chapter 2 examined the behavioural effects of THC (10 mg/kg) on WT and *Nrg1* HET mice. Two groups of animals have been tested in either the open field test (30 min), followed by the hole board test (7 min) and the elevated plus maze (5 min), or in the light dark test (10 min), followed by social interaction (10 min) and prepulse inhibition (30 min), animals returning to their home cage for 5 min between the different tests. The main results obtained from this experiment are summarised in Table 3 and show that *Nrg1* HET mice are more sensitive to the effects of THC on locomotor activity and prepulse inhibition.

Table 3. Summary of main results form Chapter 2.

Test	Chapter 2 Effect of THC (10 mg/kg)
Locomotor activity in the open field 	Hypolocomotion more important in *Nrg1* HET mice than WT mice
Anxiety in the light dark 	Decreased time spent in the light compartment only in *Nrg1* HET mice
Social interaction 	Decreased social interaction in both WT and *Nrg1* HET mice similarly
Prepulse inhibition 	Facilitation only in *Nrg1* HET mice

Chapter 3

HETEROZYGOUS NEUREGULIN 1 MICE DISPLAY GREATER BASELINE AND Δ^9-TETRAHYDROCANNABINOL-INDUCED C-FOS EXPRESSION

Neuroscience 149 (2007) 861–870

HETEROZYGOUS NEUREGULIN 1 MICE DISPLAY GREATER BASELINE AND Δ^9-TETRAHYDROCANNABINOL-INDUCED c-Fos EXPRESSION

A. A. BOUCHER,[a,b,c] G. E. HUNT,[d] T. KARL,[a,e] J. MICHEAU,[c] I. S. McGREGOR[f] AND J. C. ARNOLD[a,b]*

[a]*Schizophrenia Research Institute, Sydney, NSW 2010, Australia*

[b]*Department of Pharmacology, Bosch Institute, Bosch Building DO5, University of Sydney, Sydney, NSW 2006, Australia*

[c]*Laboratoire de Neurosciences Cognitives, Université Bordeaux I, 33405 Talence, France*

[d]*Department of Psychological Medicine, University of Sydney, Concord Hospital, Sydney, NSW 2139, Australia*

[e]*Neuroscience Research Program, Garvan Institute of Medical Research, NSW 2010, Australia*

[f]*School of Psychology, University of Sydney, Sydney, NSW 2006, Australia*

Abstract—Cannabis use may increase the risk of developing schizophrenia by precipitating the disorder in genetically vulnerable individuals. Neuregulin 1 (*NRG1*) is a schizophrenia susceptibility gene and mutant mice heterozygous for the transmembrane domain of this gene (*Nrg1* HET mice) exhibit a schizophrenia-related phenotype. We have recently shown that *Nrg1* HET mice are more sensitive to the behavioral effects of the main psychoactive constituent of cannabis, Δ^9-tetrahydrocannabinol (THC). In the present study, we examined the effects of THC (10 mg/kg i.p.) on neuronal activity in *Nrg1* HET mice and wild type-like (WT) mice using c-Fos immunohistochemistry. In the lateral septum, THC selectively increased c-Fos expression in *Nrg1* HET mice with no corresponding effect being observed in WT mice. In addition, THC promoted a greater increase in c-Fos expression in *Nrg1* HET mice than WT mice in the central nucleus of the amygdala, the bed nucleus of the stria terminalis and the paraventricular nucleus of the hypothalamus. Consistent with *Nrg1* HET mice exhibiting a schizophrenia-related phenotype, these mice expressed greater drug-free levels of c-Fos in two regions thought to be involved in schizophrenia, the shell of the nucleus accumbens and the lateral septum. Interestingly, the effects of genotype on c-Fos expression, drug-free or following THC exposure, were only observed when animals experienced behavioral testing prior to perfusion. This suggests an interaction with stress was necessary for the promotion of these effects. These data provide neurobiological correlates for the enhanced behavioral sensitivity of *Nrg1* HET mice to THC and reinforce the existence of cannabinoid-neuregulin 1 interactions in the CNS. This research may enhance our understanding of how genetic factors increase individual vulnerability to schizophrenia and cannabis-induced psychosis.

Key words: cannabinoid, c-Fos, schizophrenia, susceptibility gene, transgenic mice.

Schizophrenia is a chronic and disabling mental disorder that affects 1% of the world's population. Drugs of abuse such as cannabis increase the risk of developing schizophrenia (Linszen et al., 1994; Arseneault et al., 2004). Cannabis consumption is associated with an earlier onset of psychotic symptoms, an exacerbation of the disorder, poor treatment outcome and an increased likelihood of psychotic relapse (Degenhardt and Hall, 2002). The observation that many cannabis users do not develop schizophrenia has led to the hypothesis that cannabinoid exposure is a component cause that might unmask schizophrenia in people that have a predisposition to the disorder. Indeed, the cause of schizophrenia is likely to be multifactorial involving the interaction of susceptibility genes with environmental risk factors such as cannabis use (Bayer et al., 1999; Arseneault et al., 2004).

Human and animal studies have reported that neuregulin 1 (*NRG1*), a gene from chromosome 8p, is a candidate gene for schizophrenia (Stefansson et al., 2002; Walss-Bass et al., 2006). Nrg1 is a ligand for ErbB receptor tyrosine kinases which when bound may affect schizophrenia-related neurodevelopmental processes such as the expression and function of CNS neurotransmitter receptors, myelination, axon guidance, neuronal migration and glial differentiation (Falls, 2003). Several studies have shown that mutant mice heterozygous for the transmembrane domain of *Nrg1* (*Nrg1* HET mice) exhibit a schizophrenia-related behavioral phenotype. This includes sensorimotor gating deficits, hyperlocomotion and an increased sensitivity to environmental factors (Stefansson et al., 2002; O'Tuathaigh et al., 2006; Boucher et al., 2007; Karl et al., 2007). In addition, consistent with the neurodevelopmental theory of schizophrenia, these mice display an age-dependent phenotype and a hypofunctional glutamatergic system with a deficit in NMDA receptor expression (Stefansson et al., 2002; Karl et al., 2007). Taken together, *Nrg1* HET mice provide one of the most promising animal models of genetic vulnerability to schizophrenia.

*Correspondence to: J. C. Arnold, Department of Pharmacology, Bosch Building DO5, University of Sydney, Sydney, NSW 2006, Australia. Tel: +61-2-9036-5361; fax: +61-2-9351-2658.
E-mail address: arnie@med.usyd.edu.au (J. C. Arnold).

Abbreviations: ANOVA, analysis of variance; BNST, bed nucleus of the stria terminalis; CEA, central nucleus of the amygdala; CPU, caudate putamen; LH, lateral hypothalamus; LSV, ventrolateral septum; NAC, nucleus accumbens core; NAS, nucleus accumbens shell; *NRG1*, neuregulin 1; *Nrg1* HET, heterozygous neuregulin 1; PAG, periaqueductal gray; PB, phosphate buffer; PBH, phosphate-buffered horse serum; PFA, paraformaldehyde; PFC, prefrontal cortex; PPI, prepulse inhibition; PV, paraventricular nucleus of the thalamus; PVN, paraventricular nucleus of the hypothalamus; THC, Δ^9-tetrahydrocannabinol; VEH, vehicle; VMH, ventromedial hypothalamus; VTA, ventral tegmental area; WT, wild type-like.

0306-4522/07$30.00+0.00
doi:10.1016/j.neuroscience.2007.08.020

We have recently shown that *Nrg1* HET mice also have increased sensitivity to the acute behavioral effects of the main psychoactive constituent of cannabis, Δ^9-tetrahydrocannabinol (THC) (Boucher et al., 2007). These mice were more sensitive to the locomotor suppressant and anxiogenic effects of THC than wild type-like (WT) mice. Further, THC selectively facilitated sensorimotor gating as measured by prepulse inhibition (PPI) in *Nrg1* HET mice. This prior study provided the first evidence that heterozygous deletion of a schizophrenia susceptibility gene alters the behavioral effects of cannabinoids. Here we examine the neurobiological underpinnings of such a phenomenon by measuring THC-induced neuronal activation in *Nrg1* HET and WT mice using c-Fos immunohistochemistry.

EXPERIMENTAL PROCEDURES

Animals

Male adult (18–19 weeks) heterozygous *Nrg1* transmembrane domain mice generated from a C57BL/6 background strain were provided by Prof. Richard Harvey (Victor Chang Cardiac Research Institute, Sydney, Australia) as previously described (Boucher et al., 2007; Karl et al., 2007). Animals were pair-housed under a 12 h light/dark schedule with food and water available *ad libitum*. All research and animal care procedures were approved by the "Garvan Institute/St. Vincent's Hospital Animal Experimentation Ethics Committee" and were in agreement with the "Australian Code of Practice for the Care and Use of Animals for Scientific Purposes." Every effort was made to minimize the number of animals used and their suffering.

Drug and experimental procedures

As previously described by Boucher et al. (2007), THC (Sigma-Aldrich, Sydney, NSW, Australia) was dissolved in 4% ethanol, 1% Tween 80, and 95% saline. THC or vehicle (VEH) was administered intraperitoneally (i.p.) at 10 mg/kg in a volume of 10 ml/kg of body weight. Thirty minutes after the injection, mice were tested in various paradigms of motor coordination (wire hang, pole, beam walk and rotarod tests), explorative (open field and hole-board tests) and anxiety-related behaviors (light–dark test and elevated plus maze). Animals were habituated to these behavioral tests on two consecutive days prior to the administration of THC to limit the impact of novelty-induced c-Fos expression. Data for these results are not reported due to an insufficient number of animals being tested to provide meaningful results. Immediately following behavioral testing and approximately 90 min after injection with either VEH or THC (10 mg/kg), mice were anesthetized with halothane and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde (PFA). To assess the influence of behavioral testing on c-Fos expression, another group of animals was treated with VEH or THC (10 mg/kg) 90 min before the perfusion, however none of these animals were tested in any behavioral paradigms. We then analyzed c-Fos expression using the same protocol and analyzed the regions of interest where a differential effect between *Nrg1* HET and WT mice was observed in animals that were behaviorally tested.

Immunohistochemistry

The brains were removed and postfixed overnight in fresh PFA at 4 °C. For cryoprotection, brains were incubated in 15% sucrose until they sank (approximately 1–2 days). The brains were then placed in 30% sucrose for approximately 3 days, until they sank again. Following this, the brains were placed in the cryostat for slow freezing for 1 h at −11–17 °C and sliced at 40 μm. Tissues were stored in freezing solution (ethanediol/glycerol) at −15–20 °C until the c-Fos staining was performed. For immunohistochemistry, free floating sections were washed in phosphate buffer (PB) before being sequentially incubated in 3% hydrogen peroxide for 30 min and 3% normal horse serum for 30 min. Sections were then stained with the c-Fos primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA, rabbit polyclonal; reacts with c-Fos p62 of mouse and non–cross-reactive with FosB, Fra-1 or Fra-2) diluted 1:10,000 in phosphate-buffered horse serum (PBH) (0.1% bovine serum albumin, 0.2% Triton X-100, 2% normal horse serum) for 3 days at 4 °C. Sections were washed in PB and then incubated for 1 h in biotinylated anti-rabbit IgG secondary antibody (Vector Laboratories, Burlingame, CA, USA) diluted 1:500 in PBH. Sections were washed again before being incubated in ExtrAvidin peroxidase (Sigma, diluted 1:1000 in PBH) for 2 h. After three washes in PB, peroxidase activity was visualized by placing the tissue in nickel diaminobenzidine (0.05% diaminobenzidine tetrahydrochloride, 0.4% ammonium chloride, 2% nickel ammonium sulfate, 20% D-glucose) and glucose oxidase (Sigma, 1:1000) and the reaction was terminated after 10 min by washing in PB. Sections were stored in PB at 4 °C before being mounted onto gelatinized slides, dehydrated, xylene cleared and coverslipped. Fos immunoreactive cells (black and dark brown) were quantified by a rater blind to treatment with reference to the mouse brain atlas of Paxinos and Franklin (2000) as previously described (McGregor et al., 1998; Arnold et al., 2001). Digitized images were produced by a Leica DC500 camera using a 10× objective attached to an Olympus BX51 light microscope. Images were acquired using Leica IM1000 Image Manager (version 4) software. The only post-production enhancement was reduction of color to black and white and the adjustment of brightness and contrast equally for all images using Adobe Photoshop CS2 (version 9.0).

Data analysis

Data for c-Fos immunoreactivity were analyzed using two-way analysis of variance (ANOVA). The two factors were genotype (WT and *Nrg1* HET mice) and treatment (VEH and THC). When a significant two-way ANOVA was found, within group comparisons were made using Tukey's post hoc test (where the factors of genotype or treatment were split by the corresponding factor). These post hoc comparisons are shown in Table 1 and Table 2. To assess the effects of behavioral testing on c-Fos expression, the regions of interest were also analyzed by two-way ANOVA in animals not previously tested in behavioral paradigms. These data were additionally analyzed using a three-way ANOVA where the factors were genotype (WT and *Nrg1* HET mice), treatment (VEH and THC) and behavior (testing or no testing). A significant level of $P<0.05$ was chosen for all comparisons.

RESULTS

VEH-treated *Nrg1* HET express greater c-Fos levels in the LSV and NAS

c-Fos expression in behaviorally tested animals for all reported regions is shown in Table 1. The comparison of baseline genotype differences in behaviorally tested animals showed that VEH-treated *Nrg1* HET mice expressed significantly more c-Fos than VEH-treated WT mice in the ventrolateral septum (LSV) and in the nucleus accumbens shell (NAS) (Fig. 1). This was highlighted by two-way ANOVA with a significant overall effect of genotype in the LSV [$F(1,12)=40.18$, $P<0.01$] and in the NAS [$F(1,12)=8.9$, $P<0.05$]. Consistent with this post hoc analysis using Tukey's test showed an increased c-Fos expression in VEH-treated *Nrg1* HET compared with WT mice in the LSV ($P<0.05$) and

Table 1. Quantification of c-Fos expression in WT and *Nrg1* HET mice after behavioral testing

Region	Bregma	WT		*Nrg1* HET	
		VEH	THC	VEH	THC
Frontal regions					
PFC, medial	+1.98	24.5±7	16±1	23±6	28±6
NAS	+0.98	12±1	12±2	21±3*	22.5±5
NAC	+0.98	1.5±1	1±0.5	1±0.6	1.5±1
CPU, medial	+0.98	8±4	1±1	4±2	12±7
CPU, central	+0.98	1±1	0.5±0.5	1±0.5	1±1
CPU, dorsolateral	+0.98	0±0	1±1	1±0.5	0.25±0.25
LSV	+0.98	39±8	36±9	65±4*	99±6##
BNST, dorsolateral	+0.26	2±0.4	37±13#	2±1	52±7###
Hypothalamus					
PVN	−0.94	19±8	53.5±8#	30±4	69±3###
Lateral	−1.58	8±2	10.5±3	10±3	13±3
Ventromedial	−1.58	0.5±0.3	3.5±2	2.5±1	3±1
Thalamus					
PV	−0.94	27±4	68.5±16#	28±8	70±15#
Amygdala					
CEA	−1.34	1.5±1	39±14#	2±1	48±10##
Hippocampus					
CA1	−1.58	1±0.5	0±0	1±1	0±0
CA3	−1.58	2±1	0.25±0.25	1±1	0.5±0.3
Midbrain					
VTA	−3.28–3.40	6±3	7.5±2	7.5±2	11±1
PAG, ventrolateral	−4.60	10±3	11.5±2	10.5±2	18±5

Number of Fos-labeled cells in various brain regions of behaviorally tested WT and *Nrg1* HET mice following VEH or THC (10 mg/kg) (n=4 per group). Data are presented as means±S.E.M.

* $P<0.05$, significant effects of vehicle-treated *Nrg1* HET compared to WT mice.

# $P<0.05$, significant effects of THC compared to vehicle in *Nrg1* HET and WT mice.

$P<0.01$, significant effects of THC compared to vehicle in *Nrg1* HET and WT mice.

$P<0.001$, significant effects of THC compared to vehicle in *Nrg1* HET and WT mice.

in the NAS ($P<0.05$). A trend for an increased baseline c-Fos expression was also detected in the paraventricular nucleus of the hypothalamus (PVN) [$F(1,12)=4.49$, $P=0.056$].

THC-induced c-Fos expression in *Nrg1* HET and WT mice not tested in behavioral paradigms prior to perfusion is shown in Table 2. Interestingly, VEH-treated *Nrg1* HET mice did not express more c-Fos than VEH-treated WT mice, as shown by two-way ANOVA with no effect of genotype being observed in any region examined including the LSV and the NAS. Moreover, three-way ANOVA comparing the effect of THC on mice that were behaviorally tested or not prior to c-Fos analysis showed a genotype by behavioral testing interaction in the LSV [$F(1,19)=19.02$, $P<0.001$] and a trend in the NAS [$F(1,19)=3.63$, $P=0.07$]. These results highlight that the selective increase in c-Fos in the LSV and NAS of VEH-treated *Nrg1* HET mice was only apparent when animals were behaviorally tested prior to the perfusion.

Nrg1 HET but not WT mice display THC-induced c-Fos expression in the LSV

When examining the effect of THC treatment in behaviorally tested animals we observed that, THC selectively in-

Table 2. Quantification of c-Fos expression in WT and Nrg1 HET mice without prior behavioral testing

Region	Bregma	WT		*Nrg1* HET	
		VEH	THC	VEH	THC
NAS	+0.98	7±3	5±2	4±0	7±7
LSV	+0.98	14±6	40±3#	18.5±6.5	41±4#
BNST, dorsolateral	+0.26	0±0	13±3#	1±1	34±7#
PVN	−0.94	6±2	52±24	7±0	57±20
CEA	−1.34	0±0	28±5##	9±2	40±8

Number of Fos-labeled cells in brain regions of non-behaviorally tested WT and *Nrg1* HET mice following VEH or THC (10 mg/kg) (n=2–3 per group). Data are presented as means±S.E.M.

# $P<0.05$, significant effects of THC compared to vehicle in *Nrg1* HET and WT mice.

$P<0.01$, significant effects of THC compared to vehicle in *Nrg1* HET and WT mice.

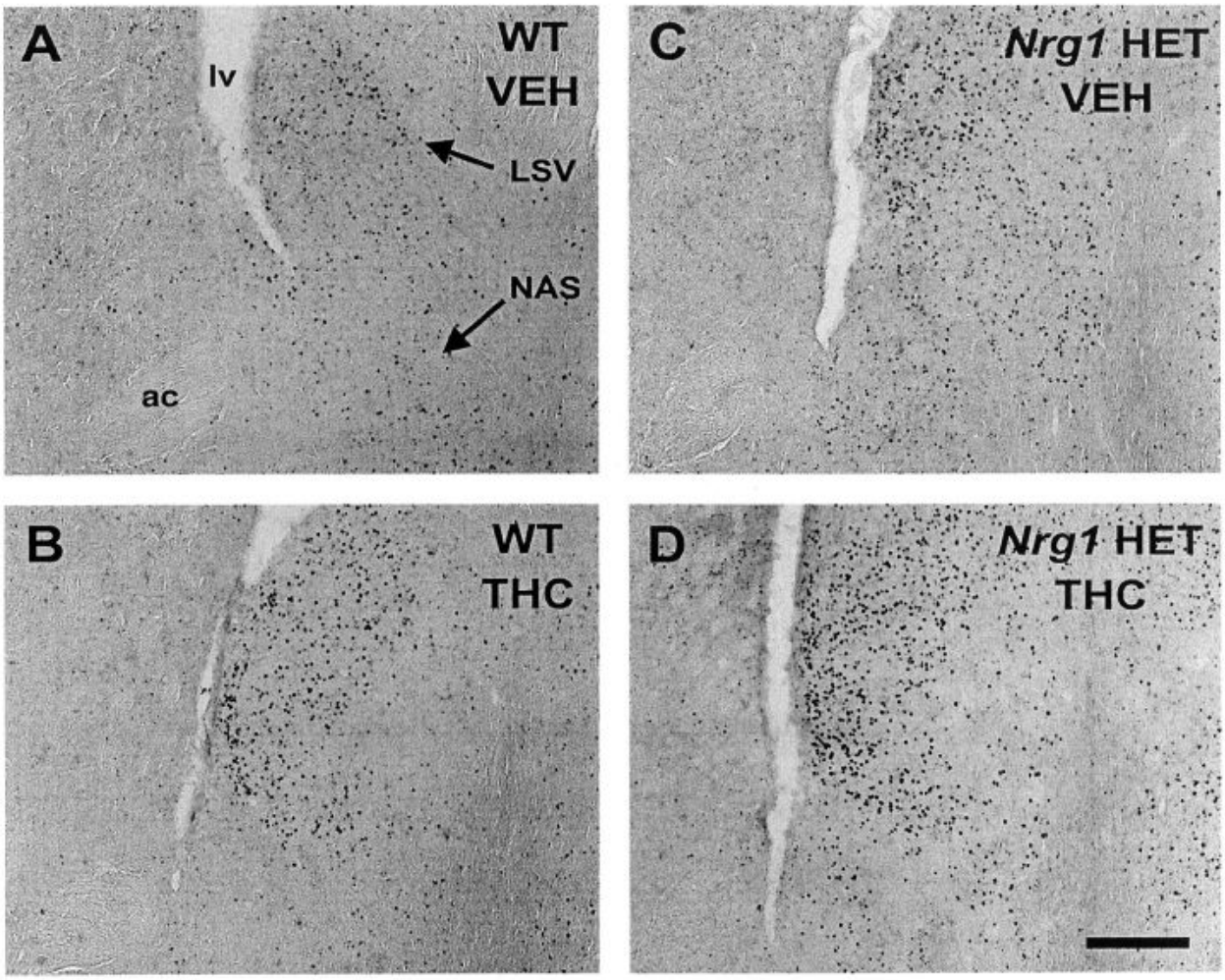


Fig. 1. Fos-labeled neurons within the LSV and the NAS in representative sections from WT mice treated with (A) VEH or (B) THC (10 mg/kg) and *Nrg1* HET mice treated with (C) VEH or (D) THC (10 mg/kg). The lateral ventricle (lv) and anterior commissure (ac) are also indicated. Scale bar=300 μm.

creased c-Fos expression in *Nrg1* HET mice with no corresponding effect being observed in WT mice in the LSV (see Table 1 and Fig. 1). This was highlighted by two-way ANOVA with a strong trend in treatment [$F(1,12)=4.72$, $P=0.05$] and a significant genotype by treatment interaction [$F(1,12)=6.95$, $P<0.05$]. Consistent with this post hoc analysis of the LSV showed THC selectively increased c-Fos expression compared with VEH only in *Nrg1* HET mice ($P<0.01$) but not in WT mice.

Two-way ANOVA of THC-induced c-Fos expression in animals not exposed to behavioral tests prior to the perfusion (see Table 2) revealed no effect of genotype and no genotype by treatment interaction in the LSV. In non-behaviorally tested animals, THC increased c-Fos expression in the LSV as supported by an effect of the treatment [$F(1,7)=26.15$, $P<0.01$]. However, no differential effect was observed as analyzed by Tukey's post hoc test where the THC-induced increase in c-Fos expression was similar for both WT and *Nrg1* HET mice (Ps<0.05 for both groups). Interestingly, when examining the effect of behavior on the results observed in the LSV, three-way ANOVA revealed a genotype by treatment by behavioral testing interaction [$F(1,19)=4.62$, $P<0.05$], highlighting that the increased sensitivity of *Nrg1* HET mice to THC-induced c-Fos in the LSV is dependent on behavioral testing.

The effects of THC on c-Fos expression in other brain regions

In behaviorally tested animals, THC increased c-Fos expression in both *Nrg1* HET and WT mice according to two-way ANOVA with a significant overall effect of treatment in the paraventricular nucleus of the thalamus (PV) [$F(1,12)=12.78$, $P<0.01$], the central nucleus of the amygdala (CEA) [$F(1,12)=24.01$, $P<0.01$], the dorsolateral part of the bed nucleus of the stria terminalis (BNST) [$F(1,12)=33.62$, $P<0.01$] and the PVN [$F(1,12)=34.49$, $P<0.01$] (see Table 1). Although no genotype by treatment interactions were observed in these regions, post hoc analysis revealed that THC exerted a greater magnitude of effect on

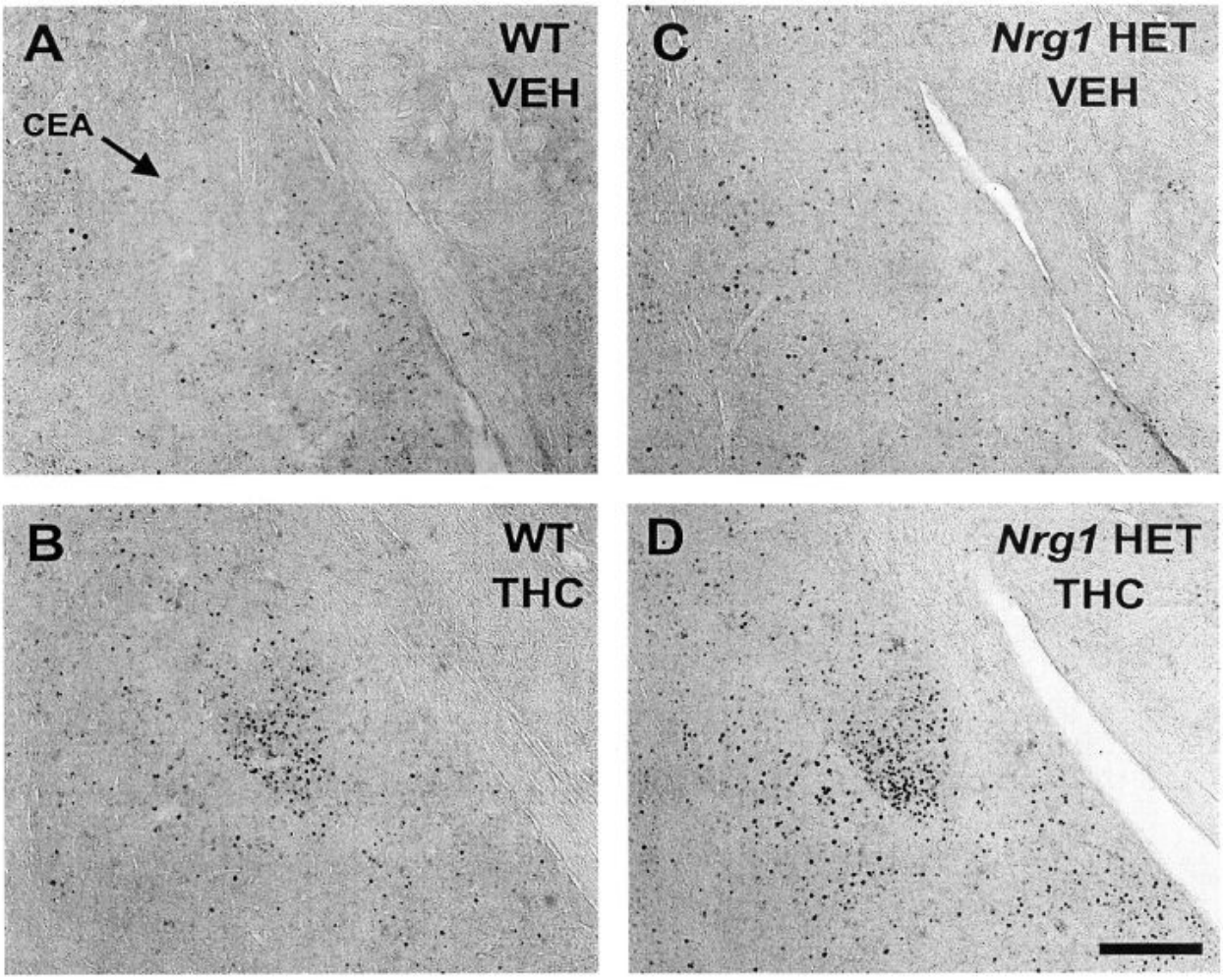


Fig. 2. Fos-labeled neurons within the CEA in representative sections from WT mice treated with (A) VEH or (B) THC (10 mg/kg) and *Nrg1* HET mice treated with (C) VEH or (D) THC (10 mg/kg). Scale bar=300 μm.

the *Nrg1* HET mice in the CEA (Fig. 2), dorsolateral BNST (Fig. 3) and PVN (Fig. 4) ($P<0.01$, $P<0.001$ and $P<0.001$ respectively) compared with WT mice ($Ps<0.05$).

Interestingly, in animals not behaviorally tested this increased magnitude of effect of THC on *Nrg1* HET compared with WT mice in these regions was not observed. According to two-way ANOVA, THC increased c-Fos expression as supported by an effect of the treatment in the CEA [$F(1,7)=28.39$, $P<0.01$], BNST [$F(1,7)=23.54$, $P<0.01$] and PVN [$F(1,7)=7.41$, $P<0.05$]. However, Tukey's post hoc test showed that in animals that have not been behaviorally tested, a greater magnitude of effect of THC in *Nrg1* HET compared with WT mice was not observed in any region (see Table 2). These results show that the increased magnitude of effects of THC on c-Fos expression in the CEA, BNST and PVN is also dependent on behavioral testing.

Other brain regions were also analyzed for c-Fos expression in behaviorally tested animals (Table 1), with THC having no effect on either *Nrg1* HET or WT mice in the medial prefrontal cortex (PFC), the NAS, the core of the nucleus accumbens (NAC), the caudate putamen (CPU), the lateral and ventromedial hypothalamus (LH and VMH, respectively), the CA1 and CA3 subregions of the hippocampus, the ventral tegmental area (VTA) and the ventrolateral periaqueductal gray (PAG).

DISCUSSION

The main finding of the present study is that THC selectively induced c-Fos in the LSV of *Nrg1* HET mice with no such effect being observed in WT mice. Interestingly, this differential effect of genotype was dependent upon behavioral experimentation as this effect was not observed when animals were exposed to THC and left in their home cage prior to perfusion. Consonant with prior research THC increased c-Fos levels according to the classic expression pattern for cannabinoids. That is, THC increased c-Fos-labeled neurons in the PV, CEA, dorsolateral BNST and PVN in both genotypes. Interestingly, the magnitude of effect of THC-induced c-Fos expression was greater in *Nrg1* HET than WT mice in the CEA, dorsolateral BNST

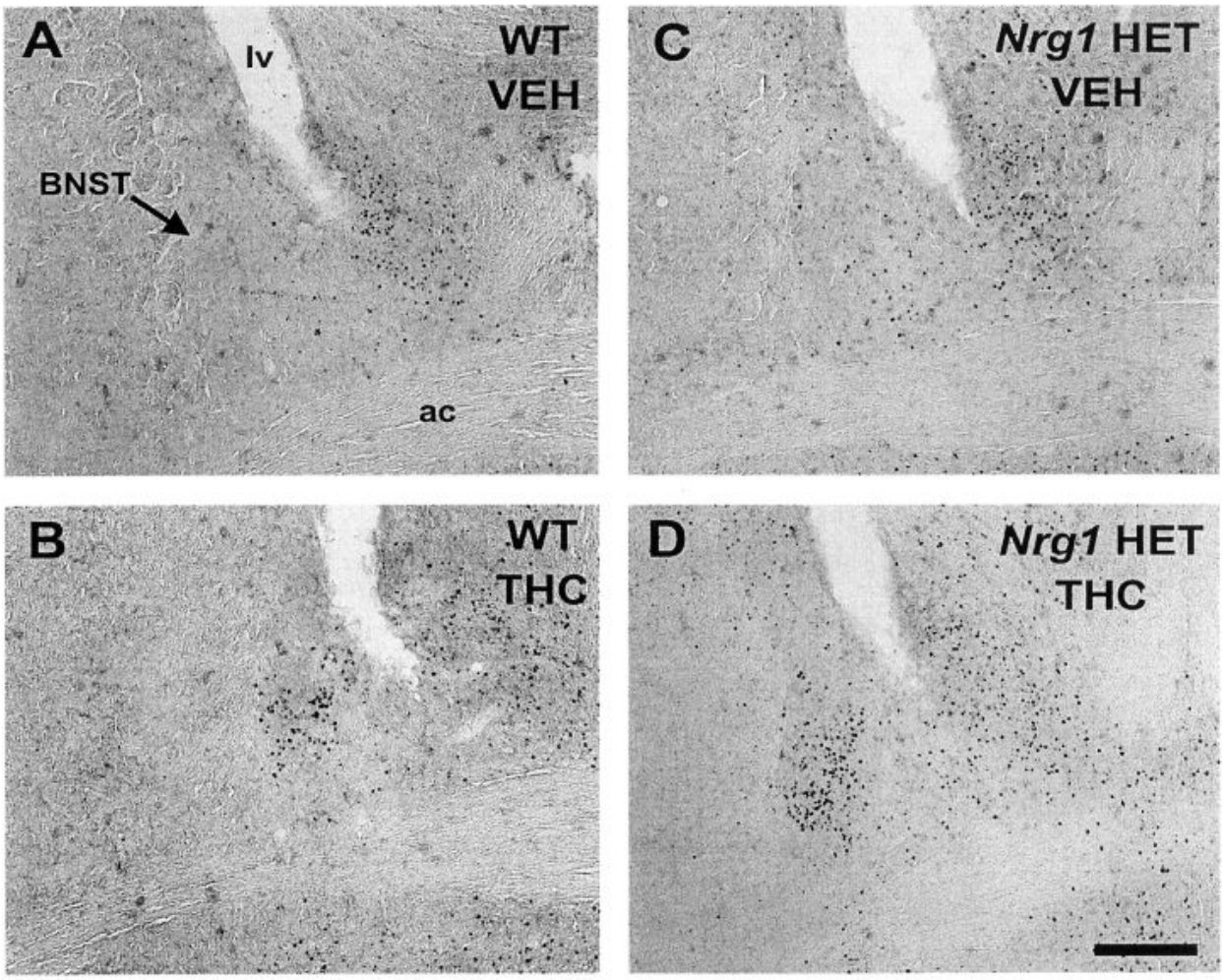


Fig. 3. Fos-labeled neurons within the dorsolateral part of the BNST in representative sections from WT mice treated with (A) VEH or (B) THC (10 mg/kg) and *Nrg1* HET mice treated with (C) VEH or (D) THC (10 mg/kg). The lateral ventricle (lv) and anterior commissure (ac) are also indicated. Scale bar=300 μm.

and PVN only in behaviorally tested animals. Supporting the notion that *Nrg1* HET mice display a schizophrenia-related phenotype, these mice showed augmented baseline c-Fos expression in both the NAS and the LSV compared with WT mice, although, once again, this effect was reliant on behavioral assessment with no genotype effect being observed in these regions in testing naïve animals.

Here we demonstrate for the first time that behaviorally tested *Nrg1* HET mice have higher baseline c-Fos expression in the NAS and LSV. As the lateral septum projects to the NAS (Risold and Swanson, 1997; Sheehan et al., 2004), enhanced c-Fos expression in these regions may indicate an abnormal LSV–NAS pathway in *Nrg1* HET mice. Given that both these areas are implicated in the etiology of schizophrenia, the current results provide further support for the notion that *Nrg1* HET mice display a schizophrenia-related phenotype (Stefansson et al., 2002; Boucher et al., 2007; Karl et al., 2007). Human studies show that excessive dopamine release in schizophrenia patients subserves the positive symptoms of the disorder and explains the effectiveness of typical antipsychotic agents that antagonize dopamine receptors (Carlsson, 1988; Egan and Weinberger, 1997). Augmented dopamine release is likely responsible for the increased c-Fos levels observed in the NAS of *Nrg1* HET mice as this region predominantly releases this neurotransmitter. This also offers a parsimonious explanation for the marked motor and exploratory hyperactivity exhibited by *Nrg1* HET mice (Stefansson et al., 2002; Boucher et al., 2007; Karl et al., 2007).

Increased c-Fos expression in the LSV and NAS of *Nrg1* HET mice compared with WT mice in behaviorally tested animals may also provide a neuronal correlate for PPI deficits observed in *Nrg1* HET mice first reported by Stefansson et al. (2002). While we did not initially repeat this finding (Boucher et al., 2007) we have recently replicated such an effect using a modified PPI design (unpublished observations). Schizophrenia patients display deficits in PPI consistent with attentional dysfunction being a symptom of the disorder. Importantly, a polymorphism in

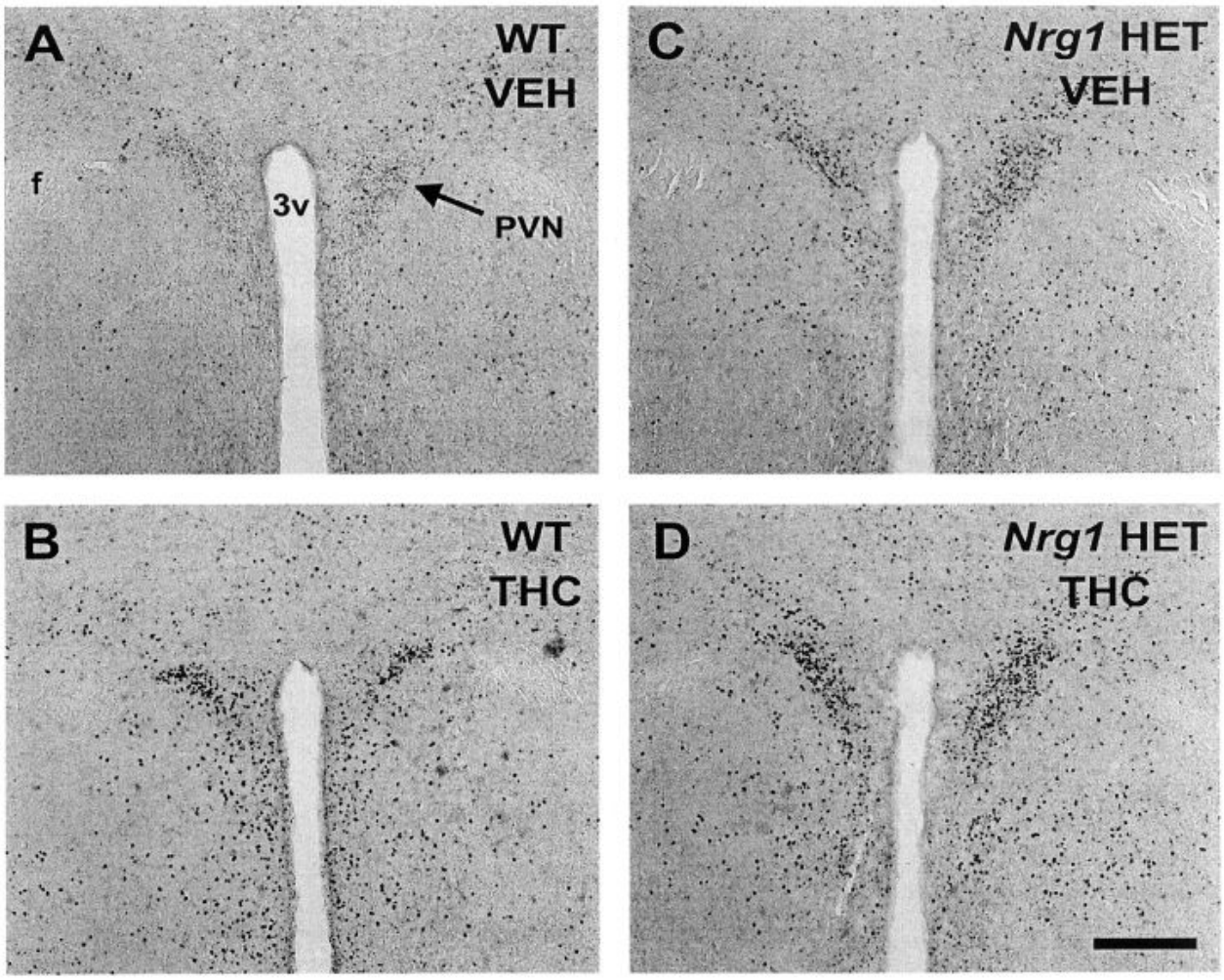


Fig. 4. Fos-labeled neurons within the PVN in representative sections from WT mice treated with (A) VEH or (B) THC (10 mg/kg) and *Nrg1* HET mice treated with (C) VEH or (D) THC (10 mg/kg). The third ventricle (3v) and the fornix (f) are also indicated. Scale bar=300 μm.

the *NRG1* gene was recently associated with PPI deficits in both healthy and schizophrenic patients (Hong et al., 2007). Further, elevated dopamine levels in the NAS are well established in mediating PPI deficits and the septum also appears to mediate sensory gating phenomena (Koch, 1996; van Luijtelaar et al., 2001). Given that the LSV and NAS are responsive to stress (Abercrombie et al., 1989; Sheehan et al., 2004), it is possible that reduced Nrg1 levels interact with the stress of behavioral testing promoting overactivity in these brain sites which might subserve the PPI deficits observed in *Nrg1* HET mice.

THC administration strongly increased c-Fos expression in the PV, CEA, dorsolateral BNST and PVN. These results are consistent with studies examining the effects of cannabinoids on c-Fos expression in both mice (Valjent et al., 2002) and rats (McGregor et al., 1998; Arnold et al., 2001). However, no effect of THC on c-Fos expression was observed in the PFC, NAS, NAC, CPU, LH, VMH, CA1, CA3, VTA and PAG. Unlike our study, Valjent et al. (2002) demonstrated THC-induced c-Fos expression in the NAS, NAC, CPU and VMH, while Derkinderen et al. (2003) reported that THC promoted c-Fos in the CA1 and CA3. Strain differences in the effectiveness of cannabinoids might explain why we did not replicate these findings in our study as we used a distinct strain to that used by Valjent et al. (2002) and Derkinderen et al. (2003) who both used CD-1 mice. Here the C57BL/6 background strain was utilized which has been previously shown to be subsensitive to the actions of cannabinoids (Onaivi et al., 1995). Genetic variation in the responsiveness of animals to cannabinoids is well-documented, for example, Arnold et al. (2001) demonstrated that Lewis rats display less cannabinoid-induced c-Fos compared with Wistar rats.

THC-induced c-Fos expression in the PV, CEA, dorsolateral BNST and PVN is likely to reflect cannabinoid action on an integrated circuit that mediates emotional, endocrine and behavioral responses to sensory information. The PV sends highly processed sensory information to the BNST and CEA (Moga et al., 1995). The BNST appears to be a rostral extension of the CEA and these areas share reciprocal connections consistent with their intimate role in mediating anxiety-related behavior (Swanson and Petrovich,

1998). Further, the CEA indirectly connects with the PVN via a relay through the BNST (Prewitt and Herman, 1998; Dong et al., 2001). Thus, THC-induced activation of a PV–CEA–BNST–PVN "stress circuit" likely subserves the well-characterized effects of cannabinoids on anxiety-related behavior and the hypothalamo-pituitary–adrenal (HPA) axis (Onaivi et al., 1995; Viveros et al., 2005; Boucher et al., 2007).

The observation that heterozygous deletion of the *Nrg1* gene renders animals more sensitive to the effects of THC in the LSV provides a neurobiological correlate for our recent data showing *Nrg1* HET mice are more sensitive to the behavioral effects of THC. Boucher et al. (2007) showed THC facilitated PPI in *Nrg1* HET but not in WT mice. This is interesting as drugs which modulate PPI in animal studies, whether they be pro-psychotic agents that impair PPI (e.g. amphetamine, phencyclidine) (Dulawa and Geyer, 1996), or anti-psychotic agents that facilitate PPI (e.g. haloperidol, clozapine) (Ouagazzal et al., 2001) all increase the expression of c-Fos in the lateral septum (Sumner et al., 2004). The lateral septum is also likely involved in the "stress circuit" outlined above sharing reciprocal connections with the PV, BNST, CEA and the PVN (Moga et al., 1995; Risold and Swanson, 1997; Sheehan et al., 2004). Further, the LSV is thought to mediate stress- and anxiety-related behavior (Dielenberg et al., 2001; Sheehan et al., 2004). Therefore, the selective effect of THC on c-Fos expression in the LSV of *Nrg1* HET mice, combined with the observation that THC exerted an increased magnitude of effect on c-Fos expression in the CEA, BNST and PVN only in behaviorally tested *Nrg1* HET mice suggests that depletion of *Nrg1* interacts with the stress of experimentation to enhance the sensitivity of an LSV–CEA–BNST–PVN circuit to the actions of THC. Such a circuit might also underlie the enhanced behavioral effects of THC on *Nrg1* HET mice including increased THC-induced anxiety-related behavior as measured in the light–dark emergence test (Boucher et al., 2007).

The enhanced sensitivity of *Nrg1* HET mice to THC-induced c-Fos expression was only observed in animals exposed to behavioral testing. Behavioral testing involves manipulations likely to stress animals such as handling and removal from the home cage. Indeed, it has been shown that rats exposed to a battery of behavioral tests exhibit higher levels of the stress hormone corticosterone compared with rats with no prior behavioral experience (Uphouse et al., 1983). Interestingly in our study, the brain regions where THC exerted greater effects upon *Nrg1* HET mice are stress-related, i.e. the LSV, CEA, BNST and PVN. The interaction between neuregulins, stress and cannabinoids is interesting given studies highlighting that such neurochemical systems independently impact upon stress systems. For example, a recent human study showed a single nucleotide polymorphism in *NRG1* interacts with environmental stress in the form of job strain to enhance the development of atherosclerosis (Hintsanen et al., 2007). Further, THC exposure increases corticosterone levels (Weidenfeld et al., 1994) and deletion of CB1 receptors decreases basal release of this hormone (Uriguen et al., 2004). Furthermore, a synergistic interaction occurs between cannabinoids and stress exposure on c-Fos expression in the CEA (Patel et al., 2005). This is consistent with human evidence reporting that the stress of mild oral surgery precipitates adverse emotional reactions in cannabis-intoxicated patients (Gregg et al., 1976).

The robust increased sensitivity of behaviorally tested *Nrg1* HET mice to THC induced c-Fos expression further supports the view that interactions occur between neuregulin and cannabinoid systems. Such an interaction may occur in this region as neuregulins, ErbB3, ErbB4 and CB1 receptors are localized in the septum (Pinkas-Kramarski et al., 1994, 1997; Tsou et al., 1998; Steiner et al., 1999). The cellular and molecular explanation for such an interaction in the CNS is unknown. Cancer research has shown that crosstalk exists between G protein–coupled receptors and ErbB receptors mediated by two known mechanisms: 1) G protein receptor–promoted metalloproteinase cleavage of membrane-tethered ErbB ligands or 2) G protein receptor activation of Src family kinases and phosphorylation of ErbB receptors (Yarden and Sliwkowski, 2001). Interestingly, cannabinoids have been shown to activate metalloproteinases (Rosch et al., 2006) and Src (Berghuis et al., 2005; He et al., 2005). Furthermore, cannabinoids induce cancer cell proliferation by metalloproteinase-mediated transactivation of the ErbB1 receptor (Hart et al., 2004).

CONCLUSION

Here we present neurobiological evidence that behaviorally tested *Nrg1* HET mice are more sensitive to the effects of THC. This was most robustly observed in the LSV where THC promoted c-Fos expression selectively in *Nrg1* HET mice with no corresponding effect being observed in WT mice. This further supports our prior behavioral research showing that interactions occur between cannabinoid and neuregulin systems. Consistent with *Nrg1* HET mice exhibiting a schizophrenia-related phenotype, these animals displayed greater baseline c-Fos expression in two regions implicated in the etiology of schizophrenia, the NAS and the LSV. The effects of genotype on c-Fos expression at baseline or following THC exposure were only observed when animals experienced behavioral testing prior to perfusion. This suggests an interaction with stress was necessary in the promotion of these effects. Taken together, these data demonstrate that heterozygous deletion of *Nrg1*—a schizophrenia susceptibility gene—alters the sensitivity of animals to the neurobehavioral effects of the main psychoactive constituent of cannabis, THC under conditions of stress. This research enhances our understanding of how genetic factors may increase an individual's vulnerability to schizophrenia and cannabis-induced psychosis.

Acknowledgments—This research was supported by: the Schizophrenia Research Institute (SRI) utilizing infrastructure funding from NSW Health; the Sylvia and Charles Viertel Charitable Foundation; a Bill Ritchie Postdoctoral Research Fellowship (awarded to T.K.); the National Alliance for Research on Schizophrenia and Depression (Young Investigator Award awarded to T.K.) and a Clive and Vera Ramaciotti Foundation Young Investigator Grant

awarded to J.C.A. A.A.B. was supported by an SRI Postgraduate Research Scholarship. We thank Prof. Richard Harvey for providing the Nrg1 HET mice and the biological testing facility staff (especially J. Fisher, M. Pickering, and K. Kerr) for their support.

REFERENCES

Abercrombie ED, Keefe KA, DiFrischia DS, Zigmond MJ (1989) Differential effect of stress on in vivo dopamine release in striatum, nucleus accumbens, and medial frontal cortex. J Neurochem 52:1655–1658.

Arnold JC, Topple AN, Mallet PE, Hunt GE, McGregor IS (2001) The distribution of cannabinoid-induced Fos expression in rat brain: differences between the Lewis and Wistar strain. Brain Res 921:240–255.

Arseneault L, Cannon M, Witton J, Murray RM (2004) Causal association between cannabis and psychosis: examination of the evidence. Br J Psychiatry 184:110–117.

Bayer TA, Falkai P, Maier W (1999) Genetic and non-genetic vulnerability factors in schizophrenia: the basis of the "two hit hypothesis." J Psychiatr Res 33:543–548.

Berghuis P, Dobszay MB, Wang X, Spano S, Ledda F, Sousa KM, Schulte G, Ernfors P, Mackie K, Paratcha G, Hurd YL, Harkany T (2005) Endocannabinoids regulate interneuron migration and morphogenesis by transactivating the TrkB receptor. Proc Natl Acad Sci U S A 102:19115–19120.

Boucher AA, Arnold JC, Duffy L, Schofield PR, Micheau J, Karl T (2007) Heterozygous neuregulin 1 mice are more sensitive to the behavioural effects of delta(9)-tetrahydrocannabinol. Psychopharmacology (Berl) 192:325–336.

Carlsson A (1988) The current status of the dopamine hypothesis of schizophrenia. Neuropsychopharmacology 1:179–186.

Degenhardt L, Hall W (2002) Cannabis and psychosis. Curr Psychiatry Rep 4:191–196.

Derkinderen P, Valjent E, Toutant M, Corvol JC, Enslen H, Ledent C, Trzaskos J, Caboche J, Girault JA (2003) Regulation of extracellular signal-regulated kinase by cannabinoids in hippocampus. J Neurosci 23:2371–2382.

Dielenberg RA, Hunt GE, McGregor IS (2001) "When a rat smells a cat": the distribution of Fos immunoreactivity in rat brain following exposure to a predatory odor. Neuroscience 104:1085–1097.

Dong HW, Petrovich GD, Swanson LW (2001) Topography of projections from amygdala to bed nuclei of the stria terminalis. Brain Res Brain Res Rev 38:192–246.

Dulawa SC, Geyer MA (1996) Psychopharmacology of prepulse inhibition in mice. Chin J Physiol 39:139–146.

Egan MF, Weinberger DR (1997) Neurobiology of schizophrenia. Curr Opin Neurobiol 7:701–707.

Falls DL (2003) Neuregulins: functions, forms, and signaling strategies. Exp Cell Res 284:14–30.

Gregg JM, Small EW, Moore R, Raft D, Toomey TC (1976) Emotional response to intravenous delta9tetrahydrocannabinol during oral surgery. J Oral Surg 34:301–313.

Hart S, Fischer OM, Ullrich A (2004) Cannabinoids induce cancer cell proliferation via tumor necrosis factor alpha-converting enzyme (TACE/ADAM17)-mediated transactivation of the epidermal growth factor receptor. Cancer Res 64:1943–1950.

He JC, Gomes I, Nguyen T, Jayaram G, Ram PT, Devi LA, Iyengar R (2005) The G alpha(o/i)-coupled cannabinoid receptor-mediated neurite outgrowth involves Rap regulation of Src and Stat3. J Biol Chem 280:33426–33434.

Hintsanen M, Elovainio M, Puttonen S, Kivimaki M, Raitakari OT, Lehtimaki T, Rontu R, Juonala M, Kahonen M, Viikari J, Keltikangas-Jarvinen L (2007) Neuregulin-1 genotype moderates the association between job strain and early atherosclerosis in young men. Ann Behav Med 33:148–155.

Hong LE, Wonodi I, Stine OC, Mitchell BD, Thaker GK (2007) Evidence of missense mutations on the neuregulin 1 gene affecting function of prepulse inhibition. Biol Psychiatry, in press.

Karl T, Duffy L, Scimone A, Harvey RP, Schofield PR (2007) Altered motor activity, exploration and anxiety in heterozygous neuregulin 1 mutant mice: implications for understanding schizophrenia. Genes Brain Behav, in press.

Koch M (1996) The septohippocampal system is involved in prepulse inhibition of the acoustic startle response in rats. Behav Neurosci 110:468–477.

Linszen DH, Dingemans PM, Lenior ME (1994) Cannabis abuse and the course of recent-onset schizophrenic disorders. Arch Gen Psychiatry 51:273–279.

McGregor IS, Arnold JC, Weber MF, Topple AN, Hunt GE (1998) A comparison of delta 9-THC and anandamide induced c-fos expression in the rat forebrain. Brain Res 802:19–26.

Moga MM, Weis RP, Moore RY (1995) Efferent projections of the paraventricular thalamic nucleus in the rat. J Comp Neurol 359: 221–238.

Onaivi ES, Chakrabarti A, Gwebu ET, Chaudhuri G (1995) Neurobehavioral effects of delta 9-THC and cannabinoid (CB1) receptor gene expression in mice. Behav Brain Res 72:115–125.

O'Tuathaigh CM, O'Sullivan GJ, Kinsella A, Harvey RP, Tighe O, Croke DT, Waddington JL (2006) Sexually dimorphic changes in the exploratory and habituation profiles of heterozygous neuregulin-1 knockout mice. Neuroreport 17:79–83.

Ouagazzal AM, Jenck F, Moreau JL (2001) Drug-induced potentiation of prepulse inhibition of acoustic startle reflex in mice: a model for detecting antipsychotic activity? Psychopharmacology (Berl) 156: 273–283.

Patel S, Cravatt BF, Hillard CJ (2005) Synergistic interactions between cannabinoids and environmental stress in the activation of the central amygdala. Neuropsychopharmacology 30:497–507.

Paxinos G, Franklin KBJ (2000) The mouse brain in stereotaxic coordinates, 2nd edition X. Burlington, MA: Academic Press.

Pinkas-Kramarski R, Eilam R, Alroy I, Levkowitz G, Lonai P, Yarden Y (1997) Differential expression of NDF/neuregulin receptors ErbB-3 and ErbB-4 and involvement in inhibition of neuronal differentiation. Oncogene 15:2803–2815.

Pinkas-Kramarski R, Eilam R, Spiegler O, Lavi S, Liu N, Chang D, Wen D, Schwartz M, Yarden Y (1994) Brain neurons and glial cells express Neu differentiation factor/heregulin: a survival factor for astrocytes. Proc Natl Acad Sci U S A 91:9387–9391.

Prewitt CM, Herman JP (1998) Anatomical interactions between the central amygdaloid nucleus and the hypothalamic paraventricular nucleus of the rat: a dual tract-tracing analysis. J Chem Neuroanat 15:173–185.

Risold PY, Swanson LW (1997) Connections of the rat lateral septal complex. Brain Res Brain Res Rev 24:115–195.

Rosch S, Ramer R, Brune K, Hinz B (2006) R(+)-methanandamide and other cannabinoids induce the expression of cyclooxygenase-2 and matrix metalloproteinases in human nonpigmented ciliary epithelial cells. J Pharmacol Exp Ther 316:1219–1228.

Sheehan TP, Chambers RA, Russell DS (2004) Regulation of affect by the lateral septum: implications for neuropsychiatry. Brain Res Brain Res Rev 46:71–117.

Stefansson H, Sigurdsson E, Steinthorsdottir V, Bjornsdottir S, Sigmundsson T, Ghosh S, Brynjolfsson J, Gunnarsdottir S, Ivarsson O, Chou TT, Hjaltason O, Birgisdottir B, Jonsson H, Gudnadottir VG, Gudmundsdottir E, Bjornsson A, Ingvarsson B, Ingason A, Sigfusson S, Hardardottir H, Harvey RP, Lai D, Zhou M, Brunner D, Mutel V, Gonzalo A, Lemke G, Sainz J, Johannesson G, Andresson T, Gudbjartsson D, Manolescu A, Frigge ML, Gurney ME, Kong A, Gulcher JR, Petursson H, Stefansson K (2002) Neuregulin 1 and susceptibility to schizophrenia. Am J Hum Genet 71: 877–892.

Steiner H, Blum M, Kitai ST, Fedi P (1999) Differential expression of ErbB3 and ErbB4 neuregulin receptors in dopamine neurons and forebrain areas of the adult rat. Exp Neurol 159:494–503.

Sumner BE, Cruise LA, Slattery DA, Hill DR, Shahid M, Henry B (2004) Testing the validity of c-fos expression profiling to aid the therapeutic classification of psychoactive drugs. Psychopharmacology (Berl) 171:306–321.

Swanson LW, Petrovich GD (1998) What is the amygdala? Trends Neurosci 21:323–331.

Tsou K, Brown S, Sanudo-Pena MC, Mackie K, Walker JM (1998) Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system. Neuroscience 83:393–411.

Uphouse L, Tilson H, Mitchell CL (1983) Long-term effects of behavioral testing on serum hormones and brain weight. Life Sci 33: 1395–1400.

Uriguen L, Perez-Rial S, Ledent C, Palomo T, Manzanares J (2004) Impaired action of anxiolytic drugs in mice deficient in cannabinoid CB1 receptors. Neuropharmacology 46:966–973.

Valjent E, Mitchell JM, Besson MJ, Caboche J, Maldonado R (2002) Behavioural and biochemical evidence for interactions between delta 9-tetrahydrocannabinol and nicotine. Br J Pharmacol 135: 564–578.

van Luijtelaar G, Fabene PF, de Bruin N, Jongema C, Ellenbroek BA, Veening JG (2001) Neural correlates of sensory gating in the rat: decreased Fos induction in the lateral septum. Brain Res Bull 54:145–151.

Viveros MP, Marco EM, File SE (2005) Endocannabinoid system and stress and anxiety responses. Pharmacol Biochem Behav 81:331–342.

Walss-Bass C, Liu W, Lew DF, Villegas R, Montero P, Dassori A, Leach RJ, Almasy L, Escamilla M, Raventos H (2006) A novel missense mutation in the transmembrane domain of neuregulin 1 is associated with schizophrenia. Biol Psychiatry 60: 548–553.

Weidenfeld J, Feldman S, Mechoulam R (1994) Effect of the brain constituent anandamide, a cannabinoid receptor agonist, on the hypothalamo-pituitary-adrenal axis in the rat. Neuroendocrinology 59:110–112.

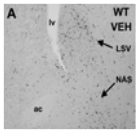
Yarden Y, Sliwkowski MX (2001) Untangling the ErbB signalling network. Nat Rev Mol Cell Biol 2:127–137.

(Accepted 24 August 2007)
(Available online 28 August 2007)

Summary of the main results at the end of Chapter 3

Chapter 3 examined the neurobiological effects of THC (10 mg/kg) on c-Fos expression using immunohistochemistry on WT and *Nrg1* HET mice. The main results obtained from this experiment are summarised in Table 3 and show that *Nrg1* HET mice are more sensitive to the effects of THC on c-Fos expression in the ventrolateral septum (LSV). In addition, this effect is dependent on the stress induced by behavioural testing.

Table 3. Summary of main results form Chapter 2 and 3.

Test	Chapter 2 Effect of THC (10 mg/kg)	Chapter 3 Effects of THC (10 mg/kg)
Locomotor activity in the open field 	Hypolocomotion more important in *Nrg1* HET mice than WT mice	
Anxiety in the light dark 	Decreased time spent in the light compartment only in *Nrg1* HET mice	
Social interaction 	Decreased social interaction in both WT and *Nrg1* HET mice similarly	
Prepulse inhibition 	Facilitation only in *Nrg1* HET mice	
Immunohistochemistry 		Increased c-Fos expression in the LSV of *Nrg1* HET mice dependent on behavioural testing

Chapter 4

DIFFERENTIAL NEUROBEHAVIOURAL EFFECTS OF REPEATED CANNABINOID TREATMENT ON HETEROZYGOUS NEUREGULIN 1 MICE

1. Introduction

Cannabis is the most widely used illicit drug in the world. Evidence suggests that cannabis exposure may precipitate schizophrenia in individuals that are genetically vulnerable to this disorder (Bayer et al., 1999). One of the most promising susceptibility genes for schizophrenia is neuregulin 1 (*NRG1*) (Stefansson et al., 2002) and animal studies have shown that mutant mice heterozygous for the transmembrane domain of *Nrg1* (*Nrg1* HET mice) exhibit a schizophrenia-related phenotype. Specifically, these animals develop an age-dependent phenotype, an increased sensitivity to environmental factors, hyperlocomotion, abnormalities in social behaviour and sensorimotor gating deficits (Stefansson et al., 2002, O'Tuathaigh et al., 2006, Boucher et al., 2007a, Karl et al., 2007). In addition to these behavioural defects, neurobiological changes have been reported where *Nrg1* HET mice displayed increased levels of serotonergic 5-HT_{2A} receptors (Dean et al., 2008) and an increased baseline c-Fos expression in brain areas implicated in schizophrenia such as the ventrolateral septum (LSV) and the shell of the nucleus accumbens (NAS) (Boucher et al., 2007b).

We have previously reported that *Nrg1* HET mice have an increased sensitivity to the acute neurobehavioural effects of cannabinoids (Boucher et al., 2007a, b). For example, the administration of the main psychoactive constituent of cannabis, Δ^9-tetrahydrocannabinol (THC), selectively facilitated prepulse inhibition (PPI) in *Nrg1* HET mice (Boucher et al., 2007a). We also showed that unlike WT animals, *Nrg1* HET mice show increased sensitivity to THC-induced c-Fos expression as measured in the LSV, and that these differential effects were dependent upon behavioural testing (Boucher et al., 2007b). Therefore, this phenomenon appears to be

dependent on a 3-way interaction between *Nrg1* deficiency, stress and cannabinoid administration.

As *Nrg1* HET mice are more sensitive to the neurobehavioural effects of THC, this study aims to investigate whether these animals adapt differently than their wild type-like (WT) littermates to the chronic administration of a cannabinoid receptor agonist. This is particularly relevant, as chronic, high dose cannabis exposure is more strongly linked to the development of schizophrenia (Linszen et al., 1994). It is a common phenomenon that animals develop tolerance to the behavioural effects of cannabinoids after repeated administration (Costa et al., 1996, Lichtman and Martin, 2005). This phenomenon is partly explained by the development of neuroadaptative processes that act to counteract the acute action of the drug. For example, desensitization and/or down regulation of cannabinoid CB1 receptors in the brain may subserve tolerance to cannabinoids. Interestingly, tolerance arises to different behavioural effects at differing rates, for example reduced sensitivity to cannabinoid-induced hypothermia occurs more rapidly than cannabinoid-promoted locomotor suppression (Gonzalez et al., 2005).

As neuregulins are neurodevelopmental factors involved in synaptogenesis, neuronal migration, glial differentiation, myelination and neurotransmitter receptor expression (Corfas et al., 2004), we aim in the present study to assess if *Nrg1* HET mice display altered neuroadaptive responses to chronic cannabinoid exposure. To achieve this, we observed the effects of repeated exposure to the synthetic analogue of THC, CP 55,940 on various physiological (body temperature), behavioural [locomotor activity, exploration, social interaction, anxiety and PPI] and neurobiological (c-Fos and Fos B/ΔFos B expression) measures.

2. Methods

2.1. Animals

Male adult (22 to 25 weeks) group-housed *Nrg1* HET mice generated from a C57BL/6 background strain were provided by Prof Richard Harvey (Victor Chang Cardiac Research Institute, Sydney Australia) as previously described (Karl et al., 2007). *Nrg1* HET mice and their control wild-type like littermates (WT) were pair-housed under a 12:12 h normal light:dark schedule [light phase: white light (illumination: 80 lx) - dark phase: red light (illumination: <2 lx)] with food and water available *ad libitum*. Microbiological monitoring revealed no infection of the SPF facility holding room - with the exception of the pathogens commonly found in commercial and research facilities, *Pasteurella pneumotropica* and *Helicobacter spp*. Male adult (age-matched ±7 days) group-housed A/J mice (Animal Resources Centre, Canning Vale, Australia) were used as standard opponents in the social interaction test. All research and animal care procedures were approved by the "Garvan Institute / St Vincent's Hospital Animal Experimentation Ethics Committee" and were in agreement with the "Australian Code of Practice for the Care and Use of Animals for Scientific Purposes".

2.2. Drug treatments

CP 55,940 (Tocris, Ellisville, USA) was dissolved in 4% ethanol, 1% Tween 80, and 95% saline. CP 55,940 or vehicle (VEH) were administered intraperitoneally (i.p.) at 0.4 mg/kg in a volume of 10 ml/kg of body weight, 30 min prior to behavioural testing.

2.3. Experimental procedure

Animals were treated once daily for 15 days. Thirty min after the injection, mice were tested for body temperature (BT) and locomotor activity in the open field (OF) every second day. Mice were additionally tested for social interaction (SI), light-dark (LD) and PPI on days 1, 7 and 15. The order of the test was always the same and separated by a 5 min rest time in the animals' home cage. For habituation purposes all test animals were transported to the testing room 1 h prior to behavioural testing (holding and test room were part of the same facility having therefore identical light, air pressure, and temperature conditions). Environmental odours were removed from the different test apparatus by cleaning the equipment after each trial with a 30% ethanol solution.

BT: Rectal temperature was measured in each mouse using a mouse rectal temperature probe (ADInstruments, Bella Vista, Australia) attached to a thermocouple (Dicksmith electronics). The probe was placed 3 cm into the rectum of the mice for approximately 5 s until the temperature was constant. BT was recorded immediately prior to, and 30 min after, the injection. Data were expressed as difference in BT between the baseline BT and BT + 30 min.

OF: Locomotor activity and explorative-like tendencies were evaluated by placing the mouse into an infrared photobeam-controlled (x-, y-, and z-axis) open field activity test chamber (43.2 cm x 43.2 cm; MED Associates Inc., USA, Vermont). Animals were tested for 10 min (illumination at floor level: 20 lx), and the total distance travelled (horizontal activity: x- and y-axis) and the vertical activity (z-axis) were recorded automatically (software settings: box size: 3; ambulatory trigger: 2; resting delay: 1000 ms; resolution: 100 ms) in the central and peripheral areas.

PPI: Patients with schizophrenia show impaired sensorimotor gating. PPI is the operational measure of sensorimotor gating, in which a weak pre-stimulus (prepulse) attenuates the startle response (Wang et al., 2003). PPI was tested in two startle chambers (SR-Lab: San Diego Instruments, San Diego, USA). Animals were habituated to the test device for three consecutive days (Day 1: 5 min; Day 2-3: 10 min) before being tested 24 h later. The protocol used was adapted from methods developed by Geyer and Swerdlow (Geyer and Swerdlow, 1998). After a 5 min acclimation period with a 70 dB background noise each session of approximately 30 min consisted of 115 trials in a pseudorandomised order: A block of 5 x 120 dB trials was presented at the beginning and end of each test session in order to observe habituation of the startle response and to scale down the initial startle response to a stable plateau. The remainder of the session consisted of the following trials of stimuli presented in a pseudorandomized order: 5 trials of startle alone trials for different startle stimuli (70 / 80 / 100 / 120 dB) and 5 trials of prepulse-startle stimulus (120 dB) trials for a variety of prepulse intensities (PI: 4, 12 or 16 dB above background noise) and intratrial intervals (prepulse-pulse intervals of 32, 64, 128, 256 or 512 ms). We chose a variable intertrial interval of averaged 15 s (range 10–20 s), prepulse duration of 20 ms and startle duration of 40 ms. Startle response was measured as the average mean amplitude. Percentage of PPI (%PPI) was calculated as [(startle response 120 dB – PPI response) x 100 / startle response 120 dB]. We also analysed the mean %PPI of all summed prepulse responses (averaged over PI and intratrial intervals) for each group.

SI: This pharmacologically validated model is widely used to measure anxiety-like behaviours (File, 1988, Kask et al., 2001). Test animals were placed together with an unfamiliar A/J standard opponent into the OF activity test chamber

(in opposite corners), where they were allowed to explore the arena and each other freely for 10 min. The behaviour of the test mouse was recorded online. The total duration in the active socio-positive behaviours such as general sniffing, anogenital sniffing, allogrooming, following and crawling over/under were recorded. Active social interaction (SI) time is inversely related to the anxiety of rodents, which is confirmed by the observation that the maximum active SI time is found when rodents are tested in a familiar test arena with a low level of illumination (File and Hyde, 1978).

LD: This paradigm mimics the natural conflict between the tendency of mice to explore a novel environment and to avoid an exposed open area (DeFries et al., 1966, Crawley, 1985). Test animals were placed into the open field chamber, which was equipped with a dark box insert for mice (covering half the area of the chamber: MED Associates Inc.). An opening located in the centre of the partition connected light (illumination: 20 lx) and dark (illumination: <2 lx) compartments. At the start of the experiment mice were placed into the bright compartment, facing the dark one. The time spent in the light compartment was recorded during a 10 min session and was used as a measure of anxiety (Costall et al., 1989, Crawley, 1999). Animals that did not explore the dark chamber for more than 10 % of the total time on day 1 were excluded from the analysis (1 *Nrg1* HET-VEH, 1 WT-VEH and 1 WT-CP 55,940).

2.4. Immunohistochemistry

We used c-Fos and Fos B/ΔFos B immunohistochemistry in diverse brain regions to assess changes in neuronal activity following repeated CP 55,940 exposure. While c-Fos is rapidly and transiently expressed with novelty and acute drug exposure, ΔFos B accumulates in the brain and persists for a long period of time as it is extremely

stable, therefore reflecting long term molecular changes indicating of neuroadaptation processes (Nestler et al., 1999). However, the anti-Fos B antibody used here is not specific to ΔFos B, a product of the Fos B gene, but recognizes the full-length Fos B isoform that is not specific to long term neuronal changes. Therefore we also measured the expression of c-Fos to assess if any Fos B/ΔFos B expression would appear without concomitant expression of c-Fos, thus supporting that the Fos B/ΔFos B expression could be due to long term changes induced by ΔFos B (Berton et al., 2007).

Immediately after behavioural testing was completed and approximately 90 minutes after the final injection of either VEH or CP 55,940 (day 15), mice were anaesthetized with halothane and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer saline. The brains were removed and postfixed overnight in fresh PFA at 4°C before being incubated in 15% sucrose, followed by 30% sucrose for cryoprotection. The brains were then placed in the cryostat for slow freezing at -17°C and the entire brain was sliced at 40 μm in the coronal plane. Brain slices were placed sequentially into three vials containing phosphate buffer (PB). Tissues were stored in freezing solution (ethanediol/glycerol) at -15°C until needed. Free floating sections were washed three times in PB then placed in a 1% hydrogen peroxide solution for 30 min and then placed in 3% normal horse serum for 30 min. Sections were then placed in vials with 3 ml of primary c-Fos antibody (Santa Cruz Biotechnology, rabbit polyclonal; reacts with c-Fos p62 of mouse and non-cross reactive with FosB, Fra-1 or Fra-2) diluted 1:10 000 in phosphate buffered horse serum (PBH) (0.1% bovine serum albumin, 0.2% Triton X-100, 2% normal horse serum) for 3 days at 4°C. Another vial was prepared in the same way as above, but stained with the Fos B/ΔFos B primary antibody (Santa Cruz

Biotechnology, rabbit polyclonal) diluted in 1:2000 in PBH overnight at 4°C**.** Afterwards, both sets of tissue were washed in PB and then incubated for 1 h in biotinylated anti-rabbit IgG secondary antibody (Vector Laboratories, Burlingame, CA) diluted 1:500 in PBH. Sections were washed again before being incubated in ExtrAvidin peroxidase (Sigma, diluted 1:1000 in PBH) for 2 h. After three washes in PB, peroxidase activity was visualised by placing the tissue in nickel diaminobenzidine (0.05% diaminobenzidine tetrahydrochloride, 0.004% ammonium chloride, 0.02% nickel ammonium sulphate, 0.2% D-glucose) and glucose oxidase (Sigma, 1:1000) and the reaction was terminated after 10 min by washing in PB. Sections where stored in PB at 4°C before being mounted onto gelatinized slides, dehydrated, xylene cleared and coverslipped. Fos immunoreactive cells (black and dark brown) were quantified by a rater blind to treatment with reference to the mouse brain atlas of Paxinos and Franklin (Paxinos and Franklin, 2000) as previously described (McGregor et al., 1998, Arnold et al., 2001b, Boucher et al., 2007b).

2.5. Data analysis

For each behavioural model, we first analysed the overall effects using repeated measures two-way analysis of variance (ANOVA). The two factors were genotype (WT and *Nrg1* HET mice) and treatment (VEH and CP 55,940) and the repeated measure was test day. When needed, we evaluated the development of tolerance in CP 55,940-treated animals or habituation to behavioural testing in VEH-treated animals, by using repeated measures two-way ANOVA split by the corresponding factor. Then when focusing on each day separately, we investigated the specific effects of the treatment and/or of the genotype using two-way ANOVA split by the corresponding

factor. In the OF, the total distance travelled was further analyzed as a % of VEH using one-way ANOVA to reveal any genotype difference on this measure.

Fos counts in each brain region were analysed using two-way ANOVA followed by Tukey's post-hoc test for within group comparisons. A significance level of $P<0.05$ was chosen for all comparisons. In the graphs and tables, data are presented as means ± standard error of the mean (SEM) and the post hoc effects are reported with symbols when appropriate (see legends).

3. Results

3.1. Body temperature and locomotor activity

The results from locomotor activity in the OF and BT are shown in Fig. 1. Repeated CP 55,940 treatment produced an overall decreased BT [Treatment: $F(1,35) = 79.22$, $P < 0.001$] (Fig. 1A). Trends for an effect of the genotype [$F(1,35) = 3.54$, $P = 0.07$] and for a genotype by treatment interaction [$F(1,35) = 3.42$, $P = 0.07$] were observed. An effect of test day [$F(7,245) = 16.89$, $P < 0.001$] and treatment by test day [$F(7,245) = 18.47$, $P < 0.001$] and genotype by treatment by test day [$F(7,245) = 2.46$, $P < 0.05$] interactions were also observed. When separating by treatment, no test day effect was observed in VEH-treated animals but a genotype by test day interaction was revealed [$F(7,126) = 2.32$, $P < 0.05$]. Tolerance to the effects of the drug on BT was highlighted by a test day effect in CP 55,940-treated WT and *Nrg1* HET animals [$F(7,119) = 27.94$, $P < 0.001$] and a difference between CP 55,940-treated *Nrg1* HET and WT mice was also observed over days [genotype: $F(1,17) = 4.64$, $P < 0.05$]. To assess the difference in development of tolerance between *Nrg1* HET mice and WT mice, we analysed genotype effects in CP 55,940-treated animals on the different days (Fig. 1A). No difference was observed between CP 55,940-treated *Nrg1* HET and WT

mice on day 1. Interestingly, a genotype effect was observed in CP 55,940-treated animals on days 3 [$F(1,17) = 11.31$, $P < 0.01$] and 5 [$F(1,17) = 5.83$, $P < 0.05$]. This highlights that while CP 55,940 promoted equivalent hypothermia in *Nrg1* HET and WT mice on day 1, the drug reduced BT less in *Nrg1* HET mice than WT mice on days 3 and 5.

In the OF, repeated exposure to CP 55,940 promoted an overall decreased distance travelled [treatment: $F(1,35) = 8.15$, $P < 0.01$] and *Nrg1* HET mice exhibited an overall higher locomotor activity [genotype: $F(1,35) = 6.77$, $P < 0.05$] (Fig. 1B). An effect of test day [$F(7,245) = 9.13$, $P < 0.001$] and a treatment by test day interaction [$F(7,245) = 22.64$, $P < 0.001$] were also observed. When separating by treatment, VEH-treated animals habituated to the OF [test day: $F(7,126) = 8.88$, $P < 0.001$] and a strong trend for a genotype effect was observed [$F(1,18) = 4.38$, $P = 0.05$]. A test day effect was observed in CP 55,940-treated animals [$F(7,199) = 17.66$, $P < 0.001$] showing that tolerance developed to the effect of the drug in the OF. When assessing genotype effects in VEH-treated animals on the different days, although no significant hyperactivity of *Nrg1* HET mice compared to WT mice was observed on day 1, a genotype effect was revealed on days 9 [$F(1,18) = 6.54$, $P < 0.05$], 11 [$F(1,18) = 4.55$, $P < 0.05$] and 15 [$F(1,18) = 7.28$, $P < 0.05$]. Interestingly, when analysing the % of change of locomotor activity between day 1 and day 15, VEH-treated *Nrg1* HET mice showed a strong trend for reduced habituation to the OF compared to WT mice (a 6.35 % versus 37.89 % reduction respectively) [$F(1,18) = 4.36$, $P = 0.05$]. This suggests that hyperactivity was unmasked when VEH-treated WT mice habituated to the OF.

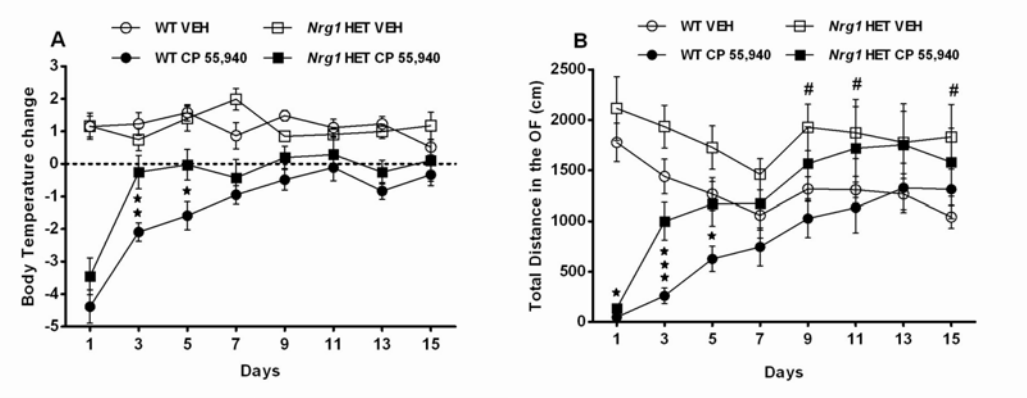


Figure 1: Tolerance to the effects of CP 55,940 (0.4 mg/kg) compared to vehicle (VEH) on (A) BT and (B) locomotor activity in the OF of WT and *Nrg1* HET mice (WT VEH, n = 12; WT CP 55,940, n = 11; *Nrg1* HET VEH, n = 8; *Nrg1* HET CP 55,940, n = 8). Data are presented as means ± SEM. Significant effects of *Nrg1* HET mice compared to WT mice in CP 55,940-treated animals are indicated by ★ ($P < 0.05$), ★★ ($P < 0.01$) and ★★★ ($P < 0.001$). Significant effects of *Nrg1* HET mice compared to WT mice in VEH-treated animals in the OF are indicated by # ($P < 0.05$).

Like for BT, we assessed differential development of tolerance between *Nrg1* HET mice and WT mice by evaluating any genotype difference within CP 55,940-treated animals on the different days. CP 55,940-induced locomotor suppression was less important in *Nrg1* HET mice than in WT mice as revealed by treatment effects on days 1 [$F(1,17) = 5.53$, $P < 0.05$], 3 [$F(1,17) = 16.62$, $P < 0.001$] and 5 [$F(1,17) = 5.23$, $P < 0.05$] (Fig. 1B). As there was a marginal difference between *Nrg1* HET mice and WT mice on day 1, we examined the rate of induction of tolerance by analysing the effect of CP 55,940 on total distance travelled as a % of the VEH group for each genotype using one-way ANOVA. No difference between *Nrg1* HET mice and WT mice was found on day 1, showing that the animals were equally affected by the drug and only travelled for 6.47 % and 2.62 % of the distance travelled by VEH-treated animals respectively. However on day 3 CP 55,940-treated *Nrg1* HET mice travelled significantly more than WT mice (51.64 % versus 17.99 % of distance travelled by VEH) [treatment: $F(1,17) = 10.72$, $P < 0.01$]. This indicates that on day 3 *Nrg1* HET mice displayed a more rapid decline in the effect of CP 55,940 on locomotor activity than WT mice. Complete tolerance developed to CP 55,940-

induced decreased locomotor activity in the OF, as treatment effects were only observed for 3 days in *Nrg1* HET mice [day 1: $F(1,14) = 38.51$, $P < 0.001$, day 3: $F(1,14) = 10.95$, $P < 0.01$] and 5 days in WT mice [day 1: $F(1,21) = 74.39$, $P < 0.001$, day 3: $F(1,21) = 37.42$, $P < 0.001$, day 5: $F(1,21) = 9.6$, $P < 0.01$].

3.2. Prepulse inhibition

In the PPI paradigm (Fig. 2A) no overall effect of the genotype, treatment or test day was observed. However, genotype by treatment [$F(1,35) = 4.49$, $P < 0.05$] and genotype by treatment by test day [$F(2,70) = 5.42$, $P < 0.01$] interactions were observed. When analysed by days of treatment, a genotype difference was observed in VEH-treated animals, with a decreased %PPI of *Nrg1* HET mice compared to WT mice on day 1 [$F(1,18) = 4.79$, $P < 0.05$], but not on days 7 and 15. An effect of CP 55,940 was observed on day 1 as the drug decreased %PPI in WT mice [$F(1,21) = 5.83$, $P < 0.05$] while it increased % PPI in *Nrg1* HET mice [$F(1,14) = 4.95$, $P < 0.05$]. However, no effect of CP 55,940 on %PPI in WT mice or in *Nrg1* HET mice was observed on days 7 and 15.

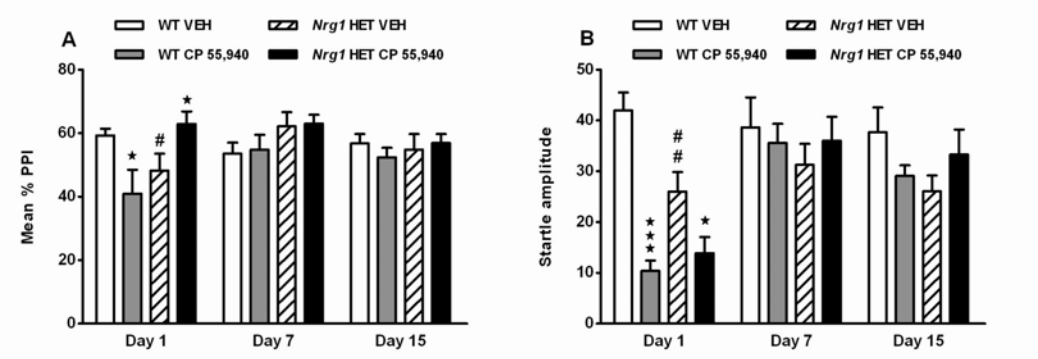


Figure 2: Tolerance to the effects of CP 55,940 (0.4 mg/kg) compared to vehicle (VEH) on (A) % PPI (mean of the different prepulse intensities) and (B) startle response of WT and *Nrg1* HET mice (WT VEH, n = 12; WT CP 55,940, n = 11; *Nrg1* HET VEH, n = 8; *Nrg1* HET CP 55,940, n = 8). Data are presented as means ± SEM. Significant effects of CP 55,940 compared to VEH within the same genotype group are indicated by ★ ($P < 0.05$) and ★★★ ($P < 0.001$). Significant effects of *Nrg1* HET mice compared to WT mice in VEH-treated animals are indicated by # ($P < 0.05$) and # # ($P < 0.01$).

During the PPI test we also measured the startle response of the animals (Fig. 2B). Over days, CP 55,940 administration decreased startle response [treatment: $F(1,35) = 4.2$, $P < 0.05$]. No effect of the genotype was observed but a significant genotype by treatment interaction was revealed [$F(1,35) = 4.14$, $P < 0.05$]. An effect of test day [$F(2,70) = 20.67$, $P < 0.001$] and a treatment by test day interaction [$F(2,70) = 21.03$, $P < 0.001$] were also reported. Tolerance to the reduced startle response was observed in CP 55,940-treated animals [test day: $F(2,34) = 36.3$, $P < 0.001$]. On the different days independently, genotype difference in VEH-treated animals was observed on day 1 where *Nrg1* HET mice showed a decreased startle response compared to WT mice [$F(1,18) = 8.93$, $P < 0.01$]. Treatment with CP 55,940 decreased startle response in *Nrg1* HET mice and WT mice on day 1 [$F(1,14) = 5.95$, $P < 0.05$ and $F(1,21) = 57.04$, $P < 0.001$ respectively], with no effect seen on days 7 and 15.

3.3. Social interaction and rearing frequency

The results from the total time in SI are shown in Fig. 3A. Repeated administration of CP 55,940 produced an overall decreased time spent in SI [$F(1,35) = 107.18$, $P < 0.001$] and the effect of the drug was in interaction with the test day [$F(2,70) = 11.11$, $P < 0.001$]. No overall genotype difference was observed over days. Depending on the treatment, we observed habituation to SI over the days in VEH-treated animals [test day: $F(2,36) = 6.11$, $P < 0.01$] and development of tolerance to the effect of the drug on SI in CP 55,940-treated mice [test day: $F(2,34) = 12.38$, $P < 0.001$]. On the different days separately, no genotype differences were observed in VEH-treated animals on any days. The development of tolerance to CP 55,940-induced decreased SI was not complete, as a significant treatment effect was observed on all days for

both WT [day 1, F(1,21) = 95.35, $P < 0.001$; day 7, F(1,21) = 49.19, $P < 0.001$; day 15, F(1,21) = 9.87, $P < 0.01$] and *Nrg1* HET mice [day 1, F(1,14) = 23.21, $P < 0.001$; day 7, F(1,14) = 21.17, $P < 0.001$; day 15, F(1,14) = 7.35, $P < 0.05$].

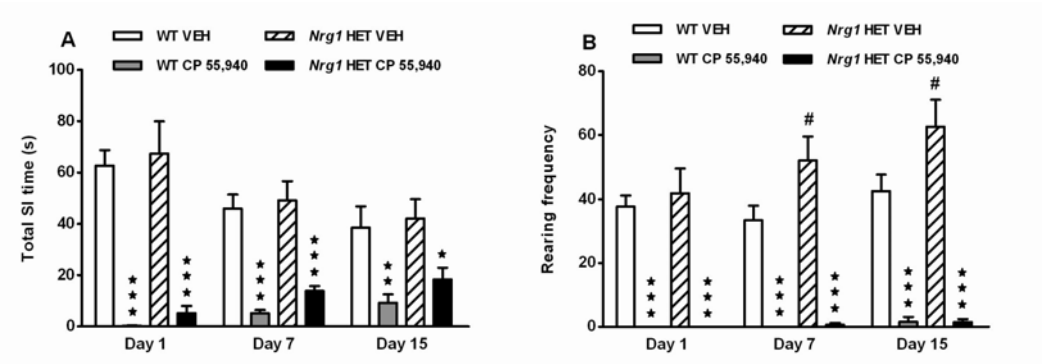


Figure 3: Tolerance to the effects of CP 55,940 (0.4 mg/kg) compared to vehicle (VEH) on (A) SI and (B) rearing frequency of WT and *Nrg1* HET mice (WT VEH, n = 12; WT CP 55,940, n = 11; *Nrg1* HET VEH, n = 8; *Nrg1* HET CP 55,940, n = 8). Data are presented as means ± SEM. Significant effects of CP 55,940 compared to VEH within the same genotype group are indicated by ★ ($P < 0.05$), ★★ ($P < 0.01$), and ★★★ ($P < 0.001$). Significant effects of *Nrg1* HET mice compared to WT mice in VEH-treated animals are indicated by # ($P < 0.05$).

Exploration-like behaviour of *Nrg1* HET and WT mice was recorded as the rearing frequency of the animals during the SI test (Fig. 3B). When repeatedly administered, CP 55,940 induced an overall decreased rearing frequency [F(1,35) = 130.6, $P < 0.001$]. Trends for a genotype effect [F(1,35) = 3.47, $P = 0.07$] and a genotype by treatment interaction [F(1,35) = 3.28, $P = 0.08$] were observed. Interestingly a significant test day effect was observed [F(2,70) = 10.19, $P < 0.001$] and treatment by test day [F(2,70) = 6.3, $P < 0.01$], genotype by test day [F(2,70) = 3.56, $P < 0.05$] and genotype by treatment by test day [F(2,70) = 3.34, $P < 0.05$] interactions were reported. In VEH-treated animals, an effect of test day [F(2,36) = 9.01, $P < 0.001$] and importantly a genotype by test day interaction [F(2,36) = 3.82, $P < 0.05$] were observed showing a differential habituation to rearing of VEH-treated *Nrg1* HET mice compared to VEH-treated WT mice. No test day effect was observed in CP 55,940-treated animals, showing that both WT and *Nrg1* HET mice did not

develop tolerance to the drug-induced suppression of decreased rearing. When analysed on the different days, genotype differences were revealed in VEH-treated animals as *Nrg1* HET mice exhibited an increased rearing frequency compared to WT mice on days 7 [$F(1,18) = 4.95$, $P < 0.05$] and 15 [$F(1,18) = 4.77$, $P < 0.05$]. CP 55,940 decreased rearing frequency on all days for both WT [day 1, $F(1,21) = 100.55$, $P < 0.001$; day 7, $F(1,21) = 47.61$, $P < 0.001$; day 15, $F(1,21) = 57.6$, $P < 0.001$] and *Nrg1* HET mice [day 1, $F(1,14) = 29.97$, $P < 0.001$; day 7, $F(1,14) = 45.85$, $P < 0.001$; day 15, $F(1,14) = 51.36$, $P < 0.001$]. Similar results were also observed on vertical activity in the OF, another measure of rearing (data not shown).

3.4. Anxiety in light-dark and open field tests

The results from the anxiety-related behaviours are shown in Fig. 4. The measure of the time spent in the light compartment of the LD (Fig. 4A) revealed that repeated CP 55,940 treatment decreased the time spent in the light over days [$F(1,32) = 17.02$, $P < 0.001$]. An effect of test day was also observed [$F(2,64) = 6.55$, $P < 0.01$] as well as trends for genotype by treatment [$F(1,32) = 3.78$, $P = 0.06$], genotype by test day [$F(2,64) = 2.97$, $P = 0.06$] and genotype by treatment by test day [$F(2,64) = 2.95$, $P = 0.06$] interactions. However, no overall genotype effect was observed. Over days, VEH-treated animals habituated to the LD independently of the genotype [test day: $F(2,32) = 8.62$, $P < 0.01$] and a trend for a test day effect was observed in CP 55,940-treated animals [$F(2,32) = 3.03$, $P = 0.06$]. Interestingly, a genotype by test day interaction was observed in CP 55,940-treated animals [$F(2,32) = 3.33$, $P < 0.05$] showing that tolerance to the anxiogenic-like effect of the drug in the LD did not appear similarly in *Nrg1* HET mice compared to WT mice. On the different days separately, no genotype difference between WT and *Nrg1* HET mice was observed in

VEH-treated animals on any days. Interestingly, CP 55,940 decreased the time spent in the light of *Nrg1* HET mice on days 1 [$F(1,13) = 9.78$, $P < 0.01$], 7 [$F(1,13) = 26.57$, $P < 0.001$] and 15 [$F(1,13) = 28.79$, $P < 0.001$], while it only decreased time in the light on day 1 in WT mice [$F(1,19) = 67.72$, $P < 0.001$].

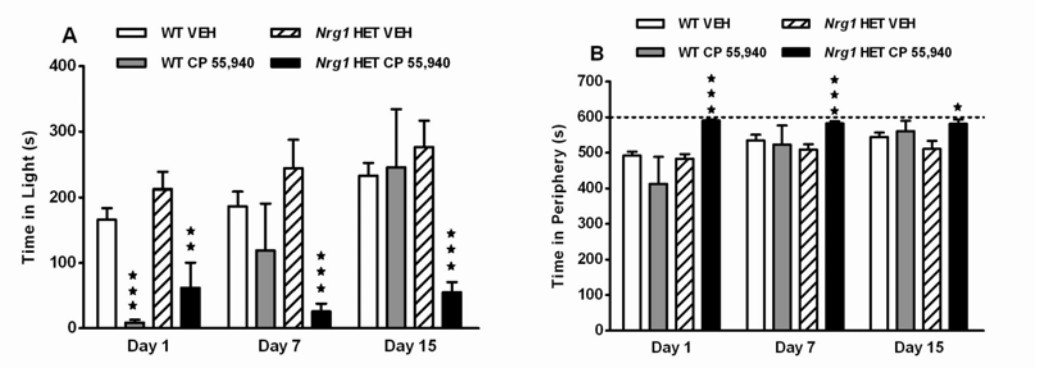


Figure 4: Tolerance to the effects of CP 55,940 (0.4 mg/kg) compared to vehicle (VEH) on (A) the light dark test (WT VEH, n = 11; WT CP 55,940, n = 10; *Nrg1* HET VEH, n = 7; *Nrg1* HET CP 55,940, n = 8) and (B) the OF in WT and *Nrg1* HET mice (WT VEH, n = 12; WT CP 55,940, n = 11; *Nrg1* HET VEH, n = 8; *Nrg1* HET CP 55,940, n = 8). Data are presented as means ± SEM. Significant effects of CP 55,940 compared to VEH within the same genotype are indicated by ★ ($P < 0.05$), ★★ ($P < 0.01$), and ★★★ ($P < 0.001$). The doted line shows the total time spent in the OF.

We also analysed the time spent in the peripheral area of the OF ("thigmotaxis") as another indicator of anxiety-related behaviour (Simon et al., 1994) (Fig. 4B). No overall effect of treatment or genotype was revealed over days, but a genotype by treatment interaction [$F(1,35) = 6.73$, $P < 0.05$] was observed. In addition a trend for an effect of test day [$F(2,70) = 2.97$, $P = 0.06$] was also reported. Independently of their genotype, VEH-treated animals showed an increased time spent in the periphery of the OF over days [test day: $F(2,36) = 8.04$, $P < 0.01$]. No tolerance was reported to the effect CP 55,940 as shown with no effect of test day and no genotype by test day interaction in CP 55,940-treated animals. Interestingly an effect of the genotype was observed [$F(1,17) = 4.94$, $P < 0.05$] showing that CP 55,940-treated *Nrg1* HET mice exhibited an increased thigmotaxis compared to CP 55,940-treated WT mice that was constant over days. When analysing the different

days separately, no genotype difference between WT and *Nrg1* HET mice was observed in VEH-treated animals on any day. Consistent with the increased thigmotaxis of CP 55,940-treated *Nrg1* HET mice reported over days, CP 55,940 only increased the time spent in the periphery of the OF in *Nrg1* HET mice on every day [day 1, $F(1,14) = 64.98$, $P < 0.001$; day 7, $F(1,14) = 19.58$, $P < 0.001$; day 15, $F(1,14) = 7.53$, $P < 0.05$] but not in WT mice.

3.5. Fos immunohistochemistry

Table 1 shows c-Fos and Fos B/ΔFos B counts in diverse brain areas. The regions where no significant genotype or treatment effects and no genotype by treatment interaction were found on Fos expression included the medial prefrontal cortex (PFC), the shell and core of the nucleus accumbens (NAC), the central and dorsolateral caudate putamen (CPU), the lateral and ventromedial hypothalamus (HT), the ventral tegmental area (VTA) and the ventrolateral periaqueductal gray (PAG) (see Table 1). CP 55,940 increased Fos B/ΔFos B expression in two regions, the paraventricular nucleus of the thalamus (PVT) [$F(1,35) = 15.7$, $P < 0.001$] and in the central nucleus of the amygdala (CEA) [$F(1,34) = 9.2$, $P < 0.01$]. Interestingly, in the LSV there was a significant genotype by treatment interaction [$F(1,34) = 5.6$, $P < 0.05$] and post-hoc analysis revealed that chronic CP 55,940 treatment increased Fos B/ΔFos B expression in the LSV in *Nrg1* HET mice ($P < 0.01$) but not in WT mice (Fig. 5).

Repeated CP 55,940 exposure decreased c-Fos expression in four brain regions; the medial caudate putamen (CPUm) [$F(1,35) = 7.8$, $P < 0.01$], the bed nucleus of the stria terminalis (BNST) [$F(1,15) = 12$, $P < 0.01$] and two hippocampal areas, the CA1 [$F(1,31) = 4.4$, $P < 0.05$] and CA3 region [$F(1,31) = 6.3$, $P < 0.05$]. CP 55,940 treatment also increased c-Fos expression in the paraventricular nucleus of the

hypothalamus (PVN) [F(1,32) = 4.2, $P < 0.05$] and in the PVT [F(1,34) = 4.5, $P < 0.05$].

Table 1. Fos B/ΔFos B and c-Fos expression.

Region	Bregma	Marker	WT		*Nrg1* HET		2-way ANOVA
			VEH	CP 55,940	VEH	CP 55,940	
PFC medial	+1.98	Fos B	22.3±3.2	24.4±3.8	22.1±3.4	21.7±4.1	
		c-Fos	29.5±6.3	22.6±4.8	24.1±6.7	12±2.7	
NAC shell	+0.98	Fos B	47.5±9.4	41.5±10.9	50.4±6.2	55.3±10.8	
		c-Fos	13.8±3.7	9.7±2.3	12.9±4	11.6±5.3	
NAC core	+0.98	Fos B	56.4±13.4	50.6±9.3	53.4±6.1	59.6±14.9	
		c-Fos	5±1.7	2.3±0.8	3.6±1.5	3.1±1.8	
CPU medial	+0.98	Fos B	41.8±13.3	39.3±6.2	36.9±6.5	52.25±11.5	
		c-Fos	17.75±4.3	8.5±2	25.6±8.9	7.25±3.7	t
CPU central	+0.98	Fos B	7.7±2.7	3.4±1.4	8.5±4.7	8.3±6.3	
		c-Fos	3.7±1.2	3.6±1.8	4.4±2.6	3±2.1	
CPU dorsolateral	+0.98	Fos B	2.1±1.2	0.8±0.3	0.8±0.6	2.3±1	
		c-Fos	1.6±0.5	2.2±0.9	1±0.7	1.9±1.3	
LSV	+0.98	Fos B	56.8±5.5	53.7±7.3	41.6±4.7	69.1±7	i
		c-Fos	67.5±9.5	86.1±10	69.75±8.5	67.1±9.1	
BNST dorsolateral	+0.26	Fos B	6.25±1.2	4.75±1.9	7.5±3.2	7.5±1.8	
		c-Fos	5.5±2.3	1.25±0.9	5.6±1	1.3±0.5	t
PVN	-0.94	Fos B	6.9±1.8	16.9±5.6	4.6±0.8	7.4±1.6	
		c-Fos	19.5±3.8	38.4±9.9	11.9±4.4	22.1±7.2	t
PVT	-0.94	Fos B	0.75±0.5	6.2±1.4	1.9±0.6	4.5±1.1	t
		c-Fos	39.1±6.9	84.1±17.6	41.7±9.5	53.9±14.4	t
CEA	-1.34	Fos B	14.9±2.7	29.3±5.5	14.3±3.2	25.5±3.3	t
		c-Fos	6.8±1.4	7.7±1.4	3.4±1.3	6.6±2.1	
HT lateral	-1.58	Fos B	2.2±0.6	3.1±0.7	1.3±0.6	2±1.1	
		c-Fos	10.4±2.3	12.7±2.6	8.1±3	9.6±3.6	
HT ventromedial	-1.58	Fos B	0.8±0.3	1.9±0.7	0.1±0.1	0.4±0.4	
		c-Fos	0.7±0.4	0.3±0.2	0.7±0.4	0.3±0.3	
CA1	-1.58	Fos B	0.1±0.1	0.1±0.1	0±0	0±0	
		c-Fos	2.2±0.7	1.4±0.4	3.3±2	0±0	t
CA3	-1.58	Fos B	1.5±0.7	0.9±0.5	1.6±0.5	0.5±0.4	
		c-Fos	5.5±1.1	1.4±0.7	4±1.7	2.2±1	t
VTA	-3.28-3.40	Fos B	0.1±0.1	0.7±0.3	0.7±0.4	0.8±0.3	
		c-Fos	3.1±0.8	5.5±1.4	3±1.5	2.5±1.3	
PAG ventrolateral	-4.60	Fos B	4.3±1.1	6.4±0.8	4.1±1.2	6.1±1.3	
		c-Fos	19±3.9	23.6±4.4	16±2.8	14.5±3.3	

Fos B/ΔFos B and c-Fos counts following 15 days repeated treatment with CP 55,940 (0.4 mg/kg) or vehicle (VEH) in WT and *Nrg1* HET mice (WT VEH, n = 12; WT CP 55,940, n = 11; *Nrg1* HET VEH, n = 8; *Nrg1* HET CP 55,940, n = 8). Data are presented as means ± SEM. Significant effect with 2-way ANOVA for treatment and interaction are indicated by t and i, respectively.

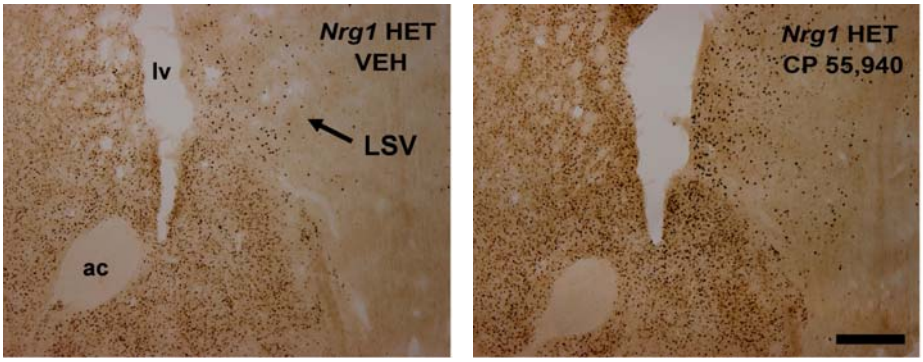


Figure 5: Fos B/ΔFos B-labelled neurons within the LSV in representative sections from *Nrg1* HET mice treated with VEH or CP 55,940 (0.4 mg/kg). The lateral ventricle (lv) and anterior commissure (ac) are also indicated. Scale bar = 300 μm.

4. Discussion

The present results show that mice heterozygous for the *Nrg1* gene display distinctive neurobehavioural responses to repeated cannabinoid exposure over days. Tolerance to CP 55,940-induced hypothermia and hypolocomotion developed more rapidly in *Nrg1* HET mice compared to WT mice. Enhanced development of tolerance was not observed for all measures, with *Nrg1* HET mice maintaining a consistent anxiogenic-like response to CP 55,940 over days, unlike WT control mice, as assessed by the time spent in the light compartment of the light dark test and the time spent in the periphery of the open field. Conversely, in both genotypes very little or no tolerance was observed to the effects of CP 55,940 in tests of SI or rearing frequency, while complete tolerance was evident within one week of cannabinoid treatment on startle response and PPI. The most striking neurobiological correlate of chronic cannabinoid treatment was that Fos B/ΔFos B was selectively increased in the LSV of *Nrg1* HET mice but not WT mice.

VEH-treated *Nrg1* HET mice displayed an overall hyperactivity that is consistent with previous studies on these mice (Stefansson et al., 2002, Boucher et al., 2007a, Karl et al., 2007). Interestingly, the hyperactivity of VEH-treated *Nrg1* HET

mice was not present on the first day of testing, but was unmasked with repeated testing as VEH-treated WT mice habituated to the OF more than *Nrg1* HET mice. These results show that *Nrg1* HET mice are deficient in habituation to the OF over days. In addition, we observed an increased rearing frequency in VEH-treated *Nrg1* HET mice compared to WT mice on days 7 and 15 in the SI tests. This shows that no habituation to such exploration-related behaviour occurred and rearing frequency even intensified in *Nrg1* HET mice over days. Taken together, these results suggest a deficit in between sessions habituation to exploration in *Nrg1* HET mice which is consistent with reduction in within session habituation showed by previous research (O'Tuathaigh et al., 2006).

Acute administration of CP 55,940 strongly decreased BT and locomotor activity of both WT and *Nrg1* HET mice. After repeated administration these classical effects of cannabinoids became less marked as tolerance developed (Little et al., 1988, Oliva et al., 2004). Interestingly, here we observed that tolerance to the locomotor suppressant and hypothermic effects of CP 55,940 developed more rapidly in *Nrg1* HET mice than in WT mice. Tolerance is a homeostatic mechanism that attempts to counter the imbalance engendered by excessive exposure of the brain to a drug. Indeed, high cannabinoid doses administered at frequent intervals will more rapidly promote tolerance than lower doses of these drugs administered less often (McKinney et al., 2008). Here we hypothesised that the more rapid tolerance induction to the locomotor suppressant and hypothermic effects of cannabinoids in *Nrg1* HET mice might be explained by their enhanced acute cannabinoid-sensitivity as highlighted by our previous findings (Boucher et al., 2007a, b). That is, *Nrg1* HET mice might invoke a more marked counteradaptive response to lessen the greater impact that the cannabinoids have on these mice acutely compared to WT mice.

Future studies could address the underlying mechanisms responsible for this. For example, cannabinoid tolerance is primarily explained by cannabinoid receptor downregulation and desensitization (see review by Gonzalez et al., 2005), therefore it would be interesting to observe if repeated cannabinoid exposure has a differential effect on these processes in *Nrg1* HET and WT mice.

Nrg1 and its receptor ErbB4 regulate many neurotransmitter receptors, including NMDA, dopamine and GABA receptors (Ozaki et al., 1997, Yurek et al., 2004, Woo et al., 2007). In our prior research showing that *Nrg1* HET mice are more sensitive to the acute neurobehavioural effects of cannabinoids, we postulated that partial deficiency of NRG1 could have engendered a reorganisation of the brain during development that may have increased the expression or efficacy of CB1 receptors. In the present study, the increased development of tolerance to CP-55,940-induced hypothermia and locomotor suppression in *Nrg1* HET mice further emphasises that interactions occur between cannabinoid and NRG1 signalling in the brain. Importantly, cannabinoid and neuregulin systems are both expressed in brain regions that mediate motor function and body temperature. For example both CB1 and NRG1-ErbB4 are present in the basal ganglia where they modulate dopamine expression (Steiner et al., 1999, van der Stelt and Di Marzo, 2003, Yurek et al., 2004), and in the preoptic nucleus (Pinkas-Kramarski et al., 1994, Steiner et al., 1999, Rawls et al., 2002), a region involved in body temperature regulation (Ishiwata et al., 2005).

PPI is the phenomenon whereby the pre-exposure to a weak acoustic stimulus reduces the startle response to a loud tone and is impaired in schizophrenia patients (Swerdlow et al., 1994). Unlike in our earlier study (Boucher et al., 2007a), here we replicated the finding of Stefansson et al. (2002) showing that on the first day of testing, acute VEH-treated *Nrg1* HET mice display deficits in PPI. However, here we

observed that these *Nrg1* HET mice present a concomitant impairment in the acoustic startle response to a loud 120 dB stimulus, highlighting that the deficit in PPI is likely confounded by a reduced startle responsivity. It is unclear why acute VEH-treated *Nrg1* HET mice displayed a reduction in startle response and PPI in the present study but no reduction in startle response or PPI in Boucher et al. (2007a). One major difference between these two studies is the experimental design used. In Boucher et al. (2007a) a counterbalanced within-subjects design was implemented, such that most animals were previously exposed to the PPI testing chambers on 1 or 2 occasions whereas in the present study, acute VEH-treated *Nrg1* HET mice were naïve to the test procedure. Therefore, we speculate that familiarity with the testing apparatus may determine whether deficits in acoustic startle and PPI are observed in VEH-treated *Nrg1* HET mice on day 1. Reinforcing this view is the fact that *Nrg1* HET mice only showed a deficit in startle response and PPI on day 1, which was not replicated on days 7 and 15 of testing using exactly the same PPI testing parameters. Thus the reduction of startle response and PPI in acute VEH-treated *Nrg1* HET mice appears highly dependent on familiarity to the testing apparatus, so that only under relatively unfamiliar circumstances will this aspect of their phenotype be revealed.

In the PPI paradigm, the first injection of CP 55,940 differentially affected the mice depending on their genotype. In WT mice, CP 55,940 promoted a PPI deficit that is consistent with prior research (Martin et al., 2003, Nagai et al., 2006). In contrast, CP 55,940 facilitated PPI in *Nrg1* HET mice, an effect reversing the PPI deficit observed in VEH-treated *Nrg1* HET mice. This is consistent with our previous study showing PPI facilitation in THC-treated *Nrg1* HET mice, even in mice that showed no cannabinoid-induced reduction in acoustic startle response (Boucher et al., 2007a). However in the present study the high dose of CP 55,940 suppressed the

acoustic startle response in both *Nrg1* HET and WT mice, confounding the differential effect we observed on day 1 on PPI. As heavy cannabis abuse is more reliably associated with worsening of symptoms in patients with schizophrenia (Linszen et al., 1994) we expected that repeated administration of CP 55,940 might alter the profile of the *Nrg1* HET mice in the PPI paradigm. However, no such effects were observed and by day 7 both genotypes became completely tolerant to the effects of CP 55,940 on acoustic startle and PPI. It is possible that dosing in adulthood, once the *Nrg1* HET mice express their characteristic phenotype, does not provide the optimal period to assess the effects of chronic cannabinoids. One study showed that PPI deficits could be revealed in adult rats that received prior repeated cannabinoid injections during puberty, however no such effect was evident when rats were exposed to the same dosing regime during adulthood (Schneider and Koch, 2003). This suggests that puberty offers a unique period of vulnerability to the adverse effects of cannabis. This is especially significant given that human users commence cannabis use at ages that often coincide with puberty and the age of onset of schizophrenia (Gonzalez-Pinto et al., 2008). Further, as Nrg1 signalling is involved in the regulation of hormonal control during puberty, future studies could assess the administration of cannabinoids in *Nrg1* HET mice in this critical age period (Ma et al., 1999).

Another behavioural model that was investigated in the present study was SI. The acute administration of CP 55,940 decreased SI both in WT and *Nrg1* HET mice similarly to our previous report using THC (Boucher et al., 2007a). After repeated treatment, little tolerance to CP 55,940-induced decreased SI was observed in *Nrg1* HET and WT mice. This is consistent with studies where, while tolerance was observed to the sedative effects of cannabinoids, the social behaviour was still

impaired by the drug (Frischknecht et al., 1982). Similarly, absence of tolerance to decreased sociability has been reported in humans (Haney et al., 1999). During the SI test, the animal's exploration was also measured as the frequency of rearing. Interestingly, no tolerance to the effects of CP 55,940 on rearing were observed after 15 days even though both WT and *Nrg1* HET mice were tolerant to the locomotor suppressant effects of CP 55,940 by day 7. Therefore, our results are consistent with prior research in rats showing that rearing is more resistant to tolerance to THC than locomotor suppression (Miczek, 1976). This result reinforces that rearing and locomotor activity are mediated by distinct neurocircuits that are differentially susceptible to the development of tolerance induced by repeated cannabinoid exposure.

In the LD test, the first administration of CP 55,940 decreased the time spent in the light compartment in both WT and *Nrg1* HET mice. This is consistent with the equivalent decrease in SI promoted by CP 55,940 in *Nrg1* HET and WT mice observed here and also the well-characterised anxiogenic-like effects of cannabinoids in rodents (Rutkowska et al., 2006). The anxiogenic effects of CP 55,940 on WT mice progressively decreased over days 7 and 15 indicative of cannabinoid tolerance. However, no such tolerance to the anxiogenic action of CP 55,940 was observed in *Nrg1* HET mice that maintained a pronounced cannabinoid-induced aversion of the light compartment over days. Importantly, as the animals were tolerant to the locomotor suppressant effects of CP 55,940 on days 7 and 15, the persistent decreased time spent in the light of *Nrg1* HET mice appears specific to increased anxiety-like behaviour and can not be accounted for cannabinoid-induced locomotor suppression. Reinforcing the observation that *Nrg1* HET mice display no tolerance to cannabinoid-induced anxiety-related behaviour is the finding that these animals showed a

consistent CP 55,940 promoted increase in time spent in the outer perimeter of the OF over days 1, 7 and 15 of dosing. Such thigmotaxic behaviour is also considered an index of anxiety as it is increased by anxiogenic agents and decreased by anxiolytic drugs (Simon et al., 1994). Taken together, these results demonstrate a long-term differential effect between *Nrg1* HET mice and WT mice in their sensitivity to anxiety-related behaviour promoted by repeated cannabinoid treatment.

The finding that CP 55,940 selectively increased thigmotaxis in *Nrg1* HET mice but not WT mice on day 1 is consistent with our earlier study showing that the anxiogenic-like effects of acute THC in the LD test were only observed in *Nrg1* HET mice but not in WT mice (Boucher et al., 2007a). However this effect was not reproduced in the OF test where both WT and *Nrg1* HET mice displayed THC-induced decreased time spent in the centre of the OF. In addition, in the present study, both *Nrg1* HET and WT mice displayed an equivalent aversion to the light compartment of the LD test. This might be explained by subtle differences between the present study and the study of Boucher et al. (2007a). Here a between-subjects design was implemented, whereas Boucher et al. (2007a) utilised a within-subjects design. This difference may have altered the baseline level of exploration in the LD on day 1 due to variation in test experience between the two cohorts of mice. As such, VEH-treated WT and *Nrg1* HET mice spent more time in the light compartment providing more room to reveal the anxiogenic effect of cannabinoids in both genotypes. Other differences between the present study and that of Boucher et al. (2007a) are that a different cannabinoid drug and dose was used here and that the animals used in the present study were approximately a month younger which may have decreased the time needed to develop a strong, drug-free anxiolytic-like phenotype in the *Nrg1* HET mice (Karl et al., 2007).

Nrg1 HET mice showed a lack of tolerance to the cannabinoid-induced anxiety-like behaviour as measured in the time spent in the light compartment of the LD test and in the centre of the OF, while simultaneously showing more rapid tolerance to cannabinoid-induced locomotor suppression and hypothermia compared to WT mice. Such findings seem contradictory however they may be triggered by different alterations occurring in the discrete neurocircuits that underlie anxiety, motor behaviour and body temperature. As previously discussed, more rapid tolerance to the locomotor suppressant and hypothermic effects of CP 55,940 in *Nrg1* HET mice might be explained by Nrg1's contribution to processes that subserve cannabinoid tolerance in motor- or temperature-related regions of the brain. However, the lack of tolerance to the anxiogenic-like effects of CP 55,940 might be explained by selective, long-term neuroadaptive changes occurring in anxiety-related circuits of *Nrg1* HET mice that are not observed in WT mice. Indeed, we report in the present study that, after 15 days repeated treatment with CP 55,940, *Nrg1* HET mice display increased Fos B/ΔFos B expression in the LSV, unlike WT mice which showed no induction of this neuronal marker.

This finding is extremely interesting given our previous data that acute THC treatment selectively increased c-Fos expression in the LSV of *Nrg1* HET mice but not WT mice (Boucher et al., 2007b). Importantly, the selective enhancement in Fos B/ΔFos B expression in *Nrg1* HET mice in the present study may not be simply a short-term response of the LSV to cannabinoid exposure as following chronic CP 55,940 treatment no significant increase in c-Fos expression was observed in the LSV of *Nrg1* HET mice. However, as our antibody does not discriminate between Fos B and ΔFos B it cannot be ruled out that this effect is due to the shorter half-life of Fos B expression. In any case, this result further supports that the LSV is an important site

of cannabinoid-promoted dysregulation in *Nrg1* HET mice which might subserve the maintenance of long-term anxiety/stress responses to chronic cannabinoid exposure in these mice. It is important to note that in our prior study (Boucher et al., 2007b), the acute effect of THC on c-Fos expression in the LSV was dependent on the stress of behavioural testing. It is possible the effect observed here with ΔFos B could also be due to cannabinoid-stress interaction, however we did not test this hypothesis directly here. An interaction between neuregulin deficiency and cannabinoid exposure is likely to occur in the septum where both ErbB4 and CB1 receptors (Tsou et al., 1998, Steiner et al., 1999) are present, however the exact mechanism by which this interaction occurs in the LSV is unknown and further studies are required to understand the molecular processes underlying such a difference.

An increased Fos B/ΔFos B expression was also observed in the CEA of both WT and *Nrg1* HET mice, and this effect was not accompanied by an increased c-Fos expression. This suggests that long-term adaptive changes could have occurred in this stress-related region. As this area is interconnected with the lateral septum (Sheehan et al., 2004), it is surprising that the differential effect observed on Fos B/ΔFos B expression in the LSV is not associated with genotype difference in the CEA. Interestingly, acute cannabinoid treatment robustly increases c-Fos expression in the CEA (Boucher et al., 2007b), however with repeated cannabinoid treatment animals were clearly tolerant to this effect with no increased c-Fos expression being observed in this area following repeated CP 55,940 administration. Interestingly, such tolerance to cannabinoid-induced c-Fos expression does not necessarily follow for all regions. For example, independent of genotype, chronic CP 55,940 administration continued to increase c-Fos expression in the PVT to a level similar to that seen with acute THC administration. However in this area, *Nrg1* HET mice tended to express less CP

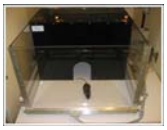
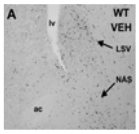
55,940-induced c-Fos, which is consistent with these animals showing greater tolerance to cannabinoid-induced c-Fos expression than WT mice. Similarly, repeated CP 55,940 treatment significantly increased c-Fos expression in the PVN, albeit less markedly than in our acute THC-induced c-Fos study (Boucher et al., 2007b). Interestingly, tolerance does not occur to some of the neuroendocrine effects of cannabinoids, such as the decreased prolactin and luteinizing hormone secretion from the pituitary gland (de Miguel et al., 1998). Further, repeated cannabinoid exposure does not promote alterations in CB1 receptor binding and mRNA levels in the hypothalamus (de Miguel et al., 1998). The persistent effect of CP 55,940 on c-Fos expression in the PVT and PVN is consistent with such findings and underscores the concept that there exists regional diversity in the responsiveness of the brain to chronic cannabinoid exposure.

These results show that *Nrg1* HET mice adapt differently to repeated cannabinoid exposure compared to WT mice. *Nrg1* HET mice displayed more rapid tolerance to the locomotor suppressant and hypothermic effects of cannabinoids. On the contrary, *Nrg1* HET mice maintained a cannabinoid-induced anxiety-like responses with repeated cannabinoid dosing as seen in the LD test, unlike WT mice. Such a differential effect on anxiety-related behaviour correlated with a selective increase in Fos B/ΔFos B in the LSV of *Nrg1* HET mice highlighting that a depletion of *Nrg1* might enhance the neuroadaptive response to chronic cannabinoid exposure in this area which is involved in stress. It also underscores that the LSV may be an important region of dysregulation in *Nrg1* HET mice exposed to cannabinoids. The present results further support that neuregulin and cannabinoid systems interact in the brain, which may provide a mechanism responsible for genetic vulnerability to cannabis-induced schizophrenia.

Summary of the main results at the end of Chapter 4

Chapter 4 examined the behavioural and neurobiological effects of repeated CP 55,940 exposure (0.4 mg/kg) on WT and *Nrg1* HET mice. This dose is comparable to the dose of 10 mg/kg THC that could not be used in this study as THC was not available at the time of the experiment. Animals were tested for body temperature and locomotor activity every 2 days and for body temperature followed by locomotor activity, social interaction, light dark and prepulse inhibitions tests on days 1, 7 and 15. The main results obtained from this experiment are summarised in Table 2 and show that *Nrg1* HET mice developed tolerance to the hypothermic and locomotor suppressant effects of the drug faster than WT mice. In addition, while tolerance developed to the anxiety-like effects of CP 55,940 in WT mice, no tolerance was observed on this behaviour in *Nrg1* HET mice. Fos B/ΔFos B expression was also measured as a marker of long term neuroadaptation and after 15 days repeated exposure CP 55,940 increased Fos B/ΔFos B expression in the ventrolateral septum (LSV) of *Nrg1* HET mice but not WT mice.

Table 2. Summary of main results form Chapter 2, 3 and 4.

Test	**Chapter 2 Effect of THC (10 mg/kg)**	**Chapter 3 Effects of THC (10 mg/kg)**	**Chapter 4 Repeated CP 55,940 (0.4 mg/kg)**	
			On day 1	Tolerance
Locomotor activity in the open field 	Hypolocomotion more important in *Nrg1* HET mice than WT mice		Strong hypolocomotion in both *Nrg1* HET and WT mice	*Nrg1* HET mice developed tolerance to the hypolocomotion faster than WT mice (similar results seen with hypothermia)
Anxiety in the light dark 	Decreased time spent in the light compartment only in *Nrg1* HET mice		Decreased time spent in the light compartment in both *Nrg1* HET and WT mice	Tolerance developed in WT mice but not in *Nrg1* HET mice
Social interaction 	Decreased social interaction in both WT and *Nrg1* HET mice similarly		Decreased social interaction in both WT and *Nrg1* HET mice	No tolerance developed
Prepulse inhibition 	Facilitation only in *Nrg1* HET mice		Facilitation in *Nrg1* HET mice, disruption in WT mice	Tolerance developed in both *Nrg1* HET and WT mice
Immunohistochemistry 		Increased c-Fos expression in the LSV of *Nrg1* HET mice		Increased Fos B/ΔFos B expression in the LSV of *Nrg1* HET mice

Chapter 5

CHRONIC TREATMENT WITH Δ^9-TETRAHYDROCANNABINOL IMPAIRS SPATIAL MEMORY AND REDUCES ZIF268 EXPRESSION IN THE MOUSE FOREBRAIN

1. Introduction

Cannabis is the most widely used illicit drug in the world. Apart from its extensive recreational use, it also has some potential therapeutic relevance (Rog et al., 2005, Smith, 2005). One limitation of cannabis use is that its consumption induces cognitive impairments (Solowij et al., 1995, Fletcher et al., 1996), as well as structural and functional brain alterations (Jentsch et al., 1998, Kanayama et al., 2004, Yucel et al., 2008). Thus, developing a better insight of the chronic effects of cannabinoids on cognition is of critical importance for understanding the adverse effects of this drug.

Long-term cannabis users exhibit neuropsychological deficits such as impairments in memory and attention (Solowij et al., 2002, Messinis et al., 2006). In laboratory animals the acute administration of the main psychoactive constituent of cannabis, Δ^9-tetrahydrocannabinol (THC), induces deficits in cognitive processes (Lichtman and Martin, 1996, Varvel et al., 2001, Da and Takahashi, 2002). While the acute effects of cannabinoids are well documented, the long term effects of the drug on learning and memory have received less attention (Nakamura et al., 1991, Hampson et al., 2003). With chronic treatment, many of the effects of cannabinoids are diminished – a phenomenon known as tolerance. Tolerance develops to the characteristic effects of cannabinoids at different rates, for example it develops more rapidly to cannabinoid-induced hypothermia than locomotor suppression (Whitlow et al., 2003, Gonzalez et al., 2005). Little is known about tolerance to the memory disruptive effects of cannabinoids, with a few human and animal studies suggesting that cannabinoid-induced memory impairment is more resistant to the development of tolerance (Nakamura et al., 1991, Haney et al., 1999, Nava et al., 2001, Solowij et al., 2002). This difference in the time-course of the development of tolerance to the effects of cannabinoids is of particular interest in animal models of behaviour given

that it is often difficult to dissociate the specificity of learning perturbations from the confounding effects of these drugs on motor activity.

The memory impairments promoted by cannabinoids appear to more strongly impact spatial memory. This hippocampus-mediated form of learning can be evaluated using animal models like the Morris water maze. Using this paradigm, different tasks can be examined to differentiate between reference and working memory (Varvel et al., 2001). Reference memory is evaluated in the place version of the water maze where the platform remains at a constant location, while working memory is usually studied by changing the location of the platform to assess behavioural flexibility, a process that is impaired with cannabinoid administration (Egerton et al., 2005, Hill et al., 2006). As memory is strongly regulated by the hippocampus, we also evaluated the chronic effects of THC on the brain by measuring zif268 expression. Zif268 (also known as krox-24, NGFI-A or egr-1) is an immediate early gene that is involved in learning and memory processes (Bozon et al., 2002). This transcription factor is abundant in the hippocampus where cannabinoids normally increase its expression (Derkinderen et al., 2003), an effect postulated to subserve the memory impairments promoted by these drugs (Lichtman et al., 1995, Egashira et al., 2002)

The doses of THC used by previous mouse studies to promote deficits in working memory range between 3 and 10 mg/kg (Varvel et al., 2001, Da and Takahashi, 2002, Varvel et al., 2005, Niyuhire et al., 2007). Here we aimed to assess if deficits in reversal learning in mice also occur using a lower dose of THC (1 mg/kg) that is more relevant to those administered by humans. Furthermore, we aimed to evaluate whether the extended administration of THC induces memory deficits that persist even after the animals have developed tolerance to the locomotor suppressant

effects of the drug. In addition, we measured the underlying neuronal substrates that may be responsible for such effects by analysing zif268 expression in the brain.

2. Methods

2.1. Subjects

Male adult C57BL/6J mice (weighing 23-28 g at the beginning of the experiment) were obtained from Charles River (Lyon, France). Animals were housed at 22-23°C under a 12 h light/dark cycle (7:00 a.m. on) with food and water available ad libitum. One week prior to the experiment, animals were separated into individual home cages and handled for 2–3 min/day during all the experiment to minimize non-specific stress. Experiments were performed in accordance with the guidelines on the ethical use of animals from the European Communities Council Directive of 24 November 1986 (86/609/EEC).

2.2. Drug treatments

THC (25 mg/ml, Sigma, France) was dissolved at 5 mg/ml in ethanol. This preparation was emulsified in a solution at 2.5 mg of polysorbate 80 (Sigma, France) /ml of ethanol. Ethanol was then evaporated at room temperature using a rotary concentrator-evaporator (SpeedVac®System, ThermoSavant) for 20 min under a pressure of 5.1 bar. The residue was resuspended in NaCl (0.9 %) and sonicated for a final THC concentration of 0.1 mg/ml. The same protocol where ethanol was used in replacement of THC was used to prepare the vehicle (Veh) solution. THC (1 mg/kg) or Veh were administered intraperitoneally (i.p.) at a volume of 10 ml/kg.

2.3. Apparatus

Learning in the Morris water maze was evaluated using a pool that measured 150 cm in diameter and 55 cm in height, and that was placed in a room maintained at 23-25 ◦C under constant lighting with many extra-maze cues posted on walls. It was filled with 21±1 ◦C water until 15 cm of the top and made opaque by addition of white non-toxic paint. A hidden platform of 13 cm in diameter was submerged 5 mm below the surface. Its location could be indicated to the animals by placing a 15 cm height and 4 cm of diameter intra-maze cue on the submerged platform. A video camera mounted above the pool was used to record swim trials. Swim speed, escape latency (time to reach the platform), time spent in the target quadrant (where the platform should be during the probe trial) and animal paths were recorded and analysed using an automated tracking system (Videotrack, France).

A different set of animals were then tested for locomotor activity using an activity meter (Imetronic, France). Animals were placed individually in the boxes of the apparatus immediately after injection with THC or Veh, for 1 h under light exposure and without food or water available.

2.4. Procedure of memory testing in the water maze

For this experiment, mice were separated into 2 groups (see Fig. 1). Animals in the non pre-treated (NPT) group were treated with daily injections of THC (1 mg/kg) or Veh 30 min before testing in the water maze on days 1-11 (behavioural testing phase). Animals in the pre-treated (PT) group received 13 daily injections of THC (1 mg/kg) or Veh before the behavioural testing phase where, like NPT animals, these animals continued to receive THC or Veh on each day of testing. Therefore, such a design yielded 4 treatment groups: PT-Veh, PT-THC, NPT-Veh and NPT-THC.

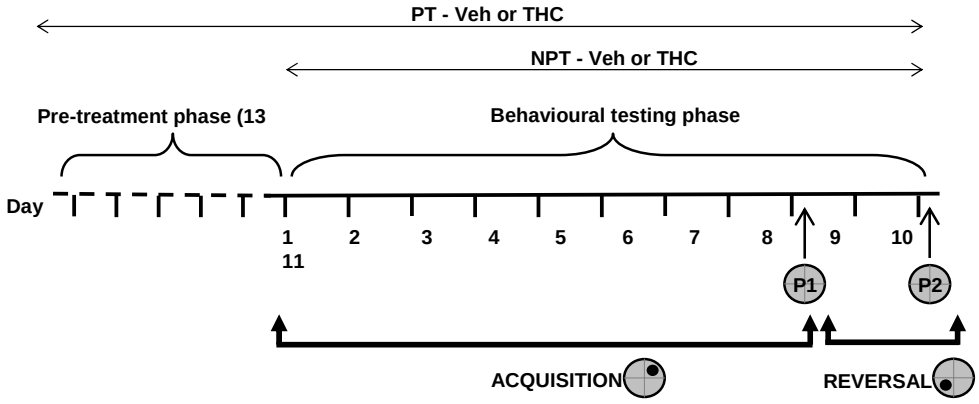


Figure 1. Protocol. Animals were separated in 2 groups: non pre-treated (NPT) mice were treated with Veh or THC (1 mg/kg) during the behavioural testing phase in the water maze. Pre-treated (PT) animals were treated Veh or THC (1 mg/kg) for 13 days before the behavioural testing phase and, like the NPT group, until the end of the experiment. The behavioural testing phase consisted in 9 days of acquisition of the spatial reference memory task where the location of the platform remained constant, followed by 2 days of reversal where the location of the platform was changed. Animals were also tested in the first probe trial (P1) at the end of the acquisition on day 9, and for the second probe trial (P2) at the end of the reversal on day 11.

On the first day of the behavioural testing phase, animals received a pre-training session consisting of 2 *cued trials* where the hidden platform was indicated by a visual intra-maze cue. Then the acquisition started and mice were tested for *spatial reference memory*. Each animal was trained to find the hidden platform kept at the same location for 1 session of 4 trials per day for 9 days. Mice were released from a random location and were allowed to swim until they found and climbed on the platform or until a maximum of 90 s has elapsed. If a mouse did not find the platform, it was gently guided to the platform by hand where it was left for 20 s and then dried and returned to its home cage placed in a heating box. Mice were trained in groups of 4 animals, allowing an intertrial interval of 15 min for each mouse. One hour after the end of the last session of the acquisition, mice were tested in a *probe trial* lasting 60 s where the platform was removed from the water maze.

After 9 days of acquisition, mice were submitted to a *reversal test* where the platform location was moved in the opposite quadrant in order to evaluate the

behavioural and cognitive flexibility of the animals. Mice were tested in 2 sessions of 4 trials separated by 24 h. One hour after the end of these reversal trials, mice were again submitted to a *probe trial* lasting 60 s where the platform was removed from the water maze.

2.5. Immunohistochemistry

Home cage control animals (Quiet cont group) did not receive any injection and were not behaviourally tested.

Ninety minutes after the last probe trial, animals were anaesthetised with an i.p. injection of Tribromoethanol (Avertin) and perfused transcardially with 0.9% saline followed by 100 ml of 4 % paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) at pH 7.4. Brains of animals kept in their home cages were also harvested as controls. The brains were removed and postfixed in PFA for 20 h. For cryoprotection, brains were placed in 30 % sucrose until they sunk. Brains were then sliced at 50 µm in a frozen microtome.

Tissue sections were stored in cryoprotectant solution at – 20 °C until the staining was performed. For immunohistochemistry, free floating sections were washed 4 times for 10 min in PB before being incubated in 0.5 % hydrogen peroxide for 30 min. After 4 washes in 0.9 % NaCl, sections were incubated in 1 % bovine serum albumin, 0.2 % Triton X100 (PB-BSA-TX) for 30 min at room temperature. Sections were then stained with the zif268 primary antibody (Santa Cruz Biotechnology) diluted at 1:5,000 in PB-BSA-TX for 24 h at 4 °C while undergoing shaking. After 4 washes in PB containing 0.2 % Triton X-100 (PB-TX), sections were incubated in biotinylated anti-rabbit secondary antibody (Jackson ImmunoResearch) diluted at 1:2,000 in PB-BSA-TX for 2 h at room temperature while undergoing

shaking. Sections were washed again 4 times in PB-TX and transferred to an avidin-biotin-peroxidase solution (Sigma, France) for 2 h at room temperature and protected from light. Sections were washed 4 times in PB and 3 times in 0.05 M Tris HCl (pH 7.5), The peroxidase activity was visualised by placing the tissue in 0.037 % diaminobenzidine tetrahydrochloride diluted in Tris-HCl and 0.015 % hydrogen peroxidase for 5 to 15 min protected from light. The reaction was terminated by 4 washes in 0.05 M Tris HCl and tissues were stored in PB containing 0.2 g/l Azide (Sigma). Sections were then mounted onto slides, air dried, dehydrated in toluene and coverslipped. Quantitative densitometry was performed on the images of digitized brain sections using Biocom Visiolab 2000 software. Each structure was counted 5 to 8 times depending on the quality of the section. Results are indicated by number of reactive cells by unit of surface.

2.6. Statistical analysis

Data from the water maze were analysed using two-way repeated measures analysis of variance (ANOVA) where the between subjects factors were treatment (Veh or THC) and group (PT or NPT), and trial or session (day) as the repeated measure. When needed, data were examined for each day separately by two-way ANOVA and further analysed using Tukey's post hoc test. Data from locomotor activity were analysed using one-way repeated measures ANOVA where the between subjects factor was treatment (Veh or THC), and day as the repeated measure. Data from immunohistochemistry were analysed using a Student t test. A significant level of $P < 0.05$ was chosen for all comparisons.

3. Results

3.1. Swim speed

As cannabinoids have sedative effects, we analysed the swim speed of the animals during all phases of the water maze test to assess if motor impairment would persist when animals were placed in the water and forced to swim (data not shown). No overall effect of THC treatment was observed on swim speed over days of testing [$F(1,44) = 1.32$; $P = 0.26$] whether the animals were pre-treated or not. When analysing the different days separately, no effect of THC on swim speed was observed as measured on the first trial of each day. As observed during the tasks, all animals were able to swim and climb onto the platform. Taken together, these results show that THC treatment did not alter the swimming capability of the animals.

3.2. Cued trials

On the first day of behavioural testing before the beginning of the acquisition task, animals received a pre-training session consisting of 2 cued trials where the platform was indicated by a visual cue (Fig. 2). A trend for THC treatment to increase escape latency was observed during the first cued trial [$F(1,44) = 2.88$, $P = 0.097$]. Tukey's post hoc test revealed that THC only reduced escape latency in NPT mice ($P < 0.05$). However, this effect was transient as it was not observed on the second trial and all animals were able to perform the task suggesting that THC did not impair visual ability and the procedural aspect of the experiment.

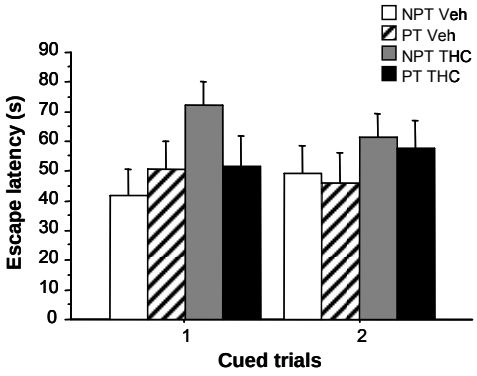


Figure 2. Escape latency in the 2 cued trials prior to the beginning of the acquisition on the first day of the behavioural testing phase in animals (n = 12 per group) pre-treated (PT) or non pre-treated (NPT) with either Veh or THC (1 mg/kg). Data are presented as means ± SEM.

3.3. Acquisition of the spatial reference memory task

Escape latencies for all sessions (average of the 4 trials per day) of acquisition and reversal are shown in Fig. 3. During acquisition, animals learnt where to find the platform as highlighted by a decreased escape latency over days [session: $F(8, 352) = 58.27$, $P < 0.001$]. Interestingly, THC increased escape latency over days [treatment: $F(1, 44) = 9.62$, $P < 0.01$] and a treatment by session interaction was also observed [$F(8, 352) = 2.29$, $P < 0.05$]. To increase the accuracy of these results, we then analysed each session separately. We observed that THC impaired acquisition of the task on days 1 [$F(1, 44) = 8.13$, $P < 0.01$], 2 [$F(1, 44) = 8.14$, $P < 0.01$] and 3 [$F(1, 44) = 5.17$, $P < 0.05$], with no difference observed on the other days. These results show that, independently of whether they received a pre-treatment or not, THC-treated animals learnt the location of the platform slower than Veh-treated animals.

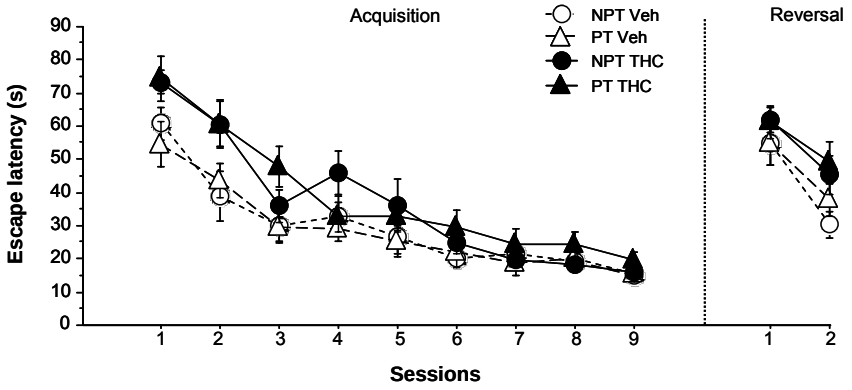


Figure 3. Escape latency for each session (4 trials per day) of acquisition of the spatial memory task (day 1-9) and reversal of the platform location (day 10-11) in animals (n = 12 per group) pre-treated (PT) or non pre-treated (NPT) with either Veh or THC (1 mg/kg). Data are presented as means ± SEM.

3.4. Reversal of the spatial memory

During the reversal phase (Fig. 3), the platform location was changed to assess learning flexibility of the animals. On the first session of the reversal on day 10, all animals showed an increased latency to reach the platform compared to the last

session of acquisition as they had to learn a new position of the platform. This is highlighted by a session effect between day 9 and 10 [$F(1, 44) = 260.83$, $P < 0.001$]. On the second session of reversal (day 11), the escape latency of all animals decreased compared to the first one, as shown by an effect of the session between days 10 and 11 [$F(1, 44) = 27.34$, $P < 0.001$]. This shows that the animals started to learn the new location of the platform. Interestingly, while no effect of THC was observed on the last days of acquisition, the reversal of the platform triggered a learning deficit in THC-treated animals during the reversal [treatment: $F(1, 44) = 5.93$, $P < 0.05$]. When analysing the sessions of reversal separately, a treatment effect was observed on day 11 [$F(1, 44) = 6.03$, $P < 0.05$], showing that THC impaired performance in the reversal phase, reducing the ability of the animals to learn the new location of the platform. Similarly to the beginning of the acquisition, THC treatment impaired the escape latency whether the animals received a pre-treatment or not.

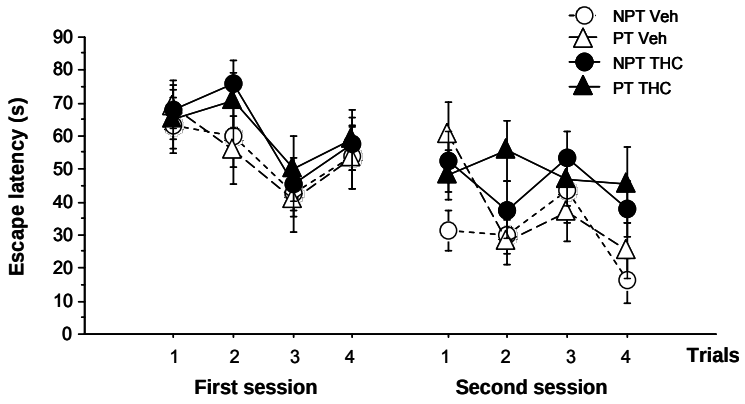


Figure 4. Detail of the 4 trials of the 2 sessions of reversal in animals (n = 12 per group) pre-treated (PT) or non pre-treated (NPT) with either Veh or THC (1 mg/kg). Data are presented as means ± SEM.

To gain a more detailed insight into our results, we then analysed the different trials of the 2 sessions of reversal separately (Fig. 4). A trend for an effect of THC treatment was observed on the second trial of the first session of reversal [$F(1, 44) = 3.01$, $P = 0.09$] but not on the first trial as animals had to find the new location of the

platform. This THC-induced impairment in performance observed on the second trial of the first day of reversal was accentuated on the second day, as highlighted by an effect of THC treatment on trial 2 [$F(1, 44) = 5.19$, $P < 0.05$] and 4 [$F(1, 44) = 5.31$, $P < 0.05$] of the second session. These results are consistent with the interpretation that THC impaired working memory during the reversal.

3.5. Probe trials

The results from the probe trials are shown in Fig. 5. The first probe trial was done 1 hour after the end of the acquisition on day 9. The platform was removed from the water maze and we measured the time spent in the target quadrant where the platform was formerly located during the acquisition. Consistent with results from escape latencies on the last days of acquisition, all animals spent the same time in the target quadrant as shown by the absence of treatment effect [$F(1, 44) = 0.12$, $P = 0.73$]. In addition, all animals were above chance level in the time spent exploring the target quadrant ($P < 0.001$), showing that all animals had learnt the location of the platform at the end of the acquisition (Fig. 5A; P1 TQ1). We also measured the latency for the first platform location crossing (Fig. 5B). In the first probe test, we observed that THC-treated mice exhibited a significant increased latency to cross the platform compared to Veh-treated animals [$F(1, 44) = 5.3$, $P < 0.05$] and this effect was independent of the treatment condition [$F(1, 44) = 2.53$, $P = 0.12$].

One hour after the end of the second session of reversal on day 11, a second probe trial was done to compare whether animals swam in the new target quadrant or in the old one where the platform was located during the acquisition. No effect of THC was observed on the time spent in the old target quadrant [$F(1, 44) = 0.47$, $P = 0.49$] that all animals explored at chance level (Fig. 5A; P2 TQ1). This shows that all

mice stopped exploring the quadrant where the platform was located during the acquisition. In the new target quadrant, THC increased the latency to first cross the location of the platform [F(1, 44) = 4.8, $P < 0.05$] (Fig. 5B; probe test 2). Supporting this result, a trend for THC to decrease the time spent in the new target quadrant was observed [F(1, 44) = 3.38, $P = 0.07$] (Fig. 5A; P2 newTQ). Importantly, Veh-treated animals spent more time (above chance level) in the new target quadrant than in the old one [F(1, 22) = 6.09, $P < 0.05$], showing that they have started to learn the new location of the platform. However, THC-treated animals were at chance level in both the new and the old quadrants. Taken together, these results show that THC impaired reversal learning in the water maze.

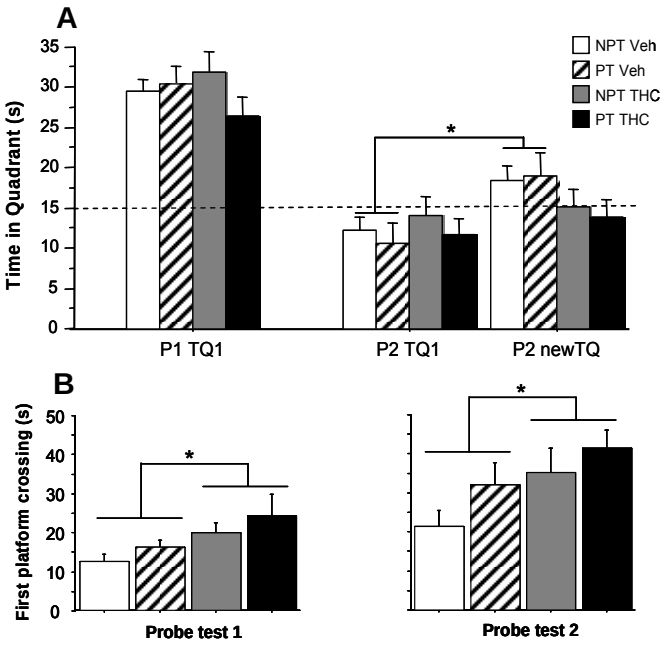


Figure 5. A) Time spent in the different target quadrants (TQ) during the 2 probe trials. The first probe (P1) occurred 1 h after the end of the acquisition (i.e. on day 9) and the second probe (P2) occurred 1 h after the end of the reversal (i.e. on day 11). B) Latency to cross the platform location for the first time in animals (n = 12) pre-treated (PT) or non pre-treated (NPT) with either Veh or THC (1 mg/kg). Data are presented as means ± SEM. (Means ± SEM). Significant effects are indicated by ⋆ (P

3.6. Locomotor activity

We also measured locomotor activity using another set of animals (Fig. 6). No difference was observed between the animals on day 0 prior to the beginning of the drug administration. Using one-way repeated measure ANOVA, we showed that THC treatment promoted an overall decreased locomotor activity over days [F(1, 54) =

5.48, $P < 0.05$]. The analysis of each days separately showed that THC reduced locomotor activity on days 2 [F(1,9) = 14.84, $P < 0.01$], 3 [F(1,9) = 5.94, $P < 0.05$] and 4 [F(1,9) = 6.84, $P < 0.05$]. From the fifth day of treatment, no difference was seen between Veh- and THC-treated mice, showing that animals became tolerant to the locomotor suppressant effect of THC.

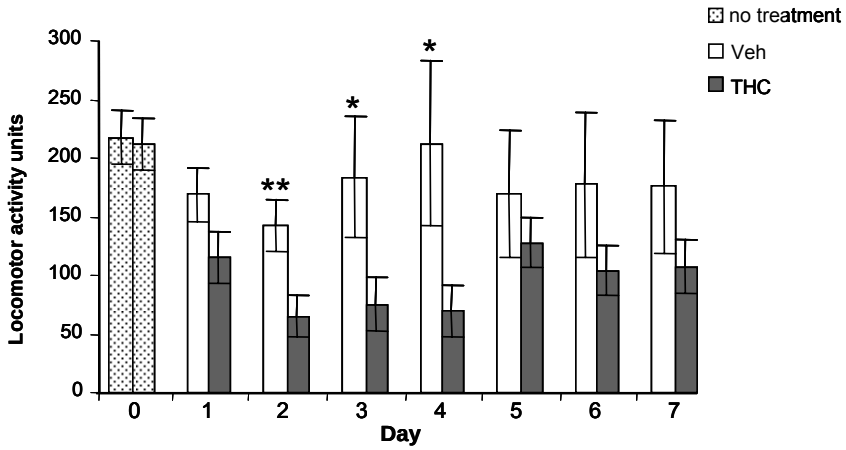


Figure 6. Tolerance to locomotor activity in animals (n = 5-6) treated with Veh or THC (1 mg/kg). Data are presented as means ± SEM. Significant effects are indicated by ⋆ (P < 0.05) and ⋆⋆ (P < 0.01).

3.7. Immunohistochemistry

The number of zif268 immunoreactive cells for the different regions are shown in Fig. 7. Analysis with Student's t-test showed that in animals that were not pre-treated (NPT-Veh compared to NPT-THC), THC decreased zif268 expression in the CA3 of the hippocampus ($P < 0.001$), in the dorsomedial ($P < 0.01$) and dorsolateral ($P < 0.05$) part of the caudate putamen and in the prefrontal cortex ($P < 0.05$). No effect of THC was observed in animals that received pre-treatment with THC (PT-Veh compared to PT-THC).

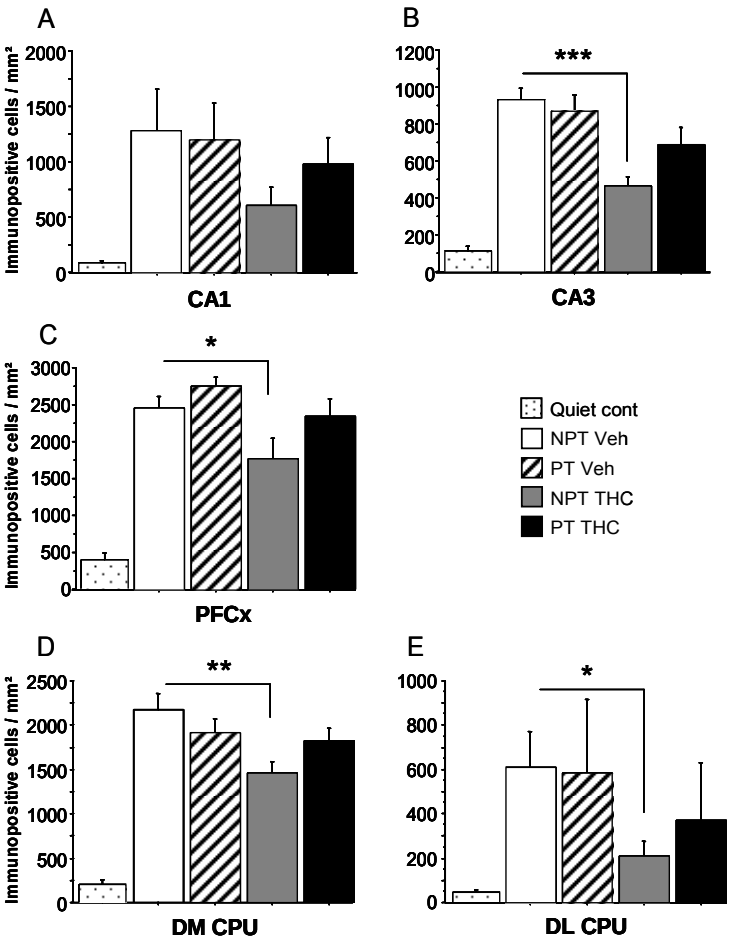


Figure 7. Number of zif268 immunopositive cells in (A) CA1 and (B) CA3 of the hippocampus, (C) the prefrontal cortex (PFCx) and (D) the dorsomedial (DM) and (E) the dorsolateral (DL) caudate putamen (CPU) in animals (n = 6 per group) that were pre-treated (PT) or non pre-treated (NPT) with either Veh or THC (1 mg/kg). Animals from "Quiet cont" group did not receive any injection and were not trained. Data are presented as means ± SEM. Significant effects are indicated by ★ ($P < 0.05$), ★★ ($P < 0.01$) and ★★★ ($P < 0.001$).

4. Discussion

The main results from the present study indicate that animals treated with a low dose of THC, whether they were pre-treated or not, showed a reduced learning of the spatial reference memory task. However, they eventually acquired the task like control animals. During the reversal phase, while both NPT and PT animals were tolerant to the locomotor suppressant effects of THC, the drug impaired cognitive

flexibility of the animals as they did not learn the new location of the platform compared to control. This highlights a long-lasting cognitive deficit in these animals, as even after 24 days of exposure to the drug, PT mice still had not developed tolerance to this effect. Immunohistochemical analysis revealed that THC decreased zif268 expression in the CA3 of the hippocampus, in the prefrontal cortex and in the dorsomedial and dorsolateral caudate putamen only in animals that did not received a pre-treatment, with no effect seen in PT animals.

At the beginning of the acquisition, animals were trained in 2 cued trials where the location of the platform was indicated by a visual cue. No significant difference between Veh- and THC-treated animals was observed, showing that all animals were able to find the platform. This result, together with the absence of difference in swim speed in animals treated with THC compared to Veh, suggests that THC did not induce any visual or locomotor impairment in mice exploring the water maze. These results are consistent with studies showing that, while cannabinoids impair learning in the place version of the water maze, no deficit was observed in the cued version of the water maze task using the visible platform (Ferrari et al., 1999, Niyuhire et al., 2007).

Following the 2 cued trials, acquisition of the reference memory task began. Animals received 4 trials per session over 9 consecutive days where the hidden platform remained in a fixed position. We observed a slower acquisition of spatial memory performance in animals treated with THC, irrespective of whether these animals were pre-treated with THC for 13 days or not. Cannabinoids agonist like HU 210 (Ferrari et al., 1999)and THC (Da and Takahashi, 2002) have previously been shown to impair learning in the place version of the water maze task. By the ninth day, no difference was observed between Veh- and THC-treated groups, showing that

all animals had learnt the task. These results demonstrate that THC impaired the acquisition of spatial reference memory, and this effect was overcome with repeated learning sessions.

For the reversal testing, the location of the platform was changed but the procedural component of the task (to swim to the platform to escape) remained unmodified. Thus animals had to extinguish the original spatial response and learn a new one by making new associations between the novel platform location and the surrounding spatial cues (Rossato et al., 2006). On the first trial of the first day of reversal (day 11) an increased time to reach the platform was observed for all animals, as they had to find the new location of the platform. Interestingly on the second trial of this day, THC-treated animals were delayed in finding the platform compared to Veh-treated animals, and this effect was accentuated on the second day of reversal (see Fig. 4). This is consistent with a deficit in working memory, as it is usually evaluated with the repeated learning of a new location of the platform. Research evaluating the effects of cannabinoids on working memory has widely reported deficits in this form of learning. In C57BL/6 mice, 3 mg/kg THC impaired working memory (Varvel et al., 2001), and this effect was reversed by the cannabinoid receptor antagonist SR141716A. In a similar paradigm to ours, THC decreased spatial working memory on the second trial of the delayed-matching-to-place in the water maze when administered at 2 and 5 mg/kg (Fadda et al., 2004). Impairment of working memory with THC (6 mg/kg) was also observed in the radial arm maze, another model that can be used to measure working memory (Lichtman and Martin, 1996). In the present study, using a relatively low dose of THC which is more comparable to human dosing, we observed a reversal deficit which suggests that THC

impaired the re-actualization of the information, this capacity of flexibility being one of the properties of working memory.

One hour following the last trial of acquisition on day 9, animals were tested in a probe trial where the platform was removed from the pool. We observed that all mice explored the target quadrant where the platform was localized during the acquisition above chance level, confirming that they successfully learnt the spatial reference memory task. However, although the difference between Veh- and THC-treated animals is quite moderate, the significantly higher latency to the first platform crossing implies a less accurate spatial response in mice that were treated with THC. On the second probe trial at the end of the reversal on day 11, Veh-treated animals spent more time in the new target quadrant than THC-treated animals, and above chance level. This illustrates that Veh-treated animals were again learning the new location of the platform faster than THC-treated mice. This further strengthens our interpretation that THC impaired behavioural flexibility in this model. As THC-treated animals did not learn the new platform location this deficit in reversal might be due to an increased perseverance of the animals in returning to the previously learnt platform location. However our results showed that THC-treated animals, that spent less time in the new target quadrant, did not spend more time in the former target quadrant where the platform was located during the acquisition, that they explored at chance level. Therefore, THC-induced deficits in reversal learning are unlikely due to perseverance and most likely explained by a specific deficit in learning the new platform location.

Interestingly during both the reference memory and the reversal tasks, no differences were observed between THC-treated PT and NPT animals, showing no differential effect of the pre-treatment on learning. With repeated administration,

tolerance to the effects of cannabinoids such as locomotor suppression, analgesia or hypothermia develop rapidly (Oliva et al., 2003), however effects on social or memory impairment appear to be more resistant to the development of tolerance (for a review, see Gonzalez et al., 2005). We analysed the effect of repeated THC exposure on locomotor activity and observed that animals developed tolerance to the locomotor suppressant effects of 1 mg/kg THC with no difference observed between Veh- and THC-treated animals after 5 days of treatment. Thus, THC-induced deficits on reference memory cannot be related to the sedative effects of cannabinoids, as NPT animals that received the drug for the first time exhibited the same level of performance as PT animals on day 1. Further, PT animals that received 13 days of pre-treatment were already tolerant to the locomotor suppressant effect of THC at the beginning of the acquisition. In conjunction with the absence of effects of the drug on swim speed, this strengthens the interpretation that this low dose of THC promoted a specific memory deficit.

While the acute effects of cannabinoids on memory are widely reported, the effects of long term cannabinoid exposure have received less attention. Interestingly, the memory deficits observed during the reversal phase were independent of whether the animals received 13 days of pre-treatment prior to the beginning of the acquisition or not. This suggests that even after 24 days of treatment for the PT group and 11 days of treatment for the NPT group, THC was still effective in impairing memory. In human studies persistent cognitive deficits were observed after repeated use of cannabis (Solowij et al., 2002, Messinis et al., 2006). Animal studies have also reported the lack of development of tolerance to cannabinoid-induced working memory impairment (Nakamura et al., 1991, Nava et al., 2001). These studies used 5 mg/kg THC, a dose higher than the one used here. Thus, results from the present

study show that memory impairments promoted by THC, even at such a low dose, are resistant to the development of tolerance.

In NPT mice, THC decreased zif268 expression in hippocampal CA3 and to a lesser extent in the CA1, which is consistent with the view that the hippocampus mediates the memory impairing effects of cannabinoids (Lichtman et al., 1995, Misner and Sullivan, 1999, Hampson and Deadwyler, 2000, Egashira et al., 2002). Interestingly, THC exposure also reduced the expression of zif268 in the prefrontal cortex. This region is another site thought to participate in cannabinoid-induced spatial memory impairment in previous studies. For example, direct administration of THC into the medial prefrontal cortex has been shown to disrupt spatial working memory (Silva de Melo et al., 2005). Further, repeated THC administration in rats caused a persistent reduction in medial prefrontal cortical dopamine turnover which was assumed to subserve in part the cognitive dysfunction observed in these animals (Verrico et al., 2003). Because memory processes are dependent on synaptic plasticity in the hippocampus and prefrontal cortex (Laroche et al., 2000, Riedel and Davies, 2005), these results suggest that the THC-induced impairment of working memory observed here could be mediated by a decreased neuronal activity in both the hippocampus and prefrontal cortex as measured by zif268.

The decreased zif268 expression in the hippocampus we observed here was more dramatically observed in NPT than in PT animals, although the latter did exhibit cognitive impairments which were sometimes even more marked as assessed by the probe tests (Fig. 5). These results suggest that between 11 and 24 days of treatment, some cellular and molecular determinants of tolerance began developing which are dissociable from the mnemonic effect of THC. Interestingly, a 2-week chronic THC treatment did not produce tolerance to CB1-mediated inhibition of extracellular

hippocampal acetylcholine and working memory, but there was a time-course difference between these two phenomena suggesting they might be dissociated (Nava et al., 2001). Similar to the effects of THC on the hippocampus, THC also decreased zif268 expression in the dorsomedial and dorsolateral caudate putamen only in NPT animals. The dorsal striatum is not reported to be implicated in spatial memory (Egashira et al., 2002), but recent reports have emphasized a role of the medial striatum in behavioural flexibility (McDonald et al., 2008). The behavioural effects of THC on striatal function seem to fit better with this interpretation as the PT animals were tolerant to the locomotor suppressant effect of THC at the time of testing and no deficits were observed in the cued trials of the water maze task, a task supposed to be mediated by the caudate putamen (Packard and Teather, 1998).

The present study examined for the first time the effects of repeated cannabinoid exposure on zif268 expression in the mouse forebrain. In prior *in situ* hybridisation studies, acute cannabinoid receptor agonist exposure increased the expression of zif268 mRNA in the CA1 and CA3 regions of the hippocampus (Derkinderen et al., 2003), cingulate cortex, fronto-parietal cortex and the caudate-putamen (Mailleux et al., 1994). Consistent with this, (Glass and Dragunow, 1995)showed in an immunohistochemical study that acute administration of a high dose of CP 55,940 (2.5 mg/kg) significantly increased zif268 in the striatum (CP 55,940 is approximately 30 times as potent as THC, see (Arnold et al., 2001a, Arnold et al., 2001b). Given that acutely cannabinoids induce zif268 expression, the decreased zif268-labelled cells we observed here indicates that zif268 expression is inversely regulated by repeated cannabinoid exposure. Indeed, such a phenomenon has been reported for the psychostimulant drug, cocaine. Acute cocaine increased zif268 expression in the rat forebrain, however with repeated administration it

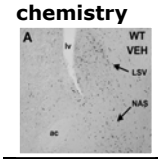
decreased basal zif268 expression (Bhat et al., 1992). We hypothesise that the diminished zif268 expression observed here following repeated THC exposure may promote changes in the zif268-regulated target genes which subserve the long-term memory impairing actions of cannabinoids.

The present study shows that THC delayed the acquisition of a reference memory task in the water maze even in animals tolerant to the locomotor suppressant effects of the drug. When submitted to the reversal of the platform, THC impaired reversal learning suggesting deficits in behavioural flexibility consistent with dysfunction in working memory. This effect was still present in animals that received up to 24 days of treatment, showing that the memory disruptive effects of THC are particularly resistant to the development of tolerance even when using a low dose more consistent with human consumption. In the selected brain regions, THC decreased zif268 immunoreactivity only in animals that did not receive a pre-treatment, suggesting that tolerance to the neuronal effects of the drug may have appeared between 11 and 24 days of treatment. In conclusion, this study showed that repeated cannabinoid exposure induced persistent memory deficits which are resistant to tolerance and may be, at least initially, regulated by diminished expression of the transcription factor zif268.

Summary of the main results at the end of Chapter 5

Chapter 5 examined the behavioural effects of repeated THC exposure (1 mg/kg) on WT mice using the water maze. The main results obtained from this experiment are summarised in Table 1 and show that THC delayed learning of a spatial reference task and importantly, after 24 days repeated exposure, THC impaired reversal learning in animal that were already tolerant to the effects of the drug on locomotor activity.

Table 1. Summary of main results form Chapter 2, 3, 4 and 5.

Test	Chapter 2 Effect of THC (10 mg/kg)	Chapter 3 Effects of THC (10 mg/kg)	Chapter 4 Repeated CP 55,940 (0.4 mg/kg)		Chapter 5 Chronic THC (1 mg/kg) WT only
			On day 1	Tolerance	
Locomotor activity in the open field 	Hypo-locomotion more important in *Nrg1* HET mice than WT mice		Strong hypo-locomotion in both *Nrg1* HET and WT mice	*Nrg1* HET mice developed tolerance to the hypo-locomotion faster than WT mice	Tolerance to the decreased locomotor activity developed after 5 days
Anxiety in the light dark 	Decreased time spent in the light compartment only in *Nrg1* HET mice		Decreased time spent in the light compartment in both *Nrg1* HET and WT mice	Tolerance developed in WT mice but not in *Nrg1* HET mice	
Social interaction 	Decreased social interaction in both WT and *Nrg1* HET mice similarly		Decreased social interaction in both WT and *Nrg1* HET mice	No tolerance developed	
Prepulse inhibition 	Facilitation only in *Nrg1* HET mice		Facilitation in *Nrg1* HET mice, disruption in WT mice	Tolerance developed in both *Nrg1* HET and WT mice	
Immunohisto-chemistry 		Increased c-Fos expression in the LSV of *Nrg1* HET mice		Increased Fos B/ΔFos B expression in the LSV of *Nrg1* HET mice	Decreased zif268 expression in CA3, PFC and CPU only in NPT mice
Memory in the water maze 					Delayed learning of spatial memory Impaired reversal learning

Chapter 6

GENERAL DISCUSSION

1. Significance of key findings and future directions

While human population studies indicate that a gene-environment interaction is likely required for the development of schizophrenia, they are restricted in the mechanistic detail they can provide in improving the understanding of this phenomenon. Indeed epidemiologists have made a call to basic scientists to assist in the quest to delineate the exact genetic and neuropharmacological basis for cannabis-induced schizophrenia (Henquet et al., 2005). The research presented in this thesis provides the first attempt to create an animal model of genetic vulnerability to cannabinoid-induced schizophrenia. This was achieved using mice heterozygous for the schizophrenia susceptibility gene *Nrg1* that display many schizophrenia-like endophenotypes. These animals were exposed to acute and chronically administered cannabinoid drugs, including the main psychoactive constituent of cannabis, THC, and their neurobehavioural responses were compared to that of WT mice. Further, an animal model of chronic cannabinoid-induced impairments in spatial working memory was also developed that might be applied to the *Nrg1* HET mice in future studies.

The results from this thesis further reinforce the nature of the *Nrg1* HET mouse phenotype and that it provides a compelling animal model of schizophrenia. For example, *Nrg1* HET mice show a hyperactive phenotype (Chapter 2) that is resistant to habituation compared to WT animals (Chapter 4). The *Nrg1* HET mice display a moderate anxiolytic-like phenotype that is task-specific as it was only observed in the LD but not in the SI test (Chapter 2). However, the locomotor hyperactivity of these mice reduces the impact of such a conclusion as it confounds anxiogenic measurement in exploration-based models like the LD task. *Nrg1* HET mice also displayed PPI deficits (Chapter 4); however, this effect was not observed in Chapter 2. A change in PPI testing parameters in Chapter 4 was necessary to reveal

the PPI deficit of *Nrg1* HET mice. Therefore, the consistency of such a phenotype is questionable and requires further research to identify the exact conditions required to reliably uncover this aspect of the *Nrg1* HET mice phenotype. On a neurobiological level we reported for the first time in Chapter 3 that *Nrg1* HET mice show enhanced c-Fos expression in schizophrenia-relevant areas of the CNS, i.e. the LSV (Sheehan et al., 2004), and the NAS (van der Stelt and Di Marzo, 2003). This is consistent with these animals having a schizophrenia phenotype, especially with respect to the increased c-Fos expression in the NAS which might be caused by increased dopamine levels in this region.

SI was measured in Chapter 2 and 4 of the present thesis but did not reveal drug-free differences between *Nrg1* HET mice and WT mice. Social withdrawal is generally observed in schizophrenia, but the absence of a social deficit in *Nrg1* HET mice suggests that this behaviour may not involve *Nrg1* processes. O'Tuathaigh and colleagues (2008) reported increased aggressive behaviours of *Nrg1* HET mice in the resident intruder test where the opponent is placed in the home cage of the tested animals. Here mice were tested in a novel environment, thus context differences could account for the absence of SI between our studies and O'Tuathaigh's, as aggressive behaviour is more robust in a territorial context. This suggests that a more confrontational environment should be used to evaluate SI in the future. In addition, O'Tuathaigh et al. (2008) reported a selective impairment of *Nrg1* HET mice in recognising social novelty. Cannabinoid treatment impairs social recognition in rodents (Schneider and Koch, 2002). Therefore it would be of high interest to evaluate if the effects of cannabinoids on social recognition would be intensified in *Nrg1* HET mice.

The data reported in Chapter 2 were the first supporting that *Nrg1* HET mice exhibit an increased sensitivity to cannabinoids in behavioural domains associated with schizophrenia-like symptoms in animals. This key finding is a breakthrough in understanding the genetic basis for cannabis-induced psychosis as it demonstrates that alteration in *Nrg1* modulates the acute actions of cannabinoids. The most striking result from Chapter 2 was that *Nrg1* HET mice expressed a greater THC-induced enhancement in PPI compared to WT mice. It is tempting to speculate that these data are consistent with the self-medication hypothesis of schizophrenia as acute THC treatment enhanced attentional function in the *Nrg1* HET mice as measured in the PPI model. To assess the neurobiological correlates of such an increased behavioural sensitivity of *Nrg1* HET mice, Chapter 3 measured neuronal activation following acute THC exposure. An increased THC-induced c-Fos expression was observed selectively in the LSV of *Nrg1* HET but not in WT mice. Taken together, these two chapters demonstrate that cannabinoid and neuregulin systems interact to modulate the sensitivity of *Nrg1* HET mice to the acute neurobehavioural effects of cannabinoids.

The increased sensitivity of *Nrg1* HET mice to the acute effects of THC might be explained by the establishment of compensatory mechanisms that might have developed during neurodevelopment in *Nrg1* HET mice. Nrg1 and its receptor ErbB4 play essential roles in neuronal migration, neurotransmitter receptor expression and myelination (Corfas et al., 2004) and are highly expressed in the developing brain suggesting that in humans like in animals, Nrg1 is involved in neuronal maturation (Pinkas-Kramarski et al., 1994, Bernstein et al., 2006). Interestingly, the presence of such compensatory mechanism was suggested in mutant mice null for the Nrg1 receptor ErbB4 (Thuret et al., 2004). Therefore, the partial deficiency of *Nrg1* could

have engendered a reorganisation of the brain during development that could have affected the expression or efficacy of different neurotransmitter systems including CB1 receptors, explaining the different sensitivity of *Nrg1* HET mice after acute THC treatment in Chapter 2 and 3. Indeed our group has preliminary data suggesting that the levels of CB1 and NMDA receptors binding show altered expression in *Nrg1* HET mice as measured by autoradiography (unpublished observations).

As human studies showed that it is more consistently the chronic use of cannabis that precipitates schizophrenia in vulnerable individuals, Chapter 4 examined the effect of repeated administration of cannabinoids. As THC could not be used in this experiment as it was unavailable at this time, the effects of the synthetic analogue of THC, CP 55,940, were evaluated on *Nrg1* HET mice. Upon repeated administration, we were expecting that chronic administration of CP 55,940 would precipitate PPI deficits in *Nrg1* HET mice. However no long-term effect of the drug was observed in PPI, wether it is facilitation or perturbation. These results show that, in the limitation of animal research, the PPI model may not be able to discriminate long term effects of cannabinoids in terms of psychotic-related symptoms. However other models revealed interesting genotype differences after repeated cannabinoid treatment. Tolerance to CP 55,940-induced locomotor suppression and hypothermia developed more rapidly in *Nrg1* HET mice than in WT mice. By contrast, while tolerance developed to the anxiogenic-like effects of CP 55,940 in WT animals, no tolerance to this anxiogenic-like effect could be observed in *Nrg1* HET mice, even after 15 days exposure to the drug. In addition, this effect was associated with an increased Fos B/ΔFos B expression in the LSV of *Nrg1* HET mice, with no corresponding effect observed in WT mice. These results provide new insight into *Nrg1*-cannabinoid interactions and show that repeated cannabinoid exposure promotes

differential behavioural effects on *Nrg1* HET and WT mice and reaffirm that the LSV may be an important site for cannabinoid-induced neuronal dysregulation in the brain of *Nrg1* HET mice.

Nrg1 and ErbB4 are also expressed in the adult brain (Steiner et al., 1999, Law et al., 2004), and neuregulins are involved in regenerative processes in the mature brain (Kerber et al., 2003). As observed in Chapter 4, *Nrg1* depletion may confer a distinct neuroadapative response to repeated treatment with CP 55,940 observed in adulthood. Interestingly, the neurobehavioural response of the *Nrg1* HET mice to repeated cannabinoid exposure varied depending on the measure being taken. The neuroadaptive processes underlying these effects are likely distinct and involve partly disparate brain circuitry. For example, an enhanced rate of CB1 receptor down-regulation or desensitisation in the substantia nigra and hypothalamus could explain why *Nrg1* HET mice develop tolerance more rapidly to the locomotor suppressant and hypothermic effects of CP 55,940 respectively. Alternatively, the sustained anxiogenic response of *Nrg1* HET mice with repeated testing might be subserved by induction of the long-term neuronal marker Fos B/ΔFos B in the LSV, an area involved in anxiety-related behaviour (Sheehan et al., 2004, Sotomayor et al., 2005, Calfa et al., 2007). Future studies are required to clearly delineate the mechanisms subserving these altered neuroadaptive responses to repeated CP 55,940 in *Nrg1* HET mice. For example, analysing CB1 receptor function using autoradiography would help to further understand the neuronal changes that occur in the different brain regions of these animals following a repeated treatment with cannabinoids.

Neurodevelopmental processes underlying brain maturation are also critical during adolescence (Schneider, 2008) - the age coinciding with the onset of schizophrenia. Indeed, adolescence is also the age-period at which people often start

experimenting with cannabis. Human studies have shown that earlier cannabis use confers greater risks for developing schizophrenia than later cannabis use (Arseneault et al., 2002). Thus, adolescence may offer a period of particular vulnerability to the adverse effects of cannabis. Consistent with this, studies have shown that cannabinoid exposure in adolescence may induce subtle but lasting neurobiological changes in adult brain circuits underlying higher thought functions (Quinn et al., 2008, Rubino and Parolaro, 2008, Schneider, 2008). A previous study by Karl and colleagues (2007) indicated that *Nrg1* HET mice have an age-dependent phenotype, with symptoms like hyperactivity being evident after 5 months of age. Therefore, it would be of particular interest to observe whether cannabinoid exposure in adolescent *Nrg1* HET mice facilitates the earlier development of the "schizophrenia-like" phenotype paralleling human evidence showing that cannabis use might precipitate schizophrenia earlier in people with vulnerability to the disorder (Barnes et al., 2006, Mauri et al., 2006).

The results from Chapter 3 and 4 of this thesis highlight that the LSV may be a particular site of dysregulation in *Nrg1* HET mice which responds aberrantly to cannabinoid exposure. It is surprising that this particular region is the recurring brain area discerning genotype-cannabinoids interaction, given that schizophrenia is most generally associated with dysfunctions in the cingulate and prefrontal corteces or the hippocampus (Mitchell et al., 2001), regions where we did not find any interactions. However, the lateral septum is involved in the integration of cognitive processes by receiving inputs from the hippocampus, as well as from the PFC and the entorhinal cortex (Sheehan et al., 2004). It also shares reciprocal projections with the hypothalamus, the amygdala and the BNST that indicate a role in relaying affective information. Further, its role in incentive motivation and schizophrenia is highlighted by its connections with the mesocorticolimbic dopaminergic system (Sheehan et al.,

2004). In addition to the modulation of these diverse functions, the lateral septum has widely been reported to be involved in the stress response. It receives vasopressin innervations from the amygdala and the BNST that are involved in social response to stress and anxiety-provoking environments (Koolhaas et al., 1998). When directly injected in the lateral septum, nicotine and serotonin receptor agonists induced anxiogenic effects in rats (Cheeta et al., 2000).

In Chapter 3, the increased sensitivity of *Nrg1* HET mice to the effects of THC on c-Fos expression in the LSV was dependent on whether the animals had been behaviourally tested, indicating that one possible reason for this effect could be that the stress associated with the procedure would be necessary to reveal this effect. Interestingly, infusion of a corticotrophin-releasing factor 2 receptor agonist directly into the lateral septum increased anxiety-related behaviour at lower doses in a high stress environment that in a low stress environment (Henry et al., 2006). This suggests that modulation of anxiety by the lateral septum is dependent on the background stress level of the animal. Thus increasing the magnitude of stress could exaggerate the effects of cannabinoids in *Nrg1* HET mice. Importantly, schizophrenia patients have an increased reactivity to psychosocial stress, and this effect has been associated with *NRG1* polymorphism (Keri et al., 2008). Taken together, these data suggest a three-way interaction between cannabinoid exposure, depletion of *Nrg1* and stress which uncovers increased schizophrenia-related neurobehavioural responses.

The persistent Fos B/ΔFos B expression promoted by CP 55,940 in the LSV might suggest that it is a key region in the regulation of drug-induced psychosis. Evidence that the lateral septum might be involved in schizophrenia processes is supported by the observations that most antipsychotic drugs increase c-Fos expression in the LSV (Pinna et al., 1999). Interesting, after repeated exposure to haloperidol no

tolerance developed to the induction of c-Fos expression in the LSV (Sebens et al., 1995). In addition, the chronic administration of clozapine increases cytochrome c oxidase expression in the septum (Prince et al., 1998). In contrast, PCP and methamphetamine that are known for inducing psychotic symptoms decrease cytochrome c oxidase activity in the septum, an effect reversed by antipsychotic drugs (Prince et al., 1997). Cytochrome c oxidase is a pivotal enzyme from mitochondria that is involved in oxidative metabolism to generate ATP by cell respiration and has a role in apoptosis (Kadenbach et al., 2000, Ow et al., 2008). Inhibited neuronal functional activity, as a consequence of reduced energy metabolism mediated by altered mitochondrial function and decreased cytochrome oxidase levels, has been proposed to influence the symptomatology of schizophrenia (Marchbanks et al., 1995). Interestingly, both cannabinoid and Nrg1-ErbB signalling have been implicated in inducing apoptosis by releasing cytochrome c (Downer et al., 2001, Rohrbach et al., 2005). In conclusion, these results suggest that cannabinoids could influence the response of the LSV of *Nrg1* deficient mice by interacting with cytochrome c oxidative processes.

The lateral septum expresses various substances to support neurotransmission. The rostral region of the lateral septum expresses neurotensin and enkephalin and caudal parts show high levels of somatostatin. However the ventral component of the lateral septum appears to predominantly express estrogen (Sheehan et al., 2004). Estrogens act on an interconnected network of hypothalamic and limbic areas, and especially on the lateral septum, where they modulate aggressive behaviour (Trainor et al., 2006). Interestingly, estrogens also regulate cannabinoid-induced presynaptic inhibition of glutamate and GABA release in the hypothalamus (Nguyen and Wagner, 2006), and stimulate interaction between estrogen receptors and the neuregulin

receptor ErbB4 as shown in breast cancer cells (Zhu et al., 2006). Variation in the estrogen receptor gene has been associated to schizophrenia and estrogens have been suggested to provide a potential treatment for schizophrenia due to their neuromodulatory and neuroprotective activities (Kulkarni et al., 2008, Weickert et al., 2008). All together these studies suggest that the interaction between cannabinoids and NRG1-ErbB4 signalling in the LSV might involve estrogen receptors.

Chapter 4 highlighted that tolerance develops at different rates dependent on the behaviour tested, however memory processes were not evaluated as it requires more elaborated protocols. Thus Chapter 5 evaluated the chronic effects of cannabinoids in a model of reversal learning in the water maze using a low dose of THC (1 mg/kg) in C57Bl/6 wild-type mice (the background strain of our *Nrg1* HET mice). In addition, an effect of 13 days pre-treatment with the drug was evaluated to observe whether memory deficits would persist after tolerance had developed to the locomotor suppressant of the drug. This allowed testing of a cognitive-specific effect of THC without locomotor confounds promoted by the sedative effects of the drug. The data reported in Chapter 5 showed that THC promoted a deficit in reversal learning which was observed even after 24 days of treatment, suggesting that tolerance did not develop to the cognitive impairing effects of THC.

There has been expanding interest in recent years of the involvement of cannabinoids in executive functions, as they modulate diverse higher-order cognitive processes including attention, inhibitory control and decision-making, time estimation, working memory and behavioural flexibility (Pattij et al., 2008). The latter represent the capacity to adapt to the environment by adjusting behavioural strategies - that is to suppress old responses and replace them with new ones. This process is impaired in cannabis users (Lamers et al., 2006) and animal models reported

impairments in attentional set shifting tasks (Egerton et al., 2005, Hill et al., 2006). To our knowledge only one study previously showed that modulation of the cannabinoid system impairs behavioural flexibility in reversal learning in the water maze (Varvel and Lichtman, 2002), thus the results from Chapter 5 provide the first evidence for a persistent deficit in reversal learning following chronic cannabinoid treatment. The protocol outlined in Chapter 5 could also provide a model of high interest to evaluate if cannabinoids trigger increased memory deficits in *Nrg1* HET mice compared to WT mice. O'Tuathaigh and colleagues (2007) reported intact spatial learning of *Nrg1* HET mice in the Barnes maze and intact spatial working memory using a spontaneous alternation task however spatial memory performance of *Nrg1* HET mice has never been evaluated in the water maze. Association of *NRG1* gene with working memory deficits have been shown in healthy individuals (Krug et al., 2008), thus it is also possible that *Nrg1* HET mice might display altered working memory compared to WT mice using the water maze and the effects of cannabinoids might be exaggerated in *Nrg1* HET mice in such a task.

While behavioural studies can give a profound insight into modelling psychiatric diseases in animals, the main limitation of these tests include the absence of consistency between experiments. Behavioural models are strongly dependent upon the procedure used, for example a change in the PPI paradigm in Chapter 4 compared to Chapter 2 revealed a PPI deficit in VEH-treated *Nrg1* HET mice. In addition, other factors can influence the outcome of behavioural testings, especially when measuring anxiety-related behaviours. We observed an increased time spent in the light compartment of the LD and in the time spent in SI of baseline VEH-treated WT and *Nrg1* HET mice on the 1st day of administration in Chapter 4 compared to Chapter 2. Such variations can be accounted for the difference in familiarity to the apparatus

(repeated testing in Chapter 2 using within-subjects comparison versus use of naïve animals in Chapter 4 using a between-subjects comparison) or the age of the animals (22-25 weeks in Chapter 4 versus 26-30 weeks in Chapter 2). Another limitation of these behavioural models is the interpretation of the effects observed. The increased locomotor activity of *Nrg1* HET mice confounded the specificity of the anxiety-like effect in the LD in Chapter 2, and the decreased startle response also confounds the PPI deficit observed in VEH-treated *Nrg1* HET mice. Thus a careful investigation of the test parameters associated with the evaluation of several models is required to increase the strength of the results. Future studies of biological correlates such as corticosterone levels measurements could be helpful to further characterize the specificity of the increased sensitivity of *Nrg1* HET mice to cannabinoid-induced anxiety-like behaviour.

When taken together, the results from the present thesis support that gene-environment interactions increase vulnerability to developing schizophrenia-like symptoms. Consistent with the main theory of the association between cannabis and schizophrenia considered in Chapter 1, cannabis would increasing psychosis in vulnerable individuals, as supported by the increased sensitivity of *Nrg1* HET mice to the neurobehavioural effects of cannabinoids observed in this thesis. This work raises the need for future investigations on gene-environment interactions, and other studies exploring other environmental causes such as pregnancy or early life stress would be of high interest to try to further understand the basis of the development of schizophrenia.

While the use of *Nrg1* HET mice provides a very promising model for schizophrenia, other genetic animal models are on offer to explore the genetic basis of cannabis-induced psychosis. One of the most interesting are those involving genetic

manipulation of the *DISC1* gene. Collectively, these mice have enlarged ventricles and exhibit behavioural impairments such as hyperactivity, abnormal spatial working memory and sociability, and an anhedonia/depression-like deficit. Further, these mice show deficits in LI and PPI that were reversed by antipsychotic treatment (Koike et al., 2006, Clapcote et al., 2007, Hikida et al., 2007, Li et al., 2007, Pletnikov et al., 2008). Other schizophrenia susceptibility genes have also been investigated, for example PRODH that induced PPI impairments (Gogos et al., 1999) and deficits in associative learning and exaggerated stress and amphetamine responses (Paterlini et al., 2005). Therefore, the use of mutant mice for schizophrenia susceptibility genes is a new and exciting tool in understanding psychiatric diseases and it would be of interest to replicate the effects of cannabinoids with other susceptibility genes. To model the polygenetic aetiology of schizophrenia, the use of mutant mice exhibiting mutation for several susceptibility genes would represent the future of genetic modelling of psychiatric disorder.

2. Conclusion

In conclusion, the present thesis provides the first evidence that partial deletion of the schizophrenia susceptibility gene *Nrg1* modulates the neurobehavioural actions of acutely and chronically administered cannabinoids. *Nrg1* HET mice appear more sensitive to the acute neurobehavioural effects of cannabinoids. Acutely THC exposure had a beneficial action on attentional function by promoting PPI facilitation in *Nrg1* HET mice. However, with repeated cannabinoid administration such an acute benefit disappeared and the *Nrg1* HET mice displayed a long-lasting anxiogenic profile that was resistant to tolerance. Conversely, *Nrg1* HET mice developed tolerance to the locomotor suppressant and hypothermic

effects of cannabinoids more rapidly than WT mice, indicating a distorted neuroadaptive response in these animals. Another major finding of the current thesis is that the lateral septum appears to be an important brain region of cannabinoid-induced dysregulation in *Nrg1* HET mice. Setting up animal models that parallel human disorders as complex as schizophrenia is necessary in understanding the exact nature of the underlying neuronal mechanisms. This research enhances our understanding of the neurobehavioural consequences of interactions between neuregulin 1 and cannabinoid systems that may provide a model for genetic vulnerability to cannabis-induced schizophrenia. These data might ultimately assist in helping to inform why individuals with genetic irregularities in the *NRG1* gene should avoid using cannabis.

Chapter 7

REFERENCES

A

Abercrombie, E. D., Keefe, K. A., DiFrischia, D. S. and Zigmond, M. J., 1989. Differential effect of stress on in vivo dopamine release in striatum, nucleus accumbens, and medial frontal cortex. J Neurochem. 52, 1655-1658.

Abood, M. E. and Martin, B. R., 1992. Neurobiology of marijuana abuse. Trends Pharmacol Sci. 13, 201-206.

Acri, J. B., Morse, D. E., Popke, E. J. and Grunberg, N. E., 1994. Nicotine increases sensory gating measured as inhibition of the acoustic startle reflex in rats. Psychopharmacology (Berl). 114, 369-374.

Albrechet-Souza, L., Oliveira, A. R., De Luca, M. C., Tomazini, F. M., Santos, N. R. and Brandao, M. L., 2005. A comparative study with two types of elevated plus-maze (transparent vs. opaque walls) on the anxiolytic effects of midazolam, one-trial tolerance and fear-induced analgesia. Prog Neuropsychopharmacol Biol Psychiatry. 29, 571-579.

Allebeck, P., Adamsson, C., Engstrom, A. and Rydberg, U., 1993. Cannabis and schizophrenia: a longitudinal study of cases treated in Stockholm County. Acta Psychiatr Scand. 88, 21-24.

Allen, R. M. and Young, S. J., 1978. Phencyclidine-induced psychosis. Am J Psychiatry. 135, 1081-1084.

Andreasson, S., Allebeck, P., Engstrom, A. and Rydberg, U., 1987. Cannabis and schizophrenia. A longitudinal study of Swedish conscripts. Lancet. 2, 1483-1486.

Arnold, J. C., Hunt, G. E. and McGregor, I. S., 2001a. Effects of the cannabinoid receptor agonist CP 55,940 and the cannabinoid receptor antagonist SR 141716 on intracranial self-stimulation in Lewis rats. Life Sci. 70, 97-108.

Arnold, J. C., Topple, A. N., Hunt, G. E. and McGregor, I. S., 1998. Effects of pre-exposure and co-administration of the cannabinoid receptor agonist CP 55,940 on behavioral sensitization to cocaine. Eur J Pharmacol. 354, 9-16.

Arnold, J. C., Topple, A. N., Mallet, P. E., Hunt, G. E. and McGregor, I. S., 2001b. The distribution of cannabinoid-induced Fos expression in rat brain: differences between the Lewis and Wistar strain. Brain Res. 921, 240-255.

Arseneault, L., Cannon, M., Poulton, R., Murray, R., Caspi, A. and Moffitt, T. E., 2002. Cannabis use in adolescence and risk for adult psychosis: longitudinal prospective study. Bmj. 325, 1212-1213.

Arseneault, L., Cannon, M., Witton, J. and Murray, R. M., 2004. Causal association between cannabis and psychosis: examination of the evidence. Br J Psychiatry. 184, 110-117.

B

Bakker, S. C., Hoogendoorn, M. L., Selten, J. P., Verduijn, W., Pearson, P. L., Sinke, R. J. and Kahn, R. S., 2004. Neuregulin 1: genetic support for schizophrenia subtypes. Mol Psychiatry. 9, 1061-1063.

Baldacara, L., Borgio, J. G., de Lacerda, A. L. and Jackowski, A. P., 2008. Cerebellum and psychiatric disorders. Rev Bras Psiquiatr. 30, 281-289.

Balerio, G. N., Aso, E. and Maldonado, R., 2006. Role of the cannabinoid system in the effects induced by nicotine on anxiety-like behaviour in mice. Psychopharmacology (Berl). 184, 504-513.

Bangalore, S. S., Prasad, K. M., Montrose, D. M., Goradia, D. D., Diwadkar, V. A. and Keshavan, M. S., 2008. Cannabis use and brain structural alterations in first episode schizophrenia--a region of interest, voxel based morphometric study. Schizophr Res. 99, 1-6.

Barnes, T. R., Mutsatsa, S. H., Hutton, S. B., Watt, H. C. and Joyce, E. M., 2006. Comorbid substance use and age at onset of schizophrenia. Br J Psychiatry. 188, 237-242.

Bayer, T. A., Falkai, P. and Maier, W., 1999. Genetic and non-genetic vulnerability factors in schizophrenia: the basis of the "two hit hypothesis". J Psychiatr Res. 33, 543-548.

Berghuis, P., Dobszay, M. B., Wang, X., Spano, S., Ledda, F., Sousa, K. M., Schulte, G., Ernfors, P., Mackie, K., Paratcha, G., Hurd, Y. L. and Harkany, T., 2005. Endocannabinoids regulate interneuron migration and morphogenesis by transactivating the TrkB receptor. Proc Natl Acad Sci U S A. 102, 19115-19120.

Bernstein, H. G., Lendeckel, U., Bertram, I., Bukowska, A., Kanakis, D., Dobrowolny, H., Stauch, R., Krell, D., Mawrin, C., Budinger, E., Keilhoff, G. and Bogerts, B., 2006. Localization of neuregulin-1alpha (heregulin-alpha) and one of its receptors, ErbB-4 tyrosine kinase, in developing and adult human brain. Brain Res Bull. 69, 546-559.

Bertoglio, L. J. and Carobrez, A. P., 2002. Anxiolytic effects of ethanol and phenobarbital are abolished in test-experienced rats submitted to the elevated plus maze. Pharmacol Biochem Behav. 73, 963-969.

Berton, O., Covington, H. E., 3rd, Ebner, K., Tsankova, N. M., Carle, T. L., Ulery, P., Bhonsle, A., Barrot, M., Krishnan, V., Singewald, G. M., Singewald, N., Birnbaum, S., Neve, R. L. and Nestler, E. J., 2007. Induction of deltaFosB in the periaqueductal gray by stress promotes active coping responses. Neuron. 55, 289-300.

Bertram, I., Bernstein, H. G., Lendeckel, U., Bukowska, A., Dobrowolny, H., Keilhoff, G., Kanakis, D., Mawrin, C., Bielau, H., Falkai, P. and Bogerts, B., 2007. Immunohistochemical evidence for impaired neuregulin-1 signaling in the prefrontal cortex in schizophrenia and in unipolar depression. Ann N Y Acad Sci. 1096, 147-156.

Bhat, R. V., Cole, A. J. and Baraban, J. M., 1992. Chronic cocaine treatment suppresses basal expression of zif268 in rat forebrain: in situ hybridization studies. J Pharmacol Exp Ther. 263, 343-349.

Black, D. W. and Boffeli, T. J., 1989. Simple schizophrenia: past, present, and future. Am J Psychiatry. 146, 1267-1273.

Boissier, J. R., Simon, P. and Lwoff, J. M., 1964. [Use of a Particular Mouse Reaction (Hole Board Method) for the Study of Psychotropic Drugs.]. Therapie. 19, 571-583.

Boksa, P., 2004. Animal models of obstetric complications in relation to schizophrenia. Brain Res Brain Res Rev. 45, 1-17.

Bortolato, M., Aru, G. N., Frau, R., Orru, M., Luckey, G. C., Boi, G. and Gessa, G. L., 2005. The CB receptor agonist WIN 55,212-2 fails to elicit disruption of prepulse inhibition of the startle in Sprague-Dawley rats. Psychopharmacology (Berl). 177, 264-271.

Bortolato, M., Campolongo, P., Mangieri, R. A., Scattoni, M. L., Frau, R., Trezza, V., La Rana, G., Russo, R., Calignano, A., Gessa, G. L., Cuomo, V. and Piomelli,

D., 2006. Anxiolytic-Like Properties of the Anandamide Transport Inhibitor AM404. Neuropsychopharmacology.

Boucher, A. A., Arnold, J. C., Duffy, L., Schofield, P. R., Micheau, J. and Karl, T., 2007a. Heterozygous neuregulin 1 mice are more sensitive to the behavioural effects of Delta9-tetrahydrocannabinol. Psychopharmacology (Berl). 192, 325-336.

Boucher, A. A., Hunt, G. E., Karl, T., Micheau, J., McGregor, I. S. and Arnold, J. C., 2007b. Heterozygous neuregulin 1 mice display greater baseline and Delta(9)-tetrahydrocannabinol-induced c-Fos expression. Neuroscience. 149, 861-870.

Bouwknecht, J. A., van der Gugten, J., Groenink, L., Olivier, B. and Paylor, R. E., 2004. Effects of repeated testing in two inbred strains on flesinoxan dose-response curves in three mouse models for anxiety. Eur J Pharmacol. 494, 35-44.

Bozon, B., Davis, S. and Laroche, S., 2002. Regulated transcription of the immediate-early gene Zif268: mechanisms and gene dosage-dependent function in synaptic plasticity and memory formation. Hippocampus. 12, 570-577.

Braga, R. J., Mendlowicz, M. V., Marrocos, R. P. and Figueira, I. L., 2005. Anxiety disorders in outpatients with schizophrenia: prevalence and impact on the subjective quality of life. J Psychiatr Res. 39, 409-414.

Bramon, E., Dempster, E., Frangou, S., Shaikh, M., Walshe, M., Filbey, F. M., McDonald, C., Sham, P., Collier, D. A. and Murray, R., 2008. Neuregulin-1 and the P300 waveform-A preliminary association study using a psychosis endophenotype. Schizophr Res. 103, 178-185.

C

Calfa, G., Bussolino, D. and Molina, V. A., 2007. Involvement of the lateral septum and the ventral Hippocampus in the emotional sequelae induced by social defeat: role of glucocorticoid receptors. Behav Brain Res. 181, 23-34.

Campbell, I., 1976. The amotivational syndrome and cannabis use with emphasis on the Canadian scene. Ann N Y Acad Sci. 282, 33-36.

Cardno, A. G. and Gottesman, II, 2000. Twin studies of schizophrenia: from bow-and-arrow concordances to star wars Mx and functional genomics. Am J Med Genet. 97, 12-17.

Carlsson, A., 1988. The current status of the dopamine hypothesis of schizophrenia. Neuropsychopharmacology. 1, 179-186.

Carr, V. J., Lewin, T. J., Neil, A. L., Halpin, S. A. and Holmes, S., 2004. Premorbid, psychosocial and clinical predictors of the costs of schizophrenia and other psychoses. Br J Psychiatry. 184, 517-525.

Caspi, A., Moffitt, T. E., Cannon, M., McClay, J., Murray, R., Harrington, H., Taylor, A., Arseneault, L., Williams, B., Braithwaite, A., Poulton, R. and Craig, I. W., 2005. 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. 57, 1117-1127.

Castner, S. A., Goldman-Rakic, P. S. and Williams, G. V., 2004. Animal models of working memory: insights for targeting cognitive dysfunction in schizophrenia. Psychopharmacology (Berl). 174, 111-125.

Chambers, R. A., Krystal, J. H. and Self, D. W., 2001. A neurobiological basis for substance abuse comorbidity in schizophrenia. Biol Psychiatry. 50, 71-83.

Chaperon, F., Soubrie, P., Puech, A. J. and Thiebot, M. H., 1998. Involvement of central cannabinoid (CB1) receptors in the establishment of place conditioning in rats. Psychopharmacology (Berl). 135, 324-332.

Chaperon, F. and Thiebot, M. H., 1999. Behavioral effects of cannabinoid agents in animals. Crit Rev Neurobiol. 13, 243-281.

Cheeta, S., Kenny, P. J. and File, S. E., 2000. Hippocampal and septal injections of nicotine and 8-OH-DPAT distinguish among different animal tests of anxiety. Prog Neuropsychopharmacol Biol Psychiatry. 24, 1053-1067.

Chen, Y. J., Johnson, M. A., Lieberman, M. D., Goodchild, R. E., Schobel, S., Lewandowski, N., Rosoklija, G., Liu, R. C., Gingrich, J. A., Small, S., Moore, H., Dwork, A. J., Talmage, D. A. and Role, L. W., 2008. Type III neuregulin-1 is required for normal sensorimotor gating, memory-related behaviors, and corticostriatal circuit components. J Neurosci. 28, 6872-6883.

Chong, V. Z., Thompson, M., Beltaifa, S., Webster, M. J., Law, A. J. and Weickert, C. S., 2008. Elevated neuregulin-1 and ErbB4 protein in the prefrontal cortex of schizophrenic patients. Schizophr Res. 100, 270-280.

Clapcote, S. J., Lipina, T. V., Millar, J. K., Mackie, S., Christie, S., Ogawa, F., Lerch, J. P., Trimble, K., Uchiyama, M., Sakuraba, Y., Kaneda, H., Shiroishi, T., Houslay, M. D., Henkelman, R. M., Sled, J. G., Gondo, Y., Porteous, D. J. and Roder, J. C., 2007. Behavioral phenotypes of Disc1 missense mutations in mice. Neuron. 54, 387-402.

Colombo, G., Orru, A., Lai, P., Cabras, C., Maccioni, P., Rubio, M., Gessa, G. L. and Carai, M. A., 2007. The cannabinoid CB1 receptor antagonist, rimonabant, as a promising pharmacotherapy for alcohol dependence: preclinical evidence. Mol Neurobiol. 36, 102-112.

Compton, D. R., Aceto, M. D., Lowe, J. and Martin, B. R., 1996. In vivo characterization of a specific cannabinoid receptor antagonist (SR141716A): inhibition of delta 9-tetrahydrocannabinol-induced responses and apparent agonist activity. J Pharmacol Exp Ther. 277, 586-594.

Corbett, R., Camacho, F., Woods, A. T., Kerman, L. L., Fishkin, R. J., Brooks, K. and Dunn, R. W., 1995. Antipsychotic agents antagonize non-competitive N-methyl-D-aspartate antagonist-induced behaviors. Psychopharmacology (Berl). 120, 67-74.

Corfas, G., Roy, K. and Buxbaum, J. D., 2004. Neuregulin 1-erbB signaling and the molecular/cellular basis of schizophrenia. Nat Neurosci. 7, 575-580.

Corvin, A. P., Morris, D. W., McGhee, K., Schwaiger, S., Scully, P., Quinn, J., Meagher, D., Clair, D. S., Waddington, J. L. and Gill, M., 2004. Confirmation and refinement of an 'at-risk' haplotype for schizophrenia suggests the EST cluster, Hs.97362, as a potential susceptibility gene at the Neuregulin-1 locus. Mol Psychiatry. 9, 208-213.

Costa, B., Parolaro, D. and Colleoni, M., 1996. Chronic cannabinoid, CP-55,940, administration alters biotransformation in the rat. Eur J Pharmacol. 313, 17-24.

Costall, B., Jones, B. J., Kelly, M. E., Naylor, R. J. and Tomkins, D. M., 1989. Exploration of mice in a black and white test box: validation as a model of anxiety. Pharmacol Biochem Behav. 32, 777-785.

Coyle, J. T., 1996. The glutamatergic dysfunction hypothesis for schizophrenia. Harv Rev Psychiatry. 3, 241-253.

Crawley, J. N., 1985. Exploratory behavior models of anxiety in mice. Neurosci Biobehav Rev. 9, 37-44.

Crawley, J. N., 1999. Behavioral phenotyping of transgenic and knockout mice: experimental design and evaluation of general health, sensory functions, motor abilities, and specific behavioral tests. Brain Res. 835, 18-26.

Cruz-Morales, S. E., Santos, N. R. and Brandao, M. L., 2002. One-trial tolerance to midazolam is due to enhancement of fear and reduction of anxiolytic-sensitive behaviors in the elevated plus-maze retest in the rat. Pharmacol Biochem Behav. 72, 973-978.

D

D'Souza, D. C., 2007. Cannabinoids and psychosis. Int Rev Neurobiol. 78, 289-326.

D'Souza, D. C., Abi-Saab, W. M., Madonick, S., Forselius-Bielen, K., Doersch, A., Braley, G., Gueorguieva, R., Cooper, T. B. and Krystal, J. H., 2005. Delta-9-tetrahydrocannabinol effects in schizophrenia: implications for cognition, psychosis, and addiction. Biol Psychiatry. 57, 594-608.

D'Souza, D. C., Perry, E., MacDougall, L., Ammerman, Y., Cooper, T., Wu, Y. T., Braley, G., Gueorguieva, R. and Krystal, J. H., 2004. The psychotomimetic effects of intravenous delta-9-tetrahydrocannabinol in healthy individuals: implications for psychosis. Neuropsychopharmacology. 29, 1558-1572.

Da, S. and Takahashi, R. N., 2002. SR 141716A prevents delta 9-tetrahydrocannabinol-induced spatial learning deficit in a Morris-type water maze in mice. Prog Neuropsychopharmacol Biol Psychiatry. 26, 321-325.

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

de Miguel, R., Romero, J., Munoz, R. M., Garcia-Gil, L., Gonzalez, S., Villanua, M. A., Makriyannis, A., Ramos, J. A. and Fernandez-Ruiz, J. J., 1998. Effects of cannabinoids on prolactin and gonadotrophin secretion: involvement of changes in hypothalamic gamma-aminobutyric acid (GABA) inputs. Biochem Pharmacol. 56, 1331-1338.

Dean, B., Karl, T., Pavey, G., Boer, S., Duffy, L. and Scarr, E., 2008. Increased levels of serotonin 2A receptors and serotonin transporter in the CNS of neuregulin 1 hypomorphic/mutant mice. Schizophr Res. 99, 341-349.

Dean, B., Sundram, S., Bradbury, R., Scarr, E. and Copolov, D., 2001. 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. 103, 9-15.

DeFries, J. C., Hegmann, J. P. and Weir, M. W., 1966. Open-field behavior in mice: evidence for a major gene effect mediated by the visual system. Science. 154, 1577-1579.

Degenhardt, L. and Hall, W., 2002. Cannabis and psychosis. Curr Psychiatry Rep. 4, 191-196.

Degenhardt, L., Hall, W. and Lynskey, M., 2001. Alcohol, cannabis and tobacco use among Australians: a comparison of their associations with other drug use and use disorders, affective and anxiety disorders, and psychosis. Addiction. 96, 1603-1614.

Degenhardt, L., Hall, W. and Lynskey, M., 2003. Testing hypotheses about the relationship between cannabis use and psychosis. Drug Alcohol Depend. 71, 37-48.

Dejaegere, T., Serneels, L., Schafer, M. K., Van Biervliet, J., Horre, K., Depboylu, C., Alvarez-Fischer, D., Herreman, A., Willem, M., Haass, C., Hoglinger, G. U., D'Hooge, R. and De Strooper, B., 2008. Deficiency of Aph1B/C-gamma-secretase disturbs Nrg1 cleavage and sensorimotor gating that can be reversed with antipsychotic treatment. Proc Natl Acad Sci U S A. 105, 9775-9780.

Denenberg, V. H., 1969. Open-field behavior in the rat: what does it mean? Ann N Y Acad Sci. 159, 852-859.

Derkinderen, P., Valjent, E., Toutant, M., Corvol, J. C., Enslen, H., Ledent, C., Trzaskos, J., Caboche, J. and Girault, J. A., 2003. Regulation of extracellular signal-regulated kinase by cannabinoids in hippocampus. J Neurosci. 23, 2371-2382.

Devane, W. A., Dysarz, F. A., 3rd, Johnson, M. R., Melvin, L. S. and Howlett, A. C., 1988. Determination and characterization of a cannabinoid receptor in rat brain. Mol Pharmacol. 34, 605-613.

Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A. and Mechoulam, R., 1992. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science. 258, 1946-1949.

Dewey, W. L., 1986. Cannabinoid pharmacology. Pharmacol Rev. 38, 151-178.

Di Marzo, V., Breivogel, C. S., Tao, Q., Bridgen, D. T., Razdan, R. K., Zimmer, A. M., Zimmer, A. and Martin, B. R., 2000. Levels, metabolism, and pharmacological activity of anandamide in CB(1) cannabinoid receptor knockout mice: evidence for non-CB(1), non-CB(2) receptor-mediated actions of anandamide in mouse brain. J Neurochem. 75, 2434-2444.

Di Marzo, V. and Petrocellis, L. D., 2006. Plant, synthetic, and endogenous cannabinoids in medicine. Annu Rev Med. 57, 553-574.

Dielenberg, R. A., Hunt, G. E. and McGregor, I. S., 2001. "When a rat smells a cat": the distribution of Fos immunoreactivity in rat brain following exposure to a predatory odor. Neuroscience. 104, 1085-1097.

Dixon, A. K., Huber, C. and Lowe, D. A., 1994. Clozapine promotes approach-oriented behavior in male mice. J Clin Psychiatry. 55, 4-7.

Dong, H. W., Petrovich, G. D. and Swanson, L. W., 2001. Topography of projections from amygdala to bed nuclei of the stria terminalis. Brain Res Brain Res Rev. 38, 192-246.

Downer, E., Boland, B., Fogarty, M. and Campbell, V., 2001. Delta 9-tetrahydrocannabinol induces the apoptotic pathway in cultured cortical neurones via activation of the CB1 receptor. Neuroreport. 12, 3973-3978.

DSM-IV, Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition. American Psychiatry Association.

Duan, J., Martinez, M., Sanders, A. R., Hou, C., Krasner, A. J., Schwartz, D. B. and Gejman, P. V., 2005. Neuregulin 1 (NRG1) and schizophrenia: analysis of a US family sample and the evidence in the balance. Psychol Med. 35, 1599-1610.

Duffy, L., Cappas, E., Scimone, A., Schofield, P. R. and Karl, T., 2008. Behavioral profile of a heterozygous mutant mouse model for EGF-like domain neuregulin 1. Behav Neurosci. 122, 748-759.

Dulawa, S. C. and Geyer, M. A., 1996. Psychopharmacology of prepulse inhibition in mice. Chin J Physiol. 39, 139-146.

Duncan, G. E., Sheitman, B. B. and Lieberman, J. A., 1999. An integrated view of pathophysiological models of schizophrenia. Brain Res Brain Res Rev. 29, 250-264.

E

Egan, M. F. and Weinberger, D. R., 1997. Neurobiology of schizophrenia. Curr Opin Neurobiol. 7, 701-707.

Egashira, N., Mishima, K., Iwasaki, K. and Fujiwara, M., 2002. Intracerebral microinjections of delta 9-tetrahydrocannabinol: search for the impairment of spatial memory in the eight-arm radial maze in rats. Brain Res. 952, 239-245.

Egerton, A., Brett, R. R. and Pratt, J. A., 2005. Acute delta9-tetrahydrocannabinol-induced deficits in reversal learning: neural correlates of affective inflexibility. Neuropsychopharmacology. 30, 1895-1905.

Ellenbroek, B. A. and Cools, A. R., 2000. Animal models for the negative symptoms of schizophrenia. Behav Pharmacol. 11, 223-233.

Ellison, G. D. and Eison, M. S., 1983. Continuous amphetamine intoxication: an animal model of the acute psychotic episode. Psychol Med. 13, 751-761.

Elvevag, B. and Goldberg, T. E., 2000. Cognitive impairment in schizophrenia is the core of the disorder. Crit Rev Neurobiol. 14, 1-21.

Emrich, H. M., Leweke, F. M. and Schneider, U., 1997. Towards a cannabinoid hypothesis of schizophrenia: cognitive impairments due to dysregulation of the endogenous cannabinoid system. Pharmacol Biochem Behav. 56, 803-807.

Espejo, E. F., 1997. Effects of weekly or daily exposure to the elevated plus-maze in male mice. Behav Brain Res. 87, 233-238.

F

Fadda, P., Robinson, L., Fratta, W., Pertwee, R. G. and Riedel, G., 2004. Differential effects of THC- or CBD-rich cannabis extracts on working memory in rats. Neuropharmacology. 47, 1170-1179.

Falls, D. L., 2003. Neuregulins: functions, forms, and signaling strategies. Exp Cell Res. 284, 14-30.

Faraone, S. V., Taylor, L. and Tsuang, M. T., 2002. The molecular genetics of schizophrenia: an emerging consensus. Expert Rev Mol Med. 4, 1-13.

Farber, N. B., Wozniak, D. F., Price, M. T., Labruyere, J., Huss, J., St Peter, H. and Olney, J. W., 1995. Age-specific neurotoxicity in the rat associated with NMDA receptor blockade: potential relevance to schizophrenia? Biol Psychiatry. 38, 788-796.

Favrat, B., Menetrey, A., Augsburger, M., Rothuizen, L. E., Appenzeller, M., Buclin, T., Pin, M., Mangin, P. and Giroud, C., 2005. Two cases of "cannabis acute psychosis" following the administration of oral cannabis. BMC Psychiatry. 5, 17.

Feng, Y., 2008. Convergence and Divergence in the Etiology of Myelin Impairment in Psychiatric Disorders and Drug Addiction. Neurochem Res.

Fernandez-Espejo, E. and Galan-Rodriguez, B., 2004. Sensorimotor gating in mice is disrupted after AM404, an anandamide reuptake and degradation inhibitor. Psychopharmacology (Berl). 175, 220-224.

Ferrari, F., Ottani, A. and Giuliani, D., 1999. Influence of the cannabinoid agonist HU 210 on cocaine- and CQP 201-403-induced behavioural effects in rat. Life Sci. 65, 823-831.

File, S. E., 1988. How good is social interaction as a test of anxiety? In: Simon, P. et al. (Eds.), Animal models of psychiatric disorders. Selected models of anxiety, depression and psychosis. Karger, Basel, München, Paris, London, New York,New Delhi, Singapore, Tokyo, and Sydney, pp. 151-166.

File, S. E., 1990. One-trial tolerance to the anxiolytic effects of chlordiazepoxide in the plus-maze. Psychopharmacology (Berl). 100, 281-282.

File, S. E. and Hyde, J. R., 1978. Can social interaction be used to measure anxiety? Br J Pharmacol. 62, 19-24.

File, S. E., Mabbutt, P. S. and Hitchcott, P. K., 1990. Characterisation of the phenomenon of "one-trial tolerance" to the anxiolytic effect of chlordiazepoxide in the elevated plus-maze. Psychopharmacology (Berl). 102, 98-101.

Fletcher, J. M., Page, J. B., Francis, D. J., Copeland, K., Naus, M. J., Davis, C. M., Morris, R., Krauskopf, D. and Satz, P., 1996. Cognitive correlates of long-term cannabis use in Costa Rican men. Arch Gen Psychiatry. 53, 1051-1057.

Flynn, S. W., Lang, D. J., Mackay, A. L., Goghari, V., Vavasour, I. M., Whittall, K. P., Smith, G. N., Arango, V., Mann, J. J., Dwork, A. J., Falkai, P. and Honer, W. G., 2003. Abnormalities of myelination in schizophrenia detected in vivo with MRI, and post-mortem with analysis of oligodendrocyte proteins. Mol Psychiatry. 8, 811-820.

Fride, E., Foox, A., Rosenberg, E., Faigenboim, M., Cohen, V., Barda, L., Blau, H. and Mechoulam, R., 2003. Milk intake and survival in newborn cannabinoid CB1 receptor knockout mice: evidence for a "CB3" receptor. Eur J Pharmacol. 461, 27-34.

Frischknecht, H. R., Sieber, B. and Waser, P. G., 1982. Effects of multiple, chronic and early hashish exposure on mating behavior, nest-building and gestation in mice. Comp Biochem Physiol C. 72, 363-368.

Fukui, N., Muratake, T., Kaneko, N., Amagane, H. and Someya, T., 2006. Supportive evidence for neuregulin 1 as a susceptibility gene for schizophrenia in a Japanese population. Neurosci Lett. 396, 117-120.

G

Gainetdinov, R. R., Mohn, A. R. and Caron, M. G., 2001. Genetic animal models: focus on schizophrenia. Trends Neurosci. 24, 527-533.

Genn, R. F., Tucci, S., Marco, E. M., Viveros, M. P. and File, S. E., 2004. Unconditioned and conditioned anxiogenic effects of the cannabinoid receptor agonist CP 55,940 in the social interaction test. Pharmacol Biochem Behav. 77, 567-573.

Georgieva, L., Dimitrova, A., Ivanov, D., Nikolov, I., Williams, N. M., Grozeva, D., Zaharieva, I., Toncheva, D., Owen, M. J., Kirov, G. and O'Donovan, M. C., 2008. Support for Neuregulin 1 as a Susceptibility Gene for Bipolar Disorder and Schizophrenia. Biol Psychiatry.

Gerlach, J., 1991. New antipsychotics: classification, efficacy, and adverse effects. Schizophr Bull. 17, 289-309.

Gerlai, R., Pisacane, P. and Erickson, S., 2000. Heregulin, but not ErbB2 or ErbB3, heterozygous mutant mice exhibit hyperactivity in multiple behavioral tasks. Behav Brain Res. 109, 219-227.

Geyer, M. A., 2008. Developing translational animal models for symptoms of schizophrenia or bipolar mania. Neurotox Res. 14, 71-78.

Geyer, M. A., Krebs-Thomson, K., Braff, D. L. and Swerdlow, N. R., 2001. Pharmacological studies of prepulse inhibition models of sensorimotor gating deficits in schizophrenia: a decade in review. Psychopharmacology (Berl). 156, 117-154.

Geyer, M. A. and Swerdlow, N. R., 1998. Measurement of startle response, prepulse inhibition, and habituation. In: Crawley, J. N. and Skolnick, P. (Eds.), Current protocols in neuroscience, New York, pp. 8.7.1-8.7.15.

Giuffrida, A., Leweke, F. M., Gerth, C. W., Schreiber, D., Koethe, D., Faulhaber, J., Klosterkotter, J. and Piomelli, D., 2004. Cerebrospinal anandamide levels are elevated in acute schizophrenia and are inversely correlated with psychotic symptoms. Neuropsychopharmacology. 29, 2108-2114.

Giuffrida, A., Parsons, L. H., Kerr, T. M., Rodriguez de Fonseca, F., Navarro, M. and Piomelli, D., 1999. Dopamine activation of endogenous cannabinoid signaling in dorsal striatum. Nat Neurosci. 2, 358-363.

Glass, M. and Dragunow, M., 1995. Induction of the Krox 24 transcription factor in striosomes by a cannabinoid agonist. Neuroreport. 6, 241-244.

Gleason, S. D. and Shannon, H. E., 1997. Blockade of phencyclidine-induced hyperlocomotion by olanzapine, clozapine and serotonin receptor subtype selective antagonists in mice. Psychopharmacology (Berl). 129, 79-84.

Gogos, J. A., Santha, M., Takacs, Z., Beck, K. D., Luine, V., Lucas, L. R., Nadler, J. V. and Karayiorgou, M., 1999. The gene encoding proline dehydrogenase modulates sensorimotor gating in mice. Nat Genet. 21, 434-439.

Golub, M. S., Germann, S. L. and Lloyd, K. C., 2004. Behavioral characteristics of a nervous system-specific erbB4 knock-out mouse. Behav Brain Res. 153, 159-170.

Gonzalez-Maeso, J., Weisstaub, N. V., Zhou, M., Chan, P., Ivic, L., Ang, R., Lira, A., Bradley-Moore, M., Ge, Y., Zhou, Q., Sealfon, S. C. and Gingrich, J. A., 2007. Hallucinogens recruit specific cortical 5-HT(2A) receptor-mediated signaling pathways to affect behavior. Neuron. 53, 439-452.

Gonzalez-Pinto, A., Vega, P., Ibanez, B., Mosquera, F., Barbeito, S., Gutierrez, M., Ruiz de Azua, S., Ruiz, I. and Vieta, E., 2008. Impact of Cannabis and Other Drugs on Age at Onset of Psychosis. J Clin Psychiatry, e1-e7.

Gonzalez, S., Cebeira, M. and Fernandez-Ruiz, J., 2005. Cannabinoid tolerance and dependence: a review of studies in laboratory animals. Pharmacol Biochem Behav. 81, 300-318.

Gottesman, II and Gould, T. D., 2003. The endophenotype concept in psychiatry: etymology and strategic intentions. Am J Psychiatry. 160, 636-645.

Graham, F. K., 1975. Presidential Address, 1974. The more or less startling effects of weak prestimulation. Psychophysiology. 12, 238-248.

Gray, J. A. and Roth, B. L., 2007. Molecular targets for treating cognitive dysfunction in schizophrenia. Schizophr Bull. 33, 1100-1119.

Gray, N. S., Pilowsky, L. S., Gray, J. A. and Kerwin, R. W., 1995. Latent inhibition in drug naive schizophrenics: relationship to duration of illness and dopamine D2 binding using SPET. Schizophr Res. 17, 95-107.

Grech, A., Van Os, J., Jones, P. B., Lewis, S. W. and Murray, R. M., 2005. Cannabis use and outcome of recent onset psychosis. Eur Psychiatry. 20, 349-353.

Gregg, J. M., Small, E. W., Moore, R., Raft, D. and Toomey, T. C., 1976. Emotional response to intravenous delta9tetrahydrocannabinol during oral surgery. J Oral Surg. 34, 301-313.

Gruber, O., Falkai, P., Schneider-Axmann, T., Schwab, S. G., Wagner, M. and Maier, W., 2008. Neuregulin-1 haplotype HAP(ICE) is associated with lower hippocampal volumes in schizophrenic patients and in non-affected family members. J Psychiatr Res.

Gu, Z., Jiang, Q., Fu, A. K., Ip, N. Y. and Yan, Z., 2005. Regulation of NMDA receptors by neuregulin signaling in prefrontal cortex. J Neurosci. 25, 4974-4984.

H

Hahn, C. G., Wang, H. Y., Cho, D. S., Talbot, K., Gur, R. E., Berrettini, W. H., Bakshi, K., Kamins, J., Borgmann-Winter, K. E., Siegel, S. J., Gallop, R. J. and Arnold, S. E., 2006. Altered neuregulin 1-erbB4 signaling contributes to NMDA receptor hypofunction in schizophrenia. Nat Med. 12, 824-828.

Hakak, Y., Walker, J. R., Li, C., Wong, W. H., Davis, K. L., Buxbaum, J. D., Haroutunian, V. and Fienberg, A. A., 2001. Genome-wide expression analysis reveals dysregulation of myelination-related genes in chronic schizophrenia. Proc Natl Acad Sci U S A. 98, 4746-4751.

Hall, D., Gogos, J. A. and Karayiorgou, M., 2004a. The contribution of three strong candidate schizophrenia susceptibility genes in demographically distinct populations. Genes Brain Behav. 3, 240-248.

Hall, J., Whalley, H. C., Job, D. E., Baig, B. J., McIntosh, A. M., Evans, K. L., Thomson, P. A., Porteous, D. J., Cunningham-Owens, D. G., Johnstone, E. C. and Lawrie, S. M., 2006. A neuregulin 1 variant associated with abnormal cortical function and psychotic symptoms. Nat Neurosci. 9, 1477-1478.

Hall, W., 1998. Cannabis use and psychosis. Drug Alcohol Rev. 17, 433-444.

Hall, W. and Degenhardt, L., 2000. Cannabis use and psychosis: a review of clinical and epidemiological evidence. Aust N Z J Psychiatry. 34, 26-34.

Hall, W., Degenhardt, L. and Teesson, M., 2004b. Cannabis use and psychotic disorders: an update. Drug Alcohol Rev. 23, 433-443.

Hall, W. and Solowij, N., 1998. Adverse effects of cannabis. Lancet. 352, 1611-1616.

Hambrecht, M. and Hafner, H., 2000. Cannabis, vulnerability, and the onset of schizophrenia: an epidemiological perspective. Aust N Z J Psychiatry. 34, 468-475.

Hampson, R. E. and Deadwyler, S. A., 2000. Cannabinoids reveal the necessity of hippocampal neural encoding for short-term memory in rats. J Neurosci. 20, 8932-8942.

Hampson, R. E., Simeral, J. D., Kelly, E. J. and Deadwyler, S. A., 2003. Tolerance to the memory disruptive effects of cannabinoids involves adaptation by hippocampal neurons. Hippocampus. 13, 543-556.

Haney, M., Ward, A. S., Comer, S. D., Foltin, R. W. and Fischman, M. W., 1999. Abstinence symptoms following oral THC administration to humans. Psychopharmacology (Berl). 141, 385-394.

Hart, S., Fischer, O. M. and Ullrich, A., 2004. Cannabinoids induce cancer cell proliferation via tumor necrosis factor alpha-converting enzyme

(TACE/ADAM17)-mediated transactivation of the epidermal growth factor receptor. Cancer Res. 64, 1943-1950.

Hashimoto, R., Straub, R. E., Weickert, C. S., Hyde, T. M., Kleinman, J. E. and Weinberger, D. R., 2004. Expression analysis of neuregulin-1 in the dorsolateral prefrontal cortex in schizophrenia. Mol Psychiatry. 9, 299-307.

He, J. C., Gomes, I., Nguyen, T., Jayaram, G., Ram, P. T., Devi, L. A. and Iyengar, R., 2005. The G alpha(o/i)-coupled cannabinoid receptor-mediated neurite outgrowth involves Rap regulation of Src and Stat3. J Biol Chem. 280, 33426-33434.

Henquet, C., Di Forti, M., Morrison, P., Kuepper, R. and Murray, R. M., 2008. Gene-Environment Interplay Between Cannabis and Psychosis. Schizophr Bull.

Henquet, C., Murray, R., Linszen, D. and van Os, J., 2005. The environment and schizophrenia: the role of cannabis use. Schizophr Bull. 31, 608-612.

Henry, B., Vale, W. and Markou, A., 2006. The effect of lateral septum corticotropin-releasing factor receptor 2 activation on anxiety is modulated by stress. J Neurosci. 26, 9142-9152.

Herkenham, M., Lynn, A. B., Little, M. D., Johnson, M. R., Melvin, L. S., de Costa, B. R. and Rice, K. C., 1990. Cannabinoid receptor localization in brain. Proc Natl Acad Sci U S A. 87, 1932-1936.

Hides, L., Dawe, S., Kavanagh, D. J. and Young, R. M., 2006. Psychotic symptom and cannabis relapse in recent-onset psychosis. Prospective study. Br J Psychiatry. 189, 137-143.

Hikida, T., Jaaro-Peled, H., Seshadri, S., Oishi, K., Hookway, C., Kong, S., Wu, D., Xue, R., Andrade, M., Tankou, S., Mori, S., Gallagher, M., Ishizuka, K., Pletnikov, M., Kida, S. and Sawa, A., 2007. Dominant-negative DISC1 transgenic mice display schizophrenia-associated phenotypes detected by measures translatable to humans. Proc Natl Acad Sci U S A. 104, 14501-14506.

Hill, M. N., Froese, L. M., Morrish, A. C., Sun, J. C. and Floresco, S. B., 2006. Alterations in behavioral flexibility by cannabinoid CB1 receptor agonists and antagonists. Psychopharmacology (Berl). 187, 245-259.

Hintsanen, M., Elovainio, M., Puttonen, S., Kivimaki, M., Raitakari, O. T., Lehtimaki, T., Rontu, R., Juonala, M., Kahonen, M., Viikari, J. and Keltikangas-Jarvinen, L., 2007. Neuregulin-1 genotype moderates the association between job strain and early atherosclerosis in young men. Ann Behav Med. 33, 148-155.

Hoffman, H. S. and Searle, J. L., 1968. Acoustic and temporal factors in the evocation of startle. J Acoust Soc Am. 43, 269-282.

Hogg, S., 1996. A review of the validity and variability of the elevated plus-maze as an animal model of anxiety. Pharmacol Biochem Behav. 54, 21-30.

Holmes, A. and Rodgers, R. J., 1999. Influence of spatial and temporal manipulations on the anxiolytic efficacy of chlordiazepoxide in mice previously exposed to the elevated plus-maze. Neurosci Biobehav Rev. 23, 971-980.

Hong, C. J., Huo, S. J., Liao, D. L., Lee, K., Wu, J. Y. and Tsai, S. J., 2004. Case-control and family-based association studies between the neuregulin 1 (Arg38Gln) polymorphism and schizophrenia. Neurosci Lett. 366, 158-161.

Hong, L. E., Wonodi, I., Stine, O. C., Mitchell, B. D. and Thaker, G. K., 2008. Evidence of missense mutations on the neuregulin 1 gene affecting function of prepulse inhibition. Biol Psychiatry. 63, 17-23.

Hosking, R. D. and Zajicek, J. P., 2008. Therapeutic potential of cannabis in pain medicine. Br J Anaesth. 101, 59-68.

Howlett, A. C., Breivogel, C. S., Childers, S. R., Deadwyler, S. A., Hampson, R. E. and Porrino, L. J., 2004. Cannabinoid physiology and pharmacology: 30 years of progress. Neuropharmacology. 47 Suppl 1, 345-358.

Huang, M., Li, Z., Dai, J., Shahid, M., Wong, E. H. and Meltzer, H. Y., 2008. Asenapine Increases Dopamine, Norepinephrine, and Acetylcholine Efflux in the Rat Medial Prefrontal Cortex and Hippocampus. Neuropsychopharmacology.

I

Ingason, A., Soeby, K., Timm, S., Wang, A. G., Jakobsen, K. D., Fink-Jensen, A., Hemmingsen, R., Berg Rasmussen, H. and Werge, T., 2006. No significant association of the 5' end of neuregulin 1 and schizophrenia in a large Danish sample. Schizophr Res. 83, 1-5.

Ishiwata, T., Saito, T., Hasegawa, H., Yazawa, T., Kotani, Y., Otokawa, M. and Aihara, Y., 2005. Changes of body temperature and thermoregulatory responses of freely moving rats during GABAergic pharmacological stimulation to the preoptic area and anterior hypothalamus in several ambient temperatures. Brain Res. 1048, 32-40.

Isoldi, K. K. and Aronne, L. J., 2008. The challenge of treating obesity: the endocannabinoid system as a potential target. J Am Diet Assoc. 108, 823-831.

Iversen, L., 2003. Cannabis and the brain. Brain. 126, 1252-1270.

Iwata, N., Suzuki, T., Ikeda, M., Kitajima, T., Yamanouchi, Y., Inada, T. and Ozaki, N., 2004. No association with the neuregulin 1 haplotype to Japanese schizophrenia. Mol Psychiatry. 9, 126-127.

J

Jablensky, A., 2000. Epidemiology of schizophrenia: the global burden of disease and disability. Eur Arch Psychiatry Clin Neurosci. 250, 274-285.

Javitt, D. C., 2007. Glutamate and Schizophrenia: Phencyclidine, N-Methyl-d-Aspartate Receptors, and Dopamine-Glutamate Interactions. Int Rev Neurobiol. 78, 69-108.

Jentsch, J. D., Verrico, C. D., Le, D. and Roth, R. H., 1998. Repeated exposure to delta 9-tetrahydrocannabinol reduces prefrontal cortical dopamine metabolism in the rat. Neurosci Lett. 246, 169-172.

Johns, A., 2001. Psychiatric effects of cannabis. Br J Psychiatry. 178, 116-122.

Jones, D., 2008. End of the line for cannabinoid receptor 1 as an anti-obesity target? Nat Rev Drug Discov. 7, 961-962.

K

Kadenbach, B., Huttemann, M., Arnold, S., Lee, I. and Bender, E., 2000. Mitochondrial energy metabolism is regulated via nuclear-coded subunits of cytochrome c oxidase. Free Radic Biol Med. 29, 211-221.

Kampman, O., Anttila, S., Illi, A., Saarela, M., Rontu, R., Mattila, K. M., Leinonen, E. and Lehtimaki, T., 2004. Neuregulin genotype and medication response in Finnish patients with schizophrenia. Neuroreport. 15, 2517-2520.

Kanayama, G., Rogowska, J., Pope, H. G., Gruber, S. A. and Yurgelun-Todd, D. A., 2004. Spatial working memory in heavy cannabis users: a functional magnetic resonance imaging study. Psychopharmacology (Berl). 176, 239-247.

Karl, T., Duffy, L., Scimone, A., Harvey, R. P. and Schofield, P. R., 2007. Altered motor activity, exploration and anxiety in heterozygous neuregulin 1 mutant mice: implications for understanding schizophrenia. Genes Brain Behav. 6, 677-687.

Karl, T., Pabst, R. and von Horsten, S., 2003. Behavioral phenotyping of mice in pharmacological and toxicological research. Exp Toxicol Pathol. 55, 69-83.

Kask, A., Nguyen, H. P., Pabst, R. and von Horsten, S., 2001. Factors influencing behavior of group-housed male rats in the social interaction test: focus on cohort removal. Physiol Behav. 74, 277-282.

Kendler, K. S., MacLean, C. J., O'Neill, F. A., Burke, J., Murphy, B., Duke, F., Shinkwin, R., Easter, S. M., Webb, B. T., Zhang, J., Walsh, D. and Straub, R. E., 1996. Evidence for a schizophrenia vulnerability locus on chromosome 8p in the Irish Study of High-Density Schizophrenia Families. Am J Psychiatry. 153, 1534-1540.

Kerber, G., Streif, R., Schwaiger, F. W., Kreutzberg, G. W. and Hager, G., 2003. Neuregulin-1 isoforms are differentially expressed in the intact and regenerating adult rat nervous system. J Mol Neurosci. 21, 149-165.

Keri, S., Kiss, I., Seres, I. and Kelemen, O., 2008. A polymorphism of the neuregulin 1 gene (SNP8NRG243177/rs6994992) affects reactivity to expressed emotion in schizophrenia. Am J Med Genet B Neuropsychiatr Genet.

Khantzian, E. J., 1997. The self-medication hypothesis of substance use disorders: a reconsideration and recent applications. Harv Rev Psychiatry. 4, 231-244.

Kim, J. S., Kornhuber, H. H., Schmid-Burgk, W. and Holzmuller, B., 1980. Low cerebrospinal fluid glutamate in schizophrenic patients and a new hypothesis on schizophrenia. Neurosci Lett. 20, 379-382.

Kim, J. W., Lee, Y. S., Cho, E. Y., Jang, Y. L., Park, D. Y., Choi, K. S., Jeun, H. O., Cho, S. H., Jang, S. Y. and Hong, K. S., 2006. Linkage and association of schizophrenia with genetic variations in the locus of neuregulin 1 in Korean population. Am J Med Genet B Neuropsychiatr Genet. 141B, 281-286.

Koch, M., 1996. The septohippocampal system is involved in prepulse inhibition of the acoustic startle response in rats. Behav Neurosci. 110, 468-477.

Koike, H., Arguello, P. A., Kvajo, M., Karayiorgou, M. and Gogos, J. A., 2006. Disc1 is mutated in the 129S6/SvEv strain and modulates working memory in mice. Proc Natl Acad Sci U S A. 103, 3693-3697.

Konrad, A., Vucurevic, G., Musso, F., Stoeter, P., Dahmen, N. and Winterer, G., 2008. ErbB4 Genotype Predicts Left Frontotemporal Structural Connectivity in Human Brain. Neuropsychopharmacology.

Koolhaas, J. M., Everts, H., de Ruiter, A. J., de Boer, S. F. and Bohus, B., 1998. Coping with stress in rats and mice: differential peptidergic modulation of the amygdala-lateral septum complex. Prog Brain Res. 119, 437-448.

Krug, A., Markov, V., Eggermann, T., Krach, S., Zerres, K., Stocker, T., Shah, N. J., Schneider, F., Nothen, M. M., Treutlein, J., Rietschel, M. and Kircher, T., 2008. Genetic variation in the schizophrenia-risk gene neuregulin1 correlates with differences in frontal brain activation in a working memory task in healthy individuals. Neuroimage.

Kulkarni, J., de Castella, A., Fitzgerald, P. B., Gurvich, C. T., Bailey, M., Bartholomeusz, C. and Burger, H., 2008. Estrogen in severe mental illness: a potential new treatment approach. Arch Gen Psychiatry. 65, 955-960.

Kumari, V., Soni, W. and Sharma, T., 2001. Influence of cigarette smoking on prepulse inhibition of the acoustic startle response in schizophrenia. Hum Psychopharmacol. 16, 321-326.

L

Lachman, H. M., Pedrosa, E., Nolan, K. A., Glass, M., Ye, K. and Saito, T., 2006. Analysis of polymorphisms in AT-rich domains of neuregulin 1 gene in schizophrenia. Am J Med Genet B Neuropsychiatr Genet. 141B, 102-109.

Lamers, C. T., Bechara, A., Rizzo, M. and Ramaekers, J. G., 2006. Cognitive function and mood in MDMA/THC users, THC users and non-drug using controls. J Psychopharmacol. 20, 302-311.

Laroche, S., Davis, S. and Jay, T. M., 2000. Plasticity at hippocampal to prefrontal cortex synapses: dual roles in working memory and consolidation. Hippocampus. 10, 438-446.

Law, A. J., Kleinman, J. E., Weinberger, D. R. and Weickert, C. S., 2007. Disease-associated intronic variants in the ErbB4 gene are related to altered ErbB4 splice-variant expression in the brain in schizophrenia. Hum Mol Genet. 16, 129-141.

Law, A. J., Lipska, B. K., Weickert, C. S., Hyde, T. M., Straub, R. E., Hashimoto, R., Harrison, P. J., Kleinman, J. E. and Weinberger, D. R., 2006. Neuregulin 1 transcripts are differentially expressed in schizophrenia and regulated by 5' SNPs associated with the disease. Proc Natl Acad Sci U S A. 103, 6747-6752.

Law, A. J., Shannon Weickert, C., Hyde, T. M., Kleinman, J. E. and Harrison, P. J., 2004. Neuregulin-1 (NRG-1) mRNA and protein in the adult human brain. Neuroscience. 127, 125-136.

Le Foll, B., Forget, B., Aubin, H. J. and Goldberg, S. R., 2008. Blocking cannabinoid CB1 receptors for the treatment of nicotine dependence: insights from pre-clinical and clinical studies. Addict Biol. 13, 239-252.

Le Pen, G., Gaudet, L., Mortas, P., Mory, R. and Moreau, J. L., 2002. Deficits in reward sensitivity in a neurodevelopmental rat model of schizophrenia. Psychopharmacology (Berl). 161, 434-441.

Lee, C. and Rodgers, R. J., 1990. Antinociceptive effects of elevated plus-maze exposure: influence of opiate receptor manipulations. Psychopharmacology (Berl). 102, 507-513.

Lee, P. R., Brady, D. L., Shapiro, R. A., Dorsa, D. M. and Koenig, J. I., 2007. Prenatal stress generates deficits in rat social behavior: Reversal by oxytocin. Brain Res. 1156, 152-167.

Leng, A., Yee, B. K., Feldon, J. and Ferger, B., 2004. Acoustic startle response, prepulse inhibition, and spontaneous locomotor activity in MPTP-treated mice. Behav Brain Res. 154, 449-456.

Leroy, S., Griffon, N., Bourdel, M. C., Olie, J. P., Poirier, M. F. and Krebs, M. O., 2001. Schizophrenia and the cannabinoid receptor type 1 (CB1): association study using a single-base polymorphism in coding exon 1. Am J Med Genet. 105, 749-752.

Levinson, D. F., 1991. Pharmacologic treatment of schizophrenia. Clin Ther. 13, 326-352.

Leweke, F. M., Gerth, C. W. and Klosterkotter, J., 2004. Cannabis-associated psychosis: current status of research. CNS Drugs. 18, 895-910.

Leweke, F. M., Giuffrida, A., Koethe, D., Schreiber, D., Nolden, B. M., Kranaster, L., Neatby, M. A., Schneider, M., Gerth, C. W., Hellmich, M., Klosterkotter, J. and Piomelli, D., 2007. Anandamide levels in cerebrospinal fluid of first-episode schizophrenic patients: impact of cannabis use. Schizophr Res. 94, 29-36.

Leweke, F. M., Giuffrida, A., Wurster, U., Emrich, H. M. and Piomelli, D., 1999. Elevated endogenous cannabinoids in schizophrenia. Neuroreport. 10, 1665-1669.

Li, T., Stefansson, H., Gudfinnsson, E., Cai, G., Liu, X., Murray, R. M., Steinthorsdottir, V., Januel, D., Gudnadottir, V. G., Petursson, H., Ingason, A., Gulcher, J. R., Stefansson, K. and Collier, D. A., 2004. Identification of a novel neuregulin 1 at-risk haplotype in Han schizophrenia Chinese patients, but no association with the Icelandic/Scottish risk haplotype. Mol Psychiatry. 9, 698-704.

Li, W., Zhou, Y., Jentsch, J. D., Brown, R. A., Tian, X., Ehninger, D., Hennah, W., Peltonen, L., Lonnqvist, J., Huttunen, M. O., Kaprio, J., Trachtenberg, J. T., Silva, A. J. and Cannon, T. D., 2007. Specific developmental disruption of disrupted-in-schizophrenia-1 function results in schizophrenia-related phenotypes in mice. Proc Natl Acad Sci U S A. 104, 18280-18285.

Lichtman, A. H., Dimen, K. R. and Martin, B. R., 1995. Systemic or intrahippocampal cannabinoid administration impairs spatial memory in rats. Psychopharmacology (Berl). 119, 282-290.

Lichtman, A. H. and Martin, B. R., 1996. Delta 9-tetrahydrocannabinol impairs spatial memory through a cannabinoid receptor mechanism. Psychopharmacology (Berl). 126, 125-131.

Lichtman, A. H. and Martin, B. R., 2005. Cannabinoid tolerance and dependence. Handb Exp Pharmacol, 691-717.

Lin, H. F., Liu, Y. L., Liu, C. M., Hung, S. I., Hwu, H. G. and Chen, W. J., 2005. Neuregulin 1 gene and variations in perceptual aberration of schizotypal personality in adolescents. Psychol Med. 35, 1589-1598.

Lindenmayer, J. P., 1995. New pharmacotherapeutic modalities for negative symptoms in psychosis. Acta Psychiatr Scand Suppl. 388, 15-19.

Linszen, D. H., Dingemans, P. M. and Lenior, M. E., 1994. Cannabis abuse and the course of recent-onset schizophrenic disorders. Arch Gen Psychiatry. 51, 273-279.

Lipska, B. K. and Weinberger, D. R., 2000. To model a psychiatric disorder in animals: schizophrenia as a reality test. Neuropsychopharmacology. 23, 223-239.

Little, P. J., Compton, D. R., Johnson, M. R., Melvin, L. S. and Martin, B. R., 1988. Pharmacology and stereoselectivity of structurally novel cannabinoids in mice. J Pharmacol Exp Ther. 247, 1046-1051.

Lubow, R. E., 2005. Construct validity of the animal latent inhibition model of selective attention deficits in schizophrenia. Schizophr Bull. 31, 139-153.

Lubow, R. E. and Moore, A. U., 1959. Latent inhibition: the effect of nonreinforced pre-exposure to the conditional stimulus. J Comp Physiol Psychol. 52, 415-419.

M

Ma, Y. J., Hill, D. F., Creswick, K. E., Costa, M. E., Cornea, A., Lioubin, M. N., Plowman, G. D. and Ojeda, S. R., 1999. Neuregulins signaling via a glial erbB-2-erbB-4 receptor complex contribute to the neuroendocrine control of mammalian sexual development. J Neurosci. 19, 9913-9927.

Mailleux, P., Verslype, M., Preud'homme, X. and Vanderhaeghen, J. J., 1994. Activation of multiple transcription factor genes by tetrahydrocannabinol in rat forebrain. Neuroreport. 5, 1265-1268.

Malone, D. T. and Taylor, D. A., 2006. The effect of Delta9-tetrahydrocannabinol on sensorimotor gating in socially isolated rats. Behav Brain Res. 166, 101-109.

Mansbach, R. S. and Geyer, M. A., 1989. Effects of phencyclidine and phencyclidine biologs on sensorimotor gating in the rat. Neuropsychopharmacology. 2, 299-308.

Mansbach, R. S., Geyer, M. A. and Braff, D. L., 1988. Dopaminergic stimulation disrupts sensorimotor gating in the rat. Psychopharmacology (Berl). 94, 507-514.

Mansbach, R. S., Rovetti, C. C., Winston, E. N. and Lowe, J. A., 3rd, 1996. Effects of the cannabinoid CB1 receptor antagonist SR141716A on the behavior of pigeons and rats. Psychopharmacology (Berl). 124, 315-322.

Manzanares, J., Uriguen, L., Rubio, G. and Palomo, T., 2004. Role of endocannabinoid system in mental diseases. Neurotox Res. 6, 213-224.

Marchbanks, R. M., Mulcrone, J. and Whatley, S. A., 1995. Aspects of oxidative metabolism in schizophrenia. Br J Psychiatry. 167, 293-298.

Martin, R. S., Secchi, R. L., Sung, E., Lemaire, M., Bonhaus, D. W., Hedley, L. R. and Lowe, D. A., 2003. Effects of cannabinoid receptor ligands on psychosis-relevant behavior models in the rat. Psychopharmacology (Berl). 165, 128-135.

Mathew, S. V., Law, A. J., Lipska, B. K., Davila-Garcia, M. I., Zamora, E. D., Mitkus, S. N., Vakkalanka, R., Straub, R. E., Weinberger, D. R., Kleinman, J. E. and Hyde, T. M., 2007. Alpha7 nicotinic acetylcholine receptor mRNA expression and binding in postmortem human brain are associated with genetic variation in neuregulin 1. Hum Mol Genet. 16, 2921-2932.

Mauri, M. C., Volonteri, L. S., De Gaspari, I. F., Colasanti, A., Brambilla, M. A. and Cerruti, L., 2006. Substance abuse in first-episode schizophrenic patients: a retrospective study. Clin Pract Epidemol Ment Health. 2, 4.

McDonald, C., Marshall, N., Sham, P. C., Bullmore, E. T., Schulze, K., Chapple, B., Bramon, E., Filbey, F., Quraishi, S., Walshe, M. and Murray, R. M., 2006. Regional brain morphometry in patients with schizophrenia or bipolar disorder and their unaffected relatives. Am J Psychiatry. 163, 478-487.

McDonald, R. J., King, A. L., Foong, N., Rizos, Z. and Hong, N. S., 2008. Neurotoxic lesions of the medial prefrontal cortex or medial striatum impair multiple-location place learning in the water task: evidence for neural structures with complementary roles in behavioural flexibility. Exp Brain Res. 187, 419-427.

McGrath, J., Saha, S., Chant, D. and Welham, J., 2008. Schizophrenia: A Concise Overview of Incidence, Prevalence, and Mortality. Epidemiol Rev.

McGregor, I. S., Arnold, J. C., Weber, M. F., Topple, A. N. and Hunt, G. E., 1998. A comparison of delta 9-THC and anandamide induced c-fos expression in the rat forebrain. Brain Res. 802, 19-26.

McKinney, D. L., Cassidy, M. P., Collier, L. M., Martin, B. R., Wiley, J. L., Selley, D. E. and Sim-Selley, L. J., 2008. Dose-related differences in the regional pattern of cannabinoid receptor adaptation and in vivo tolerance development to delta9-tetrahydrocannabinol. J Pharmacol Exp Ther. 324, 664-673.

Mechoulam, R., Ben-Shabat, S., Hanus, L., Ligumsky, M., Kaminski, N. E., Schatz, A. R., Gopher, A., Almog, S., Martin, B. R., Compton, D. R. and et al., 1995. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. Biochem Pharmacol. 50, 83-90.

Mei, L. and Xiong, W. C., 2008. Neuregulin 1 in neural development, synaptic plasticity and schizophrenia. Nat Rev Neurosci. 9, 437-452.

Meltzer, H. Y., 2002. Suicidality in schizophrenia: a review of the evidence for risk factors and treatment options. Curr Psychiatry Rep. 4, 279-283.

Meltzer, H. Y., Bastani, B., Ramirez, L. and Matsubara, S., 1989. Clozapine: new research on efficacy and mechanism of action. Eur Arch Psychiatry Neurol Sci. 238, 332-339.

Messinis, L., Kyprianidou, A., Malefaki, S. and Papathanasopoulos, P., 2006. Neuropsychological deficits in long-term frequent cannabis users. Neurology. 66, 737-739.

Meyer, D. and Birchmeier, C., 1995. Multiple essential functions of neuregulin in development. Nature. 378, 386-390.

Miczek, K. A., 1976. Mouse-killing and motor activity: effects of chronic delta9-tetrahydrocannabinol and pilocarpine. Psychopharmacology (Berl). 47, 59-64.

Mikuriya, T. H., 1969. Marijuana in medicine: past, present and future. Calif Med. 110, 34-40.

Millan, M. J., Brocco, M., Gobert, A., Joly, F., Bervoets, K., Rivet, J., Newman-Tancredi, A., Audinot, V. and Maurel, S., 1999. Contrasting mechanisms of action and sensitivity to antipsychotics of phencyclidine versus amphetamine: importance of nucleus accumbens 5-HT2A sites for PCP-induced locomotion in the rat. Eur J Neurosci. 11, 4419-4432.

Miller, P., Lawrie, S. M., Hodges, A., Clafferty, R., Cosway, R. and Johnstone, E. C., 2001. Genetic liability, illicit drug use, life stress and psychotic symptoms: preliminary findings from the Edinburgh study of people at high risk for schizophrenia. Soc Psychiatry Psychiatr Epidemiol. 36, 338-342.

Misner, D. L. and Sullivan, J. M., 1999. Mechanism of cannabinoid effects on long-term potentiation and depression in hippocampal CA1 neurons. J Neurosci. 19, 6795-6805.

Mitchell, R. L., Elliott, R. and Woodruff, P. W., 2001. fMRI and cognitive dysfunction in schizophrenia. Trends Cogn Sci. 5, 71-81.

Moga, M. M., Weis, R. P. and Moore, R. Y., 1995. Efferent projections of the paraventricular thalamic nucleus in the rat. J Comp Neurol. 359, 221-238.

Montgomery, K. C., 1958. The relation between fear induced by novel stimulation and exploratory behavior. J Comp Physiol Psychol. 48, 254-260.

Moore, T. H., Zammit, S., Lingford-Hughes, A., Barnes, T. R., Jones, P. B., Burke, M. and Lewis, G., 2007. Cannabis use and risk of psychotic or affective mental health outcomes: a systematic review. Lancet. 370, 319-328.

Munafo, M. R., Attwood, A. S. and Flint, J., 2008. Neuregulin 1 genotype and schizophrenia. Schizophr Bull. 34, 9-12.

Murray, R. M., Lappin, J. and Di Forti, M., 2008. Schizophrenia: from developmental deviance to dopamine dysregulation. Eur Neuropsychopharmacol. 18 Suppl 3, S129-134.

N

Nagai, H., Egashira, N., Sano, K., Ogata, A., Mizuki, A., Mishima, K., Iwasaki, K., Shoyama, Y., Nishimura, R. and Fujiwara, M., 2006. Antipsychotics improve Delta9-tetrahydrocannabinol-induced impairment of the prepulse inhibition of the startle reflex in mice. Pharmacol Biochem Behav. 84, 330-336.

Nakamura, E. M., da Silva, E. A., Concilio, G. V., Wilkinson, D. A. and Masur, J., 1991. Reversible effects of acute and long-term administration of delta-9-tetrahydrocannabinol (THC) on memory in the rat. Drug Alcohol Depend. 28, 167-175.

Nava, F., Carta, G., Colombo, G. and Gessa, G. L., 2001. Effects of chronic Delta(9)-tetrahydrocannabinol treatment on hippocampal extracellular acetylcholine concentration and alternation performance in the T-maze. Neuropharmacology. 41, 392-399.

Nestler, E. J., Kelz, M. B. and Chen, J., 1999. DeltaFosB: a molecular mediator of long-term neural and behavioral plasticity. Brain Res. 835, 10-17.

Newell, K. A., Deng, C. and Huang, X. F., 2006. Increased cannabinoid receptor density in the posterior cingulate cortex in schizophrenia. Exp Brain Res. 172, 556-560.

Nguyen, Q. H. and Wagner, E. J., 2006. Estrogen differentially modulates the cannabinoid- induced presynaptic inhibition of amino acid neurotransmission in proopiomelanocortin neurons of the arcuate nucleus. Neuroendocrinology. 84, 123-137.

Nicodemus, K. K., Luna, A., Vakkalanka, R., Goldberg, T., Egan, M., Straub, R. E. and Weinberger, D. R., 2006. Further evidence for association between ErbB4 and schizophrenia and influence on cognitive intermediate phenotypes in healthy controls. Mol Psychiatry. 11, 1062-1065.

Nikam, S. S. and Awasthi, A. K., 2008. Evolution of schizophrenia drugs: a focus on dopaminergic systems. Curr Opin Investig Drugs. 9, 37-46.

Niyuhire, F., Varvel, S. A., Martin, B. R. and Lichtman, A. H., 2007. Exposure to marijuana smoke impairs memory retrieval in mice. J Pharmacol Exp Ther. 322, 1067-1075.

Norton, N., Moskvina, V., Morris, D. W., Bray, N. J., Zammit, S., Williams, N. M., Williams, H. J., Preece, A. C., Dwyer, S., Wilkinson, J. C., Spurlock, G., Kirov, G., Buckland, P., Waddington, J. L., Gill, M., Corvin, A. P., Owen, M. J. and O'Donovan, M. C., 2006. Evidence that interaction between neuregulin 1 and its receptor erbB4 increases susceptibility to schizophrenia. Am J Med Genet B Neuropsychiatr Genet. 141B, 96-101.

Nyberg, J. M., Vekovischeva, O. and Sandnabba, N. K., 2003. Anxiety profiles of mice selectively bred for intermale aggression. Behav Genet. 33, 503-511.

O

O'Tuathaigh, C. M., Babovic, D., O'Sullivan, G. J., Clifford, J. J., Tighe, O., Croke, D. T., Harvey, R. and Waddington, J. L., 2007. Phenotypic characterization of spatial cognition and social behavior in mice with 'knockout' of the schizophrenia risk gene neuregulin 1. Neuroscience. 147, 18-27.

O'Tuathaigh, C. M., O'Connor, A. M., O'Sullivan, G. J., Lai, D., Harvey, R., Croke, D. T. and Waddington, J. L., 2008. Disruption to social dyadic interactions but not emotional/anxiety-related behaviour in mice with heterozygous 'knockout'

of the schizophrenia risk gene neuregulin-1. Prog Neuropsychopharmacol Biol Psychiatry. 32, 462-466.

O'Tuathaigh, C. M., O'Sullivan, G. J., Kinsella, A., Harvey, R. P., Tighe, O., Croke, D. T. and Waddington, J. L., 2006. Sexually dimorphic changes in the exploratory and habituation profiles of heterozygous neuregulin-1 knockout mice. Neuroreport. 17, 79-83.

Oades, R. D., Klimke, A., Henning, U. and Rao, M. L., 2002. Relations of clinical features, subgroups and medication to serum monoamines in schizophrenia. Hum Psychopharmacol. 17, 15-27.

Oliva, J. M., Ortiz, S., Palomo, T. and Manzanares, J., 2003. Behavioural and gene transcription alterations induced by spontaneous cannabinoid withdrawal in mice. J Neurochem. 85, 94-104.

Oliva, J. M., Ortiz, S., Palomo, T. and Manzanares, J., 2004. Spontaneous cannabinoid withdrawal produces a differential time-related responsiveness in cannabinoid CB1 receptor gene expression in the mouse brain. J Psychopharmacol. 18, 59-65.

Onaivi, E. S., Chakrabarti, A., Gwebu, E. T. and Chaudhuri, G., 1995. Neurobehavioral effects of delta 9-THC and cannabinoid (CB1) receptor gene expression in mice. Behav Brain Res. 72, 115-125.

Onaivi, E. S., Green, M. R. and Martin, B. R., 1990. Pharmacological characterization of cannabinoids in the elevated plus maze. J Pharmacol Exp Ther. 253, 1002-1009.

Onaivi, E. S., Ishiguro, H., Sejal, P., Meozzi, P. A., Myers, L., Tagliaferro, P., Hope, B., Leonard, C. M., Uhl, G. R., Brusco, A. and Gardner, E., 2006. Methods to study the behavioral effects and expression of CB2 cannabinoid receptor and its gene transcripts in the chronic mild stress model of depression. Methods Mol Med. 123, 291-298.

Ouagazzal, A. M., Jenck, F. and Moreau, J. L., 2001. Drug-induced potentiation of prepulse inhibition of acoustic startle reflex in mice: a model for detecting antipsychotic activity? Psychopharmacology (Berl). 156, 273-283.

Ow, Y. L., Green, D. R., Hao, Z. and Mak, T. W., 2008. Cytochrome c: functions beyond respiration. Nat Rev Mol Cell Biol. 9, 532-542.

Owen, M. J., Craddock, N. and O'Donovan, M. C., 2005. Schizophrenia: genes at last? Trends Genet. 21, 518-525.

Ozaki, M., Sasner, M., Yano, R., Lu, H. S. and Buonanno, A., 1997. Neuregulin-beta induces expression of an NMDA-receptor subunit. Nature. 390, 691-694.

P

Packard, M. G. and Teather, L. A., 1998. Amygdala modulation of multiple memory systems: hippocampus and caudate-putamen. Neurobiol Learn Mem. 69, 163-203.

Park, S., Puschel, J., Sauter, B. H., Rentsch, M. and Hell, D., 1999. Spatial working memory deficits and clinical symptoms in schizophrenia: a 4-month follow-up study. Biol Psychiatry. 46, 392-400.

Parlapani, E., Schmitt, A., Wirths, O., Bauer, M., Sommer, C., Rueb, U., Skowronek, M. H., Treutlein, J., Petroianu, G. A., Rietschel, M. and Falkai, P., 2008. Gene expression of neuregulin-1 isoforms in different brain regions of elderly schizophrenia patients. World J Biol Psychiatry, 1-8.

Patel, S., Cravatt, B. F. and Hillard, C. J., 2005. Synergistic interactions between cannabinoids and environmental stress in the activation of the central amygdala. Neuropsychopharmacology. 30, 497-507.

Paterlini, M., Zakharenko, S. S., Lai, W. S., Qin, J., Zhang, H., Mukai, J., Westphal, K. G., Olivier, B., Sulzer, D., Pavlidis, P., Siegelbaum, S. A., Karayiorgou, M. and Gogos, J. A., 2005. Transcriptional and behavioral interaction between 22q11.2 orthologs modulates schizophrenia-related phenotypes in mice. Nat Neurosci. 8, 1586-1594.

Pattij, T., Wiskerke, J. and Schoffelmeer, A. N., 2008. Cannabinoid modulation of executive functions. Eur J Pharmacol. 585, 458-463.

Paxinos, G. and Franklin, K. B. J., 2000. The Mouse Brain in Stereotaxic Coordinates. Academic Press; 2nd edition.

Pellow, S., Chopin, P., File, S. E. and Briley, M., 1985. Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods. 14, 149-167.

Peralta, V. and Cuesta, M. J., 1992. Influence of cannabis abuse on schizophrenic psychopathology. Acta Psychiatr Scand. 85, 127-130.

Pertwee, R. G., 2008. Ligands that target cannabinoid receptors in the brain: from THC to anandamide and beyond. Addict Biol. 13, 147-159.

Petryshen, T. L., Middleton, F. A., Kirby, A., Aldinger, K. A., Purcell, S., Tahl, A. R., Morley, C. P., McGann, L., Gentile, K. L., Rockwell, G. N., Medeiros, H. M., Carvalho, C., Macedo, A., Dourado, A., Valente, J., Ferreira, C. P., Patterson, N. J., Azevedo, M. H., Daly, M. J., Pato, C. N., Pato, M. T. and Sklar, P., 2005. Support for involvement of neuregulin 1 in schizophrenia pathophysiology. Mol Psychiatry. 10, 366-374, 328.

Pinkas-Kramarski, R., Eilam, R., Alroy, I., Levkowitz, G., Lonai, P. and Yarden, Y., 1997. Differential expression of NDF/neuregulin receptors ErbB-3 and ErbB-4 and involvement in inhibition of neuronal differentiation. Oncogene. 15, 2803-2815.

Pinkas-Kramarski, R., Eilam, R., Spiegler, O., Lavi, S., Liu, N., Chang, D., Wen, D., Schwartz, M. and Yarden, Y., 1994. Brain neurons and glial cells express Neu differentiation factor/heregulin: a survival factor for astrocytes. Proc Natl Acad Sci U S A. 91, 9387-9391.

Pinna, A., Wardas, J., Cozzolino, A. and Morelli, M., 1999. Involvement of adenosine A2A receptors in the induction of c-fos expression by clozapine and haloperidol. Neuropsychopharmacology. 20, 44-51.

Pletnikov, M. V., Ayhan, Y., Nikolskaia, O., Xu, Y., Ovanesov, M. V., Huang, H., Mori, S., Moran, T. H. and Ross, C. A., 2008. Inducible expression of mutant human DISC1 in mice is associated with brain and behavioral abnormalities reminiscent of schizophrenia. Mol Psychiatry. 13, 173-186, 115.

Postma, P., Gray, J. A., Sharma, T., Geyer, M., Mehrotra, R., Das, M., Zachariah, E., Hines, M., Williams, S. C. and Kumari, V., 2006. A behavioural and functional neuroimaging investigation into the effects of nicotine on sensorimotor gating in healthy subjects and persons with schizophrenia. Psychopharmacology (Berl). 184, 589-599.

Prewitt, C. M. and Herman, J. P., 1998. Anatomical interactions between the central amygdaloid nucleus and the hypothalamic paraventricular nucleus of the rat: a dual tract-tracing analysis. J Chem Neuroanat. 15, 173-185.

Prince, J. A., Yassin, M. S. and Oreland, L., 1997. Normalization of cytochrome-c oxidase activity in the rat brain by neuroleptics after chronic treatment with PCP or methamphetamine. Neuropharmacology. 36, 1665-1678.

Prince, J. A., Yassin, M. S. and Oreland, L., 1998. A histochemical demonstration of altered cytochrome oxidase activity in the rat brain by neuroleptics. Eur Neuropsychopharmacol. 8, 1-6.

Pulver, A. E., Lasseter, V. K., Kasch, L., Wolyniec, P., Nestadt, G., Blouin, J. L., Kimberland, M., Babb, R., Vourlis, S., Chen, H. and et al., 1995. Schizophrenia: a genome scan targets chromosomes 3p and 8p as potential sites of susceptibility genes. Am J Med Genet. 60, 252-260.

Q

Quinn, H. R., Matsumoto, I., Callaghan, P. D., Long, L. E., Arnold, J. C., Gunasekaran, N., Thompson, M. R., Dawson, B., Mallet, P. E., Kashem, M. A., Matsuda-Matsumoto, H., Iwazaki, T. and McGregor, I. S., 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.

R

Rang, H., Dale, M., JM, R. and Flower, R., 2007. Rang and Dales' Pharmacology. Churchill Livingstone Elsevier.

Rawls, S. M., Cabassa, J., Geller, E. B. and Adler, M. W., 2002. CB1 receptors in the preoptic anterior hypothalamus regulate WIN 55212-2 [(4,5-dihydro-2-methyl-4(4-morpholinylmethyl)-1-(1-naphthalenyl-carbonyl)- 6H-pyrrolo[3,2,1ij]quinolin-6-one]-induced hypothermia. J Pharmacol Exp Ther. 301, 963-968.

Riedel, G. and Davies, S. N., 2005. Cannabinoid function in learning, memory and plasticity. Handb Exp Pharmacol, 445-477.

Rimer, M., Barrett, D. W., Maldonado, M. A., Vock, V. M. and Gonzalez-Lima, F., 2005. Neuregulin-1 immunoglobulin-like domain mutant mice: clozapine sensitivity and impaired latent inhibition. Neuroreport. 16, 271-275.

Risold, P. Y. and Swanson, L. W., 1997. Connections of the rat lateral septal complex. Brain Res Brain Res Rev. 24, 115-195.

Rodgers, R. J., 1997. Animal models of 'anxiety': where next? Behav Pharmacol. 8, 477-496; discussion 497-504.

Rodgers, R. J., Evans, P. M. and Murphy, A., 2005. Anxiogenic profile of AM-251, a selective cannabinoid CB1 receptor antagonist, in plus-maze-naive and plus-maze-experienced mice. Behav Pharmacol. 16, 405-413.

Rodgers, R. J. and Johnson, N. J., 1995. Factor analysis of spatiotemporal and ethological measures in the murine elevated plus-maze test of anxiety. Pharmacol Biochem Behav. 52, 297-303.

Rodgers, R. J., Johnson, N. J., Cole, J. C., Dewar, C. V., Kidd, G. R. and Kimpson, P. H., 1996. Plus-maze retest profile in mice: importance of initial stages of trail 1 and response to post-trail cholinergic receptor blockade. Pharmacol Biochem Behav. 54, 41-50.

Rodgers, R. J., Lee, C. and Shepherd, J. K., 1992. Effects of diazepam on behavioural and antinociceptive responses to the elevated plus-maze in male mice depend upon treatment regimen and prior maze experience. Psychopharmacology (Berl). 106, 102-110.

Rodgers, R. J. and Shepherd, J. K., 1993. Influence of prior maze experience on behaviour and response to diazepam in the elevated plus-maze and light/dark tests of anxiety in mice. Psychopharmacology (Berl). 113, 237-242.

Rog, D. J., Nurmikko, T. J., Friede, T. and Young, C. A., 2005. Randomized, controlled trial of cannabis-based medicine in central pain in multiple sclerosis. Neurology. 65, 812-819.

Rohrbach, S., Muller-Werdan, U., Werdan, K., Koch, S., Gellerich, N. F. and Holtz, J., 2005. Apoptosis-modulating interaction of the neuregulin/erbB pathway with anthracyclines in regulating Bcl-xS and Bcl-xL in cardiomyocytes. J Mol Cell Cardiol. 38, 485-493.

Rosch, S., Ramer, R., Brune, K. and Hinz, B., 2006. R(+)-methanandamide and other cannabinoids induce the expression of cyclooxygenase-2 and matrix metalloproteinases in human nonpigmented ciliary epithelial cells. J Pharmacol Exp Ther. 316, 1219-1228.

Rossato, J. I., Zinn, C. G., Furini, C., Bevilaqua, L. R., Medina, J. H., Cammarota, M. and Izquierdo, I., 2006. A link between the hippocampal and the striatal memory systems of the brain. An Acad Bras Cienc. 78, 515-523.

Rouillon, F., Toumi, M., Dansette, G. Y., Benyaya, J. and Auquier, P., 1997. Some aspects of the cost of schizophrenia in France. Pharmacoeconomics. 11, 578-594.

Roy, K., Murtie, J. C., El-Khodor, B. F., Edgar, N., Sardi, S. P., Hooks, B. M., Benoit-Marand, M., Chen, C., Moore, H., O'Donnell, P., Brunner, D. and Corfas, G., 2007. Loss of erbB signaling in oligodendrocytes alters myelin and dopaminergic function, a potential mechanism for neuropsychiatric disorders. Proc Natl Acad Sci U S A. 104, 8131-8136.

Rubino, T. and Parolaro, D., 2008. Long lasting consequences of cannabis exposure in adolescence. Mol Cell Endocrinol. 286, S108-113.

Rutkowska, M., Jamontt, J. and Gliniak, H., 2006. Effects of cannabinoids on the anxiety-like response in mice. Pharmacol Rep. 58, 200-206.

S

Sams-Dodd, F., 1999. Phencyclidine in the social interaction test: an animal model of schizophrenia with face and predictive validity. Rev Neurosci. 10, 59-90.

Sams-Dodd, F., Lipska, B. K. and Weinberger, D. R., 1997. Neonatal lesions of the rat ventral hippocampus result in hyperlocomotion and deficits in social behaviour in adulthood. Psychopharmacology (Berl). 132, 303-310.

Sanders, A. R., Duan, J., Levinson, D. F., Shi, J., He, D., Hou, C., Burrell, G. J., Rice, J. P., Nertney, D. A., Olincy, A., Rozic, P., Vinogradov, S., Buccola, N. G., Mowry, B. J., Freedman, R., Amin, F., Black, D. W., Silverman, J. M., Byerley, W. F., Crowe, R. R., Cloninger, C. R., Martinez, M. and Gejman, P. V., 2008. No significant association of 14 candidate genes with schizophrenia in a large European ancestry sample: implications for psychiatric genetics. Am J Psychiatry. 165, 497-506.

Savonenko, A. V., Melnikova, T., Laird, F. M., Stewart, K. A., Price, D. L. and Wong, P. C., 2008. Alteration of BACE1-dependent NRG1/ErbB4 signaling

and schizophrenia-like phenotypes in BACE1-null mice. Proc Natl Acad Sci U S A. 105, 5585-5590.

Sawa, A. and Snyder, S. H., 2003. Schizophrenia: neural mechanisms for novel therapies. Mol Med. 9, 3-9.

Schlosser, R. G., Koch, K., Wagner, G., Nenadic, I., Roebel, M., Schachtzabel, C., Axer, M., Schultz, C., Reichenbach, J. R. and Sauer, H., 2008. Inefficient executive cognitive control in schizophrenia is preceded by altered functional activation during information encoding: an fMRI study. Neuropsychologia. 46, 336-347.

Schneider, M., 2008. Puberty as a highly vulnerable developmental period for the consequences of cannabis exposure. Addict Biol. 13, 253-263.

Schneider, M. and Koch, M., 2002. The cannabinoid agonist WIN 55,212-2 reduces sensorimotor gating and recognition memory in rats. Behav Pharmacol. 13, 29-37.

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.

Schneier, F. R. and Siris, S. G., 1987. A review of psychoactive substance use and abuse in schizophrenia. Patterns of drug choice. J Nerv Ment Dis. 175, 641-652.

Schofield, D., Tennant, C., Nash, L., Degenhardt, L., Cornish, A., Hobbs, C. and Brennan, G., 2006. Reasons for cannabis use in psychosis. Aust N Z J Psychiatry. 40, 570-574.

Sebens, J. B., Koch, T., Ter Horst, G. J. and Korf, J., 1995. Differential Fos-protein induction in rat forebrain regions after acute and long-term haloperidol and clozapine treatment. Eur J Pharmacol. 273, 175-182.

Seeman, P., 1987. Dopamine receptors and the dopamine hypothesis of schizophrenia. Synapse. 1, 133-152.

Segarra, N., Bernardo, M., Valdes, M., Caldu, X., Falcon, C., Rami, L., Bargallo, N., Parramon, G. and Junque, C., 2008. Cerebellar deficits in schizophrenia are associated with executive dysfunction. Neuroreport. 19, 1513-1517.

Sei, Y., Ren-Patterson, R., Li, Z., Tunbridge, E. M., Egan, M. F., Kolachana, B. S. and Weinberger, D. R., 2007. Neuregulin1-induced cell migration is impaired in schizophrenia: association with neuregulin1 and catechol-o-methyltransferase gene polymorphisms. Mol Psychiatry. 12, 946-957.

Sheehan, T. P., Chambers, R. A. and Russell, D. S., 2004. Regulation of affect by the lateral septum: implications for neuropsychiatry. Brain Res Brain Res Rev. 46, 71-117.

Shen, W. W., 1999. A history of antipsychotic drug development. Compr Psychiatry. 40, 407-414.

Silberberg, G., Darvasi, A., Pinkas-Kramarski, R. and Navon, R., 2006. The involvement of ErbB4 with schizophrenia: association and expression studies. Am J Med Genet B Neuropsychiatr Genet. 141B, 142-148.

Silva de Melo, L. C., Cruz, A. P., Rios Valentim, S. J., Jr., Marinho, A. R., Mendonca, J. B. and Nakamura-Palacios, E. M., 2005. Delta(9)-THC administered into the medial prefrontal cortex disrupts the spatial working memory. Psychopharmacology (Berl). 183, 54-64.

Simon, P., Dupuis, R. and Costentin, J., 1994. Thigmotaxis as an index of anxiety in mice. Influence of dopaminergic transmissions. Behav Brain Res. 61, 59-64.

Smith, P. F., 2005. The safety of cannabinoids for the treatment of multiple sclerosis. Expert Opin Drug Saf. 4, 443-456.

Snodgrass-Belt, P., Gilbert, J. L. and Davis, F. C., 2005. Central administration of transforming growth factor-alpha and neuregulin-1 suppress active behaviors and cause weight loss in hamsters. Brain Res. 1038, 171-182.

Solowij, N. and Michie, P. T., 2007. Cannabis and cognitive dysfunction: parallels with endophenotypes of schizophrenia? J Psychiatry Neurosci. 32, 30-52.

Solowij, N., Michie, P. T. and Fox, A. M., 1995. Differential impairments of selective attention due to frequency and duration of cannabis use. Biol Psychiatry. 37, 731-739.

Solowij, N., Stephens, R. S., Roffman, R. A., Babor, T., Kadden, R., Miller, M., Christiansen, K., McRee, B. and Vendetti, J., 2002. Cognitive functioning of long-term heavy cannabis users seeking treatment. Jama. 287, 1123-1131.

Sotomayor, R., Forray, M. I. and Gysling, K., 2005. Acute morphine administration increases extracellular DA levels in the rat lateral septum by decreasing the GABAergic inhibitory tone in the ventral tegmental area. J Neurosci Res. 81, 132-139.

Stanley-Cary, C. C., Harris, C. and Martin-Iverson, M. T., 2002. Differing effects of the cannabinoid agonist, CP 55,940, in an alcohol or Tween 80 solvent, on prepulse inhibition of the acoustic startle reflex in the rat. Behav Pharmacol. 13, 15-28.

Stefansson, H., Sarginson, J., Kong, A., Yates, P., Steinthorsdottir, V., Gudfinnsson, E., Gunnarsdottir, S., Walker, N., Petursson, H., Crombie, C., Ingason, A., Gulcher, J. R., Stefansson, K. and St Clair, D., 2003. Association of neuregulin 1 with schizophrenia confirmed in a Scottish population. Am J Hum Genet. 72, 83-87.

Stefansson, H., Sigurdsson, E., Steinthorsdottir, V., Bjornsdottir, S., Sigmundsson, T., Ghosh, S., Brynjolfsson, J., Gunnarsdottir, S., Ivarsson, O., Chou, T. T., Hjaltason, O., Birgisdottir, B., Jonsson, H., Gudnadottir, V. G., Gudmundsdottir, E., Bjornsson, A., Ingvarsson, B., Ingason, A., Sigfusson, S., Hardardottir, H., Harvey, R. P., Lai, D., Zhou, M., Brunner, D., Mutel, V., Gonzalo, A., Lemke, G., Sainz, J., Johannesson, G., Andresson, T., Gudbjartsson, D., Manolescu, A., Frigge, M. L., Gurney, M. E., Kong, A., Gulcher, J. R., Petursson, H. and Stefansson, K., 2002. Neuregulin 1 and susceptibility to schizophrenia. Am J Hum Genet. 71, 877-892.

Steiner, H., Blum, M., Kitai, S. T. and Fedi, P., 1999. Differential expression of ErbB3 and ErbB4 neuregulin receptors in dopamine neurons and forebrain areas of the adult rat. Exp Neurol. 159, 494-503.

Stevens, J. R., 2002. Schizophrenia: reproductive hormones and the brain. Am J Psychiatry. 159, 713-719.

Stone, J. M., Morrison, P. D. and Pilowsky, L. S., 2007. Glutamate and dopamine dysregulation in schizophrenia--a synthesis and selective review. J Psychopharmacol. 21, 440-452.

Sullivan, P. F., Kendler, K. S. and Neale, M. C., 2003. Schizophrenia as a complex trait: evidence from a meta-analysis of twin studies. Arch Gen Psychiatry. 60, 1187-1192.

Sumner, B. E., Cruise, L. A., Slattery, D. A., Hill, D. R., Shahid, M. and Henry, B., 2004. Testing the validity of c-fos expression profiling to aid the therapeutic classification of psychoactive drugs. Psychopharmacology (Berl). 171, 306-321.

Susser, E. S. and Lin, S. P., 1992. Schizophrenia after prenatal exposure to the Dutch Hunger Winter of 1944-1945. Arch Gen Psychiatry. 49, 983-988.

Swanson, L. W. and Petrovich, G. D., 1998. What is the amygdala? Trends Neurosci. 21, 323-331.

Swerdlow, N. R., Braff, D. L., Taaid, N. and Geyer, M. A., 1994. Assessing the validity of an animal model of deficient sensorimotor gating in schizophrenic patients. Arch Gen Psychiatry. 51, 139-154.

T

Tandon, R., Keshavan, M. S. and Nasrallah, H. A., 2008. Schizophrenia, "Just the Facts" What we know in 2008. 2. Epidemiology and etiology. Schizophr Res. 102, 1-18.

Tang, J. X., Chen, W. Y., He, G., Zhou, J., Gu, N. F., Feng, G. Y. and He, L., 2004. Polymorphisms within 5' end of the Neuregulin 1 gene are genetically associated with schizophrenia in the Chinese population. Mol Psychiatry. 9, 11-12.

Thiselton, D. L., Webb, B. T., Neale, B. M., Ribble, R. C., O'Neill, F. A., Walsh, D., Riley, B. P. and Kendler, K. S., 2004. No evidence for linkage or association of neuregulin-1 (NRG1) with disease in the Irish study of high-density schizophrenia families (ISHDSF). Mol Psychiatry. 9, 777-783; image 729.

Thomson, P. A., Christoforou, A., Morris, S. W., Adie, E., Pickard, B. S., Porteous, D. J., Muir, W. J., Blackwood, D. H. and Evans, K. L., 2007. Association of Neuregulin 1 with schizophrenia and bipolar disorder in a second cohort from the Scottish population. Mol Psychiatry. 12, 94-104.

Thuret, S., Alavian, K. N., Gassmann, M., Lloyd, C. K., Smits, S. M., Smidt, M. P., Klein, R., Dyck, R. H. and Simon, H. H., 2004. The neuregulin receptor, ErbB4, is not required for normal development and adult maintenance of the substantia nigra pars compacta. J Neurochem. 91, 1302-1311.

Tibbo, P., Swainson, J., Chue, P. and LeMelledo, J. M., 2003. Prevalence and relationship to delusions and hallucinations of anxiety disorders in schizophrenia. Depress Anxiety. 17, 65-72.

Tkachev, D., Mimmack, M. L., Ryan, M. M., Wayland, M., Freeman, T., Jones, P. B., Starkey, M., Webster, M. J., Yolken, R. H. and Bahn, S., 2003. Oligodendrocyte dysfunction in schizophrenia and bipolar disorder. Lancet. 362, 798-805.

Townsend, M. H. and Wilson, M. S., 2005. Comorbid anxiety disorders and divalproex sodium use among partial hospital patients with psychotic disorders. Compr Psychiatry. 46, 368-370.

Tsou, K., Brown, S., Sanudo-Pena, M. C., Mackie, K. and Walker, J. M., 1998. Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system. Neuroscience. 83, 393-411.

U

Ujike, H., Takaki, M., Nakata, K., Tanaka, Y., Takeda, T., Kodama, M., Fujiwara, Y., Sakai, A. and Kuroda, S., 2002. CNR1, central cannabinoid receptor gene, associated with susceptibility to hebephrenic schizophrenia. Mol Psychiatry. 7, 515-518.

Uphouse, L., Tilson, H. and Mitchell, C. L., 1983. Long-term effects of behavioral testing on serum hormones and brain weight. Life Sci. 33, 1395-1400.

Uriguen, L., Perez-Rial, S., Ledent, C., Palomo, T. and Manzanares, J., 2004. Impaired action of anxiolytic drugs in mice deficient in cannabinoid CB1 receptors. Neuropharmacology. 46, 966-973.

V

Valjent, E., Mitchell, J. M., Besson, M. J., Caboche, J. and Maldonado, R., 2002. Behavioural and biochemical evidence for interactions between Delta 9-tetrahydrocannabinol and nicotine. Br J Pharmacol. 135, 564-578.

Valverde, O., 2005. Participation of the cannabinoid system in the regulation of emotional-like behaviour. Curr Pharm Des. 11, 3421-3429.

van den Buuse, M., Garner, B., Gogos, A. and Kusljic, S., 2005. Importance of animal models in schizophrenia research. Aust N Z J Psychiatry. 39, 550-557.

van der Stelt, M. and Di Marzo, V., 2003. The endocannabinoid system in the basal ganglia and in the mesolimbic reward system: implications for neurological and psychiatric disorders. Eur J Pharmacol. 480, 133-150.

van Gaalen, M. M. and Steckler, T., 2000. Behavioural analysis of four mouse strains in an anxiety test battery. Behav Brain Res. 115, 95-106.

van Luijtelaar, G., Fabene, P. F., de Bruin, N., Jongema, C., Ellenbroek, B. A. and Veening, J. G., 2001. Neural correlates of sensory gating in the rat: decreased Fos induction in the lateral septum. Brain Res Bull. 54, 145-151.

Varvel, S. A., Anum, E., Niyuhire, F., Wise, L. E. and Lichtman, A. H., 2005. Delta(9)-THC-induced cognitive deficits in mice are reversed by the GABA(A) antagonist bicuculline. Psychopharmacology (Berl). 178, 317-327.

Varvel, S. A., Hamm, R. J., Martin, B. R. and Lichtman, A. H., 2001. Differential effects of delta 9-THC on spatial reference and working memory in mice. Psychopharmacology (Berl). 157, 142-150.

Varvel, S. A. and Lichtman, A. H., 2002. Evaluation of CB1 receptor knockout mice in the Morris water maze. J Pharmacol Exp Ther. 301, 915-924.

Varvel, S. A., Wiley, J. L., Yang, R., Bridgen, D. T., Long, K., Lichtman, A. H. and Martin, B. R., 2006. Interactions between THC and cannabidiol in mouse models of cannabinoid activity. Psychopharmacology (Berl). 186, 226-234.

Verma, V., Tan, C. H., Ong, W. Y., Grigoryan, G. A., Jones, C. A., Stolzberg, D., Salvi, R., Gross, K. W., Ratty, A. K. and Dawe, G. S., 2008. The chakragati mouse shows deficits in prepulse inhibition of acoustic startle and latent inhibition. Neurosci Res. 60, 281-288.

Verrico, C. D., Jentsch, J. D. and Roth, R. H., 2003. Persistent and anatomically selective reduction in prefrontal cortical dopamine metabolism after repeated, intermittent cannabinoid administration to rats. Synapse. 49, 61-66.

Viveros, M. P., Marco, E. M. and File, S. E., 2005. Endocannabinoid system and stress and anxiety responses. Pharmacol Biochem Behav. 81, 331-342.

Volkow, N. D., Fowler, J. S., Wang, G. J., Swanson, J. M. and Telang, F., 2007. Dopamine in drug abuse and addiction: results of imaging studies and treatment implications. Arch Neurol. 64, 1575-1579.

Vostrikov, V., Orlovskaya, D. and Uranova, N., 2008. Deficit of pericapillary oligodendrocytes in the prefrontal cortex in schizophrenia. World J Biol Psychiatry. 9, 34-42.

W

Walker, E. and Bollini, A. M., 2002. Pubertal neurodevelopment and the emergence of psychotic symptoms. Schizophr Res. 54, 17-23.

Walss-Bass, C., Liu, W., Lew, D. F., Villegas, R., Montero, P., Dassori, A., Leach, R. J., Almasy, L., Escamilla, M. and Raventos, H., 2006. A novel missense mutation in the transmembrane domain of neuregulin 1 is associated with schizophrenia. Biol Psychiatry. 60, 548-553.

Wang, J. H., Short, J., Ledent, C., Lawrence, A. J. and van den Buuse, M., 2003. Reduced startle habituation and prepulse inhibition in mice lacking the adenosine A2A receptor. Behav Brain Res. 143, 201-207.

Weickert, C. S., Miranda-Angulo, A. L., Wong, J., Perlman, W. R., Ward, S. E., Radhakrishna, V., Straub, R. E., Weinberger, D. R. and Kleinman, J. E., 2008. Variants in the estrogen receptor alpha gene and its mRNA contribute to risk for schizophrenia. Hum Mol Genet. 17, 2293-2309.

Weidenfeld, J., Feldman, S. and Mechoulam, R., 1994. Effect of the brain constituent anandamide, a cannabinoid receptor agonist, on the hypothalamo-pituitary-adrenal axis in the rat. Neuroendocrinology. 59, 110-112.

Weinberger, D. R. and Gallhofer, B., 1997. Cognitive function in schizophrenia. Int Clin Psychopharmacol. 12 Suppl 4, S29-36.

Whitlow, C. T., Freedland, C. S. and Porrino, L. J., 2003. Functional consequences of the repeated administration of Delta9-tetrahydrocannabinol in the rat. Drug Alcohol Depend. 71, 169-177.

WHO, 2008.). World Health Organization.

Williams, N. M., Preece, A., Spurlock, G., Norton, N., Williams, H. J., Zammit, S., O'Donovan, M. C. and Owen, M. J., 2003. Support for genetic variation in neuregulin 1 and susceptibility to schizophrenia. Mol Psychiatry. 8, 485-487.

Winterer, G., Konrad, A., Vucurevic, G., Musso, F., Stoeter, P. and Dahmen, N., 2008. Association of 5' end neuregulin-1 (NRG1) gene variation with subcortical medial frontal microstructure in humans. Neuroimage. 40, 712-718.

Wobrock, T., Ecker, U. K., Scherk, H., Schneider-Axmann, T., Falkai, P. and Gruber, O., 2008. Cognitive impairment of executive function as a core symptom of schizophrenia. World J Biol Psychiatry, 1-10.

Wong, A. H. and Van Tol, H. H., 2003. Schizophrenia: from phenomenology to neurobiology. Neurosci Biobehav Rev. 27, 269-306.

Woo, R. S., Li, X. M., Tao, Y., Carpenter-Hyland, E., Huang, Y. Z., Weber, J., Neiswender, H., Dong, X. P., Wu, J., Gassmann, M., Lai, C., Xiong, W. C., Gao, T. M. and Mei, L., 2007. Neuregulin-1 enhances depolarization-induced GABA release. Neuron. 54, 599-610.

Woo, T. U., Kim, A. M. and Viscidi, E., 2008. Disease-specific alterations in glutamatergic neurotransmission on inhibitory interneurons in the prefrontal cortex in schizophrenia. Brain Res. 1218, 267-277.

Wright, I. K., Upton, N. and Marsden, C. A., 1991. Resocialisation of isolation-reared rats does not alter their anxiogenic profile on the elevated X-maze model of anxiety. Physiol Behav. 50, 1129-1132.

Y

Yang, J. Z., Si, T. M., Ruan, Y., Ling, Y. S., Han, Y. H., Wang, X. L., Zhou, M., Zhang, H. Y., Kong, Q. M., Liu, C., Zhang, D. R., Yu, Y. Q., Liu, S. Z., Ju, G. Z., Shu, L., Ma, D. L. and Zhang, D., 2003. Association study of neuregulin 1 gene with schizophrenia. Mol Psychiatry. 8, 706-709.

Yarden, Y. and Sliwkowski, M. X., 2001. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol. 2, 127-137.

Yucel, M., Solowij, N., Respondek, C., Whittle, S., Fornito, A., Pantelis, C. and Lubman, D. I., 2008. Regional brain abnormalities associated with long-term heavy cannabis use. Arch Gen Psychiatry. 65, 694-701.

Yurek, D. M., Zhang, L., Fletcher-Turner, A. and Seroogy, K. B., 2004. Supranigral injection of neuregulin1-beta induces striatal dopamine overflow. Brain Res. 1028, 116-119.

Z

Zavitsanou, K., Garrick, T. and Huang, X. F., 2004. Selective antagonist [3H]SR141716A binding to cannabinoid CB1 receptors is increased in the anterior cingulate cortex in schizophrenia. Prog Neuropsychopharmacol Biol Psychiatry. 28, 355-360.

Zhao, X., Shi, Y., Tang, J., Tang, R., Yu, L., Gu, N., Feng, G., Zhu, S., Liu, H., Xing, Y., Zhao, S., Sang, H., Guan, Y., St Clair, D. and He, L., 2004. A case control and family based association study of the neuregulin1 gene and schizophrenia. J Med Genet. 41, 31-34.

Zhu, Y., Sullivan, L. L., Nair, S. S., Williams, C. C., Pandey, A. K., Marrero, L., Vadlamudi, R. K. and Jones, F. E., 2006. Coregulation of estrogen receptor by ERBB4/HER4 establishes a growth-promoting autocrine signal in breast tumor cells. Cancer Res. 66, 7991-7998.

Zuardi, A. W., Crippa, J. A., Hallak, J. E., Moreira, F. A. and Guimaraes, F. S., 2006. Cannabidiol, a Cannabis sativa constituent, as an antipsychotic drug. Braz J Med Biol Res. 39, 421-429.

Chapter 8

APPENDIX

ACCEPTED MANUSCRIPT FOR

BEHAVIOURAL PHARMACOLOGY

Chronic treatment with Δ^9-tetrahydrocannabinol impairs spatial memory and reduces zif268 expression in the mouse forebrain

Aurélie A. Boucher[1,2,3], Lucie Vivier[1], Mathilde Metna-Laurent[1], Laurent Brayda-Bruno[1], Nicole Mons[1], Jonathon C. Arnold[2,3], Jacques Micheau[1]

[1]CNIC, Université de Bordeaux, CNRS UMR 5228, 33405 Talence, France

[2]School of Medical Sciences (Pharmacology) and Bosch Institute, The University of Sydney, NSW 2006, Australia

[3]Schizophrenia Research Institute, Sydney, NSW 2010, Australia

Correspondance to J. Micheau, CNIC, CNRS UMR 5228, Université de Bordeaux, 33405 Talence, France. Email : j.micheau@cnic.u-bordeaux1.fr

Running head: Chronic THC on memory and zif268 expression

Source of support for the work: This work was supported by grants from the French Centre National de la Recherche Scientifique [C.N.R.S] and the Conseil Régional d'Aquitaine.

ABSTRACT

Few studies have investigated the effects of chronic cannabinoid exposure on memory performance and whether tolerance occurs to cannabinoid-induced memory impairment. Here we studied the effects of repeated exposure to Δ^9-tetrahydrocannabinol (THC: 1 mg/kg) on spatial memory and zif268 expression in mice. One group of animals were not pre-treated with THC while another group was injected with 13 daily injections of THC prior to memory testing in the Morris water maze. Both groups were administered THC throughout the memory testing phase of the experiment. THC decreased spatial memory and reversal learning, even in animals that received the THC pre-treatment and were tolerant to the locomotor suppressant effects of the drug. Zif268 immunoreactivity was reduced in the CA3 of the hippocampus and in the prefrontal cortex only in non pre-treated animals, indicating that while tolerance to the effects of cannabinoids on neuronal activity were evident, cannabinoid-promoted memory impairment in these animals persisted even after 24 days of exposure. Taken together this study demonstrates that following extended administration of THC its spatial memory impairing effects are resistant to tolerance.

Key words: cannabinoid; memory; zif268; chronic; tolerance; hippocampus

INTRODUCTION

Cannabis is the most widely used illicit drug in the world. Apart from its extensive recreational use, it also has some potential therapeutic relevance (Rog et al., 2005, Smith, 2005). One limitation of cannabis use is that its consumption induces cognitive impairments (Solowij et al., 1995, Fletcher et al., 1996), as well as structural and functional brain alterations (Jentsch et al., 1998, Kanayama et al., 2004, Yucel et al., 2008). Thus, developing a better insight of the chronic effects of cannabinoids on cognition is of critical importance for understanding the adverse effects of this drug.

Long-term cannabis users exhibit neuropsychological deficits such as impairments in memory and attention (Solowij et al., 2002, Messinis et al., 2006). In laboratory animals the acute administration of the main psychoactive constituent of cannabis, Δ^9-tetrahydrocannabinol (THC), induces deficits in cognitive processes (Lichtman and Martin, 1996, Varvel et al., 2001, Da and Takahashi, 2002). While the acute effects of cannabinoids are well documented, the long term effects of the drug on learning and memory have received less attention. With chronic treatment, many of the effects of cannabinoids are diminished – a phenomenon known as tolerance. Tolerance develops to the characteristic effects of cannabinoids at different rates, for example it develops more rapidly to cannabinoid-induced hypothermia than locomotor suppression (Whitlow et al., 2003, Gonzalez et al., 2005). Little is known about tolerance to the memory disruptive effects of cannabinoids, with a few human and animal studies suggesting that cannabinoid-induced memory impairment is more resistant to the development of tolerance (Nakamura et al., 1991, Deadwyler et al., 1995, Nava et al., 2001, Solowij et al., 2002, Hampson et al., 2003). This difference in the time-course of the development of tolerance to the effects of cannabinoids is of particular interest in animal models of behaviour given that it is often difficult to

dissociate the specificity of learning perturbations from the confounding effects of these drugs on motor activity.

The memory impairments promoted by cannabinoids appear to more strongly impact spatial memory (Ferrari et al., 1999, Varvel et al., 2001, Da and Takahashi, 2002, Fadda et al., 2004). This hippocampus-mediated form of learning can be evaluated in animals using the Morris water maze. Using this paradigm, different tasks can be examined to differentiate between reference and working memory (Varvel et al., 2001). Reference memory is evaluated in the place version of the water maze where the platform remains at a constant location, while working memory is usually studied by changing the location of the platform to assess behavioural flexibility, a process that is impaired with cannabinoid administration (Egerton et al., 2005, Hill et al., 2006). As memory is strongly regulated by the hippocampus, we also aimed to evaluate the chronic effects of THC on the brain by measuring zif268 expression. Zif268 (also known as krox-24, NGFI-A or egr-1) is an immediate early gene that is involved in learning and memory processes (Bozon et al., 2002). This transcription factor is abundant in the hippocampus where cannabinoids normally increase its expression (Derkinderen et al., 2003), an effect postulated to subserve the memory impairments promoted by these drugs (Lichtman et al., 1995, Egashira et al., 2002).

The doses of THC used by previous mouse studies to promote deficits in working memory range between 3 and 10 mg/kg (Varvel et al., 2001, Da and Takahashi, 2002, Varvel et al., 2005, Niyuhire et al., 2007). Here we aimed to assess if deficits in reversal learning in mice also occur using a lower dose of THC (1 mg/kg) that is more relevant to those administered by humans. Furthermore, we aimed to evaluate whether the extended administration of THC induces memory deficits that

persist even after the animals have developed tolerance to the locomotor suppressant effects of the drug. In addition, we measured the underlying neuronal substrates that may be responsible for such effects by analysing zif268 expression in the brain.

METHODS

Subjects

Male adult C57BL/6J mice (weighting 23-28g at the beginning of the experiment) were obtained from Charles River (Lyon, France). Animals were housed at 22-23°C under a 12 h light/dark cycle (7:00 a.m. on) with food and water available ad libitum. One week prior to the experiment, animals were separated into individual home cages and handled for 2–3 min/day during all the experiment to minimize non-specific stress. Experiments were performed in accordance with the guidelines on the ethical use of animals from the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Drug treatment

THC (25 mg/ml, Sigma, France) was dissolved at 5 mg/ml in ethanol. This preparation was emulsified in a solution at 2.5 mg of polysorbate 80 (Sigma, France) /ml of ethanol. Ethanol was then evaporated at room temperature using a rotary concentrator-evaporator (SpeedVac®System, ThermoSavant) for 20 min under a pressure of 5.1 bars. The residue was resuspended in NaCl (0.9 %) and sonicated for a final THC concentration of 0.1 mg/ml. The same protocol where ethanol was used in replacement of THC was used to prepare the vehicle (Veh) solution. THC (1 mg/kg) or Veh were administered intraperitoneally (i.p.) at a volume of 10 ml/kg.

Apparatus

Learning in the Morris water maze was evaluated using a pool that measured 150 cm in diameter and 55 cm in height, and that was placed in a room maintained at 23-25 ◦C under constant lighting with many extra-maze cues posted on walls. It was filled with 21±1 ◦C water until 15 cm of the top and made opaque by addition of white non-toxic paint. A hidden platform of 13 cm in diameter was submerged 5 mm below the surface. Its location could be indicated to the animals by placing a 15 cm height and 4 cm of diameter intra-maze cue on the submerged platform. A video camera mounted above the pool was used to record swim trials. Swim speed, escape latency (time to reach the platform), time spent in the target quadrant (where the platform should be during the probe trial) and animals paths were recorded and analysed using an automated tracking system (Videotrack, France).

A different set of animals were then tested for locomotor activity using an activity meter (Imetronic, France). Animals were placed individually in the boxes of the apparatus immediately after injection with THC or Veh, for 1 hour under light exposure and without food or water available.

Procedure of memory testing in the water maze

For this experiment, mice were separated in 2 groups (see Fig. 1). Animals in the non pre-treated (NPT) group were administered daily injections of THC (1 mg/kg) or Veh 30 min before testing in the water maze on days 1-11 (behavioural testing phase). Animals in the pre-treated (PT) group received 13 daily injections of THC (1 mg/kg) or Veh before the behavioural testing phase where, like NPT animals, these animals continued to receive THC or Veh on each day of testing. Therefore, such a design yields 4 treatment groups: PT-Veh, PT-THC, NPT-Veh and NPT-THC.

On this first day of the behavioural testing phase, animals received a pre-training session consisting of 2 *cued trials* where the hidden platform was indicated by a visual intra-maze cue. Then the acquisition started and mice were tested for *spatial reference memory*. Each animal was trained to find the hidden platform kept at the same location for 1 session of 4 trials per day for 9 days. Mice were released from random location and were allowed to swim until they found and climbed on the platform or until a maximum of 90 s has elapsed. If a mouse did not find the platform, it was gently guided to the platform by hand where it was left for 20 s and then dried and returned to its home cage placed in a heating box. Mice were trained in groups of 4 animals, allowing an intertrial interval of 15 min for each mouse. One hour after the end of the last session of the acquisition, mice were tested in a *probe trial* during 60 s where the platform was removed from the water maze.

After 9 days of acquisition, mice were submitted to a *reversal test* where the platform location was moved to the opposite quadrant in order to evaluate the behavioural and cognitive flexibility of the animals. Mice were tested in 2 sessions of 4 trials separated by 24 h. One hour after the end of these reversal trials, mice were again submitted to a *probe trial* during 60 s where the platform was removed from the water maze.

Immunohistochemistry

Home cage control animals (Quiet cont group) did not receive any injection and were not behaviourally tested.

Ninety minutes after the last probe trial, animals were anaesthetised with an i.p. injection of Avertin and perfused transcardially with 100 ml of 4 % paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) at pH 7.4. Brains of animals

kept in their home cages were also harvested as controls. The brains were removed and postfixed in PFA for 20 h. For cryoprotection, brains were placed in 30 % sucrose until they sunk. Brains were then sliced at 50 µm in a frozen microtome.

Tissues were stored in cryoconservation solution at – 20 °C until the staining was performed. For immunohistochemistry, free floating sections were washed 4 times for 10 min in PB before being incubated in 0.5 % hydrogen peroxide for 30 min. After 4 washes in 0.9 % NaCl, sections were incubated in 1 % bovine serum albumin, 0.2 % Triton X100 (PB-BSA-TX) for 30 min at room temperature. Sections were then stained with the zif268 primary antibody (Santa Cruz Biotechnology) diluted at 1:5,000 in PB-BSA-TX for 24 h at 4 °C under shaking. After 4 washes in PB containing 0.2 % Triton X100 (PB-TX), sections were incubated in biotinylated anti-rabbit secondary antibody (Jackson ImmunoResearch) diluted at 1:2,000 in PB-BSA-TX for 2 h at room temperature while undergoing shaking. Sections were washed again 4 times in PB-TX and transferred to an avidin-biotin-peroxydase solution (Sigma, France) for 2 h at room temperature and protected from the light. Sections were washed 4 times in PB and 3 times in 0.05 M Tris HCL (pH 7.5). The peroxidase activity was visualised by placing the tissue in 0.037 % diaminobenzidine tetrahydrochloride diluted in Tris-HCl and 0.015 % hydrogen peroxidase for 5 to 15 min protected from light. The reaction was terminated by 4 washes in 0.05 M Tris HCl and tissues were stored in PB containing 0.2 g/l Azide (Sigma). Sections were then mounted onto slides, air dried, dehydrated in toluene and coverslipped. Quantitative densitometry was performed on the images of digitized brain sections using Biocom Visiolab 2000 software. Each structure was counted 5 to 8 times depending on the quality of the section. Results are indicated by number of reactive cells by unit of surface.

Statistical analysis

Data from the water maze were analysed using two-way repeated measures analysis of variance (ANOVA) where the between subjects factors were treatment (Veh or THC) and group (PT or NPT), and trial or session (day) as the repeated measure. When needed, data were examined for each day separately by two-way ANOVA and further analysed using Tukey's post hoc test. Data from locomotor activity were analysed using one-way repeated measures ANOVA where the between subjects factor was treatment (Veh or THC), and day as the repeated measure. Data from immunohistochemistry were analysed using a student t test. A significant level of $P < 0.05$ was chosen for all comparisons.

RESULTS

Swim speed

As cannabinoids have sedative effects, we analysed the swim speed of the animals during all phases of the water maze test to assess if motor impairment would persist when animals were placed in the water and forced to swim (data not shown). No overall effect of THC treatment was observed on swim speed over days of testing [$F(1,44) = 1.32$; $P = 0.26$] whether the animals were pre-treated or not. When analysing the different days separately, no effect of THC on swim speed was observed as measured on the first trial of each day. As observed during the tasks, all animals were able to swim and climb onto the platform. Taken together, these results show that THC treatment did not alter the swimming capability of the animals.

Cued trials

On the first day of behavioural testing before the beginning of the acquisition task, animals received a pre-training session consisting of 2 cued trials where the platform was indicated by a visual cue (Fig. 2). A trend for THC treatment to increase escape latency was observed during the first cued trial [$F(1,44) = 2.88$, $P = 0.097$]. Tukey's post hoc test revealed that THC only reduced escape latency in NPT mice ($P < 0.05$). However, this effect was transient as it was not observed on the second trial and all animals were able to perform the task suggesting that THC did not impair visual ability and the procedural aspect of the experiment.

Acquisition of the spatial reference memory task

Escape latencies for all sessions (average of the 4 trials per day) of acquisition and reversal are shown in Fig. 3. During acquisition, animals learnt where to find the platform as highlighted by a decreased escape latency over days [session: $F(8, 352) = 58.27$, $P < 0.001$]. Interestingly, THC increased escape latency over days [treatment: $F(1, 44) = 9.62$, $P < 0.01$] and a treatment by session interaction was also observed [$F(8, 352) = 2.29$, $P < 0.05$]. To increase the accuracy of these results, we then analysed each session separately. We observed that THC impaired acquisition of the task on days 1 [$F(1, 44) = 8.13$, $P < 0.01$], 2 [$F(1, 44) = 8.14$, $P < 0.01$] and 3 [$F(1, 44) = 5.17$, $P < 0.05$], with no difference observed on the other days. These results show that, independently of whether they received a pre-treatment or not, THC-treated animals learnt the location of the platform slower than Veh-treated animals.

Reversal of the spatial memory

During the reversal phase (Fig. 3), the platform location was changed to assess learning flexibility of the animals. On the first session of the reversal on day 10, all animals showed an increased latency to reach the platform compared to the last session of acquisition as they had to learn a new position of the platform. This is highlighted by a session effect between day 9 and 10 [$F(1, 44) = 260.83$, $P < 0.001$]. On the second session of reversal (day 11), the escape latency of all animals decreased compared to the first one, as shown by an effect of the session between days 10 and 11 [$F(1, 44) = 27.34$, $P < 0.001$]. This shows that the animals started to learn the new location of the platform. Interestingly, while no effect of THC was observed on the last days of acquisition, the reversal of the platform triggered a learning deficit in THC-treated animals during the reversal [treatment: $F(1, 44) = 5.93$, $P < 0.05$]. When analysing the sessions of reversal separately, a treatment effect was observed on day 11 [$F(1, 44) = 6.03$, $P < 0.05$], showing that THC impaired performance in the reversal phase, reducing the ability of the animals to learn the new location of the platform. Similarly to the beginning of the acquisition, THC treatment impaired the escape latency whether the animals received a pre-treatment or not.

To gain a more detailed insight into our results, we then analysed the different trials of the 2 sessions of reversal separately (Fig. 4). A trend for an effect of THC treatment was observed on the second trial of the first session of reversal [$F(1, 44) = 3.01$, $P = 0.09$] but not on the first trial as animals had to find the new location of the platform. This THC-induced impairment in performance observed on the second trial of the first day of reversal was accentuated on the second day, as highlighted by an effect of THC treatment on trial 2 [$F(1, 44) = 5.19$, $P < 0.05$] and 4 [$F(1, 44) = 5.31$, P

< 0.05] of the second session. These results are consistent with the interpretation that THC impaired working memory during the reversal.

Probe trials

The results from the probe trials are shown in Fig. 5. The first probe trial was done 1 hour after the end of the acquisition on day 9. The platform was removed from the water maze and we measured the time spent in the target quadrant where the platform was formerly located during the acquisition. Consistent with results from escape latencies on the last days of acquisition, all animals spent the same time in the target quadrant as shown by the absence of treatment effect [$F(1, 44) = 0.12$, $P = 0.73$]. In addition, all animals were above chance level in the time spent exploring the target quadrant ($P < 0.001$), showing that all animals had learnt the location of the platform at the end of the acquisition (Fig.5A; P1 TQ1). We also measured the latency for the first platform location crossing (Fig. 5B). In the first probe test, we observed that THC-treated mice exhibited a significant increased latency to cross the platform compared to Veh-treated animals [$F(1, 44) = 5.3$, $P < 0.05$] and this effect was independent of the treatment condition [$F(1, 44) = 2.53$, $P = 0.12$].

One hour after the end of the second session of reversal on day 11, a second probe trial was done to compare whether animals swam in the new target quadrant or in the old one where the platform was during the acquisition. No effect of THC was observed on the time spent in the old target quadrant [$F(1, 44) = 0.47$, $P = 0.49$]. In addition, as shown in Fig.5A (P2 TQ1) all animals irrespective of treatment group explored this quadrant below chance level. This shows that all mice stopped exploring the quadrant where the platform was located during the acquisition. In the new target quadrant, THC increased the latency to first cross the location of the platform [F(1,

44) = 4.8, $P < 0.05$] (Fig 5B; probe test 2). Supporting this result, a trend for THC to decrease the time spent in the new target quadrant was observed [F(1, 44) = 3.38, $P = 0.07$] (Fig.5A; P2 newTQ). Importantly, Veh-treated animals spent more time (above chance level) in the new target quadrant than in the old one [F(1, 22) = 6.09, $P < 0.05$], showing that they have started to learn the new location of the platform. However, THC-treated animals were at chance level in both the new and the old quadrants. Taken together, these results show that THC impaired reversal learning in the water maze.

Locomotor activity

We also measured locomotor activity using another set of animals (Fig. 6). No difference was observed between the animals on day 0 prior to the beginning of the drug administration. Using one-way repeated measure ANOVA, we showed that THC treatment promoted an overall decreased locomotor activity over days [F(1, 54) = 5.48, $P < 0.05$]. The analysis of each days separately showed that THC reduced locomotor activity on days 2 [F(1,9) = 14.84, $P < 0.01$], 3 [F(1,9) = 5.94, $P < 0.05$] and 4 [F(1,9) = 6.84, $P < 0.05$]. From the fifth day of treatment, no difference was seen between Veh- and THC-treated mice, showing that animals became tolerant to the locomotor suppressant effect of THC.

Immunohistochemistry

The number of zif268 immunoreactive cells for the different regions are shown in Fig. 7. Analysis with student's t-test showed that in animals that were not pre-treated (NPT-Veh compared to NPT-THC), THC decreased zif268 expression in the CA3 of the hippocampus ($P < 0.001$), in the dorsomedial ($P < 0.01$) and dorsolateral ($P <$

0.05) part of the caudate putamen and in the prefrontal cortex ($P < 0.05$). No effect of THC was observed in animals that received pre-treatment with THC (PT-Veh compared to PT-THC).

DISCUSSION

The main results from the present study are that animals treated with a relatively low dose of THC, whether they have been pre-treated or not, had a reduced learning of the spatial reference memory task. However, they eventually acquired the task like control animals. During the reversal phase, while both NPT and PT animals were tolerant to the locomotor suppressant effects of THC, the drug impaired cognitive flexibility of the animals as they did not learn the new location of the platform compared to control. This highlights a long-lasting cognitive deficit in these animals, as even after 24 days of exposure to the drug, PT mice still had not developed tolerance to this effect. Immunohistochemical analysis revealed that THC decreased zif268 expression in the CA3 of the hippocampus, in the prefrontal cortex and in the dorsomedial and dorsolateral caudate putamen only in animals that did not received a pre-treatment, with no effect seen in PT animals.

At the beginning of the acquisition, animals were trained in 2 cued trials where the location of the platform was indicated by a visual cue. No significant difference between Veh- and THC-treated animals was observed, showing that all animals were able to find the platform. This result, together with the absence of difference in swim speed in animals treated with THC compared to Veh, suggests that THC did not induce any visual or locomotor impairment in mice exploring the water maze. These results are consistent with studies showing that, while cannabinoids impair learning in

the place version of the water maze, no deficit was observed in the cued version of the water maze task using the visible platform (Ferrari et al., 1999, Niyuhire et al., 2007).

Following the 2 cued trials, acquisition of the reference memory task began. Animals received 4 trials per session over 9 consecutive days where the hidden platform remained in a fixed position. We observed a slower acquisition of spatial memory performance in animals treated with THC, irrespective of whether these animals were pre-treated with THC for 13 days or not. Cannabinoids like HU 210 (Ferrari et al., 1999) and THC (Da and Takahashi, 2002) have previously been shown to impair learning in the place version of the water maze task. By the ninth day, no difference was observed between Veh- and THC-treated groups, showing that all animals had learnt the task. These results demonstrate that THC impaired the acquisition of the spatial reference memory, which was overcome with repeated learning sessions.

For the reversal testing, the location of the platform was changed but the procedural component of the task (to swim to the platform to escape) remained unmodified. Thus animals had to extinguish the original spatial response and learn a new one by making new associations between the novel platform location and the surrounding spatial cues (Rossato et al., 2006). On the first trial of the first day of reversal (day 11) an increased time to reach the platform was observed for all animals, as they had to find the new location of the platform. Interestingly on the second trial of this day, THC-treated animals were delayed in finding the platform compared to Veh-treated animals, and this effect was accentuated on the second day of reversal (see Fig. 4). Using acute doses of THC higher than in the present study, working memory deficits have been reported both in rats and mice (Lichtman and Martin, 1996, Varvel et al., 2001). In a similar paradigm to ours, THC decreased spatial

working memory on the second trial of the delayed-matching-to-place paradigm in the water maze when administered at 2 and 5 mg/kg (Fadda et al., 2004). In line with these data, our own study, by using a relatively low dose of THC more comparable to human dosing, demonstrated working memory impairment as evidenced by reversal deficit. Indeed, as illustrated by our data, instead of promoting perseveration responses (see below) THC appeared to alter the re-actualization of the information, this capacity of flexibility being one of the properties of working memory.

The first probe trial carried out one hour after the last acquisition trial showed that all mice explored the target quadrant above chance level, confirming that they successfully learnt the spatial reference memory task. However, although the difference between Veh- and THC-treated animals is quite moderate, the significantly higher latency to the first platform crossing implies a less accurate spatial response in THC-treated mice. On the second probe trial at the end of the reversal on day 11, Veh-treated animals spent more time in the new target quadrant than THC-treated animals, and above chance level. This illustrates that Veh-treated animals were again learning the new location of the platform faster than THC-treated mice. This further strengthens our interpretation that THC impaired behavioural flexibility in this model. As THC-treated animals did not learn the new platform location this deficit in reversal might be due to an increased perseverance of the animals in returning to the previously learnt platform location. However our results showed that THC-treated animals that spent less time in the new target quadrant did not spend more time in the former target quadrant. Therefore, THC-induced deficits in reversal learning are most likely explained by a specific deficit in learning the new platform location.

Interestingly during both the reference memory and the reversal tasks, no difference was observed between THC-treated PT and NPT animals, showing no

differential effect of the pre-treatment on learning. With repeated administration, tolerance to the effects of cannabinoids such as locomotor suppression or hypothermia develops rapidly (Oliva et al., 2003), however effects on memory impairment appear to be more resistant to the development of tolerance (for a review see Gonzalez et al., 2005). We analysed the effect of repeated THC exposure on locomotor activity and observed that animals developed, tolerance to the locomotor suppressant effects of 1mg/kg THC after 5 days of treatment. Thus, THC-induced deficits on reference memory cannot be related to the sedative effects of cannabinoids, as NPT and PT animals exhibited the same level of performance on day 1 of acquisition. In conjunction with the absence of effects of the drug on swim speed, this strengthens the interpretation that this relatively low dose of THC promoted a specific memory deficit.

While the acute effects of cannabinoids on memory are widely reported, the effects of long term cannabinoid exposure have received less attention. Interestingly, the memory deficits observed during the reversal phase were independent of whether the animals received 13 days of pre-treatment prior to the beginning of the acquisition or not. This suggests that even after 24 days of treatment for the PT group and 11 days of treatment for the NPT group, THC was still effective in impairing memory. To our knowledge, the present study is the first to report the absence of development of tolerance to cannabinoid-induced working memory impairments in mice. These results are consistent with studies using rats reporting that THC administered at 5 mg/kg impaired working memory in the radial arm maze even after 90 days of administration 6 days per week (Nakamura et al., 1991). A 2-weeks pre-treatment with the same dose of THC injected twice daily did not modify the effect of a challenging dose of 2.5 mg/kg THC on impairment of correct alternation task in the

T-maze showing that animals did not develop tolerance to working memory deficits (Nava et al., 2001). The spatial working memory impairments observed with 2 and 5 mg/kg THC-rich extracts were also observed over months of dosing with brief periods of washout (Fadda et al., 2004). When tolerance was observed, it only appeared after 30 days of exposure to relatively high doses of both THC (10 mg/kg) and the cannabinoid receptor agonist WIN 55,212-2 in delayed-match-to-sample and delayed-non match-to-sample tasks, respectively (Deadwyler et al., 1995, Hampson et al., 2003). Thus consistent with previous studies, the results from the present study show that memory impairments promoted by THC, even at such a relatively low dose, are resistant to the development of tolerance. It would be interesting to examine if tolerance could be observed to the memory impairing effects with such a low dose of THC and how many days of administration beyond the 24 days examined here, would be required.

In NPT mice, THC decreased zif268 expression in hippocampal CA3 and to a lesser extent in the CA1, which is consistent with the view that the hippocampus mediates the memory impairing effects of cannabinoids (Lichtman et al., 1995, Misner and Sullivan, 1999, Hampson and Deadwyler, 2000, Egashira et al., 2002). Interestingly, THC exposure also reduced the expression of zif268 in the prefrontal cortex. This region is another site thought to participate in cannabinoid-induced spatial memory impairment. For example, direct administration of THC into the medial prefrontal cortex has been shown to disrupt spatial working memory (Silva de Melo et al., 2005). Further, repeated THC administration in rats caused a persistent reduction in medial prefrontal cortical dopamine turnover which was assumed to subserve in part the cognitive dysfunction observed in these animals (Verrico et al., 2003). Because memory processes are dependent on synaptic plasticity in the

hippocampus and prefrontal cortex (Laroche et al., 2000, Riedel and Davies, 2005), these results suggest that the THC-induced impairment of working memory observed here could be mediated by a decreased neuronal plasticity in both the hippocampus and prefrontal cortex as measured by zif268.

The decreased zif268 expression in the hippocampus and prefrontal cortex in NPT animals vanished in PT animals, although the latter did exhibit cognitive impairments which were sometimes even more marked as assessed by the probe tests (Fig. 5). These results suggest that between 11 and 24 days of treatment, some cellular and molecular determinants of tolerance began developing which are dissociable from the memory impairing effect of THC. Interestingly, 2 week chronic THC treatment did not produce tolerance to CB1-mediated inhibition of extracellular hippocampal acetylcholine and working memory, but there was a time-course difference between these two phenomena suggesting they might be dissociated (Nava et al., 2001).

Similar to the effects of THC on the hippocampus and prefrontal cortex, THC also decreased zif268 expression in the dorsomedial and dorsolateral caudate putamen only in NPT animals. The dorsal striatum is not reported to be implicated in spatial memory (Egashira et al., 2002), but recent reports have emphasized a role of the medial striatum in behavioural flexibility (McDonald et al., 2008). The behavioural effects of THC on striatal function seem to fit better with this interpretation as the PT animals were tolerant to the locomotor suppressant effect of THC at the time of testing and no deficits were observed in the cued trials of the water maze task, a task supposed to be mediated by the caudate putamen (Packard and Teather, 1998).

The present study examined for the first time the effects of repeated cannabinoid exposure on zif268 expression in the mouse forebrain. In prior *in situ* hybridisation studies, acute cannabinoid receptor agonist exposure increased the

expression of zif268 mRNA in the CA1 and CA3 regions of the hippocampus (Derkinderen et al., 2003), cingulate cortex, fronto-parietal cortex and the caudate-putamen (Mailleux et al., 1994). Consistent with this, (Glass and Dragunow, 1995) showed in an immunohistochemical study that acute administration of a high dose of CP 55,940 (2.5 mg/kg) significantly increased zif268 in the striatum (CP 55,940 is approximately 30 times as potent as THC, see (Arnold et al., 2001a, Arnold et al., 2001b). Given that acutely cannabinoids induce zif268 expression, the decreased zif268-labelled cells we observed here indicates that zif268 expression is inversely regulated by repeated cannabinoid exposure. Indeed, such a phenomenon has been reported for the psychostimulant drug, cocaine. Acutely cocaine increased zif268 expression in the rat forebrain, however with repeated administration it decreased basal zif268 expression (Bhat et al., 1992).

In conclusion, this study showed that repeated cannabinoid exposure induced persistent memory deficits which are resistant to tolerance and may be, at least initially, regulated by diminished expression of the transcription factor zif268.

REFERENCES

Arnold, J. C., Hunt, G. E. and McGregor, I. S., 2001a. Effects of the cannabinoid receptor agonist CP 55,940 and the cannabinoid receptor antagonist SR 141716 on intracranial self-stimulation in Lewis rats. Life Sci. 70, 97-108.

Arnold, J. C., Topple, A. N., Mallet, P. E., Hunt, G. E. and McGregor, I. S., 2001b. The distribution of cannabinoid-induced Fos expression in rat brain: differences between the Lewis and Wistar strain. Brain Res. 921, 240-255.

Bhat, R. V., Cole, A. J. and Baraban, J. M., 1992. Chronic cocaine treatment suppresses basal expression of zif268 in rat forebrain: in situ hybridization studies. J Pharmacol Exp Ther. 263, 343-349.

Bozon, B., Davis, S. and Laroche, S., 2002. Regulated transcription of the immediate-early gene zif268: mechanisms and gene dosage-dependent function in synaptic plasticity and memory formation. Hippocampus. 12, 570-577.

Da, S. and Takahashi, R. N., 2002. SR 141716A prevents Δ^9-THC-induced spatial learning deficit in a Morris-type water maze in mice. Prog Neuropsychopharmacol Biol Psychiatry. 26, 321-325.

Deadwyler, S. A., Heyser, C. J. and Hampson, R. E., 1995. Complete adaptation to the memory disruptive effects of Δ^9-THC following 35 days of exposure. Neurosci Res Commun. 17, 9-18.

Derkinderen, P., Valjent, E., Toutant, M., Corvol, J. C., Enslen, H., Ledent, C., Trzaskos, J., Caboche, J. and Girault, J. A., 2003. Regulation of extracellular signal-regulated kinase by cannabinoids in hippocampus. J Neurosci. 23, 2371-2382.

Egashira, N., Mishima, K., Iwasaki, K. and Fujiwara, M., 2002. Intracerebral microinjections of Δ^9-THC: search for the impairment of spatial memory in the eight-arm radial maze in rats. Brain Res. 952, 239-245.

Egerton, A., Brett, R. R. and Pratt, J. A., 2005. Acute Δ^9-THC -induced deficits in reversal learning: neural correlates of affective inflexibility. Neuropsychopharmacology. 30, 1895-1905.

Fadda, P., Robinson, L., Fratta, W., Pertwee, R. G. and Riedel, G., 2004. Differential effects of THC- or CBD-rich cannabis extracts on working memory in rats. Neuropharmacology. 47, 1170-1179.

Ferrari, F., Ottani, A., Vivoli, R. and Giuliani, D., 1999. Learning impairment produced in rats by the cannabinoid agonist HU 210 in a water-maze task. Pharmacol Biochem Behav. 64, 555-561.

Fletcher, J. M., Page, J. B., Francis, D. J., Copeland, K., Naus, M. J., Davis, C. M., Morris, R., Krauskopf, D. and Satz, P., 1996. Cognitive correlates of long-term cannabis use in Costa Rican men. Arch Gen Psychiatry. 53, 1051-1057.

Glass, M. and Dragunow, M., 1995. Induction of the krox 24 transcription factor in striosomes by a cannabinoid agonist. Neuroreport. 6, 241-244.

Gonzalez, S., Cebeira, M. and Fernandez-Ruiz, J., 2005. Cannabinoid tolerance and dependence: a review of studies in laboratory animals. Pharmacol Biochem Behav. 81, 300-318.

Hampson, R. E. and Deadwyler, S. A., 2000. Cannabinoids reveal the necessity of hippocampal neural encoding for short-term memory in rats. J Neurosci. 20, 8932-8942.

Hampson, R. E., Simeral, J. D., Kelly, E. J. and Deadwyler, S. A., 2003. Tolerance to the memory disruptive effects of cannabinoids involves adaptation by hippocampal neurons. Hippocampus. 13, 543-556.

Hill, M. N., Froese, L. M., Morrish, A. C., Sun, J. C. and Floresco, S. B., 2006. Alterations in behavioral flexibility by cannabinoid CB1 receptor agonists and antagonists. Psychopharmacology (Berl). 187, 245-259.

Jentsch, J. D., Verrico, C. D., Le, D. and Roth, R. H., 1998. Repeated exposure to Δ^9-THC reduces prefrontal cortical dopamine metabolism in the rat. Neurosci Lett. 246, 169-172.

Kanayama, G., Rogowska, J., Pope, H. G., Gruber, S. A. and Yurgelun-Todd, D. A., 2004. Spatial working memory in heavy cannabis users: a functional magnetic resonance imaging study. Psychopharmacology (Berl). 176, 239-247.

Laroche, S., Davis, S. and Jay, T. M., 2000. Plasticity at hippocampal to prefrontal cortex synapses: dual roles in working memory and consolidation. Hippocampus. 10, 438-446.

Lichtman, A. H., Dimen, K. R. and Martin, B. R., 1995. Systemic or intrahippocampal cannabinoid administration impairs spatial memory in rats. Psychopharmacology (Berl). 119, 282-290.

Lichtman, A. H. and Martin, B. R., 1996. Δ^9-THC impairs spatial memory through a cannabinoid receptor mechanism. Psychopharmacology (Berl). 126, 125-131.

Mailleux, P., Verslype, M., Preud'homme, X. and Vanderhaeghen, J. J., 1994. Activation of multiple transcription factor genes by THC in rat forebrain. Neuroreport. 5, 1265-1268.

McDonald, R. J., King, A. L., Foong, N., Rizos, Z. and Hong, N. S., 2008. Neurotoxic lesions of the medial prefrontal cortex or medial striatum impair multiple-location place learning in the water task: evidence for neural structures with complementary roles in behavioural flexibility. Exp Brain Res. 187, 419-427.

Messinis, L., Kyprianidou, A., Malefaki, S. and Papathanasopoulos, P., 2006. Neuropsychological deficits in long-term frequent cannabis users. Neurology. 66, 737-739.

Misner, D. L. and Sullivan, J. M., 1999. Mechanism of cannabinoid effects on long-term potentiation and depression in hippocampal CA1 neurons. J Neurosci. 19, 6795-6805.

Nakamura, E. M., da Silva, E. A., Concilio, G. V., Wilkinson, D. A. and Masur, J., 1991. Reversible effects of acute and long-term administration of Δ^9-THC on memory in the rat. Drug Alcohol Depend. 28, 167-175.

Nava, F., Carta, G., Colombo, G. and Gessa, G. L., 2001. Effects of chronic Δ^9-THC treatment on hippocampal extracellular acetylcholine concentration and alternation performance in the T-maze. Neuropharmacology. 41, 392-399.

Niyuhire, F., Varvel, S. A., Martin, B. R. and Lichtman, A. H., 2007. Exposure to marijuana smoke impairs memory retrieval in mice. J Pharmacol Exp Ther. 322, 1067-1075.

Oliva, J. M., Ortiz, S., Palomo, T. and Manzanares, J., 2003. Behavioural and gene transcription alterations induced by spontaneous cannabinoid withdrawal in mice. J Neurochem. 85, 94-104.

Packard, M. G. and Teather, L. A., 1998. Amygdala modulation of multiple memory systems: hippocampus and caudate-putamen. Neurobiol Learn Mem. 69, 163-203.

Riedel, G. and Davies, S. N., 2005. Cannabinoid function in learning, memory and plasticity. Handb Exp Pharmacol, 445-477.

Rog, D. J., Nurmikko, T. J., Friede, T. and Young, C. A., 2005. Randomized, controlled trial of cannabis-based medicine in central pain in multiple sclerosis. Neurology. 65, 812-819.

Rossato, J. I., Zinn, C. G., Furini, C., Bevilaqua, L. R., Medina, J. H., Cammarota, M. and Izquierdo, I., 2006. A link between the hippocampal and the striatal memory systems of the brain. An Acad Bras Cienc. 78, 515-523.

Silva de Melo, L. C., Cruz, A. P., Rios Valentim, S. J., Jr., Marinho, A. R., Mendonca, J. B. and Nakamura-Palacios, E. M., 2005. Δ^9-THC administered into the medial prefrontal cortex disrupts the spatial working memory. Psychopharmacology (Berl). 183, 54-64.

Smith, P. F., 2005. The safety of cannabinoids for the treatment of multiple sclerosis. Expert Opin Drug Saf. 4, 443-456.

Solowij, N., Michie, P. T. and Fox, A. M., 1995. Differential impairments of selective attention due to frequency and duration of cannabis use. Biol Psychiatry. 37, 731-739.

Solowij, N., Stephens, R. S., Roffman, R. A., Babor, T., Kadden, R., Miller, M., Christiansen, K., McRee, B. and Vendetti, J., 2002. Cognitive functioning of long-term heavy cannabis users seeking treatment. Jama. 287, 1123-1131.

Varvel, S. A., Anum, E., Niyuhire, F., Wise, L. E. and Lichtman, A. H., 2005. Δ^9-THC-induced cognitive deficits in mice are reversed by the GABA(A) antagonist bicuculline. Psychopharmacology (Berl). 178, 317-327.

Varvel, S. A., Hamm, R. J., Martin, B. R. and Lichtman, A. H., 2001. Differential effects of Δ^9-THC on spatial reference and working memory in mice. Psychopharmacology (Berl). 157, 142-150.

Verrico, C. D., Jentsch, J. D. and Roth, R. H., 2003. Persistent and anatomically selective reduction in prefrontal cortical dopamine metabolism after repeated, intermittent cannabinoid administration to rats. Synapse. 49, 61-66.

Whitlow, C. T., Freedland, C. S. and Porrino, L. J., 2003. Functional consequences of the repeated administration of Δ^9-THC in the rat. Drug Alcohol Depend. 71, 169-177.

Yucel, M., Solowij, N., Respondek, C., Whittle, S., Fornito, A., Pantelis, C. and Lubman, D. I., 2008. Regional brain abnormalities associated with long-term heavy cannabis use. Arch Gen Psychiatry. 65, 694-701.

FIGURES AND LEGENDS

Figure 1. Protocol

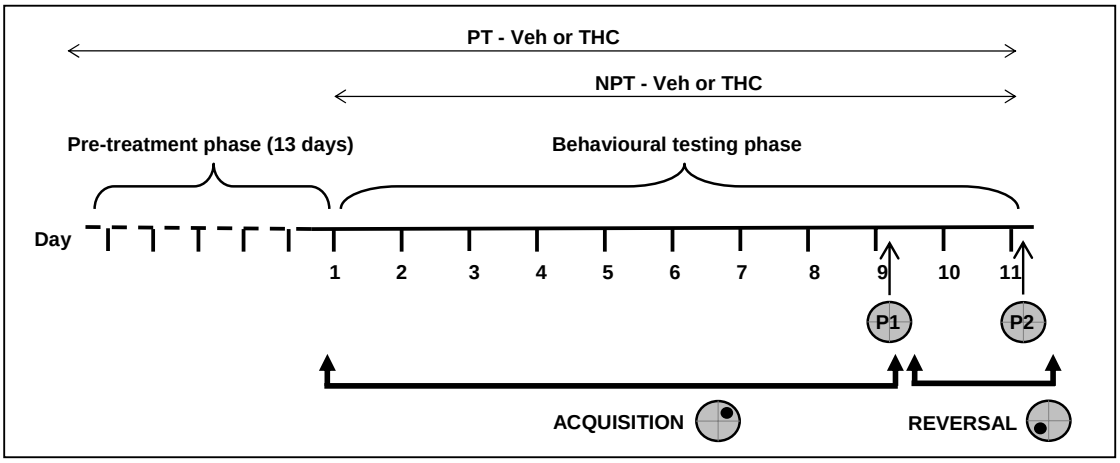


Figure 2. Cued trials.

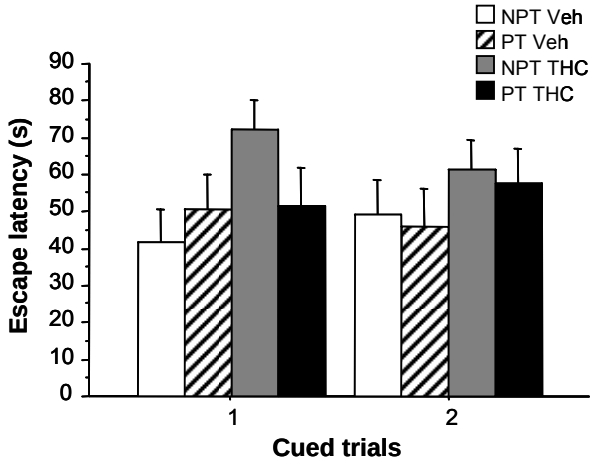


Figure 3. Spatial memory.

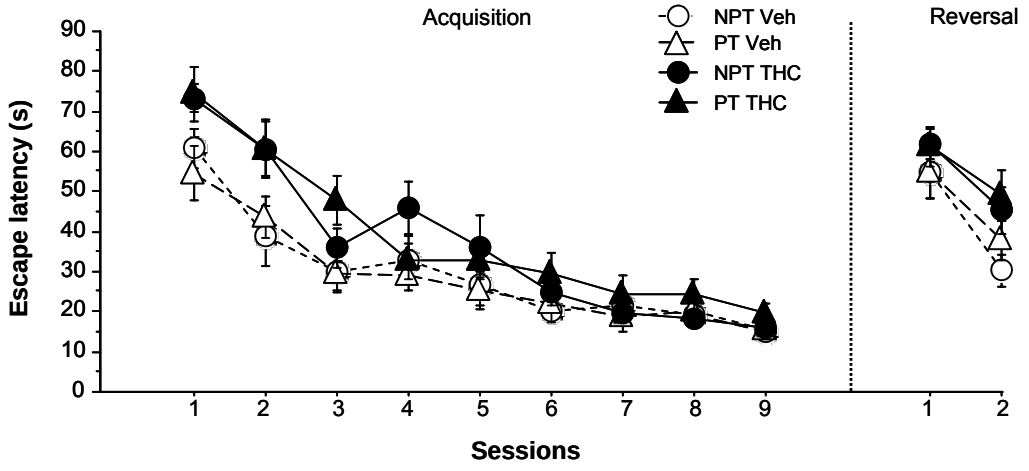


Figure 4. Reversal.

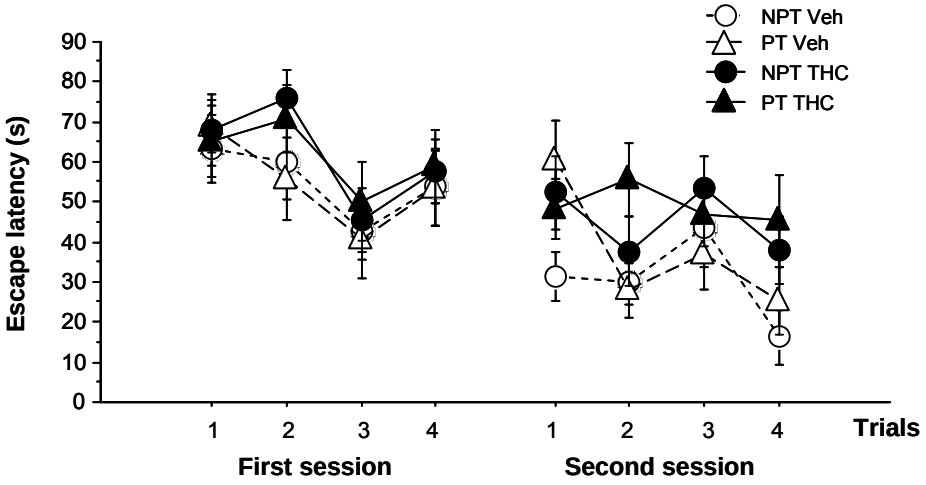


Figure 5. Probe trials.

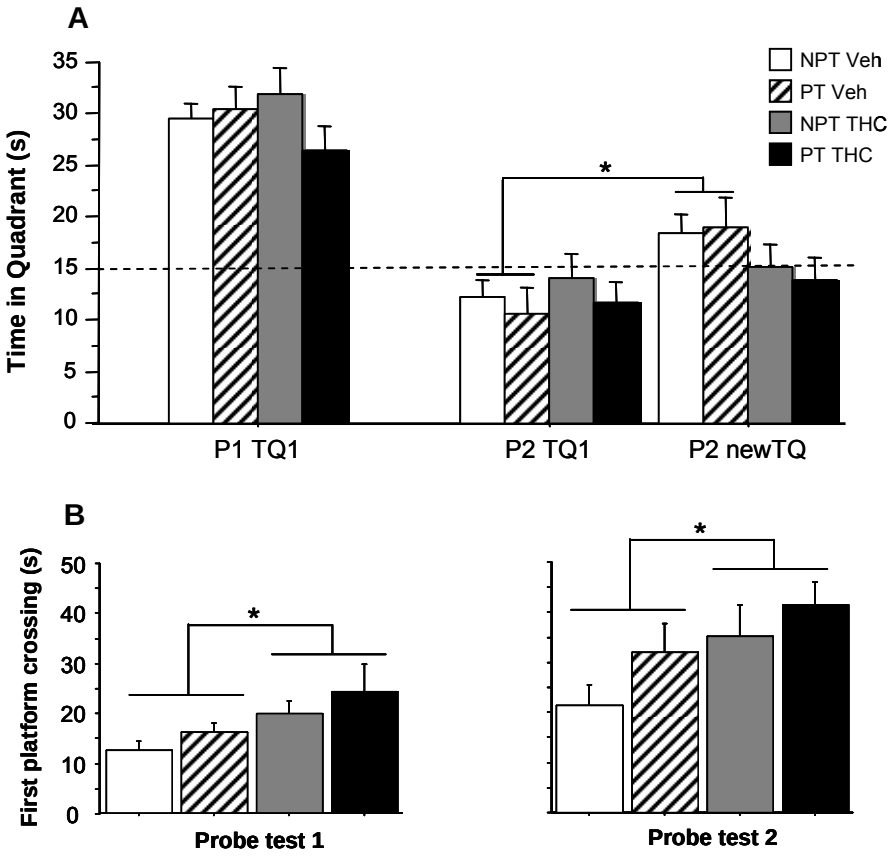


Figure 6. Locomotor activity.

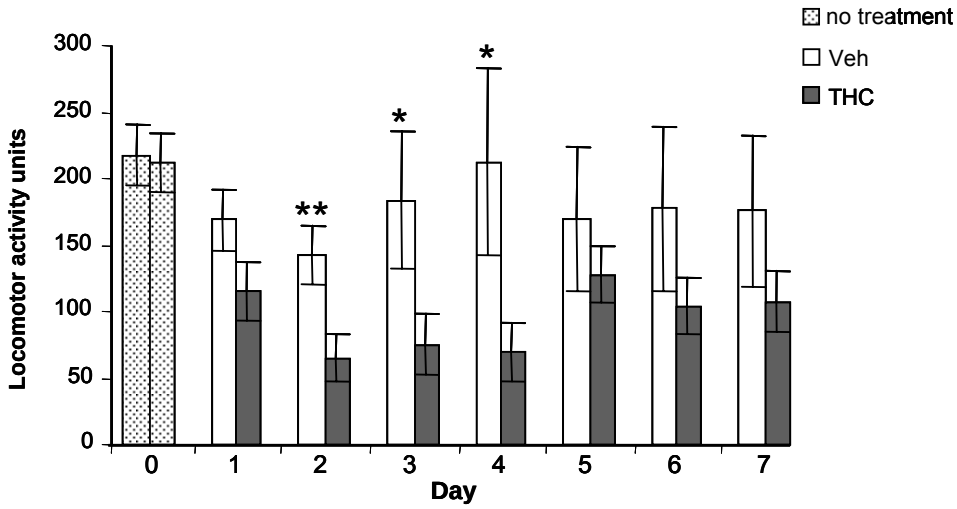
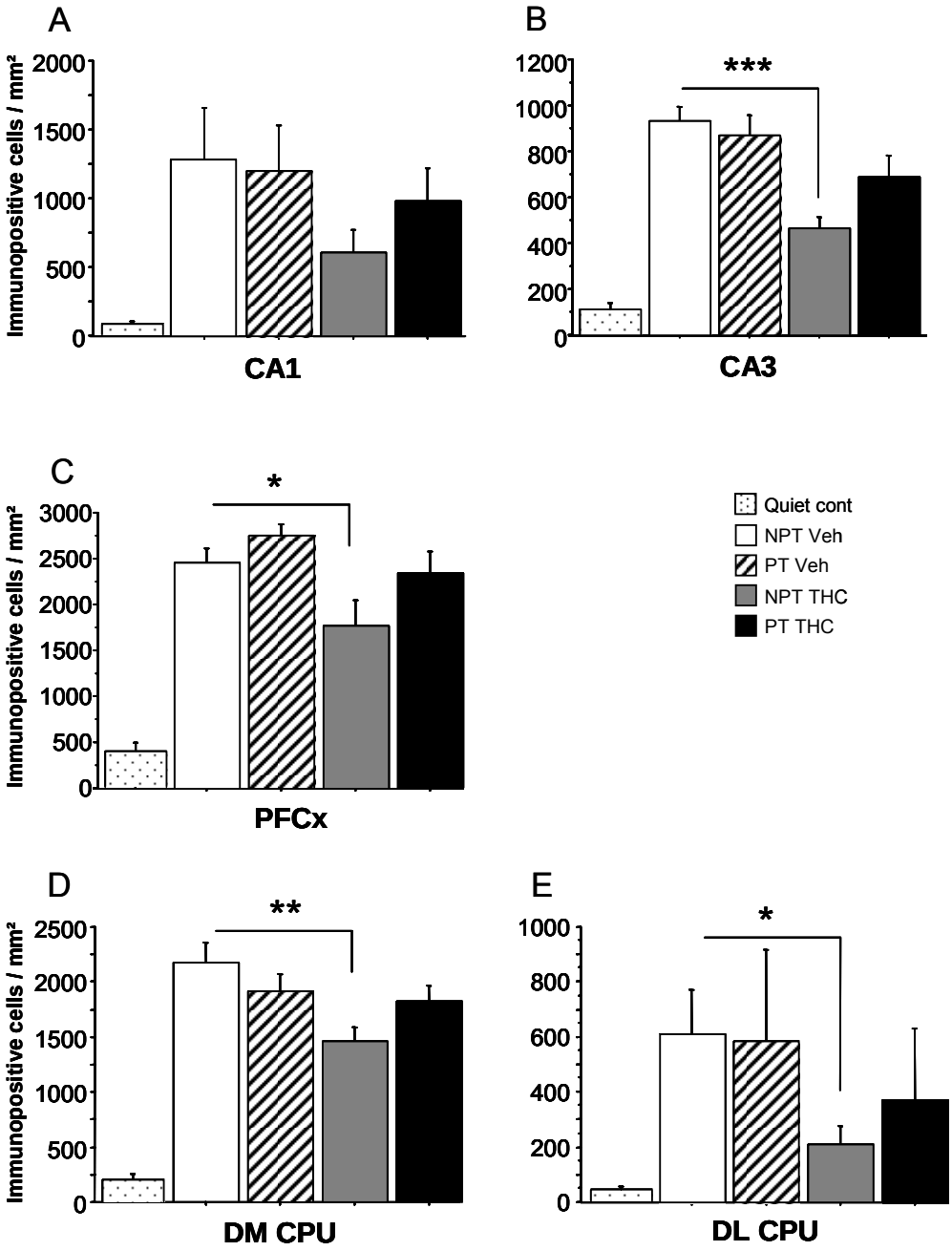


Figure 7. Zif268 immunohistochemistry.



LEGENDS.

Figure 1. Animals were separated in 2 groups: non pre-treated (NPT) mice were administered with Veh or THC (1 mg/kg) during the behavioural testing phase in the water maze (n = 12 per group). Pre-treated (PT) animals were administered Veh or THC (1 mg/kg) for 13 days before the behavioural testing phase and, like the NPT group, until the end of the experiment (n = 12 per group). The behavioural testing phase consisted in 9 days of acquisition of the spatial reference memory task where the location of the platform remained constant, followed by 2 days of reversal where the location of the platform was changed. Animals were also tested in the first probe trial (P1) at the end of the acquisition on day 9, and for the second probe trial (P2) at the end of the reversal on day 11.

Figure 2. Escape latency in the 2 cued trials prior to the beginning of the acquisition on the first day of the behavioural testing phase in animals (n = 12 per group) pre-treated (PT) or non pre-treated (NPT) with either Veh or THC (1 mg/kg). Data are presented as means ± SEM.

Fig. 3. Escape latency for each session (4 trials per day) of acquisition of the spatial memory task (day 1-9) and reversal of the platform location (day 10-11) in animals (n = 12 per group) pre-treated (PT) or non pre-treated (NPT) with either Veh or THC (1 mg/kg). Data are presented as means ± SEM.

Figure 4. Detail of the 4 trials of the 2 sessions of reversal in animals (n = 12 per group) pre-treated (PT) or non pre-treated (NPT) with either Veh or THC (1 mg/kg). Data are presented as means ± SEM.

Figure 5. A) Time spent in the different target quadrants (TQ) during the 2 probe trials. The first probe (P1) occurred 1h after the end of the acquisition (i.e. on day 9) and the second probe (P2) occurred 1h after the end of the reversal (i.e. on day 11). B) Latency to cross the platform location for the first time in animals (n = 12) pre-treated (PT) or non pre-treated (NPT) with either Veh or THC (1 mg/kg). Data are presented as means ± SEM. (Means ± SEM). Significant effects are indicated by ⋆ ($P < 0.05$). The doted line represents chance level.

Figure 6. Tolerance to locomotor activity in animals (n = 5-6) treated with Veh or THC (1 mg/kg). Data are presented as means ± SEM. Significant effects are indicated by ⋆ ($P < 0.05$) and ⋆⋆ ($P < 0.01$).

Figure 7. Number of zif268 immunopositive cells in (A) CA1 and (B) CA3 of the hippocampus, (C) the prefrontal cortex (PFCx) and (D) the dorsomedial (DM) and (E) the dorsolateral (DL) caudate putamen (CPU) in animals (n = 6 per group) were pre-treated (PT) or non pre-treated (NPT) with either Veh or THC (1 mg/kg). Animals from "Quiet cont" group did not receive any injection and were not trained (n = 6). Data are presented as means ± SEM. Significant effects are indicated by ⋆ ($P < 0.05$), ⋆⋆ ($P < 0.01$) and ⋆⋆⋆ ($P < 0.001$).