Assessment of genetic and environmental factors in animal models of schizophrenia

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ASSESSMENT OF GENETIC AND ENVIRONMENTAL FACTORS IN ANIMAL MODELS OF SCHIZOPHRENIA

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"Realists do not fear the results of their study."

-- ***Fyodor Mikhaylovich Dostoevsky (1821-1881)***

To:

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and

Fred Tibuhwa Yafesi 'Akiiki' Bitanihirwe (R.I.P. 1947 - 2011)

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LIST of ABBREVIATIONS

3-MT	3-Methoxytyramine
5-HIAA	5-Hydoxyindoleaceticacid
5-HT	5-Hydroxytryptamine/Serotonin
AKT1	V-akt murine thymoma viral oncogene homolog 1
AMPA	Alpha-amino-3-hydroxyl-5-methyl-4-isoxazole propionic acid
AMPH	Amphetamine
ANOVA	Analysis of Variance
APO	Apomorphine
ATF4	Activating transcription factor 4
BAC	Bacterial artificial chromosome
BAC-ΔC Tg	Transgenic mice expressing truncated DISC1 using BAC clones
Bcl-2	B-cell lymphoma 2
bp	Base pair
CA	Cornu ammonis
CAMK	Calmodulin-dependent protein kinase
cAMP	Cyclic adenosine monophospate
CG	Anterior cingulate
CIT	Citron
CNS	Central nervous system
COMT	Catechol-*O*-methyl tranferase
CPu	Caudate putamen
CREB	cAMP response element-binding
CS	Conditioned stimulus
D_1/D_2	$Dopamine_1/Dopamine_2$ receptor subtypes
dHPC	Dorsal hippocampus
DISC1	Disrupted-in-Schizophrenia 1
DNA	Deoxyribonucleic acid
DN-DISC1	Dominant negative – DISC1
DOPAC	Dihydroxyphenylacetic acid
DSM-IV	Diagnostic and statistical manual for mental disorders 4^{th} edition
ENU	N-ethyl-N-nitrosourea
EPS	Extra pyramidal symptoms
ErbB4	V-erb-a erythroblastic leukemia viral oncogene homolog 4

FEZ1	Fasiculation and elongation protein-zeta-1
GABA	Gamma-aminobutyric acid
GAD	Glutamic acid decarboxylase
GD	Gestation day
GSH/GSSG	Reduced glutathione/ oxidized glutathione
GSK3β	Glycogen synthase kinase 3 beta
hDISC1 mutant	Human mutant DISC1
HVA	Homovanilic acid
ICD 10	International classification of diseases 10^{th} edition
i.p.	Intraperitoneal
i.v.	Intravenous
IFN	Interferon
Il	Interleukin
IL	Infra limbic
kDA	Kilodaltons
LI	Latent inhibition
LIS1	Lissencephaly 1
LPS	Lipopolysaccharide
LTP	Long term potentiation
MHC	Major histocompatability complex
MK-801	Dizocilipine
MMN	Mismatch negativity
mPFC	Medial prefrontal cortex
mRNA	Messenger ribonucleic acid
MRI	Magnetic resonance imaging
mTOR	Mammalian target of rapamycin
NA	Noradrenaline
NAc	Nucleus accumbens
NAC	N-acetyl cysteine
N-CoR	Nuclear receptor corepressor
NDE1	Nuclear distribution protein nudE homolog 1
NDEL1	Nuclear distribution protein nudE-like 1
NK	Natural killer
NMDA	N-methyl-D-aspartate

NPE	Non-pre-exposed
NRG1	Neuregulin 1
PBS	Phosphate buffered saline
PCNT	Pericentrin
PCP	Phencyclidine
PDE	Phosphodiesterase
PE	Pre-exposed
PFC	Prefrontal cortex
PI3K	Phosphoinositol-3-kinase
PKA	Protein kinase A
PL	Prelimbic
PML	Promyelocytic leukaemia
PND	Postnatal day
Poly-I:C	Polyriboinosonic:polycribocytidilic acid
PPI	Prepulse inhibition
PSD	Post synaptic density
RNA	Ribonucleic acid
SAL	Saline
SNP	Single nucleotide polymorphism
TGF	Transforming growth factor
THC	Δ^9-tetrahydrocannabinol
TNF	Tumor necrosis factor
TNIK	TRAF2 and NCK-interacting protein kinase
tTA	Tetracycline transactivator
US	Unconditioned stimulus
vHPC	Ventral hippocampus
VitC	Ascorbic Acid
VTA	Ventral tegmental area
Y2H	Yeast two hybrid

SUMMARY

Schizophrenia is a chronic, severe and debilitating mental illness. It occurs as a sporadic and heritable disease, typically presenting in adolescence or early adulthood, and leads to great disability and distress. The prevailing hypothesis for the etiology of schizophrenia is that variations in multiple risk genes, each contributing a subtle effect, interact with each other and with environmental stimuli to impact both early and late brain development. Although a clear mechanism underlying the pathogenesis of schizophrenia remains unknown, maternal immune activation as a consequence of exposure to infection during pregnancy has become an attractive hypothesis for explaining, at least in part, the pathophysiology of schizophrenia.

The main aim of this thesis was to evaluate and compare two animal models of schizophrenia: an environmental model and a genetic model. For the environmental animal model I employed a well-established experimental mouse model of prenatal exposure to a viral-like acute phase response induced by the synthetic analogue of double-stranded ribonucleic acid, polyriboinosinic-polyribocytidilic acid (Poly-I:C). Whereas for the genetic model I used a double transgenic mouse model with an inducible expression system of mutant human Disrupted-in-Schizophrenia-1 (DISC1). In this respect, DISC1 has emerged as a strong candidate gene associated with major mental illness including depression, anxiety, bipolar disorder and schizophrenia.

The first series of experiments sought to assess the validity of the "*Critical time window hypothesis"* and to determine whether as opposed to the positive symptoms associated with immune activation during early/middle pregnancy that immune activation during late pregnancy with Poly-I:C may result in a cluster of behavioural and neuropathological changes reminiscent of the negative profile observed in schizophrenia. It was found that maternal immune activation with Poly-I:C during late pregnancy (i.e. gestation day 17) led to delay-dependent impairments in spatial working memory and

recognition memory together with a marked reduction of V-akt murine thymoma viral oncogene homolog 1(AKT1)-positive cells in the prefrontal cortex (**CHAPTER 2**). Interestingly, correlative analyses demonstrated a significant positive correlation between the number of AKT1-positive cells and cognitive performance under high storage load in the temporal domain. It was concluded that alterations in AKT1 signaling and associated cognitive dysfunctions may not only result from a genetic predisposition but may also arise from (immune associated) environmental insults.

The next series of experiments showed that immune activation during late pregnancy leads to phenotypes relevant to the negative and executive/cognitive symptoms of schizophrenia (**CHAPTER 3**). Poly-I:C mice exhibited anhedonia and abnormalities in social behaviour in addition to alterations in the locomotor and stereotyped behavioural responses to acute apomorphine treatment. Specifically, male but not female offspring born to immune challenged mothers displayed behavioural/cognitive inflexibility as indexed by the presence of an abnormally enhanced latent inhibition effect (a measure of selective attention). Prenatal immune activation during late gestation also led to numerous, partly sex-specific changes in basal neurotransmitter levels including reduced dopamine and glutamate contents in the prefrontal cortex and hippocampus. This pattern of results supports the possibility that infection-mediated interference with early fetal brain development may predispose the developing organism to the emergence of neurochemical imbalances in adulthood, which may be critically involved in the precipitation of adult behavioural and pharmacological abnormalities.

The research described in the subsequent chapters aimed at evaluating the *in vivo* functional role of DISC1 in behaviour. For this purpose, a transgenic mouse model of inducible expression of mutant human DISC1 (hDISC1 mutant) was employed to explore the effect of this gene product on schizophrenia relevant behavioural endophenotypes. The first series of experiments revealed that adult mice expressing

mutant human DISC1 are unaffected across a variety of behavioural domains, including exposure to a novel environment, selective attention and sensory motor gating in the form of prepulse inhibition of the startle response (**CHAPTER 4**). However, mice that expressed mutant human DISC1 exhibited an increased overall performance in visual attention using a two-choice visual discrimination/detection test. In addition, the locomotor activating effect of amphetamine, a behavioural measure of mesolimbic dopamine activity, was significantly enhanced in hDISC1 mutant mice. Interestingly, the locomotor activating effect of dizocilipine, a behavioural measure of glutamatergic neurotransmission, was shown to facilitate the locomotor response in female hDISC1 mutant mice. These results suggest that the expression of mutant human DISC1 contributes to some - but not all - aspects of the schizophrenia-like phenotype, mainly in the form of an increased responsiveness to psychostimulants.

In order to further dissect the cognitive effects of mutant human DISC1 expression, additional behavioural tests were conducted using this transgenic mouse model. Cognitive performance of hDISC1 mutant mice was assessed across several behavioural domains, including novel spatial recognition, spatial learning and memory, object exploration and reversal learning (**CHAPTER 5**). hDISC1 mutant mice displayed cognitive inflexibility in addition to deficits in novel spatial and object recognition memory although spatial working memory was spared. As similar deficits in recognition memory and cognitive flexibility have been found in schizophrenia, hDISC1 mutant mice may also model certain aspects of cognition and behaviour relevant to schizophrenia.

The combined results from the studies in this thesis present a complex behavioural phenotype that suggests altered neuropathological and neurochemical interactions following prenatal Poly-I:C immune activation in mice that are dynamic; demonstrating both sex and brain region specificity. Similarly, expression of mutant human DISC1 exerts differential effects on various behavioural phenotypes relevant to schizophrenia. Future studies would be required in order to elucidate whether the development of the

behavioural, neuropathological and neurochemical alterations in both these rodent models of schizophrenia follows a temporal profile similar to the emergence of the symptomology observed in schizophrenia patients and whether these anomalies may be reversed by pharmacological interventions or by alternative methods such as environmental enrichment or physical exercise. Nonetheless, the findings from this thesis provide further support for the involvement of both genetic and environmental components in schizophrenia and suggest that a deficit in either one of these elements can result in long-term behavioural and neuropathological alterations.

RÉSUMÉ

La schizophrénie est une maladie mentale chronique, grave et débilitante. C'est une maladie sporadique et héréditaire, généralement présente à l'adolescence ou l'âge adulte qui conduit à une grande invalidité et une grande détresse. L'hypothèse qui prévaut pour l'étiologie de la schizophrénie est que des variations dans les gènes à risques, qui contribuent subtilement et interagissent les uns avec les autres mais aussi avec les stimuli environnementaux et impactent à la fois le développement précoce et tardif du cerveau. Bien qu'il n'existe pas un mécanisme clair qui explique la pathogénie de la schizophrénie, l'exposition à une infection pendant la grossesse ayant pour conséquence l'activation du système immunitaire maternel est une hypothèse séduisante pour expliquer, du moins en partie, la physiopathologie de la schizophrénie.

L'objectif principal de cette thèse était d'évaluer et de comparer deux modèles animaux de la schizophrénie: un modèle environnemental et un modèle génétique. Nous avons utilisé un modèle expérimental animal environnemental bien établi chez la souris qui consiste en une exposition prénatale de type viral aiguë induite par l'analogue synthétique du double-brin d'acide ribonucléique, acide polyriboinosinique-polyribocytidilique (Poly-I:C). Pour le modèle génétique, nous avons utilisé un modèle de souris doublement transgéniques avec un système d'expression inductible de mutants humains du gène Disrupted-in-Schizophrenia-1 (DISC1). À cet égard, DISC1 a émergé comme un gène candidat qui est fortement associé à une maladie mentale grave, comme la dépression, l'anxiété, le trouble bipolaire et la schizophrénie.

La première série d'expériences a cherché à évaluer la validité de «l'hypothèse du temps critique» et de déterminer si, par opposition aux symptômes positifs associés à l'activation immunitaire pendant la grossesse précoce ou moyenne, que l'activation immunitaire en fin de grossesse avec Poly-I:C peut aboutir à une constellation de changements comportementaux et neuropathologiques rappellant le profil

négatif observé dans la schizophrénie (**CHAPITRE 2**). On a constaté que l'activation immunitaire maternelle avec Poly-I:C en fin de grossesse (soit après 17 jours de gestation) conduit à des retards temporels et des déficiences dans la mémoire de travail spatiale et la mémoire de reconnaissance avec une réduction marquée des cellules du cortex préfrontal positives pour V-akt, l'homologue murin de l'oncogène viral du thymome 1 (AKT1). Fait intéressant, les analyses de corrélations ont démontré une corrélation positive significative entre le nombre de cellules positives AKT1 et la performance cognitive sous charge de stockage élevée dans la région temporale. Il a été conclu que les altérations de la signalisation pour AKT1 et les dysfonctionnements cognitifs associés ne peuvent résulter que d'une prédisposition génétique, mais peuvent également découler de dommages environnementaux associés à l'immunité.

Une seconde série d'expériences ont montré que l'activation immunitaire pendant la grossesse tardive conduit à des phénotypes qui sont pertinents pour les symptômes négatifs et exécutif /cognitifs de la schizophrénie (**CHAPITRE 3**). Les souris exposées aux Poly-I:C montraient une anhédonie et des anomalies dans le comportement social, en plus de modifications dans les réponses locomotrices et stéréotypées de comportement due au traitement apomorphinique aiguë. Plus précisément, seul les descendants mâles, nés de mères ayant été immunitairement affectées, affichent une inflexibilité comportementale / cognitive indexée par la présence d'un effet d'inhibition latente anormalement accrue (une mesure de l'attention sélective). L'activation immunitaire prénatale en fin de gestation a également conduit à de nombreux changements, en partie liés au sexe quant aux niveaux de base en neurotransmetteurs, comprenant une réduction du contenu en dopamine et en glutamate dans le cortex préfrontal et l'hippocampe. Ces résultats tendent à soutenir la possibilité que l'infection interfère avec le développement du cerveau fœtal précoce et peut prédisposer l'organisme en développement à une émergence de déséquilibres neurochimiques à l'âge adulte, qui peuvent être impliqués de façon critique dans la précipitation chez l'adulte d'anomalies comportementales et pharmacologiques.

La recherche décrite dans les chapitres suivants visait à évaluer, in vivo, le rôle fonctionnel de DISC1 dans le comportement. À cette fin, un modèle transgénique murin d'expression inductible de mutants humains de DISC1 (hDISC1 mutant) a été utilisé pour explorer l'effet de ce produit du gène sur les endophenotypes comportementaux pertinents de la schizophrénie. La première série d'expériences a montré chez les souris adultes qui expriment le DISC1 humain mutant, qu'elles ne sont pas affectées dans des domaines comportementaux aussi divers que, l'exposition à un nouvel environnement, l'attention sélective et de déclenchement sensorimoteur sous la forme de préimpulsion d'inhibition à la réaction de sursaut (**CHAPITRE 4**). Cependant, les souris qui exprimaient le DISC1 humain mutant montraient une performance globalement accrue d'attention visuelle avec un test visuel à double choix de discrimination/detection. En outre, l'effet d'amphétamine activatrice de la locomotion, qui est aussi une mesure comportementale de l'activité dopaminergique mésolimbique, a été significativement renforcé chez les souris hDISC1 mutantes. Fait intéressant, l'effet d'activation de la locomotion de la dizocilipine, qui est une mesure comportementale de la neurotransmission glutamatergique, a montré une facilitation de la réponse locomotrice des souris femelles mutantes hDISC1. Ces résultats suggèrent que l'expression de mutants humains DISC1 contribue pour certains - mais pas tous - les aspects du phénotype de type schizophrénique, principalement sous la forme d'une réactivité accrue aux psychostimulants.

Afin de continuer à disséquer les effets cognitifs de l'expression de DISC1 humain mutant, d'autres tests comportementaux ont été réalisés en utilisant ce modèle de souris transgéniques. Les performances cognitives des souris hDISC1 mutantes ont été évaluées dans plusieurs domaines du comportement, y compris la reconnaissance spatiale, l'apprentissage et la mémoire spatiale, l'exploration et l'apprentissage objet d'inversion (**CHAPITRE 5)**. Les souris hDISC1 mutantes affichent une inflexibilité cognitive, en plus de déficits de la mémoire spatiale et dans la reconnaissance d'objets nouveaux, bien que l'espace mémoire de travail soit épargné. Comme des déficits similaires dans la mémoire de reconnaissance et de la flexibilité cognitive ont été observés dans la schizophrénie, les souris

hDISC1 mutantes peuvent ainsi modéliser certains aspects de la cognition et du comportement pertinents à la schizophrénie.

Les résultats combinés des études de cette thèse présentent un phénotype comportemental complexe qui suggère une altération des interactions neuropathologiques et neurochimiques à la suite d'une activation prénatale du système immunitaire avec les Poly-I: C chez les souris, altérations qui sont dynamiques; démontrant à la fois une spécificité due au sexe et à la région du cerveau. De même, l'expression de mutants DISC1 humains exerce des effets différentiels sur différents phénotypes comportement aux pertinents à la schizophrénie. Des études futures seraient nécessaires afin d'élucider si le développement des altérations du comportement, à la fois neuropathologiques et neurochimiques chez ces deux modèles murins de la schizophrénie suit un profil temporel similaire à l'émergence de la symptomatologie observée chez les patients schizophrènes et si ces anomalies peuvent être inversés par des interventions pharmacologiques ou par des méthodes alternatives telles que l'enrichissement de l'environnement ou l'exercice physique. Néanmoins, les résultats de cette thèse fournissent un appui supplémentaire quant à l'implication de composants génétiques et environnementaux dans la schizophrénie et suggèrent que le déficit dans un de ces éléments peut conduire à long terme à des altérations comportementales et neuropathologiques.

ZUSAMMENFASSUNG

Schizophrenie ist eine chronische, schwere und schwächende Geisteskrankheit. Es tritt als sporadische und vererbbare Erkrankung, die typischerweise präsentiert in der Adoleszenz oder im frühen Erwachsenenalter und führt zu großen Behinderung und Leid. Die vorherrschende Hypothese für die Ätiologie der Schizophrenie ist, dass Schwankungen in mehrere Risikogene, jeder trägt einen subtilen Effekt, mit einander und mit Reize aus der Umwelt zu Auswirkungen der frühen und späten Entwicklung des Gehirns zu interagieren. Obwohl eine klare Mechanismus der Pathogenese der Schizophrenie ist unbekannt, mütterliche Immunsystem-Aktivierung als Folge der Exposition gegenüber Infektionen während der Schwangerschaft ist zu einem attraktiven Hypothese zur Erklärung, zumindest teilweise, der Pathophysiologie der Schizophrenie.

Das Hauptziel der vorliegenden Dissertation war es, zwei Tiermodelle der Schizophrenie zu evaluieren und zu vergleichen: ein Umwelt- und ein genetisches Modell. Bezüglich des Umweltmodells benutzten wir ein etabliertes experimentelles Mausmodell der pränatalen Exposition gegenüber Virus-ähnlichen Akut-Phasenreaktionen induziert durch ein synthetisches Analogon der Doppelhelix der Ribonukleinsäure, namentlich Polyriboinosin-Polyribocytidilinsäure (Poly-I:C). Bezüglich des genetischen Modells benutzten wir ein doppeltes transgenes Mausmodell mit einem induzierbaren Expressionssystems der humanen Disrupted-in-Schizophrenia-1-Mutante (DISC1). In dieser Hinsicht hat sich das DISC1-Gen als ein wichtiger Kandidat herausgestellt, welches mit den häufigsten psychischen Erkrankungen wie Depression, Angst- und bipolaren Störungen und Schizophrenie assoziiert ist.

In der ersten Serie von Experimenten sollte die Validität der „Kritischen Zeitfenster Hypothese“ untersucht und bestimmt werden, ob im Gegensatz zu den mit der Immunaktivierung verbundenen Positivsymptomen während der Früh- und mittleren Trächtigkeit die Immunaktivierung während der Spätträchtigkeit assoziiert mit Poly-I:C zu einem Cluster von Verhaltens- und neuropathologischen

Veränderungen führt, der den Negativsymptomen der Schizophrenie ähnelt (**KAPITEL 2**). Es wurde herausgefunden, dass die maternale Immunaktivierung mit Poly-I:C während der Spätträchtigkeit (Gestationstag 17) zu Verzögerungs-abhängigen Beeinträchtigungen im räumlichen Arbeitsgedächtnis und des Wiedererkennungsgedächtnisses führte, verbunden mit einer deutlichen Reduktion der murinen V-akt Thymom viralen Onkogen homologen 1 (AKT1)-positiven Zellen im präfrontalen Cortex. Interessanterweise zeigten Korrelationsanalysen einen signifikanten positiven Zusammenhang zwischen der Zahl der AKT1-positiven Zellen und der kognitiven Leistungsfähigkeit unter hoher Speicherlast in der zeitlichen Domäne. Daher wurde geschlussfolgert, dass Änderungen im AKT1-Signaling und assoziierte kognitive Dysfunktionen nicht nur das Resultat einer genetischen Prädisposition sind, sondern auch auf (immun-assoziierten) Umweltschäden beruhen.

Die nächste Serie von Experimenten gezeigt, dass Aktivierung des Immunsystems während der späten Schwangerschaft zu Phänotypen, die für die negativen und Executive / kognitive Symptome der Schizophrenie führt (**KAPITEL 3**). Poly-I:C Mäuse zeigten Anhedonie und Anomalien im sozialen Verhalten zusätzlich zu Veränderungen in der Bewegungs-und stereotype Verhaltensweisen an die akute Apomorphin-Behandlung. Insbesondere angezeigt männlichen, aber nicht weiblichen Nachkommen geboren, um immun herausgefordert Mütter Verhaltenstherapie / kognitive Inflexibilität durch die Anwesenheit eines ungewöhnlich verbesserte latente Inhibition-Effekt (ein Maß für die selektive Aufmerksamkeit) indiziert. Pränatale Immunaktivierung während der späten Schwangerschaft auch zu zahlreichen, zum Teil geschlechtsspezifische Veränderungen der basalen Neurotransmitter Ebenen, einschließlich reduzierter Dopamin und Glutamat Inhalte im präfrontalen Kortex und im Hippocampus führte. Dieses Muster der Ergebnisse unterstützt die Möglichkeit, dass eine Infektion-Interferenz mit der frühen fetalen Entwicklung des Gehirns kann sich entwickelnden Organismus die Entstehung von neurochemischen Ungleichgewicht im Erwachsenenalter, die sich kritisch in die Fällung von erwachsenen Verhaltens-und pharmakologische Veränderungen einbezogen werden prädisponieren können.

Die Forschung in den nachfolgenden Kapiteln beschriebenen bei der Bewertung der in vivo funktionelle Rolle DISC1 im Verhalten abzielen. Zu diesem Zweck wurde ein transgenes Mausmodell der induzierbare Expression von mutierten Menschen DISC1 (hDISC1 Mutante) eingesetzt, um die Wirkung dieses Gens Produkt auf Schizophrenie relevanten Verhaltens Endophänotypen erkunden. Die erste Serie von Experimenten gezeigt, dass erwachsene Mäuse mit mutierten humanen DISC1 unberührt in einer Vielzahl von Verhaltens-Domänen, einschließlich der Exposition gegenüber einer neuen Umgebung, selektive Aufmerksamkeit und sensomotorischen Gating in Form von Vorpuls Hemmung der Schreckreaktion sind (**KAPITEL 4**). Jedoch zeigte Mäusen, dass mutierte menschliche DISC1 Ausdruck einer erhöhten Gesamt-Performance in der visuellen Aufmerksamkeit mit einem Zwei-Wahl visuelle Diskriminierung / Erkennung im Test. Darüber hinaus wurde am Bewegungsapparat aktivierende Wirkung von Amphetamin, eine Verhaltensänderung Maß mesolimbischen Dopamin-Aktivität, deutlich in hDISC1 mutierten Mäusen verbessert. Interessanterweise war der Bewegungsapparat aktivierende Wirkung von dizocilipine, eine Verhaltensänderung messen der glutamatergen Neurotransmission, dargestellt am Bewegungsapparat Reaktion bei weiblichen hDISC1 mutierten Mäusen zu erleichtern. Diese Ergebnisse legen nahe, dass die Expression von mutierten Menschen DISC1 einige beiträgt - aber nicht alle - Aspekte der Schizophrenie-ähnlichen Phänotyp, vor allem in der Form einer erhöhten Reaktionsfähigkeit auf Psychostimulanzien.

Um weitere sezieren die kognitiven Wirkungen von mutierten Menschen DISC1 Expression wurden zusätzliche Verhaltenstests durchgeführt unter Verwendung dieser transgenen Mausmodell. Kognitive Leistungsfähigkeit hDISC1 mutierten Mäusen wurde über mehrere Domänen Verhaltens, einschließlich neuartiger räumlicher Anerkennung, räumliches Lernen und Gedächtnis, Objekt Exploration und Umkehr Lernen beurteilt (**KAPITEL 5**). hDISC1 mutierten Mäuse zeigten kognitive Inflexibilität neben Defiziten in neue Raum-und Objekterkennung Speicher obwohl räumliche Arbeitsgedächtnis wurde verschont. Da ähnliche Defizite in Anerkennung Gedächtnis und kognitive

Flexibilität bei der Schizophrenie gefunden haben, können hDISC1 mutierten Mäusen auch Modell bestimmte Aspekte der Kognition und Verhalten relevant Schizophrenie.

Zusammenfassend beschreiben die in dieser Dissertation zusammengefassten Ergebnisse einen komplexen Verhaltensphänotyp mit veränderten neuropathologischen und -chemischen Interaktionen folgend aus einer pränatalen Poly-I:C Immunaktivierung in Mäusen, welche dynamisch ist und zudem geschlechts- und Hirnregion-spezifisch ist. Ebenso zeigt die Expression der mutierten humanen DISC1 unterschiedliche Auswirkungen auf verschiedene Verhaltensphänotypen mit Relevanz für die Schizophrenie. Zukünftige Studien wären nötig um herauszufinden of die Entwicklung der Verhaltens-, neuropathologischen und neurochemischen Veränderungen in beiden Nagetiermodellen der Schizophrenie einem zeitlichen Profilähnlich dem Auftreten der Symptome entspricht, die bei Schizophrenie Patienten beobachtet werden und ob diese Anomalien durch pharmakologische Intervention oder alternative Methoden, wie Umweltanreicherung oder physische Aktivität umgekehrt werden können. Dennoch unterstützen die Erkenntnisse dieser Arbeit weiter die Einfluss sowohl genetischer als auch Umweltfaktoren auf die Schizophrenie und lassen vermuten, dass ein Defizit in einem dieser beiden Elemente zu langfristigen Verhaltens- und neuropathologischen Veränderungen führen kann.

RIASSUNTO

La schizofrenia è stata a lungo vista come un disturbo debilitante cronico con un esito quasi inevitabilmente negativo. Si manifesta come una malattia sporadica ed ereditaria. In oltre, la schizofrenia si presenta in adolescenza o all'inizio dell'età adulta, e porta alla disabilità e grande angoscia. L'ipotesi prevalente per l'eziologia della schizofrenia è che le variazioni nei geni di rischio interagiscono tra loro e con stimoli ambientali che interrompano lo sviluppo normale del cervello. Anche se un chiaro meccanismo alla base della patogenesi della schizofrenia rimane sconosciuta, l'attivazione del sistema immunitario materno come conseguenza di esposizione a infezione durante la gravidanza è diventata una ipotesi interessante per spiegare, almeno in parte, la fisiopatologia della schizofrenia.

L'obiettivo principale di questa tesi è stato di valutare e di confrontare due modelli animali di schizofrenia: un modello ambientale e un modello genetico. Per il modello ambientale ho usato un ben definito modello sperimentale di esposizione prenatale. Questo modello stimola una risposta immunitaria con un un analogo sintetico del doppio intrecciato acido ribonucleico, polyriboinosinic-polyribocytidilic acido (Poly-I:C). Per il modello genetico ho usato un topo transgenico con una sistema che induce l'espressione del mutante umano Disrupted-in-Schizophrenia-1 (DISC1). A questo proposito, DISC1 è emerso come un forte gene candidato associata a grave malattia mentale tra cui la depressione, ansia, disturbo bipolare e la schizofrenia.

La prima serie di esperimenti ha cercato di valutare la validità del "*Critical time window hypothesis"* e stabilire se in contrasto a gli sintomi positivi della schizofrenia che sono associati con l'attivazione del sistema immunitario durante la gravidanza iniziale/mezzo, che l'attivazione della sistema immunitario durante la gravidanza in tarda con Poly-I:C potrebbe risultare in un gruppo di cambi

comportamentali e neuropatologici che assomiglia ad un profilo negativo osservatti nella schizofrenia (**CAPITOLO 2**). Si è constatato che l'attivazione della sistema immunitaria materna con Poly-I:C durante la gravidanza in tarda (ad esempio il 17º giorno di gravidanza) puo risultatare in un menomazione ritardo-dipendente nella memoria di funzionamenti spaziale e di memoria di riconoscimento insieme con una riduzione di V-akt omologo murino timoma oncogene virale (AKT1)-cellule positive nella corteccia prefrontale. È interessante notare che le analisi correlative hanno dimostrato una significativa correlazione positiva tra il numero di AKT1-cellule positive e l'esecuzione cognitivo quando il 'peso' e stato alto nel dominio temporale. Si e concluso che alterazioni nella segnalazione di AKT1 e disfunzioni cognitive non può scaturire soltanto da una predisposizione genetica, ma può anche derivare da (immunitaria associata) ambientale insulti.

Le prossimi serie di esperimenti hanno dimostrato che l'attivazione immunitaria durante gravidanza in tarda risulta in fenotipi rilevanti per i sintomi negativi ed esecutivo/cognitivi della schizofrenia (**CAPITOLO 3**). Topi Poly-I:C hanno mostrato una mancanza di soddisfazione e delle anomalie in comportamento sociale in oltre a delle alterazioni di comportamento locomatorio e stereotipico seguendo trattamento con apomorfina. In particlolare, prole di sesso maschile man non feminile nati da madri esposte ad attivazione immunitario prenatale hanno dimostrato comportamento/cognitivo inflessibile indicato dalla presenza di un anormale potenziamento di *latent inhibition* (una misura di attenzione selettiva). In oltre, l'attivazione del sistema immunitario materno durante gravidanza in tarda e risultato in numerosi cambi in parte sesso-specifici dei livelli di neurotrasmettitori compreso una riduzione di dopamina e glutammato nella corteccia prefrontale e nell'ippocampo. Questo schema di risultati sostiene la possibilità che interferenza mediata da infezioni con il sviluppò del cervello fetale potrebbe predisporre l'organismo che si sta sviluppando a l'emergere di un squilibrio neurochimico durante l'età adulta, che potrebbe essere coinvolto nella precipitazione dei comportamenti anomali durante l'età adulta.

La ricerca descritta nei capitoli succesivi ha cercato di valutare il ruolo funzionale di DISC1 nel comportamento. A questo scopo, un topo transgenico d'espressione inducibile di mutante umano Disrupted-in-Schizophrenia-1 (hDISC1 mutante) e stato impiegato per esplorare l'effetto di questo gene su dei comportamenti rilevanti alla schizofrenia. Le prime serie di esperenti hanno rivelato che topi adulti che esprimono il mutante umano DISC1 non sono inalterati attraverso una varietà di domini cognitivi, compresa l'esposizione ad un ambiente nuovo, l'attenzione seletiva e *prepulse inhibition* (**CAPITOLO 4**). Tuttavia, i topi che esprimono il mutante umano DISC1 hanno dimostrato un aumento nel esecuzione di attenzione visuale durante un test di discriminazione visiva. In oltre, l'effetto locomotorio del anfetamina, una misura comportamentale di attività dopaminergico mesolimbico, era potenziato nei topi hDISC1 mutanti. In oltre, l'effetto locomotorio di dizocilpina, una misura della neurotrasmissione glutamatergica, ha dimostrato di facilitare la locomozione in topi hDISC1 mutanti feminilie. Questi risultati suggeriscono che l'espressione del mutante umano DISC1 contribuisce ad alcuni – ma non tutti – gli fenotipi della schizofrenia – principalmente sotto forma di un aumento della risposta a psicostimolanti.

In modo di dissezionare gli effetti cognitivi del espressione di hDISC1 mutante, ulteriori test comportamentali sono stati condotti utilizzando questo topo transgenico. L'esecuzione cognitivo dei topi hDISC1 mutanti è stata valutata attraverso alcuni domini del comportamento, tra i quali il riconoscimento spaziale, l'apprendimento spaziale e memoria, l'esplorazione di un nuovo oggetto e l'inversione di apprendimento (**CAPITOLO 5**). Topi che esprimono il mutante umano DISC1 mostrano inflessibilità cognitiva in oltre a carenze in memoria di nuovo spazio e memoria di riconoscimento dei oggetti nonostante la memoria di funzionamenti spaziale è stato risparmiato. Poiché simili carenze in memoria di riconoscimento e inflessibilità sono stato osservati in pazienti con la schizofrenia, topi che esprimono il mutante umano DISC1 potrebbero modellare certi aspetti di cognizione e comportamento rilevanti alla schizofrenia.

I risultati complessivi degli studi in questa tesi presentano un fenotipo di comportamento complesso che suggerisce alterate interazioni neuropatologiche e neurochimiche seguendo l'attivazione immunitaria prenatale con Poly-I:C in topi, dimostrando sia sesso e regione specificità del cervello. Allo stesso modo, l'espressione del mutante umano DISC1 esercita effetti differenziali su vari fenotipi di comportamento rilevanti alla schizofrenia. Studi futuri saranno necessari per delucidare se lo sviluppo del comportamento, la neuropatologia, e la neurochimica alterata in entrambi questi due modelli della schizofrenia seguono un profilo temporale simile al'emergere dei sintomi osservati in pazienti con la schizofrenia, e se queste anomalie possono essere invertite con intervento farmacologico, o con altri metodi come l'arricchimento del ambiente o persino esercitazione fisica. Tuttavia, i risultati da questo tesi forniscono più sostegno per il coinvolgimento di entrambi componenti genetici e ambientali nella schizofrenia e suggeriscono che una carenza di uno o l'altro di questi elementi può risultare in un'alterazione del comportamento e neuropatologia a lungo termine.

CHAPTER 1.

INTRODUCTION

Schizophrenia is a chronic, severe and disabling psychiatric illness that affects about 1% of the population worldwide (Perala *et al*., 2007). The symptoms of the disorder can be divided into three main categories: positive symptoms (disorganized speech, hallucination and delusions), negative symptoms (blunted affect, avolition, anhedonia and social withdrawal) and cognitive deficits (impairments in memory, attention and executive function) (Carpenter, 1994; Tamminga and Holcomb, 2005) all of which may co-exist in the same individual. Although the symptoms that establish the diagnosis are usually not present until young adulthood, prodromal symptoms and endophenotypic features of cognitive and social deficits can precede psychotic illness and manifest in unaffected relatives. The total costs of treating schizophrenia are high, estimated to be US$ 62.7 billion (including US$ 22.7 billion in direct healthcare costs) in the USA for the year 2002 (Aggarwal *et al*., 2005). Notably, the World Health Organization places schizophrenia among the world's top ten causes of disability (Electronic Reference 1).

Treatments remain palliative, and diagnostic tests are not yet available despite recognized trends in patients, which include ventricular enlargement (Andreasen *et al*., 1994; Wright *et al*., 2000; Pantelis *et al*., 2005), reduced volume in the medial temporal lobe (Shenton *et al*., 1992; Arnold, 1997), white matter deficits (Uranova *et al*., 2004, 2007; Rowland *et al*., 2009) and impairments of cortical circuits in the dorsolateral prefrontal cortex (Benes *et al*., 1991; Bitanihirwe *et al*., 2009a; Lewis and Sweet, 2009; Hashimoto *et al*., 2003; Woo *et al*., 2004, 2008).

The prevailing hypothesis for the etiology of schizophrenia is that variations in multiple risk genes, each contributing a subtle effect, interact with each other and with environmental stimuli to impact both early and late brain development (Lewis and Lieberman, 2000; Lewis and Levitt, 2002, **Figure 1.1**).

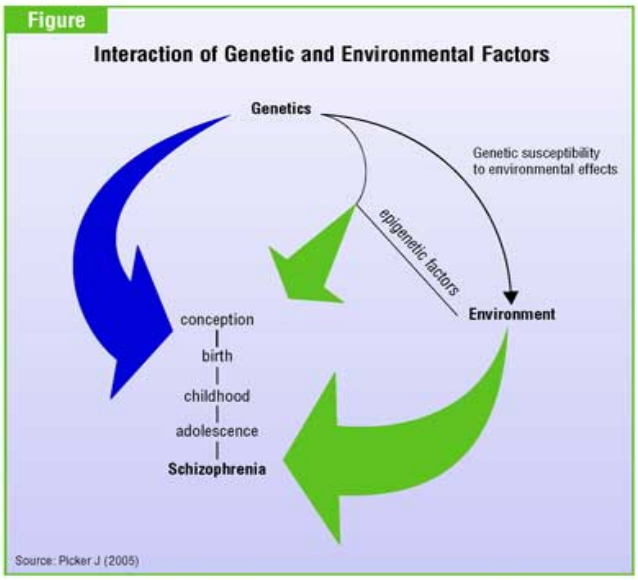

Figure 1.1: Schizophrenia: Interaction between genetic and environmental factors during neurodevelopment. (Source: Electronic Reference 2)

One of the major objectives of researchers studying diseases such as schizophrenia is to try to determine the earliest molecular changes associated with the disease. The rationale for this approach is that it reduces the confounding effects of secondary phenomena that might follow the relevant early changes. The lack of brain tissue from presymptomatic patients largely precludes such studies in human subjects. However, several early changes have been identified in animal models.

Animal models afford powerful tools to explore how genetic and potential environmental risk factors associated with schizophrenia impact brain development. Clearly no single animal model will be able to recapitulate the diverse and complex symptom profile observed in schizophrenia. Research has instead aimed to model aspects of the disorder examining links between candidate genes or environmental risk factors and adverse outcomes such as brain structures, neurophysiology and ultimately function. Animal models have therefore been used to evaluate the impact of pharmacological agents, developmental lesions, genetic manipulations and environmental risk factors in an attempt to explore potential etiologic hypotheses (Lipska and Weinberger; 2000; van den Buuse *et al*., 2005). These models

have highlighted specific developmental windows whereby genetic manipulation or an early insult can subsequently lead to neurochemical imbalances, brain architectural irregularities and aberrations in behaviour in adult animals.

The current literature review will describe two risk factors (maternal immune activation and disrupted-in-schizophrenia 1, DISC1) and how they might be implicated in schizophrenia. First I will provide a general overview of schizophrenia. I will then describe the neurodevelopmental hypothesis of schizophrenia and the epidemiological evidence implicating infection during pregnancy as a candidate risk factor for the disease. I will then highlight the susceptibility of the central nervous system (CNS) to developmental insult, discussing evidence of this in animal models, and speculate on mechanisms whereby maternal immune activation could lead to long-term changes in brain architecture, neurochemistry and behaviour. Subsequently, I will discuss the cell biology of DISC1 and I will then summarize the literature implicating DISC1 in schizophrenia. Finally, I will discuss DISC1 animal models of schizophrenia.

1.1 The conceptualization of "Schizophrenia"

In 1852 the Belgian physician Benedict Morel described the case of a young adolescent boy who had been a cheerful, outgoing individual and a good student but who gradually became melancholic and withdrawn and appeared to progressively lose his cognitive abilities. Morel believed the deterioration was a result of an arrest in brain development which he attributed to hereditary causes. He considered such cases to be irremediable and named the disorder *demence precoce* to refer to his observations that the degenerative process began early in life and progressed rapidly to dementia.

Four decades later (1896), the distinguished German psychiatrist Emil Kraeplin published the fifth edition of his text book on psychiatry in which he introduced a new nosological system. Kraeplin broadened Morel's demence precoce, incorporated Wilhelm Griesinger's notions of a predisposing diffuse cerebral pathology, included Karl Kahlbaum's catatonia and Eward Hecker's hebephrenia, added the category dementia paranoides, and concluded that all were actually subtypes of a single disorder he called *dementia praecox* (early deterioration of the intellect).

The term *schizophrenia*, which means "split mind", was introduced a decade later in 1911 by the Swiss psychiatrist Paul Eugen Bleuler. who characterised the splitting ('schizo') of affect (i.e. emotional tone) from cognition (Breier, 1999). He further identified four characteristic symptoms: autism, ambivalence, blunted affect and disturbances of volition. While these early clinicians readily accepted that schizophrenia was a brain disorder with heritable vulnerabilities, this biological conceptualization of the disorder was eclipsed by the rise of psychoanalysis in the 1930s, and the dominance for the next half century of its theoretical conceptualization that intra-psychic conflicts are the cause of psychiatric disorders (Coyle, 2006).

1.2 Clinical manifestations and standardized diagnostic methods

Considerable heterogeneity is observed among patients in the course, onset, and outcome of schizophrenia and the particular combination of symptoms observed at any time. The disorder usually starts to develop with prodromal symptoms. This consists of a heterogeneous group of symptoms that include: changes in mood; depression; peculiar feelings and thoughts; problems in professional and social situations; aggressiveness; inability to concentrate; and sleeping problems. Approximately 75 % of individuals who later go on to develop schizophrenia exhibit these symptoms, and thus they have been suggested to have predictive value for psychosis (Haddock and Lewis, 1996). However, recent data suggests that the transition rate to full blown psychosis in "ultra high risk" individuals may be as low as 16% (Yung *et al*., 2008). Following frank onset of schizophrenia, the disorder may embrace a constellation of symptoms that classically include positive symptoms, negative symptoms and cognitive deficits (**Table 1.1.).**

Positive symptoms	Negative Symptoms	Cognitive Symptoms
Psychotic episode (displacement from 'reality', inability to separate real from unreal experiences) including; *delusions* (false beliefs/judgment). *hallucinations* (strong subjective perceptions of an object or event which is non-existent that may affect any or all sensory perceptions). *disorganized speech* or *behaviour*; *thought disorder*	Social and occupational dysfunction Lack of motivation, withdrawal, loss of concentration Blunted or flat affect (loss of emotional tone or reaction) Inability to articulate	Deficits in executive functions, attention, working memory, long-term memory, social learning and intelligence.

Table 1.1: Major features of schizophrenia

Schizophrenia diagnoses are based on either the *International Classification of Diseases* (ICD), currently tenth edition (ICD-10) (World Health Organization 1992) or *Diagnostic and Statistical Manual*

for Mental Disorders (DSM), currently fourth edition (DSM-IV or DSM-IV-TR) (American Psychiatric Association 1994) (**Table 1.2**).

DSM-IV	ICD-10
At least two of the symptoms in "a" must be present for a minimum of one month. Additionally, "b" must be present for six months and criteria "c-e" must be fulfilled. a) delusions, hallucinations, bizarre behaviour and negative symptoms. b) occupational or social dysfunction c) schizoaffective or mood disorder exclusion d) disturbance must not be due to medications or drug abuse e) if a patient has a pervasive development disorder, prominent delusions or hallucinations must be present for one month	Minimum of one clear symptom, or at least two if less clear cut, listed in "a-d" or at least two of the symptoms in "e-i" should be present for one month or more. a) thought echo, thought insertions or withdrawal, and thought broadcasting b) delusion of control or passivity c) hallucinatory voices d) persistent delusions e) significant and consistent change of personal behaviour f) negative symptoms that are not due to depression or neuroleptic treatment g) persistent hallucinations accompanied by deletions or by persistent over-valued ideas consistently occurring for weeks or months h) incoherent or irrelevant speech i) catatonic behaviour

Table 1.2: Diagnostic criteria for schizophrenia according to DSM-IV and ICD-10, respectively.

A diagnosis of schizophrenia may be made if continuing signs of a disturbance have been present for at least six months, concordant with a social and occupational dysfunction for a significant period of the time since onset, provided other medical conditions and drug induced psychosis resulting from the use of amphetamine (AMPH), phencyclidine (PCP) or other psychostimulants have first been ruled out. If both schizophrenic and affective symptoms develop together and are evenly balanced, the diagnosis of schizoaffective disorder should be made, even if the schizophrenic symptoms by themselves would have justified the diagnosis of schizophrenia.

Following the first psychotic 'break' (first incident), approximately 90 % of patients suffer one or more relapses later in life (Robinson *et al*., 1999). Schizophrenia is therefore often considered a chronic disease given that the cognitive and negative symptoms usually persist for months or even years (Mueser and McGurk, 2004; Tandon *et al*., 2009).

1.3. Treatment

Schizophrenia is normally treated with a combination of psychotherapy, social adjustments as well as drug administration. The neuroleptics used to treat schizophrenic patients are usually divided in to two main categories: 'typical' and 'atypical' neuroleptics. The typical neuroleptics act by blocking the action of the neurotransmitter dopamine at the receptor level, chiefly the D_2 subtype receptor (Sawa and Snyder, 2003). The lower level of dopamine stimulation has been suggested to subvert positive symptoms. However, decreased dopamine level results in increased acetylcholine levels that may attribute to the so called extra pyramidal symptoms (EPS) (Coyle, 2006). Crucially, long-term use of these antipsychotics has been attributed to the development of a relatively irreversible neurologic syndrome known as *tardive dyskinesia*, which is characterized by writhing movements of the tongue and choreiform movements of the extremities (Coyle, 2006). As a result of such side effects a large number of patients tend to discontinue their medication. Some frequently used typical neuroleptics include chloropromazine, haloperidol, sulpride, thioridazine and stelazine (Kapur and Remington, 2001). The more recently introduced atypical neuropeltics include clozapine, risperidone, olanzapine and quetiapine. Interestingly, these compounds show a more heterogeneous binding pattern than the classical typical neuroleptics by acting on the dopamine D_4 receptor, muscarinic receptors, α-adrenergic receptors, histamine receptors and the serotonin 5-HT_{2A} receptor (Coyle, 2006). However, the exact binding patterns still have not been elucidated. The atypical neuroleptics have subversive effects on both negative and positive symptoms exhibited by schizophrenics (Kandel, 2000; Kapur and Remington, 2001). Particularly, quetiapine has been shown to improve some cognitive deficits in schizophrenia when compared to typical neurolpetics such as haloperidol (Purdon *et al*., 2001). Moreover, atypical neuroleptics do not cause severe neurological side effects nor do they result in EPS. A minority of individuals that are diagnosed with schizophrenia respond well to treatment and recover after the first break. Unfortunately many patients do not respond to treatment (Gerretsen *et al*., 2009).

1.4. Neurodevelopmental hypothesis of schizophrenia

The neurodevelopmental hypothesis of schizophrenia posits that the disorder is the result of an early brain insult, either pre or perinatal, which interferes with brain development leading to abnormalities which are expressed in the mature brain (Weinberger, 1987; Murray and Lewis, 1987). The etiology of the neurodevelopmental abnormalities in schizophrenia are postulated to stem from either the inheritance of abnormal genes, which impair brain development, or from some fetal or neonatal adversity. Unlike other genetic neurodevelopmental disorders (e.g., Down's syndrome or Fragile X syndrome), or severe gestational and birth traumas (e.g., kernicterus), there are no immediate overt manifestations of schizophrenia. Rather, most individuals appear to function normally until they enter the greatest period of risk in late adolescence and early adulthood (Lewis and Lieberman, 2000). Interestingly, prospective epidemiological studies have reported an association between adult-onset schizophrenia and delays or abnormalities in childhood or adolescent cognitive and neuromotor development. These developmental deficits are thought to be genetically programmed, consistent with the concept of schizophrenia as a neurodevelopmental disorder (Jones and Murray, 1991).

Post mortem brain studies of schizophrenia have reported cytoarchitectural abnormalities such as neuronal disarray and malpositioning, suggesting that neuronal migration and proliferation is disturbed during the gestational period (Beckmann and Senitz, 2002). The existence of neuronal changes in the absence of massive glial proliferation (gliosis) supports the idea that the cytoarchitectural pathology observed in schizophrenia is associated with damage during neurodevelopment (Benes *et al*., 1991; Arnold *et al*., 1998). Changes in neuronal structure and synapses have frequently been observed in autopsied brains from patients with schizophrenia (Selemon and Goldman-Rakic, 1999; Glantz and Lewis, 2000). These changes might represent direct neuron alterations in response to neuronal disconnectivity or compensatory mechanisms arising from earlier neurodevelopmental insults.

At least four elements might play a role in postnatal brain maturation associated with schizophrenia: maturation of dopaminergic projections, gamma-aminobutyric acid (GABA) interneuron maturation, pruning of glutamate synapses, and oligodendrocyte differentiation and myelination, each of which will be discussed below.

Maturation of dopaminergic projections: Abnormal maturation of the mesolimbic and mesocortical dopamine systems have been suggested to contribute to the etiology of schizophrenia (Goto and Grace, 2007; Meyer and Feldon, 2009). Dopaminergic projections from the ventral tegmental area (VTA) to the cortex exhibit marked postnatal maturation (Rosenberg and Lewis, 1995; Tseng *et al*., 2007; Goto and Grace, 2007). Until young adulthood, the concentration of dopamine and the staining intensity of tyrosine hydroxylase (the rate-limiting enzyme in the synthesis of dopamine from tyrosine) continue to increase in the prefrontal cortex (PFC) (Rosenberg and Lewis, 1995; Lambe *et al*., 2000). Interestingly, neuroanatomical studies in postmortem brains from patients with schizophrenia have revealed a reduction in tyrosine hydroxylase expression and dopamine levels in the PFC (Akil *et al*., 1999, 2000). Although the exact mechanism underlying such a neurochemical disturbance in schizophrenia remains unknown these observations may however reflect defects of postnatal maturation of the mesocortical dopaminergic system.

Dopaminergic neurons within the VTA have also been shown to project to the medial and ventral parts of the caudate putamen, nucleus accumbens, hippocampus, and amygdala (Meyer and Feldon, 2009; Goto and Grace, 2007). These projections from the mesolimbic dopaminergic system are not fully matured until after adolescence. The late maturation of these connections has been proposed to influence the development and integration of normal or abnormal emotional behaviour and cognition during adolescence (Goto and Grace, 2007). Given that dopamine plays a critical role in regulating cortical circuits engaged in cognitive function, abnormalities in postnatal mesolimbic and mesocortical

dopaminergic maturation may contribute to the behavioural and cognitive deficits associated with disturbed cortical neurocircuitry after puberty (Niwa *et al*., 2010).

GABA interneuron maturation: Deficits in interneurons are thought to play an important role in the pathophysiology of schizophrenia (Lewis *et al*., 2005; Lisman *et al*., 2008). Dysfunction in the parvalbumin containing fast-spiking interneurons can lead to disinhibition of pyramidal neurons in the cortex and hippocampus, as well as asynchrony of pyramidal neuron activation and cognitive impairment, all thought to be hallmarks of schizophrenia pathophysiology. Interestingly, parvalbumin neuronal circuits appear to achieve maturation, as reflected in their attaining the adult pattern of parvalbumin protein expression (Erickson and Lewis, 2002) and a concomitant increase of gamma-band oscillation power during late adolescence and early adulthood (Rojas *et al.,* 2006), which coincides with the period of time when schizophrenia symptomatology typically begins to emerge. Gamma-band oscillations are thought to provide the temporal structure that is necessary for synaptic plasticity. Therefore, deficits in gamma band oscillations may disturb the developmental synaptic reorganization process that is occurring during the period of late adolescence and early adulthood, which may in turn contribute to the onset of schizophrenia and the functional deterioration that is characteristic of the early stage of the illness (Woo *et al*., 2010).

Pruning of glutamate synapses: The intrinsic cortical circuitry undergoes substantial reorganization during the periadolescence period with a dramatic decrease in the number of synapses (Woo and Crowell, 2005). The synapses which are eliminated or 'pruned' are asymmetric and mainly glutamatergic (Bourgeois and Goldman-Rakic, 1993). The loss of synaptic connectivities in schizophrenia may be the result of "overpruning" due to dysregulation of the developmental pruning process (McGlashan and Hoffman, 2000; Woo and Crowell, 2005). Alternatively, albeit not mutually exclusively, other pathophysiologic events occurring during earlier stages of brain development may result in

"subthreshold" synaptic deficits within the intrinsic cortical circuitry (Woo and Crowell, 2005). The periadolescent pruning process magnifies these preexisting deficits, resulting in a loss in the number of synapses beyond the critical threshold, eventually compromising the functional integrity of the circuitry (McGlashan and Hoffman, 2000; Woo and Crowell, 2005). Interestingly, recent evidence has emerged implicating neuroimmune interactions in the normal elimination of synapses in the developing CNS. For example, the classical complement cascade and major histocompatability complex (MHC) class I molecules appear to regulate synaptic function and mediate elimination of synapses in the CNS (Schafer and Stevens, 2010).

Deficits in oligodendrocyte differentiation and myelination: Although patients suffering from schizophrenia exhibit myelin deficits (Uranova *et al*., 2004, 2007), a pathology that is characteristic of a demyelination process has not been observed. In fact, it has been proposed that myelin deficits in schizophrenia may arise from a partial or complete failure of myelination in late adolescence and early adulthood (Dwork, 2007). Consistent with the idea that the developmental myelination process may be deficient in patients with schizophrenia, brain imaging studies have shown that the developmental increase in white matter observed during normal brain maturation is not evident in patients with schizophrenia (Bartzokis, 2002). There is normally an age-related increase in white matter density that is consistent with greater myelination of the major fiber tracts which link various associational cortical regions, such as the PFC and the anterior cingulate cortex, with the temporal cortical region throughout adolescence (Benes, 1989). Interestingly, the period in which the normal maturation of myelinated pathways occurs coincides with the common age of onset of schizophrenia symptomatology.

1.5. Neurochemistry of schizophrenia

A number of theories of schizophrenia have been put forward, dominated for many years by neuropharmacology, that implicate aberrant neurotransmission systems. In particular, aberrant dopaminergic, glutamatergic and GABAergic sytems have been implicated in schizophrenia. In this section, I will therefore focus on how abnormal neurotransmission of dopamine, glutamate and GABA may play a pathological role in schizophrenia.

1.5.1. *Dopamine*

The classical dopamine hypothesis of schizophrenia attributes the symptoms of schizophrenia to over active dopamine (i.e. hyper-dopaminergia) neurotransmission in subcortical regions of the brain (Snyder, 1972). Support for this hypothesis comes from the clinical observation that dopamine agonists such as AMPH and cocaine can result in symptoms that resemble those observed in patients with schizophrenia. Furthermore, it has been shown that the efficacy of antispsychotic medication is proportional to their antagonistic activity at dopamine (primarily D_2) receptors (Seeman and Lee, 1975). However, given the increasing awareness of the importance of negative and cognitive symptoms of schizophrenia, and their resistance to D_2 receptor antagonism, the dopamine hypothesis has since been modified. The revised dopamine hypothesis of schizophrenia suggests an over activity of the dopaminergic system in the mesolimbic pathway may contribute to the positive symptoms of the disorder whereas a deficit of dopamine function (i.e hypo-dopaminergia) in the mesocortical pathway may result in the negative symptoms and cognitive deficits observed in schizophrenia patients (Davis *et al*., 1991).

Although post-mortem studies generally support the dopamine hypothesis (Akil *et al*., 1999, 2000), the exposure to antipsychotic medication in the patient samples has generated controversy as to whether the changes observed are a direct result of the disease process or a confounding effect of

antipsychotic medication (Clow *et al*., 1980). However, direct evidence supporting altered dopamine transmission in drug free schizophrenics has emerged with the advances in brain imagining techniques (e.g., positron emission tomography scans). For example, D_1 receptor availability measured *in vivo* was found to be increased in the PFC in drug naïve schizophrenic patients (Abi-Dargham *et al*., 2002), possibly reflecting a compensatory mechanism in response to hypodopaminergic activity. This same study reported that the increased D_1 receptor expression was a strong predictor of poor performance on the N-back task, a test of working memory. Other studies have shown that drug naïve patients with schizophrenia exhibit elevated baseline and AMPH-induced dopamine release within the striatum (Laruelle *et al*., 1996; Abi-Dargham *et al*., 2009) an observation which is believed to be associated with psychotic symptoms in schizophrenia.

More recently, a variety of genetic evidence has been put forward suggesting that genes (e.g., catechol-*O*-methyl transferase (COMT) (Egan *et al*., 2001; Shifman *et al*., 2002), dopamine receptor D_4 (DRD4) (Aguire *et al*., 2007; Lai *et al*., 2010) and V-akt murine thymoma viral oncogene homolog 1 (AKT1) (Tan *et al*., 2009; Thiselton *et al*., 2008) or specific variants of genes, that code for mechanisms involved in dopamine function may be more prevalent in people experiencing psychosis or diagnosed with schizophrenia, suggesting a definitive role for dopamine in the disease process.

1.5.2 *NMDA*

Several lines of evidence have implicated glutamatergic dysfunction in schizophrenia. The strongest evidence comes from dissociative anesthetics including PCP and ketamine which when abused induce a psychotic state (e.g. both negative and positive symptoms, as well as many of the cognitive deficits) that resembles schizophrenia (Javitt and Zukin, 1991; Newcomer and Krystal, 2001). These compounds have also been shown to strongly exacerbate symptoms in patients with schizophrenia (Malhotra *et al*., 1997). Because PCP and related compounds are antagonists of NMDA receptors, the

pharmacologic literature has been interpreted as suggesting that schizophrenia may be associated with a decrease or 'hypofunction' of NMDA-receptor activity (Coyle and Tsai, 2004).

Besides psychopharmacological models, transgenic animals have been used to evaluate the behavioural and molecular outcomes of NMDA hypofunction. NMDA receptor subunit NR_1 knockdown mice which have a global reduction of NMDA receptors, exhibit a spectrum of altered behaviours consistent with those observed in schizophrenia (e.g. attentional deficits, impaired social behaviour and cognitive symptoms) and a pattern which is similar to those induced by NMDA receptor antagonists. Similar findings have been obtained by altering the glycine binding site on the NR_1 subunit, a site that must be occupied by glycine or D-serine for the efficient opening of the NMDA channel (Clements and Westbrook, 1991).

Two lines of investigation provide direct evidence for altered NMDA function in schizophrenia. Firstly, neuroanatomical evaluation of postmortem brain tissue has shown alterations in NMDA receptor subunits and interacting post synaptic density (PSD) proteins in patients with schizophrenia (Kristiansen *et al*., 2006). Secondly, an auditory event related potential in the supra-granular layer of primary auditory cortex called mismatch negativity (MMN) is reduced in schizophrenia (Umbricht and Krljes, 2005). Because MMN expression has been shown to be dependent on NMDA receptor function, the reduction in MMN observed in schizophrenia may be an indication of NMDA hypofunction.

The potential neurobiological underpinnings of the NMDA hypofunction observed in schizophrenia are likely to be varied, given the complex genetic and environmental aspects associated with the disorder. The possibilities include elevated levels of N-acetylaspartylglutamate (Bergeron *et al*., 2005) and kynurenic acid (Stone, 1993), which reduce or anatagonize NMDA receptor function. Other mechanisms involve reduced channel expression or trafficking (Woo *et al*., 2004, 2008; Bitanihirwe *et al*., 2009a; Lau and Zukin 2007), alterations in the redox state of the NMDA channel (Choi and Lipton,

2000), or even a reduction in the concentrations of the co-agonists, glycine (Neeman *et al*., 2005) and D-serine levels (Hashimoto *et al*., 2003).

Because endogenous dysfunction of NMDA receptor-mediated neurotransmission appears to contribute to the pathophysiology of schizophrenia, potentiation of NMDA receptor function may be a useful approach for the treatment of this disorder. One potential strategy has involved the activation of mGlu2/3 receptors (Patil *et al*., 2007), which are known to form complexes with 5-HT_{2A} receptors (Gonzalez-Maeso *et al*., 2008). Another strategy is to increase synaptic levels of glycine by blocking the glycine transporter since glycine acts as a co-agonist site on the NMDA receptor. Indeed, glycine has been reported to ameliorate persistent negative symptoms of schizophrenia (Heresco-Levy *et al*., 1999). Treatment with the endogenous glycine transport inhibitor sarcosine has also been reported to reduce negative symptoms, improve cognition and further reduce positive symptoms in schizophrenic patients receiving concurrent antipsychotics (Tsai *et al*., 2004).

1.5.3. *GABA*

Postmortem studies have provided strong evidence implicating the GABA neurotransmitter system in the pathogenesis of schizophrenia. These studies have reported a lower concentration of cortical GABA (Yoon *et al*., 2010) in addition to reduced numbers of glutamic acid decarboxylase (GAD_{65} and GAD_{67}) messenger ribonucleic acid (mRNA) expressing neurons in the brains of patients with schizophrenia (Volk *et al*., 2000; Heckers *et al*., 2002). Subsequent studies have expanded on these observations by demonstrating alterations in several presynaptic components of the GABAergic system (Volk *et al*., 2001). These GABA deficits are largely restricted to the chandelier and basket subclass of GABAergic interneurons which contain the calcium binding protein parvalbumin (Lewis *et al*., 2005). These neurons synapse on the cell body or axon initial segment of glutamatergic neurons and thus are positioned to potently regulate pyramidal cell output. The reported reductions in the number of

GABAergic interneurons, have been shown to occur in various cortical regions including the PFC (Beasley *et al*., 2002; Hashimoto *et al*., 2003; Volk *et al*., 2000), anterior cingulate cortex (Benes *et al*., 1991) and in the hippocampus (Heckers *et al*., 2002). A reduction in GABA synthesis and release would therefore be expected to result in some form of compensatory mechanism. Indeed, it has been shown that the immunoreactivity for the $GABA_A$ α_2 receptor subunit is markedly increased in the postsynaptic axon initial segments of pyramidal neurons (Volk *et al*., 2002).

Further evidence supporting the existence of GABAergic deficits in schizophrenia comes from experiments using noninvasive methods such as high-field magnetic resonance spectroscopy to examine GABA concentration in the brain. These studies have reported reduced cortical GABA in the visual cortex of patients with schizophrenia (Yoon *et al*., 2010). Transcranial magnetic stimulation has been utilized as another non-invasive means to assess cortical inhibitory mechanisms within the brain. Studies using this method have shown inhibitory action to be reduced in patients with schizophrenia (Fitzgerald *et al*., 2003). These experiments, taken together with the pathophysiology, strongly suggest that the GABA system is compromised in schizophrenia.

1.6. Genetic epidemiology of schizophrenia

Schizophrenia occurs at similar prevalence rates throughout most of the world, despite the diversity of cultural, racial and socioeconomic groups (McGrath *et al*., 2004). This observation also lends weight to the concept of an inherited vulnerability with a relatively stable gene frequency. Modern studies suggest that the risk for schizophrenia is increased ~10-fold (lifetime risk 1-16%) in first-degree relatives of schizophrenic probands compared to relatives of control probands from the general population (0–2 %) (Kendler and Diehl, 1993) (**Figure 1.2**). Furthermore, family, twin and adoption studies have consistently suggested that most or nearly all of this tendency for schizophrenia to run in families is genetic in etiology, with heritability estimates as high as 0.8 (Norton *et al*., 2006). Like other common disorders, the genetics are complex (Risch, 1990).

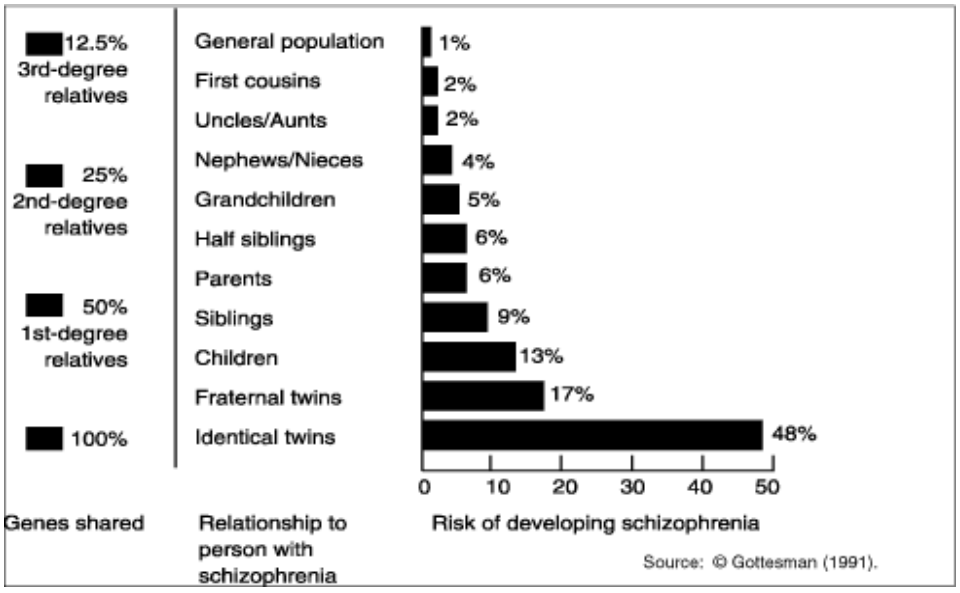

Figure 1.2: Risk of developing schizophrenia (Source: Gottesman, 1991)

1.7. The concept of the "Endophenotype" in schizophrenia research

Gottesmann and Shields were the first to describe the potential use of "endophenotypes" to describe aspects of schizophrenia that constituted "internal phenotypes" which were not as discernable as diagnostic clinical symptoms (Gottemann and Gould, 2003). Essentially, endophenotypes are discrete, quantifiable and heritable phenotypes that may be a part of a complex illness. An endophenotype may be neurophysiological, biochemical, endocrinological, neuroanatomical, cognitive, or neuropsychological phenotype (Gottesman and Gould, 2003). Criteria useful for the identification of markers in psychiatric genetics have been suggested and have been adapted to apply to endophenotypes. These criteria include that the endophenotype is associated with illness in the population, it be heritable, it possess measurement reliability, it be primarily state independent (i.e. manifestation whether illness is present or in remission), that within families, the endophenotype and illness co-segregate and the endophenotype have greater prevalence within affected families than within the general population (Gould and Gottesmann, 2006).

The rationale for the use of endophenotypes holds that if the phenotypes associated with a disorder are very specialized and represent relatively straightforward and putatively more elementary phenomena (as opposed to behavioural macros), the number of genes required to produce variations in these traits may be fewer than those involved in producing a psychiatric diagnostic entity. Endophenotypes provide a means of identifying the downstream traits or facets of clinical phenotypes, as well as the upstream consequences of genes, and, in principle, could assist in the identification of abberant genes in the hypothesized polygenic systems conferring vulnerabilities to disorders. In this context, two well-established endophenotypes in schizophrenia are sensory motor gating (Turestsky *et al*., 2007; Braff *et al*., 2008) and working memory dysfunction (Braff *et al*., 2008).

1.8. Behavioural analysis in animal models of schizophrenia

The modeling of human psychiatric disorders such as schizophrenia in animals is extremely challenging seeing as they cannot self-report hallucinations, scattered thinking, and other features of the disease. Nonetheless, one fruitful experimental approach is to focus on the behavioural and cognitive endophenotypes of schizophrenia in mice which may help to elucidate the underlying processes that are thought to characterize the disorder (Arguello and Gogos, 2006; Kellendonk *et al*., 2009). In order to assess such phenotypes, cross-species translational paradigms have been developed for the identification and characterization of neuropsychological, cognitive and psychopharmacological core dysfunctions implicated in human psychotic disorders. **Table 1.3** provides a selection of behavioural paradigms commonly used to assess schizophrenia-related abnormalities in rats and mice.

Behavioural paradigm	Neuropsychological/chemical processes involved
Open field exploration	Exploration -, anxiety related, stereotypical behaviour
Elevated Plus Maze	Anxiety behaviour
Sucrose preference test	Anhedonia
Y-Maze	Novelty preference and spatial recognition memory
Object Recognition Task	Recognition memory
Morris water maze	Spatial working memory and reference memory
Cheeseboard	Spatial working memory and reference memory
5-choice serial reaction time test	Attention
Social interaction test	Social interaction
Prepulse inhibition	Sensorimotor gating
Latent inhibition	Selective associative learning
Behavioural reaction to Amphetamine	Dopamine associated neurotransmission
Behavioural reaction to NMDA- receptor blocker	Glutamate associated neurotransmission

Table 1.3. An overview of some of the experimental paradigms used to investigate behavioural and neurochemical abnormalities in animal models.

1.9. Environmental risk factors

A number of environmental risk factors have been put forward as possible contributory causes of schizophrenia. In particular, urban birth and upbringing has been reported more frequently among individuals with schizophrenia than in the general population (Pedersen and Mortensen, 2001; Van Os *et al*., 2010). The reason of this occurrence is unclear, but exposure to deleterious factors, including infection, toxins, or malnutrition associated with urban environment have been suggested as possible mechanisms. Other evidence suggests that complications during delivery are associated with an increased risk of developing schizophrenia (Cannon *et al*., 2002). In addition to schizophrenia, these factors predispose to other disorders including mood disorders, antisocial personality disorder, learning problems and minimal brain dysfunction (syndrome) (Fatemi and Folsom, 2009).

Besides prenatal causes, a variety of factors in childhood and youth environments have been proposed to contribute to the risk of developing schizophrenia. These include exposure to cannabis (van Os and Kapur , 2009; van Os *et al*., 2010) and migration (Selten *et al*., 2007) in addition to growing up in a dysfunctional family (Wahlberg *et al*., 1997) or an ethnic minority group (van Os and Kapur, 2009; van Os *et al*., 2010). Interestingly, adoption studies have shown that individuals at high genetic risk of schizophrenia are more sensitive to problems in the rearing environment than individuals having lower genetic risk (Wahlberg *et al*., 1997; Tienari *et al*., 2004). Therefore, environmental stressors are thought to have a particularly deleterious effect on those individuals with a genetic diathesis or predisposition to a particular psychopathology (van Os *et al*., 2010).

1.10. Infections and schizophrenia

A variety of epidemiological evidence has been put forward implicating prenatal infection as a risk factor for schizophrenia. Specifically, the observation of an excess of winter-spring births (5-8%) among individuals who develop schizophrenia later in life has been interpreted as evidence of risk posed by elevated winter infections (Mortensen *et al*., 1999; Torrey *et al*., 1997). In this respect, exposure to specific infectious agents during fetal development and early development has been associated with schizophrenia as shown in **Table 1.4.** Notably, comparison between schizophrenics born to infected as opposed to non-infected mothers has recently revealed differences in anatomical (enlarged cavum septum pellucidum) and cognitive function between the two groups (Brown *et al*., 2009a,b).

Table 1.4. Infectious diseases during the prenatal period and early life associated with schizophrenia in later life

Specific infectious Agents		
Organism	**Positive Studies**	**Negative studies**
Toxoplasma gondii	Brown *et al*., 2005; Mortensen *et al*., 2007	Xiao *et al*., 2009
Herpes simplex virus type 2	Buka *et al*., 2001	Brown *et al*., 2006
Influenza viruses	Ellman *et al*., 2009 ; Brown *et al*., 2004; Adams *et al*.,1993	Torrey *et al*., 1988 Westergaard *et al*., 1999
Rubella virus	Brown and Susser, 2002	Buka *et al*., 2001; Torrey *et al*., 1988
Polio virus	Torrey *et al*., 1988; Suvisaari *et al*., 1999	Cahill *et al*., 2002
Measles virus	Torrey *et al*., 1988	O'Callaghan *et al*., 1994

There is still no clear consensus as to what period during pregnancy infection may confer the maximal risk for the offspring to develop schizophrenia. Initial studies reported an increased risk of schizophrenia when maternal infection occurred during the second trimester (Mednick *et al*., 1988; Wright *et al*., 1995; Stöber *et al*., 2002) whereas subsequent studies reported that infection during the first trimester of pregnancy is associated with the highest risk of schizophrenia in the offspring (Brown *et al*., 2001, 2004; Babulas *et al*., 2006; Sørensen *et al*., 2009). Nonetheless, these data have been interpreted to suggest that maternal infection with a wide variety of agents might potentially increase risk for schizophrenia through a mechanism whereby infection triggers innate immunity through common signaling pathways.

1.11. Mechanistic links between immune activation and schizophrenia

There is considerable agreement in the literature examining both rodents and primates about plausible mechanisms linking maternal immune activation during pregnancy to schizophrenia including downstream events such as cytokine release, oxidative stress and apoptosis in addition to abnormal zinc and iron metabolism.

1.11.1 *Cytokines*

Cytokines are pleotrophic glycoproteins often considered the 'hormones' of the immune system. They are involved in the regulation of immunologic and inflammatory responses in physiologic and pathologic conditions (Steinke and Borish, 2006). Examples of cytokines include interleukins (IL), interferons (IFN), tumor necrosis factors (TNF), transforming growth factors (TGF), and chemokines. These molecules are synthesized and secreted by a variety of cell types, including not only immune cells such as T lymphocytes, natural killer (NK) cells, dendritic cells, polymorphonuclear leukocytes, monocytes/macrophages, and microglia but also non-immune cells, such as fibroblasts, endothelial cells, adipocytes, and neurons (Ikram *et al*., 2004).

In addition to being important mediators of the cross-talk between the CNS and the immune system, cytokines also play a role in brain development and synaptic plasticity which might have implications for clinical psychiatry (Deverman and Patterson, 2009; McAfoose and Baune, 2009). In line with this notion, pathological induction of cytokines in response to maternal infections has been shown to have adverse effects on the neurodevelopment of offspring (Meyer *et al*., 2009; Deverman and Patterson, 2009; Patterson, 2009; Boksa, 2010).

Although the epidemiological relationship between *in utero* infections and schizophrenia remains unclear, the maternal cytokine-associated inflammatory response to infection may be a crucial link, as the

identity of the pathogen seems irrelevant (Gilmore and Jarskog, 1997; Brown and Derkits, 2010; Deverman and Patterson, 2009; Meyer *et al*., 2009; Patterson, 2009; Watanabe *et al*., 2010). Based on these observations, the cytokine hypothesis of schizophrenia has been conceived, predicting that perturbed cytokine signaling plays a pivotal role in the pathophysiology of this disease (Gilmore and Jarskog, 1997; Meyer *et al*., 2009; Watanabe *et al*., 2010).

1.11.2. *Oxidative stress*

The immune response can be a source of oxidative stress during infection. It is therefore possible that infection during pregnancy could act to increase maternal oxidative stress and thereby alter fetal brain development. Oxidative stress is a pathophysiological process resulting from an impairment in cellular antioxidant defense mechanisms to counterbalance and control endogenous reactive oxygen species and reactive nitrogen species generated from normal oxidative metabolism or from pro-oxidant environmental exposures (Kohen and Nyska, 2002; Berg *et al*., 2004). Notably, this process has been linked to the pathophysiology of various neurological disorders including schizophrenia (Bitanihirwe and Woo, 2011; Do *et al*., 2009; Yao and Reddy, 2011)

Infection with the bacterial endotoxin lipopolysaccharide (LPS) during pregnancy has been shown to trigger oxidative stress during fetal brain development (Lanté *et al*., 2007). Specifically, LPS treatment triggered an oxidative stress response in the hippocampus of male fetuses, evidenced by damage to proteins and in the ratio of reduced/oxidized forms of glutathione (GSH/GSSG). In contrast, none of the biochemical changes observed in males were observed in female fetuses. This report also showed that NMDA synaptic currents and long term potentiation [LTP, a phenomenon critical for learning and required to encode memories (Malenka and Nicoll, 1999)], in addition to spatial working memory in the water maze, were impaired in male but not in female offspring exposed to immune activation by LPS *in utero*. Interestingly, pretreatment with the antioxidant N-acetyl cysteine (NAC), a precursor of GSH,

prevented the LPS induced changes in the biochemical markers of oxidative stress in male fetuses, and the delayed detrimental effects in male offspring, completely restoring both LTP in the hippocampus and spatial working memory performance (Lanté *et al*., 2007). Together these findings suggest that oxidative stress in the fetal brain may participate in the neurodevelopmental damage induced by prenatal infection.

1.11.3. *Zinc*

Besides affecting the development and integrity of the immune system, zinc has a variety of effects within the CNS and these effects depend on an elaborately regulated and precisely balanced zinc concentration (Bitanihirwe and Cunningham, 2009b; Frederickson *et al*., 2005; Rink and Gabriel, 2000). In fact, recent evidence suggests that supplementation of the maternal diet with increased levels of zinc during pregnancy can prevent prenatal LPS-induced anomalies in object recognition memory in adult mouse offspring (Coyle *et al*., 2009). Increased levels of cytokines resulting from an infection process have been shown to induce acute phase proteins in the liver including the zinc-binding protein, metallothionein (Ghoshal *et al*., 2001). Interestingly, maternal and fetal zinc levels have been shown to drop following immune activation in pregnant rodents as a result of metallothionein induction (Coyle *et al*., 2009). Consistent with this idea, it has been reported that treatment of wild-type mice with LPS results in the induction of metallothionein and a fall in plasma zinc levels (Carey *et al*., 2003). However, this effect was not observed in metallothionein knockout mice that were treated with LPS (Carey *et al*., 2003). The hypozincaemia associated with LPS exposure may also result from IL-6 mediated induction of zinc transporters (Liuzzi *et al*., 2005). Notably, the birth defects associated with prenatal exposure to LPS could be abrogated with a supplementation of zinc in the maternal diet (Carey *et al*., 2003; Chua *et al*., 2006). It was therefore postulated that zinc supplementation may help prevent the behavioural deficits associated with prenatal LPS exposure by restoring zinc to normal levels necessary for appropriate CNS development (Coyle *et al*., 2009).

1.11.4. *Iron*

Iron has been shown to play a critical role in the immune system and in defense against infection (Brock and Mulero, 2000) with experimental and clinical data suggesting that there is an increased risk of infection during iron deficiency. In the context of maternal infection as a risk factor for schizophrenia, a recent study demonstrated the importance of hypoferremia (viz. a cytokine induced reduction of serum non-heme iron) in inducing behavioural and biochemical changes relevant to schizophrenia (Aguilar-Valles *et al*., 2010). The study used a rat model of localized injury induced by turpentine, which triggers the innate immune response and inflammation, in order to investigate the effects of maternal iron supplementation on the offspring's dopamine function. Offspring of turpentine treated mothers exhibited an enhanced behavioural sensitization to amphetamine following repeated exposure to this drug, when compared to control offspring. These behavioural changes were accompanied by increased baseline levels of tyrosine hydroxylase, dopamine and its metabolites, selectively in the nucleus accumbens (Aguilar-Valles *et al*., 2010). Interestingly, the behavioural and neurochemical changes were prevented by maternal iron supplementation. Given that schizophrenia is associated with increased subcortical dopamine, it is probable that abnormalities in fetal/maternal iron homeostasis may play a role in developmental processes that render the offspring more susceptible to schizophrenia.

1.11.5. *Apopotosis*

Apoptosis is a form of programmed cell death that is tightly regulated by a complex cascade of pro- and anti-apoptotic proteins. This process is pervasive in fetal brain development and eliminates injured or diseased neurons during the lifespan (Jarskog *et al*., 2004). In particular, apoptosis has been shown to be mediated by pro-inflammatory cytokines such as Il-1β (Fankhauser *et al*., 2000) and TNF-α (Rath and Aggarwal, 1999). A role for apoptosis in the etiopathogenesis of schizophrenia has been suggested in order to account for evidence of neuronal or synaptic/dendritic loss in the absence of cortical

gliosis in postmortem studies (Jarskog *et al*., 2005). Notably, analysis of the middle temporal gyrus from postmortem brain of patients with schizophrenia was shown to exhibit reduced levels of Bcl-2, an anti-apoptotic regulatory protein (Jarskog *et al*., 2000). However, a subsequent study by the same group found the levels of caspase 3, a marker of apoptosis, to be unchanged in the temporal lobe (Jarskog *et al*., 2004). It was therefore speculated that stimuli which induce apoptosis, including fetal insults (e.g. maternal infection during pregnancy), may result in the progressive neurostructural alterations in prodromal and first-episode psychosis.

1.12. Animal models of maternal infection/immune activation

The epidemiological association between maternal infection during pregnancy and enhanced risk of schizophrenia in the offspring has spurred the creation of a variety of rodent models of systemic prenatal infection or prenatal immune activation in order to characterize changes in brain function and behaviour caused by the prenatal insult (Patterson, 2009; Boksa, 2010; McAlonan *et al*., 2010; Meyer and Feldon, 2010). In these models, immune activation is induced in pregnant rats or mice by using specific viral pathogens, immune-stimulating agents or pro-inflammatory cytokines, and the long-term brain and behavioural effects of the prenatal maternal manipulations are then investigated in the offspring. Specifically, prenatal exposure to human influenza virus, the viral mimic polyriboinosinic–polyribocytidilic acid (Poly-I:C), LPS, turpentine, and the pro-inflammatory cytokine IL-6 have been shown to result in a multitude of behavioural, cognitive and pharmacological abnormalities in the resultant adult offspring (Meyer and Feldon, 2010; Patterson, 2009; Boksa, 2010; McAlonan *et al*., 2010). Consistent with the post-pubertal onset of schizophrenia most of the prenatal infection-induced behavioural deficits observed in rodents display a post-pubertal emergence.

A critical element of rodent models is that they allow the evaluation of prenatal immune activation at a specific time during pregnancy. This allows for the critical assessment and dissection of time windows in gestation during which prenatal immune activation alters specific CNS systems and provides an important initial step to elucidating the mechanisms mediating these effects. Indeed, by applying such a meticulous approach Meyer and colleagues have managed to dissect the effects by which maternal immune activation with Poly-I:C during different gestational times precipitates distinct psychopathological and neuropathological symptom clusters in the offspring. This section will therefore focus on the Poly-I:C model of immune activation.

By using the Poly-I:C model it was shown that prenatal immune activation with Poly-I:C during early/middle pregnancy (i.e. gestation day (GD) 9) leads to a behavioural profile characterized by deficits in spatial working memory (when the mnemonic load on temporal retention was high), reduced prepulse inhibition (PPI), abnormalities in selective associative learning in the form of latent inhibition (LI) and increased sensitivity to apomorphine (APO) (Meyer *et al*., 2006a,b; Vuillermot *et al*., 2010). In contrast, maternal immune activation with Poly-I:C during late pregnancy (i.e. GD17) resulted in deficits in spatial recognition memory and working memory (when the mnemonic load on temporal retention was low), potentiated response to the non-competitive NMDA-receptor antagonist dizocilpine (MK-801) and perseverative behaviour in the form of impaired discrimination reversal learning (Meyer *et al*., 2006a,b, 2008). However, similarities between GD9 and GD17 mice have been observed including an enhanced sensitivity to the locomotor stimulating effects of AMPH in addition to an abolition of the unconditioned stimulus-pre-exposure effect (Meyer *et al*., 2006b). Taken together, these prenatal time-window studies in the Poly-I:C mouse model indicate a developmental vulnerability to specific forms of behavioural and cognitive dysfunction in the offspring depending upon when immune activation is induced (**Table 1.5**)

The behavioural contrast between the effects of prenatal immune activation with Poly-I:C treatment in early/middle and late gestation may be related to differing symptom clusters of schizophrenia. Indeed, one hypothesis emerging from the prenatal time-window studies carried out in the mouse Poly-I:C model is that early/middle and late pregnancy immune challenge may capture the positive–negative dichotomy of schizophrenia (Sullivan *et al*., 2006; Meyer *et al*., 2007). Besides a dissociation in behavioural profiles, additional support for the notion that the timing of the immune challenge may prove effective in capturing the positive–negative dichotomy of schizophrenia comes from the neuroanatomical and neurochemical analysis of brains from immunologically challenged mice (**Table 1.6**). Notably, neurochemical studies have reported an increased level of dopamine in the mPFC and globus pallidus following administration of Poly-I:C on GD9 (Winter *et al*., 2009).

This observation may be relevant to the pathophysiology of the positive symptoms in schizophrenia given that their manifestation has been linked to a hyper-dopaminergic state (See section 1.5).

Synopsis of Behaviour Results		
Behaviour	***Poly-I:C GD9***	***PolyI-I:C GD17***
Open field exploration	No effect	No effect
Elevated Plus Maze	No effect*	No effect*
Reversal Learning	No effect	Impaired
Prepulse inhibition	Impaired	Impaired
Latent inhibition	Impaired – Attenuated LI	No effect
Morris Water Maze	Impaired[β]	Impaired[§]
Unconditioned Stimulus pre-exposure effect	Impaired	Impaired
Behavioural reaction to Apomorphine	Increased sensitivity	Not determined
Behavioural reaction to Amphetamine	Increased sensitivity	Increased sensitivity
Behavioural reaction to NMDA- receptor blocker	No effect	Increased sensitivity

Table 1.5. Overview of the experimental paradigms used to investigate behavioural abnormalities in the Poly-I:C model. *Unpublished observation: Meyer and Feldon. [β]When the load on mnemonic demand is high. [§]When the load on mnemonic demand is low.

Synopsis of Neuroanatomical and Neurochemical Results		
Feature	***Poly-I:C- GD9***	***Poly-I:C- GD17***
Enlarged lateral ventricles	Present[1]	No effect[1]
Brain volume	No effect[1]	No effect[1]
White matter deficits	Present[2]	Present[2]
Interneurons	Reduced parvalbumin cells	Reduced parvalbumin cells
Reelin	Reduced reelin containing neurons	Reduced reelin containing neurons
Glutamate receptors	No effect	Reduced NR_1 expression
Neurogenesis	Reduced	Reduced
Apoptosis	No effect	Increased
Dopamine levels	Increased[3]	Not determined
Serotonin levels	Reduced[3]	Not determined
Glutamate levels	No effect[3]	Not determined
GABA levels	No effect[3]	Not determined
Taurine levels	Reduced[3]	Not determined
D1 receptor expression	Reduced	No effect

Table 1.6. Comparison of the neuroanatomical and neurochemical abnormalities found in Poly-I:C GD9 and Poly-I:C GD17 mouse models. [1]Li *et al*., 2009; [2]Li *et al*., 2010, [3]Winter *et al*., 2009.

Together these findings support the use of the Poly-I:C maternal immune activation model as a means to dissect specific (i.e. early/middle and late) brain and behavioural abnormalities relevant to the positive and negative/cognitive symptoms of schizophrenia. The Poly-I:C model therefore provides a powerful tool in order to elucidate the structural and functional consequences of immune challenge during different times of gestation. Information gleaned from this research may provide important new insight into the fundamental neuropathological mechanisms underlying the segregation of positive and negative/cognitive symptoms of schizophrenia (Meyer and Feldon, 2011).

1.13. DISC1 as a risk factor for schizophrenia

Disrupted-in-Schizophrenia-1 (DISC1) has emerged as a strong candidate gene associated with major mental illness including depression, bipolar disorders and schizophrenia. Numerous studies have also reported evidence of linkage or association between DISC1 and impaired cognitive function in schizophrenic individuals. Further support implicating DISC1 in mental illness comes from animal models. Although the DISC1 protein has no known enzymatic activity it has been shown to exert its effect on multiple proteins through interaction to modulate their functional states in addition to their temporal and spatial activity. DISC1 protein interaction partners, combined with the a complex pattern of protein isoforms (James *et al*., 2004) and splice variants (Nakata *et al*., 2009) in addition to the widespread subcellular distribution of DISC1 (Chubb *et al*., 2008) have led to the suggestion that DISC1 acts as a multi-functional scaffold protein within the cell affecting multiple critical developmental pathways within the brain, disruption of which can lead to a variety of psychiatric illness phenotypes (Bradshaw and Porteous, 2011).

1.13.1. *DISC1*

DISC1 was first reported to be associated with increased risk of schizophrenia, bipolar disorder and recurrent major depression in a large Scottish pedigree via a balanced (1;11) (q42.1; q14.3) chromosomal translocation (St. Clair *et al*., 1990). The translocation mutation disrupts the DISC1 gene between exons 8 and 9 (Blackwood *et al*., 2001), as well as a non coding gene, termed DISC2. As an antisense transcript, DISC2 may modulate DISC1 expression.

1.13.2. *DISC1 protein structure*

The DISC1 gene is composed of 13 exons with an open reading frame that can encode a full-length protein consisting of 854 amino acid residues (~100 kDA) (Millar *et al.,* 2000). Because the Scottish translocation occurs between exons 8 and 9 the cytogenetic disruption would result in the loss of the C-terminal 257 amino acids of the protein.

Alternative splice variants of the DISC1 gene in human brain have shown that it can produce more than 50 splice variants in the brain (Nakata *et al*., 2009). These splice variants have been shown to result in four protein isoforms. The full-length human DISC1 protein is predicted to consist of an N-terminal region (encoded by exons 1 and 2) and an alpha-helical coiled–coil-containing C-terminal region (encoded by exons 3–13) (Millar *et al*., 2000).

1.13.3. *Expression*

DISC1 is expressed in a variety of body tissues, including testis, heart, placenta, kidneys and brain (Millar *et al*., 2000; James *et al*., 2004). The expression levels of DISC1 display pronounced developmental regulation with a high expression levels in the hippocampus and transient expression in the thalamus (Austin *et al.,* 2004). Expression of DISC1 in mouse brain has been reported to exhibit two major peaks at approximately embryonic day 13.5 and postnatal day 35 (Schurov *et al*., 2004). These periods of development correspond to the time points of active neurogenesis and onset of puberty in human development (Schurov *et al*., 2004). In a recent study, Mao and colleagues have reported the embryonic ventricular/subventricular zones of the cortex where neural progenitor cells reside to express high levels DISC1 (Mao *et al*., 2009). The knockdown of DISC1 on embryonic day 13 leads to decreased neural progenitor cell proliferation and premature neuronal differentiation in the developing cortex (Kamiya *et al*., 2005).

Post mortem studies in nonhuman primates and human brain tissue have shown DISC1 to be expressed in the dentate gyrus of the hippocampus and temporal and parahippocampal cortex (James *et al*., 2004; Lipska *et al*., 2006; Austin *et al*., 2004). Neuroanatomical studies in adult mouse brains have further shown DISC1 to be widely expressed in the cerebellum, cerebral cortex, dentate gyrus, amygdala, Cornu ammonis (CA)1-3 and olfactory bulb (Schurov *et al*., 2004).

1.13.4. *Association and linkage studies*

Over the past years a large number of linkage studies and association studies have been performed. Results from these studies suggest that DISC1 may play a role in major mental illnesses and associated cognitive functions. The strongest association was observed between an allelic haplotype, HEP3, which contains two single nucleotide polymorphisms (SNPs) (T-A allele at rs751229 and rs3738401) in a Finnish study (Hennah *et al*., 2003, 2005). This haplotype was found to be especially associated with schizophrenia in males (Hennah *et al.,* 2003). Since then a variety of studies have reported an association between DISC1 and schizophrenia in more than one ethnic group (Hodgkinson *et al*., 2004; Schumacher *et al*., 2009; Szeszko *et al*., 2008; Hashimoto *et al.,* 2006). Whereas other studies have failed to detect association between DISC1 and schizophrenia (Kim *et al*., 2008; Sanders *et al*., 2008; Devon *et al*., 2001).

Besides the genetic association studies implicating DISC1 in schizophrenia, evidence has emerged of an association between variants in the DISC1 gene and schizophrenia related endophenotypes. The Ser704Cys haplotype has been associated with schizophrenia and with the structure and function of the hippocampus (Callicott *et al*., 2005). The Ser allele was associated with altered hippocampal structure and function in healthy subjects, including reduced hippocampal gray matter volume and altered engagement of the hippocampus during several cognitive tasks assayed with functional magnetic resonance imaging (Callicott *et al*., 2005). Moreover, carriers of the Ser704/704 genotype appear to

exhibit increased activation of the PFC, especially of the left hemisphere during verbal fluency tasks, a classic test of prefrontal function (Prata *et al*., 2008). This finding has been interpreted as a manifestation of less efficient prefrontal function, with more activation needed to achieve the same behavioural output. Recently, it has been reported that white matter integrity is associated with the Ser704Cys haplotype in patients with schizophrenia (Sprooten *et al*., 2011). Other variants in DISC1 have displayed association with: visual working memory (Hennah *et al*., 2005), executive function (Chakirova *et al*., 2011) attention (Liu *et al*. 2006), sociability (Li *et al*., 2007), the P300 event related potential (Blackwood *et al*., 2001) and cognitive aging (Thomson *et al*., 2005a). There is also evidence suggesting that DISC1 is associated with anhedonia psychosis proneness measures in the general population (Tomppo *et al*., 2009). DISC1 also seems to play a role in brain physiology by associating with reduced gray matter volume in several brain regions including the superior frontal gyrus, anterior cingulated gyrus and the dorsolateral PFC (Cannon *et al*., 2005; Szeszko *et al*., 2008).

Given the phenotypical heterogeneity linked to DISC1 it is perhaps unsurprising that this gene has been associated with other neurodevelopmental disorders. Notably, DISC1 has been associated with bipolar disorder (Thomson *et al*., 2005b), schizoaffective disorder (Thompson *et al*., 2005) and depression (Hashimoto *et al*., 2006). DISC1 also displays evidence of association with autism and Asperger's syndrome (Kilpinen *et al*., 2008). Furthermore, recent reports point towards a link between DISC1 and anxiety (Harris *et al*., 2010) and chronic fatigue syndrome (Fukuda *et al*., 2010).

1.13.5. *The DISC1 interactome*

DISC1 is a multifunctional scaffold protein that interacts and regulates a multitude of proteins in a variety of subcellular compartments including the centrosome, nucleus and mitochondrion (Chubb *et al*., 2008; Ishizuka *et al*., 2006; Bradshaw and Porteous, 2011) (**Figure 1.2**). Many putative interacting proteins have been identified through protein complex (co-) immunoprecipitation and yeast two-hybrid (Y2H) screening (Millar *et al*., 2005; Miyoshi *et al*., 2003, 2004; Camargo *et al*., 2007) and where these have been examined, a large proportion have been validated by downstream experimentation (Chubb *et al*., 2008). DISC1 and the binding proteins are mainly involved in functions related to neuronal migration, neurite outgrowth, synapse function, cell cycling, intracellular transport / exocytosis and adult neurogenesis. In this section, I will discuss what is known about the biological functions of DISC1-interacting proteins, with particular attention to aspects of their biology which potentially relate to schizophrenia, through effects on neurodevelopment, neurotransmission or neurosignalling.

The best characterized DISC1 protein–interactors include lissencephaly 1(LIS1), Nuclear distribution protein nudE homolog 1 (NDE1), and Nuclear distribution protein nudE-like 1 (NDEL1). This trio form a complex with DISC1 in the centrosome (Brandon *et al*., 2004) and have been shown to be involved in mitosis, neuronal migration, and microtubule organization during brain development (Chubb *et al*., 2008). Functionally, the NDEL1-DISC1 interaction has been shown to be critical to multiple neurodevelopmental processes that are abnormal in schizophrenia including neural outgrowth (Kamiya *et al*., 2005). Interestingly, genetic association studies have shown both NDE1 and NDEL1 to be implicated in the etiology of schizophrenia (Tomppo *et al*., 2009; Hennah *et al*., 2007).

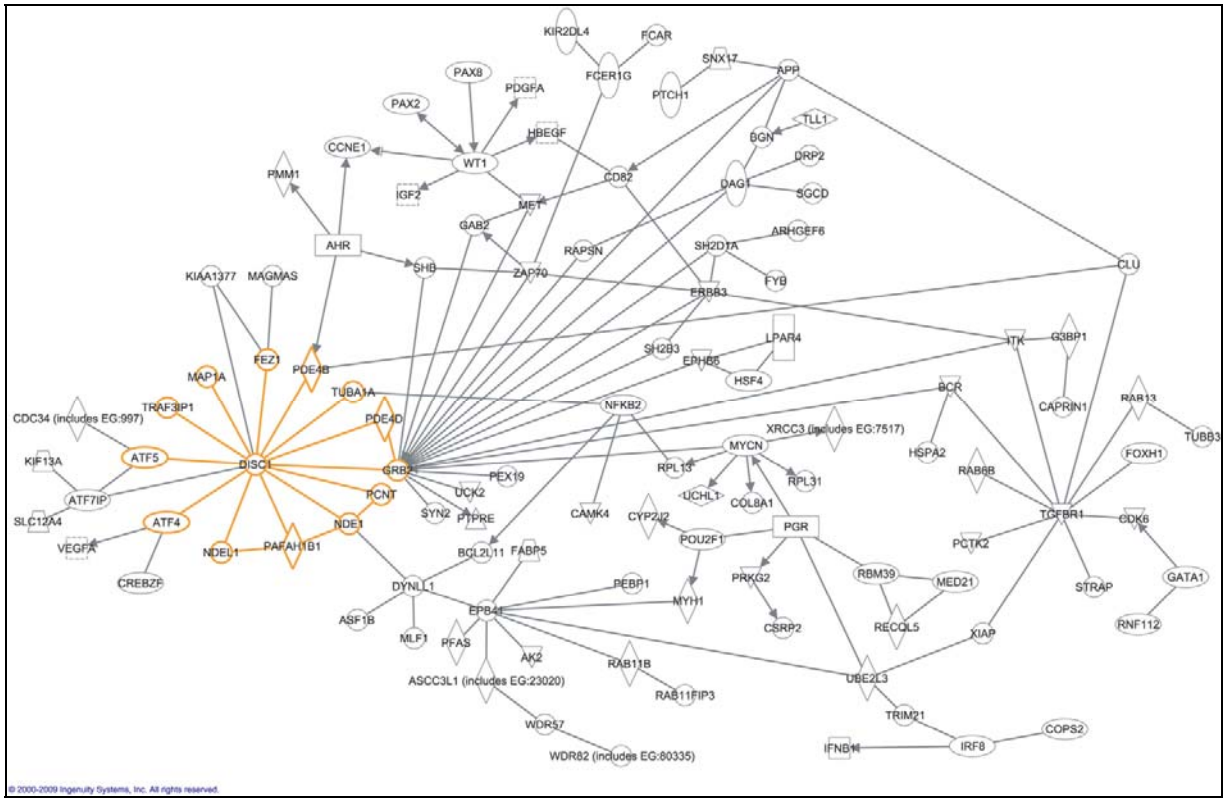

Figure 1.2. A network of genes related to DISC1 and its core interactors. (Source: Hennah and Porteous, 2009)

Independent studies have reported associations between phosphodiesterase 4B (PDE4B) and schizophrenia (Millar *et al*., 2005; Tomppo *et al*., 2009). Mammalian PDE4 isoforms comprise four distinct gene families that are homologues of DUNCE, a gene product required for synaptic plasticity, and learning and memory in *Drosophila* (Davis, 1996). DISC1 binds to PDE4B, an enzyme important in regulating the levels of the second messenger molecule cyclic adenosine monophosphate (cAMP). PDE4B also associates with the DISC1/NDEL1/NDE1/LIS1 centrosomal complex and may regulate its function through phosphorylating NDE1. Mutations within DISC1 disrupt PDE4B binding and function. Intriguingly, a DISC1 mutant mouse line has been shown to exhibit reduced binding between DISC1 and PDE4B (Clapcote *et al.,* 2007).These mice also have reduced brain PDE4B activity, most likely as a direct effect of the mutation upon PDE4B association with DISC1 (Clapcote *et al.,* 2007).

Recently it was shown that DISC1 interacts with glycogen synthase kinase 3β (GSK3β) (Mao *et al*., 2009). This study showed DISC1 to inhibit GSK3β signaling, thereby inhibiting wnt/β signaling and as a result regulating neural progenitor cell proliferation. Many signaling components in the latter pathway have been implicated in schizophrenia, including neuregulin 1 (NRG1), V-erb-a erythroblastic leukemia viral oncogene homolog 4 (ErbB4, which encodes for the receptor of NRG1), and AKT1 (Ross and Margolis, 2010). Interestingly, two independent studies have reported an association between GSK3β and schizophrenia (Souza *et al*., 2008; Scasselati *et al*., 2004).

The fasciculation and elongation protein-zeta-1 (FEZ1) has been shown to be an interacting partner of DISC1 using Y2H analysis, and co-localizes with DISC1 in growth cones in cultured hippocampal neurons (Miyoshi *et al*., 2003). Perhaps unsurprisingly, disruption of the DISC1–FEZ1 interaction has been shown to result in neuronal deficits, such as defects in axonal outgrowth (Miyoshi *et al*., 2003). Although there is biological (Lipska *et al*., 2006) and genetic (Yamada *et al*., 2004) evidence implicating FEZ1 in the pathophysiology of schizophrenia, other studies have failed to replicate the genetic association (Hodgkinson *et al*., 2007; Koga *et al*., 2007). DISC1 also interacts with the scaffold protein kendrin/pericentrin (Miyoshi *et al*., 2004), a giant protein known to localize specifically to the centrosome and which plays a role in the microtubule nucleation and aster formation. The binding of DISC1 to kendrin, is indispensible for the centrosomal localization of DISC1. Furthermore, *in vitro* evidence suggests that the DISC1-kendrin interaction is involved in centrosomal microtubule network formation (Shimizu *et al*., 2008). Intriguingly, recent studies indicate that mutations within the PCNT gene, which encodes pericentrin, are associated with primordial dwarfism (Rauch *et al*., 2008). Individuals with primordial dwarfism present with several features, including extreme proportionate short stature and small brain size relative to body size (microcephaly), suggesting PCNT to be important for neurodevelopment (Rauch *et al*., 2008). Importantly, there have been positive genetic association findings with regards to the potential involvement of PCNT in schizophrenia (Numata *et al*., 2009). Another DISC1 interactor, activating transcription factor 4 (ATF4 or CREB2),

plays a key role in the regulation of the stress response in neurons (Galehdar *et al*., 2010). Thus, the interaction between DISC1 and ATF4 has been hypothesized to play a role in the response to environmental factors relevant to schizophrenia, such as birth hypoxia and congenital infections (Jaaro-Peled *et al*., 2009). A genetic association study detected two polymorphisms within the ATF4 gene that were significantly associated with schizophrenia (Qu *et al*., 2008). Other genetic studies evaluating DISC1 interacting proteins including TNIK (encoding TRAF2 and NCK interacting kinase) and YWHAE (encoding 14-3-3 epsilon) have implicated these genes in schizophrenia (Plotkin *et al*., 2009; Ikeda *et al*., 2008), although some of these are single studies or report modest associations that await firm replication.

1.13.6. *Animal models of DISC1*

1.13.6.1. *Zebrafish*

Zebrafish (*Danio rerio*) have recently become a focus of neurobiology studies since larvae display behavioural and neuropathalogical phenotypes that are quantifiable and relate to those seen in man. Because the zebrafish affords the opportunity to perform rapid loss- and gain-of-function assays, the feasibility of screening small molecules and the ability to assay function of human gene variants rapidly it is an excellent tool for analysis of genes associated with mental health disorders (Kabashi *et al*., 2011).

Conserved domains are present in the DISC1 gene in zebrafish and mammals. Zebrafish DISC1 is expressed maternally and zygotic expression is strongest in the developing CNS (Brandon *et al*., 2009). In order to evaluate the embryonic function of DISC1, zebrafish were generated using a DISC1 morpholino (designed anti-sense oligonuceotides that are used in order to transiently decrease the expression of a particular gene) that ablates all normal DISC1 mRNA (Drerup *et al*., 2009). Knock down of DISC1 in zebrafish embryos using morpholinos resulted in defects of normal oligodendrocyte development and near total failure of olig2-positive cerebellar neuron development (Wood *et al*, 2009). Specifically, loss of DISC1 was shown to result in a persistent cranial neural crest cell medial migration, dorsal to the developing neural epithelium, and hindered migration away from the region dorsal to the neural rod (Drerup *et al*., 2009). The failure of cranial neural crest cells to migrate away from the neural rod was determined to be correlated with the enhanced expression of two transcription factors, foxd3 and sox10. Because foxd3 and members of the Sox family are expressed in the developing brain (Wegner and Stolt, 2005; Takahashi *et al*., 2009) it has been speculated that their regulation by DISC1 may be conserved in the brain. These data identify DISC1 as a crucial positive modulator of cell migration, fate determination and differentiation and confirm the efficacy of using zebrafish as a tool to explore the function of genes implicated in human mental health disorders.

1.13.6.2. *Drosophila*

Drosophila melanogaster has provided a powerful genetic system in which to elucidate fundamental cellular pathways in the context of a developing and functioning nervous system. Generation of genetically engineered flies is much easier than generation of knockout or transgenic mice. Thus, transgenic flies expressing human disease genes have been generated to examine functions of such disease factors, with the hope that these flies can be utilized as disease models.

Flies that express human DISC1 are viable and display no gross morphological changes (Sawamura *et al*., 2008). In the transgenic flies, human full-length DISC1 is expressed in a punctate pattern in the nucleus (Sawamura *et al*., 2008). The transgenic flies display disturbance in sleep homeostasis, which has been reportedly associated with CREB signaling or CRE-mediated gene transcription (Hendrick *et al*., 2001). The punctate localization of DISC1 in the nucleus is also observed in mammalian cells with colocalization with promyelocytic leukemia bodies, a nuclear compartment for gene transcription. Notably, DISC1 has been shown to modulate CRE-mediated gene transcription by interacting with ATF4/CREB2 and recruiting the nuclear receptor corepressor (N-CoR) to the transcriptional machinery. The interaction of DISC1 with N-CoR may play a key role in this modulation, because N-CoR is responsible for recruiting histone deacetylase to targeted transcriptional machinery (Jepsen *et al*., 2002). Recent studies have suggested a role for the cascades involving histone acetyltransferase and deacetylase in psychiatric conditions, including cocaine-induced plasticity, bipolar disorder, depression and schizophrenia (Tsankova *et al*., 2006; Kumar *et al*., 2005; Sharma *et al*., 2006). As nuclear DISC1 is altered in brains from subjects with psychosis as well as those with substance or alcohol abuse (Sawamura *et al*., 2005), disturbance of DISC1 with respect to recruitment of the repressor

complex to transcriptional machinery, such as that comprising CREB and ATF4/CREB2, may play an important role in a wide range of psychiatric disorders.

1.13.6.3. *Mouse models*

1.13.6.3.1 *Behavioural changes*

In order to evaluate the effects of altered DISC1 function on endophenotypes involved in schizophrenia and other mental disorders, seven different mouse models have been generated to date (Koike *et al*., 2006; Shen *et al*., 2008; Hikida *et al*., 2007; Clapcote *et al*., 2007; Pletnikov *et al*., 2008; Li *et al*., 2007). Three of these models were generated expressing a construct corresponding to the DISC1 translocation observed in the Scottish pedigree: one expressing a dominant-negative form of DISC1 (DN -DISC1) under the α-Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII) promoter limiting expression of the transgene to the forebrain (Hikida *et al*., 2007); another model made use of the Tet-Off double transgenic system under the regulation of the CAMKII promoter to generate transgenic mice with inducible expression of mutant human DISC1 (hDISC1 mutant) (Pletnikov *et al.*, 2008) and one line expressed truncated murine DISC1 using the bacterial artificial chromosome (BAC) system (BAC-ΔC Tg mice) (Shen *et al*., 2008).

Concerning behavioural abnormalities in these animal models of DISC1, the DN-DISC1 mutants display hyperactivity, deficits in sensorimotor gating (as measured by PPI) and olfactory-associated behaviour, and an anhedonia/depression-like state. However, no deficits in working memory were reported (Hikida *et al*., 2007). In contrast, BAC-ΔC Tg mice exhibit increased immobility and reduced vocalization in depression-related tests, and impairment in conditioning of LI (Shen *et al*., 2008). hDISC1

mutant mice display some sex-specific behavioural deficits: spontaneous hyperactivity in the open field and decreased social investigation and increased aggressivity in a dyadic test of social interaction, each confined to males, and impaired spatial working memory in females (Pletnikov *et al*., 2008). Interestingly, constant (i.e. prenatal and postnatal) expression of mutant human DISC1 resulted in male specific sensitivity to the locomotor stimulating effects of AMPH and dizocilpine (MK-801), whereas selective postnatal expression (i.e. from embryonic day 15 onwards) only resulted in decreased social behaviour in male mice and depression-like responses in female mice (Ayhan *et al*., 2011).

In addition to the transgenic mutants described above, two mutant lines carrying single point-mutations in the endogenous mouse DISC1 have recently been developed (Clapcote *et al.*, 2007). These mutants were identified by a systematic N-ethyl-N-nitrosourea (ENU) experiment. One DISC1 mutation (Q31L (127A/T: glutamine→ leucine)) produced a depressive-like phenotype including deficits in the forced swim test, decreased sucrose consumption (a putative index of anhedonia) and reduced social approach behaviours. Interestingly, increased immobility in the forced swim test in Q31L DISC1 mutants was reversed by the antidepressant bupropion. A second DISC1 mutation (L100P (334T/C: (leucine→ proline)) was associated with a schizophrenia-like phenotype, including profound deficits in PPI and LI in addition to deficits in working memory and hyperactivity. The deficits in PPI and LI in L100P DISC1 mutants could be pharmacologically reversed by both typical (haloperidol) and atypical (clozapine) antipsychotics. These findings demonstrate that DISC1 missense mutations in mice give rise to phenotypes related to mood disorders and schizophrenia. Even though the missense mutations provide an important link between mutations in the DISC1 gene and phenotypes related to schizophrenia, the amino acid sequences generated by these missense mutations are not conserved between humans and rodents and therefore they might not be directly comparable to the human genetic variations involved in the disease.

Another DISC1 mutant line was discovered based on the observation that the 129S6 Sv/Ev strain of inbred mice harbour a 25-bp deletion variant in exon 6 of DISC1 (D25 BP) that introduces a termination codon at exon 7, abolishes production of the full-length protein, and impairs working memory performance when transferred to the C57BL/6J genetic background (Kvajo *et al*., 2006; Koike *et al*., 2008). These mice however did not exhibit any impairment on other forms of memory (win-shift version of radial maze, short-term memory, novel object recognition, recognition memory, Morris water maze, reference memory) or on tests of associative learning (contextual conditioning).

Li and colleagues generated a transgenic DISC1 line with an inducible transgenic system in which they demonstrated that early postnatal (day 7) expression of a DISC1 C-terminal fragment, but not adult induction was associated with deficits in spatial working memory and reduced sociability (Li *et al*., 2007). Postnatal disruption of DISC1 function also resulted in depressive-like behaviour, as indexed by decreased latency to float (i.e. increased duration of immobility) in the forced swim task. This observation is particularly interesting as it is consistent with the depressive profile observed in both the DISC1 Q31L line (Clapcote *et al*., 2007) and the BAC-ΔC Tg mice (Shen *et al*., 2008).

1.13.6.3.2 *Anatomical and biochemical changes*

Enlargement of the lateral cerebral ventricles accompanied by volume decreases of various brain areas are among the most consistent structural brain imaging abnormalities observed in schizophrenia (Wright *et al*., 2000). DISC1 mouse models have similar gross abnormalities in brain structure. Translational magnetic resonance imaging (MRI) studies of transgenic mice with ENU induced missense mutations (L100P or Q31L) of DISC1 have shown these mice to exhibit enlarged ventricles in association with reduced brain volume compared with wild-types, especially in the cerebellum, cortex, entorhinal cortex, and thalamus (Clapcote *et al*., 2007). In contrast, DN-DISC1 mice (Hikida *et al*., 2007) and

hDISC1 mutant mice (Pletnikov *et al*., 2008) exhibit enlarged lateral ventricles (without a change in brain volume).

BAC-ΔC Tg mice are the only model in which both reduced cerebral cortex and enlarged lateral ventricles have been detected by histology (Shen *et al*., 2008). These mice also exhibited a thinning of layers 2-3 which may have resulted in the observed reduction in the thickness of the cortex in this model. In addition, expression of truncated murine DISC1 using the BAC vector system resulted in partial agenesis of the corpus callosum which is expected to interfere with interhemispheric communication. Notably, alterations in interhemispheric callosal connections has been found in schizophrenia (Miyata *et al*., 2007)

Previous studies have shown DISC1 to be involved in neurogenesis (Mao *et al*., 2009). Specifically, DISC1 has been shown to enhance Wnt/β-catenin-dependent neural progenitor proliferation (Mao *et al*., 2009). Within this pathway, DISC1 binds and inhibits GSK-3β, thereby inducing β-catenin/TCF/LEF-dependent gene transcription. Interestingly, Q31L, L100P and BAC-ΔC Tg mice have been reported to exhibit reduced neurogenesis (Shen *et al*., 2008; Lee *et al*., 2011). Because high levels of DISC1 are expressed in the neurogenic regions of the hippocampus, DISC1 is believed to play a role in ongoing adult neurogenesis. Although speculative, it may be that mutations in the DISC1 gene could disrupt adult neurogenesis which may be related to morphological and functional changes affecting the hippocampus in schizophrenia (Toro and Deakin, 2007).

Cell culture studies have shown DISC1 to play an important role in neurite growth (Miyoshi *et al*., 2003; Kamiya *et al*., 2005). Consistent with the observations in studies carried out in primary cell cultures, isolated embryonic cortical neurons from two DISC1 mouse models (i.e. hDISC1 mutant and BAC-ΔC Tg mice) have been shown to exhibit attenuated neurite outgrowth (Pletnikov *et al*., 2008; Shen *et al*., 2008).

Biochemical analyses of mutant DISC1 mice have shown some of these mice to exhibit biochemical deficits. Immuno-co-precipitation studies have demonstrated that DISC1 directly interacts with the multiple PDE4 isoforms (Millar *et al*., 2005). In this context, co-expression studies of DISC1 and PDE4 in cell culture have shown both Q31L and L100P DISC1 ENU-induced mutants to exhibit reduced binding to PDE4B (Clapcote *et al*., 2007). Although, it appeared that the Q31L mutants had a lower PDE4B activity, consistent with their resistance to the PDE4 inhibitor, rolipram (Clapcote *et al*., 2007). The mouse model of inducible expression of mutant human DISC1 was found to induce transient (at postnatal day (PND) 7, but not at PND 21) molecular changes including reduced protein levels of its interactors such as endogenous mouse DISC1 and LIS1(but not NDEL1) in addition to the presynaptic marker SNAP-25 (but not the postsynaptic marker PSD-95) (Pletnikov *et al*., 2008)

Increasing evidence suggests that the subset of GABA cells that contain the calcium buffering protein parvalbumin, which exhibit fast-spiking firing properties are functionally disturbed in schizophrenia (Hashimoto *et al*., 2003; Bitanihirwe *et al*., 2009a; Beasley *et al*., 2002). Consistent with the literature, a reduced number of parvalbumin- positive interneurons has been reported in the medial PFC (mPFC) of the DN-DISC1 mice (Hikida *et al*., 2007). Parvalbumin neurons have also been reported to be reduced in the hippocampus and mPFC of BAC-ΔC Tg mice (Shen *et al*., 2008). Interestingly, BAC-ΔC Tg mice were shown to exhibit a displaced pattern of parvalbumin staining in the dorsolateral prefrontal cortex with a significantly reduced number of parvalbumin cells in the inner layers (4–5) and a significant increase of parvalbumin cells in the outer layers (Shen *et al*., 2008). In contrast, no differences were detected in the numbers of calbindin- or parvalbumin-positive interneurons in the mPFC of the D25 BP mutant (Kvajo *et al*., 2008).

Cytoarchitectural abnormalities have been described in the hippocampus and the PFC of the various DISC1 models. Early postnatal day expression of the C-terminal portion of DISC1 in mice results

in reduced dendritic complexity of dentate gyrus granule cells and basal synaptic transmission although CA1 LTP was spared (Li *et al*., 2007). The D25 BP mutant was found to exhibit region-specific morphological alterations, including alterations in the organization of newly born dentate gyrus cells such as a misorientation of the apical dendrites (Kvajo *et al*., 2008). Similarly, mature granule cells were found to exhibit misoriented apical dendrites, decrease in total dendrite length, and reduced spine number. These findings were associated with deficits in the CA3/CA1 synapse which suggested a potential deficit in short term plasticity. A more subtle effect in the form of a ≈10% decrease in apical dendrite length of layer 5 pyramidal neurons was also observed in the mPFC of these mice (Kvajo *et al*., 2008). Interestingly, postmortem brain studies of schizophrenia have also reported similar dendritic abnormalities (Glantz and Lewis, 2000).

1.15. Aims and outline of the Thesis

Although schizophrenia is defined as a complex disorder resulting from an intricate interplay between both genetic and environmental factors, no study to date has attempted to evaluate or partition the contribution of these two separate factors to the development of the disorder. The present thesis aims to fill this 'void' in the literature by evaluating and comparing a *genetic* and an *environmental* animal model of schizophrenia.

The first part of this thesis involves the further behavioural and anatomical characterization of an established environmental animal model of schizophrenia. It assesses the extent to which maternal immune activation via the toll-like receptor 3 agonist and cytokine inducer Poly-I:C during pregnancy can precipitate the myriad of behavioural, cognitive and neuroanatomical deficits associated with schizophrenia in the offspring. Because not much emphasis has been placed until now on evaluating the behavioural and anatomical effects following this manipulation during the late gestation period, as opposed to the early/middle gestation period, a major drive was placed on evaluating the effects of the immune challenge during late pregnancy on the offspring. One of the key aims of the present thesis was to assess the validity of the "*Critical time window hypothesis"* and to determine whether immune activation during late pregnancy may result in a cluster of behavioural and neuropathological changes reminiscent of the negative profile observed in schizophrenia (Sullivan *et al*., 2006; Meyer *et al*., 2007) as opposed to the positive symptoms associated with immune activation during early/middle pregnancy.

The second part of the thesis involved evaluating a genetic mouse model for a well established genetic risk factor of schizophrenia, namely DISC1. The DISC1gene has been associated with several roles in the developing brain, including neuronal migration and synaptic integration. However, still little is known of the functional role of DISC1 in behaviour. In order to understand the functional significance

of DISC1, the behavioural implications associated with the expression of mutant human DISC1 in genetically modified mice was investigated.

The following specific aims were addressed in this thesis:

• The aim of **CHAPTER 2** was to evaluate whether the offspring of dams that had been challenged with Poly-I:C during late pregnancy (i.e. GD17) exhibit deficits in spatial working memory and recognition memory performance seeing as patients with schizophrenia have been shown to be impaired in both these cognitive processes (Pantelis *et al*., 2001). In addition, brains of these animals were analyzed in order to evaluate possible dopaminergic related neuroanatomical changes that may be of potential relevance to schizophrenia.

• In **CHAPTER 3**, the aim was to further determine the effects of late prenatal immune activation on cognitive function in the offspring. Since patients with schizophrenia on average report reduced pleasure (Wolf, 2006) and have been shown to exhibit deficits in social interaction (Goldberg and Schmidt, 2001) in addition to impairment in selective attention (Lubow and Kapan, 2010) a series of tests was conducted to evaluate a similar set of behavioural endophenotypes in the Poly-I:C GD17 mice. Because stereotypy (a symptom characterized by repetitive, functionless motor behaviour) is observed in patients with schizophrenia (Morrens *et al*., 2006), stereotyped behaviour was evaluated in Poly-I:C mice by using the dopamine receptor agonist apomorphine seeing as dopaminergic neurotransmission is believed to play a role in mediating this type of behaviour. Neurochemical analysis was subsequently conducted on the brains of these animals in order examine for any neurochemical changes that may be similar to those observed in schizophrenia.

• **CHAPTER 4** aimed to behaviourally characterize transgenic mice that express mutant human DISC1. Specifically, the expression of mutant human DISC1 on attentional processes including selective

attention and sustained attention was evaluated seeing as deficits in these processes are commonly seen in patients with schizophrenia and because these processes have not been evaluated previously in this genetic mouse model. In addition, the sensitivity to the locomotion-stimulating effects of psychostimulants (i.e. the dopaminergic agonist amphetamine and the NMDA receptor antagonist dizocilipine) was assessed in these mice in order to evaluate dopaminergic and glutamatergic neurotransmission in these animals, two key neurotransmitter systems known to be affected in schizophrenia.

• In **CHAPTER 5**, the aim was to further dissect the cognitive implications of mutant human DISC1 expression. Cognitive performance of hDISC1 mutant mice was assessed across several behavioural domains, including novel spatial recognition, spatial learning and memory, object exploration and reversal learning. All cognitive processes known to be affected in patients with schizophrenia.

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CHAPTER 2.

Cognitive impairment following prenatal immune challenge in mice correlates with prefrontal cortical AKT1 deficiency

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[Personal contribution to the work: Designing the research, running the behavioural tests, analysis of the behavioural data, and writing the manuscript]

ABSTRACT

Accumulating evidence indicates that genetically determined deficiency in the expression of the cytoplasmic serine-threonine protein kinase AKT1 may contribute to abnormal prefrontal cortical structure and function relevant to the cognitive disturbances in schizophrenia. However, it remains essentially unknown whether prefrontal AKT1 expression may also be influenced by environmental factors implicated in the etiology of this mental illness. One of the relevant environmental risk factors of schizophrenia and related disorders is prenatal exposure to infection and/or immune activation. The present study therefore explored whether prenatal immune challenge may lead to prefrontal AKT1 deficiency and associated changes in cognitive functions attributed to the prefrontal cortex. For these purposes, we used a well-established experimental mouse model of prenatal exposure to a viral-like acute phase response induced by the synthetic analogue of double-stranded RNA, polyriboinosinic-polyribocytidilic acid (Poly-I:C). We found that adult offspring born to Poly-I:C treated mothers showed delay-dependent impairments in spatial working memory and recognition memory together with a marked reduction of AKT1-positive cells in the prefrontal cortex. These effects emerged in the absence of concomitant changes in prefrontal catechol-*O*-methyltransferase (COMT) density. Correlative analyses further demonstrated a significant positive correlation between the number of AKT1-positive cells in distinct prefrontal cortical subregions and cognitive performance under high storage load in the temporal domain. Our findings thus highlight that schizophrenia-related alterations in AKT1 signaling and associated cognitive dysfunctions may not only be precipitated by genetically determined factors, but may also be produced by (immune-associated) environmental insults implicated in the etiology of this disabling brain disorder.

INTRODUCTION

AKT1 (V-akt murine thymoma viral oncogene homolog 1) is a cytoplasmic serine-threonine protein kinase which has been implicated in numerous biological functions such as cellular survival, growth and transcriptional regulation (Brunet *et al*., 2001; Song *et al*., 2005). In the central nervous system (CNS), AKT1 serves as a key signaling intermediate downstream of dopamine D_2 receptors and thus may play an important role for central dopaminergic signaling and regulation of dopamine-associated behaviours (Beaulieu *et al*., 2004, 2005; Lai *et al*., 2006; Ukai *et al*., 2004). Accumulating evidence suggests that alterations in AKT1 signaling may be critically involved in schizophrenia (Emamian *et al*., 2004; Tan *et al*., 2009). Several genetic studies found a significant association between genetic variation in *AKT1* and schizophrenia (Emamian *et al*., 2004; Ikeda *et al*., 2004; Norton *et al*., 2007; Tan *et al*., 2009; Thiselton *et al*., 2008; Xu *et al*., 2007); however, negative reports also exist (Ide *et al*., 2006; Liu *et al*., 2006, 2009). It has been suggested that reduced AKT1 expression may precipitate psychosis-related abnormalities by disrupting prefrontal cortical structures and functions (Emamian *et al*., 2004; Tan *et al*., 2009). In support of this hypothesis, recent investigations show that cognitive performance linked to frontostriatal circuitry and prefrontal physiology is altered in human subjects with a genetic variation in *AKT1* that has been associated with schizophrenia (Tan *et al*., 2008). Furthermore, *AKT1*-deficient mice display multiple changes in prefrontal pyramidal dendritic ultra-structure and concomitant deficits in working memory under dopamine agonists challenge (Lai *et al*., 2006). The apparent impact of reduced AKT1 expression on prefrontal cortex (PFC) structure and function fits well with the suggested role of prefrontal cortical deficits in schizophrenia in general (Goldman-Rakic, 1994; Goldman-Rakic and Selemon, 1997; Ragland *et al*., 2009; Weinberger *et al*., 1994), and with the relative contribution of deficient dopaminergic signaling to the emergence of negative and cognitive symptoms of this disorder in particular (Davis *et al*., 1991; Knable and Weinberger, 1997). Genetic disruption of AKT1 signaling may

thus be critical for abnormal PFC functions underlying especially (but not limited to) the pathogenesis of cognitive symptoms in schizophrenia and related disorders (Tan *et al*., 2009).

In addition to the genetic liability to schizophrenia, various environmental factors have been found to increase the risk of this mental illness (Dean and Murray, 2005; McDonald and Murray, 2000). Maternal infection during pregnancy is one of the relevant environmental factors that has been repeatedly demonstrated to significantly increase the risk of schizophrenia in the offspring (Brown, 2006, 2008; Brown and Susser, 2002; Fatemi, 2005; Patterson, 2007). One prevalent hypothesis suggests that infection-induced disruption of fetal neurodevelopmental processes may predispose the organism to long-lasting changes in subsequent brain and behavioural development, thereby increasing the risk of psychotic disorders in later life (Fatemi, 2005; Gilmore and Jarskog, 1997; Meyer *et al*., 2007, 2008a; Patterson, 2002). In line with this hypothesis, experimental data have shown that the long-term neuropathological consequences of prenatal immune challenge include multiple alterations in PFC neurochemistry and morphology relevant to psychotic disorders (Baharnoori *et al*., 2009; Fatemi *et al*., 1999; Gilmore *et al*., 2004; Meyer *et al*., 2008b,c; Winter *et al*., 2009). In addition, both experimental investigations in animals (Meyer *et al*., 2005, 2006, 2008c; Ozawa *et al*., 2006) and recent studies in humans (Brown *et al*., 2009) demonstrate that prenatal infection and/or immune activation induces deficits in cognitive functions linked to prefrontal cortical networks. Considering the emerging role of AKT1 signaling in normal and abnormal PFC structure and function (Lai *et al*., 2006; Tan *et al*., 2008, 2009), abnormal AKT1 expression in this brain area may be an important but yet unidentified link between prenatal immune challenge and emergence of cognitive deficits relevant to schizophrenia in adulthood.

The present study was therefore designed to test this hypothesis by exploring the putative relationship between prenatal infection-induced alterations in prefrontal cortical AKT1 expression and cognitive functions linked to the PFC. We used a well-established mouse model of prenatal viral-like infection which is based on maternal gestational administration of polyriboinosinic-polyribocytidilic acid

(Poly-I:C). Poly-I:C is a synthetic analogue of virus-specific double-stranded RNA which induces a cytokine-associated viral-like acute phase response (Kimura *et al*., 1994; Traynor *et al*., 2004). Using this experimental model system, we first characterized the long-term effects of prenatal immune challenge on adult cognitive functions known to be sensitive to manipulations targeting the PFC (Dalley et al., 2004; Delatour and Gisquet-Verrier, 1999; Goldman-Rakic, 1996; Robbins, 2000; Heidbreder and Groenewegen 2003). This included the assessment of spatial working memory and spatial novelty recognition. In a next step, we performed immunohistochemical investigations of AKT1 protein expression in the PFC of the same subjects, followed by correlative analyses between cognitive performance and prefrontal cortical AKT1 protein expression. Since genetic variants of *AKT1* have also been found to produce epistatic effects with the genetic variants encoding for catechol-*O*-methyltransferase (COMT) (Tan *et al*., 2008), an enzyme critically involved in the catabolism of cortical dopamine (Gogos *et al*., 1998; Napolitano *et al*., 1995; Yavich *et al*., 2007), we additionally included measurements of COMT protein in our immunohistochemical and correlative analyses.

METHODS

Animals

C57BL6/J mice were used throughout the study. Female and male breeders were obtained from our in-house specific pathogen free (SPF) colony at the age of 10-14 weeks. Breeding began after 2 weeks of acclimatization to the new animal holding room, which was a temperature- and humidity-controlled (21±1°C, 55±5%) holding facility under a reversed light–dark cycle (lights off: 08:00–20:00). All animals had *ad libitum* access to food (Kliba 3430, Klibamühlen, Kaiseraugst, Switzerland) and water. All procedures described in the present study had been previously approved by the Cantonal Veterinarian's Office of Zurich, and are in agreement with the Principles of Laboratory Animal Care (NIH publication No. 86-23, revised 1985). All efforts were made to minimize the number of animals used and their suffering.

Maternal immune activation during pregnancy

For the purpose of the maternal immunological manipulation during pregnancy, female mice were subjected to a timed mating procedure as described previously (Meyer *et al*., 2005). Pregnant dams on gestation day (GD) 17 received either a single injection of Poly-I:C (potassium salt; Sigma-Aldrich, Buchs, St. Gallen, Switzerland) or vehicle. Poly-I:C (5 mg/kg) was dissolved in sterile pyrogen-free 0.9% NaCl (=vehicle) solution to yield a final concentration of 1 mg/ml and was administered via the intravenous (i.v.) route at the tail vein under mild physical constraint. The dose of Poly-I:C and gestational window were chosen based on our previous studies in C57BL/6 mice (Meyer *et al*., 2005, 2006, 2008a-c). All solutions were freshly prepared at the day of administration and injected with a volume of 5 ml/kg.

Cognitive testing of offspring

All offspring were weaned and sexed on postnatal day (PND) 21. Littermates of the same sex were caged separately. All animals were maintained under *ad libitum* food and water diet unless specified otherwise. They were kept in a temperature and humidity controlled animal vivarium under a 12:12h reversed light-dark cycle as described above.

Offspring from multiple independent litters (6 Poly-I:C, 6 saline) were randomly selected for the cognitive phenotyping. Both male and female offspring were included in all the tests described below in order to assess potential sex-dependent effects of the prenatal immunological manipulation. The numbers of animals included in the control and Poly-I:C groups were *N*=11 (5♂, 6♀) and *N*=10 (5♂, 5♀), respectively. Cognitive testing commenced when the offspring reached the adult stage of development starting on postnatal day 80, and was always conducted during the dark phase of the light-dark cycle. Cognitive phenotyping of prenatally Poly-I:C-exposed and control offspring included the assessment of spatial working memory in the cheeseboard maze and spatial recognition memory in the Y-maze according to modified protocols described by Kirwan *et al*. (2005) and Sanderson *et al*. (2007). The basic procedures used for the cognitive phenotyping are described below. A detailed description of the test apparatuses and procedures can be obtained in the **Supplementary Methods**.

Spatial working memory in the cheeseboard maze

The apparatus consisted of a wooden circular board containing 32 holes which were arranged in a radial design (see **Figure 2.1a** and **Supplementary Methods**). Following the initial habituation (days 1-5) and pre-testing (day 6) phases (see **Supplementary Methods)**, working memory testing was started. The working memory task was based on the matching-to-position paradigm, in which food-

deprived animals were required to learn the novel position of a rewarded hole revealed to them on trial 1 of each day in order to navigate effectively to the same location (i.e. matching) on the subsequent trial on the same day. Hence, each test day included 2 trials. The reward remained in the same position across trials on a given test day, but took a new position on each test day. The daily change of the reward location ensured that the information acquired on a given day was irrelevant for the next day, thus taxing the flexible use of working memory (Baddeley, 2003).

To manipulate the retention demand in the temporal domain, the ITI varied between trials 1 and 2. First, a minimal ITI of 2 min was used for 2 consecutive days (days 7-8). The ITI was then increased to 5 min on the next 2 testing days (days 9-10), and then to 10 min thereafter (days 11-12). Finally, a maximal ITI of 15 min was used on the following 6 testing days (days 13-18) in order to consolidate the anticipated working memory impairment in prenatally immune challenged animals under extended ITI conditions (Meyer *et al*., 2005, 2008c). Working memory was indexed by the reduction in latency and distance moved to find the location of the reward in trial 2 relative to trial 1.

Spatial novelty preference in the Y-maze

Spatial recognition memory was evaluated by a spatial novelty preference task in the Y-maze one week following completion of working memory test in the cheeseboard. The apparatus was made of transparent Plexiglas and consisted of three identical arms radiating from a central triangle and spaced 120° from each other (see **Supplementary Methods)**. The test of spatial recognition memory in the Y-maze consisted of two phases, namely the sample and choice phases.

Sample phase: The animals were allowed to explore two arms (referred to as "start arm" and "familiar arm"). Access to the remaining arm ("novel arm") was blocked by a barrier wall door (see **Supplementary Methods)**. To begin a trial, the subjects were introduced at the end of the start arm,

facing the central triangle and were allowed to freely explore both the start and familiar arms for 5 min. The subject was then removed and kept in a holding cage during the specific retention intervals (see below) prior to the choice phase.

Choice phase: Following a specific retention interval (see below), the test subjects were introduced to the maze again. During the choice phase, the barrier wall was removed so that the animals could freely explore all arms of the maze for 2 min. On each trial, the time spent in each of the three arms was recorded. The percentage time spent in the novel arm during the choice phase was calculated by the formula [time spent in the novel arm/ (time spent in all arms)] × 100 and used as the index for spatial novelty preference. In addition, total distance moved on the entire maze was recorded and analyzed in order to assess general locomotor activity.

To manipulate the retention demand in the temporal domain, the interval between the two phases (i.e. sample and choice phases) of the Y-maze test were varied. The interval between the two phases was then increased to 15 min in accordance with the extended (15 min) retention interval used for the working memory test in the cheeseboard maze.

Immunohistochemistry

The immunohistochemical analyses were conducted in the same animals previously subjected to cognitive testing. This allowed us to correlate the cognitive measures with the immunohistochemical data of interest. For the immunohistochemical studies, all animals were perfused transcardially, and the brains were processed for the immunohistochemical stainings as described in detail in the **Supplementary Methods.** AKT1 and COMT protein were visualized and analyzed using standard immunohistochemical procedures (for a detailed description see **Supplementary Methods**).

Delineation of prefrontal cortical regions

All stereological and densitometric analyses (see below) were conducted in the three main areas of the mouse PFC, namely the anterior cingulate cortex (CG), prelimbic cortex (PL) and infralimbic cortex (IL). Each of these regions was delineated according to the "Mouse Brain in Stereotaxic Coordinates" by Franklin and Paxinos (2008). The stereological and densitometric analyses were conducted on brain sections ranging from bregma +2.2 mm to +1.7 mm (see **Figure 2.3a**).

Stereological estimates of AKT1-immunorecative cells

The number of AKT1-immunoreactive cells in the three prefrontal cortical regions of interest (CG, PL and IL) of the right brain hemisphere was determined by stereological estimations using the optical fractionator method (Gundersen *et al*., 1988). Since the expression of AKT1 protein was found to be highly enriched in the cell cytoplasm (see **Figure 2.3b**), it was feasible to precisely delineate individual AKT1-immunoreactive cells necessary for stereological estimations. A detailed description of the stereological analyses performed can be found in the **Supplementary Methods**.

Optical densitometry of COMT-immunoreactive cells

Quantification of COMT-immunoreactive cells in the three prefrontal cortical regions (CG, PL and IL) of the right brain hemisphere was achieved by means of optical densitometry using ImageJ software (ImageJ, NIH, Maryland, USA). Optical densitometry was chosen because COMT protein was found to be expressed primarily at the cell periphery of prefrontal cortical cells, thus forming a cell membrane-like expression pattern (see **Figure 2.3b**). This expression pattern readily undermined the precise delineation of individual COMT-positive cells, which would be necessary for cell quantification

by stereological analyses. A detailed description of the densitometric analyses performed can be found in the **Supplementary Methods**.

Statistical analysis

All cognitive and immunohistochemical data were analyzed using parametric ANOVA, followed by restricted ANOVA whenever appropriate as fully described in the **Supplementary Methods**. Correlative analyses between cognitive measures and immunohistochemical data were performed using first order partial correlations, partialling for prenatal treatment. Such partial correlations were used in order to control for the effects of the independent variable of prenatal treatment. Statistical significance was set at $p < 0.05$ for all analyses. All statistical analyses were conducted using the statistical software SPSS version 13.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Effects of prenatal immune challenge on spatial working memory in the cheeseboard

The critical measure of working memory in the matching-to-position paradigm of the cheeseboard task is the reduction in time spent and/or distance moved to find the location of the rewarded hole from trial 1 (when the location of the platform was essentially unknown to the subjects) to trial 2. There were no signs of working memory impairment in prenatally immune-challenged offspring relative to control offspring when the animals were tested in trials presented with a minimal (i.e., 2 min) or 5 min interval between trials 1 and 2. Hence, male and female offspring from both groups displayed a clear reduction in the latency and distance moved to find the rewarded hole in trial 2 relative to trial 1 (**Figure 2.1**). For both ITI conditions, the 2 × 2 × 2 × 2 (prenatal treatment × sex × trial × day) ANOVA of latency or distance moved only revealed a significant main effect of trials [latency (2-min delay): $F_{1,17}$ = 8.65, $P < 0.05$; distance (2-min delay): $F_{1,17}$ = 5.21, $P < 0.05$; latency (5-min delay): $F_{1,17}$ = 4.65, $P < 0.05$; distance (5-min delay): $F_{1,17}$ = 3.98, $P < 0.05$].

However, offspring born to immune-challenged mothers displayed impaired working memory when the interval interposed between trials 1 and 2 was increased to extended delays. This delay-dependent working memory deficit in Poly-I:C offspring started to appear at the 10 min delay condition and was most pronounced and statistically significant under the 15-min delay condition (**Figure 2.1**). When an ITI of 15 min was used, only control offspring showed a significant reduction in latency and distance moved from trials 1 to 2, whilst offspring born to Poly-I:C-treated mothers failed to show a comparable improvement (**Figure 2.1**). Statistical support for these impressions was obtained by the 2 × 2 × 2 × 6 (prenatal treatment × sex × trial × day) ANOVA of latency or distance moved in the 15-min test condition, which revealed a significant interaction between prenatal treatment and trial [latency: $F_{1,17}$ = 5.52, $P < 0.05$; distance: $F_{1,17}$ = 4.36, $P < 0.05$]. Subsequent ANOVA restricted to the control group

confirmed the significant reduction in the latency and distance moved to find the location of the rewarded hole in trial 2 relative to trial 1 [latency: $F_{1,9} = 5.24$, $P < 0.05$; distance: $F_{1,9} = 4.97$, $P < 0.05$]. Furthermore, a 2 × 2 × 6 (prenatal treatment × sex × day) ANOVA of latency or distance moved restricted to trial 2 revealed a significant main effect of prenatal treatment in both measures [latency: $F_{1,17} = 5.25$, $P < 0.05$; distance: $F_{1,17} = 4.51$, $P < 0.05$]. These effects of the prenatal treatment were similarly apparent across different testing days and in both male and female subjects.

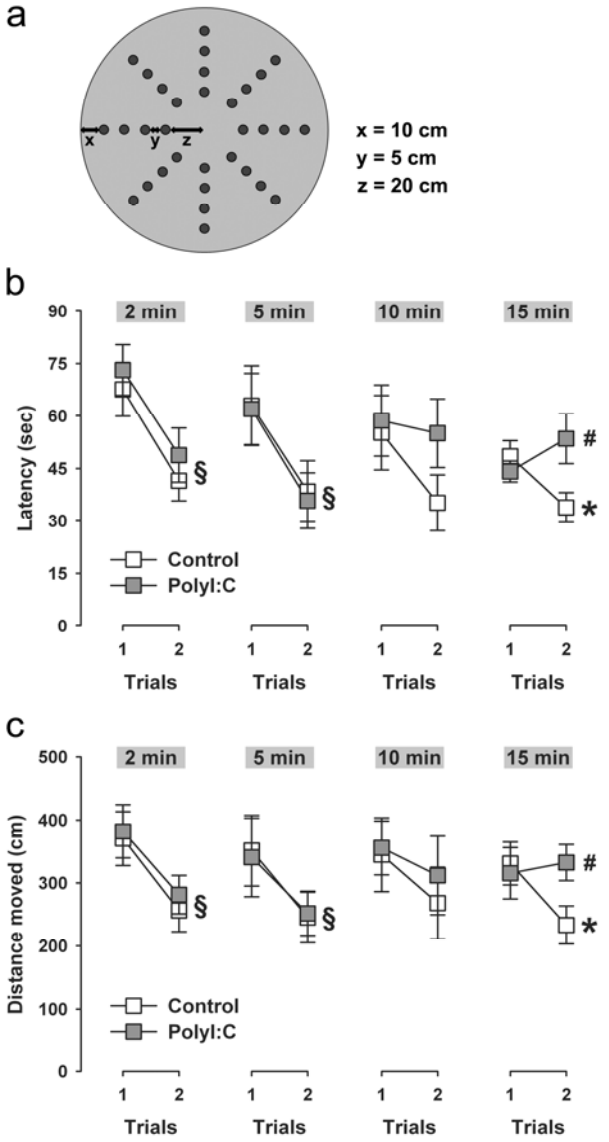

Figure 2.1. Prenatal immune activation leads to delay-dependent spatial working memory impairment in a matching-to-position version of the cheeseboard maze task. (a) Spatial working memory was assessed using a cheeseboard maze containing 32 holes arranged in a radial design. The working memory task was based on the matching-to-position paradigm, in which the animals were required to learn the novel position of a rewarded hole revealed to them on trial 1 of each day in order to navigate effectively to the same location (i.e. matching) on the subsequent trial on the same day. **(b)** The graph depicts the latency (sec) to find the position of the rewarded hole in trials 1 and 2 for each individual intertrial delays (2, 5, 10, or 15 min) and for the two prenatal treatment groups (control of Poly-I:C). The analysis of working memory performance as indexed by the latency revealed a clear delay-dependent working memory deficit in Poly-I:C offspring relative to control offspring. $^{\S}P < 0.05$, $^{*}P < 0.05$, $^{\#}P < 0.05$, reflect the significant difference between control and Poly-I:C offspring in trial 2 of the test based on the significant main effect of prenatal treatment **(c)** The graph depicts the distance moved (cm) to find the position of the rewarded hole in trials 1 and 2 for each individual intertrial delays (2, 5, 10, or 15 min) and for the two prenatal treatment groups (control of Poly-I:C). Consistent with the outcomes in the analysis of latency, prenatally Poly-I:C offspring displayed a clear delay-dependent working memory deficit in the analysis of distance moved. $^{\S}P < 0.05$,$^{*}P < 0.05$, $^{\#}P < 0.05$, reflecting the significant difference between control and Poly-I:C offspring in trial 2 of the test based on the significant main effect of prenatal treatment.All values are means ± SEM. *n*(control) = 11 (5♂, 6♀), *n*(Poly-I:C) = 10 (5♂, 5♀).

Effects of prenatal immune challenge on spatial recognition memory

The critical measure of spatial recognition memory is the relative time spent in the novel (previously unexplored) arm during the choice phase of this test. When Poly-I:C and control offspring were tested using a minimal retention interval of 2 min between the initial sample and subsequent choice phase, animals from both groups displayed a noticeable preference towards the novel spatial environment during the critical choice phase. Indeed, as depicted in **Figure 2.2a**, both Poly-I:C and control offspring spent more relative time in the novel as opposed to the previously explored arms, thus clearly performing above a chance level of 33.3%. The 2 × 2 (prenatal treatment × sex) ANOVA of the percent time spent in the novel arm (as calculated by the formula [time spent in the novel arm / time spent in all arms] × 100) did not reveal any significant main effects when the spatial novelty preference test was run using the 2-min retention interval.

However, when an extended retention interval of 15 min was interposed between the initial sample and subsequent choice phase, offspring born to Poly-I:C-challenged mothers no longer displayed a preference towards the novel arm versus the previously explored arms (**Figure 2.2a**). In contrast, control offspring continued to show a clear spatial novelty preference at this retention interval (**Figure 2.2a**). This led to a marked difference between the percent time spent in the novel arm displayed by Poly-I:C and control offspring (**Figure 2.2a**). Statistical support for these impressions was yielded by the 2 × 2 (prenatal treatment × sex) ANOVA of the percent time spent in the novel arm during the choice phase of the 15-min retention interval test, which revealed a significant main effect of prenatal treatment [$F_{1,17} = 11.59$, $P < 0.01$]. Consistent with the results obtained in the spatial working memory tests in the cheeseboard maze, the effect of prenatal Poly-I:C-induced immune challenge on spatial novelty preference disruption were evident in both male and female offspring.

The additional analyses of total distance moved during the choice phase of the 2 min and 15 min retention tests showed that general locomotor activity was highly comparable between Poly-I:C and control offspring (see **Figure 2.2b**). Similarly, locomotor activity did not differ between the two groups during the sample phase of the test (data not shown). Hence, the prenatal Poly-I:C-induced disruption of spatial novelty preference (specifically in the 15 min retention interval condition) could not be accounted for by possible confounds resulting from differences in general locomotor and exploratory activity in the Y-maze.

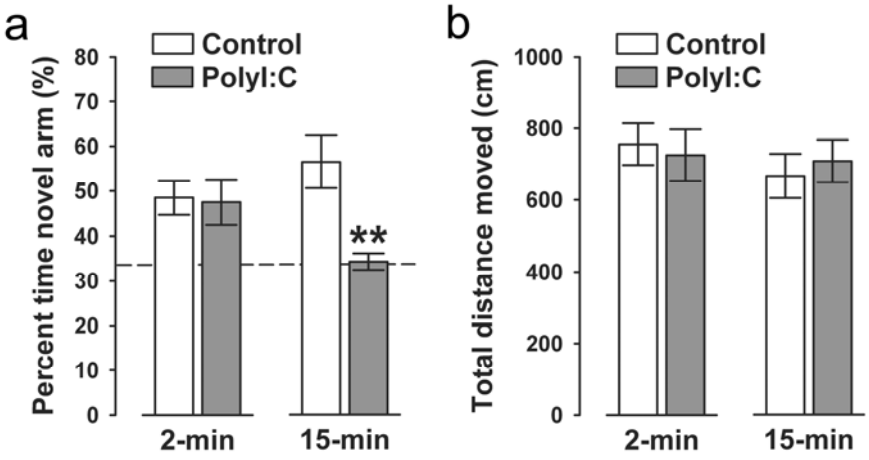

Figure 2.2. Prenatal immune activation leads to delay-dependent disruption of spatial recognition memory in the Y-maze. (a) The graph depicts the percent time spent in the novel (previously unexplored) arm as during the choice phases of the 2-min and 15-min retention interval tests. Note that both control and Poly-I:C offspring clearly performed above chance level (indicated by the dashed line) when they were tested using a minimal (2-min) retention interval. However, Poly-I:C offspring showed a marked deficit in spatial recognition memory relative to control offspring when tested under the extended (15-min) delay condition. $^{**}P < 0.01$, reflecting the significant main effect of prenatal treatment in percent time spent in the novel arm. **(b)** The graph depicts the total distance moved on the Y-maze during the choice phases of the 2-min and 15-min retention interval tests. There were no group differences in this measure at both retention interval used. All values are means ± SEM. *n*(control) = 11 (5♂, 6♀), *n*(Poly-I:C) = 10 (5♂, 5♀).

Effects of prenatal immune challenge on prefrontal cortical AKT1 and COMT protein expression

Our immunohistochemical analyses showed that AKT1 protein was abundantly expressed in all three major subregions of the PFC (i.e., in the CG, PL and IL). AKT1-positive cells were clearly identifiable by the presence of darkly stained cell bodies in these regions (see **Figure 2.3b**, **2.4b**). Most importantly, unbiased stereological estimations revealed a marked reduction in the number of AKT1-positive cells in prenatally immune-challenged offspring relative to control offspring (**Figure 2.4a**). This effect of the prenatal immunological insult emerged in both male and female subjects and was largely independent of the precise subregion investigated. The 2 × 2 (prenatal treatment × sex) ANOVA of AKT1-positive cell numbers in the CG, PL and IL revealed a significant main effect of prenatal treatment for each prefrontal cortical subregion [CG: $F_{1,16} = 24.03$, $P < 0.001$; PL: $F_{1,16} = 7.73$, $P < 0.05$; IL: $F_{1,16} = 16.40$, $P < 0.001$]. The between-subjects factor of sex never interacted significantly with the between-subjects factor prenatal treatment.

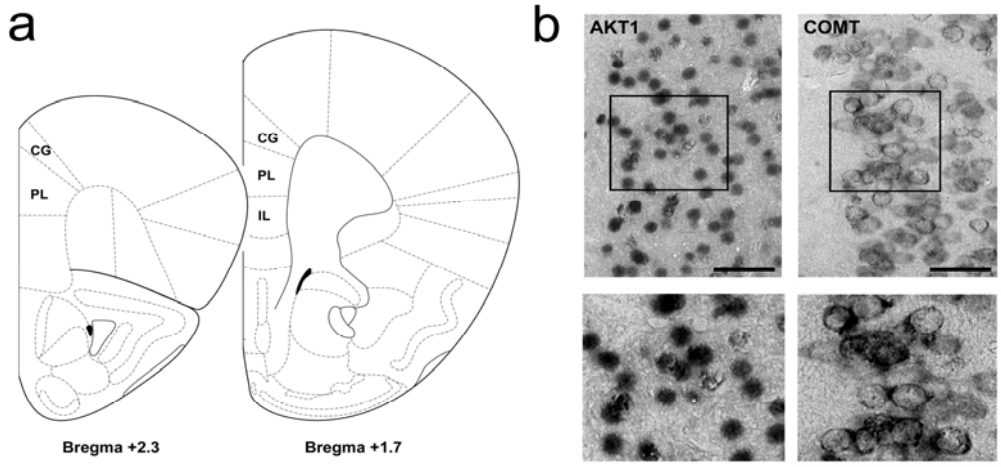

Figure 2.3. Immunohistochemical evaluation of AKT1 and COMT in the prefrontal cortex. (a) Schematic coronal brain sections delineating the prefrontal cortical areas investigated with reference to bregma (adapted from "The Mouse Brain in Stereotaxic Coordinates" by Paxinos and Franklin, 2001). Stereological and optical density measurements were performed within the boundaries shown for the cingulate cortex (CG), prelimbic cortex (PL) and infralimbic cortex (IL). All immunohistochemical analyses were performed on sections ranging from Bregma +2.3 to +1.7. **(b)** Typical immunohistochemical stainings of AKT1-positive and COMT-positive cells in the PL region. Note that AKT1 protein was primarily expressed in the cytoplasm, whereas COMT protein was expressed mainly at the cell perimeter of prefrontal cortical cells, forming a cell membrane-like expression pattern. Scale bar = 45 μm. The squares indicate the region of magnification.

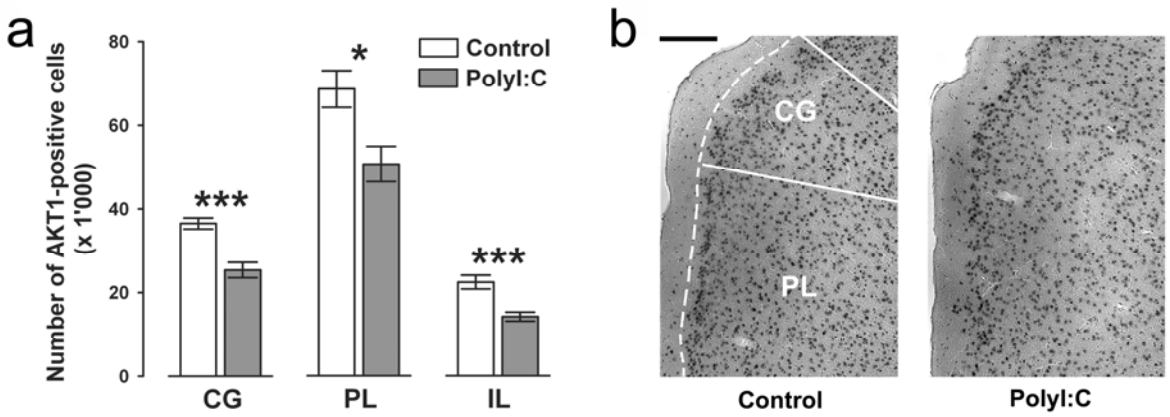

Figure 2.4. Prefrontal cortical AKT1 deficiency following prenatal immune activation. (a) Offspring born to Poly-I:C-treated mothers displayed a significant reduction of AKT1-positive cells in the cingulate cortex (CG), prelimbic cortex (PL) and infralimbic cortex (IL) compared to control offspring. **P* < 0.05 and ****P* < 0.001, based on the significant main effects of prenatal treatment on AKT1-positive cell numbers in the CG and IL. All values are means ± SEM. *n*(control) = 10 (5♂, 5♀), *n*(Poly-I:C) = 10 (5♂, 5♀). **(b)** Photomicrographs of coronal brain sections at the level of the prefrontal cortex taken from representative control and Poly-I:C offspring. The photomicrographs show AKT1-positive cells in the CG and PL subregions of the prefrontal cortex. Scale bar = 150 μm.

In addition to the stereological assessment of AKT1-positive cells, we also measured prefrontal AKT1 expression in terms of optical densitometry. Importantly, the densitometric analyses showed that similar to the outcomes in the stereological analyses, prenatal immune activation led to decreased AKT1 density in all three sub-regions of the PFC (data not shown). Hence, it is unlikely that prenatally immune challenged animals could have compensated to a reduction in AKT1-positive cell numbers with increased production of AKT1 per cell.

We also explored the effects of prenatal Poly-I:C-induced immune activation relative to prenatal control treatment on the expression of COMT in the CG, PL and IL subregions of the PFC. The densitometric analyses of COMT protein expression did not provide any evidence for a significant effect of prenatal immune activation on COMT protein expression in any of the three prefrontal cortical subregions of interest. The mean±SEM of COMT optical densities in the CG, PL and IL subregions of the PFC are provided for each experimental group in **Table 2.1**.

Prenatal treatment	Optical density of COMT protein		
	CG	PL	IL
Control	9.97±0.89	8.70±1.21	7.34±1.75
PolyI:C	13.14±1.66	10.50±1.77	9.23±1.56

Table 2.1. Summary of the relative optical densities of catechol-*O*-methyltransferase (COMT) density in prefrontal cortical regions of adult offspring born to control of Poly-I:C-exposed mothers. CG, cingulate cortex; PL, prelimbic cortex; IL, infralimbic cortex. All values refer to mean ± SEM; *n*(control) = 10 (5♂, 5♀), *n*(Poly-I:C) = 10 (5♂, 5♀).

Correlative analyses between cognitive performance and prefrontal cortical AKT1 or COMT expression

For the purpose of the correlative analyses between the cognitive measures and immunohistochemical data, we selected the longest (15 min) retention interval in each cognitive test because of the clear presence of impaired cognitive performance in prenatally immune challenged offspring when tested under extended delay conditions (see **Figure 2.1, 2.2**). Working memory performance in the cheeseboard test was indexed by the improvement in the latency (sec) to find the location of the rewarded hole from trial 1 to trial 2; and spatial recognition memory in the Y-maze test was indexed by the percent (%) time spent in the novel arm during the choice phase. These cognitive indexes were then correlated with the stereological and densitometric measures of AKT1 and COMT, respectively, using first order partial correlations controlling for prenatal treatment (control or Poly-I:C).

Working memory performance under the extended (15 min) ITI delay generally correlated positively with the number of AKT1-positive cells in all three regions of the PFC (see **Figure 2.5a**). However, after adjusting the correlations for the independent variable of prenatal treatment, only the correlation between AKT1-positive cells in the CG subregion of the PFC and working memory performance in the cheeseboard maze met criteria for significance ($r = +0.52$, df = 17, $n = 20$, $P < 0.05$; **Figure 2.5b**). Cognitive performance in the Y-maze spatial novelty preference test also positively correlated with the number of AKT1-positive cells in the prefrontal cortical areas of interest

(**Figure 2.5b**). More specifically, AKT1-positive cells in both the CG and PL subregion of the PFC showed a significant positive correlation with spatial recognition memory in the Y-maze test (CG: $r = +0.47$, df = 17, $n = 20$, $P < 0.05$; PL: $r = +0.57$, df = 17, $n = 20$, $P < 0.05$), whilst the correlation between AKT1-positive cells in the IL subregion and spatial recognition memory failed to attain statistical significance (**Figure 2.5b**).

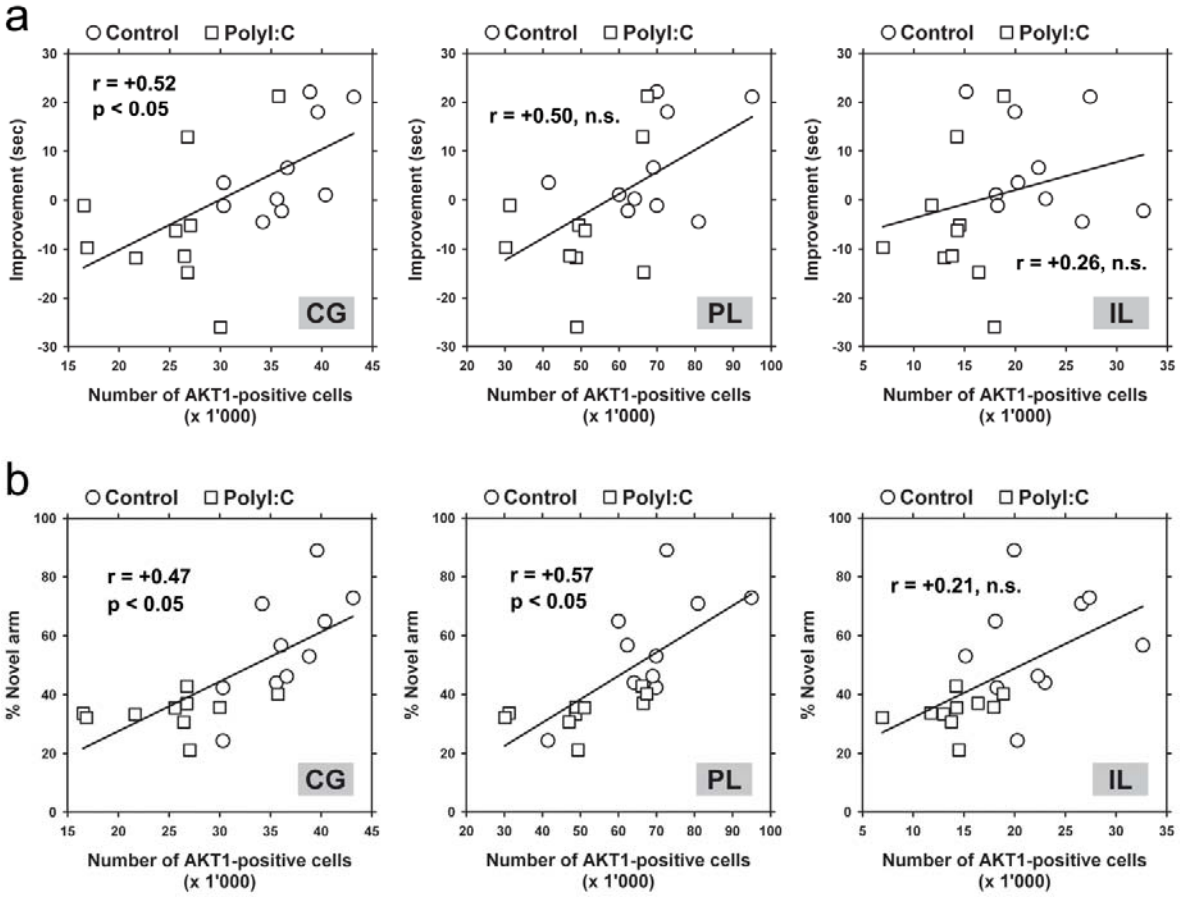

Figure 2.5. Correlations between AKT1-positive cells in the PFC and cognitive performance. Scatter plots are depicted for correlations between the cognitive measures obtained in the cheeseboard **(a)** or Y-maze **(b)** task and AKT1-positive cells in the cingulate cortex (CG), prelimbic cortex (PL) and infralimbic cortex (IL). Working memory performance in the cheeseboard maze task was indexed by the improvement in the latency (sec) to find the location of the rewarded hole from trial 1 to trial 2 under the extended (15-min) intertrial interval condition. Spatial recognition memory in the Y-maze was indexed by the percent (%) time spent in the novel arm during the choice phase of the extended (15-min) retention interval test. These cognitive indexes were correlated with the COMT optical density measures using first order partial correlations, partialling for prenatal treatment (control or Poly-I:C). The regression lines represent the partial correlative analyses adjusted for prenatal treatment. $n = 20$ (10 control, 10 Poly-I:C) and df = 17 for each correlation.

On the other hand, cognitive performance in the working memory (cheeseboard) and spatial recognition memory (Y-maze) tests generally correlated inversely with the density of COMT protein in the PFC (see **Figure 2.6**). Out of the six possible correlations, two attained statistical significance after adjustments for the independent variable of prenatal treatment: COMT density in the PL subregion showed a significant negative correlation with working memory performance as assessed in the cheeseboard maze task (r = -0.42, df = 17, n = 20, $P < 0.05$; **Figure 2.6a**), and COMT density in the IL subregion correlated significantly with spatial recognition memory as assessed in the Y-maze (r = -0.47, df = 17, n = 20, $P < 0.05$; **Figure 2.6b**).

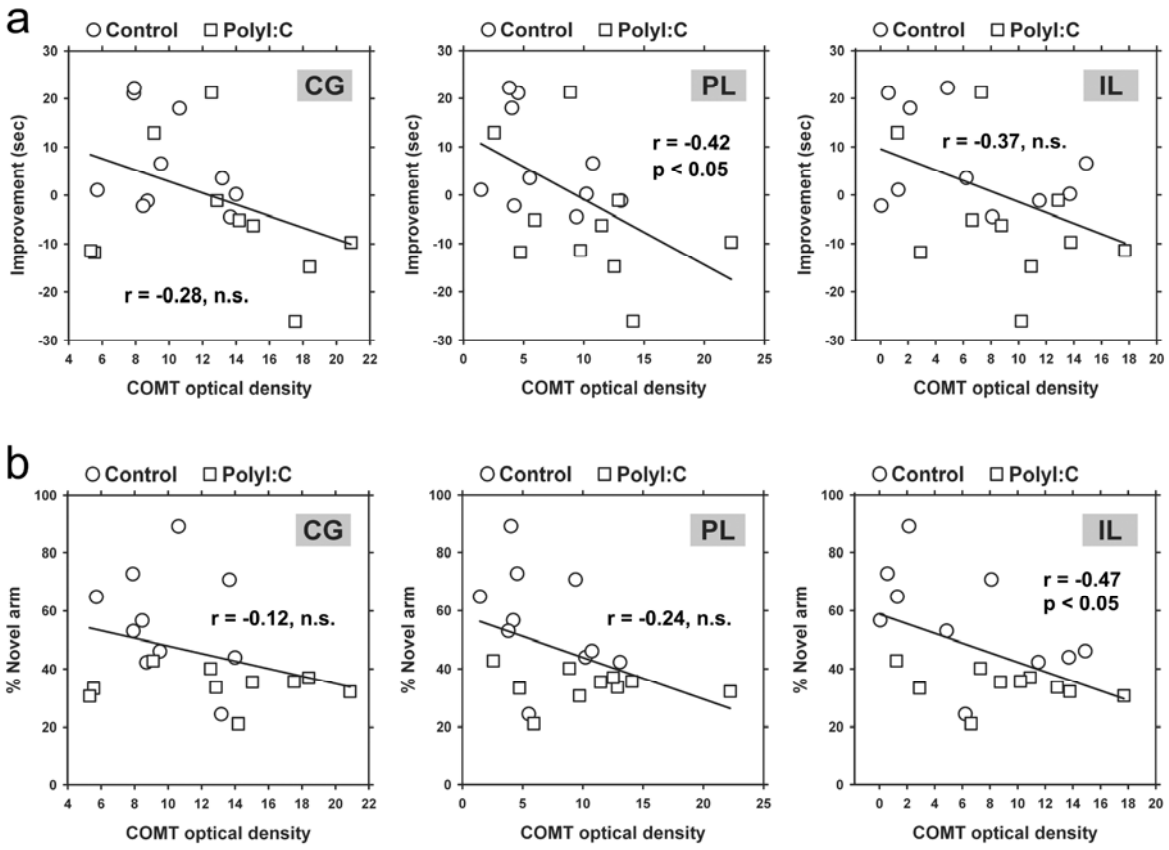

Figure 2.6. Correlations between COMT density in the PFC and cognitive performance. Scatter plots are depicted for correlations between the cognitive measures obtained in the cheeseboard **(a)** or Y-maze **(b)** task and COMT optical density in the cingulate cortex (CG), prelimbic cortex (PL) and infralimbic cortex (IL). Working memory performance in the cheeseboard maze task was indexed by the improvement in the latency (sec) to find the location of the rewarded hole from trial 1 to trial 2 under the extended (15-min) delay condition. Spatial recognition memory in the Y-maze was indexed by the percent (%) time spent in the novel arm during the choice phase of the extended (15-min) retention interval test. These cognitive indexes were correlated with the COMT optical density measures using first order partial correlations, partialling for prenatal treatment (control or Poly-I:C). The regression lines represent the partial correlative analyses adjusted for prenatal treatment. n = 20 (10 control, 10 Poly-I:C) and df = 17 for each correlation.

We also conducted partial correlations between the number of AKT1-positive cells and COMT optical density in each of the three prefrontal cortical subregions in order to explore whether there may be a significant relationship between these markers in the PFC. In all three prefrontal cortical regions, there seemed to be negative correlations between AKT1-positive cells and COMT optical density; however, none of these correlations attained statistical significance (CG: r = -0.22, df = 17, n = 20, p = 0.38; PL: r = -0.29, df = 17, n = 20, P = 0.26; CG: r = -0.24, df = 17, n = 20, P = 0.32; data not shown).

DISCUSSION

Our study provides the first experimental evidence for a significant environmental influence in the form of prenatal exposure to a viral-like acute phase response on altered AKT1 protein expression in the PFC. The present data demonstrates that prenatal Poly-I:C-induced immune challenge in mice leads to a marked reduction in the number of AKT1-positive cells in all three major subregions of the PFC (CG, PL and IL) at adult age (**Figure 2.3**). These structural changes were accompanied by the presence of both spatial working and recognition memory impairments. Consistent with the emerging role of AKT1 signaling in normal and abnormal prefrontal cortical structure and function (Lai *et al*., 2006; Tan *et al*., 2008, 2009), our findings thus highlight that abnormal AKT1 expression in the PFC may be an important cellular link between prenatal immune challenge and emergence of cognitive deficits in adulthood.

The present hypothesis of a critical link between prefrontal cortical AKT1 deficiency and emergence of cognitive impairment is further supported by the positive correlations between the number of AKT1-positive cells in the PFC and cognitive performance in spatial working memory and novelty preference tests. We found that in the extended (15 min) retention interval conditions, in which prenatally immune challenged animals displayed clear impairments in spatial working memory (**Figure 2.1**) and spatial recognition memory (**Figure 2.2**), reduced numbers of AKT1-positive cells correlated with poor performance in both cognitive tests (**Figure 2.5, 2.6**). Even though our study does not provide evidence for a causal relationship, our findings highlight the possibility that reduced AKT1 expression in the PFC undermines spatial working memory and recognition memory when the demand on temporal retention is high.

The prenatal infection-induced disruption of adult cognitive functions (**Figure 2.1, 2.2**) and prefrontal AKT1 expression (**Figure 2.4**) emerged in the absence of concomitant alterations in prefrontal

cortical COMT protein density (**Table 2.1**). This suggests that prenatal exposure to viral-like infection may not represent a significant environmental risk factor for long-term alterations in prefrontal COMT density. However, despite the lack of a significant group difference between prefrontal cortical COMT densities in prenatally immune challenged and control offspring (**Table 2.1**), we found significant inverse correlations between COMT protein density in the PFC and spatial working memory (**Figure 2.5b**) and recognition memory (**Figure 2.6b**). This inverse correlation is consistent with reports showing that enhanced COMT activity in homozygote valine-valine (VAL-VAL) allele carriers is associated with impaired executive functions and working memory in human subjects with or without overt neuropsychiatric disorders (de Frias *et al*., 2006; Diaz-Asper *et al*., 2006; Goldberg *et al*., 2003; Malhotra *et al*., 2002; Tan *et al*., 2007, 2009).

The null effect of prenatal Poly-I:C-induced immune activation on COMT protein density, together with its prominent influence on AKT1-positive cells, is indicative of a selective long-term neuropathological impact of prenatal exposure to immune activation on the prefrontal dopamine system. This is consistent with our recent demonstration that late prenatal Poly-I:C-induced immune activation in mice does not lead to changes in dopamine D_1 receptor densities in the adult PFC (Meyer *et al*., 2008a). Prefrontal D1 receptors have long been suggested to critically modulate PFC-dependent cognitive functions, including working memory (Sawaguchi and Goldman-Rakic, 1991, 1994; Williams and Castner, 2006; Williams and Goldman-Rakic, 1995). More specifically, modulation of working memory by dopamine in the PFC appears to follow an inverted U-shaped function, with too much or too little D_1 activation leading to deficient working memory (for a review, see Seamans and Yang, 2004; Williams and Castner, 2006). However, in contrast to the suggested role of prefrontal D_1 receptors in PFC-dependent cognitive functions, late prenatal immune challenge in mice leads to spatial working and recognition memory impairments in the absence of altered prefrontal D_1 receptor densities (Meyer *et al*., 2008c).

The present findings of reduced prefrontal cortical AKT1 expression may thus provide an alternative cellular mechanism by which altered dopaminergic signaling can contribute to prenatal infection-induced cogntive dysfunctions in adulthood. Indeed, AKT1 appears to be critically involved in the down-stream signaling cascade of dopamine D_2 receptor activation (Beaulieu *et al*., 2004, 2005), and dopamine D_2 receptor are suggested to play a complementary role to D_1 receptors in the regulation of working memory (Floresco and Magyar, 2006; Goldman-Rakic, 1996; Tanaka *et al*., 2004). More specifically, by reducing the barriers for new information to be established in cortical networks, dopaminergic signals conveyed through dopamine D_2 receptors are believed to enable new information to be rapidly updated and/or manipulated online. This D_2-dependent process ensures that salient new information relevant for the guidance of on-going behaviour is rapidly processed and updated online. Since an essential component of spatial working memory and recognition memory is to update relevant information for the planning and/or guidance of on-going behaviour, disturbances in dopamine D_2-related processes can also be expected to impair performance in these cognitive processes.

Our study is centered upon the impact of infection-induced AKT1 deficiency in the PFC on spatial working memory and recognition memory. In addition to the PFC, several other regions are known to be critical for these cognitive functions, especially the hippocampus (Bannerman *et al*., 2004; Kesner RP and Hopkins, 2006). We have also attempted to evaluate the effects of prenatal immune challenge on hippocampal AKT1 protein expression. However, in contrast to its abundant expression in cortical structures (see **Figure 2.3**, **2.4**), the protein levels of AKT1 were generally low in the hippocampus of adult C57BL/6 mice (data not shown). This readily undermined a reliable assessment of AKT1 protein in prenatally immune-challenged and control offspring by the chosen immunohistochemical methods. Another limitation of the present study is that we did not explore potential influences of prenatal immune activation on other components of the AKT1 signaling pathway such as phosphoinositide 3 kinases (PI3 kinases) or glycogen synthase kinase 3 beta (GSK3β), which act upstream and downstream of AKT1, respectively (Lindsley *et al*., 2008). These issues should be addressed in future studies in order to

ascertain whether prenatal viral-like immune challenge may specifically affect the (prefrontal cortical) expression of AKT1, or whether this prenatal immunological insults may negatively influence multiple components of the AKT1 signaling pathway. Furthermore, it remains to be further explored whether the reduction in prefrontal AKT1 expression following prenatal immune challenge may represent a primary immune-mediated effect (Meyer *et al*., 2008a; Wang *et al*., 2008), or conversely, whether this may represent a secondary effect emerging in response to other primary neuropathological deficits induced by the prenatal immunological insult (Meyer and Feldon, 2009). If the former is the case, then one would expect that prenatal immune challenge may lead to altered AKT1 expression already during fetal and/or neonatal stages of life. Additional longitudinal studies are warranted in order to explore the ontogenetic mechanisms of altered AKT1 expression following prenatal immune challenge.

Abnormalities in the AKT1 signaling pathway have recently been implicated in the etiopathology of schizophrenia. Several (but not all) genetic linkage studies revealed a significant association between genetic variation in AKT1 and schizophrenia (Emamian *et al*., 2004; Ikeda *et al*., 2004; Norton *et al*., 2007; Tan *et al*., 2009; Thiselton *et al*., 2008; Xu *et al*., 2007). Such AKT1 variants in schizophrenic patients appear to be associated with reduced expression of AKT1 protein and/or mRNA in cortical and subcortical brain areas (e.g., Emamian *et al*., 2004; Thiselton *et al*., 2008). Another line of support for a significant role of altered AKT1 expression in schizophrenia-related disorders is derived from recent experimental investigations in mice with *AKT1* deficiency. Compared to wild-type litter mates, AKT1-deficient mice exhibit changes in the expression of genes related to synaptic function, neuronal development, myelination, and actin polymerization, all of which are implicated in the neuropathology of schizophrenia (Lai *et al*., 2006). Furthermore, AKT1-deficient mice display multiple changes in prefrontal pyramidal dendritic ultra-structure and concomitant deficits in working memory under dopamine agonists challenge (Lai *et al*., 2006). Hence, reduced AKT1 expression may precipitate schizophrenia-related abnormalities by disrupting prefrontal cortical structures and functions (Lai *et al*., 2006; Tan *et al*., 2009). Our findings support this hypothesis by demonstrating a significant correlation between AKT1 deficiency

in the PFC and disrupted cognitive performance in spatial working memory and recognition memory tests. As reviewed in detail elsewhere (Meyer *et al*., 2007, 2009; Meyer and Feldon, 2009; Patterson, 2009), prenatal Poly-I:C-induced immune activation in mice and rats is known to capture a variety of brain and behavioural abnormalities relevant to schizophrenia. Hence, the prenatal Poly-I:C model is a heuristic neurodevelopmental animal model of schizophrenia-like pathology, which enjoys a high degree of face, construct and predictive validity (Meyer *et al*., 2007, 2009; Meyer and Feldon, 2009; Patterson, 2009) as well as intrinsic etiological significance to the disorder. Against this background, the present findings provide additional experimental support for a significant role of altered AKT1 expression in the precipitation of schizophrenia-related dysfunctions following early (infection-induced) neurodevelopmental disruption.

Notably, our experimental approach used to assess the impact of reduced prefrontal AKT1 expression readily differs from genetic linkage studies demonstrating attenuated cognitive performance in human subjects with specific AKT1 variants (Tan *et al*., 2008). In the latter, cognitive measures are typically correlated with specific AKT1 variants, whereas our experimental approach provides correlative evidence for a link between reduced prefrontal AKT1 protein (as assessed by immunohistochemical analyses of cellular AKT1 protein expression) and impaired cognitive performance in spatial working memory and recognition tests. However, it is important to emphasize that those AKT1 variants in individuals with apparent cognitive dysfunctions, including patients with schizophrenia, are associated with reduced AKT1 mRNA and/or protein levels in several brain regions, including PFC and hippocampus (see e.g., Emamian *et al*., 2004; Thiselton *et al*., 2008). Hence, our study readily provides informative results with regards to the human clinical condition, in which specific AKT1 variants are associated with reduced CNS levels of AKT1 mRNA/protein and specific cognitive impairments relevant to schizophrenia (Emamian *et al*., 2004; Tan *et al*., 2008; Thiselton *et al*., 2008).

Taken together, the present findings may be relevant for the continual identification and characterization of the cellular mechanisms involved in the precipitation of cognitive dysfunctions in schizophrenic patients especially with (but not limited to those with) prenatal infectious histories. According to the experimental results presented here, alterations in AKT1 signaling and associated cognitive dysfunctions in schizophrenia may not only be precipitated by genetically determined factors (Emamian *et al*., 2004; Tan *et al*., 2009), but may also be produced by (immune-associated) environmental insults relevant for the etiology of this disabling brain disorder. The present experimental demonstration of long-term alterations in AKT1 expression following prenatal immune activation may also encourage further evaluations of anti-inflammatory treatments in the pharmacotherapy of schizophrenia (Müller *et al*., 2004a,b; Riedel *et al*., 2005). Support for beneficial effects of add-on therapy with ant-inflammatory agents in the symptomatic treatment of schizophrenia comes from clinical trials using cyclooxygenase-2 (COX-2) inhibitors such as celecoxib, which reduce the production of pro-inflammatory cytokines and prostaglandins. Independent clinical studies have demonstrated that the combination of the antipsychotic drug risperidone and celecoxib shows a significant superiority over risperidone alone in the treatment of cognitive and positive symptoms, general psychopathology symptoms, as well as of Positive and Negative Syndrome Scale (PANSS) total scores (Müller *et al*., 2002, 2005; Akhondzadeh *et al*., 2007). Given the heuristic value of the prenatal PolyI:C model in preclinical schizophrenia research (Meyer *et al*., 2007, 2009; Meyer and Feldon, 2009; Patterson, 2009), it would be highly interesting to explore whether anti-inflammatory treatments may be effective in normalizing the neuropathological and behavioural deficits induced by prenatal immune activation. Further examination and evaluation of immune-related genetic and environmental influences on AKT1 expression may thus provide important new insight into the neural basis of psychosis-related behavior and may open a new avenue for the development of effective pharmacological treatments of cognitive symptoms in schizophrenia.

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STATEMENT OF INTEREST

All authors declare they have no conflicts of interest to disclose.

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SUPPLEMENTARY METHODS

Cognitive testing of offspring

All offspring were weaned and sexed on postnatal day (PND) 21. Littermates of the same sex were caged separately. All animals were maintained under *ad libitum* food and water diet unless specified otherwise. They were kept in a temperature and humidity controlled animal vivarium under a 12:12h reversed light-dark cycle as described above.

Offspring from multiple independent litters (6 Poly-I:C, 6 saline) were randomly selected for the cognitive phenotyping. Both male and female offspring were included in all the tests described below in order to assess potential sex-dependent effects of the prenatal immunological manipulation. The numbers of animals included in the control and Poly-I:C groups were *N*=11 (5♂, 6♀) and *N*=10 (5♂, 5♀), respectively. Cognitive testing commenced when the offspring reached the adult stage of development starting on postnatal day 80, and was always conducted during the dark phase of the light-dark cycle. Cognitive phenotyping of prenatally Poly-I:C-exposed and control offspring included the assessment of spatial working memory in the cheeseboard maze and spatial recognition memory in the Y-maze according to modified protocols described by Kirwan *et al*. (2005) and Sanderson *et al*. (2007). The same animals were first subjected to the cheeseboard maze test followed by the Y-maze test.

Spatial working memory in the cheeseboard maze

The apparatus consisted of a wooden circular board (diameter: 1.1 m, thickness: 3 cm) painted in grey. The maze was elevated 75 cm above the floor. It was positioned in a well-lit room enriched with distal spatial cues. One side of the cheeseboard maze was used for habituation to the procedure and the testing apparatus, and therefore was not drilled with any holes. The other side was drilled with 32 holes (diameter: 3.1 cm, depth: 1.3 cm) arranged in radial design (see **Figure 2.1a**). Each hole contained a

Plexiglas bottle cap which could be equipped with a visual cue (composed of a blue flag attached to a 15 cm stick) and/or a reward in the form of 75 µl freshly prepared condensed milk (Alicommerce SAS, Liebefeld-Bern, Switzerland) used in a 1:4 (milk:water) dilution. A digital camera was mounted above the cheeseboard maze. Images were captured at a rate of 5 Hz and transmitted to a PC running the Ethovision (Noldus Information Technology, Wageningen, The Netherlands) tracking system, which calculated the latency and distance moved to reach the reward on each trial.

All animals were progressively food deprived during the initial habituation phase (days 1-5) until a minimal 90% free-feeding weight was reached. Animals exhibiting excessive body weight loss were provided with a 1-gr pellet one hour before lights were turned on. The animals were habituated to the cheeseboard using the no-holes surface of the maze for 2 daily trials of 2 min each. To begin a trial, the animals were placed gently in the center of the cheeseboard under a semitransparent plastic bucket (diameter: 15cm, height: 25cm). The trial started when the bucket was removed. The inter-trial interval (ITI) was approximately 1 min. The 2 daily 2-min trials on the no-holes surface of the cheeseboard served to familiarize the animals with the test apparatus and environment, and to reduce potential confounds arising from stress induced by the procedure. On day 6, the animals were then pre-trained in the cheeseboard using the visual cue for 2 consecutive trials. One random hole was cued with the flag and rewarded with the milk solution. For both trials, each animal was left on the maze for 2 min. If it failed to reach the rewarded hole during this time, it was gently guided to the rewarded hole by the experimenter. All animals consumed the reward within the allowed maximum time of 15 sec.

Following the habituation (days 1-5) and pre-testing (day 6) phases, working memory testing was started. The working memory task was based on the matching-to-position paradigm, in which the animals were required to learn the novel position of a rewarded hole revealed to them on trial 1 of each day in order to navigate effectively to the same location (i.e. matching) on the subsequent trial on the same day. Hence, each test day included 2 trials. For both trials, each animal was left on the maze for 2 min. If it

failed to reach the rewarded hole during this time, it was gently guided to the rewarded hole by the experimenter. The reward remained in the same position across trials on a given test day, but took a new position on each test day. The daily change of the reward location ensured that the information acquired on a given day was irrelevant for the next day, thus taxing the flexible use of working memory (Baddeley, 2003). To begin a trial, the animals were placed gently in the center of the cheeseboard under a semitransparent plastic bucket as described before. The trial started when the bucket was removed. At the beginning of each testing day, each cap was brushed with a cotton stick soaked with the reward solution so as to minimize potential confounds by olfactory cues. The allocation of the rewarded holes to a specific spatial location was counterbalanced across subjects.

To manipulate the retention demand in the temporal domain, the ITI varied between trials 1 and 2. First, a minimal ITI of 2 min was used for 2 consecutive days (days 7-8). The minimal ITI referred to the time needed to clean the maze with 5% ethanol solution and to let it dry before the next trial commenced. The ITI was then increased to 5 min on the next 2 testing days (days 9-10), and then to 10 min thereafter (days 11-12). Finally, a maximal ITI of 15 min was used on the following 6 testing days (days 13-18) in order to consolidate the anticipated working memory impairment in prenatally immune challenged animals under extended ITI conditions (Meyer *et al*., 2005, 2008). During all the ITIs used (i.e., 2, 5, 10, and 15 min), the animals were placed in an opaque waiting box located in the testing room. Working memory was indexed by the reduction in latency and distance moved to find the location of the reward in trial 2 relative to trial 1 (when the location of the reward was essentially unknown to the animals).

Spatial novelty preference in the Y-maze

Spatial recognition memory was evaluated by a spatial novelty preference task in the Y-maze 1 week following completion of working memory test in the cheeseboard. The spatial novelty preference test in the Y-maze assesses short-term spatial memory and utilizes the natural tendency of rodents to explore novel over familiar spatial environments (Dellu *et al*., 1992).

The apparatus was made of transparent Plexiglas and consisted of three identical arms (50 cm × 9 cm; length × width) surrounded by 10-cm high transparent Plexiglas walls. The three arms radiated from a central triangle (8 cm on each side) and spaced 120° from each other. Access to each arm from the central area could be blocked by a removable opaque barrier wall. The floor of the maze was covered with sawdust bedding, which was changed between testing phases as well as between trials. The maze was elevated 90 cm above the floor and was positioned in a well-lit room enriched with distal spatial cues. For each retention interval tested (see below), a distinct set of extra-maze cues surrounded the Y-maze. A digital camera was mounted above the Y-maze apparatus. Images were captured at a rate of 5 Hz and transmitted to a PC running the Ethovision (Noldus Information Technology, Wageningen, The Netherlands) tracking system, which calculated the time spent and distance moved in the three arms and center zone of the Y-maze.

The test of spatial recognition memory in the Y-maze consisted of two phases, namely the sample and choice phases. The allocation of arms (start, familiar and novel arm) to a specific spatial location was counterbalanced across subjects.

● *Sample phase:* The animals were allowed to explore two arms (referred to as "start arm" and "familiar arm"). Access to the remaining arm ("novel arm") was blocked by a barrier wall door. To begin a trial, the subject was introduced at the end of the start arm, facing the central triangle and was allowed

to freely explore both the start and familiar arms for 5 min. Test timing was initiated once the subject had made an entry into the central triangular area, as detected by the Ethovision tracking system. The subject was then removed and kept in a holding cage during the specific retention intervals (see below) prior to the choice phase. The barrier door was removed and the sawdust flooring changed to avoid olfactory cues.

- *Choice phase:* Following a specific retention interval (see below), the test subject was introduced to the maze again. During the choice phase, the barrier wall was removed so that the animals could freely explore all arms of the maze for 2 min. The subject was then removed from the maze the returned to the home cage. The sawdust flooring was changed in preparation for the next trial. On each trial, the time spent in each of the three arms was recorded. The percentage time spent in the novel arm during the choice phase was calculated by the formula [time spent in the novel arm/ (time spent in all arms)] × 100 and used as the index for spatial novelty preference. In addition, total distance moved on the entire maze was recorded and analyzed in order to assess general locomotor activity.

To manipulate the retention demand in the temporal domain, the interval between the two phases (i.e. sample and choice phases) of the Y-maze test were varied. First, a minimal interval of 2 min was used. This minimal delay referred to the time needed to change the sawdust flooring between the two phases in order to avoid confounds by olfactory cues. The interval between the two phases was then increased to 15 min in accordance with the extended (15-min) retention interval used for the working memory test in the cheeseboard maze.

Immunohistochemistry

The immunohistochemical analyses were conducted in the same animals previously subjected to cognitive testing. Hence, the numbers of animals included in the control and Poly-I:C groups were *N*=11 (5♂, 6♀) and *N*=10 (5♂, 5♀), respectively. One animal (control ♀) was not included in the immunohistochemical assays because of incomplete perfusion.

Following a resting period of two weeks after completion of the cognitive testing, the animals were anesthetized with an overdose of Nembutal™ (Abbott Laboratories, North Chicago, USA) and perfused transcardially with 0.9% saline, followed by 4% phosphate-buffered paraformaldehyde solution containing 15% picric acid. The dissected brains were postfixed in the same fixative and microwaved in a citric acid buffer (pH = 4.5) for 90 s with an intensity of 480 W according to protocols established by Fritschy *et al*. (1998). The brains were then cryoprotected using 30% sucrose in phosphate buffered saline (PBS) and frozen with powdered dry ice.

Coronal sections (30 µm thick) were cut from frozen blocks on a sliding microtome and eight randomly sampled series of sections from Bregma +2.2 to +1.6 were collected, rinsed in PBS, and stored at -20 °C in antifreeze until further processing. For immunostaining, the slices were first rinsed three times for 10 min in PBS. Blocking was done in PBS, 0.3% Triton X-100, and 5% normal serum for 1 h at room temperature. The following primary antibodies were used: rabbit anti-AKT1 (Calbiochem, EMD Chemicals Inc., Darmstadt, Germany; diluted 1:3000) and rabbit anti-COMT (SDI, Newark, USA; diluted 1:5000). The primary antibody was diluted in PBS containing 2% normal goat serum and 0.3% Triton X-100 and incubated overnight at room temperature. After three washes with PBS, the sections were incubated with the biotinylated secondary goat antibody (Jackson ImmunoResearch Laboratories Inc., PA, USA), diluted 1:500 in antibody buffer for 1 h at room temperature. The sections were washed again three times for 10 min in PBS and incubated with Vectastain Kit (Vector Laboratories; Burlingame, CA

USA) diluted in PBS for 1 h. After three rinses in Tris–HCl 0.1M (pH = 7.4), the sections were stained with 1.25% 3,3-diaminobenzidine (DAB) and 0.08% H_2O_2 for 10 min, rinsed four times in PBS, dehydrated and cover-slipped with Eukid® (Kindler GmbH, Freiburg, Germany).

Stereological estimates of AKT1-immunorecative cells

The number of AKT1-immunoreactive cells in the three prefrontal cortical regions of interest (CG, PL and IL) of the right brain hemisphere was determined by stereological estimations using the optical fractionator method (Gundersen *et al*., 1988). Consistent with previous immunohistochemical studies (Sinor and Lillien, 2004), the expression of AKT1 protein was found to be highly enriched in the cell cytoplasm (see **Figure 2.3b**). Therefore, it was feasible to precisely delineate individual AKT1-immunoreactive cells necessary for stereological estimations. With the aid of the image analysis computer software Stereo Investigator (version 6.50.1; Microbrightfield, Colchester, VT, USA), every section of a one-in-six series was measured, resulting in an average of 6-7 brain sections per animal for the analysis of AKT1-immunoreactive cells in the CG and PL areas, and of 3-4 brain sections per animal for the IL area. For all prefrontal cortical areas, the following sampling parameters were used: (1) a fixed counting frame with a width of 40 µm and a length of 40 µm; and (2) a sampling grid size of 150 × 110 µm. The counting frames were placed randomly at the intersections of the grid within the outlined structure of interest by the software. The cells were counted following the unbiased sampling rule using the 40× oil lens [numerical aperture (NA), 1.3] and included in the measurement when they came into focus within the optical dissector (Howard and Reed, 2005). All stereological data were acquired and analyzed under blind conditions.

Optical densitometry of COMT-immunoreactive cells

Quantification of COMT-immunoreactive cells in the three prefrontal cortical regions (CG, PL and IL) of the right brain hemisphere was achieved by means of optical densitometry using ImageJ software (ImageJ, NIH, Maryland, USA). Optical densitometry was chosen because COMT protein was found to be expressed primarily at the cell perimeter of prefrontal cortical cells, thus forming a cell membrane-like expression pattern (see **Figure 2.3b**). This expression pattern readily undermined the precise delineation of individual COMT-positive cells, which would be necessary for cell quantification by stereological analyses. Therefore, we quantified COMT-immunoreactivity by the alternative means of optical densitometry. Digital images were acquired at a magnification of 2.5× (NA 0.075) using a digital camera (Axiocam MRc5, Zeiss, Jena, Germany) mounted on a Zeiss Axioplan microscope. Exposure times were set so that pixel brightness was never saturated. Pixel brightness was measured in the respective areas of the right brain hemisphere. In addition, pixel brightness was measured in the forceps minor corpus callosum (fmi) as background area. The background-corrected optical densities were averaged per brain region and animal. 6-7 brain sections per animal were included in the analysis of COMT-IR cells in the CG and PL areas, and of 3-4 brain sections per animal in the analysis of the IL area. All densitometric data were acquired and analyzed under blind conditions.

Statistical analysis

All cognitive data were analyzed using parametric ANOVA, followed by restricted ANOVA whenever appropriate. In the working memory test (cheeseboard), the latency and distance traveled to find the location of the rewarded hole was analyzed with the between-subjects factors of prenatal treatment and sex, and with the within-subjects factors of trials and days. The data collected for the different retention intervals of the working memory test (i.e., in the 2, 5, 10, and 15 min ITI conditions) were analyzed separately. Spatial novelty preference (indexed as percentage time spent in the novel arm

of the Y-maze) was analyzed with the between-subjects factors of prenatal treatment and sex. The data collected on the different retention intervals of Y-maze test (i.e., in the 2 and 15 min interval conditions) were analyzed separately. Stereological estimates of AKT1-positive cells and densitometric measures of COMT-immunoreactivity were analyzed using with the between-subjects factors of prenatal treatment and sex. Additional correlative analyses were also conducted in order to seek evidence for possible correlations between cognitive performance in the spatial working memory or spatial novelty preference test and levels of AKT1 or COMT protein expression in the three prefrontal cortical areas of interest. In addition, correlative analyses between AKT1 and COMT expression were conducted for each prefrontal cortical region of interest. These correlative analyses were performed using first order partial correlations for prenatal treatment. Such partial correlations were used in order to control for the effects of the independent variable of prenatal treatment. Statistical significance was set at $p < 0.05$ for all analyses. All statistical analyses were conducted using the statistical software SPSS version 13.0 (SPSS Inc., Chicago, IL, USA).

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CHAPTER 3.

Late Prenatal Immune Activation in Mice Leads to Behavioural and Neurochemical Abnormalities Relevant to the Negative Symptoms of Schizophrenia

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[Personal contribution to the work: Designing the research, running the behavioural tests, analysis of the behavioural data, and writing the manuscript]

ABSTRACT

Based on the human epidemiological association between prenatal infection and higher risk of schizophrenia, a number of animal models have been established to explore the long-term brain and behavioural consequences of prenatal immune challenge. Accumulating evidence suggests that the vulnerability to specific forms of schizophrenia-related abnormalities is critically influenced by the precise timing of the prenatal immunological insult. In the present study, we tested the hypothesis whether late prenatal immune challenge in mice may induce long-term behavioural and neurochemical dysfunctions primarily associated with the negative symptoms of schizophrenia. We found that prenatal exposure to the viral mimic polyriboinosinic-polyribocytidilic acid (Poly-I:C, 5mg/kg, i.v.) on gestation day 17 led to significant deficits in social interaction, anhedonic behaviour, and alterations in the locomotor and stereotyped behavioural responses to acute apomorphine treatment in both male and female offspring. In addition, male but not female offspring born to immune-challenged mothers displayed behaviour/cognitive inflexibility as indexed by the presence of an abnormally enhanced latent inhibition effect. Prenatal immune activation in late gestation also led to numerous, partly sex-specific changes in basal neurotransmitters levels, including reduced dopamine and glutamate contents in the prefrontal cortex and hippocampus, as well as reduced GABA and glycine contents in the hippocampus and prefrontal cortex, respectively. The constellation of behavioural and neurochemical abnormalities emerging following late prenatal Poly-I:C exposure in mice leads us to conclude that this immune-based experimental model provides a powerful neurodevelopmental animal model especially for (but not limited to) the negative symptoms of schizophrenia.

INTRODUCTION

Human epidemiological studies have repeatedly demonstrated that the risk of developing schizophrenia and related disorders is significantly enhanced following prenatal exposure to infection (Brown, 2006; Brown and Derkits, 2010; Fatemi, 2005; Patterson, 2007). This has motivated the establishment of several rodent models of prenatal infection and/or immune activation which aim at exploring this epidemiological association on experimental grounds (for a recent review, see Meyer *et al.*, 2009; Meyer and Feldon, 2009, 2010; Patterson, 2009). One such model is based on prenatal exposure to the viral mimic polyriboinosinic-polyribocytidilic acid (Poly-I:C) in rats or mice. Poly-I:C is a synthetic analogue of double-stranded RNA which elicits a cytokine-associated viral-like acute phase response in mammalian organisms (Kimura *et al.*, 1994; Traynor *et al.*, 2004). Numerous experimental investigations have provided robust evidence for the emergence of long-term functional and structural brain abnormalities following prenatal exposure to Poly-I:C-induced immune challenge in both rats and mice (reviewed in Meyer *et al*, 2009; Meyer and Feldon, 2009, 2010; Patterson, 2009). Importantly, many of the prenatal Poly-I:C-induced behavioural, cognitive and pharmacological dysfunctions in adult offspring are directly implicated in schizophrenia and other psychosis-related disorders, including abnormalities in sensorimotor gating, selective attention, working memory and sensitivity to psychostimulant drugs. At least some of these functional abnormalities can be normalized by acute and/or chronic antipsychotic drug treatment (Meyer *et al.,* 2010; Ozawa *et al.*, 2006; Shi *et al.*, 2003; Zuckerman *et al.*, 2003; Zuckerman and Weiner, 2005) and are dependent on post-pubertal maturational processes (Meyer *et al.*, 2006c; 2008a; Ozawa *et al.*, 2006; Vuillermot *et al.*, 2010; Wolff and Bilkey, 2008; Zuckerman *et al.*, 2003). Taken together, the prenatal Poly-I:C model in rats and mice is characterized by a high level of face, construct and predictive validity for schizophrenia-like psychopathology, and it successfully mimics the developmental delay of symptom onset (Fatemi and Folsom, 2009; Meyer and Feldon, 2010; Rapaport *et al.*, 2005; Weinberger, 1987).

Using the Poly-I:C model of prenatal immune activation in mice, we have recently shown that the vulnerability to specific forms of schizophrenia-related functional and structural abnormalities is critically influenced by the precise timing of the prenatal immunological challenge (Li *et al.,* 2009; Meyer *et al.*, 2006a,b, 2007, 2008a). For example, abnormalities in selective attention in the form of latent inhibition (LI) disruption (Meyer *et al.*, 2006a) and sensorimotor gating impairment in the form of reduced prepulse inhibition (PPI; Li *et al.,* 2009; Meyer *et al.*, 2008a) are part of a symptom cluster characteristic for prenatal Poly-I:C-induced immune activation in early/middle gestation. On the other hand, emergence of perseverative behaviour in the form of impaired discrimination reversal learning (Meyer *et al.*, 2006b), spatial working memory deficits under low storage load in the temporal domain (Meyer *et al.*, 2008a), and marked potentiation of the sensitivity to *N*-methyl-D-aspartate (NMDA)-receptor antagonism by acute dizocilpine (MK-801) treatment (Meyer *et al.*, 2008a) are more readily seen following prenatal Poly-I:C exposure in late gestation compared to identical prenatal immune challenge in early/middle gestation. Based on this, it has been suggested that late prenatal immune activation in mice may mimic some critical functional abnormalities relevant to the negative symptoms of schizophrenia (Sullivan *et al.*, 2006; Meyer *et al.*, 2009). However, experimental support for this hypothesis is relatively sparse thus far, and the extent to which late prenatal immune activation may induce long-term behavioural abnormalities relevant to the negative symptoms of schizophrenia clearly warrants further exploration.

Therefore, the present study tested the hypothesis that prenatal Poly-I:C-induced immune activation in late gestation may lead to multiple behavioural abnormalities relevant to the negative symptoms of schizophrenia. These symptoms refer to features that are normally present in healthy subjects but are reduced or absent as a result of the disease process (Crow, 1980). Besides others, negative symptoms include social interaction deficits, presence of repetitive stereotyped behaviours, anhedonia and behavioural and/or cognitive inflexibility (Blanchard and Cohen, 2006; Crider, 1997; Crow, 1980; Foussias and Remington, 2008; McGlashan and Fenton, 1992; Morrens *et al.,* 2006). In keeping with our working hypothesis, our phenotypic characterization of the effects of late prenatal immune challenge thus

comprised tests which have been designed to assess such behavioural functions in translational rodent models (Arguello and Gogos, 2006; Crawley, 2007; Meyer and Feldon, 2010). In addition to the functional phenotyping, we also explored the neurochemical correlates of behavioural dysfunctions following prenatal immune challenge. This was achieved by means of neurochemical analyses of central monoamines and their metabolites, as well as of excitatory and inhibitory amino acids in post-mortem brain tissue of prenatally immune challenged and control offspring.

MATERIALS AND METHODS

Animals

C57BL/6 mice were used throughout the study. Female and male breeders were obtained from our in-house specific pathogen free (SPF) colony at the age of 10-14 weeks. Breeding began after 2 weeks of acclimatization to the new animal holding room, which was a temperature- and humidity-controlled (21±1°C, 55±5%) holding facility under a reversed light–dark cycle (lights off: 08:00–20:00). All animals had *ad libitum* access to food (Kliba 3430, Klibamühlen, Kaiseraugst, Switzerland) and water. All procedures described in the present study had been previously approved by the Cantonal Veterinarian's Office of Zurich and are in agreement with the principles of laboratory animal care in the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication No. 86-23, revised 1985). All efforts were made to minimize the number of animals used and their suffering.

Maternal immune activation in late gestation

For the purpose of the maternal immunological manipulation in late gestation, female mice were subjected to a timed mating procedure as described previously (Meyer *et al.*, 2005). Pregnant dams on gestation day (GD) 17 received either a single injection of Poly-I:C (potassium salt; Sigma-Aldrich, Buchs, St. Gallen, Switzerland) or vehicle. Poly-I:C (5 mg/kg) was dissolved in sterile pyrogen-free 0.9% NaCl (= vehicle) solution to yield a final concentration of 1 mg/ml and was administered via the intravenous (i.v.) route at the tail vein under mild physical constraint. The dose of Poly-I:C was chosen based on our previous studies in C57BL/6 mice (e.g., Meyer *et al.*, 2006a,b, 2008a). All solutions were freshly prepared on the day of administration and injected with a volume of 5 ml/kg.

The selected gestational window (i.e., GD 17) in mice corresponds roughly to the middle-to-late second trimester of human pregnancy, respectively, with respect to developmental biology and percentage

of gestation from mice to human (Clancy *et al.,* 2001, 2007). This gestational stage is identical to the one selected in our previous experimental studies examining the impact of the precise timing of prenatal immune activation on postnatal brain dysfunctions (Li *et al.,* 2009; Meyer *et al.*, 2006a,b, 2008b).

Phenotypic characterization of offspring

All offspring were weaned and sexed on postnatal day (PND) 21. Littermates of the same sex were caged separately. All animals were maintained under *ad libitum* food and water, and were kept in a temperature and humidity controlled animal vivarium under a 12:12 h reversed light-dark cycle as described above.

Offspring from multiple independent litters (8 Poly-I:C, 8 saline) were randomly selected for the behavioural and pharmacological tests. Both male and female offspring were included in all the tests described below in order to assess potential sex-dependent effects of the prenatal immunological manipulation. A first cohort of Poly-I:C and control offspring (n=16 [8 males, 8 females] in each experimental group) was subjected to behavioural testing in the following test order: (1) social interaction test, (2) sucrose preference test, and (3) latent inhibition (LI) test (see below). All animals were allowed a 1-week resting period between each test. The first cohort of Poly-I:C and control offspring was then used for post-mortem neurochemical analyses (see below) following a resting period of 4 weeks upon conclusion of the LI test. A second cohort of Poly-I:C and control offspring (n=12 [6 males, 6 females] in each experimental group) was used for the assessment of basal and apomorphine-induced stereotypies (see below). All behavioural testing commenced when the offspring reached the adult stage of development (i.e., PND 80 onwards).

Social interaction test

Social behaviour is commonly referred to behaviour that takes place in a social context and results from the interaction between and among individuals (of the same species). Since mice (like most other rodents) are highly social animals, social interaction can be efficiently studied under experimental conditions (Crawley, 2007). Social interaction was assessed by analyzing the relative exploration time between an unfamiliar congenic mouse and an inanimate dummy object. A detailed description of the test apparatus and procedures used in the social interaction test can be obtained in the *Supplementary Materials and Methods.*

Sucrose preference test

Anhedonic behaviour was assessed by a standard sucrose preference test using methods adapted from previous studies (Hayward *et al.,* 2006; Slattery *et al.,* 2007). The sucrose preference test is based on the observation that rodents typically show a preference for a sweet sucrose solution when presented with a free choice between the sucrose solution and water. A reduction in this preference is commonly considered as an indication of anhedonia (Cryan and Mombereau, 2004; Hayward *et al.,* 2006; Slattery *et al.,* 2007) and conversely an increase may suggest hyperhedonia. A detailed description of the apparatus and experimental procedures used can be obtained in the *Supplementary Materials and Methods*. Sucrose preference was indexed by a percentage score [sucrose consumption / (total liquid consumption) × 100%] obtained on two consecutive test days (see *Supplementary Materials and Methods*). In addition, we also recorded and analyzed total fluid consumption during the habituation and test phases (see *Supplementary Materials and Methods*) in order to compare the general fluid intake between the two prenatal treatment groups.

Latent inhibition (LI) test

Behavioural/cognitive inflexibility was studied by assessing the presence of LI persistence. LI is a selective learning procedure, in which prior repeated pre-exposures to the to-be-conditioned stimulus (CS) alters the subsequent development of the conditioned response (CR) following explicit parings between the same CS and an unconditioned stimulus (US) (Weiner, 2003). A significant LI effect (i.e. impaired conditioning following repeated inconsequential pre-exposures to the CS) is typically manifest when the organism is subjected to a high number of CS-pre-exposures before CS-US conditioning (Weiner, 2003; Weiner and Arad, 2009). In contrast, presentation of a low number of CS-alone stimuli during the pre-exposure phase readily prevents the expression of LI in normal subjects (Weiner, 2003; Weiner and Arad, 2009). However, organisms which tend to show behavioural and/or cognitive inflexibility are expected to express the LI effect under parametric conditions that are insufficient for control animals to display LI (Weiner, 2003; Weiner and Arad, 2009).

LI persistence was studied in a two-way active avoidance procedure using four identical two-way shuttle boxes. The test apparatus used has been fully described before (Meyer *et al.*, 2006c). A white noise stimulus served as the CS, and electric foot shock served as the US. In order to facilitate the anticipated expression of LI persistence in prenatally immune challenged offspring relative to control offspring, we used a LI procedure which has been shown in preliminary studies to result in minimal or no LI in adult control C57BL/6 mice (Meyer, Feldon: unpublished observations). The test procedures consisted of two phases: pre-exposure and conditioning, conducted 24 hrs apart. Equal numbers of offspring from both prenatal treatment (Poly-I:C or vehicle) conditions were allocated to one of the two conditions: CS-pre-exposed (CS-PE) and non-pre-exposure (NPE). In the pre-exposure phase, CS-PE subjects were presented with 40 pre-exposures to a 5-s white noise CS according to a random interstimulus interval schedule, whereas NPE subjects were confined to the chamber for an equivalent period of time without any stimulus presentation. On conditioning day, the subjects were returned to the

same shuttle boxes and received a total of 100 avoidance trials. A detailed description of the test procedures can be found in the *Supplementary Materials and Methods.*

To index conditioned avoidance learning, the mean response latency performed on successive 10-trial blocks were analyzed (see *Supplementary Materials and Methods*). In addition, the mean numbers of spontaneous shuttles during the inter-trial intervals (ITIs) were recorded and analyzed in order to account for basal locomotor activity during the conditioning phase.

Basal and apomorphine-induced stereotyped behaviour

Behavioral stereotypy is defined as uniform, repetitive and compulsive actions within a restricted pattern which often have no obvious goals or end-points. Acute treatment with the mixed dopamine D1/D2 receptor agonist apomorphine (APO) is well known to induce a dose-dependent enhancement of stereotyped behaviours in mice, including repetitive climbing, wall-leaning, sniffing and gnawing (Protais *et al*, 1976; Cabib and Puglisi-Allegra, 1985; Tirelli and Witkin, 1994). Acute APO treatment can thus be used to probe the sensitivity to drug-induced stereotyped behavioural responses.

Basal and APO-induced stereotyped behaviours were assessed by analyzing horizontal and vertical movements and behaviours in a wire mesh cylinder following vehicle (vitamin C solution, VitC) or APO (5 mg/kg, s.c.) administration. A detailed description of the test apparatus and experimental procedures used can be found in the *Supplementary Materials and Methods*.

Post-mortem neurochemistry

Levels of monoamines (noradrenaline, NA; dopamine, DA; serotonin [=5-hydroxytryptamine], 5-HT) and their metabolites (dihydroxyphenylacetic acid, DOPAC; homovanillic acid, HVA;

5-hydroxyindoleacetic acid, 5-HIAA; 3-methoxytyramine, 3-MT), amino acids (γ-aminobutyric acid, GABA; glutamate; aspartate; glycine) and the sulfonic amino acid taurine were determined using high performance liquid chromatography (HPLC) according to procedures established before (Peleg-Raibstein *et al*, 2005, 2008) and described in detail in the *Supplementary Materials and Methods*. Following decapitation and dissection of the brain, coronal sections were prepared using razorblade cuts along the following coordinates with respect to bregma: anterior-posterior +2.3 to+1.3, +1.3 to +0.3, –0.1 to –0.6, –1.2 to –2.2, and –2.8 to –3.8. Discrete brain regions were then collected using a micropunch needle (1 mm in diameter) generating micropunches for the medial prefrontal cortex (mPFC), amygdala (AMY), caudate-putamen (CPu), nucleus accumbens (NAc), dorsal hippocampus (dHPC), and ventral hippocampus (vHPC) (for a detailed description, see *Supplementary Materials and Methods*). Monoamines and amino acids were determined using amperometric electrochemical and fluorescence detectors, respectively (see *Supplementary Materials and Methods* for detailed information).

Statistical analysis

Behavioural and neurochemical data were analyzed using parametric analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) *post hoc* group comparisons or restricted ANOVA whenever appropriate. Behavioural measures obtained in the analysis of stereotyped responses to APO (i.e., scores for climbing, leaning and sniffing behaviour) were first subjected to square-root (sqrt) transformation in order to better conform the data to the normal distribution assumption of parametric ANOVA. Statistical significance was set at $P < 0.05$. Analyses were conducted using the Statistical Package for the Social Sciences (SPSS) software (version 13.0) implemented on a personal computer running the Windows XP operating system.

RESULTS

Late prenatal immune challenge leads to reduced social interaction

The relative exploration time between an unfamiliar congenic mouse and an inanimate dummy object was used to assess social interaction in adult control and Poly-I:C offspring. The relative exploration time was recorded during a 5-min test period and analyzed as a function of 1-min bins. As shown in **Figure 3.1**, control offspring born to vehicle-treated mothers displayed a clear preference towards the unfamiliar mouse, indicating intact social interaction. On the other hand, offspring exposed to prenatal Poly-I:C treatment in late gestation displayed no such preference (**Figure 3.1**). The Poly-I:C-induced disruption of social interaction was evident in both male and female offspring.

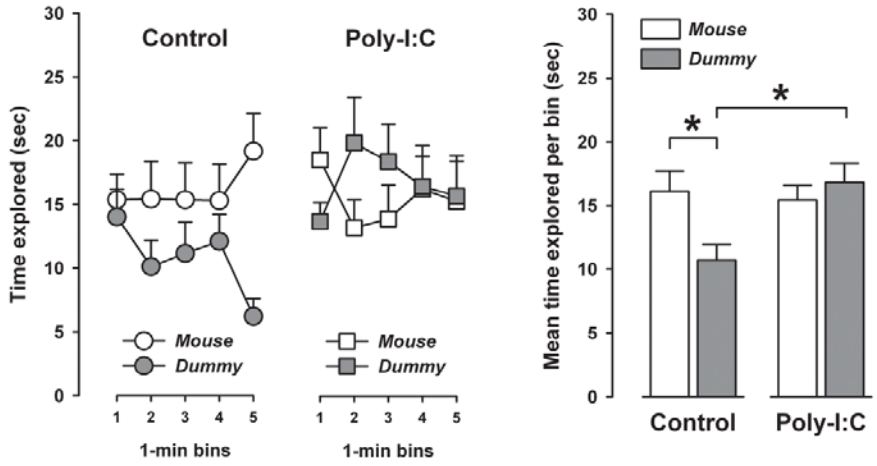

Figure 3.1. Social interaction deficits following late prenatal immune challange. Social interaction was assessed by analyzing the relative exploration time between an unfamiliar congenic mouse ("mouse") and an inanimate dummy object ("dummy"). The line plots depict the relative exploration time as a function of successive 1-min bins, and the bar plot shows the mean exploration time per bin. Control offspring born to vehicle-treated mothers displayed a significant preference towards the unfamiliar congenic mouse, indicating intact social interaction. On the other hand, offspring exposed to prenatal Poly-I:C treatment in late gestation displayed no such preference. *$P < 0.05$, based on restricted ANOVAs. All values are means ± SEM.

Statistical support for these impressions was obtained by the 2 × 2 × 2 × 5 (prenatal treatment × sex × object ×1-min) repeated-measures ANOVA of exploration time, which yielded a significant main effect of treatment ($F_{1,28} = 4.56$, $P < 0.05$) and its interaction with object ($F_{1,28} = 6.07$, $P < 0.05$).

Additional 2 × 2 × 5 (sex × object × 1-min) repeated-measures ANOVA of exploration time restricted to control and Poly-I:C offspring were then conducted to further confirm the presence or absence of a significant preference towards the live unfamiliar mouse *versus* the inanimate dummy object. By revealing a significant main effect of object ($F_{1,14}$ = 8.23, P < 0.05), the ANOVA restricted to control offspring confirmed a significant preference towards the live mouse. In contrast, the main effect of object (or its interaction with bins) was far from significant in the restricted ANOVA of exploration time in Poly-I:C offspring [F<0.5]. Additional 2 × 2 × 5 (prenatal treatment × sex × 1-min) repeated-measures ANOVAs of exploration time restricted to the two objects (i.e., live mouse and inanimate dummy object) were also conducted. The ANOVA restricted to the inanimate dummy object revealed a significant main effect of prenatal treatment ($F_{1,28}$ = 9.28, P < 0.05), reflecting the significant increase in dummy object exploration exhibited by Poly-I:C offspring compared to control offspring (**Figure 3.1**). The ANOVA restricted to the live mouse did not reveal any significant main effects or interactions.

Late prenatal immune challenge leads to anhedonic behaviour in the sucrose preference test

Prenatal Poly-I:C-induced immune activation on GD17 did not significantly affect basal water intake during the habituation phase of the sucrose preference test. The 2 × 2 × 3 (prenatal treatment × sex × days) repeated-measures ANOVA of water consumption during the initial 72-hrs habituation phase did not reveal any significant main effects or interactions. The overall daily mean±SEM of water intake during this phase was 7.67±0.24 ml.

Similarly, the 2 × 2 × 2 (prenatal treatment × sex × days) repeated-measures ANOVA of total fluid (water and sucrose solution) consumption during the subsequent 48-hrs test period did not reveal any significant main effects or interactions, indicating that the amount of total fluid consumed during the two test days was highly comparable between the two prenatal treatment groups and sex (**Figure 3.2b**).

The overall mean±SEM of total fluid (water and sucrose solution) intake during the test phase was 6.36±0.18 ml.

However, prenatal Poly-I:C exposure significantly reduced sucrose preference (as indexed by the percentage score of sucrose consumption) compared to prenatal control treatment (**Figure 3.2a**). This specific effect of the prenatal immunological manipulation was equally seen at each of the two test days and emerged in both male and female offspring. Statistical support for these impressions was yielded by the 2 × 2 × 2 (prenatal treatment × sex × days) repeated-measures ANOVA of percentage sucrose intake, which revealed a significant main effect of prenatal treatment ($F_{1,28} = 7.89$, $P < 0.01$). No other main effects or interactions reached statistical significance.

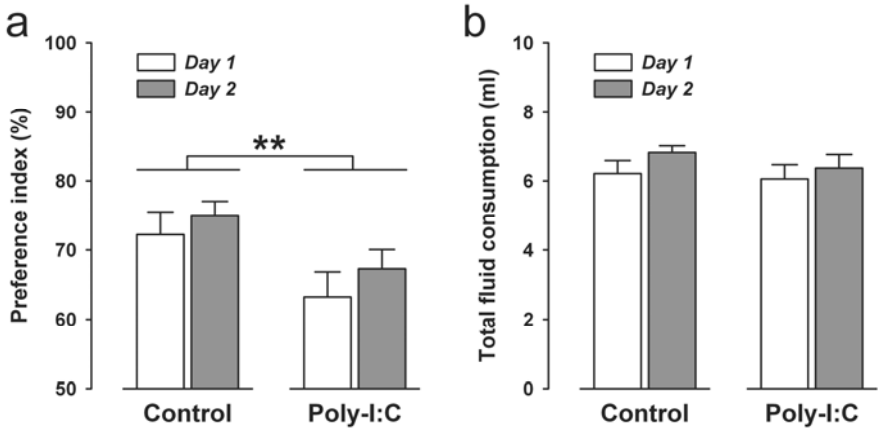

Figure 3.2. Presence of anhedonic behaviour following late prenatal immune challenge. Anhedonic behavior was assessed using a 2-days (48 hrs) sucrose preference test, in which animals had free choice between regular tap water and a 0.5% sucrose solution. **(a)** The graph depicts sucrose preference as indexed by a percentage score [sucrose consumption / (total liquid consumption) × 100%] obtained on two consecutive test days (day 1 and 2). Adult offspring exposed to prenatal Poly-I:C treatment in late gestation displayed a significant reduction in the sucrose preference index compared to adult control offspring. $^{**}P < 0.01$, signifying the main effect of prenatal treatment in the corresponding repeated-measures ANOVA. **(b)** The graph shows the total fluid (water + sucrose solution) consumption on the two consecutive test days. Prenatal Poly-I:C treatment did not affect total fluid intake across the two test days. All values are means ± SEM.

Late prenatal immune challenge leads to abnormally enhanced latent inhibition (LI)

Conditioned avoidance learning was assessed by analyzing the mean response latency performed on successive 10-trials blocks. Acquisition of the conditioned response was evident by a significant decrease in response latency as a function of blocks, leading to a significant main effect of blocks [$F_{9,216} = 35.03$, $P < 0.001$] in the 2 × 2 × 2 × 10 (prenatal treatment × sex × pre-exposure × 10-trials block) repeated-measures ANOVA of response latency. As expected, pre-exposures to a low number of the white-noise CS did not induce a LI effect (i.e., a decrease in conditioned responding following repeated CS-pre-exposures prior to conditioning) in offspring born to control mothers (**Figure 3.3**). Hence, the response latency between control NPE and PE subjects were highly comparable (**Figure 3.3**). However, prenatal Poly-I:C exposure led to a sex-dependent enhancement of the LI effect: Whereas the LI effect was also clearly absent in female offspring born to Poly-I:C-treated mothers, a marked LI effect was present in male Poly-I:C offspring (**Figure 3.3**). This pattern of results led to a significant treatment × sex × pre-exposure interaction in the 2 × 2 × 2 × 10 (prenatal treatment × sex × pre-exposure × 10-trials block) repeated-measures ANOVA of response latency ($F_{1,24} = 6.72$, $P < 0.05$). An additional 2×10 (pre-exposure × 10-trials block) ANOVA restricted to male Poly-I:C subjects provided further statistical support for the presence of significant LI effect by revealing a significant main effect of pre-exposure ($F_{1,6} = 5.87$, $P < 0.05$).

As depicted in **Figure 3.3**, the emergence of a significant LI effect in male Poly-I:C offspring was attributable to retarded learning in CS-PE subjects relative to CS-PE control offspring. Statistical support for this impression was yielded by a 2 × 10 (prenatal treatment × 10-trials block) ANOVA restricted to male CS-PE subjects, which revealed a significant main effect of prenatal treatment ($F1,6 = 12.30$, $P < 0.05$) and a significant two-way interaction ($F_{9,54} = 2.72$, $P < 0.05$). On the other hand, an additional 2×10 (prenatal treatment × 10-trials block) ANOVA restricted to male NPE subjects did not provide any evidence for a significant effect of the prenatal manipulation on conditioned learning in NPE subjects.

Hence, on the background of absent LI in male control offspring, the presence of significant LI in male Poly-I:C offspring exclusively stemmed from a specific effect of the prenatal manipulation on conditioned learning in CS-PE subjects.

Locomotor activity as indexed by the spontaneous ITI shuttles during conditioning decreased in all animals as a function of successive 10-trial blocks, leading to a significant main effect of blocks in the 2 × 2 × 2 × 10 (prenatal treatment × sex × pre-exposure × 10-trials block) repeated-measures ANOVA of ITI shuttles ($F_{9,216} = 119.11$, $P < 0.001$). This was not significantly influenced by the prenatal treatment or stimulus pre-exposure conditions. However, female offspring generally displayed a higher number of ITI shuttles compared to male offspring regardless of the prenatal treatment and stimulus pre-exposure conditions. This led to a significant main effect of sex ($F_{1,24} = 8.27$, $P < 0.01$) and its interaction with 10-trial blocks ($F_{9,216} = 3.93$, $P < 0.01$). The mean±SEM of ITI shuttles per 10-trials block was 1.70±0.30 and 0.93±0.21 in female and male offspring, respectively.

Since our findings of a sex-specific enhancement of the LI effect in male Poly-I:C offspring (**Figure 3.3**) was based on a small number of NPE and CS-PE subjects (4 male subjects per pre-exposure condition and prenatal treatment group), we re-assessed the effects of prenatal Poly-I:C exposure on LI enhancement in an independent experiment. As outlined in the ***Supplementary Data***, we were able to successfully replicate our initial findings in a second independent cohort of male Poly-I:C and control offspring (see ***Supplementary Data***). The successful replication suggests that our initial findings of LI enhancement in male Poly-I:C offspring (**Figure 3.3**) can not be accounted for by spurious findings and/or possible litter effects, but rather represents a robust effect of the prenatal treatment on behavioural/cognitive perseveration in selective associative learning.

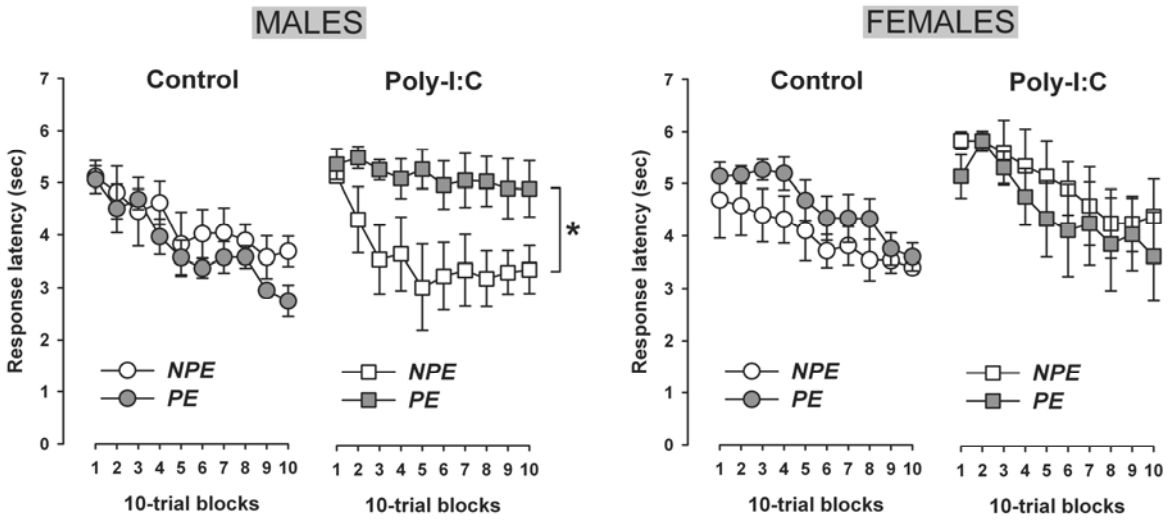

Figure 3.3. Presence of abnormally enhanced latent inhibition (LI) following late prenatal immune challenge. The LI effect was studied in a two-way active avoidance procedure, in which pre-exposed (PE) subjects were presented with a low number of the to-be-conditioned stimulus (CS) prior to conditioning, whereas non-pre-exposed (NPE) subjects were not pre-exposed to the CS. The graph depicts the response latency as a function of blocks of 10 trials in male and female offspring. Male but not female offspring exposed to prenatal Poly-I:C treatment in late gestation displayed a significant LI effect (i.e., reduced response latency in PE relative to NPE subjects) under parametric conditions, in which control offspring did not display LI. $*P < 0.05$, signifying the significant main effect of pre-exposure in the corresponding ANOVA restricted to male Poly-I:C offspring. All values are means ± SEM.

Late prenatal immune challenge leads to altered locomotor and stereotyped behavioural responses to acute apomorphine (APO) treatment

Vertical locomotor activity. Vertical locomotor activity in the wire mesh cylinder was recorded and analyzed separately for the initial vehicle (VitC) and subsequent APO phase. **Figure 3.4a** shows that adult Poly-I:C offspring appeared to display increased levels of vertical locomotor activity compared to adult control offspring in the initial vehicle (VitC) phase. However, the 2 × 2 × 6 (prenatal treatment × sex × 5-min bin) repeated-measures ANOVA of vertical locomotor activity following VitC administration failed to attain a significant main effect or interaction involving the between-subjects factor of prenatal treatment. However, a significant difference between Poly-I:C and control offspring was obtained in terms of vertical locomotor activity following acute APO administration, with APO-treated Poly-I:C offspring displaying a general increase in vertical locomotor activity compared to APO-treated control offspring (**Figure 4a**). This effect of prenatal Poly-I:C exposure emerged similarly in male and female

offspring. Statistical support for these impressions was obtained by the 2 × 2 × 12 (prenatal treatment × sex × 5-min bin) repeated-measures ANOVA of vertical locomotor activity following APO administration, which revealed a significant main effect of prenatal treatment ($F_{1,19}$ = 5.80, P < 0.05). Vertical locomotor activity was generally increased in APO-treated female mice compared to APO-treated male mice regardless of the prenatal treatment conditions. This led to a significant main effect of sex ($F_{1,19}$ = 4.01, P < 0.05). However, the between-subjects factor of sex did not interact significantly with the factor of prenatal treatment (F < 1). The mean±SEM of vertical locomotor activity displayed per 5-min bin was 281.60±14.18 and 193.48±8.92 in female and male offspring, respectively.

Climbing. Climbing behaviour was also analyzed separately for the initial vehicle (VitC) and subsequent APO phase. There were no significant differences between Poly-I:C and control offspring in terms of climbing behaviour during the initial vehicle phase (**Figure 3.4b**). Administration of APO led to a marked increase in climbing behaviour (**Figure 3.4b**). Most interestingly, Poly-I:C offspring displayed a faster onset of APO-induced enhancement in climbing behaviour in comparison with control offspring (**Figure 3.4b**). Indeed, whilst climbing behavior in APO-treated Poly-I:C offspring approached peak levels immediately after APO treatment, climbing behaviour in APO-treated control offspring reached peak levels only by 15 min following the APO injection (see **Figure 3.4b**). Statistical support for these impressions was obtained by the 2 × 2 × 11 (prenatal treatment × sex × sampling interval) repeated-measures ANOVA of climbing behaviour following APO administration, which yielded a significant main effect of sampling interval ($F_{10,190}$ = 4.63, P < 0.001) and its interaction with prenatal treatment ($F_{10,190}$ = 3.08, P < 0.01). Additional 2 × 2 (prenatal treatment × sex) ANOVAs restricted to each of the 11 sampling intervals were then performed in order to further verify the presence or absence of significant group differences in climbing behaviour at specific post-injection intervals. By revealing a significant main effect of prenatal treatment ($F_{1,19}$ = 7.77, P < 0.05), the ANOVA restricted to the first sampling interval confirmed that Poly-I:C offspring displayed a faster onset of APO-induced climbing behaviour

compared to controls (see **Figure3.4b**). This time-dependent effect of the Poly-I:C treatment emerged in both male and female offspring.

Leaning. In agreement with the outcomes in the analysis of climbing behaviour (**Figure 3.4b**), leaning behaviour was highly comparable between Poly-I:C and control offspring during the initial VitC phase (**Figure 3.4c**). Acute APO treatment led to a temporary increase in leaning behaviour: It led to an initial increase, which was most pronounced during the first 10 min following APO administration, but subsided afterwards (**Figure 3.4c**). This led to a highly significant main effect of sampling interval ($F_{10,190}$ = 5.50, P < 0.001) in the 2 × 2 × 11 (prenatal treatment × sex × sampling interval) repeated-measures ANOVA of leaning behaviour following APO administration. Interestingly, the APO-induced temporary increase in leaning behaviour appeared to be blunted in Poly-I:C offspring relative controls (**Figure 3.4c**), as indicated by the prenatal treatment × sampling time interaction approaching statistical significance ($F_{10,190}$ = 1.82, P = 0.059). Additional 2 × 2 (prenatal treatment × sex) ANOVAs restricted to each of the 11 sampling intervals were then performed in order to further verify the presence or absence of significant group differences in leaning behaviour at specific post-injection intervals. These analyses confirmed that Poly-I:C offspring displayed reduced leaning behaviour relative to controls specifically during the first sampling interval, as supported by the presence of a significant main effect of prenatal treatment ($F_{1,19}$ = 4.89, P < 0.05) in the ANOVA restricted to the first sampling interval. This specific effect of the Poly-I:C treatment emerged in both male and female offspring.

Sniffing. **Figure 3.4d** depicts sniffing behaviour displayed by Poly-I:C and control offspring during the initial vehicle (VitC) and subsequent APO phase. Prenatal Poly-I:C treatment appeared to increase the amount of sniffing during the VitC phase compared to sniffing displayed by VitC-treated control offspring (**Figure 3.4d**). However, the main effect of prenatal treatment in the 2 × 2 × 5 (prenatal treatment × sex × sampling interval) repeated-measures ANOVA of sniffing following VitC administration only attained statistical trend level ($F_{1,19}$ = 4.31, P = 0.0516). As depicted in **Figure 3.4d**,

APO administration led to a noticeable reduction in the amount of sniffing relative to sniffing measured in the initial VitC phase. The APO-induced reduction in sniffing displayed by Poly-I:C offspring appeared to be maximal at the first and last sampling interval, whilst the APO-induced decrease in sniffing displayed by control offspring was maximal between sampling intervals 2 and 6 (**Figure 3.4d**). These patterns of results led to a significant main effect of sampling interval ($F_{10,190} = 2.78$, $P < 0.01$) and its interaction with prenatal treatment ($F_{10,190} = 2.60$, $P < 0.01$). Additional 2 × 2 (prenatal treatment × sex) ANOVAs restricted to each of the 11 sampling intervals were then conducted in order to seek statistical support for the presence or absence of significant group differences in sniffing behaviour at specific post-injection intervals. However, these analyses did not reveal any significant main effects of prenatal treatment or its interaction with the between-subjects factor of sex.

Grooming and biting/gnawing. The amount of grooming and biting/gnawing behaviour in Poly-I:C and control offspring was generally very low, both following VitC and APO administration; and no significant group differences were revealed in the corresponding statistical analyses (data not shown).

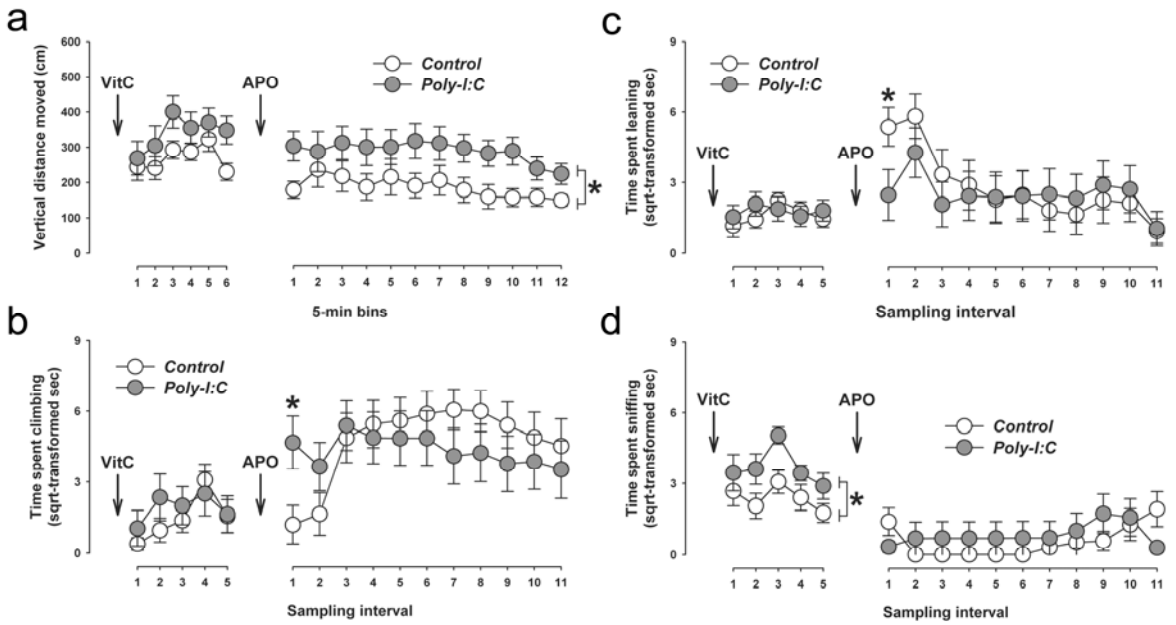

Figure 3.4. Late prenatal immune activation leads to altered locomotor and stereotyped behavioural responses to systemic apomorphine (APO) treatment. **(a)** The graph depicts locomotor activity as indexed by the vertical distance moved following systemic ascorbic acid (VitC, = vehicle) treatment and following systemic APO (5 mg/kg, s.c.) treatment. Vertical distance moved is plotted as a function of 5-min bins. Adult offspring subjected to prenatal Poly-I:C exposure in late gestation displayed an overall significant increase in the vertical distance moved following APO treatment relative to APO-treated offspring born to control mothers. *$P < 0.05$, signifying the significant main effect of prenatal treatment in the corresponding repeated-measures ANOVA. **(b)** The graph shows the time spent climbing on successive sampling intervals (1-min observation period every 5 minutes) following VitC and APO treatment. Adult Poly-I:C offspring displayed a significant increase in the time spent climbing specifically during the first observation period compared to APO-treated control offspring. *$P < 0.05$, indicating the significant main effect of prenatal treatment in the corresponding ANOVA restricted to the first observation period following APO treatment. **(c)** The graph depicts the time spent leaning on successive sampling intervals (1-min observation period every 5 minutes) following VitC and APO treatment. Adult Poly-I:C offspring displayed a significant decrease in the time spent leaning specifically during the first observation period compared to APO-treated control offspring. *$P < 0.05$, signifying the significant main effect of prenatal treatment in the corresponding ANOVA restricted to the first observation period following APO treatment. **(d)** The graph depicts the time spent sniffing on successive sampling intervals (1-min observation period every 5 minutes) following VitC and APO treatment. There were no significant group differences in the time spent sniffing neither in the initial VitC phase nor in the subsequent APO phase. All values are means ± SEM.

Late prenatal immune challenge leads to multiple changes in central monoamine contents

All monoamines (NA, DA and 5-HT) were detectable in all brain regions of interest and were analyzed by 2 × 2 (prenatal treatment × sex) ANOVAs. **Figure 3.5** depicts the monoamine levels (NA, DA and 5-HT) measured in distinct brain regions of Poly-I:C and control offspring, and **Table 3.1** summarizes the corresponding metabolites (DOPAC, HVA, 3-MT, and 5-HIAA) and neurotransmitter/metabolite ratios (DOPAC/DA, HVA/DA, 3-MT/DA, 5-HIAA/5-HT) in Poly-I:C and control animals.

Noradrenaline (NA). Prenatal Poly-I:C exposure significantly increased the levels of NA in the dHPC relative to levels in control offspring, and this effect emerged in both male and female offspring (**Figure 3.5a**). ANOVA of NA in the dHPC revealed a main effect of prenatal treatment ($F_{1,27} = 9.45$, $P < 0.01$). In addition, the analysis of NA contents in the AMY revealed a significant prenatal treatment × sex interaction ($F_{1,27} = 5.02$, $P < 0.05$), indicating a sex-specific effect of the prenatal treatment on this measure. Subsequent ANOVAs restricted to each sex showed that female but not male Poly-I:C offspring displayed a significant ($F_{1,14} = 4.19$, $P < 0.05$) increase in NA levels in the AMY (**Figure 3.5a**). No other main effects or interactions attained statistical significance in the analyses of NA levels.

Dopamine (DA). The DA contents were significantly reduced in the mPFC and vHPC of Poly-I:C offspring compared to control offspring (**Figure 3.5b**). These effects were apparent in both male and female subjects. Statistical support for these interpretations was yielded by the corresponding ANOVAs, which yielded significant main effects of prenatal treatment [mPFC: $F_{1,27} = 6.68$, $P < 0.05$; vHPC: $F_{1,25} = 7.25$, $P < 0.05$]. No other main effects or interactions attained statistical significance in the analyses of DA contents.

Serotonin (5-HT). Prenatal Poly-I:C exposure led to sex-specific changes of 5-HT contents in the AMY: Female but not male Poly-I:C offspring displayed significantly enhanced 5-HT contents in the AMY relative to female control offspring (**Figure 3.5c**). ANOVA of 5-HT contents in the AMY revealed a significant main effect of prenatal treatment ($F_{1,27} = 4.60$, $P < 0.01$) and its interaction with sex [$F_{1,27} = 8.98$, $P < 0.01$]. The subsequent ANOVAs of AMY 5-HT contents restricted to male and female subjects further confirmed the significant increase in female Poly-I:C offspring compared to female control offspring (main effect of prenatal treatment in females: $F_{1,14} = 11.16$, $P < 0.01$). On the other hand, prenatal Poly-I:C treatment significantly reduced 5-HT levels in the vHPC of male but not female subjects relative to prenatal control treatment (**Figure 3.5c**). Statistical support for this interpretation was yielded by the ANOVA of 5-HT levels in the vHPC, which revealed a significant interaction between prenatal treatment and sex ($F_{1,26} = 10.83$, $P < 0.01$); and by the subsequent ANOVAs restricted to each sex, which confirmed the presence of a significant main effect of prenatal treatment in males ($F_{1,13} = 16.43$, $P < 0.01$). No other main effects or interactions attained statistical significance in the analyses of 5-HT contents.

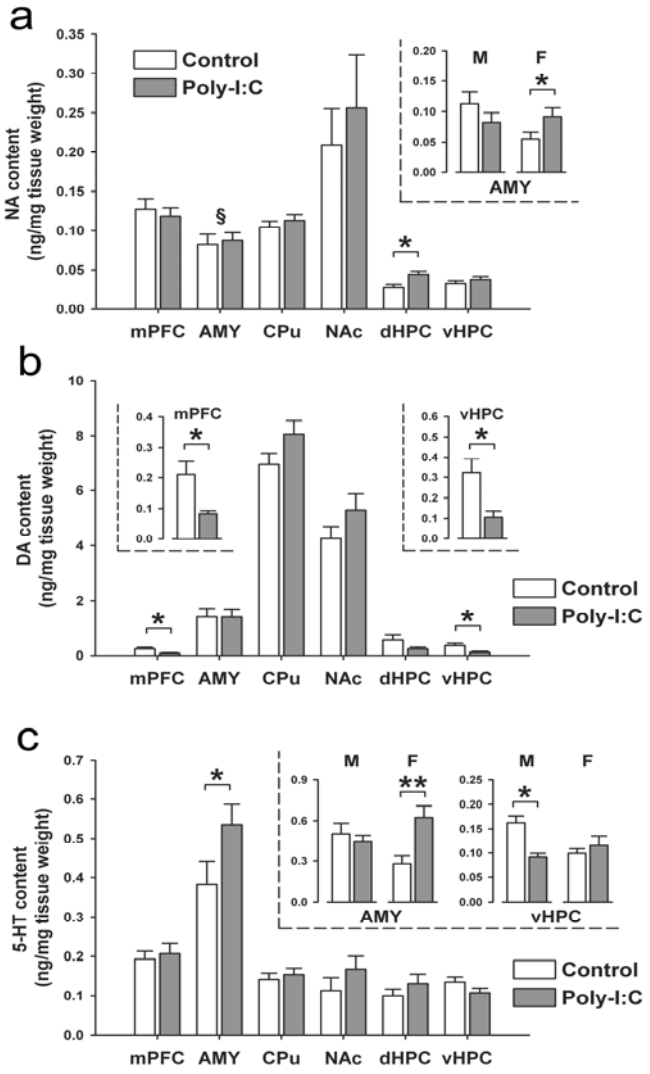

Figure 3.5. Effects of late prenatal immune challenge on basal monoamine levels. Levels of noradrenalin (NA), dopamine (DA) and serotonin (5-HT) were determined in post-mortem brain tissue using high performance liquid chromatography (HPLC). Monoamine contents were measured in the medial prefrontal cortex (mPFC), amygdala (AMY), caudate putamen (CPu), nucleus accumbens (NAc), dorsal hippocampus (dHPC), and ventral hippocampus (vHPC). All monoamine levels are expressed as ng per mg fresh tissue weight. **(a)** NA contents in adult control and Poly-I:C offspring. **P* < 0.05, based on the corresponding ANOVA. Symbol (§) signifies the presence of a significant interaction between prenatal treatment and sex, as further shown by the inlet depicting NA levels in male (M) and female (F) offspring separately. **(b)** DA contents in adult control and Poly-I:C offspring. **P* < 0.05, based on the corresponding ANOVA. The inlets provide DA levels in the mPFC and vHPC at higher magnification. **(c)** 5-HT contents in adult control and Poly-I:C offspring. **P* < 0.05, based on the corresponding ANOVA. The inlets show the sex-specific effects of prenatal Poly-I:C exposure on 5-HT levels in the AMY and vHPC. All values are means ± SEM.

The two excitatory amino acids, glutamate and aspartate, were also detectable in all brain regions examined (mPFC AMY, CPu, NAc, dHPC, and vHPC). The levels of glutamate and aspartate measured in Poly-I:C and control offspring are shown in **Figure 3.6**. For each brain region, glutamate and aspartate levels were analyzed using 2 × 2 (prenatal treatment × sex) ANOVAs.

		DOPAC	HVA	3-MT	DOPAC/DA	HVA/DA	3-MT/DA	5-HIAA	5-HIAA/5HT
mPFC									
Control	Male	0.13±0.02	0.14±0.02	0.02±0.00	0.79±0.11	0.99±0.15	0.16±0.05	0.13±0.02	0.58±0.03
	Female	0.19±0.06	0.15±0.03	0.03±0.01	1.06±0.28	0.92±0.21	0.17±0.04	0.14±0.02	0.94±0.10
PolyI:C	Male	0.06±0.01	0.11±0.01	0.01±0.00	0.76±0.13	1.47±0.21*	0.21±0.05	0.13±0.02	0.55±0.03
	Female	0.10±.015	0.11±0.01	0.01±0.00	1.36±0.19	1.35±0.14*	0.17±0.05	0.14±0.02	0.92±0.10
AMY									
Control	Male	0.30±0.07	0.31±0.05	0.09±0.02	0.50± 0.09	0.40±0.06	0.11±0.02	0.29±0.04	0.55±0.08
	Female	0.80±0.24	0.48±0.10	0.17±0.04	0.52±0.08	0.32±0.04	0.11±0.02	0.40±0.04	0.92±0.06
PolyI:C	Male	0.34±0.09	0.30±0.05	0.11±0.02	0.34±0.08	0.35±0.08	0.15±0.06	0.25±0.03**	0.64±0.08
	Female	0.67±0.18	0.45±0.09	0.15±0.04	0.53±0.08	0.39±0.06	0.12±0.01	0.24±0.05**	0.70±0.10
CPu									
Control	Male	0.96±0.12	0.70±0.08	0.25±0.02	0.13±0.01	0.10±0.01	0.04±0.00	0.13±0.02	0.77±0.03
	Female	2.21±0.30	0.76±0.05	0.30±0.01	0.36±0.06	0.12±0.01	0.05±0.00	0.09±0.01	0.78±0.04
PolyI:C	Male	1.05±0.05	0.79±0.07	0.30±0.02	0.13±0.01	0.09±0.01	0.04±0.00	0.12±0.02	0.71±0.03
	Female	2.21±0.43	0.90±0.08	0.32±0.02	0.12±0.01	0.13±0.01	0.04±0.01	0.12±0.02	0.87±0.06
NAc									
Control	Male	1.07±0.14	0.72±0.07	0.22±0.02	0.21±0.03	0.13±0.01	0.04±0.00	0.13±0.02	0.72±0.13
	Female	1.74±0.48	0.63±0.05	0.19±0.05	0.61±0.13	0.21±0.03	0.06±0.01	0.05±0.02	2.39±0.81
PolyI:C	Male	1.02±0.16	0.75±0.11	0.22±0.03	0.17±0.02	0.12±0.01	0.04±0.00	0.08±0.03	0.41±0.06
	Female	2.10±0.57	0.81±0.19	0.25±0.06	0.17±0.02	0.17±0.02	0.05±0.01	0.10±0.04	0.79±0.15
dHPC									
Control	Male	0.27±0.06	0.16±0.03	0.07±0.02	0.75±0.25	0.45±0.14	0.16±0.06	0.12±0.01	0.98±0.12
	Female	0.26±0.10	0.12±0.03	0.07±0.02	1.42±0.26	0.89±0.24	0.36±0.07	0.10±0.02	1.44±0.15
PolyI:C	Male	0.17±0.04	0.12±0.03	0.06±0.01	0.76±0.11	0.50±0.09	0.21±0.05	0.13±0.02	0.96±0.06
	Female	0.14±0.01	0.10±0.01	0.04±0.00	1.22±0.23	0.88±0.18	0.41±0.10	0.14±0.02	1.38±0.18
vHPC									
Control	Male	0.16±0.03	0.11±0.02	0.06±0.01	0.41±0.05	0.29±0.05	0.15±0.02	0.13±0.01	0.83±0.06
	Female	0.17±0.05	0.10±0.02	0.06±0.01	1.05±0.16	0.81±0.21	0.34±0.08	0.12±0.02	1.14±0.09
PolyI:C	Male	0.08±0.02	0.06±0.01	0.03±0.00	0.78±0.16	0.80±0.16	0.35±0.08	0.09±0.01	1.02±0.07
	Female	0.10±0.02	0.08±0.01	0.04±0.00	1.63±0.29	1.45±0.31	0.59±0.17	0.15±0.02	1.20±0.09

Table 3.1. Mean±SEM vales in (ng/mg tissue) of monoamine metabolites and monamine/metabolite ratios in the distinct brain regions of adult control offspring and offspring subjected to prenatal Poly-I:C treatment during late gestation.

Abbreviations: AMY, Amygdala, CPu, caudate putamen; DA, dopamine; dHpc, dorsal hippocampus; DOPAC, dihydrophenylaceticaced; HVA, homovanillic acid; mPFC, medial prefrotntal cortex; NAc, nucleus accumbens; vHPC, ventral hippocampus; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine; 3-MT, 3-methoxytyramine. Prenatal Poly-I:C exposure significantly enhanced 5-HIAA contents specifically in the AMY but not in the other brain areas investigated. **P<0.01, based on the ANOVA of 5-HIAA levels in the AMY of male and female offspring.

Glutamate. Prenatal Poly-I:C exposure induced a sex-specific reduction of glutamate levels in the mPFC relative to prenatal control treatment: As depicted in **Figure 3.6a**, the levels of glutamate were markedly reduced in the mPFC of male but not female Poly-I:C offspring relative to male control offspring. Statistical support for this impression was obtained in the corresponding ANOVA, which yielded a significant main effect of prenatal treatment ($F_{1,28}$ = 10.95, P < 0.05) and its interaction with sex ($F_{1,28}$ = 5.19, P < 0.05). The subsequent ANOVAs of mPFC glutamate contents restricted to male and female subjects further confirmed the significant decrease in male Poly-I:C offspring compared to male control offspring (main effect of prenatal treatment in males: $F_{1,14}$ = 20.71, P < 0.001). In addition to its effects on glutamate levels in the mPFC, prenatal Poly-I:C treatment also significantly decreased glutamate contents in the dHPC (**Figure 3.6a**), and this effect similarly emerged in both males and females (**Figure 3.6a**). The corresponding ANOVA of glutamate levels in the dHPC revealed a significant main effect of prenatal treatment ($F_{1,28}$ = 12.79, P < 0.01). No other main effects or interactions attained statistical significance in the analyses of glutamate contents in the AMY, CPu, NAc, and vHPC.

Aspartate. Similar to its sex-dependent effects on glutamate levels in the mPFC (**Figure 3.6a**), prenatal Poly-I:C exposure induced a significant reduction of aspartate levels in the mPFC of male but not female offspring compared to male control offspring (**Figure 3.6b**). This pattern of results led to a significant ($F_{1,28}$ = 8.28, P < 0.01) interaction between prenatal treatment and sex in the ANOVA of aspartate contents in the mPFC. The additional ANOVA restricted to male offspring confirmed the significant decrease in mPFC aspartate levels in male Poly-I:C offspring relative to control ($F_{1,14}$ = 17.84, P < 0.001). Similar to the sex-independent effects on glutamate levels in the dHPC (**Figure 3.6a**), prenatal Poly-I:C treatment also markedly decreased aspartate contents in the dHPC of both male and female offspring relative to prenatal control treatment (**Figure 3.6b**). The corresponding ANOVA of aspartate contents in the dHPC revealed a significant main effect of prenatal treatment ($F_{1,28}$ = 12.88, P < 0.01). No other main effects or interactions attained statistical significance in the analyses of aspartate contents in the AMY, CPu, NAc, and vHPC.

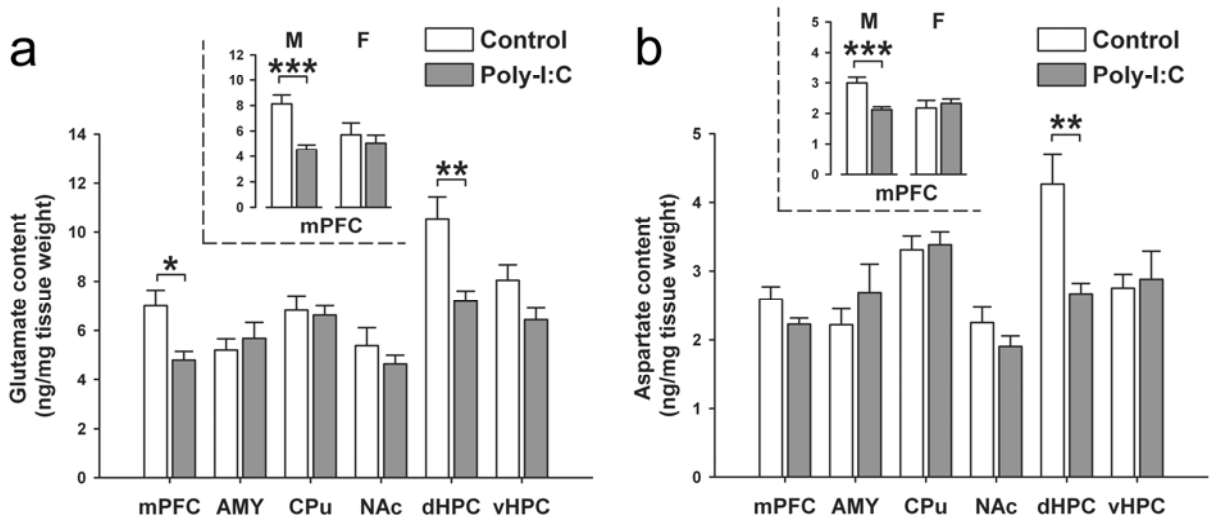

Figure 3.6. Effects of late prenatal immune challenge on basal levels of excitatory amino acids. Levels of the excitatory amino acids glutamate and aspartate were determined in post-mortem brain tissue using high performance liquid chromatography (HPLC). The contents of these excitatory amino acids were measured in the medial prefrontal cortex (mPFC), amygdala (AMY), caudate putamen (CPu), nucleus accumbens (NAc), dorsal hippocampus (dHPC), and ventral hippocampus (vHPC). All levels are expressed as ng per mg fresh tissue weight. **(a)** Glutamate contents in adult control and Poly-I:C offspring. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, based on corresponding ANOVAs. The inlet shows the sex-specific effects of prenatal Poly-I:C exposure on glutamate levels in the mPFC of male (M) and female (F) offspring. **(b)** Aspartate contents in adult control and Poly-I:C offspring. **$P < 0.01$ and ***$P < 0.001$, based on corresponding ANOVAs. The inlet shows the sex-specific effects of prenatal Poly-I:C exposure on aspartate levels in mPFC of male (M) and female (F) offspring. All values are means ± SEM.

Late prenatal immune challenge leads to changes in the basal levels of inhibitory amino aids

Detectable levels of the two inhibitory amino acids, glycine and γ-aminobutyric acid (GABA), and the amino sulfonic acid taurine, were present in all brain regions of interest (mPFC AMY, CPu, NAc, dHPC, and vHPC). The levels of glycine, GABA and taurine measured in Poly-I:C and control offspring are shown in **Figure 3.7**, and were analyzed using 2 × 2 (prenatal treatment × sex) ANOVAs for each individual brain region.

<u>*Glycine.*</u> Poly-I:C offspring displayed a significant reduction of glycine levels specifically in the mPFC in comparison with mPFC glycine levels in control offspring (**Figure 3.7a**). This effect of the Poly-I:C treatment emerged similarly in male and female offspring, as supported by the significant main effect of prenatal treatment ($F_{1,28} = 12.17$, $P < 0.01$) in the ANOVA of mPFC glycine contents. No other main effects or interactions attained statistical significance in the analyses of glycine levels.

GABA. Prenatal Poly-I:C exposure led to a sex-specific reduction of GABA levels in the dHPC relative to prenatal control treatment: As depicted in **Figure 3.7b**, the levels of GABA were markedly decreased in the dHPC of female but not male Poly-I:C offspring relative to female control offspring. Statistical support for this impression was obtained in the corresponding ANOVA, which yielded a significant main effect of prenatal treatment ($F_{1,28} = 6.03$, $P < 0.05$) and sex ($F_{1,28} = 4.43$, $P < 0.05$), as well as a significant interaction between prenatal treatment and sex ($F_{1,28} = 5.98$, $P < 0.05$). An additional ANOVA restricted to female subjects further confirmed the significant reduction of GABA levels in the dHPC of female Poly-I:C offspring compared to female control offspring ($F_{1,14} = 7.81$, $P < 0.05$). No other main effects or interactions attained statistical significance in the analyses of GABA contents in the AMY, CPu, NAc, and vHPC.

Taurine. Male but not female Poly-I:C offspring showed a significant decrease in taurine levels in the mPFC compared to mPFC taurine contents measured in male control animals (**Figure 3.7c**). Statistical support for this sex-specific effect of prenatal Poly-I:C exposure was obtained in the ANOVA of mPFC taurine levels, which revealed a significant main effect of prenatal treatment ($F_{1,28} = 8.69$, $P < 0.01$) and sex ($F_{1,28} = 4.40$, $P < 0.05$), as well as a significant interaction between prenatal treatment and sex ($F_{1,28} = 4.21$, $P < 0.05$). Subsequent ANOVAs restricted to male and female subjects verified the

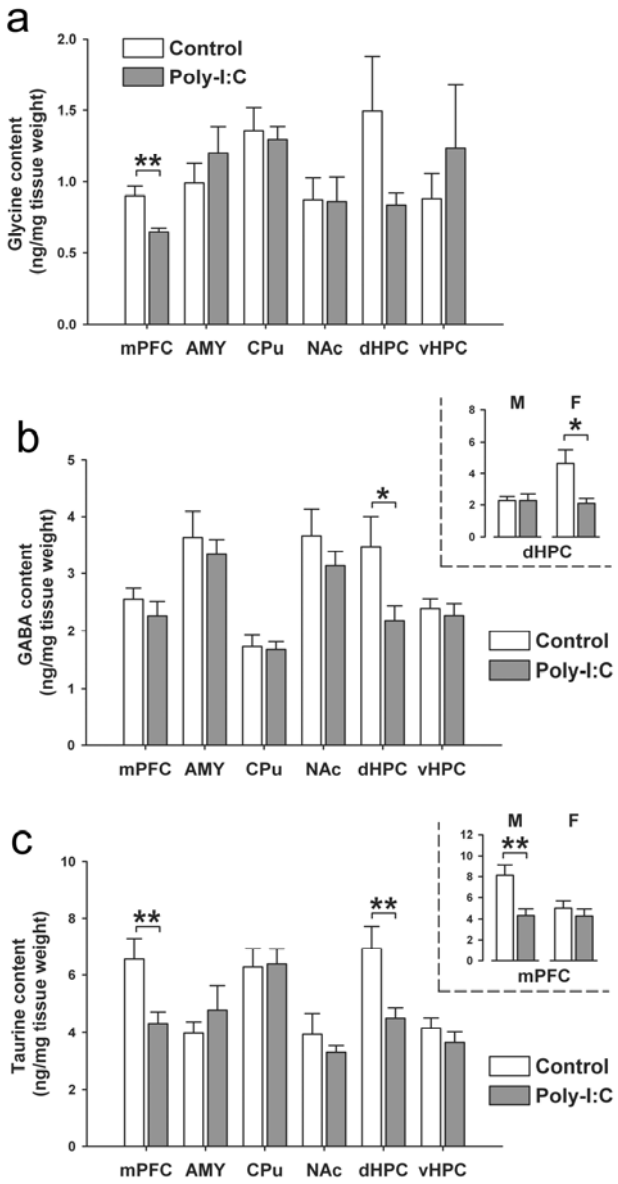

Figure 3.7. Effects of late prenatal immune challenge on basal levels of inhibitory amino acids. Levels of the inhibitory amino acids glycine, γ-aminobutyric acid (GABA) and taurine were determined in post-mortem brain tissue using high performance liquid chromatography (HPLC). The contents of these inhibitory amino acids were measured in the medial prefrontal cortex (mPFC), amygdala (AMY), caudate putamen (CPu), nucleus accumbens (NAc), dorsal hippocampus (dHPC), and ventral hippocampus (vHPC). All levels are expressed as ng per mg fresh tissue weight. **(a)** Glycine contents in adult control and Poly-I:C offspring. $^{**}P < 0.01$, based on the corresponding ANOVA. **(b)** GABA contents in adult control and Poly-I:C offspring. $^{*}P < 0.05$, based on corresponding ANOVAs. The inlet shows the sex-specific effects of prenatal Poly-I:C exposure on GABA levels in the dHPC of male (M) and female (F) offspring. **(c)** Taurine contents in adult control and Poly-I:C offspring. $^{**}P < 0.01$, based on corresponding ANOVAs. The inlet shows the sex-specific effects of prenatal Poly-I:C exposure on taurine levels in the mPFC of male (M) and female (F) offspring. All values are means ± SEM.

significant reduction of taurine levels in the mPFC of male Poly-I:C offspring compared to male control offspring ($F_{1,14}$ = 10.04, P < 0.01). On the other hand, prenatal Poly-I:C treatment significantly reduced taurine contents in the dHPC in both male and female offspring relative to prenatal control treatment (**Figure 3.7c**). This led to a significant ($F_{1,28}$ = 8.09, P < 0.01) main effect of prenatal treatment in the ANOVA of taurine levels in the dHPC. No other main effects or interactions attained statistical significance in the analyses of taurine contents in the AMY, CPu, NAc, and vHPC.

DISCUSSION

The present experimental investigation in mice demonstrates that exposure to a viral-like acute phase response by Poly-I:C treatment in late gestation leads to long-term deficits in social interaction, anhedonic behaviour in the sucrose preference test, presence of abnormally enhanced LI in associative learning, and alterations in the locomotor and stereotyped behavioural responses to acute APO treatment. These effects extend our previous observations that late prenatal Poly-I:C-induced immune challenge in mice is capable of inducing cognitive abnormalities in the form of deficient spatial working and recognition memory (Bitanihirwe *et al*., 2010; Meyer *et al.,* 2008b; 2010), retarded discrimination reversal learning (Meyer *et al.,* 2006b) and enhanced behavioural sensitivity to acute treatment with the indirect dopamine receptor agonist amphetamine and the NMDA-receptor antagonist MK-801 (Meyer *et al.,* 2008b). The present study thus identified several novel phenotypes which are part of a pathological symptom cluster characteristic for prenatal Poly-I:C-induced immune activation in late gestation. Our detailed post-mortem neurochemical analyses further shows that the behavioural and pharmacological abnormalities emerging following late prenatal immune challenge are associated with various changes in basal neurotransmitter levels and their metabolites. This confirms the expectation that the appearance of multiple behavioural and pharmacological abnormalities following prenatal immune challenge is paralleled by multiple neurochemical disturbances.

In one of our previous attempts to characterize the long-term brain and behavioural consequences of prenatal Poly-I:C exposure in mice, we found that late prenatal immune challenge did not significantly affect the LI effect under parametric conditions which were effective to produce robust LI in adult control offspring (Meyer *et al.,* 2006a). Here we show, however, that adult offspring of mothers treated with Poly-I:C in late gestation display LI under parametric conditions which are insufficient to induce the LI effect in control offspring. Hence, late prenatal Poly-I:C exposure in mice leads to abnormally enhanced LI under such conditions. According to the "switching model" of LI proposed by Weiner (1990, 2003), the development of LI involves the acquisition of two independent and conflicting contingencies in pre-

exposure and conditioning, which then compete for expression during conditioning. In order to show LI, the organism must remain under the control of the information acquired in pre-exposure. Since during the pre-exposure phase the CS is not followed by a significant event, the information acquired in pre-exposure relates to a stimulus-no event contingency. In contrast, absence of LI would indicate that the organism switches to respond according to the new stimulus-event (i.e., CS-US) contingency present during conditioning. According to these theoretical accounts, manipulations which retard switching and thus induce behavioural/cognitive perseveration facilitate the expression of LI under parametric conditions in which control subjects fail to show LI. It follows that the abnormally enhanced LI effect manifested in prenatally Poly-I:C-treated offspring may readily be accounted for by such perseveration effects, so that CS-pre-exposed Poly-I:C subjects remain under the control of information acquired in pre-exposure (stimulus-no event) and therefore display impaired learning during the critical CS-US conditioning phase. The comparison between conditioned learning in CS-pre-exposed Poly-I:C and control offspring further supports this possibility and suggests that the presence of LI in Poly-I:C offspring exclusively stemmed from a specific effect of the prenatal manipulation on conditioned learning in CS-PE subjects (see **Figure 3.3**). Furthermore, the current interpretation of perseveration effects in LI are in agreement with our previous findings showing that prenatal Poly-I:C-induced immune activation in late gestation leads to perseverative behaviour in the form of retarded discrimination reversal learning (Meyer *et al.,* 2006b).

Several studies in humans have shown that LI is disrupted in acutely-ill schizophrenic patients with marked positive symptoms (reviewed in Weiner, 2003; Lubow, 2005). Hence, under parametric conditions in which healthy human subjects show a significant LI effect, acutely psychotic patients fail to do so (reviewed in Weiner, 2003; Lubow, 2005). On the other hand, the presence of abnormally enhanced LI positively correlates with the severity of negative symptoms in (chronically-ill) patients with schizophrenia (Cohen *et al.,* 2004; Rascle *et al.,* 2001). Based on these findings in humans and parallel experimental investigations in rats and mice (Gaisler-Salomon and Weiner, 2003; Gaisler-Salomon *et al.*, 2008; Lipina *et al.,* 2005), it has been suggested that presence of abnormally enhanced LI may provide a

correlate of a specific aspect of negative symptomatology, namely, behavioural or attentional perseveration (Weiner, 2003; Weiner and Arad, 2009). Against this background, the presence of abnormally enhanced LI in adult offspring subjected to prenatal Poly-I:C treatment in late gestation suggests that this prenatal immunological manipulation can induce specific behavioural/cognitive abnormalities directly implicated in the negative symptoms of schizophrenia. Interestingly, this LI perseveration effect only emerged in male but not female offspring born to Poly-I:C-treated mothers, indicating that the male sex is more vulnerable than the female sex to develop such behavioural/cognitive abnormalities. This sex-dependent effect of prenatal Poly-I:C treatment is consistent with the clinical impressions that male schizophrenic patients seem to exhibit a more severe clinical profile compared to female patients (Aleman *et al.*, 2003; Flor-Henry, 1990), especially in terms of negative symptomatology and cognitive deficits (Roy *et al.*, 2001). However, the efficacy of prenatal Poly-I:C treatment to induce sex-specific abnormalities appears to be specific to the behavioral and/or cognitive processes involved in LI, since maternal Poly-I:C exposure induced social interaction deficits and anhedonic behavior similarly in male and female offspring.

Using a social interaction test which is based on the relative exploration of an unfamiliar live mouse *versus* a inanimate dummy object, we found that whilst control offspring spent significantly more time exploring the live mouse, prenatally Poly-I:C-treated offspring displayed no preference towards either objects (**Figure 3.1**). Hence, offspring born to Poly-I:C-exposed mothers spent equal amounts of time exploring the live mouse and the inanimate dummy object, indicating a marked disruption of normal social behavior. This deficit cannot be accounted for by reduced exploration activities *per se* because if anything, Poly-I:C offspring displayed an overall increase in the total time exploring the two presented objects (i.e. live mouse and dummy object). Rather, the lack of a clear preference towards the live mouse *versus* the dummy object may suggest the presence of a genuine social interaction deficit in offspring born to immune-challenged mothers. This suggestion is consistent with previous reports of social interaction deficits emerging in adult mice exposed to Poly-I:C in middle gestation (GD 12; Smith *et al.*, 2007) or to the bacterial endotoxin lipopolysaccharide (LPS) in late gestation (GD 17; Hava *et al.*, 2006).

Notably, deficient social interaction is one of the hallmark negative symptoms in schizophrenia (Foussias and Remington, 2008; McGlashan and Fenton, 1992; Schooler and Spohn, 1982). Therefore, the presence of genuine social interaction deficits revealed in Poly-I:C offspring provides further direct support for our proposal that late prenatal immune challenge in mice can induce long-term behavioral abnormalities relevant to the negative symptoms of this disorder.

To the best of our knowledge, the present study is the first to demonstrate significant long-term effects of prenatal immune activation in the sucrose preference test of anhedonia. This test has been shown to be a sensitive experimental paradigm to evaluate hedonic aspects of feeding behavior in mice (Cryan and Mombereau, 2004; Hayward *et al.*, 2006; Pothion *et al.*, 2004). Indeed, when presented with a free choice between sucrose solution and water, mice (and other rodent species) typically show a marked preference for the sucrose solution. Consistent with this natural tendency of rodents, we found that both Poly-I:C and control offspring preferred the sucrose solution over the water solution during the choice test phase: The preference index for sucrose consumption was clearly above a level of 50% indifference in both prenatal treatment groups (**Figure 3.2**). Most importantly, however, prenatally immune-challenged offspring showed a significant (~15%) reduction in the sucrose preference index relative to control offspring. Since a reduction in this preference index is often indicative of anhedonic behavior (Cryan and Mombereau, 2004; Hayward *et al.,* 2006; Pothion *et al.,* 2004), our findings suggest that late prenatal exposure to a viral-like acute phase response may facilitate the expression of anhedonia in adulthood. We deem it unlikely that the presence of anhedonic behavior in prenatally Poly-I:C-treated offspring may be attributable to enhanced sucrose neophobia. This is because we used a 48-hrs sucrose preference test procedure which readily precludes any effects of sucrose neophobia (Hayward *et al.,* 2006). Furthermore, since the total fluid (water and sucrose solution) intake during the test phase was highly comparable between control and Poly-I:C offspring (**Figure 3.2**), the presence of anhedonic behavior seen in prenatally immune-challenged offspring can also not be explained by possible alterations in general fluid consumption. The prenatal Poly-I:C-induced reduction in sucrose preference thus readily reflects a genuine effect of the prenatal immunological manipulation on anhedonic behavior. Anhedonia has often

been discussed to be another hallmark feature of negative symptoms in schizophrenia (Foussias and Remington, 2008; Horan *et al.*, 2008; Juckel *et al.*, 2003; McGlashan and Fenton, 1992). Hence, late prenatal Poly-I:C treatment in mice also successfully captures this specific aspect of behavioral pathology seen in schizophrenic patients with marked negative symptoms.

Another previously unexplored pathological phenotype emerging following late prenatal Poly-I:C treatment in mice is alterations in the locomotor and stereotyped behavioral reactions to acute APO treatment (see **Figure 3.4**). Consistent with previous reports in C57BL/6 mice (Cabib and Puglisi-Allegra, 1985; Protais *et al.*, 1976), we found that acute APO treatment at the selected dose (5 mg/kg, s.c.) led to a pronounced increase in repetitive climbing (**Figure 3.4b**). Most importantly, we revealed that adult Poly-I:C offspring displayed a faster onset of APO-induced climbing compared to APO-treated control offspring (**Figure 3.4b**). T his effect was paralleled by a temporary attenuation of leaning behavior in APO-induced Poly-I:C offspring relative to APO-treated controls (**Figure 3.4c**). The opposite direction between the effects of the Poly-I:C treatment on APO-induced climbing and leaning behavior is not surprising because repetitive climbing and leaning are two behavioral categories which per definition cannot co-exist and thus compete for expression. Hence, Poly-I:C offspring displayed a faster onset of APO-induced climbing at the expense of reduced expression of leaning behavior. Poly-I:C and control offspring also differed in terms of the vertical locomotor response to acute APO treatment, with APO-treated Poly-I:C offspring showing an overall increase in vertical movements relative to APO-treated control offspring (**Figure 3.4a**). Taken together, our findings suggest that prenatal Poly-I:C treatment in late gestation is effective in enhancing the sensitivity to acute APO treatment both in terms of stereotyped behavioral responses as well as vertical locomotor activity. Whilst the former effect is primarily reflected by a difference in response onset, the latter represents an overall potentiation of the drug's effect on vertical locomotor activity measures. In relation to the clinical pathology in humans, it is interesting to note that several (but not all) studies have reported enhanced sensitivity of schizophrenic patients to systemic APO treatment, as assessed by APO-stimulated peripheral growth hormone (GH) responses (Hirschowitz *et al.*, 1986; Meltzer *et al.*, 1984; Zemlan *et al.*, 1986). Interestingly, APO-induced GH

responses appear to positively correlate with psychosis ratings and negative symptom scale scores (Meltzer *et al.,* 1984). This clinical impression agrees well with the present interpretation that (1) experimentally induced prenatal immune challenge in mice leads to long-term behavioral and pharmacological changes relevant to the negative symptoms of schizophrenia, and (2) that this experimentally induced pathological symptom cluster includes enhanced sensitivity to acute APOI treatment.

Another major finding of the present study is that late prenatal immune activation leads to long-term neurochemical changes in multiple neurotransmitter systems (**Figure 3.5-7**). However, the prenatal Poly-I:C-induced changes in neurotransmitter and metabolite levels were, at least in part, dependent on the precise brain area examined, suggesting that distinct brain structures may have a differential vulnerability for prenatal infection-induced imbalances in basal neurotransmitter levels. This interpretation is consistent with previous experimental attempts to characterize the long-term neurochemical consequences of prenatal immune challenge during early fetal development in mice (Winter *et al.*, 2009) and of prenatal exposure to human influenza in mice (Fatemi *et al.,* 2008; Winter *et al.,* 2008). Furthermore, we found that some of the Poly-I:C-induced neurochemical changes are clearly sex-specific, highlighting a differential vulnerability of the male and female sex to long-term neurochemical abnormalities emerging following late prenatal immune challenge.

Besides other significant changes, the neurochemical abnormalities manifest in prenatally immune challenged offspring are characteristic of marked hypodopaminergic (**Figure 3.5b**) and hypoglutamatergic (**Figure 3.6a**) states in prefrontal and hippocampal regions. Converging evidence derived from studies in humans and translational animal models suggest that such hypodopaminergic and hypoglutamatergic states in prefrontal and hippocampal areas may be critically involved especially in the precipitation of negative and cognitive symptoms of schizophrenia (reviewed in Davis *et al.*, 1991; Goff and Coyle, 2001; Javitt, 2007; Knable and Weinberger, 1997; Tamminga, 2006). Hence, the results obtained in the analyses of DA and glutamate provide imperative evidence that the long-term

abnormalities induced by late prenatal immune challenge in mice involve neurochemical changes highly relevant for the pathophysiology of negative symptoms. Considering the effects of late prenatal Poly-I:C exposure on behavioral and pharmacological abnormalities discussed above, the present results demonstrate that this experimental model system successfully captures both functional and neurochemical abnormalities implicated especially in (but not limited to) the negative symptoms of schizophrenia.

A given behavioural, cognitive or pharmacological trait is typically regulated by multiple interconnected brain structures and neurotransmitter system, so that neurochemical disturbances at many sites within a complex neuronal circuitry can give rise to a similar pathological phenotype (Meyer and Feldon, 2010). Likewise, abnormalities in one particular brain area or neurotransmitter system are likely to have an impact on a wide range of behavioral, cognitive and pharmacological functions. For these reasons, our experimental approach does not readily provide straightforward information as to what extent distinct neurochemical abnormalities may contribute to specific behavioral abnormalities in prenatally immune challenged subjects. Additional pharmacological and/or in-vivo microdialysis approaches would be needed to address this issue in more detail. On speculative grounds, however, we deem it likely that the identified neurotransmitter changes in Poly-I:C offspring may, at least in part, provide a neurochemical basis for the emergence of behavioral pathology described here. For example, reduced glutamatergic signaling may be critically involved in the emergence of abnormally enhanced LI. This suggestion would be consistent with the seminal work by Weiner and colleagues showing that impaired glutamatergic signaling induced by MK-801 treatment leads to a similar pattern of LI enhancement in rats and mice (Gaisler-Salomon and Weiner, 2003; Gaisler-Salomon *et al.*, 2008; Lipina *et al*., 2005). Interestingly, the effects of prenatal immune challenge on glutamatergic changes appear more pronounced in males compared to female subjects (**Figure 3.6a**), and these effects parallel the sex-dependent effects of prenatal immune challenge on LI persistence (**Figure 3.3**). On the other hand, a reduced dopaminergic tone may be involved in the expression of anhedonia, based on the suggested role of dopamine in reward-related behavior (Berridge and Robinson, 1998; Cagniard *et al.,* 2006; Wise, 2008), as well as in the development of social interaction deficits (Cabib *et al.*, 2000; Fernandez Espejo, 2003).

In summary, the constellation of behavioral and neurochemical abnormalities induced by late prenatal Poly-I:C exposure in mice reported here and before (Meyer *et al.*, 2006a,b, 2008a) leads us to conclude that this immune-based experimental model provides a powerful neurodevelopmental animal model especially for (but not limited to) the negative symptoms of schizophrenia. This model can successfully capture a wide spectrum of behavioral and neurochemical abnormalities implicated in the negative symptoms of this disorder. Importantly, it incorporates neurodevelopmental perspectives and etiological significance. In support for the latter suggestion, it is interesting to note that the levels of negative symptoms such as anhedonia have found to be exaggerated in schizophrenic patients who were born in winters with high infectious disease rates (Watson *et al.,* 1987). Taken together, the late prenatal Poly-I:C model in mice represents a clear improvement over the limited success and etiological relevance of traditional pharmacological or lesion-based experimental approaches in modeling brain and behavioral abnormalities relevant to the negative symptoms of schizophrenia (Ellenbroek and Cools, 2000). Since current pharmacotherapy of negative symptoms remains unsatisfactory (Buchanan *et al*, 2007; Remington *et al.,* 2010), the late prenatal Poly-I:C model in mice may provide fruitful new avenues for the establishment and evaluation of novel antipsychotic compounds in attempts to improve pharmacological treatments for the negative symptoms of schizophrenia.

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DISCLOSURE

All authors declare they have no conflicts of interest to disclose.

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SUPPLEMENTARY MATERIAL AND METHODS

Animals

C57BL/6 mice were used throughout the study. Female and male breeders were obtained from our in-house specific pathogen free (SPF) colony at the age of 10-14 weeks. Breeding began after 2 weeks of acclimatization to the new animal holding room, which was a temperature- and humidity-controlled (21±1°C, 55±5%) holding facility under a reversed light–dark cycle (lights off: 08:00–20:00). All animals had *ad libitum* access to food (Kliba 3430, Klibamühlen, Kaiseraugst, Switzerland) and water. All procedures described in the present study had been previously approved by the Cantonal Veterinarian's Office of Zurich and are in agreement with the principles of laboratory animal care in the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication No. 86-23, revised 1985). All efforts were made to minimize the number of animals used and their suffering.

Maternal immune activation in late gestation

For the purpose of the maternal immunological manipulation in late gestation, female mice were subjected to a timed mating procedure as described previously (Meyer *et al*, 2005). Pregnant dams on gestation day (GD) 17 received either a single injection of Poly-I:C (potassium salt; Sigma-Aldrich, Buchs, St. Gallen, Switzerland) or vehicle. Poly-I:C (5 mg/kg) was dissolved in sterile pyrogen-free 0.9% NaCl (= vehicle) solution to yield a final concentration of 1 mg/ml and was administered via the intravenous (i.v.) route at the tail vein. The dose of Poly-I:C was chosen based on our previous studies in C57BL/6 mice (e.g., Meyer *et al.*, 2006a,b, 2008). All solutions were freshly prepared on the day of administration and injected with a volume of 5 ml/kg. All animals were returned to their home cages immediately after the injection procedure.

The selected gestational window (i.e., GD 17) in mice corresponds roughly to the middle-to-late second trimester of human pregnancy, respectively, with respect to developmental biology and percentage of gestation from mice to human (Clancy *et al,* 2001, 2007). This gestational stage is identical to the one selected in our previous experimental studies examining the impact of the precise timing of prenatal immune activation on postnatal brain dysfunctions (Li *et al,* 2009; Meyer *et al*, 2006a,b, 2008).

For the purpose of the i.v. injections, each animal's tail was first bathed in 40°C water in order to better visualize and dilate the tail veins. Subsequently, the animals were mildly restrained using a non-restrictive Plexiglas restrainer (561-RC, PLAS Labs, MI 48906, USA) and the substance was injected either at the right or left tail vein. The success of the injection was verified according to three main criteria: (1) resistance of the injection (this refers to the fact that an incomplete hit of the vein prevents the intravenous administration of the full volume of substance), (2) temporary replacement of blood by the injected substance (this refers to the fact that a successful injection at the tail vein can be visualized by the temporary replacement of blood by the injected substance), (3) appearance of a blood drop following retraction of the needle (this refers to the fact that following successful i.v. injection, a blood drop appears at the injection site following retraction of the needle). In addition to these criteria, we have previously verified the effectiveness of intravenous Poly-I:C administration in mice in terms of the elicited cytokine responses in maternal and fetal tissue (see e.g., Meyer *et al.,* 2006b).

Phenotypic characterization of offspring

All offspring were weaned and sexed on postnatal day (PND) 21. Littermates of the same sex were caged separately. All animals were maintained under *ad libitum* food and water, and were kept in a temperature and humidity controlled animal vivarium under a 12:12 h reversed light-dark cycle as described above.

Offspring from multiple independent litters (8 Poly-I:C, 8 saline) were randomly selected for the behavioural and pharmacological tests. Both male and female offspring were included in all the tests described below in order to assess potential sex-dependent effects of the prenatal immunological manipulation. A first cohort of Poly-I:C and control offspring (*N*=16 [8 males, 8 females] in each experimental group) was subjected to behavioural testing in the following test order: (1) social interaction test, (2) sucrose preference test, and (3) latent inhibition (LI) test (see below). All animals were allowed a 1-week resting period between each test. The first cohort of Poly-I:C and control offspring was then used for post-mortem neurochemical analyses (see below) following a resting period of 4 weeks upon conclusion of the LI test. A second cohort of Poly-I:C and control offspring (*N*=12 [6 males, 6 females] in each experimental group) was used for the assessment of basal and apomorphine-induced stereotypies (see below). All behavioural testing commenced when the offspring reached the adult stage of development (i.e., PND 80 onwards).

Social interaction test

Social behaviour is commonly referred to behaviour that takes place in a social context and results from the interaction between and among individuals (of the same species). Since mice (like most other rodents) are highly social animals, social interaction can be efficiently studied under experimental conditions (Crawley, 2007).

The social interaction test apparatus was made of Plexiglas and consisted of three identical arms (50 cm × 9 cm; length × width) surrounded by 10-cm high Plexiglas walls. The three arms radiated from a central triangle (8 cm on each side) and spaced 120° from each other. The Plexiglas walls of apparatus were covered by brown self-adhesive paper, and the floor of the maze was covered with sawdust bedding, which was changed between each individual habituation and test trial. Two out of the three arms contained a rectangular wire grid cages (13 cm × 8 cm × 10 cm, length × width × height; bars horizontally and vertically spaced 9 mm apart). The third arm did not contain a metal wire cage and served as the start

zone (see below). The allocation of metal wire cages to the different arms was counterbalanced across experimental groups.

All animals were first habituated to the test apparatus on two consecutive days. This served to familiarize the test animals with the apparatus and to reduce novelty-related locomotor hyperactivity which may potentially confound social interaction during the critical test phase. The rectangular wire cages (located at the end of two arms) were left empty during the habitation phase. Each test mouse was gently placed in the start arm and allowed to explore the apparatus for 10 min on each habituation day.

The critical test phase took place one day after the second habituation day. During the test phase, one metal wire cage contained an unfamiliar C57BL/6 mouse ("stranger mouse"); and the other wire cage contained an inanimate object ("dummy mouse"), which was made of black LEGO™ bricks and took the shape of a mouse. The allocation of the "stranger mouse" and "dummy mouse" to the two wire cages was counterbalanced across experimental groups. To start a test trial, the test mouse was gently placed in the start arm and allowed to explore freely for 5 min. The relative time (in sec) spent exploring the two wire cages, which either contained the unfamiliar "stranger mouse" or inanimate "dummy mouse", was taken to index social interaction. Exploration was recorded using the Ethovision XT (Noldus Information Technology, Wageningen, The Netherlands) tracking system and was defined as nose detection within a 1-cm interaction zone adjacent to the metal wire cages. In addition, total distance traveled in the three arms and central zone of the test apparatus were also recorded in order to obtain measures of general locomotor activity.

Sucrose preference test

Anhedonic behaviour was assessed by a standard sucrose preference test using methods adapted from previous studies (Hayward *et al.,* 2006; Slattery *et al.,* 2007). The sucrose preference test is based on the observation that rodents typically show a preference for a sweet sucrose solution when presented with

a free choice between the sucrose solution and water. A reduction in this preference is commonly considered as an indication of anhedonia (Cryan and Mombereau, 2004; Hayward *et al.,* 2006; Slattery *et al.,* 2007) and conversely an increase may suggest hyperhedonia.

The apparatus consisted of Macrolon cage type II cages (14×18×34.5 cm) with modified grid tops, which allowed the efficient placement or removal of the drinking tubes. Each cage was provided with two drinking tubes made from 15 ml polypropylene test tubes (Sarstedt, Germany) and equipped with an air-tight screw top. The liquid contained inside the tubes was accessible through an opening of 2.5 mm diameter made at the end of the tubes, which was small enough to prevent leakage. The placement and removal of the drinking tubes was enabled by two acrylic rings (2 cm in inner diameter) mounted in between the metal grids of the cage top. The tubes could be fitted smoothly into the ring, remaining in place with the tube cap resting on top of the ring. When the two drinking tubes were in place, a distance of 4 cm separated the two drinking tubes, at a level of approximately 5 cm above the cage floor that was covered with sawdust. The placement was such that the animals could easily switch drinking from one tube to the other.

All animals were moved from grouped to single housing throughout the period of this experiment. They were first familiarized with drinking water from the two tubes. Water consumption was measured every 24 hrs at 14.00 h for 3 days (habituation phase). The sucrose preference test then began on the next day and lasted for 48 hrs (test phase). During the test, the subjects were allowed free constant access to liquid from the two drinking tubes and food located on the cage top. One drinking tube was filled with a 0.5% sucrose solution and the other with water. The selected concentration of the sucrose solution (i.e., 0.5% sucrose solution) has been shown in preliminary studies to provide a robust but not maximal sucrose preference in C57BL/6 mice (Meyer and Feldon: unpublished observation; see also Pothion *et al.,* 2004).

The amount of liquid consumed was measured daily at 14.00 h, when the two tubes were carefully removed and weighed. The relative left-right positions of the sucrose and water tubes were counterbalanced within each group and across the two testing days. After the first measurement on test day 1, the tubes were refilled and replaced in alternate positions for test day 2. The use of a 48-hrs testing period allowed us to preclude any effects of neophobia and/or artifactual bias toward one side. Sucrose preference was indexed by a percentage score [sucrose consumption / (total liquid consumption) × 100%]. In addition, we also recorded and analyzed total fluid consumption during the habituation and test phases in order to compare the general fluid intake between the two prenatal treatment groups.

Latent inhibition test

Behavioural/cognitive inflexibility was studied by assessing the presence of LI persistence. LI is a selective learning procedure, in which prior repeated pre-exposures to the to-be-conditioned stimulus (CS) alters the subsequent development of the conditioned response (CR) following explicit parings between the same CS and an unconditioned stimulus (US) (Weiner, 2003). A significant LI effect (i.e. impaired conditioning following repeated inconsequential pre-exposures to the CS) is typically manifest when the organism is subjected to a high number of CS-pre-exposures before CS-US conditioning (Weiner, 2003; Weiner and Arad, 2009). In contrast, presentation of a low number of CS-alone stimuli during the pre-exposure phase readily prevents the expression of LI in normal subjects (Weiner, 2003; Weiner and Arad, 2009). However, organisms which tend to show behavioural and/or cognitive inflexibility are expected to express the LI effect under parametric conditions that are insufficient for control animals to display LI (Weiner, 2003; Weiner and Arad, 2009).

LI persistence was studied in a two-way active avoidance procedure using four identical two-way shuttle boxes. The test apparatus used has been fully described before (Meyer *et al.*, 2006c). A 80-dB_A white noise delivered by a speaker served as the CS, and electric foot shock (0.3 mA) delivered through stainless steel rods served as the US. The test procedures consisted of two phases: pre-exposure and

conditioning, conducted 24 h apart. Equal numbers of offspring from both prenatal treatment (PolyI:C or vehicle) conditions were allocated to one of the two conditions: CS-PE and non-pre-exposure (NPE).

In the pre-exposure phase, the CS-PE subjects were placed in the appropriate test chambers and presented with 40 pre-exposures to a 5-s noise CS according to a random interstimulus interval schedule of 40±15 sec. The NPE subjects were confined to the chamber for an equivalent period of time without any stimulus presentation. The total number of shuttle responses performed during the pre-exposure session was recorded.

On conditioning day, the subjects were returned to the same shuttle boxes and received a total of 100 avoidance trials presented with an intertrial interval (ITI) of 40±15 sec. A trial began with the onset of the noise CS. If the animal shuttled within 5 sec of CS onset, the CS was terminated and the animal avoided the electric shock (US) on that trial. Avoidance failure led immediately to an electric foot shock presented in coincidence to the CS. This could last for a maximum of 2 sec but could be terminated by a shuttle response during this period (that is, an escape response). To index conditioned avoidance learning, the mean response latency performed on successive 10-trials blocks were analyzed. In addition, the mean numbers of spontaneous shuttles during the ITIs were recorded and analyzed in order to account for basal locomotor activity during the conditioning phase.

Basal and apomorphine-induced stereotypic behaviour

Behavioural stereotypy is defined as uniform, repetitive and compulsive actions within a restricted pattern which often have no obvious goals or end-points. Acute treatment with the mixed dopamine D1/D2 receptor agonist apomorphine (APO) is well known to induce a dose-dependent enhancement of stereotyped behaviours in mice, including repetitive climbing, wall-leaning, sniffing and gnawing (Protais *et al.*, 1976; Cabib and Puglisi-Allegra, 1985; Tirelli and Witkin, 1994). Acute APO treatment can thus be used to probe the sensitivity to drug-induced stereotyped behavioural responses.

Basal and APO-induced stereotyped behaviours were assessed in a wire mesh cylindrical cage (diameter, 14 cm; height, 20 cm; mesh size, 0.1 cm). The wire mesh cylinder was located in a testing room under dim diffused lighting (35 lux as measured in the center of the cylinder). A digital camera was mounted directly above the cylinder. This camera transmitted images (captured at a rate of 5 Hz) to a PC running the Ethovision (Noldus Information Technology, Wageningen, The Netherlands) tracking system for the purpose of assessment of horizontal locomotor activity. An additional digital video camera was mounted perpendicular to the cylinder to record vertical movements and behaviours (see below). The output of the perpendicular camera was transmitted to a PC running the Windows XP operating system and the WINTV-2000 program for data acquisition.

The animals were first injected with vehicle (vitamin C solution, VitC) and then gently placed in the center of the cylinder. They were then allowed to explore freely for 30 min, during which horizontal and vertical movements and behaviours were recorded by the two cameras. This phase of test served to familiarize the animals to the test apparatus and to assess potential differences between Poly-I:C and control offspring in terms of horizontal and vertical movements and behaviours under basal conditions. Following the 30-min VitC period, the animals were briefly removed from the apparatus and injected with APO solution. They were then immediately placed back to the wire mesh cylinder and allowed to explore freely for 60 min, during which horizontal and vertical movements and behaviours were recorded again. This phase of test served to assess potential differences between Poly-I:C and control offspring in terms of horizontal and vertical movements and behaviours following acute APO challenge.

APO (Sigma-Aldrich) was dissolved in 1% sterile ascorbic acid (VitC, pH 3.2) to achieve the desired dose of 5 mg/kg selected based on preliminary findings in C57BL/6 mice showing that APO at this dose leads to robust climbing behaviour without producing any floor effects on this parameter (Bitanihirwe, Meyer: unpublished observation). The selected dose of APO conformed to doses commonly employed in studies of a similar nature in C57BL/6 mice (see e.g., Cabib and Puglisi-Allegra, 1984;

Tirelli and Witkin, 1994). APO and vehicle (VitC) solutions were freshly prepared on the day of testing and administered via the subcutaneous (s.c.) route with an injection volume of 5 ml/kg.

For each animal, horizontal locomotor activity was measured automatically with the aid of the Ethovision (Noldus Information Technology, Wageningen, The Netherlands) tracking system. Stereotyped behaviours were scored manually by the analysis of video tapes. This was achieved by means of a time-sampling technique performed by an observer who was blind to the experimental groups. Behavioural scores were obtained every 5 min for 1 min. Hence, for the initial VitC phase, there was a total of 5 observations (at 5, 10, 15, 20, and 25 min, each lasting 1 min) beginning 5 min after the VitC injection; and for the subsequent APO phase, a total of 11 observations were made (at 5, 10, 15, 20, 25, 30, 35, 40, 45, 40 and 55 min, each lasting 1 min) beginning 5 min after the APO injection.

The following behavioural categories were established *a priori* on the basis of pilot observations and analyzed for each animal and test phase:

Climbing: The wire of the test chamber wall was grasped with all four paws. No distinction was made between climbing in which the mice remained stationary and climbing in which they moved.

Leaning: Body rearing with at least one forepaw against the wall; episodes with both forepaws and one hind limb on the wall were also considered leaning.

Biting/gnawing: Incisors over the wire, often accompanied by the typical jaw movements of biting and gnawing. This behaviour was displayed while climbing, leaning or having all paws on the floor.

Sniffing: Sniffing was scored when the animal showed uninterrupted sniffing for at least 3 s during the 1 min sampling period.

Grooming: The grooming routine involved a mouse scratching itself with the hind feet, then perhaps 'washing' its face or fur with its hands and grooming with the teeth.

Post-mortem neurochemistry

Levels of monoamines (noradrenaline, NA; dopamine, DA; serotonin [=5-hydroxytryptamine], 5-HT) and their metabolites (dihydroxyphenylacetic acid, DOPAC; homovanillic acid, HVA; 5-hydroxyindoleacetic acid, 5-HIAA; 3-methoxytyramine, 3-MT), amino acids (γ-aminobutyric acid, GABA; glutamate; aspartate; glycine) and the sulfonic amino acid taurine were determined using high performance liquid chromatography (HPLC) according to procedures established before (Peleg-Raibstein *et al.*, 2005, 2008). Poly-I:C and control offspring were killed and dissected in random order. The brains were extracted from the skull within 1 min after decapitation and immediately frozen on dry ice and then were stored at -80 °C until dissection of the brain. For dissection, the frozen brain was placed ventral side up on an ice-chilled plate covered with filter paper and was cut with a razor blade into 1 mm thick coronal sections. The slices were placed on an ice cold dissection plate for the removal of discrete brain regions, using a 1 mm micropunch for the caudate-putamen (CPu), medial prefrontal cortex (mPFC), nucleus accumbens (NAc), amygdala (AMY), dorsal hippocampus (dHPC), and ventral hippocampus (vHPC). Coronal sections were prepared along the following coordinates with respect to bregma: anterior-posterior +2.3 to+1.3, +1.3 to +0.3, –0.1 to –0.6, –1.2 to –2.2, and –2.8 to –3.8. Tissue punches from the left and right hemispheres of each brain area of interest were combined, weighed and placed in 1.5 ml polypropylene microcentrifuge tubes containing ice cold 300 µl 0.4M $HClO_4$ and homogenized using ultrasound. After centrifugation at 10,000×g for 20 min at 4 ˚C, the clear supernatant layers were removed into a 1 ml syringe and filtered through a 0.2 µm nylon filter to separate the insoluble residue. This solution was immediately frozen and stored at −80 ˚C until injection onto the HPLC system. For all brain regions, with the exception of CPu and NAc, an aliquot of 50 µl was injected in the HPLC system. Due to the much higher concentration of dopamine in the CPu and NAc, only 20 µl was injected into the column.

Chromatographic conditions for detection of monoamines and metabolites: A HPLC system coupled with an amperometric electrochemical detector (Decade II; Antec, Leyden, The Netherlands) was used to determine concentrations of dopamine (DA), dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 3-methoxytyramine (3-MT), 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA). The samples were injected via a refrigerated autoinjector (ASI-100, Dionex, CA, USA) equipped with a 100 µl injection loop. The samples were separated on a reversed-phase column (125mm×3mm YMC column, Nucleosil 120-3 C 18, YMC Europe GmbH, Germany). An HPLC pump (P680, Dionex, CA, USA) connected to a pulse damper and a degasser was used to pump the mobile phase (see below) throughout the system. The working potential of the electrochemical glassy carbon flow cell (VT-03; Antec, Leyden, Netherlands) was +0.70V versus an ISAAC reference electrode. A chromatography workstation (Chromeleon, Dionex, Olten, Switzerland) was used for data acquisition and calculations. The mobile phase consisted of 250 ml of HPLC-grade acetonitrile, 5 l of aqueous solution containing 0.27mM sodium ethylendiammoniumtetraacetate ($C_{10}H_{14}N_2O_8Na_2 \bullet 2H_2O$), 0.43mM triethylamine ($C_6H_{15}N$), 8 mM potassium chloride, and 0.925 mM octanesulphonic acid ($C_8H_{17}O_3SNa$) which acted as an ion pairing reagent. The pH was adjusted to 2.95 by adding concentrated phosphoric acid. This was pumped through the system at a flow rate of 0.4 ml/min. The position and height of the peaks of the endogenous components were compared with samples of external calibrating standard solutions.

Chromatographic conditions for detection of amino acids: A chromatography workstation (Chromeleon, Dionex, Olten, Switzerland) controlled a high pressure gradient pump (P 580, Dionex), a column oven (STH 585, Dionex), a fluorescence detector (RF 2000, Dionex) and an autosampler with cooling module (CMA/200, CMA-Microdialysis, Sweden) to analyze aspartate, glutamate, γ-aminobutyric acid (GABA), glycine, and taurine on a reversed-phase column (125mm×3mm YMC column, Nucleosil 120-3 C 18, YMC Europe GmbH, Germany). Ten µl of sample was automatically derivatized with 5 µl ortho-phthaldialdehyde/2-mercaptoethanol (OPA complete reagent, Sigma) using an autosampler set at 4°C to obtain a fluorescent isoindole derivative which was detectable with the

fluorescent detector applying an excitation wavelength of 340 nm and an emission wavelength of 450 nm. Mobile phase A consisted of 20 mM sodium acetate dissolved in methanol: water (30% methanol), adjusted to a pH of 6.00 using acetic acid. After 7 min a step gradient changed the eluent from mobile phase A to mobile phase B (100% methanol) to flush the column, which was maintained at 30°C for temperature stabilization. Both mobile phases A and B were delivered at a flow rate of 0.4 ml/min. Subsequently the column was re-equilibrated with mobile phase A over the next 30 min. The area of the amino acids peaks was calibrated with external standard solutions.

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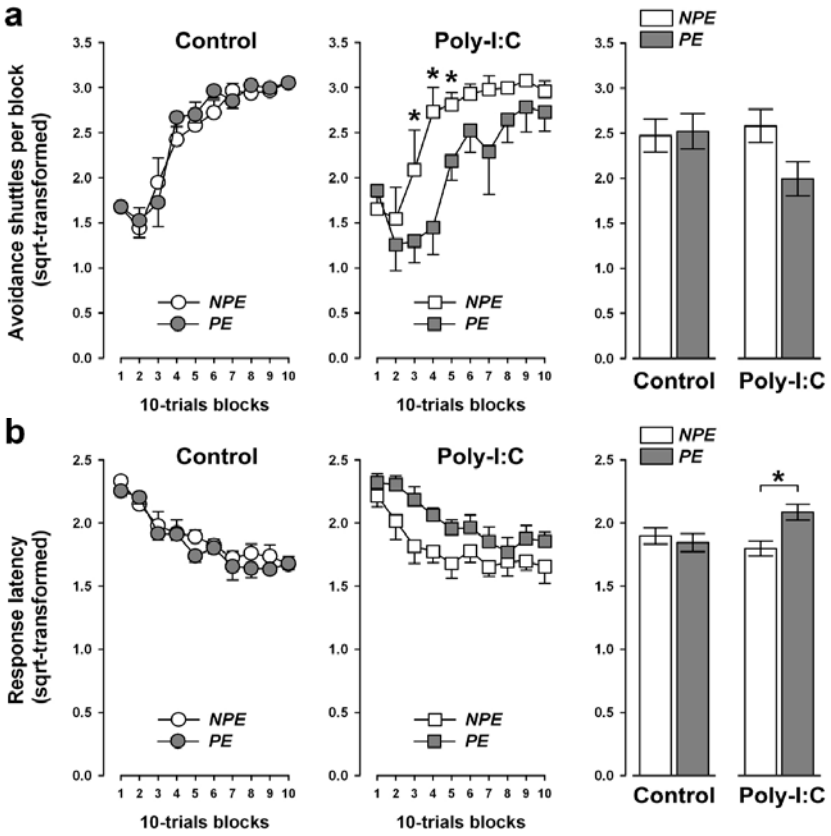
SUPPLEMENTARY DATA



Supplementary Figure 1. Presence of abnormally enhanced latent inhibition (LI) following late prenatal immune challenge. Pregnant C57BL/6 mice on gestation day 17 were treated with the viral mimic Poly-I:C (5 mg/kg, i.v.) or corresponding control vehicle (sterile pyrogen-free 0.9% NaCl) solution as fully described in the *Supplementary Materials and Methods*. The resulting male offspring were subjected to LI testing when they reached the adult stage of development (postnatal day 90). The LI effect was studied in a two-way active avoidance procedure, in which an $80dB_A$ white noise stimulus served as the to-be-conditioned stimulus (CS), and electric foot shock (0.3 mA) served as the unconditioned stimulus (US) (see *Supplementary Materials and Methods* for a detailed description of the test apparatus and procedures used). Pre-exposed (PE) subjects were presented with 40 CS-pre-exposures prior to conditioning, whereas non-pre-exposed (NPE) subjects were not pre-exposed to the CS. **(a)** The line plots depict the number of avoidance shuttles (square-root [sqrt] transformed) as a function of blocks of 10 trials, and the bar plots depict the mean number of avoidance shuttles (square-root [sqrt] transformed) per block performed during the CS-US conditioning phase of the test. CS-pre-exposures did not induce a LI effect (i.e., a decrease in conditioned responding following repeated CS-pre-exposures prior to conditioning) in offspring born to control mothers. However, a marked LI effect was present in male Poly-I:C offspring, as evident by the reduction in the conditioned avoidance responses performed by PE Poly-I:C subjects relative to NPE Poly-I:C subjects. $*P < 0.05$, signifying the significant main effect of pre-exposure in corresponding one-way ANOVAs performed at individual 10-trials blocks restricted to Poly-I:C offspring; restricted ANOVAs were performed following presence of a significant three-way interaction in the initial 2 × 2 × 10 (prenatal treatment × pre-exposure × 10-trials block) ANOVA of conditioned avoidance shuttles [$F(9,180) = 2.06$, $P < 0.05$]. **(b)** The line plots depict the response latency (square-root [sqrt] transformed sec) as a function of blocks of 10 trials, and the bar plots depict the mean response latency (square-root [sqrt] transformed sec) per block performed during the CS-US conditioning phase of the test. Consistent with the outcomes in the analysis of avoidance shuttles, pre-exposures to the CS prior to conditioning did not induce a LI effect in offspring born to control mothers. Hence, the response latency between control NPE and PE subjects were highly comparable. In contrast, a significant LI effect (i.e., a decrease in response latency following repeated CS-pre-exposures prior to conditioning) was present in male Poly-I:C offspring. $*P < 0.05$, signifying the significant main effect of pre-exposure in the 2 × 10 (pre-exposure × 10-trials block) ANOVA of response latency restricted to Poly-I:C offspring [$F(1,10) = 4.04$, $P < 0.05$]; restricted ANOVAs were performed following presence of a significant interaction between prenatal treatment and pre-exposure [$F(1,20) = 4.83$, $P < 0.05$] in the initial 2 × 2 × 10 (prenatal treatment × pre-exposure × 10-trials block) ANOVA of response latency. The number of subjects in each prenatal treatment group and pre-exposure condition was 6 males. All values are means ± SEM.

CHAPTER 4.

Information Processing and Psychostimulant Effects in Mice that Express Mutant Human DISC1.

With Mikhail Pletnikov[#], Joram Feldon and Urs Meyer

[Personal contribution to the work: Designing the research, running the behavioural tests, analysis of the behavioural data, and writing the chapter]

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ABSTRACT

Disrupted-in-Schizophrenia-1 (DISC1) has emerged as a strong candidate gene associated with major mental illness including depression, anxiety, bipolar disorders and schizophrenia. Numerous studies have also reported evidence of linkage or association between DISC1 and impaired cognitive function in schizophrenic individuals. In order to further understand the *in vivo* functions of DISC1 we set out to evaluate schizophrenia-relevant behaviours with a particular emphasis on cognitive information processing in mice that express mutant human DISC1 (hDISC1). In addition, we examined the locomotor activating effects of psychomimetics in these mice. Beginning at 3-4 months of age, male and female hDISC1mutant mice were tested on a battery of tests to evaluate locomotor activity, sensorimotor functioning, selective attention, sustained attention and sensitivity to psychomimetics. Spontaneous locomotor activity of hDISC1 mutant mice in the open-field was not significantly different from controls. Similarly, sensory motor gating and selective attention in the form of latent inhibition were spared by the genetic manipulation. Although compared with control mice, hDISC1mutant mice exhibited increased visual attention in a two-choice visual discrimination/detection test. The locomotor activating effect of amphetamine, a behavioural measure of mesolimbic dopamine activity, was significantly enhanced in hDISC1mutant mice. Specifically, the locomotor activating effect of dizocilipine, a behavioural measure of glutamatergic neurotramsmission, was shown to facilitate the locomotor response in female hDISC1 mutant mice. The behavioural effects of psychomimetic drugs coupled with the results from the visual discrimination task suggest that the expression of hDISC1 contributes to some - but not all - aspects of the schizophrenia-like phenotype and as such may represent an especially useful model for studying the neuronal basis of sustained attention and enhanced psychomimetic sensitivities.

INTRODUCTION

Disrupted-in-schizophrenia-1 (DISC1) is a coiled-coil protein encoded by the DISC1 gene located on chromosome 1q42.1 in humans. It was first reported to be associated with increased risk of schizophrenia and mood disorders in a large Scottish pedigree via a balanced (1;11) (q42.1; q14.3) chromosomal translocation (St. Clair *et al*., 1990; Blackwood *et al*., 2001). Subsequent linkage and association studies have linked DISC1 gene variations to schizophrenia in diverse populations (Hamshere *et al*., 2005; Liu *et al*., 2006; Macgregor *et al*., 2004).

DISC1 protein is abundantly expressed in the hippocampus and the cerebral cortex (Porteous *et al*., 2006), two brain areas critically implicated in schizophrenia and related disorders (Harrison, 2004; Heckers, 1997). As a multi-functional scaffold protein, DISC1 has been shown to interact with multiple proteins required for cell growth, movement, signal transduction, neural positioning, axonal growth and transport (Jaaro-Peled *et al*., 2009). Because the roles of DISC1 in the developing brain include neuronal migration (Kamiya *et al*., 2005) and integration (Duan *et al*., 2007), the mutant protein product of the DISC1 translocation appears to act in a dominant negative manner and as such may interfere with any of these critical neurophysiological processes precipitating the cognitive and behavioural abnormalities associated with schizophrenia (Bradshaw and Porteous, 2011; Sawa and Snyder, 2005).

Initial investigations of transgenic mouse models in which expression of a dominant-negative form of DISC1 (DN-DISC1) is expressed under the alpha-calmodulin kinase II (CAMKII) promoter have emphasized the effect of the carboxy-terminal truncated isoform of DISC1 on brain function. Besides hyperactivity in an open-field, these mice exhibited sensory-motor gating deficits in addition to an impairment in learning a water maze task (Hikida *et al*., 2007). DN-DISC1 mice also exhibited enlarged ventricles and a modest reduction in parvalbumin interneurons, two hallmark features of schizophrenia (Jaaro-Peled *et al*., 2010). Using the mutagen *N*-nitroso-*N*-ethylurea (ENU), Clapcote and colleagues managed to induce two point mutations in exon 2 of murine DISC1 that lead to the manifestation of

multiple pharmacological and behavioural phenotypes relevant to schizophrenia and depression (Clapcote *et al*., 2007). Furthermore, Kvajo and colleagues described a mouse mutant carrying two termination codons (in exon 7 and 8), which leads to the expression of a truncated DISC1 transcript. These mice exhibit working memory deficits associated with impaired dendritic growth and reduced number of spines in mature granule cells of the dentate gyrus (Kvajo *et al*., 2008). Moreover, Shen and colleagues employed a bacterial artificial chromosome (BAC) approach to generate transgenic DISC1 mice with a ~148 kb artificial chromosome. These transgenic mice display a wide variety of schizophrenia-related phenotypes including enlarged lateral ventricles, reduced cerebral cortex and deficits in latent inhibition (Shen *et al*., 2008). More recent studies with inducible expression systems, which express mutant human DISC1 (hDISC1) or the carboxy-terminal portion of DISC1 in a temporal and spatial pattern similar to wild-type mice (Ayhan *et al*., 2011; Li *et al*., 2007; Pletnikov *et al*., 2008) have supported these earlier observations and have also reported some age- and gender-dependent impairments in behavioural phenotypes reminiscent of schizophrenia (Ayhan *et al*., 2011; Pletnikov *et al*., 2008). These results suggest that mutant DISC1 has negative effects on a variety of cognitive and neuroanatomical outcomes in mice.

However, still little is known of *in vivo* functional role of hDISC1 in behaviour. Specifically, it is not known whether expression of the mutant human DISC1 product affects information processing in the form of selective attention, sensory motor gating or sustained visual attention. Latent inhibition is a measure of selective attention by which learning about the consequences of a stimulus is retarded if that stimulus has been experienced (during a pre-exposure (PE) stage) without prior reinforcement (Lubow and Moore, 1959). The LI model of selective attention has been demonstrated with a variety of learning paradigms and across many different species (Lubow, 2005). In this respect, numerous studies have demonstrated that LI can be used to index dysfunctional attentional processing in pathological groups. Interestingly, non-medicated schizophrenic patients with positive symptoms have been shown to exhibit disrupted LI (Gray *et al*., 1995; Swerdlow *et al*., 1996). This effect has been suggested to stem from high levels of distraction, such that during the PE stage attention to the pre-exposed stimulus is maintained

rather than reduced. Conversely, when LI is potentiated, this may indicate an inability to shift from a previously learned association to a new one as is often observed in schizophrenic patients with negative symptoms (Rascle *et al*., 2001). Patients with schizophrenia have also been shown to exhibit deficits in sustained visual attention as measured by the Continuous Performance Test (CPT) (Kurtz *et al*., 2001). An impairment in the performance on the CPT has been consistently found in patients with schizophrenia across various stages of the illness (Liu *et al*., 2002) as well as in first-degree relatives of patients with schizophrenia (Chen *et al*., 1998; Grove *et al*., 1991). Impairment in sustained attention has therefore been suggested to represent a stable or 'trait' deficit in patients with schizophrenia. Another major feature of information processing deficits in schizophrenia patients is deficient sensorimotor gating. Theoretically, this process allows for the filtering of unimportant stimuli and the corresponding allotment of attentional resources to salient environmental cues (Braff and Geyer, 1990; Braff *et al*., 2001). Prepulse inhibition (PPI) is a cross species phenomenon often used to assess sensory motor gating. Specifically, PPI refers to pre-attentive process in which a weak-pre-stimulus or prepulse suppresses the response to a subsequent startling stimulus (Graham, 1975). Deficits in sensory motor gating as assessed by PPI are therefore believed to lead to the sensory flooding and subsequent cognitive fragmentation observed in patients with schizophrenia (Braff and Geyer, 1990; Braff *et al*., 2001).

It follows that in the present study we focused on assessing the role of mutant human DISC1 expression on information processing (i.e. LI, sustained attention and PPI) seeing as they have not been evaluated in depth in mice that express the mutant DISC1 product. In addition, we assessed the integrity of the dopamine and glutamatergic systems, both known to be affected in schizophrenia, by evaluating amphetamine and dizocilopine-induced hyperlocomotion in hDISC1 mice.

MATERIALS and METHODS

Animals and Husbandry

The present study was conducted on 3 genotypes x 2 sexes = 6 groups of mice. hDISC1 mutant mice were generated using the Tet-Off double transgenic system (Clontech, Palo Alto, CA, USA) as previously described (Pletnikov *et al*., 2008; Pletnikov 2009). Briefly, the gene of interest is cloned under control of the tetracycline responsive element (TRE), whose activation is dependent on binding of tetracycline-controlled transcriptional activator (tTA). Binding of tTA to TRE, and consequently transcription of the downstream gene (i.e. DISC1), can be prevented by interaction of tTA with tetracycline (See **Figure 4.1**). DISC1 and tTA (The Jackson Laboratory, Bar Harbor, ME) mice were mated heterozygously. In the present study, double transgenic (DISC1/tTA) mice were included in the mutant group (referred to as hDISC1 mutants), whereas their single tTA transgenic and single DISC1 littermates were included in the control groups (referred to as DISC1 controls and tTA controls, respectively). The offspring were weaned, ear marked and housed in sex-matched groups of two to six per cage with *ad libitum* food (food pellets, Kliba 3430, Klibamühlen, Kaiseraugst, Switzerland) and water at postnatal day 21. Behavioural testing commenced when they reached 3–4 months of age. The animals had never experienced any form of behavioural testing prior to the studies described here, and the order of the testing was designed to proceed from the least stressful to the more stressful tests. Each genotype consisted of subjects derived from multiple independent litters. The vivarium temperature was maintained at 21 ± 1°C, with humidity at 55 ± 5%. The holding facility was maintained under a reversed light-dark cycle (lights off: 700 to 1900h). All procedures described in the present study had been approved previously by the Zurich Cantonal Veterinary Office and are in agreement with the *Principles of Laboratory Animal Care* (National Institutes of Health publication number 86-23, revised 1985).

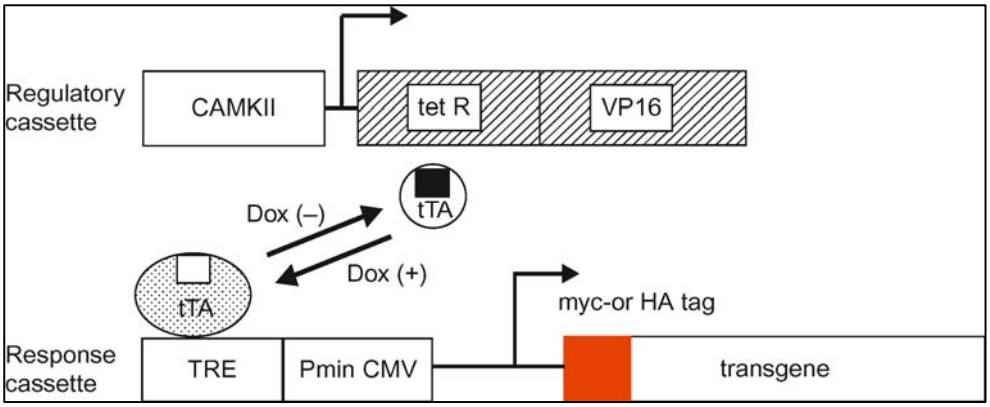

Figure 4.1. Schematic representation of the Tet-Off system. A Tet system is composed of two distinct expression cassettes, one containing the regulatory protein (tTA) under the control of a cell-type-specific promoter (e.g., the CAMKII promoter) and the second response cassette containing the TRE fused to a minimal promoter (e.g., CMV), regulating the expression of the transgene that is usually fused to a tag for easy detection of transgene expression. The chimeric protein tTA consists of the TetR fused to the viral transactivator VP16. In the absence of Dox, the tTA protein binds to the TRE within the compound promoter Pmin CMV to allow the expression of the transgene. In the presence of Dox, the tTA changes its conformation, gets detached from the TRE and no transgene expression is observed. (Source: Pletnikov, 2009)

Genotyping and PCR

Genotypes of the mice were determined by polymerase chain reaction (PCR) analyses of the mouse tail according to protocols established before (Mikhail Pletnikov, personal communication). PCR primers for genotyping were as follows: forward-5′GACCTCCATAGAAGACACCGGGAC-3′, reverse-5′TGAGCTGAATCCCAAAGTGCGCCG-3′ for the hDISC1 mutant allele. Whereas forward-5′-CGCTGTGGGGCATTTTACTTTAG-3′ and reverse-5′-CATGTCCAGATCGAAATCGTC-3′ primers were used for the tTA allele. The amplicon from the hDISC1 product is ~500 kb, and the tTA product is ~450 kb. PCR products were separated on a 1.5% agarose gel. The bands were visualized using ethidium bromide staining and ultraviolet transillumination.

Behavioural experiments

Open-field exploration

A four-unit open field consisting of a white box divided into four separate square arenas (*L* x *W* x *H* = 40 × 40 × 35cm) was used, allowing four animals to be tested concurrently. They were located in a testing room under dim diffused lighting, providing an illumination intensity of ~ 35 lux as measured in the center of the arenas. To begin, the animals were gently placed in the center of the arena and allowed to explore for 60 minutes. The mouse's movements were tracked and recorded automatically via the Ethovision tracking system (Noldus Information Technology, Wageningen, The Netherlands). For the purpose of data collection, the arena was conceptually partitioned into two zones: a center zone (measuring 13.5 x 13.5 cm^2) in the middle of the arena and a peripheral zone occupying the remaining area. These measures were analyzed as a function of 5 minute bins with the between-subjects factors of genotype and sex. The open field was cleaned between animals of any feces or urine and wiped down with a dilute solution of ethanol.

Prepulse inhibition

To assess for prepulse inhibition of the acoustic startle response, each mouse was tested with the SR-LAB startle apparatus (San Diego Instruments, San Diego, CA, USA). The test apparatus and procedures have been fully described before (Yee *et al*., 2005). Each session was initiated with a 2 minute habituation phase before the first trial began. The first 6 trials consisted of 6 startle-alone trials, comprising 2 trials of each of the 3 possible pulse (100, 110, or 120 dB_A) intensities. These trials served to habituate and stabilize the animals' startle response and were not included in the analysis. Subsequently, the animals were presented with 10 blocks of discrete test trials. Each block consisted of the following: 3 pulse-alone trials (100, 110, or 120 dB_A), 3 prepulse-alone trials (+6, +12, or +18 dB_A above background), 9 possible combinations of prepulse-plus-pulse trials (3 levels of prepulse × 3 levels

of prepulse), and 1 no stimulus. The 16 discrete trials within each block were interleaved in a pseudorandom order with a variable inter-trial interval of 15 s (ranging from 10 to 20 s). For each of the 3 pulse intensities (100, 110, or 120 dB_A), PPI was indexed by the percentage inhibition of the startle response at each level of prepulse intensity by the following formula: % PPI = [1 – (mean reactivity on prepulse-plus-pulse trials/mean reactivity on pulse-alone trials)] x 100%.

Two-choice visual discrimination task

Food restriction procedure. Food restriction was gradually introduced by progressively reducing the available feeding time across 5 days (12 hr, 8 hr, 4 hr, 3 hr, 1 hr). At the same time, the animals were familiarized with the liquid reward in the home cage to minimize food neophobia. Afterwards, daily food rations were calculated as a function of each animal's weight loss/gain from the previous day in order to maintain a stable weight of not less than 85% of the ad lib weight. Drinking water was freely available throughout.

Habituation and magazine training. Visual attention was assessed in a set of four operant chambers as previously described (Bitanihirwe *et al*., 2011). Two magazines, each equipped with a stimulus light inside, were mounted within the panel wall, above floor level. A nose-poke into the magazines was detected by an infrared photocell beam placed at the magazine's entrance. Each magazine was equipped with a liquid dipper dispenser capable of delivering 0.01ml of liquid reward (20% condensed milk solution, Milch Lait, Schweizer Milchproduzenten, Switzerland). A retractable lever was centered between the two magazines with a stimulus light mounted directly above the lever. To limit positional bias, two additional transparent partitioning walls were positioned apart, one on either side of the retractable lever. The animals were therefore required to take a detour to move between the lever and either magazine. The data were analyzed by the Graphic State (Version 1.013) tracking system. The animals were habituated to the operant chambers over four consecutive days and acclimatized to

consuming the liquid reward from the dippers. The dipper of one of the two magazine trays was programmed to be raised and illuminated by the magazine light until a nose poke into the magazine was detected, after which time the dipper remained raised for 3 s allowing access to the liquid reward. This procedure was repeated, with a variable inter-trial interval (ITI) of 15±5 s and a pseudorandom schedule under which one of the two reinforcer magazines was illuminated. The lever remained retracted and the house light was switched on during the entire session, which ended either when 18 min had elapsed or 60 rewards had been collected. All animals collected at least 40 rewards on the last day.

Lever press shaping. Shaping training began 24 hrs after the fourth habituation session. During shaping the animals were trained for six days to press the lever to obtain the liquid reward. Each trial began with the presentation of the lever. A single lever-press was followed by its immediate retraction and the illumination of the magazine tray of one randomly selected magazine, until a nose poke into the magazine was detected, at which time the dipper was raised for 3 s allowing access to the liquid reward. The next trial began after a 10 s interval. Only one magazine (left or right) was used for each animal's daily session, with access to the other magazine blocked by a metal plate. Training resumed on the following day with the other magazine tray. This minimized the possible development of a side bias. On each day, half of the animals were trained using the left magazine and the remaining animals using the right magazine. The house light remained on throughout the session, which ended after 24 trials were completed or when 25 minutes had elapsed. All animals had completed at least 19 trials on the last training day.

Visual discrimination. Visual discrimination training began 24 hrs following the completion of the shaping process. Access to both magazines was now available. With the house light on and the lever retracted, the animals were introduced into the chamber. The first trial began 5 s later, initiated with the presentation of the lever. One lever press resulted in the retraction of the lever, followed immediately by the random illumination of one of the two magazine lights for a maximum duration of 30 s. A nose poke into the illuminated magazine was rewarded by a 3 s access to the liquid reward (correct response) in the

continual presence of the magazine light. Failure to respond within the 30 s (an omission) or an (incorrect) nose poke response into the non-illuminated magazine was followed immediately by a 30 s time-out period when all stimulus lights (including the house light) were switched off and the lever remained retracted. The next trial then began after a 10 s ITI. A session ended after 48 trials (completed or omitted) or when 50 min had elapsed. Four performance variables were calculated at the end of each session: (i) *Choice accuracy* was defined by the formula: *correct responses* / (*correct responses* + *incorrect responses*) × 100%, (ii) *Omission rate* was defined as *omissions* / *all initiated trials* × 100%, (iii) *Choice latency* was defined as the time between lever press and nose-poke (correct or incorrect) excluding omissions and (iv) *Initiation latency*, defined as the time elapsed between presentation of the lever and the lever press.

Test of visual discrimination with variable signal duration. Once the animals had achieved high accuracy (≥80% on two consecutive days) in the basic visual discrimination task described above, the duration of the visual signal was reduced to 10, 2, 1 or 0.5 s. The animals were still allowed a maximum of 30 s to perform a nose poke response following the presentation of the visual signal, before an omission would be recorded. Throughout the maximum number of 48 trials (i.e. twelve trials at each stimulus duration regardless of omission), every block of 4 trials randomly featured one of the four possible signal durations. A single attention session lasted for a maximum of 50 minutes.

Latent inhibition

Latent inhibition (LI) is a process of learning to ignore irrelevant stimuli and is manifested as slower learning about a stimulus that has been pre-exposed without consequence (PE), compared to learning about the same stimulus without pre-exposure (NPE) (Lubow and Moore, 1959). Abnormality in LI may assume the form of an attenuation (against the presence of strong LI in controls) or abnormal

persistence (against the absence of LI in controls) (Weiner, 2003). We therefore sought to assess these two levels of LI by adjusting the conditioning and pre-exposure parameters in two separate tests for LI.

Conditioned freezing paradigm. In order to assess for disrupted LI (i.e. under extensive pre-exposure conditions) mice were assessed in a conditioned freezing paradigm using two sets of four chambers as previously described (Meyer *et al*., 2005). The test procedures consisted of four phases: PE, conditioning, context test, and tone test. Animals were randomly allocated to either the pre-exposed or non-pre-exposed condition. Pre-exposed animals received 40 presentations of a 30 s tone stimulus at a variable interstimulus interval of 40 ± 30 s, while NPE animals were confined to the chamber for an equivalent time. Conditioning followed immediately at the end of PE without removing the animals from the chambers. Conditioning contained three discrete trials of tone–shock pairings. Each trial began with the 30 s tone stimulus followed immediately by the delivery of a 1 s footshock (0.25 mA). Each trial was preceded and followed by a 180 s interval. The tone test was conducted another 24, 48 and 72 h later in the same test chamber to assess the conditioned response to the tone and its extinction. Following a 180 s acclimatization period, the tone was continuously presented for 180 s, and freezing (immobility) in the presence of the tone stimulus was evaluated. Freezing was expressed as the percentage time freezing for all test sessions.

Two-way active avoidance paradigm. The presence of persistent LI (by restricting the number of pre-exposures before the conditioning phase) was assessed using active avoidance paradigm. The active avoidance task captures elements of both classical and instrumental conditioning, in which the animals learn to perform a specific operant act in response to a tone stimulus to avoid the delivery of an aversive footshock. The apparatus consisted of four identical two-way shuttle boxes as fully described before (Yee *et al*. 2006). Electric shocks (0.3 mA) could be delivered through the grid floor. A white noise stimulus (an 86 dB_A tone) served as the conditioned stimulus (CS) and electric foot shock served as the

unconditioned stimulus (US). The test procedures consisted of two phases: pre-exposure and conditioning, conducted 24 h apart. Subjects were allocated to one of the two conditions: CS-PE and NPE. In the pre-exposure phase, CS-PE subjects were presented with 40 pre-exposures to the CS according to a random interstimulus interval schedule of 40 ± 15 s, whereas NPE subjects were confined to the chamber for an equivalent period of time without any stimulus presentation. On the conditioning day, the subjects were returned to the same shuttle boxes and received a total of 100 avoidance trials presented with an intertrial interval of 40 ± 15 s. To index conditioned avoidance learning, the number of avoidance shuttles across successive 10-trial blocks was analyzed. In addition, the mean numbers of spontaneous shuttles during the ITIs were recorded and analyzed to measure or control for basal locomotor activity during the conditioning phase.

Locomotor response to psychomimetic drugs in the open field

The tests for psychomimetic (amphetamine (AMPH) or dizocilipine (MK-801))-induced hyperlocomotor activity was conducted in the open-field apparatus described above. Animals were allocated between two testing rooms and amongst eight arenas. Additionally, animals were counterbalanced with respect to genotype and drug conditions. In order to acclimatize the animals to the open field, they were placed in the center of the arena and allowed to explore freely for 30 minutes. At the end of this time period, the animals were removed from the apparatus and injected with sterile 0.9% NaCl (saline) solution. They were then immediately returned to the same arenas and allowed to explore for another 30 minutes. Subsequently, the animals were briefly removed from the apparatus once more, administered with amphetamine (1mg/kg) or MK-801(0.3 mg/kg), and returned to the same arenas again. The locomotor responses to the acute drug challenge were then monitored for a period of 60 minutes. Amphetamine (Sigma-Aldrich, Germany) and MK-801(Sigma-Aldrich, Germany) were dissolved in isotonic 0.9% NaCl solution to achieve the desired concentration for injection. Psychostimulants were administered via the intraperitoneal route. Dosages were selected based on previous studies in hDISC1

mutant mice (Ayhan *et al*., 2011). The volume of injection was 5 ml/kg. The AMPH and MK-801 solutions were freshly prepared on the day of testing.

Statistical Analyses

All data were analyzed by parametric analysis of variance (ANOVA) using the between-subject factors, genotype, in combination with drug and pre-exposures as appropriate. Additional within-subject factors (e.g. 5 minute bins, ten-trial blocks) were also included according to the nature of the considered dependent variables. The raw locomotor activity scores for the psychostimulant assessment were subjected to a (square root) transformation in order to normalize the data and to reduce skewness, thereby enabling the use of paramateric statistical analyses to assist interpretation of the statistical outcomes. Significant effects were further investigated by Fisher's least significant difference (LSD) post hoc comparisons or restricted ANOVAS whenever appropriate. Statistical analyses were conducted using the Statistical Package for the Social Sciences (SPSS) software (version 13.0) implemented on a personal computer running the Windows XP operating system. Statistical significance was set at $P < 0.05$.

RESULTS

Locomotor activity: Open-Field

To examine locomotor activity in test mice in response to a novel environment, animals were placed into an open-field arena for 60 minutes and their movements were recorded. The three groups showed clear habituation in terms of distance moved over the course of the experimental session ($F_{11,594}$ = 30.855; P = 0.001; **Figure 4.2**) with peak activity occurring in the first 5 minute period and declining thereafter. This effect was present in both male and female subjects. A significant main interaction effect of genotype x bins x sex was detected for the total distance travelled ($F_{22,594}$ = 1.766; P = 0.017) within the centre of the open field. However, subsequent post hoc analysis in both males ($F_{11,297}$ = .864, P = 0.643) and females ($F_{11,297}$ = 1.436, P = 0.096) did not reveal any interactions of genotype with bins for the total distance travelled within the centre of the open field.

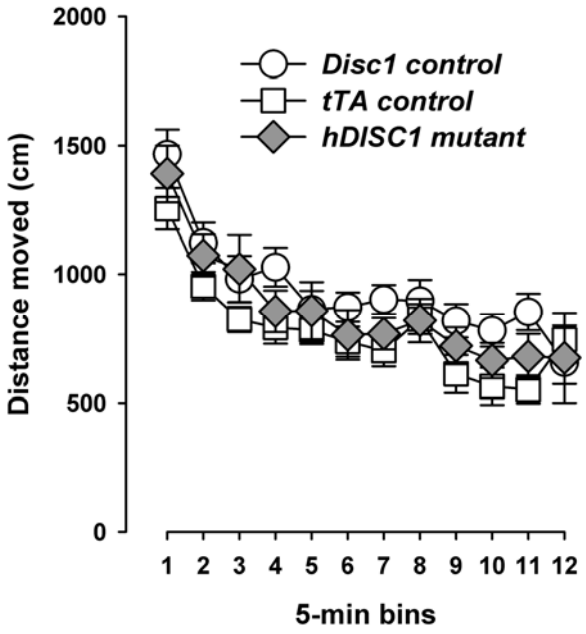

Figure 4.2. Spontaneous locomotor activity in the open field. The line plot shows distance moved (cm) as a function of 5-min bins, and the bar plot depicts the mean distance traveled (cm) per bin. hDISC1 mutant, n = 20; DISC1, n = 20; tTA, n = 20. No significant differences in locomotor activity were detected between genotypes. The three groups showed clear habituation in terms of distance moved over the course of the experimental session ($P <$ 0.001). All values are means±SEM.

Sensory motor gating: Pre-pulse Inhibition

In all groups, the percent PPI was related to the prepulse intensity (prepulse intensity: $F_{(2,106)} = 77.817$, $p < 0.001$) and the pulse intensity (pulse intensity: $F_{2,106} = 5.100$, $p < 0.0077$). As shown in **Figure 4.3**, all three genotypes displayed comparable levels of PPI as a function of the prepulse-intensity, evidenced by the lack of a significant effect of genotype ($F_{2,53} = 0.63$, $P = 0.939$) or prepulse-intensity × genotype interaction ($F_{4,106} = 0.857$, $P = 0.4924$). Interestingly, we detected a significant three way interaction of prepulse × sex × genotype ($F_{4,106} = 2.705$, $P = 0.034$). Subsequent post-hoc analyses revealed a significant interaction of prepulse × genotype ($F_{4,54} = 2.626$, $P = 0.0445$) and pulse × genotype ($F_{4,54} = 2.725$, $P = 0.0387$) in the females subjects. Sex specific analysis of the three pre-pulses at each pulse intensity in females did not reveal any significant effect of prepulse x genotype

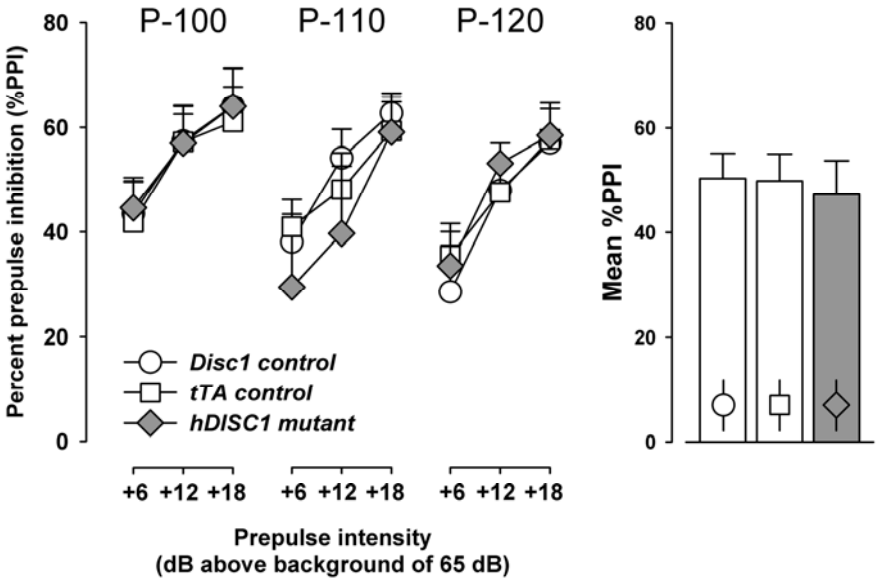

Figure 4.3. Prepulse inhibition is expressed in terms of percent inhibition calculated with reference to the respective pulse-alone trials of a given pulse intensity. hDISC1 mutant, $n = 19$; DISC1, $n = 20$; tTA, $n = 20$.

(p-100 = $F_{4,54} = .508$, $P = 0.7298$; p-110 = $F_{4,54} = 1.646$, $P = 0.1761$; p-120 = $F_{4,54} = 1.484$, $P = 0.2198$). Similarly, analysis of the three pre-pulses at each pulse intensity did not reveal any significant effect of genotype (p-100 = $F_{2,27} = .199$, $P = 0.8204$; p-110 = $F_{2,27} = 2.052$, $P = 0.2480$; p-120 = $F_{2,54}$ =

.628, $P = 0.5411$). Restricted analysis in males did not reveal any effects of genotype or interactions with prepulse ($F_{4,52} = .624$, $P = 0.6474$) or pulse ($F_{4,52} = 0.045$, $P = 0.9961$). It is therefore likely that this higher level interaction may be due to within subject fluctuations in response to the prepulse intensity specifically within the female subjects.

Startle reactivity to pulse-alone trials was not significantly altered in all groups (**Table 4.1**). The 3 × 2 × 3 (genotype × sex × pulse level) ANOVA of startle reactivity did not yield significant effects involving the between-subjects factor of genotype ($F_{4,106} = 1.439$, $P = 0.226$). Similarly, no effect was observed in prepulse-alone trials. A 3 × 2 × 4 (genotype × sex × prepulse level) ANOVA of reactivity to prepulse-alone trails (including background reactivity) did not yield significant effects involving the between-subjects factor of genotype ($F_{6,159} = 0.401$, $P = 0.877$; **Table 4.1**).

Table 4.1. Mean startle reactivities to prepulse and pulse-alone startle stimuli.

	DISC1 Control (***n*** =20)	**hDISC1 Mutant** (***n*** = 19)	**tTA Control** (***n*** =20)
Background	4.840 ± 1.018	5.783 ± 1.046	5.020 ± 1.018
pp6	7.090 ± 1.756	7.174 ± 1.804	6.130 ± 1.756
pp12	9.675 ± 1.878	10.259 ± 1.930	11.915 ±1.878
pp18	15.080 ± 3.534	17.622 ± 3.631	16.005 ± 3.534
pA	34.445 ± 9.049	50.642 ± 9.294	56.805 ± 9.046
pB	65.645 ±11.287	84.611 ±11.597	98.835 ± 11.287
pC	93.950 ±15.730	120.016 ±16.161	125.880 ±15.730

Mean startle magnitude in arbitrary units (± SEM) to prepulse (+6, +12, or +18 dB_A) and pulse (100, 110, or 120 dB_A) acoustic stimuli. pp = prepulse; p = pulse

Sustained Attention: Two-choice visual discrimination task

To examine sustained attentional abilities in mutant mice, mice were first trained to acquire a simple two-choice visual discrimination rule, upon which the contribution of attentional processes to task performance could be altered by varying the duration of the relevant discriminatory stimulus. Following 11 days of training, during which the to-be-attended-to stimulus cue remained illuminated for the entire length of each trial, baseline visual discrimination performance in the three groups of mice, as calculated

by averaging each group's mean percent correct score during the final training session, was 93.75% ± 2.34%, 89.84% ± 6.88% and 88.65% ± 4.03% in DISC1, hDISC1 mutant and tTA groups, respectively. In addition to measuring choice accuracy, reaction time was assessed by calculating the latency to lever press in addition to the latency to choose as measured from the time when the discriminatory signal was initiated to the nose poke (excluding trials with omission). Upon reaching criterion on the visual discrimination task, average initiation latencies (1.615s± 0.259s, 1.907s± 0.251s and 1.523s ± 0.259s in DISC1, hDISC1 mutant and tTA groups, respectively; $F_{2,49}$ = 0.624, P = 0.540) and choice response latencies (1.107s± 0.75s, 1.145s± 0.73s and 1.217s ± 0.75s in, DISC1, hDISC1 mutant and tTA groups, respectively; $F_{2,49}$ = 0.552, P = 0.579) during the final training session were not statistically different. A session comprised of mixed stimulus-duration trials were then administered, and the results are depicted in **Figure 4.4**. As expected, choice accuracy was found to decline in the three groups of mice in conjunction with a decrease in stimulus duration ($F_{3,147}$ = 89.792, P = 0.001). A significant genotype effect was observed, as tTA and DISC1 animals made fewer correct responses compared with hDISC1 mutant animals ($F_{2,49}$ = 6.550, P = 0.003), however, the interaction between stimulus duration and genotype was itself non-significant ($F_{6,147}$ = 0.346, P = 0.911). No effect of sex ($F_{1,49}$ = 1.162, P = 0.286) was detected. Although there was no statistical difference detected between the 3 groups of mice when latency to lever press was evaluated ($F_{2,49}$ = 1.398, P = 0.257), the average reaction times across the three groups of mice did however show a monotonic reduction with increasing signal duration. For all three groups of mice there was a clear effect of signal duration ($F_{3,147}$ = 8.418, P = 0.001), with response latencies increasing as the duration of the stimulus cue was shortened, but there was no significant interaction between genotype and stimulus duration ($F_{6,147}$ = 0.946, P = 0.464).

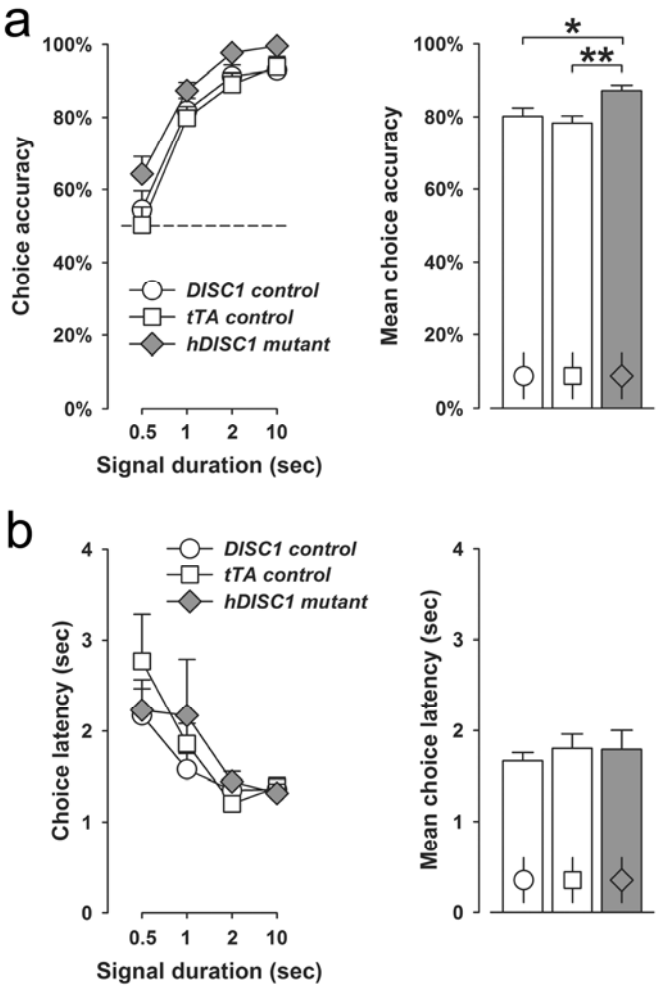

Figure 4.4. Attentional performance in the two-choice visual discrimination task. (a) Choice accuracy for each stimulus-duration trial type expressed as a percentage of correct responses to the cued magazine. Dashed line represents chance-level performance (i.e. 50%). * P< 0.05 and ** P< 0.01 statistical significance. (b) Reaction time expressed as a latency to make a nose poke response following illumination of the stimulus cue light. hDISC1 mutant, n = 19; DISC1, n = 18; tTA, n = 18.

Latent Inhibition: Condition freezing paradigm

Patients with schizophrenia often have deficits in LI (Rascle *et al*., 2001), a form of selective associative learning. In the conditioned freezing paradigm used to assess LI here, the development of conditioned freezing was evaluated across the three tone–footshock pairings. Latent inhibition was evident by elevated freezing levels in the non-pre-exposed animals relative to the pre-exposed animals (pre-exposure: $F_{1,42}$ = 5.517, P = 0.001). The within-subject analysis showed significant effects of trials, ($F_{2,84}$ = 25.036, P = 0.001) supporting the acquisition of the freezing response.

The expression of conditioned freezing in response to the CS-tone was evaluated 24, 48 and 72 hrs after conditioning within the same test chambers. The presence of LI in all groups was demonstrated across the three days of assessment by a clear reduction in the amount of freezing to the tone in the pre-exposed animals relative to the non-pre-exposed ones (pre-exposure: $F_{1,42} = 5.362$, $P = 0.026$). Significant effects of bins ($F_{11,462} = 4.948$, $P = 0.001$) and days ($F_{2,84} = 46.403$, $P = 0.001$) were detected supporting within session extinction and day-to-day extinction, respectively. Notably, ANOVA did not reveal any significant main effects or interactions involving the genotype variable (bins x genotype x pre-exposure: $F_{22,462} = 1.172$, $P = 0.268$ or bins x sex x genotype x pre-exposure: $F_{22,462} = .705$, $P = 0.836$) suggesting that expression of mutant human DISC1 did not influence the expression of LI in the condition freezing paradigm (**Figure 4.4**).

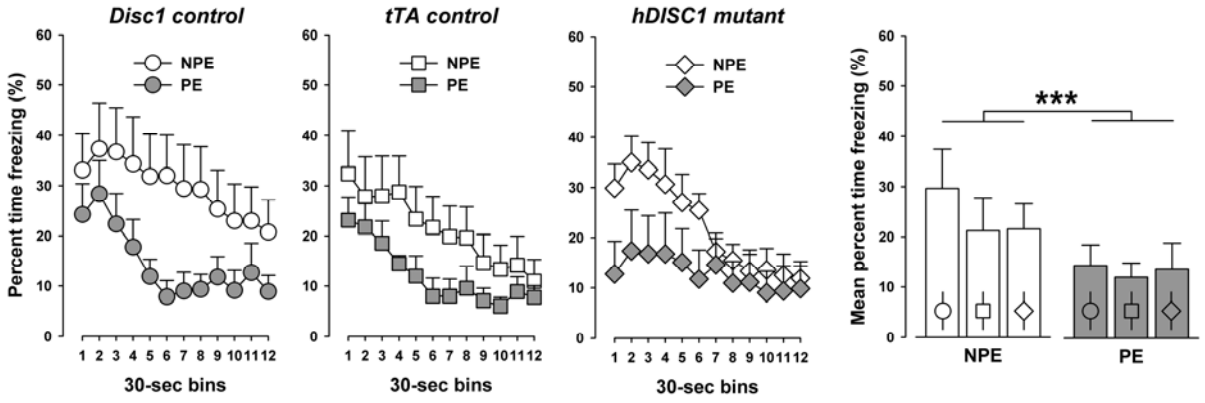

Figure 4.5. Latent inhibition (LI) measured in the conditioned freezing paradigm collapsed over 3 days. Freezing in the pre-exposed (PE) subjects was lower than in the non-pre-exposed (NPE) subjects, constituting the LI effect. *** P< 0.001 statistical significance. hDISC1 mutant, $n = 7$ PE, 10 NPE; DISC1, $n = 10$ PE, 9 NPE; tTA, $n = 10$ PE, 8 NPE

Latent Inhibition: Two way active avoidance

Because there was no evidence of disrupted LI in the hDISC1 mutant mice we decided to screen these mice for a potential abnormality in the opposite pole of LI (i.e. persistent LI) using another associative learning paradigm (i.e., active avoidance). Test mice were assessed under experimental parameters known to be sensitive to LI expression. Specifically, presentation of a low number of CS-alone stimuli during the pre-exposure phase readily prevents the expression of LI in normal subjects (Weiner, 2003; Weiner and Arad, 2009). However, organisms which tend to show behavioural and/or cognitive inflexibility are expected to express the LI effect under parametric conditions that are insufficient for control animals to display LI (Weiner, 2003; Weiner and Arad, 2009). Acquisition of the conditioned avoidance response was evident in both groups as indicated by the increase in the number of avoidance responses over successive 10-trial blocks (blocks: $F_{9,414} = 133.221$, $P = 0.001$). In agreement with the results obtained in the open-field test, spontaneous locomotor activity (as measured by the number of spontaneous shuttles performed during the ITI periods) did not differ between genotypes. An absence of the LI effect (reflected by similar avoidance responding in the NPE subjects and PE subjects) was evidenced by a lack of a significant main effect of pre-exposure ($F_{1,46} = 0.427$, $P = 0.5167$; **Figure 4.6**). ANOVA revealed a significant main effect of genotype that stemmed from what appears to be a lower number of active avoidance responses in DISC1 control group compared to the other two groups ($F_{1,46} = 5.166$, $P = 0.0099$). Further statistical analyses did not detect any interactions involving the genotype variable.

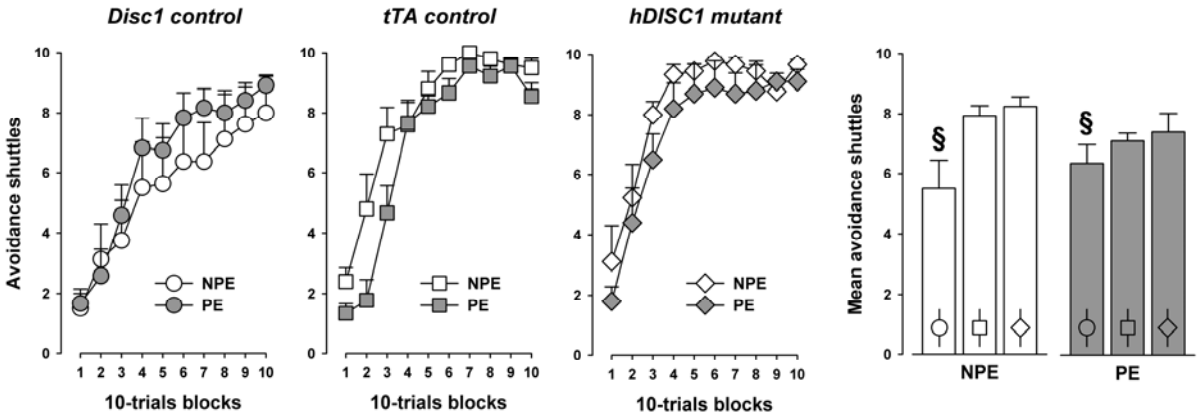

Figure 4.6. Latent inhibition measured in the conditioned active avoidance paradigm. **(a)** The line plots show the numbers of avoidance shuttles performed by non-pre-exposed (NPE) or pre-exposed (PE) animals. hDISC1 mutant, n = 10 PE, 9 NPE; DISC1, n = 10 PE,10 NPE; tTA, n = 9 PE, 10 NPE **(b)** The bar plot depicts the mean avoidance shuttles performed by NPE or PE subjects. § P< 0.05 statistical significance. All values are means±SEM.

Motor stimulating effects of acute amphetamine or dizocilipine treatment

To assess whether hDISC1 mutant mice possess a psychotic-like phenotypic profile, we tested the locomotor stimulant effect of the indirect dopamine-receptor agonist AMPH – a drug known to exacerbate and induce psychotic symptoms in humans (Laruelle *et al*., 1996). Locomotor activity for the baseline period (bins: $F_{5,115} = 20.498$, $P = 0.001$) and after vehicle (saline) injection (bins: $F_{5,115} = 1.226$, $P = 0.301$) was similar between genotypes. Systemic administration of AMPH resulted in markedly increased locomotor activity in all groups (bins: $F_{11,\ 253} = 12.020$, $P = 0.001$). However, the drug-induced hyperactivity was significantly more pronounced in mutant animals (bins × treatment: $F_{22,253} = 1.922$, $P = 0.009$; **Figure 4.7**), particularly in the first half of the 60 minute test session. Post-hoc analysis restricted to each of the 5 minute bins revealed that hDISC1 mutant mice exhibited a statistically significant (p values < .05) enhanced locomotor reaction to AMPH in the first, second, third, fourth and seventh 5-minute bins after drug injection in comparison with tTA controls but not DISC1 controls. No other main effects or interactions attained statistical significance.

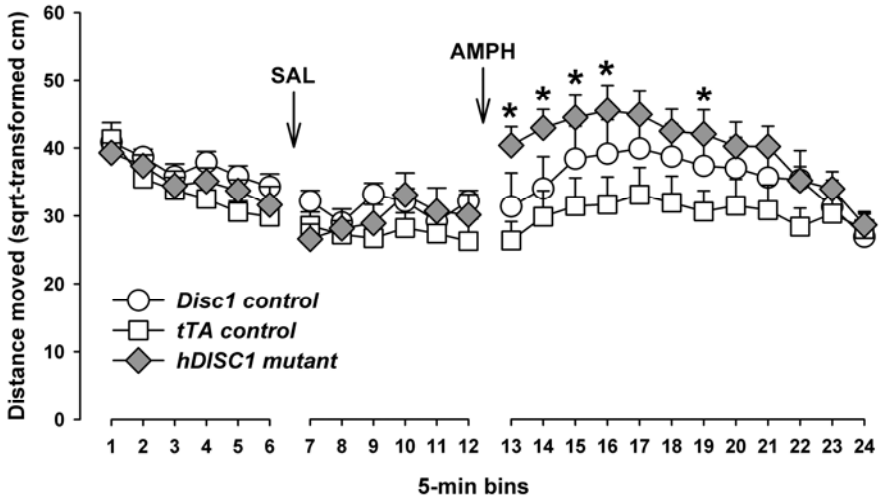

Figure 4.7. Locomotor reaction to systemic treatment with amphetamine (AMPH). The line plot shows locomotor activity in the open field expressed as distance traveled (cm) per 5-min bin during the initial habituation phase, following saline (SAL) administration, and following acute AMPH (1 mg/kg, i.p.) treatment. hDISC1 mutant mice and DISC1 displayed a significant overall increase in the distance moved following amphetamine treatment compared to drug-challenged tTA mice. All values are means±SEM. *P< 0.05 statistical significance is based on Fisher's LSD post hoc comparisons between the three genotypes. hDISC1 mutant, $n = 9$; DISC1, $n = 10$; tTA, $n = 10$

We also evaluated the behavioural sensitivity of the hDISC1mutant mice to treatment with the non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist MK-801 because patients with schizophrenia are known to display enhanced sensitivity to glutamatergic drug challenge (Krystal *et al.,* 1994; Lahti *et al.,* 2001). Genotype did not significantly affect spontaneous locomotor activity (bins: $F_{5,120} = 2.178$, $P = 0.061$). Locomotor response to saline injection was not significantly different between the three groups (bins: $F_{5,120} = 0.579$, $P = 0.716$) although there appeared to be increased activity in the hDISC1 mutant group (genotype: $F_{2,24} = 3.314$, $P = 0.054$). Analysis of locomotor response following a challenge with MK-801 revealed a significant main effect of bins ($F_{11, 264} = 16.612$, $P = 0.001$), bins x sex ($F_{11, 264} = 2.234$, $P = 0.013$) , bins × genotype interactions ($F_{22, 264} = 1.994$, $P = 0.006$) and bins × sex × genotype interactions ($F_{22, 264} = 2.043$, $P = 0.005$) but no main effect of genotype ($F_{2, 24} = 2.581$, $P = 0.097$). ANOVA split by sex revealed a significant main effect of bins (Females: $F_{11, 132} = 9.137$, $P = 0.001$; Males: $F_{11, 132} = 9.686$, $P = 0.001$) and bins × genotype interaction (Females: $F_{22, 132} = 2.441$, $P = 0.001$; Males: $F_{22, 132} = 1.630$, $P = 0.049$) in both sexes. Subsequent post-hoc analysis restricted to each of the 5 minute bins revealed that female hDISC1 mutant mice exhibited a statistically significant (p values < .05) enhanced locomotor reaction to MK-801 challenge in the third, fourth, fifth, sixth, seventh

and eighth 5 minute bins after drug injection in comparison with DISC1 controls. Although restricted analysis of the second bin revealed a significant difference in locomotor activity that was restricted to a difference between hDISC1mutant mice as compared to tTA control mice. In contrast, a significant difference in the locomotor activity following MK-801 challenge in male mice was only detected in the second 5 minute bin. This effect was restricted to enhanced locomotor activity in hDISC1mutant mice as compared to tTA control mice.

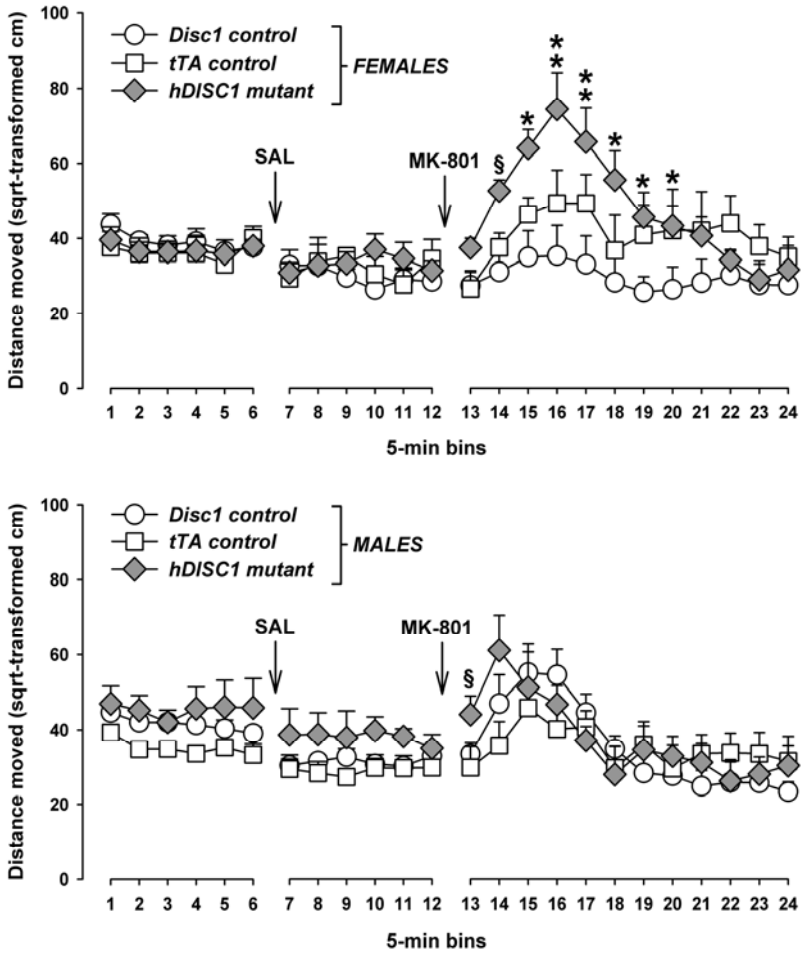

Figure 4.8. Locomotor reaction to systemic treatment with MK-801. The line plot shows locomotor activity in the open field expressed as distance traveled (cm) per 5-min bin during the initial habituation phase, following saline (SAL) administration, and following acute dizocilipine (0.3 mg/kg, i.p.) treatment. Female hDISC1 mutant mice displayed a significant overall increase in the distance moved following MK-801 treatment compared to drug-challenged DISC1 and tTA mice. All values are means±SEM. * P< 0.05 and ** P< 0.01 statistical significance is based on Fisher's LSD post hoc comparisons between the three genotypes. § P< 0.05 between hDISC1 mutant and control and tTA control. hDISC1 mutant, Males *n* = 5; Females *n* = 5, DISC1, Males *n* = 5; Females *n* = 5; tTA, Males *n* = 5; Females *n* = 5.

DISCUSSION

Using a double transgenic mouse model of inducible expression of mutant human DISC1, we have studied the impact of mutant human DISC1 expression on behaviours and psychomimetic responses relevant to schizophrenia. To date, six other independent DISC1 mouse models have been created (Clapcote *et al*., 2007; Hikida *et al*., 2007; Kvajo *et al*., 2008; Li *et al*., 2007; Shen *et al*., 2008) and their behaviours analyzed (Clapcote *et al*., 2007; Hikida *et al*., 2007; Ibi *et al*., 2010; Koike *et al*., 2006; Kvajo *et al*., 2008; Li *et al*., 2007; Lipina *et al*., 2010, 2011; Pogorelov *et al*., 2011; Shen *et al*., 2008). We found that hDISC mutant mice display normal habituation in the open-field and exhibit intact LI in both poles (i.e. attenuated and persistent LI). Sensory motor gating in the form of PPI was also spared. Compared with control mice, hDISC1 mutant mice exhibited increased sustained visual attention in a two-choice visual discrimination/detection test and displayed an increased sensitivity to psychomimetics acting on dopaminergic function. Specifically, response to the locomotor stimulating effects of MK-801 were facilitated in female hDISC1 mutant mice.

Heightened sensitivity to the induction of hyperactivity by psychotomimetic drugs, such as the dopamine agonist AMPH, has been considered an endophenotypic measure related to the positive symptoms of schizophrenia (Arguello and Gogos, 2006). Consistent with a recent study, hDISC1 mutant mice show enhanced susceptibility to AMPH-induced hyperactivity (Ayhan *et al*., 2011). This pattern of enhanced AMPH-induced locomotor activity is reminiscent the increased susceptibility to the disorganizing effects of AMPH observed in individuals with schizophrenia (Laruelle *et al*., 2003; Szeszko *et al*., 1999). Locomotor hyperactivity in response to AMPH is generally thought to be mediated by increased release of dopamine in mesocorticolimbic brain areas. Specifically, dopaminergic projections to the nucleus accumbens are believed to comprise the major neurphysiological substrate of AMPH induced locomotion (Costall and Naylor, 1977; Creese and Iversen, 1975).

The role of hDISC1 in the dopamine system appears to be complex since Ayhan *et al.* (2010) reported sex and region specific reductions in the levels of dopamine in hDISC1 mutant mice. Specifically, male hDISC1 mutant mice exhibited a reduction in dopamine and its metabolites in the frontal cortex. These observations are interesting given the intricate role that dopamine plays in schizophrenia. Notably, the cognitive and negative symptoms of schizophrenia have been linked to a hypodopaminergic state in the mesocortical projections whereas the positive symptoms of schizophrenia have been linked to enhanced dopamine transmission in the mesolimbic projections (Laruelle *et al*., 2003). Recently, Lipina *et al.* (2010) have shown in ENU-induced missense DISC (viz. L100P (334T/C); leucine → proline) mutants that not only do these mice exhibit a facilitation of the psychostimulant effect of AMPH in the open field but that these mice also exhibit increased expression of striatal dopamine D_2 receptors (Lipina *et al*., 2010). Interestingly, administration of the D_2 receptor antagonist haloperidol reversed the psychostimulant effects of AMPH in the mutant mice. Further, Niwa *et al*. (2010) reported that *in utero* knockdown of DISC1 in pyramidal neurons leads to selective abnormalities in postnatal mesocortical dopaminergic maturation in addition to behavioural abnormalities including sensitivity to methamphetamine. They also observed decreased dopamine content in the medial prefrontal cortex (Niwa *et al*., 2010). Although speculative, it may be that that hDISC1 directly affects dopaminergic transmission (either within the frontal cortex or in other brain areas that supply dopaminergic afferents) which may result in the emergence of abnormal behaviour.

This study also showed that female hDISC1 mice were more sensitive to MK-801-induced locomotor sensitivity. In addition, there were no differences between hDISC1 mice and control mice on baseline locomotion or following saline injection suggesting that the effect of the injection procedure could not have acted as a stressor to increase locomotor activity. The interaction between hDISC1 and sex on MK801-induced behaviour may have been the result of sex differences on pharmacokinetic mechanisms (Andine *et al*., 1999). Sex differences in MK-801-induced behaviour have been reported previously in the present transgenic DISC1 mouse model (Ayhan *et al*., 2011). In contrast to the present findings, the only significant differences observed following MK-801 challenge in the study by Ayhan *et*

al. were in male hDISC1 mutant mice. Consistent with the hDISC1 expression profile used in the present study, the psychostimulant effects of MK-801 observed in the study by Ayhan *et al.* were only detected in the group that expressed the mutant product throughout life (i.e. prenatal and postnatal expression of hDISC1). It may be possible that differences in data acquisition procedure (activity chambers with infrared beams as opposed to the in house Ethovision tracking system) and protocols adopted for the experiments mean may have affected the result turn out. Specifically, as opposed to the infrared light beam system which detects beams broken by the animal so that the software can determine the location of the rodent within the arena the Ethovision™ system allows precise video tracking of the mouse position throughout the experiment. Despite these differences, both these observations attest the dangers of generalizing conclusions to both sexes based upon data obtained in a single sex only.

It is thought that the MK-801-induced locomotor hyperactivity in rodents may be mediated by indirect facilitation of dopaminergic transmission in the nucleus accumbens (Kretchmer, 1999; Mathe *et al*., 1996) and the ventral tegmental area (Murase *et al*., 1993) caused by inhibition of the NMDA receptor and subsequent release of dopamine neurons from the inhibitory control of the glutamatergic system (Svensson *et al*., 1992). However, locomotor hyperactivity induced by NMDA antagonism in rodents is largely independent of dopaminergic activation. It follows that dopamine neurotransmission may not be necessary to sustain locomotor activation by this class of drugs. For example, phencyclidine-induced locomotor hyperactivity is temporally dissociated from its effect on dopamine release (Adams and Moghaddam, 1998). Given that DISC1 regulates the expression and function of several neurotransmitter receptor targets implicated in psychostimulant sensitization, including NMDA (Hayashi-Takagi *et al*., 2010; Wang *et al*., 2010) and dopamine (Marley and von Zastrow, 2010; Pogorelov *et al*., 2011), the mechanism underlying the enhanced locomotor response observed here in hDISC1 mutants is unclear. However, a sensitized dopamine system has been proposed to underlie the emergence of psychotic symptoms (the 'endogenous sensitization' hypothesis of schizophrenia) (Abi-Dargham *et al*., 1998; Glenthøj and Hemmingsen, 1997; Peleg-Raibstein *et al*., 2009). These findings suggest that perturbations

in DISC1 function may contribute to the development or expression of a more marked and possibly more persistent sensitization response, a neural process that has been linked to schizophrenia.

In recent years it has become increasingly clear that the deficits which characterize schizophrenia are often accompanied by dysfunction in certain aspects of attentional processing (Braff, 1993). Translational behavioural paradigms developed to examine this cognitive domain have therefore proven indispensible when applied to animal models of schizophrenia and related disorders. However, the assessment of attentional functioning in rodents has typically involved the use of behavioural tests (e.g. 5-choice serial reaction time task) requiring several weeks to months of training (Patel *et al.,* 2006; Pattij *et al*., 2007; Young *et al*., 2004). We report here that, compared with the control mice, hDISC1 mutant mice display better performance on a two-choice discrimination task that measures aspects of sustained visual attention. Training and testing on this task can be completed within only 2 weeks, and performance has been shown to be sensitive to both glutamatergic and cholinergic blockade (Bitanihirwe *et al*., 2011). All groups of mice readily acquired the visual discrimination rule, but hDISC1mutant mice appeared to show a better overall performance. This observation was reflected by a higher response accuracy in hDISC1 mutants across all the four different stimulus durations relative to the two control groups (**Figure 4.4**). These data provide the first evidence of hDISC1 on visual attentional functioning in mice. In this respect, it is worth noting that the increased sustained attention or 'hypervigilance' effects observed in hDISC1 mutant mice recapitulate some of the changes of visuospatial attention that have been reported in human subjects with anxiety disorder (Vasey *et al*., 1995, 1996). Notably, anxiety leads to increased sustained attention or hypervigilance for threat, a greater level of threat detection leads to increased anxiety which, in turn, leads to increased hypervigilance (Dalgleish *et al*., 2001). Although schizophrenia has been associated with the inability to filter out stimuli (Braff, 1993), the increased sustained attention observed in the hDISC1 mutant mice may actually represent a signature of anxiety vulnerability rather than schizophrenia. In line with this notion, recent evidence has implicated DISC1 in anxiety (Harris *et al*., 2010).

Prepulse inhibition is a neurobiological measure used to investigate sensory gating, the process of filtering irrelevant sensory information through cortico-striato-thalamo-cortical pathways (Swerdlow *et al*. 2001). The cognitive fragmentation often associated with psychotic episodes in schizophrenia patients are thought to arise from a deficit in PPI processing (Braff *et al*., 2001). Even though PPI deficit is not a unique endophenotype of schizophrenia, PPI is a translatable readout between human and animals to assess biological processes in psychiatric disorders. Mice that express mutant human DISC1 were therefore assessed for sensory motor gating deficits using this paradigm. Consistent with the original findings by Pletnikov *et al.* (2008) hDISC1 mutant mice displayed a normal startle response compared to control mice at all tested acoustic stimulus intensities (**Figure 4.3**). They also showed a normal PPI across all prepulse intensities used (**Figure 4.3**). Unlike the present findings, three other studies using different transgenic DISC1 mice have reported deficits in PPI (Clapcote *et al*., 2007; Hikida *et al*., 2007; Shen *et al*., 2008). This discrepancy might be due to the differences in methodology used to generate the transgenic mice in addition to an influence of the different genetic backgrounds on which the mice were created. Clapcote and colleagues generated spontaneous ENU-induced mutations in the DISC1 gene of C57BL/6 mice (Clapcote *et al*., 2007). In contrast, Hikida and colleagues generated a transgenic DISC1 mouse line in the C57Bl/6 background expressing a dominant negative form of DISC1 behind the ectopic CAMKII promoter limiting the expression of the transgene to the forebrain (Hikida *et al*., 2007). The Shen mouse model was generated under a C57Bl/6 BAC construct for gene targeting, thus using a mouse mutant DISC1 corresponding to the human mutation under the mouse DISC1 promoter and mimicking the native mouse DISC1 expression (Shen *et al*., 2008). However, the present DISC1 mouse line were generated using an inducible expression system and bred on a complex hybrid B6;SJL;CBA background.

In order to further assess attentional processing in hDISC1 mutant mice we conducted two LI paradigms to examine selective attention. Latent inhibition was evaluated using the conditioned freezing paradigm and active avoidance. In both tasks, hDISC1 mutant mice demonstrated performances comparable to control mice, suggesting intact LI in hDISC1 mutant mice. Two separate studies examined

the role of DISC1 in selective attention using ENU-induced missense DISC1 mutants (Clapcote *et al*. 2007) and BAC expressing DISC1 mice (Shen *et al*., 2008). In contrast to the results in hDISC1 mutant mice, these mice displayed deficits in LI, suggesting impaired selective attention in these mice (Clapcote *et al*., 2007; Shen *et al*., 2008).

One important difference between the present study and the previous studies evaluating the inducible mutant hDISC1 mouse model is the inclusion of the single transgenic DISC1 mice as an additional control group. In the original study by Pletnikov and colleagues they opted to solely use the tTA mice as a control group because the expression of the tTA protein can potentially affect mouse brain and behaviour development (McKinney *et al*., 2008). Even though the study by Pletnikov and colleagues did not reveal any significant differences between single DISC1 transgenic mice and wild-type mice in behavioural tests we felt it necessary to add this group as another control measure in order to dissect any potential behavioural differences between the single transgenic mice and the double mutant hDISC1 mouse that may result from a direct genetic manipulation. The performance of the tTA and DISC1 control groups were mainly similar across most behavioural tests suggesting that the genetic manipulation did not appear to affect these mice as compared to the hDISC1 mutants. In fact, the only difference that was observed between the control mice groups was during the psychomimetic drug challenges suggesting potential differences to pharmacological sensitivity of these single transgenic mice. These findings provide a rationale for the evaluation of the genetic manipulation on the phenotype of both the single transgenic animals used to generate a double transgenic mutant and their inclusions as control groups for a more effective comparison.

In conclusion, the results of this study indicate that hDISC1 mutant mice exhibit abnormalities on certain behavioural measures relevant to schizophrenia and mood disorders such as anxiety, including sensitivity to psychomimetics and hypervigilance, respectively. However, expression of mutant human DISC1 does not appear to affect information processing in the forms of LI and sensory motor gating. These results therefore suggest that hDISC1 mutant mice may not be ideal for studying information

processing deficits but that this model may prove more useful for investigating the neural basis of anxiety and enhanced psychomimetic drug sensitivities.

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CHAPTER 5.

Cognitive Flexibility and Executive Function in Mice that Express Mutant Human DISC1.

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[Personal contribution to the work: Designing the research, running the behavioural tests, analysis of the behavioural data, and writing the chapter]

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ABSTRACT

Disrupted-in-Schizophrenia-1 (DISC1), which has been implicated in the development of major mental illness including depression, anxiety, bipolar disorders and schizophrenia, is expressed widely throughout the brain and influences key neurodevelopmental processes such as neurite outgrowth, neurite extension and neuronal migration. The mutant human DISC1 (hDISC1 mutant) mouse exhibits a behavioural phenotype relevant for schizophrenia research, characterized by the development of locomotor hyperactivity, social withdrawal and working memory impairment. These behavioural deficits were accompanied by a massive enlargement of the cerebral ventricular system. As only limited data are available on cognitive performance of hDISC1 mutant mice, we conducted an assessment of these mice in a variety of paradigms, including spatial recognition memory in the Y-maze, spatial working memory on the cheeseboard, recognition memory using the novel object recognition task and cognitive flexibility in an operant based discrimination reversal learning task. Mice that expressed mutant human DISC1 displayed deficits in spatial recognition memory, object recognition memory and reversal learning although spatial working memory as assessed on the cheeseboard appeared spared. These findings indicate that DISC1 plays an integral role in cognition and present further behavioural validation of this genetic mouse model for the schizophrenia candidate gene DISC1.

INTRODUCTION

The Disrupted-in-Schizophrenia-1 (DISC1) gene is localized on chromosome 1q42, and was first identified in a large Scottish family with a high loading of major mental illnesses (St. Clair *et al*., 1990) Specifically, the (1;11)(q42.1;q14.3) translocation allele of the DISC1 gene associates with bipolar affective disorder, recurrent major depression and schizophrenia (Blackwood *et al*., 2000; Millar *et al*., 2001). Subsequent linkage and association studies have linked DISC1 gene variations to schizophrenia in diverse populations (Hamshere *et al*., 2005; Liu *et al*., 2006; Macgregor *et al*., 2004). Interestingly, DISC1 mRNA can be found in schizophrenia-relevant brain areas such as the prefrontal cortex and hippocampus—in both humans (Kirkpatrick *et al*., 2006; Lipska *et al*., 2006) and rodents (Schurov *et al*., 2004). Within the nervous system, DISC1 influences key neurodevelopmental processes such as synapse formation (Duan *et al*., 2007), axon guidance (Chen *et al*., 2011), progenitor proliferation, (Mao *et al*., 2009; Ishizuka *et al*., 2011) neuronal migration (Kamiya *et al*., 2005; Ishizuka *et al*., 2011), and the regulation of expression/activation of receptors e.g. N-methyl-D-aspartic acid (NMDA), all of which are thought to be involved in schizophrenia (Wang *et al*., 2010; Hayashi-Takagi *et al*., 2010). DISC1 also appears to be involved in neural plasticity via a number of mechanisms affecting glutamatergic neurotransmission (Hayashi-Takagi *et al*., 2010). Its involvement in processes such as the regulation of components of the post synaptic density and consequently of synapse form and function (Wang *et al*., 2010) in addition to its role in the structural change of dendritic spines—a key mechanism underlying long term potentiation (a synaptic model of memory)—suggests that DISC1 plays a role in cognitive processes (Hayashi-Takagi *et al*., 2010). Importantly, schizophrenia patients experience deficits in cognition, executive function, and memory processes (Heinrichs and Vaz, 2004; Heinrichs *et al*., 1998). Further, memory impairment is a robust and stable feature of schizophrenia that includes deficits in free recall, cued recall and recognition, and does not appear affected by potential moderating factors such as illness duration or severity of psychosis (Aleman *et al*., 1999).

The molecular mechanism of the DISC1 translocation is still uncertain and remains controversial. Possible outcomes include haplo-insufficiency or a dominant/negative product, or both, of the C-terminally truncated mutant protein encoded from exons 1 to 8 (Sawa and Snyder, 2005). In this context, mutant DISC1 has been shown to disrupt formation of the DISC1–NUDEL–LIS1 protein complex, leading to abnormalities in neuronal migration (Kamiya *et al*., 2005), decreased complexity of dendrite arbors and neurite outgrowth (Pletnikov *et al*., 2007), reminiscent of the findings from postmortem schizophrenia brain samples (Buchanan and Carpenter, 1997; Harrison, 2004; McCullumsith *et al*., 2004). Importantly, the mouse model for mutant human DISC1 (hDISC1mutant) shows a schizophrenia-relevant behavioural phenotype, which is characterized by sex specific changes in working memory deficits, sensitivity to psychotimulants (Ayhan *et al*., 2011; **CHAPTER 4**), social interaction (Pletnikov *et al*., 2008; Ayhan *et al*., 2011), hypervigilance (**CHAPTER 4**), increased susceptibility to environmental risk factors such as prenatal immune activation (Abazyan et *al*., 2010) and decreased levels of cortical dopamine levels (Ayhan *et al*., 2011). Furthermore, mutant human DISC1 exerts differential effects on behavioural and neuroanatomical phenotypes, depending on the stage of development at which the protein is expressed during neurodevelopment (Ayhan *et al*., 2011). Despite cognitive deficits being one of the key features of schizophrenia in humans, there has been limited characterization of cognitive abilities in the hDISC1 mutant mouse model (Arguello and Gogos, 2010). To extend the understanding of the role mutant human DISC1 plays in cognitive processes, we examined the hDISC1 mutant mouse model in a battery of cognitive tasks. Using a comprehensive behavioural test battery, which included the Y-maze, an operant based discrimination reversal learning task, cheeseboard and object recognition we investigated the impact of mutant human DISC1 on spatial recognition memory, cognitive flexibility, spatial learning and memory and recognition memory in adult mice.

MATERIALS and METHODS

Animals and Husbandry

The generation of hDISC1 mutant mice has been described previously (Pletnikov *et al*., 2008; Pletnikov, 2009). Briefly, the gene of interest is cloned under control of the tetracycline responsive element (TRE), whose activation is dependent on binding of tetracycline-controlled transcriptional activator (tTA) (See **Figure 5.1**). Binding of tTA to TRE, and consequently transcription of the downstream gene (i.e. DISC1), can be prevented by interaction of tTA with tetracycline. DISC1 and tetracycline-transactivator (tTA) (The Jackson Laboratory, Bar Harbor, ME) mice were mated heterozygously. In the present study, double transgenic DISC1/tTA mice were included in the mutant group (referred to as hDISC1 mutants), whereas their single tTA transgenic and single DISC1 littermates were included in the control groups. Genotypes were determined after weaning (postnatal day 21) by polymerase chain reaction amplification (primers for DISC1 heterozygous mice: forward-5′GACCTCCATAGAAGACACCGGGAC-3′ and reverse-5′TGAGCTGAATCCCAAAGTGCGCCG-3′; for tTA heterozygous mice: Whereas forward-5′-CGCTGTGGGGCATTTTACTTTAG-3′ and reverse-5′-CATGTCCAGATCGAAATCGTC-3′). The mice had free access to food (Kliba 3430, Klibamühlen, Kaiseraugst, Switzerland) and water except during the discrimination reversal learning and cheeseboard tasks, during which mice were restricted to 1 hr of food per day to maintain 85% of their body weight. Each genotype consisted of subjects derived from multiple independent litters. The vivarium temperature was maintained at 21 ± 1°C, with humidity at 55 ± 5%. The holding facility was maintained under a reversed light-dark cycle (lights off: 700 to 1900h) with all behavioural tests being conducted during the dark cycle. All procedures described in the present study had been approved previously by the Zurich Cantonal Veterinary Office and are in agreement with the *Principles of Laboratory Animal Care* (National Institutes of Health publication number 86-23, revised 1985).

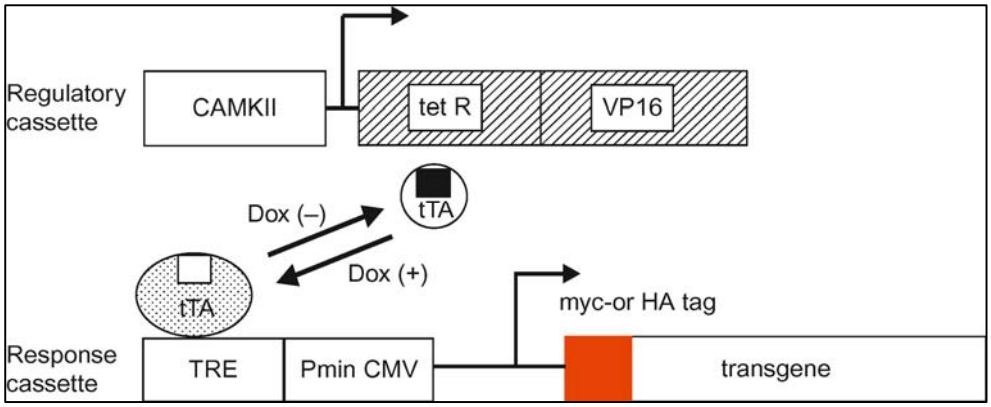

Figure 5.1. Schematic representation of the Tet-Off system. A tetracycline (Tet) system is composed of two distinct expression cassettes, one containing the regulatory protein (i.e. tetracycline transactivator, tTA) under the control of a cell- type-specific promoter (e.g., the CAMKII promoter) and the second response cassette containing the tetracycline responsive element (TRE) fused to a minimal promoter (e.g., cytomegalovirus, CMV), regulating the expression of the transgene that is usually fused to a tag for easy detection of transgene expression. The chimeric protein tTA consists of the tetracycline repressor (TetR) fused to the viral transactivator VP16. In the absence of Doxycycline (Dox), the tTA protein binds to the TRE within the compound promoter PminCMV to allow the expression of the transgene. In the presence of Dox, the tTA changes its conformation, gets detached from the TRE and no transgene expression is observed. (Source: Pletnikov, 2009)

Spatial novelty preference in the Y-maze

The apparatus was made of transparent Plexiglas and consisted of three identical arms radiating from a central triangle and spaced 120º from each other. The test of spatial recognition memory in the Y-maze consisted of two phases, namely the sample and choice phases.

Sample phase. The animals were allowed to explore two arms (referred to as 'start arm' and 'familiar arm'). Access to the remaining arm ('novel arm') was blocked by a barrier wall door. To begin a trial, the subjects were introduced at the end of the start arm, facing the central triangle and were allowed to freely explore both the start and familiar arms for 5 min. The subject was then removed and kept in a holding cage during the specific retention intervals (see below) prior to the choice phase.

Choice phase. Following a specific retention interval (see below), the test subjects were reintroduced to the maze. During the choice phase, the barrier wall was removed so that the animals could freely explore all arms of the maze for 2 min. On each trial, the time spent in each of the three arms was

recorded. The percentage time spent in the novel arm during the choice phase was calculated by formula: (1) *time spent in the novel arm/time spent in all arms x 100* and used as the index for spatial novelty preference. In addition, total distance moved on the entire maze was recorded and analyzed in order to assess general locomotor activity. To manipulate the retention demand in the temporal domain, the interval between the two phases (i.e. sample and choice phases) of the Y-maze test were varied. To start off a minimal inter-trial interval (ITI) of 2 min was assessed. The interval between the two phases was then increased to 15 min and finally 30 min.

Spatial working memory in the cheeseboard maze

Food restriction procedure. Food restriction was gradually introduced by progressively reducing the available feeding time across 5 days (12 hr, 8 hr, 4 hr, 3 hr, 1 hr). At the same time, the animals were familiarized with the liquid reward (20% condensed milk solution, Milch Lait, Schweizer Milchproduzenten, Switzerland) in the home cage to minimize food neophobia. Afterwards, daily food rations (food pellets, Kliba 3430, Klibamühlen, Kaiseraugst, Switzerland) were calculated as a function of each animal's weight loss/gain from the previous day in order to maintain a stable weight of not less than 85% of the ad lib weight. Drinking water was freely available throughout.

Training and working memory assessment with variable inter trial intervals. The apparatus consisted of a wooden circular board containing 32 holes which were arranged in a radial design (Llano *et al*., 2010). Following the initial habituation (days 1–5) and pretesting (day 6) phases, working-memory testing was started. The working memory task was based on the matching-to-position paradigm, in which food-deprived animals were required to learn the novel position of a rewarded hole revealed to them on trial 1 of each day in order to navigate effectively to the same location (i.e. matching) on the subsequent trial on the same day. Hence, each test day included two trials. The reward remained in the same position across trials on a given test day, but took a new position on each test day. The daily change of the reward

location ensured that the information acquired on a given day was irrelevant for the next day, thus taxing the flexible use of working memory (Baddeley, 2003).

To manipulate the retention demand in the temporal domain, the ITI varied between trials 1 and 2. First, a minimal ITI of 2 min was used for seven consecutive days (days 7–12), the ITI was then increased to 15 min on the next seven testing days (days 13–19) in order to assess working-memory function in the test groups. Working memory was indexed by the reduction in latency and distance moved to find the location of the reward in trial 2 relative to trial 1. The interval between the two phases was then increased to 15 min in accordance with the extended (15 min) retention interval used for the working memory test in the Y-maze.

Object recognition

Object recognition memory was evaluated 1 week following completion of working-memory test in the cheeseboard maze. The procedure described by Singer *et al.,* (2007) was followed to test novel object recognition memory in mice. Briefly, a rectangular arena ($L \times W \times H$: 61 × 40 × 22 cm) made of transparent Plexiglas was placed in a quiet room. The arena rested on a table top elevated 100 cm above the floor in the center of a testing room with an ambient light level of ~100 lux. First, mice of each genotype were individually placed in the arena for 10 min each day for 2 days with two identical objects (objects with various shapes) to acclimatize them with test condition. On the test day, the mice were placed in the arena with two new identical objects and allowed to explore the objects for 10 min (Familiarization phase). The mice were then removed and placed back into a holding cage for 2 min or 2 hr (retention time) while the objects were replaced with two new objects; one of the new objects was identical in shape to the objects used during the familiarization phase and the other was of a novel shape. The mice were then reintroduced to the arena after a retention time of 2 min or 2 hr and allowed to explore the objects for 6 min (Testing phase) (**Figure 5.2.**). Behavioural activity during the familiarization and testing phases were videotaped and analyzed by an observer. A mouse was considered

to be involved in object exploration when the animal had at least one forepaw on the object or was actively engaged in sniffing or licking the object.

Preferential exploration of the novel objects in the tests was expressed using a discrimination ratio: exploration time of novel object ÷ sum of both novel and familiar object exploration times * 100. Rodents have a natural tendency to explore novel objects in their surrounding compared to objects already familiar. Increased time of exploration of novel object, i.e., a value significantly different from 50% (chance level), reveals the rodent analogue of declarative memory.

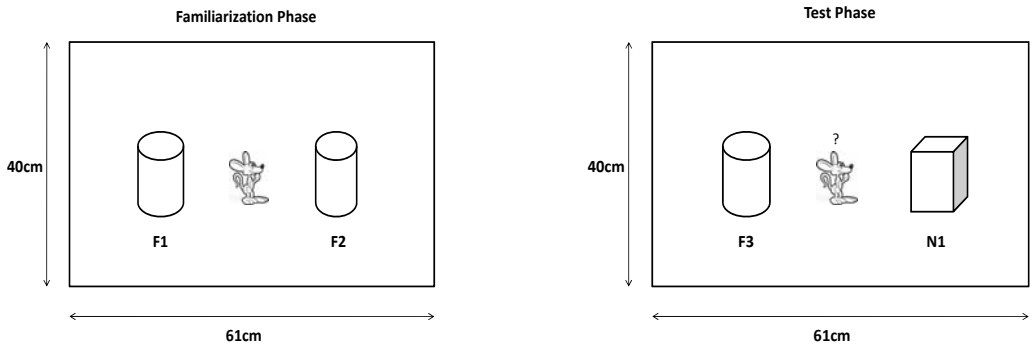

Figure 5.2. Schematic representation of the novel object recognition experimental procedure. The mouse was allowed to explore two identical objects (F1 and F2) for 10 min on the Familiarization phase. The test trial was conducted 2 min–2 hr after the acquisition trial. During the test phase two new objects; one of the new objects was identical in shape (F3) to the objects used during the familiarization phase and the other was of a novel shape (N1).The preference index, indicating the time spent exploring the novel object, was expressed as the ratio of the total time spent exploring both objects.

Discrimination reversal

Apparatus. Four operant chambers (Model E10-10, Habitest System, Coulbourn Instruments, Allentown, PA, USA) were used, each placed inside a ventilated and sound-attenuated box. The chambers measured 33 cm in height with a Plexiglas wall positioned 13 cm away from the panel wall, creating a floor measuring 13 × 25 cm. A 2.8 W house light was installed in the middle of the panel wall, 23 cm above the steel grid floor and pointing upwards at the ceiling to provide diffused ambient illumination inside the chamber. Two reward dispensing magazines and one retractable lever were mounted within the panel wall. The retractable lever (Model H21-03 M) was 2.4 cm above the grid floor, positioned in-between the two magazines. When extended into the chamber, it was illuminated by a 2.8 W stimulus light mounted 10 cm directly above it. The two identical magazines (Model H14-22M-20) were positioned 13.2 cm apart from each other, 2 cm above floor level. A 1.4 W stimulus light was installed inside each magazine. When switched on, this provided a discrete illumination of the inside of the magazine and served as the signal cue in the discrimination reversal task. Nose-poke into the magazines was detected by an infrared photocell beam placed at the magazine's entrance. Each magazine was equipped with a liquid dipper dispenser (Model H14-05R) capable of delivering 0.01 ml of liquid reward (20% dilution of the commercial condensed milk, Milch Lait, Schweizer Milchproduzenten, Switzerland). Stimulus and operant contingency was controlled by Coulbourn Instruments Graphic State (Version 1.013) software, which also stored all session's data in separate time-stamp events files.

Food restriction procedure. During the course of the experiment, the animals were maintained on a 23 hr food deprivation regime, with their body weight reduced to no less than 85% of their ad lib weight. Water was available throughout.

Habituation. The animals were habituated to the operant chambers over four consecutive days and acclimatized to consuming the liquid reward from the dippers. The dipper of one of the two magazine trays was programmed to be raised and illuminated by the magazine light until a nose poke into the

magazine was detected, after which time the dipper remained raised for 3 s allowing access to the liquid reward. This procedure was repeated, with a variable inter-trial interval (ITI) of 15±5 s and a pseudorandom schedule under which one of the two reinforcer magazines was illuminated. The lever remained retracted and the house light was switched on during the entire session, which ended either when 18 min had elapsed or 60 rewards had been collected. All animals collected at least 40 rewards on the last day.

Lever press shaping. Shaping training began 24 hrs after the fourth habituation session. During shaping the animals were trained for six days to press the lever to obtain the liquid reward. Each trial began with the presentation of the lever. A single lever-press was followed by its immediate retraction and the illumination of the magazine tray of one randomly selected magazine, until a nose poke into the magazine was detected, at which time the dipper was raised for 3 s allowing access to the liquid reward. The next trial began after a 10 s interval. Only one magazine (left or right) was used for each animal's daily session, with access to the other magazine blocked by a metal plate. Training resumed on the following day with the other magazine tray. This minimized the possible development of a side bias. On each day, half of the animals were trained using the left magazine and the remaining animals using the right magazine. The house light remained on throughout the session, which ended after 24 trials were completed or when 25 minutes had elapsed. All animals had completed at least 19 trials on the last training day.

Acquisition training. Next, the animals were trained to earn a reward by making a nose-poke into the magazines in a trial-discrete manner. A trial began with the presentation of the lever, and the pressing of the lever triggered the illumination of both of the magazines. The identities (left or right) of the positive magazine were counterbalanced within each group. Upon a single nose-poke in to one of the magazines, the mouse was rewarded by a 3 s access to the liquid reward only if the assigned positive magazine was selected. Otherwise, the trial ended without any other consequence, and the session entered into an ITI of 20 s. If the animal failed to make a nose-poke within 30 s of the illumination of the

magazines, the trial would be aborted and the session entered into an ITI. The session ended when the animals completed 16 trials. The criterion of successful discrimination was set at 14 or more correct choices across three consecutive days.

Reversal learning. Following acquisition training the reward valence of the two magazines was reversed. Reversal learning continued until the animals again achieved the criterion performance (i.e. 14 or more correct choices on three consecutive days). Errors to criterion in the acquisition and in the reversal phase were taken as the dependent measures.

Statistical Analyses

All data were analyzed by parametric analysis of variance (ANOVA) using the between-subject factors, genotype, in combination with drug and pre-exposures as appropriate. Additional within-subject factors were also included according to the nature of the considered dependent variables. To assist interpretation of the statistical outcomes, significant effects were further investigated by restricted ANOVAS whenever appropriate. Statistical analyses were conducted using the Statistical Package for the Social Sciences (SPSS) software (version 13.0) implemented on a personal computer running the Windows XP operating system. Statistical significance was set at $P < 0.05$.

RESULTS

Y-maze

To examine spatial recognition memory in hDISC1 mutant, DISC1 and tTA control mice, the relative time spent in the novel (previously unexplored) arm during the choice phase of the Y-maze task across the three delays was assessed. The 3 x 3 x 2 (delays x genotype x sex) ANOVA of the percent time spent in the novel arm [as calculated by formula (1) above] revealed a significant main effect of delays representing a decline in spatial recognition performance in the three groups of mice as the delay between trials was increased ($F_{2,108} = 3.245$, $P = 0.043$). A significant genotype effect was observed ($F_{2,54} = 4.370$, $P = 0.017$), as hDISC1 mutant mice appeared to display a reduced preference towards the novel arm as opposed to the previously explored arms with increasing delays when compared with DISC1 and tTA control animals (**Figure 5.3a**). However, this assumption was not supported by a significant interaction between delays and genotype ($F_{4,108} = 0.425$, $P = 0.790$). There was no effect of either sex ($F_{1,54} = 0.518$, $P = 0.475$) or interaction between sex and genotype ($F_{2,54} = 1.415$, $P = 0.252$) in the Y-maze task.

The additional analyses of total distance moved during the choice phase of the 2-min, 15-min and 30-min retention tests showed that general locomotor activity was highly comparable between the three genotypes (**Figure 5.3b**). Similarly, locomotor activity did not differ between the two groups during the sample phase of the test. Hence, the disruption of spatial novelty preference observed in the hDISC1 mutant mice compared to the DISC1 and tTA control groups could not be accounted for by possible confounds resulting from differences in general locomotor and exploratory activity in the Y-maze.

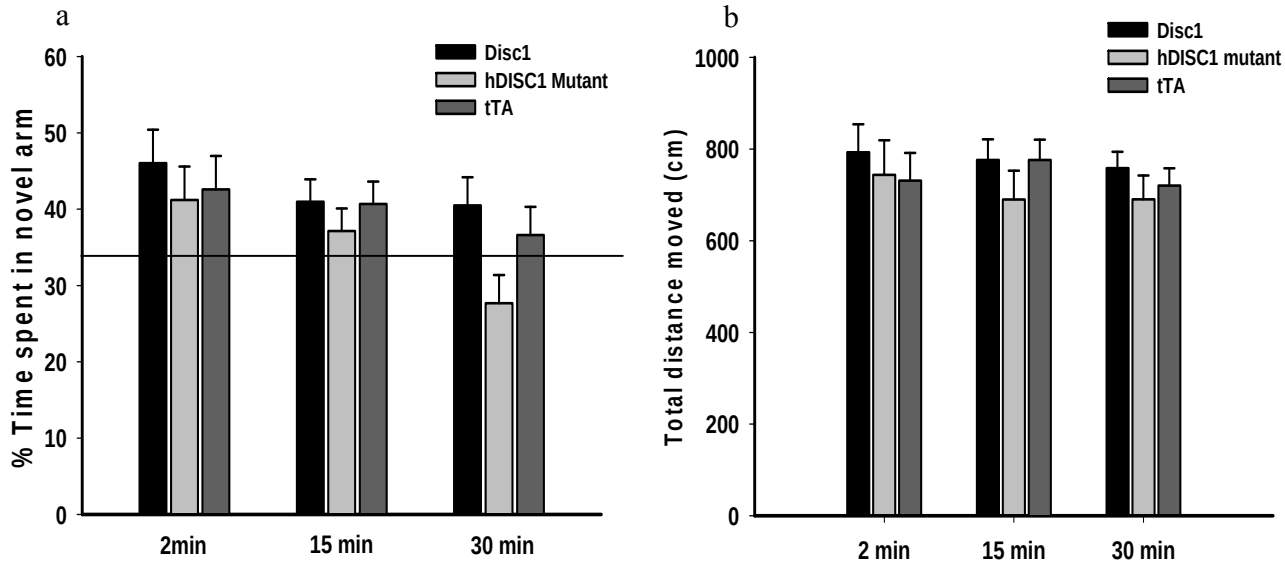

Figure 5.3. Expression of mutant human DISC1 leads to a disruption of spatial recognition memory in the Y-maze. (a) The graph depicts the percent time spent in the novel (previously unexplored) arm during the choice phases of the 2 min, 15 min and 30 min retention interval tests. Note that all groups clearly performed above chance level (indicated by the line) when they were tested using a minimal and 15 retention interval. However, hDISC1 mutant mice appear to exhibit poorer performance in spatial recognition memory relative to the control groups at the 30 min interval. The line indicates chance level (i.e. 33%) (b) The graph depicts the total distance moved on the Y-maze during the choice phases of the 2 min, 15 min and 30 min retention interval tests. There were no group differences in this measure at all three retention intervals used. All values are means±SEM. DISC1 control, n = 20 (10 male, 10 female), hDISC1 mutant n = 20 (10 male, 10 female), tTA control, n = 20 (10 male, 10 female).

Spatial working memory in the cheeseboard maze

The critical measure of working memory in the matching-to-position paradigm of the cheeseboard task is the reduction in time spent and/or distance moved to find the location of the rewarded hole from trial 1 (when the location of the platform was essentially unknown to the subjects) to trial 2.

The 3 x 2 x 2 x 7 x 2 (genotype x sex x trials x days x delay) ANOVA of latency to retrieve the liquid reinforcer reward revealed a significant interaction of delays and treatment (latency: $F_{2,23}$ =2.482, P = 0.016) although this interaction did not reach statistical significance for the factor of distance travelled (distance : $F_{2,23}$ =4.975, P = 0.106). Restricted analysis of the function of delay showed that there were no

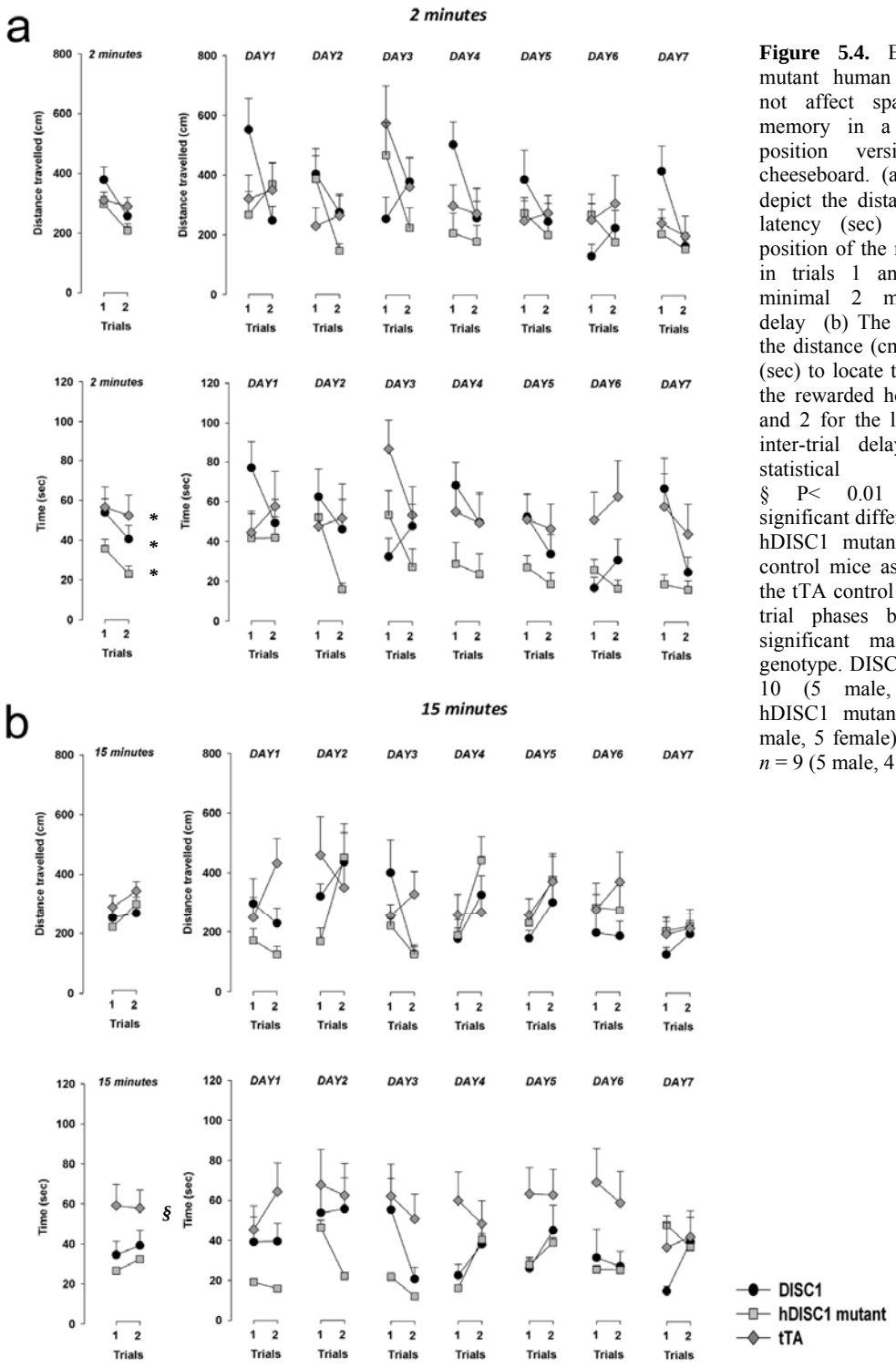

Figure 5.4. Expression of mutant human DISC1 does not affect spatial working memory in a matching-to-position version of the cheeseboard. (a) The graphs depict the distance (cm) and latency (sec) to find the position of the rewarded hole in trials 1 and 2 for the minimal 2 min inter-trial delay (b) The graphs depict the distance (cm) and latency (sec) to locate the position of the rewarded hole in trials 1 and 2 for the longer 15 min inter-trial delay. *P< 0.05 statistical significance. § P< 0.01 reflect the significant difference between hDISC1 mutant and DISC1 control mice as compared to the tTA control group in both trial phases based on the significant main effect of genotype. DISC1 control, *n* = 10 (5 male, 5 female), hDISC1 mutant *n* = 10 (5 male, 5 female), tTA control, *n* = 9 (5 male, 4 female).

signs of working-memory impairment in hDISC1 mutants relative to the two control groups when the animals were tested in trials presented with a minimal 2 min interval between trials 1 and 2. Hence, male

and female offspring from all three groups displayed a clear reduction in the latency to find the rewarded hole in trial 2 relative to trial 1 (**Figure 5.4a**). The 3 x 2 x 2 x 7 (genotype x sex x trial x day) ANOVA of latency only revealed a significant main effect of trials (latency: $F_{1,138}$ =12.421, P = 0.002).

Subsequent restricted analysis of the 3 genotypes at the 15 min delay showed that when a longer ITI was interposed none of the groups showed a significant reduction in latency from trials 1 to 2. Statistical support for these impressions was obtained by the 3 x 2 x 2 x 7 (genotype x sex x trials x days) ANOVA of latency in the 15-min test condition which revealed no significant effect of trials ($F_{1,23}$ = 0.763, P = 0.392) or interactions between trials and genotype ($F_{2, 23}$ = 0.421, P = 0.661). Interestingly, a significant genotype effect was observed ($F_{2,23}$ = 5.692, P = 0.010), as tTA mice took statistically longer to retrieve the liquid reward compared with hDISC1 mutant and DISC1 control animals across both trial phases (**Figure 5.4b**).

Object recognition

Object recognition memory was assessed at two different delays: 2 min or 2 hr. Two separate tests were conducted for each delay condition to enhance reliability (Hale and Good, 2005). The data were averaged across the two tests of each delay condition before being submitted to statistical analysis. All groups exhibited a positive preference for the novel object at the shortest retention delay of 2 min (exploration time: $F_{1,23}$ = 22.872, P = 0.001, **Figure 5.5a**), but only the DISC1 and tTA mice were able to maintain this up to a delay of 2 hr (**Figure 5.5b)**. This phenotype was similarly observed in male and female mutant mice. These impressions were confirmed by a 3 x 2 x 2 (Genotype x Sex x Delays) ANOVA of the discrimination ratio, which yielded a significant effect of genotype ($F_{1,23}$ = 13.807, P = 0.001) and a significant effect of genotype and delays ($F_{1,23}$ = 11.461, P = 0.001). Separate ANOVAs restricted to each delay condition indicated that the hDISC1 mutant and control mice differed significantly only in the 2-hr delay condition ($F_{2,23}$ = 23.443, P = 0.001). Neither the factor sex nor its interaction attained statistical significance.

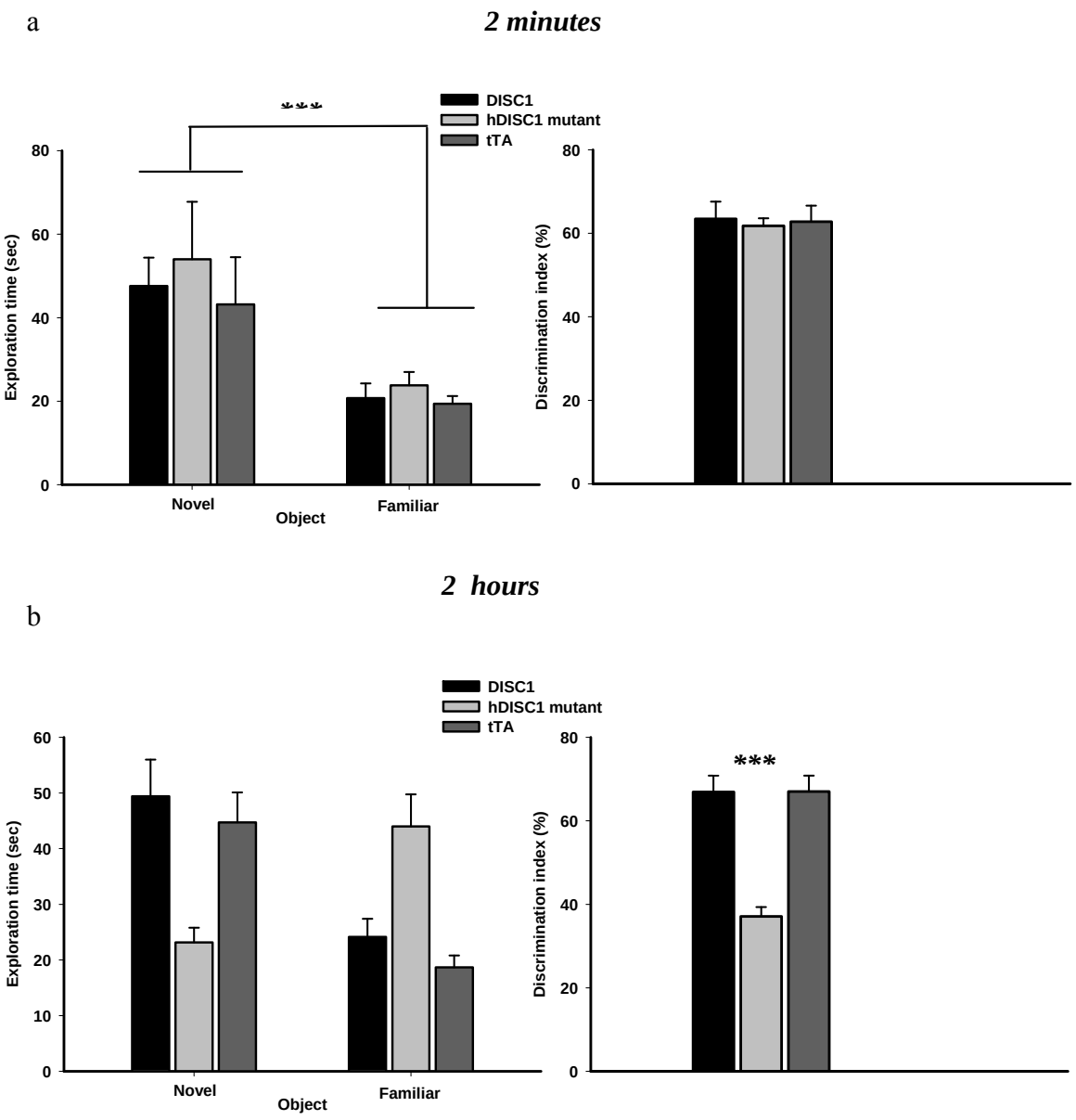

Figure 5.5. Performance of DISC1, tTA and mutant mice in the novel object recognition task assessed over a 2 min (a) and a 2 hr (b) retention interval. Mutant mice exhibit a significantly reduced preference for the novel object at the two hour retention interval in comparison to the DISC1 and tTA control mice. *** P< 0.001 statistical significance. DISC1 control, *n* = 10 (5 male, 5 female), hDISC1 mutant *n* = 10 (5 male, 5 female), tTA control, *n* = 9 (5 male, 4 female).

Reversal learning

Discrimination reversal learning was assessed using an operant task in which animals had to learn to nose poke in to one of the two illuminated magazines, the animals were then required to learn to

switch to the previously unrewarded magazine. A deficit in the reversal phase, but not in the acquisition phase, is indicative of perseverative behaviour, as has been implicated in schizophrenia (Ridley, 1994). A 3 x 2 x 2 (genotype x sex x phase) ANOVA revealed a significant effect of phase ($F_{1,21}$ =8.934, P = 0.007) and an interaction of genotype and phase ($F_{2,21}$ =4.536, P = 0.023). Notably, behavioural perseveration was evident in the hDISC1 mutants and DISC1 control groups but not the tTA control group (**Figure 5.6)**. hDISC1 mutant and DISC1 control animals were uniquely impaired in the reversal phase. One-way ANOVA of the number of errors to criterion in the reversal phase yielded a clear main effect of genotype ($F_{2,21}$ = 3.538; P = 0.047) that is solely attributed to the perseverative performance of the hDISC1 mutant (phase: $F_{1,8}$ = 55.613; P = 0.001) and DISC1 control (phase: $F_{1,7}$ = 6.681; P = 0.0362) groups as compared to the tTA control group (phase: $F_{1,6}$ = 0.382; P = 0.559). No group difference was detected in the equivalent measure at the acquisition phase ($F_{2,21}$ = 2.036; P = 0.155), supporting the interpretation that the deficits observed in hDISC1 mutants and DISC1 control mice did not stem from a general learning impairment.

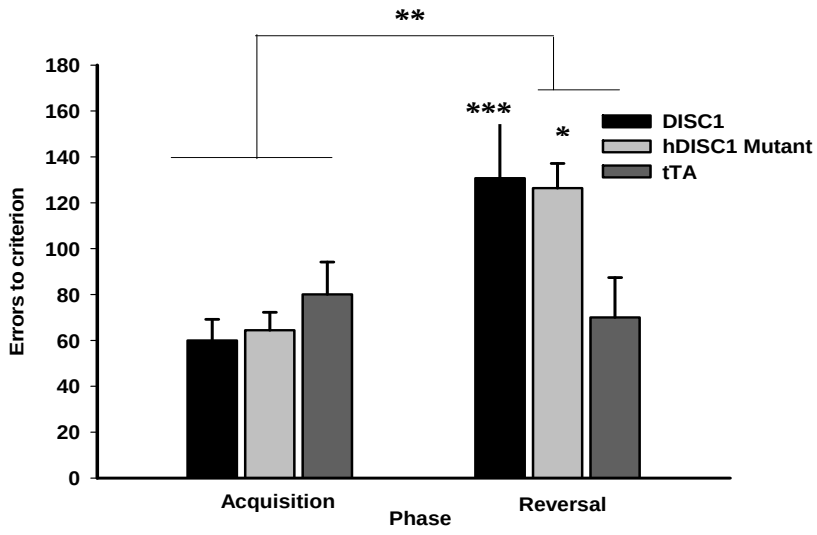

Figure 5.6. Performance in the acquisition and reversal phase of the discrimination reversal experiment is indexed by the number of errors required to achieve criterion performance. hDISC1 mutants and DISC1 control mice exhibited preservative behaviour. Restricted analysis contrasting their acquisition and reversal performance revealed that hDISC1 mutant and DISC1 control mice exhibit cognitive inflexibility. In contrast tTA control mice exhibited intact reversal performance. *P<0.05, **P<0.01, ***P<0.001 statistical significance. DISC1 control, n = 9 (5 male, 4 female), hDISC1 mutant n = 10 (5 male, 5 female), tTA control, n = 8 (5 male, 3 female)

DISCUSSION

We report here that expression of mutant human DISC1 leads to domain-specific cognitive deficits in spatial recognition, cognitive flexibility and recognition memory in mice. These deficits were apparent in distinct behavioural tasks, i.e., tests sensitive to novel spatial preference, discrimination-reversal learning and object recognition memory. However, learning impairments were not universal, as hDISC1 mutant mice showed intact spatial working memory abilities on the cheeseboard task.

Cognitive assessment of mice in the Y-maze revealed that mutant mice exhibited a deficit in the novel spatial recognition version of the Y-maze, indicating a deficit in spatial recognition memory in these transgenic mice. Although there appeared to be a trend towards a reduction in time spent exploring the novel arm in the hDISC1 mutant mice compared to the DISC1 and tTA controls as longer delays were interposed between trials this observation could not be confirmed statistically (**Figure 5.3a**). Despite the lack of a significant interaction the data does suggest a decrease in the mnesic trace of spatial information in hDISC1 mutant mice. Although potential confounds cannot be totally excluded, the deficit appeared to be specific, as the difference in time spent exploring the novel arms could not be accounted for by distance travelled or speed differences between groups. The impairment in spatial recognition in hDISC1 mutant mice observed here have not been previously demonstrated. This finding indicates that mutant human DISC1 plays a specific role in spatial recognition memory formation in mice.

Declarative memory assessment of mutant human DISC1 expression in the novel object recognition test showed that hDISC1 mutant animals spent significantly less time exploring the novel object compared to the control groups after the 2 hr retention interval indicating impairment in long-term object recognition memory in mutants. In this context, the hippocampus has been assumed to be critical for the encoding and expression of relationships between items of memory (Eichenbaum, 1999). It also

plays an integral role in the short-term consolidation of spatial information in addition to supervising the consolidation of spatial memory in the entorhinal cortex before some transfer occurs to the neocortex for permanent storage (Cho and Kesner, 1996). Besides the hippocampus, other brain regions of the medial temporal lobe are believed to be essential for declarative memory (Squire and Zola, 1996). Cognitive deficits such as impaired declarative memory are believed to be the most enduring feature of schizophrenia, and are believed to be a better predictor of quality of life than positive and negative symptoms (Sharma and Antonova, 2003; Williams *et al*., 2008). Deficits in cognitive functions related to prefrontal cortex and hippocampus/medial temporal lobe (recognition memory and other forms of declarative memory) have all been described in schizophrenia subjects (Cirillo and Seidman, 2003; Pelletier *et al*., 2005). Thus, deficit in object recognition memory in this simple model of declarative memory dependent on hippocampal and parahippocamapal functions (Moses *et al*., 2005; Murray *et al*., 2000) may be consistent with reports showing association of variations in DISC1 to human cognitive performance (Calicott *et al*., 2005; Prata *et al*., 2008; Takahashi *et al*., 2009; Chakirova *et al*., 2011).

Reversal learning using an operant task showed that hDISC1 mutant and the DISC1 groups exhibit cognitive inflexibility. While the hDISC1 mutant and DISC1 control mice readily learned the discrimination rule, when the reinforcement contingencies were inverted, the hDISC1 mutant and DISC1 control mice were impaired in their ability to inhibit the previously reinforced behavioural strategy. Notably, reversal learning requires flexible switching between cues within the same modality or perceptual dimension, and taxes a variety executive functions mediated by the prefrontal cortex, including working memory, attention and response inhibition (Roberts, 2006), all processes known to be affected in schizophrenia. In this context, the capacity for our in house operant discrimination reversal task to concurrently assess reversal learning in addition to discrimination provides an excellent internal control, not only for learning and behavioural performance generally, but also because discrimination is impaired in some cases of schizophrenia. For example, discrimination and reversal learning has been reported to be significantly impaired in chronic hospitalized schizophrenic patients with frontal lobe damage (Pantelis *et*

al., 1999). The impairment in reversal learning in hDISC1 mutant mice observed here has not been previously demonstrated and therefore suggests that DISC1 exerts a significant role in cognitive flexibility in mice.

The cognitive impairments seen in hDISC1 mutant mice were not universal, as no genotype differences in spatial working memory were seen on the cheeseboard. Indeed, mutant mice showed significantly better performance on the cheeseboard task than the tTA control group. The intact spatial learning and memory on the cheeseboard is not consistent with a previous investigation of hDISC1 mutant mice utilizing the Morris water maze (Pletnikov *et al*., 2008). This study showed a sex specific deficit of spatial working memory in female hDISC1 mutant mice. This discrepancy may be due to differences in methodology. For example while the water maze paradigm requires the animal works to escape from a mildly aversive situation (i.e. water), on the cheeseboard the animal works for a food reward. Furthermore, it is possible that the in house reverse dark/light cycle system which promotes the behavioural experimention of animals during the dark phase of the cycle (seeing as *mus musculus* is a nocturnal species) may have played a role in these differences. In this respect, the reverse light cycle allows the investigator to test the mouse during its night phase, when mice are most active, rather that during its day phase, when mice normally sleep (Crawley, 2007). Another potential discrepancy may result from the mixed strain on which the hDISC1 mutant were bred. Notably, performance on the cheeseboard has been validated in house by using the C57BL/6 strain (Llano *et al*., 2010; **CHAPTER 2**) so it may be possible that the mixed strain on which hDISC1 mutant mice have been generated may have affected the performance on the cheeseboard task. In this respect, it important to emphasize that strain differences in general behavioural activity have been reported before (Carola *et al*., 2002; Contet *et al*., 2001; Sik *et al*., 2003).

The DISC1 gene is temporally and spatially expressed during development in multiple populations of neurons (James *et al*., 2004) but not in astrocytes (Shurov *et al*., 2004). It is broadly

expressed in olfactory bulb, cortex, hippocampus, hypothalamus, cerebellum and brain stem (Shurov *et al*., 2004; Austin *et al*., 2003; Kirkpatrick *et al*., 2006). Specifically, DISC1 is expressed throughout the cortical layers in addition to axonal terminals and postsynaptic targets (Kirkpatrick *et al*., 2006). Thus, DISC1 could impact many of the key neural systems that regulate recognition memory and executive function. Furthermore, DISC1 has critical roles in regulating the physiology of the neocortex and hippocampus through promoting neurotransmission (Wang *et al*., 2010; Hayashi-Takagi *et al*., 2010; Niwa *et al*., 2010) and corticogenesis (Mao *et al*., 2009; Singh *et al*., 2010). Currently, there is little information about whether hDISC1mutants have anatomical or physiological defects in other regions of the forebrain where DISC1 is expressed. While they show sex specific reductions in levels of dopamine and its metabolites in the hippocampus and prefrontal cortex (Ayhan *et al*., 2011), future analyses of additional hDISC1 forebrain components could be important in elucidating their abnormal behaviours.

In conclusion, hDISC1 mutant mice demonstrated task-specific impairments in cognition, including deficits in spatial novelty preference, cognitive flexibility and recognition of a previously encountered object. These findings suggest that DISC1 plays an integral role in executive function and memory performance, and provide further evidence that hDISC1 mutant mice represent a valid model for investigating the gene's impact on schizophrenia-relevant cognitive domains. Given the complexity of the neural mechanisms by which DISC1 affects the neurodevelopment and neurotransmitter systems, including glutamate and dopamine, (Ayhan *et al*., 2011; Abazyan et *al*., 2010; Niwa *et al*., 2010; Wang et *al.,*2010; Hayashi-Takagi *et al*., 2010; Marley and von Zastrow, 2010; Pogorelov *et al*., 2011), further investigations are necessary to delineate the task-specific learning and memory deficits in hDISC1 mutant mice and their underlying causal mechanisms in more detail.

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CHAPTER 6.

GENERAL DISCUSSION

The main goal of the present thesis was to evaluate and characterize an environmental and a genetic mouse model of schizophrenia. The experimental background for the environmental model was provided by a series of studies in the literature showing that immune activation during late gestation may capture behavioural and neuroanatomical outcomes relevant to the cognitive and negative symptoms observed in schizophrenia (Meyer *et al*., 2006, 2008). The cellular mechanisms associated with the maternal immune response have been suggested to affect normal fetal programming that may result in the aberrant cognitive and neuropathological outcomes observed in the offspring (Patterson, 2009; Meyer *et al*., 2009a; Boksa, 2010). Thus, one of the aims of the thesis was to further assess the validity of the "*Critical time window hypothesis"* by evaluating the effects of immune activation during late pregnancy and whether the manipulation during this specific time point may modulate behavioural, neurochemical and neuropathological effects related to the negative and cognitive symptoms of schizophrenia in the offspring.

Besides environmental factors a number of genes have been suggested to contribute to the genesis of schizophrenia. To this end, DISC1 has emerged as a strong candidate gene associated with major mental illness including depression, anxiety, bipolar disorder and schizophrenia. Specifically, the DISC1 gene has been shown to influence many aspects of CNS function, including neurodevelopment, neurosignaling, and synaptic functioning, all of which are thought to be affected in schizophrenia (Bradshaw and Porteous, 2011). Therefore, a second series of studies were conducted to evaluate the behavioural implications associated with the expression of mutant human DISC1 in transgenic mice.

6.1 *Environmental aspect*

Prenatal exposure to infection has been proposed as a risk factor for schizophrenia based on various epidemiological data (Brown, 2011; Brown and Derkits, 2010). However, disentangling the potential mechanisms and effects of exposure to infection during pregnancy that result in schizophrenia remain challenging. Therefore the adoption of translational approaches that capitalize on findings from epidemiologic research has considerable potential for integrating etiologic and pathophysiologic research in schizophrenia (McAlonan *et al*., 2010). In this context, studies in animals have clearly demonstrated that prenatal immune activation using influenza virus or Poly-I:C causes phenotypes that appear to resemble brain and behavioural anomalies observed in schizophrenia (Meyer *et al*., 2006, 2008; Fatemi *et al*., 2011, Asp *et al*., 2009; Beraki *et al*., 2005; Short *et al*., 2010). The research presented in this thesis extends these findings to show that maternal immune challenge during the late gestation period has specific long term effects on neurochemical, neuropathological and behavioural readouts in the offspring.

A correlation between neuropathology and cognitive function in Poly-I:C mice was observed in the present thesis. Specifically, spatial recognition and working memory performance in mice that were exposed to immune activation during late pregnancy was shown to correlate positively with the expression level of the serine/threonine-protein kinase AKT1 in the mPFC (**CHAPTER 2**). Another interesting observation was that there were several alterations in neurochemical content and behaviour that were dependent on sex and brain region. For example, the male offspring of dams that were exposed to immune challenge during late pregnancy exhibited cognitive inflexibility in addition to a reduced level of glutamate and aspartate in the mPFC (**CHAPTER 3**).

The research presented in this thesis also showed that Poly-I:C mice exhibited alterations in the locomotor and stereotyped behavioural responses to acute APO treatment in the form of increased locomotor activity and climbing behaviours. These mice also exhibited anhedonia and abnormalities in social behaviour (**CHAPTER 3**). These findings were accompanied by alterations in the dopaminergic,

serotoninergic and GABAergic systems. Although no direct mechanisms have been established in this thesis, taken together; these results suggest that immune activation during late pregnancy alters various neurochemical systems that may in turn lead to alterations in behavioural and cognitive function.

6.1.1 *Immune activation and the "critical time window hypothesis"*

Prenatal immune activation at a specific time point during pregnancy may interfere with particular developmental processes occurring in fetal brain at that time point (Meyer *et al*., 2007). Therefore, it has been suggested that immune activation at different gestational periods may result in distinct long-term psychopathology and neuropathology because the maternal immune response may affect specific neurodevelopmental events occurring in the developing fetus, which may subsequently determine the specificity of the long-term neuropathological and psychopathological outcome (Meyer *et al*., 2007). In this context, the critical time window hypothesis postulates that maternal immune activation during early/middle pregnancy may result in a behavioural cluster of endophenotypes and neuropathological changes relevant to the positive symptomology of schizophrenia, whereas immune challenge during late pregnancy may capture the negative symptomology of schizophrenia (Sullivan *et al*., 2006; Meyer *et al*., 2007).

In order to assess the validity of the critical time window hypothesis in terms of the positive-negative symptom dichotomy of schizophrenia, the present thesis sought to evaluate the specificity of the behavioural and anatomical effects following the maternal immune challenge during the late gestation period. Therefore, experiments were designed to evaluate if as opposed to the positive symptoms associated with immune activation during early/middle pregnancy (Meyer *et al*., 2006; Vuillermot *et al*., 2010) whether immune activation during late pregnancy may result in a cluster of behavioural and neuropathological changes reminiscent of the negative profile observed in schizophrenia. In order to probe the validity of the critical window hypothesis from a perspective of the negative symptomology of schizophrenia, the long-term effects of immune activation were solely assessed in Poly-I:C GD17 adult

mice. One limitation of this approach was that offspring were not evaluated prior to puberty and thus the progressive onset of any behavioural and pathological aberrations could not be 'unmasked'. Another limitation of this approach is the specificity of the behavioural and neuroanatomical changes in terms of the timing of the immunological challenge in the offspring seeing as only GD17 mice were assessed in the present thesis. Nonetheless, consistent with a dissociation between the behavioural and neuroanatomical outcomes in the offspring of dams that are exposed to immune activation during early/middle pregnancy as opposed to late pregnancy are the recent unpublished findings from our laboratory that have shown that maternal immune activation with Poly-I:C on GD17 but not GD9 results in a delay-dependent impairment in spatial recognition memory in the Y-maze (Richetto, Bitanihirwe, Feldon and Meyer, See **Figure 6.1**). Additionally, the analysis of postmortem brain neurochemical content in GD9 mice (Winter *et al*., 2009) using an identical immune activation procedure (i.e. 5mg/kg Poly-I:C i.v.) to the one used in the present thesis was shown to exhibit a very different neurochemical profile (e.g. increased cortical dopamine and no changes in glutamate levels) to what was reported in GD17 mice (i.e. decreased cortical dopamine and glutamate levels, **CHAPTER 3**). This suggests that despite inducing inflammation in the fetal system (Meyer *et al*., 2006), the maternal immunological manipulations are capable of disturbing another critical aspect in the developing fetus, namely the structure and function of the neurochemical system. In order to gain further insight into the possible neuroimmunological mechanisms underlying the segregation of positive and negative symptoms in schizophrenia from a neurodevelopmental perspective, additional assessment of the structural and functional consequences resulting from *in utero* immune challenge will be necessary.

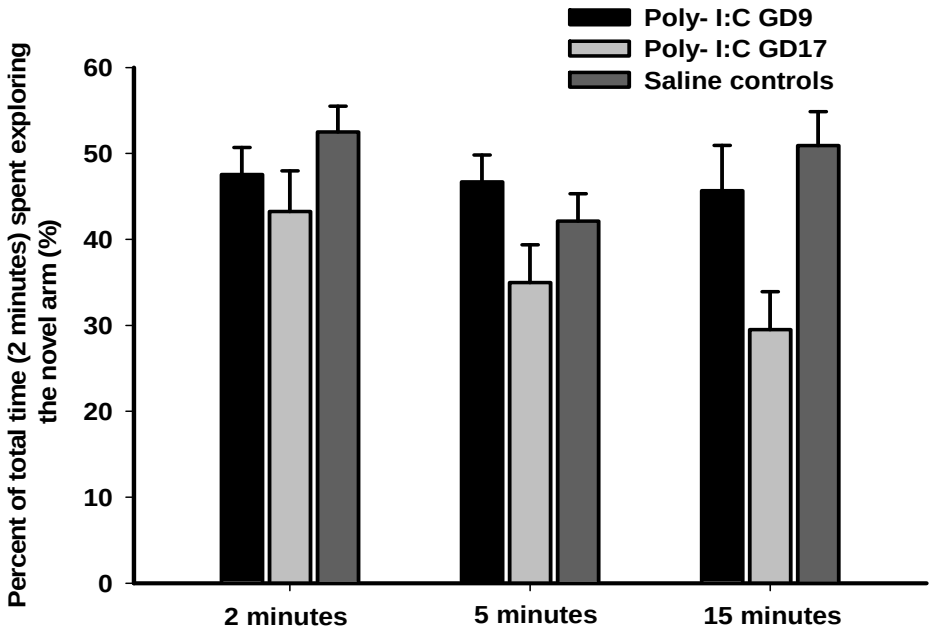

Figure 6.1 Prenatal immune activation during late pregnancy leads to a disruption of spatial recognition memory in the Y-maze in Poly-I:C GD17 mice. The graph depicts the percent of time (2 minutes) spent in the novel (previously unexplored) arm during the choice phase at the 2 minute, 5 minute and 15 minute retention interval tests.

6.1.2. *Reduced AKT1 expression - A potential downstream effect of hypodopaminergia?*

A marked impairment of spatial recognition and working memory in adult mice that were exposed to prenatal immune activation in late gestation was observed in the present thesis (**CHAPTER 2**). Decreased expression of AKT1 was detected in the PL, IL and CG of Poly-I:C mice. The impairment of spatial working memory performance exhibited a statistically significant positive correlation (r = +0.52, $P < 0.05$) with the expression of AKT1 in the CG of Poly-I:C mice during the longest retention interval (i.e. 15 minutes). Similarly, spatial recognition memory performance in the Y-maze test was positively correlated with the number of AKT1-positive cells in both the CG (r = +0.47, $P < 0.05$) and PL (r = +0.57, $P < 0.05$) subregions of the mPFC during the 15 minute interval. As a result it was presumed that cognitive impairment of spatial recognition and working memory might be associated with low AKT1 expression in Poly-I:C mice brain.

AKT1 regulates the trafficking of presynaptic dopamine transporters (Garcia *et al*., 2005) and decreased AKT1 protein levels have been reported in peripheral blood mononuclear cells (Blasi *et al*., 2011) and postmortem prefrontal cortices (Emamian *et al*., 2004) of patients with schizophrenia. Notably, AKT1 function has been coupled to dopaminergic signaling and downstream effects on prefrontal cellular structure and animal models of schizophrenia (Balu *et al*., 2010; Beaulieu *et al*., 2007; Lai *et al*., 2006; Emamian *et al*., 2004). Strikingly, the decreased expression of AKT1 in the mPFC of Poly-I:C mice reported in this thesis (**CHAPTER 2**) was mirrored by a reduced level of dopamine in the same brain region (i.e. the mPFC) of these mice (**CHAPTER 3**). Reduced dopaminergic content (i.e. "hypo-dopaminergia") and neurotransmission in the PFC is a consistent finding in schizophrenia (Abi-Dargham, 2003). Because dopamine is known to play an important role in the modulation of cognitive function (Robbins, 2003) and hedonic response (Sarchiapone *et al*., 2006; Wise, 2008) it may be possible that the reduction of dopamine observed in this thesis may contribute to the anhedonia phenotype observed in Poly-I:C mice. Similarly, it may be possible that the reduced dopamine levels in Poly-I:C mice may have contributed to the social abnormalities observed in these mice seeing as it has been reported that dopamine lesions by means of the neurotoxin 6-hydroxydopamine in the mPFC of rats results in social interaction deficits (Espejo *et al*., 2003; Li *et al*., 2010). In this respect, a tantalizing possibility is that reduced AKT1 signaling may impinge on putative dopaminergic processes which may in turn impact prefrontal structure and prefrontal function during executive cognition processes as observed in the present thesis.

6.1.3. *Cognitive inflexibility in male Poly-I:C mice*

Latent inhibition has been reported to be disrupted in schizophrenia patients (Lubow and Kaplan, 2010). The LI phenomenon has been shown to be modulated by both attentional and associative mechanisms (Lubow, 2005; Weiner, 2003) with empirical evidence suggesting that the positive symptoms associated with schizophrenia result in an attenuation of LI expression whereas the negative symptoms associated with schizophrenia produce a persistent LI effect (Weiner, 2003, Weiner

and Arad, 2009). An interesting finding from the present thesis was that acute prenatal injection of Poly-I:C resulted in an abnormally persistent LI in male but not female offspring (**CHAPTER 3**). Analyses of the response latency revealed that the emergence of the LI effect in male Poly-I:C offspring stemmed from a delayed learning response in CS-PE subjects relative to CS-PE control offspring. Interestingly, a recent study has reported a similar sex specific effect resulting in cognitive inflexibility during a set-shifting task in male but not female rats treated with Poly-I:C during late gestation (GD15, Zhang *et al*., 2011).

The lack of effect following prenatal immune activation on LI in female Poly-I:C mice is interesting in light of the human literature showing that male patients with schizophrenia perform worse than female patients with schizophrenia on tests of executive function such as the Wisconsin card sorting test (Goldstein *et al*., 1998; Longenecker *et al*., 2010; Seidman *et al*., 1997). In line with these reports, the present observation of persistent LI in the offspring of dams that were challenged with Poly-I:C during late pregnancy validate the utility of this model as an effective system for evaluating the cognitive deficits associated with the negative symptoms observed in patients with schizophrenia.

6.1.4. *Male Poly-I:C mice and excitatory neurotransmission*

The changes in excitatory neurotransmitter levels observed in Poly-I:C mice was region and sex specific (**CHAPTER 3**). Notably, male Poly-I:C mice exhibited changes in the levels of glutamate and aspartate that were restricted to the mPFC. The mPFC in the rodent consists of 4 subdivisions which are functionally and anatomically distinct from one another (Vertes, 2004). The various subdivisions of the mPFC appear to serve separate and distinct functions (Dalley *et al*., 2004). For instance, dorsal regions of mPFC (medial agranular cortex and anterior cingulated cortex) have been implicated in various motor behaviours, while ventral regions of mPFC (PL and IL) have been associated with diverse emotional, cognitive, and mnemonic processes (Dalley *et al*., 2004). Although speculative, it is possible that the

changes in excitatory neurotransmission observed in Poly-I:C male mice may actually be more restricted to the ventral regions rather than the dorsal regions of the mPFC. This disturbance may represent a neural mechanism for the behavioural cognitive inflexibility in the form of persistent LI in male Poly-I:C mice given that the NMDA receptor antagonist MK-801 has been reported to induce a similar pattern of abnormally persistent LI in rodents (Gaiser–Salomon *et al*., 2008).

Although excitatory neurotransmission was disturbed in both the hippocampus and mPFC, it is not apparent if there may have been any alteration in NMDA receptor expression in Poly-I:C mice which would suggest an adaption or compensation to decreased excitatory neurotransmission. However, evidence supporting the effect of maternal administration of Poly-I:C on excitatory neurotransmission in the offspring has been shown in the form of a decrease in the expression of the NMDA-receptor subunit 1 (NR_1) in the hippocampus of the adult offspring (Meyer *et al*., 2008). All NMDA receptors contain at least one obligatory NR_1 subunit combined with other kinds of NMDA subunits (i.e. NR_2 and NR_3) (Cull-Candy *et al*., 2001). Notably, the NR_1 subunit is widely expressed in the adult mammalian brain throughout pre- and postnatal development and has been shown to be important for the normal physiology of the NMDA receptor system (Babb *et al*., 2005). Perhaps relevant to the findings reported in the present thesis is that the effect of prenatal Poly-I:C-induced immune challenge on hippocampal NR_1 expression is observed only if the immunological manipulation is conducted in late (GD17) but not early/middle (GD9) gestation (Meyer *et al*., 2008). Together with the results presented in this thesis (**CHAPTER 3**) it appears that excitatory neurotransmitter systems may be more susceptible to a disruption of function as a consequence of immune activation during late pregnancy as opposed to early/middle gestation.

6.1.5. *Apomorphine -induced stereotyped behaviour in adult Poly-I:C mice*

Enhanced locomotor response to the non-selective dopamine agonist APO was observed in Poly-I:C mice relative to APO-treated control offspring (**CHAPTER 3**). In addition, Poly-I:C mice

exhibited a pronounced increase in repetitive climbing with what appeared to be a faster onset of APO-induced climbing in the Poly-I:C group compared with APO-treated control offspring. This effect was paralleled by a temporary attenuation of leaning behaviour in APO-induced Poly-I:C offspring relative to APO-treated controls. Perhaps surprisingly, we did not find any changes in the levels of dopamine or in dopamine turnover in the striatum (nucleus accumbens and caudate putamen) of adult Poly-I:C-mice seeing as it is believed to be important in mediating stereotypies (Canales and Graybiel, 2000). Nonetheless, it is plausible that prenatal Poly-I:C injection during late pregnancy may result in maturation-dependent alterations in the dopaminergic system which may subsequently result in altered adult dopamine mediated behaviour (Meyer and Feldon, 2009b).

6.2 *Genetic aspect*

The results presented in this thesis suggest that mice which express mutant human DISC1 may provide a useful model for modeling certain aspects of the schizophrenia phenotype. For example, hDISC1 mutant mice show altered sensitivity to psychomimetic drugs but exhibit intact sensory motor gating and selective attention in the form of LI (**CHAPTER 4**). Interestingly, the hDISC1 mutant mice also exhibited hypervigilance in a visual discrimination task (**CHAPTER 4**). These mice also exhibit cognitive inflexibility in addition to deficits in object recognition and spatial recognition memory (**CHAPTER 5**). Together, these behaviours are evocative of some of the cognitive anomalies observed in patients with mood disorders and psychoses.

6.2.1. *Information processing and sustained attention in hDISC1 mutant mice*

Prepulse inhibition was assessed in **CHAPTER 4** of the present thesis but did not reveal and significant differences between mice that express the mutant human DISC1 protein product and control mice (DISC1 and tTA controls). Impairment of sensory motor gating is generally observed in schizophrenia, but the absence of a PPI deficit in hDISC1 mutant mice suggests that this behaviour may

not involve DISC1 mediated processes. Although consistent with the original observation of a null effect of mutant human DISC1 expression on sensory motor gating in these transgenic mice by Pletnikov and colleagues (2008), three research groups have however reported deficits in PPI using different transgenic DISC1 mice models (Clapcote *et al*., 2007; Hikida *et al*., 2007; Shen *et al*., 2008). The potential basis for differences in sensory motor gating may be due to the differences in methodology used to generate the transgenic mice in addition to an influence of the different genetic backgrounds on which the mice were created.

Because LI is a unique paradigm that can reveal the two poles of irregularities across the schizophrenic spectrum (Lubow, 2005; Weiner, 2003), two LI paradigms were conducted under specific experimental parameters in order to examine whether mutant human DISC1 may either attenuate or potentiate the expression of LI in the double transgenic mouse model. In this aspect, the conditioned freezing paradigm was used to assess for the presence of attenuated LI whereas the active avoidance paradigm was utilized in order to assess any potential evidence of persistent LI. The null findings from both these experiments suggest that the expression of mutant human DISC1 does not alter LI in both poles. In contrast to the results presented in this thesis LI was found to be altered in ENU-induced missense DISC1 mutants (Clapcote *et al*., 2007) and BAC expressing DISC1 mice (Shen *et al*., 2008), suggesting impaired selective attention in these mutant mice (Clapcote *et al*., 2007; Shen *et al*., 2008). This discrepancy might be partly due to differences in the strategies used to generate the transgenic mice as discussed above or important procedural differences in the paradigms used to assess LI. As opposed to the LI paradigms used in the present thesis the study by Clapcote and colleagues used a conditioned emotional response procedure (Clapcote *et al*., 2007), whereas selective attention was evaluated by Shen and colleagues using LI of the conditioned fear response (Shen *et al*., 2008). Interestingly, Pothuizen and colleagues have shown that depending on the association learning paradigm selected that lesions in the nucleus accumbens shell may disrupt or enhance the expression of LI (Pothuizen *et al*., 2006). Although speculative, it may be possible that the specific DISC1 manipulation employed for behavioural evaluation

may play a role in the expression or abolition of the conditioned stimulus pre-exposure effect depending on the paradigm selected.

It is notable that, although sensory motor gating and selective attention was intact, sustained attention was increased in hDISC1 mutant mice. This key finding demonstrates that expression of mutant human DISC1 may modulate the neural substrates associated with anxiety disorders. Notably, clinical anxiety has been suggested to arise from a biased information-processing system, including a bias in attention (Beck and Clark, 1997). Anxiety leads to increased sustained attention or hypervigilance for threat, a greater level of threat detection leads to increased anxiety which, in turn, leads to increased hypervigilance (Dalgleish *et al*., 2001). In this respect, it is worth noting that the increased sustained attention observed in hDISC1 mutant mice recapitulate some of the changes of visuospatial attention that have been reported in human subjects with anxiety disorder (Vasey *et al*., 1995, 1996). Although schizophrenia has been associated with the inability to filter out stimuli (Braff, 1993), the increased sustained attention observed in the hDISC1 mutant mice may actually represent a signature of anxiety vulnerability rather than schizophrenia.

6.2.2. *Behavioural response to psychomimetic drug challenge in hDISC1 mutant mice*

The most interesting result stemming from **CHAPTER 4** was that hDISC1 mutant mice exhibited an increased sensitivity to the locomotor activating effect of psychomimetics. Specifically, hDISC1 mutant mice showed an elevated AMPH response in addition to a female specific increase in locomotor response to MK-801. Heightened sensitivity to the induction of hyperactivity by psychotomimetic drugs, such as AMPH and MK-801, has been considered an endophenotypic measure related to the positive symptoms of schizophrenia (Arguello and Gogos, 2006). Mice that express mutant human DISC1 displayed enhanced sensitivity to AMPH-induced hyperactivity, as previously reported (Ayhan *et al*., 2011). This pattern of enhanced AMPH-induced locomotor activity is reminiscent of the increased susceptibility to the disorganizing effects of AMPH observed in individuals with schizophrenia (Laruelle

et al., 2003; Szeszko *et al*., 1999). An increase in the sensitized behavioural response to AMPH has been reported previously in a distinct ENU-induced missense DISC1 mutant (Lipina *et al*., 2010). These mutant mice were also shown to express an increased level of striatal dopamine D_2 receptors (Lipina *et al*., 2010). Interestingly, administration of the D_2 receptor antagonist haloperidol was shown to abrogate the psychostimulant effects of AMPH in the mutant mice.

Acute administration of MK-801 resulted in a stimulatory response that was greater in female hDISC1 mutants. Sex differences in AMPH and MK-801-induced behaviour have been reported previously using the same DISC1 mouse model (Ayhan *et al*., 2011). Unlike the pychostimulant effects reported in this thesis, the study by Ayhan and colleagues reported male hDISC1 mutants to exhibit a more prominent phenotypic effect resulting from the administration of MK-801 and AMPH. It may be possible that the sex specific discrepancy between the present results and the study by Ayhan and colleagues may arise from differences in methodology. For example, as opposed to the study by Ayhan and colleagues the experiments described in this thesis were conducted under a reverse dark/light cycle with all behavioural experiments being carried out during the dark phase of the cycle. Because mice are nocturnal creatures and are therefore more active during the dark phase (Hossain *et al*., 2004), it has been suggested that dark phase testing may prove a more effective means of assessing behaviour test situations (i.e. *ethological* validity). It is also possible that differences in data acquisition procedure (activity chambers with infrared beams as opposed to the in house Ethovision tracking system) and protocols adopted for the experiments may have affected the result turn out. Despite these differences, both these observations attest the dangers of generalizing conclusions to both sexes based upon data obtained in a single sex only.

Disrupted in schizophrenia-1 has been shown to regulate the expression and function of several neurotransmitter receptor targets implicated in psychostimulant sensitization, including dopamine (Marley and von Zastrow, 2010; Pogorelov *et al*., 2011) and glutamate (Hayashi-Takagi *et al*., 2010; Wang *et al*., 2010). Even so, the neurobiological mechanism underlying the enhanced locomotor response reported in

hDISC1 mutants remains uncertain. In this respect, it has been proposed that neural sensitization of the mesolimbic dopamine system may explain how an episodic, subcortical hyperactivity can act on a basic dopaminergic and glutamatergic hypofunction to produce psychotic symptoms (Abi-Dargham *et al*., 1998; Glenthøj and Hemmingsen, 1997; Peleg-Raibstein *et al*., 2009). The increased sensitivity to the locomotor stimulating effects of AMPH and MK-801 observed in hDISC1 mutant mice reported in this thesis may therefore stem from the development or expression of a more marked and possibly more persistent sensitization response to psychostimulants.

6.2.3. *Cognitive and executive function in hDISC1 mutant mice*

Cognitive assessment of mice in the novel object recognition memory test showed that hDISC1 mutant mice spent significantly less time exploring the novel object compared to the control groups following the 2 hour retention interval indicating potential impairment in long-term object recognition memory in hDISC1 mutants (**CHAPTER 5**). Because all three genotypes spent a similar amount of time exploring both objects during the familiarization phase of the task it is unlikely that the decreased exploration of the novel object is due to differences in the general locomotor activity between the different mice groups. Recognition memory for novel versus familiar objects primarily involves the medial temporal lobe, particularly the perirhinal cortex (Brown and Aggleton, 2001). Thus, a deficit in object recognition memory in the hDISC1 mutant transgenic mice may represent a potential deficit of hippocampal and parahippocamapal functions (Moses *et al*., 2005; Murray *et al*., 2000). This observation may be consistent with the clinical reports indicating that allelic variation within the DISC1 gene is associated with altered hippocampal structure and declarative memory performance in human subjects (Callicot *et al*., 2005).

Using a two-trial Y maze task it was shown that hDISC1 mutant mice exhibited an impairment in spatial recognition memory (**CHAPTER 5**). These deficits in hDISC1 mutants are attributable to dysfunctions in memory processes on the basis that impaired locomotor activity in the two-trial spatial recognition test in the Y-maze could not be mistaken for a memory deficit because the test is based on the choice between a novel place and familiar places and the percentage of time spent in exploration is used as the measure (Dellu *et al.*, 2000). Moreover, the absence of preference for the novel arm in the hDISC1 mutants could not be accounted for by distance travelled or speed differences between groups.

Analysis of the cheeseboard data in hDISC1 mutants revealed that the rate of learning as indexed by the reduction in latency and distance moved to find the location of the reward in trial 2 relative to trial 1 across two delays (2 minutes and 15 minutes) was unaltered (**CHAPTER 5**). The finding of intact spatial working memory in hDISC1 mutant mice is inconsistent with the results of a recent study showing that mice which express the mutant human DISC1 protein impairs their performance in a spatial working memory task (Pletnikov *et al*., 2008). This discrepancy may however result from the difference in methodologies used to assess spatial learning and memory. Notably, even though the cheeseboard task has been validated in house with the C57BL/6 strain (Llano *et al*., 2010; **CHAPTER 2**), it may still be subject to considerable variability. In contrast to the water-based Morris maze, which examines similar memory processes, mice do not go directly toward the escape position but, may often inspect other holes briefly prior to visiting the correct rewarded hole. Furthermore, it is possible that the mixed strain on which the hDISC1 mutant mice were bred may have affected performance on the cheeseboard seeing as strain differences in general behavioural activity have been reported before (Carola *et al*., 2002; Contet *et al*., 2001; Sik *et al*., 2003). Another potential cause of discrepancy may stem from the in house reverse dark/light cycle system as described above.

Cognitive flexibility was assessed using a food reinforced operant discrimination reversal task. At the completion of extensive operant training, reversal learning was assessed. DISC1 control, tTA control and hDISC1 mutant mice exhibited equivalent performance during acquisition of this task. However,

marked differences were observed during the reversal phase of this experiment (**CHAPTER 5**). More specifically, when the reinforcement contingencies were inverted, the hDISC1 mutant and DISC1 control mice were impaired in their ability to alter their response patterns and redirect behaviour to stimuli previously indicating no reinforcement. These findings are indicative of an inability in both hDISC1 mutant and DISC1 control mice to inhibit the previously reinforced behavioural strategy (i.e. perserveration). However, following extended training all mice achieved an equivalent level of discrimination performance. These results provide evidence for the involvement of DISC1 in learning and memory.

In summary, the behavioural findings discussed here demonstrate that DISC1 can exert a significant role in behavioural plasticity. Although analysis of spatial working memory in mice expressing mutant human DISC1 suggested that this cognitive process was spared, these transgenic mice did however exhibit spatial recognition memory deficits in addition to an impairment in recognition memory. It was also determined that mutant human DISC1 plays a role in cognitive flexibility. Together these findings lend themselves to the hypothesis that the mutant human DISC1 protein product underlies cognitive deficits associated with psychiatric disorders and thereby represents an additional target for the development of pharmacotherapies that could attenuate the learning and memory impairments associated with these disorders.

6.3. *Partitioning the genetic and environmental contributions to schizophrenia*

There were several differences between the behavioural phenotypes of the genetic (hDISC1 mutant) and the environmental (Poly-I:C GD17) mouse models evaluated in the present thesis (**Table 6.1**) including, a sex specific enhancement of LI and a delay dependent effect of spatial working memory on the cheeseboard task which were observed in Poly-I:C GD17 mice but not in hDISC1 mutants. In contrast, there were no differences detected in novelty-induced locomotion in the open field, social interaction, cognitive flexibility or in sensory motor gating performance between both groups of mice. In addition, no differences were observed in general measures of anxiety as assessed on the elevated plus

maze, although there was a task specific difference in anxiety between the two models, with the hDISC1 mutant mice exhibiting hypervigilance in the visual discrimination task relative to Poly-I:C GD17 mice. Nonetheless, both groups exhibited an impairment in spatial recognition and spatial working memory in the Morris water maze, although this impairment was restricted to the females in the hDISC1 mutant group. In addition, a sensitivity to psychostimulants acting on the glutamatergic and the dopaminergic system was observed in both groups of mice although this effect was sex specific in the hDISC1 mutant group (**Table 6.1**).

Synopsis of Behaviour Results		
Behaviour	***Poly-I:C- GD17***	***hDISC1 Mutant***
Open field exploration	No effect	No effect
Spontaneous locomotion	Not determined	Increased locomotion[1]
Elevated Plus Maze	No effect*	No effect
Sucrose preference test	Impaired	Not determined
Y-Maze	Impaired	Impaired
Olfactory Test	Not determined	No effect[1]
Object recognition	Not determined	Impaired
Reversal Learning	Impaired	Impaired
Social interaction test	Impaired	Impaired[2]
Forced swim test	Not determined	Impaired[2]
Tail suspension test	Not determined	Impaired[1]
Dyadic social interaction	Not determined	Impaired[2]
Prepulse inhibition	No effect	No effect
Latent inhibition	[§]Impaired – Enhanced LI	No effect
Morris Water Maze	Impaired	Impaired[1ξ]
Cheeseboard	Impaired	No effect
Sustained Attention	No effect**	Superior performance
Behavioural reaction to Apomorphine	Increased sensitivity	Not determined
Behavioural reaction to Amphetamine	Increased sensitivity	Increased sensitivity[2]
Behavioural reaction to NMDA- receptor blocker	Increased sensitivity	Increased sensitivity[2]

Table 6.1. Overview of the experimental paradigms used to investigate behavioural abnormalities in the Poly-I:C and hDISC1 models. *Unpublished observation: Meyer and Feldon. ** Unpublished observation Bitanihirwe, Feldon and Meyer. [§] Male specific persistent LI. [ξ]Female specific impairment in working memory performance. [1]Pletnikov *et al*., 2008; [2]Ayhan *et al*., 2011.

Besides the behavioural profiles described above, specific differences have been detected between neurochemical and neuroanatomical phenotypes between the two mice models (**Table 6.2**). Notably, white matter deficits and a female specific increase in serotonin levels were restricted to Poly-I:C GD17 mice whereas enlarged lateral ventricles were solely observed in the hDISC1 mutant mice (Pletnikov *et al*., 2008). However, both hDISC1 mutant mice and Poly-I:C GD17 mice showed a remarkable similarity in a reduced number of parvalbumin-positive cells in addition to a reduction of dopamine levels, although this was region and sex specific in the hDISC1 mutant mice (**Table 6.2**).

Synopsis of Neuroanatomical and Neurochemical Results		
Feature	***Poly-I:C- GD17***	***hDISC1 Mutant***
Enlarged lateral ventricles	No effect[3]	Present[1]
Brain volume	No effect[3]	No effect[2]
White matter deficits	Present[3]	Not determined
Interneurons	Reduced parvalbumin cells	Reduced parvalbumin cells[2]
Reelin	Reduced	Not determined
Glutamate receptors	Reduced NR1 expression	Not determined
Neurogenesis	Reduced	Not determined
Pyramidal neurons	Not determined	Reduced neurite outgrowth[1]
Dopamine levels	Reduced	Reduced[2]*,§
Serotonin levels	Increased**	No effect[2]
Glutamate levels	Reduced	Not determined
GABA levels	Reduced	Not determined

Table 6.2. Comparison of the neuroanatomical and neurochemical abnormalities found in Poly-I:C GD17 and hDISC1 mutant mouse models. *Observed in the PFC of male hDISC1 mutants, §This reflects a female specific effect in the hippocampus. **Female specific increase in the amygdala and nucleus accumbens. [1]Pletnikov *et al*., 2008; [2]Ayhan *et al*., 2011; [3]Li *et al*., 2009

In summary, while there is an overlap in some of the phenotypes in both groups of mice there are also some specific differences between the two groups. By virtue of the distinct phenotypes of the Poly- I:C GD17 and hDISC1 mutant mice (**Table 6.1.** and **Table 6.2.**), it appears that immune activation with Poly-I:C during late pregnancy in mice may represent a superior animal model for evaluating the negative and cognitive symptoms of schizophrenia. In contrast, expression of the mutant human DISC1

product may represent a more efficient model for evaluating the neuronal basis of the cognitive symptoms of schizophrenia and mood disorders such as depression and anxiety.

6.4. *The PI3K–AKT–mTOR Pathway and Schizophrenia: A convergence point?*

Recent gene polymorphism and gene expression studies have revealed differences in schizophrenia that seem to converge within common signaling pathways that are necessary for proper development and function of both the nervous system and the immune system. One such pathway is the mammalian target of rapamycin (mTOR) pathway (Wullschleger *et al*., 2006). Specifically, the mTOR pathway is a key pathway controlling protein synthesis, immune function and brain growth, and disruptions in this pathway could lead to developmental abnormalities associated with (and perhaps causing) schizophrenia. In this respect, single-gene mutations that affect the mTOR pathway have been implicated in schizophrenia (Emamian *et al*., 2004; Norton *et al*., 2007; Tan *et al*., 2009). Furthermore, a number of other genes including DISC1 that are linked to or feed into this pathway have been found by employing genetic manipulation and protein interaction studies (Enomoto *et al*., 2009; Kim *et al*., 2009; Mao *et al*., 2009). Given the central role of this pathway, it is possible that many other rare gene variants could also exist that affect signaling through this pathway. Although these genetic differences are associated with a common pathway, they may exert an effect at different points and thus alter function and development in varied ways that could manifest as different endophenotypes within schizophrenia. Strikingly, many of the immune alterations seen in schizophrenia could result from dysregulation of the mTOR pathway. One of the earliest immune observations seen in schizophrenia was a hyposensitivity to foreign antigens (Vaughn *et al*., 1949). Other investigators have seen similar results in response to T-cell mitogens (Matloubi *et al*., 2007). As a central regulator of cell growth and metabolism, the mTOR pathway is intrinsically involved in both T-cell activation and anergy (i.e. a state of unresponsiveness) (Mondino and Mueller, 2007), and hence dysfunction in this pathway could result in the T-cell abnormalities previously seen in schizophrenia. In addition, cell survival is heavily regulated by the

mTOR pathway (Castedo *et al*., 2002). Dysregulated apoptosis in neuronal cells (Jarskog *et al*., 2005) has been described in schizophrenia, which suggests that mechanisms controlling cell survival are altered.

AKT1 is a major upstream regulator in the mTOR pathway, and mutations in this protein are associated with higher rates of schizophrenia (Emamian *et al*., 2004; Norton *et al*., 2007; Tan *et al*., 2009; Thiselton *et al*., 2008; Xu *et al*., 2007). Notably, AKT1 has recently been shown to support T-cell function and to induce regulatory T-cell formation (Pierau *et al*., 2010). Although speculative, it may be possible that the reduced expression of AKT1 detected in Poly-I:C GD17 mice (**CHAPTER 2**) may hamper the normal immune response and as a result explain a link between AKT1 and the immune dysfunction observed in schizophrenia.

Altogether, the evidence discussed here demonstrates that pathological alterations in the mTOR pathway results in neuronal and immunological deficits relevant to schizophrenia. In the context of the present thesis, the characterization of a core molecular pathway with a focus on DISC1 and other schizophrenia susceptibility genes such as AKT1 may lead to a profound advance in understanding schizophrenia and the development of therapeutic interventions.

6.5. *Gene-Environment interactions – A synthesis*

There have been numerous studies suggestive of interactions between environmental and genetic factors in schizophrenia. These include interactions between schizophrenia candidate genes (AKT1, metabotropic glutamate receptor 3, brain derived neurotrophic factor (BDNF) and dysbindin) and obstetric complications (Nicodemus *et al*., 2008). A similar gene-environmental interaction has been reported between cannabis use and COMT (Caspi *et al*., 2005). However, epidemiological studies in humans have not been able to address the mechanisms whereby gene-environment interactions occur. Several approaches to evaluate the gene-environment interaction in animal models of schizophrenia have been proposed including exposure of COMT and NRG1 knockout mice to the main psychoactive

constituent of cannabis, Δ^9-tetrahydrocannabinol (THC), which has been linked to an increased risk of developing schizophrenia (Linszen *et al.,* 1994; Luzi *et al*., 2008; van Os, 2010). Notably, mice with knockout of the COMT gene which were treated chronically with THC during adolescence were found to exhibit abnormalities in exploratory activity, spatial working memory, and anxiety (O'Tuathaigh *et al*., 2010) compared to COMT mice that had not been exposed to THC. In a similar context, heterozygous NRG1 transmembrane-domain knockout mice that were exposed to THC showed a decreased spontaneous locomotor activity and exploration in the open field test, an enhancement in PPI and a task specific anxiogenic profile compared to NRG1 mice that had not been exposed to THC (Boucher *et al.,* 2007). Together these findings provide important evidence suggesting that an interaction between genetic susceptibility factors (i.e. COMT and NRG1) and environmental factors (i.e. exposure to THC) over a particular stage of development results in the expression of schizophrenia-related endophenotypes.

Based on the evidence linking DISC1 to mental disease two separate studies have evaluated the interactions of DISC1 and maternal immune activation with Poly-I:C on behavioural phenotypes relevant to mental illness. The first study employed the DN-DISC1 mouse model (Hikida *et al*., 2007). These mice exhibited altered behaviour during an object recognition task, deficits in short term memory and fear conditioning in addition to social interaction and increased sensitivity to MK-801 (Ibi *et al*., 2010). Furthermore, the combined genetic and immune activation effects within these mice resulted in a specific reduction in parvalbumin containing inhibitory interneurons and an increase in the number of BrdU- (a marker for cell proliferation) positive cells in the hippocampus of young adult mice compared to unchallenged DN-DISC1 mice (Ibi *et al*., 2010). The second study specifically used male mice that expressed mutant human DISC1 and which were exposed to prenatal immune activation with Poly-I:C on GD9 (Abazyan *et al*., 2010). This study found that prenatal immune activation interacted with mutant human DISC1 and resulted in behavioural abnormalities that were not present in unchallenged hDISC1 mutant mice including elevated anxiety, depression-like responses, an altered pattern of sociability, and attenuated reactivity to stress. These behaviours were associated with decreased enlargement of lateral ventricles, reduced volumes of the amygdala and periaqueductal gray matter, and decreased density of

dendritic spines on granule cells of the hippocampus (Abazyan *et al*., 2010). These studies provide a thorough demonstration of the interaction of the DISC1 genetic insult in combination with a prenatal environmental insult and how their interaction can result in the neuroanatomical and behavioural deficits associated with schizophrenia and related disorders.

6.6. *Rodent models are a powerful resource for testing therapeutic strategies*

The availability of a variety of genetic rodent models of schizophrenia in addition to a number of different models of maternal immune activation/infection for schizophrenia has provided powerful tools for preclinical testing of therapeutic strategies. Notably, compounds can be tested in the animals before onset of disease and data can be accumulated fairly rapidly. In the context of this thesis, pharmacological evidence suggests that administration of the non-adenosine tri phosphate competitive GSK-3β inhibitor, 4-benzyl-2-methyl-1,2,4,-thiadiazolidine-3,5-dione (SB-216763) can reverse PPI and LI deficits as well as normalizing the hyperactivity of DISC1-L100P mutants (Lipina *et al*., 2011). Because DISC1 binds to and regulates GSK-3β it has been suggested that a decrease in DISC1 function in adult mice leads to higher GSK-3β activity and that this plays a critical role in the manifestation of these abnormal behavioural phenotypes. In a separate study, antipsychotic treatment with haloperidol was shown to abrogate the hyperactivity, deficits in PPI, LI and the psychostimulant effects of AMPH in the same mutant line (Lipina *et al*., 2010; Clapcote *et al*., 2007).

` Encouraging results have also been reported whereby anti-psychotics were administered to the offspring of dams that were exposed to some form of immune activation during pregnancy. Pharmacological intervention was initiated either at the time of behavioural testing or for several weeks to mimic the time course required for anti-psychotic efficacy in humans. Acute single injections with haloperidol, chlorpromazine or clozapine just prior to behavioural testing were shown to reverse PPI deficits in mice prenatally exposed to influenza (Shi *et al*., 2003) and to reverse changes in PPI (Borrell *et*

al., 2002; Romero *et al*., 2007), LI and reversal learning (Zuckerman *et al*., 2003; Zuckerman and Weiner, 2005) due to prenatal LPS or Poly-I:C in rats. Ozawa and colleagues showed that a 2 week treatment with clozapine, but not haloperidol, prior to testing reversed a deficit in novel object recognition in mice exposed prenatally to Poly-I:C (Ozawa *et al*., 2006). Similarly, Meyer and colleagues conducted a chronic clozapine treatment regime that lasted 3 weeks in adult mice that were exposed to Poly-I:C immune challenge on GD17. The chronic administration of clozapine significantly improved the prenatal Poly-I:C-induced working memory deficits while at the same time, it negatively affected working memory performance in adult offspring born to control mothers (Meyer *et al*., 2010a). In another study, Meyer and colleagues exposed mice prenatally to Poly-I:C on GD9 and then treated them with haloperidol, clozapine or a specific serotonin re-uptake inhibitor anti-depressant (fluoxetine) for 4 weeks during periadolescence (P35–P65) followed by behavioural testing at adulthood (P90–P120). Behavioural changes caused by prenatal Poly-I:C were reversed by some of these drug treatments: PPI deficits were reversed by clozapine and fluoxetine, LI deficits were reversed by haloperidol and clozapine, increased AMPH induced locomotion was reversed by haloperidol and fluoxetine while increased MK-801-induced locomotion was reversed only by haloperidol (Meyer *et al*., 2010b). Two elegant studies by Piontkewitz and colleagues using the Poly-I:C model in rats (GD15) have shown that they could prevent the structural abnormalities associated with schizophrenia (i.e. enlarged ventricles, and smaller hippocampus) by treating the offspring of mothers who received Poly-I:C with clozapine and risperidone during adolescence (Piontkewitz *et al*., 2009, 2010). This was paralleled by prevention of disrupted LI and hypersensitivity to AMPH. The mechanism by which anti-psychotic drugs reverse effects of prenatal immune activation are not yet clear. It is speculated that this may be related to any of the multiple actions of anti-psychotic drugs including the ability to block dopamine D_2 receptors, provide neuroprotection or enhance demethylation of gene promoters (Lieberman *et al*., 2008; Guidotti *et al*., 2009).

Because of the increasing evidence suggesting that regular exercise may alleviate symptoms of schizophrenia (Gorczynski and Faulkner, 2010) a recent study by Wolf and colleagues (2010) sought to

evaluate whether the behavioural (PPI deficits) and neuroanatomical (reduced neurogenesis accompanied by shorter telomeres and reduced telomerase activity in neural precursor cells in the hippocampus) phenotypes in the offspring of mice exposed to immune activation with Poly-I:C on GD15 could be reversed by using a voluntary exercise regimen (i.e. a running wheel within the cage). According to this study, voluntary exercise, a known stimulus of adult hippocampal neurogenesis, in the progeny of immune challenged dams could rescue the phenotype in behaviour, adult neurogenesis, and cellular senescence. The enhanced hippocampal neurogenesis in the progeny from Poly-I:C treated mothers that were subjected to voluntary exercise resembles the effect observed in mice that are exposed to environmental enrichment (Fabel *et al*., 2009). Environmental enrichment has been reported to mitigate a variety of cognitive and behavioural anomalies relevant to schizophrenia in mice such as hyperactivity and PPI deficits (McOmish *et al*., 2008). The biological bases for the beneficial effects of exercise and environmental enrichment are poorly understood but are believed to be multifactorial, likely including angiogenesis and blood flow, circulating hormones and cytokines, and local neuronal activity and oscillations (Fabel and Kempermann, 2008). Interestingly, a study has shown that BDNF plays a crucial role in modulating telomerase activity in hippocampal neurons (Fu *et al*., 2002). Although speculative, it is possible that the reported exercise induced expression of BDNF within the hippocampus (Adlard *et al*., 2005) may have a direct influence on telomerase activity which may in turn mediate cell survival and function. Together these findings are suggestive that regular exercise may represent an alternative treatment for alleviating some of the behavioural and neuroanatomical anomalies associated with schizophrenia.

6.7. *Future Directions*

Besides evaluating genetic interplay and gene-environment interactions, future studies of schizophrenia will emphasize complex interplay between environmental factors. For example, recent preliminary work conducted by Giovanoli within our laboratory sought to evaluate the potential interactions between two independent environmental factors that have been linked to schizophrenia. This experiment involved prenatal exposure to immune activation with Poly-I:C (1 mg/kg i.v.) on GD9 as the first environmental factor whereas the second environmental factor constituted a model of sub-chronic stress exposure in peri-pubertal life, which is based on the observation that exposure to physical and/or psychological stressors during early postnatal life also seems to play a role in schizophrenia (Morgan *et al*., 2007; Schreier *et al*., 2009). Specifically, the sub-chronic process involved a protocol in which mice were exposed to 5 distinct stressors applied on alternate days starting on postnatal day 30. The protocol consisted of electric foot shock (day 1), restraint stress (day 3), forced swimming (day 5), water deprivation (day 7) and multiple changes of home cages (Day 9). It was therefore hypothesized that exposure to some forms of sub-chronic stress during peri-pubertal life would exacerbate the schizophrenia phenotype in Poly-I:C offspring. Indeed, adult offspring of mothers exposed to Poly-I:C and which were also exposed to the sub-chronic stress regime demonstrated reduced locomotor activity in the open field, deficits in PPI and behavioural sensitivity to acute treatment with AMPH (Giovanoli, Feldon and Meyer, Unpublished observations). These results support the plausibility for synergistic interactions between two environmental factors, which have been individually implicated in the etiology of schizophrenia. In this respect, it may prove interesting to evaluate the combined effect of prenatal immune activation and chronic exposure to THC as another combined environmental model of schizophrenia

Although recent studies have evaluated the combined effect of the DISC1 genetic manipulation with immune activation on the offspring (Ibi *et al*., 2010; Abayhan *et al*., 2011) it would prove interesting from the perspective of this thesis to investigate the combined effect of the expression of the mutant human DISC1 product with immune activation on GD17 (seeing as the combined effects has only been evaluated

on GD9). Similarly, it would prove insightful to investigate the behavioural and neuroanatomical effects of THC exposure in hDISC1 mutant mice. Additionally, in order to get a clearer perspective as to the onset of the neurochemical, neuropathological and behavioural anomalies associated with immune activation on GD17 described in this thesis, it will be important to assess these animals prior to puberty. Finally, it would prove interesting to assess if the anomalies described in the genetic and environmental mouse models in this thesis may be reversed by pharmacological intervention, environmental enrichment or even by an exercise regime.

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CHAPTER 7.

CONCLUSION

Although the current thesis does not identify any mechanism central to the altered behavioural profile observed in mice exposed to Poly-I:C during late pregnancy or transgenic mice that express mutant human DISC1, it is clear that both these factors can affect the normal development of the CNS. Core features of schizophrenia such as decreased glutamatergic and dopaminergic cortical levels were observed in Poly-I:C mice. Furthermore, diverse schizophrenia-related endophenotypes including enhanced LI, anhedonia, alterations in the locomotor and stereotyped behavioural responses to acute apomorphine treatment in addition to deficits in spatial recognition, social interaction and working memory deficits were observed in Poly-I:C mice. In contrast to the environmental manipulation, specific features of schizophrenia such as spatial working memory impairment, sensory motor gating deficits and abnormal LI were not observed in hDISC1 mutant mice. However, expression of mutant human DISC1 was shown to affect cognitive flexibility, recognition memory and spatial recognition memory. These mice also exhibited hypervigilance and altered behavioural responses to psychostimulant drugs. The prenatal immune activation and DISC1 mice models presented in this thesis thus highlight the susceptibility of the CNS to developmental insult which may be mediated by these two separate factors. However, it is becoming increasingly recognized that rodent models, including those involving environmental manipulations or putative schizophrenia-related genes, are accompanied by a number of inherent limitations including the compensatory/adaptive effects initiated by homoeostatic processes during development (Vedeniapin, 2010; Crusio, 2004). Despite their merits, it must be emphasized that an environmental manipulation or a mutation for a specific susceptibility gene is unlikely to give rise to a phenotype that encompasses *all* aspects of schizophrenia. Such manipulations should therefore not be considered to model schizophrenia, but, rather, to model the functional roles of environmental aspects or genetic factors associated with the risk of schizophrenia. Given the complex nature of this disorder, clinical and experimental animal studies applying a gene-environment approach would seem the most promising method for further elucidating the multifactorial etiology of schizophrenia (van Os *et al*., 2010). In this respect, as the molecular and cellular basis of schizophrenia become better understood, it may be possible to develop rational treatments that target the actual cause of the disease. If so, one can

look forward to a time when treatments will not just alleviate symptoms but actually prevent the disease or halt its progression.

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APPENDIX

Sensorimotor gating and vigilance-dependent choice accuracy: a within-subject correlative analysis in wild-type C57BL/6 mice.

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[Personal contribution to the work: Designing the research, running the behavioural tests, analysis of the behavioural data and writing the manuscript]

ABSTRACT

Deterioration in attention and related processes is an early sign in schizophrenia predictive of disease development. Amongst the various translational paradigms for assessing attention in rodents, it is not known if they are equivalent in detecting individual differences. Answers here are pertinent to their use in the general human population for identifying individuals at high risk of developing schizophrenia. The present study employed a within-subject approach to examine in mice two common paradigms for assessing attention that differ markedly in their implementation. An operant-based two-choice visual discrimination task (2-CVDT) that depends on effortful attention to brief visual cues was contrasted with prepulse inhibition (PPI) of the acoustic startle reflex, a well-established test of pre-attentive gating whereby processing of a startle-eliciting stimulus is inhibited by a preceding weak prepulse stimulus. Here, we revealed a correlation showing that individual mice with low PPI tended to perform poorly in the 2-CVDT in terms of choice accuracy but not response speed. This specific positive correlation suggests that the two readouts might be regulated via common attentional mechanisms, which might be critically dependent on normal muscarinic and *N*-methyl-D-asparate receptor functions. As demonstrated here, blockade of either receptor type by scopolamine or dizocilpine impaired 2-CVDT performance at doses that have been shown to disrupt PPI in mice. Further studies contrasting these two paradigms would be warranted to characterize the possible underlying psychological constructs that give rise to this correlation and to clarify whether the two paradigms may effectively capture schizophrenia-related cognitive deficits belonging to orthogonal domains.

INTRODUCTION

Dysfunction in attention is central to a number of psychiatric conditions, including schizophrenia (Cornblatt and Kelip, 1994). In a recent review on the measurement and treatment research to improve cognition in schizophrenia (MATRICS) initiative, Young *et al*. have emphasized the importance of furthering our understanding and characterization of the translational paradigms within separate cognitive domains to facilitate the development of novel pharmacotherapy against such impairments, the treatment of which remains unsatisfactory (Young *et al*., 2009). Within the domain of attention, the most consistently reported form of deficiency amongst schizophrenia patients is sustained attention or vigilance (Riccio *et al*., 2002), which refers to the continuous allocation of attentional resources for the detection of rare events for prolonged periods of time (Chudasama and Robbins, 2004; Robbins, 2002). The prototypical translational paradigm emphasized by the MATRICS (Young *et al*., 2009) is the 5-choice serial reaction time task (5-CSRTT) originally developed by Robbins for rats (Robbins, 2002).

The 5-CSRTT is a powerful paradigm for assessing a variety of attentional processes and has been considered as a rat analogue of the continuous performance test (CPT) in humans (Robbins, 2002). Its application in rats has been well established, and it has also been successfully translated to mice (Humby *et al*., 1999). As in all operant-based task, appropriate pretraining is necessary to ensure stable and consistent performance. It is not uncommon that training up to 3–4 months is necessary to reach 70–80% accuracy in rats (Carli *et al*., 1983; Granon *et al*., 1998; Bari *et al*., 2008) or mice (de Bruin *et al*., 2006; Humby *et al*., 1999; Patel *et al*., 2006; Pattij *et al*.,2007; Young *et al*., 2004). The recent development of a simpler version of the 5-CSRTT by Dillon and colleagues (Dillon *et al*., 2009; Kornecook *et al*., 2010) including its application to mice (which sometimes are less adaptive for operant training than rats) suggests its application as a high-throughput alternative. The two-choice visual discrimination task (2-CVDT) developed by Dillon *et al*. retains the critical element for assessing

sustained attention or vigilance (Dillon *et al*., 2009). The task requires the animal to learn to choose between two (viz., left vs right magazines) rather than five reward magazines according to a visual discriminatory signal, which can be progressively reduced to impose increasing demand on sustained visual attention. The reduction in possible choices (from 5 to 2) certainly reduces the attentional demand on spatial distinction. Additional differences and simplifications of the task design introduced in the 2-CVDT, however, have rendered it less suitable to dissect the distinct processes implicated in response inhibition/selection than the original 5-CSRTT. But, such simplifications are pertinent to the task being less demanding on mice, allowing high level of performance to be achieved within a shorter period of time (Dillon *et al*., 2009).

Although not included in the MATRICS, another common paradigm related to early sensory gating control – prepulse inhibition (PPI) of the acoustic startle reflex – has been discussed as a valuable complementary paradigm (Young *et al*., 2009). While the 5-CSRTT taxes sustained attention and inhibitory control, PPI is widely accepted as an operational measure of pre-attentional sensory gating (Graham, 1980) whereby perception of a weak (non-startling "prepulse") stimulus impedes the response to a shortly succeeding intense (startle eliciting "pulse") stimulus (Buckland *et al*., 1969; Hoffman and Searle, 1968). PPI does not require prior training; it can be easily and similarly implemented in animals and humans in a single test session (Swerdlow *et al*., 1999). PPI deficiency is consistently observed in schizophrenia patients, and reportedly also in their healthy relatives, suggesting that it may represent an endophenotype of the disease (Turetsky *et al*., 2007). A link between schizotypy personality scale and PPI deficiency has also been revealed in the general human population (Kumari *et al*., 2005).

However, the relationship between PPI deficits and the distinct domains of cognitive impairment identified in schizophrenia remains poorly specified (Hagan and Jones, 2005; Young *et al*., 2009). The limited studies in healthy controls have suggested potential associations between PPI and some forms of attention, execution time and/or planning speed in various tasks (Young *et al*., 2009). Here, we examined if individual differences in PPI expression within a homogeneous cohort of C57BL/6 mice might predict

sustained attention performance measured in the 2-choice version of Robbin's original 5-CSRTT (Dillon *et al*., 2009; Kornecocook *et al*., 2010). Few studies have directly addressed this, and the outcome would be instructive in deciding the extent to which PPI might parallel, or be orthogonal to, the identified domain of schizophrenia cognitive deficit represented by the 5-CSRTT.

Despite the obvious differences in their implementation, training and motivational demand, 5-CSRTT performance and PPI expression are both tightly regulated by brain dopaminergic, cholinergic and glutamatergic activity (Robbins, 2002; Ukai *et al*., 2004; Yee and Feldon, 2009; Yee *et al*., 2004 Young *et al*., 2009), and the two tests are similarly sensitive to strain differences in mice (Greco *et al*., 2005; Patel *et al*., 2006; Singer *et al.,* 2009). Psychologically, such a link might be anticipated by recent findings that the direct reaction to the prepulse stimulus positively correlated with the PPI magnitude in mice (Yee *et al*., 2005) . Given that the prepulse precedes the pulse stimulus in time, it is tempting to speculate a causal relationship behind such a correlation (Yee *et al*., 2004). Importantly, greater PPI has been found in healthy humans when they were instructed to pay attention to the prepulse stimulus (Fillion *et al*,1993, 1994, 1998). Hence, although PPI occurs unconsciously (Blumenthal, 1995) its expression can be influenced by effortful sustained attentional demands.Given the pivotal role of prepulse perception in the generation of the PPI effect, parallels might be drawn between the sensitivity to detect the prepulse stimulus and the brief visual discriminatory signal used to guide correct response in the 5-CSRTT or its two-choice adaptation.

In addition, we investigated the pharmacological properties of the 2-CVDT for possible parallelism with the known sensitivity of PPI to NMDAR/glutamatergic and muscarinic receptor blockade. First, we tested the effect of the N-methyl-D-asparate receptor (NMDAR) blocker dizocilipine (MK-801). Answers here are not only relevant to its comparison with PPI but also with the original 5-CSRTT, given that MK-801 readily disrupts performance in both tests (Paine and Carlezon, 2009; Paine *et al*., 2007; Yee *et al*., 2004). Secondly, we evaluated the effects of the muscarinic cholinergic receptor antagonist, scopolamine, at a dose that disrupts PPI in C57Bl6 mice (Ougazzal *et al*., 2001; Yeomans *et*

al., 2010). Although the efficacy of scopolamine to impair choice accuracy in the 2-CVDT has been reported previously (Dillon *et al*., 2009), the demonstration fell short of confirming its dependency on the central action of the drug. At the end of our study, we therefore compared the effects of scopolamine with its methyl analogue – methylscopolamine, which does not cross the blood–brain barrier, in addition to saline control. Confirmation on this critical finding would consolidate the relevance of central muscarinic activity to all three tests: PPI, 5-CSRTT, and its simplified two-choice version.

The present study thus provides further characterization of the 2-CVDT performance in mice and its possible correlation with PPI expression, as well as its sensitivity to glutamatergic and cholinergic treatments that are known to impair attentional processing. The data would be instrumental to future applications of the 2-CVDT as an additional paradigm to the original 5-CSRTT to index attention and vigilance.

MATERIALS and METHODS

Subjects

Subjects were 30 naïve male C57BL/6 mice obtained from our in-house specific pathogen free colony, 12 weeks old at the start of the study. They were caged singly in Macrolon type III cages (Techniplast, Milan, Italy), housed inside a temperature (21 ◦C) and humidity (55%) controlled vivarium under a reversed 12:12 h day/night cycle (lights off: 07.00–19.00 h). During all experiments, the animals were maintained on a restrictive food diet. All tests were conducted in the dark phase. The procedures described here are in agreement with the Principles of Laboratory Animal Care (NIH publication No. 86-23, revised 1985), and had been approved by the Cantonal Veterinary Office of Zurich.

Food restriction schedule

Food restriction was gradually introduced by reducing the available feeding time across 5 days (12 h, 8 h, 4 h, 3 h, 3 h). At the same time, the animals were familiarized with the liquid reward in the home cage to minimize food neophobia. Afterwards, daily food rations (food pellets, Kliba 3430, Klibamühlen, Kaiseraugst, Switzerland) were calculated as a function of each animal's weight loss/gain from the previous day in order to maintain a stable weight of not less than 85% of the ad lib weight. Drinking water was freely available throughout.

Experimental design

The study was conceived in two parts, conducted in the same cohort of mice (Table 1). First, they were tested on the two-choice visual discrimination task (2-CVDT; [Dillon *et al*., 2009]) followed by a test of prepulse inhibition (PPI). Then, a series of pharmacological tests were performed on the 2-CVDT.

SEQUENCE OF EXPERIMENTATION		Duration (days)	Final sample size
PART 1:	• **2-choice visual discrimination task**		
	Habituation and magazine training	4	30
	Lever press shaping	6	30
	Baseline discrimination	4	30
	Variable signal duration	3	30
	• **Prepulse inhibition**	1	30
PART 2 [c]:	• **Effects of MK-801 on 2-choice visual discrimination**		
	Saline vs 0.075 vs 0.15mg/kg, signal duration = 0.5 – 10s	1[a]	9/10/10
	Saline vs 0.075 vs 0.15mg/kg, signal duration = 0.1 – 1s	1[a]	10/10/10
	Saline vs 0.3 mg/kg, signal duration = 0.1 – 1s	1[a,b]	13/13
	• **Effects of scopolamine on 2-choice visual discrimination**		
	Saline vs methylscopolamine 1mg/kg vs scopolamine 1mg/kg, signal duration = 0.1 – 1s	1[d]	10/9/10

[a] Animals were subdivided into three drug conditions, balanced for previous task performance in Part 1. Subsequent subdivisions were counterbalanced for previous drug experience.
[b] Preceded by one day of injection-free baseline test, and followed by three days of drug- and test-free period.
[c] Initially, the experiment was designed to include a higher dose of 1mg/kg, but this was stopped as soon as it was clear that this dose was too high and seriously affected motor behaviour. Testing therefore continued with only the lower dose of 0.3 mg/kg. One animal in the methylscopolamine condition stopped responding and was dropped from the final analysis.
[d] One animal in the methylscopolamine condition stopped responding and was dropped from the final analysis.

Apparatus

Operant boxes - The 2-choice visual discrimination task was performed in four operant chambers (Model E10-10, Habitest System, Coulbourn Instruments, Allentown, PA, USA), each placed inside a ventilated and sound-attenuated box. The chamber measured 33 cm in height, with a panel wall 25 cm wide. A Plexiglas wall was positioned 13 cm away from the panel wall, allowing a floor space of 13 × 25 cm. A 2.8 W house light was installed in the middle of the panel wall, 23 cm above the steel grid floor, pointing upward at the ceiling, providing diffused illumination. Two magazines(Model H14-22M-20), 13.2 cm apart, were mounted within the panel wall, 2 cm above floor level. Each magazine was equipped with a 1.4 W stimulus inside. Nose-poke into the magazines was detected by an infrared photocell beam placed at the magazine's entrance. Each magazine was equipped with a liquid dipper dispenser (Model

H14-05R) capable of delivering 0.01ml of liquid reward (20% condensed milk solution, Milch Lait, Schweizer Milchproduzenten, Switzerland). A retractable lever (Model H21-03M) was positioned in-between the two magazines, 2.4 cm above the grid floor. A 2.8-W stimulus light was mounted 10 cm directly above the lever. To limit positional bias, two additional 5-cm wide transparent partitioning walls were positioned 6 cm apart, one on either side of the retractable lever. The animals were therefore required to take a detour to move between the lever and either magazine. The operant boxes were controlled by Graphic State (Version 1.013) implemented on a PC connected to all four boxes via a control interface. A miniature digital camera was installed within each operant chamber allowing for on-line monitoring and a record for subsequent examination when necessary.

Acoustic startle reaction boxes - Prepulse inhibition was assessed using four acoustic startle chambers for mice (SR-LAB, San Diego Instruments, San Diego, CA, USA) as fully described elsewhere (Yee *et al*., 2004a,b). Each startle chamber comprised a non-restrictive cylindrical enclosure made of clear Plexiglas attached horizontally on a mobile platform, which was in turn resting on a solid base inside a sound-attenuated isolation cubicle. A high-frequency loudspeaker mounted directly above the animal enclosure inside each cubicle produced a continuous background noise of 65 dB_A and the various acoustic stimuli in the form of white noise. Vibrations of the Plexiglas enclosure caused by the whole-body startle response of the animal were converted into analogue signals by a piezoelectric unit attached to the platform. These signals were digitized and stored by a computer. A total of 130 readings were taken at 0.5-ms intervals (i.e., spanning across 65 ms), starting at the onset of the startle stimulus in pulse-alone and prepulse-plus-pulse trials, and at the onset of the prepulse stimulus in prepulse-alone trials (see below for definition of trial types). The average amplitude (in arbitrary units) over the 65ms was used to determine the stimulus reactivity.

Two-choice Visual Discrimination Task

Habituation and magazine training - Over four consecutive days, the animals were habituated to the operant chambers and acclimatized to consuming the liquid reward from the dipper in the magazine. The dipper of one of the two magazine trays was programmed to be raised, accompanied by the illumination of the magazine light until a nose poke into the magazine was detected, at which time the dipper was raised for 3s allowing access to the liquid reward. This was repeated after a variable 15s ±5s inter-trial interval (ITI). The lever was retracted and the house light remained on during the entire session, which ended when 18 min had elapsed or 60 rewards had been collected. All animals collected at least 40 rewards on the last day.

Lever press shaping - Next, the animals were trained to press lever to gain the opportunity to obtain the liquid reward for six days. Each trial began with the presentation of the lever. A single lever-press was followed by its immediate retraction and the illumination of the magazine tray at one of randomly selected magazine, until a nose poke into the magazine was detected, at which time the dipper was raised for 3s allowing access to the liquid reward. The next trial then began 10 s later. Only one magazine (left or right) was used per daily session, with access to the other magazine blocked off by a metal plate. Training resumed on the following day with the other magazine tray. This minimized the possible development of a side bias. On each day, half of the animals were trained with the left magazine with the remaining ones using the right magazine. The house light remained on throughout the session, which ended after 24 trials were completed or when 25 min had elapsed. All animals had completed at least 19 trials on the last training day.

Visual discrimination - Access to both magazines was now available. With house light on and the lever retracted, the animals were introduced into the chamber. The first trial began 5 s later. A trial was initiated with the presentation of the lever. One lever press resulted in the retraction of the lever, followed immediately by the random illumination of one of the two magazine lights for a maximum

duration of 30s. A nose poke into the illuminated magazine was rewarded by a 3-s access to the liquid reward (correct response) in the continual presence of the magazine light. Failure to response in the 30s (an omission) or an (incorrect) nose poke response into the non-illuminated magazine was followed immediately by a 30 s time-out period when all stimulus lights (including the houselight) were switched off and the lever remained retracted. The next trial then began after a 10 s ITI. A session ended after 48 trials (completed or omitted) or when 50min had elapsed. Four performance variables were calculated at the end of each session: (i) *Choice accuracy* was defined by the formula: *correct responses* / (*correct responses* + *incorrect responses*) × 100%, (ii) *Omission rate* was defined as *omissions* / *all initiated trials* × 100%, (iii) Latency to choose (*choice latency*) was defined as the time between lever press and nose-poke (correct or incorrect) excluding omissions, and (iv) Latency to lever press (*initiation latency*), defined as the time elapsed between presentation of the lever press and the lever press. There was no upper limit programmed for initiation latency.

Test of visual discrimination with variable signal duration - Once the animals had achieved high accuracy (≥80% on two consecutive days) in the basic visual discrimination task described above, the duration of the visual signal indicating where nose poke response should be directed in order to obtain reward was reduced to 10, 2, 1 or 0.5s. The animals were still allowed a maximum of 30s to perform a nose poke response following the presentation of the visual signal, before an omission would be recorded. Amongst the maximum number of 48 trials (regardless of omission), every block of 4 trials featured one of the four possible signal durations. Testing lasted for 3 daily sessions, with each session lasting for a maximum of 50 min.

Prepulse inhibition of acoustic startle reflex

The animals were still maintained on food deprivation during this experiment. The test procedure was identical to that described by Yee *et al*. (2004). The animals were presented with a series of discrete trials comprising a mixture of four types of trials. These included pulse-alone trials, prepulse-pulse trials, prepulse-alone trials, and trials in which no discrete stimulus other than the constant background noise (denoted as 'no-stimulus' trials). Intensity of the prepulse stimulus s varied amongst 69, 73, 77, 81, and 85 dB_A, which corresponded respectively to 4, 8, 12, 16, and 20 decibel units above the constant background noise at 65 dB_A. The duration of prepulse stimuli was 20 ms, and the pulse stimulus 40ms. The stimulus onset asynchrony (SOA) of the prepulse and pulse stimuli on prepulse-pulse trials was 100 ms.

After the animals were placed into the Plexiglas enclosure of the startle chamber, they were allowed 2 min of acclimatization before delivery of the first trial. The first six trials consisted of startle-alone trials, which served to habituate and stabilize the animals' startle response. Subsequently, the animals were presented with 12 blocks of discrete test trials. Each block consisted of one trial of each of the following trial types: pulse-alone, prepulse-plus-pulse trials of each of the five levels of prepulse, prepulse-alone of each of the five levels of prepulse, and no stimulus (ie background alone). The session was concluded with the final block of six consecutive startle-alone trials, which were compared with the very first six trials for assessing startle habituation. Successive trials were separated by a variable 15±5 s ITI.

Prepulse inhibition (PPI) refers to the attenuation of the startle response to the pulse stimulus when a prepulse stimulus preceded the presentation of the pulse. A percent PPI (%PPI) index was used to calculate the degree of startle inhibition at each prepulse intensity: %PPI = [(pulse-alone)−(prepulse-plus-pulse)]/(pulse-alone)×100%]. An average %PPI score was computed for individual animals across the five possible prepulse intensities. In addition, to index the direct reaction to the prepulse (prepulse-alone

trials), a linear regression line was used to fit the average reactivity score (log-transformed) from no-stimulus trials to increasing levels of prepulse, and the slope of the fitted line was used to represent direct reactivity to the prepulse stimulus.

Pharmacological experiments

Design and duration of the drug experiments are summarized in Table 1. All drugs were dissolved in sterile saline solution to achieve the desired concentration for injection via the intraperitoneal route at a volume of 5ml/kg, 20 min before testing. All solutions for injection were freshly prepared on the day of testing. MK-801, scopolamine and methylscopolamine were obtained from Sigma–Aldrich, Germany.

Statistical Analyses

All data were analyzed by parametric analysis of variance (ANOVA). To assist interpretation of the statistical outcomes, significant effects were further investigated by supplementary restricted analyses applied to subsets of the data included in the overall ANOVA, or by pair-wise comparisons based on the associated mean-square error terms taken from the overall ANOVA. All statistical analyses were carried out using SPSS for Windows (version 13, SPSS Inc. Chicago IL, USA). "Type III" ANOVAs as specified by SPSS were consistently used throughout the present report. To better conform to the homogeneity and normality assumptions of parametric ANOVA a natural logarithmic data transformation was performed on the reactivity scores obtained in the PPI experiment (see Csomor *et al*. 2008) as well as on the response and initiation latencies derived from the two-choice visual discrimination experiment. Pearson's product moment correlations were conducted to evaluate possible associations between behavioural indexes obtained in the PPI experiment and performance in the initial (drug free) phase of the visual discrimination task. A p-value of <0.05 was consistently taken the yardstick for statistical significance.

RESULTS

Pre-training & discrimination learning

Habituation, magazine training, and lever-press shaping were successful and all animals acquired the necessary responses. Next, the animals were trained on the visually guided discrimination using visual signals lasting for a maximum of 30s. Within four days, all animals had achieved criterion performance at 80% accuracy or above across two consecutive days, with an omission rate lower than 15%. On the last day choice accuracy and omission rate were 99% (±0.49) and 9.4% ± (2.5), respectively. The mean choice (magazine nose poke) latency was6.8 sec (±0.6), and the mean initiation (lever press) latency was 16.8 sec (±0.8) on the last day of discrimination training.

Performance under variable signal duration

Accuracy **-** As illustrated in **Figure 1A**, choice accuracy generally improved as a function of increasing signal duration. Accuracy was high, at >90% correct, when the signal duration was from 2 to 10s, and choice accuracy on such trials was stable across the three days of tests. Reducing the signal duration to 1s led to a moderate reduction in choice accuracy, but the animals demonstrated improvement over days at this condition. Further shortening of the signal to 0.5s was associated with a notable deterioration of choice accuracy: performance on day 1 was close to chance (50%) level, but then gradually increased over the next two days approaching 70% correct on the third day. These interpretations were supported by 3 × 4 (days × signal duration) repeated measures ANOVA of percent accuracy which yielded a significant main effect of signal duration [$F(3,87)=128.79$, $p<0.001$] and its interaction with days [$F(6,174)=2.47$, $p<0.05$]. The main effect of days was not statistically significant [$p>0.18$].

Omission - This measure essentially reflected the accuracy measure. When choice accuracy appeared high, omission was low, and vice versa (**Figure 1B**). Thus, omission rate decreased as a function of day, and this decrement was more pronounced with shorter signal duration. A days by signal duration ANOVA of percent omission revealed a significant main effect of signal duration [$F(3,87)=56.68$, $p<0.001$] and days [$F(2,58)=11.46$, $p<0.001$] as well as their interaction [$F(6,174)=5.96$, $p<0.01$].

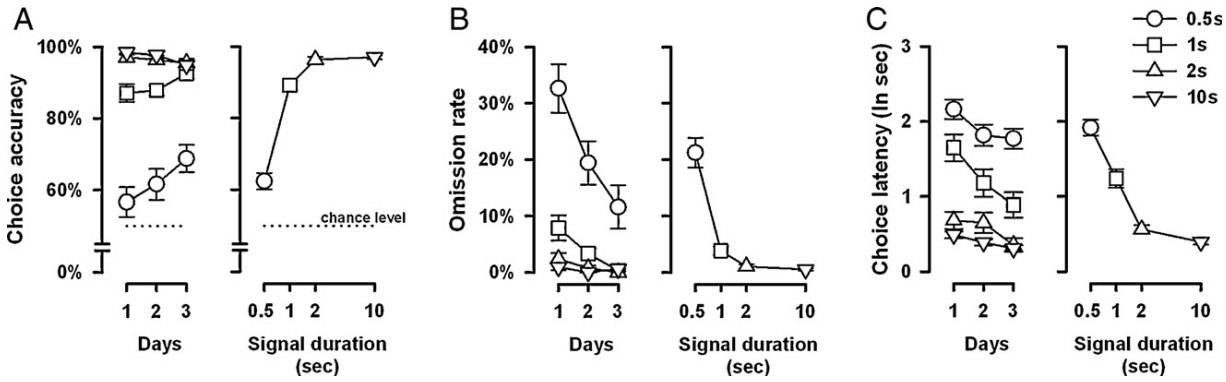

Figure 1. Two-choice visual discrimination with variable signal duration. Each plot consists of two panels: left panel depicts performance expressed as separate lines corresponding to each signal duration (0.5, 1, 2, 10 s) and plotted as a function of days, and the right panel depicts performance as a function of increasing signal duration collapsed across days. Three performance measures are shown: A. choice accuracy, B. omission rate, and C. choice latency. All values refer to mean values ±SE (n = 30). Signal duration (in s) is plotted in the abscissa using a logarithmic scale in all plots, whereas days are illustrated using a linear scale.

Choice latency - The latency to choose as measured from the time when the discriminatory signal was initiated to the nose poke (excluding trials of omission) showed a clear decrease over training days across regardless of signal duration (**Figure 1C**). Amongst the four signal durations, choice latency showed a monotonic reduction with increasing signal duration. In support of these interpretation, a days by signal duration ANOVA of logarithmically transformed choice latency yielded a significant effect of days [$F(2,58)=11.08$, $p<0.001$] and of signal duration [$F(3,87)=94.58$, $p<0.001$], but not their interaction [$p>0.12$].

Initiation latency - The latency to press the lever to initiate the presentation of the signal was averaged across all trials (including omission trials). No distinction was made between stimulus duration because the stimulus had yet to occur. There was a significant but marginal monotonic reduction of initiation latency over days, decreasing from 7.0±0.4s on day 1, 6.1±0.4s on day 2, to 5.3±0.4s on day 3. Repeated measures ANOVA of logarithmically transformed initiation latency yielded a clearly significant days effect [$F(2,58)=13.84$, $p<0.001$].

Prepulse Inhibition of Acoustic Startle Reaction

Prepulse inhibition

As expected, startle reactivity was progressively weakened by the presence of prepulse on prepulse-pulse trials compared with pulse-alone trials. This constitutes the prepulse inhibition (PPI) effect, and is readily appreciable by the conventional measure of percent inhibition (%PPI, **Figure 2A**) as well as the analysis of reactivity scores (**Figure 2B**) on prepulse-pulse trials compared with pulse-alone trials. Repeated measures ANOVA of both measures yielded as expected a highly significant effect of prepulse intensity [%PPI: $F(4,116)=75.49$, $p<0.001$, logarithmically transformed reactivity scores: $F(5,145)=164.18$, $p<0.001$].

Reactivity on prepulse-alone and no-stimulus trials

Increasing intensity of the prepulse stimulus when present alone also induced appreciable reactivity in a monotonic fashion (**Figure 2C**), leading to a significant main effect of prepulse intensity [$F(5,145)=58.78$, $p<0.001$]. The linear trend of this effect accounted for 91.1% of the variance attributed to the overall main effect of prepulse intensity, supporting our choice to use the individual linear slope to index (log-transformed) reactivity to prepulse stimuli. The mean value ± SEM of this index was 0.31±0.03.

Startle habituation

Comparison of startle reactivity on the first and final blocks of 6 pulse-alone trials provided no evidence for startle habituation, consistent with our impression in C57BL/6 mice in the past (Yee et al., 2005). Indeed, only 6 out of 30 animals showed a numerical tendency of startle habituation. The effect of blocks was not significant [p>0.1] in the repeated measures ANOVA of logarithmically transformed reactivity scores.

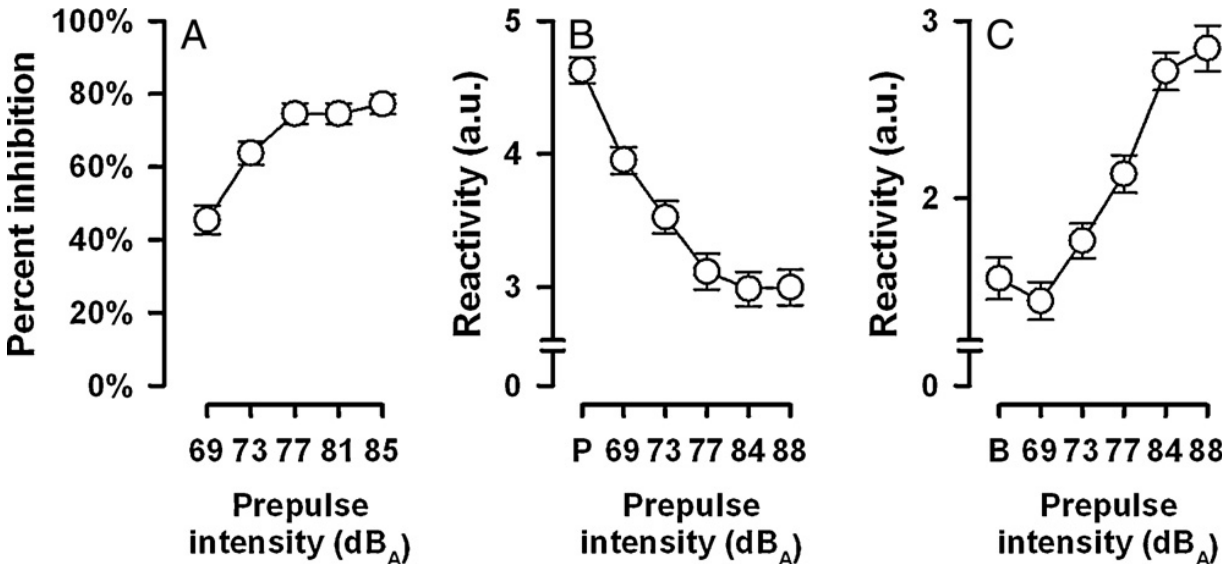

Figure 2. PPI and prepulse-elicited reactivity. The behavioural indices illustrated are: A. percent inhibition of startle response as a function of increasing prepulse intensity. B. Startle reactivity to the pulse stimulus on pulse-alone trials (P) and prepulse-plus-pulse trials of increasing intensity. C. Reactivity obtained on no-stimulus trials with background (B) noise only, and on prepulse-alone trials. All values refer to mean values±SE (n = 30).

Correlation between visual discrimination and PPI performance

Pearson's product moment corrections were performed on the four performance indices obtained from the visual discrimination task and four behavioural indices in the PPI experiment, and the statistical outcomes are illustrated in the correlation matrix presented in **Table 2**. Four statistically significant associations were revealed. Two of these were between tests, and both involved the choice accuracy

measure in the discrimination task, which correlated significantly with PPI expression (as indexed by %PPI, **Figure 3A**) and with the sensitivity/reactivity to the prepulse stimulus in prepulse-alone trials (**Figure 3B**). Correlation between these two PPI-related behavioural indices was also significant (**Figure 3C**). Lastly, the two reaction time measures in the discrimination task: choice latency and initiation latency were also found to be significantly correlated (**Figure 3D**).

Table 2 Correlation matrix between behaviour indices on visual discrimination and prepulse inhibition. The Pearson's product moment coefficient is illustrated in each cell, with the associated two-tailed probability level in parenthesis (n = 30, df = 28). Significant correlations are emphasized by a gray underlay.

	startle reactivity	%PPI	prepulse reactivity	choice accuracy	omission rate	choice latency	Initiation latency
habituation index	0.199 (0.292)	-0.226 (0.229)	0.041 (0.828)	-0.016 (0.931)	-0.244 (0.194)	-0.054 (0.779)	-0.357 (0.053)
startle reactivity		0.103 (0.586)	0.042 (0.827)	0.103 (0.587)	-0.059 (0.757)	-0.201 (0.288)	0.073 (0.701)
%PPI			**0.409** (0.025)	**0.372** -0.043	0.17 (0.368)	-0.122 (0.52)	0.189 (0.316)
prepulse reactivity				**0.461** (0.01)	-0.265 (0.157)	-0.051 (0.789)	0.11 (0.564)
choice accuracy					-0.269 (0.15)	-0.037 (0.846)	-0.061 (0.749)
omission rate						0.278 (0.138)	0.313 (0.092)
choice latency							**0.375** (0.041)

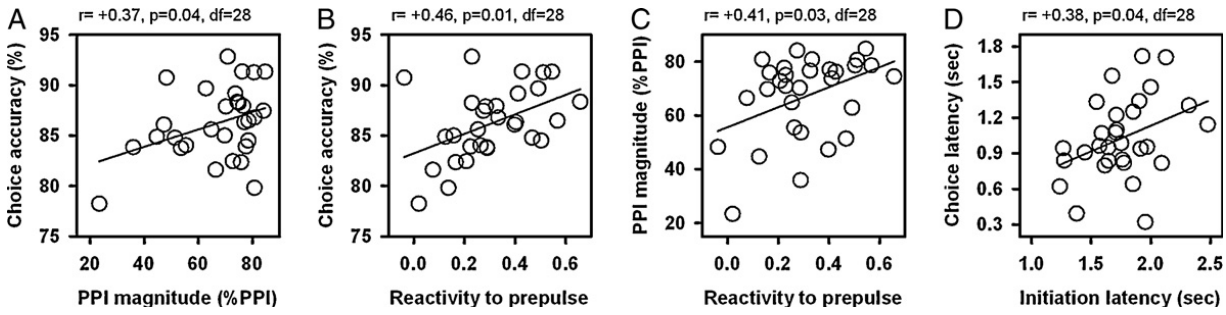

Figure 3. Scatter plots of the four significant correlative associations identified. Overall choice accuracy was associated with prepulse inhibition magnitude (A) as well as prepulse-elicted reactivity (B). These two behavioural indices from the PPI experiment were themselves correlated with each other (C). Within the visual discrimination task, the latency to initiate a trial (lever press) and the latency to choose (nose poke) correlated significantly with each other (D). The straight line in each scattered plot depicts the fitted linear regression line. n = 30, df = 28.

Pharmacological Experiments

NMDA receptor blockade by MK-801

0 vs 0.075 vs 0.15 mg/kg at signal duration 0.5−10s. One animal in the vehicle-saline condition stopped responding on this day and was dropped from the final analysis. The final group size was saline=9, 0.075 mg/kg = 10, and 0.15 mg/kg =10. Systemic injection of MK-801 at either dose did not resulted in any appreciable effect in any of the dependent measures [all F'<1] of the discrimination task as confirmed by separate 3 × 4 (treatment × signal duration) split-plot ANOVAs. Choice accuracy showed the expected dependency on signal duration [$F(3,81)=21.93$, $p<0.001$] (**Figure 4A**), and so did omission rate [$F(3,81)=14.33$, $p<0.001$] (**Figure 4B**) and choice latency [$F(3,81)=18.49$, $p<0.001$]] (**Figure 4C**). The initiation latency was also not affected by drug treatment as indicated by the lack of a significant effect in a one-way ANOVA of ln-transformed initiation latency. The mean initiation latency was: saline=2.06s; 0.075 mg/kg=2.74s, 0.15mg/kg=2.52s (SE =0.36s).

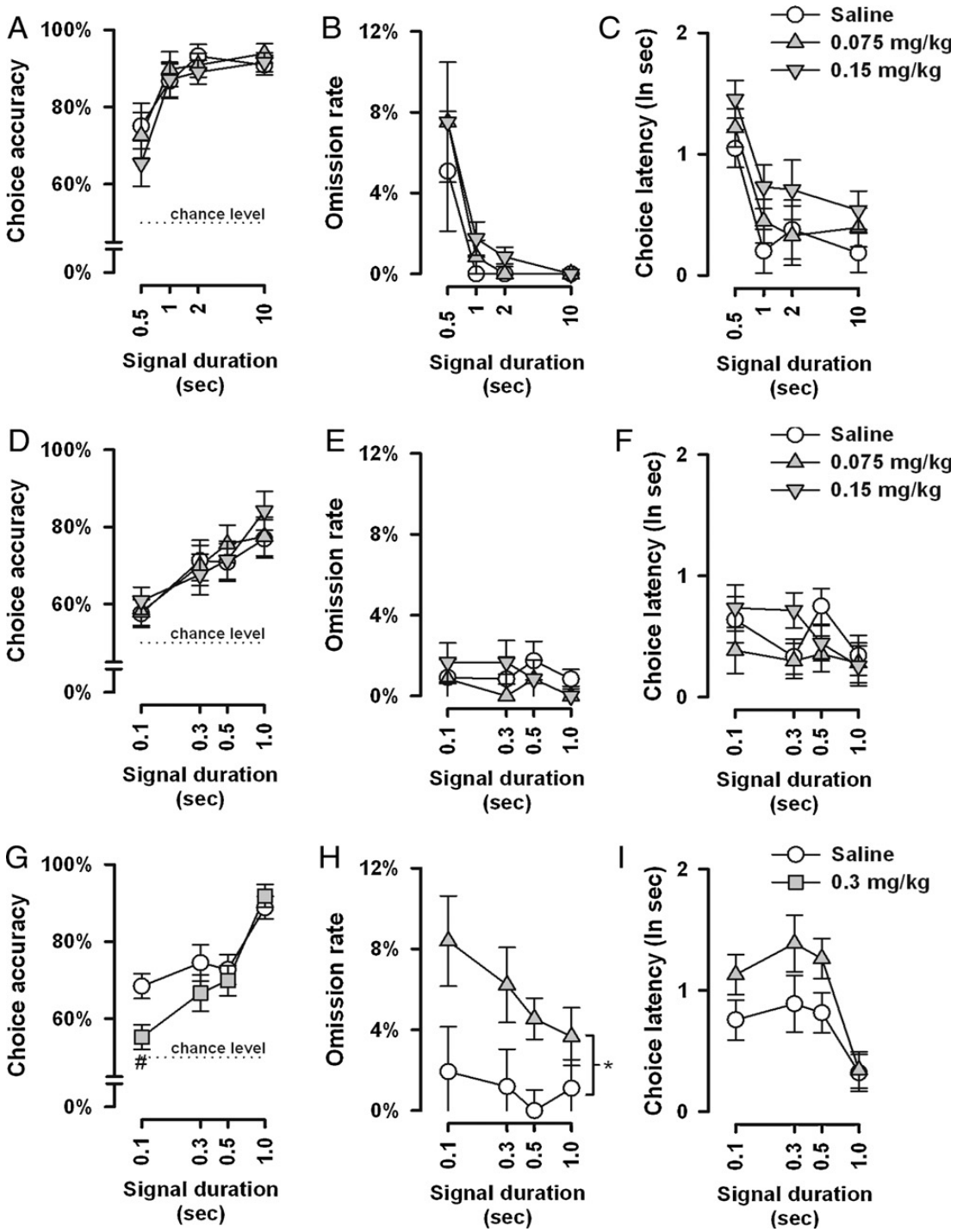

Figure 4. The acute effects of systemic MK-801 on visual discrimination performance. Three consecutive experiments were performed differing in doses of MK-801 and therange of signal duration employed (see text). Choice accuracy (A, D and G), omission rate (B, E, H) and choice latency (C, F and I) are illustrated for each experiment. All values refer to mean values ±SE. Signal duration (in s) is plotted in the abscissa using a logarithmic scale in all plots. * indicates a significant main effect of treatment ($p < 0.05$). # denotes a significant treatment effect ($p = 0.008$) identified in a restricted analysis confined to the signal duration of 0.1 s.

0 vs 0.075 vs 0.15 mg/kg at signal duration 0.1−1s - By reducing the duration of the signal further, overall performance was lowered and a greater sensitivity to the signal duration was demonstrated in terms of choice accuracy (**Figure 4D**). However, MK-801 at these two doses remained ineffective, not only in affecting choice accuracy but also in terms of omission rate (**Figure 4E**), choice latency (**Figure 4F**), or initiation latency [saline=2.3s, 0.075mg/kg=2.4s, 0.15mg/kg=2.6s, SE=0.28s]. Separate ANOVAs only yielded a significant effect of signal duration in choice accuracy [$F(3,81)=21.58$, $p<0.001$]. None of the other measures showed such sensitivity. No animals were dropped from the final analysis.

0 vs 0.3 mg/kg at signal duration 0.1−1s - Next, a higher dose of MK-801 was attempted. Initial attempt to include another higher dose at 1mg/kg was aborted due to motor side effects that impeded performance. The remaining animals were distributed equally into two groups (n=13). At 0.3 mg/kg, MK-801 began to show appreciable behavioural effect. This was clearly seen in the omission rate (**Figure 4H**), yielding a main effect of treatment [$F(1,24)=5.69$. $p<0.05$]. This effect was apparent across different signal duration. The interaction between treatment and signal duration was not significant [$p=0.39$], but a sensitivity to signal duration was observed [$F(3,72)=2.86$, $p<0.05$]. Choice accuracy appeared to be somewhat affected by MK-801 at this dose, but it was restricted to the shortest signal duration at 0.1s (**Figure 4G**). ANOVA of choice accuracy, however, did not yield an effect of treatment [$p=0.17$], and the critical treatment by signal duration interaction was only tentative [$F(3,72)=2.49$, $p=0.07$]. However, when the data set was restricted to this condition only, a highly significant treatment effect emerged [$F(1,24)=8.40$, $p=0.008$], which would remained significant with Bonferroni correction. The impact of MK-801 here was limited also on choice latency (**Figure 4I**), analysis of which only yielded a significant effect of signal duration [$F(3,72)=15.00$, $p<0.001$]. Similarly, one-way ANOVA of ln-transformed initiation latency failed to reveal a significant treatment effect. The mean initiation latency was: saline = 3.70 s, 0.3 mg/kg = 5.13 s (SE = 0.52 s).

Muscarinic receptor blockade by scopolamine

Scopolamine (1mg/kg, i.p.) was highly effective in disrupting choice accuracy, and this effect was apparent regardless of signal duration (**Figure 5A**). ANOVA of choice accuracy revealed a significant effect of treatment [$F(2,26)=10.10$. $p<0.001$], and of signal duration [$F(3,37)=10.43$, $p<0.001$]; there was no evidence for their interaction [$F<1$]. The drug also exerted a parallel effect on choice latency: it extended the latency to choose across all signal duration (**Figure 5C**). The trial initiation latency was however only very weakly affected, albeit in a similar direction, by scopolamine (saline=3.9±0.6s, methylscopolamine=4.0±0.6s, scopolamine=5.9±0.6s); this effect was far from statistical significance [$p=0.73$]. Omission rate was not affected, and remained relatively low (**Figure 5B**). Analysis of omission rate did not reveal any significant effect. Overall, methyscopolamine and vehicle control group were essentially indistinguishable from each other in all measures. Post-hoc comparisons of all significant treatment effects indicated that methylscopolamine and saline controls never differed significantly from each other, and both control groups differed from the scopolamine-treated animals [all p's<0.5], strongly suggesting that the effects were mediated by the central action of scopolamine.

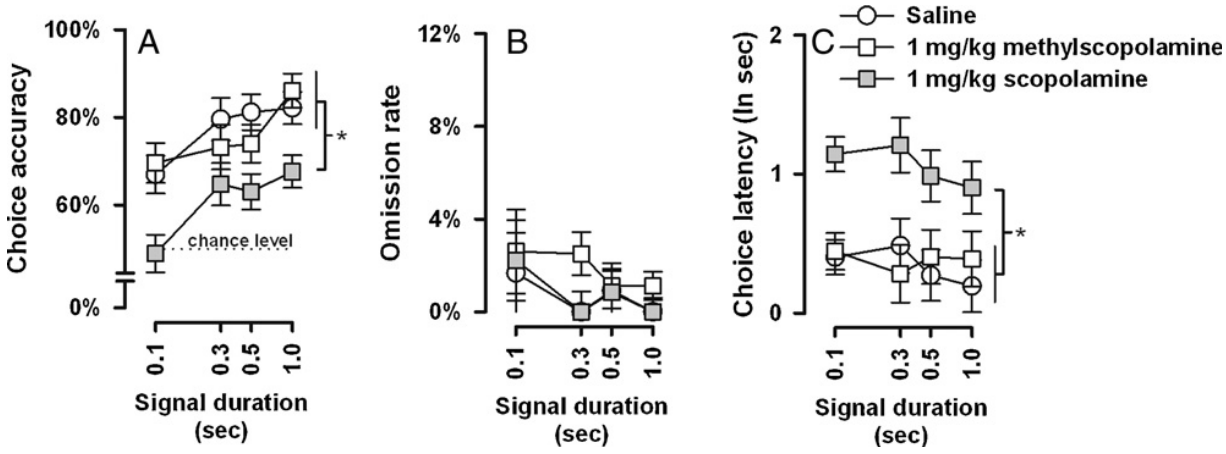

Fig. 5. The acute effects of systemic scopolamine on visual discrimination performance. Choice accuracy (A), omission rate (B) and choice latency (C) are illustrated as a function of increasing signal duration (in s), which was plotted using a logarithmic scale in the abscissa. All values refer to mean values ±SE. * Refers to a significant difference between scopolamine and the two control groups (methylscopolamine and saline).

DISCUSSION

The present study demonstrated that individual difference in sensorimotor gating as indexed by PPI of the acoustic startle reflex correlated with vigilance as reflected by choice accuracy in the two-choice version of Robbins's 5-CSRTT (R^2=14%). This was further accompanied by a correlation between this vigilance measure and the propensity to react to the prepulse stimulus in the PPI test (R^2=21%), which would be anticipated by the known positive relationship between prepulse reactivity and PPI magnitude (R^2=17%, see Yee and Feldon 2009). Although these correlations accounted for about one fifth or less of the variance in each case, their emergence is meaningful. They suggest a relationship between sensorimotor gating function and vigilance within the normal population (C57BL/6 mice). This relationship is demonstrated in only one strain of inbred mice here, and its possible generalization to other common strains, as well as other species, including humans would be warranted. Notably, the correlations were exclusively seen with the measure of choice accuracy but not reaction time. Although both measures were responsive to decreasing signal duration, their overall variations did not seem to be related within-subjects. This might further suggest that the correlations demonstrated were unlikely mediated by individual variation in motor activity. On the other hand, the significant correlation that emerged between initiation latency (the lever press required to initiate the signal) and choice latency (time to perform the nose poke choice response after presentation of the signal) suggest that both are linked to individual difference in general speed of motor execution (R^2=14%).

The correlative relationship between sensorimotor gating and vigilance revealed here might suggest that the two behavioural variables are mediated via common attentional mechanisms. Despite the common view that PPI is mediated via pre-attentive automatic information processing and thus occurs unconsciously (Blumenthal, 1995) there is clear evidence for conscious attentional modulation of PPI. That is, PPI is enhanced in healthy humans when attention is directed to detecting the prepulse stimulus (e.g. Filion and Poje, 2003; Heekeren *et al*., 2004; Thorne *et al*., 2005; Fillion *et al*., 1993, 1994, 1998). This form of attentional control over PPI can be explained by Graham's protection of processing hypothesis (Graham, 1975) according to which on-going processing of the prepulse stimulus is protected

against interference by the forthcoming pulse stimulus. One might therefore propose that similar attentional processes underlie the ability to detect the prepulse stimulus and the brief visual discriminatory signal used to guide correct response in the 5-CSRTT (see **Fig. 3** and **Table 2**).

Further evidence for a potential parallelism between PPI expression and sustained attention comes from clinical observations that deficits in both domains frequently co-exist in several neuropsychiatric disorders including schizophrenia (Scholes and Martin-Iverson, 2009, 2010) and animal studies showing that the two measures are similarly sensitive to a number of brain and pharmacological manipulations (Young *et al*., 2004; Gould *et al*., 2005).

Schizophrenia patients consistently exhibit deficits in attentional processes and (Cornblatt and Keilp, 1994; Chudasama and Robbins, 2004) and vigilance has been identified by the MATRICS program (Young *et al*.. 2009) as one of seven cognitive domains that are most commonly impaired in schizophrenia. Likewise, PPI deficits have been repeatedly demonstrated in schizophrenia patients since the early work by Braff *et al*. (1978) (for a review see Braff *et al*., 2001). Loss of PPI in schizophrenia is widely believed to reflect deficient sensorimotor gating that result from a disruption of preattentive information processing leading to sensory flooding and cognitive fragmentation (Braff and Geyer, 1990). More recent data however question this view. In fact, it has been found that schizophrenia patients only exhibited impaired PPI when they were instructed to attend to the prepulse stimulus but showed normal PPI under experimental conditions that ensured the participants were ignoring the auditory stimuli (Dawson *et al*., 1993, 2000; Martin-Iverson *et al*., 2009; Scholes and Martin-Iverson, 2009, 2010). Therefore, it has been hypothesized that PPI deficiency in schizophrenia might stem from deficits in selective/sustained attention rather than from dysfunction of preattentive gating (Dawson *et al*., 1993, 2000; Hazlett *et al*., 2007; Kedzior and Martin-Iverson, 2007). This might also be indicative of a common mechanism underlying the PPI deficits and attentional impairments in schizophrenia. Thus far, there is weak evidence that PPI expression is related to a specific cognitive domain (Young *et al*., 2009). However, these studies did not include classical attentional paradigms (Young *et al*., 2009). Further

investigations of a potential link between PPI and attention in both schizophrenia patients as well as the normal population are therefore highly warranted. On the other hand, PPI has recently been reported to positively correlate with measures of planning in healthy controls such that subjects with the lowest PPI showed the longest response times in selected cognitive tests from the Cambridge Neuropsychological Test Automated Battery (CANTAB; Bitsios *et al*., 2006; Csomor *et al*., 2008; Giakoumaki *et al*., 2006). Notably, no such link was detected in the present study since the PPI magnitude neither correlated with choice latency nor initiation latency indicating that animals with low PPI levels did not exhibit longer reaction times than subjects with high PPI levels. This, however, does not exclude the possibility of a potential relationship between PPI and reaction time in C57BL/6 mice in other cognitive tasks.

Another line of evidence for a potential parallelism between PPI and sustained attention comes from a mouse model. The DBA/2 mouse strain consistently exhibits poor PPI and weak startle reactivity relative to other inbred mouse strains (Bortolato *et al*., 2007; Bullock *et al*., 1997; Logue *et al*., 1997; McCaughran *et al*., 1997; Olivier *et al*., 2001; Singer *et al*., 2009). Due to this intrinsic PPI deficit, the DBA/2 strain has been considered as an predictive animal model for detecting antipsychotic drug activity (Olivier *et al*., 2001). We have previously confirmed that the PPI deficiency in DBA/2 relative to C57BL/6 mice was independent of the strain difference in baseline startle reactivity (Singer *et al*., 2009). This is of relevance given that the two strains were also found to differ in the 5-CSRTT with the DBA/2 strain performing worse than the C57BL/6 strain particularly in terms of choice accuracy (Greco *et al*., 2005; Patel *et al*., 2006).

Finally, PPI and vigilance are similarly sensitive to a number of pharmacological manipulations including the disruptive effects of NMDAR and cholinergic antagonists. PPI disruption induced by non-competitive NMDAR antagonists such as MK-801 and PCP is a well-established finding in rodents (Bast *et al*., 2000; Swerdlow and Geyer, 1998; Yee *et al*., 2004). More recently, systemic MK-801 has been reported to decrease accuracy and increase omission in the 5-CSRTT in rats (Paine *et al*., 2007; Paine and Carlezon, 2009). These effects of MK-801 on 5-CSRTT performance could be partially reversed by low

doses of the atypical antipsychotic but were resistant to the typical antipsychotic haloperidol which even exacerbated the effects of MK-801 at high doses (Paine and Carlezon, 2009). This pharmacological profile is similar to that of NMDAR antagonist-induced disruption of PPI which is also reversible by atypical and resistant to typical antipsychotics (Geyer *et al*., 2001). In the present study, we have evaluated for the first time the effect of MK-801 in the 2-choice version of the 5-CSRTT in mice, and found similar effects. MK-801 impaired choice accuracy and increased omission at a dose of 0.3 mg/kg. Notably, the impairment on accuracy was only seen at the lowest stimulus duration (0.1s) when the attentional demand was the highest. This pattern indicates that the effect of MK-801 was mediated via direct interference with the neural elements underlying attention and did not stem from changes in sensory, motor, or motivational factors. The fact that MK-801 neither affected initiation latency nor choice latency strengthens this interpretation. It is also unlikely that the increase in omission was responsible for the effect on accuracy because MK-801 consistently increased the number of omitted trials across all stimulus durations.

Both 5-CSRTT performance and PPI expression are also robustly disrupted by muscarinic cholinergic antagonists such as scopolamine (De Bruin *et al*., 2006; Fendt *et al*., 1999; Hohnadel *et al*., 2007; Humby *et al*., 1999; Jones and Shannon, 2000; Pattij *et al*., 2007; Singer *et al*. submitted; Tadros *et al*., 2009; Ukai *et al*., 2004). The sensitivity of the 2-choice version of the 5-CSRTT to the disruptive effect of scopolamine has recently been demonstrated (Dillon *et al*. 2009). Here, we replicated this finding by showing that systemic scopolamine (1 mg/kg) clearly reduced choice accuracy across all stimulus durations, and demonstrated that this effect solely stemmed from its impact on the CNS because methylscopolamine, the quaternary analogue of scopolamine, that does not cross the blood–brain barrier and therefore is only effective in blocking peripheral muscarinic receptors, did not produce any effect in all dependent measures. Consistent with Dillon *et al*.'s report scopolamine also delayed choice latency (Dillon *et al*., 2009). However, we did not replicate the increase in omission rate reported by these authors when they increased the dose of scopolamine to 0.2 mg/kg. It is imperative to point out that we had adopted the task design by Dillon and colleagues here in allowing the animals 30 s to respond before an

omission would be registered. This is substantially longer than the 5-s holding time limit typically imposed in the 5-CSRTT, and thus the present task might be less sensitive in capturing potential effects on omissions. A shorter response window might be a useful modification of the present 2-CDVT. Nevertheless, it should also be noted that scopolamine has often been found to affect different behavioural measures of the 5-CSRTT and consensus on the precise pattern of effects has yet to be established (Jakala *et al*., 1992; Jones and Higgins, 1995; Jones *et al*. 1995; Mirza and Stolerman, 1998; Robbins, 2002).

In summary, these pharmacological studies provide further credence to the hypothesis that PPI and vigilance is impaired by hypofuncion of either NMDAR-mediated glutamatergic or central muscarinic cholinengic activity. This is of relevance to the hypothesized dysfunction of these neurotransmitter systems in the pathology of schizophrenia (Coyle, 2006; Crook *et al*., 2001; Dean *et al*., 2002; el-Mallakh *et al*., 1991; Olney *et al*., 1999; Raedler *et al*., 2003) and might therefore suggest that attentional deficits and loss of PPI in schizophrenia patients may mediated via common glutamatergic and/or cholinergic mechanisms.

CONCLUSIONS

Different lines of evidence support a close link between PPI expression and sustained attention. The present study revealed that the two behaviours not only correlated with each other but also with the propensity to react to prepulse stimulus in normal C57BL/6 mice. This further suggests that the attentional processes underlying vigilance are also central to the modulation of PPI. Thus, the 5-CSRTT, or its 2-choice analogue, provides an important supplement to the analysis of PPI which might help to dissociate whether changes in PPI by a given manipulation stem from an effect on pre-attentive gating or conscious attentional control of PPI. Importantly, these data underscore the utility of the 2-choice version of the 5-CSRTT as a novel and efficient means of assessing sustained attention in mice in a relatively high-throughput manner, which might provide a useful tool in the testing of new pharmacological agents in addition to the characterization of animal models.

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Singer P, Feldon J, Yee BK (unpublished observations). Disruption of prepulse inhibition by scopolamine and its attenuation by typical and atypical antipsychotic drugs in C57BL/6 mice. *Psychopharmacology*.

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Served as a social contact to people within Aberdeen who suffered from mental health problems. ***Pillar Aberdeen, Aberdeen, United Kingdom*** Oct 2003 - Aug 2004

PRESENTATIONS

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German Endocrine Brain Immune Network (Oral Presentation) "Late prenatal immune activation in mice leads to behavioural and neurochemical abnormalities relevant to the negative symptoms of schizophrenia." Essen, Germany – **April 2010**
Schizophrenia International Research Society (Poster Presentation) "Cognitive impairment following prenatal immune challenge in mice correlates with prefrontal cortical AKT1 deficiency." Florence, Italy – **April 2010**
Swiss Society for Neuroscience Conference (Poster Presentation) "Maternal Immune Activation in Late Pregnancy Alters Adult Behaviour: An Informal Model of Schizophrenia." Fribourg, Switzerland – **March 2009**
Schizophrenia International Research Society (Poster Presentation) "Glutamatergic modulation of parvalbumin messenger RNA containing neurons via the *N*-methyl-D-aspartate receptor in the prefrontal cortex in schizophrenia." Venice, Italy – **June 2008**
Department of Psychiatry (Oral Presentation) "Altered Gene Expression in Schizophrenia" Presented to fellow students, scientists and clinicians at The Johns Hopkins University School of Medicine, USA – **August 2006**

PUBLICATIONS

B.K.Y. Bitanihirwe, S.N. Dubroqua, P. Singer, J. Feldon, BK. Yee (2011) Sensorimotor gating and vigilance-dependent choice accuracy: a within-subject correlative analysis in wild-type C57BL/6 mice. *Behavioural Brain Research,* **217**(1):178-187.
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B.K.Y. Bitanihirwe, M.P. Lim, T-U.W. Woo (2010) *N*-Methyl-D-Aspartate Expression in Parvalbumin-Containing Inhibitory Neurons in the Prefrontal Cortex in Bipolar Disorder. *Bipolar Disorders*, **12**:95-101.
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K. Ishizuka, N. Cascella, S. Lin, D. Schretlen, M. Morita, K. Tajinda, C. Hookway, **B. Bitanihirwe**, S. Kong, A. Sawa (2008) Towards the establishment of a biological indicator for schizophrenia (in Japanese). *Kyushu Neuropsychiatry*, **53**(1)104-105.

PUBLISHED ABSTRACTS

Yee B.K.; Peleg-Raibstein, D.; Hauser, J.; Singer, P.; Dubroqua, S.; **Bitanihirwe, B.**; Llano Lopez, L.; Gargiulo, P.A.; Feldon, J., (2010) Is PPI cognitive? What have we learned from a correlative approach? *International Behavioral Neuroscience Society*
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