Strathclyde Institute of Pharmacy and Biomedical Sciences

Impact of Environmental Risk Factors for Schizophrenia on the Developing Brain: Characterisation of the Effects of PolyIC and THC on Functional Neural Systems and Behaviour

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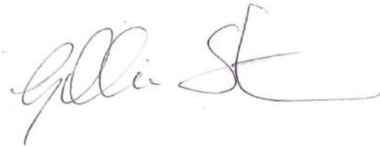
Signed: 

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ABSTRACT

Cannabis abuse can produce deficits in cognition and has been implicated as a 'late' environmental risk factor in the pathogenesis of the poly-factorial disorder schizophrenia. Evidence suggests an age-related susceptibility to the deleterious effects of cannabis as early onset of use may increase the vulnerability of the brain to the adverse consequences of cannabis abuse. Animal models are crucial for exploration of mechanistic and causative theories, and long-term behavioural consequences of adolescent cannabis abuse in a controlled experimental environment. This thesis evaluates the vulnerability of the adolescent/peripubertal brain to Δ^9-tetrahydrocannabinol (THC), the principal psychoactive constituent of cannabis, and explores the potential interplay between this schizophrenia-related 'late' environmental risk factor and an 'early' environmental risk factor (prenatal infection - maternal immune activation (MIA)) on functional neural systems and behaviours relevant to schizophrenia.

Cannabinoid CB_1 receptor ontogeny (activated in the brain by the receptor ligand THC) within important cognitive substrates, the prefrontal cortex (PFC) and hippocampus, was investigated to delineate a period of neurodevelopmental vulnerability for peripubertal THC treatment. CB_1 receptor ligand binding revealed that the PFC and hippocampus follow differential late maturational trajectories throughout the peripubertal period. The 'vulnerability window' for peripubertal THC treatment was defined as post-natal day (PD) 35-56 to encompass the dynamic peripubertal ontogenetic patterns of the CB_1 receptor in both these regions. Furthermore, age-related alterations in cerebral metabolism and regional functional connectivity profiles were evident in the hippocampus and important neuromodulatory nuclei including the ventral tegmental area, dorsal raphe, locus coeruleus and the diagonal band of Broca.

Acute THC administration (5mg/kg) produced hypometabolism in the thalamus and an altered functional connectivity profile between thalamic nuclei and the PFC, hippocampus and the nucleus accumbens. THC-induced anomalistic neural activity was

evident in key neuromodulatory nuclei and produced perturbed functional connectivity within acetylcholine, noradrenaline, and dopamine neural pathways. Acute THC treatment resulted in alterations in cerebral metabolism in the amygdala and aberrant functional connectivity profiles between amygdaloid nuclei and the hippocampus, PFC and nucleus accumbens. There appeared to be an age-related sensitivity to THC in several thalamic, neuromodulatory and amygdaloid nuclei.

Peripubertal low-dose intermittent THC (3.5mg/kg, 3 times a week), mimetic of light, recreational adolescent cannabis use, produced long-term cognitive inflexibility, as measured by the attentional-set shifting task, perturbed cerebral metabolism in the dorsolateral orbital cortex and the nucleus accumbens core and altered functional coupling between both these regions and neural substrates subserving reward-related learning including prefrontal, septal and amygdala subfields. High-dose daily THC (7mg/kg) throughout the peripubertal period, mimetic of heavy daily cannabis abuse, did not precipitate any schizophrenia-related behaviours in adulthood. MIA induced by prenatal exposure to the immune-stimulating agent polyriboinosinic-polyribocytidilic acid (PolyIC) did not produce any schizophrenia-related phenotypes in adulthood. However, prenatal PolyIC exposure produced residual hypermetabolism within discrete components of the prefrontal cortex dorsolateral orbital and cingulate cortices and hypometabolism within the CA3 subfield of the hippocampus. The functional connectivity signatures of all these regions indicated a unified MIA effect of aberrant mesocorticolimbic functional coupling in adulthood. Furthermore, chronic intermittent treatment with low-dose THC during the peripubertal period caused an increase in sensitivity to amphetamine (indicative of aberrant mesolimbic dopamine transmission) in PolyIC-treated offspring compared to PBS-treated offspring, suggestive of a synergistic effect of these two environmental risk factors.

In conclusion, the findings presented in this thesis have provided clear evidence of dose-specific detrimental effects of 'adolescent' THC exposure on behaviour and the functional neural systems that may underpin these deficits which impact on behaviour and neural systems into adulthood.

ABBREVIATIONS

ANATOMICAL

Agranular insular cortex	AIC
Anterodorsal thalamus	AD
Anteromedial thalamus	AM
Anteroventral thalamus	AV
Auditory cortex	Aud C
Basolateral amygdala	BLA
CA1 stratum moleculare	CA1 Mol
CA1 stratum oriens	CA1 Oriens
CA1 stratum radiatum	CA1 Rad
CA2 stratum moleculare	CA2 Mol
CA2 stratum oriens	CA2 Oriens
CA2 stratum radiatum	CA2 Rad
CA3 stratum moleculare	CA3 Mol
CA3 stratum oriens	CA3 Oriens
CA3 stratum radiatum	CA3 Rad
Central amygdala	CA
Centromedial thalamus	CM
Cingulate cortex	Cg
Dentate gyrus	DG
Dorsal raphe	DR
Dorsal subiculum	dSub
Dorsolateral orbital cortex	DLO
Dorsolateral striatum	DlStr
Dorsal tegmental nucleus	DTN
Entorhinal cortex	Ento C
Globus pallidus	GP
Horizontal diagonal band of Broca	VDB
Infralimbic cortex	IL
Inferior colliculus	Inf Coll
Interpeduncular nucleus	IPN
Lateral geniculate nucleus	Lat Gen
Lateral habenula	LHb
Lateral hypothalamus	Lat Hypo
Lateral intermediate septum	LSi
Lateral orbital cortex	lO
Laterodorsal septum	LSD
Locus coeruleus	LC
Mammillary body	Mam Body
Medial geniculate nucleus	Med Gen
Medial habenula	MHb

Medial orbital cortex	mO
Medial septum	MS
Median raphe	MR
Mediodorsal thalamus	MD
Motor cortex	Motor C
Molecular layer	Mol L
Nucleus accumbens core	NacC
Nucleus accumbens shell	NacS
Parietal cortex	Par
Parataenial thalamus	PT
Paraventricular thalamus	PV
Posterior thalamus	Post T
Prefrontal cortex	PFC
Piriform cortex	Pir
Prelimbic cortex	PrL
Pontine nucleus	PN
Retrosplinial cortex	Retro C
Reticular thalamus	Rt
Secondary motor cortex	M2
Somatosensory cortex	SS
Substantia nigra pars reticulata	SNR
Substantia nigra pars compacta	SNC
Subthalamic nucleus	Subthal N
Subiculum	Sub
Septohippocampal nucleus	Shi
Ventral orbital cortex	vO
Visual cortex	Visual C
Ventral pallidum	VP
Ventromedial striatum	vmStr
Vertical diagonal band of Broca	VDB
Ventral tegmental area	VTA
Ventroanterior thalamus	VA
Ventrolateral thalamus	VL
Ventromedial posterior thalamus	VMP
Ventral subiculum	vSub

MISCELLANEOUS

Δ^9-tetrahydrocannabinol	THC
2-arachidonylglycerol	2-AG
2-[1-^{14}C]-deoxy-D-glucose	2DG
5-choice serial reaction time task	5-CSRTT
Acetylcholine	ACh
Analysis of variance	ANOVA
Attentional set -shifting task	ASST
Cannabidiol	CBD
Cannabinoid(s)	CB(s)
Catechol-O-methyltransferase	COMT
Complex discrimination	CD
Confidence interval	CI
Depolarisation-induced Suppression of Excitation	DSE
Depolarisation-induced Suppression of Inhibition	DSI
Discrimination index	DI
Disrupted in schizophrenia-1	DISC-1
Dominant-negative mutant *Disc-1*	DN-*Disc-1*
Dopamine	DA
Endocannabinoid(s)	eCB (s)
Extradimensional set-shift	ED
First reversal of discrimination phase	Rev1
Gamma-aminobutyric acid	GABA
Gestation day	GD
Glutamate	Glu
High-dose daily THC (7mg/kg)	THC B
Intradimensional set-shift	ID
Knock out	KO
Latent inhibition	LI
Lateralized reaction time task	LRTT
Lipopolysaccharide	LPS
Local cerebral glucose uptake	LCGU
Low-dose intermittent THC (3.5mg/3times a week)	THC A
Material immune activation	MIA
Messenger RNA	mRNA
Methylazoxymethanol acetate	MAM
Neuroregulin-1	NRG-1
Noradrenaline	NA
Novel object recognition	NOR
Partial least squares regression	PLSR
Phencyclidine	PCP
Phosphate buffed saline	PBS
Polyriboinosinic-polyribocytidilic acid	PolyIC

Postnatal day	PD
Prepulse inhibition	PPI
Regional optical density	ROD
Region(s) of interest	RoI(s)
Second reversal discrimination phase	Rev1
Serotonin	5-HT
Simple discrimination	SD
Sodium chloride-sodium citrate buffer	SSC
Third reversal discrimination phase	Rev3
Variable importance for the projection	VIP
Whole brain-tissue average	WBA
Wisconsin card sorting task	WCST

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CHAPTER ONE

General Introduction

Currently, cannabis is the most commonly used illicit drug worldwide with a particularly high prevalence rate noted amongst adolescents (Home Office, 2012). However, great controversy surrounds this drug as public debate into the legalisation of the drug for therapeutic use continues. The therapeutic properties of cannabis have long been exploited such as its role as an antiemetic, analgesic and anticonvulsant (reviewed by Hollister, 1986; Robson, 2001; Baker *et al*., 2003). These benefits are unquestionably overshadowed by the potential deleterious effects of long term cannabis use on mental health. Epidemiological evidence suggests that cannabis use can induce deficits in various aspects of cognition including executive function and working memory and has been implicated as a risk factor for schizophrenia (Solowij et al., 2002; Arseneault et al., 2004; Henquet et al., 2005; Cohen et al., 2008; D'Souza et al., 2009; Morrison et al., 2009; Crean et al., 2011). Furthermore, there is increasing evidence of age related susceptibility to the deleterious effects of cannabis as early onset of use may increase vulnerability to the adverse consequences and lead to more severe sequelae than recreational use in adulthood (Andréasson *et al*., 1987; Ehrenreich *et al*., 1999; Pope *et al*., 2003; Ajdacic-Gross *et al*., 2007; Miettunen *et al*., 2008; Sugranyes *et al*., 2009; Fontes *et al*., 2011). Moreover, the recent move by the UK government to upgrade cannabis from a Class C to a Class B drug further sparked the controversy surrounding the drug with strong opposition to the reclassification of cannabis being voiced by Professor David Nutt, the former chair of the Advisory Council on the Misuse of Drugs (Nutt *et al.*, 2007; Nutt, 2009). It is therefore necessary to gain a greater understanding of the deleterious effects of cannabis that underlie the potential vulnerability of the adolescent brain to cannabis abuse.

1.1 |The Cannabinoid System in the Brain

1.1.1 |Exogenous Cannabinoids

The *Cannabis sativa* plant contains over 60 active constituents known as cannabinoids (CBs). The most researched of these CBs is a highly lipophilic substance known as Δ^9-tetrahydrocannabinol (THC). First isolated and synthesised in 1964 by Gaoni and Mechoulam, THC was identified as the principal psychoactive constituent of the *Cannabis sativa* plant (Gaoni and Mechoulam, 1964). CBs are highly lipophilic compounds that can readily cross the blood brain barrier to exert their effects. Other naturally occurring CBs include Δ^8-tetrahydrocannabinol, cannabinol and cannabidiol (CBD). The last of these, CBD, differs from THC, Δ^8-tetrahydrocannabinol and cannabinol, as it does not exhibit psychotomimetic properties. In fact, CBD appears to have antipsychotic effects as it has been shown to attenuate the psychotogenic effects of THC (Bhattacharyya *et al*., 2010). Synthetic CB compounds such as WIN 55212-2, CP 55, 940 and HU-210 are also available for research purposes.

1.1.2 |Pharmacokinetics of Cannabinoids

Smoke inhalation is the most common and efficient route of THC administration. Following inhalation, THC is rapidly absorbed into the bloodstream via the lungs and is redistributed throughout the body and readily crosses the blood-brain barrier and enters the brain. Peak plasma concentrations are usually reached at approximately the same time as smoking cessation dependent on smoking technique. The bioavailability of THC via smoke inhalation varies between 10-30% (Iversen, 2000).

Oral ingestion of THC in fat-containing foods is a common yet less reliable route of THC administration. Absorption is slower following this route of administration with lower, more delayed peak THC levels observed. Peak plasma concentrations are reached approximately 1-4 hours following ingestion. Bioavailability is also greatly

reduced due to first pass metabolism resulting in an average THC bioavailability of less than 10% (Iversen, 2000).

In humans, THC concentrations within plasma and body tissues exhibit a biphasic pattern of decline (Ashton, 2001). Initial peak THC plasma concentrations drop quite rapidly, 1-6 hours dependent on route of administration. However, THC and other fat-soluble metabolites subsequently accumulate in fatty tissues reaching peak levels in 4-5 days (Agurell *et al*., 1986). The subsequent slow plasma redistribution of THC and its metabolites from fat sequestration greatly elongates its half-life to up to 7 days (Maykut, 1985).

THC metabolism takes place in the liver where it is primarily metabolized into 11-hydroxy-THC (11-OH-THC) and up to 20 other metabolites. 11-OH-THC, which exhibits equipotent psychoactive properties with THC, and the other metabolites are either gradually excreted in the urine (35%) or in the gut (65%), where they can be subsequently re-absorbed into the bloodstream. Following re-absorption, 11-OH-THC is then eventually metabolized to the non-psychoactive 11-nor-9-carboxy-THC and eventually eliminated completely from the body (Adams and Martin, 1996; Agurell *et al*., 1986).

Collectively, the pharmacokinetic properties of THC - both the sequestration in fat and production of active metabolites - greatly extend the pharmacological effects of THC in the body.

1.1.3 |Pharmacodynamics of Cannabinoids

To date, two $G_{i/o}$ protein coupled CB receptors have been identified in mammalian tissue - CB_1 and CB_2 receptors. Both the CB_1 and CB_2 receptors belong to the family of the seven *trans*-membrane spanning receptors. Of these receptors, CB_1 receptors are primarily located in the central nervous system whilst CB_2 receptors are typically

expressed within immune and haemopoietic tissues (Herkenham *et al*., 1991; Galiègue *et al*., 1995; Pertwee, 1997).

THC exhibits a lower affinity for CB_1 and CB_2 receptors as compared to the CB receptor affinity of potent synthetic receptor agonists such as WIN 55212-2, CP 55, 940 and HU-210. THC also displays a low efficacy at these receptors indicating it to be a partial agonist for both the CB receptors (Pertwee, 2006, Pertwee, 2008).

The anatomical localization of CB_1 receptors within the brain has been visualised using various different techniques including *in situ* hybridisation to detect mRNA, autoradiographic and immunohistochemical labelling of the receptors themselves (Iversen, 2003). CB_1 receptors are heterogeneously dispersed throughout the brain with particularly high concentrations found in the cerebral cortex, hippocampus, lateral caudate putamen, substantia nigra pars reticulata, globus pallidus, entopeduncular nucleus, molecular layer of the cerebellum and ependymal and sub-ependymal zones at the centre of the olfactory bulb. This heterogeneous distribution pattern of CB_1 receptors correlates well with the cognitive and motor effects of CBs. Conversely, low concentrations of CB_1 receptors are observed within the brainstem (Herkenham *et al*., 1991; Pertwee, 1997). As this brain region plays a vital role in the modulation of cardiovascular and respiratory function, a low binding CB_1 binding profile could account for the low toxicity and lack of lethality of cannabis. CB_1 receptors are predominantly located on pre-synaptic terminals, a feature that may play a crucial role in unmasking the physiological mechanism/s responsible for THC-induced alterations in cognition. This will be further discussed in sections 1.1.4 and 1.1.5.

1.1.4 |Endogenous Cannabinoid System in the Brain

The endocannabinoid (eCB) system is a modulatory system composed of CB receptors and lipid mediators known as endocannabinoids (eCBs) that bind to these receptors.

Following the discovery of the two $G_{i/o}$ protein -coupled CB receptors, CB_1 and CB_2 receptors, the search for endogenous ligands for these receptors began. The first eCB discovered was anandamide (*N*-arachidonylethanolamine), a long chain fatty acid amide, closely followed by the discovery of 2-arachidonylglycerol (2-AG) , a long chain fatty acid ester (Devane *et al*., 1992; Mechoulam *et al*., 1995). Other eCBs subsequently discovered include virodhamine (*O*-arachidonoyl ethanolamine), *N*-arachidonoyl ethanolamine (NADA) and nolodin ether (2-arachidonoyl glyceryl ether) (Hanus *et al.,* 2001; Huang *et al.,* 2002; Porter *et al.,* 2002). The two most researched of these endogenous ligands are anandamide and 2-AG and like exogenous CBs, these ligands target CB receptors within the CNS (Devane *et al.,* 1992; Mechoulam *et al.,* 1995).

In the brain, eCBs function as neuromodulators, however, unlike conventional neurotransmitters, they are not stored in vesicles but synthesised on demand from lipid precursors in the post-synaptic cell. Following depolarisation of the post-synaptic cell, eCBs are synthesised and released into the synapse where they act on pre-synaptically located CB receptors. The effect of these lipid mediators is transient and they are deactivated by uptake into the cell and metabolised. Metabolism of anandamide and 2-AG occurs mainly by enzymatic hydrolysis by fatty acid amide hydrolase and monoacylglycerol lipase (2-AG only) (Grotenhermen, 2006).

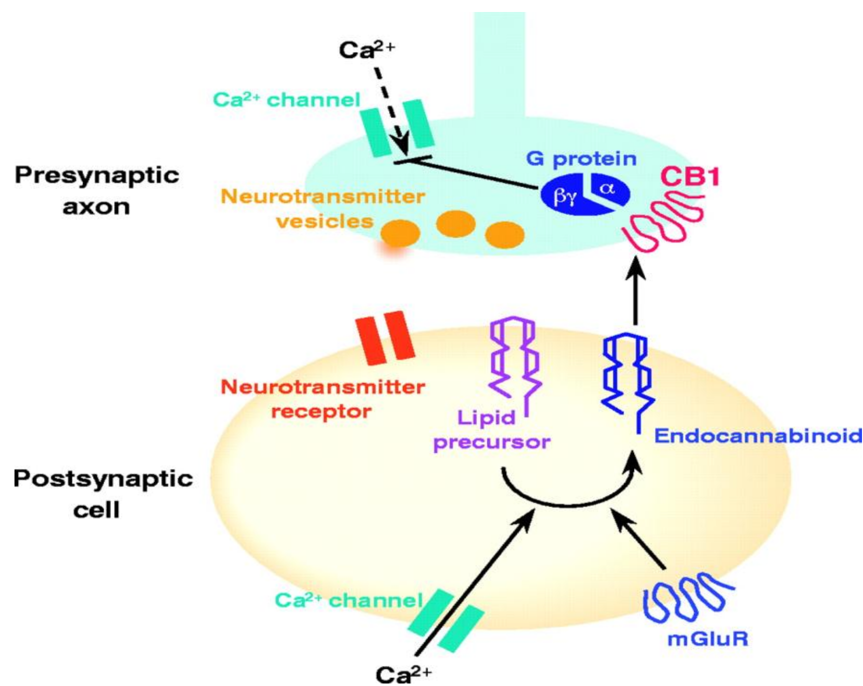

Figure 1.01 Retrograde signalling by eCBs. Depolarisation of the post-synaptic cell opens voltage-dependent Ca^{2+} channels. The influx in Ca^{2+} leads to activation and synthesis of eCBs from lipid precursors. eCBs then leave the post-synaptic cell and activate pre-synaptic CB_1 receptors. Activation of the G-protein of CB_1 receptor directly inhibits pre-synaptic Ca^{2+} influx and decreases the release of vesicles of neurotransmitter (taken from Wilson and Nicoll, 2002).

The discovery of these endogenous CBs has facilitated the elucidation of the pharmacological actions and pathophysiological properties of exogenous CBs such as THC which had long remained enigmatic.

1.1.5 |Endocannabinoid-mediated Synaptic Plasticity

CB_1 receptors are coupled to several different signal transduction pathways including adenylate cyclase, MAP kinase and ion channels. The involvement of these specific transduction pathways in eCB-mediated inhibition of neurotransmission is believed to contribute to the physiological functioning of this receptor as a modulator of synaptic plasticity (Ameri, 1999). Synaptic plasticity can be defined as the dynamic regulation

of synaptic strength and efficacy in response to environmental or internal stimuli (Gerdeman and Lovinger, 2003). eCBs play a pivotal role as retrograde signalling molecules in the facilitation of the physiological basis of learning and memory- synaptic plasticity.

Depolarisation-induced Suppression of Inhibition (DSI) is a form of fast transient retrograde signalling from postsynaptic neurons back to the pre-synaptic terminals of the inhibitory cells that innervate them (Iversen, 2003). This is a form of short term synaptic plasticity. Briefly, Ca^{2+} influx into the post-synaptic cell causes cellular depolarisation which subsequently induces the synthesis of CBs. These eCBs are released into the synapse where they bind to the pre-synaptic CB receptors resulting in pre-synaptic inhibition of release of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) (Fig 1.01). This phenomenon was first observed within the hippocampus and cerebellum (Pitler and Alger, 1992; Vincent *et al*., 1992). Within the cerebellum, pre-synaptic inhibition of release of the excitatory neurotransmitter glutamate (Glu) has also been demonstrated and is referred to as depolarisation-induced suppression of excitation (Kreitzer and Regehr, 2001). DSI has subsequently been shown to be a widespread phenomenon occurring in the basal ganglia, cortex and amygdala (Bodor *et al*., 2005; Katona *et al*., 1999; Kreitzer and Regehr 2001; Mátyás *et al*., 2006; Pitler and Alger 1992; Vincent *et al*., 1992)

Long-term potentiation and its opposing process long-term depression can be defined as a long lasting enhancement or reduction in synaptic strength respectively. These processes are greatly involved in synaptic plasticity (Gerdeman and Lovinger, 2003). The exact role of eCBs in these processes remain unclear, however, they are thought to be facilitated by eCB-mediated long lasting inhibition of neurotransmission. (Mackie, 2006).

The eCBs thus function as neurotransmitter modulators, controlling both the long and short term release of neurotransmitters from both inhibitory and excitatory neurons.

Exogenous CBs such as THC do not mimic endogenous CBs specific 'on demand' release and rapid inactivation but rather they cause non-specific long lasting widespread activation of CB_1 receptors resulting in persistent inhibition of neurotransmitter release from CB_1 receptor containing neurons. As a result exogenous CBs can disrupt the physiological functioning of the eCB system (Iversen, 2003).

1.2 |Cannabis Use in Society

1.2.1 |Potency of THC in Cannabis Products

In the *Cannabis sativa* plant, THC content is highest in the flowering tops, reducing in a downward pattern towards the leaves, lower leaves, stems, and seeds of the plant. Traditional herbal cannabis (mean THC content 8.4%; range 0·3–22%) is prepared from the dried flowering tops and leaves; hashish (mean THC content 5.9%; range 1.3–27.8%) is composed of dried cannabis resin and compressed flowers (Home Office, 2008). In recent years, sophisticated cultivation techniques have lead to the emergence of the highly potent sinsemilla (from the Spanish sin semilla—without seeds) onto the streets. Sinsemilla also known as 'skunk' is produced by hydroponic cultivation. This farming method involves artificial control of 'daylight' length, propagation of female cuttings and prevention of fertilization resulting in the production of the highly potent sinsemilla (mean THC content 16.2%; range 4.1-46%) (Home Office, 2008; King *et al*., 2005).

In a recent study carried out by the Home Office in 2008, it was reported that in England and Wales, following seizure of 2921 cannabis samples, 80.8% proved to be herbal cannabis and 15.3% cannabis resin. Further microscopic analysis of approximately two-thirds of the herbal cannabis samples seized revealed that 97% of these were sinsemilla. Furthermore, the study reported a marked increase in the proportion of herbal cannabis seized in recent years. In 2002, herbal cannabis

represented less than one third of police cannabis seizures, however, this figure has dramatically increased to over 80%. Moreover, the investigators found that in nearly all herbal cannabis samples the CBD content was less than 0.1% (Home Office, 2008). As mentioned in section 1.1.1, CBD appears to have potential antipsychotic properties as it has been shown to alleviate the psychotogenic effects of THC (Bhattacharyya *et al*., 2010). Thus, extremely low levels of CBD coupled with stark increases in THC potency in street cannabis may pose a real threat to public health.

1.2.2 |Patterns of Cannabis Abuse

Currently, cannabis is the most commonly used illicit drug worldwide with a particularly high prevalence amongst adolescents. Cannabis use is highest amongst the 16-29 year age bracket (Home Office, 2012). In the 2011/12 British Crime Survey of 15.7% of 16-24 year olds reported having used cannabis in the last year (Home Office, 2012). The peak years for initiation into cannabis use are 14-18years with over half of cannabis users having tried cannabis for the first time by the age of 16 years (Independent Drug Monitoring Unit, 2005). In 2011, cannabis was the mostly commonly used illicit drug among 11 to 15 year old secondary school students surveyed in England. An age-related increase in prevalence of use was evident with 0.2% of 11 year olds and 48.8% of 15 year olds reported having tried cannabis and 7.6% of 15 year olds reported having taking cannabis within the last year (Home Office, 2011). A similar survey of 37,307 13 and 15 year old school pupils in Scotland found that 17% of 15 year olds and 3% of 13 year olds had used cannabis at least once. Of these pupils, 13% of the 15 year old boys and 8% of 15 year old girls had reported using cannabis in the last month (SALSUS, 2010).

It is thus evident that cannabis abuse amongst adolescents is being initiated from an extremely young age. These statistics, coupled with emerging evidence suggesting an increased vulnerability of the adolescent brain to cannabis, due to late maturative

processes such as structural and functional brain re-modelling, are a growing concern for society (Casey *et al*., 2005). This will be discussed further in section 1.4.

1.3 |Role of Cannabinoids in Cognitive Dysfunction

1.3.1 |Acute Effects of Cannabis on Human Cognition

The effects of acute cannabis exposure have been investigated in several human studies. The general consensus amongst these studies is that acute cannabis use results in transient dose-related deficits in various neuropsychological batteries measuring aspects of memory and attention (Lundqvist, 2005; Ranganathan and D'Souza, 2006). These findings suggest that acute cannabis use induces alterations in hippocampal-dependent cognitive processing such as the attending to and encoding of information and is congruent with high CB_1 receptor densities in this region (Herkenham *et al*., 1991). Interestingly, a recent study investigating the effects of acute exposure to high potency cannabis found that high potency cannabis induces deficits in executive functions akin to those seen in subjects following chronic cannabis use (Ramaekers *et al*., 2006). The term executive functions refer to a group of superior abilities of organization and integration of regions of the brain responsible for higher cognition such as the prefrontal cortex (Roberts *et al*., 1998). These include the ability to plan and set goals, self regulation, cognitive flexibility and effective execution and feedback. Ramaekers and colleagues found that subjects exposed to high levels of THC showed impaired performances in the Tower of London task, a decision making task that measures planning and executive function. These findings are highly topical as the concentration of THC present in street bought cannabis is significantly increasing (Ramaekers *et al*., 2006).

1.3.2 |Residual Effects of Cannabis on Human Cognition

Heavy chronic cannabis use has been shown to affect attentional/executive functions and frontal lobe function (Pope and Yurgelun-Todd 1996). Pope and Yurgelun-Todd (1996) found decreased mental flexibility, increased perseveration and reduced learning amongst heavy cannabis using college students. Furthermore, they suggest that abilities to shift and/or sustain attention, functions associated with the prefrontal cortex, were most affected. There is also evidence for a dose-dependent relationship between the severity of cognitive deficits and cannabis intake as measured by the Wisconsin card sorting task (WCST), a task that measures mental flexibility (Bolla *et al*., 2002). However, the issue of cognitive deficits induced by heavy long term cannabis abuse is controversial as some studies have failed to find persistent, long term impairments of cognition following abuse (Pope *et al.,* 2001; Pope *et al*., 2002). Conversely, Solowij and her group found that long-term cannabis users show impairments in memory and attention that endure beyond the period of intoxication and worsen with increasing years of regular cannabis use (Solowij *et al*., 2002).

One possible reason for equivocal findings amongst the different studies could be due differential THC and CBD content in cannabis consumed or may be due to the fact that cannabis is potentially more toxic to susceptible populations e.g. adolescents or those of a particular genetic predisposition, and thus lead to more severe sequelae. This concept will be discussed in greater detail in section 1.4.

1.3.3 |Human Imaging Studies

Several human imaging studies have shown subnormal cerebral blood flow in long term cannabis users following a cessation period of less than a week (Mathew *et al*., 1986; Mathew *et al*., 1989; Lundqvist *et al*., 2002). Block and colleagues (2002) demonstrated base-line hypoactivity amongst young frequent cannabis users. This study showed an overall lowering of brain activity (9%) of users compared to control subjects (Block *et*

al., 2002). Furthermore, a study using positron emission tomography imaging found persistent metabolic alterations in prefrontal regions amongst heavy users abstinent for 25 days (Eldreth *et al*., 2004). Several studies have also been carried out using cognitive challenge paradigms. Pope and Yurgelun-Todd, using functional magnetic resonance imaging, investigated the effects of chronic cannabis use on performance of visual working memory tasks. They found diminished activation of the dorsolateral prefrontal cortex (PFC) compared to control subjects. A decrease in activation was still present, albeit less pronounced, after 28 days of washout (Pope and Yurgelun-Todd, 1996). Moreover, blood flow during performance of verbal memory recall tasks is decreased in the PFC amongst frequent cannabis users (Block *et al*., 2002). Interestingly, Jager and colleagues found reduced hippocampal and dorsolateral PFC activation among frequent cannabis users whilst performing an associative learning task. However, this aberrant activation did not impair performance on the task (Jager *et al*., 2007). Collectively, these findings are strongly indicative that chronic cannabis use induces notable functional alterations in regions involved in higher cognitive function such as the PFC and hippocampus.

1.3.4 |Limitations of Human Studies

Findings derived from human studies investigating the effects of cannabis use are often subject to debate due to methodological discrepancies and poor experimental design. It is often difficult for researchers to disentangle confounding factors in order to determine the underlying mechanisms. Such confounding factors include polydrug use, family history of substance abuse disorders, comorbid psychiatric disorders and in addition, cognitive deficits among cannabis users may be attributable to acute or sub-acute cannabis withdrawal. There is a difficulty reliably quantifying the total amount of cannabis consumed by subjects. Thus, preclinical studies are necessary to address these methodological issues. Preclinical studies allow for controlled drug dosing regimes enabling researchers to assess dose related drug effects on brain function. Furthermore,

they allow for systematic behavioural testing at designated time points following drug treatment to examine the persistence of effects in the absence of drug.

1.3.5 |Preclinical Studies Investigating the Effects of Cannabinoids on Cognition

The effects of CBs on various aspects of learning and memory have long been recognised. It has been consistently demonstrated in preclinical studies that CBs can induce acute but not residual deficits in various behavioural tasks for learning and short term memory that require hippocampal interaction in rodents (reviewed by Lichtman *et al*., 2002). CBs have been shown to impair performance in both spatial working memory tasks such as the T, 8-arm radial and Morris water maze tasks and non-spatial working memory tasks such as the delayed match to sample or the delayed non-match to sample tasks (Ferrari *et al*., 1999; Hampson and Deadwyler, 1998; Jentsch *et al*., 1997; Lichtman and Martin, 1996; Mallet and Beninger 1998; Miyamoto *et al*., 1995).

Attention refers to the ability to evaluate and allocate priority to incoming environmental stimuli and disregard irrelevant stimuli (Pattij *et al*., 2008). Behavioural tests such as the lateralized reaction time task (LRTT) and 5-choice serial reaction time task (5-CSRTT) are popular methods of measuring visuospatial attention in rodents. Intermittent chronic treatment with THC over a 14 day period in adult rats has been shown to produce impairments in the LRTT that persisted for 14 days following last THC treatment. To date, results from some studies using synthetic CBs have been ambiguous; Arguello and Jentsch (2004) found deficits in the LRTT following acute challenges with synthetic CB WIN 55212-2 whilst a corresponding study using 5-CSRTT failed to elicit similar deficits in visuospatial attention (Arguello and Jentsch 2004; Pattij *et al*. 2007).

Cognitive flexibility refers to successful adaptation of behaviour in response to a changing environment (Pattij *et al*., 2008). The attentional set-shifting task (ASST) is

commonly utilized to measure several aspects of cognitive flexibility including the ability to form an attentional set and reverse stimulus-reward associations (Birrell and Brown, 2000). In 2005, Egerton *et al*. demonstrated that acute administration of THC produced dose-related deficits in reversal learning, as measured by the ASST. These behavioural deficits were associated with deficits in neural activity (as measured by immediate early gene expression) in the orbitofrontal cortex and interconnecting striatal regions (Egerton *et al*., 2005).

1.4 |Vulnerability of the Adolescent Brain to Cannabinoids

1.4.1 |Brain Development throughout Adolescence

Throughout adolescence the human brain undergoes several developmental changes and cognitive maturation which collectively lead to greater cognitive efficacy. Recent advances in neuroimaging techniques have made it possible to track these changes throughout adolescence. Regions of the brain responsible for basic functioning such as the motor and sensory cortices are first to mature, followed by temporal and parietal cortices which are associated with basic language skills and spatial attention. Last to mature are higher order association areas involved in executive functions including decision making and planning, such as the PFC (Casey *et al*., 2005)(Fig 1.02).

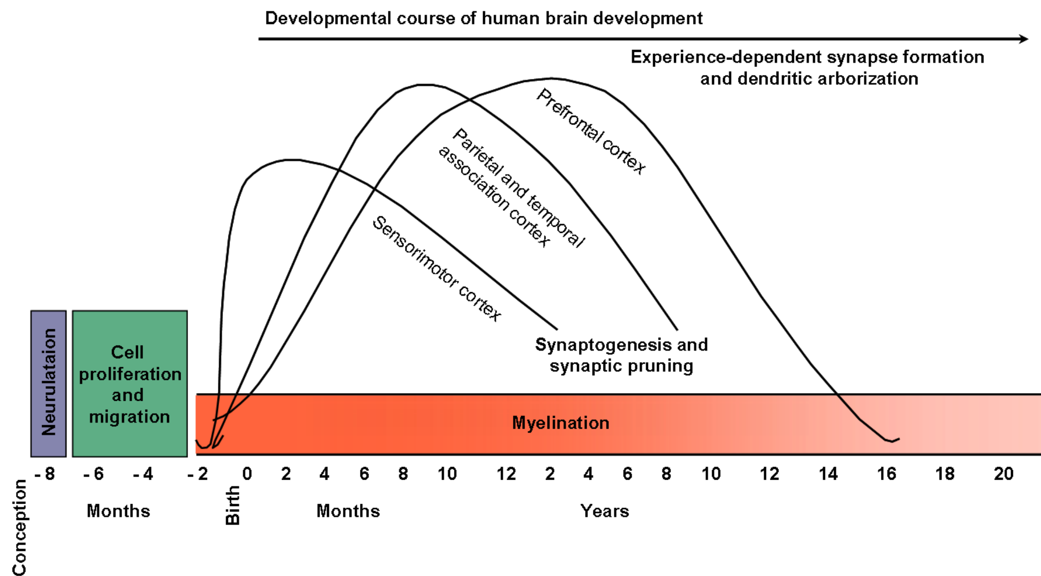

Figure 1.02 Patterns of Maturation in the Human Brain. Areas involved with basic functioning such as the sensorimotor cortex develop first, followed by the parietal and temporal association cortices and lastly, areas associated with higher cognitive processing such as the PFC are last to mature (adapted from Casey *et al*., 2005).

Alterations to both white and grey matter occur in the form of myelination and synaptic pruning respectively. Adaptations in grey matter are of particular interest as imaging studies have shown an overall increase in grey matter density in childhood and adolescence followed by postpubertal loss (Sowell *et al*., 2003). Grey matter density is an indirect measure of the composition of glia, vasculature and neuronal dendritic and synaptic processes within the brain. A reduction in grey matter density is primarily induced by synaptic pruning of neurons (Casey *et al.,* 2005). Synaptic pruning is a process involving the removal of excess synapses. It has been postulated that the pattern of pruning is determined by synapse use: connections that are employed to respond to the environment are retained and strengthened whilst synapses which are rarely used are eliminated, thus creating dedicated neural networks resulting in an increase in the efficacy of cognitive processing (Lichtman and Colman 2000; Luna and Sweeney 2004).

As mentioned earlier, grey matter maturation is a patterned process in which areas involved in higher cognitive functioning such as the PFC and hippocampus are last to undergo maturation (Casey *et al*., 2005; Giedd *et al*., 1999; Gogtay *et al*., 2004). Moreover, throughout the adolescent period an augmentation in risk-taking and novelty seeking phenotypes is commonly observed (Spear, 2000). These phenotypic observations correlate with an increase in adolescent neural activation within the ventral striatum, the reward centre of the brain, compared to adulthood (Sowell *et al*., 1999). It is postulated that throughout the adolescent period there is a shift in anatomical control of behaviour, from limbic- to PFC-mediated behaviour with an increase in inhibitory connections between the two regions i.e. a shift from affective-driven behaviour to more regulated cognitive-driven behaviour (Nelson *et al*., 2005; Viveros *et al*., 2011). Thus, cannabis abuse during this epoch of vulnerability could have long-term detrimental effects on the structural re-modelling of the brain.

1.4.2 |Effects of Adolescent Cannabis Use on Cognition

Studies investigating the adverse effects of adolescent cannabis exposure on cognition have yielded some very interesting results. Wilson and colleagues compared 29 long-term cannabis users (cannabis use commencing before 17 years of age) with 28 users who had initiated use at age 17 or later. Using magnetic resonance imaging they showed that early-onset users had a lower percentage of grey matter and a higher percentage of white matter, relative to whole-brain volume, than the late-onset users. The greatest difference in grey matter was noted in the frontal lobe region of the brain (Wilson *et al*., 2000). Schweinsburg and colleagues (2008) found that abstinent adolescent cannabis users exhibited reduced dorsolateral PFC activation during a spatial working memory task. This reduced activation did not however impair performance on the task (Schweinsburg *et al*., 2008). Abnormal cerebellar morphometry and subtle perturbances in PFC volume have also been observed in abstinent adolescent cannabis users (Medina *et al*., 2009; Medina *et al*., 2010). These findings suggest that cannabis

use during adolescence does cause disruption, of some sort, to the brain maturation process.

In a study conducted by Pope *et al.* (2003), it was found, in a battery of neuropsychological tests, that 69 early-onset heavy cannabis users (who began smoking before the age of 17) tested following a two week abstinence period, performed significantly worse than the late-onset users and controls. However, these early-onset users also displayed a lower baseline IQ compared to those of the late-onset users and controls (Pope *et al*., 2003). Fried and colleagues tested the effects of cannabis use on IQ in a group of 70 young people. This was achieved by subtracting each person's IQ score at nine years of age (before drug use) from their score at age 17-20 years. They found that current heavy cannabis use caused a significant dose-dependent decline in IQ. However, the group failed to show any long lasting consequences of cannabis abuse on global intelligence. Although there were no notable long lasting consequences of cannabis abuse on global intelligence, it was beyond the scope of their study to investigate the effects on particular cognitive domains associated with attention and executive functions (Fried *et al*., 2002). Interestingly, Hanson and group demonstrated enduring deficits in attention processing speeds in abstinent adolescent cannabis users (Hanson *et al*., 2010). Moreover, a recent study carried out by Fontes *et al.* (2011) found that early-onset chronic cannabis abuse, before the age of 15, leads to greater cognitive impairments in executive functioning compared to both late-onset users and controls. These findings suggest an age-dependent relationship exists between age of onset of cannabis abuse and the scale of impact on neurocognitive functioning (Fontes *et al*., 2011).

1.4.3 |Preclinical Studies Investigating the Effects of Peripubertal Exposure to Cannabinoids

Some animal studies have been carried out investigating the long-lasting consequences of peripubertal (broadly corresponding to the adolescent period in humans) exposure to various different CBs on cognition.

Schneider and Koch (2003) found that chronic peripubertal but not adult treatment with the synthetic CB agonist WIN 55,212-2 leads to long lasting deficits in recognition memory, a type of short-term memory (Schneider and Koch, 2003). In a study carried out by O'Shea and colleagues (2006), similar impairments were reported in the novel object recognition task following peripubertal treatment with the synthetic CB agonist CP-55,940 (O'Shea *et al.,* 2006). Again, these deficits were not evident in adults treated with CP-55,940. Furthermore, repeated peripubertal exposure to THC has been shown to produce persistent deficits in both object recognition memory and spatial working memory (Quinn *et al*., 2008; Rubino *et al*., 2009)

Preclinical studies investigating the long-term effects of CBs are scarce; however, of those carried out it appears that peripubertal exposure to CBs has a more pronounced effect on cognition compared to exposure in adulthood (Schneider and Koch, 2003; Quinn *et al*., 2008; Rubino *et al*., 2009).

As mentioned earlier, one of the growing concerns of cannabis abuse is the association between adolescent cannabis use and the precipitation of schizophrenia in adulthood (Andréasson *et al*., 1987; Arseneault *et al*., 2004; Kuepper *et al*., 2011). In the following sections, I summarise the symptomatology, pathology of and treatment strategies for this neuropsychiatric disorder prior to discussing the range of risk factors, including cannabis abuse, that are implicated in the pathogenesis of this disease.

1.5 |Schizophrenia

Schizophrenia is a severe and chronic neuropsychiatric disorder. Describing it first in 1887, Dr. Emile Kraepelin used the term 'dementia praecox' to describe symptomatology of a progressively deteriorating psychotic disorder. Whilst some of Kraepelin's original clinical observations are congruent with those seen amongst schizophrenic patients, it was the Swiss psychiatrist Eugene Bleuler who coined the term 'schizophrenia' which is nowadays used to describe a reframed and substantially different disease concept than that of 'dementia praecox'. The aetiology of schizophrenia remains ambiguous but it is well accepted that its cause is multi-factorial with several risk factors of genetic, environmental and neurodevelopmental origin being implicated. A recent systematic review of epidemiological evidence found that the median lifetime prevalence of schizophrenia was four in 1000 persons. Furthermore, concerning sex differences and the prevalence of schizophrenia, males are slightly more at risk compared to females with a prevalence ratio of 1.4:1 respectively (McGrath *et al*., 2008). Schizophrenia typically manifests itself in late adolescence/early adulthood, occurring a few years earlier in males than in females (Häfner *et al*., 1998).

To date, there are no reliable biomarkers available for the diagnosis of schizophrenia. Instead, clinical diagnosis is based on the presence of a defined set of patterned symptoms. The Diagnostic and Statistical Manual of Mental Disorder IV (DSM-IV) and the International Classification of Diseases 10th revision (ICD-10) are the diagnostic guidelines followed in the USA and Europe respectively. According to the ICD-10, diagnosis of schizophrenia is made depending upon the presence of one or more symptoms for a minimum duration of one month. Due to the heterogeneous nature of symptom presentation amongst patients, the disorder can be further classified into paranoid, hebephrenic, catatonic, undifferentiated and residual sub-types (ICD-10).

1.5.1 |Symptomatology of Schizophrenia

Symptoms are typically classified into one of three categories; positive, negative and cognitive symptoms. Positive symptoms reflect an excess in normal functioning and include auditory hallucinations and grandiose, persecutory or reference delusions. Conversely, negative symptoms reflect a loss in normal functioning and include symptoms such as blunted affect, alogia, anhedonia, avolition, apathy and motor disturbances (ICD-10). Cognitive dysfunction is a core symptom among schizophrenic patients. Impairments in key cognitive domains such as speed of processing, attention/vigilance, working memory, verbal learning and memory, visual learning and memory, reasoning and problem solving and social cognition are common among schizophrenic patients (Nuechterlein *et al*., 2004). Moreover, the extent of cognitive dysfunction is a predictor of functional outcome in society (Green, 1996).

1.5.2 |Neurotransmitter System Abnormalities

Schizophrenia is undoubtedly a spectrum disorder, with fundamental heterogeneity in symptom manifestation a key feature of the disorder. Thus, it is inferred that the overt symptomatology of schizophrenia would be precipitated by a widespread molecular disruption in the neural circuits subserving specific affected neurofunctional domains. Dysregulation of multiple neurotransmitter systems including the GABA, Glu and dopamine (DA) systems have been implicated (reviewed by Carlsson *et al.*, 1999).

Aberrant DA neurotransmission has long been implicated as a key factor in the pathogenesis of schizophrenia (reviewed by Stone *et al.*, 2007). Subcortical hyperdopaminergia coupled with prefrontal hypodopamingeria is now the accepted dual-faceted pattern of DA dysregulation in the schizophrenic brain (Davis *et al*., 1991). This dual-faceted pattern was first postulated by Davis and colleagues, who hypothesised that elevated mesolimbic DA transmission at D_2 receptors in subcortical regions accounted for the positive symptoms observed in schizophrenia. Moreover,

decreased mesocortical DA transmission at D_1 receptors in the PFC may account for cognitive impairments and negative symptoms associated with the disorder (Davis *et al*., 1991). This theory was supported by the fact that acute amphetamine administration, which enhances DA transmission, can induce transient psychosis in healthy volunteers and also exacerbates the psychotic symptoms of schizophrenic patients (Angrist *et al*., 1974, Angrist *et al*., 1980). Furthermore, typical antipsychotics (D_2 receptor antagonists) display a high affinity for the DA D_2 receptor and ameliorate the positive symptoms. However, these antipsychotics exhibit little efficacy on treating the negative and cognitive symptoms of schizophrenia (Miyamoto *et al*., 2004). Moreover, a caveat to the interpretation of the aforementioned data obtained from human imaging and amphetamine administration studies is the potential confound of stress introduced by these experimental procedures. Thus, such DA dysregulation taking place under minimal stress remains to be elucidated in a clinical setting (reviewed by Carlsson *et al.*, 1999).

Post-mortem studies of schizophrenic patients have revealed a reduction in the neurotransmitter GABA, the enzyme responsible for its synthesis, glutamate decarboxylase, and GABA transporter-1 (Perry *et al*., 1979; Sherman *et al*., 1991; Volk *et al*., 2001). Furthermore, it appears to be a particular sub-type of GABA-ergic interneuron, the fast-spiking parvalbumin-positive interneurons, that seem to be primarily affected in schizophrenia (Woo *et al*., 1998) This GABA-ergic hypofunction has been observed in several brain regions including the PFC, hippocampus, nucleus accumbens and the thalamus (Perry *et al*., 1979; Sherman *et al*., 1991; Akbarian *et al*., 1995; Volk *et al*., 2001; Heckers *et al*., 2002).

Alterations in the glutamatergic system have also been reported amongst schizophrenic patients (reviewed by Stone *et al.*, 2007). These pathophysiological findings include decreased Glu levels in the cerebrospinal fluid and elevated brain levels of the neuropeptide N-acetylaspartylglutamate, which exhibits antagonistic properties to Glu, amongst schizophrenic patients (Tsai *et al*., 1995; Faustman *et al*., 1999). Furthermore,

antagonism of the glutamatergic N-methyl-D-aspartic acid or N-Methyl-D-aspartate (NMDA) receptor with phencyclidine (PCP) and ketamine can induce both negative symptoms and transient psychosis, further implicating disrupted glutamatergic transmission in the pathogenesis of schizophrenia (Javitt and Zukin, 1991; Krystal *et al*., 1994).

Thus, it is evident that multiple DA systems alongside the several other neurotransmitter systems are involved in the pathophysiology of schizophrenia. This diverse pharmacology further convolutes the potential treatment strategies for this spectrum disorder.

1.5.3 |Neuroanatomical Brain Abnormalities

Currently, no clear clinical neuropathologies have been determined in order to aid in the diagnosis of schizophrenia. However, whilst there appears to be an absence in signature diagnostic abnormalities, several neuroanatomical anomalies are present amongst schizophrenics. Ventricular enlargement, particularly in the lateral and third ventricles, and cortical volume reduction has been observed amongst schizophrenic patients (Degreef *et al*., 1992; Schlaepfer *et al*., 1994).

Cytoarchitectural abnormalities such as reduced cell body size in pyramidal neurons of the hippocampus and PFC have also been reported. In keeping with these observations, reduced dendritic arborisation and dendritic spines in the same neuronal populations has been observed (reviewed by Harrison, 2005). Furthermore, a reduction in number and functionality of oligodendrocytes, which are integral for myelination and maintenance of synaptic integrity, has been reported amongst schizophrenic patients (Uranova *et al*., 2001; Hof *et al*., 2003).

Abnormalities in white matter tracts, especially those interconnecting frontal and temporal lobes, have also been reported in schizophrenic patients. Andreason *et al*. (1994) reported specific regional abnormalities in white matter integrity in the thalamus

and related circuitry (Andreasen *et al.,* 1994). Perturbations in white matter tract integrity have also been demonstrated among schizophrenic patients in the corpus callosum (Wang *et al.,* 2011). Moreover, Fitzsimmons and colleagues (2009) showed bilateral disruption of the integrity of the fornix (white matter tract connecting the hippocampus to the septum, anterior thalamus and mammillary bodies) in schizophrenia (Fitzsimmons *et al.,* 2009).

Despite these findings, the debate surrounding neuroanatomical abnormalities associated with schizophrenia continues as many studies have failed to replicate the findings above. Furthermore, one major caveat in the search for 'signature' diagnostic neuropathologies is that changes in morphology in the schizophrenic brain may well be consequence of treatment or artefacts of tissue processing (reviewed by Harrison, 1999; Iritani, 2007).

1.6 |Treatment of Schizophrenia

Current pharmacological treatment strategies for schizophrenia consist of two classes of antipsychotic drugs; typical (first generation) and atypical (second generation) antipsychotics. Both treatment strategies have differential abilities to alleviate the several pathological dimensions of schizophrenia and both are accompanied by side-effects.

1.6.1 |Typical Antipsychotics

The use of antipsychotics for the treatment of schizophrenia first came to the fore in the 1950s with the advent of the first antipsychotic chlorpromazine. Chlorpromazine and many other typical antipsychotics subsequently discovered exhibit a high binding affinity for the DA D_2 receptor. This shared pharmacological property is the mechanism underlying their efficacy in the treatment of the positive symptoms of

schizophrenia. Typical antipsychotics have proven much less effective in the treatment of the negative and cognitive symptoms of schizophrenia (Miyamoto *et al*., 2005).

Whilst the antipsychotic efficacy of these drugs is thought to be mediated through mesolimbic antagonism of the D_2 receptor, D_2 receptor antagonism in the nigrostriatal pathway, which controls motor function, can lead to adverse side-effects, known as extra-pyramidal symptoms (reviewd by Reynolds, 2004). These side effects include motor disturbances such as parkinsonism, akathisia, dystonia and tardive dyskinesia (Uchida *et al*., 2011).

1.6.2 |Atypical Antipsychotics

Atypical antipsychotics exhibit rich pharmacological profiles encompassing multiple receptor interactions including DA (D_1,D_2,D_3 and D_4), serotonin (5-HT) (5-HT_{1A}, 5-HT_{2C}, 5-HT_6 and 5-HT_7), muscarinic, cholinergic and histamine receptors (Miyamoto *et al*., 2005). This rich pharmacology is believed to mediate atypical antipsychotics' ability to treat the both the positive symptoms of schizophrenia in the absence of severe neurological side-effects such as extra-pyramidal symptoms (reviewed by Miyamoto *et al*., 2005; Reynolds, 2004). Furthermore, the first atypical antipsychotic discovered, clozapine, has proven successful in medicating treatment-resistant patients (Kane *et al*., 1988; Conley and Kelly, 2001). However, despite the initial enthusiasm for atypical antipsychotic drugs, recent clinical evidence provided by the CATIE studies does not support the view that atypical antipsychotics show improved clinical efficacy for the treatment of schizophrenia (Lieberman, 2007). Moreover, this study demonstrated that adverse side-effects associated with atypical antipsychotic treatment such as weight gain, type-II diabetes mellitus and sexual dysfunction greatly reduced patient adherence to antipsychotic treatment (Dixon *et al*., 2000; Allison and Casey, 2001; Lieberman, 2007; Uçok and Gaebel, 2008).

1.7 |Risk Factors of Schizophrenia

To date, to attribute causality to one particular risk factor of schizophrenia has proven difficult. Rather, it is now accepted that the pathogenesis of schizophrenia is multi-factorial and a consequence of complex interplay between putative genetic and environmental risk factors.

1.7.1 |Genetic Factors

The hereditary component of schizophrenia is a well established phenomenon. Numerous family, twin and adoption studies have confirmed the 'familial' component of schizophrenia. The risk of developing schizophrenia increases in accordance to increased shared genes i.e. the degree of biological relatedness to the patient determines relative risk. Siblings or dizygotic twins share approximately 50% of genes which translates to an approximate risk of 9% of developing schizophrenia. Moreover, as monozygotic twin share 100% of their genes, their % risk escalates to 50% (Kety and Rosenthal, 1968; Kendler *et al*., 1993; Cardno *et al*., 1999; Tsuang *et al*., 2001). It is evident that whilst the aetiology of schizophrenia may be genetically influenced it is not genetically determined, thus, the complex interplay between genetic predisposition and exposure to environmental insults plays an integral role in the precipitation of schizophrenia.

Advances in genetic technology have further elucidated the genetic architecture of schizophrenia. An abundance of candidate genes have been identified as susceptibility genes for the development of schizophrenia. The most investigated of these include the catechol-O-methyltransferase (COMT), neuroregulin-1 (NRG-1), dysbindin and disrupted in schizophrenia-1 (DISC-1) genes (Egan *et al*., 2001; Millar *et al*., 2000; Stefansson *et al*., 2002; Straub *et al*., 1995). The discovery of these genes has further elucidated the role genetic mechanisms play in the evolution of schizophrenia. Furthermore, they could possibly serve as diagnostic biomarkers for the detection of

potentially vulnerable populations. Current research aims to understand the neurobiological role of these genes. Importantly, these vulnerability genes display a diverse range in function; the COMT gene is associated with dopaminergic transmission whilst other genes such as DISC-1 and NRG-1 impact on glutamatergic transmission, providing further evidence for neuropathology of multiple neurotransmitter systems in schizophrenia (reviewed by Harrison, 2005). More recently, large scale genome wide association studies have consistently demonstrated the association of schizophrenia with genetic markers in the major histocompatibility complex (6p22.1)-containing genes (involved in immune-related functions) including NOTCH4 and histone protein loci (reviewed by Tiwari *et al.,* 2010). Thus, the diverse polygenic nature of schizophrenia means further research is required to fully grasp the implications of genetic perturbations in the pathogenesis of schizophrenia.

1.7.2 |Environmental Risk Factors

The putative role of environmental risk factors in the aetiopathogenesis of schizophrenia has long been overshadowed by a somewhat preferential sway in scientific interest towards the genetic liability of schizophrenia but also by methodological limitations in experimental design of studies investigating environmental risk factors.

In recent years, however, the role of environmental risk factors in the precipitation of schizophrenia is gaining increasing interest. Both 'early' environmental risk factors, such as obstetric complications, prenatal exposure to infectious agents (see section 1.7.3) and urbanicity, and 'late' environmental risk factors such as cannabis abuse (see section 1.7.4) have been implicated.

A number of studies have been carried out investigating the relationship between obstetric complications and schizophrenia. Based on a Finnish birth cohort of over 11,000 subjects, Jones and colleagues (1998) found that low birth weight (<2500 g) and the combination of low birth weight and preterm delivery were more common among

the schizophrenic subjects. Furthermore, of the 125 individuals that survived severe perinatal brain damage, 5% of these subjects subsequently developed schizophrenia (Jones *et al*., 1998). In a meta-analysis carried out by Geddes *et al*. (1999), significant associations were revealed between obstetric complications such as premature rupture of membranes, premature birth and resuscitation or incubator use and the development of schizophrenia (Geddes *et al*., 1999).

Many studies have illustrated a relationship between urbanicity and an increased risk of schizophrenia. Lewis and colleagues investigated this relationship among a cohort of 49,191 Swedish male conscripts and found that conscripts who later developed schizophrenia were 1.65 times likely to have been reared in an urban rather than a rural setting (Lewis *et al*., 1992). In a large scale population-based cohort study of 1.89 million Danish people, Pedersen and Mortensen (2001) reported that the risk of developing schizophrenia increases with the number of years spent in areas of higher urbanisation but also increases with escalating degrees of urbanisation. This study also disentangled the effect of urbanicity at birth from an effect of urbanicity during upbringing in order to postulate potential vulnerable age periods. They found a significant relationship between urban upbringing and not urban birth on the development of schizophrenia in adulthood (Pedersen and Mortensen, 2001).

1.7.3 |Prenatal Exposure to Infectious Agents

Several epidemiological studies have illustrated a relationship between 'early' neurodevelopmental disruption, prompted by exposure to prenatal infectious agents, and the development of schizophrenia in adulthood (Torrey *et al.,* 1977; Nasrallah and McCalley-Whitters, 1984; O'Callaghan *et al.,* 1991; Brown et al., 2001; Brown *et al.,* 2004; reviewed by Brown *et al.,* 2010). In 2004, Brown and colleagues showed that exposure to the influenza virus during the first trimester of pregnancy increased the risk of development of schizophrenia in affected offspring by 7-fold (Brown *et al*., 2004). Furthermore, prenatal exposure to the rubella virus increases the risk of development of

schizophrenia by up to 20-fold in affected offspring (Brown *et al*., 2001; Brown *et al*., 2006). Bacterial and parasitic infections such as bronchopneumonia (O'Callaghan *et al*., 1994; Sørensen *et al.,* 2009) and toxoplasmosis (Brown *et al*., 2006; Mortensen *et al.,* 2007) respectively have also been shown to increase the risk of schizophrenia in affected progeny.

Throughout prenatal and neonatal life, vital neurodevelopmental processes such as neurogenesis, neural migration, axonogenesis and dendrogenesis take place (O'Rahilly and Muller, 1987; Bayer *et al.,* 1993; Huang *et al.,* 2009). Thus, disruption of these processes may result in maldevelopment of the brain and may precipitate the manifestation of schizophrenia in adulthood. This concept forms the basis of the 'neurodevelopmental' hypothesis of schizophrenia. Perturbed neurodevelopment, (ventricular enlargement, decreased cortical volume and abnormal white matter tract integrity), aberrant dermatoglyphics and the presence of minor physical abnormalities in patients with schizophrenia indicate the occurrence of an 'early' developmental disturbance (Degreef *et al.,* 1992; Andreasen *et al.,* 1994; Avila *et al.,* 2003; Lloyd *et al.,* 2008; Kito *et al.,* 2009; Wang *et al.,* 2011) (discussed further in section 4.1). The mechanism by which prenatal infection can precipitate schizophrenia in adulthood remains elusive. However, due to the heterogeneity of infectious agents implicated in the disease's pathogenesis it has been hypothesised that a common maternal immunological response to pathogens i.e. elevation in pro-inflammatory cytokine levels rather than the pathogen itself is involved in the aetiology of schizophrenia (reviewed by Pearce, 2001; Fatemi *et al.,* 2009; Meyer *et al.,* 2009).

1.7.4 |Cannabis Abuse

It has been well documented that cannabis use can lead to transient dose-dependent toxic, so called drug-induced psychoses (Johns *et al*., 2001). However, the nature of the relationship between cannabis consumption and the development of chronic, in particular schizophrenic, psychoses remains contentious. The attribution of causality of

cannabis consumption to the development of schizophrenia based on clinical evidence has proven difficult due to confounds such as comorbid psychiatric illnesses entangled in the experimental design of epidemiological studies (reviewed by Arseneault *et al*., 2004).

The potential role of the eCB system in the pathogenesis of schizophrenia has recently come to light. Elevated levels of the eCB anandamide in the cerebrospinal fluid of patients with schizophrenia have been reported, this, accompanied with a post mortem study demonstrating increased binding of synthetic CB_1 receptor antagonist in the dorsolateral PFC of schizophrenic patients are indicative of the potential aetiological role of the eCB system in schizophrenia (Leweke *et al*., 1999; Dean *et al.*, 2001).

The potential risk factor that cannabis poses to the development of schizophrenia was first suggested by Andréasson and colleagues in 1987. In a longitudinal study carried out on a cohort of 45,570 Swedish conscripts aged between 18-20 years, Andreasson *et al*. (1987) found that heavy consumption of cannabis (more than 50 times) before the age of 18 years resulted in a 6.7 fold increase in the risk of developing schizophrenia in later life suggesting that cannabis use acts as an independent risk factor for the development of the disease (Andréasson *et al.,* 1987). Recent studies have produced concurring results. Arseneault *et al*. reviewed the relevant epidemiological data on this subject and concluded that cannabis consumption is associated with a two-fold increase in the development of schizophrenia. Moreover, it is suggested that early adolescent-onset cannabis use is associated with a higher risk, with those who have used cannabis by the age of 15 being four times more likely to have a diagnosis of schizophreniform disorder at age 26 compared to controls (Arseneault *et al*., 2002, Arseneault *et al*., 2004).

As mentioned in section 1.4, adolescence is both a critical and extremely vulnerable period of cerebral development thus disruption by exogenous CBs such as THC can have long lasting detrimental sequelae. This age-related neurodevelopmental

vulnerability coupled with the emerging pattern of initiation of cannabis abuse in early adolescence (see section 1.2.2) is a growing public health concern as there is strong evidence implicating adolescent cannabis abuse as an important risk factor for the development of schizophrenia. It is therefore of great interest to understand the potential pathological mechanisms evoked by THC, the main psychoactive constituent of cannabis, in the adolescent brain in relation to schizophrenia. Preclinical models offer a tractable system to investigate mechanisms and test hypotheses.

Here, I briefly review the pharmacological, neurodevelopmental, maternal immune activation and gene x environment animal models with relevance to schizophrenia. Importantly, there has been little attempt to utilise THC in schizophrenia-related animals models despite it being a strong environmental risk factor.

1.8 |Animal Models of Schizophrenia

As noted in section 1.3.4, preclinical studies provide a crucial controlled experimental setting to model human diseases. Whilst schizophrenia is undoubtedly a selectively human disorder, animal models are a critical component of schizophrenia research as they enable the exploration of numerous mechanistic and causative theories in a controlled environment in order to gain greater insight into the pathogenesis and potential treatment strategies for schizophrenia.

1.8.1 |Pharmacological Models

Amphetamine and PCP have long been employed as pharmacological models of schizophrenia by reason of their well established psychotogenic properties.

Chronic treatment with amphetamine, which enhances DA neurotransmission, in escalating doses results in a long term sensitized state that can produce a number of

behavioural phenotypes relevant to schizophrenia (see Table 1.01) (Robinson and Becker, 1986). These behavioural phenotypes include disruption of sensorimotor gating, as measured by the prepulse inhibition (PPI) of the acoustic startle test, and enhanced locomotor activity in response to amphetamine (Tenn *et al*., 2003). This model has also illustrated disruption to several cognitive domains implicated in schizophrenia such as attention/vigilance and problem solving and reasoning. Kapur and colleagues (2007) demonstrated long-term impairments in performance in the 5-CSRTT, a PFC-dependent behavioural task used to measure visual attention. Amphetamine sensitization led to increased omissions and reduced response accuracy when animals were challenged with shorter stimulus durations (Kapur *et al*., 2007). Deficits in other PFC-dependent tasks such as the ASST, in particular the extradimensional set-shift (ED) and reversal learning discrimination phases of the task, have also been reported in this model (Fletcher *et al*., 2005; Featherstone *et al*., 2008).

PCP is a non-competitive NMDA receptor antagonist which is commonly employed as a pharmacological model of schizophrenia-related phenotypes. The rationale for exploitation of this NMDA antagonist in rodent models of schizophrenia was based on observations noting its capacity to induce both the positive, negative and cognitive symptoms of schizophrenia in humans (Javitt and Zukin, 1991; Krystal *et al*., 1994).

Acute PCP treatment produces several schizophrenia-related phenotypes such as hyperlocomotion, reduced PPI and deficits in set-shifting in the ASST (Table 1.01) (Mansbach and Geyer, 1989; Sams-Dodd, 1996; Egerton *et al*., 2005; Kalinichev *et al*., 2008). Egerton and colleagues (2005) also showed decreased expression of the neuronal activation marker *zif-268* in the infralimbic region of the PFC corresponding to observed deficits in set-shifting following acute PCP treatment (Egerton *et al*., 2005). Sub-chronic treatment with PCP has been shown to produce enhanced sensitivity to amphetamine administration, a measure of aberrant mesolimbic DA transmission, coupled with and potentially propagated by long-lasting reductions in cortical DA utilisation (Jentsch *et al*., 1998). This pattern of neural substrate dysfunction is a core

pathological feature of schizophrenia (Davis *et al*., 1991). Furthermore, this sub-chronic treatment regime has been shown to induce cognitive inflexibility, as measured by the ED discrimination phase of the ASST, which was ameliorated by the cognitive enhancer modafinil (Egerton *et al*., 2008; Dawson *et al*., 2010).

1.8.2 |Neurodevelopmental Models

As outlined in section 1.7, developmental disruption during the critical neurodevelopmental epoch of prenatal and neonatal life significantly increases the risk of developing schizophrenia in adulthood. Observations of subtle neuroanatomical abnormalities such as ventricular enlargement, decreased cortical volume and aberrant white matter tract integrity alongside perturbed to biological markers of fetal development (the presence of abnormal dermatoglyphic profiles and minor physical abnormalities such as reduced facial symmetry and abnormalities relating to eye and ear development) amongst schizophrenic patients has led to the hypothesis that deviation from normal prenatal and neonatal development plays a key role in the pathogenesis of schizophrenia (Degreef *et al*., 1992; Schlaepfer *et al*., 1994; Avilo *et al.,* 2003; Lloyd *et al.,* 2008; Kito *et al.,* 2009; Wang *et al.,* 2011) (for further discussion, see section 4.1). Environmental and chemical manipulations during critical phases of neurodevelopment are commonly utilized to produce neurodevelopmental models of schizophrenia.

Exposure to the DNA-alkyating agent methylazoxymethanol acetate (MAM) can produce behavioural abnormalities relevant to schizophrenia (Table 1.01). Administration of MAM, which disturbs the proliferation and migration of neuronal precursor cells, on gestation day (GD) 17, results in the post-pubertal emergence of a broad spectrum of behavioural deficits. These include impairments in latent inhibition (LI) and PPI, impaired performance in ED set-shifting, deficits in reversal learning, enhanced sensitivity to psychotropic drugs such as PCP and amphetamine and decreased social interaction (Featherstone *et al*., 2007; Flagstad *et al*., 2005; Lodge and Grace 2009; Moore *et al*., 2006). However, the timing of MAM administration appears

to be critical factor in the experimental design, as administration of MAM prior to GD15 produces gross neurodevelopmental abnormalities which are not in keeping with those observed amongst schizophrenic patients (Moore *et al*., 2006).

Neonatal lesioning (PD7) of the ventral hippocampus (corresponding to the anterior hippocampus in humans) by local injection of the excitotoxin, ibotenic acid has been shown to produce behavioural phenotypes relevant to schizophrenia in adulthood, including hyperlocomotor activity in response to amphetamine, impaired PPI and cognition and decreased social interaction (Lipska *et al.,* 1994; Lipska, 2004; Tseng *et al.,* 2009). However, the neonatal lesion model has a relatively high mortality rate of approximately 15% and lower construct validity compared to other animal models (Richtand *et al.,* 2006; Pratt *et al.,* 2012)

Isolation rearing is an example of an environmental manipulation paradigm that models particular aspects of schizophrenia. In this model, following weaning, animals are reared in single housing where they are exposed to minimal handling and are not in physical contact with conspecifics, thus exposing animals to an early-life adverse event, an implicated risk factor of schizophrenia. Long-term behavioural effects of isolation rearing include spontaneous hyperactivity, impairments in PPI (strain-specific) and object recognition memory (Table 1.01; Bakshi *et al*., 1998; Bianchi *et al*., 2006). Moreover, alongside these behavioural alterations, social isolation produces neuroanatomical changes such as reduced medial PFC volume as measured by magnetic resonance imaging (Schubert *et al*., 2009). Interestingly, this model may also produce behavioural responses consistent with a depression-like phenotype (reviewed by Fone and Porkess, 2008).

1.8.3 |Maternal Immune Activation Models

Maternal immune activation (MIA) is another commonly employed neurodevelopmental model of schizophrenia. MIA using the bacterial endotoxin,

lipopolysaccharide (LPS), has been shown to induce deficits in schizophrenia-related phenotypes in adulthood. In rats, Borrell and colleagues have shown that maternal LPS administration produces deficits in the PPI behavioural paradigm (Borrell *et al*., 2002), Moreover, enhanced locomotion in response to systemic administration of low dose amphetamine, a measure of aberrant mesolimbic dopaminergic transmission and a key pathological feature of schizophrenia, is apparent in offspring of LPS-infected mothers (Fortier *et al*., 2003). Meyer and co-workers have investigated the effects of maternal exposure to polyriboinosinic-polyribocytidilic acid (PolyIC), a synthetic analogue of viral double-stranded RNA, on several behavioural paradigms in mice in adulthood and have yielded some very interesting results. They showed dose dependent effects of prenatal administration of PolyIC such as reduced exploratory behaviour, reduced PPI and increased sensitivity to acute systemic low dose amphetamine administration compared to vehicle-treated offspring (Meyer *et al*., 2005). Zuckerman and Weiner (2004) demonstrated that prenatal PolyIC treatment to rats produced enhanced locomotor activity in response to acute systemic administration of the NMDA receptor antagonist MK-801 and excessive behavioural switching, a schizophrenia-related cognitive phenotype, as manifested in loss of LI and rapid reversal learning. Furthermore, these cognitive deficits were alleviated following acute administration of the antipsychotic drug clozapine (Zuckerman & Weiner 2004).

1.8.4 |Gene x Environment Models

As mentioned in section 1.7, it is now accepted that the manifestation of schizophrenia in adulthood is a consequence of exposure to a conglomeration of risk factors, genetic and/or environmental in nature, throughout critical epochs of neurodevelopment. In order to gain a greater understanding as to how multiple risk factors impact on one another and attempt to disentangle their apparent interactive complexity, gene x environment animal models are beginning to emerge.

The COMT gene plays an integral role in the degradation of DA, particularly in the PFC as it encodes the COMT enzyme which degrades catecholamines such as DA. Dysregulation of this gene has been implicated in schizophrenia (Egan *et al*., 2001). A longitudinal birth cohort study showed that individuals who had a specific single nucleotide polymorphism in their COMT gene and subsequently smoked cannabis throughout adolescence were more likely to develop schizophrenia in adulthood compared to those who did not use cannabis (Caspi *et al*., 2005).

On the premise of these findings, O'Tuathaigh and colleagues developed a gene x environment model rodent model to explore the potential interplay between THC and the COMT gene on the development of schizophrenia-related phenotypes in adulthood. The group employed a COMT knock out (KO) animal model alongside an adolescent THC dosing regime in order to mimic the gene x environment interaction. The group found that adolescent exposure to THC induced greater levels of exploratory behaviour, greater impairment in spatial working memory, and a stronger anti-anxiety effect in COMT knockouts compared to wild types (O' Tuathaigh *et al*., 2010). One may have expected that given the implied effect of the COMT gene on psychosis, a COMT KO would reduce rather than augment the deleterious effects of THC. However, the group concluded that their observed effect may reflect a compensatory mechanism in the COMT mutants. Thus, this gene x environment model does indicate the existence of an, albeit unexpected, interactive effect between the two risk factors but the mechanisms responsible for this interaction remains elusive.

The DISC-1 gene encodes for the synaptic protein DISC-1. This protein plays a role in critical neurodevelopmental events such as neural migration and differentiation (Bradshaw and Porteous, 2011). Translocation of the DISC-1 gene has been implicated in schizophrenia (Millar et al., 2000; Blackwood et al., 2001). Several different strains of genetically manipulated mice that exhibit partial loss of DISC-1 function have been created (Pratt *et al*., 2012).

Several strains of genetically manipulated *Disc-1* mutant mice demonstrate mild phenotypes relevant to schizophrenia such as increased exploratory behaviour and decreased social interaction (Hikida *et al*., 2007; Pletnikov *et al*., 2008). Deficits in PPI and LI have also been observed but these findings have not always been successfully replicated (Clapcote *et al*., 2007; Pletnikov *et al*., 2008). Furthermore, these behavioural deficits were accompanied by selective neurodevelopmental abnormalities commonly observed in schizophrenic patients, such as enlargement of lateral ventricles and reduced immunoreactivity of cortical parvalbumin-positive interneurons (Hikida *et al*., 2007; Pletnikov *et al*., 2008).

Based on the observed ability of particular *Disc-1* mutant mouse models to produce subtle schizophrenia-related phenotypes, Ibi and colleagues investigated the effect of exposure to an environmental risk factor such as neonatal immune activation in a genetically liable mouse model on phenotypic aberrations in adulthood. They showed a synergistic effect of combined neonatal PolyIC treatment in dominant-negative mutant *Disc-1* (DN- *Disc-1*) mice resulting in deficits in object recognition memory and also enhanced sensitivity to MK-801 induced hyperactivity. These deficits were not evident in DN- *Disc-1* or PolyIC treated groups (Ibi *et al*., 2010) . This study provides further evidence for the role of gene x environment interactions on the precipitation of schizophrenia-related phenotypes in adulthood.

Table 1.01 Comparative overview of current animal models with relevance to schizophrenia

Animal Model	Basal and Drug-induced Locomotor Activity	Sensorimotor Gating	Cognition	Social Interaction
Gestational MAM (GD 17)				
(Moore *et al.,* 2006; Featherstone *et al.,* 2007; Lodge *et al.,* 2009)	↑ LMA in novel environment ↑ sensitivity to amphetamine- and NMDA antagonist-induced LMA	↓ PPI (appears at puberty)	Impaired spatial working memory (Morris water maze) Cognitive inflexibility (ASST) Impaired LI	↓ social interaction (appears at puberty)
Maternal Immune Activation				
(Borrell *et al.,* 2002; Zuckerman and Weiner, 2004; Meyer *et al.,* 2005; Ozawa *et al.,* 2006)	↓ exploratory behaviour ↑ sensitivity to amphetamine- and NMDA antagonist-induced LMA	↓ PPI (appears at puberty)	Impaired LI Deficits in NOR	ND
Post-weaning Social Isolation				
(Bakshi *et al.,* 1998; Lapiz *et al.,* 2003; Bianchi *et al.,* 2006; Fone and Porkess, 2008, McLean *et al.,* 2010)	↑ LMA in novel environment ↑ sensitivity to amphetamine-induced LMA	Post-pubertal ↓ PPI (strain-specific)	Deficits in NOR Cognitive inflexibility (ASST)(females)	↑ agression and total social interaction
Neonatal Ventral Hippocampal Lesion				
(Lipska *et al.,* 2004; Tseng *et al.,* 2009)	↑ LMA in novel environment ↑ sensitivity to amphetamine-induced LMA	Post-pubertal ↓ PPI	Impaired memory acquisition (T-maze and Morris water maze) Cognitive inflexibility, deficits in reversal learning (ASST)	↑ agression ↓social interaction
Amphetamine Model				
(Tenn *et al.,* 2003; Featherstone *et al.,* 2007; Kapur *et al.,* 2007; Featherstone *et al.,* 2008)	↑ sensitivity to amphetamine-induced LMA	↓ PPI (dependent on dosage regimen) at puberty)	Deficits in visual attention (5-CSRTT) Cognitive inflexibility (ASST)	No effect
PCP Model				
(Sams-Dodd, 1996; Egerton *et al.,* 2005; Egerton *et al.,* 2008; Kalinchev *et al.,* 2008; Neill *et al.,* 2010)	↑ sensitivity to amphetamine- and NMDA antagonist-induced LMA	No sustained deficit in PPI	Deficits in NORT Cognitive inflexibility (ASST)	No effect (rodents)

Table 1.01 Comparative overview of current animal models with relevance to schizophrenia
(adapted from Jones et al., 2011) LMA, locomotor activity; PPI, LI, latent inhibition; NMDA, N-methyl-D-aspartate/glutamate receptor; prepulse inhibition task; NOR, novel object recognition task; ASST, attentional set-shifting task; 5-CSRTT, 5-choice serial reaction time task; ND, not determined

1.9 |Summary and Aims of Thesis

Clinical evidence indicates that the adolescent brain is particularly vulnerable to the deleterious effects of cannabis. Initiation of cannabis use (peak years 14-18 years) coincides with a critical period of brain maturation whereby neurodevelopmental processes such as plastic and structural remodelling allow for refinement and integration of certain brain regions and neural circuitry (Huttenlocher, 1990; Giedd *et al*., 1999; Gogtay *et al*., 2004; Independent Drug Monitoring Unit, 2005). Cannabis consumption throughout this 'vulnerability window' has been shown to increase susceptibility to the adverse cognitive perturbances in executive function and working memory associated with exposure to THC (Pope and Yurgelun-Todd, 1996; Ehrenreich et al., 1999; Pope et al., 2003; Harvey and Porter, 2006; Hanson et al., 2010). Moreover, early-onset of cannabis use has been strongly implicated as a 'late' environmental risk factor for the development of schizophrenia in adulthood (Andréasson *et al*., 1987; Arseneault *et al*., 2002; Henquet *et al*., 2005; Miettunen *et al.*, 2008) As it is now accepted that the pathogenesis of schizophrenia is multi-factorial and a consequence of complex interplay between putative genetic and/or environmental risk factors, exposure to 'early' environmental risk factors such as prenatal infection (more specifically, MIA) followed by exposure to a 'late' environmental insult such as cannabis abuse may lead to the precipitation of schizophrenia in adulthood. However, interpretation of findings derived from human studies are often subject to debate due to the methodological discrepancies, ethical constraints and confounding factors such as polydrug use, differential drug doses and comorbid psychiatric disorders inextricably incorporated into their experimental design.

Despite the clinical evidence indicating the negative impact of adolescent cannabis abuse, preclinical studies investigating the deleterious effects of CBs are sparse. Of those available, many research groups have focussed on the individual effects of peripubertal exposure to synthetic CBs such as WIN 55212-2 and CP-55,940. As these synthetic CBs exhibit differential pharmacological profiles to THC, extrapolation of the

findings of such preclinical studies to humans is therefore limited. Furthermore, the time-frame of exposure to these CBs deviates greatly between research groups, with some defining the period just prior to sexual maturation as the peripubertal period (PD40-65). As the rationale for the 'vulnerability window' of adolescence is based on the time-frame of neurodevelopmental maturation taking place throughout adolescence rather than sexual maturation; extrapolation of these findings to humans is again limited. With regard to current animal models incorporating multiple risk factors of schizophrenia, most research groups have focused on the impact of gene x environment interactions rather than explore the potential interactive effects of exposure to 'early' and 'late' environmental insults on the precipitation of schizophrenia in adulthood.

Therefore, the overall aim of this thesis is to investigate the vulnerability of the adolescent brain to the deleterious effects of THC.

Three specific aims will therefore be pursued in the work contained in this thesis:

1) To determine a pubertal 'vulnerability window' of the PFC and hippocampus to the deleterious effects of THC.

2) To identify the neural substrates and functional systems sensitive to developmental disruption by exposure to THC.

3) To investigate the individual effects of and potential interplay between exposure to the schizophrenia-related environmental risk factors maternal immune activation and peripubertal exposure to THC on functional brain imaging, CB_1 receptor expression levels and the precipitation of behavioural perturbances relevant to schizophrenia.

CHAPTER TWO

Ontogenetic Mapping of CB_1 Receptors within Discrete Components of the Prefrontal Cortex and Hippocampus

2.1 |Introduction

Studies investigating the ontogeny of CB_1 protein expression (Cnr1, official symbol assigned by HUGO Gene Nomenclature Committee for rodent CB_1 protein) have demonstrated the presence of CB_1 binding early in post-natal development. Rodriquez de Fonseca and colleagues (1993) employed membrane homogenate radioligand binding assays to map the CB_1 receptor neurodevelopmental pattern in rats. They detected specific binding in the forebrain from as early as post-natal day (PD) 2. Subsequently, from PD10, when quantification of receptors in more defined areas such as the striatum, limbic forebrain and ventral mesencephalon was possible, CB_1 receptor binding levels increased across all regions measured until maximum receptor density levels were reached at PD30-40. This was followed by a subsequent decline to adult values at PD70, possibly arising from synaptic pruning which may occur from PD30-40 onwards (Rodríguez de Fonseca *et al.*, 1993). In parallel with protein measurements, McLaughlin and group (1994) quantified the ontogenetic development of CB_1 receptor messenger RNA (mRNA) (*Cnr1*, official symbol assigned by HUGO Gene Nomenclature Committee for rodent CB_1 gene) expression in the brain using the *in situ* hybridisation technique. *In situ* hybridisation is a molecular technique involving the localisation and detection of a specific transcript, in this case CB_1 receptor mRNA (RNA molecule that codes for the CB_1 receptor protein) by hybridising the complementary strand of a nucleotide probe to the sequence of interest. Complementary to this,

McLaughlin and group (1994) mapped the ontogeny of CB_1 receptor binding, in order to determine whether the ontogeny of mRNA expression levels translated to the developmental pattern of CB_1 receptor binding levels. They found that CB_1 mRNA expression reached adult levels as early as PD3 whilst CB_1 binding progressively increased across the time-points measured (PD3, 10 and 21) until reaching adult levels at PD 70 (McLaughlin *et al*., 1994). These findings are somewhat incongruent with previously published animal and human studies as this study failed to demonstrate an age-related decline in receptor density levels mediated by synaptic pruning (Rodríguez de Fonseca *et al*., 1993; Gogtay *et al*., 2004; Lenroot and Giedd, 2006). A possible explanation for this may be due to the methodological design used by McLaughlin *et al*. (1994) who used whole brain samples and limited time-points which may have masked the emergence of a region-specific ontogenetic pattern of CB_1 receptor binding levels. However, these findings are indicative of an ontogenetic delay between CB_1 receptor mRNA expression and CB_1 receptor protein expression.

The eCB system has the capacity to modulate a diverse range of cognitive and emotional processes through activation of the heterogeneously distributed CB_1 receptors (Reviewed by Zanettini *et al*., 2011). Interference to the physiological functioning of the eCB system by exogenous CBs such as THC, during critical phases of neurodevelopment such as the adolescence/peripubertal period, results in cognitive and emotional dysfunction (Schneider and Koch, 2003; O'Shea *et al*., 2004; Rubino *et al*., 2009; Tuathaigh *et al*., 2010). Whilst a number of studies have administered CBs throughout the peripubertal period, the length and time-frame of the treatment regime employed varies greatly between research groups, with some groups defining the peripubertal period as the period just prior to sexual maturity whilst other research groups have based their treatment regime on the aforementioned published CB_1 receptor ontogeny literature. However, a pertinent point to consider is that whilst some preclinical data has been published on the ontogeny of CB_1 receptors, to date no preclinical study has provided a detailed account of the ontogenetic development of CB_1 receptors within discrete subfields of the PFC and hippocampus from the post-natal period through to

adulthood. As these important neural substrates undergo a developmental transition throughout the peripubertal period, disruption of which is believed to underpin the adverse sequelae associated with peripubertal THC exposure, it is therefore of importance to map the ontogenetic development of these said neural substrates. This information will then allow informed decisions about the timing of THC treatments to be made in order to facilitate maximal impact of peripubertal THC treatment on these brain structures.

2.2 |Aim and Rationale

The aim of this study was to map the ontogeny of CB_1 receptor mRNA expression and receptor binding levels in multiple subfields of important cognitive substrates, the PFC and hippocampus, from the neonatal period through to adulthood in order to define a window of developmental vulnerability and thus form the basis of future experimental THC treatment regimes throughout this thesis. *In situ* hybridisation and $[^3H]$SR141761A binding assays were employed in order to quantify the levels of CB_1 receptor mRNA and binding levels respectively.

2.3 |Methods

2.3.1 |Animals

Time-mated pregnant Lister-hooded rats (Harlan, UK), sperm-positive on a specific day (GD1), were used in this study. Male offspring from these rats were sacrificed at 9 different time-points on PD0 (n=3/group), PD3 (n=3/group), PD10 (n=3/group), PD25 (n=4/group), PD3 (n=5/group), PD42 (n=6/group), PD50 (n=6/group), PD60 (n=8/group) and PD70 (n=8/group). Pregnant dams and subsequent offspring were housed under standard conditions on a 12 hr/12 hr light/dark cycle (lights on 07:00hr). Animals received food and water *ad libitum* in the home cage. Room temperature (21°±2°C) and humidity (45–55%) were kept constant throughout. All experiments were performed in accordance with the Animals (Scientific Procedures) Act 1986.

2.3.2 |Euthanasia

Animals were killed by cervical dislocation on the following time-points, PD0, PD3, PD10, PD25, PD35, PD42, PD50, PD60 and PD70. Brains were rapidly removed and frozen in isopentane (cooled to -42° C) and then coated in M-1 embedding medium (Thermo Scientific Ltd). Brains were stored at -80 ^{o}C until required.

2.3.3 |Ontogeny of CB_1 Receptor Messenger RNA Expression

In situ hybridisation was carried out in order to define the ontogenetic pattern of CB_1 mRNA expression (*Cnr1*) in discrete subfields of the PFC and hippocampus from the neonatal period through to adulthood (PD70).

2.3.3.1 |Cryotomy and Fixation of Cerebral Sections

20µm coronal sections were cut at a temperature of -20 ^{o}C using a rotary microtome in a cryostat (Leica CM 1850). Sections were collected at coronal levels 9 and 63 in accordance to Paxinos & Watson's atlas of the rat brain (6th Edition) and thaw-mounted onto poly-L-lysine coated mRNAase free microscope slides. Typically two sections from each animal were collected onto each slide. Parallel sections were collected for measurement of CB_1 receptor expression (section 2.3.4). Sections were allowed to dry at room temperature followed by fixation in 4% depolymerised paraformaldehyde (Sigma-Aldrich Inc, UK) in 1 x phosphate buffered saline (PBS) for 5 mins. Slides were then rinsed in 1xPBS and dehydrated through a graded series of ethanol solutions (70-100% AR grade). Following dehydration sections were stored in 100% AR grade ethanol at 4 ^{o}C until required.

2.3.3.2 |Probe Design and Labelling

A 42-mer oligonucleotide probe with a sequence complementary to bases 4-45 (GGT GAT GGT ACG GAA GGT GGT GGT GTC TGC AAG GCC ATC TAG GAT) of the CB_1 receptor mRNA was obtained from Cruachem Ltd to allow quantification of mRNA expression using *in situ* hybridisation (Berrendero *et al.,* 1999). *In situ* hybridisation was carried out according to the procedure previously described by Egerton *et al.* (2005) with slight modifications (Egerton *et al.,* 2005). The oligonucleotide probe was 3' end radiolabelled with [^{33}P]dATP (specific activity 1250Ci/mmol, Perkin-Elmer Ltd). This was achieved by combining 2µl [^{33}P]dATP and 1.5µl of probe with terminal deoxynucleotidyl transferase kit (Roche Bioscience Ltd, UK). The mixture was incubated for one hour at 37 ^{o}C to allow the labelling reaction to occur. The enzyme reaction was terminated by the addition of 40µl of DEPC treated water and unincorporated nucleotides were removed from the reaction mixture using a QIAquick nucleotide removal kit (Qiagen Ltd, UK). After probe elution, the extent of probe

labelling was measured by liquid scintillation counting; probes labelled with activity ranging from 100,000-300,000d.p.m/μl were used for *in situ* hybridisation.

2.3.3.3 |Hybridisation of Radiolabelled Probes to Sections

Sections were removed from storage and allowed to air-dry for 30 mins. 4μl of labelled probe (5ng/μl) mixed with 200μl of hybridisation buffer (50% deionised formamide, 20% 20x standard saline citrate (pH7), 5% 0.5M sodium phosphate (pH7), 1% 0.1M sodium pyrophosphate, 2% 5mg/ml polyadenylic acid and 10% dextran sulphate) (1 probe:50 hybridisation buffer) was applied to each slide and then covered in parafilm. A control 'cold probe' slide containing 16μl unlabelled probe, 4μl of labelled probe (5ng/μl) mixed with 200μl of hybridisation buffer i.e.an excess of unlabelled versus labelled probe was added one per autoradiographic film. Tissue was hybridised overnight at 42 ^{o}C. Following hybridisation, slides were briefly immersed in 1xsodium chloride-sodium citrate buffer (SSC) solution at room temperature and parafilm was removed. Sections were then incubated at 60 ^{o} C in pre-warmed 1xSSC for 30 mins Sections were then washed in 1xSSC and 0.1and 0.1xSSC and dehydrated through immersion in a graded series of ethanol solutions (70-100% AR grade). Washes lasted approx. 20s for each solution. Sections were allowed to air-dry, then exposed to autoradiographic film (Kodak Biomax MR1) and following an exposure period of 5 days films were developed according to manufacturer's instructions. All sections for each brain level examined were placed alongside one and other on the same film to avoid the possibility of temporal changes being due to film variation.

2.3.3.4 |Quantification of CB_1 Receptor Messenger RNA Expression

Regional CB_1 mRNA expression was quantified using a MCID densitometry system (MCID Imaging Research, St. Catharines, Ontario, Canada). Bilateral quantitative regional optical density (ROD) measurements were taken from duplicate sections from each animal in discrete brain areas as anatomically defined according to Paxinos &

Watson's atlas of the rat brain (6th Edition) - prelimbic cortex, infralimbic cortex, ventral and lateral orbital cortices, agranular insular cortex, anterior cingulate cortex and the CA1-3 and dentate gyrus regions of the hippocampus (see Fig 2.03-4). Sample boxes were placed bilaterally in a particular brain area and an average ROD measurement for this area was calculated. Typically, 2-4 measurements were made per region from two sections. Non-specific hybridisation was quantified using the 'cold probe' section for each film used and were deducted from ROD measurements accordingly.

2.3.4 |Ontogeny of CB_1 Receptor Binding

Receptor ligand autoradiography was employed in order to define the ontogenetic pattern of CB_1 binding in discrete components of the PFC and hippocampus from the neonatal period through to adulthood.

2.3.4.1 |Cryotomy of Cerebral Sections

20μm coronal cerebral sections were cut at a temperature of -20 ^{o} C using a rotary microtome in a cryostat (Leica CM 1850). Sections were collected adjacent to those collected for measurement of mRNA expression at coronal levels 9 and 63 in accordance to Paxinos & Watson's atlas of the rat brain (6th Edition) and thaw-mounted onto poly-L-lysine coated microscope slides. Sections were allowed to dry at room temperature and subsequently stored in air tight boxes at -20 ^{o} C until required.

2.3.4.2 |CB_1 Receptor Ligand Binding Assay

Receptor binding was performed on cerebral sections (in duplicate). Slides were gradually brought to room temperature over a one hour period whilst remaining in air tight storage boxes. Slides were placed in the pre-heated incubator to humidify and equilibrate to the incubation temperature (37 ^{o} C). Sections were subsequently incubated at 37°C for two hours with 2nM [^{3}H]SR141761A (50 Ci/mmol, American Radiolabeled Chemicals, Inc.) in binding buffer (50mM Tris-HCl, pH 7.4, 5% bovine serum albumin).

Non-specific binding was assessed in adjacent cerebral sections incubated in parallel in the presence of 2µM unlabelled HU-210. After incubation, sections were placed in the first wash solution (50mM Tris-HCl, pH 7.4, 1% bovine serum albumin) for four hours at 4°C. Sections were then transferred into the second wash solution (50mM Tris-HCl, pH 7.4, 0.5% formaldehyde) for 5 mins at room temperature. They were then briefly dipped in distilled water to remove buffer salts. Sections were dried in a cool airstream for one hour. Slides were left overnight in a slide box containing silica gel to ensure sections were completely dry. Slides were exposed to autoradiographic film (Kodak Biomax XAR) together with [^{3}H] standards (American Radiolabeled Chemicals, Inc.) and following an exposure period of 12 weeks films were developed according to manufacturer's instructions.

2.3.4.3 |Quantification of CB_1 Receptor Binding

Regional CB_1 receptor binding was quantified using the MCID densitometry system (MCID Imaging Research, St. Catharines, Ontario, Canada). Bilateral optical density measurements were taken from duplicate sections from each animal in discrete brain areas as anatomically defined according to Paxinos & Watson's atlas of the rat brain (6th Edition) - prelimbic cortex, infralimbic cortex, ventral and lateral orbital cortices, agranular insular cortex, anterior cingulate cortex and the CA1-3 and dentate gyrus regions of the hippocampus (see Fig 2.03-4). Sample boxes were placed bilaterally in a particular brain area and converted to fmol of [^{3}H]SR141716A bound per mg of tissue equivalent using autoradiographic standards. Specific binding was calculated by subtracting non-specific binding from total binding (estimated from the presence or absence of unlabelled SR141716A, respectively). For each brain, a mean level of [^{3}H]SR141716A binding density was calculated in the different discrete brain regions analysed.

2.3.5 |Statistical Analysis

CB_1 receptor binding and mRNA expression data for each discrete brain area were analysed individually using general linear repeated measures ANOVA models (Factor One: CB_1 mRNA expression/CB_1 receptor binding, Factor Two: time). In all cases, statistical significance was defined as $p<0.05$. Data were expressed as mean ± SEM.

2.4 |Results

2.4.1 |Ontogeny of CB_1 Receptor mRNA Expression

CB_1 receptor mRNA expression levels were quantified using *in situ* hybridisation. CB_1 receptor mRNA expression was quantified in 10 cerebral structures, four hippocampal (CA1-CA3 and the dentate gyrus) and 6 cortical (prelimbic, cingulate, agranular insular, infralimbic, ventral orbital and lateral orbital cortices) Regions of interest (RoI) over 8 time-points from PD0-PD70. Levels of mRNA expression within the hippocampus and PFC for all time-points are summarised in Table 2.01. In all discrete subfields of the hippocampus measured, the highest mRNA expression levels were detected at PD0 (Fig 2.01A-D and Fig 2.03). In the PFC, due to methodological issues, mRNA levels were not quantified on PD0, thus, the highest mRNA expression levels were detected at PD3 in the prelimbic, cingulate, agranular insular, infralimbic and lateral orbital cortices (Fig 2.02A-F and Fig 2.03). Within the ventral orbital cortex, peak mRNA expression was present on PD10. These peaks of mRNA expression were succeeded by a steady decline followed by a plateau of mRNA expression in both the hippocampus and PFC with low levels of mRNA expression present from PD25 or PD35 onwards. This variation in age on mRNA expression was confirmed by general linear model repeated measures ANOVA analysis (Hippocampus-CA1 $F_{(7,21)}=32.59$, $p<0.001$; CA2 $F_{(7,21)}=20.30$, $p<0.001$; CA3 $F_{(7,21)}=41.06$, $p<0.001$; dentate gyrus $F_{(7,14)}=11.85$, $p<0.001$; PFC-prelimbic cortex $F_{(7,14)}=13.61$, $p<0.001$; cingulate cortex $F_{(7,14)}=23.60$, $p<0.001$; infralimbic cortex $F_{(7,14)}=21.99$, $p<0.001$; agranular insular cortex $F_{(7,14)}=9.76$, $p<0.001$; ventral orbital cortex $F_{(7,14)}=7.96$, $p<0.001$ and lateral orbital cortex $F_{(7,14)}=16.47$, $p<0.001$).

2.4.2 |Ontogeny of CB_1 Receptor Binding

CB_1 receptor binding levels were quantified using the selective radiolabelled CB_1 antagonist [^{3}H]SR141716A binding assay. [^{3}H]SR141716A binding was quantified in

10 cerebral structures, four hippocampal and 6 cortical RoIs over 6 time-points from PD25-PD70. Whilst CB_1 binding assay was carried out on hippocampal and cortical sections from PD0 to PD70, the degree of specific binding detected in both levels prior to PD25 corresponded to that of background and was therefore unquantifiable. High levels of [^{3}H]SR141716A were observed in the hippocampal formation i.e. CA1, CA2, CA3 and the dentate gyrus (Fig 2.01A-D and Fig 2.04). Moderate [^{3}H]SR141716A binding was observed in prefrontal cortices i.e. prelimbic, ventral orbital, lateral orbital, agranular insular, infralimbic and cingulate cortices (Fig 2.02A-F and Fig 2.04). [^{3}H]SR141716A binding for all time-points is summarised in Table 2.02.

In the hippocampus, specific [^{3}H]SR141716A binding was first detected on PD25. General linear model repeated measures ANOVA analysis revealed a significant effect of age on specific [^{3}H]SR141716A binding levels from PD25 until PD70 in the CA1-3 subfields of the hippocampus ($F_{(5,20)}$=3.58, p=0.018; $F_{(5,20)}$=5.82, p=0.002 and $F_{(5,20)}$=4.81, p=0.005 respectively) whilst approaching significance in the dentate gyrus ($F_{(5,20)}$=2.41, p=0.08). In the hippocampal subfields CA1, CA2 and dentate gyrus, [^{3}H]SR141716A binding levels increased from PD25 until maximum binding levels were reached on PD42 whilst peak binding levels within the CA3 subfield were measured at PD25. When maximal binding was reached in all four hippocampal subfields, receptor binding markedly decreased until PD50, at which point, binding levels began to increase until PD70, the final observational time-point.

In the PFC, similar to the hippocampus, specific [^{3}H]SR141716A binding was first detected on PD25. General linear model repeated measures ANOVA analysis revealed a significant effect of age on specific [^{3}H]SR141716A binding levels from PD25 until PD70 in all subfields of the PFC measured (prelimbic cortex $F_{(5,15)}$=22.17, p<0.001; cingulate cortex $F_{(5,10)}$=9.88, p<0.001; infralimbic cortex $F_{(5,10)}$=9.88, p<0.001; agranular insular cortex $F_{(5,10)}$=15.96, p<0.001; lateral orbital cortex $F_{(5,10)}$=8.52, p<0.001 and ventral orbital cortex $F_{(5,15)}$=22.95, p<0.001). Among all components of the PFC a congruent pattern of binding was observed, a progressive increase in binding from PD25

until highest binding levels were reached (prelimbic, cingulate, ventral and lateral orbital cortices-PD60; infralimbic cortex-PD42 and agranular insular cortex-PD50), succeeded by a decline in binding levels on PD70, the last observational time-point in this study.

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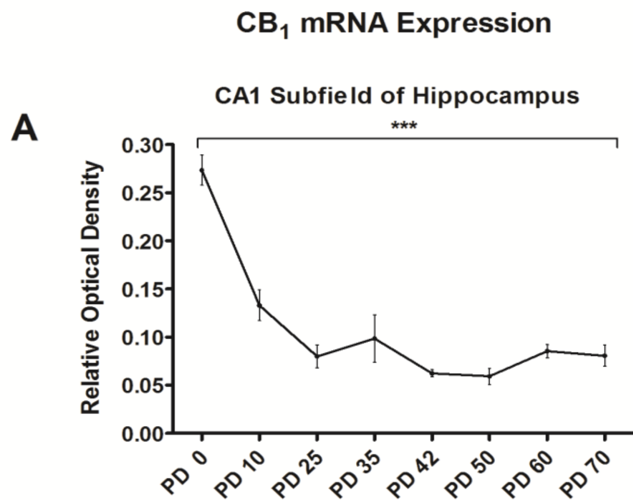
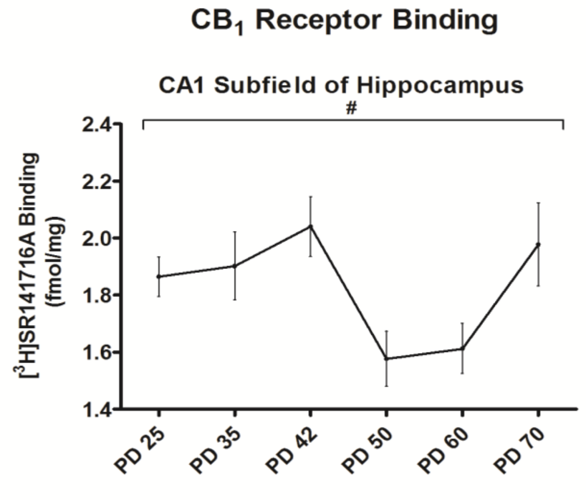
CB_1 mRNA Expression | **CB_1 Receptor Binding**

A





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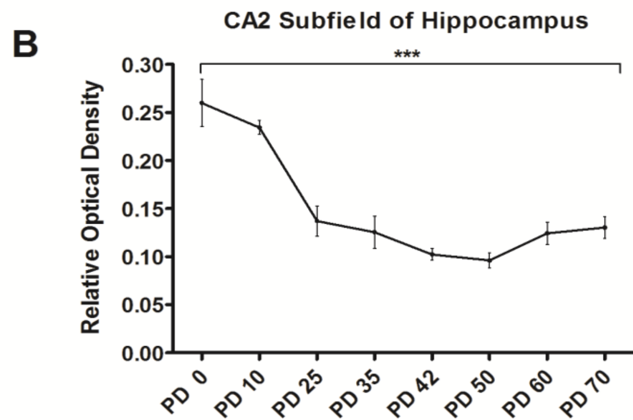

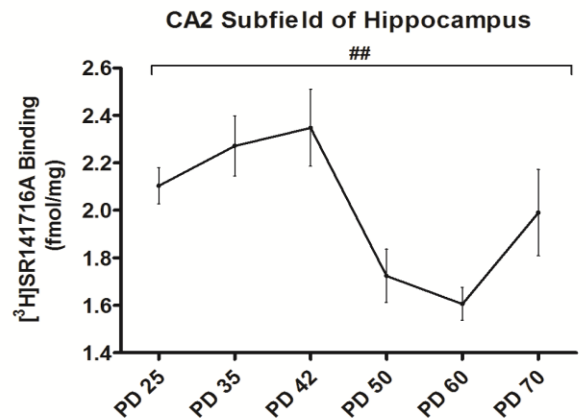

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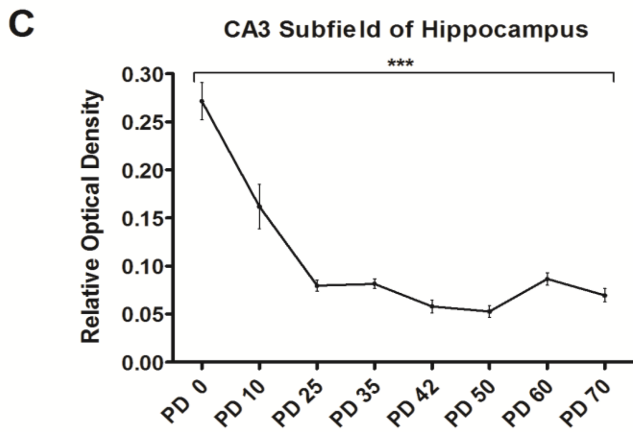

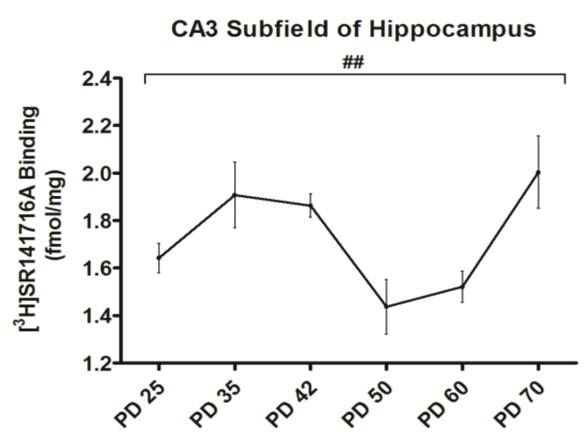

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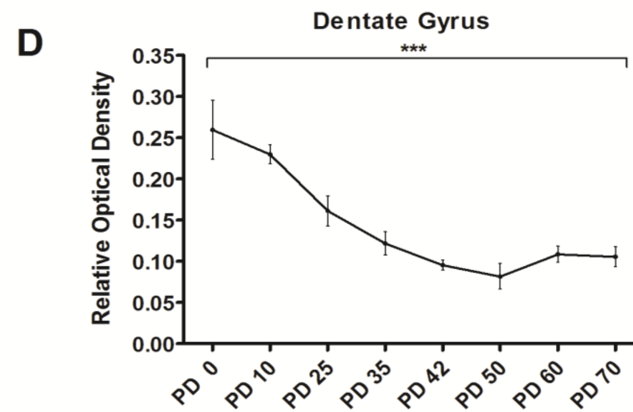


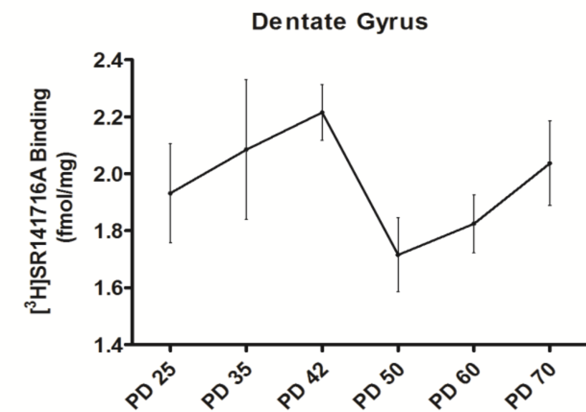
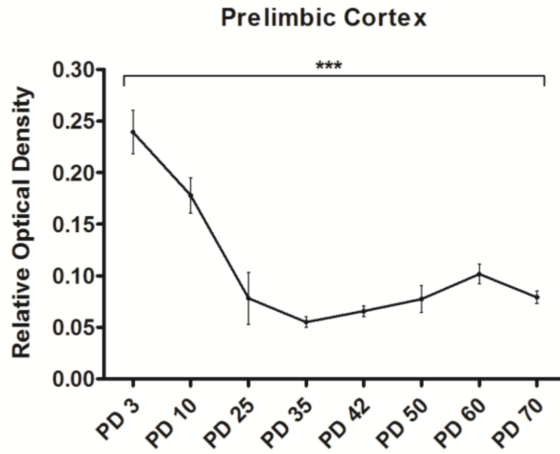


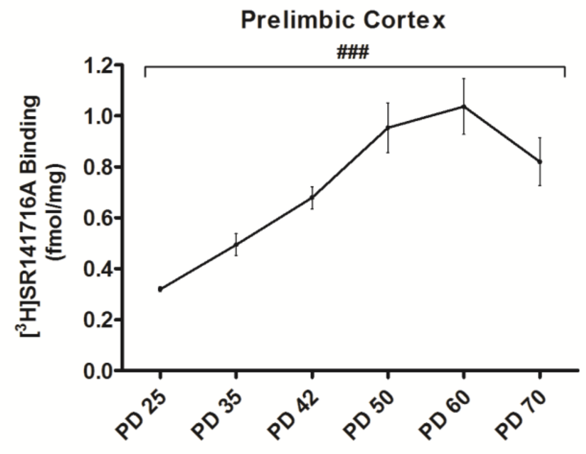
Figure 2.01A-D Ontogeny of CB_1 receptor mRNA expression and binding in discrete subfields of the hippocampus. Graphs on left-hand side represent mean ± SEM CB_1 receptor mRNA expression in discrete subfields of the hippocampus measured in relative optical density (ROD) units at 8 different time-points from PD0 until PD70 (n=3-8/group). Graphs on right-hand side represent mean ± SEM specific $[^3H]$SR141716A binding in discrete components of the hippocampus measured at 6 different time-points from PD25 until PD70 (n=5-8/group). Specific CB_1 receptor binding in the hippocampus prior to PD25 corresponded to that of background and was therefore unquantifiable. Data were analysed using univariate repeated measures general linear ANOVA models. ***$p<0.001$ denotes a significant main effect of age on CB_1 receptor mRNA expression. #$p<0.05$, ##$p<0.01$ and ###$p<0.001$ denotes a significant main effect of age on CB_1 receptor binding.

CB_1 mRNA Expression

CB_1 Receptor Binding

A





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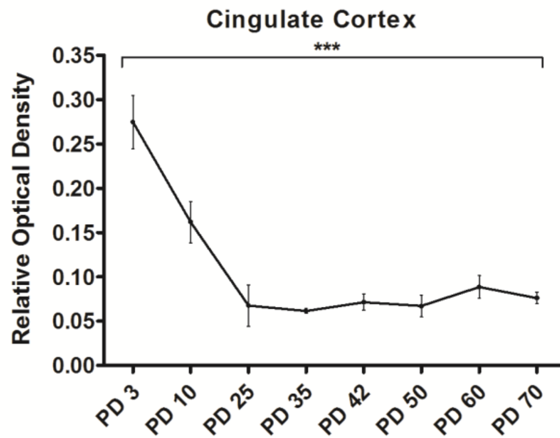

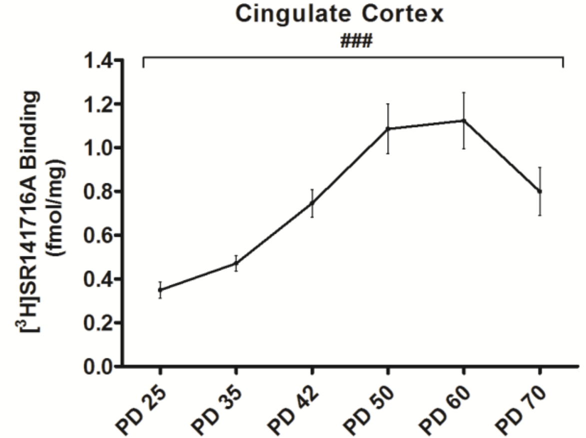

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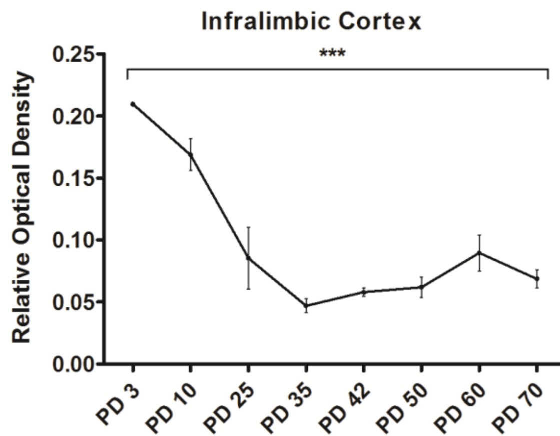

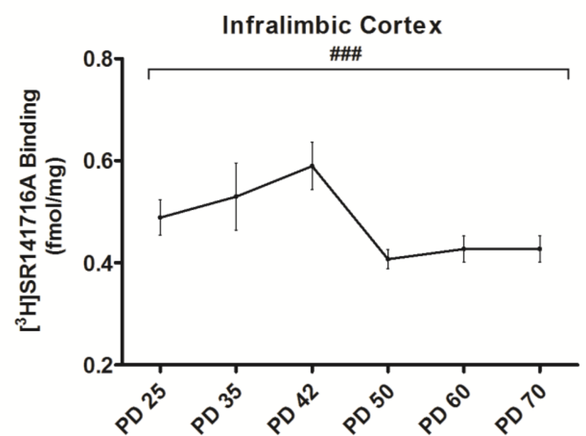

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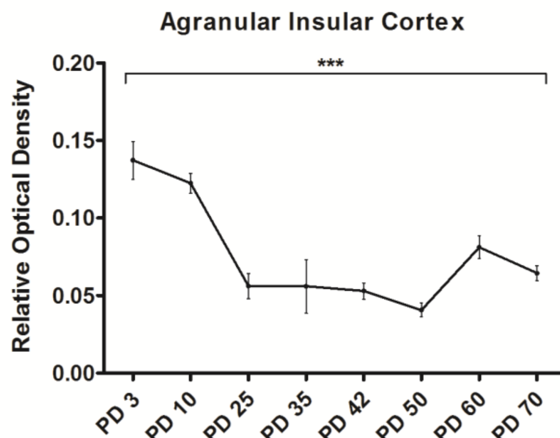
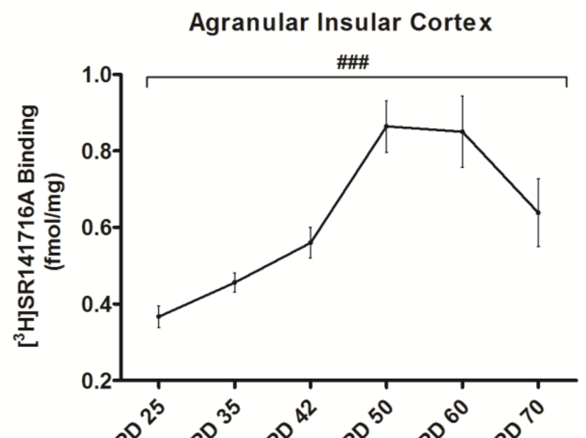




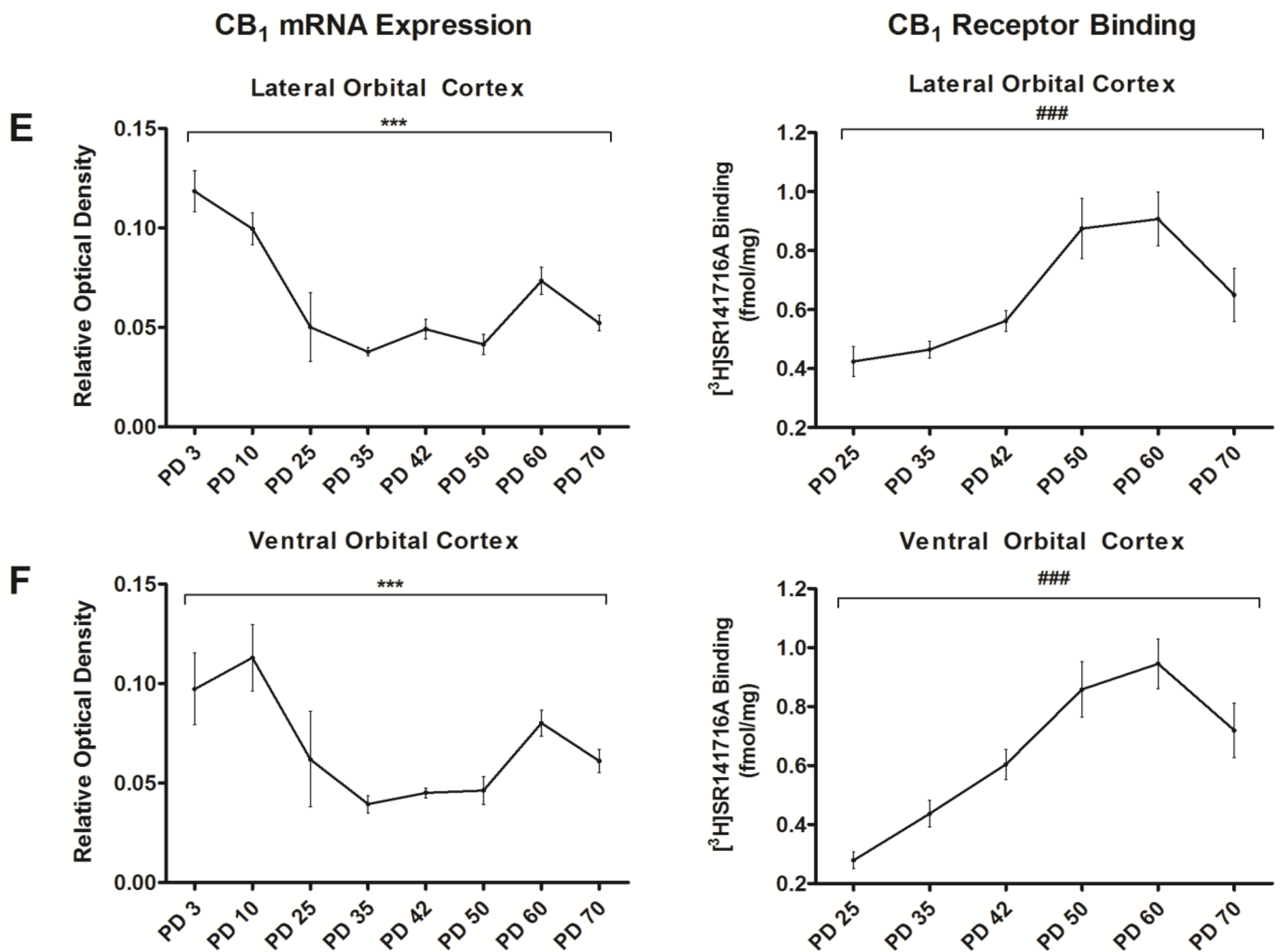


Figure 2.02A-F Ontogeny of CB_1 receptor mRNA expression and binding in discrete subfields of the PFC. Graphs on left-hand side represent mean ± SEM CB_1 receptor mRNA expression in discrete subfields of the PFC measured in relative optical density (ROD) units at 8 different time-points from PD3 until PD70 (n=3-8/group). Graphs on right-hand side represent mean ± SEM specific [^{3}H]SR141716A binding in discrete components of the PFC measured at 6 different time-points from PD25 until PD70 (n=5-8/group). Specific CB_1 receptor binding in the PFC prior to PD25 corresponded to that of background binding and was therefore unquantifiable. Data were analysed using univariate repeated measures general linear ANOVA models. ***$p<0.001$ denotes a significant main effect of age on CB_1 receptor mRNA expression. #$p<0.05$, ##$p<0$ .01 and ###$p<0.001$ denotes a significant main effect of age on CB_1 receptor binding.

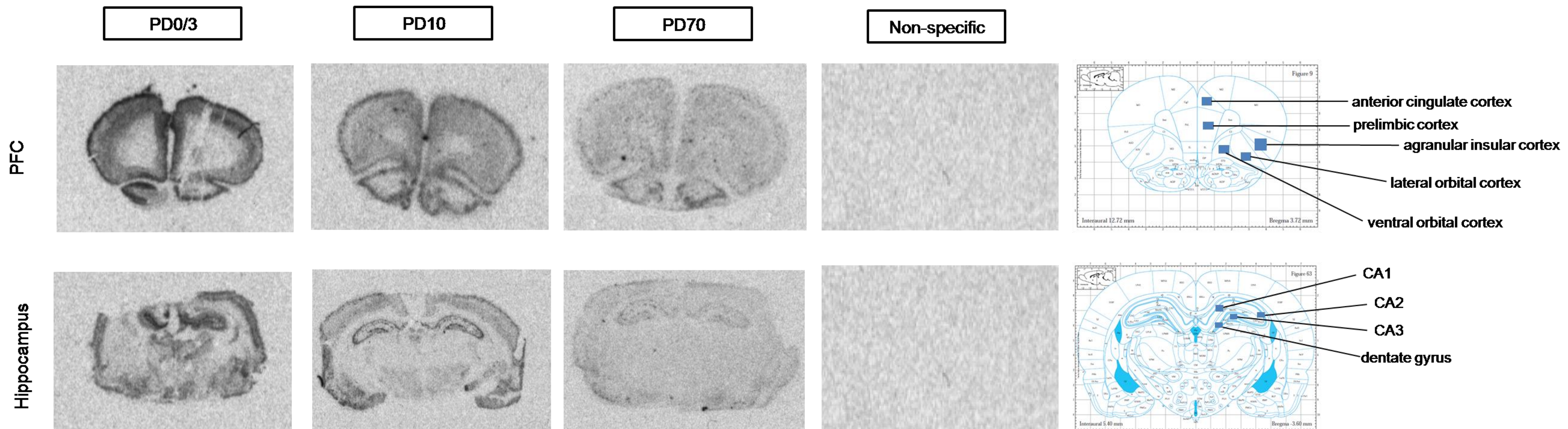


Figure 2.03 Autoradiograms illustrating the ontogeny of CB_1 receptor mRNA expression in the prefrontal cortex and hippocampus. These representative autoradiogram images are illustrating the ontogeny of CB_1 receptor mRNA expression in discrete subfields of the PFC and hippocampus from the neonatal period (PD0/3) through until adulthood (PD70). Levels of non-specific binding of radiolabelled oligonucleotide are also illustrated. Measurements of discrete brain areas were taken as anatomically defined according to Paxinos & Watson's atlas of the rat brain (6th Edition) as illustrated in sections on the far right panel.

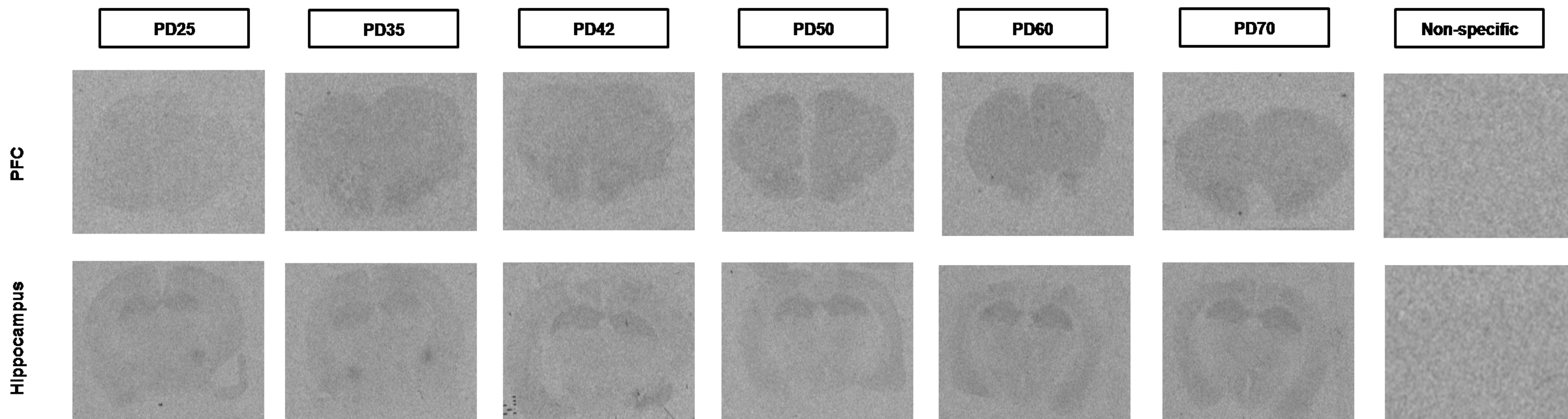


Figure 2.04 Autoradiograms illustrating the ontogeny of CB_1 receptor binding in the prefrontal cortex and hippocampus. These representative autoradiogram images are illustrating the ontogeny of specific [^{3}H]SR141716A binding in discrete subfields of the PFC and hippocampus at 6 different time-points from PD25 until PD70. Levels of non-specific binding of [^{3}H]SR141716A radioligand are also illustrated. Measurements of discrete brain areas were taken as anatomically defined according to Paxinos & Watson's atlas of the rat brain (6th Edition) as illustrated in Fig 2.03.

Table 2.01 Ontogeny of CB_1 receptor mRNA expression from PD0 to PD70

	Day 0	Day 3	Day 10	Day 25	Day 35	Day 42	Day 50	Day 60	Day 70
	mean ± SEM	mean ± SEM	mean ± SEM	mean ± SEM	mean ± SEM	mean ± SEM	mean ± SEM	mean ± SEM	mean ± SEM
Cortex									
*** Prelimbic (PrL)	- ± -	0.24 ± 0.02	0.18 ± 0.04	0.08 ± 0.05	0.06 ± 0.01	0.07 ± 0.01	0.08 ± 0.03	0.10 ± 0.02	0.08 ± 0.02
*** Cingulate (Cg)	- ± -	0.27 ± 0.03	0.16 ± 0.06	0.07 ± 0.05	0.06 ± 0.01	0.07 ± 0.02	0.07 ± 0.03	0.09 ± 0.03	0.08 ± 0.02
*** Infralimbic (IL)	- ± -	0.21 ± 0.01	0.17 ± 0.03	0.09 ± 0.05	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.02	0.09 ± 0.03	0.07 ± 0.02
*** Agranular Insular (AIC)	- ± -	0.14 ± 0.02	0.12 ± 0.02	0.06 ± 0.02	0.06 ± 0.04	0.05 ± 0.01	0.04 ± 0.01	0.08 ± 0.02	0.06 ± 0.01
*** Ventral Orbital (vO)	- ± -	0.10 ± 0.03	0.11 ± 0.04	0.06 ± 0.05	0.04 ± 0.01	0.05 ± 0.01	0.05 ± 0.02	0.08 ± 0.02	0.06 ± 0.02
*** Lateral Orbital (lO)	- ± -	0.12 ± 0.02	0.10 ± 0.02	0.05 ± 0.03	0.04 ± 0.00	0.05 ± 0.01	0.04 ± 0.01	0.07 ± 0.02	0.05 ± 0.01
Hippocampus									
*** CA1	0.27 ± 0.02	- ± -	0.13 ± 0.03	0.08 ± 0.03	0.10 ± 0.05	0.06 ± 0.01	0.06 ± 0.02	0.09 ± 0.02	0.08 ± 0.03
*** CA2	0.26 ± 0.03	- ± -	0.23 ± 0.01	0.14 ± 0.04	0.13 ± 0.03	0.10 ± 0.01	0.10 ± 0.02	0.12 ± 0.03	0.13 ± 0.03
*** CA3	0.27 ± 0.02	- ± -	0.16 ± 0.05	0.08 ± 0.01	0.08 ± 0.01	0.06 ± 0.02	0.05 ± 0.01	0.09 ± 0.02	0.07 ± 0.02
*** Dentate Gyrus (DG)	0.26 ± 0.04	- ± -	0.23 ± 0.02	0.16 ± 0.04	0.12 ± 0.03	0.10 ± 0.02	0.08 ± 0.03	0.11 ± 0.02	0.11 ± 0.03

Table 2.01 Ontogeny of CB_1 receptor mRNA expression from PD0 to PD70 in discrete subfields of the PFC and hippocampus. Values represent mean ± SEM CB_1 receptor mRNA expression measured in relative optical density (ROD) units at 8 different time-points from PD0 until PD70 (PD0 (n=3/group), PD3 (n=3/group), PD10 (n=3/group), PD25 (n=4/group), PD3 (n=5/group), PD42 (n=6/group), PD50 (n=6/group), PD60 (n=8/group) and PD70 (n=8/group)). Data were analysed using univariate repeated measures general linear ANOVA models. ***p <0.001 denotes a significant main effect of age on CB_1 receptor mRNA expression. – denotes measurements not taken.

Table 2.02 Ontogeny of CB_1 receptor binding from PD25 to PD70

	Day 25 mean ± SEM	Day 35 mean ± SEM	Day 42 mean ± SEM	Day 50 mean ± SEM	Day 60 mean ± SEM	Day 70 mean ± SEM
Cortex						
### Prelimbic (PrL)	0.32 ± 0.01	0.49 ± 0.04	0.68 ± 0.04	0.95 ± 0.10	1.04 ± 0.11	0.82 ± 0.09
### Cingulate (Cg)	0.49 ± 0.03	0.53 ± 0.07	0.59 ± 0.05	0.41 ± 0.02	0.43 ± 0.03	0.41 ± 0.02
### Infralimbic (IL)	0.28 ± 0.03	0.44 ± 0.05	0.60 ± 0.05	0.86 ± 0.09	0.95 ± 0.08	0.72 ± 0.09
### Agranular Insular (AIC)	0.42 ± 0.05	0.46 ± 0.03	0.56 ± 0.04	0.87 ± 0.10	0.91 ± 0.09	0.65 ± 0.09
### Ventral Orbital (vO)	0.37 ± 0.03	0.46 ± 0.02	0.56 ± 0.04	0.86 ± 0.07	0.85 ± 0.09	0.64 ± 0.09
### Lateral Orbital (lO)	0.35 ± 0.04	0.47 ± 0.03	0.75 ± 0.06	1.09 ± 0.11	1.12 ± 0.13	0.80 ± 0.11
Hippocampus						
# CA1	1.86 ± 0.07	1.90 ± 0.12	2.04 ± 0.11	1.58 ± 0.10	1.61 ± 0.09	1.98 ± 0.14
## CA2	2.10 ± 0.08	2.27 ± 0.13	2.35 ± 0.16	1.72 ± 0.11	1.61 ± 0.07	1.99 ± 0.18
## CA3	1.64 ± 0.06	1.91 ± 0.14	1.86 ± 0.05	1.44 ± 0.11	1.52 ± 0.06	2.00 ± 0.15
Dentate Gyrus (DG)	1.93 ± 0.17	2.08 ± 0.25	2.21 ± 0.10	1.72 ± 0.13	1.82 ± 0.10	2.04 ± 0.15

Table 2.02 Ontogeny of CB_1 receptor binding from PD25 to PD70 in discrete subfields of the PFC and hippocampus. Values represent mean ± SEM specific [^{3}H]SR141716A binding measured at 6 different time-points from PD25 until PD70 (PD25 (n=4/group), PD3 (n=5/group), PD42 (n=6/group), PD50 (n=6/group), PD60 (n=8/group) and PD70 (n=8/group)). Specific CB_1 receptor binding in both the hippocampus and PFC prior to PD25 corresponded to that of background binding and was therefore unquantifiable. Data was analysed using univariate repeated measures general linear ANOVA models. #p < 0.05, ##p <0 .01 and ###p <0.001 denotes a significant main effect of age on CB_1 receptor binding.

2.5 |Discussion

In the present study, quantification of CB_1 receptor mRNA expression and binding levels at 8 different developmental time-points from the neonatal period through to adulthood served to provide a detailed ontogenetic profile of the CB_1 receptor in discrete components of the hippocampus and PFC. The findings of this study indicate the presence of distinct ontogenetic trajectories of mRNA expression and CB_1 receptor binding levels in discrete components of the hippocampus and PFC.

In the hippocampus, maximal mRNA expression levels were detected early in neonatal development on PD0, followed by a progressive decline in mRNA expression levels. Adult mRNA levels were reached and maintained from PD25 and PD35 onwards in the CA1-3 and dentate gyrus subfields of the hippocampal formation respectively. Similar to the hippocampus, peak CB_1 receptor mRNA expression levels were detected in the neonatal period on PD3 in 5 of the 6 subdivisions of the PFC measured, namely, the prelimbic, cingulate, infralimbic, agranular insular and lateral orbital cortices. Within the ventral orbital cortex, maximum mRNA expression was detected on PD25. The findings in this study of high mRNA expression levels present early in neurodevelopment are in keeping with previous literature (McLaughlin *et al*., 1994; Berrendero *et al*., 1999). Furthermore, the ontogeny of CB_1 receptor binding levels within the discrete hippocampal and prefrontal subfields exhibited a differential developmental pattern to that of CB_1 mRNA expression levels. Whilst high mRNA expression levels are evident in the PFC and hippocampus in the neonatal period, specific quantifiable binding was not detected until PD25 in all four hippocampal and 6 prefrontal subfields measured, suggestive of an ontogenetic delay in the expression of CB_1 receptors within these neural substrates.

Within the prelimbic, cingulate, agranular, ventral and lateral orbital cortices, binding levels progressively increased from PD25 reaching a maximum on PD50 or PD60 and decreasing afterwards until reaching adult values (PD70). In the infralimbic cortex, CB_1

receptor binding increased from PD25 until reaching maximal binding capacity at PD42 followed by a decline in binding, with adult values reached from PD50 onwards. In the present study, the ontogenetic pattern observed within the PFC, a progressive increase in binding capacity throughout the peripubertal period and subsequent decline in receptor density to adult values (PD70), is akin to the developmental trajectory within the human brain, that of synapse and receptor overproduction followed by regressive elimination and refinement of neural circuitry. The late maturation of the PFC parallels the ontogeny of PFC-dependent cognitive development (Huttenlocher, 1979; Gogtay *et al*., 2004; Lenroot and Giedd, 2006). Interestingly, the hippocampus demonstrated a differential ontogenetic binding profile to the PFC. Within the CA1, CA2 and DG subfields of the hippocampus, CB_1 receptor binding levels peaked at PD42; this was followed by a decline in binding capacity, and subsequent increase in binding levels until adult (PD70) binding levels were reached. In the CA3 subfield, binding levels increased from PD25 to PD35, followed by a decline in binding capacity from PD35 until PD50, and subsequent increase in binding levels until maximum binding levels were reached at PD70. Whilst limited preclinical literature detailing the ontogeny of the CB_1 receptor binding levels in specific structures such as the hippocampus exists, a study carried out by Belue and colleagues showed that CB_1 receptor binding levels within the hippocampus increased across PD7 to PD14 until adult levels (PD60) were reached at PD21 (Belue *et al*., 1995). Conversely, the findings of the present study indicate that dynamic changes in the binding capacity of the CB_1 receptor take place throughout the peripubertal period. Thus, this present study has elucidated a distinct developmental trajectory pattern within the subfields of the hippocampus that was potentially masked in the previously mentioned study due to the limited number of comparative time-intervals employed. One possible explanation for this novel finding is that changes in the degree of efficacy of G-protein-coupled signalling mechanisms take place throughout the peripubertal period and as a consequence led to the altered binding capacity of the CB_1 receptor demonstrated in this present study. This hypothesis is supported by a recent study carried out by Moore and group (2010) where they showed CB_1 agonist-stimulated incorporation of $[^{35}S]GTP\gamma S$ in the hippocampus was greater in adults compared to

peripubertal animals indicating that CB_1 receptors are less functionally coupled to G-proteins in the peripubertal period (Moore *et al*., 2010).

There are, however, some methodological points to consider within the experimental design of this study. Firstly, due to the number of time-points encompassed in the experimental design group sizes in each time-point were limited. Furthermore, with regard to $[^3H]$SR141716A binding, samples were placed to film for a period of 12 weeks which subsequently led to a large degree of background binding. Both of these factors could have led to variability within the data from certain time points. Moreover, due to the size and developmental stage of the brain during the early post-natal time-points it proved technically difficult to collect anatomically accurate sections of the PFC and hippocampus. As a consequence, group sizes for early time-points were greatly reduced, which in turn lead to increased variability within the data. In future, the inclusion of larger group sizes at each PD time point would help in reducing the noise within the data. In addition, it would also be of interest to extend the time frame of ontogenetic analysis beyond PD70 in order to determine the plateau stage of binding in adulthood within the hippocampus and PFC.

2.6 |Conclusions

The findings in the present study have elucidated distinct ontogenetic trajectories of mRNA expression and CB_1 receptor binding levels in discrete components of the hippocampus and PFC. Furthermore, the detailed ontogenetic profiles of the CB_1 receptor in the hippocampus and PFC generated in this study have delineated a window of developmental vulnerability within both these cognitive substrates and thus formed the basis of future experimental THC treatment regimes throughout this thesis. The time-frame of peripubertal neurodevelopmental vulnerability of the CB_1 receptor to the deleterious effects of THC was defined as PD35-56 since this time-window encompassed escalating and peak levels of CB_1 protein expression in both the PFC and hippocampus.

CHAPTER THREE

Identification of Neural Substrates Sensitive to Developmental Disruption by Acute THC Exposure

3.1 |Introduction

In both the human and rodent brain, CB_1 receptors are heterogeneously distributed with moderate to high binding profiles observed in discrete neural areas within the cerebral cortex, hippocampus, PFC and basal ganglia. CB_1 receptors are also present in lower numbers in thalamic nuclei (Herkenham *et al*., 1991; Glass *et al*., 1997; Pertwee, 1997). This heterogeneous distribution pattern of CB_1 receptor throughout a number of neural systems correlates with the diverse range of psychological, behavioural and motor effects mediated through THC administration. In humans, acute cannabis exposure is characterised by transient intoxication and euphoria which may be followed by drowsiness or a depressive state. Other immediate consequences of cannabis consumption include perturbances in motor control, sensory functions and cognitive processing such as memory and learning (reviewed by Adams and Martin, 1996). Whilst some psychological manifestations, such as euphoria or heightened sensitivity to external stimuli, arising from cannabis use cannot be readily measured in a preclinical settings, preclinical studies have cultivated a parallel pattern of motor and cognitive perturbances. In the rodent, acute administration of CBs can suppress locomotor activity and impair performance in both spatial and non-spatial working memory tasks (Miyamoto *et al*., 1995; Adams and Martin, 1996; Lichtman and Martin, 1996; Jentsch *et al*., 1997; Hampson and Deadwyler, 1998; Mallet and Beninger, 1998; Ferrari *et al*., 1999). As noted in section 1.2.2, cannabis is the most commonly used illicit drug worldwide with a particularly high prevalence amongst adolescents (peak years of initiation between 14-18 years) (Independent Drug Monitoring Unit, 2005; Home

Office, 2012). Clinical studies have demonstrated that cannabis abuse can lead to enduring impairments in memory, attention and executive functions, and there is growing evidence indicating an increased vulnerability of the adolescent brain to these adverse sequelae such as associated with cannabis abuse (Pope and Yurgelun-Todd, 1996; Pope *et al*., 2001, 2003; Block *et al*., 2002; Iversen, 2005). Moreover, cannabis use is implicated as an environmental risk factor for schizophrenia, with early onset of cannabis consumption increasing the risk of developing schizophrenia in adulthood (Andréasson *et al*., 1987; Arseneault *et al*., 2002, Arseneault *et al*., 2004). The adolescent period is a critical neurodevelopmental epoch; maturative processes such as such the dynamic ontogeny of the CB_1 receptor (as demonstrated in chapter two), and global cerebral structural and functional remodelling take place throughout this period (Huttenlocher, 1979; Block *et al*., 2002; Pope *et al*., 2003; Gogtay *et al*., 2004; Casey *et al*., 2005; Lenroot and Giedd, 2006). Disruption of these critical neurodevelopmental events may play a key role in induction of THC-induced psychopathological effects.

The neural systems that underpin the increased vulnerability of the adolescent brain to the adverse consequences of cannabis use are not clearly understood. Whilst receptor binding and mRNA studies provide important information on the neuroanatomical location of receptor expression they do not give any insight into the functional neural systems that are undergoing developmental maturation throughout adolescence and thereby may differentially respond to THC compared to THC exposure in adulthood. Such a systems level analysis can be achieved using functional brain imaging techniques such as 2-[1-^{14}C]-deoxy-D-glucose (2DG) autoradiography. This preclinical *in vivo* imaging technique, enables quantification of glucose metabolic activity as an indicator of neural activity within discrete brain regions simultaneously in a controlled experimental environment. In comparison to other functional imaging techniques such as functional magnetic resonance imaging, 2DG autoradiography offers superior spatial resolution and circumvents the experimental necessity to anesthetise test animals as 2DG autoradiography can be performed on freely moving animals. Moreover, quantification of glucose metabolism as a measure of functional activity overcomes experimental

caveats associated with functional mapping using immediate early gene markers such as *c-fos* or *zif268* (*c-fos* and *zif268* genes are rapidly up-regulated following neural stimulation) including difficulty in quantification of down-regulation in neural activity due to low basal levels of gene expression (*c-fos*) and expression specificity (*zif268* is not expressed on all neurons; primarily expressed on a subset of excitatory neurons) (Farivar *et al.*, 2004). Whilst 2DG autoradiography measures each brain region as an independent variable, further analysis of 2DG data using partial least squares regression (PLSR) multivariate analysis, a mathematical model that assumes that brain function reflects the synchronous activity of groups of brain regions rather than independent activity of any single neural substrates, facilitates the elucidation of functional connectivity patterns of brain regions. Together, these important analytical tools can be employed to elucidate the neural substrates affected by acute THC administration and to probe the developmental mechanisms underlying age-related sensitivity to THC.

3.1.1 |Theoretical Basis for 2-[1-^{14}C]-deoxy-D-glucose Autoradiography

Glucose is the brain's primary source of energy, providing the energy required for the range of complex biochemical processes that take place within the brain. Indeed, glucose metabolism is directly linked to neuronal activity (Sokoloff *et al*., 1977). 2DG autoradiography is a brain imaging technique used to assess functional activity in discrete brain regions through measurement of brain glucose metabolism levels. As energy consumption is directly related to functional activity, quantification of local glucose utilisation allows for the mapping of regional brain activation (Sokoloff *et al*., 1977). This technique is a useful analytical tool in preclinical experiments for the assessment of alterations in cerebral activity following drug administration (Brett *et al*., 2001; Cochran *et al*., 2003; Dawson *et al*., 2010; Dawson *et al*., 2011).

2DG is a structural analogue of glucose, differing only by the presence of a hydrogen atom on the second carbon as compared to a hydroxyl group in glucose. 2DG is transported through the blood-brain barrier by the same saturable carrier as glucose and

subsequently competes with glucose for the enzyme hexokinase within cerebral tissues. This enzyme is responsible for the phosphorylation of both substrates to their respective hexose-6-phosphates. Thus, 2DG exhibits identical biochemical behaviour to glucose until it reaches this point in the glycolytic pathway where the presence of the hydrogen on the second carbon atom prevents it from being metabolised any further. Glucose-6-phophate is converted to fructose-6-phosphate by phosphohexose isomerase and metabolized further via the glycolytic and tricarboxylic acid cycle pathways. However, the anomalous structure of 2DG prevents the isomerisation of the compound by the enzyme phosphohexose isomerase to fructose-6-phoshate. Cessation of 2DG glycolysis results in its accumulation in the brain tissues allowing subsequent quantification of the accumulated radiolabelled 2DG as a measure of glucose metabolism (Sokoloff *et al*., 1977) (Fig 3.01). Although 2DG is not a candidate molecule for isomerisation by phosphohexose isomerase, it does have an affinity for the enzyme and if present in high enough concentrations can competitively inhibit glucose-6-phosphate and lead to a toxic hypoglycaemia (Horton *et al*., 1973). Thus, radiolabelled 2DG is administered in tracer amounts to allow for autoradiographical detection of metabolically active without disrupting these processes.

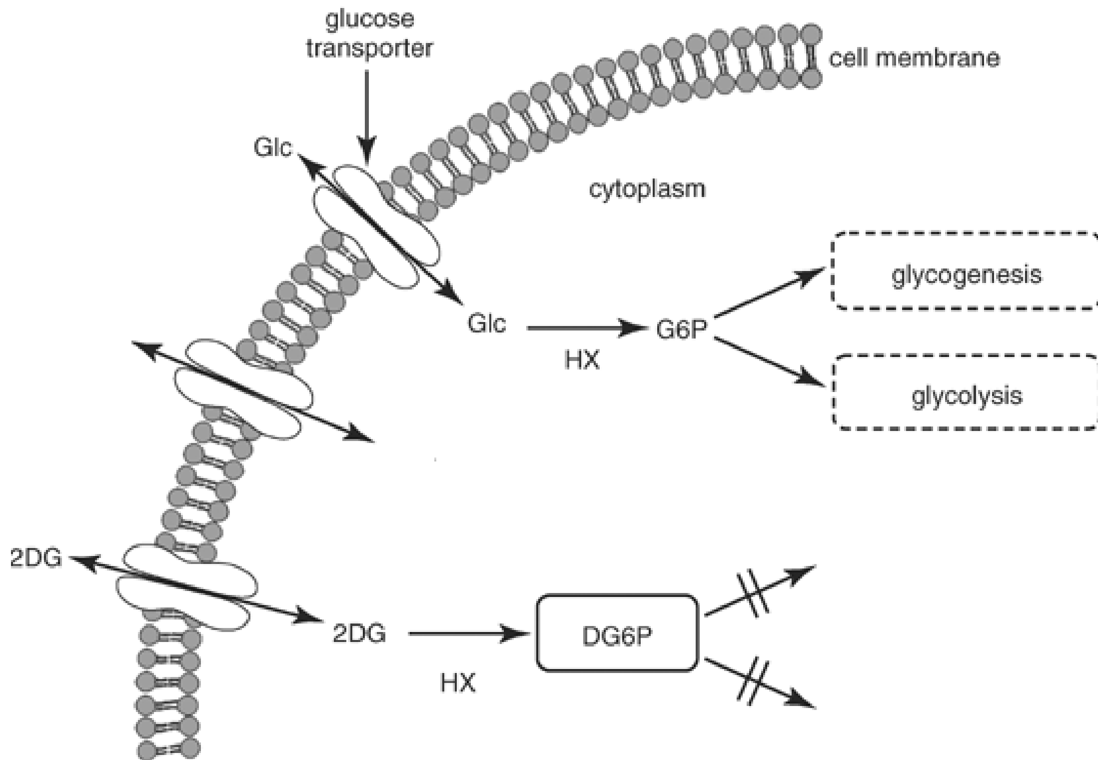


Figure 3.01 Schematic diagram of glucose and 2-Deoxyglucose tissue uptake and metabolism. Both glucose (Glc) and 2-Deoxyglucose (2DG) are taken up into tissue via glucose transporter and subsequently phosphorylated by hexokinase (HX) to the respective hexose-6-phosphates. Glucose-6-phophate (G6P) is converted to fructose-6-phosphate by phosphohexose isomerase and metabolised further via glycolytic pathways whilst deoxyglucose-6-phosphate (DG6P) cannot be phosphorylated by phosphohexose and is therefore trapped in the tissue (adapted from Yamamoto *et al*., 2011).

The fully quantitative 2DG autoradiography experimental technique involves intermittent monitoring of arterial radiotracer and glucose levels via intravascular cannulation throughout a 45 minute experimental period. Acquisition of these experimental variables allows for the calculation of local cerebral glucose utilisation (LCGU) using an operational equation developed by Sokoloff *et al*. (1977) based on the biochemical model of glucose metabolism. Fully quantitative 2DG autoradiography is a powerful *in vivo* neuroimaging technique as application of the operational equation allows for delineation of LCGU in absolute terms and enables quantitative localisation of radiolabelled tracer in order to determine neural activity in discrete brain regions. However, due to the nature of the experimental set-up, the spectrum of experimental settings to which the technique can be applied is limited. For example, the requirement to generate profiles of plasma glucose and 2DG concentrations using intravascular cannulation precludes certain types of experiment such as behavioural experiments or the use of very small animals such as mice as there is a danger of hypovolaemic stress resulting from repeated blood sampling (Dawson *et al*., 2008). The process of intravascular cannulation may elevate stress levels in the experimental animals and therefore compromise results obtained. For such reasons, recent studies have employed a modified version of this technique that circumvents the necessity for intravascular cannulation by obtaining a terminal trunk blood sample to quantify concentrations of radiolabelled tracer and glucose. Thus, plasma variables (particularly plasma glucose) alongside whole brain tissue average (WBA) ^{14}C concentrations (a measure of global cerebral ^{14}C uptake) can be quantified to ensure the experimental manipulation does not alter these experimental variables in a manner which would confound interpretation of experimental results (Dawson *et al*., 2008, Dawson *et al*., 2010, Dawson *et al*., 2011).

3.1.2 |Theoretical Basis for Functional Connectivity Analysis Using the Partial Least Squares Regression Mathematical Model

Quantification of overt glucose metabolism using 2DG autoradiography functions as an indicator of local neural activity within a discrete brain region. The PLSR mathematical model assumes that correlated activity between brain regions reflects their synchronous activity or functional connectivity. For this reason, the PLSR analytical approach is commonly employed in clinical human neuroimaging studies in order to elucidate connectivity patterns of brain regions to a defined 'seed' region across a broad spectrum of experimental conditions (Kilpatrick *et al*., 2006; Diaconescu *et al*., 2010) More recently, the PLSR algorithm has been applied to 2DG preclinical data to investigate the effects of psychomimetic drugs on brain functional connectivity (Dawson *et al*., 2010; Dawson *et al*., 2011).

PLSR is a type of quantitative multivariate analysis which models the relationship between two data matrices, X and Y. This multivariate linear regression model is particularly suited to analysing functional connectivity in the brain as it can model the influence of multiple, collinear 'predictor' (X) variables upon a given dependent (Y) variable (Wold *et al*., 2001; Dawson *et al*., 2010; Dawson *et al*., 2011). Thus, PLSR enables the modelling of the functional connectivity relationship between a 'seed' brain region (dependent variable) and all of the other brain regions analysed ('explanatory' variables). The model generates a statistical output known as variable importance for the projection (VIP) statistic which is a summary measure of the importance of an x-variable (brain region) in relation to the both Y and X variables i.e. the VIP statistic can be considered to reflect the functional coupling between the defined 'seed' brain region and the other brain regions of interest. Small VIP values, <0.8 are considered to make a small contribution, 0.8-1 a moderate contribution and large VIP values, larger than 1, are considered to make large contribution to determining the values in the X and Y matrices (Dawson *et al*., 2010, Dawson *et al*., 2011).

3.2 |Aims and Objectives

The aim of this study was to test the hypothesis that the adolescent brain shows altered functional activity compared to the adult brain rendering it more sensitive to the deleterious effects of THC.

The specific objectives of this study were to investigate:

a) Developmental hypothesis: Important neural events take place throughout the peripubertal period; is this reflected by altered cerebral metabolism and neural system connectivity in peripubertal rats relative to adult rats?

b) THC-induced alterations in brain function: What discrete neural substrates are affected by acute THC administration and how does THC alter neural system connectivity?

c) THC sensitivity hypothesis: Are peripubertal animals (PD35) more sensitive to the effects of THC compared to adult animals (PD70)?

These objectives were achieved through employment of 2DG imaging in combination with functional connectivity analysis using PLSR.

3.3 |Methods

3.3.1 |Animals

20 peripubertal (PD35) and 20 adult (PD70) male Lister-hooded rats (Harlan, UK) were housed under standard conditions on a 12 hr/12 hr light/dark cycle (lights on 06:00hr). They received food and water *ad libitum* in the home cage. Room temperature ($21^{o}\pm2^{o}$C) and humidity (45–55%) were kept constant throughout. The experiment was performed in accordance with the Animals (Scientific Procedures) Act 1986.

3.3.2 |THC Preparation

Δ^{9}-tetrahydrocannabinol amorphous resin (Tocris Biosciences) was dissolved in ethanol and subsequently re-suspended in 0.9% saline solution, as described below, to prevent the introduction of a confounding factor of alcohol into the experimental design. THC solution was prepared as described by Pertwee *et al*. (1992). Briefly, the THC solution was mixed with 1% of Tween 80 (Sigma-Aldrich Inc. UK) by weight and ethanol was removed by evaporation under a stream of nitrogen. THC was re-suspended in 0.9% saline solution in a series of aliquots of increasing volume to ensure that the dispersion of THC was homogenous. After each aliquot was added the mixture was vortexed. The resultant suspension was protected from light as it is light sensitive and stored at -20°C until needed.

3.3.3 |Drug Administration

Animals from both age groups were randomly allocated into treatment groups (n=10/group) and were subsequently administered either vehicle (1% Tween 80 in 0.9% saline) or 5mg/kg THC preparation intraperitoneally in a volume of 1ml/kg. The four treatment groups were as follows:

1) Peripubertal animals (PD35) whom received acute THC (5mg/kg) treatment.

2) Peripubertal animals (PD35) whom received vehicle treatment.

3) Adult animals (PD70) whom received acute THC (5mg/kg) treatment.

4) Adult animals (PD70) whom received vehicle treatment.

All groups were administered treatment 30 mins prior to initiation of 2DG experimental procedure to allow for absorption of THC. The dose and timing of THC administration used in this study was based on that used in similar preclinical brain imaging study carried out in our laboratory (Brett *et al*., 2001).

3.3.4 | ^{14}C-2-Deoxyglucose Autoradiography

Local cerebral glucose utilisation (LCGU) was determined 30 mins after treatment with 5mg/kg THC or vehicle. 125µCi/kg of *2*DG, specific activity 50mCi/mmol, 0.1Ci/ml (Perkin-Elmer Ltd) was prepared along with 0.1ml heparinised 0.9% saline and injected intraperitoneally before animals were returned to their home cage. 45 mins after isotope injection animals were decapitated and a terminal blood sample collected by torso inversion in heparinised weigh-boats. The brain was rapidly removed and frozen in isopentane (cooled to -42° C), coated in M-1 embedding medium and stored at –80 ^{o} C until required. Blood samples were centrifuged for one minute at 13,000rpm to separate the plasma and aliquots were removed for the determination of plasma glucose (10µl) and ^{14}C (20µl) concentrations by semi-automated glucose oxidase assay (Beckman) and liquid scintillation analysis (Packard), respectively.

20µm coronal sections were cut at a temperature of -20 ^{o} C using a rotary microtome in a cryostat (Leica CM 1850). Three consecutive sections were collected every 200µm, thaw mounted onto slide covers and rapidly dried on a hot plate (70°C). Coverslides were exposed to autoradiographic film (Kodak Biomax MR1) together with ^{14}C standards (40–1069 nCi/g tissue equivalents; Amersham International) and following an exposure period of 5 days films were developed according to the manufacturer's

instructions. Autoradiographic images were analyzed using the MCID densitometry system (MCID Imaging Research, St. Catharines, Ontario, Canada). The local isotope concentration for each RoI was derived from the optical density of autoradiographic images relative to that of the co-exposed ^{14}C standard. 68 anatomically distinct RoIs were measured with reference to Paxinos & Watson's atlas of the rat brain (6th Edition). LCGU in each RoI was determined as the ratio of ^{14}C present in that region relative to the average ^{14}C concentration in the whole brain of the same animal, referred to as the 2DG uptake ratio. WBA ^{14}C levels were determined as the average ^{14}C concentration across all sections in which a RoI was measured.

2DG data for all experimental groups were analyzed using univariate general linear ANOVA models. When analysing changes in the rate of metabolism between experimental groups anatomically discrete brain regions were assumed to represent independent variables within each measure and no correction was applied for multiple comparisons (McCulloch *et al*., 1982). Bonferroni *post-hoc* comparisons were carried out to determine whether there was a differential liability to THC between age groups. Plasma variables for the 2DG study were analyzed using univariate general linear ANOVA model. Significance was set at $p<0.05$ throughout.

3.3.5 | Functional Connectivity Analysis Using Partial Least Squares Regression Model

The PLSR model was used to define functional brain connectivity signatures by modelling the relationship between the rate of metabolism, as determined by the 2DG uptake ratio, in a specified 'seed' brain region (the dependent variable) and all the other RoI measured ('explanatory' variables; 68 brain regions in each analysis) as previously described by Dawson *et al*. (2011). For each experimental factor (Age and Treatment) as no significant treatment x age was evident, data was merged to increase statistical power of factors and PLS models were generated using the PLSR module in XLSTAT 2009 (Addinsoft, New York, NY). The variable importance for the projection (VIP)

statistic was used as a measure of the functional connectivity of each explanatory brain region to the 'seed' brain region and the standard deviation of the VIP statistic for each explanatory brain region was estimated by the re-sampling method jack-knifing ('leave-one-out' procedure). A significant functional connection between brain regions was considered to exist if the 95% confidence interval (CI) for the VIP statistic exceeded 0.8, because this threshold denotes a considerable contribution of an explanatory variable to the dependent variable in PLSR models (Wold *et al*., 2001). Statistical differences in the VIP for each explanatory RoI to a given 'seed' brain region between experimental groups were analysed using Student's *t*-tests with Bonferroni correction applied for multiple comparisons. Significance was set at $p<0.05$ throughout.

In this study, when investigating age-related effects on functional connectivity, 9 'seed' brain regions were analysed, namely, CA1 stratum moleculare, radiatum and oriens, dentate gyrus, vertical and horizontal diagonal band of Broca, nucleus accumbens core, locus coeruleus and dorsal raphe. These regions were chosen as 'seed' regions as ANOVA analysis revealed age-related alterations in the overt rate of metabolism within these RoIs (see section 3.4.1.1). In order to determine the effects of acute THC treatment on functional connectivity 8 'seed' regions were analyzed, which were anteromedial, anteroventral, reticular and mediodorsal thalamic nuclei, basolateral and medial amygdala, nucleus accumbens core and locus coeruleus. These select regions were chosen as 'seed' regions because within these RoIs, acute THC administration significantly altered overt glucose metabolism (see section 3.4.1.2).

3.4 |Results

3.4.1 |Semi-Quantitative ^{14}C-2-Deoxyglucose Autoradiographic Imaging

Terminal plasma variables and WBA^{14}C levels from animals in each age (PD35 and PD70, n=10/gp) and treatment group (acute THC (5mg/kg) and vehicle (1% Tween 80 in 0.9% saline), n=10/gp) were analysed using general linear univariate ANOVA models (Table 3.01). ANOVA analysis did not reveal any significant differences in plasma glucose levels between experimental groups. Acute THC treatment did not significantly affect WBA^{14}C, plasma glucose or ^{14}C levels. ANOVA analysis revealed a significant effect of age on plasma ^{14}C levels ($F_{(1,35)}$=11.83, p=0.002), with plasma ^{14}C levels significantly less in peripubertal animals relative to adult animals. Furthermore, statistical analysis revealed a significant difference in WBA^{14}C concentrations between age groups ($F_{(1,31)}$=40.00, $p<0.001$), with peripubertal animals demonstrating lower global WBA^{14}C levels compared to adult animals. As plasma glucose levels were unaffected and plasma ^{14}C levels were lower in peripubertal animals, this suggests that there was no age-related differential ability of 2DG to enter the brain from the plasma. However, the significant difference in WBA^{14}C levels between the two age groups suggests that global glucose metabolism was significantly lower in peripubertal animals compared to adult animals. In this study, cerebral metabolism in discrete RoIs is calculated using the 2DG uptake ratio (ratio of ^{14}C present in a RoI relative to the average ^{14}C concentration in the whole brain of the same animal), thus taking into consideration variations in WBA^{14}C levels between experimental animals.

3.4.1.1 |Age-related Differential Patterns of Regional Cerebral Metabolism

Significant age-dependent increases and decreases in LCGU were observed on a region-dependent basis, in 17 of 68 regions analysed. The effects of age on all 68 RoI measured are detailed in Table 3.02.

Cerebral metabolism in several subfields of the hippocampal formation was significantly higher in adult animals (PD70) relative to peripubertal animals (PD35) including three strata of the CA1 layer, namely CA1 stratum moleculare ($F_{(1,29)}$=6.67, p=0.015), radiatum ($F_{(1,29)}$=5.61, p=0.025) and oriens ($F_{(1,29)}$=5.67, p=0.024) and the dentate gyrus ($F_{(1,31)}$=14.19, p<0.001, Fig 3.02A-B). Conversely, adult animals had significantly decreased rate of cerebral glucose uptake within the septohippocampal nucleus ($F_{(1/31)}$=4.55, p=0.041, Fig 3.02A). Among adult rats, two cortical layers, the somatosensory ($F_{(1,31)}$=5.73, p=0.023) and piriform cortices exhibited enhanced glucose metabolism relative to peripubertal rats ($F_{(1,28)}$=10.93, p=0.003, Fig 3.02A). This pattern of age-dependent increase in cerebral metabolism was further evident within three nuclei of the basal ganglia, the subthalamic nucleus ($F_{(1,31)}$=7.68, p=0.009), globus pallidus ($F_{(1,31)}$=4.74, p=0.037) and the ventromedial striatum $F_{(1,32)}$=5.67, p=0.023) in addition to the inferior colliculus ($F_{(1,26)}$=14.15, p<0.001) and the anteroventral thalamus ($F_{(1,30)}$=5.38, p=0.027, Fig 3.02A). An increase in cerebral glucose metabolism was also evident in adult animals in principal cholinergic and noradrenergic nuclei, namely the vertical ($F_{(1,29)}$=4.1, p=0.05) and horizontal limb of the diagonal band of Broca ($F_{(1,28)}$=8.72, p=0.006) and the locus coeruleus ($F_{(1,26)}$=5.35, p=0.029, Fig 3.02A). However, glucose metabolic activity was reduced in principal dopaminergic and serotonergic nuclei in adult animals (PD70) relative to peripubertal animals (PD35), specifically the ventral tegmental area ($F_{(1,29)}$=17.45, p<0.001) and dorsal raphe respectively ($F_{(1,26)}$=4.44, p=0.045, Fig 3.02A).

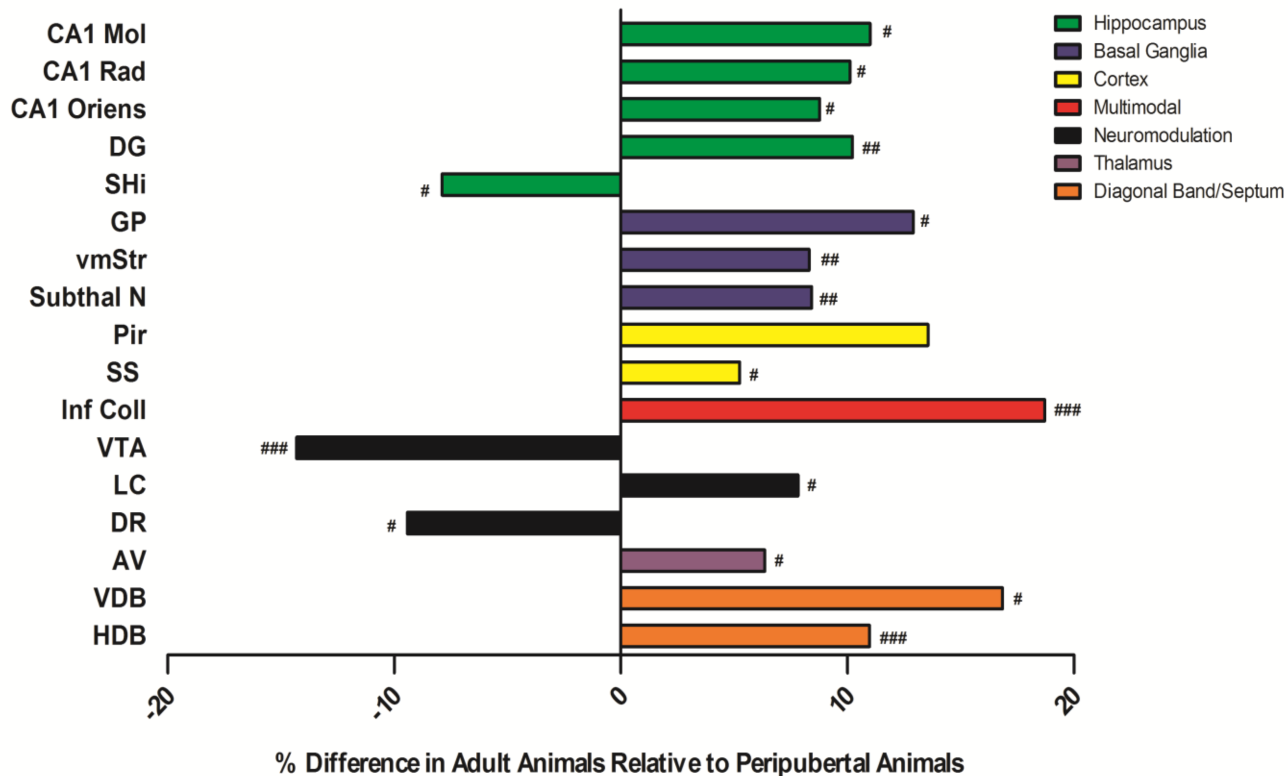
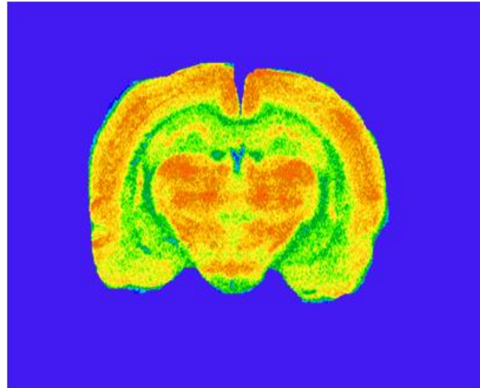
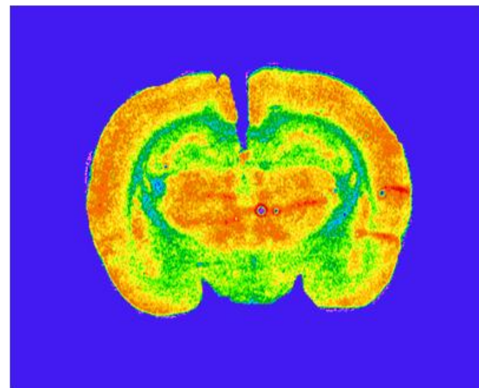


Figure 3.02A Age-dependent differences in overt cerebral metabolism in adult animals (PD70) relative to peripubertal animals (PD35). Data from all experimental groups was analysed using univariate general linear ANOVA models. In adult animals, cerebral glucose metabolism was significantly decreased in the septohippocampal nucleus (SHi), ventral tegmental area (VTA) and dorsal raphe nucleus (DR) relative to peripubertal animals. Cerebral glucose metabolism was significantly increased in the CA1 stratum moleculare (CA1 Mol), CA1 stratum radiatum (CA1 Rad), CA1 stratum oriens (CA1 Oriens), dentate gyrus (DG), globus pallidus (GP), ventromedial striatum (vmStr), subthalamic nucleus (Subthal N), piriform (Pir) and somatosensory (SS) cortices, inferior colliculus (Inf Coll) and locus coeruleus (LC), anteroventral thalamus, vertical (VDB) and horizontal diagonal band of Broca (HDB) in adult rats compared to peripubertal rats. As there was no significant evidence for treatment x age interactions in any of these regions, data are shown as % difference in pooled adult data relative to peripubertal data irrespective of treatment. #$p<0.05$, ##$p<0.01$ and ###$p<0.001$ denotes a significant main effect of age on cerebral glucose metabolism. Raw data are shown in Table 3.02.

Peripubertal (PD35)

Adult (PD70)





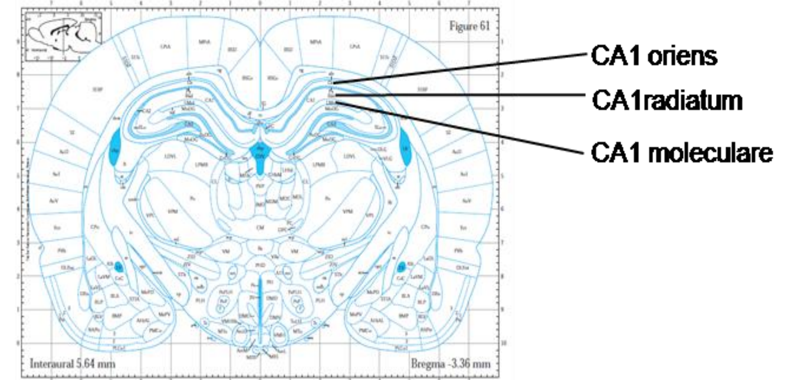


Figure 3.02B Autoradiograms illustrating an age-related increase in cerebral metabolism in the CA1 region of the hippocampus in adult animals. relative to peripubertal animals These representative autoradiogram images illustrate an age-related significant increase in cerebral metabolism (as measured by LCGU) in discrete strata of the CA1 region of the hippocampus in adult animals (PD70) relative to peripubertal animals (PD35). Measurements of discrete brain areas were taken as anatomically defined according to Paxinos & Watson's atlas of the rat brain (6th Edition) as illustrated in sections on the far right panel.

3.4.1.2 |Effects of Acute THC Administration on Regional Cerebral Metabolism

Acute THC administration led to both increases and decreases in LCGU on a region-dependent basis, in 15 of 68 regions analysed. The effects of acute THC treatment on all 68 RoI measured are detailed in Table 3.02

Acute THC administration induced hypometabolism in four thalamic nuclei including the reticular ($F_{(1,30)}=16.14$, $p<0.001$) and mediodorsal thalamus ($F_{(1,30)}=22.38$, $p<0.001$) and two anterior thalamic structures, namely the anteromedial ($F_{(1,30)}=12.18$, $p=0.002$) and anteroventral thalamus ($F_{(1,30)}=12.84$, $p<0.001$, Fig 3.03A-B). Acute exposure to THC significantly decreased glucose uptake within the nucleus accumbens core ($F_{(1,32)}=5.32$, $p=0.028$, Fig 3.03A). THC-induced hypometabolism was also evident in the somatosensory cortex ($F_{(1,31)}=7.95$; $p=0.008$), subthalamic nucleus ($F_{(1,31)}=8.11$, $p=0.009$) and the visual relaying centre, the lateral geniculate nucleus ($F_{(1,31)}=14.92$, $p<0.001$, Fig 3.03A). Of the two amygdala nuclei that exhibited significant alterations in LCGU following acute THC treatment, opposing effects of THC on LCGU was observed. Cerebral metabolism within the basolateral amygdala was significantly reduced by acute THC administration ($F_{(1,31)}=12.87$, $p<0.001$, Fig 3.03A). Conversely, within the medial amygdala, acute systemic administration of 5mg/kg THC led to increased cerebral metabolism ($F_{(1,31)}=8.42$, $p=0.007$, Fig 3.03A). Among the septal nuclei analysed, acute THC treatment significantly increased LCGU in two septal structures, the medial ($F_{(1,31)}=4.513$, $p=0.042$) and lateral intermediate nuclei $F_{(1,31)}=5.98$, $p=0.02$, Fig 3.03A). THC-induced hypermetabolism was observed within the midbrain RoIs, the inferior colliculus ($F_{(1,26)}=10.75$, $p=0.003$, Fig 3.03A) and interpeduncular nucleus ($F_{(1,31)}=6.9$, $p=0.014$, Fig 3.03A). Acute THC administration produced a hypermetabolic state within a principal source of noradrenergic innervation in the forebrain, the locus coeruleus ($F_{(1,26)}=4.79$, $p=0.03$, Fig 3.03A).

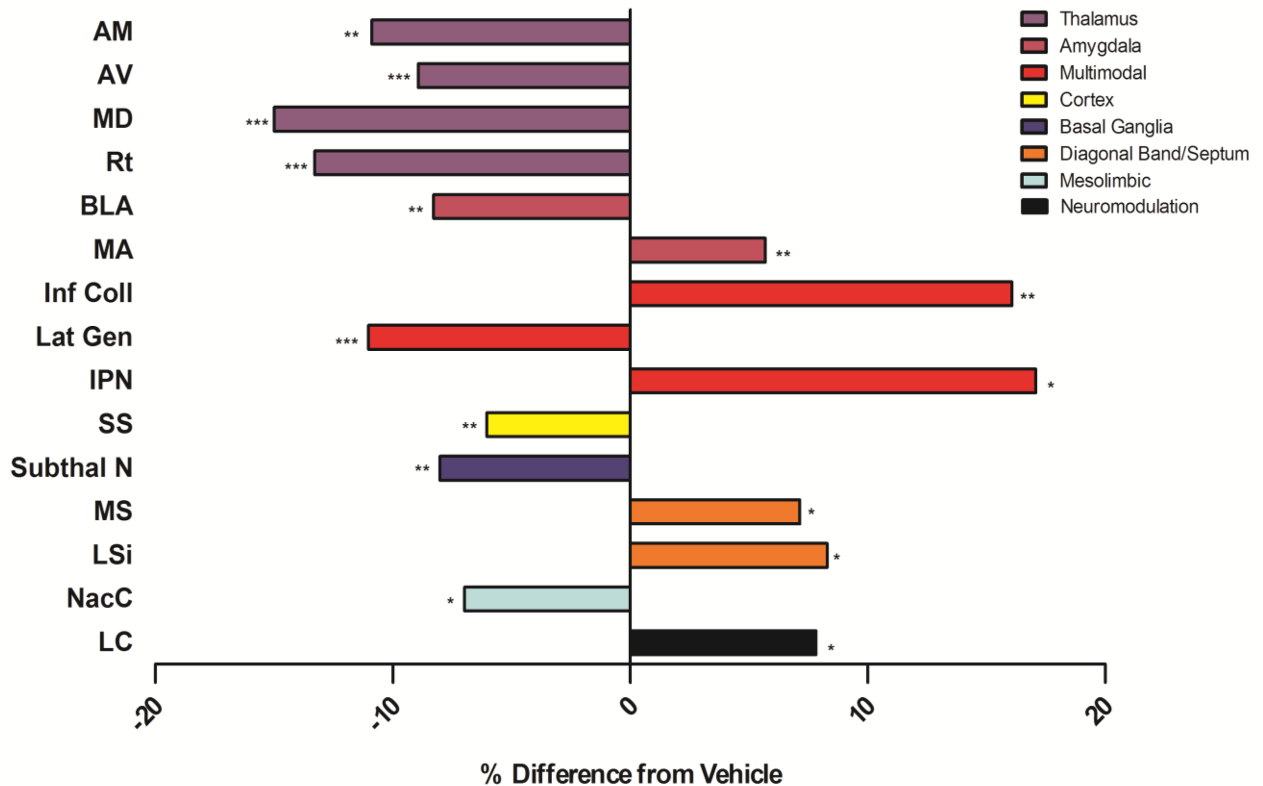
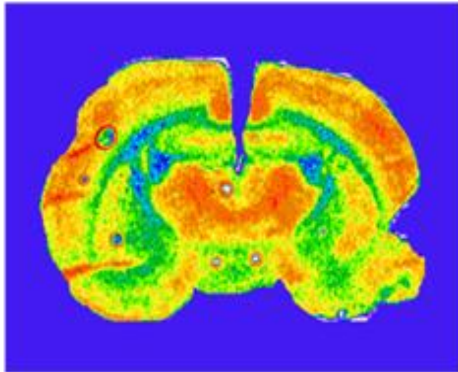
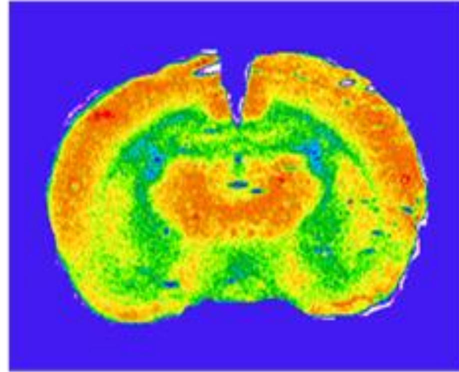


Figure 3.03A THC-induced alterations in overt cerebral metabolism. Data from all experimental groups was analysed using univariate general linear ANOVA models. Acute THC treatment significantly decreased cerebral glucose metabolism in the anteromedial (AM), anteroventral (AV), mediodorsal (MD) and reticular (Rt) thalamic nuclei, basolateral amygdala (BLA), somatosensory cortex (SS), lateral geniculate nucleus (Lat Gen), subthalamic nucleus (Subthal N) and nucleus accumbens core (NacC). Acute THC treatment significantly enhanced cerebral glucose metabolism in the medial amygdala (MA), inferior colliculus (Inf Coll), interpeduncular nucleus (IPN), medial (MS) and lateral intermediate septum (LSi) and locus coeruleus (LC). As there was no significant evidence for a treatment x age interaction in any of these regions, data are shown as % difference in THC-treated animals relative to controls regardless of age. *p<0.05, **p<0.01 and ***p<0.001 denotes a significant main effect of acute THC (5mg/kg) treatment on cerebral glucose metabolism. Raw data shown in Table 3.02.

Peripubertal Vehicle Treatment

Peripubertal Acute THC Treatment





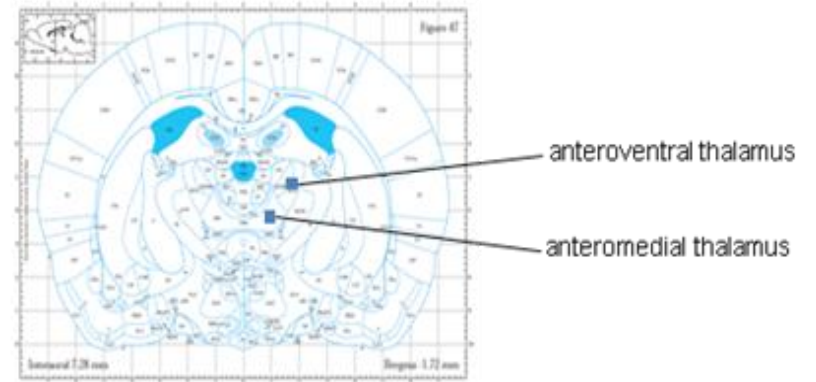


Figure 3.03B Autoradiograms illustrating THC-induced hypometabolism in the anterior thalamus in peripubertal animals. These representative autoradiogram images illustrate a significant THC-induced hypometabolism (as measured by LCGU) in the anteroventral and anteromedial thalamus in peripubertal animals (PD35). Measurements of discrete brain areas were taken as anatomically defined according to Paxinos & Watson's atlas of the rat brain (6th Edition) as illustrated in sections on the far right panel.

3.4.1.3 |Regional Age-dependent Sensitivity to Acute THC Administration

No age x treatment interactions were present in any of the 68 RoI investigated. However, visual inspection of the data and clear regional effects of THC upon glucose use indicated that there was an age-related response of THC. Thus, to probe the presence of an age-related liability to THC, differences in cerebral metabolism between THC-treated animals relative to respective age control was investigated using Bonferroni *post-hoc* comparisons.

In three of four thalamic nuclei significantly affected by acute THC administration the THC-induced reduction in glucose metabolism appeared to be more pronounced in peripubertal animals (anteromedial thalamus, $p<0.05$; mediodorsal and reticular thalamus ($p<0.001$, Fig 3.04) compared to adult animals (reticular thalamus $p<0.05$, Fig 3.04). In contrast to the thalamic nuclei, THC-induced hypometabolism in the nucleus accumbens core and lateral geniculate nucleus appears to be more pronounced in adult animals ($p<0.05$, Fig 3.04) relative to peripubertal animals. In the subthalamic nucleus, hypometabolism following acute THC exposure appeared to be more pronounced in peripubertal animals ($p<0.05$, Fig 3.04) compared to adult animals. There were opposing age-related differences in sensitivity to THC among the basolateral and medial amygdala nuclei. In the basolateral amygdala, THC treatment appeared to be more pronounced effect in peripubertal animals ($p<0.001$, Fig 3.04) as compared to adult animals. In the medial amygdala, adult animals were more sensitive to the effects of THC ($p<0.05$, Fig 3.04) compared to peripubertal animals. THC-induced hypermetabolism appeared to be more prominent in adult animals ($p<0.05$, Fig 3.04) relative to peripubertal animals in the lateral intermediate septum. In the inferior colliculus the THC-induced hypermetabolic state appeared to be more pronounced in adult animals ($p<0.05$, Fig 3.04) relative to peripubertal animals. THC-induced hypermetabolism appears to more prominent in peripubertal animals ($p<0.05$, Fig 3.04) compared to adult animals.

Figure 3.04

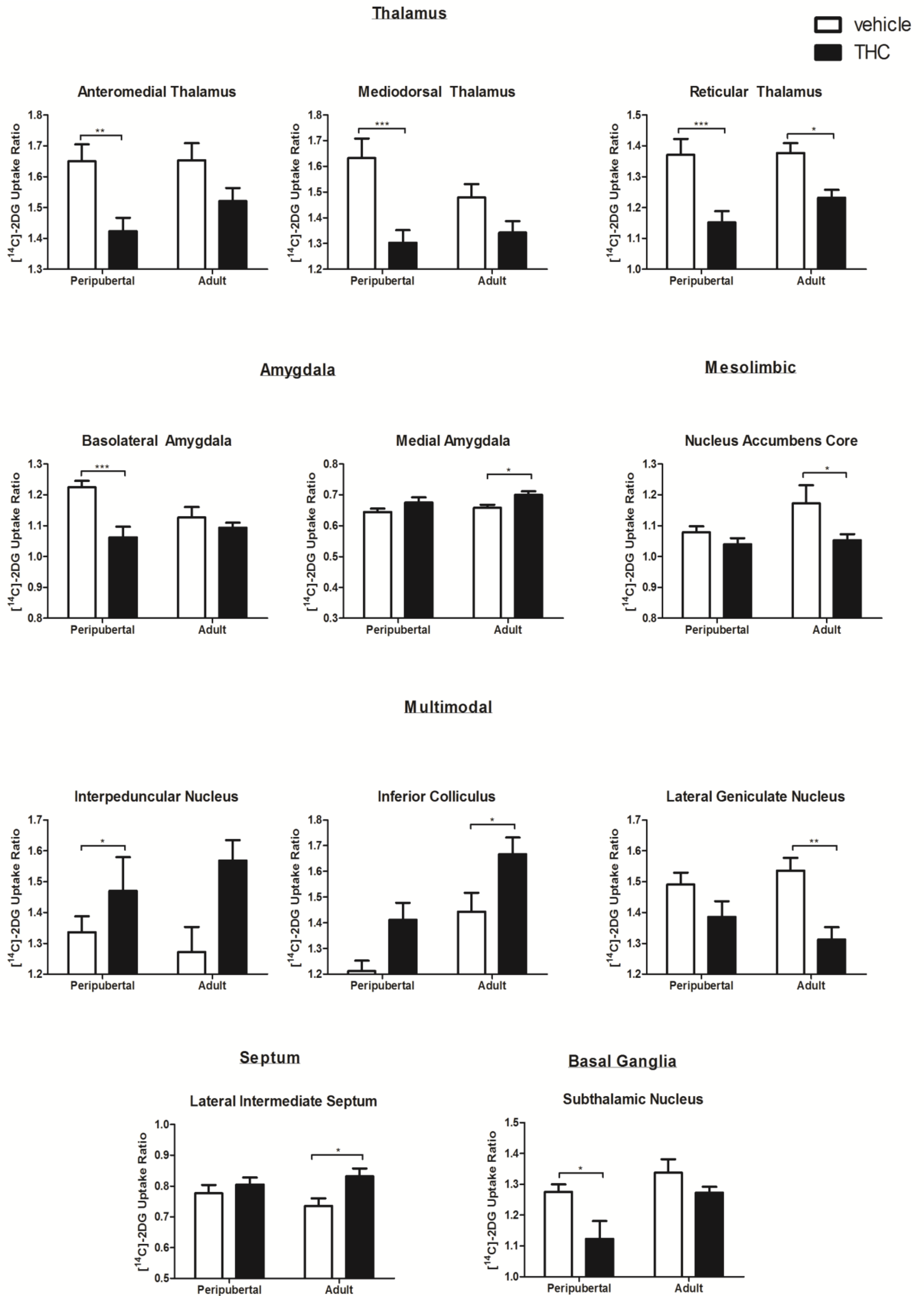


Figure 3.04 Regional age-dependent sensitivity to acute THC administration. Data from all experimental groups in each discrete RoI was analysed using univariate general linear ANOVA models. Bonferroni *post-hoc* analysis was carried out to probe the presence of age-dependent developmental sensitivity in RoIs significantly affected by THC treatment. In the anteromedial, mediodorsal and reticular thalamic nuclei, basolateral amygdala and subthalamic nucleus, THC-induced hypometabolism appeared to be more pronounced in peripubertal animals relative to adult animals. In the lateral geniculate nucleus THC-induced hypometabolism appeared to be more pronounced in adult animals relative to peripubertal ones. THC-hypermetabolism in the medial amygdala, inferior colliculus and lateral intermediate septum appeared to be more pronounced adult animals relative to peripubertal ones whilst in the interpeduncular nucleus, THC-induced hypermetabolism appeared to be more pronounced in peripubertal animals relative to adult animals. *p<0 .05, **p<0.01 and ***p<0.001 denotes a significant difference in cerebral metabolism between THC-treated animals relative to respective control.

Table 3.01 Terminal plasma parameters and whole brain average ^{14}C levels

	Peripubertal Animals		Adult Animals	
	vehicle	THC (5m/kg)	vehicle	THC (5m/kg)
	mean ± SEM	mean ± SEM	mean ± SEM	mean ± SEM
Plasma Glucose (mmol/L)	8.42 ± 0.80	8.42 ± 0.66	8.78 ± 1.06	8.23 ± 0.92
## Plasma ^{14}C (nCi/ml)	30.09 ± 14.04	41.28 ± 25.87	52.63 ± 19.68	75.43 ± 34.66
### WBA ^{14}C (nCi/gm)	196.16 ± 40.67	195.97 ± 72.36	304.56 ± 33.55	305.04 ± 46.75

Table 3.01 Terminal plasma parameters and whole brain average ^{14}C levels from all experimental groups. Data are shown as mean ± SEM. Data was analysed using univariate general linear ANOVA models. Acute THC treatment did not significantly affect WBA^{14}C, plasma glucose or ^{14}C levels suggesting that THC treatment did alter the ability of 2DG to enter the brain from the plasma. Plasma ^{14}C and WBA^{14}C levels were significantly lower in peripubertal animals as compared to adult animals. These findings suggest that whilst 2DG uptake into the brain was not reduced in peripubertal animals, the rate of glucose metabolism is significantly lower in the brain of peripubertal animals as compared to adult. *$p<0.05$ denotes a significant effect of acute THC (5mg/kg) treatment. ##$p<0.01$ and ###$p<0.001$ denotes a significant main effect of age.

Table 2 Effects of Acute THC Treatment and Age on Cerebral Metabolism in Peripubertal and Adult Animals

	Peripubertal Animals		Adult Animals	
	Control	THC (5m/kg)	Control	THC (5m/kg)
	mean ± SEM	mean ± SEM	mean ± SEM	mean ± SEM
Cortex				
Medial Orbital (mO)	1.27 ± 0.12	1.18 ± 0.07	1.27 ± 0.04	1.17 ± 0.04
Ventral Orbital (vO)	1.53 ± 0.07	1.41 ± 0.06	1.54 ± 0.04	1.51 ± 0.03
Lateral Orbital (lO)	1.47 ± 0.07	1.38 ± 0.05	1.47 ± 0.04	1.42 ± 0.03
Dorsolateral Orbital (DLO)	1.11 ± 0.11	1.08 ± 0.02	1.04 ± 0.03	1.09 ± 0.03
Prelimbic (PrL)	1.16 ± 0.03	1.17 ± 0.05	1.15 ± 0.03	1.15 ± 0.02
Infralimbic (IL)	1.24 ± 0.03	1.23 ± 0.06	1.20 ± 0.05	1.09 ± 0.03
Cingulate (Cg)	1.17 ± 0.02	1.18 ± 0.01	1.21 ± 0.03	1.21 ± 0.02
Primary Motor (M1)	1.11 ± 0.03	1.14 ± 0.04	1.11 ± 0.02	1.14 ± 0.02
Secondary Motor (M2)	1.17 ± 0.03	1.18 ± 0.03	1.12 ± 0.03	1.14 ± 0.02
Agranular Insular (AIC)	1.10 ± 0.02	1.13 ± 0.04	1.07 ± 0.02	1.06 ± 0.01
## Piriform (Pir)	1.29 ± 0.05	1.37 ± 0.04	1.50 ± 0.04##	1.50 ± 0.05##
Motor (Motor C)	1.33 ± 0.03	1.30 ± 0.02	1.39 ± 0.04	1.32 ± 0.02
** # Somatosensory (SS)	1.51 ± 0.04	1.41 ± 0.03**	1.60 ± 0.03#	1.50 ± 0.02**#
Retrosplenial (Retro C)	1.16 ± 0.04	1.19 ± 0.07	1.32 ± 0.07	1.19 ± 0.02
Parietal (Par)	1.26 ± 0.03	1.24 ± 0.04	1.29 ± 0.02	1.28 ± 0.02
Auditory (Aud C)	1.38 ± 0.04	1.28 ± 0.08	1.33 ± 0.08	1.32 ± 0.05
Entorhinal (Ento C)	0.80 ± 0.03	0.80 ± 0.02	0.82 ± 0.04	0.88 ± 0.02
Visual (Visual C)	1.16 ± 0.07	1.34 ± 0.02	1.35 ± 0.06	1.40 ± 0.05
Thalamus				
Anterodorsal (AD)	1.09 ± 0.03	1.09 ± 0.07	1.14 ± 0.03	1.16 ± 0.03
*** # Anteroventral (AV)	1.44 ± 0.03	1.31 ± 0.03***	1.53 ± 0.04#	1.40 ± 0.03***#
** Anteromedial (AM)	1.65 ± 0.05	1.42 ± 0.04**	1.65 ± 0.05	1.52 ± 0.04**
*** Reticular (Rt)	1.37 ± 0.05	1.15 ± 0.04***	1.38 ± 0.03	1.23 ± 0.02***
*** Mediodorsal (MD)	1.63 ± 0.07	1.30 ± 0.05***	1.48 ± 0.05	1.34 ± 0.04***
Hypothalamus				
Lateral Hypothalamus (Lat Hypo)	0.77 ± 0.02	0.80 ± 0.02	0.73 ± 0.02	0.78 ± 0.01
Amygdala				
** Basolateral (BLA)	1.22 ± 0.02	1.06 ± 0.03**	1.13 ± 0.03	1.09 ± 0.02**
** Medial (MA)	0.64 ± 0.01	0.67 ± 0.02**	0.66 ± 0.01	0.70 ± 0.01**
Central (CA)	0.85 ± 0.01	0.82 ± 0.02	0.81 ± 0.02	0.82 ± 0.02
Basal Ganglia				
# Globus Pallidus (GP)	0.85 ± 0.05	0.77 ± 0.03	0.96 ± 0.06#	0.87 ± 0.04#
Ventral Pallidium (VP)	0.77 ± 0.03	0.79 ± 0.02	0.82 ± 0.04	0.78 ± 0.02
Dorsolateral Striatum (dlStr)	1.36 ± 0.02	1.32 ± 0.03	1.44 ± 0.06	1.34 ± 0.03
# Ventromedial Striatum (vmStr)	1.29 ± 0.03	1.24 ± 0.03	1.43 ± 0.07#	1.33 ± 0.02#
Substantia Nigra pars reticulata (SNR)	0.83 ± 0.01	0.88 ± 0.05	0.88 ± 0.03	0.80 ± 0.01
Substantia Nigra pars compacta (SNC)	1.09 ± 0.02	1.17 ± 0.10	1.14 ± 0.04	1.08 ± 0.02
** ## Subthalamic Nucleus (Subthal N)	1.28 ± 0.02	1.12 ± 0.05**	1.34 ± 0.04##	1.27 ± 0.02**##
Hippocampus				
## Dentate Gyrus (DG)	0.67 ± 0.04	0.74 ± 0.03	0.83 ± 0.02##	0.80 ± 0.01
# CA1 Stratum Moleculare (CA1 Mol)	1.00 ± 0.03	1.03 ± 0.05	1.18 ± 0.06#	1.08 ± 0.02##
# CA1 Stratum Radiatum (CA1 Rad)	0.79 ± 0.02	0.84 ± 0.04	0.95 ± 0.05#	0.85 ± 0.01#
# CA1 Stratum Oriens (CA1 Oriens)	0.79 ± 0.02	0.79 ± 0.03	0.91 ± 0.04#	0.82 ± 0.02#
CA2 Stratum Moleculare (CA2 Mol)	1.01 ± 0.03	1.01 ± 0.06	1.13 ± 0.06	0.99 ± 0.02
CA2 Stratum Radiatum (CA2 Rad)	0.76 ± 0.02	0.81 ± 0.05	0.83 ± 0.04	0.77 ± 0.01
CA2 Stratum Oriens (CA2 Oriens)	0.77 ± 0.02	0.79 ± 0.05	0.83 ± 0.04	0.79 ± 0.01
CA3 Stratum Moleculare (CA3 Mol)	1.02 ± 0.04	1.01 ± 0.05	1.14 ± 0.06	1.00 ± 0.01
CA3 Stratum Radiatum (CA3 Rad)	0.81 ± 0.02	0.86 ± 0.05	0.99 ± 0.07	0.84 ± 0.02
CA3 Stratum Oriens (CA3 Oriens)	0.77 ± 0.02	0.83 ± 0.06	0.92 ± 0.07	0.81 ± 0.01
Subiculum (Sub)	0.99 ± 0.02	0.94 ± 0.01	1.01 ± 0.03	1.04 ± 0.03
# Septohippocampal Nucleus (Shi)	0.69 ± 0.02	0.73 ± 0.03	0.64 ± 0.03#	0.67 ± 0.02#
Septum				
Laterodorsal Septum (LSD)	0.60 ± 0.02	0.64 ± 0.02	0.60 ± 0.02	0.62 ± 0.02
* Lateral Intermediate Septum (LSi)	0.78 ± 0.03	0.81 ± 0.02*	0.74 ± 0.02	0.83 ± 0.02*
* Medial Septum (MS)	0.98 ± 0.03	1.07 ± 0.04*	1.04 ± 0.02	1.09 ± 0.03*
Diagonal Band of Broca				
# Vertical Band (VDB)	0.90 ± 0.04	1.08 ± 0.06	1.09 ± 0.05#	1.11 ± 0.04#
## Horizontal Band (HDB)	0.86 ± 0.03	1.00 ± 0.05	1.08 ± 0.06##	1.09 ± 0.04##
Mesolimbic				
* Nucleus Accumbens Core (NacC)	1.08 ± 0.02	1.04 ± 0.02*	1.17 ± 0.06	1.05 ± 0.02*
Nucleus Accumbens Shell (NacS)	0.92 ± 0.03	0.99 ± 0.03	1.00 ± 0.05	0.97 ± 0.04
### Ventral Tegmental Area (VTA)	0.92 ± 0.03	0.87 ± 0.04	0.75 ± 0.02###	0.78 ± 0.03###
Neuromodulatory				
# Dorsal Raphe (DR)	1.25 ± 0.07	1.12 ± 0.05	1.09 ± 0.02#	1.07 ± 0.03#
Median Raphe (MR)	1.23 ± 0.02	1.16 ± 0.03	1.22 ± 0.01	1.23 ± 0.02
* # Locus Coerulus (LC)	0.69 ± 0.01	0.73 ± 0.01*	0.73 ± 0.03#	0.79 ± 0.02*#
Mutimodal				
Medial Habenula (MHb)	0.89 ± 0.04	0.86 ± 0.03	0.86 ± 0.04	0.89 ± 0.04
Lateral Habenula (LHb)	1.13 ± 0.07	1.04 ± 0.05	1.24 ± 0.07	1.20 ± 0.08
Medial Geniculate (Med Gen)	1.21 ± 0.05	1.26 ± 0.11	1.47 ± 0.15	1.30 ± 0.02
*** Lateral Geniculate (Lat Gen)	1.49 ± 0.04	1.39 ± 0.05***	1.54 ± 0.04	1.31 ± 0.04***
** ### Inferior Colliculus (Inf Coll)	1.21 ± 0.04	1.41 ± 0.06**	1.44 ± 0.07###	1.67 ± 0.06**###
Mamillary Body (Mam Body)	1.61 ± 0.07	1.66 ± 0.22	1.78 ± 0.08	1.52 ± 0.02
* Interpeduncular Nucleus (IPN)	1.34 ± 0.05	1.47 ± 0.1*	1.27 ± 0.08	1.57 ± 0.06*
Pontine Nucleus (PN)	0.98 ± 0.04	1.05 ± 0.02	1.01 ± 0.02	1.00 ± 0.02
Dorsal Tegmental Nucleus (DTN)	1.30 ± 0.04	1.27 ± 0.05	1.31 ± 0.04	1.27 ± 0.06

Table 3.02 Effects of acute THC treatment and age on overt regional cerebral metabolism in peripubertal (PD35) and adult animals (PD70). Data was analysed using univariate general linear ANOVA models. Data represents mean ± SEM 2DG uptake ratio. *p<0.05, **p<0.01 and ***p<0.001 denotes a significant main effect of acute THC (5mg/kg) treatment on cerebral glucose metabolism. #p<0.05, ##p<0.01 and ###p<0.001 denotes a significant main effect of age on cerebral glucose metabolism.

3.4.2 |Regional Functional Connectivity Analysis Using Partial Least Squares Regression

In order to elucidate age-related and THC-induced alterations in regional functional connectivity the mathematical model PLSR was employed.

3.4.2.1 |Age-related Alterations in Regional Functional Connectivity

Age-related alterations in regional functional connectivity were investigated in 9 'seed' brain regions, namely, CA1 stratum moleculare, radiatum and oriens, dentate gyrus, vertical and horizontal diagonal band of Broca, nucleus accumbens core, locus coeruleus and dorsal raphe. These regions were chosen as 'seed' regions as ANOVA analysis revealed age-related alterations in the overt rate of metabolism within these RoIs.

Hippocampus

In adult animals, the functional coupling of the stratum oriens of the CA1 subfield of the hippocampus was significantly reduced to the stratum moleculare and radiatum of the CA1 subfield compared to peripubertal animals. Similarly, the functional connectivity of the CA1 stratum radiatum to the dentate gyrus was reduced in adult animals. Conversely, there was a significant enhancement in functional connectivity of all three strata of the CA1 to discrete strata of the CA2 and CA3 hippocampal subfields in adult animals. In adult animals, there was a significant enhancement of the functional coupling of the CA1 with the mammillary body but a significant reduction in functional connectivity with the interpeduncular nucleus. There was an opposing effect of age on the functional connectivity of the CA1 moleculare and radiatum on two components of the substantia nigra, with a decline of functional coupling of the CA1 moleculare to two subfields of the substantia nigra (substantia nigra pars compacta and pars reticulata) and an enhancement of the functional coupling of the CA1 radiatum to both the substantia nigra pars reticulata and pars compacta along with two other basal ganglia nuclei, ventral pallidum and dorsolateral striatum, in adult animals relative to peripubertal

animals. Age-related alterations in functional connectivity between all three strata of the CA1 subfield and discrete cortical areas were evident, with both increases and decreases in functional coupling seen on a subfield-dependent basis. There was a decline in functional coupling between the CA1 oriens and the inferior colliculus, medial septum and anterodorsal thalamus in adult animals relative to peripubertal animals. Furthermore, there was an age-related decline in functional coupling between the CA1 radiatum and important neuromodulatory nuclei, the vertical diagonal band of Broca and median raphe, acetylcholine (Ach) and 5-HT nuclei respectively (Fig 3.05A-C).

Within the dentate gyrus of the hippocampal formation, a decline in functional coupling with discrete strata of CA2 and CA3 subfields of the hippocampus and the anatomically-related medial temporal lobe structure, the entorhinal cortex, was present in adult animals relative to peripubertal animals. Furthermore, there was an age-related decrease in functional coupling between the dentate gyrus and the GABA-ergic thalamic nucleus, the reticular thalamus (Fig 3.05D).

Fig 3.05A-B Significant age-related alterations in functional connectivity in the stratum moleculare and stratum radiatum of the CA1 region of the hippocampus respectively

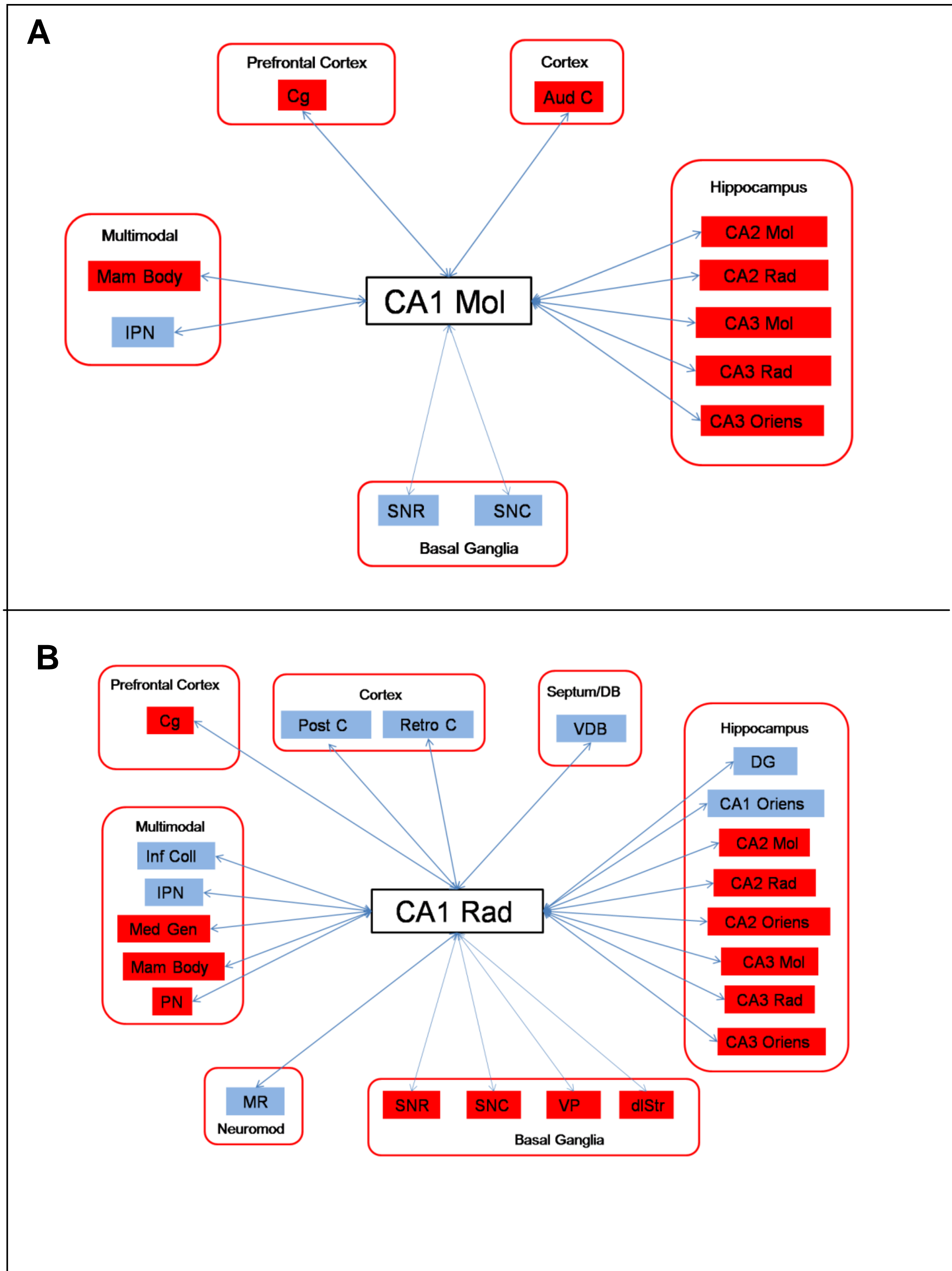


Fig 3.05C-D Significant age-related alterations in functional connectivity in the CA1 stratum oriens and dentate gyrus respectively

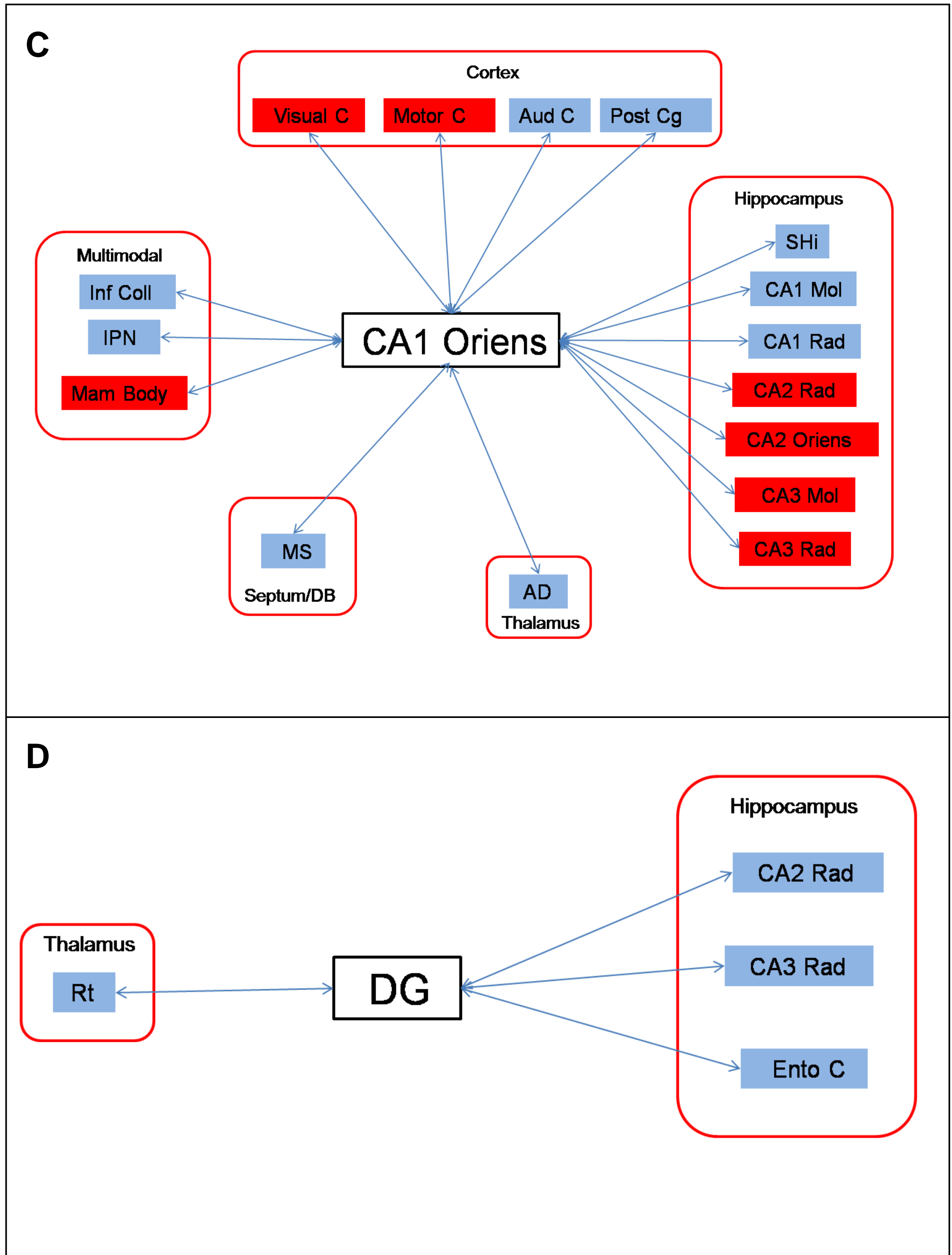


Figure 3.05A-D Summary diagrams of significant age-related alterations in functional connectivity in components of the hippocampal formation. CA1 stratum moleculare (CA1 Mol), CA1 stratum radiatum (CA1 Rad), CA1 stratum oriens (CA1 Oriens) and the dentate gyrus (DG) were defined as 'seed' regions significantly affected by age. Functionally connected regions to the 'seed' region were defined as regions where the 95% CI of the VIP statistic exceeded 0.8 in either experimental group. Age-related alterations in functional connectivity were analysed using Student's *t*-test followed by Bonferroni post-hoc correction for multiple comparisons. Significance was set at $p<0.05$. Red and blue boxes denote a significant increase and decrease respectively in the strength of a given functional connection in adult animals relative to peripubertal animals (for key to brain structures see abbreviation list).

Diagonal Band of Broca

Both the vertical and horizontal limbs of the diagonal band of Broca exhibit an age-related decline in functional coupling with multiple hippocampal subfields including discrete strata of the CA1, CA2 and CA3 subfields accompanied by an enhancement of functional coupling with the subiculum (Fig 3.06A-B). Furthermore, the coupling of both these cholinergic nuclei with the mammillary body and inferior colliculus is decreased but enhanced within the lateral geniculate and dorsal tegmental nuclei in adult animals. In adult animals, there is an increase in functional connectivity of the vertical diagonal band of Broca with the serotonergic nucleus the median raphe. Conversely, there is an age-related decline in functional coupling of the horizontal diagonal band of Broca to the serotonergic nucleus the dorsal raphe. Functional coupling of the vertical diagonal band of Broca was increased to the ventral pallidum whilst a decrease of functional coupling was observed between the horizontal diagonal band and ventral pallidum in adult animals relative to peripubertal ones. There was an age-related decrease in functional coupling of both the vertical and horizontal diagonal band of Broca and the midbrain basal ganglia substrate, the substantia nigra compacta in adult rats relative to peripubertal rats. Age-related alterations in functional coupling of both components of the diagonal band of Broca were present in multiple anterior thalamic nuclei and cortical areas, with both increases and decreases in functional connectivity occurring on a subfield-dependent basis. In adult animals, there was an increase in the functional connectivity of the vertical diagonal band of Broca to two cholinergic septal structures, the laterodorsal and lateral intermediate septum. There was an increase in the functional coupling between the horizontal diagonal band of Broca and the vertical diagonal band of Broca but a decline in functional connectivity to the medial septum in adult animals compared to peripubertal animals. In adult animals, there was a significant increase in the functional connectivity of the horizontal diagonal band of Broca to lateral hypothalamus but coupling was reduced to both the pontine and lateral habenular nuclei (Fig 3.06A-B).

Fig 3.06A-B Significant age-related alterations in functional connectivity in the horizontal and vertical diagonal band of Broca respectively

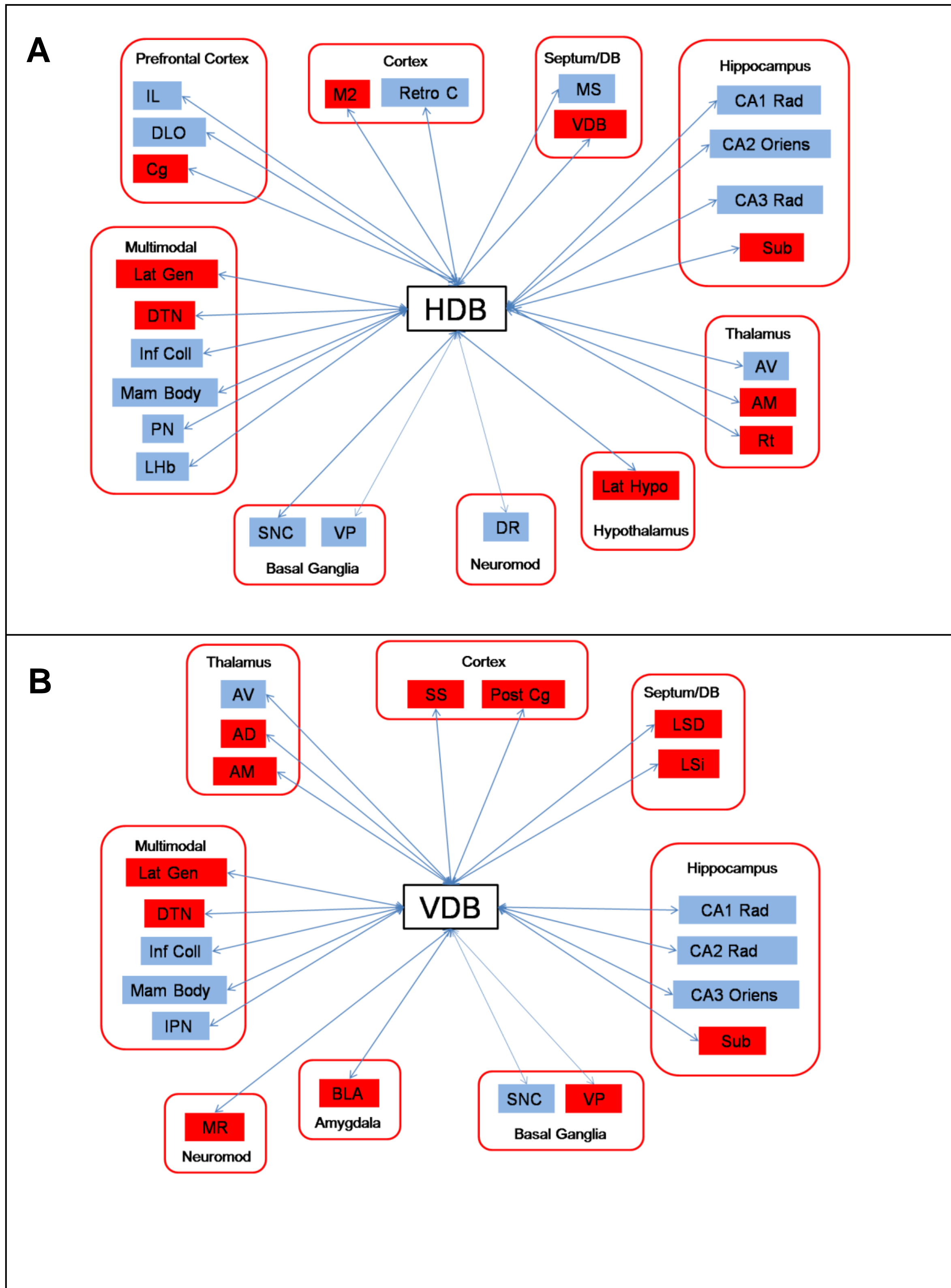


Figure 3.06A-B Summary of significant age-related alterations in functional connectivity in the diagonal band of Broca. Vertical diagonal band of Broca (VDB) and horizontal diagonal band of Broca (HDB) were defined as 'seed' regions significantly affected by age. Functionally connected regions to the 'seed' region were defined as regions where the 95% CI of the VIP statistic exceeded 0.8 in either experimental group. Age-related alterations in functional connectivity were analysed using Student's *t*-test followed by Bonferroni post-hoc correction for multiple comparisons. Significance was set at $p<0.05$. Red and blue boxes denote a significant increase and decrease respectively in the strength of a given functional connection in adult animals relative to peripubertal animals (for key to brain structures see abbreviation list).

Ventral Tegmental Area

There was an age-related decline in functional coupling between the dopaminergic nucleus the ventral tegmental area and two components of the hippocampal formation, namely the subiculum and entorhinal cortex, and two nuclei of the amygdaloid complex, the basolateral and medial amygdaloid nuclei (Fig 3.07A). Furthermore, the functional coupling of the ventral tegmental area to the lateral hypothalamus, secondary motor cortex, substantia nigra pars compacta, anteromedial thalamus and two auditory processing structures, the medial geniculate and inferior colliculus nuclei, was decreased in adult rats compared to peripubertal animals. Conversely, there was an enhancement in the functional connectivity of the ventral tegmental area to two cholinergic nuclei, namely, the lateral intermediate septum and the vertical diagonal band of Broca, and two discrete subfields of the orbital cortex, the ventral and lateral orbital cortices. Moreover, a similar age-related increase in functional coupling was present in the retrosplenial cortex and dorsal tegmental nucleus (Fig 3.07A).

Dorsal Raphe Nucleus

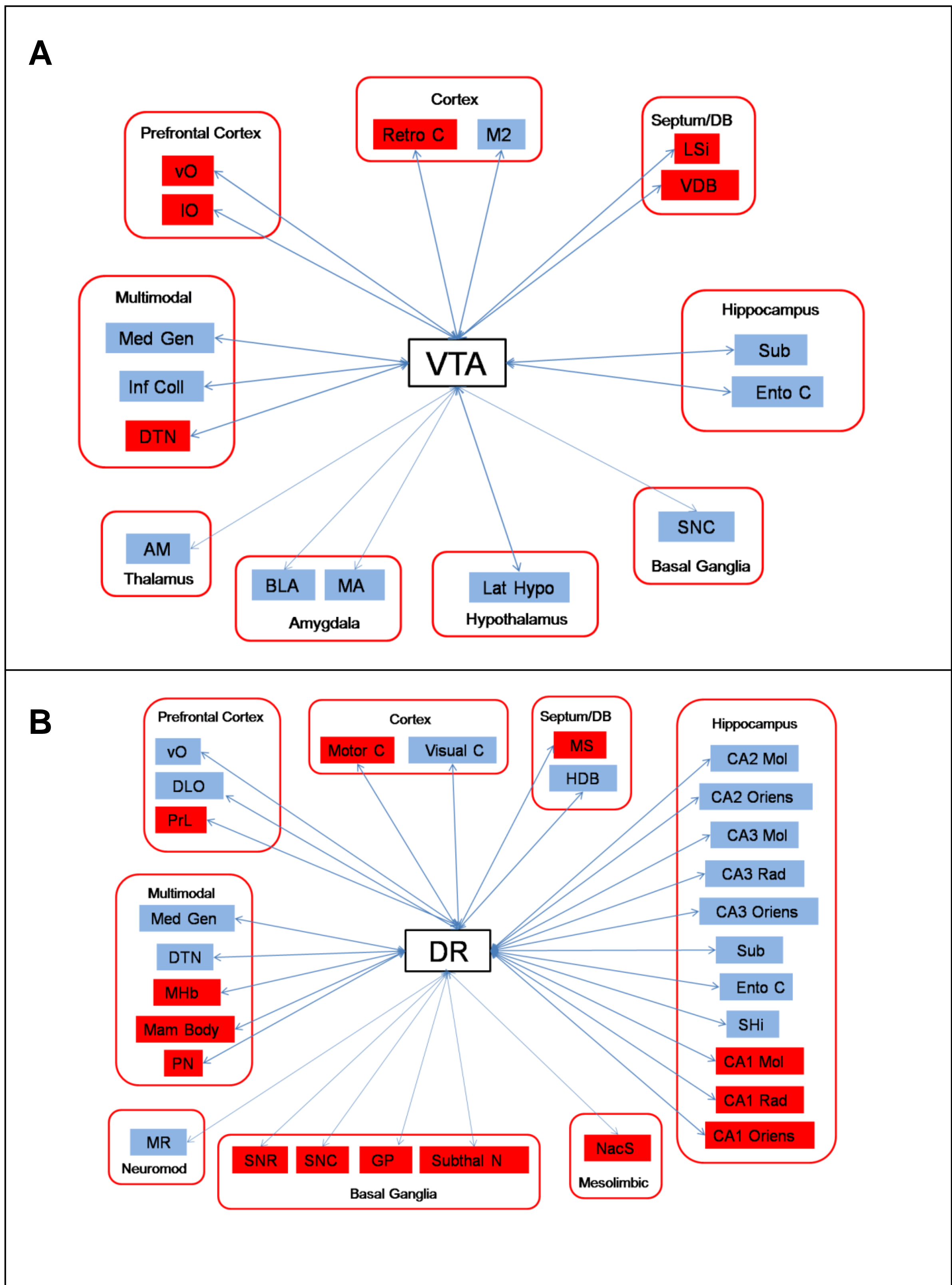
Dorsal raphe nucleus functional connectivity to multiple subfields of the hippocampal formation was altered in adult animals relative to peripubertal animals (Fig 3.07B). There was an age-related decline in functional coupling between the dorsal raphe nucleus to multiple discrete layers of the CA2 and CA3 hippocampal subfields and to the subiculum, entorhinal cortex and septohippocampal nucleus. In contrast, dorsal raphe nucleus coupling with three discrete strata of the CA1 hippocampal subfield was enhanced in adult animals compared to peripubertal animals. A similar age-related increase in functional connectivity of the dorsal raphe nucleus to multiple basal ganglia nuclei, namely, substantia nigra (substantia nigra pars compacta and pars reticulata), globus pallidus and subthalamic nucleus was present in adult animals compared to peripubertal animals. In adult animals, dorsal raphe nucleus functional coupling was also increased to the medial habenula, motor cortex, mammillary body, pontine nucleus

and nucleus accumbens shell but decreased to the visual cortex, medial geniculate and dorsal tegmental nucleus. Age-related alterations in functional coupling of the dorsal raphe nucleus and the PFC was present in adult animals relative to peripubertal animals, with decreased coupling to two orbital subfields, ventral and dorsolateral orbital cotices, but enhanced coupling of the dorsal raphe nucleus to prelimbic cortex. In adult animals, there was a decline in functional connectivity between the dorsal raphe and serotinergic nucleus, the median raphe nucleus. Age-related functional connectivity alterations between the dorsal raphe nucleus and the cholinergic system were evident with increased functional coupling to the medial septum and decreased connectivity to the horizontal diagonal band of Broca apparent in adult animals (Fig 3.07B).

Locus Coeruleus

Functional coupling of the noradrenergic locus coeruleus and multiple components of the hippocampal formation, namely, CA1 moleculare, CA2 oriens, CA3 radiatum and the subiculum was decreased in adult animals relative to peripubertal animals (Fig 3.07C). This decline in functional connectivity was also present in two cortical areas, the anterior cingulate and primary motor cortices, and the vertical diagonal band of Broca. In contrast, the functional coupling of the locus coeruleus was enhanced to the medial amygdala. In the locus coeruleus, a complex functional coupling profile was observed within the various components of the basal ganglia. An age-related enhancement in functional coupling was observed in the pallidum (globus pallidus and ventral pallidum) and ventromedial striatum whilst a decline in functional connectivity was evident in the two components of the substantia nigra complex (substantia nigra pars compacta and pars reticulata) (Fig 3.07C).

Fig 3.07A-B Significant age-related alterations in functional connectivity in the ventral tegmental area and dorsal raphe nucleus respectively



↑ Functional connection

↓ Functional connection

Fig 3.07C Significant age-related alterations in functional connectivity in the locus coeruleus nucleus

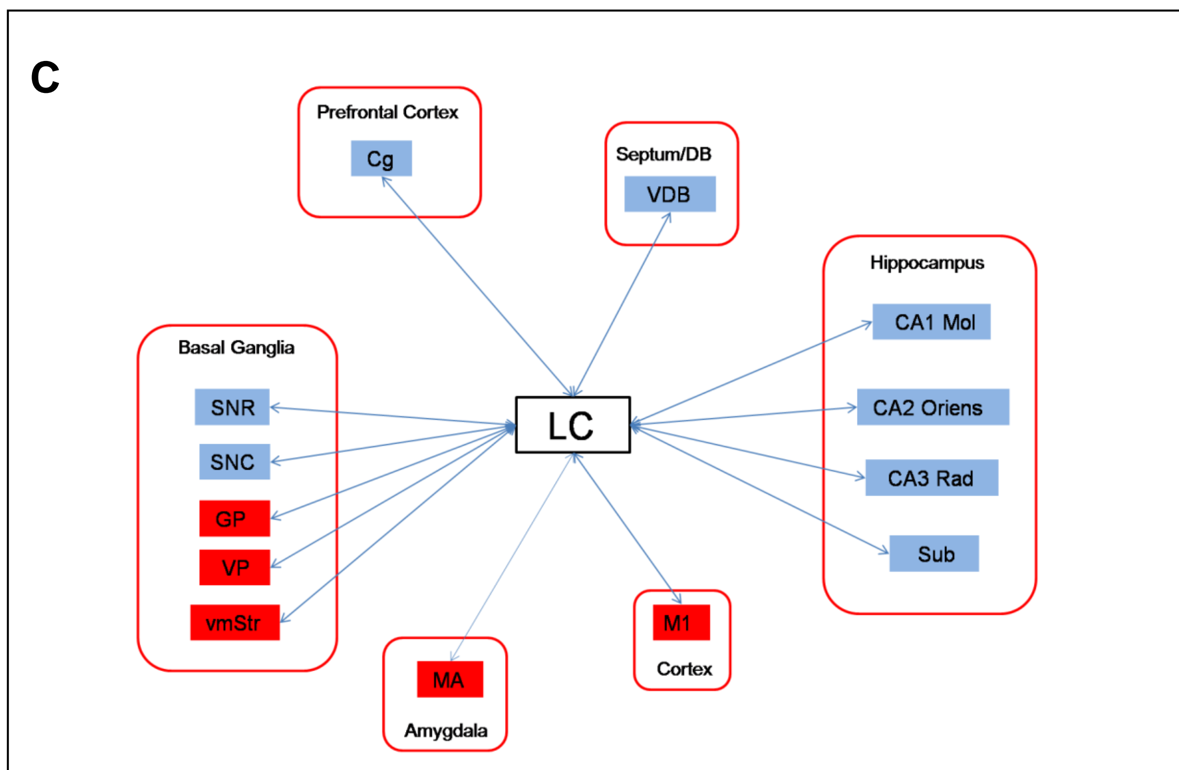


Figure 3.07A-C **Summary of significant age-related alterations in functional connectivity in the ventral tegmental area, dorsal raphe nucleus and locus coeruleus.** Neuromodulatory substrates the ventral tegmental area (VTA), dorsal raphe nucleus (DR) and locus coeruleus (LC) were defined as 'seed' regions significantly affected by age. Functionally connected regions to the 'seed' region were defined as regions where the 95% CI of the VIP statistic exceeded 0.8 in either experimental group. Age-related alterations in functional connectivity were analysed using Student's *t*-test followed by Bonferroni post-hoc correction for multiple comparisons. Significance was set at $p<0.05$. Red and blue boxes denote a significant increase and decrease respectively in the strength of a given functional connection in adult animals relative to peripubertal animals (for key to brain structures see abbreviation list).

3.4.2.2 |Effects of Acute THC Administration on Regional Functional Connectivity

The effects of acute THC treatment on functional connectivity were analysed in 8 'seed' regions; these were the anteromedial, anteroventral, reticular and mediodorsal thalamic nuclei, basolateral and medial amygdala, nucleus accumbens core and locus coeruleus. These regions were chosen as 'seed' regions because within these RoIs, acute THC administration significantly altered overt glucose metabolism

Thalamus

Acute THC treatment significantly attenuated functional connectivity of the reticular thalamic nucleus to three subfields of the anterior thalamic nucleus, namely the anterodorsal, anteroventral and anteromedial thalamus (Fig 3.08A). Similarly, acute THC administration enhanced functional coupling of the reticular thalamus to the serotonergic substrate the median raphe. In contrast, the coupling of the reticular thalamus to two cholinergic septal structures, the medial and laterodorsal septal nuclei, was significantly decreased following acute systemic administration of THC (5mg/kg). THC administration led to a reduction in functional coupling of the reticular thalamus to the lateral geniculate nucleus, inferior colliculus and the mammillary body and enhanced functional coupling with the lateral habenula. Acute THC treatment significantly altered the functional coupling of the reticular thalamus to multiple cortical substrates on a subfield-dependent basis. Furthermore, THC induced a complex pattern of altered functional connectivity of the reticular thalamus to components of the hippocampus, amygdala and basal ganglia, with both increased and decreased functional coupling evident on a region-dependent basis (Fig 3.08A).

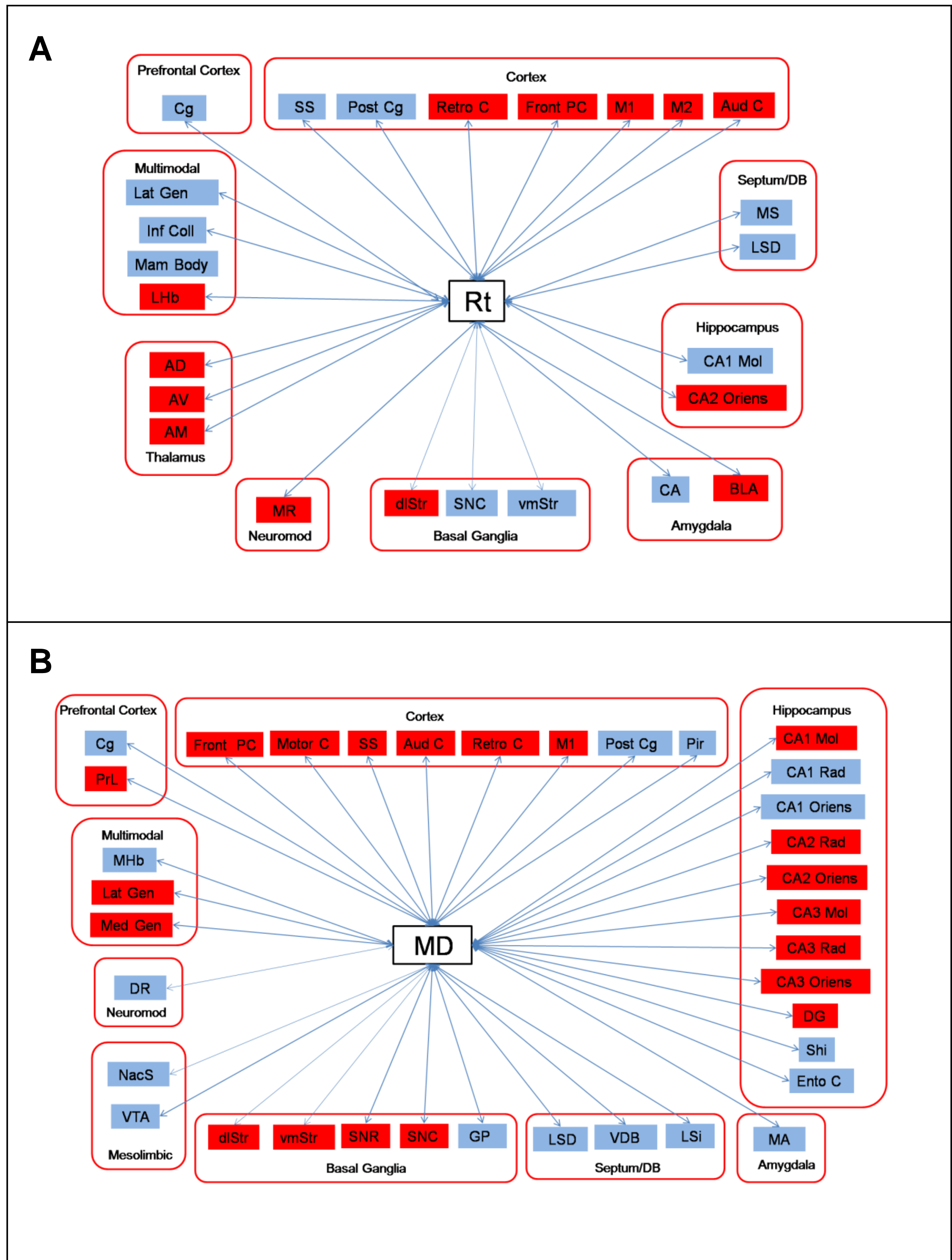
Acute systemic THC administration altered the functional connectivity of the mediodorsal thalamus to components of the DA, 5-HT and ACh neurotransmitter systems (Fig 3.08B). THC administration led to a reduction in functional coupling of

the mediodorsal thalamus from mesolimbic dopaminergic neural substrates, including the nucleus accumbens shell and ventral tegmental area. In addition THC treatment significantly reduced functional connectivity of the mediodorsal thalamus to cholinergic nuclei, the vertical diagonal band of Broca, laterodorsal and lateral intermediate septum, and the 5-HT-releasing dorsal raphe nucleus. Acute THC enhanced functional connectivity between the mediodorsal thalamus and multiple nuclei of the basal ganglia including two striatal (ventromedial and dorsolateral striatum) and two subfields of the substantia nigra (pars compacta and pars reticulata) whilst decreasing functional coupling with the globus pallidus. A similar THC-induced decrease in functional connectivity was present in the medial habenula, medial amygdala and dorsal raphe whilst THC increased functional coupling of the mediodorsal thalamic nucleus to the lateral and medial geniculate nuclei. Functional connectivity of the mediodorsal thalamus to multiple cortical and hippocampal subfields was altered in a complex way, with both increases and decreases seen on a subfield-dependent basis (Fig 3.08B).

Both the anteromedial and anteroventral thalamic nuclei exhibited a significant THC-induced enhancement of functional connectivity with each other and to other thalamic nuclei, specifically, the reticular and anterodorsal thalamus (Fig 3.08C-D). THC treatment altered the functional coupling of the anteromedial and anteroventral thalamic nuclei with multiple subfields of hippocampus but in an opposing manner. THC decreased functional coupling of the anteromedial thalamus with the CA1, CA2, CA3 subfields and the entorhinal cortex whilst coupling of anteroventral thalamus to discrete layers of the CA1 and CA2 was functionally enhanced as a result of THC administration. Both the anteromedial and anteroventral exhibited THC-induced altered functional connectivity with several basal ganglia nuclei. Both thalamic nuclei exhibited a decline in functional coupling with the pallidum (globus pallidus and ventral pallidum) as a result of acute THC administration. Furthermore, within the anteromedial thalamus, THC treatment resulted in decreased functional coupling to substantia nigra (substantia nigra pars reticulata and compacta) and increased connectivity with the dorsolateral striatum. Conversely, acute systemic THC administration decreased functional

connectivity between the anteroventral thalamic nucleus and the dorsolateral and ventromedial striatum. Both thalamic nuclei showed a decrease in functional connectivity with the mesolimbic reward-related neural substrate, the nucleus accumbens core and became functionally enhanced with habenular nuclei (lateral and medial habenula) as a result of acute THC treatment. THC altered functional connectivity of the anteromedial and anteroventral thalamic nuclei with the multiple subfields of the cortex. THC treatment also altered the functional coupling of the anteroventral thalamus to the PFC, with decreased functional coupling seen in the cingulate, medial and lateral orbital cortices and a functional enhancement evident in the prelimbic cortex. Furthermore, a THC-induced enhancement in functional connectivity with the mammillary body, medial geniculate and basolateral amygdala was present in the anteroventral thalamic nucleus. THC increased functional connectivity of the anteromedial thalamus to the medial raphe and dorsal tegmental nucleus whilst decreasing functional coupling with the lateral hypothalamus, inferior colliculus, medial amygdala and interpeduncular and pontine nuclei (Fig 3.08C-D).

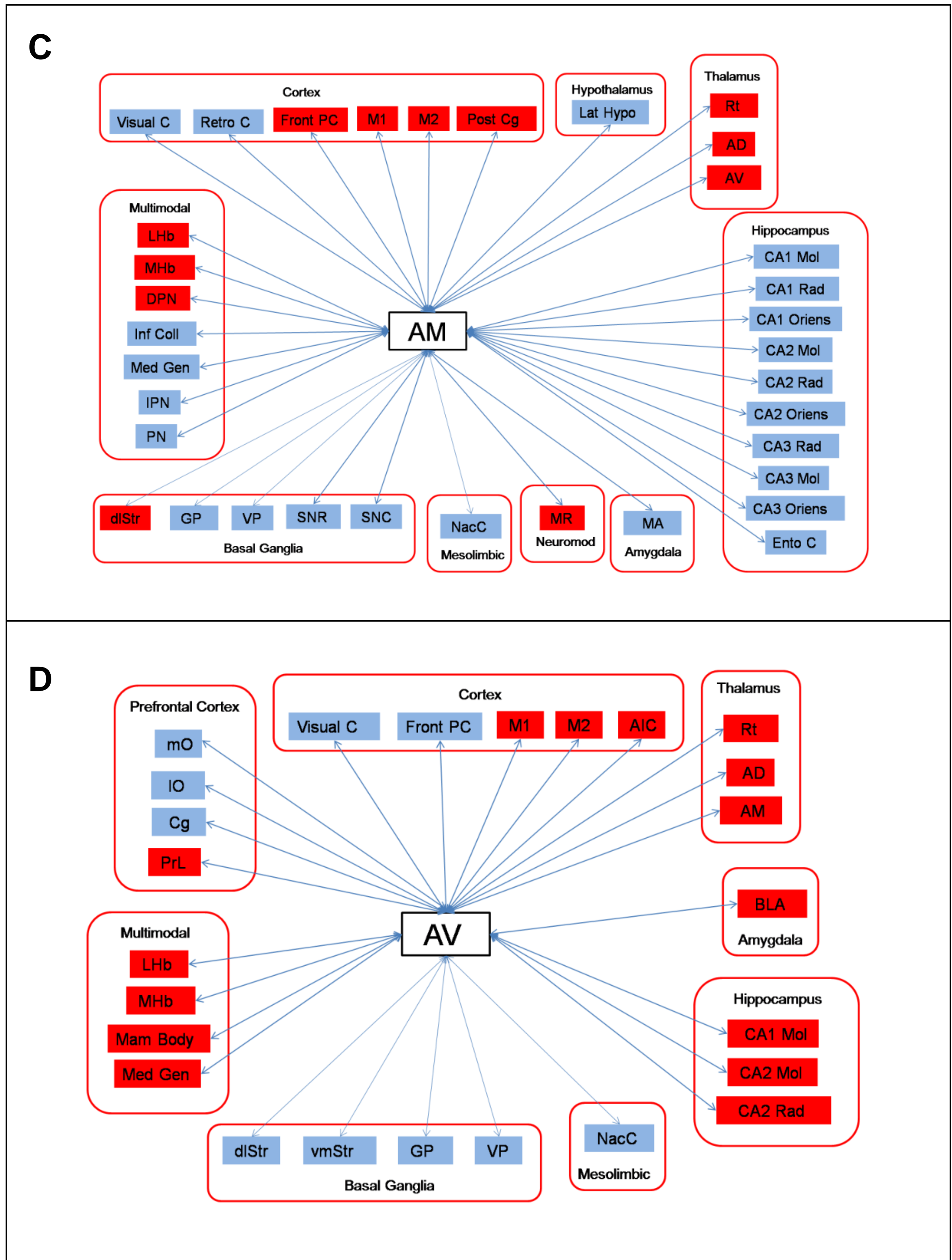
Fig 3.08A-B Significant THC-induced alterations in functional connectivity in the reticular and mediodorsal thalamic nuclei respectively



↑ Functional connection (blue)

↓ Functional connection (red)

Fig 3.08C-D Significant THC-induced alterations in functional connectivity in the anteromedial and anteroventral thalamic nuclei respectively



↑ Functional connection

↓ Functional connection

Figure 3.08A-D Summary of significant THC-induced alterations in functional connectivity in the reticular, mediodorsal anteromedial and anteroventral thalamic nuclei. Reticular (Rt), mediodorsal (MD), anteromedial (AM) and anteroventral (AV) thalamic nuclei were defined as 'seed' regions significantly affected by THC. Functionally connected regions to the 'seed' region were defined as regions where the 95% CI of the VIP statistic exceeded 0.8 in either experimental group. THC-induced alterations in functional connectivity were analysed using Student's *t*-test followed by Bonferroni post-hoc correction for multiple comparisons. Significance was set at $p<0.05$. Red and blue boxes denote a significant increase and decrease respectively in the strength of a given functional connection in THC-treated animals relative to controls (for key to brain structures see abbreviation list).

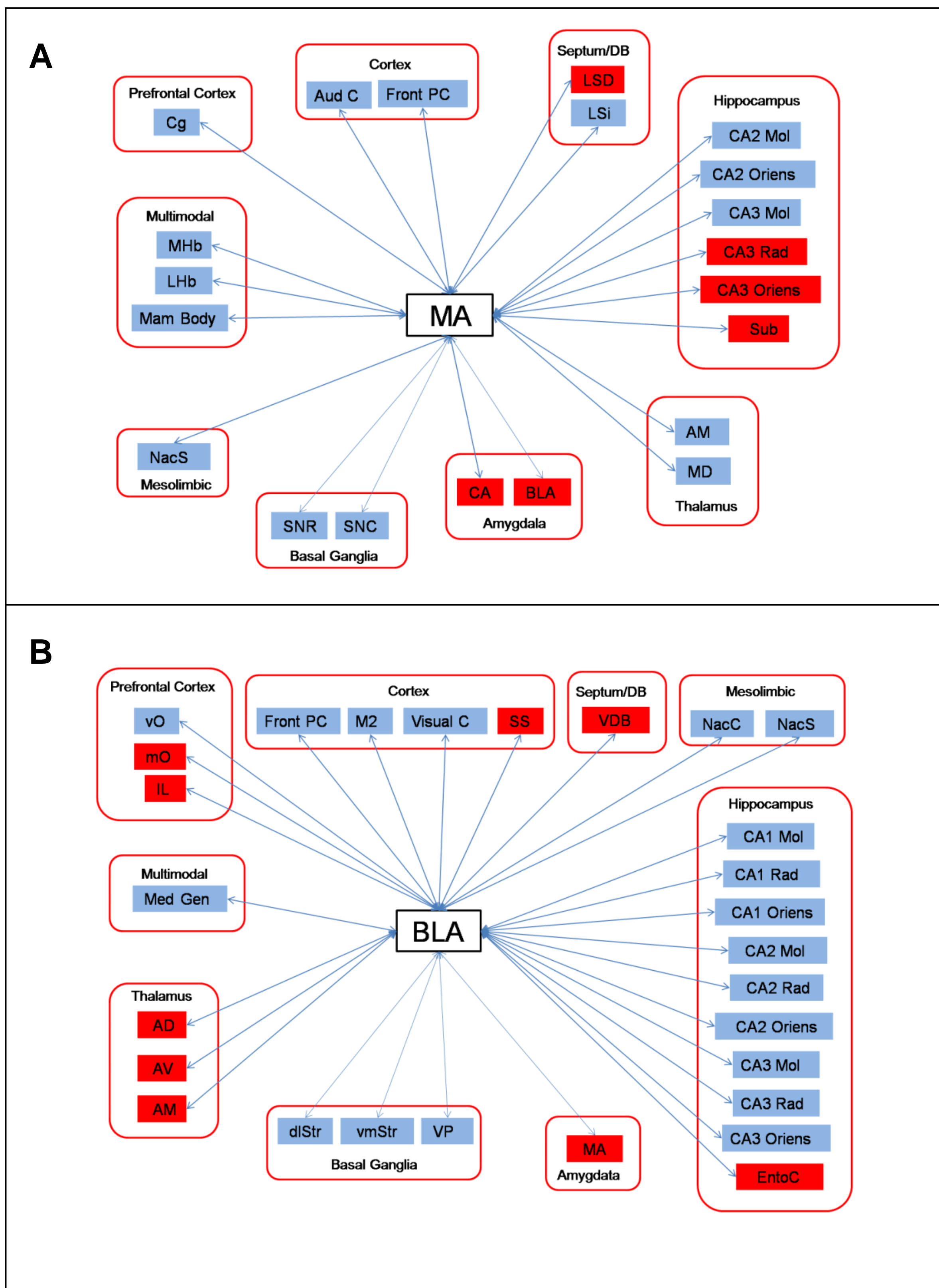
Amygdala

Acute THC treatment reduced the functional coupling of both the basolateral and medial amygdala with the nucleus accumbens shell (Fig 3.09A-B). Furthermore, THC significantly decreased the functional connectivity of the basolateral amygdala with the nucleus accumbens core. The functional connectivity of both the basolateral and medial amygdala with basal ganglia nuclei was significantly decreased by THC. THC administration significantly decreased functional coupling of the medial amygdala with both the substantia nigra pars compacta and pars reticulata and decreased functional coupling of the basolateral amygdala with two striatal subfields (dorsolateral and ventromedial striatum) and the ventral pallidum. Both the medial and basolateral amygdala showed a reciprocal enhancement of functional coupling as a result of THC administration. Furthermore, THC treatment significantly enhanced the functional connectivity of the basolateral amygdala with another amygdaloid subdivision, the central amygdala. THC decreased the functional coupling of the medial amygdala with the cingulate cortex whilst THC altered the functional connectivity of the basolateral amygdala with components of the PFC, with both increases and decreases seen on a subfield-dependent basis.

Acute THC administration also had a contrasting effect on some aspects of connectivity to the two amygdala subdivisions, with THC enhancing the functional connectivity of the basolateral amygdala to three anterior thalamic nuclei (anterodorsal, anteroventral and anteromedial thalamic nuclei) whilst THC treatment resulted in functional dissociation between the medial amygdala and the anteromedial and medial thalamic nuclei. THC increased functional coupling of the medial amygdala to two subdivisions of the lateral septum (laterodorsal and lateral intermediate septum) whilst significantly decreasing functional coupling with the auditory and frontal parietal cortices, two habenular nuclei (lateral and medial habenula) and mammillary body. THC treatment altered functional coupling of the basolateral amygdala with multiple cortical layers and decreased functional connectivity with the medial geniculate nucleus. THC treatment

altered functional coupling of both the basolateral and medial amygdala with multiple hippocampal subfields in a complex manner, with both increases and decreases seen on a subfield-dependent manner (Fig 3.09A-B).

Fig 3.09A-B Significant THC-induced alterations in functional connectivity in the medial and basolateral nuclei of the amygdala respectively



↑ Functional connection

↓ Functional connection

Figure 3.09A-B Summary of significant THC-induced alterations in functional connectivity in the basolateral and medial amygdala nuclei. Basolateral (BLA) and medial (MA) amygdala were defined as 'seed' regions significantly affected by THC. Functionally connected regions to the 'seed region' were defined as regions where the 95% CI of the VIP statistic exceeded 0.8 in either experimental group. THC-induced alterations in functional connectivity were analysed using Student's *t*-test followed by Bonferroni post-hoc correction for multiple comparisons. Significance was set at $p<0.05$. Red and blue boxes denote a significant increase and decrease respectively in the strength of a given functional connection in THC-treated animals relative to controls (for key to brain structures see abbreviation list).

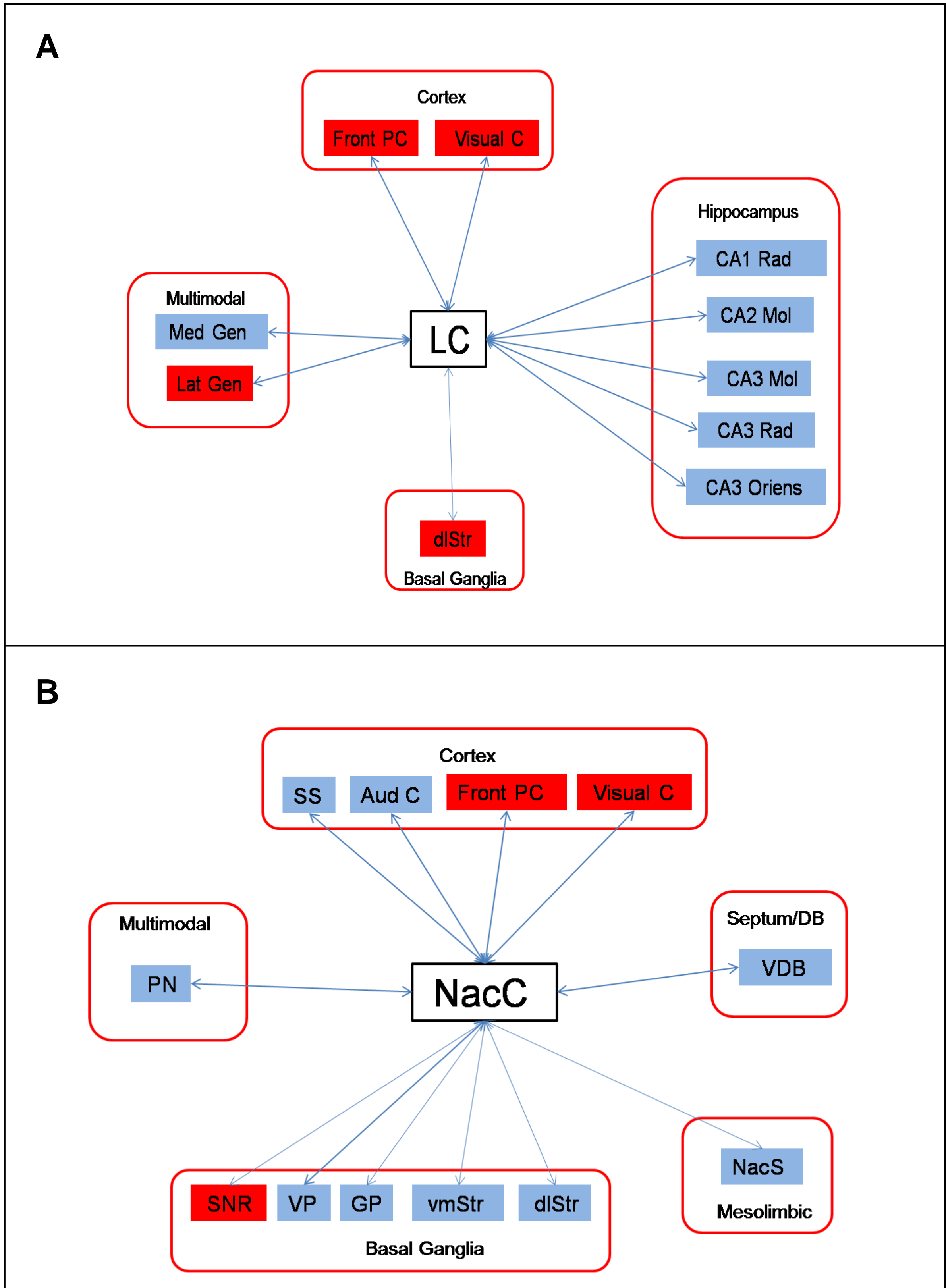
Locus Coeruleus

Acute systemic THC administration led to a functional dissociation between the locus coeruleus, the principal NA-producing nucleus located in the pons, and several discrete strata of the CA1, CA2 and CA3 subdivisions of the hippocampal formation (Fig 3.10A). In contrast, THC treatment increased functional coupling of the locus coeruleus to two cortical areas (frontal parietal and visual cortices) and the dorsolateral striatum. THC treatment had opposing effects on the functional coupling of the locus coeruleus to geniculate nuclei; THC decreased the functional coupling of the locus coeruleus to the auditory relay substrate the medial geniculate nucleus whilst THC increased functional connectivity with the visual relaying centre, the lateral geniculate nuclei (Fig 3.10A).

Nucleus Accumbens Core

Acute systemic THC treatment significantly decreased functional connectivity between the nucleus accumbens core and shell (Fig 3.10B). Acute THC treatment decreased functional coupling of the nucleus accumbens core to multiple subfields of the basal ganglia including two striatal subfields (ventromedial and dorsolateral striatum) and the pallidum (globus pallidus and ventral pallidum). By contrast, functional coupling to the substantia nigra reticulata was significantly increased following acute THC administration. An acute THC-induced decrease in functional coupling was present in the pontine nucleus and the vertical diagonal band of Broca. Functional connectivity of the mediodorsal thalamus to cortical layers was altered on a subfield-dependent basis (Fig 3.10B).

Fig 3.10A-B Significant THC-induced alterations in functional connectivity in the locus coeruleus and nucleus accumbens core respectively



↑ Functional connection

↓ Functional connection

Figure 3.10A-B Summary of significant THC-induced alterations in functional connectivity in the nucleus accumbens core and locus coeruleus. Neuromodulatory structures the nucleus accumbens core (NacC) and locus coeruleus (LC) were defined as 'seed' regions significantly affected by THC. Functionally connected regions to the 'seed region' were defined as regions where the 95% CI of the VIP statistic exceeded 0.8 in either experimental group. THC-induced alterations in functional connectivity were analysed using Student's *t*-test followed by Bonferroni post-hoc correction for multiple comparisons. Significance was set at $p<0.05$. Red and blue boxes denote a significant increase and decrease respectively in the strength of a given functional connection in THC-treated animals relative to controls (for key to brain structures see abbreviation list).

3.5 |Discussion

3.5.1 |Age-related Differential Patterns of Cerebral Metabolism and Functional Connectivity

The results of this study indicate the presence of age-related differences in glucose metabolism and regional functional connectivity in neural systems associated with cognitive and reward processing and neuromodulation in adult animals (PD70) relative to peripubertal animals (PD35). Throughout the adolescent period the brain undergoes a dynamic phenotypic transition from affective-driven behaviour to more regulated cognitive-driven behaviour mediated through important plastic and structural remodelling that allows for refinement and integration of functionally associated neural substrates and neural architecture (Gogtay *et al*., 2004; Nelson *et al*., 2005). The heteromodal nature of this cognitive development means that ontogenetic changes are on-going at differential rates in discrete neural substrates. Thus, the nature of the experimental design of this study lends itself to act as a comparative snapshot of differential cerebral activity at PD35 and PD70 respectively.

Hippocampus

The findings of this study demonstrate that multiple hippocampal subfields undergo an age-dependent difference in cerebral metabolism with LCGU levels significantly higher in adult animals (PD70) relative to peripubertal animals (PD35). These regions included the multiple layers of the CA1 (CA1 stratum moleculare, CA1 stratum radiatum, CA1 stratum oriens) and the dentate gyrus. Hypometabolism in these neural structures at the peripubertal time-point of PD35 may reflect the functional immaturity of these regions. These findings are in keeping with clinical imaging studies that support the late maturative trajectory of the hippocampus (Giedd *et al*., 1999; Casey *et al*., 2005). Moreover, functional connectivity analysis of these regions revealed a decrease in functional coupling among discrete strata of the CA1 subfield but enhancement in

functional connectivity of each stratum of the CA1 subfield with multiple layers of CA2-CA3 subfields consistent with the developmental integration between hippocampal subfields during the PD35-PD70 developmental period. Conversely, the dentate gyrus becomes functionally disconnected with other hippocampal regions in adulthood. This functional connectivity data may indicate the presence of age-dependent alterations in the functional organisation between the multiple components of the hippocampal formation. In agreement with this, Uhlhaas and group found that during the adolescent period, there is an interval of destabilisation in neural synchrony followed by a re-organisation of synchronisation patterns within the hippocampus (Uhlhaas *et al*., 2009). Major developmental changes in the hippocampus during the peripubertal-adult period are also supported by observations of altered cholinergic (Fig 3.06A-B), noradrenergic (Fig 3.07C) and serotinergic (Fig 3.07B) functional connectivity during this period, supporting a re-organisation of hippocampus functioning throughout this transitional period. Thus, the findings of this study provide new evidence for age-related functional re-organisation and late maturation of the hippocampus.

Basal Ganglia

Another interesting finding of this study is the increase in metabolic activity in multiple basal ganglia nuclei in adulthood. The globus pallidus, ventromedial striatum, and subthalamic nucleus exhibited a developmental change in glucose metabolism between the peripubertal period (PD35) and adulthood (PD70). This finding may seem somewhat unexpected as the timescale of maturational trajectories is generally governed by the degree of complexity of functioning associated with the neural substrate i.e. as the basal ganglia plays a pivotal role in motor function, maturation of these brain regions would be expected to take place early in development. However, these findings are in keeping with a human magnetic resonance imaging study carried out by Sowell and colleagues, where they found a post-adolescent decrease in grey matter in the putamen and globus pallidus indicative of late maturation of these regions (Sowell *et al*., 1999). A possible explanation for this is that specific basal ganglia nuclei may follow a similar

maturational trajectory to higher cognitive brain areas due to the supportive involvement of basal ganglia nuclei in the cognitive-driven behaviour. This explanation is supported by a study carried out by Menon and colleagues where they demonstrated simultaneous increase in neural activity of specific basal ganglia nuclei, namely the globus pallidus and putamen, alongside PFC activation following the introduction of a working memory component to a movement task suggestive of an increase in motor sequencing demands during memory-guided movement (Menon *et al*., 2000).

<u>Ventral Tegmental Area and Brain Reward Circuitry</u>

In this study, in the ventral tegmental nucleus, there is an age-related decline in activity in this region with a decrease in LCGU levels in adult rats relative to peripubertal rats. The ventral tegmental area is a key node in the cerebral reward pathway and primary source of dopaminergic innervations to the ventral striatum (Swanson, 1982). As mentioned earlier in this section, there are important shifts in behavioural patterns throughout the adolescent period, whereby there is a decline in affect-driven behaviour and an increase in more controlled cognitive-driven phenotypy. These phenotypic observations correlate with the developmental pattern of increased in neural activation in PFC and hippocampus accompanied by a decline in neural activation within the ventral striatum, the reward centre of the brain (Sowell *et al*., 1999; Gogtay *et al*., 2004; Lenroot and Giedd, 2006). Thus, the decrease in cerebral glucose metabolism in the ventral tegmental area in adult animals is in agreement with the aforementioned previous findings of age-related reductions in neural activation in this reward-processing pathway. Furthermore, functional connectivity analysis of the ventral tegmental area revealed developmental alterations in functional communication with the subiculum, orbital cortex and amygdala, key neural correlates in the acquisition of stimulus-reward associations (Everitt *et al*., 1991; O'Doherty, 2004; Martin-Fardon *et al*., 2008) Collectively, cerebral metabolism and functional connectivity data relating to the ventral tegmental area shown in this study of decreased neural activation in the ventral tegmental area alongside age-related alterations in functional coupling with the

amygdala, orbital cortex and subiculum provide further evidence for the neurodevelopmental maturation of the DA system (Teicher *et al*., 1995; Moll *et al*., 2000; Tarazi and Baldessarini, 2000; Weickert *et al*., 2007; Rothmond *et al*., 2012).

Neuromodulatory Nuclei

Data available on the ontogenetic mapping of neural systems is sparse, with most research exploring this phenomenon focusing on the development of dopaminergic brain systems (Teicher *et al*., 1995; Moll *et al*., 2000; Tarazi and Baldessarini, 2000; Weickert *et al*., 2007; Rothmond *et al*., 2012). However, this study has demonstrated age-dependent alterations in glucose metabolism in important neuromodulatory nuclei of 5-HT, NA and ACh neurotransmitter systems, namely the dorsal raphe nucleus, locus coeruleus and diagonal band of Broca respectively. Within the dorsal raphe nucleus, the primary source of cerebral serotonergic input, glucose metabolism is decreased in adult animals relative to peripubertal animals. Whilst very little research has been carried out on the ontogeny of the 5-HT system, the results in this study of decreased activity within the dorsal raphe nucleus of adult rats is in keeping with ontogenetic behavioural data published by Darmani and colleagues (1996). They examined the ontogeny of psychopharmacological sensitivity to the 5-HT$_{2A}$ agonist, (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) in mice and observed a developmental peak in DOI-induced head twitches and ear scratching at PD28 (range, PD22-PD35), followed by a dramatic decline in both behaviours until no significant behavioural response to DOI was obtained in adulthood (Darmani *et al*., 1996). The findings of this study, alongside the behavioural data of Darmini and group suggest a developmental down-regulation of the cerebral 5-HT activity akin to those evident in the DA system.

Conversely, both the locus coeruleus and the diagonal band of Broca exhibited an age-related increase in cerebral glucose metabolism in adult animals relative to peripubertal ones. The locus coeruleus and diagonal band of Broca project noradrenergic and cholinergic nerve bundles to the hippocampus and both NA and Ach play important

modulatory roles in hippocampal-dependent cognition (Sara, 2009; Huh *et al*., 2010). Thus, the potential increase in glucose metabolism in neuromodulatory regions may be governed by the late developmental maturation of the hippocampus. Interestingly, a congruent pattern in functional connectivity among all of these neuromodulatory nuclei is that of age-related alterations in functional connectivity profiles between these neuromodulatory nuclei and multiple subfields of the hippocampus. These findings provide further evidence for the late maturational trajectory and functional synchronisation of the hippocampus.

The data from this study provide new evidence that the peripubertal period is a critical neurodevelopmental epoch with age-related differences in neural activation and functional communication present in neural substrates involved with cognitive and reward processing and neuromodulation.

3.5.2 |THC-induced Alterations in Cerebral Metabolism and Functional Connectivity

This study demonstrated that acute THC administration (5mg/kg) produced overt alterations in cerebral metabolism and brain systems connectivity in neural substrates associated with learning and memory, sensory, auditory, visual and emotional processing and neuromodulation. Furthermore, this study presents the novel finding of a THC-induced developmental sensitivity on cerebral glucose metabolism in select neural correlates affected by THC administration.

Thalamus and Cognition-relevant Circuitry

Acute THC administration induced a significant reduction in glucose metabolism within four thalamic structures, namely, the mediodorsal, reticular and anteromedial and anteroventral thalamic nuclei. Furthermore, acute THC administration increased functional coupling of all four affected thalamic nuclei with each other, suggesting a prominent role for CBs in the regulation of thalamic inter-communication. Another functional connectivity pattern seen across all four thalamic nuclei was altered functional coupling with neural substrates subserving multiple cognitive domains, including the PFC, hippocampus and nucleus accumbens (Floresco *et al*., 1999; Floresco *et al*., 2006; Floresco *et al*., 2009; Burgess *et al*., 2002; Vertes, 2005; Kouneiher *et al*., 2009).

There is strong evidence for a role of thalamic nuclei in cognitive processes. With regard to the mediodorsal thalamus and cognition, transient inactivation of the mediodorsal thalamus by bilateral lidocaine injections selectively impairs performance on the delayed spatial win-shift radial-arm maze foraging task, a behavioural assay used to measure spatial working memory (Floresco *et al*., 1999). In addition it has also been shown that both transient disconnection of PFC from the mediodorsal thalamus, and disconnection of the PFC from the nucleus accumbens impaired performance in this task

(Floresco *et al*., 1999). Furthermore, Floresco and colleagues (2003) using extracellular single-unit recordings have demonstrated a modulatory role of the mediodorsal thalamus in hippocampal-PFC information processing finding that activation of mediodorsal thalamus afferents exerted a pronounced inhibitory effect on hippocampal-evoked firing in PFC neurons (Floresco and Grace, 2003). Thus, the mediodorsal thalamus is an integral component of an interconnected neural circuit alongside the PFC, hippocampus and nucleus accumbens which collectively subserve certain aspects of spatial working memory processing. In this study acute THC administration significantly reduced glucose metabolism in the mediodorsal thalamus and led to aberrant functional connectivity profiles of the mediodorsal thalamus with components of the hippocampus, PFC and nucleus accumbens. Indeed, previous behavioural studies have confirmed the ability of acute THC to impair performance in spatial working memory tasks, the findings of this study suggest that THC-induced disruption of serial information transmission along this interconnected neural system may underlie perturbed performance on such working memory tasks (Molina-Holgado *et al*., 1995; Lichtman and Martin, 1996; Varvel *et al*., 2001).

Lesioning of the complete anterior thalamus (that is, all of the anteroventral, anterodorsal and anteromedial thalamic nuclei due to the close anatomical proximity of the structures) results in deficits in hippocampal-dependent allocentric (place-related) spatial memory in behavioural assays such as the rewarded forced alternation task in the T-maze task, 8-arm radial and water maze tasks (Aggleton *et al*., 1996; van Groen *et al*., 2002; Mitchell and Dalrymple-Alford, 2006). In the present study, acute THC administration led to a hypometabolic state within both the anteromedial and anteroventral thalamus. Furthermore, this study demonstrated for the first time aberrant functional coupling of both the anteromedial and anteroventral thalamus with multiple components of the hippocampus. The anterior thalamus is a key nodal point for an extended hippocampal system and both the anteromedial and anteroventral thalamic nuclei have important roles in learning (Aggleton *et al* 1996; Aggleton and Pearce, 2001; van Groen *et al*., 2002; Mitchell and Dalrymple-Alford, 2006). In agreement with

the novel findings of this study, acute THC administration has previously been shown to produce allocentric spatial memory deficits associated with anterior thalamus and hippocampal system damage, suggesting that acute THC administration may induce aberrant functioning of these key neural systems underlying allocentric learning (Lichtman *et al*., 1995; Aggleton *et al*., 1996; Aggleton and Pearce, 2001; Varvel *et al*., 2001).

The reticular thalamic nucleus is a thin lamina of GABA-ergic neurons that encapsulates the thalamus. Whilst this thalamic nucleus receives collateral inputs from both thalamocortical and corticothalamic fibres it projects exclusively to other thalamic reticular nuclei, gating the flow of information to the thalamus and thus modulating thalamic activity (McAlonan and Brown, 2002). Activation of CB_1 receptors located on GABA-ergic cells of the reticular thalamus have been shown to dynamically regulate thalamic synchrony (Sun *et al*., 2011). In this present study systemic acute THC administration led to a reduction in cerebral metabolism in the reticular thalamic nucleus and an enhancement of functional coupling of this thalamic nucleus to the anterior thalamus. Thus, direct activation of CB_1 receptors in the reticular thalamus might contribute to the enhanced reticular-anterior thalamic coupling evident after acute THC administration in this study. Moreover, this study demonstrated that acute THC treatment produced altered functional coupling of the reticular thalamus to multiple cortical, hippocampal, basal ganglia and septal subfields. Thus, although the neuronal efferents of the reticular thalamus are destined solely for the thalamus, through perturbed thalamic synchrony, acute THC administration may result in indirect but nevertheless widespread deviance of functional coupling of the reticular thalamus to important cognitive neural substrates.

Interestingly, in this study it was shown that acute THC administration led to altered functional connectivity of the mediodorsal thalamus to multiple components of the basal ganglia complex. This subcortical group of structures, which have a high CB_1 receptor binding profile, play a key role in regulation of motor behaviour (Bhatia and Marsden,

1994). The basal ganglia nuclei and the mediodorsal thalamus are functionally linked as the mediodorsal thalamus plays an integral role in the mediation of subcortical information going to the cortex in order to regulate motor behaviour (McFarland and Haber, 2002) However, in this study, despite the dense CB_1 receptor expression among basal ganglia nuclei, acute THC administration led to an alteration in glucose metabolism in only one of the basal ganglia structures measured, the subthalamic nucleus. These findings are in agreement with a previous study carried out by our group that showed minimal overt effects of acute THC on cerebral metabolism in basal ganglia nuclei (Brett *et al*., 2001). Similar to the mediodorsal thalamic nucleus, acute THC administration reduced glucose metabolism within the subthalamic nucleus. Thus, this study demonstrates that although acute THC administration does not appear to induce large scale overt changes glucose metabolism in basal ganglia nuclei, functional connectivity analysis using the partial least squares regression analysis enabled the elucidation of covert THC effects within the connectivity of the basal ganglia network. Such deviations in basal ganglia functionality resulting from acute THC treatment are in accordance with both preclinical and clinical phenotypic effects of acute THC, including reduced locomotor activity and impairments in perceptual motor control and impulsivity respectively (Navarro *et al*., 1993; Ramaekers *et al*., 2006).

Neural Substrates of Sensory Processing

Acute systemic THC treatment also produced significant alterations in cerebral glucose utilisation in neural systems important for sensory processing including the sensorimotor cortex and auditory and visual stimuli processing substrates, the inferior colliculus and lateral geniculate nucleus, respectively. Changes in sensory stimulus processing in these discrete brain regions may contribute to perturbances in sensory perception observed in humans (Peters *et al*., 1976).

Amygdala and Neural Circuitry Subserving Emotional Behaviour

In both humans and animals, acute THC intoxication transiently modulates subjective anxiety and fear levels (Onaivi *et al*., 1990; Wachtel *et al*., 2002; D'Souza *et al*., 2004). The eCB system plays a pivotal role in the regulation of emotional behaviour. Knock-out of the CB_1 receptor effects multiple facets of emotional-related behaviour in mice, resulting in increased aggressive behaviour as measured in the resident intruder task, anxiogenic-like responsiveness in the light/dark box task and an increased sensitivity to exhibit depressive-like tendencies in the chronic unpredictable stress behaviour assay (Martin *et al*., 2002). Neural substrates rich in CB_1 receptors, such as the amygdala, hippocampus and cortex, are integral components of the complex neural circuitry that governs emotional behaviour (Herkenham *et al*., 1991; Glass *et al*., 1997; Pessoa, 2010). In this study, acute THC administration had significant overt albeit opposing effects of cerebral glucose metabolism in two nuclei of the amygdala, namely the basolateral and medial amygdala. Within the basolateral amygdala, acute systemic THC administration significantly reduced metabolic activity and led to a decrease in functional connectivity with multiple discrete regions of the hippocampus, the nucleus accumbens, basal ganglia and an altered functional connectivity profile with components of the PFC. Conversely, acute systemic THC administration significantly enhanced LCGU in the medial amygdala and induced aberrant functional coupling of the medial amygdala to the hippocampus, lateral septum and a decrease in functional coupling with the nucleus accumbens and cingulate cortex. Interestingly, acute THC treatment increased functional connectivity between the medial amygdala and the basolateral and central amygdala. The medial amygdala plays a modulatory role in aggression and lesioning this nucleus decreases aggressive behaviour (Vochteloo and Koolhaas, 1987). The observation that acute THC administration modulates aggressive behaviour is in agreement with the THC-induced hypermetabolism in this amygdaloid nucleus as found in this study (McDonough *et al*., 1972; Kilbey *et al*., 1977). The basolateral amygdala has been implicated as a fundamental component of fear processing and learning, specifically; it functions in the processing and storing of associations to aversive stimuli

(Zald, 2003). The findings in this study of reduced glucose metabolism in the basolateral amygdala are in agreement with a human imaging and behavioural study carried out by Phan and colleagues, where they demonstrated a THC-mediated reduction in amygdala reactivity and subsequent attenuation in the reactivity levels of subjects in response to threatening stimuli (threatening facial expressions) (Phan *et al.*, 2008). Thus, this study provides new evidence that acute THC administration leads to alterations in glucose metabolism and functional coupling in neural correlates involved in the modulation of differential aspects of emotional behaviour.

Neuromodulatory Nuclei

Acute systemic THC administration (5mg/kg) led to altered glucose metabolism in multiple neuromodulatory nuclei; specifically, THC induced a hypermetabolic state in the medial septal nucleus and locus coeruleus whilst reducing metabolic activity in the nucleus accumbens core.

The medial septal nucleus is reciprocally connected with the hippocampal formation and, through modulation of ACh release to the hippocampus, plays an important role in spatial navigation and memory processing (Mizumori *et al.*, 1990; Leutgeb and Mizumori, 1999). Acute THC administration has been shown to reduce extracellular hippocampal ACh levels and simultaneously induce spatial working memory deficits (Nava *et al.*, 2000, Nava *et al.*, 2001). Thus, reduced neuronal activity, as supported by reduced glucose metabolism, in the medial septal nucleus demonstrated in this study may contribute to THC-induced deficits in hippocampal-dependent tasks by disrupting hippocampal ACh levels.

In this study, acute THC administration induced a hypometabolic state in the nucleus accumbens core and altered functional coupling with multiple dopaminergic components of the basal ganglia. For example, THC reduced functional connectivity between the nucleus accumbens core and the striatum (ventromedial and dorsolateral striatum) and

the pallidum (globus pallidus and ventral pallidum) whilst enhancing functional coupling with the substantia nigra. The mesolimbic DA system plays a fundamental role in the mediation of a broad spectrum of THC-induced effects. Whilst increased mesolimbic DA levels are associated with the reward-driven, euphoric and hallucinatory properties of acute THC intoxication, inactivation of the nucleus accumbens core, a major efferent of the PFC, hippocampus and amygdala, severely disrupts cognitive processing as measured by set-shifting ability indicating that the nucleus accumbens core plays a pivotal role in the maintenance of novel set shifting strategies and in inhibition of irrelevant responses (Drevets *et al*., 2001; Floresco *et al*., 2006). Moreover, Egerton *et al*. (2003) found that acute THC administration (5mg/kg) impaired performance when rats were required to form reverse-stimulus reward associations. These behavioural deficits were accompanied by altered immediate early gene expression (marker of neuronal activity) in the dorsolateral striatum and nucleus accumbens core (Egerton *et al*., 2005). Interestingly, whilst in this study THC reduced glucose metabolism within the nucleus accumbens core, conversely, acute THC administration has been shown to interact with this dopaminergic substrate, resulting in increased DA efflux (Chen *et al*., 1990). An important point to note is the low dose (0.1-1mg/kg) used to facilitate the aforementioned augmentation in DA levels within the nucleus accumbens core, therefore, due to the biphasic pharmacokinetic profile of THC, it is possible that a differential modulatory dopaminergic effect may be mediated at a higher dose, such as that used in this experiment (5mg/kg) (Taylor and Fennessy, 1977). Thus, it is clear that the nucleus accumbens alongside other components in the mesolimbic DA system are pivotal neural substrates whose functioning is disrupted by acute THC exposure.

The neurotransmitter noradrenaline (NA) plays a prominent modulatory role in multiple facets of cognition including attention and memory processing (reviewed by Berridge and Waterhouse, 2003). The locus coeruleus is the sole source of NA to both the hippocampus and PFC, critical neural substrates involved in attention and memory processing (Berridge and Abercrombie, 1999). Acute THC administration has been

shown to increase the firing of locus coeruleus noradrenergic neurons (Muntoni *et al.*, 2006). In contrast, whilst acute THC administration stimulates noradrenergic activity in the locus coeruleus, CB_1 receptor-mediated inhibition of NA release is evident in the hippocampus (Schlicker *et al.*, 1997). The results of the present study are agreement with the aforementioned studies as THC-induced hypermetabolism was evident within the locus coeruleus whilst functional connectivity analysis revealed a THC-induced decline in functional communication between the locus coeruleus and the hippocampus. Collectively, the novel findings of this study of altered neural activity in key neuromodulatory nuclei coupled with perturbed functional communication with the neural architecture of the NA, DA and Ach pathways provide new evidence supporting aberrant THC-induced neurotransmission that may underpin cognitive impairments observed following acute THC treatment.

Enhanced Liability to THC in Peripubertal Animals

A particularly pertinent point of discussion for this study is the novel finding of an apparent age-dependent differential liability to THC among select neural substrates significantly affected by acute THC treatment. Such a developmental sensitivity was evident within three out of four of these thalamic nuclei, namely, the anteromedial, reticular and mediodorsal thalamic nuclei. Within these three thalamic structures, the THC-induced reduction in glucose metabolism appeared to be pronounced among peripubertal animals as compared to adult animals suggestive of an age-related differential liability to THC resulting in differential rates of THC-induced neural activation within these substrates. As mentioned earlier in this section, these thalamic nuclei play fundamental roles in cognitive processing, thus, an enhanced vulnerability of the peripubertal brain to THC could lead to increased liability to the adverse cognitive sequelae associated with acute THC exposure. This developmental sensitivity hypothesis is in agreement with recent behavioural data that indicated age-dependent differential effects of THC on learning. For example, Cha *et al*. (2006) found that acute THC-induced impairments in both spatial and non-spatial memory were more

pronounced in peripubertal (PD30-32) animals compared to adult animals (Cha *et al*., 2006). In addition to enhanced thalamic sensitivity to THC in peripubertal animals, we also observed a potential developmental pattern of sensitivity for both the nucleus accumbens core and the basolateral amygdala. Within the nucleus accumbens core, THC-induced reduction in glucose metabolism appeared to be more pronounced in adults, whilst in the basolateral amygdala, THC-mediated hypometabolism was more pronounced in peripubertal animals. Adolescent brain development is a transitional period in which there is a shift in anatomical control of behaviour, from limbic to PFC-mediated behaviour with an increase in inhibitory connections between the two regions i.e. a shift from affective-driven behaviour to more regulated cognitive-driven behaviour (Nelson *et al*., 2005). The basolateral amygdala directly projects to the nucleus accumbens and both structures play pivotal roles in emotion-driven behaviour. The basolateral amygdala is involved with the recognition of emotionally relevant stimuli whilst the nucleus accumbens and mesolimbic DA system is implicated with reward mechanisms (Johnson *et al*., 1994). Thus, the novel finding of differential age-dependent sensitivity to THC within these regions could potentially lead to differential affective and reinforcing sequelae. Indeed, Quinn and colleagues have provided evidence for such an effect as they demonstrated that peripubertal rats find repeated THC administration less aversive than adult rats as measured by the place-conditioning behavioural assay (Quinn *et al*., 2008). Moreover, Ellgren and group (2007) demonstrated that treatment with THC throughout the peripubertal period (PD29-48) significantly increased opiate self-administration in adulthood (Ellgren *et al*., 2007). Collectively, the novel data provided by this study of age-dependent differential liability to THC, with LCGU being a measure of neuronal activity, in tandem with the behavioural data of several other research groups, provide preclinical support that the peripubertal brain is more vulnerable to adverse sequelae associated with acute THC treatment compared to adults.

Overall, both the cerebral metabolism and functional connectivity data generated in this study provide new evidence that acute exposure THC leads to diverse and widespread

aberrations in neural activity and functional communication throughout multiple complex neural systems. Furthermore, the novel data provided in this study suggests that the peripubertal brain appears to exhibit a differential liability to the acute effects of THC as compared to the brain in adulthood.

3.6 |Conclusions

The data in this chapter present multiple important findings regarding the differential THC-induced and age-dependent effects on cerebral metabolism and functional connectivity signatures in the brain. The novel cerebral metabolism and functional connectivity data provided in this study indicate that acute exposure THC leads to diverse and widespread perturbances in neural activity and functional coupling throughout multiple complex neural systems. The elucidation of a differential developmental liability to the acute effects of THC, in tandem with the findings of age-related differences in neural activation and functional connectivity in brain regions associated with cognitive and reward processing and neuromodulation, provides new evidence supporting the theory of vulnerability of the adolescent brain during this critical neurodevelopmental epoch and thus indicates that adolescent exposure to THC may induce more adverse sequelae compared to exposure in adulthood.

CHAPTER FOUR

Characterisation of a ‘Two-Hit’ Environment-environment Model with Relevance to Schizophrenia

4.1 |Introduction

Numerous putative risk factors, both genetic and environmental in nature, have been implicated in the pathogenesis of schizophrenia (section 1.7). It is now accepted that schizophrenia is best conceptualised as a disorder of multi-factorial pathogenesis and a consequence of complex interplay between putative genetic and/or environmental risk factors. Over the past two decades, the neurodevelopmental hypothesis of schizophrenia has gained increasing momentum as an explanatory theory of the aetiology of this complex disorder. This model posits that clinical manifestation of schizophrenia is a consequence of perturbances to neurodevelopmental processes that take place long before the onset of clinical symptoms and is caused by a combination of environmental and/or genetic factors (Rapoport *et al*., 2005). Keshavan first proposed the ‘two-hit’ model of schizophrenia, suggesting that the emergence of schizophrenia in adulthood is a result of maldevelopment during two critical phases of neurodevelopment, namely, early and adolescent brain development. According to this model, perturbation of early developmental processes in the brain results in disrupted functioning in specific neuronal networks. These perturbations manifest as subtle behavioural alterations evident as pre-morbid signs and symptoms commonly seen in pre-symptomatic schizophrenia patients (Larson *et al*., 2010; Klosterkötter *et al*. 2001). Subsequently, in adolescence, dysregulation of synaptic pruning may lead to aberrant synaptic remodelling and may account for the post-adolescent emergence of the overt symptoms of the disorder (Keshavan 1999; Keshavan and Hogarty 1999).

This hypothesis has led to emergence of gene x environment preclinical models of schizophrenia (section 1.8.4). However, to date, very little preclinical research has been carried out investigating the interactive effects of 'early' and 'late' environmental risk factors on the precipitation of schizophrenia-related behaviours in adulthood. In the past, preclinical studies have focused on the replication of individual environmental risk factors, such as prenatal infection (section 1.7.3) or adolescent cannabis abuse (section 1.4.3), in an attempt to elucidate the pathological processes and schizophrenia-related behavioural phenotypes evoked as a consequence of exposure to these risk factors.

Maternal Infection – 'Early' Environmental Risk Factor

Maternal infection has been strongly implicated as an 'early' environmental risk factor of schizophrenia (Brown *et al*., 2001, Brown *et al*., 2010; Brown, 2006, section 1.7.3). More specifically, immunogen-induced elevation in maternal cytokines (maternal immune activation) is believed to be the common mechanistic pathway lending to the pathogenic nature of maternal infection. PolyIC is a synthetic analogue of double stranded viral RNA commonly employed as an immune stimulating agent in preclinical investigations of prenatal infection. Following systemic administration PolyIC is detected by the pathogen recognising receptor toll-like receptor three in the mammalian immune system and subsequently stimulates an immune response in the form of cytokine release. During infection, pro-inflammatory cytokines have been shown to cross the placenta and enter the foetal environment (Urakubo *et al*., 2001). Introduction of cytokines to the foetal environment can result in neurotoxicity as cytokines such as tumour necrosis factor-α and interleukins 6 and 1-β have been shown to decrease survival of dopaminergic and serotonergic neurons (Jarskog *et al*., 1997). Thus, the developing foetal brain is particularly vulnerable as throughout this period vital neurodevelopmental processes such as neurogenesis, neural migration, axonogenesis and dendrogenesis take place (O'Rahilly & Muller 1987), disruption of which by maternal infection may lead to long-term developmental abnormalities. Previous preclinical studies have demonstrated the ability of MIA to induce neuropathologies

such as hyperdopaminergia in mesolimbic regions of the brain accompanied by cytoarchitectural alterations. Alongside these pathophysiological changes MIA has been shown to produce schizophrenia-related phenotypes such as behavioural switching, deficits in PPI and enhanced sensitivity to psychostimulants (Wolff and Bilkey 2008; Zuckerman *et al*. 2003; Zuckerman and Weiner 2005).

Adolescent Cannabis Abuse – 'Late' Environmental Risk Factor

Adolescent cannabis abuse has been strongly implicated as a 'late' environmental risk factor of schizophrenia (Andréasson *et al*., 1987; Pope *et al*., 2001; Arseneault *et al*., 2004, section 1.7.4). Preclinical studies investigating the deleterious effects of peripubertal exposure to CBs have shown enduring deficits in object recognition memory, PPI and spatial working memory following peripubertal treatment with CBs (Schneider and Koch, 2003; Quinn *et al*., 2008; Rubino *et al*., 2009). Very little preclinical literature is available on the effects of peripubertal THC exposure; however, Quinn and colleagues have shown that peripubertal THC administration produces long-term impairments in object recognition memory (Quinn *et al*., 2008)

As outlined above, preclinical studies have investigated the deleterious effects of both MIA and cannabis abuse individually. It is therefore of great interest to determine the interactive effects of exposure to these 'early' and 'late' environmental risk factors. Here, I characterise the impact of prenatal PolyIC treatment followed by peripubertal/adolescent THC treatment to model the combined effects of MIA and cannabis abuse to create a novel 'two-hit' neurodevelopmental model with translational relevance to schizophrenia.

A variety of behavioural paradigms with varying translational potential were employed in the characterisation of preclinical models with relevance to schizophrenia (Pratt *et al*., 2012). The behavioural assays employed in this present study are described below.

4.1.1 |Schizophrenia-related Behavioural Paradigms

4.1.1.1 |Prepulse Inhibition of the Acoustic Startle Reflex

PPI refers to the reduction in magnitude of an acoustic startle response when a startle-eliciting pulse stimulus is shortly preceded by a weak prepulse stimulus. This cross-species phenomenon is regarded as a pre-attentive filtering mechanism also referred to as sensorimotor gating (Geyer, 2006). The paradigm is thought to test the cognitive domain of attention/vigilance as defined by the Measurement and Treatment Research to Improve Cognition in Schizophrenia (MATRICS) initiative (Nuechterlein *et al.,* 2004). In humans, within the PPI paradigm, the eye blink reflex of oculomotor muscles in response to an 'air puff' is measured using electromyography whilst in rodents stabilimeter chambers are used to quantify startle responses to acoustic stimuli. Whilst the sensory modalities vary between species the phenomenon of PPI is conserved (Swerdlow & Geyer 1998).

PPI is known to be regulated by several neurotransmitters that are thought to elicit their effects by modulating activity in the cortico-limbic-striatopallidal circuit. For example, the DA agonist apomorphine has been shown to induce significant impairments in PPI that can be reversed by both the typical and atypical antipsychotics haloperidol and clozapine respectively (Mansbach *et al*., 1988; Swerdlow & Geyer, 1993). Similar impairments have been observed following systemic administration of the indirect DA agonist amphetamine and the non-competitive NMDA receptor antagonist PCP (Swerdlow *et al*., 1990; Keith *et al*., 1991). Some studies have shown that PCP-induced effects on PPI are not reversed by treatment with the D_2 receptor antagonist haloperidol suggesting that its ability to modulate PPI may be independent of its effects on DA transmission (Mansbach *et al*., 1988; Keith *et al*., 1991). The cholinergic system has also been implicated; the muscarinic receptor antagonist scopolamine causes a disruption of PPI that can be reversed by haloperidol (Jones and Shannon, 2000; Ukai *et*

al., 2004; Jones *et al*., 2005). Thus, multiple neurotransmitter systems are recruited and interact with one another in a complex manner to modulate PPI.

Deficient sensorimotor gating is thought to result in sensory flooding and cognitive fragmentation. This neurological abnormality is present amongst patients suffering from Huntington's disease and Obsessive-compulsive disorder (Swerdlow *et al*., 1993; Swerdlow *et al*., 1995). PPI dysfunction is also commonly observed in schizophrenic patients (Braff *et al*., 2001). Furthermore, the PPI test is a routinely used behavioural paradigm in preclinical studies to determine schizophrenia-related phenotypes (Jones *et al*., 2011; Meyer *et al*., 2005)

4.1.1.2 |Novel Object Recognition Paradigm

The NOR paradigm was first described by Ennaceur and Delacour in 1988. The paradigm is based on the natural tendency of rodents to demonstrate an exploratory preference to a novel object when simultaneously presented with both a novel and familiar object. This paradigm is based on a spontaneous behaviour and does not require prior training (Ennaceur and Delacour, 1988). The paradigm is thought to test the cognitive domain of visual learning and recognition memory as defined by the MATRICS initiative (Clark et al., 2000; Nuechterlein *et al.,* 2004). Deficits in a human analogue of the NOR paradigm, the 2-D object recognition test, are seen among schizophrenic patients (Clare *et al*., 1993).

Performance on the NOR paradigm is governed by medial temporal lobe structures, in particular the perirhinal cortex. Bilateral excitotoxic lesions to perirhinal and postrhinal cortices results in impairments in the NOR paradigm (Winters *et al*., 2004). Furthermore, transient lidocaine-induced inactivation of the perirhinal cortex during the 3 distinct phases of the NOR paradigm, the sample phase (encoding), the retention interval (consolidation) and the choice phase of the paradigm (retrieval) led to impaired performance on the test (Winters and Bussey, 2005).

4.1.1.3 |Attentional Set-shifting Task

The ASST was developed by Birrell and Brown (2000) and is analogous to the human WCST, a test that is commonly performed poorly by schizophrenic patients (Wobrock *et al*., 2009). Within the task, there are various discrimination phases designed to test the animal's cognitive capabilities, including rule acquisition, reversal learning, ability to form an attentional set and also exhibit inhibitory control. Collectively, the task is used to probe functioning within the cognitive domain of reasoning/problem solving as defined by the MATRICS initiative (Nuechterlein *et al.,* 2004).

The ASST is a PFC-dependent task with lesions to the medial PFC producing ED impairments and orbital cortex lesions producing deficits in reversal learning (Birrell and Brown, 2000; McAlonan and Brown, 2003). A similar underlying pathology is observed amongst schizophrenic patients performing the WCST, with hypofrontality of the dorsolateral PFC and orbital frontal cortex pathology linked to impairments in ED and reversal learning respectively (Bunney and Bunney, 2000; Shad *et al*., 2006)

4.1.1.4 |Locomotor Activity in a Novel Environment

Manifestation of positive symptoms such as hallucinations and delusions are uniquely human phenotypes that cannot be assessed in rodents. However, the rodent behavioural assay of hyperlocomotion in a novel environment is thought to reflect psychomotor agitation, a positive symptom common amongst schizophrenic patients, although the strength of evidence to support this view is limited (reviewed by Arguello and Gogos, 2006).

Regulation of locomotor activity is a DA dependent process and involves the mesolimbic DA system. Local administration of DA into the terminal areas of the mesolimbic system results in hyperlocomotion. Pretreatment with the D_2 antagonist haloperidol prior to DA administration greatly reduces DA-induced augmentation in locomotion, directly implicating this receptor subtype in the regulation of

hyperlocomotion and supporting the potential of this task as a translational tool for identifying antipsychotic compounds (Pijnenburg *et al*., 1976). Hyperlocomotion in a novel environment has been demonstrated in several animal models with relevance to schizophrenia (Lipska and Weinberger, 2000)

4.1.1.5 |Sensitivity to Amphetamine-induced Hyperlocomotion

As mentioned in section 1.5.2, acute amphetamine administration can induce transient psychosis in healthy volunteers and also exacerbates the psychotic symptoms of schizophrenic patients (Angrist *et al*., 1974, Angrist *et al*., 1980). Furthermore, systemic amphetamine administration leads to significantly greater increase in DA outflow in schizophrenia patients as compared to control subjects (Laruelle *et al*., 1996; Breier *et al*., 1997) indicative of aberrant mesolimbic DA transmission. These clinical observations form the basis of the rodent behavioural correlate, sensitivity to amphetamine-induced hyperlocomotion. Response to systemic amphetamine administration is a commonly employed measure of mesolimbic DA functionality with enhanced sensitivity to amphetamine indicating a dysregulation of DA signalling within the mesolimbic system.

4.2 |Aims and Objectives

The aim of this study was to test the hypothesis that a combination of two environmental challenges during 'early' and 'late' neurodevelopment will precipitate schizophrenia-like phenotypes in adulthood.

The specific objectives of this study were to:

a) Determine the individual effects of, and potential interplay between, prenatal PolyIC treatment (mimetic of prenatal infection) and peripubertal THC exposure (mimetic of adolescent cannabis abuse) on the precipitation of translational schizophrenia-related behaviours in rodents.

b) Investigate the potential differential liability of two THC treatment regimes designed to mimic patterns of social cannabis abuse: high-dose daily (mimetic of heavy daily cannabis abuse) and low-dose intermittent (mimetic of light recreational cannabis abuse) THC administration.

c) Characterise the effects of prenatal PolyIC treatment and peripubertal THC exposure on CB_1 receptor expression levels, brain functioning (as indicated by cerebral metabolism) and regional functional connectivity in adulthood, in order to identify potential mechanisms underlying the observed effects of these environmental challenges on schizophrenia-relevant behaviours.

These objectives were achieved through employment of several schizophrenia-related behavioural paradigms, [^{3}H] SR141761A binding assays, 2DG imaging and functional connectivity analysis using partial least squares regression analysis.

4.3 |Methods

4.3.1 |PolyIC Preparation and Administration

Polyriboinosinic-polyribocytidilic acid potassium salt (Sigma-Aldrich Ltd) was dissolved in 1 Molar phosphate buffered saline (PBS) to make a 4mg/ml PolyIC solution as previously described by Zuckerman *et al*. (2003). In accordance with this protocol, 4mg/kg of PolyIC or an equivalent volume of PBS (vehicle) was administered on GD15, the height of neurogenesis in the fetal rat brain. This dose of PolyIC (4mg/kg) administered intravenously on GD15 has previously been shown to provoke an systemic immune response resulting in increased levels of TNF-α and IL-6 (Dalton *et al*., 2012). Thus, please note that throughout this thesis, the terms prenatal PolyIC treatment and MIA will be used interchangeably.

Naive Lister-hooded rats (Harlan, UK) were mated at approximately 10 weeks old with GD1 defined as the first day after copulation. On GD15 pregnant dams were anaesthetised with a gaseous mix of 5% halothane/isoflorane (Merial Animal Health Ltd), 30% oxygen and 65% nitrous oxide and received either a single intravenous tail-vein injection of PolyIC (4mg/kg) or an equivalent volume of PBS (vehicle). Following PolyIC administration, dams showed a significant reduction in weight gain for the first day following PolyIC treatment compared to vehicle-treated dams (data not shown). Litter size was unaffected by prenatal PolyIC treatment (data not shown).

4.3.2 |Animals

On postnatal day (PD) 21, the resulting offspring were weaned and sexed and the male offspring were housed four to a cage. 72 Lister-hooded male rats from these offspring were housed under standard conditions on a 12 hr/12 hr light/dark cycle (lights on 07:00hr). Animals were allocated pseudorandomly into one of three groups for peripubertal treatment from PD35-56. Each experimental group contained no more than

two rats from the same litter. They received food and water *ad libitum* in the home cage, except for two weeks prior to and during the ASST. During this period, animals were maintained on a diet of 15-17gm of standard laboratory rat chow per animal per day; drinking water was available *ad libitum* throughout. Monitoring of body weight was performed regularly (usually every second day) to ensure animals remained within 85% of *ad libitum* body weight. Room temperature ($21^{o}\pm2^{o}C$) and humidity (45–55%) were kept constant throughout. All experiments were performed in accordance with the Animals (Scientific Procedures) Act 1986.

4.3.3 |THC Treatment Regimes

THC treatment regimes were based on frequency of use and THC consumption levels data obtained from the 'Cannabis Use in Britain' report published by the Independent Drug Monitoring Unit in 2005. We employed two different treatment regimes designed to mimic differential patterns of cannabis abuse in society i.e. heavy daily versus light recreational use. In order to extrapolate these data on human THC consumption levels into our animal study, drug dose scaling was carried out to determine the dosages required for peripubertal male Hooded-Lister rats aged PD 35-56. The principle of interspecies drug dose scaling stated mathematically is: $D_{human}=D_{animal}(W_{human}/W_{animal})^{0.7}$, where D=dose of drug in milligrams (mg) and W=weight in kilograms (McCann and Ricaurte, 2001). Treatment regimes consisted of either 3.5mg/kg THC preparation three times per week (THC A) (mimicking light intermittent recreational cannabis abuse) or 7mg/kg THC preparation daily (THC B) (mimicking heavy daily cannabis abuse) or vehicle (1% Tween 80 in 0.9% saline). Treatment groups (n=12/group) were as follows:

(1) Vehicle only: prenatal PBS with peripubertal vehicle treatment (PBS + vehicle),

(2) THC A only: prenatal PBS with low-dose intermittent peripubertal THC (3.5mg/kg, 3 times a week) treatment (PBS + THC A),

(3) THC B only: prenatal PBS with high-dose daily peripubertal THC (7mg/kg) treatment (PBS + THC B),

(4) PolyIC only: prenatal PolyIC with peripubertal vehicle treatment (PolyIC + vehicle),

(5) PolyIC and THC A: prenatal PolyIC with low-dose intermittent peripubertal THC (3.5mg/kg, 3 times a week) treatment (PolyIC + THC A),

(6) PolyIC and THC B: prenatal PolyIC with high-dose daily peripubertal THC (7mg/kg) treatment (PolyIC + THC B).

All treatment groups received either a daily injection of vehicle or THC in accordance with the protocol.

4.3.4 |THC Administration

THC preparation has been previously described in section 3.3.2. Chronic peripubertal treatment began on PD35 for a period of 21 days (as determined in chapter two) where animals received either 7mg/kg THC preparation daily (THC B) or 3.5mg/kg THC preparation three times per week (THC A) intraperitoneally in a volume of 2ml/kg. Control animals received injection vehicle (1% Tween 80 in 0.9% saline) intraperitoneally in a volume of 2ml/kg everyday over the 21day treatment regime. All treatment groups received either a daily injection of vehicle or THC in accordance with protocol.

4.3.5 |Behavioural Testing

Behavioural testing of PPI, locomotor activity in a novel environment and sensitivity to amphetamine-induced hyperlocomotion was carried out on PD30-32, prior to peripubertal treatment with THC and in adulthood which was defined as PD70 onwards. Due to time constraints the ASST and NOR paradigm were omitted from behavioural

testing during PD30-32 (Fig 4.01). In adulthood (PD70+), behavioural tasks were carried out in the following sequential order; locomotor activity in novel environment/sensitivity to amphetamine-induced hyperlocomotion, NOR, PPI and lastly, ASST. Of the first three behavioural paradigms measured, the least aversive behavioural paradigms were carried out first (amphetamine-induced hyperlocomotion and NOR) followed by PPI. ASST was carried out last due to length of time required to test all treatment groups (habituation phase (approx 1-2hrs) and testing phase (approx 2-5 hrs) per animal). Thus, in order to minimise the difference in age of animals when performing ASST and the different behavioural paradigms minimal inter-test intervals were used.

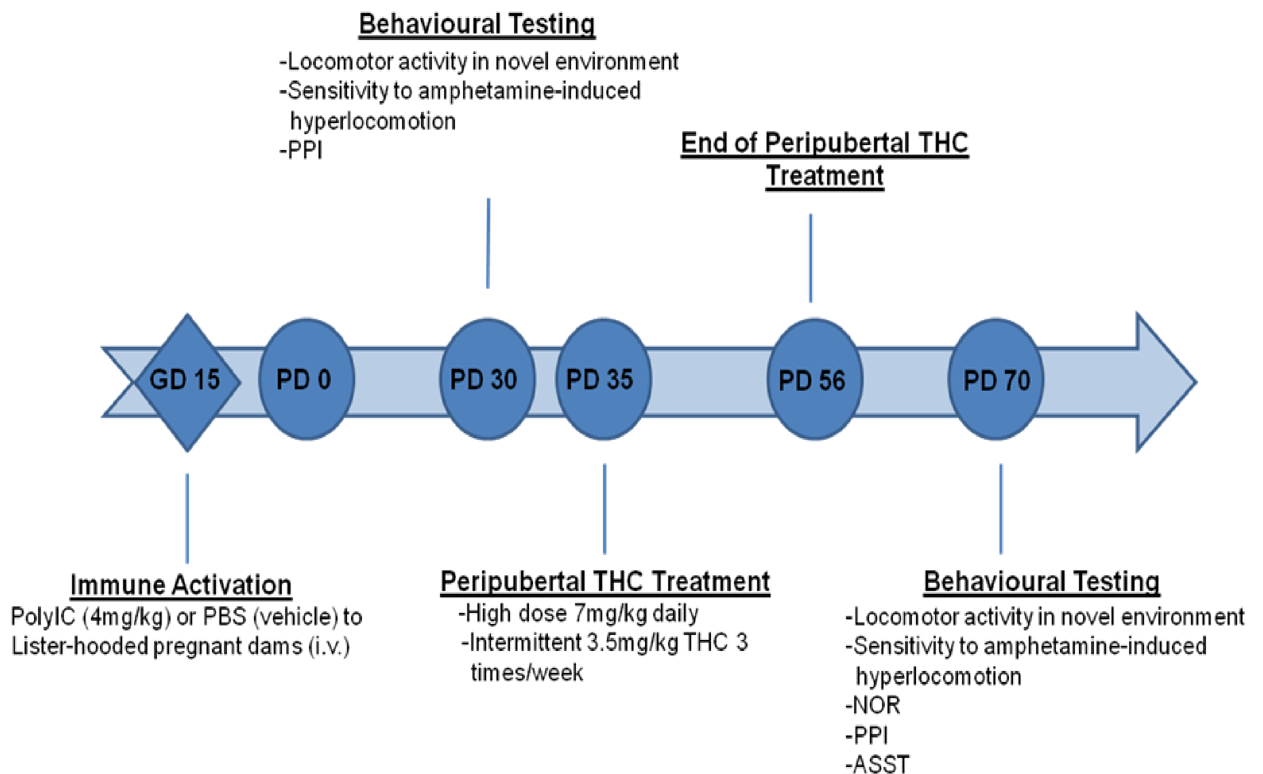


Figure 4.01 Schematic representation of time-line of behavioural experiments. On PD30-32, PPI, locomotor activity in novel environment and amphetamine-induced hyperlocomotion were measured in animals prior to peripubertal THC treatment. Amphetamine-induced hyperlocomotion, NOR, PPI and ASST behavioural paradigms were carried out on adult animals in all treatment groups (PD70+).

4.3.5.1 |Prepulse Inhibition Test

The PPI paradigm was carried out in four identical sound-attenuated chambers supplied by San Diego Instruments (California, USA). The PPI paradigm was carried out over a two consecutive days. On the day prior to PPI testing rats were habituated to the PPI procedure and PPI testing was carried out the following day.

Each test session began with a 5 minute acclimatisation period in which animals were placed in test chambers and exposed to the 65 decibel (dB) background noise. They were then tested on three blocks of trials. The first and third block of trials consisted of 6 pulse-alone trials, where the startle reflex was induced by a 40ms 120dB pulse stimulus. These blocks served to show initial startle responses and habituation to startle over the test period. The second block of trials, the test block, contained 52 trials. 10 of these were no-stimulus trials, 12 were pulse stimulus-alone trials, and in the remaining 30 trials the 40ms 120dB pulse stimulus was preceded by a 20ms prepulse stimulus of white noise at 4, 8 or 16dB above the 65dB background noise. The startle stimulus was presented 100ms after the prepulse and the recording duration lasted for 65ms following the startle stimulus. The startle response was acquired using MED Associates Startle software (SOF-825) which records the displacement of the piezoelectric platform over the 65ms immediately following the onset of the startle stimulus. The startle response is calculated as the average startle response of the animal across the 65ms recording duration following the startle stimulus. All trials were presented in a pseudo-random order. Each complete test session lasted 30 mins. % PPI for each prepulse intensity was calculated using the formula: [(pulse alone)-(prepulse and pulse)/(pulse alone)x100].

4.3.5.2 |Locomotor Activity in a Novel Environment

The test apparatus consisted of two infrared-translucent acrylic arenas (100cm x 100cm x 40 cm) which were placed on top of infrared light sources and positioned directly

underneath an infrared-sensitive camera. Movement within the arena was recorded and analysed using the tracking system EthoVISION Pro, Version 3.0 with the dependent measure in this paradigm being locomotor activity, assessed as total distance travelled by the animals in 5 minute time bins determined over a period of 20 mins.

Animals were removed from home cages and placed into the centre of the testing arenas for a 20 min period prior to injection (section 4.3.4.4). During this drug-free period the animals' natural locomotor response evoked by being placed in a novel environment was assessed. In addition, the use of this 20 minute habituation period provides a stable baseline that allows the assessment of amphetamine-induced hyperlocomotion without the confounding influences of individual variability in hyperlocomotion as a result of being placed in an novel environment.

4.3.5.3 |Sensitivity to Amphetamine-induced Hyperlocomotion.

Following the 20 minute habituation period, animals were briefly removed from the arena and injected with 1mg/kg d-amphetamine (Sigma-Aldrich Ltd) dissolved in a 0.9% saline solution or an equivalent volume of 0.9% saline solution (vehicle) subcutaneously in a volume of 1ml/kg. Dose and route of administration of d-amphetamine employed in this experiment were based on results obtained following experimental optimisation of the parameters of this behavioural paradigm in our laboratory (unpublished finding). Animals were immediately returned to arenas and remained there for a further 30 mins following drug administration. All animals received a single injection of d-amphetamine and 0.9% saline on randomly assigned alternate (Day 1 or 2) days. Arenas were cleaned with 10% Decon and dried between test sessions.

4.3.5.4 |Novel Object Recognition Paradigm

The test apparatus for the NOR paradigm consisted of four open black acrylic arenas (40cm x 40cm x 40cm). The experimental protocol used in this study was adapted from

Bevins and Besheer (2006). The objects to be discriminated were made of either brass or wood. A pilot study was carried out in another set of animals to ensure animals did not show differential preference to these objects. The heights of the objects were approximately the same (brass ring-5cm, wooden doorknob-3cm) and objects were secured onto arenas using blu-tack to prevent displacement by the animals (Fig 4.02). Objects were positioned 10cm away from the walls of the box, in opposite corners. After each trial, objects were rinsed in 10% Decon followed by water, and arenas were cleaned using 70% ethanol to dissipate odour trails. Positioning of familiar and novel objects were counterbalanced between the left and right positions to prevent location bias.

On the day prior to testing, animals were placed in the test arenas for 15 mins to ensure habituation prior to testing. During the first phase (sample phase) of the NOR paradigm, rats were placed in testing arena and exposed to two identical objects for a 10 minute exploratory period. The animal was removed from arena and returned to its home cage for a one hour inter-trial interval. Following the inter-trial interval, animals were returned to testing arena to undergo the final phase (choice phase) of the test. Animals were exposed to one familiar and one novel object for a 10 minute exploratory period (Fig 4.02). All experiments were video recorded for subsequent behavioural analysis.

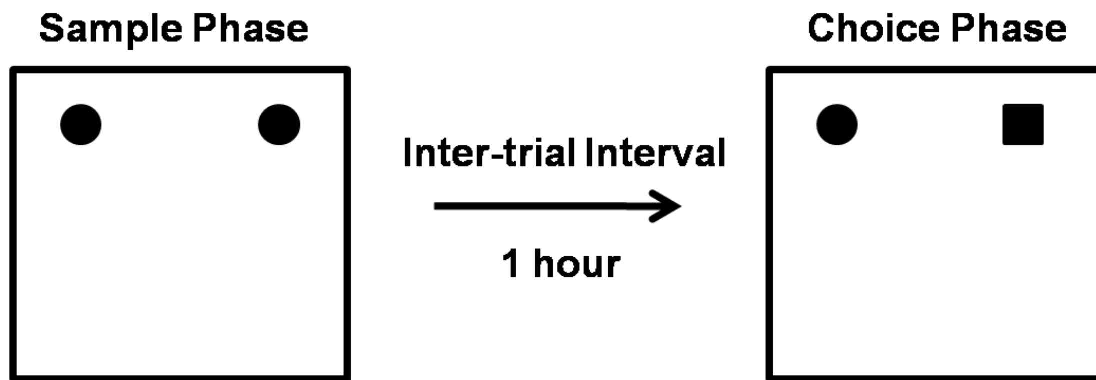


Figure 4.02 Diagrammatic representation of NOR paradigm layout.

Object interaction was scored as intentional contact with objects by sniffing, licking or touching the objects or contact directed at the object within 2cm distance of the objects. Object interaction time for each object in each trial was manually scored.

Performance on the paradigm was calculated in the form of discrimination index (DI) score. The DI is a measure of the proportion of total exploratory time spent exploring the novel object. Thus, this measure takes into consideration individual variations in total exploratory time. DI was calculated as (novel object interaction time-familiar object interaction time)/ (total object interaction time). Thus, a DI of 0 represents no discrimination made between novel and familiar object, a DI of below 0 represents a preference for the familiar object and a DI above 0 represents a preference for the novel object.

4.3.5.5 |Attentional Set Shifting Task

The test apparatus for the ASST consisted of an adapted homecage (40x70x18cm) in which one third of the cage was divided into two sections (left and right) by Plexiglas panels. Ceramic digging bowls (7cm internal diameter and 4cm depth) were placed in

these two sections and a removable Plexiglas divider separated these sections from the rest of the cage to restrict access to bowls between trials. A smaller divider was also used to restrict access to either one of the two sections when required. The digging bowls were filled with various digging media that could be scented and could conceal the food reward, which was one-half of a Honey Nut Cheerio (1/2 HNC; Nestle, Surrey, UK).

During the habituation period, which took place the day before testing, animals were trained to dig for food reward (1/2 HNC) in the ceramic bowls which were filled with sawdust from animals homecage. Once digging behaviour was reliably established, animals were exposed to all exemplars to be used during the testing paradigm to allow for habituation to the various different odours and textures used during the task. In each trial, both bowls were baited and presented on both sections to allow for side bias. Next, rats were trained to perform two simple discriminations (SD) within each perceptual dimension to be used during the test period (medium and odour).

Exemplars in the first SD differed with regard to the odour of the medium (mint vs. oregano) whilst the second SD differed with regard to the texture of the medium (paper shreddings vs. polystyrene). The rats were trained to criterion performance levels of 6 consecutive correct trials on each SD and the exemplars were not used again during the experiment.

As previously described by Birrell and Brown (2000) the testing procedure consisted of a series of discrimination phases (Table 4.01) all carried out in a single test session. Exemplar combinations and pairing used in this task are outlined in Table 4.02. For all discrimination phases of this task, the first 4 trials were regarded as exploratory trials in which the animal was allowed dig in both bowls, and a digging error was recorded if the first dig occurred in the unbaited (incorrect) bowl. In all subsequent trials, following an incorrect dig the trial was terminated by blocking access to and retrieval of reward from the baited bowl (punishment trial) and an error was recorded. For each phase of the

ASST a criterion of 6 correct consecutive digs was necessary to progress to the following phase. On the first discrimination phase of the testing period rats performed a simple discrimination SD between two bowls that differed along a single perceptual dimension (medium). When animals correctly completed the number of trials to criterion, testing progressed to the compound discrimination stage (CD). Within this discrimination phase a second, irrelevant, dimension was introduced but the correct and incorrect exemplars relevant within the SD were maintained. Next was the first reversal discrimination stage (Rev1), in which the exemplars and the dimensions were unchanged from the CD, but the previously un-rewarded exemplar was now baited and *vice versa*. The next discrimination phase was the intra-dimensional shift (ID); animals were presented with new exemplars of both the relevant and irrelevant perceptual dimensions, with the previously rewarded dimension in the SD, CD and Rev1 phases remaining the relevant dimension. The ID was followed by a second reversal (Rev2), similar to Rev1 in which the exemplars remained the same as in the ID stage but were reversed. Next, animals were tested on the ED stage of the task. As in the ID, the rat was presented with new exemplars from both the relevant and irrelevant dimensions. However contrary to the ID phase, the previously relevant dimension of medium texture was no longer rewarded and the previously irrelevant dimension of odour became the relevant dimension. The final discrimination phase was the third reversal discrimination (Rev3) where, as in Rev1 and 2, the exemplars and dimensions were unchanged from the previous discrimination (ED), but the previously relevant exemplar was now irrelevant and *vice versa*. Presentation of sets of exemplar pairs were pseudorandomly ordered throughout the task.

Table 4.01 Behavioural testing discrimination order

Discrimination	Dimensions		Exemplar Combinations	
	Relevant	Irrelevant	+	-
SD	Medium		**M1**	M2
CD	Medium	Odour	**M1**/O1	M2/O2
			M1/O2	M2/O1
Rev1	Medium	Odour	**M2**/O1	M1/O2
			M2/O2	M1/O1
ID	Medium	Odour	**M3**/O3	M4/O4
			M3/O4	M3/O3
Rev2	Medium	Odour	**M4**/O3	M3/04
			M4/O4	M3/O3
ED	Odour	Medium	**O5**/M5	O6/M6
			O5/M6	O6/M5
Rev3	Odour	Medium	**O6**/M5	O5/M6
			O6/M6	05/M5

Note: CD, compound discrimination; ED, extradimensional shift; ID, intradimensional shift; Rev, reversal; SD, simple discrimination. Table indicates the sequence of discriminations and exemplar combinations used when shifting set from medium texture to odor at the extradimensional discrimination stage. On every trial, except the SD, the pair of stimuli presented differed along both the relevant and the irrelevant dimensions. The baited exemplar (correct) is shown in bold and is paired with either exemplar from the irrelevant dimension. The combination of exemplars into positive (+) and negative (−) stimuli and their left-right position of presentation in the testcage was a pseudorandom series (Birrell and Brown 2000).

Table 4.02 Exemplar combinations employed in ASST

Medium Pairs	Odour Pairs
Coarse Tea vs Fine Tea	Cinnamon vs Ginger
Sand vs Grit	Sage vs Paprika
Coarse Sawdust vs Fine Sawdust	Tumeric vs Cloves

4.3.6 |Euthanasia

On completion of behavioural testing animals were killed by cervical dislocation. Brains were rapidly removed and frozen in isopentane (cooled to -42° C) and then coated in M-1 embedding medium (Thermo Scientific Ltd). Brains were stored at -80 ^{o} C until required.

4.3.7 |Ligand Binding Assay for CB_1 Receptors Using [^{3}H]SR141761A

Ligand binding autoradiography for [^{3}H]SR141761A CB_1 receptor binding was carried out on brain samples obtained from all 6 experimental groups following cessation of behavioural testing in adulthood. Receptor autoradiography was carried out as described in section 2.3.4.

4.3.8 | ^{14}C-2-Deoxyglucose Autoradiographic Imaging and Functional Connectivity Analysis

A separate set of experimental animals were used for this study. The experimental protocol for PolyIC administration and peripubertal treatment was repeated as described previously in section 4.3.1-4. However, as the main behavioural effects of THC

treatment were observed following low-dose intermittent THC treatment (THC A - 3.5mg/kg, administered three times a week), only the low-dose intermittent THC treatment regime was employed throughout the peripubertal period.

4.3.8.1 | ^{14}C-2-Deoxyglucose Autoradiographic Imaging

On PD70, the day in which adult testing commenced in the behavioural study, the 14C-2-Deoxyglucose experimental procedure was carried out on all experimental groups as previously detailed in section 3.3.4.

4.3.8.2 |Functional Connectivity using Partial Least Square Regression Analysis

Brain region functional connectivity was analysed using PLSR was carried out as described in section 3.3.5. Select 'seed' brain regions were chosen from RoIs in which prenatal PolyIC treatment or peripubertal THC treatment significantly altered overt cerebral metabolism (LCGU).

4.3.9 |Statistical Analysis

Behavioural Studies

Data obtained from behavioural paradigms were analysed using Student's *t*-tests, general linear repeated measures or univariate ANOVA models followed by Bonferroni *post-hoc* analysis where appropriate.

2DG Autoradiography

Data was analysed using general linear univariate ANOVA models followed by Bonferroni *post-hoc* analysis where appropriate. When analysing changes in the rate of metabolism between experimental groups anatomically discrete brain regions were assumed to represent independent variables within each measure and no correction was applied for multiple comparisons (McCulloch *et al*., 1982).

Functional Connectivity Using Partial Least Square Regression Analysis

Functional connectivity data generated using the PLSR module in XLSTAT was subsequently analysed using Student's *t*-tests. Bonferroni correction was applied for multiple comparisons made.

In all cases, statistical significance was defined as $p<0.05$. Data were expressed as mean ± SEM.

4.4 |Results

4.4.1 |Behavioural Effects of Maternal Immune Activation and Peripubertal THC Treatment

4.4.1.1 |Prepulse Inhibition Test

The PPI paradigm was employed to assess the individual effects of prenatal PolyIC exposure (MIA) and of differential peripubertal THC treatment regimes on sensorimotor gating. In addition, the potential interplay between these two environmental risk factors on the precipitation of this schizophrenia-related behavioural phenotype was determined. Sensorimotor gating was assessed during the prepubertal period (PD30-32) prior to THC treatment and in adulthood (PD70+) two weeks following cessation of THC treatment.

Startle Amplitude

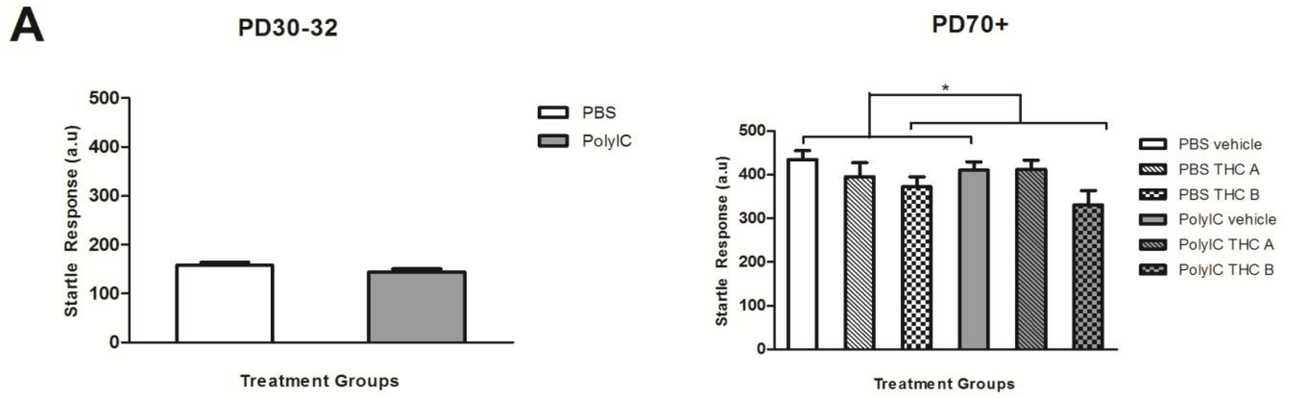
During the prepubertal period (PD30-32), startle response to the 120dB startle alone trials was not affected by MIA with no significant difference in amplitude of response observed amongst PolyIC-treated offspring compared to PBS-treated offspring (Fig 4.03A). This pattern of response to MIA was conserved in adulthood (PD 70+). Low-dose intermittent THC (3.5mg/kg/3 times a week-THC A) administration during the peripubertal period did not significantly affect startle amplitude (Fig 4.03A). In contrast, a differential response to the 120dB startle alone trials was observed amongst animals that received peripubertal treatment with THC as ANOVA analysis showed an overall effect of peripubertal THC treatment ($F_{(2,66)}=4.15$, $p=0.02$) on startle response. *Post-hoc* analysis showed that daily treatment with high-dose (7mg/kg) THC (THC B) during the peripubertal period led to a significant reduction in startle amplitude compared to vehicle-treated rats ($p<0.05$, Fig 4.03A).

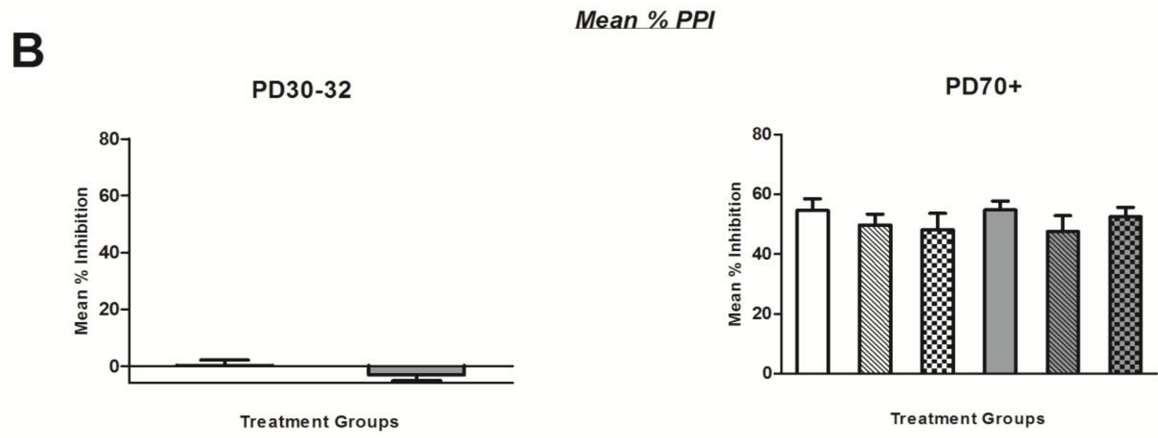
PPI of the Acoustic Startle Response

Prepubertal animals (PD30-32) from both experimental groups (PolyIC-treated and PBS-treated animals) do not appear to exhibit PPI (Fig 4.03B).

Neither prenatal PolyIC treatment nor peripubertal THC treatment significantly affected mean % PPI (average PPI at startle amplitudes of 4dB, 8dB and 16dB) in adulthood (PD 70+, Fig 4.03B). To probe whether prenatal PolyIC exposure or peripubertal treatment with THC exerted an effect at individual pre-pulse intensities, the magnitude of inhibition was analysed at startle amplitudes of 4dB, 8dB and 16dB for all treatment groups. In contrast to prepubertal animals, adult rats showed clear PPI as a significant effect of prepulse intensity ($F_{(2,132)}=387$, $p<0.0001$) was present in all treatment groups as % PPI increased with increasing prepulse intensities. % PPI across all prepulse amplitudes, 4dB, 8dB and 16dB, was not affected by prenatal PolyIC exposure or peripubertal treatment with THC (Fig 4.03B).

Startle Response





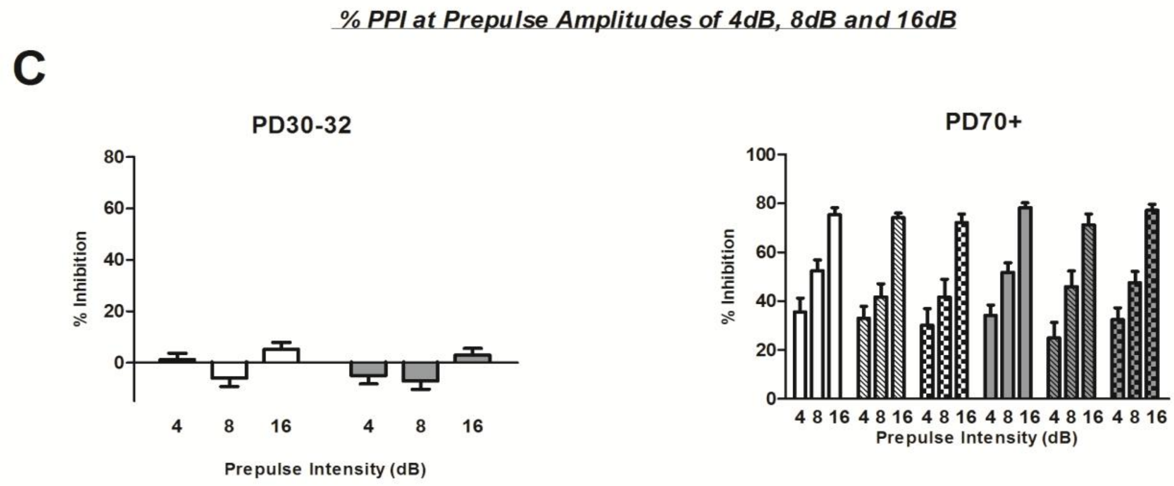


Figure 4.03A-C Effects of prenatal PolyIC exposure and peripubertal THC exposure on startle response, mean % PPI and % PPI at prepulse amplitudes of 4dB, 8dB and 16dB in prepubertal and adult animals. The startle response to 120dB startle only trials (Figure 4.03A) and mean % PPI across all three prepulse intensities (Figure 4.03B) and % PPI at prepulse amplitudes of 4dB, 8dB and 16dB were assessed on PD30-32 (graphs on left-hand side, n=36/group) and PD 70+ (graphs on right-hand side, n=12/group) in all treatment groups. Data represents mean ± SEM. Data was analysed using unpaired Student's *t*-tests (PD30-32) and general linear ANOVA models followed by Bonferroni *post-hoc* analysis (PD70+). In prepubertal animals there was no effect of prenatal PolyIC treatment (MIA) on startle response (measured in arbitrary units (a.u)) compared to PBS-treated (control) animals whilst in adult animals ANOVA analysis showed an overall effect of peripubertal THC treatment ($F_{(2,66)}=4.15$, $p=0.02$) on startle response. *Post-hoc* analysis showed that daily treatment with high-dose (7mg/kg) daily THC (THC B) during the peripubertal period led to a reduction in startle amplitude compared to vehicle-treated rats ($p<0.05$). This effect was not apparent in animals who received low-dose intermittent peripubertal THC (3.5mg/kg/three times a week) treatment (THC A) (Figure 4.03A). In the PPI of acoustic startle test, prepubertal animals (both PolyIC-treated and PBS-treated (controls) animals) did not appear to exhibit PPI (Fig 4.03B-C, left-hand side of graph). In adult animals, mean % PPI (average PPI at startle amplitudes of 4dB, 8dB and 16dB) and % PPI at startle amplitudes of 4dB, 8dB and 16dB were not affected by either prenatal PolyIC treatment or peripubertal treatment with THC treatment (Fig 4.03B-C, right-hand side of graph). * denotes a significant reduction in acoustic startle response in animals treated with high- dose daily THC (THC B) throughout the peripubertal period as compared to vehicle-treated animals ($p<0.05$).

4.4.1.2 |Locomotor Activity in a Novel Environment

Locomotor activity in a novel environment was assessed to investigate the individual effects of prenatal PolyIC treatment (MIA) and differential peripubertal THC treatment regimes on spontaneous locomotion in a novel environment and to determine the potential interplay between these two environmental risk factors on hyperlocomotion. Locomotor activity was assessed during the prepubertal period (PD30-32) prior to THC treatment and in adulthood (PD70+), two weeks following cessation of THC treatment. Locomotor activity was analysed on day one during the habituation phase prior to administration of amphetamine.

There was a significant effect of time on distance travelled during the habituation phase in both prepubertal ($F_{(3,102)}$=318.01, $p<0.001$) and adult animals ($F_{(3/198)}$=444.24, $p<0.001$) with distance travelled significantly falling over time (Fig 4.04, left-hand panel, graph on the left-hand side) and (4.05A-C left-hand panel, graph on left-hand side). This time-dependent decrease in locomotor activity indicates that animals had gradually habituated to their environmental surroundings.

During the prepubertal period, there was no significant difference in the distance travelled during the 20 minute habituation period between PolyIC-treated offspring compared to PBS-treated offspring (Fig 4.04, left-hand panel, graph on left-hand side). Similarly, prenatal PolyIC treatment did not lead to increased locomotor activity in response in a novel environment in adulthood. Furthermore, peripubertal THC treatment did not significantly affect distance travelled during the 20 minute habituation period therefore did not induce hyperlocomotion in response to a novel environment in adulthood (4.05A-C left-hand panel, graph on right-hand side respectively)

4.4.1.3 |Sensitivity to Amphetamine-induced Hyperlocomotor Activity

Enhanced sensitivity to amphetamine-induced hyperlocomotion was assessed to investigate the individual effects of prenatal PolyIC and differential peripubertal THC treatment regimes on mesolimbic DA regulation. In addition, the potential interplay between these two environmental risk factors on mesolimbic DA transmission was investigated. Testing was carried out during the prepubertal period (PD30-32) prior to THC treatment and in adulthood (PD70+), two weeks following cessation of THC treatment.

In prepubertal animals, locomotor activity in the saline-treated condition was unaffected by prenatal PolyIC treatment (Fig 4.04, right-hand panel, graph on left-hand side). Similarly, in adulthood, locomotor activity did not differ as a result of prenatal PolyIC or peripubertal THC treatment in the saline-treated condition (Fig 4.05A-C, right-hand panel, graph on left-hand side).

Systemic amphetamine administration (1mg/kg) resulted in an increase in locomotor activity in both prepubertal and adult animals (Fig 4.04 and 4.05A-C, right-hand panel, graph on left-hand side). This effect was observed across all treatment groups. As no significant effect of time was observed across the 5 minute time bins during the testing phase in any experimental groups, time bin data was collapsed to represent distance travelled (cm) following in the 30 minute testing phase. Statistical analysis was carried out on total increase in locomotor activity following amphetamine administration (relative to saline locomotor activity) data in the 30 minute testing period. During the prepubertal period, prenatal PolyIC treatment did not lead to enhanced sensitivity to amphetamine (Fig 4.04, graph on right-hand side). Interestingly, prenatal PolyIC exposure followed by intermittent treatment with low-dose THC (THC A) during the peripubertal period enhanced sensitivity to acute systemic amphetamine administration compared to PBS-treated offspring (pre- Bonferroni correction $p=0.028$) (Fig 4.05B, graph on right-hand side). This MIA effect was not apparent in groups which received

vehicle (Fig 4.05A, graph on right-hand side) or high-dose daily THC during the peripubertal period (Fig 4.05C, graph on right-hand side). However, when the significance levels were corrected for multiple comparisons the synergic effect of MIA combined with peripubertal low-dose intermittent THC treatment no longer reached significance ($p=0.08$) (Fig 4.05B, graph on right-hand side).

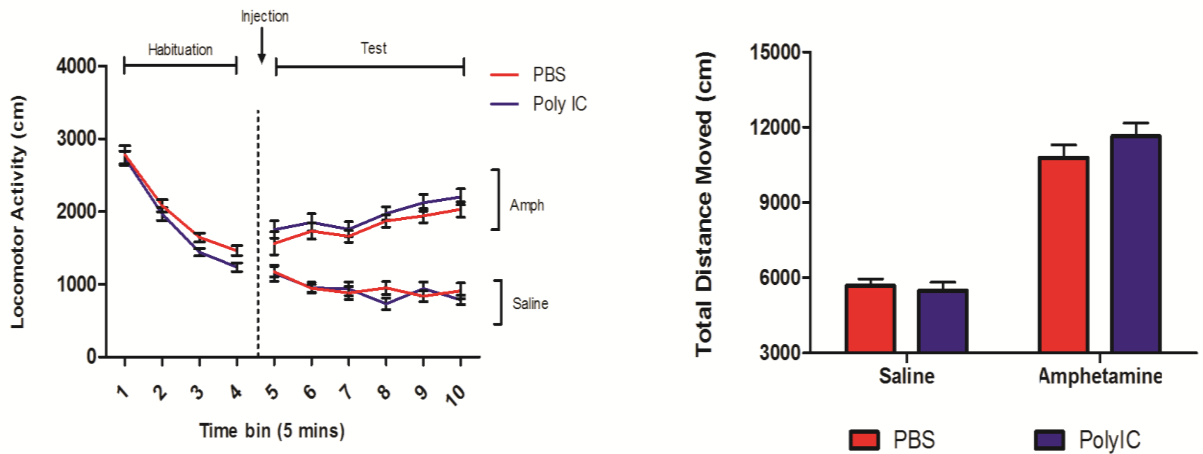
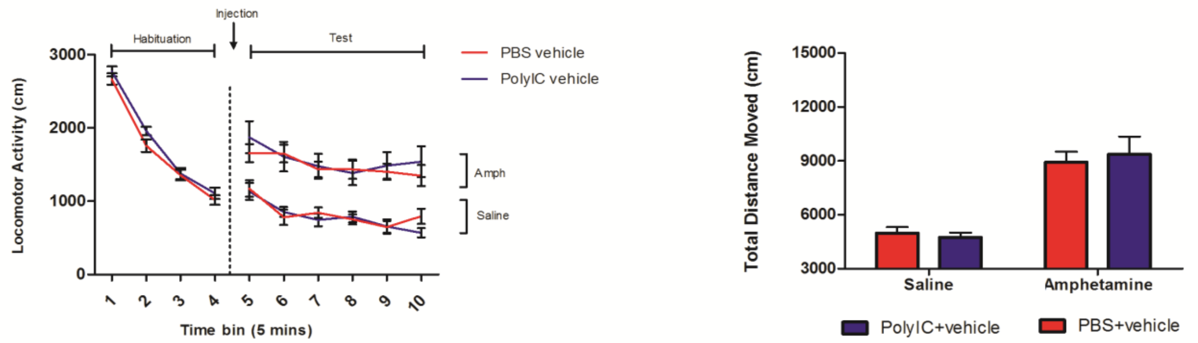
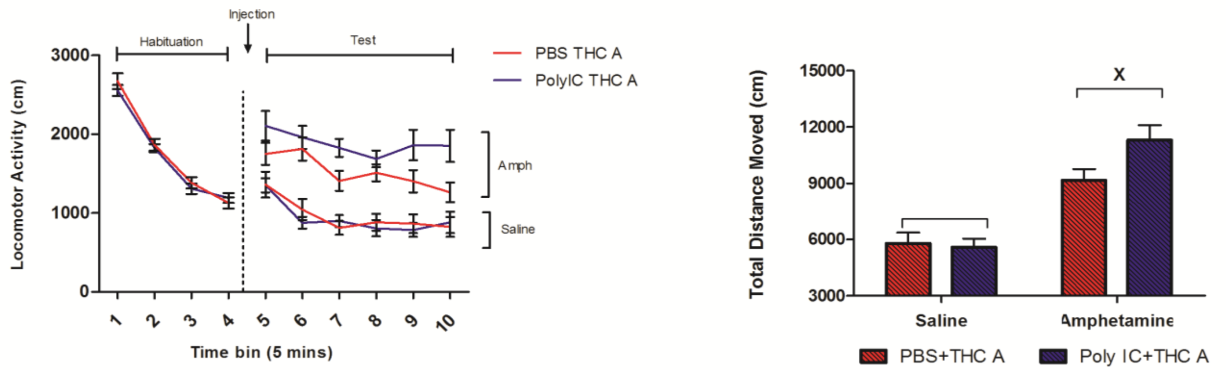


Figure 4.04 Effect of prenatal PolyIC treatment on amphetamine-induced hyperlocomotor activity in prepubertal animals. Locomotor activity was assessed on PD30-32 in prepubertal animals treated on GD15 with either PolyIC (MIA) or PBS (controls) prior to peripubertal THC treatment (n=18/group). Graph on left-hand side: Activity was measured during a 20 minute habituation phase (Habituation, left panel of graph) and a 30 minute testing phase (Test) immediately following s.c. injection of 0.9% saline (Saline, lower data sets on right panel of graph) or 1mg/kg amphetamine (Amph, upper data sets on right panel of graph). Data represents distance travelled in 5 minute time bins ± SEM. As no significant effect of time was observed across the 5 minute time bins during the testing phase data was collapsed to represent total distance travelled (cm) in the 30 minute testing phase (graph on right-hand side). Data represents total distance travelled in 30 minute testing phase ± SEM. Total increase in distance travelled following amphetamine administration data was analysed using Student's *t*-tests. Prenatal PolyIC treatment did not affect locomotion in response to a novel environment nor did MIA significantly enhance sensitivity to amphetamine compared to control animals.

A **Effects of prenatal PolyIC treatment on amphetamine-induced hyperlocomotor activity in adult animals**



B **Effects of prenatal PolyIC treatment and exposure to intermittent low-dose THC (3.5mg/kg) throughout the peripubertal period on amphetamine-induced hyperlocomotor activity in adult animals**



C **Effects of prenatal PolyIC treatment and exposure to high-dose THC (7mg/kg) daily throughout the peripubertal period on amphetamine-induced hyperlocomotor activity in adult animals**

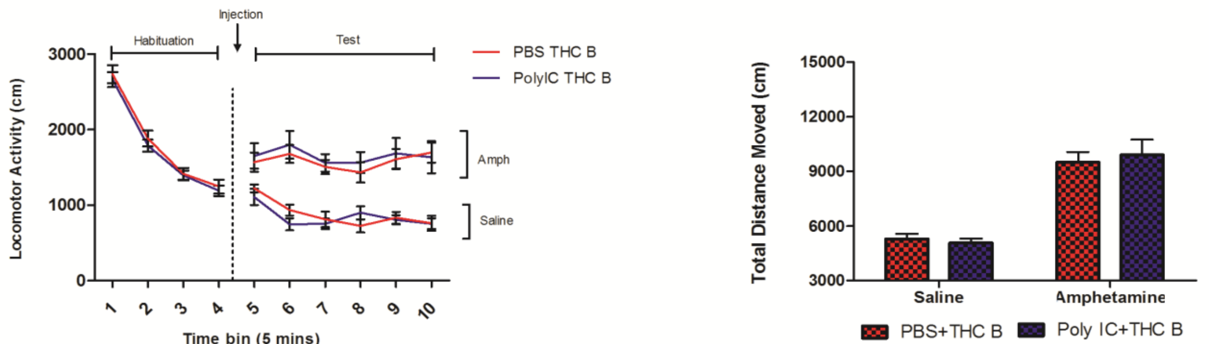


Figure 4.05A-C Effect of prenatal PolyIC exposure and peripubertal THC treatment on amphetamine-induced hyperlocomotor activity in adult animals. Locomotor activity was assessed for all treatment groups (n=12/group) in adult animals aged PD70+. Graphs on left-hand side: Activity was measured during a 20 minute habituation phase (Habituation, left panel of graph) and a 30 minute testing phase (Test) immediately following s.c. injection of 0.9% saline (Saline, lower data sets on right panel of graph) or 1mg/kg amphetamine (Amph, upper data sets on right panel of graph). Data represents distance travelled in 5 minute time bins ± SEM. No significant effect of prenatal PolyIC treatment (MIA) or peripubertal THC treatment on spontaneous locomotor activity during the habituation phase was observed (general linear model repeated measures ANOVA). As no significant effect of time was observed across the 5 minute time bins during the testing phase (general linear model repeated measures ANOVA), data was collapsed to represent total distance travelled (cm) in the 30 minute testing phase (graph on right-hand side). Data represents total distance travelled in 30 minute testing phase ± SEM. Total increase in distance travelled following amphetamine administration data was using Student's *t*-tests with Bonferroni correction applied for multiple comparisons. Statistical analysis revealed offspring of prenatally PolyIC-treated dams which received intermittent peripubertal treatment with low-dose (3.5mk/kg) THC (THC A) showed a trend towards enhanced sensitivity to amphetamine compared to PBS-treated offspring (denoted by X, pre Bonferroni correction p=0.028, post Bonferroni correction p=0.084) (Fig. 4.05B). This effect was not apparent in PolyIC-treated offspring who received peripubertal high-dose (7mg/kg) daily THC (THC B, Fig. 4.05C) or vehicle treatment (Fig. 4.05A) compared to the respective PBS-treated offspring.

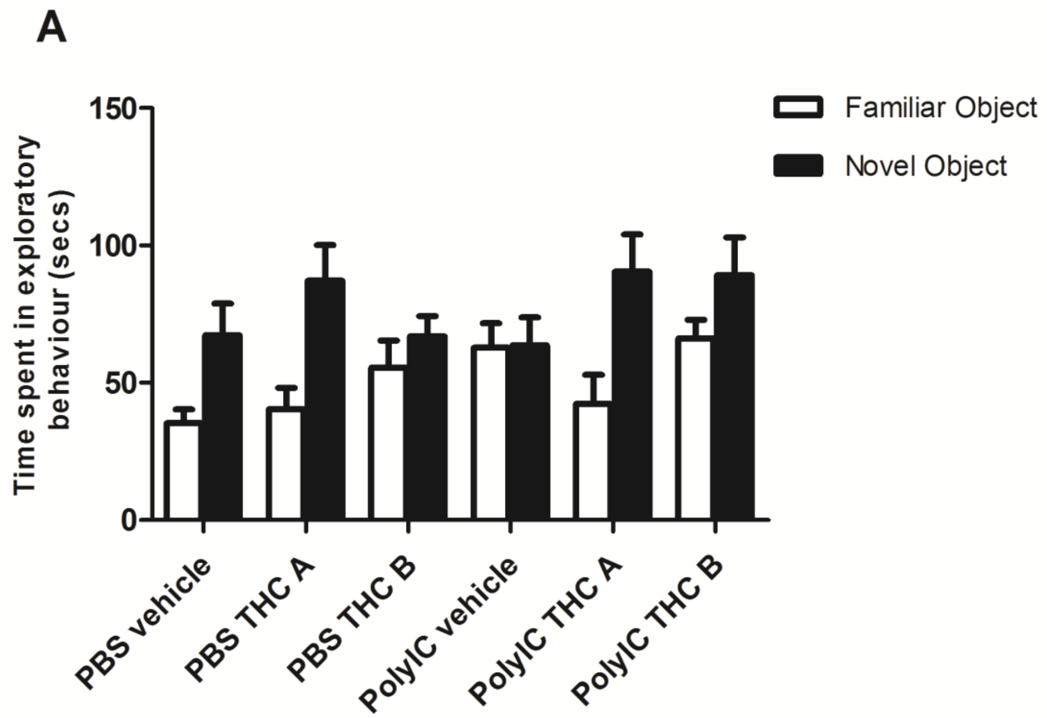
4.4.1.4 |Novel Object Recognition Paradigm

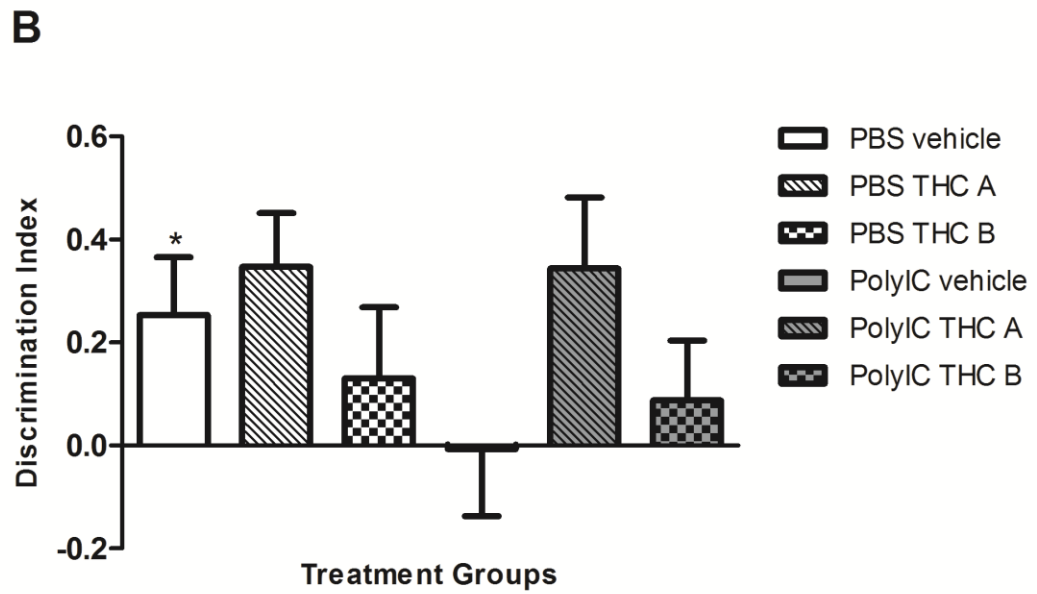
The NOR paradigm was employed to assess the individual effects of prenatal PolyIC exposure (MIA) and differential peripubertal THC treatment regimes on recognition memory and to determine the potential interplay between these two environmental risk factors on object recognition memory. Recognition memory of all treatment groups was assessed in adulthood (PD70+).

Total exploratory time during the choice phase of the paradigm was significantly affected by MIA. Exploratory time spent with both novel and familiar objects collectively was significantly increased in PolyIC-treated offspring ($F_{(1,66)}=5.25$, $p=0.025$). Total exploration time was not affected by peripubertal treatment with THC (Fig 4.06A and Table 4.03).

These exploration times were subsequently used to calculate the DI for all treatment groups. Performance on the NOR paradigm was quantified using DI. The DI is a measure of the proportion of total exploratory time spent exploring the novel object. Thus, this measure takes into consideration individual variations in total exploratory time. DI was calculated as (novel object interaction-familiar object interaction/ total object interaction time). Thus, a DI of 0 represents no discrimination made between novel and familiar object, a DI of below 0 represents a preference for the familiar object and a DI above 0 represents a preference for the novel object. PBS-treated animals that received vehicle treatment during peripubertal period (control group) successfully discriminated the novel object from the familiar one during the choice phase and spent significantly longer exploring the novel object ($p=0.045$) indicating the innate presence of recognition memory in control animals. Although it would appear that PolyIC-treated offspring do not favour exploration of the novel object over the familiar object, the DI was not statistically different from other groups. Recognition memory was not significantly affected by either prenatal PolyIC exposure or peripubertal treatment with

THC (Fig 4.06 B). No significant PolyIC x THC interaction was evident in the NOR paradigm.





* denotes a significant novel object discrimination in control animals (p<0.05)

Figure 4.06A-B Effect of prenatal PolyIC exposure and peripubertal THC treatment on time spent exploring novel and familiar objects and discrimination index respectively in NOR paradigm in adult animals. Figure 4.06A represents total exploratory time (secs) spent with both novel and familiar objects for all treatment groups (n=12/group) in the choice phase of the NOR paradigm in PD 70+ animals. These times were used to calculate the DI for all groups (DI calculated as (novel object interaction-familiar object interaction/ total object interaction time)) (Figure 4.06B). Data represents mean ± SEM. DI data from PBS-treated animals who received vehicle during peripubertal treatment (control-control) was analysed using a one sample Student's *t*-test. This revealed that control animals successfully discriminated the novel object from the familiar one and spent significantly longer exploring this object (* denotes a significant novel object discrimination ($p<0.05$)). Data from all treatment groups was analysed using general linear univariate ANOVA model. ANOVA analysis showed no significant effect of either prenatal PolyIC exposure (MIA) or peripubertal treatment with THC. THC A refers to animals treated with low-dose intermittent THC (3.5mg/kg 3 times a week) throughout the peripubertal period and THC B refers to animals treated with high-dose daily THC (7mg/kg) treatment throughout the peripubertal period.

Table 4.03 Total exploration time (sec) of all experimental groups in choice phase of NOR paradigm

Pretreatment	Peripubertal Treatment	Total Exploration Time (secs)
PBS	vehicle	102.7±9.1
PBS	THC A	127.6±16
PBS	THC B	122.4±7.1
PolyIC	vehicle	126.5±8.5*
PolyIC	THC A	132.7±12.2*
PolyIC	THC B	155.3±10.6*

* denotes a significant increase in total exploration time (novel and familiar objects) in the choice phase of the NOR in PolyIC-treated offspring as compared to PBS-treated offspring ($p<0.05$).

4.4.1.5 |Attentional Set-shifting Task

The ASST task was employed to assay the individual effects of prenatal PolyIC exposure (MIA) and differential peripubertal THC treatment regimes on various aspects of cognition and to explore the potential interaction between these two environmental risk factors on the development of residual cognitive impairments in rule acquisition, reversal learning, ability to form an attentional set and set-shifting capabilities. Performance of all treatment groups in the ASST was assessed in adulthood (PD70+).

Repeated measures ANOVA revealed a significant effect of the discrimination phase ($F_{(6,354)}=61.50$, $p<0.001$), suggesting that overall performance of the animals was dependent on the discrimination phase in question. There was a significant effect of treatment ($F_{(2,59)}=6.122$, $p=0.004$) and a significant pretreatment x treatment interaction ($F_{(2,59)}=3.436$, $p=0.025$) was also found.

To probe these effects further each discrimination phase was analysed individually using general linear univariate ANOVA models.

Within the SD and CD phases of the task, no significant differences in number of trials required to reach criterion was observed following prenatal PolyIC exposure or peripubertal THC treatment suggesting that rule acquisition was unaffected by these two factors individually. However, a significant pretreatment (PolyIC) x treatment (THC) interaction was observed within the SD phase ($F_{(2,65)}=3.3$, $p=0.04$). *Post-hoc* analysis revealed that MIA followed by intermittent treatment with low-dose THC (THC A) during the peripubertal period improved performance in the SD phase as compared to PBS-treated offspring ($p<0.05$). This MIA effect was not apparent in groups which received either vehicle ($p>0.05$) or high-dose daily THC during the peripubertal period ($p>0.05$) following MIA. This interaction was no longer apparent on progression to the CD phase of the task (Fig 4.07).

Performance on the various reversal learning phases of the task was not affected by either prenatal PolyIC treatment or peripubertal THC treatment (Fig 4.07).

In order to validate the ED phase of the ASST, the formation of an attentional set must be apparent amongst control animals. To confirm this, performance on ID shift phase versus ED phase in PBS-treated animals that received vehicle during peripubertal period was analysed using a paired Student's *t*-test. This revealed that control animals found the ED phase significantly more difficult than the ID shift phase, indicating the formation of an attentional set (p=0.0002) (Fig 4.07).

A significant effect of peripubertal THC treatment ($F_{(2,64)}=3.6$, p=0.033) was revealed within the ED phase of the task. Animals who received peripubertal low-dose (3.5mk/kg) intermittently THC treatment (THC A) exhibited a significantly impaired performance ($p<0.05$) on this phase of the task compared to-vehicle treated animals (Fig 4.07-8) irrespective of prenatal exposure to PolyIC (MIA). High-dose daily THC (7mg/kg) (THC B) did not impair performance on the ED phase of task. Prenatal PolyIC exposure did not significantly affect performance on the ED phase of the task.

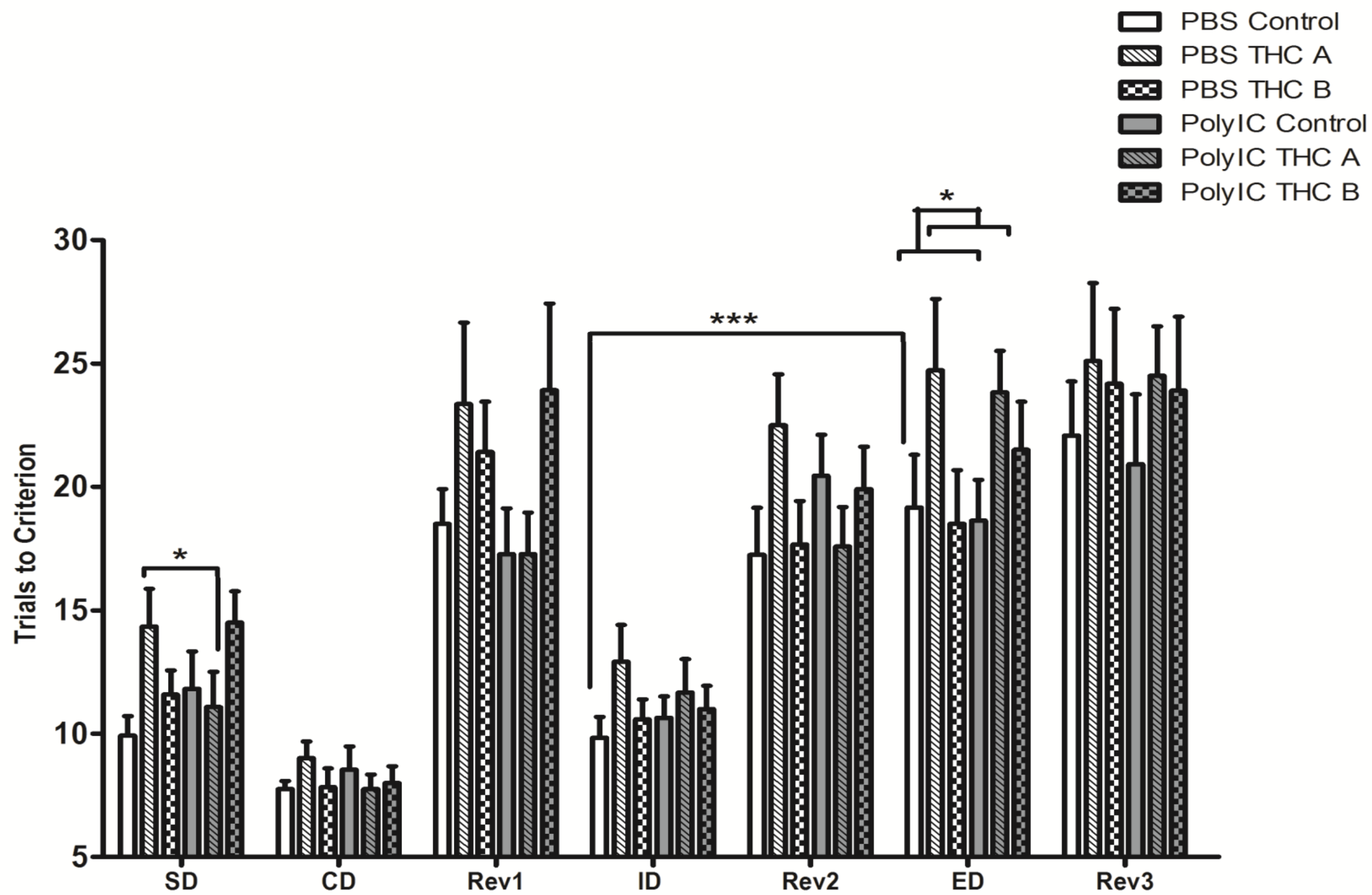

PBS Control
PBS THC A
PBS THC B
PolyIC Control
PolyIC THC A
PolyIC THC B
Trials to Criterion
30
25
20
15
10
5
*
***
*
SD
CD
Rev1
ID
Rev2
ED
Rev3

Figure 4.07 Effect of prenatal PolyIC exposure and peripubertal THC treatment on performance on the various discrimination phases of ASST in adult animals. Number of trials taken to reach criterion in discrimination phases of ASST for all treatment groups (n=10-12/group) was assessed in PD 70+ animals (SD, simple discrimination; CD, compound discrimination; Rev, reversal; ID, intradimensional shift; ED, extradimensional shift). Values represent Mean ± SEM trials to criterion. Performance on ID shift phase versus ED phase in PBS-treated animals that received vehicle during peripubertal period was analysed using a paired Student's *t*-test. This showed that control animals found the ED phase significantly more difficult than the ID shift phase, indicating the formation of an attentional set (p=0.0002). Analysis of each individual discrimination phase was carried out using a general linear univariate ANOVA model followed by *post-hoc* analysis using t-test with Bonferroni correction. A significant pretreatment x treatment interaction was revealed within the SD phase ($F_{(2,65)}$=3.3, p=0.04). *Post-hoc* analysis revealed that prenatal PolyIC exposure following intermittent treatment with low-dose THC (THC A) during the peripubertal period improved performance in the SD phase compared to PBS-treated offspring (p<0.05). This PolyIC effect was not apparent in groups which received vehicle (p>0.05) or high-dose daily THC during the peripubertal period (p>0.05). A significant effect of peripubertal treatment ($F_{(2,64)}$=3.6, p=0.033) was revealed within the ED phase of the task. Peripubertal low-dose (3.5mk/kg) intermittently THC-treated animals (THC A) exhibited a significantly impaired performance (p<0.05) on this phase of the task compared to-vehicle treated animals. * denotes a significant difference in number of trials to criterion necessary to complete ID and ED phase of task among control animals.

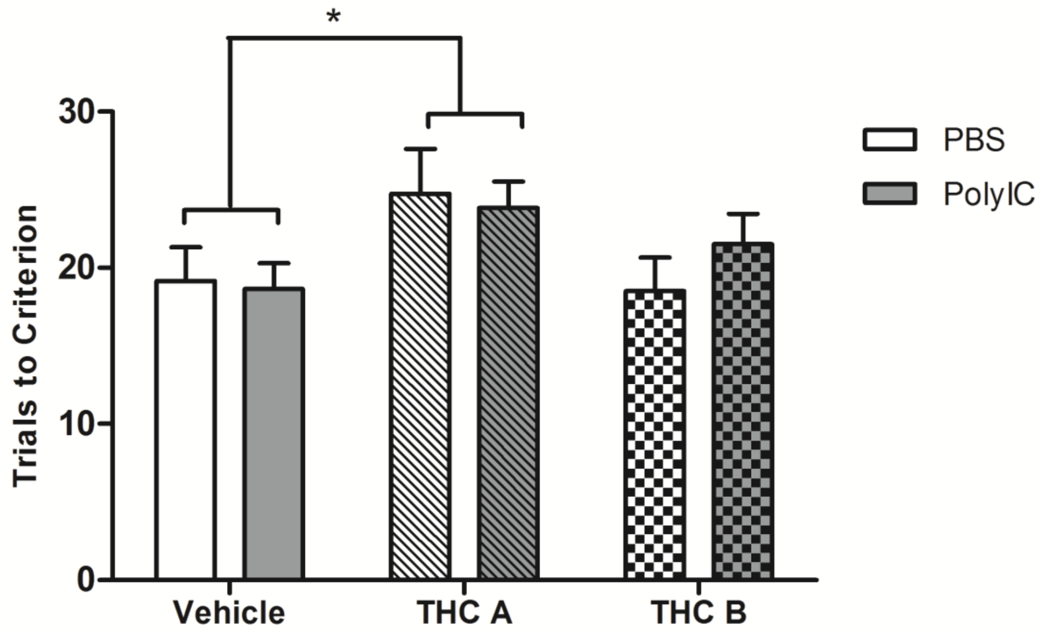


Figure 4.08 Effect of prenatal PolyIC exposure and peripubertal THC treatment on performance on the extradimensional set shift phase of ASST in adult animals. Data represents mean ± SEM trials to criterion. A significant effect of treatment ($F_{(2,64)}=3.6$, $p=0.033$) was revealed within the ED phase of the task. Peripubertal low-dose (3.5mk/kg) intermittently THC-treated animals (THC A) exhibited a significantly impaired performance ($p<0.05$) on this phase of the task compared to vehicle-treated animals irrespective of prenatal exposure to PolyIC (MIA). High-dose daily THC (THC B) did not significantly impair performance on ED phase of task.

4.4.2 |Effects of Maternal Immune Activation and Peripubertal THC Treatment on CB_1 Receptor Binding

CB_1 receptor binding levels were quantified using the selective radiolabelled CB_1 antagonist [^{3}H]SR141716A binding assay. [^{3}H]SR141716A binding was quantified in 18 cerebral structures including several cortical, hippocampal, striatal and thalamic RoI. High levels of [^{3}H]SR141716A were observed in the hippocampal formation i.e. CA1, CA2, CA3 and the dentate gyrus (Fig 4.09C). Moderate [^{3}H]SR141716A binding was observed in prefrontal and motor cortices i.e. prelimbic, ventral orbital, lateral orbital, agranular insular, infralimbic, cingulate, primary and secondary motor cortices (Fig 4.09A). A similar moderate binding profile was present in subdivisions of the striatum and nucleus accumbens i.e. ventromedial and dorsolateral striatum, nucleus accumbens core and shell (Fig 4.09B). The lowest level of [^{3}H]SR141716A binding among the 18 structures analysed was detected in the mediodorsal thalamic nucleus and retrosplenial cortex (Fig 4.09C). [^{3}H]SR141716A binding data for all experimental groups in summarised in Table 4.04.

To determine the effects of prenatal PolyIC exposure (MIA) and peripubertal THC treatment on [^{3}H]SR141716A binding for all 18 structures were analysed by general linear ANOVA model followed by Bonferroni *post-hoc* analysis where appropriate.

Prenatal PolyIC treatment did not significantly affect [^{3}H]SR141716A binding in any of the 18 cerebral structures examined. The administration of THC throughout the peripubertal period resulted in altered [^{3}H]SR141716A binding in only one structure of the 18 cerebral structures examined; namely the ventromedial striatum ($F_{(5,54)}=4.07$, $p=0.023$, Fig 4.09B). Post-hoc analysis revealed a significant increase in [^{3}H]SR141716A binding within the ventromedial striatum in animals who received low dose THC treatment intermittently (THC A) throughout the peripubertal period as compared to vehicle-treated animals ($p<0.05$, Fig 4.09B).

Table 4.04. Effects of prenatal PolyIC exposure and peripubertal THC treatment on specific CB_1 receptor binding in adulthood.

	PBS-treated Offspring			PolyIC-treated Offspring		
	vehicle	THC A	THC B	vehicle	THC A	THC B
	mean ± SEM	mean ± SEM	mean ± SEM	mean ± SEM	mean ± SEM	mean ± SEM
Cortex						
Ventral Orbital (vO)	1.57 ± 0.51	1.24 ± 0.47	1.32 ± 0.35	1.17 ± 0.32	1.89 ± 0.72	1.91 ± 0.56
Lateral Orbital (lO)	1.34 ± 0.46	1.17 ± 0.52	1.37 ± 0.37	0.99 ± 0.33	1.79 ± 0.62	1.78 ± 0.56
Prelimbic (PrL)	1.62 ± 0.51	1.79 ± 0.27	1.55 ± 0.33	1.37 ± 0.29	2.31 ± 0.56	2.38 ± 0.54
Infralimbic (IL)	1.71 ± 0.42	1.58 ± 0.33	1.46 ± 0.33	1.28 ± 0.26	2.04 ± 0.57	2.17 ± 0.53
Cingulate (Cg)	1.54 ± 0.34	2.02 ± 0.32	1.85 ± 0.35	1.66 ± 0.36	2.53 ± 0.32	2.07 ± 0.22
Primary Motor (M1)	1.40 ± 0.45	1.34 ± 0.44	1.22 ± 0.32	1.06 ± 0.29	1.73 ± 0.61	1.79 ± 0.49
Secondary Motor (M2)	1.76 ± 0.54	1.55 ± 0.49	1.33 ± 0.36	1.17 ± 0.29	1.98 ± 0.67	2.16 ± 0.57
Agranular Insular (AIC)	1.23 ± 0.42	1.32 ± 0.34	1.35 ± 0.35	0.99 ± 0.26	1.54 ± 0.50	2.07 ± 0.54
Retrosplenial (Retro C)	0.95 ± 0.35	0.81 ± 0.19	1.17 ± 0.19	0.84 ± 0.14	0.95 ± 0.29	0.54 ± 0.12
Thalamus						
Mediodorsal (MD)	0.89 ± 0.24	0.87 ± 0.27	0.94 ± 0.16	0.85 ± 0.15	1.28 ± 0.29	0.59 ± 0.13
Basal Ganglia						
Dorsolateral Striatum (dlStr)	3.34 ± 0.71	2.72 ± 0.49	2.40 ± 0.34	1.97 ± 0.44	3.28 ± 0.45	2.70 ± 0.41
* Ventromedial Striatum (vmStr)	1.80 ± 0.43	2.49 ± 0.39*	1.70 ± 0.28	1.37 ± 0.28	2.48 ± 0.31*	1.92 ± 0.28
Hippocampus						
Dentate Gyrus (DG)	2.51 ± 0.67	3.52 ± 0.58	3.97 ± 0.45	3.07 ± 0.31	3.80 ± 0.64	3.08 ± 0.47
CA1	2.97 ± 0.57	3.58 ± 0.64	3.95 ± 0.45	2.85 ± 0.32	3.89 ± 0.59	2.85 ± 0.44
CA2	2.73 ± 0.51	2.79 ± 0.43	3.46 ± 0.38	2.70 ± 0.23	3.62 ± 0.49	2.52 ± 0.36
CA3	2.54 ± 0.61	2.56 ± 0.40	2.81 ± 0.35	2.12 ± 0.16	2.77 ± 0.36	2.13 ± 0.35
Mesolimbic						
Nucleus Accumbens Core (NacC)	2.29 ± 0.53	2.37 ± 0.50	1.81 ± 0.37	1.60 ± 0.42	2.47 ± 0.47	1.69 ± 0.29
Nucleus Accumbens Shell (NacS)	2.37 ± 0.63	1.73 ± 0.39	1.57 ± 0.33	1.34 ± 0.31	1.56 ± 0.22	1.47 ± 0.26

Table 4.04 Effects of prenatal PolyIC exposure and peripubertal THC administration on CB_1 receptor binding in adulthood. Data represents mean ± SEM specific [^{3}H]SR141716A binding (fmol/mg tissue) in adult animals. Prenatal exposure to PolyIC (MIA) did not affect CB_1 receptor binding in adulthood. Low-dose intermittent THC (THC A) administration throughout the peripubertal period led to a significant residual increase in CB_1 receptor binding in the ventromedial striatum. * denotes a significant increase in CB_1 receptor binding ($p<0.05$).

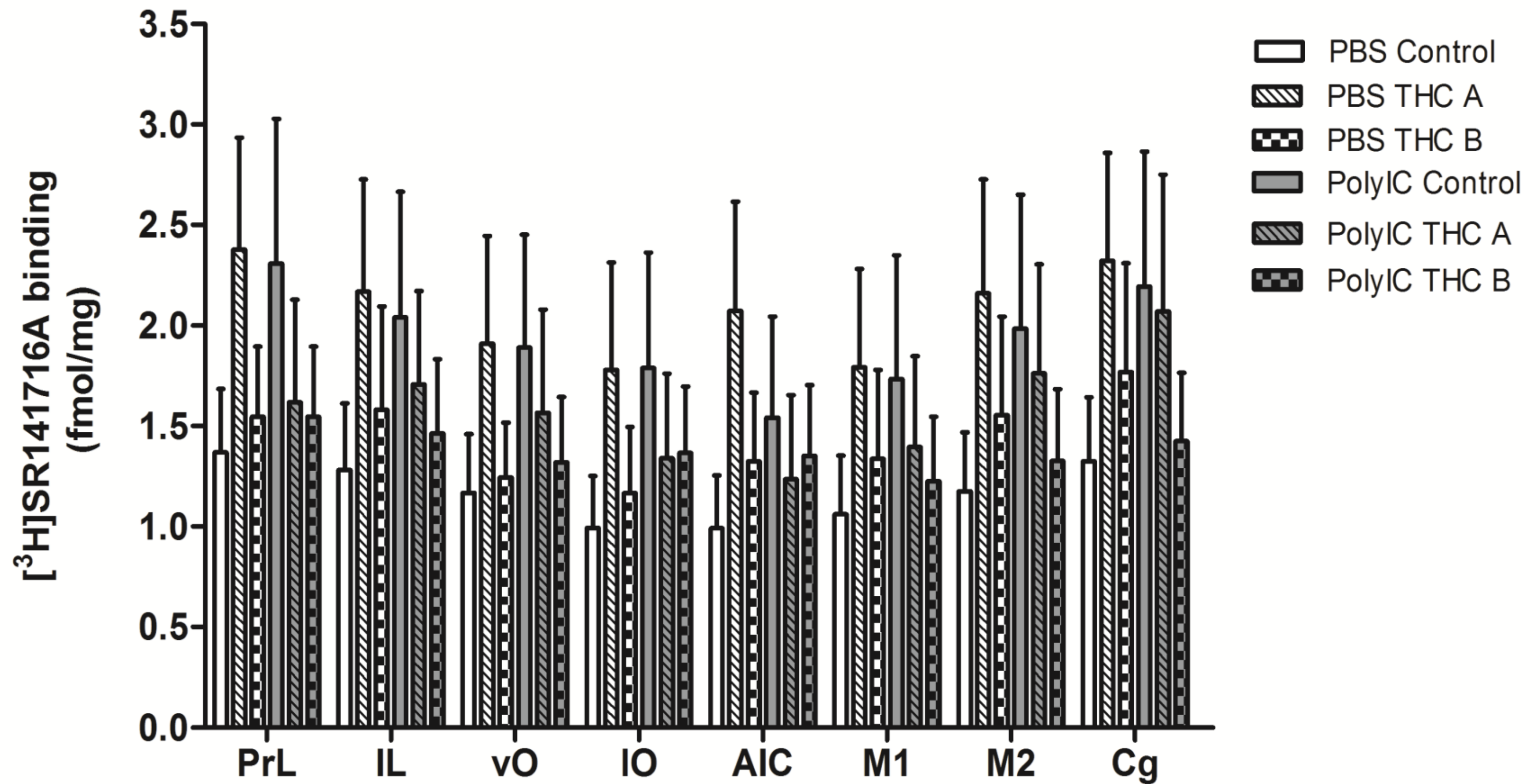


Figure 4.09A Effect of prenatal PolyIC exposure and peripubertal THC treatment on specific CB_1 receptor binding in prefrontal and motor cortices. Values represent mean ± SEM specific [^{3}H]SR141716A binding. No significant effect of prenatal PolyIC treatment (MIA) or peripubertal THC treatment on CB_1 receptor binding levels was observed within the prelimbic (PrL), infralimbic (IL), ventral (vO) and lateral orbital (lO), agranular insular (AIC), primary (M1) and secondary (M2) motor cortices.

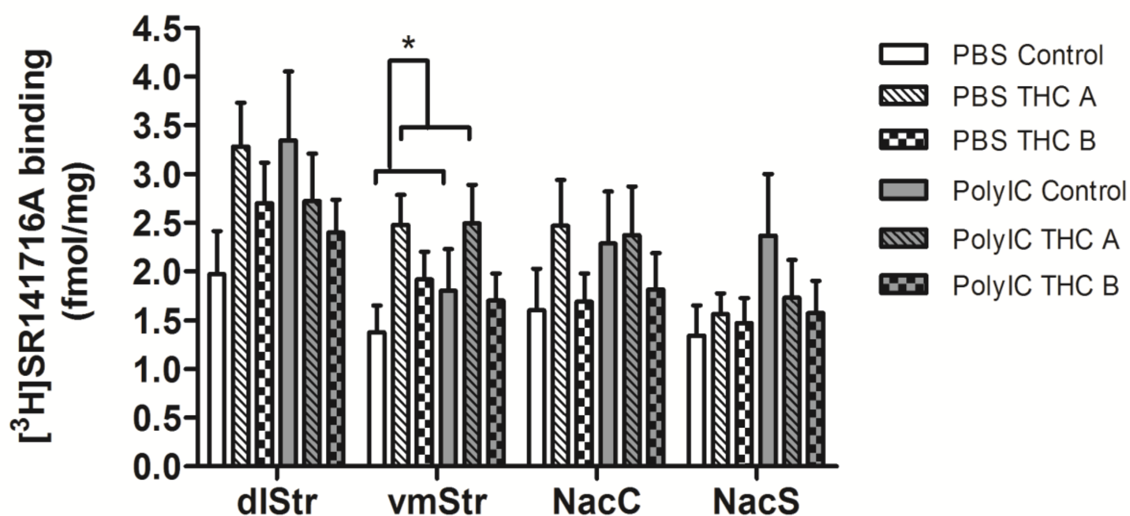


Figure 4.09B Effect of prenatal PolyIC exposure and peripubertal THC treatment on specific CB_1 receptor binding in mesolimbic brain structures Values represent mean ± SEM specific [^{3}H]SR141716A binding. CB_1 binding was significantly increased in the ventromedial striatum (vmStr) as a result of low-dose intermittent THC treatment (THC A) throughout the peripubertal period ($F_{(5,54)}$=4.07, p=0.023). This effect was not evident in the dorsolateral striatum (dlStr) or the nucleus accumbens core and shell (NacC and NacS respectively). Prenatal PolyIC treatment (MIA) did not affect CB_1 receptor binding in mesolimbic brain structures.

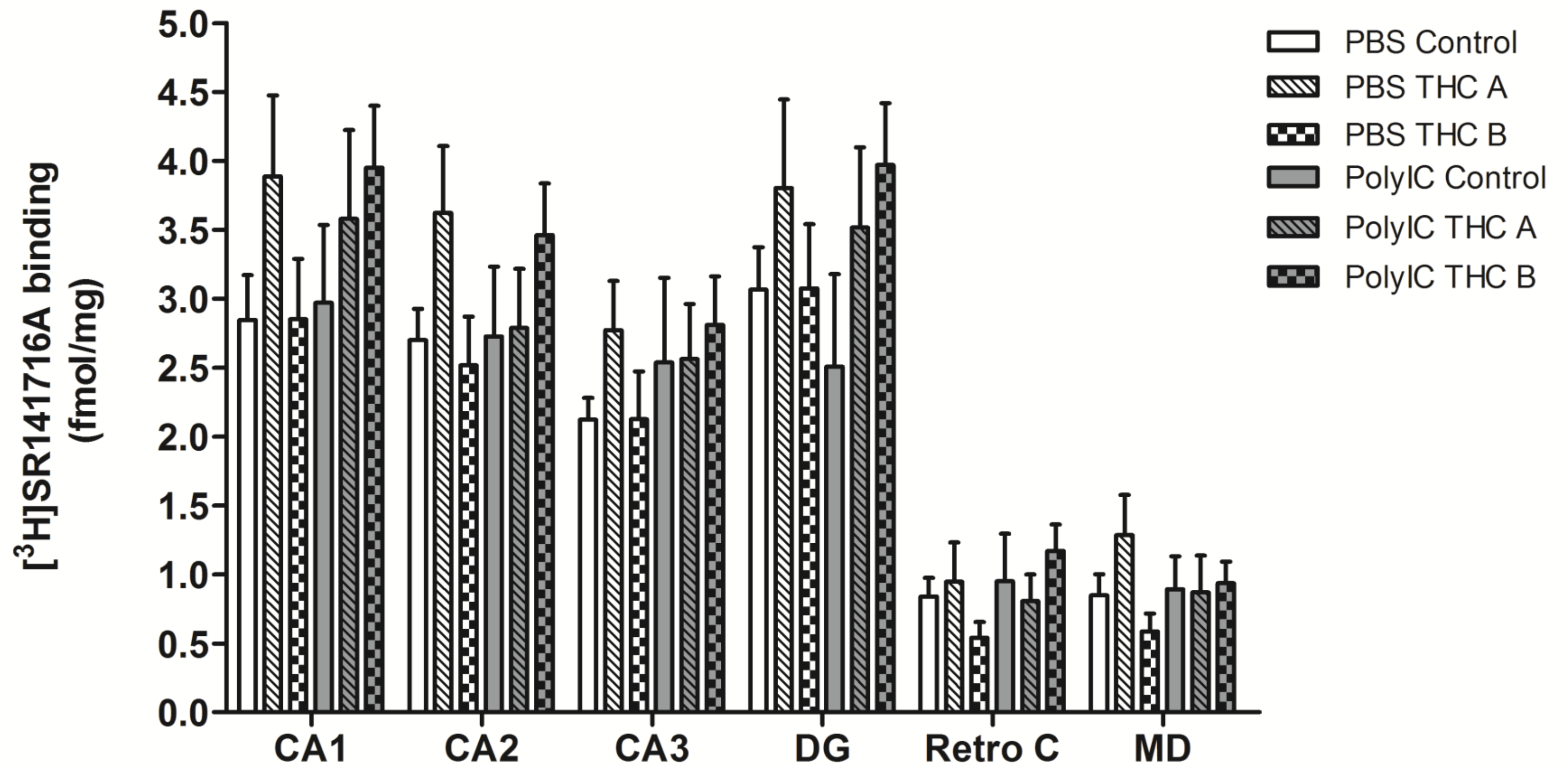


Figure 4.09D Effect of prenatal PolyIC exposure and peripubertal THC treatment on specific CB_1 receptor binding in hippocampal formation, retrosplenial cortex and mediodorsal thalamus. Values represent mean ± SEM specific [^{3}H]SR141716A binding. No significant effect of prenatal PolyIC exposure (MIA) or peripubertal THC treatment on CB_1 receptor binding levels was observed within the CA1, CA2, CA3 and dentate gyrus (DG) subdivisions of the hippocampal formation, the retrosplenial cortex (Retro C) and the mediodorsal thalamus (MD).

4.4.3 |Effects of Maternal Immune Activation and Peripubertal THC Treatment on Regional Cerebral Metabolism

Terminal plasma variables and WBA ^{14}C levels from animals in each treatment group were analysed using general linear univariate ANOVA models (Table 4.05). There were no significant differences in plasma ^{14}C or plasma glucose levels between experimental groups. There was also no significant difference in WBA^{14}C concentrations between experimental groups suggesting that neither prenatal PolyIC exposure (MIA) nor peripubertal THC treatment (PD 35-56) with low-dose intermittent (3.5mg/kg 3 times a week) altered plasma parameters or WBA^{14}C levels in a way in which may confound the interpretation of the ^{14}C-2DG uptake ratio as a reflection of cerebral metabolism.

Prenatal PolyIC exposure and peripubertal THC treatment led to both significant increases and decreases in LCGU on a region-dependent basis in four of the 66 RoI analysed. Prenatal PolyIC treatment led to altered LCGU in three RoI within specific sub-divisions of the hippocampus and PFC. Peripubertal THC treatment significantly affected LCGU in only two out of 66 cerebral structures examined namely the dorsolateral orbital cortex and the nucleus accumbens core. One significant prenatal PolyIC x peripubertal THC treatment interaction was observed in the primary motor cortex. The effects of prenatal PolyIC exposure and peripubertal THC treatment on all 66 RoI measured are detailed in Table 4.06.

Hypermetabolism was observed in PolyIC-treated offspring compared to PBS-treated offspring within the dorsolateral orbital cortex ($F_{(1,34)}$=5.41, p=0.026, Fig 4.10A) and cingulate cortex ($F_{(1,36)}$=4.35, p=0.044, Fig 4.10A-B). Conversely, a significant reduction in LCGU was observed in the CA3 subdivision of the hippocampus in PolyIC-treated offspring compared to PBS-treated offspring ($F_{(1,38)}$=7.63, p=0.009, Fig 4.10A). Furthermore, a significant pretreatment x treatment interaction was observed within the primary motor cortex ($F_{(1,36)}$=7.01, p=0.012). *Post-hoc* analysis revealed that prenatal

PolyIC exposure followed by intermittent treatment with low dose THC (THC A) during the peripubertal period significantly increased glucose uptake in this RoI compared to PolyIC-treated offspring whom received vehicle treatment throughout the peripubertal period ($p<0.05$, Fig 4.12).

Of the two RoI that exhibited significant alterations in LCGU following peripubertal THC treatment, opposing effects of THC on LCGU was observed. A similar pattern of PolyIC-induced hypermetabolism in the dorsolateral orbital cortex was observed following peripubertal treatment with low-dose intermittent THC treatment ($F_{(1,34)}=6.80$, $p=0.013$, Fig 4.11). Peripubertal THC treatment induced significant hypometabolism in the nucleus accumbens core ($F_{(1,38)}=6.92$, $p=0.012$, Fig 4.11).

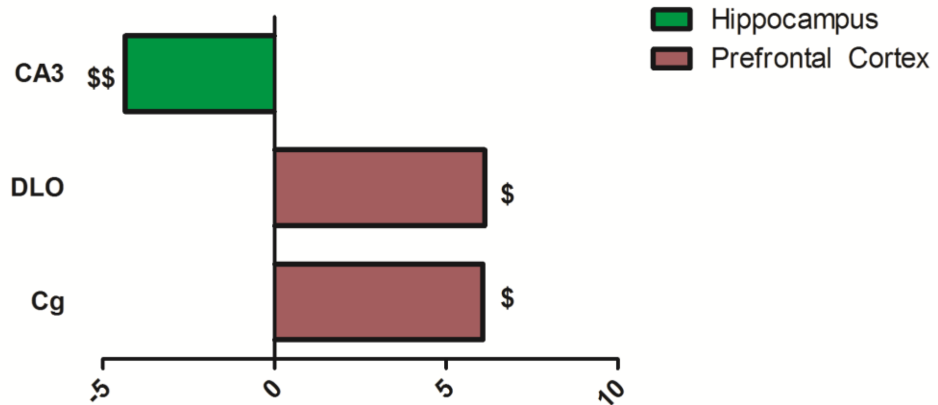
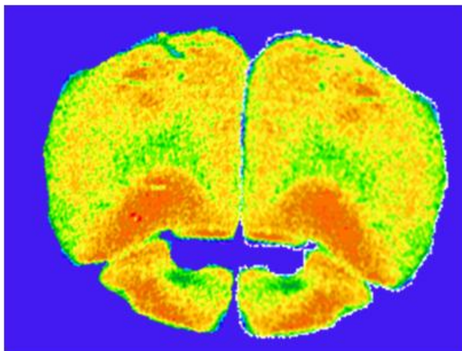
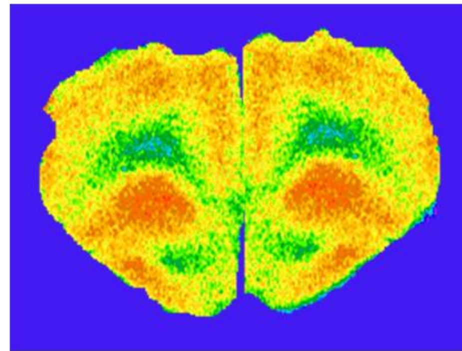


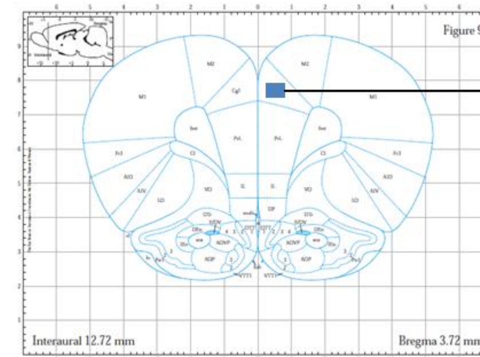
Figure 4.10A Percentage differences in overt cerebral metabolism in adult PolyIC-treated offspring relative to adult PBS-treated offspring. Data from all experimental groups were analysed using univariate general linear ANOVA models. In PolyIC-treated animals (MIA), cerebral glucose metabolism was significantly increased in the dorsolateral orbital cortex (DLO) and anterior cingulate cortex (Cg) relative to PBS-treated offspring (control). PolyIC-treated animals exhibited significant hypometabolism in the CA3 region of the hippocampus relative to PBS-treated animals. As there was no significant evidence for prenatal PolyIC exposure x peripubertal THC treatment interactions in any of these regions, data are shown as % difference in pooled PolyIC data relative to PBS data irrespective of THC treatment. \$p < 0.05 and \$\$p < 0.01 denotes a significant main effect of prenatal PolyIC exposure on cerebral glucose metabolism. Raw data are shown in Table 4.06.

PBS-treated Offspring



PolyIC-treated Offspring





anterior cingulate cortex

Figure 4.10B Autoradiograms illustrating PolyIC-induced hypermetabolism in the anterior cingulate cortex These representative autoradiogram images illustrate significant hypermetabolism (as measured by LCGU) in the anterior cingulate cortex in PolyIC-treated offspring relative to PBS-treated offspring (control). Measurements of anterior cingulate cortex were taken as anatomically defined according to Paxinos & Watson's atlas of the rat brain (6th Edition) as illustrated in sections on the far right panel.

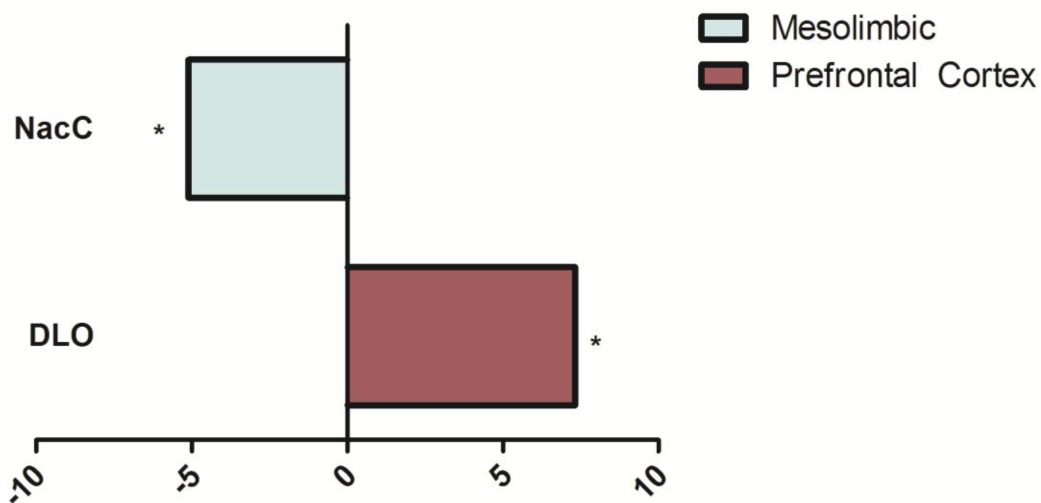


Figure 4.11A Significant differences in overt cerebral metabolism in THC-treated animals compared to vehicle-treated animals. Data from all experimental groups were analysed using univariate general linear ANOVA models. Animals who received low-dose intermittent THC treatment (THC A) throughout the peripubertal period exhibited significant hypermetabolism in the dorsolateral orbital cortex (DLO) and hypometabolism in the nucleus accumbens core (NacC) as compared to vehicle-treated animals. As there was no significant evidence for prenatal PolyIC exposure x peripubertal THC interactions in any of these regions, data are shown as % difference in pooled THC data relative to vehicle data irrespective of prenatal treatment with PolyIC. $*p<0.05$ denotes a significant main effect peripubertal THC treatment on cerebral glucose metabolism. Raw data are shown in Table 4.06.

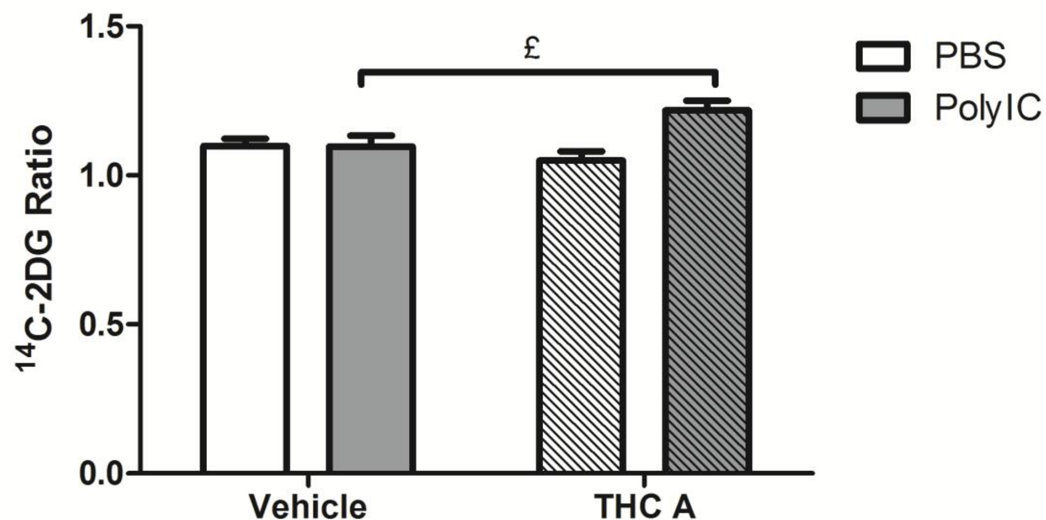


Figure 4.12 Significant interactive effect of prenatal PolyIC administration and peripubertal THC treatment on cerebral metabolism in the primary motor cortex. Data from all experimental groups were analysed using univariate 2 factor general linear ANOVA model. £ $p<0.05$ denotes a significant prenatal PolyIC exposure (MIA) x peripubertal THC treatment interaction in the primary motor cortex. *Post-hoc* analysis revealed that PolyIC-treated animals who subsequently received low-dose intermittent THC treatment throughout the peripubertal period exhibited significant hypermetabolism in the primary motor cortex as compared to PolyIC-treated animals who received vehicle throughout the peripubertal period. Raw data are shown in Table 4.06.

Table 4.05 Terminal plasma parameters and whole brain average ^{14}C levels

	PBS-treated Offspring		PolyIC-treated Offspring	
	vehicle	THC A	vehicle	THC A
	mean ± SEM	mean ± SEM	mean ± SEM	mean ± SEM
Plasma ^{14}C (nCi/ml)	49.04 ± 5.96	44.81 ± 7.41	52.06 ± 4.64	37.92 ± 3.79
Plasma Glucose (mg/ml)	8.47 ± 0.30	9.30 ± 0.31	9.13 ± 0.22	9.11 ± 0.24
WBA ^{14}C (nCi/gm)	219.01 ± 16.11	240.81 ± 28.68	202.29 ± 21.14	215.62 ± 14.36

Table 4.05 Terminal plasma parameters and whole brain average ^{14}C levels from all experimental groups. Data are shown as mean ± SEM. Data was analysed using univariate general linear ANOVA models. No significant differences in WBA ^{14}C, plasma glucose or plasma ^{14}C levels were observed in any experimental groups suggesting that prenatal PolyIC exposure (MIA) or low-dose intermittent THC treatment (THC A) did not alter the ability of 2DG to enter the brain from the plasma.

Table 4.06. Effects of prenatal PolyIC exposure and peripubertal THC treatment on cerebral metabolism

	PBS-treated Offspring						PolyIC-treated Offspring					
	vehicle			THC A			vehicle			THC A		
	mean	±	SEM	mean	±	SEM	mean	±	SEM	mean	±	SEM
Cortex												
Medial Orbital (mO)	1.43	±	0.03	1.40	±	0.03	1.46	±	0.04	1.45	±	0.07
Ventral Orbital (vO)	1.85	±	0.05	1.77	±	0.06	1.81	±	0.03	1.80	±	0.05
Lateral Orbital (lO)	1.85	±	0.05	1.80	±	0.07	1.89	±	0.03	1.87	±	0.04
*$ Dorsolateral Orbital (DLO)	1.12	±	0.03	1.16	±	0.03*	1.15	±	0.03$	1.30	±	0.05*$
Prelimbic (PrL)	1.17	±	0.02	1.14	±	0.03	1.19	±	0.03	1.20	±	0.05
Infralimbic (IL)	1.13	±	0.04	1.19	±	0.02	1.18	±	0.03	1.14	±	0.05
$ Cingulate (Cg)	1.22	±	0.03	1.12	±	0.04	1.22	±	0.04$	1.26	±	0.04$
£ Primary Motor (M1)	1.10	±	0.03	1.05	±	0.03	1.10	±	0.04	1.22	±	0.03
Secondary Motor (M2)	1.21	±	0.03	1.17	±	0.03	1.26	±	0.03	1.26	±	0.05
Agranular Insular (AIC)	1.09	±	0.03	1.11	±	0.04	1.08	±	0.02	1.17	±	0.02
Piriform (Pir)	1.47	±	0.05	1.38	±	0.04	1.39	±	0.04	1.42	±	0.03
Retrosplenial (Retro C)	1.39	±	0.02	1.39	±	0.04	1.41	±	0.04	1.32	±	0.03
Auditory (Aud C)	1.46	±	0.06	1.45	±	0.04	1.47	±	0.04	1.37	±	0.05
Entorhinal (Ento C)	0.82	±	0.02	0.88	±	0.03	0.85	±	0.02	0.85	±	0.03
Visual (Visual C)	1.31	±	0.05	1.44	±	0.07	1.44	±	0.05	1.32	±	0.07
Thalamus												
Anterodorsal (AD)	1.26	±	0.03	1.25	±	0.02	1.27	±	0.06	1.22	±	0.05
Anteroventral (AV)	1.55	±	0.04	1.56	±	0.03	1.57	±	0.04	1.45	±	0.06
Anteromedial (AM)	1.44	±	0.06	1.39	±	0.08	1.49	±	0.06	1.35	±	0.09
Reticular (Rt)	1.36	±	0.04	1.33	±	0.03	1.31	±	0.03	1.26	±	0.04
Mediodorsal (MD)	1.46	±	0.05	1.55	±	0.04	1.55	±	0.05	1.49	±	0.05
Ventroanterior Thalamus (VA)	0.82	±	0.01	0.82	±	0.02	0.78	±	0.01	0.81	±	0.02
Ventrolateral Thalamus (VL)	0.82	±	0.02	0.82	±	0.02	0.79	±	0.02	0.80	±	0.02
Centromedial Thalamus (CM)	0.79	±	0.01	0.78	±	0.02	0.75	±	0.02	0.73	±	0.01
Ventromedial Posterior Thalamus (VMP)	0.93	±	0.03	0.91	±	0.02	0.90	±	0.03	0.88	±	0.02
Posterior Thalamus (Post T)	0.82	±	0.03	0.79	±	0.02	0.80	±	0.02	0.81	±	0.02
Parataenial Thalamus (PT)	1.04	±	0.02	1.05	±	0.03	1.02	±	0.03	1.05	±	0.02
Paraventricular (PV)	1.36	±	0.04	1.36	±	0.03	1.30	±	0.03	1.33	±	0.03
Hypothalamus												
Lateral Hypothalamus (Lat Hypo)	0.76	±	0.02	0.76	±	0.02	0.74	±	0.02	0.75	±	0.03
Amygdala												
Basolateral (BLA)	1.24	±	0.04	1.30	±	0.02	1.25	±	0.04	1.26	±	0.06
Medial (MA)	0.62	±	0.01	0.61	±	0.01	0.58	±	0.02	0.71	±	0.08
Central (CA)	0.95	±	0.02	0.97	±	0.04	0.94	±	0.04	1.05	±	0.05
Basal Ganglia												
Globus Pallidus (GP)	0.83	±	0.02	0.80	±	0.01	0.80	±	0.02	0.77	±	0.02
Ventral Pallidium (VP)	0.74	±	0.02	0.71	±	0.03	0.77	±	0.03	0.71	±	0.03
Dorsolateral Striatum (dlStr)	1.33	±	0.01	1.25	±	0.02	1.28	±	0.02	1.26	±	0.03
Ventromedial Striatum (vmStr)	1.26	±	0.03	1.26	±	0.04	1.25	±	0.03	1.28	±	0.03
Substantia Nigra pars reticulata (SNR)	0.78	±	0.02	0.81	±	0.02	0.78	±	0.02	0.80	±	0.04
Substantia Nigra pars compacta (SNC)	1.10	±	0.02	1.18	±	0.02	1.18	±	0.03	1.18	±	0.05
Subthalamic Nucleus (Subthal N)	1.22	±	0.03	1.27	±	0.04	1.24	±	0.03	1.30	±	0.03
Hippocampus												
Dentate Gyrus (DG)	0.82	±	0.03	0.79	±	0.02	0.80	±	0.02	0.81	±	0.02
CA1	0.82	±	0.01	0.82	±	0.02	0.78	±	0.01	0.81	±	0.02
CA2	0.82	±	0.02	0.82	±	0.02	0.79	±	0.02	0.80	±	0.02
$$ CA3	0.79	±	0.01	0.78	±	0.02	0.75	±	0.02$$	0.73	±	0.01$$
Dorsal Subiculum (dSub)	1.04	±	0.04	1.06	±	0.03	1.08	±	0.03	1.02	±	0.04
Ventral Subiculum (vSub)	0.92	±	0.04	0.97	±	0.02	0.97	±	0.02	0.89	±	0.04
Molecular Layer (Mol L)	1.04	±	0.02	1.05	±	0.03	1.02	±	0.03	1.05	±	0.02
Septohippocampal Nucleus (Shi)	0.59	±	0.02	0.57	±	0.03	0.56	±	0.02	0.59	±	0.04
Septum												
Laterodorsal Septum (LSD)	0.65	±	0.02	0.67	±	0.01	0.62	±	0.02	0.64	±	0.03
Lateral Intermediate Septum (LSi)	0.76	±	0.02	0.79	±	0.02	0.76	±	0.02	0.79	±	0.02
Medial Septum (MS)	1.06	±	0.05	1.04	±	0.02	1.07	±	0.04	1.02	±	0.04
Diagonal Band of Broca												
Vertical Band (VDB)	1.09	±	0.05	1.07	±	0.02	1.04	±	0.05	1.07	±	0.04
Horizontal Band (HDB)	1.07	±	0.05	1.06	±	0.02	1.03	±	0.04	1.09	±	0.03
Mesolimbic												
* Nucleus Accumbens Core (NacC)	1.18	±	0.02	1.08	±	0.03*	1.10	±	0.02	1.08	±	0.01*
Nucleus Accumbens Shell (NacS)	1.00	±	0.05	0.93	±	0.05	0.93	±	0.04	0.94	±	0.04
Ventral Tegmental Area (VTA)	0.80	±	0.03	0.76	±	0.03	0.78	±	0.03	0.79	±	0.03
Neuromodulatory												
Dorsal Raphe (DR)	1.14	±	0.04	1.14	±	0.02	1.10	±	0.03	1.08	±	0.03
Median Raphe (MR)	1.10	±	0.03	1.10	±	0.02	1.16	±	0.02	1.11	±	0.02
Locus Coerulus (LC)	0.93	±	0.02	0.91	±	0.02	0.92	±	0.02	0.92	±	0.04
Mutimodal												
Medial Habenula (MHb)	0.89	±	0.04	0.92	±	0.03	0.93	±	0.03	0.92	±	0.06
Lateral Habenula (LHb)	1.31	±	0.03	1.41	±	0.05	1.39	±	0.04	1.31	±	0.04
Medial Geniculate (Med Gen)	1.41	±	0.07	1.44	±	0.08	1.46	±	0.08	1.48	±	0.08
Lateral Geniculate (Lat Gen)	1.45	±	0.03	1.54	±	0.04	1.54	±	0.03	1.48	±	0.03
Inferior Colliculus (Inf Coll)	1.19	±	0.02	1.20	±	0.04	1.24	±	0.04	1.19	±	0.01
Mamillary Body (Mam Body)	1.51	±	0.04	1.51	±	0.06	1.57	±	0.06	1.56	±	0.09
Interpeduncular Nucleus (IPN)	1.04	±	0.03	1.10	±	0.03	1.12	±	0.05	1.11	±	0.06
Pontine Nucleus (PN)	1.05	±	0.03	0.99	±	0.04	1.06	±	0.04	1.04	±	0.05
Dorsal Tegmental Nucleus (DTN)	1.19	±	0.03	1.21	±	0.03	1.15	±	0.03	1.21	±	0.05

Table 4.06 Effects of prenatal PolyIC exposure and peripubertal THC treatment on overt regional cerebral metabolism in adult animals (PD70). Data was analysed using univariate general linear ANOVA models. Data represents mean ± SEM ^{14}C-2DG uptake ratio. *p<0.05 denotes a significant main effect of low-dose intermittent THC treatment (THC A) on cerebral glucose metabolism. $p<0.05, $$p<0.01 denotes a significant main effect of prenatal PolyIC administration (MIA) on cerebral glucose metabolism. £p<0.05 denotes a significant prenatal PolyIC exposure x peripubertal THC treatment interaction in the primary motor cortex. *Post-hoc* analysis revealed that PolyIC-treated animals who subsequently received low-dose intermittent THC treatment throughout the peripubertal period exhibited significant hypermetabolism in the primary motor cortex as compared to PolyIC-treated animals who received vehicle throughout the peripubertal period.

4.4.4 |Functional Connectivity Analysis Using Partial Least Squares Regression

In order to elucidate alterations in regional functional connectivity as a result of prenatal PolyIC treatment (MIA) and exposure to low-dose intermittent THC throughout the peripubertal period, the multivariate analysis PLSR was employed (Dawson *et al*., 2010). Discrete brain regions that exhibited significant alterations in cerebral metabolism as a result of either prenatal PolyIC or peripubertal THC treatment were considered as 'seed' regions.

4.4.4.1 |PolyIC-induced Alterations in Regional Functional Connectivity

The functional connectivity of the dorsolateral orbital cortex and anterior cingulate cortex together with the CA3 subdivision of the hippocampus were considered as 'seed' regions when characterising PolyIC-induced (MIA) alterations in regional functional connectivity as these three regions showed significant long-term alterations in cerebral metabolism as a result of prenatal PolyIC exposure (section 4.4.3). PolyIC-induced alterations in the connectivity of these regions are summarised in Fig 4.13A-C.

In the dorsolateral orbital cortex a widespread reduction in functional coupling between significantly connected brain regions was observed with the one exception of the anterior cingulate cortex, where prenatal PolyIC treatment led to an increase in functional connectivity to the dorsolateral orbital cortex. MIA resulted in decreased functional coupling with two subfields of the hippocampus, namely, CA1 and ventral subiculum. This loss in functional connectivity was also observed in stimulus processing centres such as the auditory and piriform cortices, ventromedial striatum, ventral tegmental area, posterior thalamus and lateral geniculate nucleus. Functional connectivity between dorsolateral orbital cortex and other components of the PFC was altered in a complex manner with MIA leading to both an increase and decrease in

functional connectivity with the anterior cingulate and prelimbic cortices respectively (Fig 4.13A).

Coupling between the anterior cingulate cortex and other components of the PFC, namely the ventral orbital and prelimbic cortices, was significantly increased as a result of MIA. MIA led to increased coupling between the anterior cingulate cortex and the medial septum, ventral tegmental area and motor processing regions such as the primary motor cortex and the pontine nucleus. In contrast, MIA resulted in decreased functional connectivity between the molecular layer of the hippocampus and lateral habenular nucleus. A complex pattern of functional coupling was observed in the thalamus, with increases and decreases in functional connectivity observed on a subfield-dependent basis. Increases in functional coupling was observed in multiple subdivisions of the amygdala, namely, the basolateral and medial nuclei of the amygdala (Fig 4.13B)

In the CA3 subdivision of the hippocampus, MIA led to widespread reduction in functional coupling between significantly connected brain regions with the one exception of the ventromedial posterior thalamus. MIA led to a loss in coupling between CA3 and an associated component of the hippocampal formation, the dentate gyrus. MIA significantly decreased CA3 connectivity to the basolateral amygdala, substantia nigra reticulata, lateral hypothalamus and lateral orbital and primary motor cortices. Similarly, there was a significant reduction in functional coupling of the medial and lateral habenular nuclei and their afferent projection the interpeduncular nucleus from CA3 as a result of prenatal PolyIC exposure on GD15. Similar to the functional connectivity of the anterior cingulate cortex, a complex pattern of functional coupling was observed between the CA3 subfield of the hippocampus and the thalamus, with increases and decreases in functional connectivity observed on a subfield-dependant basis (Fig 4.13C).

Fig 4.13A-B Significant PolyIC-induced alterations in functional connectivity in the dorsolateral orbital and cingulate cortices respectively

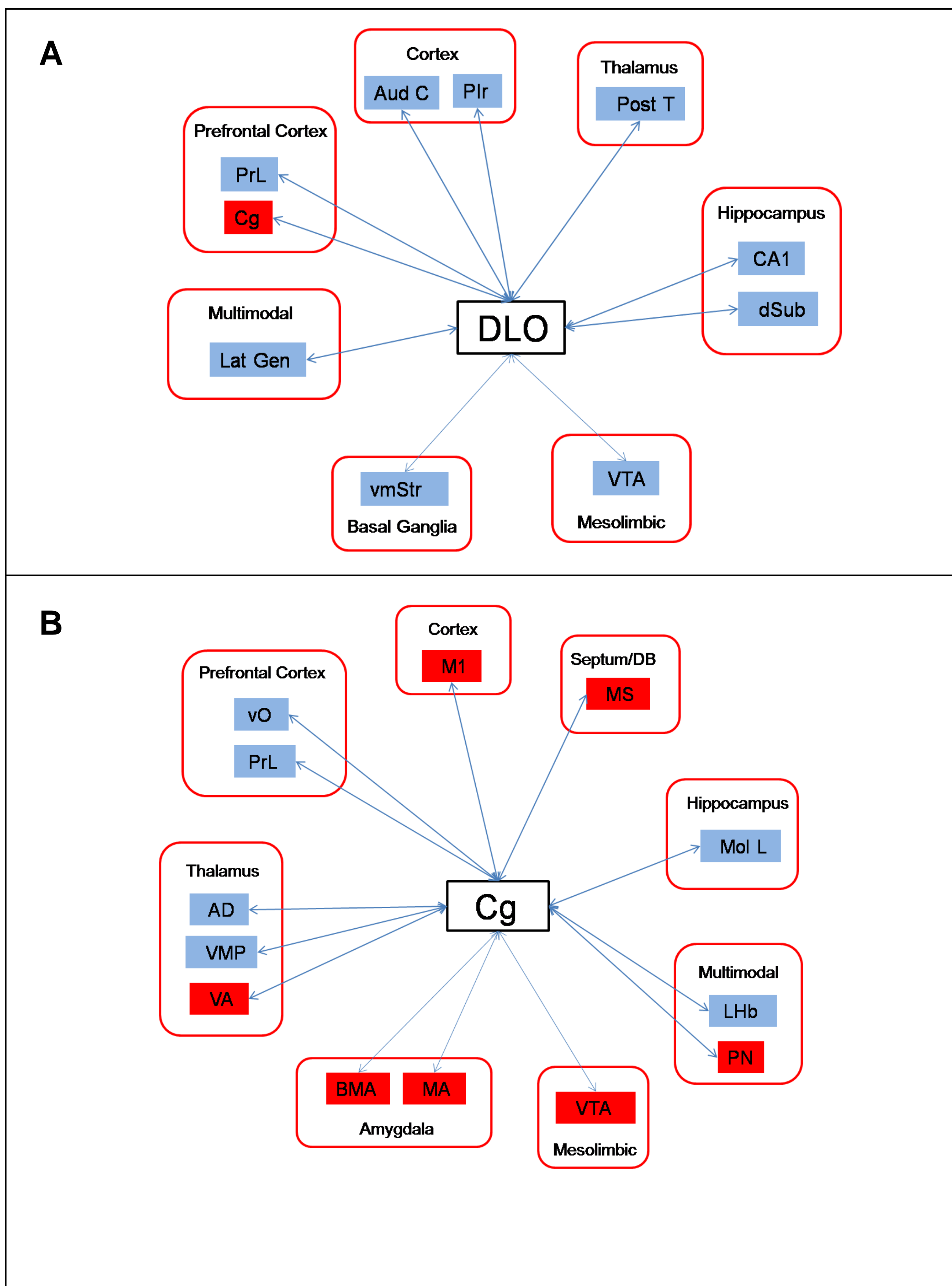


Fig 4.13C Significant PolyIC-induced alterations in functional connectivity in the CA3 subfield of the hippocampus

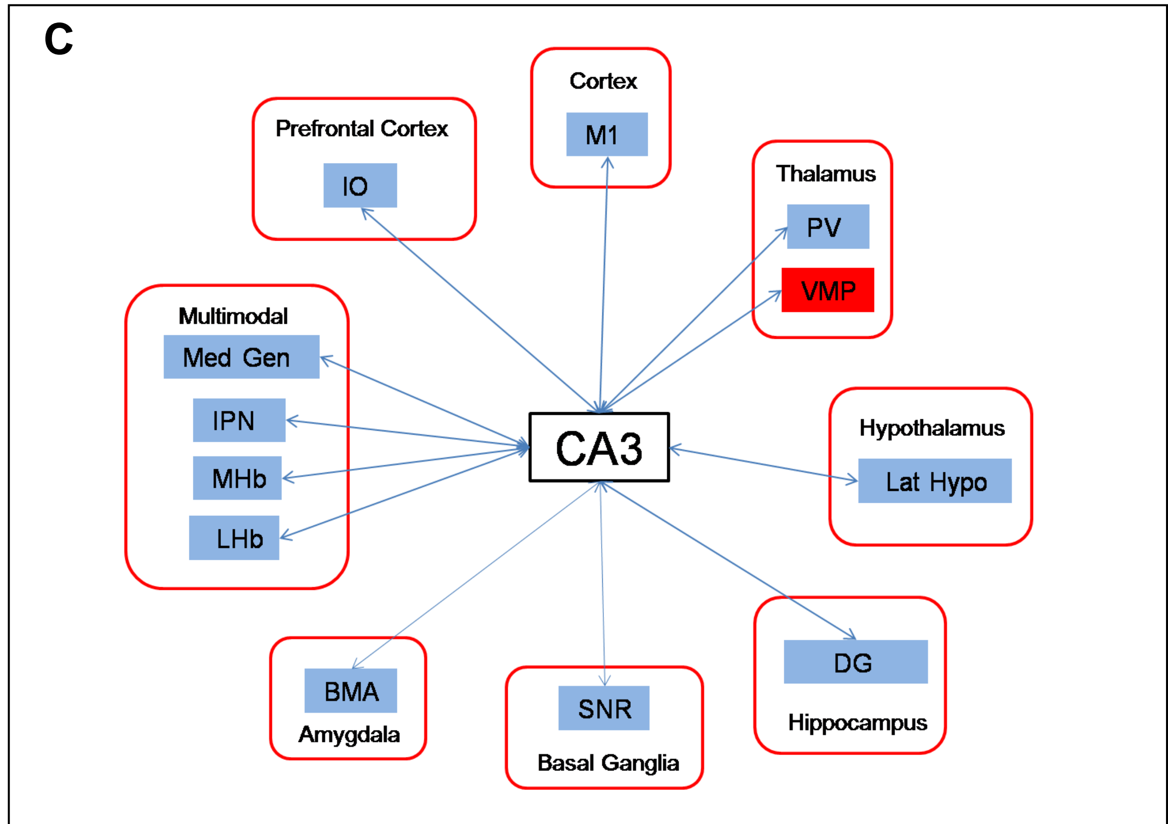


Figure 4.13A-C Summary of significant PolyIC-induced alterations in functional connectivity in the dorsolateral and cingulate cortices and the CA3 subdivision of the hippocampus. Dorsolateral orbital (DLO) and cingulate (Cg) cortices and the CA3 subdivision of the hippocampus were defined as 'seed' regions significantly affected by prenatal PolyIC treatment (MIA). Functionally connected regions to the 'seed region' were defined as regions where the 95% CI of the VIP statistic exceeded 0.8 in either experimental group. Alterations in functional connectivity arising from prenatal PolyIC exposure were analysed using Student's *t*-test followed by Bonferroni post-hoc correction. Significance was set at $p<0.05$. Red and blue boxes denote a significant increase and decrease respectively in the strength of a given functional connection in PolyIC-treated offspring (for key to brain structures see abbreviation list).

4.4.4.2 |THC-induced Alterations in Regional Functional Connectivity

The functional connectivity of nucleus accumbens core and the dorsolateral cortex were considered as 'seed' regions when characterising THC-induced alterations as these two regions showed significant alterations in cerebral metabolism as a result of peripubertal THC treatment. THC-induced alterations in the connectivity of these regions are summarised in Fig 4.14A-B.

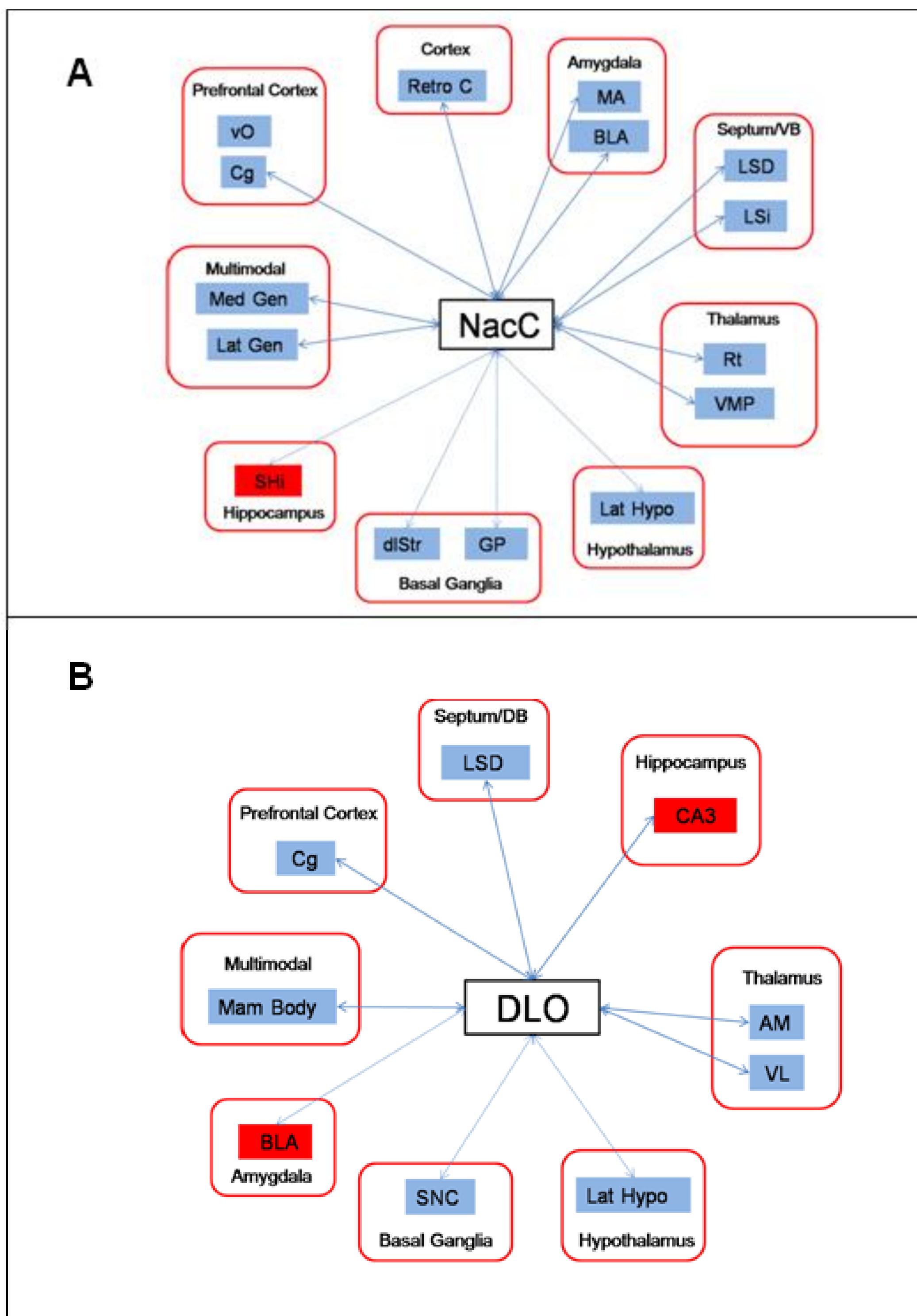
In the nucleus accumbens core, low-dose intermittent THC treatment (3.5mg/kg, 3 times a week) throughout the peripubertal period (THC A) led to a widespread decrease in functional connectivity between significantly connected brain regions with the one exception of the septohippocampal nucleus. THC treatment led to a decrease in functional coupling in the nucleus accumbens with the basolateral and medial nuclei of the amygdala, dorsal and intermediate portions of the lateral septum and ventral orbital and anterior cingulate components of the PFC. Similarly, the nucleus accumbens core was significantly functionally disconnected with the auditory and visual thalamic processing centres, the medial and lateral geniculate nuclei respectively, lateral hypothalamus and the reticular and ventromedial posterior thalamic nuclei. Functional connectivity was also decreased in the specific subfields of the basal ganglia, namely, the dorsolateral striatum and the globus pallidus (Fig 4.14A).

The dorsolateral orbital cortex exhibited altered cerebral metabolism in response to both MIA and THC treatment, however, two distinctly different patterns of altered functional connectivity were observed as a result of exposure to these two environmental risk factors. THC treatment led to an increase in functional coupling between dorsolateral orbital cortex and the basolateral amygdala and the CA3 subdivision of the hippocampus. Decreased functional activity was observed in the dorsolateral septum, anterior cingulate cortex, lateral hypothalamus, substantia nigra reticulata and mammillary body. Within the thalamus, low-dose intermittent THC treatment

throughout the peripubertal period led to decreased connectivity in the anteromedial and ventrolateral nuclei with the nucleus accumbens core (Fig 4.14B).

In summary, prenatal PolyIC exposure and peripubertal THC treatment produced distinct aberrant functional connectivity profiles. Prenatal exposure to PolyIC on GD15 led to altered functional coupling of discrete components of the PFC and hippocampus with multiple substrates of the mesolimbic system. Peripubertal THC treatment with low-dose intermittent THC produced residual perturbances in the functional coupling of the dorsolateral orbital cortex and nucleus accumbens core with neural correlates of reward-related and goal-directed behaviour.

Fig 4.14A-B Significant THC-induced alterations in functional connectivity in the nucleus accumbens core and dorsolateral orbital cortex respectively



↑ Functional connection

↓ Functional connection

Figure 4.14A-B Summary of significant THC-induced alterations in functional connectivity in the nucleus accumbens core and dorsolateral orbital cortex. Nucleus accumbens core (NacC) and dorsolateral orbital cortex (DLO) were defined as 'seed' regions significantly affected by peripubertal treatment with low-dose intermittent THC (THC A). Functionally connected regions to the 'seed region' were defined as regions where the 95% CI of the VIP statistic exceeded 0.8 in either experimental group. MIA-induced alterations in functional connectivity were analysed using Student's *t*-test followed by Bonferroni post-hoc correction. Significance was set at $p<0.05$. Red and blue boxes denote a significant increase and decrease respectively in the strength of a given functional connection in THC-treated animals (for key to brain structures see abbreviation list).

4.5 |Discussion

4.5.1 |Effects of Maternal Immune Activation and Peripubertal THC Treatment on Schizophrenia-related Phenotypes

4.5.1.1 |Effects of Maternal Immune Activation and Peripubertal THC Treatment on Sensorimotor Gating

Startle Reactivity

Startle reactivity, as measured by the magnitude of startle in response to 120dB startle alone trials within the PPI task, was unaffected by prenatal PolyIC treatment (MIA) with no significant differences in startle reactivity evident in either prepubertal (PD30-32) or adult (PD70) PolyIC-treated offspring as compared to PBS-treated (control) offspring. By contrast, startle reactivity was modified by peripubertal THC treatment in a differential manner dependent on the treatment regime used in this study. Thus, animals that received high-dose daily THC (THC B), mimetic of heavy daily cannabis use in humans, throughout the peripubertal period (PD35-56), exhibited reduced startle reactivity. This effect was not present in animals that received low-dose intermittent THC treatment (THC A), mimetic of light recreational cannabis use in humans. These animals exhibited similar levels of startle reactivity to vehicle-treated animals. The acoustic startle response is an innate protective mechanism that leads to rapid contraction of the musculature in response to an unexpected stimulus (Koch and Schnitzler, 1997). Thus, a decrease in startle reactivity as a result of high-dose daily peripubertal THC treatment may be potentially indicative of a subtle anxiogenic phenotype present amongst this experimental group. This finding is expected given that CBs appear to exhibit a bidirectional effect on anxiety dependent on CB concentration, low doses of CBs are associated with anxiolytic-like behaviours and high doses associated with anxiogenic-like behaviour (Berrendero and Maldonado, 2002; Marín *et al.*, 2003).

Sensorimotor Gating

Sensorimotor gating was not significantly affected by prenatal PolyIC exposure (MIA) or either of the peripubertal THC treatment regimes used in this study. No differences in % PPI to the acoustic startle at the various prepulse stimulus intensities were observed between any experimental groups. Moreover, no interactive effects of these two environmental risk factors were observed in the PPI task.

In this study, the effects of MIA on sensorimotor gating were assessed in both prepubertal and adult offspring. This experimental design was incorporated to establish whether MIA-induced effects were present in both age groups or were subject to a maturational delay characterised by post-pubertal emergence of phenotypic discrepancies akin to the pattern of delayed onset of symptoms amongst schizophrenic patients (Häfner *et al*., 1998). Measurement of PPI in both prepubertal and adult animals led to an interesting finding regarding the impact of age on PPI. Whilst adult animals across all experimental groups exhibit increasing %PPI in accordance to the increasing amplitude of the pre-pulse, prepubertal animals do not appear to exhibit any PPI at any of the thresholds measured. This finding suggests that performance on this task is governed in a maturational manner and the complex neural systems underpinning this behavioural parameter may still be developing in prepubertal animals. Thus, this finding provides further evidence of the late maturation and potential vulnerability of important neural systems governing schizophrenia-related behaviours.

In contrast to previous work investigating the effects of MIA on PPI (Ozawa *et al*., 2006; Wolff and Bilkey, 2008), this present study failed to demonstrate MIA-induced sensorimotor deficits in adult offspring. This discrepancy may be the result of differences in experimental design such as gender and strain differences. For example, Wolff and Bilkey (2008) demonstrated a sensorimotor gating deficit induced by PolyIC at a dose of 4mg/kg, as used in this study; however, female Sprague-Dawley rats were

used whilst in the present study male Hooded-Lister rats were used (Wolff and Bilkey, 2008).

Another pertinent point to discuss is the potential effects of long-term daily handling, as necessitated by the experimental design, during the peripubertal period. The disparities in our findings may be a result of an enrichment effect of peripubertal handling of all experimental animals. To avoid a potential handling effect, all experimental groups were handled and injected with THC or vehicle where appropriate daily from PD35-56. However, studies have shown that regular post-weaning handling can attenuate % PPI deficits induced by exposure to the environmental stress of isolation rearing (Krebs-Thomson *et al.*, 2001). Thus, the potential negative impact of prenatal PolyIC treatment may have been counteracted by an environmental enrichment effect of daily handling preventing the emergence of an overt phenotypic deviation in adulthood.

Similar to MIA, peripubertal THC treatment did not produce any residual effects on sensorimotor gating in adulthood. Although animals treated with high-dose daily THC exhibited differential startle reactivity, this affect was not mirrored in %PPI, as neither high-dose daily THC nor low-dose intermittent THC treatment throughout the peripubertal period affected %PPI in adulthood. The findings in this study are not in keeping with a previous study that demonstrated long lasting deficits in schizophrenia-related PPI following chronic CB peripubertal treatment with the synthetic CB agonist WIN 55,212-2 (Schneider and Koch 2003). One possible explanation for this variation in experimental outcome may be due to the time frame of CB administration. For example, Schneider and Koch (2003) define the peripubertal period as PD40-65 based on the timescale of sexual maturation in a rat. However, in this study, THC treatment period was based on a potential vulnerability window of the peripubertal brain relating to the maturational trajectories of the CB_1 receptor in cerebral areas associated with higher cognitive processing such as the PFC and hippocampus (as defined in chapter two). Furthermore, differences in the pharmacological properties of CBs utilized for peripubertal treatment may explain discrepancies in the CB effect as Schneider and

Koch employed the synthetic CB agonist WIN 55,212-2 whilst THC exhibits only partial agonism to the CB_1 receptor.

In summary, neither MIA nor peripubertal THC treatment resulted in any residual impairment in sensorimotor gating. Furthermore, combined exposure to both these 'early' and 'late' environmental factors, investigated for the first time in this study, do not produce any lasting effects on sensorimotor gating. Thus, although the findings of this study do indicate a late maturational trajectory of this behavioural phenotype, it would appear that the complex neural systems governing sensorimotor gating are not developmentally sensitive to the deleterious effects of either MIA or peripubertal THC treatment.

4.5.1.2 |Effects of Maternal Immune Activation and Peripubertal THC Treatment on Mesolimbic DA Transmission

As with the PPI task, prenatal PolyIC treatment (MIA) and peripubertal THC exposure failed to alter locomotor response in a novel environment. Prenatal PolyIC treatment did not lead to aberrant locomotor activity in response to a novel environment in prepubertal or adult animals. Consistent with this, peripubertal THC treatment did not significantly affect total distance travelled during the 20 minute habituation period therefore did not induce altered locomotion in response to a novel environment in adulthood. Habituation of all experimental groups to the novel environment of the open field arenas took place over the 20 minute testing period.

Studies investigating the effects of MIA on this behavioural assay have produced variable results with some groups noting a hypolocomotor state in MIA offspring whilst others have found no residual effect of MIA on locomotor activity in response to a novel environment (Meyer *et al*., 2005; Ozawa *et al*., 2006; Zuckerman and Weiner, 2005). Thus, the unaltered spontaneous locomotion observed in this study is in keeping with published results by both Osawa and colleagues and Zuckerman and group.

Previous studies have reported enhanced sensitivity of PolyIC-treated offspring to drugs capable of inducing hyperlocomotion (Ozawa *et al*., 2006; Zuckerman and Weiner, 2005). In this study we found no evidence to suggest that prenatal PolyIC treatment enhanced hyperlocomotor responses to amphetamine. The divergence of the results in this study compared to these published studies may be due to variations in experimental design. For example, although Ozawa and colleagues employed the immune activator PolyIC as the source of MIA, PolyIC was administered to pregnant mice at a dose of 5mg/kg over a 6 day consecutive period (GD12-GD17). Precise timing and duration of gestational PolyIC administration has been shown to directly influence the behavioural parameters affected in offspring and may consequently explain the difference in results between the studies (Meyer and Feldon, 2011). In parallel, Zuckerman *et al*. (2005)

observed sensitisation to stimulant-induced hyperlocomotor activity using the NMDA receptor antagonist MK-801 in PolyIC-treated offspring. However, as this study used the indirect DA agonist amphetamine to induce hyperlocomotion; discrepancies in results may be due to recruitment of differential neurotransmitter systems eliciting the observed sensitised hyperlocomotor response.

Interestingly, this study demonstrates the novel finding that PolyIC-treated offspring who subsequently received low-dose intermittent THC treatment during the peripubertal period appeared to show an enhanced sensitivity to amphetamine-induced hyperlocomotion compared to PBS-treated offspring. The indirect DA agonist amphetamine has been shown to induce psychosis in healthy individuals (Angrist *et al.*, 1980) and to also worsen psychotic symptoms amongst schizophrenic patients (Snyder *et al.*, 1974). This behavioural paradigm is commonly used to test for aberrant mesolimbic DA transmission, a key pathological feature of schizophrenia (Breier *et al.*, 1997). A possible interpretation of the apparent synergistic effect between MIA and peripubertal THC treatment on amphetamine-induced hyperlocomotion in our study is that MIA induces aberrations in normal brain development that fail to manifest in the form of a phenotypic discrepancy. However, subsequent THC treatment during the vulnerable peripubertal maturative period can result in the manifestation of an overt schizophrenia-related phenotype, in adulthood, as demonstrated in this study by an increased sensitivity to amphetamine administration. Interestingly, here we see a dose-specific effect of THC in that it is low-dose intermittent treatment (THC A) (mimetic of light recreational cannabis abuse), and not high-dose daily THC treatment (THC B) (mimetic of daily heavy cannabis abuse) during the peripubertal period that results in the emergence of this schizophrenia-related behavioural phenotype in adulthood. This dose-specific effect could reflect the formation of tolerance in response to high doses of THC on a regularly basis but this protective tolerance may not be evoked following low-dose intermittent THC treatment (discussed further in sections 4.5.1.4).

To summarise this section, prenatal exposure to PolyIC and peripubertal THC treatment did not individually produce any significant effects on mesolimbic DA transmission, as measured by locomotor responses in response to novel environment and acute exposure to amphetamine. Interestingly, this study presents the novel finding of a synergistic effect of combined exposure to these ‘early’ and ‘late’ environmental risk factors, as PolyIC-treated offspring who received low-dose intermittent THC treatment during the peripubertal period appeared to show an enhanced sensitivity to amphetamine-induced hyperlocomotion compared to PBS-treated offspring.

4.5.1.3 |Effects of Maternal Immune Activation and Peripubertal THC Treatment on Recognition Memory in Adulthood

Prenatal exposure to PolyIC (MIA) and peripubertal THC treatment did not result in any long-lasting impairments in recognition memory as measured by performance on the NOR task. These findings contrast with previous research investigating the residual effects of exposure to these environmental risk factors, as both MIA manipulation and peripubertal treatment with CBs have been previously shown to induce recognition memory deficits in adulthood.

It is important to note that in the NOR task, PBS-treated offspring who subsequently received vehicle throughout the peripubertal period (control-control animals) demonstrated a statistically significant preference for the novel object over the familiar object in the choice phase of the test. Thus, these animals demonstrated intact object recognition memory. This finding validates the performance measures generated by this paradigm.

Previous studies have found that MIA leads to the post-peripubertal emergence of overt phenotypic alterations in recognition memory (Ozawa *et al*., 2006; Wolff *et al*., 2011). Furthermore, it has been previously demonstrated that peripubertal but not adult treatment with synthetic CBs such as CP-55,940 and WIN 55,212-2 produces long term impairments in the NOR task (Schneider and Koch, 2003; O'Shea *et al*., 2004). Moreover, Quinn *et al*. (2008) showed that THC (5mg/kg) administration on alternate days throughout PD34-55 produced residual deficits in object recognition memory (Quinn *et al*., 2008). Conversely, in a study carried out by Cha *et al*. (2006), chronic peripubertal treatment with THC (5mg/kg) daily throughout PD30-50 did not produce any long lasting deficits in the non-spatial water-maze task in adulthood (Cha *et al*., 2006). This behavioural task is used as a measure of working memory akin to the object recognition memory task. Thus, as mentioned in section 4.5.1.1, variation of results in this study as compared to previous studies may again be due to other factors related to

experimental design such as handling effects, differential treatment regimes and differences in the particular CB_1 receptor agonists used.

In summary, prenatal exposure to the immune-stimulating agent PolyIC and peripubertal THC treatment did not produce any perturbations in object recognition memory in adulthood. Moreover, combined exposure to both these ‘early’ and ‘late’ environmental risk factors did not result in any synergistic aberrations in recognition memory. The findings from the NOR task suggest that the neural substrates principally involved in this behavioural parameter do not exhibit a developmental sensitivity to the potential deleterious effects of either MIA or peripubertal THC administration.

4.5.1.4 |Effects of Maternal Immune Activation and Peripubertal THC Treatment on Cognitive Flexibility in Adulthood

The ASST is used to probe various components of cognitive flexibility. The various discrimination phases are designed to test the animal's cognitive capabilities including rule acquisition, reversal learning, ability to form an attentional set and also abilities to exhibit inhibitory control. The long term effects of prenatal PolyIC exposure (MIA) or peripubertal THC treatment on various aspects of cognitive flexibility, as measured using the ASST, have not previously been investigated.

Within the SD discrimination phase of the task, animals are required to discriminate between two stimuli in the same perceptual dimension (e.g. odour) and learn the discrimination rule. In CD phase of the task the animal must maintain the acquired rule following the subsequent introduction of irrelevant dimensions. MIA or peripubertal THC treatment do not result in any long-lasting impairments in discrimination learning with all experimental groups achieving similar performance levels on the CD discrimination phase of the ASST. Interestingly, within the SD phase, it appeared that MIA following intermittent treatment with low-dose THC (THC A) during the peripubertal period improved performance in the SD phase compared to PBS-treated offspring, as measured by number of successive trials required to reach criterion, but this effect was no longer evident in the more difficult CD phase. This suggests that this initial interaction did not impact discrimination learning.

Performance on the reversal discriminations and ID shift phase is reliant on the ability to shift attention between exemplars within the same perceptual dimension e.g. odour or medium. Within the reversal stages of the task, exemplars and relevant dimensions remain the same but the discrimination rule is reversed whilst within the ID shift phase novel exemplars are introduced but the relevant dimension remains the same. Thus, the reversal stages measure the ability to switch stimulus-reward associations whilst ID phase measures the ability attend to novel exemplars and subsequently abstract the

previously learned rule and apply it to the novel context. Neither MIA nor peripubertal THC treatment affected performance on these discrimination phases. Thus, neither of these environmental factors altered capacity for attentional flexibility towards different stimuli within the same attentional dimension.

During the ED phase of the ASST, the animal is required to shift attentional set from a previously rewarded perceptual dimension (e.g. medium) to a new perceptual dimension (e.g. odour) and disregard the previously learned association, thus, measuring the animal's ability to form a new attentional set and exhibit inhibitory control. It has been shown that the PFC plays a critical role in the mediation of such attentional shifts in rats (Birrell and Brown, 2000). In this present study, PBS-treated offspring who subsequently received vehicle throughout the peripubertal period (control-control animals) found the ED phase significantly harder than the ID phase, demonstrating that attentional set was formed. This finding validates the performance measures generated by this task.

This study has demonstrated the novel finding that low-dose intermittent THC treatment during the peripubertal period induces cognitive inflexibility in adulthood. Interestingly, these deficits were not apparent in animals who received high daily doses of THC during the peripubertal period. These dose-specific effects of THC on cognitive flexibility are in keeping with those observed in the amphetamine sensitisation task, where PolyIC-treated offspring who received low dose intermittent THC treatment (not high-dose daily THC) during the peripubertal period appeared to show an enhanced sensitivity to amphetamine-induced hyperlocomotion compared to PBS-treated offspring. A potential explanation for these treatment-dependent effects is that due to the highly dynamic nature of cerebral development throughout the peripubertal period (as demonstrated in chapter two), higher order association areas such as the PFC and hippocampus are particularly vulnerable to the deleterious effects of THC. Exposure to THC during this critical epoch of neurodevelopment may perturb maturational trajectories resulting in overt deficits in cognitive flexibility in adulthood.

The finding that this schizophrenia-related phenotype was induced by low-dose intermittent THC treatment (mimetic of light recreational cannabis abuse) but not high-dose daily THC treatment (mimetic of heavy daily cannabis abuse) was surprising given that heavy chronic cannabis abuse in adulthood has been associated with decreased mental flexibility, increased perseveration and reduced learning (Pope and Yurgelun-Todd, 1996). However, as adolescent brain development is a highly dynamic period, we suggest that high levels of THC on a daily basis may evoke an adaptive neuroprotective tolerance resulting in desensitisation of CB_1 receptors and thus protecting the brain from the deleterious effects of THC. However, the results of this study suggest that this adaptive feature may not be evoked following low infrequent doses of THC leaving the brain more vulnerable to its adverse effects. This potential neuroprotective mechanism is supported by a recent study by Burston and colleagues in which they found that high peripubertal doses of THC (10mg/kg) administered twice daily resulted in desensitisation of CB_1 receptors in the PFC (Burston *et al*., 2010).

Overall these behavioural data provide new evidence that exposure to environmental insults during critical epochs of neurodevelopment produce both synergistic and individual deleterious effects on schizophrenia-related behaviours in adulthood. Furthermore, the peripubertal brain appears to exhibit a dose-specific liability to the deleterious effects of THC. However, the results obtained from the several behavioural assays employed in this study suggest an overall lack of a combined effect of exposure to 'early' and 'late' environmental risk factors on the precipitation of schizophrenia-related phenotypes in adulthood.

4.5.2 |Residual Dose-specific Effects of Peripubertal THC Treatment on CB_1 Receptor Binding

In order to gain a potential mechanistic insight into the behavioural perturbances observed in this study the effects of prenatal PolyIC exposure (MIA) and peripubertal THC treatment on CB_1 receptor expression were explored.

Levels and patterns of CB_1 receptor binding reported in this present study are in keeping with previously reported literature. A high CB_1 receptor binding profile was observed in the various components of the hippocampal formation. Moderate levels of CB_1 receptor binding were detected in striatal, prefrontal and mesolimbic regions whilst the lowest binding levels were observed in the retrosplenial cortex and mediodorsal thalamus (Herkenham *et al*., 1991).

In this present study, MIA did not produce any long lasting effects on $[^3H]$SR141716A binding. However, low-dose intermittent (3.5mg/kg, 3 times a week) peripubertal THC treatment (THC A) led to an increase in $[^3H]$SR141716A binding within the ventromedial striatum suggesting that up-regulation of the CB_1 receptor in this region had taken place. This effect was not apparent following high-dose daily THC treatment throughout the peripubertal period. This dose-specific up-regulation of the CB_1 receptor appears to be a residual effect as an extensive washout period did follow peripubertal THC treatment.

Previous studies investigating the neurobiological consequences of chronic CB exposure have yielded conflicting results. Burston and colleagues demonstrated that peripubertal high-dose THC treatment (10mg/kg) administered twice daily through PD30-39 led to the down-regulation and desensitisation of CB_1 receptors. Interestingly, they found that peripubertal male animals were less sensitive to the desensitisation effects of THC on CB_1 receptors as compared to adult male rats in the PFC and hippocampus (Burston *et al*., 2010). This suggests that the adolescent brain may be more sensitive to the

deleterious effects of THC. However, it is important to note that in the study by Burston and colleagues, measurement of CB_1 down-regulation and desensitisation took place immediately after the cessation of THC treatment preventing the potential normalisation of CB_1 receptor pharmacological properties following THC treatment. Sim-Selley *et al.* (2006) demonstrated that while long term administration of SR141716A led to region-dependent down-regulation and desensitisation of the CB_1 receptor this effect was no longer apparent following a 14 day drug wash-out period. These findings suggest that the CB_1 receptor undergoes adaptive regulation following discontinuation of CB administration (Sim-Selley *et al.*, 2006). In keeping with these findings, Ellgren and group found that low-dose intermittent treatment (1.5mg/kg/three times a week) throughout PD29-49 did not result in any lasting alterations in CB_1 receptor expression or efficacy when measured in adulthood (Ellgren *et al.*, 2007). These findings are in agreement with the findings of our present study showing that high-dose daily peripubertal THC treatment did not result in any long lasting changes in CB_1 receptor binding. Thus, discordance of results between studies may reflect the varying magnitude and duration of CB_1 receptor adaptation following clearance of the CB_1 receptor agonist.

To date, this study is the first to report an up-regulation of CB_1 receptors as a result of peripubertal THC treatment. Down-regulation and/or desensitisation of CB_1 receptors in response to THC administration is thought to represent a neuroprotective tolerance mechanism. This tolerance phenomenon is thought to be particularly prevalent within motor and limbic structures and has been shown to consequently result in behavioural tolerance with respect to the effect on locomotor activity (Rodríguez de Fonseca *et al.*, 2005). Thus, a possible explanation for this divergent result is that due to the nature and time-course of the low-dose intermittent peripubertal THC treatment regime employed in this experiment, this potential neuroprotective mechanism failed to initiate, resulting in aberrant synaptic pruning within the ventromedial striatum and consequently an atypical binding profile in adulthood. The ventromedial striatum is an integral part of the mesolimbic DA system; thus, the emergence of a residual aberrant CB_1 binding

profile within this region following peripubertal low-dose intermittent THC treatment is in keeping with the observed synergistic effects of MIA and low-dose intermittent THC treatment regime on enhanced responses to amphetamine reported in this present study (section 4.4.1).

4.5.3 |PolyIC-induced Alterations in Cerebral Metabolism and Regional Functional Connectivity

In this present study, it has been demonstrated for the first time that prenatal PolyIC exposure (MIA) leads to long-term alterations in cerebral metabolism and functional connectivity within the dorsolateral and anterior cingulate cortices and the CA3 subdivision of the hippocampus. MIA resulted in a residual hypometabolic state in the CA3 but hypermetabolism in the dorsolateral orbital and anterior cingulate cortex. Lesions to the ventral hippocampus have been shown to produce increased mesolimbic DA responsivity to stressful and pharmacological stimuli (Lipska *et al*., 1993). Furthermore, hippocampal cell loss leads to an upregulation in DA receptor binding in the nucleus accumbens (Bardgett *et al*., 1995). MIA-induced disruption to hippocampal development was highlighted by Zuckerman *et al*. (2003). They found cytoarchitectural abnormalities in the form of pyknosis, an indicator of nuclear destruction, within the CA1-3 subdivisions of the hippocampus in PolyIC-treated offspring (Zuckerman *et al*., 2003). Therefore, the observed MIA-induced hippocampal hypometabolism in this present study may be a consequence of aberrant gestational neuronal development in the hippocampus. Furthermore, this CA3 hypometabolism may in turn be responsible for producing hypermetabolic sequelae in the specific cortical subfields, all of which are directly or indirectly functionally connected to components of the DA mesolimbic system.

Although this study failed to demonstrate a direct effect of MIA on mesolimbic DA transmission potentially due to experimental confounds, the concept of MIA-induced aberrant mesolimbic DA transmission is supported by the finding in this study of a trend towards behavioural synergy between MIA and peripubertal THC treatment resulting in enhanced sensitivity to amphetamine, a behavioural index for deviant mesolimbic DA transmission (section 4.4.1.2). A similar interactive effect between MIA and

peripubertal THC treatment on glucose uptake was observed within the primary motor cortex, MIA followed by intermittent treatment with low-dose THC (THC A) during the peripubertal period significantly increased glucose uptake in this RoI as compared to PolyIC-treated offspring who received vehicle treatment throughout the peripubertal period.

Functional connectivity analysis revealed altered functional coupling between the 'seed' regions and several other components of the mesolimbic system. Within the anterior cingulate cortex, MIA led to increased coupling with distinct nuclei of the amygdala, a prominent emotional processing centre of the mesolimbic system. Conversely, within the CA3 region, MIA induced a decrease in functional coupling with the basolateral nucleus of the amygdala. Altered functional connectivity between all 'seed' regions and subfields of the PFC and hippocampus was observed in PolyIC-treated offspring. Moreover, both the anterior cingulate and dorsolateral orbital cortex exhibited MIA-induced hypermetabolism displayed significantly altered functional coupling with the ventral tegmental area, the origin of dopaminergic cell bodies of the mesolimbic system.

Collectively, these novel cerebral metabolism and functional connectivity data suggest that MIA can lead to disruption to the mesolimbic system in adulthood.

4.5.4 |Peripubertal THC-induced Alterations in Cerebral Metabolism and Regional Functional Connectivity

Throughout adolescence, the brain undergoes important plastic and structural remodelling that allows for refinement and integration of certain brain regions and neural circuitry (Gogtay *et al*., 2004). In particular, this vulnerable developmental epoch is associated with cognitive modifications and acquisition of executive functions as the brain transitions in anatomical control of behaviour, from limbic to PFC-mediated behaviour with an increase in inhibitory connections between the two regions i.e. a shift from affective-driven behaviour to more regulated cognitive-driven behaviour (Nelson *et al*., 2005). Furthermore, within reward-related neural systems, an excess of DA receptors have been found in prepubertal rats which subsequently decrease following age-related synaptic pruning (Tarazi and Baldessarini, 2000). As synaptic pruning is experience-dependent, dysregulation of synaptic pruning mediated by THC treatment may result in abnormal plasticity in reward-related learning processes (Spear, 2000; Ellgren *et al*., 2007). Moreover, such disruption of maturational trajectories may subsequently disrupt the transitional shift in DA balance from mesolimbic to mesocortical systems (Guerri and Pascual, 2010).

In the present study, low-dose intermittent THC treatment, mimetic of light recreational cannabis abuse, throughout the peripubertal period led to long-term modifications in cerebral metabolism with the nucleus accumbens core and dorsolateral orbital cortex, specifically hypometabolism in the nucleus accumbens core and hypermetabolism in the dorsolateral orbital cortex. It has been previously demonstrated that inactivation of the nucleus accumbens core, a major efferent of the PFC, hippocampus and amygdala, severely disrupts set-shifting ability without interfering with rule acquisition indicating the nucleus accumbens core plays a pivotal role in maintenance of novel set shifting strategies and inhibition of irrelevant responses (Floresco *et al.*, 2006). Furthermore, tonic levels of DA release in the nucleus accumbens regulate limbic and cortical drive (Goto and Grace, 2005). Thus, aberrations in DA modulation within the nucleus

accumbens can affect information processing leading to impairments in set-shifting behaviours (Goto and Grace, 2005). In accordance with these previous findings, this study shows for the first time that low-dose intermittent THC treatment throughout the peripubertal period leads to both hypometabolism in the nucleus accumbens core and cognitive inflexibility in adulthood.

Interestingly, a residual THC-induced hypermetabolic state was observed within the dorsolateral orbital cortex. This region also plays a fundamental role in mediating cognitive flexibility, in particular, reversal of stimulus-reward associations. Deactivation of this neural substrate leads to impaired reversal learning in the ASST (McAlonan and Brown, 2003). Thus, it is surprising that THC-induced disruption of this RoI did not affect the reversal learning stages of the ASST in the present study. Whilst deficits in reversal learning reflect maintenance of a stimulus-reward association now deemed irrelevant (perseveration), the opposite of this behaviour is known as overswitching, which is the replacement of an adaptive response without a relevant cue. Both perseveration and overswitching are common behavioural abnormalities amongst schizophrenic patients (Yogev *et al.*, 2004) Therefore, a possible behavioural parameter that could be employed to probe any potential negative outcome of hypermetabolism in the dorsolateral orbital cortex would be the LI task. LI refers to the ability to ignore irrelevant stimuli and this task in commonly employed as a behavioural index of excessive behavioural switching (Moser *et al.*, 2000).

Both the dorsolateral orbital cortex and more so the nucleus accumbens core play pivotal roles in reward-related learning. Reward-related learning is the ability to learn from reinforcement, feedback or reward and drive future behaviour based on a predicted outcome (Parkinson *et al.*, 2000). The nucleus accumbens core is regarded as the limbic-motor interface as it modulates reward-related behaviour by integrating hippocampus-dependent contextual information and amygdala-dependent affective information with PFC-dependent cognitive functions to determine an appropriate behavioural outcome (Goto and Grace 2005; Mogenson *et al.*, 1980). Furthermore, the

orbital cortex has been implicated in the coding of stimulus reward value (O'Doherty, 2004).

Peripubertal THC treatment resulted in a significant reduction in connectivity between nucleus accumbens core and two amygdaloid nuclei, the basolateral and medial amygdala. The nucleus accumbens core receives direct innervations from the amygdala; these are particularly strong from the basolateral amygdala (Zahm, 1999). Conversely, peripubertal THC treatment led to increased functional coupling between the dorsolateral orbital cortex and the basolateral amygdala. Thus, peripubertal THC alters the functional coupling of PFC-amygdala-nucleus accumbens neural circuitry in a complex manner. The amygdala is strongly implicated in emotional processing and is involved in both negative and positive affective learning. In negative affective learning, the amygdala recognises negative emotions such as fear, and associates environmental stimuli with aversive emotional responses. In positive affective learning, the amygdala interacts with several cortical and subcortical structures including the PFC, particularly medial and orbital parts, and the nucleus accumbens respectively, to associate a level of gain of reward to stimuli (Baxter and Murray, 2002). Excitotoxic lesions of the basolateral amygdala result in impairments in reward-related learning, as measured by conditioned place preference to sucrose (Everitt *et al*., 1991).

Coupling between the nucleus accumbens core and both septal (dorsolateral and lateral intermediate septa) and prefrontal structures (prelimbic and anterior cingulate cortices) were significantly reduced as a result of intermittent treatment with low-dose THC throughout the peripubertal period. Similarly, THC treatment caused a functional disconnection between the dorsolateral orbital cortex and the dorsolateral septum and anterior cingulate cortex. Both the lateral septum and anterior cingulate cortex have been implicated as important neural substrates subserving stimulus-reward learning (Parkinson *et al*., 2000; Sheehan *et al*., 2004). The lateral septum acts as node for the integration of cognitive information received from the PFC, entorhinal cortex and hippocampus with affective information arriving from the amygdala and hypothalamus,

and subsequently relaying this information to cerebral areas directly responsible for initiation of appropriate behavioural responses (Sheehan *et al*., 2004). Similarly, the anterior cingulate cortex is thought to be an important node region of the mesocorticostriatal circuit recruited in stimulus-reward learning. The anterior cingulate cortex has converging connections with the nucleus accumbens and is involved in processing informational aspects of emotion and motivation (Parkinson *et al*. 2000). Lesioning of the anterior cingulate cortex results in impairments in conditioned learning (Bussey *et al*., 1997).

These novel findings of residual altered functional connectivity in neural correlates of reward-related learning resulting from peripubertal low-dose infrequent THC administration suggests that the developing brain is extremely vulnerable to the deleterious effects of THC. A possible explanation for these findings is that THC directly impacts on normal maturational trajectories through dysregulation of synaptic pruning and thereby causes abnormal synaptic plasticity resulting in altered functional connectivity in regions recruited in reward-related learning and goal-directed behaviour. As mentioned earlier, throughout adolescent brain development there is a transition between affective-driven behaviour to more refined cognitive-driven behaviour (Nelson *et al*., 2005). Thus, THC-induced alterations throughout this vulnerable epoch could have long lasting negative behavioural consequences. Deficits in reward-related learning are common amongst schizophrenic patients (Sevy *et al*., 2007) and may reflect inability to appropriately represent the value of outcomes and plans and thereby potentially lead to a state of avolition (Gold *et al*., 2008).

Furthermore, another potential implication of the observed altered functional connectivity resulting from intermittent THC administration throughout the peripubertal period is the formation of an aberrant affective-driven association between THC and its rewarding properties. These findings could have direct implications in ‘gateway hypothesis’ of cannabis. The basis of this hypothesis is that adolescent cannabis exposure increases the risk of other illicit drug use in adulthood (Lynskey *et al*., 2006).

Table 4.07 Chapter Four Summary

Test Day	PD30	PD70+				
Treatment	PolyIC	PolyIC	THC A	THC B	PolyIC + THC A	PolyIC + THC B
Test Measure						
PPI	No PPI at PD30	-	-	-	-	-
Amphetamine-induced LMA		-	-	-	↑	-
NOR	ND	-	-	-	-	-
ASST	ND	-	Impaired ED compared to vehicle	-	Impaired ED compared to vehicle	-
Binding	ND	-	↑ vmStr	-	↑ vmStr	-
LCGU	ND	↑ DLO + Cg ↓ CA3	↑ DLO ↓ NacC	ND	↑ M1	ND

Table 4.07 Chapter Four Summary Prepubertal animals (PD30) did not appear to exhibit any PPI. Following PPI assessment in adulthood, no significant affect of neither prenatal PolyIC treatment nor peripubertal exposure to THC A (low-dose intermittent THC) or THC B (high-dose daily THC) treatment regimes was evident. Peripubertal low-dose intermittent THC (THC A) treatment in PolyIC-treated animals led to increased hyperlocomotion in response to amphetamine compared PBS-treated (control) offspring. Low-dose intermittent THC exposure produced enduring impairments in the ED phase of the ASST compared to vehicle-treated animals irrespective of prenatal treatment. Intermittent peripubertal exposure to low-dose THC led to increased CB_1 receptor binding in the ventromedial striatum (vmStr) in adulthood compared to vehicle-treated animals. PolyIC-treated offspring exhibited hypometabolism in the dorsolateral orbital (DLO) and anterior cingulate (Cg) cortices and hypometabolism in the CA3 region of the hippocampus. Low-dose intermittent THC treatment led to increased cerebral metabolism in the DLO and the nucleus accumbens core (NacC) in adulthood whilst a PolyIC x THC interactive effect was evident in the primary motor cortex (M1). – denotes no difference from respective controls or not significant interactive effect between combined environmental risk factors. ND denotes not determined.

4.6 |Conclusions

The data in this chapter present multiple important findings regarding the differential effects of exposure to 'early' and 'late' environmental risk factors on behavioural phenotypes, CB_1 receptor expression, brain functioning (as indicated by altered cerebral metabolism) and regional functional connectivity in adulthood.

During the peripubertal period, the brain exhibits differential liability to the deleterious effects of THC with low-dose intermittent treatment, mimetic of light recreational use, but not high-dose daily THC treatment, giving rise to pronounced perturbances in schizophrenia-related cognitive flexibility in adulthood. The possibility of an environment-environment interaction warrants further investigation given that low-dose intermittent THC treatment to PolyIC-treated offspring enhanced responses to amphetamine compared to PBS-treated offspring. Enduring aberrant CB_1 receptor binding levels in the ventromedial striatum following peripubertal treatment with low-dose intermittent THC provides further evidence indicating a dose-specific developmental disruption evoked by THC treatment. Furthermore, findings from this study indicate that MIA and peripubertal THC treatment lead to differential long-term alterations in both cerebral metabolism and regional functional connectivity, thus aiding the elucidation of the neural substrates recruited and affected by exposure to these environmental risk factors. However, despite the impact of these 'early' and 'late' environmental risk factors on functional neural systems, the behavioural consequences of their combined exposure was minimal in the behavioural assays employed. Collectively, these behavioural, CB_1 receptor binding, 2DG imaging and regional functional connectivity data suggest that different neural systems display distinct sensitivity loads to environmental challenges throughout neurodevelopment.

CHAPTER FIVE

General Discussion

5.1 |Summary and General Discussion

Rationale

Cannabis is the most commonly used recreational drug worldwide. The principal psychoactive constituent of cannabis, THC, can induce acute and residual impairments in various aspects of cognition including executive function and working memory (reviewed by Hall and Solowij 1998). Cannabis abuse is particularly prevalent amongst adolescents (Home Office, 2012). Initiation of cannabis use (peak years 14-18 years) coincides with a critical epoch of neurodevelopment whereby marturative processes such as plastic and structural remodelling allow for refinement and integration of certain brain regions and neural circuitry (Huttenlocher, 1990; Giedd *et al*., 1999; Gogtay *et al*., 2004; Independent Drug Monitoring Unit, 2005). Thus, exposure to THC during this critical epoch of neurodevelopment may perturb maturational trajectories and potentially result in enduring cognitive aberrations in adulthood. This hypothesis is supported by clinical evidence suggesting an age-related susceptibility to the deleterious effects of cannabis as early-onset of use may increase vulnerability to the adverse cognitive sequelae associated with exposure to THC (Ehrenreich *et al*., 1999; Pope *et al*., 2003). Moreover, this concept of critical 'vulnerability windows' of neurodevelopment forms the basis of the neurodevelopmental hypothesis of schizophrenia. This model posits that the emergence of schizophrenia in adulthood is a result of maldevelopment evoked by exposure to a combination of environmental and/or genetic factors during two critical phases of neurodevelopment, namely, prenatal/neonatal and adolescent brain development (Keshavan 1999; Keshavan and Hogarty 1999). Epidemiological studies have strongly implicated both maternal infection and cannabis abuse as important 'early'

and ‘late’ environmental risk factors in the pathogenesis of schizophrenia (Andréasson *et al*., 1987; Brown *et al*., 2001; Pope *et al*., 2001; Arseneault *et al*., 2004; Brown, 2006). However, interpretation of findings derived from human studies are often subject to debate due to the methodological discrepancies, ethical constraints and confounding factors such as polydrug use, differential drug doses and comorbid psychiatric disorders inextricably incorporated into their experimental design. Therefore, animal models are crucial for exploration of mechanistic and causative theories, and long-term behavioural consequences of both MIA (as a model of maternal infection) and peripubertal THC (mimetic of cannabis abuse) exposure in a controlled experimental environment.

The overall aims of this thesis were to

1) Identify the neural substrates and functional systems sensitive to developmental disruption by exposure to THC.

2) Investigate the individual effects of and potential interplay between exposure to the schizophrenia-related environmental risk factors, MIA and peripubertal exposure to THC on brain functioning, regional functional connectivity networks, CB_1 receptor levels and the precipitation of behavioural perturbances relevant to schizophrenia.

In this thesis, the ontogeny of CB_1 receptor within important cognitive substrates, the PFC and hippocampus, was investigated to delineate a period of neurodevelopmental vulnerability and thus form the time-frame for the peripubertal THC treatment regimes employed throughout this thesis. As demonstrated in chapter two, both the PFC and hippocampus follow differential maturational trajectories throughout the peripubertal period. Therefore, the ‘vulnerability window’ for peripubertal THC treatment was defined as PD35-56, so to encompass the dynamic peripubertal ontogenetic patterns of the CB_1 receptor in both the hippocampus and PFC. Furthermore, in chapter three, the *in vivo* imaging technique 2DG autoradiography and functional connectivity analysis using

PLSR, were used to further elucidate neural substrates and functional networks undergoing maturational development in the peripubertal period and thus vulnerable to developmental disruption. In this study, multiple subfields of the hippocampus exhibited age-dependent differential patterns in both cerebral metabolism and inter-hippocampal functional connectivity. These findings are thought to reflect functional immaturity of the hippocampus in the peripubertal period (PD35) and are consistent with both the ontogenetic findings of chapter two and clinical imaging studies that report the late maturative trajectory profile of the hippocampus (Giedd *et al*., 1999; Casey *et al*., 2005). Within the PFC, no overt differences in cerebral metabolism were evident between peripubertal and adult animals. However, age-related differences in functional coupling of the PFC with the hippocampus and important neuromodulatory nuclei such as the ventral tegmental area, dorsal raphe nucleus and locus coeruleus were evident, further supporting the late maturational trajectory profile of the PFC seen in chapter two. Moreover, an age-related reduction in cerebral activity was revealed in the ventral tegmental area, a key node in the cerebral reward pathway and primary source of dopaminergic innervations to the ventral striatum (Swanson, 1982). Peripubertal hypermetabolism within the ventral tegmental area was accompanied by developmental alterations in functional coupling with the subiculum, orbital cortex and amygdala, key neural correlates in the acquisition of stimulus-reward associations (Everitt *et al*., 1991; O'Doherty, 2004; Martin-Fardon *et al*., 2008). These findings correlate with the adolescent phenotypic transition from affective-driven behaviours to more cognitive-driven behaviour and provide further evidence for the late ontogenetic trajectory of the DA system (Teicher *et al*., 1995; Spear, 2000; Tarazi and Baldessarini, 2000).

The acute effects of THC on brain functioning and regional functional activity in the brain were clearly apparent (chapter three). Acute THC administration (5mg/kg) induced a hypometabolic state and altered functional connectivity within multiple subfields of the thalamus, suggesting a prominent role of THC in the regulation of thalamic inter-communication. Moreover, systemic THC evoked an altered functional connectivity profile between thalamic nuclei and the PFC, hippocampus and nucleus

accumbens, key neural substrates that underpin successful functionality of multiple cognitive domains (Squire, 1992; Floresco *et al*., 1999; Floresco *et al*., 2006; Floresco *et al*., 2009; Burgess *et al*., 2002; Kouneiher *et al*., 2009). THC-induced disruption of cerebral metabolism and functional connectivity within these regions may underlie the overt phenotypic cognitive perturbances previously reported following acute THC administration (Miyamoto *et al*., 1995; Hampson and Deadwyler, 1998; Mallet and Beninger, 1998; Bolla *et al*., 2002; Curran *et al*., 2002; Ramaekers *et al*., 2009). Moreover, this study elucidated anomalistic neural activity in key neuromodulatory nuclei, namely, the medial septal nucleus, locus coeruleus and nucleus accumbens core, and perturbed functional communication with the neural architecture of the ACh, NA, and DA pathways providing new evidence supporting aberrant THC-induced neurotransmission following acute THC treatment (Chen *et al*., 1990; Carta *et al*., 1998; Hernández-Tristán *et al*., 2000; Nava *et al*., 2000). Acute systemic THC administration transiently modulates subjective anxiety and fear levels (Onaivi *et al*., 1990; Wachtel *et al*., 2002; D'Souza *et al*., 2004). Here, this study found that acute THC treatment resulted in overt alterations in cerebral metabolism in both the basolateral and medial amygdala and produced aberrant functional connectivity profiles between these amygdaloid nuclei and the hippocampus, PFC and nucleus accumbens. These neural substrates are integral components of the complex neural circuitry that regulates emotional behaviour, the disruption of which reported in this study (chapter three), may underlie THC-evoked dysregulation of emotional behaviour (Herkenham *et al*., 1991; Glass *et al*., 1997; Pessoa, 2010). The THC-induced alterations in cerebral metabolism and functional connectivity data reported in this thesis provide new evidence that acute exposure THC leads to widespread perturbances in neural activity and functional networks throughout multiple complex neural systems.

An important finding to emerge from this thesis is the presence of age-dependent differential liability to THC among select neural substrates significantly affected by acute THC treatment (chapter three). THC-induced hypometabolism appeared to be more pronounced in peripubertal animals relative to adult animals in the basolateral

amygdala and a number of thalamic nuclei measured. These findings suggest an enhanced liability to acute THC administration in peripubertal animals. This pattern of differential liability to the effects of acute THC administration was also evident in the nucleus accumbens core whereby THC-induced hypometabolism appeared to be more pronounced in adult animals relative to peripubertal animals. The findings of altered sensitivity in amygdaloid, thalamic and accumbens regions provide some insight into the neural systems that underpin the altered emotional and cognitive behavioural responses observed following THC administration and are in agreement with recently published behavioural studies demonstrating differential effects of THC on behavioural assays assessing both memory and emotion-driven behaviour in peripubertal animals compared to adult animals (Cha *et al*., 2006; Quinn *et al*., 2008). The novel data provided by this study of age-dependent differential liability to THC, alongside the aforementioned behavioural data, provide preclinical support that the peripubertal brain is developmentally sensitive to the adverse sequelae associated with acute THC treatment compared to adults.

The vulnerability of the peripubertal brain to THC was further explored in chapter four. This experiment investigated the effects of exposure to 'early' and 'late' schizophrenia-related environmental risk factors, and the potential interplay of these factors on the precipitation of schizophrenia-related behaviours in adulthood. Behavioural paradigms employed in this study tested sensorimotor gating, behavioural flexibility, object recognition memory and sensitivity to amphetamine (measure of aberrant mesolimbic DA transmission). To further dissect the potential effects of peripubertal exposure to THC, two differential treatment regimes were employed in this study to mimic social cannabis use amongst adolescents. These regimes spanned the peripubertal 'vulnerability window' (PD35-56) as defined in chapter two, and included a high-dose daily (7mg/kg) THC regime (designed to mimic heavy daily cannabis use) and low-dose intermittent (3.5mg/kg/3 times/wk) THC regime, (designed to mimic light recreational cannabis use). This study revealed a dose-related effect of peripubertal THC exposure as low dose intermittent THC treatment during the peripubertal period induced cognitive

inflexibility in adulthood; an effect which was not apparent in animals who received high daily doses of THC treatment. This dose-specific liability to THC was also evident in the amphetamine sensitisation task. In this task, animals who were exposed to PolyIC on GD15 and subsequently received low dose intermittent THC treatment during the peripubertal period exhibited a trend towards enhanced sensitivity to the effects of acute systemic amphetamine administration compared to PBS treated offspring, indicative of a synergistic effect of environmental risk factors resulting in aberrant mesolimbic DA transmission. A possible interpretation of this potential synergistic effect is that prenatal PolyIC treatment induces aberrations in normal brain development that does not result in an overt phenotypic perturbance. However, subsequent THC treatment during the vulnerable peripubertal maturative period precipitates the emergence of an overt schizophrenia-related phenotype in adulthood, thus supporting the neurodevelopmental hypothesis of schizophrenia (Keshavan, 1999; Keshavan and Hogarty, 1999). A dose-related effect of peripubertal THC treatment was also evidenced on a molecular level in this study, with low-dose intermittent THC treatment resulting in residual upregulation of the CB_1 receptor in the ventromedial striatum suggestive of aberrant synaptic pruning within the ventromedial striatum resulting in an atypical binding profile in adulthood. The dose-related liability of peripubertal THC treatment observed in this study may reflect an adaptive neuroprotective tolerance induced by high-dose daily THC treatment (mimetic of heavy daily cannabis abuse) resulting in desensitisation of CB_1 receptors and thus protecting the brain from the deleterious effects of THC; such an adaptive feature may not be evoked following low infrequent doses of THC (mimetic of light recreational cannabis abuse) leaving the brain more vulnerable to its adverse effects. Given the limited effects of THC on CB_1 receptor levels it would therefore be of interest to investigate the enduring effects of these differential THC treatment regimes on the efficacy of G-protein-coupled signalling mechanisms to probe the presence of any potential dose-related neuroprotective mechanisms.

Previous behavioural studies have demonstrated the ability of prenatal PolyIC treatment to produce overt schizophrenia-related phenotypes in adult offspring, such as

impairments in exploratory behaviour, sensorimotor gating and enhanced sensitivity to amphetamine (Meyer *et al*., 2005; Ozawa *et al*., 2006; Wolff and Bilkey, 2008). Whilst this present study did not replicate these behavioural effects, possibly due to the potential environmental enrichment effect of daily handling of animals throughout the peripubertal period, a necessary experimental factor in the peripubertal THC treatment design of this study, 2DG autoradiography and PLSR analysis did reveal altered cerebral metabolism in the dorsolateral orbital and anterior cingulate cortices and the CA3 subdivision of the hippocampus and perturbed functional connectivity profiles of these regions with subfields of the amygdala, PFC, hippocampus and mesolimbic DA system. These novel findings indicate that MIA did produce enduring pathological effects in brain functioning and functional connectivity networks across a diverse range of neural systems although these perturbances were not detected as an overt aberrant phenotypy.

In tandem with the behavioural observations of cognitive inflexibility, low-dose intermittent THC treatment throughout the peripubertal period led to long-term modifications in cerebral metabolism within the nucleus accumbens core and dorsolateral orbital cortex, key neural correlates of cognitive flexibility and reward-related learning. Moreover, this peripubertal treatment regime induced enduring aberrations in the functional connectivity profiles of the nucleus accumbens core and dorsolateral orbital cortex with neural substrates subserving reward-related learning and goal-directed behaviour, including the amygdala, lateral septum. A potential pathological mechanism that might explain the perturbed behavioural, CB_1 receptor binding, and functional network profiles following low-dose intermittent THC administration throughout peripubertal period is as follows: THC could directly impact on normal maturational trajectories through dysregulation of synaptic pruning thereby causing abnormal synaptic plasticity in regions recruited in reward-related learning and goal-directed behaviour. The adolescent brain then undergoes a shift in the control of behaviour from affective-driven behaviour to more refined cognitive-driven behaviour. Thus, THC-induced alterations throughout this ‘vulnerability window’ could have long lasting negative behavioural consequences (Nelson *et al*., 2005).

In summary, this thesis has provided clear evidence of dose-related detrimental effects of ‘adolescent’ THC exposure on behaviour and the functional neural systems that may underpin these deficits. There appears to be limited interplay between combined exposure to the ‘late’ environmental risk factor THC and the ‘early’ environmental risk factor MIA on the precipitation of schizophrenia-like behaviours in adulthood. These findings provide important information on the potential long-term deleterious effects of recreational cannabis consumption on the developing adolescent brain.

5.2 |Future Directions

The research presented in this thesis has generated many interesting questions that warrant further investigation. It would be of interest to replicate the behavioural study presented in this thesis, employing a micro-pump drug delivery system for peripubertal THC treatment. This experimental modification would circumvent the necessity for daily handling of all experimental animals throughout the peripubertal period, thereby eliminating the potential experimental confounds evoked by daily handling. This experimental alteration could potentially reveal behavioural perturbances evoked by prenatal PolyIC treatment, which may have been masked by an environmental enrichment effect of daily handling in this study. Moreover, given the potential environmental enrichment effect of daily handing throughout the peripubertal period observed in this study, in tandem with clinical evidence indicating the benefits of environmental enrichment, it would also be very interesting to study the potential role of environmental enrichment in the diminution of the emergence of schizophrenia-related phenotypes in adulthood in a preclinical setting (Raine *et al*., 2003). Based on aberrant cerebral activity and functional connectivity profiles in neural structures subserving cognitive flexibility, reward learning and goal-directed behaviours elicited by low-dose intermittent THC treatment in this present study, it would be desirable to expand the scope of behavioural phenotypes employed to assess the effects of this treatment regime

on such behaviours. For example, it would be advantageous to explore the effects of peripubertal THC treatment on behavioural paradigms such as the LI task (a measure of behavioural switching) and the progressive-ratio task (a measure of goal-directed behaviour and avolition). The possibility of a dose-dependent neuroprotective mechanism evoked by peripubertal THC treatment also warrants further exploration. This theory could be probed using GTPγS autoradiography to determine the differential effects of high-dose daily peripubertal THC treatment (designed to mimic heavy daily cannabis use) and low-dose intermittent peripubertal THC treatment (designed to mimic light recreational cannabis use) on the efficacy of G-protein-coupled signalling mechanisms. Finally, investigation of the effects of low-dose intermittent THC exposure throughout the peripubertal period in genetically modified mice, carrying risk genes for schizophrenia, for example *Disc-1* mutant mice, would be of interest to further explore the impact of exposure to multiple risk factors on the precipitation of schizophrenia.

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