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The effects of clozapine and haloperidol on astrocyte function and morphology in schizophrenia

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Table of Contents

Declaration and Statement of Plagiarism i
Acknowledgments ii
Table of Contents iii
List of Figures and Tables vi
List of Abbreviations vii
Scientific Abstract viii
Lay Abstract ix
Aims and Hypothesis x
Value of Research xi
Outputs xii

Introduction 1
1. Schizophrenia – General Overview 2
1.1. Broad overview on symptomatology and epidemiology 2
1.2. Aetiology – gene and environment 2
1.3. Gross and microscopic changes in the brain 3
1.4. Current hypotheses on pathophysiology 3
1.4.1. Dopamine 3
1.4.2. Glutamate 4
1.4.3. Other neurotransmitters 4
1.5. Neuroinflammation 4
2. Astrocyte dysfunction in schizophrenia 5
2.1. Introduction to astrocyte morphology 5
2.2. Introduction to astrocyte function 5
2.2.1. The role of astrocytes in neurotransmitter homeostasis 5
2.2.2. The tripartite hypothesis 6
2.2.3. Possible role for maintaining the Blood Brain Barrier (BBB) 6
2.3. Alterations in astrocyte function and morphology seen in schizophrenia 6
2.3.1. Evidence of change in astrocyte morphology 6
2.3.2. Alteration in astrocyte energy metabolism 7
2.3.3. Alteration in synaptic transmission of glutamate 7
2.4. The emerging role of astrocytes in neuroinflammation 8
2.4.1. Reactive astrogliosis 8
2.4.2. S100B as marker of astroglial activation in schizophrenia 8
3. Dopamine 9
3.1. Introduction to the dopamine receptor 9
3.2. Dopamine receptor expression in astrocytes, CNS, and PNS 9
3.3. Dopamine receptor structure and function 9
3.3.1. Structure 9
3.3.2. Function 10
3.4. Mechanisms of dopamine signalling 10

3.4.1. Mechanisms of GPCR signalling 10
3.4.2. Regulation of dopamine receptor signalling 10
3.4.3. Role of Akt in D2R-mediated psychosis and cognitive dysfunction in schizophrenia 11
3.5. Interactions between dopamine and other signalling pathways 11
3.5.1. Regulation of Mitogen Activated Protein Kinases (MAPK) by D1-class and ionotropic glutamate receptors 11
3.5.2. Serotonergic regulation of dopamine neurotransmission 12
4. Antipsychotics 12
4.1. Neuropharmacology of antipsychotics 12
4.1.1. Typical antipsychotics 12
4.1.2. Atypical antipsychotics 13
4.2. Current evidence on effect of antipsychotics on astrocyte function 13
4.3. Effects of antipsychotics on inflammation 14
Methods and Materials 23
5. Materials 24
5.1. Compounds 24
5.2. Antibodies 24
6. Methods 24
6.1. Cell culture 24
6.2. Enzyme Linked Immunoabsorbent Assay (ELISA) 25
6.3. MTT assay 25
6.4. Immunocytochemistry 25
6.5. Analysis of immunofluorescent staining 26
6.6. SDS-PAGE and Western immunoblotting 26
6.7. Statistical analysis 27
Results 31
7. Results 32
7.1. Treatment of human astrocytes with IL-17A/TNFα-induces expression of IL-6. 32
7.2. Clozapine attenuates IL-17A/TNFα-induced levels of IL-6 in human astrocytes. 32
7.3. IL-17A/TNFα -induced levels of IL-6 are decreased by Haloperidol. 33
7.4. Clozapine and Haloperidol do not alter human astrocyte cell viability. 33
7.5. Clozapine and Haloperidol do not significantly alter astrocyte morphology, under conditions of inflammation. 33
7.6. Clozapine and Haloperidol increase ERK phosphorylation in astrocytes. 34
Discussion 43
8. Summary of findings 44
8.1. TNFα and IL-17A induce IL-6 expression in human astrocytes. 44
8.2. Clozapine and haloperidol decrease IL-6 expression in human astrocytes 45
8.3. The interaction between IL-6 and dopamine receptor signalling 45
8.4. Clozapine and haloperidol do not alter human astrocyte morphology and survival 46
8.5. Changes in ERK 1/2 phosphorylation induced by clozapine as compared to haloperidol . 46
9. Future studies and limitations 47
10. Conclusion 48

References **50**

Appendix **70**

Appendix 1 69

Appendix 2 70

List of Figures and Tables

Introduction

Table 1.1 Summary of major genes implicated in schizophrenia
Table 1.2 Properties of each dopamine receptor class subtype
Table 1.3 Pharmacological effects of clozapine and haloperidol

Figure 1.1 Epigenetic model of schizophrenia
Figure 1.2 Model of the tripartite synapse
Figure 1.3 Representation of the progression of different stages of reactive astrogliosis
Figure 1.4 Early and late dopamine receptor signalling
Figure 1.5 Regulation of ERK 1/2 by D1, D2 and glutamate receptors

Material and Methods

Table 1.1 List of Compounds
Table 1.2 List of Antibodies
Figure 1.1 Cellular extension quantification

Results

Figure 3.1 Treatment of human astrocytes with IL17A/TNFα-induces expression of IL-6
Figure 3.2 Clozapine attenuates TNFα/IL17A-induced IL-6 expression in human astrocytes
Figure 3.3 Haloperidol attenuates the expression of IL-6 induced by IL17A/TNFα in human astrocytes
Figure 3.4 Clozapine and haloperidol do not alter astrocyte cell survival
Figure 3.5 Astrocyte morphology is not altered by clozapine
Figure 3.6 Astrocyte morphology is not altered by haloperidol
Figure 3.7 Clozapine and Haloperidol increase ERK phosphorylation in astrocytes

Discussion

Figure 1.1 Molecular pathway linking dopamine, TNFα, IL17A receptors with IL-6

List of Abbreviations

Abbreviation	Definition
Akt	Protein kinase B
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BMI	Body mass index
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
CSF	Cerebrospinal fluid
DISC-1	Disrupted in schizophrenia-1 gene
GABA	Gamma-amino butyric acid
GDP	Guanosine 5'-diphosphate
GRK	G-protein coupled receptor kinase
GTP	Guanosine triphosphate
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IL	Interleukin
INF	Interferon
L-DOPA	L-3,4-dihydroxyphenylalanine
MAPK	Mitogen activated protein kinase
NF- κB	Nuclear factor kappa-light chain enhancer of activated B cells
NGF	Nerve growth factor
OPD	o-Phenylenediamine dihydrochloride
PKA	Protein kinase A
PP2A	Protein phosphatase 2
PYGM	Glycogen phosphorylase
RH	Rel homology domain
S100B	S-100 calcium binding protein B
TGF	Transforming growth factor
TNF	Tumour necrosis factor

Scientific Abstract

Schizophrenia is a chronic debilitating psychiatric disorder affecting approximately 1% of the population worldwide. The disorder is characterised by positive symptoms, negative symptoms, and clinical deficits in cognition. Current therapeutic agents, known as antipsychotics, all display some level of dopamine antagonism thereby reducing symptoms of psychosis. It has been suggested that abnormal astrocyte function is involved in aberrant neurotransmitter signalling and neuroinflammation seen in schizophrenia. The effect of antipsychotics on astrocyte cell function is still being explored. Using a human astrocyte culture model, this study aims to determine the effect of typical and atypical antipsychotics, haloperidol and clozapine, on cytokine levels, the intracellular signalling molecule Extracellular Signal-Regulated kinase 1/2 (ERK 1/2), astrocyte morphology and survival. Human astrocytes were cultured and pre-treated with clozapine or haloperidol for 1h, then stimulated with TNFα/IL-17A for 18h. Cytokine analysis was carried out using Enzyme Linked Immunoabsorbent Assay (ELISA). Changes in astrocyte morphology was analysed using immunocytochemistry, with glial fibrillary acidic protein (GFAP) and vimentin protein expressions labelling astrocytes. The results of this study showed that both clozapine and haloperidol attenuated TNFα/IL-17A induced expression of IL-6 in human astrocytes. Under pro-inflammatory conditions, pre-treatment with both clozapine and haloperidol did not change astrocyte morphology. Furthermore, an MTT assay showed that both clozapine and haloperidol did not affect cell viability. To quantify changes in extracellular signal-regulated kinases 1 and 2 (ERK 1/2), astrocytes were exposed to clozapine or haloperidol for 15 mins and processed for western immunoblotting. This study demonstrated that haloperidol, but not clozapine, significantly increased ERK 1/2 phosphorylation in human astrocytes. Taken together, we are able to ascertain that clozapine and haloperidol change the role of astrocytes in inflammation and cellular signalling in schizophrenia.

Lay Abstract

Schizophrenia is a chronic severe mental disorder that affects approximately 1% worldwide population. The disorder is characterised by distortions in perception, emotions and language, which manifest into hallucinations (i.e. hearing voices) and erratic behaviour. The exact cause of schizophrenia is unknown, although altered levels of the neurotransmitter dopamine is implicated. The treatment used in schizophrenia, known as antipsychotics, work by changing levels of neurotransmitter dopamine in the brain, but their exact mechanism is unknown. It has been suggested that astrocytes, a specialised cell type in the brain that controls essential complex functions and supports neurotransmission in the brain, may be involved in the development of schizophrenia. Therefore, this study aims to examine the effect of certain antipsychotics, clozapine and haloperidol, on astrocyte function. The results of this current study showed that both clozapine and haloperidol have a protective effect, where both antipsychotics induce an anti-inflammatory effect on astrocytes. Addtionally, this study found that antipsychotics do not change the shape of astrocytes and do not adversely affect their survival. Taken together, we suggest that antipsychotics may have a beneficial effect on astrocytes, which may influence the treatment of symptoms associated with schizophrenia.

Aims and Hypothesis

We hypothesise that both clozapine (an atypical antipsychotic) and haloperidol (a typical antipsychotic), are able to alter astrocyte morphology as well as astrocyte function, in terms of its role in neuroinflammation and regulation of neurotransmitter signalling molecules.

Based on the above hypothesis, the aims of this study are as follows:

- To examine the direct effects of clozapine and haloperidol on the pro-inflammatory cytokine release in astrocytes
- To investigate the impact of clozapine and haloperidol on astrocyte survival
- To identify the effects of clozapine and haloperidol on astrocyte cellular processes
- To explore the role of clozapine and haloperidol on intracellular dopamine signalling molecules in astrocytes

Value of Research

Schizophrenia has a high prevalance and incidence rate, affecting more than 23 million individuals worldwide according to current WHO statistics. Additionally, schizophrenia is associated with considerable disability for the individual, often affecting access to employment, housing and healthcare. The aetiopathophysiology of schizophrenia is complex, and largely unknown. It is likely that there is a complex interplay between genetic and environmental factors that contribute to altered neurotransmission, of which dopamine overactivity is thought to play a major role during symptoms of psychosis. Although it is established that antipsychotics exert its therapeutic effects via dopamine antagonism, its effects on glial cells is still being elucidated. Astrocytes are specialised macroglia and are the most abundant cell type in the CNS. Astrocytes are in constant communication with neurons; increased neuronal activity leads to homeostatic changes in astrocytes, which include augmented metabolic activity and synthesis of lactate, clearance of neurotransmitters and buffering extracellular K+. Furthermore, astrocytes play a role in regulating cytokines and other immune molecules in the brain, of which are also thought to be dysregulated in schizophrenia. Although there has been some evidence of astrocyte dysfunction in schizophrenia, there has been limited studies on the effect antipsychotics have on astrocyte function and morphology. This study aims to create a model of neuroinflammation in order to investigate the effect of clozapine and haloperidol on astrocytes and cellular signalling in schizophrenia.

Outputs

Papers

Ang, T., Dev, K.K., Sharma, K. The effects of antipsychotics on astrocyte function and morphology in schizophrenia. Journal of Neuropharmacology, 2019 (in preparation)

Presented Posters

Ang, T., Dev, K.K., The role of astrocytes in schizophrenia. 7th Annual Meeting Frontiers in Neurology Ireland, Dublin, Ireland, November 2017. (Appendix 1)

Ang, T., Dev, K.K., The effect of antipsychotics clozapine and haloperidol on astrocyte function and morphology. 11th FENS Forum of Neuroscience, Berlin, Germany, July 2018. (Appendix 2)

Introduction

1. Schizophrenia – General Overview

1.1. Broad overview on symptomatology and epidemiology

Schizophrenia is a psychiatric disorder characterised by its phenomenology: positive symptoms (i.e. thought disorder, hallucinations, delusions), negative symptoms (i.e. blunted affect, reduced motivation, poverty of thought) and clinically significant deficits in cognition (including memory, attention and executive functions) (Yamamuro et al., 2015). In addition, symptoms need to be associated with reduced occupational and social functioning in order to fulfil diagnostic criteria, as per DSM-5 (Diagnostic and statistical manual of mental disorders, Fifth Edition) (American Psychiatric Association, 2013). The prevalence of schizophrenia reaches approximately 1% worldwide, with incidence approaching 1.5 per 10,000 per annum (McGrath et al., 2008). Incidence is often higher in men, at a ratio of 1.4:1 (Abel et al., 2010). Typically, disease onset occurs during late adolescence (18-25 years), with females diagnosed slightly later (American Psychiatric Association, 2013). Regrettably, schizophrenia is associated with a high suicide rate, responsible for 10% of all completed suicides (Arsenault-Lapierre et al., 2004) and affects approximately 5% of those with schizophrenia (Hor and Taylor, 2010).

1.2. Aetiology – gene and environment

Schizophrenia is a highly heritable disease, with a higher monozygotic than dizygotic concordance rate (40-50% vs. 10-15%), suggesting a significant genetic component to overall risk (Cannon et al., 1998; Kringlen, 2000). Genome-wide association studies (GWAS) have found that schizophrenia is a polygenetic disorder, where an accumulation of multiple risk alleles leads to increased risk in this disease entity (Harrison and Weinberger, 2005). Pre-GWAS candidate gene studies have identified specific genes linked to schizophrenia **(Table 1.1)**. Their roles in influencing pathophysiology have been linked to altered synpatic function, disordered dopamine and glutamate transmission, and abnormal metabolic and immune functions (Schwab and Wildenauer, 2013). Nevertheless, a meta-analysis suggested that historical candidate genes have not proven to be of particular value in determining the genetic basis of schizophrenia, largely owing to poor statistical power (Lewis and Moghaddam, 2006). The largest GWAS to date has identified 108 schizophrenia-associated loci that include genes coding for the dopamine receptor (*DRD2*) as well as glutamatergic neurotransmission and synaptic plasticity (e.g. *GRM3, GRIN2A, SRR, GRIA1*), of which are notable due to their implications in aetiology and treatment of schizophrenia (Schizophrenia Working Consortium, 2014). Copy number variants (CNVs) are structural alterations in genes that contribute to human variability, where lines of DNA can be deleted or duplicated (Thapar and Cooper, 2013). CNVs associated with schizophrenia are uncommon in the population although carry significant risk (odds ratios 2–60) and interestingly, overlap with loci contributing to paediatric neurodevelopmental conditions (Marshall et al., 2017). Most of CNVs accounted are de-novo mutations, which would contribute to the relatively stable prevalence of schizophrenia (Rees et al., 2012). The most frequent CNV being a deletion on the long arm of chromosome 22 (22q11) (Stefansson et al., 2008). The 22q11 microdeletion occurs at a frequency of 0.3% of all schizophrenia cases.

The fact that monozygotic concordance rate is less than 100% suggests environmental risk factors are at play. These factors include a variety of insults during perinatal and early life, summarised in

the table below **(Figure 1.1)**. There have been studies looking at the gene and environment interaction, for example, cannabis use in relation to polymorphisms in genes involved in dopaminergic pathways (van Winkel, 2011) and polymorphisms in COMT increasing vulnerability stress-induced psychosis (van Winkel et al., 2008). Advances in statistical genetics, using polygenic profile scoring and Mendelian Randomization scoring tools, will allow us to further elucidate the link between genetics and environmental risk factors in schizophrenia (Marshall et al., 2017).

1.3. Gross and microscopic changes in the brain

Once known as dementia praecox, schizophrenia is now established as a neurodevelopmental disorder (Weinberger, 1987). Structural abnormalities likely arise long before first episode psychosis (Vita et al., 2006). Gross changes in the brain include ventricular enlargement and reduced volume in the medial temporal lobe (Lewis and Lieberman, 2000). Additionally, decreased grey matter density and cortical thinning has been found in the cingulate cortex (Heckers et al., 1990; Bouras et al., 2001; Williams et al., 2014).

Although more prominent changes are seen in grey matter compared to white matter in magnetic resonance imaging (MRI) studies (Bernstein et al., 2015), other neuroimaging studies and genetic analyses show alterations in the microstructure of white matter in schizophrenia. These include decreased anisotropy in the cingulum bundle in chronic schizophrenia (Kubicki et al., 2003), defects in myelin composition (Du et al.; Bernstein et al., 1992), and decreased dendritic spine density on prefrontal cortical pyramidal neurons (Glantz and Lewis, 2000). Moreover, hyperpruning of collateral axons in the prefrontal cortex is seen to contribute to the manifestation of schizophrenia (Nieuwenhuis et al., 2012). These findings are replicated in the hippocampus: decreased pyramidal neuronal size and changes in the dendritic tree contribute to neuronal dysfunction, which is associated with cognitive decline in schizophrenia (Kolomeets and Uranova, 2010). Whether abnormal structural connectivity is a primary factor in the pathology of schizophrenia or a consequence of cortical neuronal dysfunction, remains a question (Bernstein et al., 2015). Increasing evidence has suggested astroglial involvement in changes in white matter and synaptic abnormalities in schizophrenia, as discussed below.

1.4. Current hypotheses on pathophysiology

Interestingly, schizophrenia appears to be a condition that affect humans exclusively, thus limiting use of animal models (Taylor A, 2009). Therefore, our understanding of the pathophysiology of schizophrenia has been largely based on post-mortem studies, clinical observational studies and genetic studies.

1.4.1. Dopamine

Altered neurotransmitter levels have been found in schizophrenia. Excess dopamine signalling in the striatal and/or mesolimbic areas of the brain is thought to be responsible for the positive symptoms seen in schizophrenia (Carlsson, 1988). This hypothesis was born from the success of antipsychotic medication, most of which act to block dopaminergic D_2 receptors. Dopamine excess is contributed by presynaptic dopaminergic dysfunction, where increased dopamine synthesis and release is seen in patients with schizophrenia. Excess dopamine signalling in the striatal area is associated with the

development of psychosis (Kapur et al., 2005; Howes et al., 2009). Interestingly, dopamine release increases in patients who develop psychosis, and rises further with progression of psychosis (Howes et al., 2012). Contrastingly, deficits in D_1 receptor signalling in the prefrontal cortex is associated with negative symptoms (Goldman-Rakic et al., 2004). Dopamine is not the only neurotransmitter involved in the pathophysiology of schizophrenia. This is suggested by the fact that clozapine, a weak D_2 antagonist, has superior efficacy to typical antipsychotics, which are potent D_2 antagonists (Wahlbeck et al., 2000). Furthermore, the hypothesis does not explain the entire clinical picture of schizophrenia, as current antipsychotics have limited effect on negative and cognitive symptoms.

1.4.2. Glutamate

Another major neurotransmitter implicated in schizophrenia is glutamate. N-methyl-D-aspartate (NMDA) antagonists such as phencyclidine (PCP) and MK-801 (dizocilpine) have been shown to induce and exacerbate acute psychotic and negative symptoms, by preventing flow of ions such as Ca^{2+} (Steinpreis, 1996; Stone, 2009). Furthermore, studies have found increased levels of kynurenic acid, an endogenous NMDA receptor antagonist, in both cerebrospinal fluid and certain areas of the brain of schizophrenic patients (Schwarcz et al., 2001; Erhardt et al., 2007). This suggests deficient glutamate signalling is involved in schizophrenia, although there is debate as to whether this is due to abnormal glutamate pathway signalling or a defect in the glutamate receptor (Katsel et al., 2011).

1.4.3. Other neurotransmitters

Abnormalities in levels of other neurotransmitters have been proposed to affect cognitive functioning in schizophrenia. Decreased GABA in the dorsolateral prefrontal cortex (DLPFC) leads to impaired synchronisation in pyramidal cells, which is linked to deficits in working memory (Lewis and Moghaddam, 2006).

There is also recent evidence that the cholinergic system is involved in schizophrenia. Reduced cortical levels of muscarinic M_1 receptors have been found in schizophrenic patients, hypothesised to affect working memory by disrupting information between the cortex and hippocampus (Scarr and Dean, 2008; Karam et al., 2010). Interestingly, there is a higher proportion of smokers in schizophrenia patients than the average population, possibly as a method to self-medicate and amend deficits in sensory and cognitive processing (Mobascher and Winterer, 2008). This is supported by up-regulation of high affinity neuronal nicotinic acetylcholine receptors (nAChRs) and reduced levels of α7-nAChR in the hippocampus and frontal cortex found in schizophrenia (Ochoa and Lasalde-Dominicci, 2007).

1.5. Neuroinflammation

There is emerging interest in neuroinflammation and its involvement in the pathophysiology of schizophrenia. Early stresses, including maternal infection with certain viruses **(Figure 2)**, have been shown to induce an inflammatory response, where oxidative stress, release of pro-inflammatory cytokines and priming of glial cells, ultimately disrupts normal brain maturation (Meyer, 2013). Fitting with this model, altered secretion patterns of cytokines is thought to occur in schizophrenia, including an increase in pro-inflammatory IL-6 levels (van Kammen et al., 1999; O' Connell and Dev, 2014). Additionally, an increased prevalence of positive autoantibody titres are seen in patients with first-episode psychosis compared to controls, including anti-cardiolipin IgG and anti-NMDA receptor, which were independent of anti-psychotic medication (Ezeoke et al., 2013). Autoantibodies may

contribute to psychosis by cross-reacting directly with CNS antigens (e.g. anti-NMDA receptor) or via cytokine mediated aberrant dopaminergic transmission (Zalcman et al., 1994; Ezeoke et al., 2013).

2. Astrocyte dysfunction in schizophrenia

2.1. Introduction to astrocyte morphology

In terms of anatomic distribution, astrocytes are mapped out in a well-organised, non-overlapping manner, where no part of the CNS is without astrocytes or similar cells (Sofroniew and Vinters, 2010). Historically, astrocytes have been divided into protoplasmic or fibrous cell types. Protoplasmic astrocytes have a globoid distribution of finely branching processes from numerous stem branches, whereas fibrous cell types are characterised by long fibre-like processes (Ramón y Cajal, 1909). In the grey matter, interconnecting processes of protoplasmic astrocytes form gap junctions. Branching processes from a single protoplasmic astrocyte in the hippocampus or cortex is estimated to be in contact with over 100,000 different synapses, forming exclusive territories of functional islands (Bushong et al., 2002; Halassa et al., 2007).

2.2. Introduction to astrocyte function

Astrocytes are the most abundant cell type in the brain, making up five times the population of neurons in the brain (Sofroniew and Vinters, 2010). They are specialised macroglia, which form a group of glial cells that arise from the ectoderm cell line. Astrocytes are considered non-neuronal neurons. They express K+ and Na+ ion channels that induce inward currents, but do not propagate action potentials like neurons (Nedergaard et al., 2003; Sofroniew and Vinters, 2010). However, astrocytes are able to display excitability through regulated increases in intracellular Ca^{2+}, via release from intracellular stores or triggered by neurotransmitter release **(Figure 1.2)** (Charles et al., 1991; Sofroniew and Vinters, 2010). This enables astrocytes to communicate with neighbouring astrocytes, via gap junctions, or neurons (Nedergaard et al., 2003). Astrocytes are in constant communication with neurons; increased neuronal activity leads to homeostatic changes in astrocytes, which include augmented metabolic activity and synthesis of lactate, clearance of neurotransmitters and buffering extracellular K+ (Yamamuro et al., 2015).

2.2.1. The role of astrocytes in neurotransmitter homeostasis

Astrocytes supply energy for neuronal activity (Sofroniew and Vinters, 2010; Elsayed and Magistretti, 2015). Astrocytic processes come into contact with blood vessels, take up glucose, and feed this to various neural elements (axons, synapses, neural perikarya) in grey and white matter (Sofroniew and Vinters, 2010). Lactate is produced as an end product of aerobic glycolysis during glucose uptake from blood vessels; it is released by astrocytes to be taken up by neurons (Chuquet et al., 2010; Magistretti and Allaman, 2015). Astrocyte-neuron lactate transport is shown to be involved in induction of memory formation and maintain long-term potentiation (Suzuki et al., 2011; Yang et al., 2014). Moreover, it has been strongly suggested that astrocytes are the principal storage sites for glycogen in the CNS, utilised during periods of hypoglycaemia and high neuronal activity. Glycogenolysis allows short-term, rapid local supply of ATP to areas of increased energy demand (Brown and Ransom, 2007; Suh et al., 2007).

2.2.2. The tripartite hypothesis

Astrocytes are involved in synaptic development and plasticity. The relationship between astrocytes and neurons during synaptic transmission has been conceptualised as the "tripartite synapse" **(Figure 1.2A)** (Newman, 2003; Perea et al., 2009; Sofroniew and Vinters, 2010; Verkhratsky et al., 2013). There are three elements to this model: the pre-synapse, the post-synapse and the glial cell **(Figure 1.2A)** (Mitterauer, 2005). In this model, astrocytes act as regulators of neuronal activity, where they can up- or down-regulate neurotransmitters. Astrocytes can also directly affect the post-synaptic neuron, causing an excitatory or inhibitory response (Newman, 2003; Mitterauer, 2005). In addition, a specific group of astrocytes uses exocytosis, via vesicular glutamate transporter (vGluT), to release glutamate into the synaptic cleft upon activation (Jourdain et al., 2007). In this way, astrocytes are able to modify neuronal activity and influence synaptic plasticity during learning and maintaining memories (Araque and Perea, 2004; Yang et al., 2009). In the glutamatergic synapse, removal of excess glutamate by glutamate transporters on astrocytic processes prevents build up of excitotoxic neurotransmitter in the synaptic cleft, thereby delivering a system of neuroprotection (Choi, 1987; Rothstein et al., 1996).

2.2.3. Possible role for maintaining the Blood Brain Barrier (BBB)

The BBB consists of cerebral capillary endothelial tight junctions that act as a diffusion barrier to exclude certain blood-bourne molecules based on polarity and size, the surface of which are covered mostly by astrocytic end-feet (Kacem et al., 1998). The mechanism by which astrocytes maintain BBB structural integrity is controversial. Some in vitro studies have suggested astrocytes to have barrier properties by influencing polarity of the BBB (Beck et al., 1984; Takano et al., 2006). Astrocytes are also able to control cerebral blood flow by changing vessel diameter in response to increased neural activity, via molecular mediators such as prostaglandin, arachadonic acid and nitrous oxide (NO) (Takano et al., 2006; Iadecola and Nedergaard, 2007). Taking into account its role in BBB function, dysfunction in astrocytes causes increased permeability in the endothelial barrier, resulting in the influx of peripheral immune cells and pathogens (Sofroniew, 2015). This demonstrates the role of astrocytes in regulation of inflammation in the CNS, as implicated in schizophrenia.

2.3. Alterations in astrocyte function and morphology seen in schizophrenia

2.3.1. Evidence of change in astrocyte morphology

An overall decrease in glial cell density is seen in schizophrenia, in contrast to an increase in glial cell density in bipolar disorder and major depressive disorder (Cotter et al., 2001). Studies have shown significantly decreased astrocyte anisotropy in the anterior cingulate cortex (ACC) and subgenual cingulate cortex (SCC) involved in mood and cognition (Haznedar et al., 1997; Kubicki et al., 2003). Interestingly, reduction in astrocyte density in this region is contributed only by a decrease in fibrillary astrocytes, with no change in gemistocytic astrocytes. This implies that dysfunction of the SCC may be primarily or partially due to the specific role of fibrillary astrocytes, which is now understood to involve glutamate regulation (Williams et al., 2014). The morphology of astrocytes is also altered. A study using electron microscopy found swollen and dystrophic astrocytes in areas of neuronal loss in the prefrontal cortex of schizophrenic patients (Oifa and Uranova, 1991). Increased expression of capthesin B and D is shown in swollen cortical astrocytes, correlating with findings of

upregulated autophagy in schizophrenia, which has a role in synaptic and dendritic function (Bernstein et al., 2015; Merenlender-Wagner et al., 2015). There are also abnormalities in astrocyte cell organelles. Mitochondrial deficits, accumulation of lipofuscin and reduction in vacuoles contribute to astrocyte dysfunction, and may also progress with duration of illness (Kolomeets and Uranova, 2010).

2.3.2. Alteration in astrocyte energy metabolism

Impaired working memory, an important clinical feature of the disease process in schizophrenia, is suggested to be mediated by a reduction in glutamatergic activity and energy metabolism in the DLPFC (Lewis and Moghaddam, 2006; Molina et al., 2009). During periods of high neuronal activity where energy demand exceeds glucose supply, glycogenolysis in astrocytes provides lactate as a transient source of energy, thus coupling glucose utilisation to glutamatergic neurotransmission in the brain (Pellerin and Magistretti, 1994; Brown and Ransom, 2007). Glycogenolysis is regulated by the key enzyme glycogen phosphorylase, of which the glial isoform is PYGM (Pfeiffer-Guglielmi et al., 2003). This is activated by allosteric modification by isoform A of Ras-related C3 botulinum toxin substrate 1 (RAC1) in T cells (Arrizabalaga et al., 2012). Decreased levels of PYGM and RAC1 were found in post-mortem DLPFC of schizophrenia patients. Notably, PYGM protein levels inversely correlated with duration of illness, suggesting it may be a dynamic biomarker of progression of disease in chronic schizophrenia (Pinacho et al., 2016). RAC1 is the only regulator of PYGM that is altered, and may be involved in other processes such as regulation of neuronal migration and growth, thus may affect abnormal neural circuits seen in the DLPFC in schizophrenia (Govek et al., 2005).

2.3.3. Alteration in synaptic transmission of glutamate

Excitatory amino acid transporters 1 and 2 (EAAT1, EEAT2) are the main regulators of glutamate uptake and transport glutamate into astrocytes for conversion to glutamine by glutamine synthase (Valjent et al.) **(Figure 1.2B)**. EAAT and GS are localised to perisynaptic astrocytes (Katsel et al., 2011). EAAT1 (GLT1) is the main regulator of EEAT2 (GLAST) (Nicholson et al., 2014). There is evidence of decreased expression of glutamate transporters EAAT1 and EAAT2 in the parahippocampus and DLPFC in schizophrenia (Verkhratsky et al., 2013). GS expression is also reduced, in the superior temporal gyrus and deep layers of ACC in schizophrenia (Steffek et al., 2008; Katsel et al., 2011). This correlates to a decrease in astrocyte number in the deep but not superficial layer of ACC seen in schizophrenia (Katsel et al., 2011). This suggests that a deficiency in astrocyte proteins may play a role in defective glutamate transmission due to reduced recycling of glutamate.Altered glutamate transmission in schizophrenia could be result of abnormalities in D-serine. D-serine is a co-agonist to glutamate at the NMDA receptor, fundamental for its activation (Johnson and Ascher, 1987). It is considered a gliotransmitter released by astroyctes and its availability may be controlled by astrocytes independent of neuronal activity (Papouin et al., 2017). Decreased plasma levels of D-serine are found in schizophrenia patients, suggesting it could be a measurable biomarker for schizophrenia. The percentage of D-serine among total serine was significantly lower than healthy controls, therefore suggesting decreased conversion of L-serine to D-serine by serine racemase (Hashimoto et al., 2003). Furthermore, polymorphisms have been identified in serine racemase associated with a risk of paranoid schizophrenia (Morita et al., 2007). The well-established *DISC-1* mutation disrupts binding to serine racemase and increased ubiquination and degradation of serine racemase, leading to decreased endogenous levels of D-serine. Mouse models with *DISC-1* mutation are observed to have increased sensitivity to an NMDA

antagonist and schizophrenia-like symptoms (Ma et al., 2013). High dose D-serine was studied as a treatment to patients with schizophrenia, and was found to significantly improve persistent positive and negative symptoms, as well as neurocognitive symptoms (Kantrowitz et al., 2010).

2.4. The emerging role of astrocytes in neuroinflammation

2.4.1. Reactive astrogliosis

Reactive astrogliosis is a progressive, context-specific process of potential molecular, cellular and functional changes that occur to astrocytes in response to CNS insults (Sofroniew, 2009). Reactive astrocytes change their morphology and gene expression, ultimately expressing major histocompatibility complex antigens (MHA) required for T-cell activation, secreting cytokines and altering BBB permeability (Dong and Benveniste, 2001; Elsayed and Magistretti, 2015). Reactive astrogliosis is often used as a pathological sign of CNS injury or disease (Sofroniew and Vinters, 2010). The changes that occur range from mild-moderate to severe with glial scar formation **(Figure 1.3)** (Sofroniew, 2009). Astrogliosis involves proliferation of astrocytes, and can be marked by increased glial fibrillary acidic protein (GFAP) expression (Sofroniew and Vinters, 2010). Large arrays of molecular mediators are known to trigger reactive astrogliosis, including growth factors and cytokines. Reactive astrocytes play a pro-inflammatory role after CNS damage, while concurrently restricting inflammatory cell spread between areas of CNS damage and healthy tissue, thereby limiting the inflammatory process **(Figure 1.3)** (Sofroniew, 2009). There is conflicting evidence for GFAP- reactive astrocytosis in schizophrenia. The majority of studies show significantly decreased GFAP- reactive astrocytes in the cingulate and prefrontal cortex of patients with schizophrenia (Rajkowska et al., 2002; Webster et al., 2005; Toro et al., 2006), supporting the evidence describing an overall decreased glial cell density in schizophrenia (Cotter et al., 2001). Contrastingly, a post-mortem study observed increased GFAP levels in the DLPFC of patients with schizophrenia compared to controls, which may be explained by a specific dysfunction in GFAP and/or partial activation of astrocytes, despite unchanged astrocyte numbers (Feresten et al., 2013). Further studies need to be undertaken to determine whether changes in GFAP is related to other variables such as duration of illness, stage of illness, and treatment.

2.4.2. S100B as marker of astroglial activation in schizophrenia

S100B is often regarded as a marker of astroglial activation. S100B is a Ca^{2+} binding protein expressed by astrocytes in the CNS. At low concentrations, it is thought to regulate proliferation and differentiation of neurons and glia. It also functions as a neurotrophic factor, regulating synaptogenesis and neurotransmitter function (Rothermundt et al., 2009; Steiner et al., 2011). Excessive levels of S100B protein promotes an inflammatory response via expression of nitric oxide synthase or pro-inflammatory cytokines leading to neuronal dysfunction and apoptosis (O'Connell et al., 2013). Increased levels of S100B are seen in the CSF and in serum of patients with schizophrenia compared to healthy controls (Rothermundt et al., 2004; Rothermundt et al., 2009; Steiner et al., 2011; O'Connell et al., 2013). This is supported by our previous study which found that higher serum S100B levels was found in females compared to males, possibly due to an increased BMI, which may result from altered S100B release from adipocytes (O'Connell et al., 2013). It is important to note that peripheral S100B may be unreliable as a measure of astrocyte pathology in schizophrenia as S100B is released from many cell types. These include immune cells (T-cells and natural killer cells)

and adipocytes, suggesting S100B has a role in altered immune response and metabolic activity (Miuller and Schwarz, 2007; Steiner et al., 2010). Nevertheless, elevated serum levels of S100B correlate closely with CSF levels both in healthy controls and in schizophrenia, suggesting that increased levels contribute mainly to increased secretion from astrocytes (Rothermundt et al., 2009). This indicates astroglial activation occuring as part of or in respone to the disease process in schizophrenia, although whether reactive astrogliosis occurs needs to be further deineated.

3. Dopamine

3.1. Introduction to the dopamine receptor

The unifying feature of all antipsychotics is that they display a varying degree of dopamine antagonism, therefore, inhibit dopamine transmission and dopamine-dependent functions. There are four major dopaminergic pathways in the CNS: nigrostriatal, mesolimbic, mesocortical and tuberoinfundibular (Andén et al., 1964). Each dopamine system is implicated in different CNS functions; for example, dysfunction in the nigrostriatal pathway is associated with motor deficits in Parkinson's disease, whereas the mesocortical pathway mediates cognitive processes and the mesolimbic pathway in reward feedback (Alex and Pehek, 2007). Dopamine is a monoamine neurotransmitter that exerts its physiological functions via G-protein coupled receptors (GPCR) – D1, D2, D3, D4, D5 **(Table 1.2)** (Beaulieu and Gainetdinov, 2011). There are 2 major classes of dopamine receptors, divided into D_1-class receptors (includes D1 and D5 subtypes) and D_2-class receptors (D2, D3, D4 subtypes) (Tiberi et al., 1991; Clifford et al., 2000). The classification is based on shared biochemical, pharmacological, structural and genetic properties between each class subtype.

3.2. Dopamine receptor expression in astrocytes, CNS, and PNS

Dopamine receptors are widely expressed in the periphery and CNS. The receptor subtype expressed in different areas of the CNS is summarised in **Table 1.2**. D_2-class receptors are found on both pre- and post-synaptic dopaminergic neurons, whereas D_1-class receptors are found exclusively on post-synaptic dopamine-target cells (e.g. GABAergic medium spiny neurons in the striatum) (Sokoloff et al., 2006; Rankin and Sibley, 2010; Rondou et al., 2010). Previous studies have determined that dopamine receptors are expressed in astrocytes (Hansson and Ronnback, 1988; Bal et al., 1994; Zanassi et al., 1999). Direct evidence of D1, D3, D4 and D5 receptors has been shown in astrocytes within the basal ganglia, whereas high expression of D2 receptors is found in astrocytes of the prefrontal cortex **(Table 1.2)** (Miyazaki et al., 2004; Mladinov et al., 2010). In the periphery, each receptor subtype is found in varying degrees in the sympathetic ganglia, adrenal glands, kidneys, heart, blood vessels and gastrointestinal tract (Beaulieu and Gainetdinov, 2011).

3.3. Dopamine receptor structure and function

3.3.1. Structure

All dopamine receptor subtypes have seven transmembrane-spanning domains and are coupled to G-proteins, which mediate intracellular signalling. The specific G-protein that is coupled to each receptor subtype is shown in **Table 1.2**. In its inactive form, the G-protein is made up of α, β and γ

subunits that form an inactive trimeric complex, where the α subunit is bound to GDP (Beaulieu and Gainetdinov, 2011). The D_1 and D_2-receptor classes differ in genetic structure. Only D_2-class receptors have introns within their genetic coding regions, which leads to the formation of genetic slice variants for each receptor subtype that differ anatomically, pharmacologically and in their signalling properties. Most notably, alternative splicing of D2 receptors leads to the development of 2 major receptor variants – D2S (D2-short) and D2L (D2-long). Both variants differ in structure, by 29 amino acids in the third intracellular loop, and in receptor function (Clifford et al., 2000; De Mei et al., 2009). D4 receptors have the highest affinity for antipsychotic drugs, particularly clozapine. Interestingly, polymorphic variants in D4 receptors have varying affinity for clozapine, however, possession of these variants does not relate to an increased incidence of schizophrenia (Wong and Van Tol, 2003).

3.3.2. Function

In the central nervous system, dopamine receptor functions include regulation of cognitive function, affect, sleep pattern, feeding, and voluntary movement. Dopamine effect on locomotor activity has been an area extensively explored, and this is mainly mediated via D1, D2 and D3 receptors (Missale et al., 1998; Sibley, 1999). These receptors are also involved in reward and reinforcement mechanisms (Missale et al., 1998; Beaulieu and Gainetdinov, 2011). D1 and D2 receptors in the prefrontal cortex are critical in learning and executive functions such as maintaining working memory (Goldman-Rakic et al., 2004). As mentioned, clinical effect of antipsychotic drugs on D2 receptors suggest that these receptors are involved in the biochemistry of psychosis (Snyder et al., 1970; Roth et al., 2004). Little is known about the specific physiological functions of D3-5 receptors. In the peripheral nervous system, dopamine has wide-ranging effects which include sympathetic, immune and hormonal regulation (Beaulieu et al., 2015).

3.4. Mechanisms of dopamine signalling

3.4.1. Mechanisms of GPCR signalling

Dopamine receptors are known to be G-protein coupled receptors (GPCRs). Upon activation of the trimeric GPCR complex by a dopamine receptor agonist, GDP is released from its guanine nucleotide binding site on the α-subunit, leading to the dissociation of the α-subunit from its βγ-subunit counterpart **(Figure 1.4)** (Beaulieu and Gainetdinov, 2011). This allows the individual subunits to stimulate separate signalling pathways. Generally, dopamine receptors are classified based on biochemical observations of the receptor's ability to modulate adenylate cyclase (AC) activity and subsequent cAMP production. D_1-class dopamine receptors are known to stimulate AC and upregulate cAMP production via coupling to $G\alpha_{s/olf}$ receptors, whereas D_2-class dopamine receptors inhibit AC and reduce cAMP production via $G\alpha_{i/o}$ family of G-proteins **(Figure 1.4)** (Beaulieu and Gainetdinov, 2011).

3.4.2. Regulation of dopamine receptor signalling

Regulator proteins affect the downstream signalling effects of a DR agonist. The RGS (regulators of G-protein signalling) family of proteins act to reduce G-protein signalling **(Figure 1.4)**. This occurs through GTP hydrolysis, where the RGS protein binds to the GTP-bound α-subunit via the RH domain or RGS box, thereby reducing the lifespan of the active GTP-α subunit and βγ-complexes (Dohlman and Thorner, 1997; Dohlman, 2009). Regulation of dopaminergic GPCR signalling by the GPCR

kinases (GRK)/arrestin system can lead to the promotion of G-protein independent signalling or inhibition of the G-protein coupled signalling. Desensitisation of the GPCR can occur through phosphorylation by GRK at the -COOH terminal of the GPCR, which then recruits and binds the adaptor protein arrestin, and inhibits GPCR activation (Beaulieu and Gainetdinov, 2011). Additionally, the GRK system promotes receptor endocytosis through binding of arrestin to clathrin via adaptor protein β-adaptin **(Figure 1.4)** (Laporte et al., 2002). Alternatively, the GRK/arrestin system can enhance G-protein independent signalling; arrestins serve as signalling mediators that promote scaffolding of various proteins (Akt, MAPK) to induce cellular signalling (Luttrell and Lefkowitz, 2002; Shenoy and Lefkowitz, 2005; Beaulieu et al., 2007).

3.4.3. Role of Akt in D2R-mediated psychosis and cognitive dysfunction in schizophrenia

Akt, also known as protein kinase B, is a serine/threonine kinase involved in the control of growth factor-mediated cell survival and metabolism, including metabolic functions of insulin (see reviews mentioned in (Chen et al., 2001)). It is activated via phosphatidylinositol-mediated signalling to the plasma membrane and phosphorylation at threonine 308 and serine 473 by phosphatidylinositol-dependent kinase 1 (PDPK1) and rictor-mammalian target of rapamycin (mTOR) respectively (Jacinto et al., 2006). Dopamine receptors regulate Akt by forming a complex made of Akt, β-arrestin 2, and PP2A **(Figure 1.4)**. Dopamine signalling leads to dephosphorylation of Akt by PP2A (Beaulieu et al., 2005). It has been shown that inactivation of Akt after D2 receptor stimulation enhances amphetamine-induced psychosis in a mouse model of schizophrenia (Beaulieu et al., 2007). There is evidence suggesting altered sensory-gating mechanism in first episode psychosis is associated with reduced Akt phosphorylation regulated by *Neuregulin-1* (Kim et al., 2009). Akt activation leads to inactivation of two related serine/threonine kinases, GSK-3α and GSK-3β. Inhibition of GSK-3 activity affects dopamine-related behaviours, such as decreased locomotor function (Beaulieu et al., 2005). Additionally, a translocation between Chromosome 1 and 11 was associated to many psychiatric phenotypes, including schizophrenia, expressed in a large Scottish family (St Clair et al., 1990). The Disrupted in Schizophrenia (*DISC-1*) gene locus plays a role in brain development through its regulation of GSK-3β, and suppression of this gene inhibits GSK-3β activity, leading to decreased neural progenitor proliferation (Mao et al., 2009). This correlates with the fact that *DISC-1* risk alleles are associated with changes in working, short and long-term memory (Emamian, 2012). This role that GSK-3β plays in schizophrenia is supported by studies suggesting reduction in phosphorylated GSK-3β in the frontal cortex of patients with schizophrenia, compared to healthy controls (Emamian et al., 2004; Karege et al., 2007).

3.5. Interactions between dopamine and other signalling pathways

3.5.1. Regulation of Mitogen Activated Protein Kinases (MAPK) by D1-class and ionotropic glutamate receptors

Extracellular-signal regulated kinases 1 and 2 (ERK1 and ERK2) are cellular components involved in cellular proliferation and differentiation, and downstream effects of ERK 1/2 include the regulation of synaptic plasticity (Miyazaki et al., 2004). Both D_1 and D_2-class receptors are known to regulate MAPK extracellular-signal regulated kinases 1 and 2 (ERK1 and ERK2) (Beom et al., 2004; Beaulieu and Gainetdinov, 2011). D_1-class receptors promote activation and subsequent signalling of ERK proteins, whereas D_2-class receptors (in particular D3 receptors) inhibit ERK signalling (Zhang et al.,

2004). D1 receptor-mediated activation of ERK is co-dependent on stimulation of ionotropic glutamate receptors, where downstream signaling pathways of both receptors converge (Valjent et al., 2005). ERK activity is regulated by mitogen-activated protein kinase (MEK) in the NMDA pathway, and striatal enriched tyrosine phosphatase (STEP). The STEP protein inhibits ERK activity whereas the MEK protein promotes its activity, thereby causing the overall activity of ERK to remain at equilibrium. When D_1-class receptors are activated, this results in increase in cAMP and subsequent upregulation of PKA activity, leading to stimulation of DARPP-32 (32 kDa dopamine and cAMP-regulated phosphoprotein) and inactivation of the STEP regulator protein PP1 (protein phosphatase 1). By inhibiting PP1 activity, there is no longer inhibition of ERK by STEP, as PP1 can no longer activate STEP **(Figure 1.5)**. Thus, activation of ERK by MAP2K is permitted (Valjent et al., 2005). ERK phosphorylation is crucially involved in the activation of multiple transcription factors **(Figure 1.5)** and subsequently regulates gene expression of dopamine-related behaviours (Brami-Cherrier et al., 2005). An in-vivo study showed that persistent DARRP-32 dependent ERK activation and phosphorylation of downstream transcription factor histone H_3 is associated with dyskinesia in L-DOPA treated mice, and that inhibition of ERK phosphorylation prevented the induction of dyskinesia (Santini et al., 2007). Elucidating the mechanism behind this association may be pivotal in understanding how to control movement disorders associated with antipsychotic use.

3.5.2. Serotonergic regulation of dopamine neurotransmission

Recent studies have suggested that serotonin (5-HT) is a regulator of dopamine neurotransmission in all major dopaminergic pathways (Alex and Pehek, 2007). Dopaminergic neurons are innervated by 5-HT neurons that start from the medial and dorsal nuclei raphe, where there may be direct synaptic contact observed in the midbrain (Nedergaard et al., 1988). Specifically, cortical 5-HT_{1A} receptors is able to modulate dopamine activity by acting on glutamatergic projections regulating dopamine neurons in the mesocortical pathway (Díaz-Mataix et al., 2005). Hypofunction of dopaminergic neurons in the mesocortical pathway is linked to reduced activity in the prefrontal cortex, which is associated with negative symptoms and cognitive deficits observed in schizophrenia (Weinberger, 1987; Alex and Pehek, 2007).

4. Antipsychotics

4.1. Neuropharmacology of antipsychotics

4.1.1. Typical antipsychotics

Antipsychotics are the mainstay drugs used in the management of psychosis. Typical antipsychotics, including haloperidol **(Table 1.3)**, are clinically effective in resolving psychotic symptoms due to their ability to occupy >70% D2 receptors. However, inhibiting D2 signalling in mesocortical and nigrostriatal pathways lead to significant side effects seen with long-term typical antipsychotic use, such as extrapyramidal symptoms and tardive dyskinesia, thus limiting compliance (Hensler et al., 2013). Other common side effects are shown in **Table 1.3**. Although studies have described progressive cognitive decline with long-term haloperidol treatment, the CATIE trial has shown no advantage of typical antipsychotic perphenazine over atypical antipsychotics in neurocognitive improvements (Keefe et al., 2007; Goff et al., 2011).

4.1.2. Atypical antipsychotics

Atypical, or second-generation antipsychotics are able to bind to a wide range of receptors in the CNS. Although atypical antipsychotics including the prototypical clozapine **(Table 1.3)** have <70% D2 receptor occupancy, they have similar therapeutic effect to typical antipsychotics on positive symptoms, with an arguably better influence on negative symptoms and cognition. This therapeutic effect is thought to occur through their high affinity to 5-HT receptors. Clozapine is a full agonist to 5-HT_{1A} receptors and an inverse agonist to 5-HT_{2A} receptors **(Table 1.3)**. 5-HT_{1A} receptor activation increases cortical dopamine neurotransmission, as evidenced by preclinical in vivo studies that demonstrate atypical antipsychotics require functional activation of 5-HT_{1A} receptors (expressed in frontal and cingulate cortices) to stimulate dopaminergic activity in the mesocortical pathway (Díaz-Mataix et al., 2005). Given that dopamine deficits in the prefrontal cortex is associated with negative symptoms and cognitive decline in schizophrenia, it may be that atypical antipsychotics act through 5-HT_{1A} receptors to exert their therapeutic effect. Conversely, 5-HT_{2A} receptor blockade prevents stimulation of receptors on the pyramidal neurons in the prefrontal cortex, thus preventing dopaminergic activity in the VTA (Analía et al., 2005). This may provide a hypothesis to how atypical antipsychotics have a similar clinical efficacy to typical antipsychotics, despite lower affinity for dopamine receptors. Furthermore, clozapine has a 10-fold affinity for D4 as compared to D2 or D3 receptor subtypes, which may explain its reduced extrapyramidal side effects in relation to typical antipsychotics (Ogawa, 1995).

4.2. Current evidence on effect of antipsychotics on astrocyte function

The effect of prolonged medication is a difficult confounding factor to delineate in post-mortem studies. All antipsychotic drugs display some level of dopamine D2 antagonism (Karam et al., 2010). Because astrocytes express D2 receptors, long-term administration of antipsychotics may affect the role of astrocytes in the pathology of schizophrenia (O'Connell et al., 2013). Chronic exposure of haloperidol and olanzapine in Macaque monkeys lead to significant decrease in astrocyte number and density (Konopaske et al., 2008). Furthermore, C6 astrocytes treated with haloperidol and clozapine lead to reduced levels of S100B, which varied according to therapeutic dose (Steiner et al., 2010). This stands in contrast to findings of increased S100B found in CSF and serum of patients with schizophrenia (Rothermundt et al., 2004; Steiner et al., 2008; Rothermundt et al., 2009; Steiner et al., 2011; O'Connell et al., 2013). This could be mechanistically explained by the unique relationship between S100B and D2 receptors. S100B binds to the third cytoplasmic loop of the D2 receptor and enhances receptor stimulation of ERKs and inhibition of adenylate cyclase (Liu et al., 2008). Therefore, the S100B/D2 protein complex could serve as the therapeutic target for antipsychotic medication. Clozapine, but not haloperidol, is shown to enhance astrocytic exocytosis of D-serine and subsequent upregulated glutamate transmission via NMDA activation (Tanahashi et al., 2012). This has potential to be further explored. D-serine itself has been studied as a potential therapeutic target, but direct administration of D-serine leads to nephrotoxicity (Orozco-Ibarra et al., 2007). However, there is promising evidence that enhancing D-serine levels using an astrocytic α7nAChR partial agonist (EVP 6124) increases NMDA activity, via 'wakefulness-dependent cholinergic activity', to improve cognitive deficits (Papouin et al., 2017).

4.3. Effects of antipsychotics on inflammation

An altered immune system could contribute to the aetiopathology of schizophrenia. An unregulated immune response may be able to trigger or change the clinical course of schizophrenia, by way of complex mechanisms involving altered neurotransmission and synaptic plasticity (E. et al., 2012). In general, studies consistently show that antipsychotics have some anti-inflammatory effect in schizophrenia, where there is a decrease in IFN-γ and IL-1β and no change in other cytokines (IL-2, IL-4, IL-10, IL-1RA, sIL-6R, TGF-β1 and TNF-α), although clozapine shows an increase in IL-6 (Tourjman et al., 2013). Clinically, long-term clozapine use is associated with agranulocytosis, a life-threatening side effect characterised by abnormally low serum levels of neutrophils. This occurs in the minority of patients (1-3%), and peaks at about 3 months (Alvir et al., 1993). Inconclusive data on immunomodulatory effects seen with atypical antipsychotics such as clozapine and risperidone may be contributed by these drugs' propensity to induce metabolic syndrome and its subsequent effect on cytokine release (Hinze-Selch et al., 2000; Zhang et al., 2005; O' Connell and Dev, 2014).

Gene	Position	Hypothesised function	Phenotype/behaviour in SZ	References
Disrupted in schizophrenia (DISC1)	1q42	Neurite development, synaptogenesis and synaptic plasticity. Also interacts with NUDEL – microtubule organisation and cellular migration	Deficits in working memory* Abnormal neuronal migration and development of cerebral cortex*	(Karam et al., 2010)
Neuregulin 1 (NRG1)	8p12-21	Myelination, glial cell development, migration of radial cells during cortical development, neuronal plasticity, dopamine/5HT/monoamine transporter expression	Altered social behaviour and memory deficits* Decreased inhibitory interneurons and increased ventricular volume*	(Karam et al., 2010)
Catechol-O-methyl transferase (COMT)	22q11	Catecholamine metabolism (including dopamine)	VAL/VAL allele associated with greater risk of psychosis in cannabis users	(Henquet et al., 2006)
D-amino acid oxidase activator (DAO/G30)/D-amino acid oxidase (DAO)	12q24.11	DAO breaks down D-serine, the NMDA co-factor found in astrocytes	Contributes to efficiency of glutamate gating at NMDA ion channel, increasing risk of SZ	(Corvin et al., 2007)
Proline dehydrogenase (PRODH)	22q11.2	L-proline is a direct modulator of glutamatergic transmission. Also initiates apoptosis.	Regional alterations of GABA, glutamate and dopamine. Also deficits in sensorimotor gating* Interaction between COMT and PRODH, where COMT upregulation compensates for increased dopamine signalling caused by PRODH deficiency	(Karam et al., 2010)
Dystrobrevin binding protein 1 (dysbindin/DTNBP1)	6p27	Promotes axon stability, synaptic vesicle trafficking and neurotransmitter release. Modulates prefrontal cortical activity via D2 pathway.	Decreased cognitive response control	(Fallgatter et al., 2010)
Zinc finger protein 804A (ZNF804A)	2q32.1	Possible role as transcription factor	Identified in GWAS with slightly below genome-wide significance level	(O'Donovan et al., 2008) (Schwab and Wildenauer, 2013)
RAC-alpha serine/threonine protein kinase (AKT1)	14q32.33	Role in metabolism, cell stress, cell cycle regulation. Regulates neuronal cell size, cell survival, synaptic plasticity	Decreased Akt-1 in hippocampus and frontal cortex	(Karam et al., 2010)
Glycogen synthase kinase 3 (GSK-3)	3q13.33	Regulates glucose metabolism and synaptic plastivity	Decreased GSK-3 in frontal cortex	(Karam et al., 2010)

Table 1.1. Summary of major historical candidate genes implicated in schizophrenia (*based on animal models)

	D1	D2	D3	D4	D5
Class subtype	D1-class	D2-class			D1-class
Gene	DRD1	DRD2	DRD3	DRD4	DRD5
Chromosomal locus	5q35.1	11q23.1	3q13.3	11p15.5	4p16.1
No. of introns	None	6	5	3	None
No. of amino acids	446	D2S, 414 D2L, 443	400	387	477
Principle transduction	$G\alpha_s$, $G\alpha_{olf}$	$G\alpha_i$, $G\alpha_o$	$G\alpha_i$, $G\alpha_o$	$G\alpha_i$, $G\alpha_o$	$G\alpha_s$, $G\alpha_q$
Expression in CNS	Str, NA, SN, olf b, amygdala, FC, PFC, hip*, cer*, thal*, hypot*, ret	Str, NA, SN, olf t, SN, VTA, hypot, PFC, septum, amygdala, hip, cortical areas, ret, pituitary	NA, olf t, IoC, striatum, SNpc*, VTA*, hip*, septum*, cortical areas	FC, amygdala, hip, hypot, GP, SNpr, thal, ret	PFC, PMC, CC, EC, SN, hypot, hip, DG, CN*, NA*
Expression in astrocytes	Mc, Str	Str*, PFC	Str	Mc, Str	Mc, Str

Table 1.2. Genetic, biological and anatomical properties of each dopamine receptor class subtype. *Lower level of expression. Based on data gathered in review (Beaulieu and Gainetdinov, 2011).

(CC – cingulate cortex, Cer – cerebellum, DC – dentate gyrus, EC – entorhinal cortex, FC – frontal cortex, GP – globus pallidus, Hip – hippocampus, Hypot – hypothalamus, IoC – islands of Calleja, NA – nucleus accumbens, Mc – mesencephalon, Olf b – olfactory bulb, Olf t – olfactory tubercle, PFC – prefrontal cortex, PMC – premotor cortex, SN – substantia nigra, SNpc – substantia nigra pars compacta, SNpr – substantia nigra pars reticulate, Str – striatum of basal ganglia, Thal – thalamic area, VTA – ventral tegmental area)

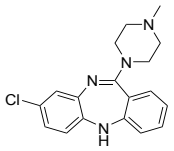
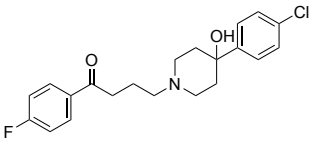
	Clozapine		Haloperidol	
Pharmacological class	Atypical antipsychotic (1st generation)		Typical antipsychotic (2nd generation)	
Chemical structure				
Receptor activity	Dopamine	D2, D3, D4, D5	Dopamine	D1, D2, D3, D4
	5-HT	1A, 1B, 1E, 1F, 2A, 2B, 2C, 5C, 6, 7	5-HT	1A, 1D, 2A, 2B, 7
	Histamine	H1, H3, H4	Histamine	-
	Musc.	M1	Musc.	-
	Adr.	α1A, α1B	Adr.	-
Primary receptor targets (in order of highest affinity)	5-HT2A	Inverse agonist (pKi 7.6 – 9.0)	D4	Antagonist (pKi 8.7 – 8.8)
			D2	Antagonist (pKi 7.4 – 8.8)
	D2	Antagonist (pKi 5.6 – 6.9)	D3	Antagonist (pKi 7.5 – 8.6)
			5-HT2A	Antagonist (pKi 6.7 – 7.3)
Common side effects	Anorexia, hypersalivation, anticholinergic symptoms, malaise, speech disorders		Depression, weight change	
Uncommon side effects	Agranulocytosis		Dyspnoea, oedema	

Table 1.3. Pharmacological effects of clozapine and haloperidol. Side effects based on information provided by the British National Formulary (Joint Formulary Comittee, 2018). Data on clozapine receptor activity based on previous studies (Sunahara et al., 1991; Lahti et al., 1993; Egan et al., 1998; Yoshio et al., 2001; Booth et al., 2002; Knight et al., 2004). Data on haloperidol receptor activity based on previous studies (Sokoloff et al., 1990; Sokoloff et al., 1992; Lahti et al., 1993; Freedman et al., 1994; MacKenzie et al., 1994; Tice et al., 1994; Mierau et al., 1995; Egan et al., 1998; Shahid et al., 2009).

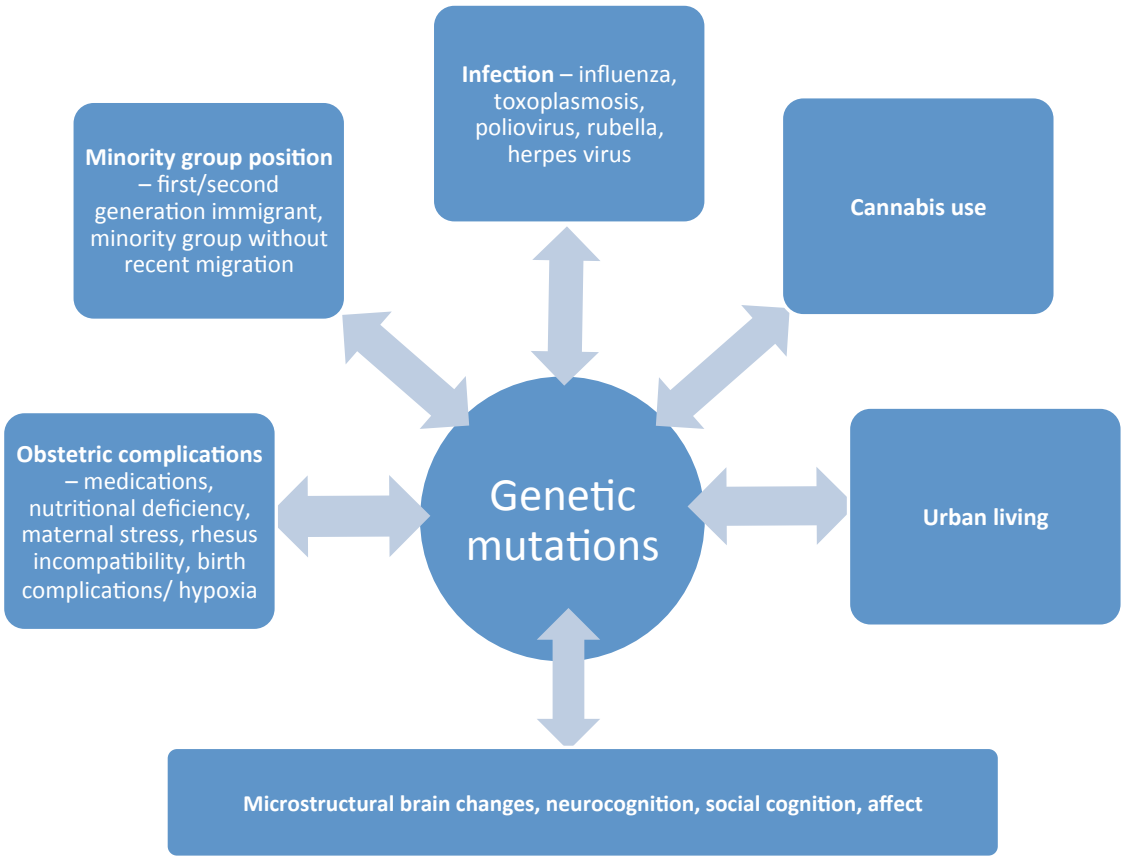


Figure 1.1. Epigenetic model of schizophrenia (van Os et al., 2010)**.** *Arrows reflect the interaction between different environmental factors and the genetic mutations, which produces the phenotype of schizophrenia*

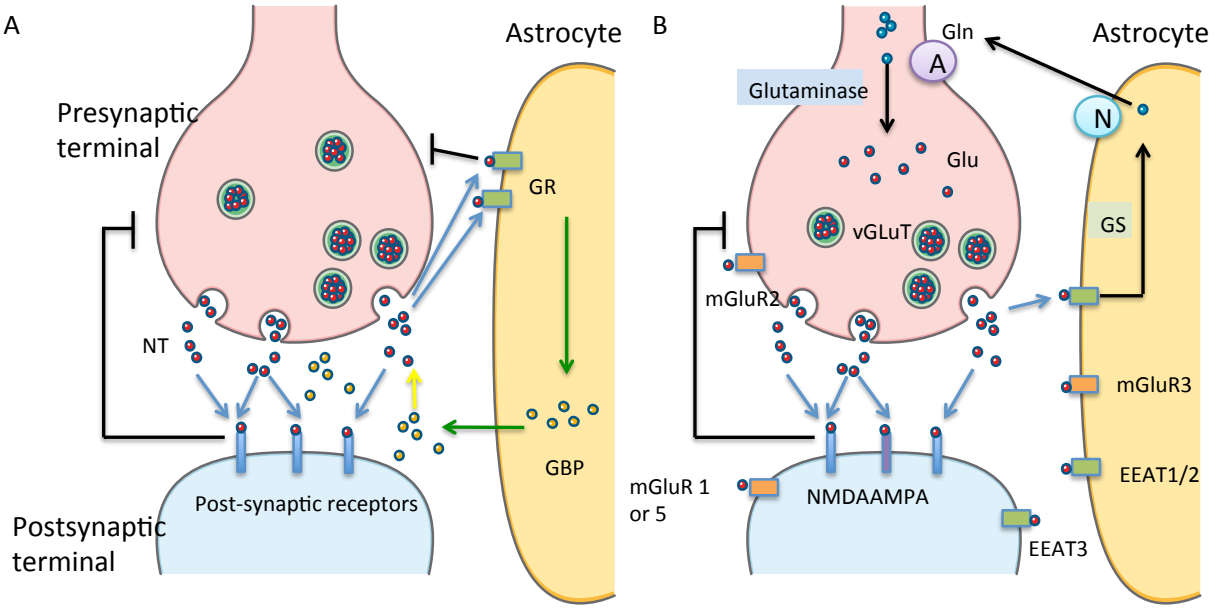


Figure 1.2. Model of the tripartite synapse and astrocyte regulation of glutamate neurotransmission. A) The neurotransmitter (NT) is released from the pre-synaptic terminal and binds to the post-synaptic receptor and glial receptor (GR). Simultaneously, there is a basal level of glial binding protein (GBP) in the synaptic cleft, which binds to the neurotransmitter. This inactivates free neurotransmitters, which are then unable to bind to post-synaptic receptors. During periods where there is a high concentration of neurotransmitter release, the activated glial receptor increases production of glial binding protein into the synaptic cleft (Mitterauer, 2005). **B)** Glutamate (Glu) is synthesised from glutamine (Gln) by glutaminase. It is then packaged into vesicles by vesicular glutamate transporters, and then exocytosed into the synaptic cleft via SNARE complex proteins. Glutamate then binds onto ionotropic receptors (NMDA or AMPA) and metabotropic receptors (mGlu1-5) on the post-synaptic terminal, presynaptic terminal and glial cell. Glutamate is cleared from the synaptic cleft through excitatory amino acid tranporters (EEAT) 1 and 2 on the astrocyte, and to a lesser extent, EEAT3 transporters on the post-synaptic neuron. In the astrocyte, it is converted back to glutamine by glutamine synthetase (GS). Glutamine is then released from the astrocyte by System N transporters and taken up back into the presynapse by System A Na+-coupled amino acid transporters (Popoli et al., 2012).

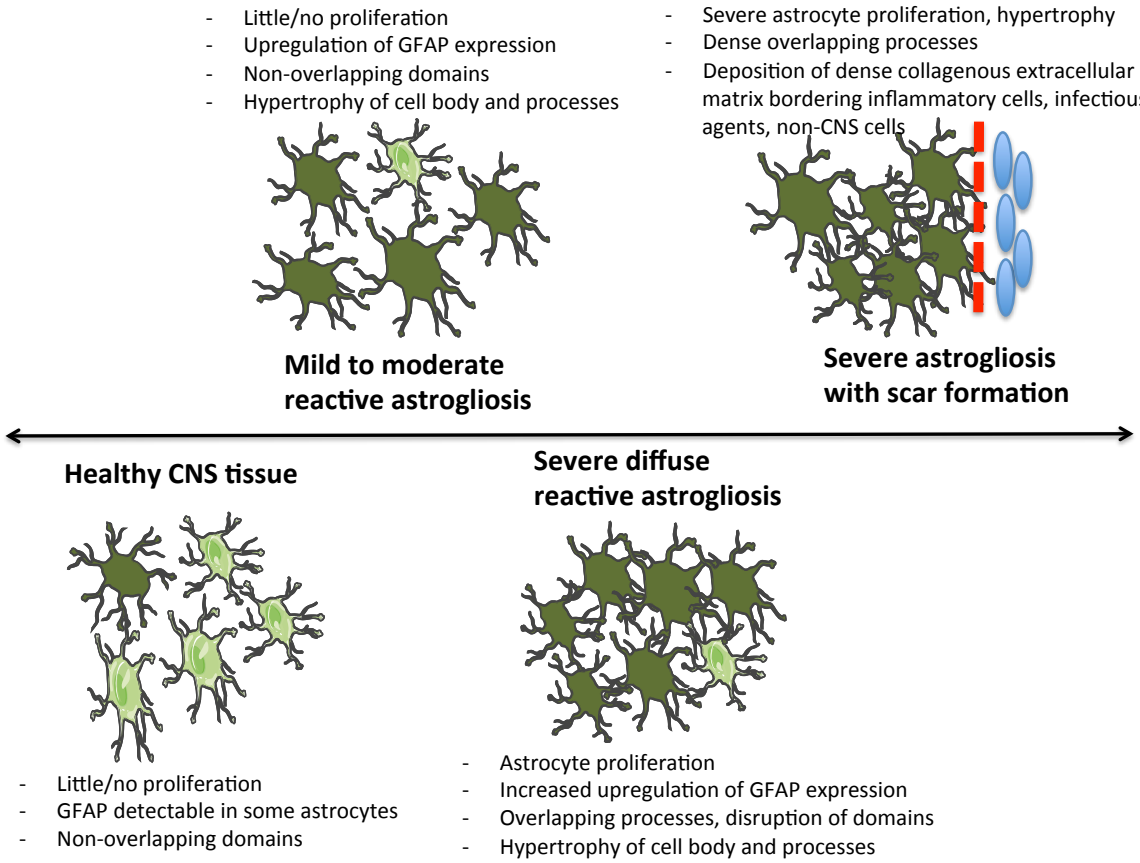


Figure 1.3. Representation of the progression of different stages of reactive astrogliosis. Mild/moderate astrogliosis is a reversible process involving variable changes of molecular expression and function, triggered by mild non-penetrative non-contusive trauma, viral/bacterial infections or in areas that are removed from focal CNS lesions. Severe diffuse reactive astrogliosis surrounds areas of focal CNS lesions, infections, and as result of chronic neurodegeneration. Because there is prolonged reorganization of tissue architecture, this process is often irreversible. Severe reactive astrogliosis with compact glial scar formation occurs at the forefront of inflammation in the CNS. Mature glial scars act as an obstacle to axon regeneration and protect healthy CNS tissue from inflammatory and foreign cells (Sofroniew and Vinters, 2010).

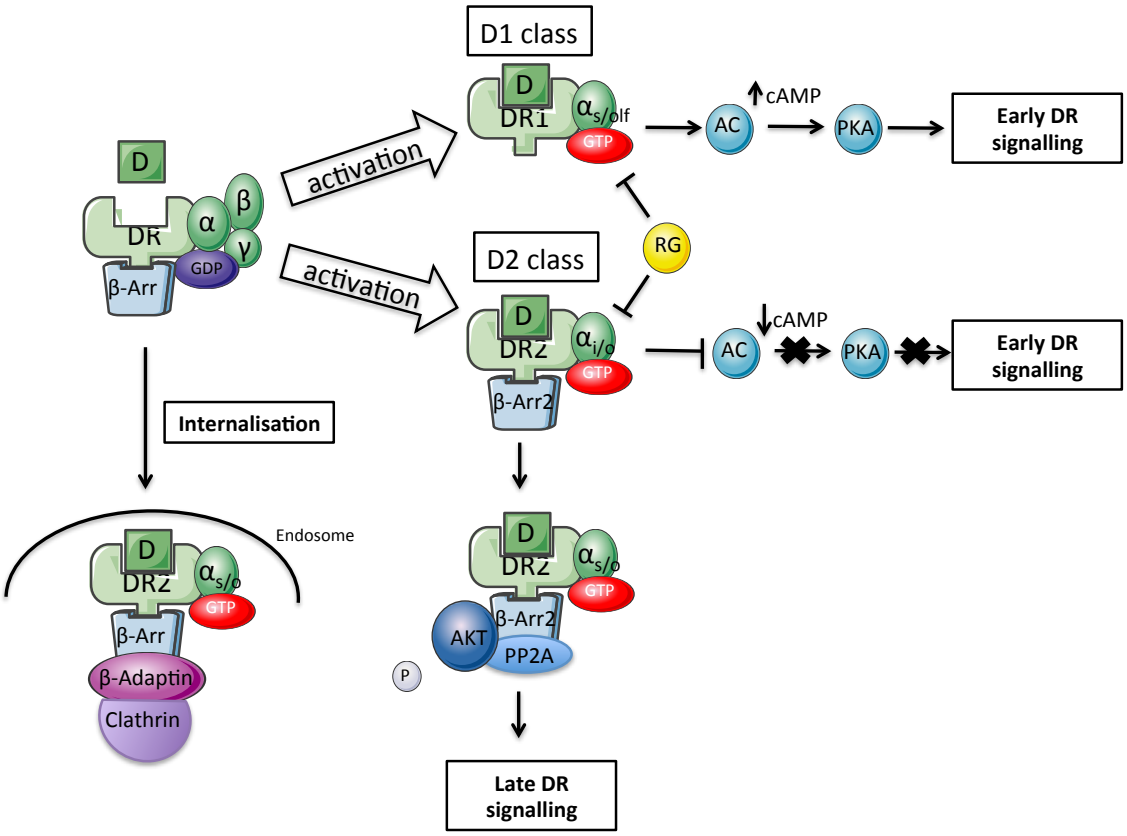


Figure 1.4. Early and late dopamine receptor signalling. Activation of the dopamine receptor leads to differing downstream signalling for D_1- and D_2-class subtypes. In the early phase of dopamine signalling, the dopamine receptor signalling is inhibited by RG inactivating G-proteins. Additionally, recruitment of β-arrestins and clathrin-mediated internalisation further inactivates G-protein mediated signalling. In the late phase of dopamine signalling, the dopamine receptor forms a complex with β-arrestin, PP2A and AKT causing dephosphorylation of AKT and GSK-3 mediated signalling, and results in a longer-lasting signalling response (Beaulieu and Gainetdinov, 2011).

(AC – adenylate cyclase, β-Arr - β-arrestin, DR – dopamine receptor, PKA – protein kinase A, RG – Regulators of G-protein family of proteins)

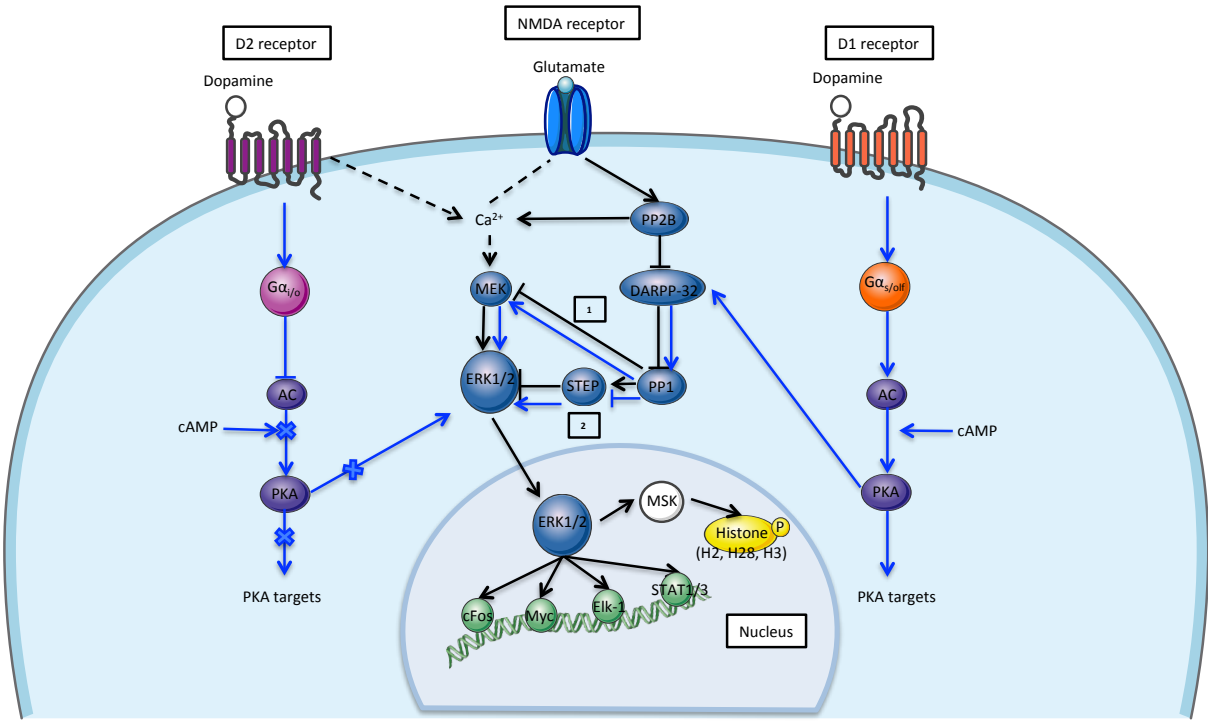


Figure 1.5. Regulation of ERK 1/2 by D1, D2 and glutamate receptors. D1 receptors upregulate ERK 1/2 whereas D2 receptors downregulate ERK 1/2, thereby regulating its translocation into the nucleus and activation of numerous transcription factors shown. ERK 1/2 can be regulated via **1)** MEK activation or **2)** DARPP-32 regulation of PP1 and STEP (Beaulieu and Gainetdinov, 2011).

Blue arrows indicate dopamine signalling, black arrows indicate glutamate signalling, dotted lines show pathway where other signalling molecules not shown.

Methods and Materials

5. Materials

5.1. Compounds

All compounds used are summarised in **Table 2.1**. Clozapine and haloperidol were both prepared as 20mM stock in dimethyl sulfoxide (DMSO, Sigma; D8418). The cytokines TNFα (R&D system; 421-ML) and IL-17A (Peprotech; 300-01A-50G) were reconstituted as per manufacture's guidelines and prepared as 100μg/ml stock solutions. Hydrogen peroxidase (H_20_2, Sigma; 216763) and all above compounds were diluted into their working concentrations in serum-free media prior to every treatment.

5.2. Antibodies

All antibodies used for immunocytochemistry and western immunoblotting are summarised in **Table 2.2**. Primary antibodies used include: mouse anti-total Extracellular signal-Related Kinase 1/2 (ERK 1/2, Millipore; 05-1152), rabbit anti-phosphorylated ERK 1/2 (Millipore; 05-797R), rabbit anti-actin (Abcam, ab8227), mouse anti-vimentin (Santa-Cruz; sc-373717), rabbit anti-glial fibrillary acidic protein (GFAP, Abcam; ab7260). Secondary antibodies used include: goat HRP-conjugated anti-mouse (Sigma; A8924), goat HRP-conjugated anti-rabbit (GE Healthcare; NA934), donkey Alexa 488 anti-rabbit (Alexa; A11008), donkey Alexa 549 anti-mouse (Jackson Immunoresearch; 115-506-068).

6. Methods

6.1. Cell culture

All in vitro cell culture was performed using stringent aseptic techniques. All cell culture preparations were undertaken under the laminar flow hood (FASTER) and any equipment used was autoclaved and sprayed with 70% ethanol before use. An antifungal agent (Aqua Resist; 4627000) was added to water in incubators and water baths to minimise the incidence of infection.

Human cortical astrocytes derived from male foetal brains at 21 weeks gestation were purchased from ScienCell Research Laboratory USA (1800, Lot No. 9063 and 11065). Cells were stored in liquid nitrogen and thawed under provider's instructions. Cells were maintained in DMEM/F12 (Fisher; 10770245) supplemented with 10% foetal bovine serum (Sigma; F7524) and 1% penicillin/streptomycin (Sigma; F7524) +/- 1% astrocyte growth supplement (ScienCell; 1852) in T-75 flasks in a humidified incubator (37^{o}C, 5% CO_2). Cells were cultured in T-75 flasks and media changed every 2-3 days. When 80-100% confluent, cells were sub-cultured by incubating the astrocyte layer with 6ml trypsin/EDTA (SAFC Biosciences; 59418C) for 5 minutes at 37^{o}C, then trypsin was inactivated with complete medium. The cell suspension was then collected, centrifuged and the cell pellet was resuspended in 1ml complete media. Cells were counted using a haemocytometer then plated onto 12-well plates at 100,000 cells per well. For treatment, cells were grown for 72h until 80-100% confluent before serum-starved for 4h and treated as per figure legend. Cell pellets were used to quantify protein levels of ERK 1/2 using Western immunoblotting. Media surrounding cells was used to measure protein levels of IL-6 by ELISA (R&D systems).

6.2. Enzyme Linked Immunoabsorbent Assay (ELISA)

Protein levels of IL-6 in cell culture supernatant were measured using ELISA kits (R&D systems; DY008) and carried out according to manufacture's protocol. Human IL-6 standard (R&D systems; DY008-840115) concentrations ranged from 9.38 – 600pg/ml. 96-well ELISA plates were coated with capture antibody (R&D systems; DY008-840113) diluted in PBS and left overnight at room temperature. The coated plate was washed three times in wash buffer (PBS-T) (0.05% Tween 20 (Sigma; P7949) in PBS, pH7.2-7.4) before blocking with reagent diluent (1% bovine serum albumin (BSA, Roche; 10015546) in PBS) for 1 hour at room temperature. The wash step was repeated. 50µl of standards diluted in reagent diluent and 50µl of samples were added to the wells in duplicate. At this stage the plates were left to incubate overnight at 4^{o}C. The wash step was repeated and detection antibody (R&D systems; DY008-840114) diluted in reagent diluent was added and left for 2h at room temperature. The plate was then washed again, before streptavidin-HRP (R&D systems; DY008-893975) in reagent diluent was added for 20 minutes at room temperature away from direct light. Substrate solution (OPD peroxidase substrate made with OPD (Sigmafast; SLBS6449) and buffer with urea H_2O_2 (Sigmafast; SLBS6451) in dH_2O) was added to each well for 20 minutes at room temperature, protected from direct light. At this point, stop solution (2N H_2SO_4, FisherScientific; 7664-93-9) was added to halt colour reaction. The optical density in each well was determined using a microplate reader (Labsystem Multiskan) at 490nm. A standard curve was produced using average absorbance values plotted against the concentrations of human IL-6 standard wells. Average absorbance values from sample wells were plotted against the standard curve, and protein concentrations of IL-6 in pg/ml were determined.

6.3. MTT assay

Human astrocytes were seeded onto 24-well plates at 70,000 cells per well and cultured for 72h until 90-100% confluent. The cells were serum starved for 4h before treatment with clozapine or haloperidol at different concentrations (10nM, 100nM, 1µM, 10µM, 100µM) for 18h. The media with compounds was then removed and the wells were washed briefly with PBS. Serum-free media was added to each well, and 0.2% Triton X-100 in PBS (Triton-X, Sigma; T9284) was added to positive control wells for 10 minutes. At this stage, 80µl of 5mg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT, Thermo Fischer; 1967002) was added to each well and incubated for 3.5h at 37^{o}C. The media supplemented with MTT was then removed and 250µl of DMSO was added to each well. The plate was covered with foil and placed on a rocker for 15 minutes to ensure the crystals dissolved in DMSO. The samples were read at 550nm and absorbance was interpreted as a percentage of control.

6.4. Immunocytochemistry

Human astrocytes were plated in 12-well plates lined with 18mm coverslips (VWR) and treatments were carried out as per figure legend. Coverslips were washed with 70% ethanol for 1 hour and then dried out under the laminar flow hood for 1 hour before being placed into 12-well plates. The cells were washed twice with PBS then fixed with 4% paraformaldehyde (PFA, Sigma; F1635) in PBS, pH 7.4. This was followed by 3 washes in PBS. The cells were permeabilised and non-reactive sites were blocked in blocking buffer (0.1% Triton-X, 1% BSA in PBS) for 1 hour at room temperature. All steps

as described above were carried out on ice. The cells were then incubated with primary antibodies anti-vimentin antibody (Santa Cruz; sc-373717) and anti-GFAP antibody (Abcam; ab7260) diluted in blocking buffer overnight at 4 ^{o}C. The wash steps were repeated. This was followed by incubation with appropriate secondary antibody diluted in blocking buffer for 1 hour at room temperature. The wash steps were repeated. Cells were counterstained with Hoescht 34580 nuclear stain (1:500 in PBS, Sigma-Aldrich) for 15-30 minutes at room temperature. The coverslips were mounted onto microscope slides antifade reagent (SlowFade Gold Antifade reagent, Life technology; S36936) and the edges were sealed with nail varnish. Cells were visualised using a fluorescent microscope (Olympus BX51 upright microscope) under 20x objective. Images were captured and optimised using the imaging software cellSens. Images were analysed using ImageJ software (https://imagej.nih.gov/ij/download.html).

6.5. Analysis of immunofluorescent staining

Images of cells were analysed using ImageJ software. In order to quantify the effect of clozapine/haloperidol +/- TNFα/IL-17A on vimentin/GFAP expression, the number of astrocyte projections were counted as per example shown in **Figure 2.3**. A 50µm concentric circle was drawn around the cell nucleus using the plugin "Concentric Circle" (https://imagej.nih.gov/ij/plugins/concentric-circles.html), and the number of astrocyte projections that extended outside this circle was counted for each cell. A total of 20-30 cells were analysed per treatment group, and the average number of projections was calculated separately for both GFAP and vimentin – stained astrocytes. In order to quantify the effect of clozapine/haloperidol +/- TNFα/IL-17A on astrocyte survival, the number of DAPI-stained nuclei were counted per 2400x1800 pixel view. A total of 3x images per treatment group were counted and averaged out separately for clozapine and haloperidol-treated cells.

6.6. SDS-PAGE and Western immunoblotting

Cell pellets were resuspended in Radioimmunoprecipitation (RIPA) buffer (150mM NaCl (Sigma; A3014), 1% Triton-X, 0.1% sodium dodecylsulphate (SDS, ThermoFisher; S/5200/53), 50mM Tris (ThermoFisher, S/5200/53) at pH8.0) and protease inhibitor (Roche; 11697498001), and stored in -20^{o}C until used. Protein levels in samples were determined using BCA protein assay kit (ThermoFisher; 23250). Tris-Glycine sample buffer (0.125M Tris-HCl at pH6.8, 50% glycerol (Sigma; G2289), 25% SDS, 10% β-mercaptoethanol (Sigma; MC6250), 11.5% distilled H_20, 0.01g bromophenol blue (Sigma; B0126) was added (1:4 dilution) and samples were denatured at 90^{o}C for 10 minutes. Electrophoresis was carried out on 10% SDS-polyacrylamide gels. Semi-dry electrophoresis transfer was completed using polyvinylidene difluoride microporous membrane (PVDF, Immobilon P, Millipore) activated in methanol (purchased at Solvent store, TBSI, TCD). The PVDF membrane was then incubated in blocking buffer (5% non-fat milk in 0.05% PBS-T) for 1-2h at room temperature. The membrane was washed in PBS-T (0.05% Tween 20 in PBS) 3 x 10 minutes at room temperature before incubating with primary antibody overnight at 4^{o}C. The wash step was repeated, and the membrane was incubated with secondary antibody for 1-2h at room temperature. All antibodies were diluted in either blocking buffer or 5% BSA in PBS-T, according to manufacture's protocol. Membranes were developed by incubating briefly in chemiluminescent HRP substrate

(ThermoScientific; 34580) and imaged using Fujifilm LAS-3000 Intelligent dark box. Densitometry measurement using ImageJ software was used to quantify the intensity of individual bands.

6.7. Statistical analysis

All data was compiled using Microsoft Excel. Statistical analysis was carried out using GraphPad Prism 7.0 software package (GraphPAD Software Inc.). One-way analysis of variance (one-way ANOVA) and post-hoc Dunnett's test for multiple comparisons was used to compare means from 2 or more groups, where a control group was present. Where no control group was present and all possible pairs of means need to be compared, post-hoc Newman Keuls multiple comparisons test was applied instead of Dunnett's test. When two treatment groups with equal sample sizes were compared, a paired one-tailed t-test was used. Individual statistical tests are stated in each figure legend. The significance levels (or alpha levels) were set at $p < 0.05$, $p < 0.01$ and $p < 0.001$. Graphical data is represented in a box and whiskers plot +/- minimum and maximum value.

Compound	Details	Working concentration
Clozapine	Sigma, C6305	10nM - 100uM
Haloperidol	Sigma, H1512	10nM - 100uM
IL17A	R&D system, 421-ML	10 - 100ng/ml
TNF-α	Peprotech, 300-01A-50G	10ng/ml
H_2O_2	Sigma, 216763	0.5mM

Table 2.1 List of compounds

PRIMARY ANTIBODIES						
Target	**Mol. Weight**	**Host**	**Supplier**	**Cat No.**	**Stored**	**Dilution**
tERK	p44/p42	Mouse	Millipore	05-1152	-20°C	1/2000
pERK	p42/p44	Rabbit	Millipore	05-797R	-20°C	1/1000
Actin	p45	Rabbit	Abcam	ab8227	-20°C	1/2500
Vimentin	Polyclonal	Mouse	Santa Cruz	sc-373717	-20°C	1/1000
GFAP	Polyclonal	Rabbit	Abcam	ab7260	-20°C	1/1000

SECONDARY ANTIBODIES					
Target	**Host**	**Supplier**	**Cat No.**	**Stored**	**Dilution**
HRP-conjugated anti-mouse	Goat	Sigma	A8924	-20°C	1/5000
HRP-conjugated anti-rabbit	Goat	GE Healthcare	NA934	-4°C	1/2000
Alexa 488 anti-rabbit	Donkey	Alexa	A11008	-4°C	1/1000
Alexa 549 anti-mouse	Donkey	JIR	115-506-068	-4°C	1/1000

Table 2.2 List of antibodies

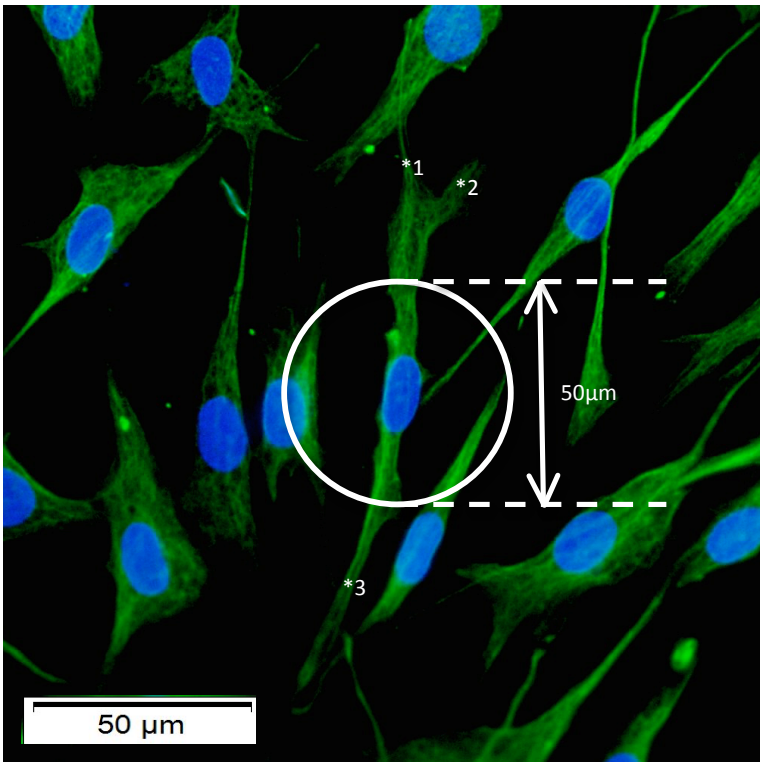


Figure 1. Cellular extension quantification. Images were analysed using ImageJ. A 50µm concentric circle was drawn around the cell nucleus using the plugin "Concentric Circle", and the number of astrocyte projections outside this circle was counted for each cell. In the example shown above, there are a total of 3 astrocyte processes extending beyond the concentric circle, thus the total count for this cell is 3. A total of 20-30 cells were analysed per treatment group, and the average number of projections was calculated separately for both GFAP and vimentin – stained astrocytes.

Results

7. Results

7.1. Treatment of human astrocytes with IL-17A/TNFα-induces expression of IL-6.

Previously, our study has shown that human astrocytes express IL-17A receptors, and in vitro treatment with IL-17A induces an increase in protein levels of IL-6, which is synergistically increased by the addition of TNFα (Elain et al., 2014). Here, we confirmed these findings demonstrating that increased levels of IL-17A induce a concentration-dependent increase in IL-6 (**Figure 1**). Human astrocytes were treated with increasing amounts of IL-17A (10ng/ml, 20ng/ml, 50ng/ml, 80ng/ml, 100ng/ml) and a fixed concentration of TNFα (10ng/ml) for 18h. Protein levels of IL-6 in the media were then analysed by ELISA (**Figure 1A**). Astrocytes treated with IL-17A/TNFα were compared to untreated cells, and showed a statistically significant increase in levels of IL-6 with increasing concentrations of IL-17A (10ng/ml, 327.6+/-104.2pg/ml; 20ng/ml, 363.8+/-93.0pg/ml; 50ng/ml, 550.3+/-129.1pg/ml; 80ng/ml, 440.3+/-116.3pg/ml; 100ng/ml, 447.7+/-92.6pg/ml, one-way ANOVA and post-hoc Newman-Keuls test, *$p < 0.05$, **$p < 0.01$) (**Figure 1B**). The maximum increase in IL-6 was found using 50ng/ml of IL-17A (550.3+/-129.1pg/ml) compared to control (14.8+/-7.1pg/ml, one-way ANOVA and post-hoc Newman-Keuls test, **$p < 0.01$). This concentration of IL-17A (50ng/ml) with TNFα (10ng/ml) was used to induce IL-6 expression in subsequent experiments, unless otherwise indicated. This data confirms findings from previous studies demonstrating that a pro-inflammatory response was induced by IL-17A/TNFα in human astrocytes.

7.2. Clozapine attenuates IL-17A/TNFα-induced levels of IL-6 in human astrocytes.

We and others have shown that altered secretion patterns of cytokines, including an increase in serum pro-inflammatory IL-6, is seen in patients with schizophrenia (van Kammen et al., 1999; O' Connell and Dev, 2014). Studies have also shown that antipsychotic treatment may play a role in regulating cytokines levels, perhaps providing subtle anti-inflammatory effects, although these results remain controversial (Pollmacher et al., 1996; Monteleone et al., 1997; Borovcanin et al., 2013). Here, we aimed to directly examine the effect of the antipsychotic clozapine on pro-inflammatory response in astrocytes. Human astrocytes were pre-treated with clozapine and then treated with TNFα (10ng/ml) supplemented with high (50ng/ml) or low (10ng/ml) concentrations of IL-17A (**Figure 2A**). In agreement with previous data, higher concentrations of IL-17A (50ng/ml)/TNFα induced an increase in IL-6 (1351.0+/-103.8 pg/ml vs. control 20.9+/-2.0pg/ml) compared to lower concentrations of IL17A (10ng/ml)/TNFα (252.9+/-41.9pg/ml vs. control 6.5+/-2.3pg/ml, one- way ANOVA and post hoc Dunnett's multiple comparison test, ***$p < 0.001$). In clozapine-treated groups, a dose-dependent decrease in the levels of IL-6 were observed, with 10μM clozapine showing an inhibition of 29.8% +/- 6.5% (947.8+/-192.2pg/ml vs. 1351+/-103.8g/ml, paired t test, $^{+}p < 0.05$). This effect of clozapine was more observable when using the lower concentration of IL-17A (10ng/ml)/TNFα, with a significant decrease in IL-6 release of 47.2% +/- 8.6% with 10μM clozapine (133.5+/-20.2pg/ml vs. 252.9+/-41.9pg/ml, paired t-test, $^{++}p < 0.01$) (**Figure 2B**). In summary, these results show that clozapine decreases IL-6 release from astrocytes, where the findings are more pronounced when using a low concentration (10ng/ml) as compared to a higher concentration (50ng/ml) of IL-17A/TNFα. This finding corroborates the idea that clozapine may have anti-inflammatory properties.

7.3. IL-17A/TNFα -induced levels of IL-6 are decreased by Haloperidol.

To further investigate whether effects of clozapine on levels of IL-6 were specific to this drug, we next aimed to examine the effects of a typical antipsychotic, haloperidol, on IL-6 release from astrocytes. Human astrocytes were cultured and treated with TNFα (10ng/ml) supplemented with high (50ng/ml) or low (10ng/ml) concentrations of IL-17A (**Figure 3A**). Similar to previous experiments, both high (50ng/ml) and low (10ng/ml) concentrations of IL-17A resulted in a significant increase in IL-6 compared to control (IL-17A 50ng/ml, 340.9+/-28.7pg/ml vs. 43.3+/-8.6pg/ml; IL-17A 10ng/ml, 60.0+/-17.4pg/ml vs. 13.9+/-1.3pg/ml, one- way ANOVA and post hoc Dunnett's multiple comparison test, **p < 0.01, ***p < 0.001). The data also showed haloperidol attenuated the levels of IL-6 induced by high concentrations of IL-17A (50ng/ml)/TNFα (10nM, 299.1+/-32.3pg/ml; 100nM, 262.8+/-24.2pg/ml; 1μM, 266.4+/-27.0pg/ml; 10μM, 270.3+/-17.6pg/ml vs. TNFα/IL-17A only, 340.9+/-28.7pg/ml, paired t-test, $^{+}p < 0.05$, $^{++}p < 0.01$) (**Figure 3B**). In conditions using a low concentration of IL-17A (10ng/ml)/TNFα, the pre-treatment of human astrocytes with haloperidol also inhibited levels of IL-6, although these effects were not as pronounced as that observed for clozapine (10nM, 24.6+/-2.8pg/ml; 100nM, 24.5+/-6.0pg/ml; 1μM, 24.8+/-3.5pg/ml; 10μM, 23.5+/-5.0pg/ml vs. TNFα/IL17A only, 60.0+/-17.4pg/ml, paired t-test, $^{+}p < 0.05$) (**Figure 3B**). Overall haloperidol appeared to exert a lesser effect on IL-17A/TNFα-induced levels of IL-6 in astrocytes compared to clozapine. These data are, nevertheless, in line with previous studies that have demonstrated a decrease in peripheral IL-6 levels following haloperidol treatment (Borovcanin et al., 2013; Handley et al., 2016).

7.4. Clozapine and Haloperidol do not alter human astrocyte cell viability.

In order to confirm the reduction in IL-6 caused by clozapine and haloperidol was not due to changes in human astrocyte cell viability or cell survival, an MTT assay was conducted. In this experiment, human astrocytes were treated with increasing concentrations of clozapine or haloperidol (10nM, 100nM, 1μM, 10μM, 100μM) for 18h (**Figure 4A**). For all concentrations of clozapine, there was no significant change in cell survival as compared to control (10nM, 10.3+/- 1.7%; 100nM, 3.8+/-0.6%; 1μM, 7.3+/-1.2%; 10μM, 2.1+/-0.3%; 100μM 4.0+/-0.7%, one-way ANOVA and post-hoc Dunnett's multiple comparison test) (**Figure 4B**). These effects were replicated in haloperidol-treated astrocytes, where all concentrations of haloperidol did not affect cell survival (10nM, 8.64+/-2.00%; 100nM, 12.35+/-2.86%; 1μM, 2.44+/-0.56%; 10μM, -0.61+/-0.14%; 100μM, 4.29+/-1.00%, one-way ANOVA and post-hoc Dunnett's multiple comparison test) (**Figure 4C**). In contrast, and as a positive control, 0.2% Triton-X100 (0.2% Tx) treated cells induced significant reduction in cell survival compared to non-treated control (81.79+/-13.55%, one-way ANOVA and post-hoc Dunnett's multiple comparison test, ***p < 0.001) (**Figure 4**). This suggests that the antipsychotics clozapine and haloperidol do not cause astrocyte cell death in-vitro and moreover their attenuation of IL-6 levels induced by IL-17A/TNFα is not due to changes in cell viability.

7.5. Clozapine and Haloperidol do not significantly alter astrocyte morphology, under conditions of inflammation.

There is evidence of decreased astroglial density in specific regions of the CNS in schizophrenia, as compared to other CNS disorders (Cotter et al., 2001). There is limited morphological data on

antipsychotic exposure to astrocytes in conditions of a neuroinflammatory environment. Following the results of our previous experiments, we investigated the effects of antipsychotics, in the presence of TNFα/IL-17A, on astrocyte morphology. Human astrocytes were treated with low dose IL-17A (10ng/ml) with TNFα (10ng/ml) and 10μM of clozapine or haloperidol for 18h. Cells were also treated separately with 0.3% H_20_2 as a positive control for 18-24h. After treatment, the cells were stained for vimentin or glial fibrillary acidic protein (GFAP) and counter-stained with DAPI. The images were studied qualitatively, where: 1) the number of astrocyte projections for each treatment group was counted for both vimentin and GFAP-labelled astrocytes, 2) the number of DAPI-stained nuclei were counted per field view. Analysis of vimentin-stained astrocytes showed there was no significant difference in average number of astrocyte projections between treatment groups TNFα/IL-17A (1.4+/-0.1 projections per cell), TNFα/IL-17A + clozapine (1.1+/-0.1 projections per cell) (**Figure 5B**), or TNFα/IL-17A + haloperidol (1.2+/-0.1 projections per cell) (**Figure 6B**) and control (1.3+/-0.1 projections per cell). In contrast, astrocytes treated with 0.3% H_20_2 for 18-24h (as positive control) showed a significant decrease in astrocyte projections compared to the non-treated control (0.3+/-0.3 projections vs. 1.3+/-0.1 projections, one-way ANOVA and post-hoc Dunnett's multiple comparisons test, **p < 0.01, ***p < 0.001) (**Figure 5B + 6B**). Similarly, analysis of GFAP-stained astrocytes showed there was also no significant difference in average number of astrocyte processes between treatment groups TNFα/IL-17A (2.1+/-0.2 projections per cell), TNFα/IL-17A + clozapine (2.1+/-0.2 projections per cell) (**Figure 5B**), or TNFα/IL-17A + haloperidol (2.1+/-0.2 projections per cell) (**Figure 6B**) and control (2.0+/-0.6 projections per cell, one-way ANOVA and post-hoc Dunnett's multiple comparisons test).

Quantification of total cell number per field view at 20x objective revealed that there was no significant difference between treatment groups. When compared to clozapine-treated group (106.7+/-12.6 cells per view), there was no difference in cell count with TNFα/IL-17A treated group (107.7+/-11.9 cells per view) and control (106.7+/-7.9 cells per view, one-way ANOVA and post-hoc Dunnett's multiple comparisons test) (**Figure 5C**). Similarly, when compared to haloperidol-treated group (139.0+/-11.7 cells per view), there was no significant difference in cell count with TNFα/IL-17A treated group (145.7+/-31.5 cells per view) and control (136.3+/-30.8 cells per view, one-way ANOVA and post-hoc Dunnett's multiple comparisons test) (**Figure 6C**). This suggests that both clozapine and haloperidol do not induce overt changes in astrocyte morphology, or impact on cell survival in the presence of TNFα/IL-17A, providing further support that the attenuation of IL-6 protein expression in antipsychotic-treated astrocytes is not due to astrocyte cell death.

7.6. Clozapine and Haloperidol increase ERK phosphorylation in astrocytes.

Given that both D1 and D2 dopamine receptor subtypes are expressed in astrocytes and regulate ERK 1/2 signalling (Miyazaki et al., 2004; Beaulieu and Gainetdinov, 2011) and that this signalling pathway plays a role in regulating the levels of cytokines such as IL-6 (Yang et al., 2003; Parrado et al., 2012), we examined if clozapine and haloperidol regulated ERK1/2 signalling in human astrocytes. In this study, human astrocytes were exposed to varying concentrations of clozapine or haloperidol (10nM, 100nM, 1μM, 10μM, 100μM) for 15 minutes, and cell pellets were processed for Western blotting using total and phosphorylated ERK 1/2 antibodies (**Figure 6A**). Astrocytes treated with 100μM clozapine showed no significant increase in the levels of phosphorylated ERK 1/2 compared to control (75.8+/-11.6% vs. 122.4+/-10.8%, paired t-test)(**Figure 6B**). Although, there is a

general trend towards an increase in phosphorylated ERK 1/2 compared to total ERK with clozapine-treated astrocytes vs. non-treated astrocytes. Contrastingly, in haloperidol treated astrocytes, we observed a significant increase in phosphorylated ERK 1/2 compared to control (45.7+/-7.6% vs. 151.3+/-25.4%, paired t test, *p < 0.05) (**Figure 6C**). We found that haloperidol increased phosphorylation of ERK 1/2 in astrocytes to a greater extent than clozapine. These findings are in keeping with previous studies which demonstrated that the typical antipsychotic stimulate phosphorylation of ERK 1/2 (Pozzi et al., 2003). This data suggests that drugs such as haloperidol and clozapine not only regulate neuronal function, but also target astrocytes to regulate pro-inflammatory signalling molecules and intracellular signalling pathways.

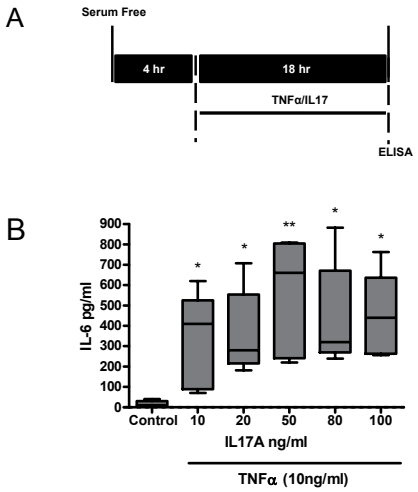


Figure 1. Treatment of human astrocytes with IL17A/TNFα-induces expression of IL-6. Human astrocytes were serum-starved for 4h then treated with TNFα (10ng/ml) and IL17A (10, 20, 50, 80, 100ng/ml) for 18h. A statistically significant increase in IL-6 was observed with all doses of IL17A. 50ng/ml of IL17A induced the maximum increase in IL-6 (0.55ng/ml). Data presented as mean +/- SEM, one way ANOVA and post-hoc Newman-Keuls test (n=3) *p<0.05, **p<0.01

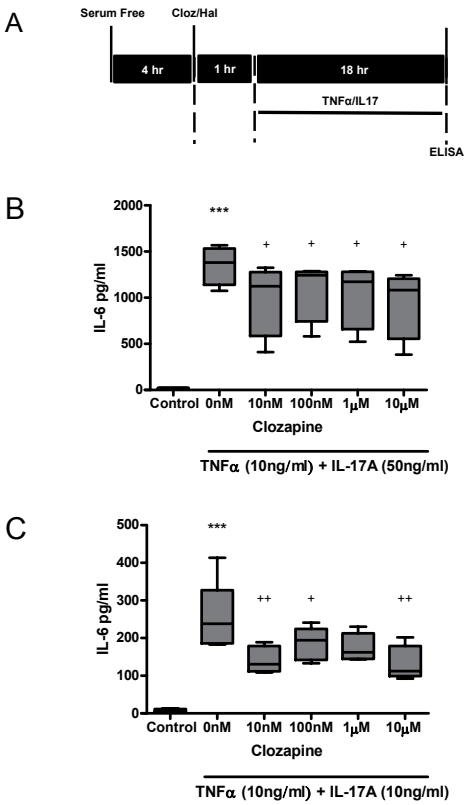


Figure 2. Clozapine attenuates TNFα/IL17A-induced IL-6 expression in human astrocytes. A) Human astrocytes were treated as per time-points shown. Cells were serum-starved for 4h before pre-treatment with clozapine or haloperidol (10nM, 100nM, 1μM, 10μM). Cells were then treated with TNFα (10ng/ml) + high dose (50ng/ml) or low dose (10ng/ml) IL17A, in addition to clozapine, for a further 18h. Media surrounding cells was used to quantify protein levels of IL-6 using ELISA. **B)** High dose IL17A (50ng/ml) + TNFα (10ng/ml) caused a significant increase in IL-6 release from human astrocytes as compared to control, which was significantly attenuated by 10μM of clozapine. **C)** Low dose IL17A (10ng/ml) + TNFα (10ng/ml) stimulated a significant rise in IL-6 release from human astrocytes. Notably, high dose clozapine (10μM) induced a slightly greater reduction in IL-6 release. Data presented as mean +/- SEM, one way ANOVA and post-hoc Dunnett's test compared to control (n=5) ***$p<0.001$; paired one-tailed t-test compared to 0nM Clozapine (i.e. TNFa/IL17A treated only) +$p<0.05$, ++$p<0.01$.

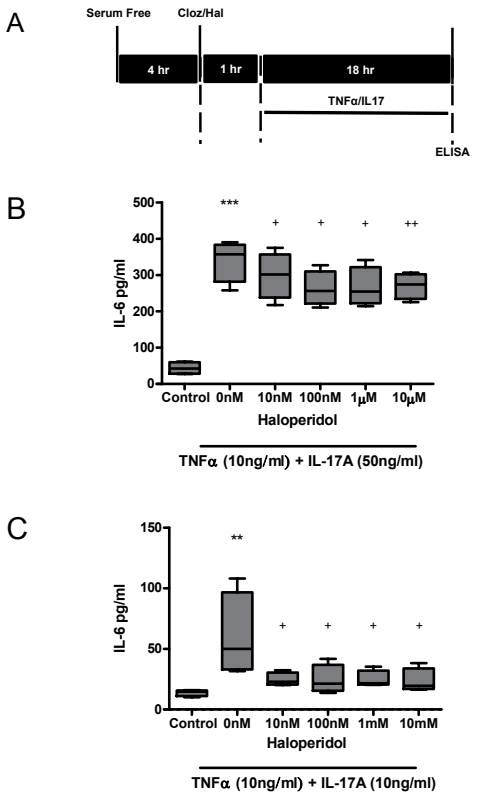


Figure 3. Haloperidol attenuates the expression of IL-6 induced by IL17A/TNFα in human astrocytes. A) Human astrocytes were treated with high dose IL17A (50ng/ml) and low dose IL17A (10ng/ml) + TNFα (10ng/ml) and haloperidol (10nM, 100nM, 1μM, 10μM) as per time-points shown. **B,C)** Both concentrations of IL17A (50ng/ml or 10ng/ml) + TNFα (10ng/ml) caused a significant rise in IL-6 release from human astrocytes. The effects of haloperidol on levels of IL-6 induced by the treatment of human astrocytes with **B)** a high concentration of IL17A (50ng/ml) + TNFα (10ng/ml) or **C)** a low concentration of IL17A (10ng/ml) + TNFα (10ng/ml), is shown. Data presented as mean +/- SEM, one way ANOVA and post-hoc Dunnett's test (n=5) **p<0.01, ***p<0.001; paired one-tailed t-test compared to 0nM Haloperidol (i.e. TNFa/IL17A treated only) +p<0.05, ++p<0.01.

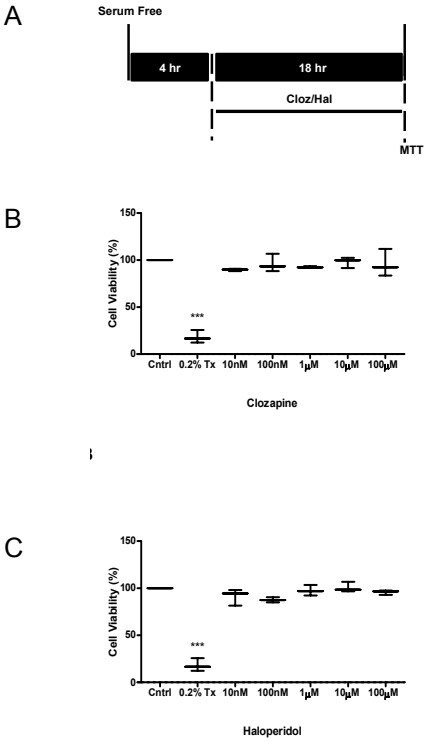


Figure 4. Clozapine and haloperidol do not alter astrocyte cell survival. A) Human astrocytes were treated with clozapine or haloperidol (10nM, 100nM, 1µM, 10µM, 100µM) for 18h or 0.2% Triton-X for 10 minutes before MTT was added for 3.5h. **B)** All concentrations of clozapine did not significantly reduce cell viability, in contrast to 0.2% Triton-X treated cells, which significantly decreased cell viability compared to control. **C)** Similarly, all concentrations of haloperidol did not significantly reduce cell viability, in contrast to 0.2% Triton-X treated cells, which significantly decreased cell viability compared to control. Data presented as mean +/- SEM, one way ANOVA and post hoc Dunnett's test for multiple comparisons (n=3) ***p<0.001.

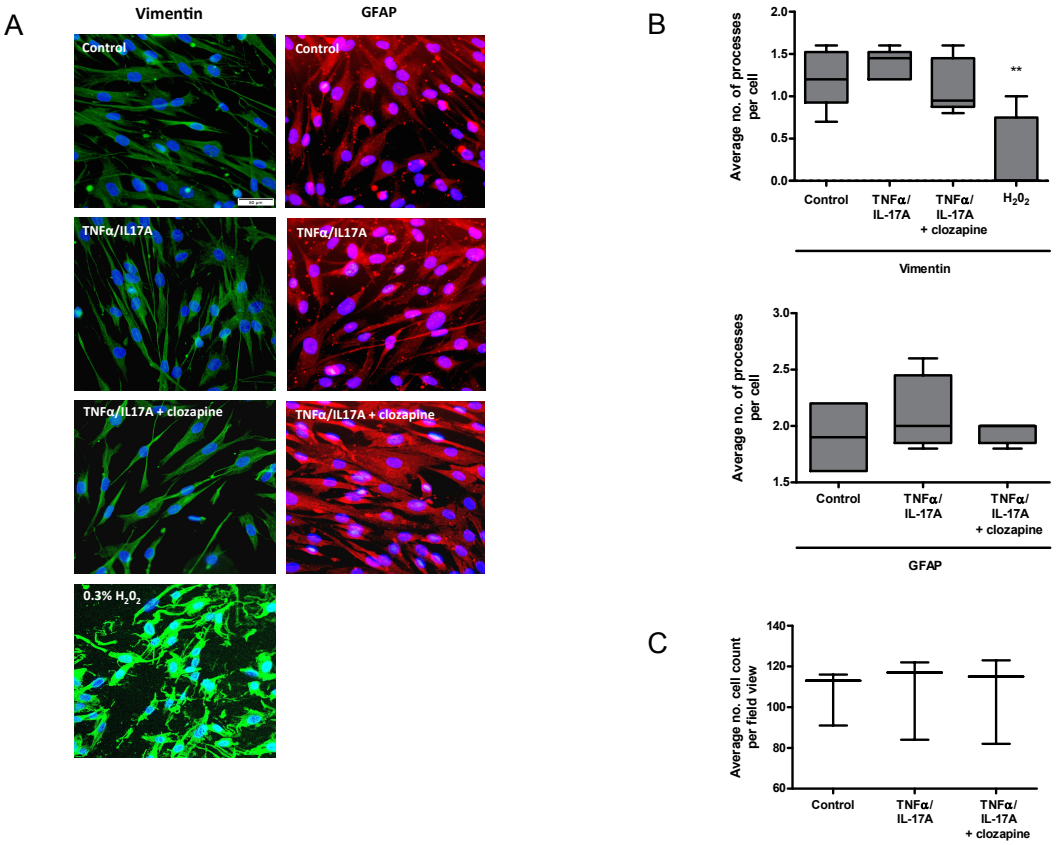


Figure 5. Astrocyte morphology is not altered by clozapine. For treatment, human astrocytes were treated with low dose IL17A (10ng/ml) with TNFα (10ng/ml) and 10μM of clozapine for 18h. Cells were also treated separately with 0.3% H_20_2 as a positive control for 18-24h. **A)** After treatment, cells were stained for vimentin/GFAP + DAPI and imaged under a fluorescent microscope. **B)** In both vimentin and GFAP – stained astrocytes, no significant difference between treatment groups and non-treated control was observed, except for H_20_2 treatment, which significantly reduced average number of astrocyte projections. **C)** No significant difference was seen between treatment groups and non-treated control for the average number of cells counted per field view. Data presented as mean +/- SEM, one way ANOVA and post hoc Dunnett's test for multiple comparisons (n=3) **p<0.01

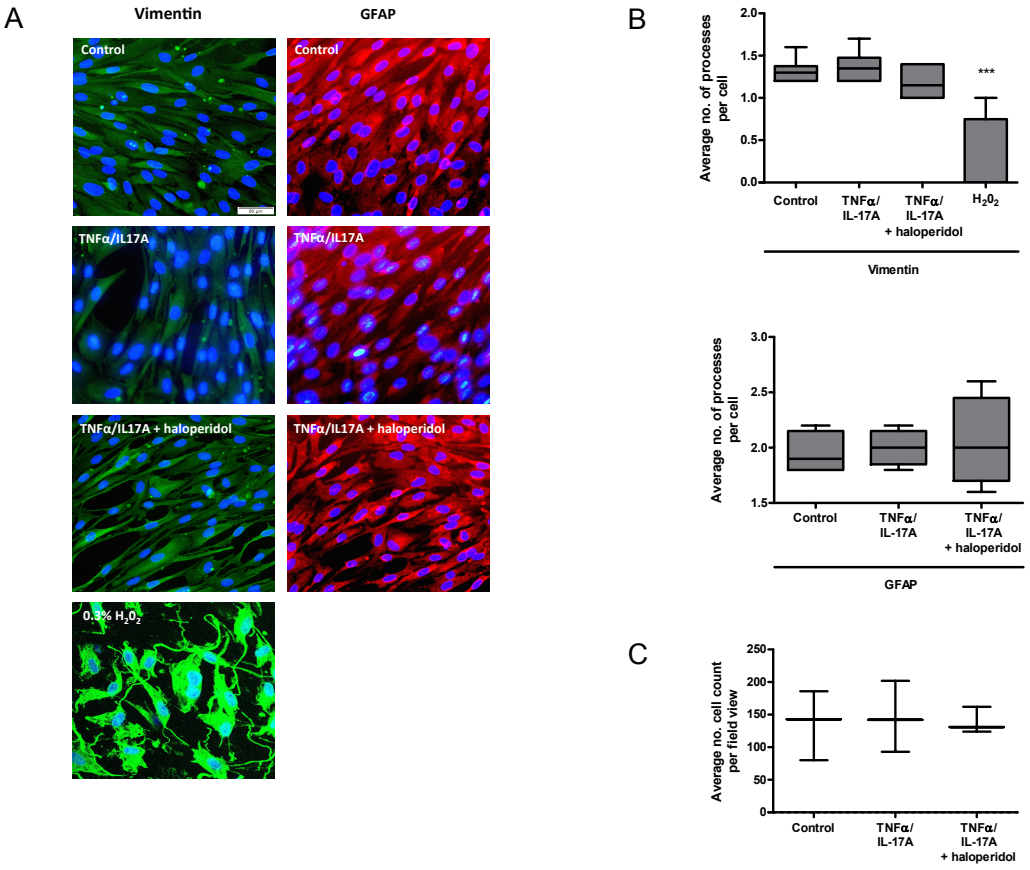


Figure 6. Astrocyte morphology is not altered by haloperidol. For treatment, human astrocytes were treated with low dose IL17A (10ng/ml) with TNFα (10ng/ml) and 10µM of haloperidol for 18h. Cells were also treated separately with 0.3% H_20_2 as a positive control for 18-24h. **A)** After treatment, cells were stained for vimentin/GFAP + DAPI and imaged under a fluorescent microscope. **B)** In both vimentin and GFAP – stained astrocytes, no significant difference between treatment groups and non-treated control was observed, except for H_20_2 treatment, which significantly reduced average number of astrocyte projections. . **C)** No significant difference was seen between treatment groups and non-treated control for the average number of cells counted per field view. Data presented as mean +/- SEM, one way ANOVA and post hoc Dunnett's test for multiple comparisons (n=3) ***p<0.001

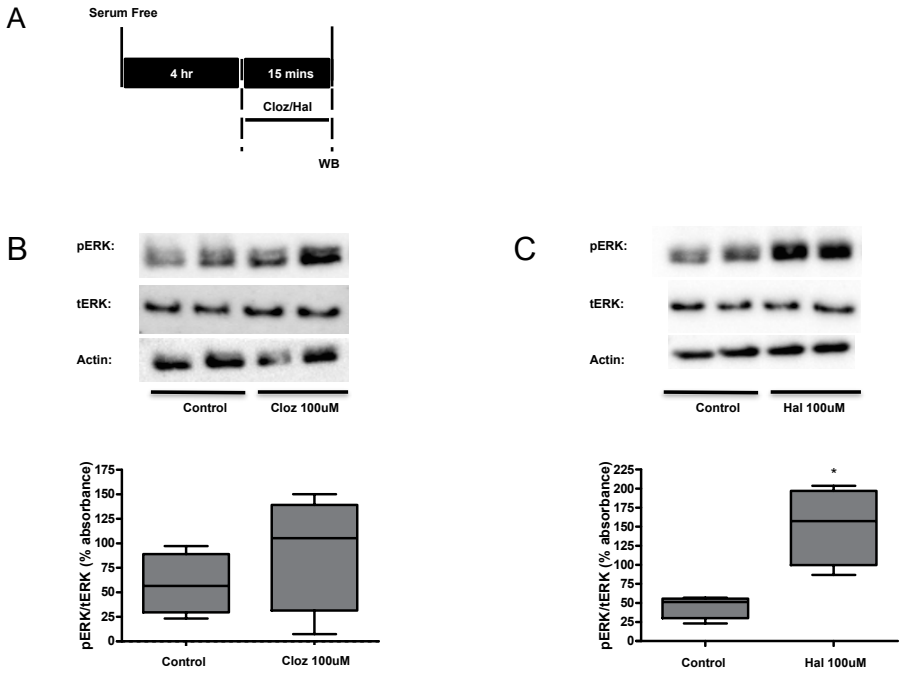


Figure 7. Clozapine and Haloperidol increase ERK phosphorylation in astrocytes. A) Human astrocytes were serum-starved for 4h before treatment with **B)** clozapine or **C)** haloperidol (10nM, 100nM, 1µM, 10µM, 100µM) for 15 minutes. The cell pellet was used to quantify total and phosphorylated levels of ERK 1/2 using Western blot. Data presented as mean +/- SEM, paired two-tailed t-test (n=4) *p<0.05

Discussion

8. Summary of findings

Schizophrenia is a debilitating chronic psychiatric disorder typified by recurrent psychoses. Changes in astrocyte morphology and function have been implicated in the pathogenesis of schizophrenia (Rothermundt et al., 2004; Bernstein et al., 2015). In addition, studies have suggested that antipsychotics may directly modulate the function of astrocytes, thus contributing to the treatment of positive symptoms, negative symptoms and cognitive deficits associated with schizophrenia (Konopaske et al., 2008; Tanahashi et al., 2012; Papouin et al., 2017). In the current study, we examined the effects of clozapine (the prototypical atypical antipsychotic) and haloperidol (a typical antipsychotic) on cytokine levels, the intracellular signalling molecule pERK, astrocyte morphology and survival. Previously, serum studies have shown increased pro-inflammatory IL-6 in patients with schizophrenia (van Kammen et al., 1999; O' Connell and Dev, 2014). Here we explore the effects of clozapine and haloperidol on IL-6 expression in human astrocytes. After 18h, clozapine attenuated IL-6 expression from TNFα/IL-17A-treated astrocytes. The effect was exaggerated using a lower dose of IL-17A (10ng/ml)/TNFα (10ng/ml) compared to a higher dose of IL-17A (50ng/ml). Haloperidol also had a similar suppressive effect on TNFα/IL-17A-mediated IL-6 expression. This suggests that both atypical and typical antipsychotics have a subtle anti-inflammatory effect in human astrocytes. This anti-inflammatory effect is supported by previous studies which show antipsychotics have an effect on IL-6 (Tourjman et al., 2013), but in disaccord with others (Pollmacher et al., 1996; Maes et al., 1997). Previously, there has been evidence of astroglial cell loss in schizophrenia, which may be contributed by antipsychotic exposure (Cotter et al., 2001; Konopaske et al., 2008). In our study, an MTT assay showed that both clozapine and haloperidol did not significantly change cell viability compared to non-treated control. Furthermore, we found that clozapine and haloperidol did not change astrocyte morphology by way of process proliferation or retraction in both GFAP and vimentin-expressed astrocytes. In order to examine the antipsychotic effects on signalling molecules in astrocytes, we investigated the effect of clozapine and haloperidol on ERK 1/2. We found that haloperidol was able to phosphorylate ERK to a greater extent than clozapine. This could be explained by the greater D2 receptor activation induced by haloperidol compared to clozapine, as ERK is regulated by the $G\alpha_{i/o}$-coupled receptor (Karam et al., 2010).

In summary, three noteworthy points emerged from this study: i) clozapine and haloperidol attenuated TNFα/IL-17A stimulated IL-6 expression in human astrocytes, ii) clozapine and haloperidol do not change cell survival and do not change astrocyte morphology, iii) haloperidol increased ERK 1/2 phosphorylation to a greater extent than clozapine. Taken together, we are able to ascertain that clozapine and haloperidol change the role of astrocytes in inflammation and cellular signalling in schizophrenia **(Figure 4.1)**.

8.1. TNFα and IL-17A induce IL-6 expression in human astrocytes.

IL-6 is a multifunctional pro-inflammatory cytokine, which amongst other immune and haematological cells, is produced by astrocytes and cortical neurons (Kishimoto et al., 1992). In the CNS, IL-6 is involved in cellular signalling, co-ordination of neuroimmune responses after injury, neuronal growth and survival and release of NGF from cultured astrocytes (Frei et al., 1989; Gruol and Nelson, 1997). Furthermore, IL-6 promotes the survival of astrocytes, where loss of signalling results in apoptosis and T-cell dysregulation in the CNS (Haroon et al., 2011). Previously, our study showed that IL-17A receptors are expressed in human astrocytes, and that IL-17A and TNFα

synergistically increase IL-6 expression in astrocytes via the NF- κB pathway (Elain et al., 2014) **(Figure 4.1)**. In this study, we found that 50ng/ml of IL-17A + 10ng/ml of TNFα induced the maximal expression of IL-6 in human astrocytes, and the dose-response plateaus at higher concentrations of IL-17A. This may be explained by the fact that IL-17 stimulates IL-6 secretion in an auto-regulatory manner (Erta et al., 2012). Therefore, it can be speculated that at 50ng/ml, there may be a negative feedback process that suppresses levels of IL-6 production from astrocytes, although more experiments will have to be done in order to prove this. Previous studies have documented increased pro-inflammatory serum and CSF levels of IL-6 in patients with schizophrenia (van Kammen et al., 1999; Garver et al., 2003; O' Connell and Dev, 2014). This change in IL-6 level is contingent upon duration of illness (Ganguli et al., 1994), stage of illness (Naudin et al., 1996) and age of patient (Maes et al., 1994). By exposing human astrocytes to TNFα and IL-17A, this acts as a basic inflammatory model of schizophrenia through which we investigated the effects of antipsychotics on astrocyte expression of IL-6.

8.2. Clozapine and haloperidol decrease IL-6 expression in human astrocytes

Treatment of patients with antipsychotics may alter serum and CSF levels of IL-6, which may in turn affect the treatment of positive symptoms associated with schizophrenia. A recent meta-analysis showed that antipsychotics, including clozapine and haloperidol, generally produce anti-inflammatory effects in schizophrenia (Tourjman et al., 2013). This study, amongst others, showed that clozapine and haloperidol have no effect on serum IL-6 levels in patients with schizophrenia (Maes et al., 1994; Pollmacher et al., 1997; Tourjman et al., 2013). In contrast, other studies suggested that a briefer exposure to clozapine increases serum levels of IL-6 (Pollmacher et al., 1996; Maes et al., 1997). To our knowledge, there are no studies that examine direct effects of antipsychotics on CSF levels of IL-6. Nevertheless, haloperidol has been found to inhibit IL-6 induced S100β secretion in glioma cells; thereby suggesting that haloperidol may indirectly suppress IL-6 levels in the CNS (de Souza et al., 2013). Our current study explored the effects of clozapine and haloperidol on IL-6 expression in human astrocytes. We concluded that both clozapine and haloperidol had subtle anti-inflammatory effects on human astrocytes, where both antipsychotics attenuated TNFα/IL-17A-induced IL-6 expression. These subtle effects may have therapeutic importance on altered neurotransmission seen during the course of illness in patients with schizophrenia.

8.3. The interaction between IL-6 and dopamine receptor signalling

IL-6 is known to promote dopaminergic and 5-HT activity in the hippocampal and prefrontal cortex (Zalcman et al., 1994). Although dopaminergic over-activity in the hippocampus is not directly associated with the pathophysiology of schizophrenia, there has been evidence suggesting that increased hippocampal activity may drive dopamine neurotransmission in the striatum via hippocampal connections to the limbic basal ganglia (Mikell et al., 2009). Dopamine hyperactivity in the striatum is essential in the generation of psychotic symptoms that characterise schizophrenia, and moreover is associated with prodromal psychotic symptoms (Kapur et al., 2005; Howes et al., 2009). Therefore, reduction of IL-6 by clozapine and haloperidol may contribute to reducing dopaminergic hyperactivity in the striatal area, thus reducing symptoms of psychosis. Furthermore,

decreased 5-HT in the mesolimbic area from a reduction in IL-6, contributes to decreased dopamine signalling, as 5-HT is known to be a regulator of dopamine via GABA signalling (Alex and Pehek, 2007). On the other hand, a reduction in IL-6 will cause little to no improvement in cognitive symptoms, given that decreasing IL-6 release will not promote dopamine activity in the prefrontal area. Changes in dopamine receptor signalling may also influence levels of IL-6 in the CNS. A growing number of GPCRs have been implicated in NF- κB activation, including the D2 receptor (Ye, 2001; Yang et al., 2003). D2 receptors are thought to activate NF- κB by enabling Gβγ-mediated recruitment and activation of tyrosine protein kinase, c-Src (Yang et al., 2003). Because both clozapine and haloperidol display some level of D2 antagonism (Karam et al., 2010), we therefore hypothesise that the reduction in IL-6 is mediated via inhibition of the NF- κB pathway **(Figure 4.1)**.

8.4. Clozapine and haloperidol do not alter human astrocyte morphology and survival

Decreased glial cell density and astrocyte loss has been described in patients with schizophrenia (Haznedar et al., 1997; Cotter et al., 2001; Kubicki et al., 2003). The loss in astrocytes may be contributed by chronic exposure to antipsychotics, as previously shown (Konopaske et al., 2008). Nevertheless, our study using cell viability assays showed that both clozapine and haloperidol do not adversely affect cell survival in the 18h exposure time we investigated. Furthermore, average cell count did not change significantly between clozapine or haloperidol-treated, TNFα/IL-17A-treated and non-treated human astrocytes. We conclude that clozapine and haloperidol-induced reduction in IL-6 is not due to astrocyte cell death. Additionally, we assessed the qualitative effect of clozapine or haloperidol on astrocyte-specific protein expression in a pro-inflammatory environment. GFAP and vimentin are both intermediate filament proteins in astrocytes, increased levels of which suggest proliferation of astrocytes following injury or inflammation (Pekny et al., 2014). Both increased and decreased levels of GFAP in the prefrontal cortex of patients with schizophrenia have been described (Rajkowska et al., 2002; Toro et al., 2006; Feresten et al., 2013). An in-vitro study showed that administration with clozapine and haloperidol did not change GFAP or vimentin levels in rat cingulate cortices (Feresten et al., 2013), whereas another study showed that clozapine, but not haloperidol, decreased GFAP levels in rat prefrontal cortices (Fatemi et al., 2008). Here, using primary human astrocytes, we found that the addition of clozapine or haloperidol did not change number of processes in GFAP or vimentin-stained human astrocytes, compared to TNFα/IL-17A-treated and non-treated cells. Additionally, there was no increase in cell number between treated and non-treated groups. Taken together, our findings suggest that under conditions of stress, both clozapine and haloperidol do not induce reactive astrocytosis by way of astrocyte proliferation.

8.5. Changes in ERK 1/2 phosphorylation induced by clozapine as compared to haloperidol

ERK phosphorylation and activation mediates synaptic connectivity and plasticity, a process dysregulated in schizophrenia (Harrison and Weinberger, 2005). Furthermore, there is evidence suggesting ERK supports the regulation of NF- κB activation via D2 receptor signalling (Yang et al., 2003), thereby potentially affecting IL-6 and other cytokine synthesis **(Figure 4.1)**. There is opposing evidence on the effect of clozapine and haloperidol on ERK phosphorylation, depending on time of drug exposure, cell and tissue type, in-vivo or in-vitro administration. For example, clozapine has a

biphasic effect on mouse striatal ERK phosphorylation between 20 minutes-24h, as opposed to haloperidol, which caused an increase in striatal pERK 1 at 480 minutes and decrease in pERK 2 at 60 minutes (Pereira et al., 2012). Moreover, in-vivo administration of clozapine reduces haloperidol-induced increase in ERK phosphorylation in mouse dorsal striatum (Pozzi et al., 2003), where the opposite effect is seen in rat dorsal striatum (Ahmed et al., 2008), but in cultured hippocampal neurons, haloperidol upregulates ERK phosphorylation (Yang et al., 2004). Here, we examined further how the antipsychotics clozapine and haloperidol regulated levels of pERK1/2 in human astrocytes **(Figure 4.1)**. We found that haloperidol increased ERK phosphorylation in astrocytes at 15 minutes. A previous study showed that protein phosphorylation induced by haloperidol mimicked that of eticlopride, a selective D2 receptor antagonist (Pozzi et al., 2003). Therefore it is reasonable to suggest that haloperidol acts to block the inhibition normally produced by D2 receptors on ERK phosphorylation. Clozapine, as compared to haloperidol, did not change levels of ERK phosphorylation in astrocytes, although we found a general trend towards an increase in pERK. This may be related to the ability for clozapine to activate multiple receptors, thus affecting other signalling pathways involved in the phosphorylation of ERK (Brunello et al., 1995). Alternatively, clozapine may have a delayed effect on ERK phosphorylation compared to haloperidol. ERK phosphorylation can be stimulated by growth factors such as the EGF ligand and BDNF, via tyrosine kinase activation (Valjent et al., 2005). Clozapine may delay an increase in ERK phosphorylation due to activation via EGF receptor signalling. In contrast, the increase in ERK phosphorylation produced by haloperidol is EGF receptor-independent (Pereira et al., 2012).

9. Future studies and limitations

There are a number of future studies that could potentially follow on from the above experiments, as briefly outlined below:

- It may be worthwhile to examine the actions of clozapine and haloperidol on IL-6 through the NF- κB pathway, namely, to examine how these antipsychotics affect nuclear translocation of NF- κB in astrocytes using immunocytochemistry, and corroborate findings by quantifying protein levels of NF- κB using Western immunoblotting.
- The number of antipsychotics we used was limited to clozapine and haloperidol, thus represented a limit of this study. To expand our results on antipsychotic effect on the astrocyte's role in inflammation, it may be worthwhile to examine effects of other antipsychotics (i.e. olanzapine, risperidone, quetiapine) on IL-6 expression in human astrocytes.
- Another limitation of this study includes the use of an astrocyte culture model. Although this allows us to directly examine the role of antipsychotics in astrocytes, we cannot examine antipsychotic effect on astrocytes in relation to other cell types. A future initiative would be to assess the role of antipsychotics in a brain slice model. This would allow us to compare the effect of antipsychotics on the morphology of astrocytes, oligodendrocytes and microglia compared to neuronal cells, as well as compare the findings in this study to the effect of antipsychotics on IL-6 release from TNFα/IL-17A treated brain slices.
- Interestingly, there has been evidence of aberrant myelination in the white matter of patients diagnosed with schizophrenia (Davis et al., 2003). Furthermore, a previous study showed that quetiapine, an atypical antipsychotic, was able to alleviate demyelination induced by cuprizone (Zhang et al., 2008). A possible future study would be to examine the effects of antipsychotics on myelin levels in an ex-vivo cerebellar slice model, where we

explore the ability of antipscyhotics to reverse astrocyte and oligodendrocyte loss induced by the toxin psychosine, as previously established with fingolimod (O'Sullivan and Dev, 2015).

10. Conclusion

Astrocyte dysfunction has been described in the pathophysiology of schizophrenia. It was initially hypothesised that antipsychotics act to change astrocyte function and morphology, thus influencing treatment of schizophrenia. In particular, the atypical antipsychotic clozapine has superior therapeutic efficacy compared to other antipsychotics (Lewis et al., 2006; McEvoy et al., 2006), suggesting that clozapine has an alternative signalling profile that allows activation of multiple intra-neural pathways (Pereira et al., 2012). Crucially, this study found that clozapine and haloperidol attenuated TNFα/IL17A-induced IL-6 expression in human astrocytes, thus demonstrating a subtle anti-inflammatory effect. Both clozapine and haloperidol did not affect astrocyte survival and did not induce astrogliosis, as indicated by GFAP and vimentin staining. Haloperidol, but not clozapine, induced significant increase in ERK phosphorylation, which may contribute to changes in IL-6 levels via NF- κB activation. Further studies are required to establish whether these findings are consistent amongst the full spectrum of antipsychotic drugs used in the treatment of schizophrenia.

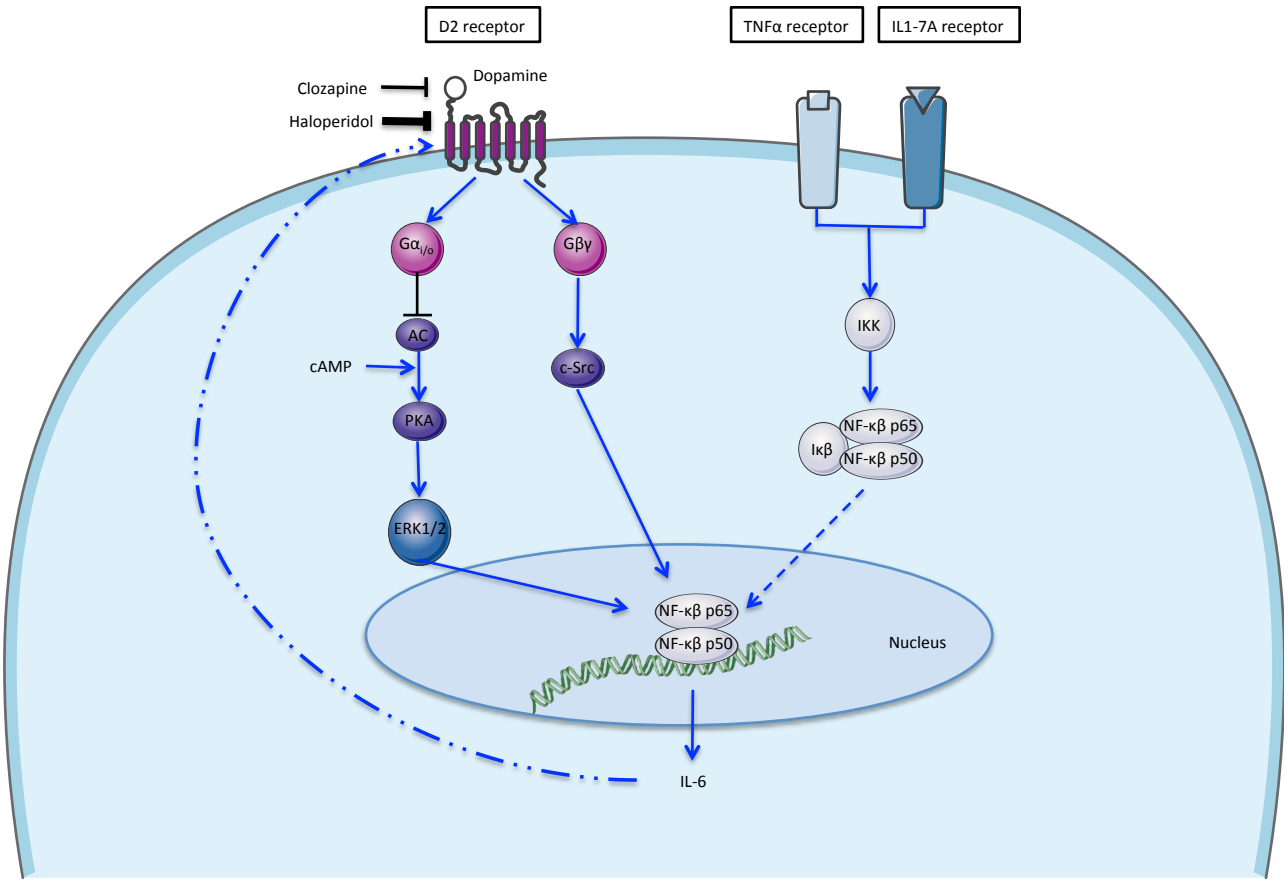


Figure 4.1 Molecular pathway linking dopamine, TNFα, IL17A receptors with IL-6. Clozapine and haloperidol are antagonists of the D2 receptor, with haloperidol possesing higher receptor affinity (as shown). The dopamine receptor regulates NF- κβ activation via G-βγ mediated c-Src activation and G-α mediated inhibition of ERK 1/2. Additionally, TNFα/IL-17A activates NF- κβ via IKK activation. This leads to IL-6 synthesis, which in turn, activates the dopamine receptor.

Blue arrows indicate activation; black lines indicate inhibition/antagonism; dashed arrow indicates nuclear translocation; dotted-dashed arrow indicates possible activation (mechanism as yet unknown). (AC – adenylate cyclase, D2 receptor – dopamine 2 receptor subtype, IκB – inhibitor of κB kinase, IKK – inhibitor of κB kinase enzyme complex, IL-6 – interleukin 6, IL-17A – interleukin 17A, NF-κB – nuclear factor κ light chain enhancer of activated B cells, PKA – protein kinase A, TNFα - tumour necrosis factor α)

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Appendix

Appendix 1

A Guiding Star: current evidence on the role astrocytes play in schizophrenia

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INTRODUCTION

Astrocytes are heterogeneous multi-functional non-neuronal neurons. Previously considered as functionally silent "scaffolding" material to neurons (1), astrocytes are now known to be the most functionally diverse glial cell type in the brain (2). They have a role in regulating synaptic activity and neurotransmitter release, neuronal migration, mediating immune responses, repair processes during CNS injury and providing nutrients to neighbouring cells (3). Because of their multi-functional role in the CNS, there is particular interest in the role of astrocytes in many psychiatric disorders, including schizophrenia.

Schizophrenia (SZ) is a debilitating chronic psychiatric disorder typified by recurrent psychoses. It is a complex disease linked to multiple genetic and environmental associations (4). The mainstay therapeutic agents are known as "antipsychotics", e.g. clozapine and haloperidol, all of which display some level of dopamine (D2) antagonism. There are many models which offer explanation to some of the symptoms produced in schizophrenia, however not the syndrome of SZ as a whole.

1. A working hypothesis – what do we know about the pathophysiology of SZ ?

Structural changes:
- Ventricular enlargement
- Cortical thinning in ACC
- Hyperpruning of collateral axons

Neurotransmitter hypothesis:
- Excess dopamine (DA) signalling
- Deficient glutamate signalling
- Decreased GABA levels and cortical M1 receptors

Neuroinflammatory model:
- Linked to early life stresses (infection)
- Increased proinflammatory cytokines
- Positive autoantibody titres

2. The role astrocytes play – how do astrocytes contribute to the working hypothesis?

i. Structural changes in astrocytes

- Overall decrease in glial cell density in SZ (5)
- Astrocyte loss is more pronounced in white matter (6) and the anterior cingulate cortex (ACC), involved in regulation of mood and cognition (7-9)
- Swollen and dystrophic astrocytes in areas of neuronal loss in the prefrontal cortex (10)
- Organelle abnormalities: mitochondrial deficits, accumulation of lipofuscin and reduction in vacuoles contribute to astrocyte dysfunction (11)

iii. Functional role of astrocytes in abnormal glutamate signalling

- **D-serine** is a gliotransmitter released by astrocytes and a co-agonist to glutamate at the NMDA receptor (18,19)
- Decreased plasma levels of D-serine are found in SZ patients (19)
- Mouse models with DISC1 mutation are observed to have increased sensitivity to an NMDA antagonist and have schizophrenia-like symptoms (20)
- Clozapine, but not haloperidol, is shown to enhance astrocytic exocytosis of D-serine and upregulate glutamate transmission via NMDA activation (21)

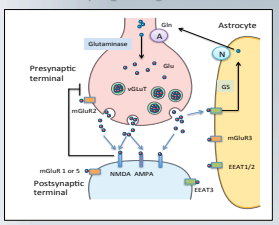

Figure 2. Regulation of glutamate transmission. Glutamate is cleared from the synaptic cleft through excitatory amino acid tranporters (EEAT) 1 and 2 on the astrocyte, and to a lesser extent, EEAT3 transporters on the post-synaptic neuron In the astrocyte, it is converted back to glutamine by glutamine synthetase (GS). Glutamine is then released from the astrocyte by System N transporters and taken up back into the presynapse by System A Na+-coupled amino acid transporters (26)

Non-functioning glial proteins in the tripartite synapse:
- Decreased expression of astrocytic glutamate transporters EAAT1 and EAAT2 in the parahippocampus and DLPFC in SZ (23)
- GS expression is also reduced, in the superior temporal gyrus and deep layers of ACC in SZ (24)
- This leads to reduced glutamate signalling

iv. A note on reactive astrogliosis

- Pro-inflammatory cytokines are increased in SZ (14) and these are known to trigger reactive astrogliosis.
- A higher density of immune-reactive astrocytes is found in post-mortem brains of patients with paranoid schizophrenia (25)

ii. S100B role in excess dopamine signalling

- **S100B** is a Ca^{2+} binding protein expressed by astrocytes
- Increased levels of S100B are seen in the cerebrospinal fluid and serum of patients with SZ compared to controls (12-15)
- S100B enhances downstream dopamine signalling (Fig. 1)
- C6 astrocytes treated with haloperidol and clozapine lead to reduced levels of S100B, according to therapeutic dose (16)

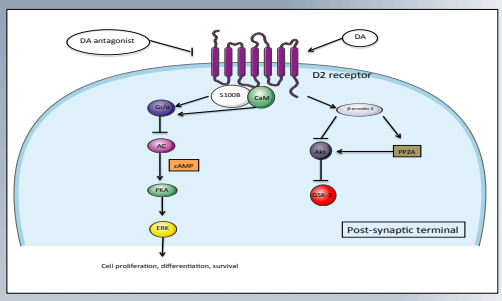

Figure 1. S100B binding to D2 receptor enhances dopamine signalling. Dopamine binds onto the D2 receptor, which is both a G-protein coupled and G-protein independent receptor. S100B attaches to the 3rd intracytoplasmic loop (D2-IC3) of D2 receptor where it is coupled to Gi/0 protein, causing inhibition of adenylate cyclase (AC) and upregulation of ERK via cAMP signalling. ERKs are mitogen-activated protein kinases that transmit signalling from outside the cell to the cell nucleus to perform a variety of functions. Of note, calmodulin (CaM) also binds to the amino terminal of third cytoplasmic loop, upregulating dopamine signalling (adapted from 17)

CONCLUSION

Astrocytes have a potentially important role in the pathophysiology of schizophrenia. It is difficult to delineate whether astroglial dysfunction correlates directly to the symptoms produced in schizophrenia or as part of neuronal abnormalities seen in schizophrenia. Additionally, the effects of long-term antipsychotic therapy on astrocytes may contribute to findings and need to be carefully separated. This is a potential avenue for further research in schizophrenia.

REFERENCES
- See Annex I attached
- Must read references: Verkhratsky et al., Astrogliopathology. The Neuroscientist. 2013; Karam et al. Signaling pathways in schizophrenia: emerging targets and therapeutic strategies. Trends in pharmacological sciences. 2010

Appendix 2

A Guiding Star: The effect of antipsychotics on human astrocyte function and morphology

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TRINITY COLLEGE DUBLIN

Introduction

Schizophrenia (SZ) is a chronic debilitating disorder affecting approximately 1% of worldwide population (1). SZ is a psychiatric disorder characterised by its phenomenology: positive symptoms (i.e. thought disorder, hallucinations, delusions), negative symptoms (i.e. blunted affect, reduced motivation, poverty of thought) and clinically significant deficits in cognition (2).

Excess dopamine (DA) signalling in the striatal and/or mesolimbic areas of the brain is thought to be responsible for the positive symptoms seen in SZ (3). This hypothesis was born from the success of antipsychotic medication, most of which act to block dopaminergic D_2 receptors. To this day, antipsychotics (**Figure 1**) remain the mainstay treatment of schizophrenia, however, current antipsychotics have limited effect on negative and cognitive symptoms.

Astrocytes have a potentially important role in the pathophysiology of SZ. It is difficult to delineate whether astroglial dysfunction correlates directly to the symptoms produced in SZ or as part of neuronal abnormalities seen in SZ. Additionally, the effects of long-term antipsychotic therapy on astrocytes may contribute to findings and need to be carefully separated. Because astrocytes express D2 receptors, long-term administration of antipsychotics may affect the role of astrocytes in the pathology of SZ (4). This is a potential avenue for further research in psychiatry.

Figure 1. **A)** Atypical antipsychotic – selective monoaminergic antagonist, highest affinity with D_4, 5-HT_2, α_1 and $_2$, H_1 receptors **B)** Typical antipsychotic – significant antidopaminergic effect, weaker anticholinergic effect

Methods and Materials

Human cortical astrocytes derived from male foetal brains at 21 weeks gestation were purchased from ScienCell Research Laboratory USA (1800, Lot No. 9063 and 11065). Cells were plated onto 12-well plates at 100,000 cells per well. For treatment, cells were grown for 72h until 80-100% confluent before serum-starved for 4h and treated as per **Figure 2A**.

Media surrounding cells was used to measure protein levels of IL6 by ELISA (R&D systems). ELISA was carried out according to manufacture's protocol. For immunocytochemistry, human astrocytes were plated in 12-well plates lined with 18mm coverslips (VWR) and treatments were carried out as per **Figure 2A**. The cells were stained for GFAP and vimentin, then counterstained with a nuclear stain (Hoescht 34580) and visualised using a fluorescence microscope (Olympus BX51 upright microscope). Images were analysed using ImageJ and statistics carried out using GraphPad Prism 7.

Results

This study confirms that human astrocytes treated with increasing concentration of IL17A enhanced TNFα-induced expression of IL6 in a dose-dependent manner, as per our previous study (5). A statistically significant increase in IL6 was observed with higher doses of IL17A (**Figure 2B**). It has been shown that altered secretion patterns of cytokines, including pro-inflammatory IL-6, is seen in SZ (6). Our study highlights the role of antipsychotics in IL6 release from astrocytes. At a higher dose of IL17A (50ng/ml), both clozapine and haloperidol do not significantly attenuate IL17A/TNFα-induced IL6 release from astrocytes (**Figure 3**). At the lower concentration of IL17A (10ng/ml), the highest dose of clozapine (10uM) causes a statistically significant decrease in IL6 release, however there is no difference in haloperidol-treated astrocytes (**Figure 4**). In terms of changes to astrocyte morphology, there is no significant change in number of astrocyte processes with TNFα (10ng/ml)/IL17A (10ng/ml) treatment and with the addition of clozapine/haloperidol (10uM) (**Figure 5**).

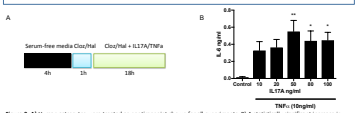

Figure 2. **A)** Human astrocytes were treated as per timepoint shown for all experiments. B) A statistically significant increase in IL6 was observed with higher doses of IL17A - 50ng/ml of IL17A induced the maximum increase in IL6 (0.55ng/ml). Data presented as mean +/- SEM, one way ANOVA and post-hoc Dunnett's test (n=3)

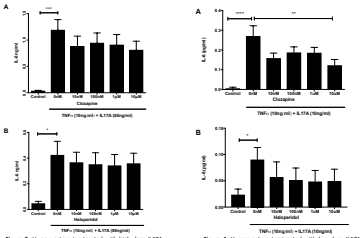

Figure 3. Human astrocytes treated with high dose IL17A (50ng/ml) with TNFα and **A)** clozapine or **B)** haloperidol as per timepoint shown in Figure 2A. Data presented as mean +/- SEM, one way ANOVA and post-hoc Dunnett's test (n=5)

Figure 4. Human astrocytes treated with low dose IL17A (10ng/ml) with TNFα and **A)** clozapine or **B)** haloperidol as per timepoint shown in Figure 2A. Data presented as mean +/- SEM, one way ANOVA and post-hoc Dunnett's test (n=5)

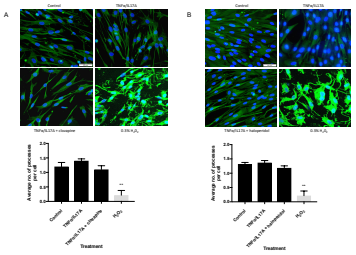

Figure 5. Human astrocytes were seeded at 1.0×10^5 to 2.0×10^5 and treated with low dose IL17A (10ng/ml) with TNFα and 10uM of **A)** clozapine or **B)** haloperidol as per timepoint shown in Figure 2A, then separately with 0.3% H_2O_2 for 24h. Data presented as mean +/- SEM, one way ANOVA and post-hoc Dunnett's test (n=3)

Discussion

There are limited in-vitro studies on the effect antipsychotics have on cytokine levels in the CNS. This study has shown that high dose clozapine (10uM) significantly decreases IL6 release from human astrocytes, although in contrast, haloperidol does not have any significant effect on IL6 levels in-vitro. This could be explained by the differing pharmacology of both drugs. Clozapine is a selective monoaminergic antagonist, acting on many dopamine and 5-HT receptor subtypes. In contrast, haloperidol mainly acts as a dopaminergic antagonist in the mesocortical pathway. Dopamine and 5-HT receptor signalling involves activation of the NF-kB pathway, leading to increased pro-inflammatory cytokine release from astrocytes (7). It can be postulated that because clozapine acts to block both dopamine and 5-HT receptors, it has a greater effect on NF-kB inactivation than haloperidol. Morphologically, there is no change in vimentin expression between treatment groups and control. In contrast to another study (8), this suggests that astrogliosis has not been induced by TNFα/IL17A, and antipsychotics made no difference to immunoreactivity of cells.

Conclusions

This study has shown that clozapine, but not haloperidol, reduces IL6 release from human astrocytes. We also show that antipsychotics do not affect astrocyte number and morphology.

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