Characterisation of GABAergic Interneurons in a Dual-Hit Neurodevelopmental Model for Schizophrenia

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Abstract

Schizophrenia is a debilitating mental illness distinguished by positive, negative, and cognitive symptoms. Pathogenesis begins in early neurodevelopment and leads to excitatory-inhibitory imbalance that is poorly managed by current treatments. Preclinical models used to understand the disease and evaluate novel therapeutics should therefore consider GABAergic deficits. One approach to improve preclinical models' face, construct, and predictive validity incorporates dual neurodevelopmental 'hits'. For example, 'PCP-Iso' rats that undergo neonatal administration of phencyclidine, to disrupt the development of glutamatergic circuitry, and then post-weaning isolation, to mimic adolescent social stress, exhibit more significant behavioural deficits than either of the single hit models. PCP-Iso may also have better predictive validity than their single-hit counterparts.

A previous finding with the same cohort of PCP-Iso rats used in this study had fewer calbindin-positive GABA interneurons in the hippocampus. The current study expanded on these previous findings and investigated changes to additional populations of GABAergic interneurons, specifically parvalbumin, somatostatin, calbindin, and Iba-1-positive cells in subregions of the frontal cortex and hippocampus. Animal models of schizophrenia and post-mortem human studies have reported increased inflammatory elements such as elevated cytokines and microglial activation. As a result, microglial activation states were also investigated in subregions of the hippocampus as expressed by Iba-1-positive cells.

A cohort of 41 male Lister-hooded rats received saline (1ml/kg s.c.) or PCP (10mg/kg) before rearing in groups or isolation. They underwent novel object discrimination three times before brains were collected for free-floating immunohistochemistry on 60μm coronal sections. Images were obtained from consistently placed regions of interest in the frontal cortex and hippocampus for each biomarker except calbindin, which only the frontal cortex was used due to hippocampal findings being published previously. Cell counts and intensity of immunoreactivity were determined with ImageJ. Microglia activation states were determined through manual examination of Iba-1-positive cells visible in images obtained during immunohistochemistry protocol.

Immunohistochemical findings in the frontal cortex of single-hit and dual-hit rats showed similar reductions of parvalbumin immunoreactivity, with wider deficits seen in PCP-Iso of select subregions of the frontal cortex. This study aims to enhance the field of animal models of schizophrenia and further characterise the face and predictive validity of the dual-hit model approach.

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Introduction

1.1 Overview

Schizophrenia is a debilitating mental illness that is distinguished by hallucinations, sporadic behaviour, and decreased cognition, among other symptoms. Current antipsychotic medications only address positive symptoms, and even these are often treatment resistant, but produce limited improvement in cognitive and negative symptoms. The poor management of symptoms contributes to the socioeconomic difficulties faced by schizophrenia patients, justifying increased preclinical research to improve understanding of the neurobiological aetiology of the disorder (Patel et al. 2014) which is the basis of the work in this thesis. Although schizophrenia is commonly characterised by overactive dopamine and excessive glutamatergic dysfunction, the exact causes are complex and multi-faceted for each individual (McCutcheon et al. 2020). This complication limits the ability of developing targeted treatment options, forcing those suffering from schizophrenia to have a severely limited quality of life. Failure to successfully control the vast category of symptoms associated with schizophrenia often prevents patients from leading a 'normal' life (Strassnig et al. 2018). Increased effectiveness in treatment can allow for patients to reintegrate into society and lead a more favourable life. Therefore, it is critical to develop clinically accurate techniques for researching neural networks involved with schizophrenia to provide a more effective gateway to develop improved treatment of cognitive and negative deficits.

1.1.1 Aetiology and Symptoms

The precise cause of schizophrenia is unknown, but replicative studies pertaining to several differing theories indicate that it is likely a multi-causation event (Figure 1). Numerous risk factors for developing schizophrenia have been identified from the study of associated genetics, environmental, and obstetric complications (Goldstone 2020). Although there is no specific phenotype or biological test to indicate there is a single gene that causes schizophrenia, genetic predispositions to have been found through family studies. Morbid risk of developing schizophrenia increases as relation increases. For example, a dizygotic twin has an approximate 17% increased risk of developing schizophrenia if their twin counterpart has schizophrenia, while the monozygotic twin has a 47% increased morbid risk (McDonald and Murphy 2003). Although international collaboration efforts of genome-wide association studies (GWAS) have made sufficient headway in analysing large quantities of in-depth phenotype data, GWAS have been unable to explain the majority of schizophrenia heritability (Dennison et al. 2020). In addition to genetic factors, birth complications have also been linked to an increased risk for schizophrenia. Obstetric complications, such as emergency caesarean section, bleeding, birth asphyxia, and low birth weight all carry an increased predisposition to developing schizophrenia in adolescence and early adulthood (DiPiro et al. 2017; Lewis and Levitt 2002). Disruptions that cause extreme stress during foetal neurodevelopment, particularly within the second trimester, have also been identified when researching potential obstetric causes of

schizophrenia (Rector et al. 2011). Environmental and social factors have also been linked to developing symptoms of schizophrenia, particularly in those who are more susceptible to the disorder. Environmental stressors such as childhood trauma, living in an urban area, and social isolation have all been identified as risk factors in schizophrenia research. Social aspects such as discrimination and economic hardship have also been shown to induce paranoid thinking (Mueser and Jeste 2008).

The symptoms of schizophrenia are classified in three core domains: positive, negative, and cognitive. Positive symptoms are the most well-recognised set of symptoms and include erratic behaviour, hallucinations, disorganised speech, and delusional thinking. Negative symptoms consist of social withdrawal, impulsivity, difficulties in planning, and a lack of motivation. Cognitive impairments in schizophrenia are characterised by an overall decline in attention and executive functions, such as working memory and impaired attention (Gohil and Carramusa 2014). These symptoms can vary in severity and change overtime but often have a dire effect on an individual's lifestyle and ability to prosper.

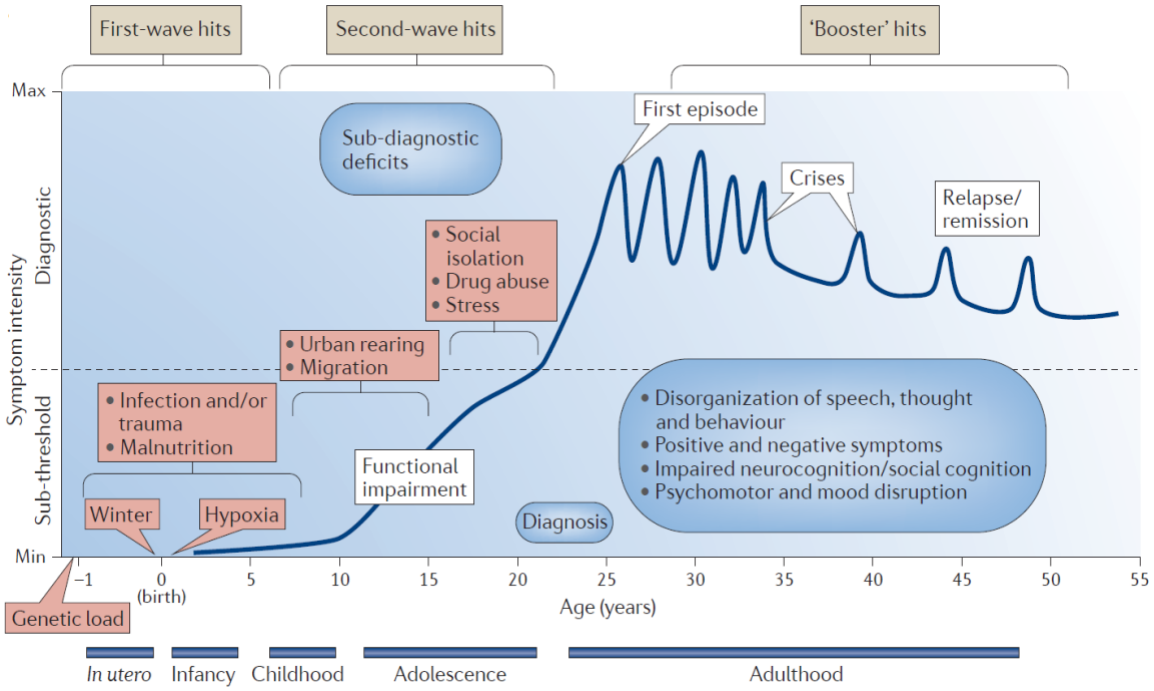


Figure 1. Onset and progression of schizophrenia in relation to risk factors and developmental landmarks (Source: Millan et al. 2016).

1.1.2 Demographics and Costs

Due to the complexity and overlap of schizophrenia with other disorders, it is a challenge to accurately provide demographic figures for the prevalence of schizophrenia. It is estimated that 1 in 300 people worldwide suffer from schizophrenia, affecting men slightly more than women (Institute of health Metrics and Evaluation (IHME) 2019; McGrath et al. 2008). An additional discrepancy noted between men and women with schizophrenia is the age of onset. Symptoms are typically displayed during the mid-to-early twenties in males while women more commonly

express symptoms in their late twenties (IHME 2019; American Psychiatric Association 2013). Although there may be other potential reasons for this disparity it has been postulated that oestrogen may contribute to this dimorphism. For instance, women with low oestrogen levels are more susceptible to schizophrenia and respond poorly to antipsychotic medication (Brand et al. 2021).

Although schizophrenia has been found to be prevalent equally across the world, cultural and socioeconomical background are important factors in both the incidence of development of schizophrenia as well as influencing likely diagnosis and treatment (DiPiro et al. 2017; American Psychiatric Association 2013). Symptoms such as hallucinations can vary person-to-person and be influenced by their respective backgrounds, further adding to the complexity of the disease. Socioeconomic background has been found to be a risk factor for schizophrenia. Recent longitudinal studies have indicated that individuals of lower-income have higher instances of schizophrenia than their non-low-income counterparts, though more studies are required to establish a firm causal relationship (Lee et al. 2018; Hakulinen et al. 2020; Werner et al. 2007).

Like other health-related issues, schizophrenia can be costly to both individuals affected by the disease as well as the community around them. The cost associated with schizophrenia can be direct costs, such as medication, and indirect costs, such as social services or law enforcement. The combined loss of productivity pertaining to schizophrenia is significantly higher relative to other mental and physical disabilities (Desai et al. 2013). As mentioned previously, schizophrenia can prevent individuals from leading a productive lifestyle. Schizophrenia is within the top fifteen leading causes of disability worldwide (Vos et al. 2017). As a result, a significant portion of the homeless population has a major psychiatric disorder, such as schizophrenia (Ayano et al. 2019). Aiding vulnerable populations such as individuals with schizophrenia requires significant funding for shelters, career programmes, health institution costs, social care, and more. It is estimated that the annual cost of schizophrenia to society and the public sector reaches £11.8 billion and £7.2 billion, respectively (Murray 2012). Access to effective treatment to disorders like schizophrenia will alleviate some of the financial burden faced by both the community and the individuals.

Unfortunately, the cost of schizophrenia extends beyond communal and personal fiscal means. Individuals with schizophrenia have been linked to an increased possibility of premature death, averaging a shorter lifespan of approximately 20 years than the general population (Olfson et al. 2015; Murray 2012). The mental and monetary toll of treatment for schizophrenia also hinders the potential quality of life for both patients and their families, bolstering the need for effective treatment options.

1.2 The Dopamine Hypothesis

In addition to socioeconomic, environmental, and genetic factors, schizophrenia is also associated by physical attributes in the brain. Large numbers of individuals with schizophrenia

have enlarged ventricles, accompanied by shrinkage of surrounding brain tissue (Harrison and Weinberger 2005). However, such changes are not diagnostic and can only be shown by comparing ventricular size in large cohorts of patents with schizophrenia from control patients. Changes in synapses and neurotransmitter systems have also been studied, particularly in glutamate and mesocorticolimbic dopamine systems. The dopamine hypothesis was developed after the drug chlorpromazine serendipitously prevented positive symptoms in schizophrenia. Chlorpromazine was later found to be a blocker of dopamine, specifically the D_2 receptor.

Evidence of the dopamine hypothesis includes several positron emission tomography (PET) studies which report that those with schizophrenia had increased D_2 receptors as well as elevated striatal dopamine release in response to amphetamines in untreated, acutely psychotic patients (Abi-Dargham et al. 2000; Laruelle and Abi-Dargham 1999). These PET findings suggest dysregulation of dopamine neurons and increased synaptic dopamine concentration (Harrison 2000). Some post-mortem studies have found changes in pre- or post-synaptic markers for dopamine in schizophrenia patients as well as abnormalities in gamma-aminobutyric acid (GABA) markers in the prefrontal cortex and hippocampus (Coyle 2004). Additionally, some individuals who display psychosis have elevated antibodies against the N-methyl-D-aspartate receptors (NMDAR), allowing stronger comprehension of the neurobiological basis of psychosis (Ellul et al. 2017). The dopamine hypothesis soon had challenges, however, once newly developed antipsychotic drugs such as clozapine had little to no effect on D_2 receptors, indicating there is more to the disease than excess dopamine (Bear et al. 2020).

The original dopamine hypothesis is now considered to be inadequate on its own and considerable evidence, as reviewed below, suggests changes to other neurotransmitters including glutamate and GABA also contribute to the deficits seen in schizophrenia. This ultimately led researchers to develop the glutamate hypothesis.

1.3 The Glutamate Hypothesis

Indirect evidence that schizophrenia encompasses more than dopamine dysfunction includes the production of side-effects by illicit phencyclidine (PCP) and ketamine usage which resemble symptoms seen in schizophrenia. PCP and ketamine were used as anaesthetics in the 1970s, but patients reported side-effects that mirrored positive symptoms of schizophrenia including hallucinations and paranoia. Neither PCP nor ketamine bind to dopamine receptors or directly alter dopamine neurotransmission. In contrast, both PCP and ketamine are non-competitive NMDA receptor antagonists.

Genetic associations with disrupted NMDA signalling in rodent models have shown behavioural and neurobiological changes mirroring those observed in schizophrenia. The gene encoding dystrobrevin-binding-protein-1 (*DTNBP1*, or dysbindin), for example, is responsible for the regulation of exocytosis, vesicle biogenesis, and receptor trafficking involved in excitatory synaptic neurotransmission (Papaleo et al. 2012; Ji et al. 2009). Reduced expression

of dysbindin has been found in the dorsolateral prefrontal cortex and hippocampus in post-mortem studies of patients with schizophrenia, seemingly attributing to negative symptoms (Papaleo et al. 2012; Weickert et al. 2004; Weickert et al. 2008). Dysbindin knock-out mice show to be hyperactive in open field tests and hyper-responsive to amphetamine-induced locomotion, with these effects being reversed by D_2 receptor antagonist quinpirole, but not the antagonist, eticlopride (Papaleo et al. 2012; Cox et al. 2009). Studies with knock-out mice have also shown alterations in pyramidal neuron NMDAR as well as cognitive deficits accompanied with significant decreases in parvalbumin staining and protein levels, suggesting an excitatory-inhibitory imbalance (Trantham-Davidson and Lavin 2019).

The scope of this project focused on the glutamate hypothesis, in which the dysfunction of glutamate is seen as a potential treatment target. Specifically, hypo-functioning NMDAR, located on GABA-positive interneurons, leading to excess dopamine, and potentially causing the positive symptoms seen in schizophrenia (Figure 2). Glutamate is an excitatory neurotransmitter whose action is often regulated by interconnected inhibitory GABAergic interneurons. As a consequence, GABAergic interneuron dysfunction can lead to excess glutamate, creating an imbalance between these excitatory and inhibitory circuitries (Javitt et al. 2012; Kaar et al. 2019). The symptoms encompassed in the glutamate hypothesis include positive and cognitive, such as hallucinations and working memory deficiencies, respectively. This observation led to the suggestion that targeting both these domains simultaneously by drugs that modify glutamate or GABAergic function (potentially as adjuncts to existing antipsychotic medication) could alleviate the unmet therapeutic need in schizophrenia.

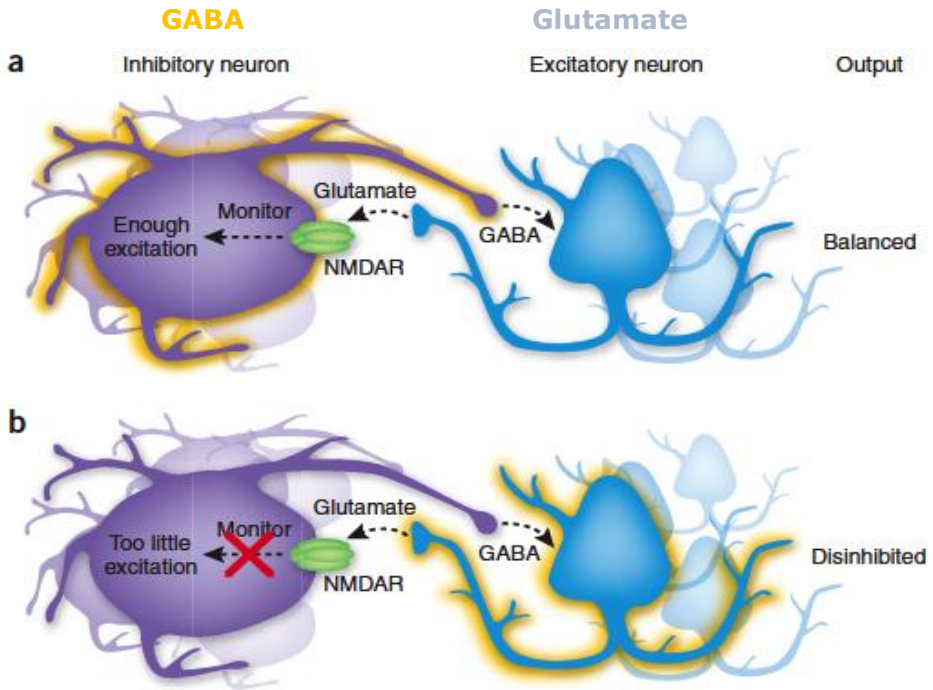


Figure 1. A schematic of the glutamate hypothesis for schizophrenia. (**a**) N-methyl-D-aspartate receptor (NMDAR) modulating excitatory neuronal activity under normal conditions, e.g., in a healthy control. (**b**) Decreased NMDAR signalling disrupts modulation causing inhibitory neurons to downregulate output and disinhibition of excitatory neurons, leading to imbalance between inhibitory and excitatory circuitry (Source: Gordon et al. 2010).

A wide array of human and animal studies have been conducted to measure overall GABA concentrations within the brain in schizophrenia patients and stress-induced animal models, establishing a trend of decreased GABA levels in schizophrenia patients and various animal models related to stress or NMDAR antagonists. The majority of the studies dictate decreased levels of GABA or GABAergic neurons, as well as increased inflammatory markers (Table 1). Measuring brain regional GABA levels are commonly accomplished via magnetic resonance spectroscopy (MRS) in humans, and enzyme-linked immunosorbent assay (ELISA) or microdialysis in animals. Administration of PCP or ketamine in animal models have produced robust neurobiological changes that closely mirror findings in patients with schizophrenia and has continued to be a preferred method to disrupt circuitry.

Table 1. A collection of rat and human studies regarding concentration of neuronal GABA levels.

Organism	Study	Summary	Findings
Rats	Cui et al. 2020	Anxiety-inducing in rats with varying aluminium treatments vs. controls via ELISA assays	↓GABA in hippocampus and frontal cortex in treated rats vs. controls ↑IL-6, IL-1β, TNF-α in hippocampus and frontal cortex in treated rats vs. controls
	Napolitano et al. 2014	MRS study on ketamine and isolated rats vs. controls	↓GABA in isolated rats after ketamine in anterior cingulate/medial PFC
	Amitai et al. 2012	Single injection vs. repeated injections of PCP in PFC via in vivo microdialysis & effects of clozapine on these conditions	↓PV, GAD-67 PCP effects reversed by clozapine
	Zhu et al. 2004	TTX, BFA, and PCP on releases of glutamate, GABA, and monoamine in PFC via microdialysis	PCP: ↓GABA, ↓ K+ -evoked GABA releases TTX: ↓GABA release BFA + TTX: normalises ↓GABA by PCP
Humans	Goto et al. 2010	MRS GABA/Cr concentrations in left basal ganglia and frontal lobe in medicated early-stage SZ subjects vs. HC at baseline and six months of antipsychotic treatments	Left basal ganglia: ↓GABA/Cr at baseline and six-month mark Frontal lobe: ND
	Kozhuharova et al. 2021	MRS GABA levels in medical PFC in low and high schizotypy subjects based on the Schizotypal Personality Questionnaire	↓GABA in high schizotypy vs low schizotypy

	Marsman et al. 2014	7T MRS GABA/Cr ratios in PFC and POC of HC vs. SZ	PFC: ↓GABA/Cr in SZ POC: ND
	Rowland et al. 2013	MRS GABA of younger and older SZ subjects and age-matched HC in AC and CSO	AC: ↓GABA in older SZ vs older HC ND in younger SZ CSO: ND in any groups

Abbreviations: GABA, gamma-aminobutyric acid; IL-6, interleukin 6; IL-1β, interleukin 1 beta; TNF- α, tumour necrosis factor alpha; MRS, magnetic resonance spectroscopy; PV, parvalbumin; GAD-67, glutamate decarboxylase 67; Cr, creatine; HC, healthy controls; SZ, schizophrenia patients; TTX, tetrodotoxin; Brefeldin-A, BFA; PCP; phencyclidine; ND, no significant difference; POC, parieto-occipital cortex; AC, anterior cingulate; CSO, centrum semiovale.

Examining levels of GABA can extend to investigating specific changes in multiple GABA-positive interneurons that are separately identified by examination of the protein content as well as their location and morphology via immunohistochemistry (IHC). Using this approach, post-mortem human studies have revealed alterations in specific GABAergic interneurons within specific brain areas in schizophrenia and in the same brain regions in animal models as will be reviewed below. Different sub-populations of GABAergic interneurons have been found to contain either parvalbumin, calretinin, somatostatin, or calbindin proteins, as described in more detail in section 1.3.1. Many of these studies particularly report changes in parvalbumin, somatostatin, and calbindin in patients with schizophrenia (Table 2). Parvalbumin and calbindin are calcium-binding proteins that play a role in calcium signalling, a critical component of action potential repolarisation within neurons. Parvalbumin also contributes to gamma-oscillations, which are vital for memory function (Klausberger et al. 2005). Somatostatin is classified as a neuropeptide that regulates working memory and cognitive function (Song et al. 2021). Calretinin, also a calcium-binding protein, has largely been unchanged in post-mortem human studies in schizophrenia patients. Levels of calbindin were diverse across various regions. Parvalbumin and somatostatin levels were largely decreased in patients with schizophrenia while calretinin was unchanged throughout (Table 2). Consequently, this justifies the rationale for specifically examining changes in parvalbumin, somatostatin, and calbindin containing GABA interneurons in rodent models for schizophrenia in the same brain regions where changes have been identified from human studies in this thesis.

Table 2. A compilation of post-mortem human studies regarding GABAergic biomarkers in schizophrenia.

Study	**Age (mean±SD)**	**Med**	**Chronic**	**Sex (M/F)**	**Region**	**CB**	**PV**	**CR**	**SST**
Curley et al. 2011	47±9	Yes	Yes	5/0	Frontal Cortex	ND	↓	ND	ND
Lanz et al. 2019	45.1±8.5	Yes	Yes	10/9	Hippocampus	ND	↓	ND	ND
Fung et al. 2014	42.6	Yes	Yes	26/9	PFC	↑	–	–	↓
Cotter et al. 2002	44.5±13	Yes	Yes	9/6	ACC	↓	–	–	ND
Hashimoto et al. 2003	43±12	Yes	Yes	12/3	PFC	ND	↓	–	ND

Hashimoto et al. 2008	47.6	Yes	Yes	10/2	Frontal Cortex	ND	↓	–	↓
Wang et al. 2011	23.6±6.8	ND	ND	5/4	Hippocampus	–	↓	ND	↓
Konradi et al. 2011	54.8±14.4	ND	ND	6/7	Hippocampus	ND	↓	ND	↓
Daviss and Lewis 1995	52.0±18.8	ND	ND	3/1	PFC	↑	ND	–	ND

Abbreviations: ND, not determined or available; Med, at least one subject was on medication for schizophrenia; PFC, prefrontal cortex; ACC, anterior cingulate caudate; CB, calbindin; PV, parvalbumin; CR, calretinin; SST, somatostatin.

1.3.1 GABA Interneuron Classification

A formal classification system for GABA interneurons has been a challenge to establish due to their diversity across species and brain location. In 2008, The Petilla Interneuron Nomenclature Group, a representative group of researchers, held a conference to establish a classification system for GABA interneurons within the cerebral cortex. This system has been widely used as a starting block to further investigate GABA interneurons and their specific roles and has been applied to other areas of the brain such as the hippocampus. Criteria for classification of sub-populations of GABA interneurons is divided into three categories: morphological, molecular, and electrophysiological characteristics (Petilla Interneuron Nomenclature Group et al. 2008).

Morphological criteria highlight cellular attributes such as soma size, dendrites, axons, and synapses, and rely on interneurons performing specific functions based on their location and connections to nearby cells (Figure 3). GABA interneurons have received nomenclature based on the specialised connection between a neuron's axons and the dendrites of another, creating different cell classes. Such classes consist of chandelier and pyramidal cells, veridically oriented, neuroglia form, basket, and martinotti interneurons resulting from identification of the spread of its collaterals. For example, chandelier and neurogliaform cells are compact and rarely extend beyond the layer that the soma is located. Because of this, only neighbouring neurons are believed to be inhibited by GABA release. Similarly, basket cell axons spread widely horizontally but less so vertically, ensuring lateral inhibition of neighbouring cells (Zaitsev et al. 2009).

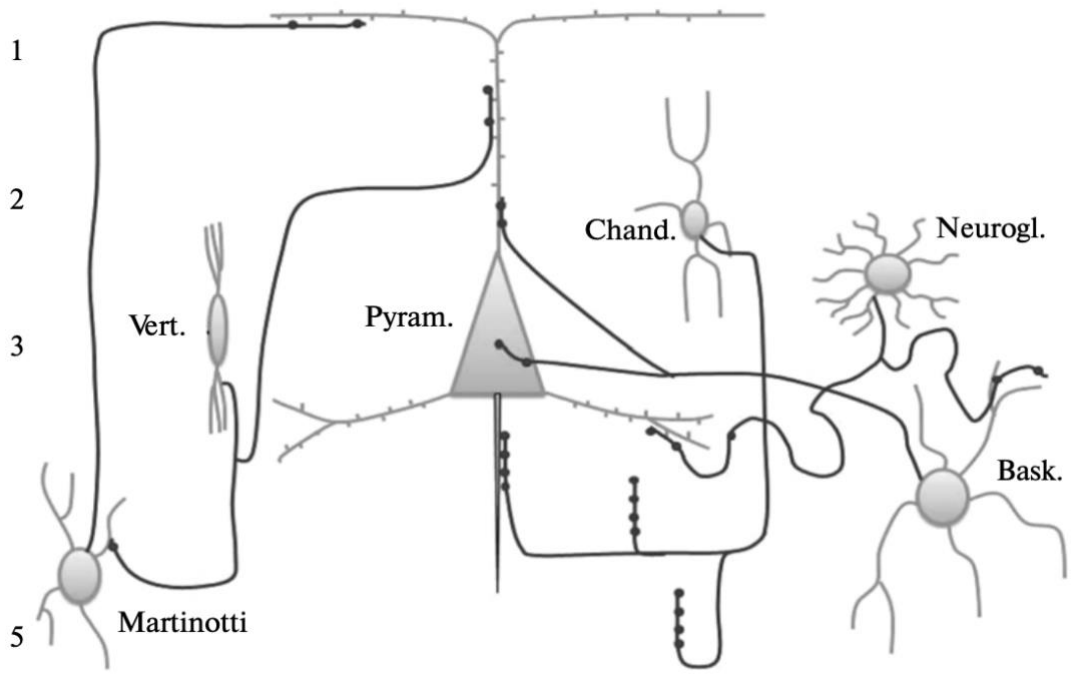


Figure 3. A schematic showcasing the connections and differences between classes of GABA interneurons and pyramidal cells within the cerebral cortex. Digits indicate cortical layers (Source: Zaitsev et al. 2013). Abbreviations: Pyram., pyramidal cell; Vert., vertically oriented interneuron; Chand., chandelier cell; Neurogl., neurogliaform interneuron; Bask., basket interneuron; Martinotti, Martinotti interneuron.

Molecular criteria focuses on characteristics related to ion channels, neurotransmitters, peptides, and other features. Some GABA interneurons have been found to produce neuromodulary peptides including somatostatin or vasoactive intestinal polypeptide (VIP), as well as co-expressing proteins such as calbindin, somatostatin VIP and calretinin (Markram et al. 2004) which can be visualised as markers for these neurons in brain tissue. There has been variation reported on molecular criteria across species and some morphological criteria has been linked with certain molecular criteria in a species. For example, basket interneurons and chandelier cells often express parvalbumin in rats (Kawaguchi and Kubota 1997).

Electrophysical criteria is determined in relation to the neuron action potential, particularly the speed of which a neuron can depolarise and repolarise and their spikes thresholds. For instance, several classifications of spikes exist, namely fast-spiking, regular-spiking, burst-spiking, late-spiking, and irregular-spiking (Figure 4). Regular-spiking neurons typically generate one action potential at the threshold of depolarisation while fast-spiking interneurons respond to thresholds within 10 to 20 Hz. Burst-spiking neurons can produce up to five spikes of approximately 100 Hz. In contrast in delay-spiking neurons spikes are not produced until after the action potential, and irregular-spiking can produce several spikes of high frequency that is followed by random single action potentials (Zaitsev et al. 2013; Ascoli et al. 2008).

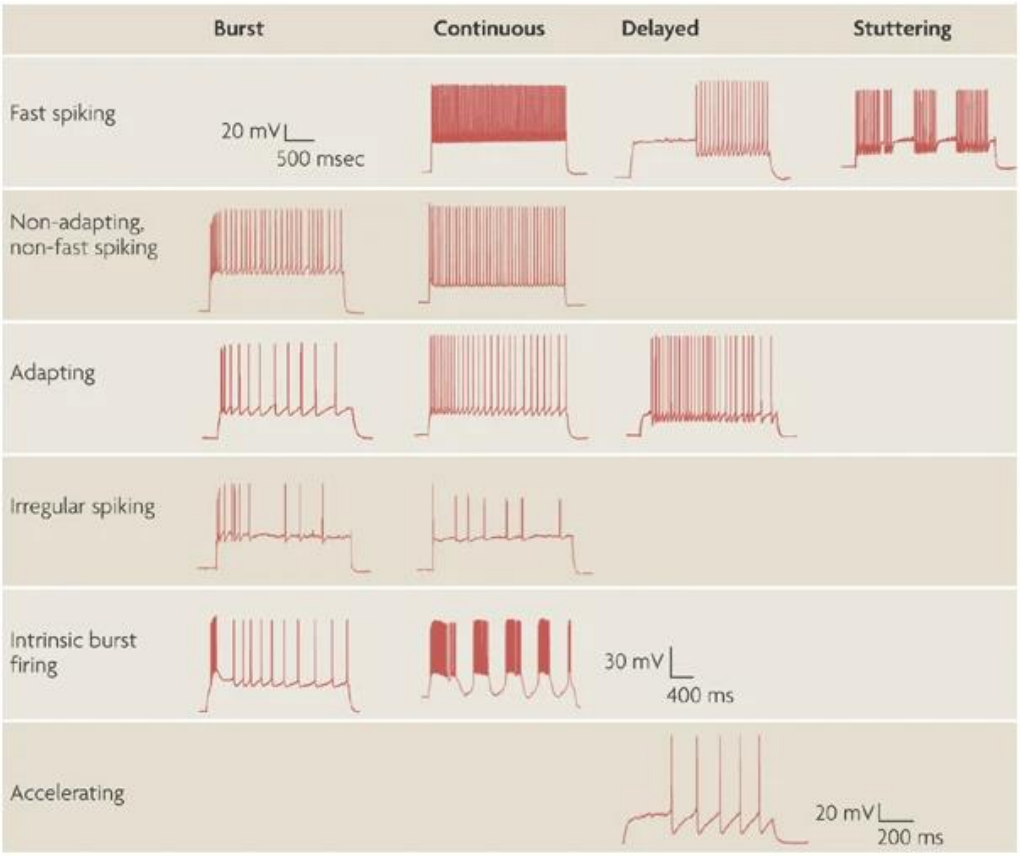


Figure 4. Cortical interneuron firing patterns. Samples are representative of standardized intrasomatic step-current injections in the rat neocortex. Step-onset patterns are organised into columns and steady-state patterns into rows. Blank areas and boxes containing only scale bars represent firing patterns that have not been characterized in neocortical interneurons. The top left scale bar refers to the traces in the first four rows and the scale bars in the fifth and sixth rows refer to those respective rows (Source: Ascoli et al. 2008).

Categorising GABA interneurons based upon these criteria varies by multiple factors, which contributes to the ongoing quest to establish firm nomenclature for GABA interneurons to better organise the vast diversity. Despite this challenge, the categories enable the ability to recognise characteristic changes in GABA neurons that are present in certain diseases. For example, differing GABA interneuron electrophysiology within the hippocampus has been reported to impact feedback loops, and parvalbumin-positive basket cell types affect gamma-band oscillations, which can create cognitive symptoms in schizophrenia such as impaired working memory and attention (Markram et al. 2004; Freund 2003).

1.3.2 Neuroinflammation

Various studies regarding neuroinflammation have provided insight into the complexity of glutamate and its involvement in schizophrenia (Laskaris et al. 2016). Neuroinflammation is caused by the triggering of the immune system in the central nervous system (CNS) involving both cytokine release and microglial activation. Neuroinflammation is vital for protection and maintenance within the brain but can quickly become overactive in perpetually stressful conditions and is then thought to contribute to various mental disorders (Kwon and Koh 2020).

Cytokines signal the immune system to activate (through the resident immune cells in the brain; microglia) and can either be pro- or anti-inflammatory in nature. Interestingly, microglia house receptors for the neurotransmitters, GABA and glutamate, explaining the rationale for investigating changes in microglial activation as a potential downstream consequence of changes in GABA in this project.

Post-mortem human studies on schizophrenia have extensively studied microglial activation and various cytokines, generally finding increased levels in microglial activation and pro-inflammatory cytokines (Table 3). Ionized calcium binding adaptor molecule 1 (Iba-1) is a common marker used to characterise microglial activation and resting states (Imai et al. 1996). Iba-1 is a reliable way to classify neuroinflammation regarding microglia and became an additional biomarker of interest and has shown have increased activation in patients with schizophrenia (Hercher et al. 2014; Schnieder et al. 2014; Table 3). Similarly, one of the most consistent observations from patients with schizophrenia is elevated interleukin-6 (IL-6) (Mondelli et al. 2011; Hayes et al 2014; Wu et al. 2019; Table 3).

Table 3. A compilation of studies regarding biomarkers and microglial activation in schizophrenia patients.

Study	Subjects	Findings
Mondelli et al. 2011	49 first-episode psychosis patients (DSM-IV criteria) and 30 HC, aged 18 to 65 years	↓BDNF; ↑IL-6; ↑TNF-α;
Hayes et al. 2014	35 HCs, 45 SZ, 15 ARMS. None medicated, aged 24 to 27 years	↓IL-6r; ↓Fibrinogen; ↓ACE ↑IL-8; ↑MCP-2
Wu et al. 2019	44 HC and 44 SZ, mean age 31.25 and 34.32 years respectively. All SZ patients were medicated	– IL-1β, IFN-γ, TGF-β ↑IL-2, IL-6, IL-8, IL-10
Bloomfield et al. 2016	Cohorts: 14 Ultra-High Risk + 14 healthy controls; 14 traditional schizophrenia patients + 14 healthy controls. None medicated	↑microglial activation in SZ (both cohorts) in frontal + temporal lobes
van Berckel et al. 2008	10 recent-onset SZ with 10 healthy controls, mean age 23 years. All SZ subjects were on medication	↑microglial activation density, ↓grey matter
Hafizi et al. 2017	19 untreated SZ with first-episode psychosis (14 antipsychotic naive), 20 HC	No significant differences were found
Doorduin et al. 2009	7 recovering SZ with no treatment for 1-2 weeks and 8 HC matched for age and sex	↑binding in SZ in hippocampus –whole-brain grey matter
Hercher et al. 2014	SZ and Bipolar Disorder patients who died by suicide (n=10), other causes (n=30), and HC (n=20)	↑microglial activation in SZ subjects
Schnieder et al. 2014	SZ (n=6), SZ suicide (n=2), affective disorder (n=8), affective disorder suicide (n=4) HC (n=18), HC suicide (n=3)	↑microglial activation density in subjects who died by suicide

Abbreviations: HC, healthy controls; BDNF, brain-derived neurotrophic factor; SZ, schizophrenia patients; ARMS, at-risk mental status; IL-6r, interleukin-6 receptor; ACE, angiotensin-converting enzyme; MCP-2, monocyte chemotactic protein 2.

1.4 Treatment Options for Schizophrenia

The current medication for schizophrenia is with antipsychotic drugs (APDs). The first generation APDs (haloperidol and chlorpromazine) were discovered by serendipity to have a beneficial effect on psychosis in schizophrenia patients during the 1950s. Subsequently, investigation of their pharmacological properties led to the development of the second generation APDs such as clozapine and olanzapine. Both these categories of antipsychotics are potent dopamine D_2 receptor antagonists and are thought to target dopaminergic hyperfunction in the mesolimbic pathway and consequently reduce positive symptoms. A third generation of ADPs, such as aripiprazole and cariprazine, are not D_2 antagonists, but rather D_2 partial agonists. All existing APDs have a rich pharmacology binding to many other monoamine receptors, although typical antipsychotics tend to produce less serotonin (5-HT_{2A}) receptor antagonism which was one feature incorporated in early atypical antipsychotics to attempt to produce less tardive dyskinesia and improve effect on negative and cognitive symptoms. Numerous side-effects of typical and atypical antipsychotics include severe brain fog, metabolic symptoms, hyperprolactinaemia, and tardive dyskinesia with prolonged use, which are poorly tolerated by patients (Stahl 2000; Gray and Roth 2007). Although timely pharmacological therapy combined with cognitive behavioural therapy are vital for a promising prognosis, the low tolerance and effectiveness of current medications created a need for a new approach for schizophrenia treatment (Patel et al. 2014; DiPiro et al. 2017). Over the last two decades drugs have been trialed (both in preclinical models and clinical trials) against several (non- D_2 receptor) pharmacological targets. In particular, drugs have been developed to indirectly enhance glutamate function as an alternate treatment strategy which might have improved impact against cognitive and social deficits than existing APDs but without producing such a wide list of side-effects, as described briefly below. The vast majority of these promising treatment options for schizophrenia have halted prior to reaching phase III clinical trials as they showed no greater benefit than existing therapies, often despite positive preclinical data.

Promising treatment options that have recently undergone clinical trials include α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-receptor-positive modulators (Ampakines), Group II metabotropic glutamate receptor (mGluR2/3) agonists, Glycine Transporter 1 (GlyT-1) inhibitors, and serotonergic 5-HT_6 receptor antagonists.

AMPA receptor function as a means for brain plasticity and memory. Ampakines act as positive modulators for the AMPA receptor, which ultimately increase hippocampal long-term potentiation which have been found to assist with memory formation and consolidation (Zeng et al. 2021; O'Neill et al. 2004). Pursual of ampakines was chosen to develop an effective treatment option that aided cognitive symptoms of schizophrenia. Although this treatment showed promise in several rodent models and the drug was generally well-tolerated in early

human trials, patients reported impaired short-term memory, headaches, and nausea, leading to its failure in clinical trials (Wezenberg et al. 2007).

Activation of mGluR2/3 receptors by agonists in the prefrontal cortex was proposed to improve cognitive performance in schizophrenia patients and offer the first non-dopaminergic treatment for the disorder and were seen as an additional hopeful opportunity to mitigate cognitive symptoms (Li et al. 2015). Many animal trials were conducted with mGluR2/3 agonists and resulted in the alleviation of symptoms caused by PCP conditions and of rats reared in social isolation (Jones et al. 2011). Findings included improved working memory, motor repetition, and locomotion (Moghaddam and Adams 1998). Due to the promising animal model results, mGluR2/3 agonists treatment was included in Phase III clinical trials in 2012. Unfortunately, the trial failed to meet adequate requirements on the positive and negative syndrome scale (Maksymetz et al. 2017).

As previously discussed, considerable evidence suggests that glutamatergic hypofunction at the excitatory NMDAR may contribute to several symptoms seen in schizophrenia. Illicit use of the non-competitive NMDAR antagonist PCP produces a wide range of symptoms like schizophrenia, and therefore brought NMDAR antagonists to the forefront of schizophrenia research (Javitt and Zukin 1991). NMDAR activation requires simultaneous occupancy of the glycine modulatory site by endogenous glycine (or D-serine) as a co-agonist that enables NMDAR activation. Drugs which could elevate glycine (or dD-serine) were thus seen as potential therapeutic targets to treat schizophrenia. GlyT-1 is a glycine transporter which reduces synaptic levels of glycine, indirectly reducing occupancy of the glycine site, thus causing a reduction in NMDAR function. Meanwhile GlyT-1 inhibitors block glycine from binding to NMDAR, enhancing NMDAR function, and act as an antipsychotic that aids in persistent negative and cognitive symptoms in schizophrenia (Heresco-Levy and Javitt 2004; Hashimoto 2010). The first selective brain penetrant GlyT-1 inhibitor produced by Roche showed initial promise but subsequently failed in phase III clinical trials (Kingwell 2014). GlyT-1 continues to undergo phase II and phase III clinical trials but has yet to pass phase III (Kingwell 2014; Boehringer Ingelheim 2022).

Serotonergic, or 5-hydroxytryptamine (5-HT), mechanisms in atypical antipsychotic drugs have been studied for decades in relation to schizophrenia and hold a promising treatment option to improve cognitive function (Meltzer 1999; Barnes et al. 2021). Several 5-HT antagonists have been identified over the years, but 5-HT_6 antagonists were some of the most consistent to improve in learning and memory in a large array of preclinical models (Woods et al. 2012; King et al. 2008). This could in part be due to the antagonist decreasing levels of GABA, which decreased glutamate, altering synaptic plasticity (King et al. 2008; de Bruin and Kruse 2015). Despite the promising outlook of 5-HT antagonists, few treatments have graduated beyond phase III clinical trials due to deficits in preclinical models (Hutson et al. 2017).

1.5 Animal Models for Schizophrenia

Due to the complexity of mental disorders, valid, predictive, preclinical models are crucial for drug development. Animal models offer the ability to closely monitor disease progression, in comparison to human trials, as well as the ability to perform invasive protocols to gain an understanding of the underlying neurobiological aetiology. Several post-mortem human studies have been conducted examining changes in GABAergic interneurons in patients with schizophrenia (Table 2), but the use of animals allow for conditions to be created progression monitored and novel therapeutics to be tested to restore normality in model for schizophrenia prior to progression to clinical trials. Face validity, predictability, constructive validity, and anatomical similarities are key components to assess the utility of any animal model in which are all likely required to see any translational relevance of studies on drug efficacy. Although animal models can include a wide array of species, rats are commonly used as they offer many similarities to the neuroanatomy, neurobiology and behavioural sequalae to the human disorder and were utilised for this project.

Although rats have limitations to studying a human CNS condition, such as interpretation of behaviours for complex human emotions and thoughts, the use of rats has an economic advantage due to their small size, rapid gestation and development, and ability to produce many offspring at once (Bryda 2013). Additionally, rats share many key traits with humans regarding memory, reward-seeking, and behaviour within social settings which are an integral part to studying neuroscience, particularly in schizophrenia (Bicks et al. 2015). These behaviours are relayed through different regions of the brain, allowing for targeted anatomical analysis.

Regions of interest (ROI) in rats and humans overlap significantly, allowing for strong face validity of the model. Neuroanatomical similarities among the frontal cortices and hippocampus are common sites for schizophrenia studies. The prefrontal cortex oversees decision-making, executive function, and social cognition while the hippocampus is responsible for working memory in humans and rodents (Bicks et al. 2015; Bizon et al. 2012). Similar to humans, rats are sociable creatures and inducing a change in their environment, such as social isolation, can impact the developing frontal cortex and mimic several core symptoms of schizophrenia.

Rats, also like humans, have a natural desire to explore and investigate objects which allow a plethora of investigative techniques to test cognition. One of these cognitive tests is novel-object discrimination (NOD), which was used in this project as an index of visual learning and memory which is often impaired in schizophrenia. Specifically, NOD is a technique to assess visual recognition in an animal and is efficient, low-stress, and appropriate for the detection of neuropsychological changes following various methodologies (Lueptow 2017). Rats are presented with an object that is familiar and an object that is new as a two-trial step in a well habituated arena, and a rat's investigation of both objects is timed and compared to assess the memory of the first trial based on their preferential exploration of a novel over a familiar object. NOD is one of several methods to achieve face and predictive validities in rodents (Jones et al.

2011). Many animal models for schizophrenia have been developed to enhance construct, face, and predictive validity through different methodologies with tests such as NOD to assess changes in behaviour and cognition. These methods can encompass a multitude of techniques but are ultimately classified into four categories: acute pharmacological, genetic, lesion, and neurodevelopmental manipulations. Each of these models have their own strengths and weaknesses that contribute diverse findings within the field.

Acute pharmacological studies utilise drug administration of NMDAR antagonists such as ketamine or PCP. Long-term administration of NMDAR antagonists can produce psychosis in humans and amplified symptoms in schizophrenia patients. This feature supported chronic use of NMDAR antagonists in rodent to model symptoms of schizophrenia (Krystal et al. 1994; Lahti et al. 1995). PCP-induced symptoms include behaviours thought to resemble positive symptoms, such as locomotor hyperactivity, but also replicate negative and cognitive deficits such as social withdrawal, and cognitive impairments in appropriate tasks (Featherstone et al. 2008; Mouri et al. 2007). Although it is a promising methodology regarding cost and time, there are issues, for instance, with constructive validity of the model as most patients with schizophrenia have not received repeated pharmacological challenges with an NMDAR antagonist.

Increased understanding of the genetic basis of schizophrenia has given rise to many genetic manipulation models in transgenic mice. Genetic models can entail knock-out or knock-in mutations to determine how a specific gene contributes to a particular phenotype of schizophrenia. Genetic models offer a streamlined approach to mitigating specific symptoms and understanding how those symptoms are controlled by genes. Symptoms from genetic models have been reversed with antipsychotic drugs, leading to strong predictive validity (Clapcote et al. 2007; Hikida et al. 2007). These advantages are met with high costs and time consumption, however, and do not mirror the complexity of disease as schizophrenia is not caused by a single genetic variance.

Lesion models include bilateral ventral hippocampal ibotenic acid administration to neonatal rats which leads to representation of all three categories of symptoms seen in schizophrenia, creating strong face validity (Sams-Dodd et al. 1997; Jones et al. 2011). The face validity of lesion models is further confirmed by ventral ventricular enlargement that is seen in first-episode schizophrenia patients (Lipska et al. 1993). Lesion models also have the advantage of offering insight to basic pathophysiology of schizophrenia. However, recent studies suggest that lesion models have poor predictive and constructive validity because of inconsistent findings of antipsychotic reversal as well as the fact that schizophrenia is not directly caused with such marked hippocampal neuronal loss (Levin and Christopher 2006; Lipska and Weinberger 1994; Rueter et al. 2004).

Neurodevelopmental models aim to limit growth in pre- or neo-natal animals to disrupt neuronal migration. Specifically, this model targets neurons undergoing rapid rates of neurogenesis at the time of intervention, so targeting specific regions based on the age of the

animal in which neurodevelopment is disrupted (Moore et al. 2006). Neurodevelopment disruption can be caused by a wide range of factors, such as maternal infection, malnutrition, and hypoxia. This model provides excellent face validity as it directly mirrors risk factors known to enhance development of symptoms such as obstetric complications or exposure to early-life adverse events like social neglect. Although neurodevelopmental models allow replication of schizophrenia-like symptoms, infection and malnutrition can lead to unwanted additional symptoms such as motor deficits in addition to an increased risk of high mortality rates. Another well-established neurodevelopmental model is isolation. Post-weaning social isolation in rodents into individual cages cause neuroanatomical, neurochemical, and behavioural changes that emerge post-puberty, resembling core features of schizophrenia (Jones et al. 2011). Changes in brain regional cytokines have been found with isolated rats, as well as increased aggression, reduced prefrontal cortex volumes, and deficits in NOD (Shortall et al. 2018; Fone and Porkess 2008; Lapiz et al. 2003).

1.5.1 The Dual-Hit Model

In recognition that schizophrenia may be the consequence of the interaction between multiple risk factors resulting in a critical level of developmental alteration required to produce sustained abnormalities, several studies have now combined previous single-hit strategies to try and produce a wider array of behavioural and neurochemical alterations that might more completely reflect the complex changes seen in schizophrenia. One such approach to advancing the preclinical model of schizophrenia is to utilise two neurodevelopmental hits. The first hit is aimed to prime the rodent to become increasingly responsive to additional stress, and then follow this by a 'second hit', in early adulthood or adolescence, similar to that often reported by patients with schizophrenia (Guerrin et al. 2021). This is referred to as the 'dual-hit' model. Hits can encompass conditions such as dietary restriction, early maternal weaning, social isolation, and obstetric complications typically thought to be risk factors. With this in mind, the group at Nottingham have successfully combined neonatal PCP administration with subsequent social isolation of rat pups (PCP-Iso rats) and shown that this replicates the hyperactivity and cognitive deficits seen with either treatment alone but in addition also produces a greater impairment in social interaction and concomitant ultrasound vocalisation than seen with either treatment alone. This suggests that this PCP-Iso model has enhanced face and predictive validity compared to previously described single-hit techniques (Gaskin et al. 2014; Watson et al. 2016; Shortall et al. 2020). Therefore, this dual treatment strategy was adopted in the current study with the aim of characterising the impact of treatment on frontal cortical and hippocampal GABA interneurons which has not been previously reported.

The dual-hit model is a promising approach to better understand the disease and evaluate potential treatments, but it is important to acknowledge its limitations. Although the ability to design multiple variables can allow freedom for researchers to investigate specific subtypes of schizophrenia, it can make it difficult to study the underlying mechanisms of the disease. Further investigation is required to understand the cellular and molecular mechanisms behind multiple combinations of hits, but it is a propitious method for preclinical modelling with strong

face, predictive, and construct validity (Gaskin et al. 2016; Monte et al. 2017; Guerrin et al. 2021). Increased comprehension of schizophrenia within preclinical models could result in an increased success rate of treatment options beyond phase III clinical trials, ultimately leading to more effective treatments that nullify the symptoms and promote societal belonging for patients. The adoption and further characterisation of the dual-hit model methodology has the potential to promote findings in a multitude of disciplines beyond schizophrenia. The replicated success of a dual-hit model approach would pioneer unexplored territory within neurological research, and open opportunities to discover underlying causes of mental illnesses pertaining to schizophrenia and potentially other disorders with cognitive deficits.

1.6 Aim of Project and Hypothesis

Given the importance of further characterising the dual-hit model and the role of GABAergic interneurons in the symptoms of schizophrenia, the aim of this project was to investigate the change in GABAergic interneurons in a dual-hit neurodevelopmental model for schizophrenia in rats. This was accomplished through automated cell counting and qualitative immunoreactivity in selected ROIs, including subregions of the frontal cortex and hippocampus. Additionally, microglial activation states were classified in the images collected from IHC sections and cross-corelation of any changes examined in the same rats to establish and potential causal link. In summary, the specific biomarkers to assist in characterisation development of the dual-hit neurodevelopmental established in this study were: parvalbumin, somatostatin, calbindin, and Iba-1.

Hypotheses: Based on the literature discussed, we hypothesize that:

1. The number of GABA-positive interneurons containing either parvalbumin, somatostatin or calbindin in the frontal cortex and hippocampus would be reduced in the PCP-Iso rats compared to single-hit and control groups.
2. The presence of Iba-1 will be increased in the PCP-Iso rats compared to single-hit and control groups.
3. The morphology of Iba-1 cells (microglia) will contain higher numbers of activated states in PCP-Iso rats compared to the single-hit and control groups.

2 Methods

2.1 Animals and Experimental Design

All procedures were conducted in accordance with the Animal (Scientific Procedures) Act, 1986 under Home Office authority, and are reported in correspondence with the ARRIVE guidelines (Percie du Sert et al. 2020) with approval from the University of Nottingham Local Ethical Committee. Data were obtained by trained observers unaware of neurodevelopmental history or any acute treatment.

A previously described (Shortall et al. 2020) cohort of 41 male Lister-hooded rats from postnatal day (PND) 3 (Charles River UK) were maintained under controlled conditions (21 ± 2 °C, 55 ± 10% humidity, 12-h light-dark cycle; on at 07:00 h) in individually ventilated cages (GR1800 Double-Decker; Tecniplast, UK) with sawdust bedding, standard environmental enrichment (cardboard play tube, wooden chew block and paper nest material). The rats had unlimited access to food and water unless stated.

Rodents were randomised (by drawing lots) to receive either vehicle saline (1mL/kg s.c.) or PCP (10mg/kg, Sigma-Aldrich) on PND 7, 9, and 11, and were subsequently reared in groups of three or four per cage (Gr) or isolation (Iso) following weaning age (PND 21). After weaning, cages (Gr: 32 × 51 cm, Iso: 25 × 42 cm) contained only sawdust with grid lids to ensure maintenance of visual, olfactory, and auditory contact (Fone and Porkess 2008). Handling was restricted to a single weekly cage change and body weight measurement until behavioural testing.

They underwent novel object discrimination (NOD) three separate times at one-to-two-week intervals (PND 57-80) following acute i.p. administration of 0.5% methylcellulose 1% Tween-80 vehicle (1mL/kg), the 5-HT_6 receptor antagonist SB-399885 (10mg/kg, Sigma-Aldrich) or $mGlu_7$ antagonist MMPIP (10mg/kg, Tocris (Bristol, UK)), on separate days (n=13-14 per neurodevelopmental condition). Each treatment was administered in pseudorandom order over the behavioural testing window. Rats were killed by concussion and decapitation on PND 79-80 after the final NOD. One hemisphere was collected for frozen sectioning and the other for ELISA assays, then stored at -80°C.

2.2 Immunohistochemistry

Brains were fixed in 4% paraformaldehyde followed by 30% sucrose (each overnight at 4°C) and frozen in isopentane on dry ice. 60µm coronal sections were obtained throughout the frontal cortex and dorsal hippocampus via a freezing microtome (Anglia Scientific, Cambridge, UK) and stored at -20°C in antifreeze (30% ethylene glycol (Fisher Scientific, 0918489) and 30% glycerol (Honeywell, 49770) in 0.1M Phosphate Buffered Saline (PBS; Oxoid, BR0014G).

Free-floating immunohistochemistry was performed on six sections from each rat from the frontal cortex (Bregma 5.20 to 4.00) and hippocampus (Bregma -2.56 to -5.80) (Paxinos and Watson 2006; Kjonigsen et al. 2011). Sections were washed (4 x 5min) in phosphate-buffered saline (PBS) to remove antifreeze and then incubated for one hour in 2% normal goat serum (Abcam, ab7481) in buffer 1 solution (0.5% bovine serum albumin (BSA, Sigma-Aldrich), 05482; 0.3% Triton-X100 (Sigma-Aldrich), in PBS) to minimise non-specific binding of the primary antibody within the tissue. They were then incubated overnight at 4°C in rabbit polyclonal antibodies (Abcam) against parvalbumin (ab11427, 1:1000), somatostatin (ab108456, 1:500), calbindin (ab108404, 1:500) or Iba-1 (Wako 019-19741, 1:2000). Sections

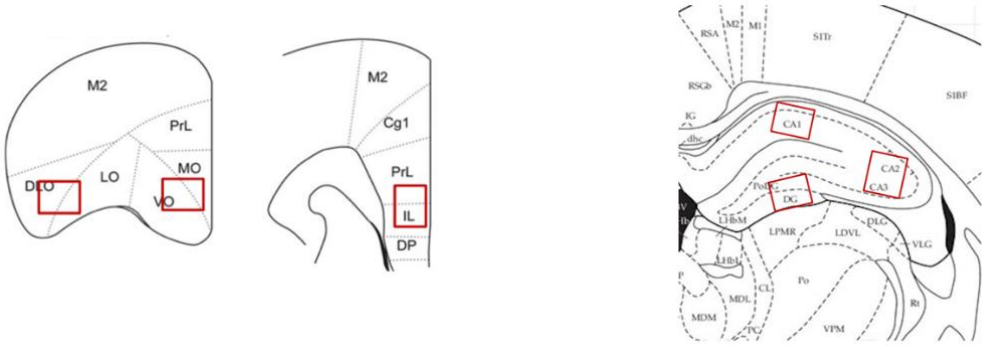
were washed (3 x 5min) in buffer 2 solution (0.15% BSA and 0.1% Triton-X100 in PBS) to prevent non-specific binding of the secondary antibody in the tissue. The tissue was then incubated for one hour with a goat anti-rabbit Alexa-Fluor 568 secondary (ab175471, 1:500). Sections were then washed (2 x 5min) in buffer 2 then (2 x 5min) wash in PBS before mounting on labelled gelatinised slides. Sections were washed with PBS and counterstained with DAPI (Sigma-Aldrich, D9564, 1:2000) for 30 seconds and washed twice with dH_2O. The slides were coverslipped with DABCO (Sigma-Aldrich, 101053951; in glycerol and PBS) then stored at 4°C.

Prior to the main body of IHC work, an optimisation study was carried out to determine appropriate primary antibody concentration and demonstrated the specificity of resulting staining, blocking necessity, and commonly accepted negative controls (Hewitt et al. 2014). The optimisation contained a variety of conditions and dilutions for primary antibodies. The conditions were primary only, secondary only, tissue only (to ensure visualisation of endogenous autofluorescence) as well as the combination of primary and secondary antibodies at various concentrations based on manufacturer recommendations and previous publications (Tsivion-Visbord et al. 2020; Murayama et al. 2020; Shortall et al. 2020; Heldt et al. 2014). The antibodies were diluted in buffer 1 solution. Tissue for optimisation was from vehicle control rats.

2.3 Microscopy

Images were obtained (Nikon EFD-3 microscope, Spot Insight camera and software) from consistently placed regions of interest (ROIs) at x10 magnification in the medial/ventral orbitofrontal (MO/VO), lateral/dorsolateral orbitofrontal (LO/DLO), and prelimbic/infralimbic (PrL/IL) cortices of the frontal cortex as well as the CA1, CA2/3, and dentate gyrus (DG) of the hippocampus (Figure 5) for up to six sections of tissue per animal for each ROI and biomarker. The staining intensity and cell counts obtained from the sections were averaged per animal for analysis, and an image for each ROI was obtained for all animals in this study.

All images were quantified in Fiji/ImageJ v2.0 (NIH, USA) using automated cell counting thresholds and mean intensity of immunoreactivity for all antibodies. To accomplish accurate automated cell counting, the images were converted to grey scale and the background was subtracted, then converted to binary through an adjusted threshold to allow Fiji/ImageJ v2.0 to clearly define and count cells present in the image (Figure 6). Microglial activation states were classified manually by examining visible microglia in Iba-1 images and dividing them into resting, activated, and ameboid states as described in a previous publication (Cotel et al. 2015). Resting state microglia were characterised by thin cell bodies and expansive microglial arms, activated states contained a larger cell body and microglia were thicker and arms closer to the cell body, and ameboid states were described to have a large cell body with very short or absent microglial arms. Representative images of each biomarker were obtained on a confocal microscope at x40 magnification (Zeiss 880) to better understand staining morphology (Figure 6).



A) Frontal Cortex **B) Hippocampus**

Figure 5. Regions of interest (ROI) photographed are indicated by red squares in **(A)** the medial/ventral orbitofrontal (MO/VO), lateral/dorsolateral orbitofrontal (LO/DLO), and prelimbic/infralimbic (PrL/IL) cortices of the frontal cortex as well as **(B)** the CA1, CA2/3, and dentate gyrus (DG) of the hippocampus. Images were taken at x10 magnification on a Nikon EFD-3 microscope with Spot Insight camera and software.

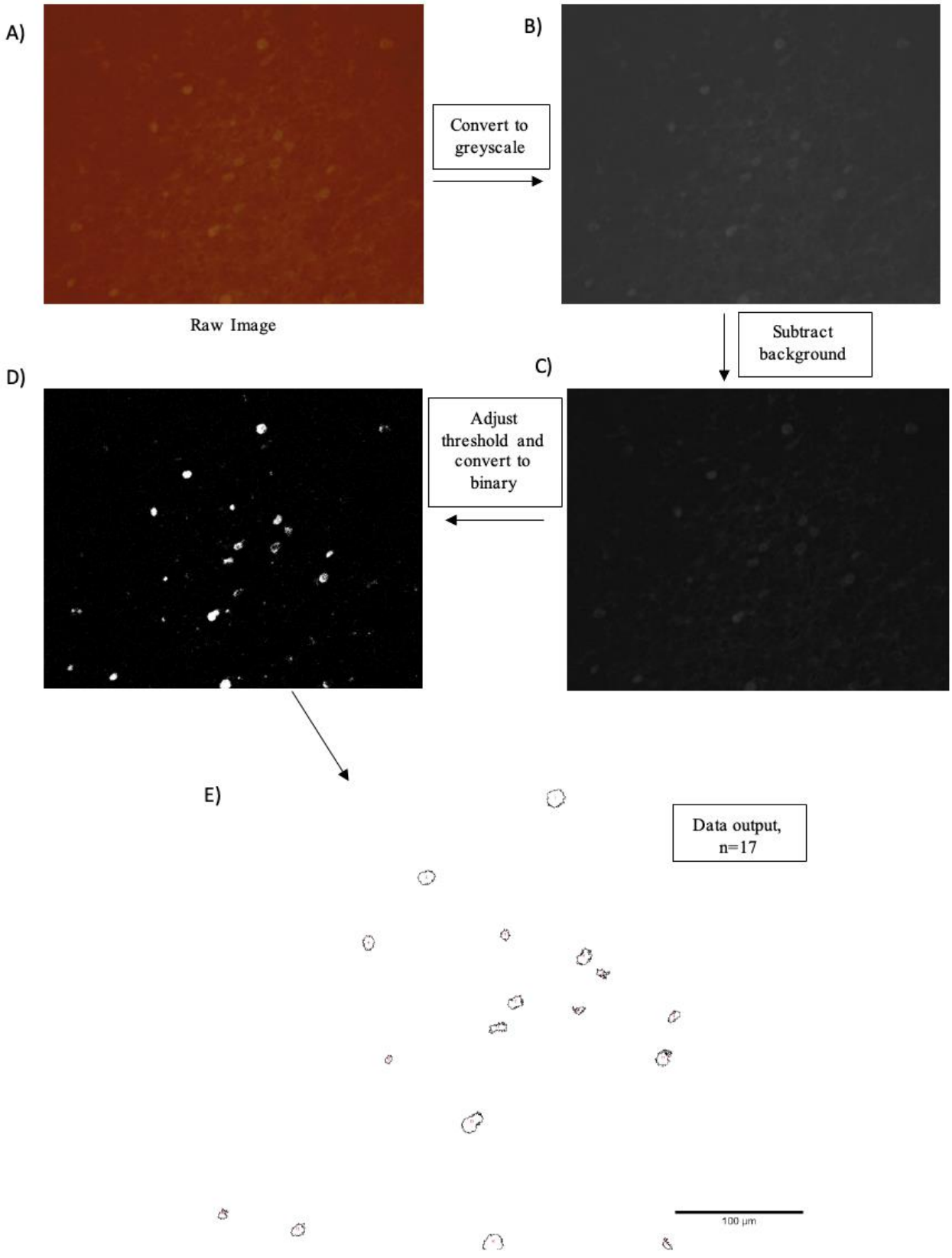


Figure 6. The process of automated cell counting in Fiji/ImageJ v2.0. **(A)** The raw image, **(B)** the image converted to grey scale, **(C)** subtracted background of the image, **(D)** threshold adjustment and binary conversion, **(E)** data output. Each image was taken at x10 magnification on a Nikon EFD-3 microscope with Spot Insight camera and software. Scale bar = 100µm.

2.4 Statistical Analysis

Statistical analysis of cell count, staining intensity, and Iba-1 morphology classification were conducted in GraphPad Prism v9.3.1. Data was tested for normality with the D'Agostino and Pearson or Kolmogorov-Smirnov omnibus tests. Following normality testing, data was analysed using a 2-way analysis of variance (ANOVA) between neurodevelopmental condition and ROI with Tukey's post hocs, or a nonparametric Kruskal-Wallis with Dunn's post-hoc. Hippocampal cell counts for each biomarker, hippocampal calbindin intensity of immunoreactivity, frontal cortical parvalbumin cell counts, and somatostatin frontal cortical data failed normality tests and underwent nonparametric analysis. Iba-1 morphology data was converted into percentages of each classification state and averaged per animal. Automated cell count validation was accomplished through an unpaired, two-tailed t-test between automated counts and counts garnered from manual counts in the PrL/IL and DG of each biomarker (n=12, with n=4 per neurodevelopmental condition, Appendix I). Pearson's r correlation analyses were also performed between immunohistochemical and discrimination ratio (exploration of novel/total choice trial object exploration) data for each biomarker, though these tests were found insignificant and are reported elsewhere (Appendix II). Data are represented as mean±SEM for immunoreactivity and microglial activation states or median±IQR for cell counts. $P<0.05$ was considered to be statistically significant.

3 Results

3.1 Optimisation Study

The optimisation study revealed that the free-floating IHC method provided adequate staining when the primary and secondary antibodies were combined, while the presence of only one of these antibodies provided little to no binding and nearly matched tissue only conditions (Figure 7).

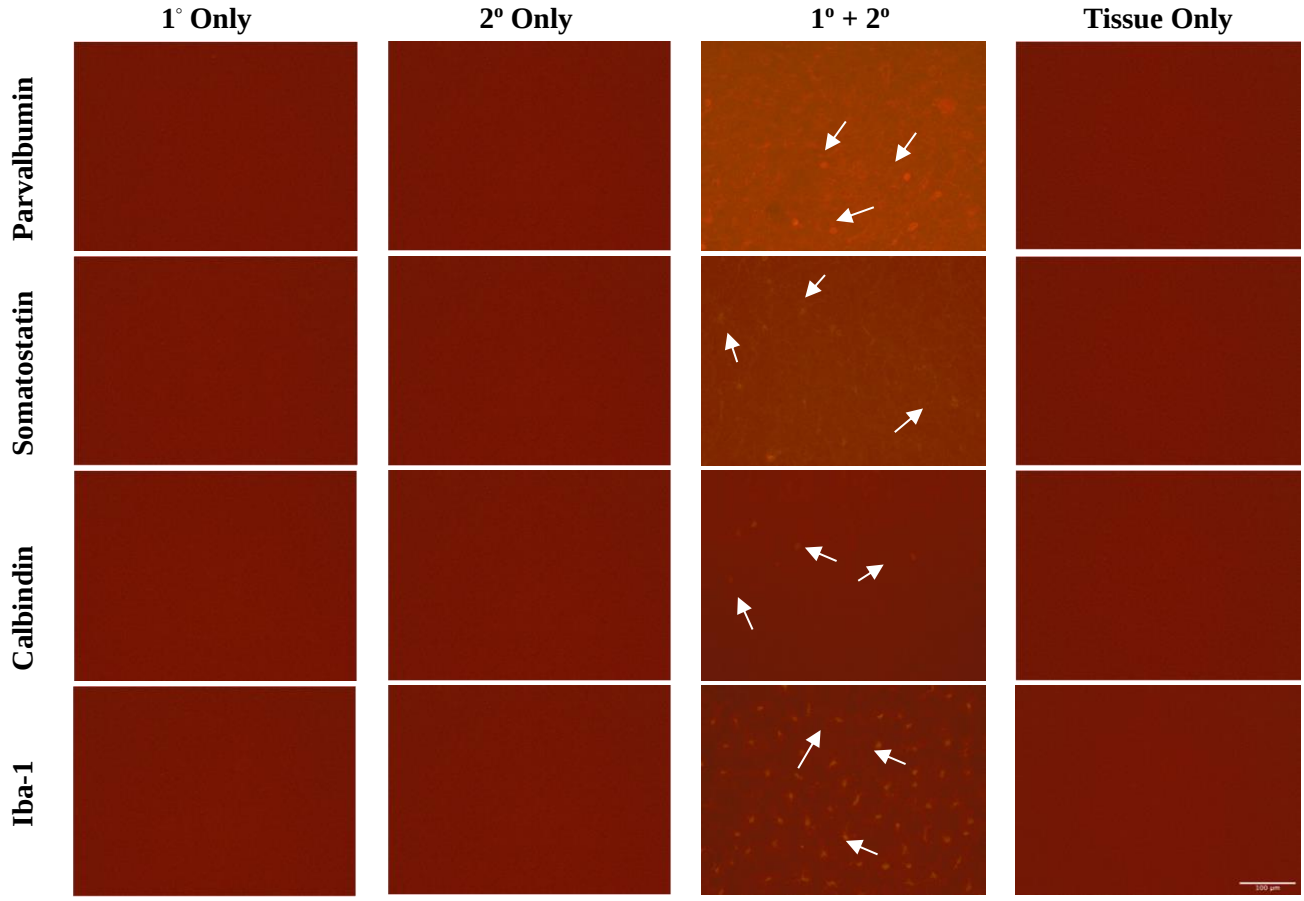


Figure 7. Images of the optimisation study conducted on tissue from negative control rats. Conditions for each biomarker included primary antibody only, secondary antibody only, primary and secondary antibodies, and tissue only with no antibodies present. Arrows indicate examples of biomarker-positive cells. Scale bar = 100µm.

3.2 Morphology of Staining

Typical markers associated with Each biomarker was examined under a confocal microscope to retrieve differences in immunoreactivity intensity and morphology. Parvalbumin, calbindin, and Iba-1 were present in cell bodies and cytoplasm. In addition to this, Iba-1 was represented in the microglia to demonstrate microglia activation. Somatostatin was localised to the surrounding fibres and notably dimmer regarding immunoreactivity (Figure 8).

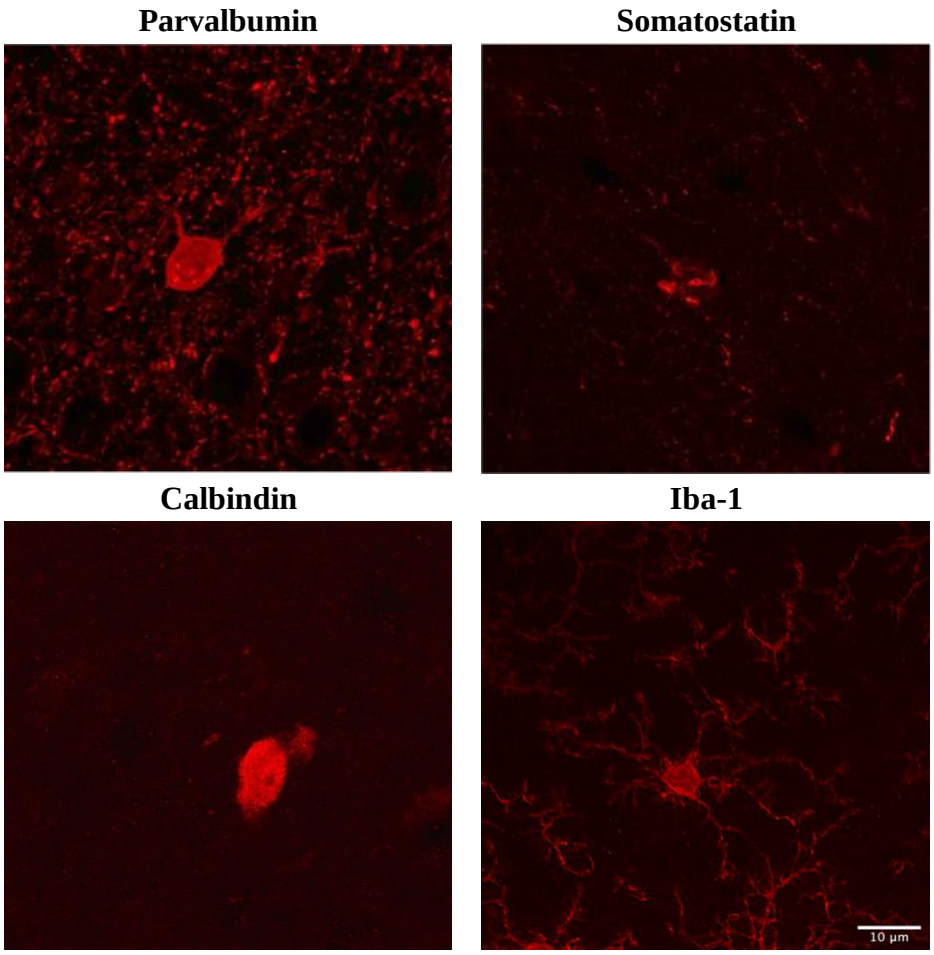


Figure 8. Representative images for each biomarker, taken via confocal microscope at x40 magnification (Zeiss 880). Scale bar = 10µm.

3.3 Immunohistochemistry

3.3.1 Parvalbumin

There were main effects of neurodevelopmental condition on parvalbumin immunoreactivity throughout the frontal cortex ($F_{(2,36)}$=11.40, P=0.0001, Figure 9A) and parvalbumin-positive cell counts in the PrL/IL (Kruskal-Wallis statistic=13.35, P=0.0013, Figure 9B). Compared to Veh-Gr controls, single-hit Veh-Iso and dual-hit PCP-Iso both exhibited reductions in parvalbumin immunoreactivity (-4 to -6%; P<0.05, Figure 9A) and counts (-58 to -62%; P<0.01, Figure 9B) within the PrL/IL. However, PCP-Iso showed additional decreases in parvalbumin immunoreactivity in the MO/VO (-7%; P<0.01) and LO/DLO (-5%; P<0.05, Figure 9A).

Additionally, there were main effects of neurodevelopmental condition on parvalbumin immunoreactivity in the DG of the hippocampus ($F_{(2,\ 36)}$=4.184, P=0.0232, Figure 9C). Compared to single-hit Veh-Iso, dual-hit PCP-Iso exhibited increases in parvalbumin immunoreactivity (+5%; P<0.05, Figure 9C). No significant effects were found regarding parvalbumin hippocampal cell counts (Figure 9D).

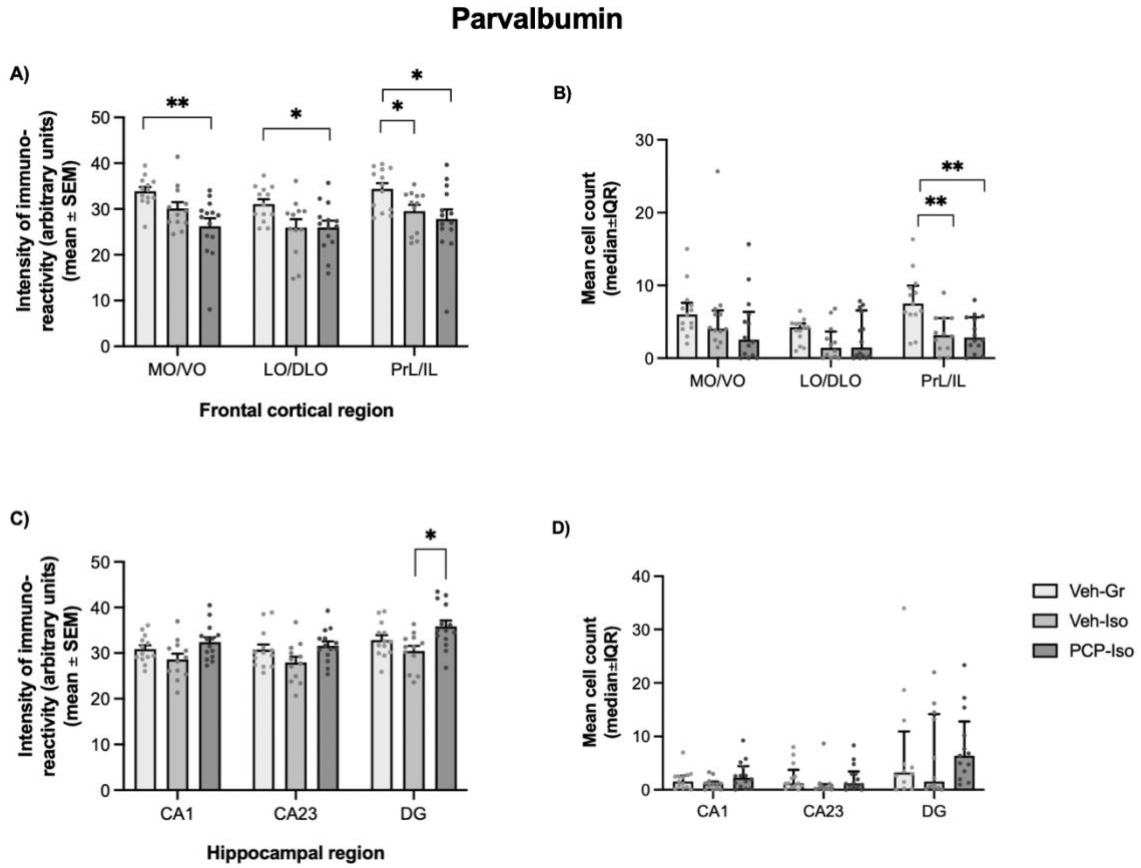


Figure 9. Impact of neonatal PCP and isolation rearing on frontal cortical and hippocampal parvalbumin immunoreactivity and cell counts. **(A)** Parvalbumin intensity of immunoreactivity in the frontal cortices (mean±SEM), **(B)** parvalbumin mean cell count in the frontal cortices (median±IQR), **(C)** parvalbumin intensity of immunoreactivity in the hippocampus (mean±SEM), **(D)** parvalbumin mean cell count in the hippocampus (median±IQR). Male Lister hooded rats that received saline (1 mL/kg s.c.; Veh) or PCP (10 mg/kg) on PND 7, 9, and 11 were housed in groups (Gr) or isolation (Iso) from weaning on PND 21, then underwent novel object discrimination (NOD) three separate times at one-to-two-week intervals (PND 57-80) following acute i.p. administration of 0.5% methylcellulose 1% Tween-80 vehicle (1mL/kg), SB-399885 (10mg/kg) or MMPIP (10mg/kg) on separate days before frontal cortical and hippocampal tissue collection on PND 79-80 (n = 12-14 per neurodevelopmental condition) (*P<0.5, **P<0.01 Tukey or Dunn's post hoc). Abbreviations: MO/VO, the medial/ventral orbitofrontal; LO/DLO, lateral/dorsolateral orbitofrontal; PrL/IL, prelimbic/infralimbic; CA, cornu ammonis; DG, dentate gyrus.

3.3.2 Somatostatin

There was an effect of neurodevelopmental condition on somatostatin. Dual-hit PCP-Iso conditions had a higher mean cell count than single-hit Veh-Iso (Kruskal-Wallis statistic=8.442, P=0.0127, Figure 10B) in the LO/DLO. No significant effects were found regarding the frontal cortical immunoreactivity or hippocampal regions.

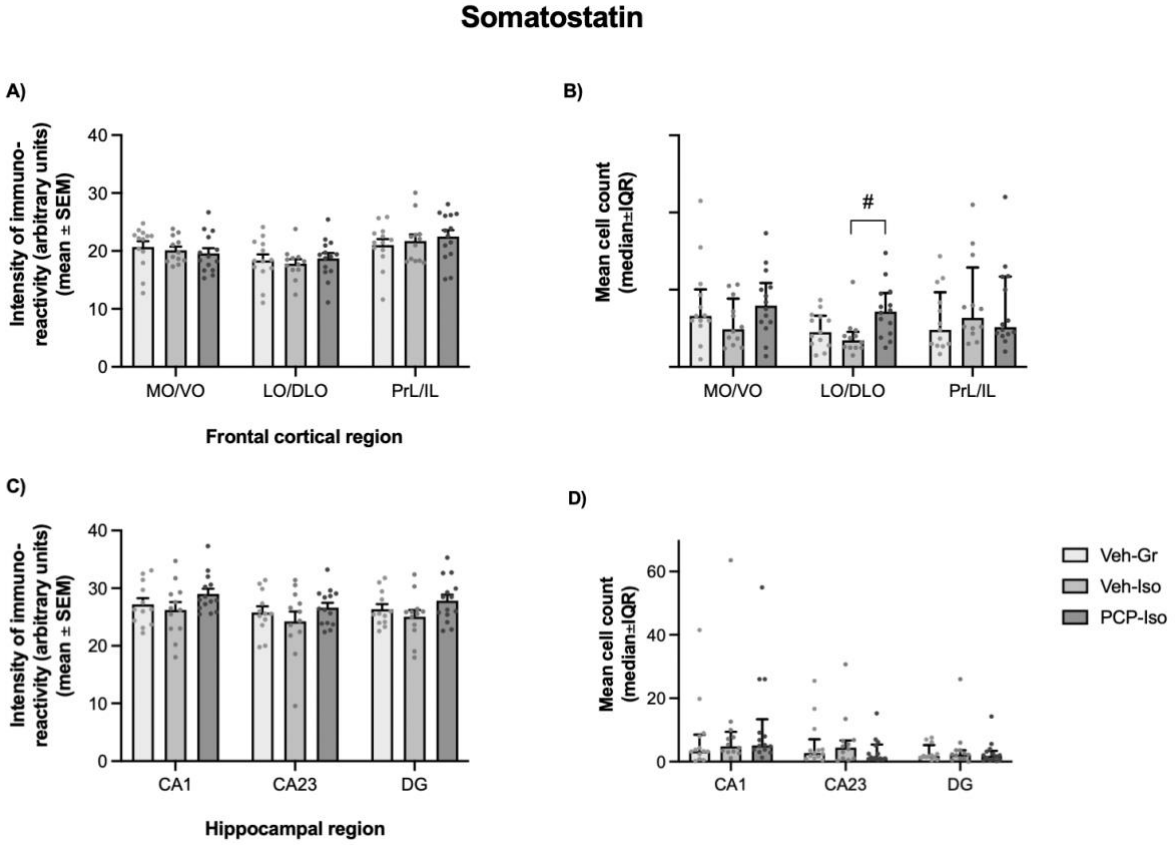


Figure 10. Impact of neonatal PCP and isolation rearing on frontal cortical and hippocampal somatostatin immunoreactivity and cell counts. **(A)** Somatostatin intensity of immunoreactivity in the frontal cortices (mean±SEM), **(B)** somatostatin mean cell count in the frontal cortices (median±IQR), **(C)** somatostatin intensity of immunoreactivity in the hippocampus (mean±SEM), **(D)** somatostatin mean cell count in the hippocampus (median±IQR). Male Lister hooded rats that received saline (1 mL/kg s.c.; Veh) or PCP (10 mg/kg) on PND 7, 9, and 11 were housed in groups (Gr) or isolation (Iso) from weaning on PND 21, then underwent novel object discrimination (NOD) three separate times at one-to-two-week intervals (PND 57-80) following acute i.p. administration of 0.5% methylcellulose 1% Tween-80 vehicle (1mL/kg), SB-399885 (10mg/kg) or MMPIP (10mg/kg) on separate days before frontal cortical and hippocampal tissue collection on PND 79-80 (n = 12-14 per neurodevelopmental condition, #P<0.05 Veh-Iso vs. PCP-Iso, Kruskal-Wallis statistic). Abbreviations: MO/VO, the medial/ventral orbitofrontal; LO/DLO, lateral/dorsolateral orbitofrontal; PrL/IL, prelimbic/infralimbic; CA, cornu ammonis; DG, dentate gyrus.

3.3.3 Calbindin

No significant effects were found regarding calbindin immunoreactivity and cell counts in the frontal cortex among different neurodevelopmental conditions (Figure 11).

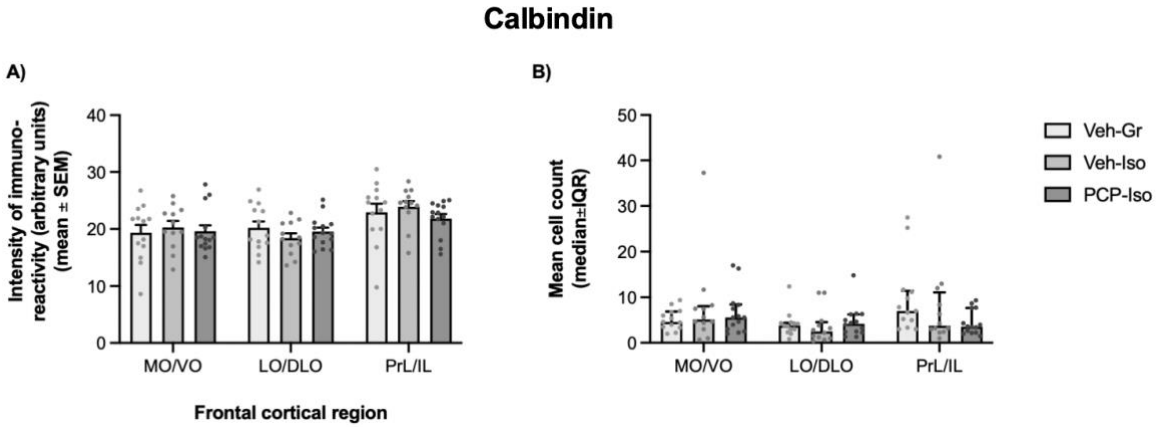


Figure 11. Impact of neonatal PCP and isolation rearing on frontal cortical calbindin immunoreactivity and cell counts. **(A)** Calbindin intensity of immunoreactivity in the frontal cortices (mean±SEM), **(B)** calbindin mean cell count in the frontal cortices (median±IQR). Male Lister hooded rats that received saline (1 mL/kg s.c.; Veh) or PCP (10 mg/kg) on PND 7, 9, and 11 were housed in groups (Gr) or isolation (Iso) from weaning on PND 21, then underwent novel object discrimination (NOD) three separate times at one-to-two-week intervals (PND 57-80) following acute i.p. administration of 0.5% methylcellulose 1% Tween-80 vehicle (1mL/kg), SB-399885 (10mg/kg) or MMPIP (10mg/kg) on separate days before frontal cortical and hippocampal tissue collection on PND 79-80 (n = 12-14 per neurodevelopmental condition). Abbreviations: MO/VO, the medial/ventral orbitofrontal; LO/DLO, lateral/dorsolateral orbitofrontal; PrL/IL, prelimbic/infralimbic.

3.3.4 Iba-1

No significant effects regarding Iba-1 immunoreactivity and cell counts found among the neurodevelopmental conditions in the frontal cortex or hippocampus (Figure 12). There were also no significant findings regarding Iba-1 morphology classification of activation states among differing neurodevelopmental conditions (Figure 13).

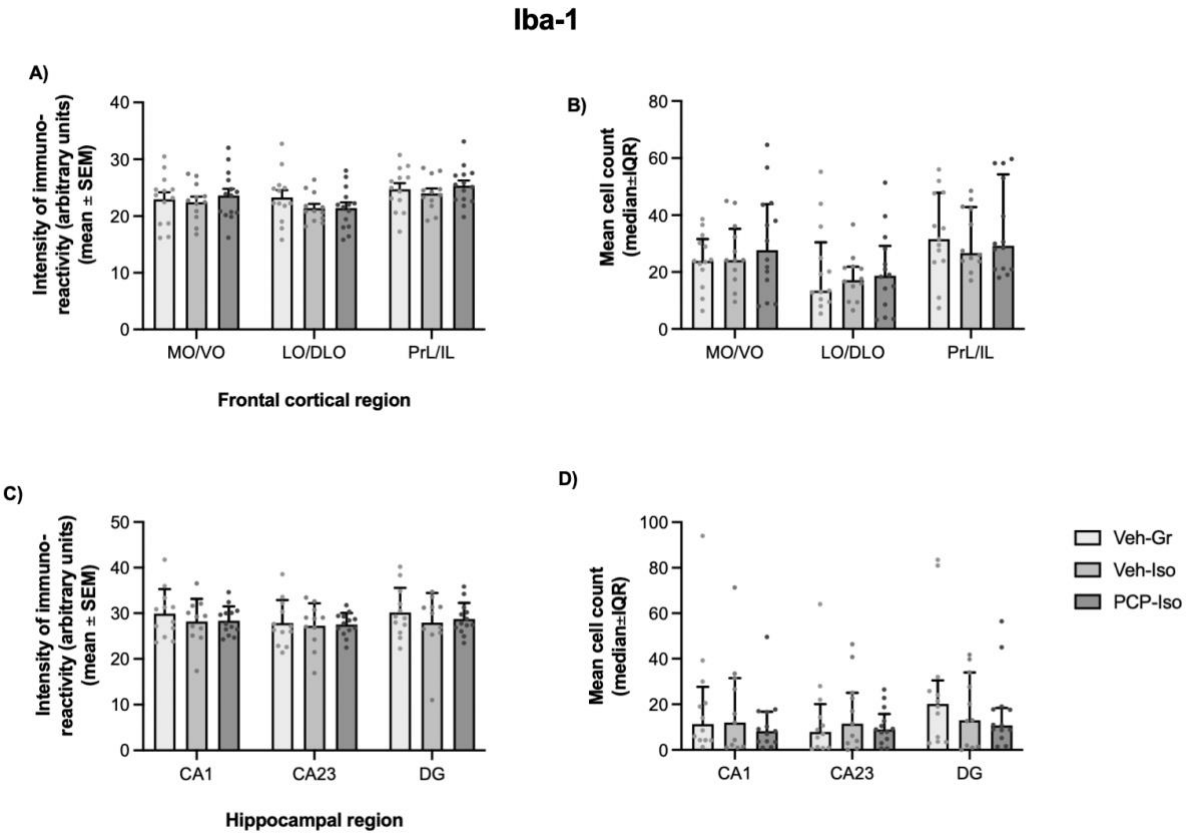


Figure 12. Impact of neonatal PCP and isolation rearing on frontal cortical and hippocampal Iba-1 immunoreactivity and cell counts. **(A)** Iba-1 intensity of immunoreactivity in the frontal cortices (mean±SEM), **(B)** Iba-1 mean cell count in the frontal cortices (median±IQR), **(C)** Iba-1 intensity of immunoreactivity in the hippocampus (mean±SEM), **(D)** Iba-1 mean cell count in the hippocampus (median±IQR). Male Lister hooded rats that received saline (1 mL/kg s.c.; Veh) or PCP (10 mg/kg) on PND 7, 9, and 11 were housed in groups (Gr) or isolation (Iso) from weaning on PND 21, then underwent novel object discrimination (NOD) three separate times at one-to-two-week intervals (PND 57-80) following acute i.p. administration of 0.5% methylcellulose 1% Tween-80 vehicle (1mL/kg), SB-399885 (10mg/kg) or MMPIP (10mg/kg) on separate days before frontal cortical and hippocampal tissue collection on PND 79-80 (n = 12-14 per neurodevelopmental condition). Abbreviations: MO/VO, the medial/ventral orbitofrontal; LO/DLO, lateral/dorsolateral orbitofrontal; PrL/IL, prelimbic/infralimbic; CA, cornu ammonis; DG, dentate gyrus.

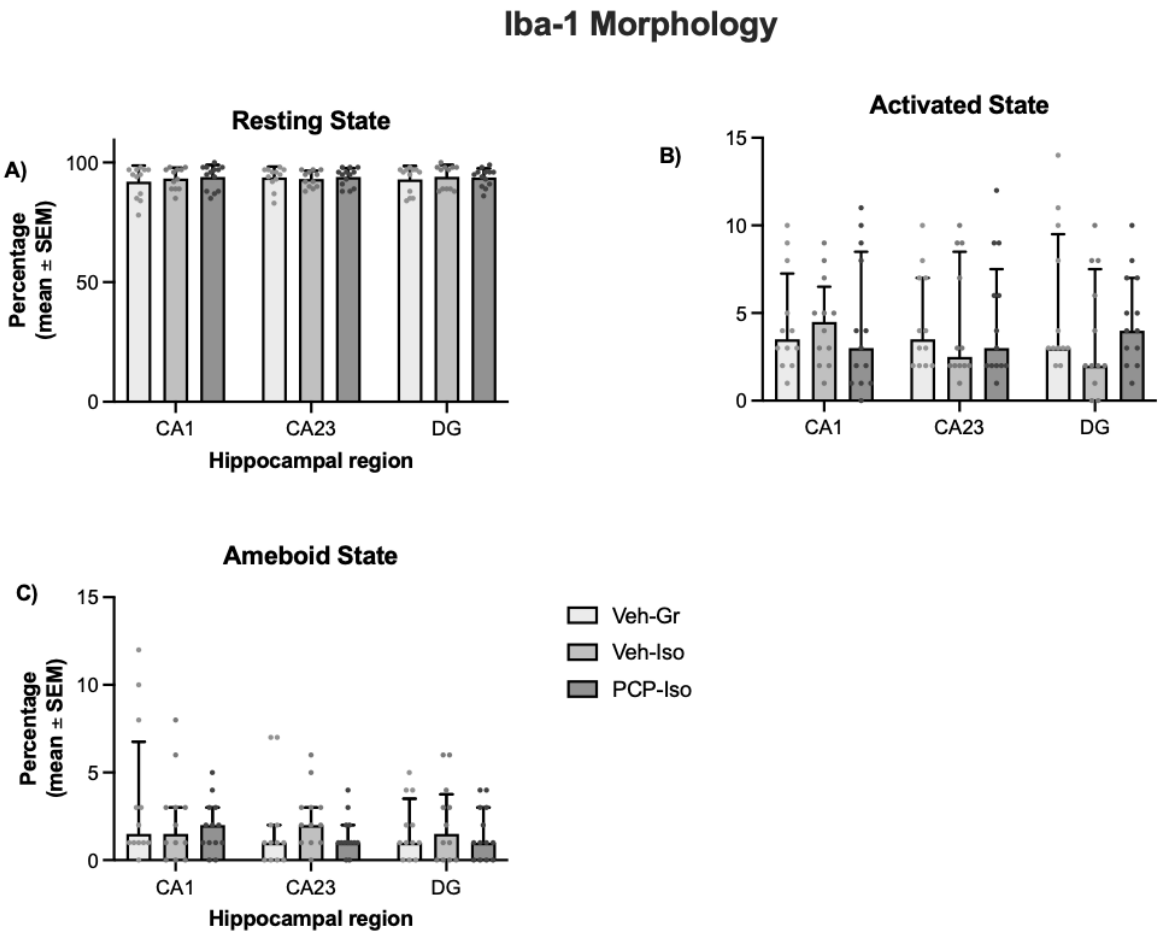


Figure 13. Impact of neonatal PCP and isolation rearing on hippocampal Iba-1 microglial activation states. **(A)** Percentage of Iba-1 microglia in resting state in the hippocampus (mean±SEM), **(B)** percentage of Iba-1 microglia in activated state in the hippocampus (mean±SEM), **(C)** Iba-1 microglia in ameboid state in the hippocampus (mean±SEM). Male Lister hooded rats that received saline (1 mL/kg s.c.; Veh) or PCP (10 mg/kg) on PND 7, 9, and 11 were housed in groups (Gr) or isolation (Iso) from weaning on PND 21, then underwent novel object discrimination (NOD) three separate times at one-to-two-week intervals (PND 57-80) following acute i.p. administration of 0.5% methylcellulose 1% Tween-80 vehicle (1mL/kg), SB-399885 (10mg/kg) or MMPIP (10mg/kg) on separate days before frontal cortical and hippocampal tissue collection on PND 79-80 (n = 12-14 per neurodevelopmental condition). Abbreviations: MO/VO, the medial/ventral orbitofrontal; LO/DLO, lateral/dorsolateral orbitofrontal; PrL/IL, prelimbic/infralimbic; CA, cornu ammonis; DG, dentate gyrus.

4 Discussion

4.1 Key Findings

The main GABAergic deficits observed in the current study were reduced parvalbumin expression in the PrL/IL of both the single-hit Veh-Iso and dual-hit PCP-Iso models with further reduction in MO/VO and LO/DLO parvalbumin only evident in the PCP-Iso model. On this basis I have accepted my original hypothesis regarding more extensive parvalbumin deficits the frontal cortex of PCP-Iso.

However, there were no effects of neurodevelopmental condition on frontal cortical somatostatin, calbindin, or Iba-1 expression, nor any changes to the chosen GABAergic or microglial markers in the hippocampus. On the basis of these negative findings, I must reject my hypothesis regarding somatostatin, calbindin, and Iba-1 expression and microglia activation.

4.2 Neuroanatomy

The prefrontal cortex is a region associated with modulating higher executive functions. Specifically, the PrL/IL partially make up the medial prefrontal cortex in rats and has been attributed to decision making based on reward potential (Bolton et al. 2018). The orbitofrontal cortex, comprised of the MO/VO and LO/DLO, is responsible for functions such as reversal learning and identity discrimination (Izquierdo 2017). Alterations in GABAergic neurotransmission in these subregions lead to disruption in executive functions as seen in schizophrenia, particularly pertaining to goal-directed behaviours and information processing (Mederos et al. 2021; Fatemi et al. 2017). The hippocampus is responsible for memory formation, retention, and retrieval. The hippocampus became an area of interest here due to a link between glutamate hypofunction and declarative memory deficits within schizophrenia (Tamminga et al. 2010). GABAergic alterations in the hippocampus, particularly involving parvalbumin, have shown to disrupt network synchronisation and memory deficits (Klausberger et al. 2005; Verret et al. 2012). Investigations of these regions within the frontal cortex as well as the hippocampus provide a holistic view into potential links between symptoms and disease neurobiology of schizophrenia.

During optimisation of current immunohistochemistry protocols, staining was observed in sections incubated in the combination of both primary and secondary antibodies but absent in negative control sections incubated with either primary or secondary antibody alone or those exposed to buffers lacking either antibody. Further confidence in the nature of the staining observed is provided by similarities reported for each of my biomarkers. The pattern and distribution of immunoreactive cells within the frontal cortex and hippocampus of parvalbumin- and calbindin-positive cells observed herein match previous descriptions of

bountiful and evenly distributed immunofluorescence in nuclei and cell bodies (Tamamaki et al. 2003; Zhao et al. 2013). The occurrence of somatostatin-positive cell bodies with abundant fibre staining also matches other findings (McDonald and Mascagni 2002). Extensive Iba-1 staining was present throughout cell bodies and processes across the tissue, allowing us to clearly visualise morphology of individual microglial cells and characterise their activation states (Laskaris et al. 2016; Murray et al. 2019). Similar findings were obtained with current rabbit anti-parvalbumin (Abcam, ab11427) and other anti-Iba-1 antibodies in the PrL/IL, orbitofrontal cortex, and hippocampus of mice (Reichelt et al. 2021). Characterisation of microglial activation through images of Iba-1 has been obtained with current anti-Iba-1 polyclonal antibody (Wako 019-19741) using tissue from mice in the hippocampus, cerebral cortex, hypothalamus as well as other areas (Mori et al. 2000). Somatostatin has been visualised throughout the frontal cortex of mice featuring the antibody used in this study (Espinosa et al. 2019), as well as subregions in the hippocampus with a different rabbit anti-somatostatin antibody (Ortiz et al. 2020). Similar visualisation of calbindin as seen here has been evident with mouse anti-calbindin monoclonal antibody using tissue from PrL/IL in rats (Osterop et al. 2015). The specific calbindin antibody used in this study has successfully provided visualisation of Purkinje cells in mice (Béchet et al. 2020) and rats (Nuryyev et al. 2017).

4.3 Single-Hit Observations

Decreased frontal cortical parvalbumin, which is broadly consistent with our findings in Veh-Iso has been previously reported in single-hit models of schizophrenia of isolation reared rats (Todorović et al. 2019). Similar decreases of parvalbumin expression have also been described in the PrL/IL subregions of male C57BL/6 mice after five weeks of isolation from PND 21 (Ueno et al. 2017). Previous work has described reduced parvalbumin in the hippocampus of isolation reared female and male Sprague Dawley rats (Harte et al. 2007; Powell et al. 2015). These changes were evident at 11 and eight weeks of isolation, respectively, and appear to parallel reduced post-mortem reports of parvalbumin- and somatostatin-positive interneurons in the hippocampus seen in patients with schizophrenia (Table 1).

In the current study, male Lister-Hooded rats underwent 11 weeks of isolation from PND 21 before tissue collection for immunohistochemistry. Weaning ages, cage size, and isolation length are parallel with the studies reported above with minimal variation. All studies allowed access to food and water *ad libitum* throughout the experiments, apart from Powell et al. 2015, in which food restrictions were applied prior to reversal-learning tests. The status of environmental enrichment was widely unreported, though all publications reported minimising animal suffering similar to this study. Verification of expected methodology in this model therefore lead to surprising negative findings for parvalbumin, somatostatin, and Iba-1 in the hippocampus and somatostatin, calbindin, and Iba-1 in the frontal cortex.

Reduced hippocampal parvalbumin expression has been found in a single-hit model using male Sprague Dawley rats after administration of NMDAR antagonist MK-801 from PND 5 to 15 (Li et al. 2015). Our findings also contrast with previous reports of reduced somatostatin in an

NMDAR antagonist model (Murueta-Goyena et al. 2020). Additionally contrasting with our findings, calbindin deficits have been reported in the frontal cortex of rats after chronic injection of methamphetamine, but frontal cortical calbindin findings remain sparse among animal models of schizophrenia (Veerasakul et al. 2016). Our findings regarding Iba-1 also fail to match current literature, as most reports state increased microglial density and activation following chronic antipsychotic administration (Cotel et al. 2015) and maternal immune activation (Zhang et al. 2019). Despite these potentially promising findings, single-hit neurodevelopmental models including isolation rearing have faced criticism for robustness, reproducibility, and predictive validity and current findings in this single-hit model further support desire for continued refinements and improvements.

4.4 Dual-Hit Effects

Wider deficits of frontal cortical parvalbumin in dual-hit PCP-Iso than Veh-Iso, as reported here, may be more representative of changes in schizophrenia (Gilabert-Juan et al. 2013). Additive effects of dual neurodevelopmental 'hits' have been seen across multiple models for schizophrenia. These studies range from gestational administration of polyinosinic:polycytidylic acid (PIC) followed by cannabinoid exposure in adolescence or peripubertal stress (Dalton et al 2012; Monte et al. 2017), to maternal separation with isolation rearing, or peripubertal stress, and early adulthood corticosterone administration (Vargas et al. 2016; Novak et al. 2013; Hill et al. 2014). Although these specific studies do not report on any of our biomarkers, previous models utilising early-life stress factors have reported deficits in parvalbumin expression (Jiang et al. 2013; Woodward and Coutellier 2021).

Not all combinations of the dual-hit model are additive, however. Studies have reported that the first hit can seemingly provide resilience for the second hit. This has been seen in various combinations, including maternal separation accompanied with isolation, neonatal AMPA/kainite agonism, and high fat diet (Ellenbroek and Cools 2002; Marriott et al. 2016; Arcego et al. 2016) as well as maternal immune activation with isolation rearing (Goh et al. 2020). Resilience in animal studies does not necessarily discount the merit of the dual-hit model approach. In fact, these findings parallel with the idea that lifetime adversity faced in humans is more beneficial than a complete lack of negative experiences, though there is very little known about what specific timing and circumstances promote resilience versus ill-effects (Seery et al. 2013; Daskalakis et al. 2013). Ultimately, dual-hit models allow investigation into complex interactions of early-life risk factors and how they contribute to the neurobiology of schizophrenia and other mental disorders.

4.5 Limitations and Improvements

There was variability in background intensity between slices, which could have been a combination of natural variation between animals and technical discrepancies during lab work. Multiple individuals contributed to the immunohistochemical staining of parvalbumin and somatostatin in the frontal cortex and hippocampus, which could have resulted in potential

differences in background as well as numbers of slices available from each animal. The target was six slices per animal, but in some cases were as low as three due to tissue damage during sectioning, staining, or mounting. Each individual contributing had a balanced allocation of tissue from each neurodevelopmental condition. Additionally, lab work was conducted simultaneously using the same buffer and antibody solutions to minimise variability as much as possible. Calbindin and Iba-1 IHC protocol and images collection were conducted by one individual, ensuring minimal variability in data retrieval for those biomarkers.

Throughout all the IHC work, only one marker was visualised at a time. To confirm the detected parvalbumin- and calbindin-positive cells were GABAergic, double staining could have been preferred using a further GABA-selective marker such as GAD_{67}, which is the enzyme that catalyses the decarboxylation of glutamate to GABA. Biomarkers such as parvalbumin, calbindin, and somatostatin are not always present in GABAergic cells, and double staining would have allowed us to further differentiate between the GABAergic and non-GABAergic cells. However, the staining appeared to match consistently throughout literature based in GABAergic cells as mentioned in section 5.2, thus making this a minor limitation.

There was no single optimal method regarding cell counting. Consistent threshold and particle size values were chosen for each marker to minimise the chances of background staining being incorrectly identified as immunoreactive cells. However, these conservative settings could have resulted in some lower intensity parvalbumin, somatostatin, calbindin, and Iba-1 positive cells not being included in the counts, and in a small number of sections with highest background there may have been some non-biomarker positive features incorrectly included. Furthermore, for somatostatin staining, which was more evident in fibres than cell bodies, it is not possible to determine whether multiple detected features are in fact different dendrites from the same cell body and should therefore be recorded as a single rather than multiple cell counts. To verify the automated detection settings were not introducing excessive artifacts, manual cell counting was also performed across all biomarkers in the frontal cortex and hippocampus and compared to the automated values, to which there were no significant differences found (Appendix I). This provides confidence that the automated cell counting reported is useable. Regarding the high background staining, slices thinner than 60μm could have assisted in focus challenges experienced with the microscope, but would have increased the risk of physical damage of the tissue during staining. Additionally, changes to blocking methodology such as increasing the BSA or detergent concentrations could be considered, though this poses a risk of over-blocking and damaging the tissue, respectively.

It is worth mentioning there are claims that blocking buffer is no longer needed due to improving antibody developments (Buchwalow et al. 2011). Although antibodies have vastly improved in the previous decades, the Buchwalow et al. study primarily utilised human tissue and the limited tissue that was used from rodents did not include the brain. Additionally, the publication did not provide visualisation of tissue with blocking buffer compared to without blocking buffer and instead provided images with no blocking buffer only. Ultimately, the time

and materials required for the blocking step were minimal and considered to have no harm for this study.

A single-hit PCP-Gr neurodevelopmental condition was not a part of the study design. Although the Veh-Iso neurodevelopmental condition satisfied the 'single-hit' aspect of the experiment design, not including a PCP-Gr condition prevented any determination of whether the additional changes seen in the PCP-Iso condition were additive or synergistic.

This cohort of rats showcased cognitive impairments as expected, particularly in PCP-Iso groups, as stated in a previous report (Shortall et al. 2020). Specifically, PCP-Iso groups exhibited an extremely robust deficit in NOD tests of visual recognition memory, which appears more reliable than that in single hit Veh-Iso, which is evident in 100% of nine PCP-Iso cohorts, compared to 70% of Veh-Iso cohorts (Shortall et al. 2020; Fone and Porkess 2008). Although we validated there was a behavioural deficit in terms of visual recognition memory via NOD, there were no behavioural testing to determine working memory, sensory-motor gaiting, reasoning, or problem-solving and therefore do not know how closely the cohort replicate the negative and social deficits or represent positive symptoms of schizophrenia associated with these tests.

4.6 Relationship Between Behavioural and Immunohistochemical Findings

Although current immunohistochemical findings do not prove that NOD deficits in Veh-Iso or PCP-Iso groups are caused by parallel frontal cortical parvalbumin deficits, they do suggest the cognitive deficits are not due to altered frontal cortical somatostatin, calbindin, and Iba-1, or hippocampal parvalbumin, somatostatin, or Iba-1 expression, or altered microglial states.

It is postulated that abnormalities in frontal cortical and hippocampal GABAergic function cause cognitive impairments seen in schizophrenia, though there are conflicting views as to whether GABAergic dysfunction causes impairments seen in NOD tests. Deficits in NOD have been reversed in several studies by atypical APD as well as ligands, such as 5-HT_7 antagonists, following sub-chronic administration of NMDAR antagonists (Rajagopal et al. 2014). A study also reported NOD deficits following sub-chronic PCP administration were attenuated by reducing $GABA_A$ receptor stimulation in the ventral hippocampus in rats (Neugebauer et al. 2018) as well as a deficit of both NOD and parvalbumin immunoreactivity in the PrL of male Lister hooded rats that was unaffected by concurrent administration of risperidone, an atypical APD (McKibben et al. 2010).

These studies point to GABAergic dysfunction of the hippocampus and frontal cortex impacting NOD. Our findings of reduced frontal cortical parvalbumin and hippocampal calbindin make it unclear as to whether cognitive deficits are caused by a sole biomarker. Due to a lack of significant findings regarding NOD choice trial ratios and parvalbumin staining

correlations in this study, the cognitive deficits are likely caused by reduced hippocampal calbindin, though further investigation is recommended.

4.7 Future Work

One of the most consistent observations from patients with schizophrenia is elevated interleukin-6 (IL-6) (Mondelli et al. 2011, Hayes et al 2014, Wu et al. 2019 in Table 3, Section 1.3.2). As a result, current research is examining inflammatory cytokine IL-6 levels in the hippocampus of Veh-Iso and PCP-Iso, as well as levels of GABA in the frontal cortex and hippocampus using commercially available ELISA kits. Utilising GABA ELISA kits will further determine whether the loss of parvalbumin and calbindin seen in this cohort are a loss of individual cells or a loss of protein expression, as well as if dual staining in IHC protocol is necessary for future studies to characterise the impact of GABAergic expression in these models.

The principle finding in the current study was frontal cortical parvalbumin deficits in Veh-Iso and PCP-Iso. Future studies should examine whether cognitive and social deficits observed in PCP-Iso can be reversed by compounds that regulate firing of parvalbumin-positive interneurons to identify causal links between interneuron marker loss and cognition. Potassium voltage gated ion channels (Kv3) are commonly accompanied with parvalbumin cells and allow them to fire at high frequencies and synchronise neural activity. Evidence suggests that positive modulation of Kv3 channels assist in the normalisation of parvalbumin interneurons, creating a potential treatment option for cognitive symptoms of schizophrenia (Yanagi et al. 2014). AUT6 and AUT9, novel Kv3 channel modulators, have reversed cognitive deficits in a sub-chronic PCP model of schizophrenia in rats (Leger et al. 2015; Harte et al. 2014). Exploring Kv3 channel modulators such as AUT6 and AUT9 in a PCP-Iso model could be insightful in establishing whether parvalbumin and cognitive deficits observed in this model can be reversed through modulation.

4.8 Concluding Remarks

We have found reduced parvalbumin expression in the frontal cortex of Veh-Iso and PCP-Iso rats with a more extensive parvalbumin deficit in PCP-Iso. This finding mirrors the previously reported pattern of hippocampal calbindin deficits in the same cohort of rats (Shortall et al. 2020). The PCP-Iso had no changes to hippocampal parvalbumin or frontal cortical calbindin, nor to somatostatin or Iba-1 in either region. The PCP-Iso model may have relevance to a subtype of schizophrenia with region-specific changes to different subsets of GABAergic interneurons. To establish causal links between interneuron marker loss and cognition, we suggest future work to encompass KV 3.1 in a PCP-Iso model of schizophrenia. This study ultimately provided further characterisation of face and predictive validity of the dual-hit model of schizophrenia in the hopes to develop enhanced preclinical models that result in pharmacological success.

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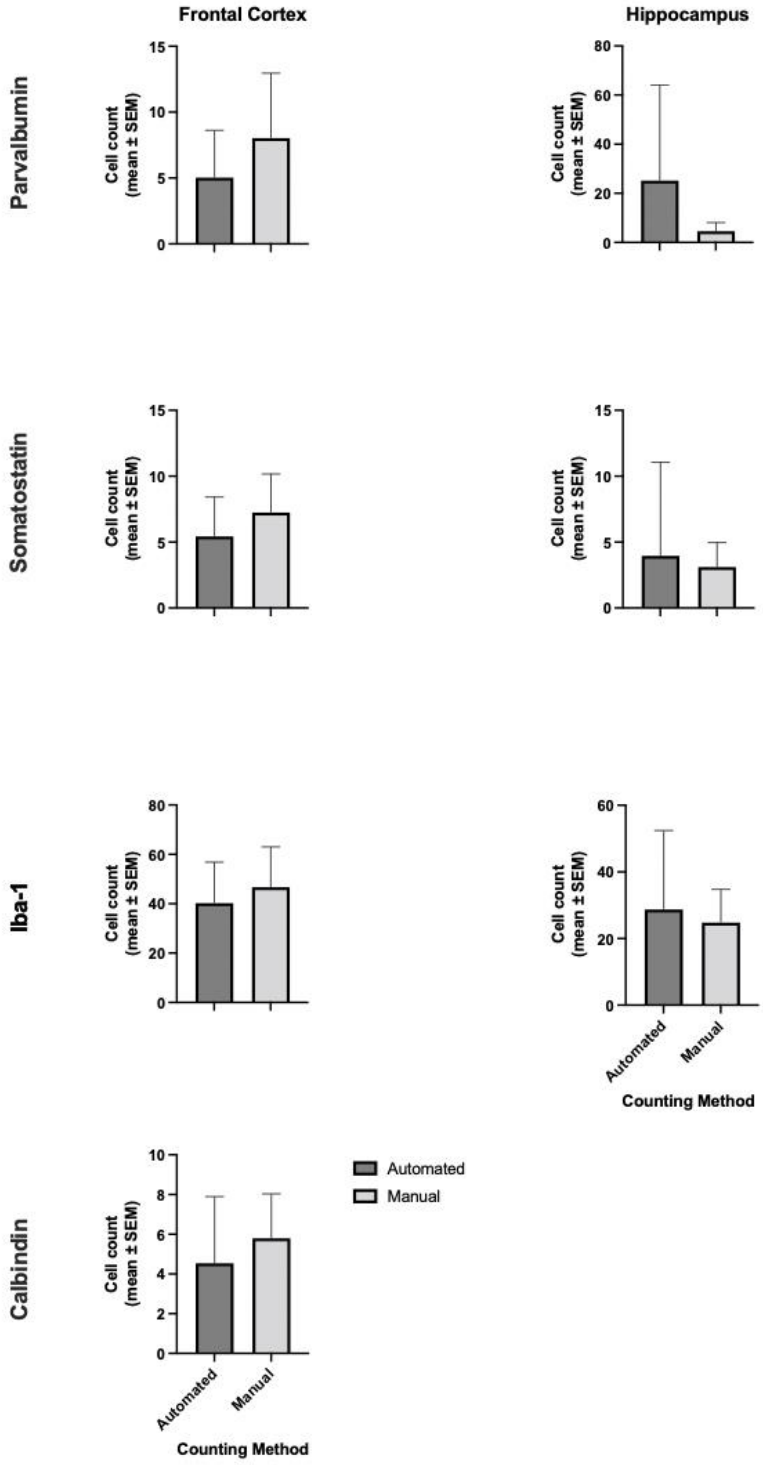
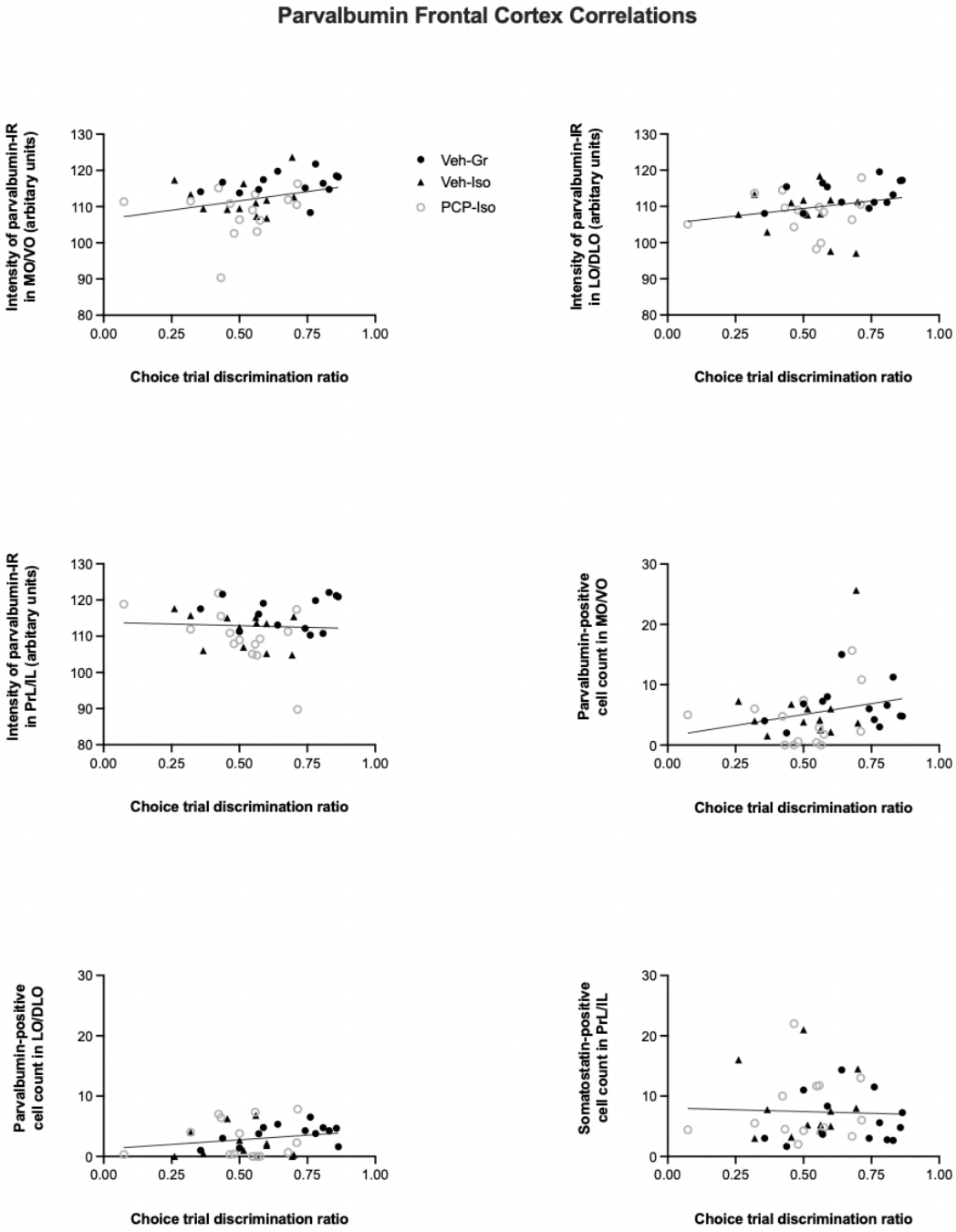
Appendix I – Further validation of the automated cell counting procedure

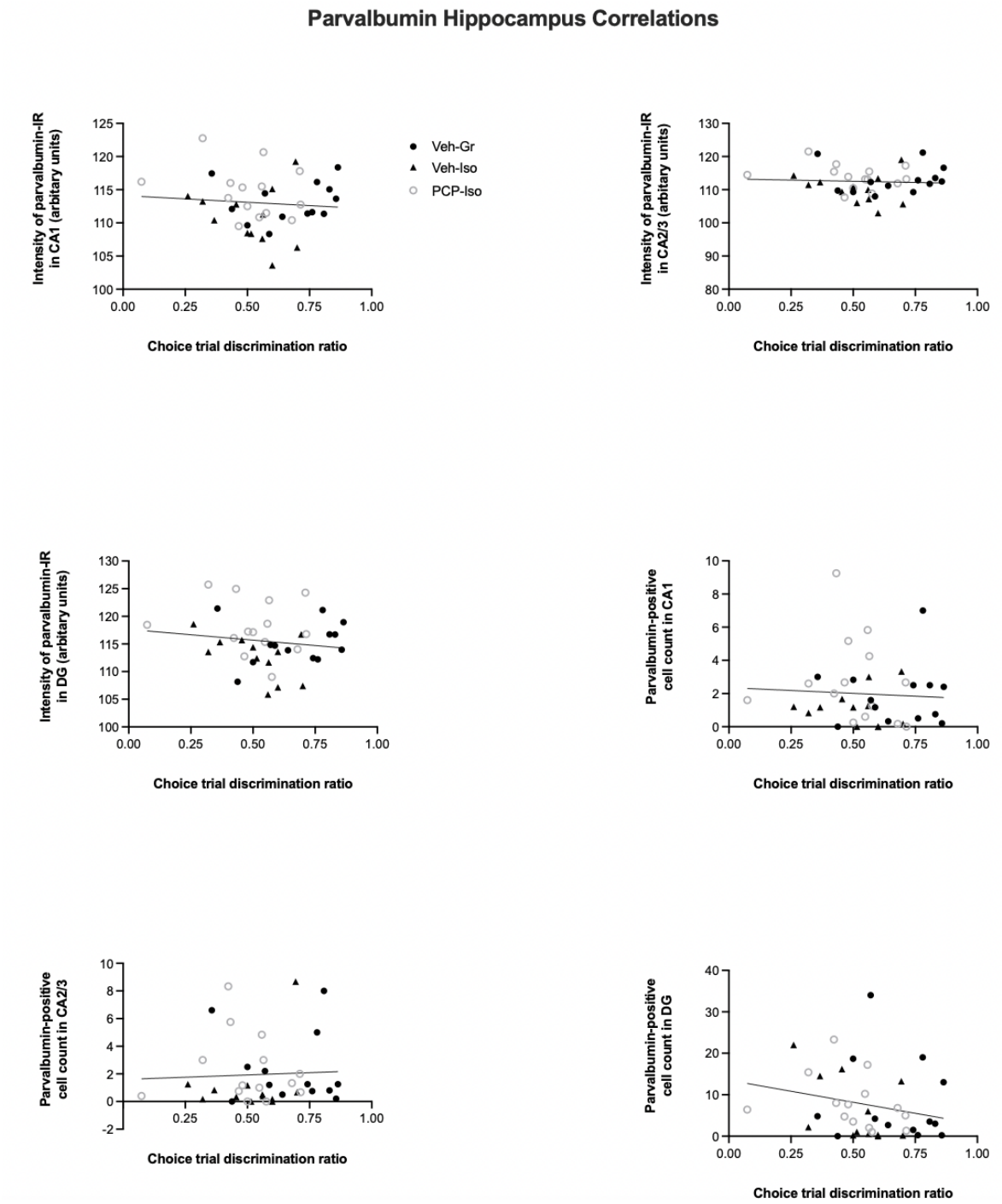


Appendix Figure 1. Comparison of automated and manual cell counts, which were independently obtained for each biomarker in the PrL/IL of the frontal cortex and DG of the hippocampus (with the exception of calbindin which was only examined in the frontal cortex as hippocampal data are published in Shortall et al. 2020). Animals for validation were chosen by a random subset comprising of an equal number of rats from each neurodevelopmental condition (n=12 rats, n=4 per neurodevelopmental condition). No significant differences were obtained between manual and automated counts (unpaired t-test, 2-tailed).

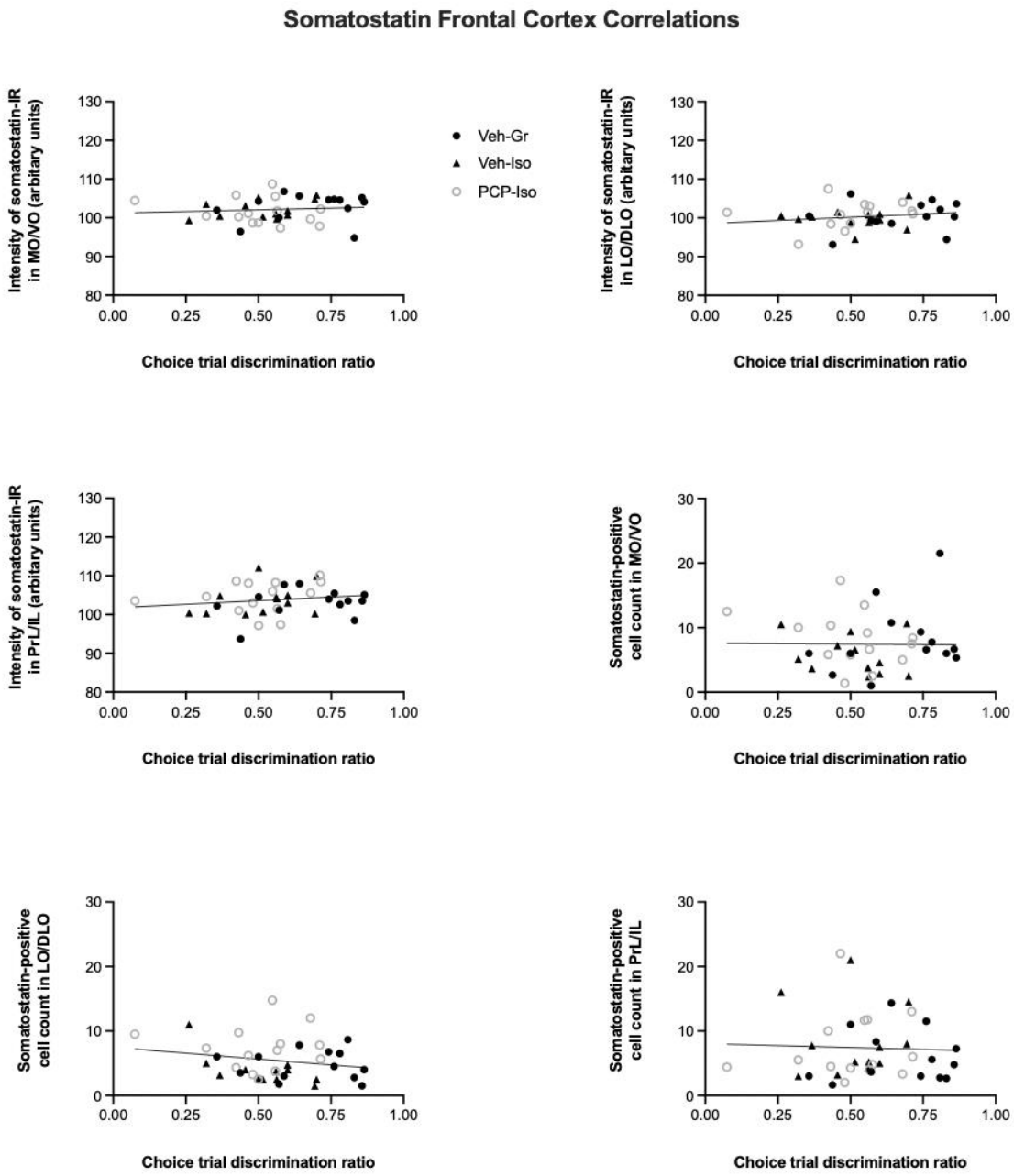
Appendix II – NOD Correlations



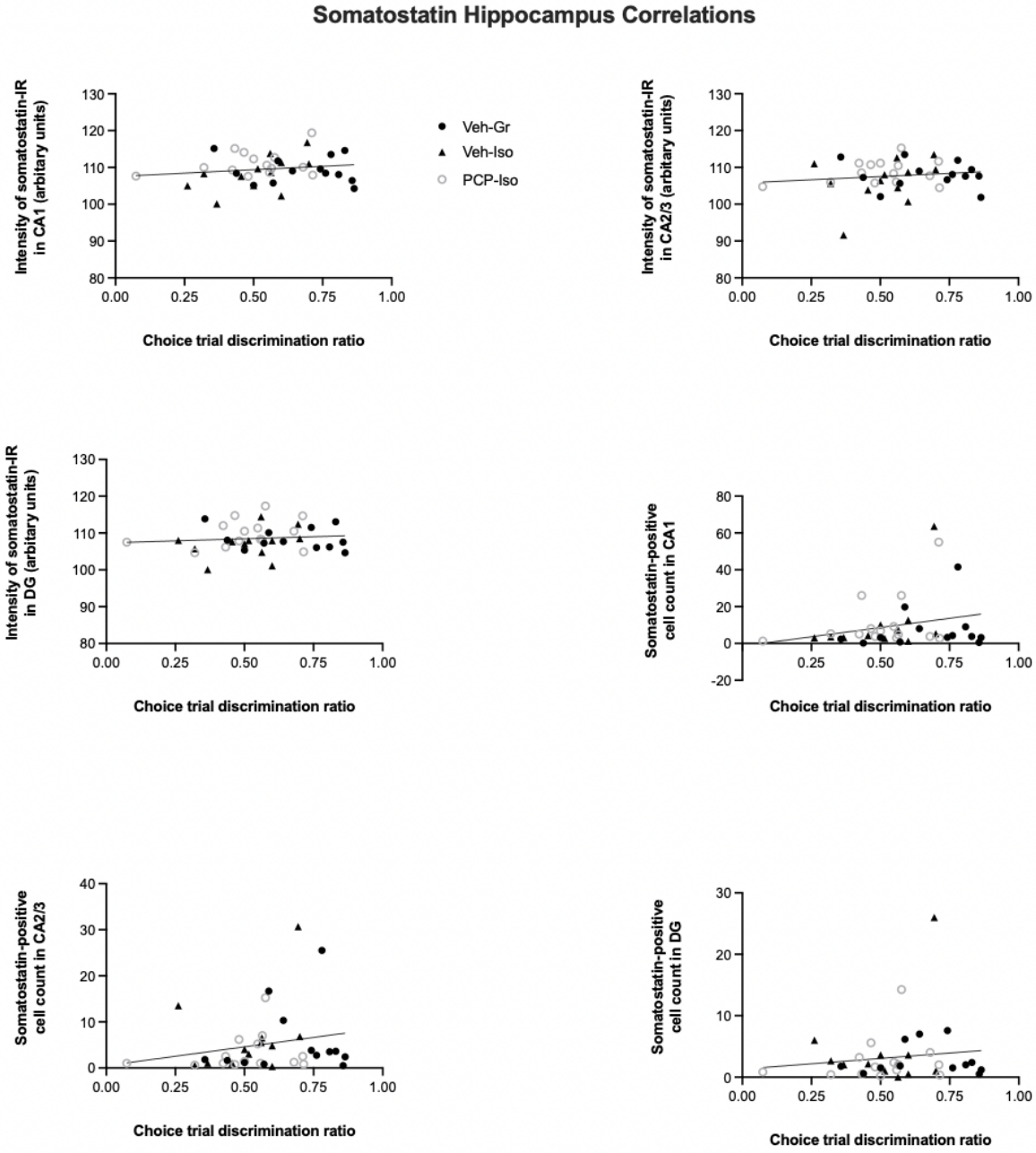
Appendix Figure 2. Correlation analysis of parvalbumin and novel object discrimination (NOD) ratios in the frontal cortex. Male Lister hooded rats that received saline (1 mL/kg s.c.; Veh) or PCP (10 mg/kg) on PND 7, 9, and 11 were housed in groups (Gr) or isolation (Iso) from weaning on PND 21, then underwent NOD three separate times at one-to-two-week intervals (PND 57-80) following acute i.p. administration of 0.5% methylcellulose 1% Tween-80 vehicle (1mL/kg), SB-399885 (10mg/kg) or MMPIP (10mg/kg) on separate days before frontal cortical and hippocampal tissue collection on PND 79-80 (n = 12-14 per neurodevelopmental condition). Abbreviations: IR, immunoreactivity; MO/VO, the medial/ventral orbitofrontal; LO/DLO, lateral/dorsolateral orbitofrontal; PrL/IL, prelimbic/infralimbic.



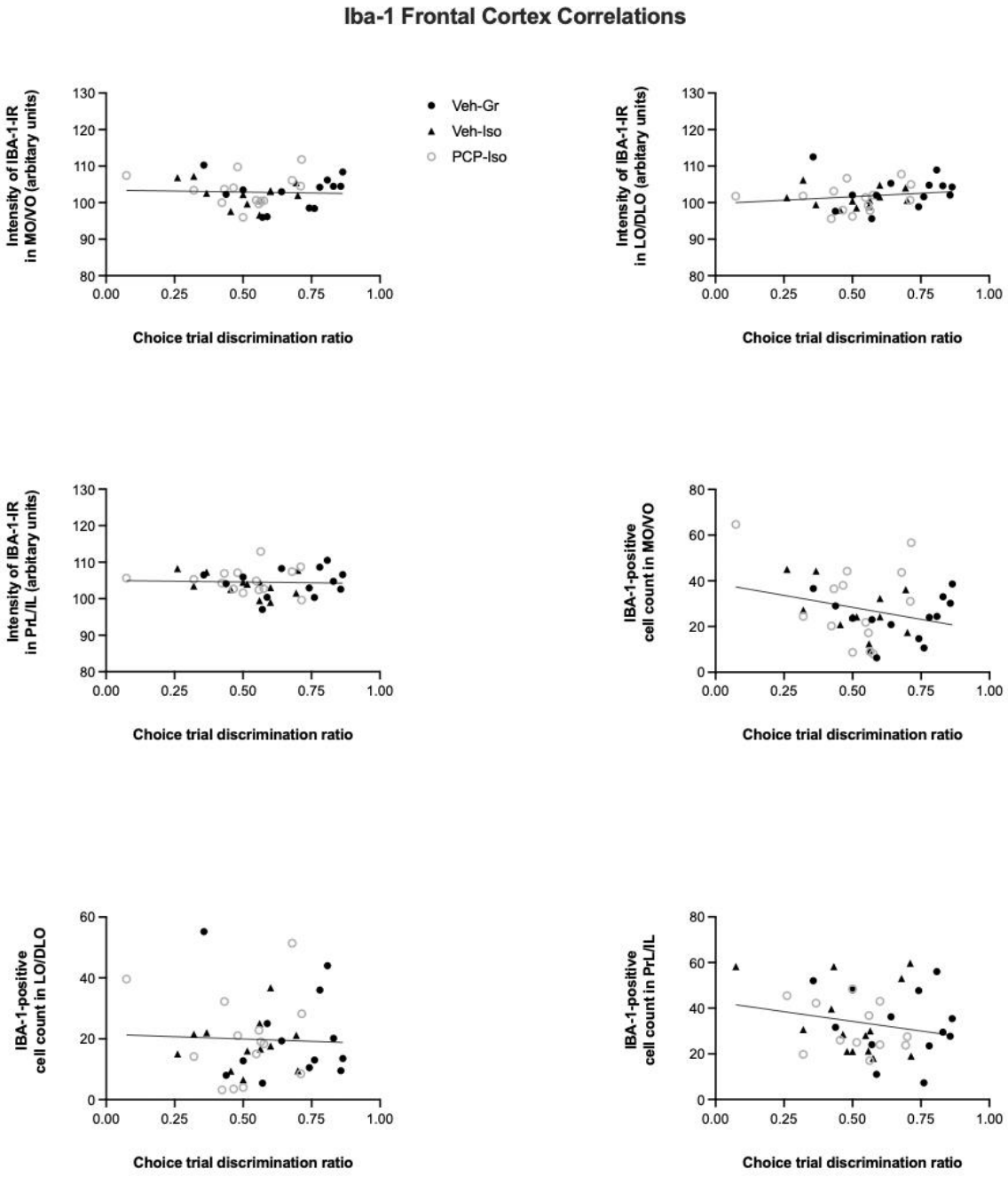
Appendix Figure 3. Correlation analysis of parvalbumin and novel object discrimination (NOD) ratios in the hippocampus. Male Lister hooded rats that received saline (1 mL/kg s.c.; Veh) or PCP (10 mg/kg) on PND 7, 9, and 11 were housed in groups (Gr) or isolation (Iso) from weaning on PND 21, then underwent NOD three separate times at one-to-two-week intervals (PND 57-80) following acute i.p. administration of 0.5% methylcellulose 1% Tween-80 vehicle (1mL/kg), SB-399885 (10mg/kg) or MMPIP (10mg/kg) on separate days before frontal cortical and hippocampal tissue collection on PND 79-80 (n = 12-14 per neurodevelopmental condition). Abbreviations: IR, immunoreactivity; CA, cornu ammonis; DG, dentate gyrus.



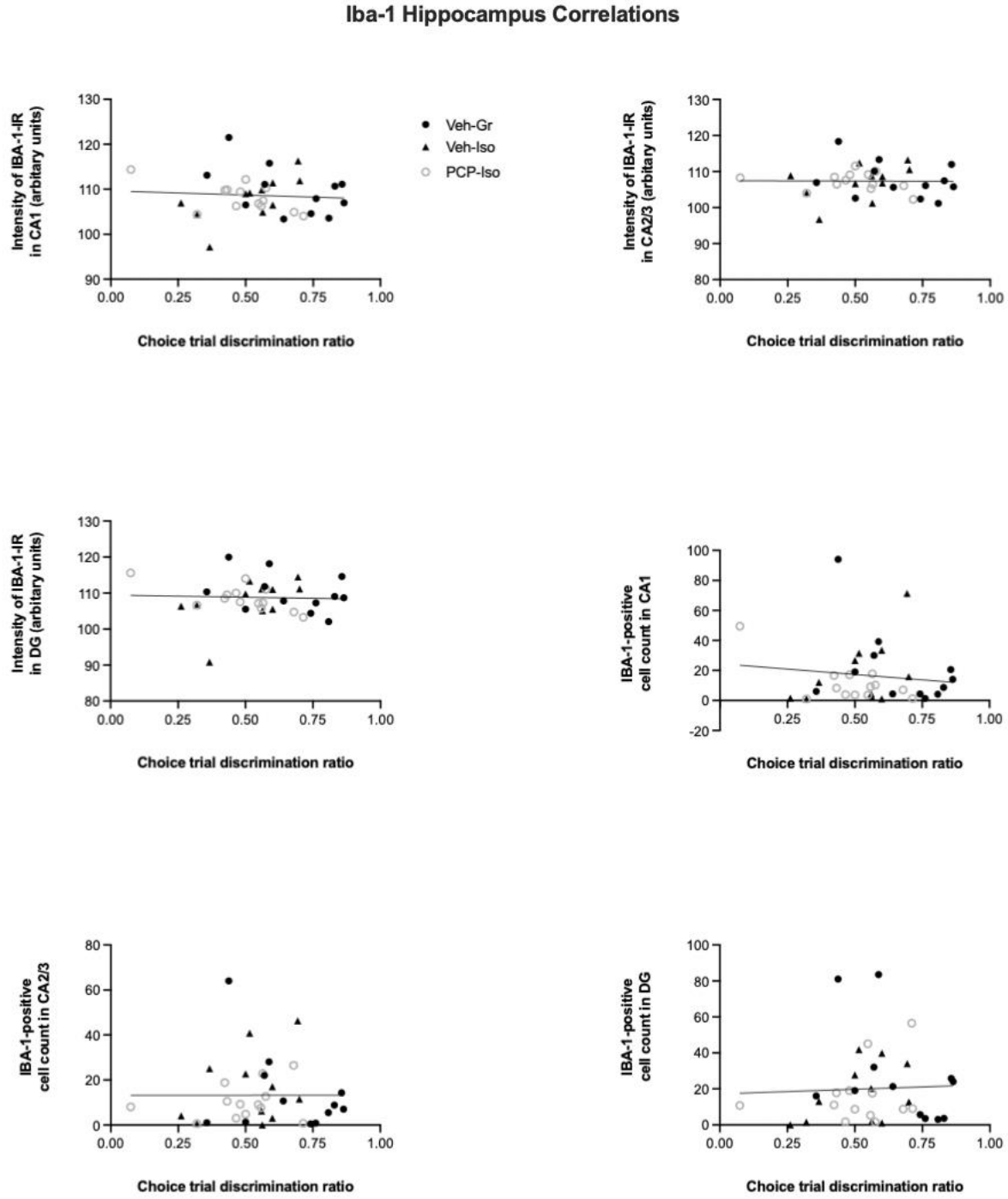
Appendix Figure 4. Correlation analysis of somatostatin and novel object discrimination (NOD) ratios in the frontal cortex. Male Lister hooded rats that received saline (1 mL/kg s.c.; Veh) or PCP (10 mg/kg) on PND 7, 9, and 11 were housed in groups (Gr) or isolation (Iso) from weaning on PND 21, then underwent NOD three separate times at one-to-two-week intervals (PND 57-80) following acute i.p. administration of 0.5% methylcellulose 1% Tween-80 vehicle (1mL/kg), SB-399885 (10mg/kg) or MMPIP (10mg/kg) on separate days before frontal cortical and hippocampal tissue collection on PND 79-80 (n = 12-14 per neurodevelopmental condition). Abbreviations: IR, immunoreactivity; MO/VO, the medial/ventral orbitofrontal; LO/DLO, lateral/dorsolateral orbitofrontal; PrL/IL, prelimbic/infralimbic; CA, cornu ammonis; DG, dentate gyrus.



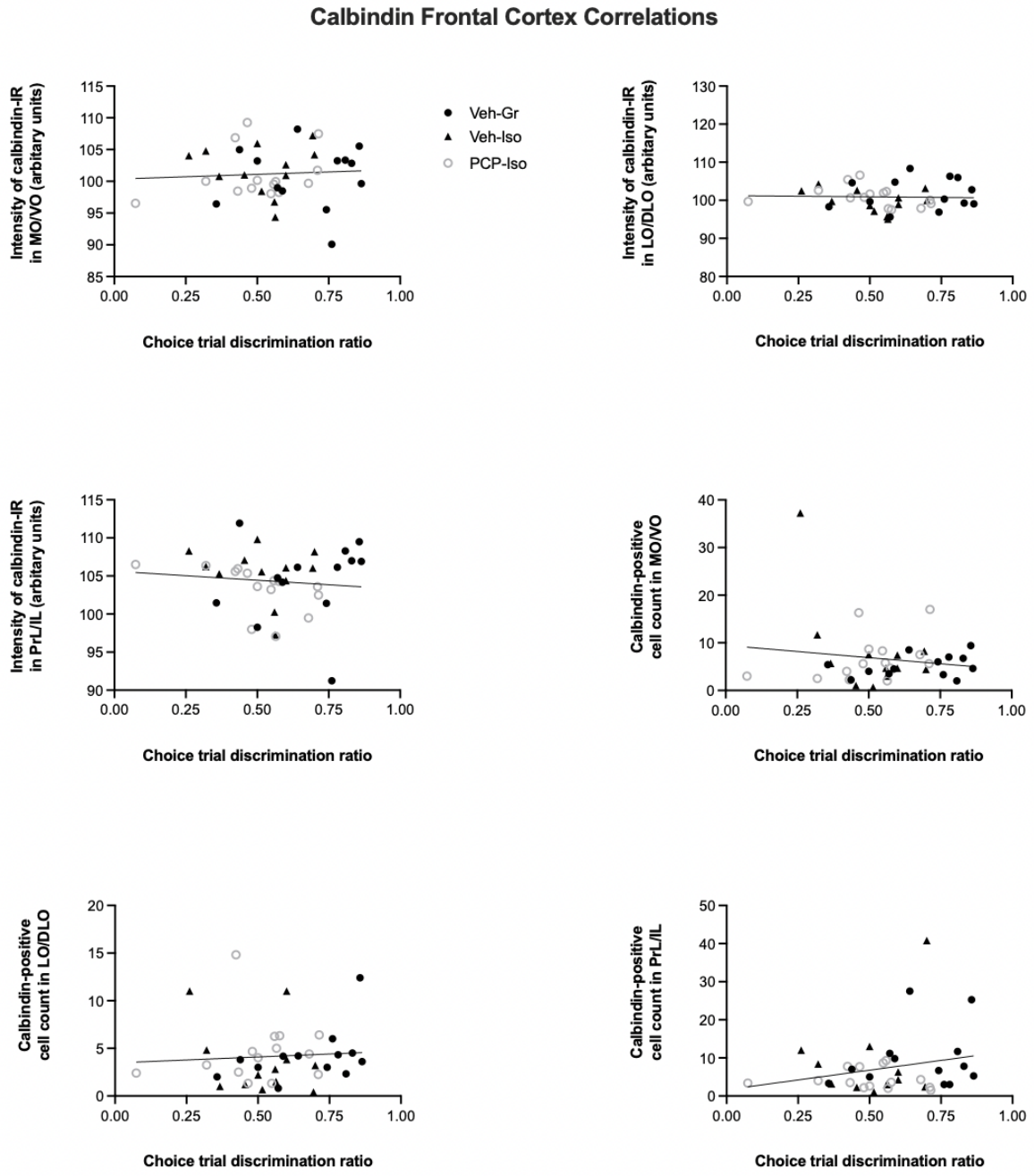
Appendix Figure 2. Correlation analysis of somatostatin and novel object discrimination (NOD) ratios in the hippocampus. Male Lister hooded rats that received saline (1 mL/kg s.c.; Veh) or PCP (10 mg/kg) on PND 7, 9, and 11 were housed in groups (Gr) or isolation (Iso) from weaning on PND 21, then underwent NOD three separate times at one-to-two-week intervals (PND 57-80) following acute i.p. administration of 0.5% methylcellulose 1% Tween-80 vehicle (1mL/kg), SB-399885 (10mg/kg) or MMPIP (10mg/kg) on separate days before frontal cortical and hippocampal tissue collection on PND 79-80 (n = 12-14 per neurodevelopmental condition). Abbreviations: IR, immunoreactivity; CA, cornu ammonis; DG, dentate gyrus.



Appendix Figure 6. Correlation analysis of Iba-1 and novel object discrimination (NOD) ratios in the frontal cortex. Male Lister hooded rats that received saline (1 mL/kg s.c.; Veh) or PCP (10 mg/kg) on PND 7, 9, and 11 were housed in groups (Gr) or isolation (Iso) from weaning on PND 21, then underwent NOD three separate times at one-to-two-week intervals (PND 57-80) following acute i.p. administration of 0.5% methylcellulose 1% Tween-80 vehicle (1mL/kg), SB-399885 (10mg/kg) or MMPIP (10mg/kg) on separate days before frontal cortical and hippocampal tissue collection on PND 79-80 (n = 12-14 per neurodevelopmental condition). Abbreviations: IR, immunoreactivity; MO/VO, the medial/ventral orbitofrontal; LO/DLO, lateral/dorsolateral orbitofrontal; PrL/IL, prelimbic/infralimbic.



Appendix Figure 7. Correlation analysis of Iba-1 and novel object discrimination (NOD) ratios in the hippocampus. Male Lister hooded rats that received saline (1 mL/kg s.c.; Veh) or PCP (10 mg/kg) on PND 7, 9, and 11 were housed in groups (Gr) or isolation (Iso) from weaning on PND 21, then underwent NOD three separate times at one-to-two-week intervals (PND 57-80) following acute i.p. administration of 0.5% methylcellulose 1% Tween-80 vehicle (1mL/kg), SB-399885 (10mg/kg) or MMPIP (10mg/kg) on separate days before frontal cortical and hippocampal tissue collection on PND 79-80 (n = 12-14 per neurodevelopmental condition). Abbreviations: CA, cornu ammonis; DG, dentate gyrus.



Appendix Figure 8. Correlation analysis of calbindin and novel object discrimination (NOD) ratios in the frontal cortex. Male Lister hooded rats that received saline (1 mL/kg s.c.; Veh) or PCP (10 mg/kg) on PND 7, 9, and 11 were housed in groups (Gr) or isolation (Iso) from weaning on PND 21, then underwent NOD three separate times at one-to-two-week intervals (PND 57-80) following acute i.p. administration of 0.5% methylcellulose 1% Tween-80 vehicle (1mL/kg), SB-399885 (10mg/kg) or MMPIP (10mg/kg) on separate days before frontal cortical and hippocampal tissue collection on PND 79-80 (n = 12-14 per neurodevelopmental condition). Abbreviations: MO/VO, the medial/ventral orbitofrontal; LO/DLO, lateral/dorsolateral orbitofrontal; PrL/IL, prelimbic/infralimbic.

Appendix III – Abbreviations

5HT – Serotonin

5-HT_6 – Serotonergic receptor

AC – Anterior cingulate

AMPA –α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

Ampakines – (AMPA)-receptor-positive modulators

APD – Antipsychotic drugs

BFA – Brefeldin-A

CA – Cornu ammonis

CB – Calbindin

CNS – Central nervous system

CR – Creatine

CSO – Centrum semiovale

D_2 – Dopamine

DG – Dentate gyrus

Dysbindin – Dystrobrevin-binding-protein-1

ELISA – Enzyme-linked immunosorbent assay

GABA – Gamma-aminobutyric acid

GAD_{67} – Glutamate decarboxylase 67

GlyT-1 – Glycine Transporter 1

GWAS – Genome-wide association studies

HC – Healthy controls

Iba-1 – Ionized calcium binding adaptor molecule 1

IHC – Immunohistochemistry

IHME – Institute of Health Metrics and Evaluation

i.p –Intraperitoneal

IR – Immunoreactivity

Kv3 – Potassium voltage gated ion channels

IL6 – Interleukin 6

ILβ – Interleukin beta

LO/DLO – Lateral/dorsolateral orbitofrontal

Med – At least one subject on medication for schizophrenia

MO/VO – Medial/ventral orbitofrontal

MRS – Magnetic resonance spectroscopy

mGluR2/3 – Group II metabotropic glutamate receptor

ND – No significant difference or not determined

NMDAR – N-methyl-D-aspartate receptors

NOD – Novel object discrimination

PCP – Phencyclidine

PET – Positron emission tomography

PIC – Polyinosinic:polycytidylic acid

PFC – Prefrontal cortex

PND – Post-natal day

POC – Parieto-occipital cortex

PrL/IL – Prelimbic/infralimbic corices

PV – Parvalbumin

ROI – Region of interest

s.c. – Subcutaneous

SST – Somatostatin

SZ – Schizophrenia patients

TNF-α – Tumour necrosis factor alpha

TTX – Tetrodotoxin

VIP – Vasoactive intestinal polypeptide