Mechanisms involved in the reorganization of prefrontal cortical circuits following neonatal lesion of the ventral hippocampus– relevance to schizophrenia.

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LIST OF ABBREVIATIONS

8-oxo-dG	8-Oxo-2'-deoxyguanosine
AAV8	Adeno-associated virus 8
ASR	Acoustic startle response
ASST	Attention set-shifting test
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
BrdU	Bromodeoxyuridine
C1q	Complement component 1q
C3	Complement component 3
C4	Complement component 4
CA	Cornu Ammonis
CaMKII	Calcium/calmodulin-dependent protein kinase II
CD	Compound discrimination
CD11b	Cluster of differentiation 11b
CD34	Cluster of differentiation 34
CD45	Cluster of differentiation 45
CD68	Cluster of differentiation 68
CDR	Compound discrimination reversal
CNS	Central nervous system
Cox2	Cyclooxygenase2
CSF	Cerebrospinal fluid
Cx3CR1	Fractalkine or CX3C chemokine receptor 1
DAB	Diaminobenzidine
dB (A)	decibels (A)
DG	Dentate gyrus
DH	Dorsal hippocampus
DISC1	Disrupted-in-schizophrenia 1
DLPFC	Dorsolateral prefrontal cortex

DRD1	Dopamine receptor D1
dTA	Diphtheria toxin
DTNBP1	Dysbindin-1
EDR	Extra-dimensional shift reversal
EDS	Extra-dimensional shift
EDTA	Ethylenediaminetetraacetic acid
EGR3	Early growth response gene 3
EPM	Elevated plus-maze
fMRI	Functional magnetic resonance imaging
GABA	Gamma-aminobutyric acid
GAD67	Glutamic acid decarboxylase67
GSH	Glutathione
GWAS	Genome wide association study
H2O2	Hydrogen peroxisde
HLA-DR	Human leukocyte antigen DR
HPC	Hippocampus
Hz	Hertz
IBA1	Ionized calcium binding adaptor molecule 1
ICAM1	Intercellular adhesion molecule-1
IDR	Intra-dimensional shift reversal
IDS	Intra-dimensional shift
IL	Infralimbic
IL-10	Interleukin-10
IL-13	Interleukin-13
IL-1RA	Interleukin-1 receptor antagonist
IL-1β	Interleukin- 1β
IL-6	Interleukin-6
iNOS	Nitric oxide synthase
LPS	Lipopolysaccharide
LTD	Long-term depression

LTP	Long-term potentiation
MIA	Maternal immune activation
miRNA	MicroRNA
mPFC	medial prefrontal cortex
NABH4	Sodium borohydride
NAC	N-acetyl cysteine
NAc	Nucleus accumbens
NADPH	Nicotinamide adenine dinucleotide phosphate
NDT	neonatal diphtheria toxin
NMDAR	N-methyl-D-aspartate receptor
Nrf2	Nuclear factor-erythroid 2-related factor 2
Nrf-2	nuclear factor-erythroid 2-related factor 2
NRG1	Neuregulin1
nVH	Neonatal ventral hippocampus
NVHL	Neonatal ventral hippocampus lesion
OFC	Orbitofrontal cortex
OGG1	8-Oxoguanine DNA Glycosylase
PBS	Phosphate-buffered saline
PCP	Phencyclidine
PD or P	Postnatal day
PET	Positron emission tomography
PFA	Paraformaldehyde
PFC	Prefrontal cortex
Poly I:C	Polyinosinic:polycytidylic acid
PP	Prepulses
PPI	Prepulse inhibition
PrL	Prelimbic
p-Smad-2/3	Phospho-Smad-2/3
PV	Parvalbumin
qRT PCR	Quantitative real-time polymerase chain reaction

ROD	Relative optical density
ROS	Reactive oxygen species
S1	Stranger 1
S2	Stranger 2
SD	Simple discrimination
SEM	Standard error of the mean
SI	Social interaction
Sod1	Superoxide Dismutase 1
Sod2	Superoxide Dismutase 2
SSC	Somatosensory cortex
TB	Tris buffer
TGF-β1	Transforming growth factor-β1
TNF-α	Tumor necrosis factor-α
Trem2	Triggering Receptor Expressed On Myeloid Cells 2
TRIS	2-Amino-2-(hydroxymethyl)propane-1,3-diol
t-Smad 2/3	Total-Smad 2/3
VH	Ventral hippocampus
α-1AR	α-1 adrenergic receptor

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ABSTRACT

Early developmental insults during the perinatal period have been known to cause neurodevelopmental disorders such as schizophrenia. A crucial role of developing ventral hippocampus (VH) on schizophrenia-related adult behavioural and cellular deficit has been demonstrated using neonatal ventral hippocampus lesion (NVHL) model. In this model ibotenic acid induced excitotoxic lesion of the VH during the first postnatal week leads to post-pubertal emergence of a range of schizophrenia- related cognitive, social and sensorimotor deficits in the NVHL animals. The behavioural deficits were also associated with altered synaptic transmission and plasticity in the medial prefrontal cortex (mPFC) of adult NVHL animals. Neuroinflammation in the prefrontal cortex has been implicated in schizophrenia and has also been suggested to mediate some of the behavioural and cellular deficits observed as a result of NVHL. In my thesis, I hypothesised neuroinflammation as a main cause of NVHL-induced cellular and behavioural aberrations and explored different cellular and molecular underpinnings contributing to neuroinflammation. We used NVHL model for the first part of our study, measured schizophrenia-related behaviours, neuroinflammation-related cellular and molecular components such as microglia, oxidative stress, pro- and anti- inflammatory factors and microRNAs (miRNAs) in the mPFC during adulthood. In the first series of experiments we revealed a development specific alteration in microglial function and phagocytic activity, increased expression of mRNA levels of complement molecules (C3 and C1q) and reduced anti-oxidative stress indicators in the mPFC of NVHL animals. Interestingly, neonatal suppression of microglial phagocytic activity by minocyline attenuated adult NVHL induced behaviours such as hyperlocomotion, deficits in prepulse inhibition (PPI) of startle, anxiety-like behaviour and memory. Our second set of experiments using adult NVHL animals revealed an increased level of pro- inflammatory (IL-1β) and reduced level of anti-inflammatory (TGF-β1) cytokines in the mPFC. Notably, neonatal supplementation of recombinant TGF-β1 prevented the appearance of adult behaviours such as hyperlocomotion, deficits in PPI of startle, social interaction (SI) and spine loss in the mPFC of NVHL animals. Further, our third set of experiments revealed deficit in cognitive flexibility and cellular alterations such as increased expression of miRNAs (miR-134 and miR-137) and oxidative stress marker (8-oxo-dG) in the mPFC of NVHL animals. Since NVHL model uses the indiscriminate excitotoxicity of the ibotenic acid to lesion the VH, the specific role of the neonatal VH neuronal populations on adult behavioral and cellular deficits

cannot be inferred from this model. In order to address this limitation, we used a viral ablation method to ablate the excitatory neurons in the neonatal VH. In this method, an adeno-associated viral 8 (AAV8) vector expressing diphtheria toxin A (dTA) or a control virus without dTA was microinfused bilaterally in the VH of C57Bl6/J CaMKII-cre mice at neonatal age P12. Ablating the neonatal VH excitatory neurons using the viral based method altered some aspects of adult social and cognitive behaviors in the dTA virus-injected group. We also found altered expression of genes involved in presynaptic function (synaptophysin) and synaptic pruning (complement C4 & C1q) in the mPFC of adult dTA virus-injected group. Our data shows that an impairment in the development of the VH leads to increase of inflammation and oxidative stress in the mPFC thereby affecting schizophrenia-related behavioral and cellular functions. Collectively, our results call for further examination of regulators of neuroinflammation and their mechanism for better understanding of schizophrenia pathophysiology and development of novel drug targets.

RÉSUMÉ

Des altérations développementales tôt au cours la période périnatale sont reconnues de mener au développement de troubles de neurodéveloppementaux tel que la schizophrénie. Un rôle crucial de l'hippocampe ventral (HV) en développement de changements comportementaux et cellulaires chez l'adulte associés à la schizophrénie a été démontré en utilisant un modèle de lésion néonatale de l'hippocampe ventral (LNHV). Dans ce modèle lésion de l'HV par excitotoxicité induite par l'acide iboténique pendant la première semaine postnatale conduit à l'émergence post-pubère d'une série de déficits cognitifs, sociaux et sensorimoteurs liés à la schizophrénie chez les animaux LNHV. Les déficits comportementaux étaient également associés à une transmission synaptique et à une plasticité synaptique altérées dans le cortex préfrontal médian (PF) d'animaux adultes atteints de LNHV. La neuroinflammation du cortex préfrontal a été impliqué dans la schizophrénie et a aussi été suggéré comme impliqué indirectement dans certains des comportements et des déficits cellulaires observés après une LNHV. Dans ma thèse, j'ai émis l'hypothèse que la neuroinflammation était la principale cause des aberrations cellulaires et comportementales induites par une LNHV et ai exploré différents processus cellulaires et moléculaires contribuant à la neuroinflammation. Au cours de la première série d'expériences, nous avons utilisé le modèle LNHV et identifié le développement d'altérations spécifiques de la fonction microgliale et de l'activité phagocytaire, une augmentation de l'expression des niveau d'ARNm des molécules du complément et une réduction de certains indicateurs de stress antioxydant du PF des animaux ayant subis une LNHV. La suppression néonatale des activités phagocytaires des microglies par traitement à la minocyline atténue les comportements adultes induits par une LNHV, dont l'hyperactivité motrice, les altérations de l'inhibition (PPI) du réflexe de sursaut, des comportements liés à l'anxiété et des déficits de mémoire. Notre second volet d'expériences utilisant des animaux adultes soumis à une LNHV a identifié une augmentation du niveau de cytokines pro-inflammatoire (IL-1β) et une réduction de cytokines anti-inflammatoire (TGF-β1) du PF. Notamment, la supplémentation du recombinant TGF-β1 empêche l'apparition de certains comportements chez l'adulte et de la perte de l'épine dendritique du PF des animaux ayant subis une LNHV. De plus, notre troisième volet d'expériences a souligné une perte de la flexibilité cognitive ainsi que des altérations cellulaires dont l'augmentation de l'expression de microARNs (miARNs) et de marqueurs de stress oxydant du PF des animaux ayant subi une LNHV. Afin d'évaluer le rôle spécifique des

neurones de l'HV néonatal dans les changements comportementaux et cellulaires nous avons utilisé une méthode virale. Dans cette méthode, un vecteur viral adéno-associé 8 exprimant la toxine diphtérique A (dTA) ou un virus témoin sans dTA a été micro-injecté de manière bilatérale dans le VH de souris C57Bl6 / J CaMKII-cre au jour néonatal P12. L'ablation des neurones excitateurs de l'HV néonatal à l'aide de la méthode virale a modifié certains aspects des comportements sociaux et cognitifs chez l'adulte dans le groupe recevant le virus de la dTA. Nous avons également constaté une altération de l'expression des gènes impliqués dans la fonction présynaptique et dans l'élagage synaptique du PF du groupe adulte infecté par le virus dTA. Nos données montrent qu'une altération du développement de l'HV entraîne une augmentation de l'inflammation et du stress oxydant dans la PF, affectant ainsi les fonctions comportementales et cellulaires liées à la schizophrénie. Dans l'ensemble, nos résultats mettent de l'avant le besoin d'un examen plus approfondi des régulateurs de la neuroinflammation et de leurs mécanismes pour mieux comprendre la physiopathologie de la schizophrénie et permettre le développement de nouvelles cibles médicamenteuses.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

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Our lab and others have been using a neurodevelopmental model of schizophrenia to understand the effect of early life insults on schizophrenia-related behavioural and cellular deficits. Recent studies provide evidence for a crucial role of neuroinflammation contributing to schizophrenia pathophysiology. My thesis identified different neuromodulators and miRNAs that may cause impairment in the maturation of the PFC as a result of NVHL (Chapter II, III and IV). Further we developed a new model using viral based method to ablate VH excitatory neurons in the neonates and look at its effect on adult schizophrenia-related behavioral and cellular functions (Chapter VI).

CONTRIBUTION OF AUTHORS

Chapter I

Antoneta Teresa Joseph wrote the general introduction and Dr. Lalit Srivastava helped in editing.

Chapter II

Dr. Chin Wai Hui performed microglia morphology and function-related experiments, data analysis and wrote part of the manuscript. Dr. Sanjeev Bhardwaj generated the sham and lesion animals, performed behavioural experiments and helped with manuscript writing. Antoneta Teresa Joseph performed experiments measuring complement molecules and other inflammatory factors, data analysis and wrote part of the manuscript relevant to complement molecules and inflammation. Kaushik Sharma, Kanchan Bisht and Katherine Picard helped Dr. Hui in performing experiments. Dr. Marie-Ève Tremblay and Dr. Lalit Srivastava contributed to the development of research idea, designed the experiments and helped with manuscript writing.

Chapter III

Antoneta Teresa Joseph conceived, designed and performed the experiments and wrote the first draft of the manuscript. Dr. Sanjeev Bhardwaj helped in performing behavioural experiments and manuscript writing. Dr. Lalit Srivastava contributed to the development of research idea and implementation, discussion of the results and part of the manuscript writing.

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Chapter VI

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CHAPTER I

GENERAL INTRODUCTION

Schizophrenia is a complex debilitating disorder with a lifetime risk of around 1% (Coyle, Balu, Benneyworth, Basu, & Roseman, 2010). It is one of the leading causes of disability in Canada and has a huge impact on global economy (Knapp, Mangalore, & Simon, 2004; Lora et al., 2012). Symptoms most commonly associated with schizophrenia are positive symptoms including hallucinations and delusions, negative symptoms including deficits in motivation, volition and cognitive impairments in different domains of memory (working memory and episodic memory), attention and executive function (van Os, Linscott, Myin-Germeys, Delespaul, & Krabbendam, 2009; Leung & Chue, 2000). A diagnosis of schizophrenia is based on the criteria laid down in the Diagnostic and Statistical Manual of Mental Disorders (DSM). According to the most recent fifth edition of DSM (DSMV) a patient is required to have at least one of the following symptoms: delusions, hallucinations, or disorganized speech coupled with social or occupational dysfunction for at least six months (5th ed.; DSM-5; American Psychiatric Association). Schizophrenia symptoms emerge normally during late adolescence or in early adulthood, although cognitive deficits precede the illness, are progressive and persist throughout the course of the disorder (Sorensen, Mortensen, Parnas, & Mednick, 2006). The late incidence of schizophrenia can be explained by the protracted maturation of brain circuits implicated in this disease, particularly during adolescence (Quinlan et al., 2018). The pathophysiology of schizophrenia is complex and involves a combined effect of genetic and environmental factors which increases the susceptibility of the disease (O'Donnell, 2011; Giovanoli et al., 2013). Poor and incomplete understanding of the pathophysiology of schizophrenia has significantly affected the development of optimal and efficacious antipsychotic drugs (Schizophrenia Working Group of the Psychiatric Genomics, 2014). Thus, one of the important steps to improve therapeutic treatments is to identify critical mechanisms contributing towards the etiology and the development of the disease (Marcotte, Pearson, & Srivastava, 2001; Lipska, 2002).

Prefrontal cortex (PFC) and Hippocampus (HPC) in schizophrenia

The brain regions associated with cognitive deficits in schizophrenia are believed to be medial temporal lobes, HPC and the PFC (Goldberg & Weinberger, 1996; Minzenberg, Laird, Thelen, Carter, & Glahn, 2009; Reichenberg & Harvey, 2007; Lesh, Niendam, Minzenberg, & Carter, 2011). Genetic evidence linking the above-mentioned brain regions to the schizophrenia

pathology have been shown in a schizophrenia genome wide association study (GWAS) (Schizophrenia Working Group of the Psychiatric Genomics, 2014). This study identified 108 genetic risk loci, mapped these variants onto sequences from different tissue samples and found increased association of these genetic variants of schizophrenia in the cortical and hippocampal brain regions. PFC is a prime brain region regulating cognitive behavioural outcomes such as executive function, working memory, behavioural flexibility and goal directed behaviour (Sakurai et al., 2015). Deficits in these PFC related behavioural outcomes have been observed consistently among schizophrenia patients. Neuroimaging studies have shown structural and functional changes in the PFC of schizophrenia patients (Lewis & Gonzalez-Burgos, 2008; Voets et al., 2008); Winterer et al., 2006). In-vivo imaging study using positron emission tomography (PET), reported reduced amphetamine-induced dopamine release in the DLPFC of schizophrenia patients compared to matched healthy controls (Slifstein et al., 2015). Functional magnetic resonance imaging (fMRI), revealed increased cortical activation and a dysregulation of information processing in the PFC among the schizophrenia patients (Winterer et al., 2006). Structural changes reported by voxel-based morphometry include a reduction of grey matter in the PFC among schizophrenia patients compared to healthy matched controls (Voets et al., 2008). Post-mortem studies in schizophrenia by (Glantz & Lewis, 2000) have provided additional support for this hypothesis by showing decreased dendritic spine density in the layer 3 pyramidal neurons of the dorso-lateral prefrontal cortex (DLPFC) in patients with schizophrenia. Apart from the pyramidal neurons, synaptic inhibition mediated by cortical γ-aminobutyric acid (GABA) neurons is also essential for maintaining cortical activity (Glantz & Lewis, 2000). Reduced expression of GABA-synthesizing enzyme glutamic acid decarboxylase 67 (GAD67) mRNA has been observed in the DLPFC parvalbumin (PV) interneuron in schizophrenia post mortem samples (Akbarian & Huang, 2006). In addition to this, a reduced expression of GAD67 is also observed in other inhibitory interneurons such as cholecystokinin and calretinin within the PFC. Several lines of evidence suggest that the deficit in GAD67 mRNA expression may represent a primary factor in GABA neuron dysfunction in schizophrenia (Gonzalez-Burgos, Fish, & Lewis, 2011). Consistent with GABAergic reduction in PV neurons, PFC-related gamma activity was shown to be reduced during cognitive task performance in schizophrenia patient group as compared to the control (Cho, Konecky, & Carter, 2006).

Cognitive control can be guided through neural pathways within the PFC and other brain structures (E. K. Miller & Cohen, 2001). One such brain structure that has found a lot of attention in schizophrenia pathology is the HPC. The HPC can be divided into five regions: the cornu ammonis (CA) including CA1–4 and the dentate gyrus (DG). Based on the dorsal to ventral axis, the curved hippocampus can be divided into dorsal hippocampus (DH) and VH in rodents corresponding to a posterior to anterior axis in primates and humans (Strange, Witter, Lein, & Moser, 2014). Anterior hippocampus is a region that has been consistently implicated in human schizophrenia. The DH is predominantly involved in spatial learning and memory whereas the VH modulates executive control, emotion and goal-directed behaviors (Fanselow & Dong, 2010; Howland, Harrison, Hannesson, & Phillips, 2008). Theta oscillations in the HPC act in co-ordination with the gamma oscillations (30–100 Hz) in the PFC to control working memory function (Lisman et al., 2008; Pastalkova, Itskov, Amarasingham, & Buzsaki, 2008). In rodents, these behaviors are controlled by the VH through excitatory monosynaptic glutamatergic projections from the ventral CA1/subiculum to the pre- and infralimbic regions of mPFC, and to other limbic regions such as the nucleus accumbens (NAc) and amygdala (Carr & Sesack, 1996; Brockmann, Poschel, Cichon, & Hanganu-Opatz, 2011). This HPC-PFC connectivity facilitates the co-ordination of the brain activity to mediate cognitive function (Thierry, Gioanni, Degenetais, & Glowinski, 2000).

A number of neuroimaging studies and meta-analyses have demonstrated consistent volume reduction and change in the shape of the hippocampi of schizophrenia patients (Csernansky et al., 2002; Vita, De Peri, Silenzi, & Dieci, 2006). Some evidence showed that the volume of the anterior but not the posterior hippocampus is smaller in schizophrenia has also been established (Goldman et al., 2007; L. Wang, Joshi, Miller, & Csernansky, 2001). This volume reduction has been associated with gray matter reduction and implicated in both alteration of functional connectivity and cognitive processing (Shah et al., 2018). The first episode psychosis patient shows a reduced hippocampal volume as compared to the controls, but the time point at which the reduction starts is still unclear (Steen, Mull, McClure, Hamer, & Lieberman, 2006; Vita et al., 2006).

Hippocampal volume reduction has been found to be associated with CA1 hyperactivity and schizophrenia-related behavioural deficits (Grimm et al., 2018; Lander et al., 2019). Reduced

expression of glutamate-metabolizing enzyme was correlated with increased pyramidal neuronal excitability within the CA1 which has been suggested to alter synaptic function and lead to hippocampal volume loss (Lander et al., 2019). Together with this, an imbalance in the GABAergic neurotransmission has also been implicated in schizophrenia. For example, GABAergic interneuron marker (PV) is shown to be reduced in all hippocampus regions (Hashimoto et al., 2003; Zhang & Reynolds, 2002). Further, abnormal activation within the HPC has been found to alter HPC-DLPFC functional connectivity during working memory task in the schizophrenia patients as compared to the healthy controls (Meyer-Lindenberg et al., 2005).

Neurodevelopmental hypothesis of Schizophrenia

The neurodevelopmental theory of schizophrenia states that genetic factors or early life adversities can affect perinatal brain development, resulting in brain dysfunction later in life that leads to a predisposition for developing schizophrenia (Weinberger, 1987; Welham, Isohanni, Jones, & McGrath, 2009). Thus, maternal stress, malnutrition, infection or immune activation, or obstetric complications (such as hypoxia) during birth are some of the factors that increase the risk of developing schizophrenia (Lewis & Levitt, 2002). Support for the neurodevelopmental hypothesis in explaining the etiology of schizophrenia has been provided by the age of onset of schizophrenia, which is during late adolescence or early adulthood. This age of onset corresponds to the phase of brain development at which the PFC is attaining maturity by undergoing synaptic pruning and interconnection between the neurons. This process also involves a decrease in cortical gray matter and presentation of cognitive deficits (Tseng, Chambers, & Lipska, 2009). Longitudinal studies on early onset of schizophrenia reported gray matter loss localized to prefrontal and temporal cortices during adolescent years (Greenstein et al., 2006). Additionally, cortical loss of grey matter has also been associated with working memory dysfunction among adolescent and adult schizophrenia patients (Douaud et al., 2007).

A number of schizophrenia risk genes including DRD2, GRIN2A, DISC1, RGS4, GRM3, and CACNA1C have been linked to predisposing the brain to developmental abnormalities leading to adult onset behavioral deficits (Harrison & Weinberger, 2005; Schizophrenia Working Group of the Psychiatric Genomics, 2014). These genes have critical role in the processes involved in the development of the brain including cell migration, axonal outgrowth, synaptogenesis, cell

proliferation, and myelination (Jones, Watson, & Fone, 2011). In rodents, recent data suggest that peak spine density in the PFC is present at postnatal day (PD31), decreasing thereafter until early adulthood (PD60) (Gourley, Olevska, Warren, Taylor, & Koleske, 2012). This indicates a role of synaptic pruning and the balance of excitation and inhibition in the maturation of PFC circuitry and its significance in neurodevelopmental disorders such as schizophrenia.

Animal models of schizophrenia.

A major challenge in the development of convincing animal models is modeling the symptomology of psychiatric disorders that are often unique to humans. Correlating the behavioural deficits seen in an animal model to add up to a recognized human psychiatric disorder and using it to develop treatment is also another challenge. These are the key reasons for lack of advancements in drug efficacies in psychiatric disorder like schizophrenia (Winship et al., 2019). In the field of psychiatric disorders, although a model would not be able to recapitulate the entire features of a disorder, an effective animal model would provide an insight into construct, face and predictive validities. Construct validity focuses on developing animal models which try to mimic known genetic and/or environmental factors. Face validity in an animal model is determined by behavioural symptoms or phenotypes based on the shared neurobiological mechanisms. Predicting the efficacy of a treatment and validating novel drugs are some of the factors by which predictive validity of an animal model is determined (Nestler & Hyman, 2010).

Animal models for schizophrenia have been largely categorised into developmental, drug-induced, lesion and genetic based manipulations. These models encompass either one or more of the following features to represent the pathophysiology of schizophrenia: post-pubertal onset of schizophrenia-like behaviors that have rodent correlates such as the prepulse inhibition of startle (PPI). The models also show PFC dysfunction, loss of hippocampal and cortical connectivity, limbic dopamine dysregulation, cortical glutamatergic hypofunction, vulnerability to stress, abnormal response to reward, social withdrawal and cognitive impairment (Jones et al., 2011; Marcotte et al., 2001; Tseng et al., 2009).

Pharmacological animal models of schizophrenia include acute or chronic administration of amphetamine, phencyclidine (PCP) and ketamine to rodents. These pharmacological models have stemmed from the findings which showed that a dysregulation of dopamine (amphetamine model) and glutamate (PCP and ketamine model) systems were implicated in schizophrenia pathology (Angrist, Sathananthan et al. 1974; Javitt & Zukin, 1991). The amphetamine model is based on manipulation of the dopaminergic system, and exhibit psychosis-like behavioral changes and hyperactivity induced by enhancing mesolimbic dopamine function (R. M. Murray, Lappin, & Di Forti, 2008). However, it should be noted that this model does not recapitulate negative or cognitive symptoms of schizophrenia (Jones et al., 2011). The PCP and ketamine models alter the glutamatergic network by blocking the N-methyl-D-aspartate receptors (NMDAR) (Konradi & Heckers, 2003). Both acute and chronic PCP administration causes social withdrawal and impairment of both PPI and cognitive flexibility (Featherstone, Rizos, Kapur, & Fletcher, 2008; Egerton et al., 2008). This model recapitulates some aspects of negative and cognitive symptoms of schizophrenia.

Besides the genes identified in schizophrenia GWAS study, there are also a number of candidate genes and chromosomal loci which were previously identified as conferring predisposition for schizophrenia, e.g., dysbindin-1 (DTNBP1), Disrupted-in-schizophrenia 1 (DISC1), Neuregulin 1 (NRG1) and deletion of chromosome 22q11 (Millar et al., 2000); (Law et al., 2006; Papaleo et al., 2012; Bassett et al., 2003). Several mouse models have been developed based on these genes. Loss of DTNBP1 in a mouse model has been found to alter excitatory synaptic neurotransmission (Karlsgodt et al., 2011) and lead to behavioral deficits like hyperactivity, cognitive deficits and altered response to psychostimulant drugs (Bhardwaj et al., 2009). Reduced hippocampal and PFC dendritic spine density indicating altered neurotransmission has also been observed in DISC1and NRG1knockout animals (Ting et al., 2011; Jaaro-Peled et al., 2009). Some of the common behavioural abnormalities in these two models include hyperactivity, PPI deficit and reduced social interaction (SI) (Harrison & Law, 2006; Fazzari et al., 2010). LgDel/+mouse model of 22q11 Deletion Syndrome reported cortical volume reduction and altered cortical connectivity. These morphological and functional changes were correlated with reduced cognitive function in LgDel/+ mice (Meechan et al., 2015).

Neonatal ventral hippocampus lesion (NVHL) model

NVHL model has been widely used to study the effect of early developmental insults to the ventral hippocampus (VH) on schizophrenia-related adult behavioural deficits (Lipska, 2002; Tseng et al., 2009). In this model, bilateral ibotenic acid-induced excitotoxic lesioning of the VH is carried out at PD 7 rats. PD 7-9 is a critical period marked by peak axonal and dendritic growth in the rat hippocampal development (Minkwitz, 1976). The first postnatal week also corresponds to human fetal brain at late third trimester (Dobbing & Sands, 1979), when putative developmental insults are postulated to occur in schizophrenia. The VH sends monosynaptic excitatory projections from the ventral CA1/subiculum to excitatory as well as inhibitory neurons of the pre- and infralimbic regions of mPFC and to other limbic regions such as NAc and amygdala (Thierry et al., 2000). It has also been shown that the theta-gamma coupled oscillations drive the communication between VH and PFC and facilitate the VH-PFC connectivity as early as the first postnatal week (Brockmann et al., 2011). Early developmental disruption of the VH-PFC connectivity in the NVHL animals affected cognition, SI, motivation, locomotor activity and sensori-motor gating during adulthood (Tseng et al., 2008; Lipska et al., 1995). The schizophrenia-like behaviours in the NVHL animals emerge only after puberty (Lipska, 2002), whereas the prodromal cognitive deficits emerge before puberty and continues into adulthood (Marquis, Goulet, & Dore, 2008). Cognitive deficits are some of the important aspect of schizophrenia and may hold key understanding of the pathophysiology of this disorder.

A number of histological, cellular, neurochemical and electrophysiological abnormalities have been observed in the PFC of NVHL animals. Cellular changes within PFC included decreased dendritic length and spine density of layer 3 pyramidal neurons (Flores, 2005). An increase of α-1 adrenergic receptor (α-1AR) levels in PFC, which impairs α-1AR mediated behavioral response has been observed in this model (Bhardwaj, Quirion, & Srivastava, 2004). NVHL animals, in line with the dopamine and glutamate hypothesis of schizophrenia, have been shown to exhibit altered DA/ glutamate interaction in the mPFC pyramidal neurons that show increased firing response following stimulation of the ventral tegmental area (O'Donnell, Lewis, Weinberger, & Lipska, 2002; Tseng & O'Donnell, 2007).

NVHL rats have been reported to display schizophrenia-like behaviours including locomotor hyper-responsiveness to psychostimulants and DA agonists (Chambers & Taylor, 2004; Flores,

Barbeau, Quirion, & Srivastava, 1996; Lipska et al., 1995). The lesioned animals also show defects in PV interneuron maturation during adolescence, which could be one of the key factors leading to improper development of PFC (Tseng & O'Donnell, 2007). Adult NVHL rats exhibit enhanced sensitivity to NMDAR antagonists (Al-Amin, Shannon Weickert, Weinberger, & Lipska, 2001) and deficits in sensorimotor gating measured by PPI (Lipska et al., 1995). PPI is modulated by the cortico-striatal-pallido-thalamic circuitry involving the PFC, HPC and a number of other related brain regions (Swerdlow, Geyer, & Braff, 2001). Individuals with schizophrenia, on average, show reduced PPI compared to healthy individuals (Braff, Grillon, & Geyer, 1992; Meincke et al., 2004). It has also been suggested that a reduced PPI results in a cognitive overload which is represented by a number of cognitive deficits (Kumari et al., 2008).

As discussed in the above section, cognitive impairments are some of the core behavioral deficits observed in schizophrenia. The NVHL animals demonstrate several cognitive deficits which include impairments in behavioural flexibility and working memory (Gruber et al., 2010; Lipska, 2002; Marquis et al., 2008). Interestingly, cognitive flexibility was reported to improve in NVHL rats treated with a metabotropic glutamate receptor agonist (Gruber et al., 2010), suggesting excessive glutamatergic transmission may be partly responsible for cognitive deficits in these animals. The working memory impairments that are observed in these animals are not seen in animals that receive equivalent VH lesions in adulthood (Lipska & Weinberger, 2002). NVHL animals also show impairment in executive function, which are high order cognitive processes involved in the maintenance and control of behavior. It is also very interesting to note that out of the two components of putative rodent executive functions, namely set shifting and reversal learning, only set shifting which is primarily an mPFC driven, is impaired in NVHL animals (Placek, Dippel, Jones, & Brady, 2013).

One of the factors which affects the degree of daily functioning among schizophrenia patients is the inability to process social information. This could be classified as social cognitive impairment and forms a part of negative symptomology of schizophrenia (Fett et al., 2011; Green, Horan, & Lee, 2015). The brain regions involved in processing this behavior include the PFC, striatum and amygdala (van Kerkhof, Damsteegt, Trezza, Voorn, & Vanderschuren, 2013). Studies in NVHL animals have shown SI deficits at both pre- (PD 35) and post- pubertal (PD 65) ages (Vazquez-Roque et al., 2012).

Neuroinflammation in schizophrenia

Neuroinflammation is believed to be a key factor in the development of schizophrenia and its neuropathology (Muller, 2018). Epidemiological studies have implicated a crucial role of influenza infection during pregnancy (third trimester) in the etiology of schizophrenia (Brown & Derkits, 2010; Erlenmeyer-Kimling et al., 1994). Elevated levels of inflammatory cytokines in the maternal serum during early pregnancy (second and third trimesters) have been associated with increased ventricular and decreased cortical volumes in the offspring's (Ellman et al., 2010). Pro-inflammatory cytokines such as interleukin (IL)-1β and IL-6 have been found to be increased in the cerebrospinal fluid (CSF) of schizophrenia patients as compared to the control patients (Muller et al., 2004). Further, prenatal infection has also been found to increase the susceptibility of schizophrenia (Lydholm et al., 2019). Prenatal immune activation in animals using lipopolysaccharide (LPS) or Polyinosinic:polycytidylic acid (poly I:C) has been shown to cause schizophrenia like behavioral deficits in offspring (Reisinger et al., 2015). This maternal immune activation (MIA) model shows an elevation of pro-inflammatory cytokines in offspring starting at adolescence until adulthood (Basta-Kaim et al., 2012). Offspring's from the poly I:C MIA rodent model, exhibit some core schizophrenia-related behavioural deficits including reduced social interaction, PPI, working memory and cognitive function (Reisinger et al., 2015; Knuesel et al., 2014). Both behavioral and cellular deficits observed in the offspring's after maternal infections is attributed to synaptic dysfunction in the hippocampus (Oh-Nishi, Obayashi, Sugihara, Minamimoto, & Suhara, 2010). One of the common factors that links neuroinflammation and synaptic dysfunction is the resident immune cells of the central nervous system, microglia.

Microglia

Microglia are best known to regulate immune response in the central nervous system (Laskaris et al., 2016). Microglia provide a rapid and efficient response for counteracting pathogenic and traumatic injuries (Blank & Prinz, 2013). In addition to its classical role, recent discoveries have identified the role of microglia in brain development and synaptic plasticity (Tay, Savage, Hui, Bisht, & Tremblay, 2017). Microglia actively remodel neuronal circuits in an activity- and experience-dependent manner, through phagocytic elimination (pruning) of axon terminals and

dendritic spines (Hong et al., 2016).This important task is thought to performed through fractalkine, which is highly expressed by neurons, and its only known receptor Cx3cr1, which is expressed in the brain exclusively on microglia (Zhan et al., 2014). Expression of synaptic pruning related complement proteins such as C1q and C4 have been identified at immature spines and have been reported to work synergistically with microglia to regulate synaptic pruning in the PFC (Sekar et al., 2016; Schafer et al., 2012). Alteration in microglia pruning function has been associated with altered circuit development and schizophrenia-related behaviors such as social interaction working and spatial memories in rodents (Kim & Cho, 2017; Lane et al., 2017; Giovanoli et al., 2013). Using PET imaging, activation of microglia was predominantly observed in the frontal cortex and temporal regions among patients with schizophrenia as compared with healthy controls (Marques et al., 2019).

Prenatal immune activation has been reported to render abnormal phenotype to microglia and the post-pubertal behavioral deficit in PFC and hippocampus of the offspring (Giovanoli et al., 2013). An abnormal morphology to microglia which could be characterised by hypertrophy of the cell body and processes and increased cellular proliferation (Levine, Enquist, & Card, 1998). This abnormal morphology has been shown to increase the secretion of pro-inflammatory cytokines like TNF alpha, IL-1β, and IL-6 and oxidative stress markers from microglia (Q. Gao et al., 2010; Giovanoli et al., 2013). An increase in inflammatory factors in the hippocampal microglia has also been observed as a result of LPS injection (Bedoui, Neal, & Gasque, 2018). The pro-inflammatory cytokines have been reported to cause abnormality in cortical dendritic development in prenatal infection model (Gilmore, Fredrik Jarskog, Vadlamudi, & Lauder, 2004). Pro-inflammatory cytokines have been proposed as a potential mechanism influencing glutamatergic, dopaminergic, and serotonergic neurotransmission (Drexhage et al., 2011).

Pro and anti-inflammatory cytokines

Pro and anti-inflammatory cytokines are positive and negative mediators of inflammation respectively (Spulber, Bartfai, & Schultzberg, 2009). An increase of pro-inflammatory cytokines, including interleukin (IL)-1β, IL-6 and TNFα was reported both peripherally and centrally in select brain regions (PFC, amygdala, and striatum) in an animal model of inflammation. IL-1β is a key trigger of neuroinflammation whereas interleukin-1 receptor antagonist (IL-1RA) prevents

neuroinflammation entering into a chronic state. Anti-inflammatory cytokines belonging to the transforming growth factor beta (TGF-β) family as well as IL-10 and IL-1RA suppress inflammation and exert a beneficial or neuroprotective action (Dobolyi, Vincze, Pal, & Lovas, 2012; Adzic et al., 2018). An increase of pro- and decrease of anti-inflammatory factors have been found to have an adverse effect on the development of the brain (C. M. Forrest, Khalil, Pisar, Darlington, & Stone, 2013). For example, conditional deletion of TGF-β signalling within midbrain was found to impair the development of dopamine neurons in a mouse model (Chleilat et al., 2018). Additionally, an increase of pro-inflammatory cytokine IL-1β was reported to affect the brain morphology and function (Spulber et al., 2009). Predominantly IL-1 β, IL-6, TNFα and TGF-β1 are secreted by microglia and have been suggested to play a role in maintaining synaptic homeostasis with in the brain. For example, TGF-β signalling has been found to play a role in promoting quiescent phenotype of microglia and thereby regulating the synaptic pruning function of microglia (Abutbul et al., 2012). Additionally, inhibiting microglial inflammatory response by TGF-β1 has also been found to ameliorate dopamine neuronal loss (Chen, Liu, Cao, Qiu, & Peng, 2017). In addition to its role in regulating synaptic pruning, anti-inflammatory cytokine IL-1RA infusion in the brain was found to ameliorate schizophrenia-related behavioural deficits such as social interaction and cognition (Konsman et al., 2008; Yamato et al., 2014). Additionally, TGF-β signaling has also been known to modulate neuronal activity within CA1 region of hippocampus and thereby regulate schizophrenia-related behaviours such as PPI and hyperactivity (Sun et al., 2010). Interestingly, components of TGF-β signaling are implicated in a number of psychiatric disorders including schizophrenia, bipolar, anxiety and mood disorders (Massague, 2012; Behrens et al., 2007; Lin et al., 2009). Down regulation of TGF-β signaling in the cortex of schizophrenia patients has also been observed previously (Iwamoto & Kato, 2006). Additionally, a polymorphism in TGF-β1 gene has been implicated in schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics, 2014).

Complement proteins

Classical complement proteins are expressed in both neuronal and glia cells and are critical for circuit formation and remodelling during early postnatal brain development (Stevens et al., 2007; Huberman, Feller, & Chapman, 2008). Complement upregulation has been associated with

neuroinflammation and exacerbated phagocytic microglia activity (Wyss-Coray & Mucke, 2002). Recent studies reveal increased expression of complement molecule C4 in post-mortem brain samples from schizophrenia patients (Sekar et al., 2016). C4 has been known to promote microglial phagocytosis through C3 activation and contribute to the excessive removal of synapses (Stevens et al., 2007). Besides C3, complement component (C1q) the initiating protein of the classical complement cascade has been found to modulate cognition by regulating synapse elimination (Stephan, Barres, & Stevens, 2012). Thus, providing evidence towards the association of C1q dependent elimination of the synapse with cognitive decline. Furthermore, C1q has been also found to regulate microglia mediated synaptic pruning via TGF-β signaling pathway (Chu et al., 2010). Inhibition of C1q, C3 or the complement receptor, reduces the number of phagocytic microglia as well as the extent of early synapse loss. This synaptic loss has been associated with abnormal activity within the hippocampus and suggested to be a mediator of excessive pruning (Hong, Dissing-Olesen, & Stevens, 2016). Further evidence for this has been provided by using C1qA knock-out mice, which showed increased excitatory synaptic connectivity in the cortex as a result of C1qA deletion (Chu et al., 2010). Overall complement protein expression in combination with microglia function has been found to be altered in a number of psychiatric disorders including schizophrenia (Monji, Kato, & Kanba, 2009; Havik et al., 2011).

Oxidative stress

A balance of pro- and anti-oxidant radicals within the central nervous system (CNS) maintains the cellular integrity while, an imbalance leads to increase in reactive oxygen species (ROS) causing oxidative stress (Fendri et al., 2006). A significant reduction of antioxidant enzymes such as glutathione peroxidase reductase were found in the brain of schizophrenia patients as compared to controls (Yao, Leonard, & Reddy, 2004). A number of studies have shown the role of oxidative stress in altering the postnatal brain maturation (O'Donnell, 2011; Giovanoli et al., 2013; Moller et al., 2013). Oxidative stress can affect cellular processes like cell signaling and neuronal excitability, which could adversely affect neuronal and interneuronal (PV) phenotypes contributing to the pathology of schizophrenia (Johnson et al., 2013). Redox alterations or oxidative stress are associated with positive, negative and cognitive symptoms in schizophrenia

(Do, Cabungcal, Frank, Steullet, & Cuenod, 2009; Cabungcal et al., 2014). A common player that has been identified to cause oxidative stress in these studies is glutathione (GSH). GSH is an endogenous antioxidant which is involved in decreasing the level of ROS and balancing oxidative stress in the PFC (Do et al., 2000). Thus, a decrease in GSH leads to an increase in oxidative stress as observed in both peripheral tissues and post-mortem samples of schizophrenia patients (Gawryluk, Wang, Andreazza, Shao, & Young, 2011). GSH precursor, N-acetyl cysteine (NAC) has been shown to reverse schizophrenia-related electrophysiological, morphological, and behavioral anomalies (Moller et al., 2013). Together with this, a number of clinical studies also support the use of NAC as an adjunctive therapeutic agent (Berk et al., 2008; Lavoie et al., 2008). For example, in a randomized double-blinded study, administration of NAC was reported to significantly improve auditory sensory processing in schizophrenia patients compared to placebo treated patients (Lavoie et al., 2008). Further evidence of increased level of oxidative stress markers has been provided by post mortem schizophrenia study (Yao et al., 2004). The pro-inflammatory cytokine, IL-6 activates the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) enzyme leading to an increase in ROS (Powell, Sejnowski, & Behrens, 2012). Increased oxidative stress has been known to lead to abnormal microglia morphology and its inflammatory responses (S. H. Choi, Aid, Kim, Jackson, & Bosetti, 2012). Further, in a microglia cell culture experiment it was shown that LPS-induced NADPH oxidase enzyme activation could be inhibited by an anti-inflammatory factor (TGF-β1) (Qian et al., 2008). Thus, suggesting the role of TGF-β1 in neuroprotection modulated by NADPH oxidase enzyme and microglia. Cognitive impairments have been observed in young rats as a result of increased oxidative stress (Fukui et al., 2002). Interestingly, some aspects of cognitive and developmental cortical deficits were observed to be rescued by reducing oxidative stress with an antioxidant treatment either during juvenile or adolescent stage in NVHL animals (Cabungcal et al., 2014). NADPH mediated increase in the oxidative stress, resulted in schizophrenia-related behavioral alterations and dysfunction of inhibitory interneurons in the PFC (Behrens et al., 2007; C. Wang et al., 2003).

Role of microRNAs in regulating neuroinflammation- relevance to schizophrenia

MicroRNAs (miRNAs) are noncoding RNAs (~22 nucleotide) evolutionarily conserved, identified and expressed in different species (Griffiths-Jones, Grocock, van Dongen, Bateman, & Enright, 2006) and tissues respectively (Bartel, 2004). Their main function is regulating gene expression by post transcriptional repression, mRNA degradation and deadenylation (Filipowicz, Bhattacharyya, & Sonenberg, 2008; Eichhorn et al., 2014). MiRNAs have been known to regulate gene expression in different regions of the brain and mediate neuronal development and circuit refinement (McNeill & Van Vactor, 2012; Schratt et al., 2006). Several miRNAs have been implicated in the mechanism modulating the inflammatory target genes (Mehta et al., 2015). Increased expression of miR-301b was found to lead to activated microglia which was followed by an increase in inflammatory factors including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and cyclooxygenase-2 (COX-2) in hippocampus. This miR-301b driven increased inflammatory overload was also associated with cognitive impairments (Tang et al., 2019). Using LPS activated-microglia the study has identified two miRNAs, miR-155 and miR-34a which have been found to maintain redox homeostasis and a balance between the different inflammatory and anti-inflammatory factors (Juknat, Gao, Coppola, Vogel, & Kozela, 2019). MiR-101a facilitated the differentiation of microglia-like cells expressing cluster of differentiation 11b, ionized calcium binding adaptor molecule 1 (IBA1), CX3C chemokine receptor 1 (Cx3CR1), and Triggering Receptor Expressed On Myeloid Cells 2 (Trem-2). In response to LPS microglia cells that are transfected with miR-101a leads to the production of IL-6 (Saika et al., 2017). The expression of an anti-inflammatory growth factor (TGF-β1) and multiple components of the TGF-β signaling pathway has been observed to be regulated by multiple miRNAs (Davis, Hilyard, Lagna, & Hata, 2008; Saika et al., 2017). For example, miR-21 has been found to activate TGF-β/Smad signaling pathway in an invitro cell culture and regulate TGF-β1 expression (Yu et al., 2019). Thus, these studies indicate a critical role of miRNAs in neuroinflammation and circuit refinement via microglia.

Perinatal period is a critical phase of brain development and any exposure to infection during this period has been associated with schizophrenia-related behavioral and circuit development impairments (Deng, 2010; Dobbing & Sands, 1979). Recent study identified miR-137 locus to be significantly associated with schizophrenia-related cellular deficits such as loss of synaptic

functions (He et al., 2018). MiR-137 has also been found to play a role in neuronal maturation, differentiation and cognitive function (Mahmoudi & Cairns, 2017). Altered expression of a number of miRNAs have been observed within the brain and peripheral samples of schizophrenia patients. For example, miR-181 has been found to be increased in the temporal lobe and DLPFC (Beveridge et al., 2008), and in blood (Shi et al., 2012). Global blood plasma level analysis in schizophrenia patients showed an upregulation of miR-130b and miR-193a-3p as compared to controls or other psychiatric patients (Wei et al., 2015). The functional targets of these miRNAs include a number of genes that have been observed to be altered in schizophrenia patients, such as brain-derived neurotrophic factor (BDNF), the dopamine receptor D1 (DRD1), NRG1, neurotrophic tyrosine kinase receptor and early growth response gene 3 (EGR3) (Stefansson et al., 2002; Jones et al., 2011). Pyramidal neuronal cells isolated from the superior temporal gyrus of post-mortem brains from schizophrenia and control patients identified several miRNAs that were differentially expressed such as miR-132 and miR-30b (B. H. Miller et al., 2012; Santarelli, Beveridge, Tooney, & Cairns, 2011; Mellios et al., 2012). Additionally, RNA-seq analysis of the granule cells from hippocampus of post-mortem schizophrenia patients exhibited an alteration in miR-182 signaling in schizophrenia patients (Kohen, Dobra, Tracy, & Haugen, 2014).

Further evidence of the role of miRNA in schizophrenia pathophysiology is shown by studies showing the effect of antipsychotics on mRNA expression. In an animal model, haloperidol and clozapine have been found to lead to a decrease in miR-219 level and regulating the function of NMDAR (Kocerha et al., 2009). In a human study, the two upregulated miRNAs (miR-130b and miR-193a-3p) in the plasma of schizophrenia patients were suppressed after 1 year of treatment with aripiprazole and risperidone (Wei et al., 2015). Several lines of evidence suggest that the miRNA plays a critical role in the effectiveness of the drug (Tahamtan, Teymoori-Rad, Nakstad, & Salimi, 2018). Thus, suggesting miRNA as a key target to study drug responses and as a potential biomarker for early detection and proper management of the psychiatric disorder (Alural, Genc, & Haggarty, 2017).

RATIONALE AND AIMS

The main goal of my doctoral thesis was to understand the mechanisms in the PFC that drive NVHL related cellular and behavioural aberrations. A number of mechanisms have been implicated in the abnormal development of the prefrontal cortical circuits and schizophrenia behavior. In our study we explored the role of neuroinflammation in PFC circuit development and schizophrenia-related behaviors. Epidemiological studies have long hinted at a role of immune dysregulation in schizophrenia. Recent genome wide association study found strong schizophrenia genetic association to the major histocompatibility complex locus and short non-coding miRNA 137; both of which have been found to be associated with increasing inflammation in the brain. Further evidence on the role of neuroinflammation in schizophrenia is provided by PET study, which reported an increase in the density of resident immune cells, microglia in schizophrenia patient. Microglia are best known to regulate immune response in the brain, recent discoveries have identified the role of microglia in brain development and synaptic plasticity. Microglia are thought to regulate synaptic pruning by modulating the expression of complement proteins, fractalkine and their receptor Cx3cr1 in the PFC. Interestingly, microRNAs have been found to modulate the expression of genes related to microglia homeostasis and oxidative stress. An alteration in miRNA expression may lead to altered microglia morphology and result in an increased expression of pro-inflammatory cytokines, oxidative stress marker and complement components molecules therefore contributing to neuroinflammation. Therefore, we believe that neuroinflammation is the main cause of the prefrontal cortex-related cellular behavioural abnormalities induced by NVHL and explored different factors contributing to neuroinflammation in the following aims.

Aim1. To investigate the role of prefrontal cortex microglia in the development of behavioural and cellular abnormalities induced by NVHL.

Aim 2. To investigate the role of prefrontal cortex anti- and pro- inflammatory cytokines in the development of behavioural and cellular abnormalities induced by NVHL.

Aim 3. To assess the role of miRNA and its effect on oxidative stress in NVHL induced neuroinflammation in mPFC.

Aim 4. To investigate the effect of neonatal ablation of ventral hippocampus excitatory neurons on the development of adult behavioral and mPFC cellular functions.

CHAPTER II

Microglia in the developing prefrontal cortex of rats show dynamic changes following neonatal disconnection of the ventral hippocampus.

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Abstract

Impaired ventral hippocampal (VH)-prefrontal cortex (PFC) connectivity is implicated in many cognitive and behavioral disorders. Excitotoxic neonatal VH (nVH) lesion in rat pups has been shown to induce synaptic pruning in the PFC as well as behavioral changes of relevance to developmental neuropsychiatric disorders. In the current study, we hypothesized that microglia, immune cells required for proper brain development and plasticity, may play a role in the development of abnormal behaviors in the nVH-lesioned animals. Ibotenic acid-induced nVH lesion was induced in postnatal day (P)7 male rats. Developmental changes in microglial density, morphology, ultrastructure and gene expression were analyzed in the PFC at P20 and P60. Our results revealed increased microglial reactivity and phagocytic activity in the lesioned rats at P20. Increased mRNA levels of C3 and C1q, complement molecules involved in synaptic pruning, were concomitantly observed. Diminished, but maintained, microglial reactivity and reduced antioxidative defenses were identified in lesioned rats at P60. Behavioral deficits were significantly reduced in the adolescent rats by suppressing microglial reactivity by a one-week minocycline treatment immediately after the lesion. These results suggest that early-life disconnection of the VH has long-lasting consequences for microglial functions in distant connected structures. The alterations in microglia may underlie synaptic reorganization and behavioral deficits observed following neonatal VH disconnection.

Introduction

Anatomical connectivity and molecular architecture of the hippocampal formation reveals a dorsoventral gradient with clear distinctions between the dorsal and ventral poles (Fanselow & Dong, 2010). Functions of the hippocampus are also suggested to be organized along the same dorsoventral axis; however, it is generally believed that the dorsal hippocampus is predominantly involved in spatial learning and memory, while the ventral hippocampus (VH; anterior hippocampus in the primates and humans) modulates executive control as well as emotional and goal-directed behaviors (Floresco, Seamans, & Phillips, 1997; Strange et al., 2014). The VH influences these behaviors via monosynaptic excitatory projections from the ventral CA1/subiculum to pre- and infra-limbic regions of the medial PFC (mPFC) as well as to other limbic regions such as the hypothalamus, nucleus accumbens (NAc) and amygdala (Jay & Witter, 1991; Thierry et al., 2000). In the development of communication between the hippocampus and PFC, the first postnatal week is critically important (Brockmann et al., 2011); postnatal days (P)7–9 are marked by peak axonal and dendritic growth in the rat hippocampal development (Minkwitz, 1976). During the first postnatal week, network oscillations in the neonatal rat PFC are driven by VH activity, while the hippocampus and PFC are functionally coupled via theta- and gamma-band oscillations (Brockmann et al., 2011).

A large number of studies, from our group and others, have provided evidence that excitotoxic lesion of the VH at the end of first postnatal week leads to post-pubertal emergence of a range of cognitive, social and sensorimotor problems, accompanied by marked synaptic reorganization in the PFC (for reviews see (Marcotte et al., 2001; Tseng et al., 2009). Striking similarities in the nature and temporal course of neonatal VH (nVH) lesion-induced behaviors to schizophrenia has led to the suggestion that nVH lesioned (NVHL) animals serve as a heuristic model to test the developmental hypothesis of schizophrenia because this cognitive disorder is postulated to arise from a faulty development of temporolimbic structures. However, it should be added that a disruption in hippocampal-prefrontal/limbic pathways is hypothesized to be a common pathophysiological mechanism in diverse behavioral disorders (Godsil, Kiss, Spedding, & Jay, 2013). Accordingly, nVH lesion may represent a unique paradigm to study mechanisms of PFC dysfunction arising from a developmentally compromised brain, without regard to psychiatric diagnosis.

The removal of presumably abnormal mPFC neurons from the adult rats with nVH lesions appears to normalize some of the behavioral and neuronal deficits suggesting that intrinsic mPFC neurons that have developed in the context of abnormal hippocampal connectivity participate in the abnormalities (Goto & O'Donnell, 2004; Lipska, al-Amin, & Weinberger, 1998). The delayed appearance of abnormal behavior is also consistent with protracted maturation of PFC circuits during adolescence (Kolb & Nonneman, 1976). The idea that mPFC abnormality mediates cognitive deficits is also supported by the finding that NVHL rats show a pattern of spatial working memory deficit that is similar to rats with adult lesion of the mPFC (Lipska & Weinberger, 2002).

We have previously provided evidence of altered synaptic connectivity in the PFC of NVHL rats, e.g., the lesioned animals have decreased dendritic spine density in layer 3 and 5 pyramidal neurons of the mPFC accompanied with an imbalance in excitatory and inhibitory synaptic transmission (Flores, 2005; Ryan, Bhardwaj, Tse, Srivastava, & Wong, 2013). Of note, a reduction in the dendritic spines on cortical pyramidal neurons is one of the most replicated post-mortem findings in schizophrenia (Glausier & Lewis, 2013). Synaptic abnormality in NVHL is also confirmed in studies showing cortical overexcitation and abnormal neural synchrony (Lee et al., 2012) and alpha-1 adrenergic receptor-mediated synaptic plasticity in the mPFC (Bhardwaj, Tse, Ryan, Wong, & Srivastava, 2014).

Taken together, there is strong evidence for a role of developing VH inputs in shaping adult neural circuit and functions of the PFC; however, the local mechanisms within the PFC that drive aberrant neural development are poorly understood. Based on our findings that neural activity shapes microglia behavior (Bisht et al., 2016; Tremblay, Lowery, & Majewska, 2010), we reasoned that a loss of developing VH inputs to the PFC might cause microglial alterations in NVHL animals, which may be responsible for the synaptic abnormality and abnormal behaviors observed in these animals. Microglia provide a rapid and efficient response for counteracting pathogenic and traumatic injuries (Blank & Prinz, 2013). In addition to this classical role, recent discoveries have demonstrated a pivotal role of these immune cells in circuit refinement and synaptic plasticity in the developing and adult brain (Tay, Savage, Hui, Bisht, & Tremblay, 2017). Collectively, findings show that microglia actively remodel neuronal circuits in an activity- and experience-dependent manner, through phagocytic elimination (pruning) of axon

terminals and dendritic spines. It has been reported that an impairment of neuron-microglia communication in Cx3cr1 gene-deleted mice leads to altered brain functional connectivity and behaviors associated with autism and related disorders (Zhan et al., 2014).

In the following set of studies, we aimed to quantitatively analyze microglial density, ultrastructure and gene expression in the mPFC of NVHL animals at two developmental time-points. Our results reveal that microglia in the PFC show reactive morphologies and increased phagocytic activities, accompanied by increased expression of complement molecules quite early, i.e., two weeks after the nVH lesion. The microglial alterations persist with a reduction of antioxidative defense during adulthood. Further, suppression of microglial reactivity by neonatal minocycline treatment was able to rescue some of the schizophrenia-related behavioral deficits in the adult rats. Taken together, our data provide a potential mechanism by which the development of prefrontal circuits is compromised following early damage to the VH. Our findings also indicate that modulation of microglial reactivity may serve as a therapeutic approach to prevent behavioral symptoms in disorders with developmentally-altered synaptic connectivity.

Materials and Methods

Neonatal ventral hippocampal lesion

Animal care and surgical procedures were according to the guidelines of Canadian Council of Animal Care and were approved by McGill University Animal Care Committee. Timed pregnant Sprague–Dawley rats were obtained from Charles River Canada and gave birth in our animal facility. nVH lesion was performed on P7 male pups according to our previously described procedures (Flores et al., 1996; Ryan et al., 2013). Pups were anesthetized by hypothermia (by covering in crushed ice for 18–20 min) and secured on a modified platform fixed to Kopf stereotaxic apparatus. The ‘lesion’ group received bilateral infusion of 0.3 µl ibotenic acid (Sigma; 10 µg/µl in 0.1 M phosphate-buffered saline (PBS) over a period of 2 min using a 30-gauge needle connected to an infusion pump, while the ‘sham’ group received the same volume of PBS (coordinates: AP −3.0 mm from Bregma, ML ±3.5 mm from midline, and DV −5.0 mm from dura). Following this, the skin was sutured using Vetbond tissue glue and the pups' ears

were tagged. After surgery, pups were placed on a heating pad until full recovery and returned to their respective mothers where they remained until weaning (P21).

Tissue harvest

The schematic representation of the experimental design is shown in Fig. 1A. Briefly, for RT-PCR, at both pre-weaning (P20) and post-pubertal (P60) ages, sham-operated and nVH lesioned rats (n = 6 each/time point), were sacrificed by cervical dislocation and their brains were removed. mPFC, including infralimbic (IL) and prelimbic (PrL) regions, were punched and the tissue of both hemispheres from each animal was pooled and stored at −80 °C until use.

For confocal microscopy, separate cohort of 16 animals (4 sham and 4 nVH lesion rats at both P20 and P60) were anesthetized with ketamine/xylazine cocktail and transcardially perfused with ice-cold PBS followed by 4% paraformaldehyde (PFA; EMS, Hatfield, PA, USA). Brain was harvested from the skull and post-fixed in 4% PFA at 4 °C overnight. After cryoprotection in 15% and 30% sucrose, 30 μm coronal sections containing both IL and PrL regions were collected using a freezing microtome. The sections were stored in cryoprotectant solution at −20 °C until histological studies.

For electron microscopy (EM), a new cohort of 16 animals (4 sham-operated and 4 nVH lesion rats at both P20 and at P60 age) was anesthetized using ketamine/xylazine cocktail and perfused with 3.5% acrolein and 4% PFA (Bisht et al., 2016). Fifty-micron transverse sections containing both IL and PrL regions were collected using a vibratome and stored in cryoprotectant solution at −20 °C until ultrastructural studies.

Quantitative real-time PCR (RT-PCR)

Medial PFC tissues were homogenized in QIAzol lysis reagent (#79306, Qiagen, Hilden, Germany) and total RNA was extracted according to the manufacturer's protocol. Subsequently, 1 μg of total RNA was reverse transcribed into cDNA using the iScript cDNA synthesis kit (#170-8891, BioRad, Hercules, CA, USA). Real-time PCR was performed with the SsoAdvanced universal SYBR Green supermix kit (BioRad) in a Lightcycler 480II (Roche,

Basel, Switzerland). Relative expression was calculated with the 2−ΔΔCT method using β-Actin for normalization as previously described (Yuan, Reed, Chen, & Stewart, 2006).

Fluorescent immunohistochemistry (IHC) and confocal microscopy

Sections were incubated in 0.1M citrate buffer at 90 °C for 8–10 min to retrieve antigens. After the slides had cooled down, they were washed and blocked in 10% donkey serum with 0.3% Triton X-100 in PBS (50 mM, pH 7.4) for 1 h at room temperature. All primary and secondary antibodies were diluted in the same blocking buffer. Sections were incubated with anti-IBA1 antibody (1:1000, #019-19741, Wako) at 4 °C overnight, rinsed in PBS, and then with Alexa Fluor 488 secondary antibodies (1:500, Thermo-scientific, Waltham, MA, USA) for 2 h at room temperature. Sections were washed in PBS, counter-stained with DAPI (1:20000, Thermo-scientific) and mounted with anti-fading media (H-1000, Vector Laboratories, Burlington, Ontario, Canada) under a glass coverslip.

Using a Quorum WaveFX Spinning disc confocal microscope, microglial imaging was performed in the IL and PrL regions (Bregma 2.2 to 3.2 according to Paxinos, George, and Charles Watson. The rat brain in stereotaxic coordinates: hard cover edition. 2006). Z-stacks were acquired at 10x magnification (density, spacing, and clustering analysis) or 20x magnification (morphological analysis) with an ORCA-R2 camera (Hamamatsu, 1344 × 1024 pixels) in 2 areas per region of interest in each of 2 tissue sections per animal. Each stack contained ∼25 slices (1 μm each for 10x) or ∼50 slices (0.5 μm each for 20x). Focus stacking was performed in Volocity software (Version 5.4, PerkinElmer, Woodbridge, Ontario, Canada).

For caspase-3 immunostaining, free-floating sections were washed with PBS and incubated with primary antibody directed against cleaved caspase- 3 (1:1000; rabbit; Millipore, Cat# 3623) for 2 h at RT and then overnight at 4 °C. The sections were labeled with Alexafluor 488 anti-rabbit donkey secondary antibody (1:300; Molecular Probes) for an hour at RT. The sections were examined using 488 nm excitation filter of a Zeiss Imager M1 microscope for fluorescent imaging. Caspase-3 staining was quantified using ImageJ software.

Analyses of microglial density, spacing, clustering and morphology

Quantitative analysis was conducted blind to the experimental conditions to assess the density, spacing, clustering, and morphology of microglia as previously described (Milior et al., 2016; Tremblay, Zettel, Ison, Allen, & Majewska, 2012). The analysis was performed on collapsed z-stacks with maximum projection using the ImageJ software (National Institutes of Health). To determine cellular density and spacing, the center of each microglial cell body was marked with a dot using the paintbrush tool. The 'analyze particles' function was used to automatically record cell numbers and spatial coordinates, in order to determine the nearest neighbor distance for each cell with the nearest neighbor distance plugin. Cellular density was determined by dividing the total number of cells by the total surface area of the acquired pictures measured in mm2 for each animal. A spacing index was calculated as the square of the average nearest neighbor distance multiplied by microglial density per animal. Microglial clusters, which consist of two or more microglial cells within 15 μm one from another, were also examined.

To analyze morphology, a total of 18–20 microglial cells per animal were analyzed. Only cells whose cell body and proximal processes were perfectly in focus were included in the analysis. Every IBA1-immunopositive microglia in a picture was analyzed before moving on to the next picture as to not introduce selection bias. For each microglia, the soma area was determined by drawing a line around the cell body by using the freehand selection tool. The arborization area was determined with the polygon selection tool to connect the most distal extremities of every process. The soma and arborization areas were calculated in pixels and converted into micrometers. Cell body circularity and solidity were shown in the shape descriptor of the measurement tool in Image J and was expressed in arbitrary value. Morphological index was calculated using the formula: soma area/arborization area (Tremblay, 2011).

Tissue preparation and immunoperoxidase staining for electron microscopy (EM)

Brain sections containing the IL and PrL regions (Bregma 2.2 to 3.2 according to Paxinos, George, and Charles Watson. The rat brain in stereotaxic coordinates: hard cover edition. 2006) were chosen for EM analysis. Sections were first washed in PBS, then quenched with 0.3% hydrogen peroxide (H2O2) and 0.1% sodium borohydride (NaBH4). Afterwards, they were

incubated for 1 h in blocking buffer (10% fetal bovine serum, 3% bovine serum albumin, 0.01% Triton X-100) and overnight with primary rabbit anti-IBA1 antibody (1/1000, Wako, #SAF5299) at 4 °C. The next day, brain sections were incubated for 2 h with goat anti-rabbit secondary antibody conjugated to biotin (1/300, #111-065-003, Jackson ImmunoResearch, West Grove, PA, USA) and for 1 h with avidin-biotin complex Vectastain solution (1:100, Vector Laboratories, #PK-6100). Sections were developed in a TRIS buffer (TB, 0.05 M; pH 8) solution containing 0.05% diaminobenzidine (DAB) and 0.015% H2O2. Sections were then incubated 30 min in 1% osmium tetroxide for lipid fixation, dehydrated in ascending concentrations of ethanol followed by propylene oxide, and embedded-flat with Durcupan resin between ACLAR films (EMS) for 72 h at 55 °C.

The IL and PrL regions were excised from the ACLAR films and cut at 75-80 nm of thickness using an ultramicrotome (Leica Ultracut UC7). IBA1-positive microglial processes were randomly imaged at 6800x using a transmission electron microscope (FEI Tecnai Spirit G2) operating at 80 kV and equipped with an ORCA-HR digital camera (Hamamatsu; 10 MP).

Analyses of microglial ultrastructural features

Ultrastructural observations were conducted at the tissue–resin border, where the penetration of antibodies and staining intensity is maximal (Tremblay et al., 2010). An average of 200 IBA1-immunopositive microglial process profiles per animal were analyzed. The area, perimeter, and shape descriptors measurements circularity and solidity were used to assess changes in morphology with ImageJ. Direct contacts with synaptic clefts and myelinated axons were counted for each microglial process. Autophagosomes devoid of contents (termed vacuoles) or containing cellular materials in the process of being digested (termed cellular inclusions) were counted on a microglial process profile basis (Tremblay et al., 2010). The analysis was performed blind to the experimental conditions.

Minocycline treatment and behavioral testing

The design and timeline for this experiment is shown in Fig. 1B. A new cohort of rats received nVH lesion surgeries at P7. The day after surgery, i.e. on P8, sham and nVH lesioned pups within each litter received an injection of saline or minocycline (Sigma; 40 mg/kg; i.p.), once a day for the next 7 days. This procedure resulted in four groups of animals: sham-saline, lesion-saline, sham-minocycline and lesion-minocycline (n = 5–8 each group). Behavioral testing was done from P55 onwards under dim light (50 lux) conditions during the light phase of the light:dark cycle. The same cohort of animals was used for all behavioral tests and the tests were performed starting with the least stressful, in the order presented below. Tests were separated by at least 72 h.

Spontaneous locomotor activity

Spontaneous locomotor activity was assessed as previously described by us (Bhardwaj et al., 2012). Acrylic arenas (AccuScan Instruments, Inc., Columbus, OH, USA) 40 × 40 × 30 cm (l × w × h) equipped with infrared sensors were used to assess locomotion. Data collection was performed using the Versamax Software (version 4.0, 2004; AccuScan Instruments, Inc.). Animals were brought from their home environment to the testing room and immediately placed into the activity boxes where their spontaneous activity and habituation were monitored and recorded in the novel environment during 60 min. For each animal, the total horizontal distance traveled (cm) was measured over 10 min intervals and used for analysis.

Prepulse inhibition (PPI) of the acoustic startle response (ASR)

Sensorimotor gating deficit, measured through PPI, is considered a translational endophenotype of schizophrenia (Turetsky et al., 2007). It was assessed according to our previously described procedures (Bhardwaj et al., 2012). We used a commercially available system (SR-LAB; San Diego Instruments, San Diego, CA) with sound-attenuating chambers, each equipped with a cylindrical plexiglas animal enclosure and a small electric fan generating a 70 decibels (dB) background noise and providing ventilation. Sound pressure levels (dB(A) weighting) were measured at the position of the rat's ears. Broadband noise pulses were presented via a speaker positioned directly above the animal. An accelerometer affixed to the animal enclosure frame was used to detect and transduce motion resulting from the animals' response. Noise pulse

parameters were controlled using SR-LAB software, which also recorded responses. Animals were acclimated to the enclosure for 5 min before being tested during 37 discrete trials. On the first two trials, the magnitude of the startle response to a 120-dB pulse was measured. These first two startling pulses were presented to habituate the animals to the testing procedure and thus were omitted from the data analysis. Subsequent trials were included in the analysis. On the subsequent 35 trials, the startle pulse was either presented alone or 100 ms after the presentation of a 30 ms prepulse. ASR to the pulse was measured following trials with prepulse (PP) intensities of 3, 6, 9, 12 and 15 dB above the background noise. Prepulses were varied randomly between the trials, and each prepulse was presented five times; animals were randomly presented with the startle pulse alone during 10 trials. The average inter-trial interval was 15 s (range, 5–30 s). The startle responses were determined automatically by the SR-LAB analysis suite. Startle magnitude was calculated as the average of the startle responses to the pulse-alone trials. PPI was calculated according the formula: %PPI = 100 − (startle response for prepulse + pulse trials)/startle response for pulse alone trials × 100%. For analysis of data, averaged percent reduction in PPI was calculated and compared between groups.

Anxiety-like behavior in elevated plus-maze (EPM)

VH-PFC connectivity has been shown to be critical for anxiety-related behaviors as measured in the EPM (Padilla-Coreano et al., 2016). Animals were tested as previously described using a standard EPM made from black plexiglass with two enclosed and two open arms, as well as a central square (Bhardwaj et al., 2012). Rats were placed in the central square of the four arms and their behaviors were videotaped for 5 min. The videos were analyzed by a treatment-blind observer for the number of entries into the open and closed arms (defined as the number of head pokes made into the open or closed arms from the central platform), as well as the time spent in the open/closed arms.

Temporal order memory

This task was conducted in a Y-shaped apparatus as described (Reid, Jacklin, & Winters, 2014). The apparatus was made of opaque plexiglass arms at 120° angle from each other (15.5 cm long, 5 cm wide and 15 cm high). Duplicate copies of objects made of plastic, ceramic or glass were used. All objects were affixed to the floor of the apparatus with a reusable adhesive putty to prevent them from being displaced during testing. Rats were habituated to the Y-maze by 5 min

exposures to the empty maze once a day for two days. Behavioral testing began 24 h after the second habituation. Each trial consisted of 2 sample phases and 1 choice (test) phase, separated by a retention delay. In the 1st sample phase (called remote phase), two identical objects were placed, one at each end of the exploration arm. Animals were released at the end of the third arm and allowed to explore the other two arms with objects for 5 min. The animals were returned to their home cage and, after an hour, were exposed to the 2nd sample phase (recent phase) in which a different set of identical objects was placed into the two exploration arms of the Y-maze. Animals were allowed to explore the new objects for 5 min after which they returned to their home cage for 1-h (retention delay). Following this, the animals were given the choice phase in which the exploration arms were each loaded with one object from one of the 2 sample phases (remote versus recent). Animals were again allowed to explore the arms for 5 min. The time spent exploring the two objects was video recorded for all the phases and then scored by an experimenter blind to the treatment condition. Discrimination ratio during the choice phase of test was calculated using the following formula: (Time exploring remote object) – (Time exploring recent object)/Total Exploration Time (remote + recent).

Three days after the last behavior testing, the animals were sacrificed by rapid decapitation and their mPFC regions were excised and frozen as mentioned above in section 2.2. The tissue was processed for quantitative real-time polymerase chain reaction (q-RT PCR) analyses of select microglial and other genes as explained in section 3.6.

Histological examination

Animals were euthanized by decapitation and their brains were removed and frozen. Thirty-five μm coronal sections at the level of the VH were mounted on pre-coated microscope slides and stained with cresyl violet staining solution (0.5%) for verification of the lesion location. Only animals with bilateral lesions confined to the ventral hippocampus were included in the nVH lesion group.

Statistics

All reported values are mean ± standard error of the mean (S.E.M.). Data were analyzed using Prism (GraphPad, Version 5). Two-way ANOVA with Bonferroni post-hoc test was used to determine interactions between lesion and time in the morphological, ultrastructural, and molecular data. In some cases, when a main effect of the lesion was significant without lesion x time interaction, we did an exploratory and limited pairwise post-hoc comparison consistent with our a priori hypothesis. Two-way or three-way ANOVAs with tukey's post-hoc tests were used to determine interaction between lesion and minocycline treatment in the behavioral data. $p < 0.05$ was considered statistically significant.

Results

Lesion verification

Consistent with our prior experience, surgical procedures resulted in about 15–20% mortality. As reported earlier (Bhardwaj et al., 2014) nVH-lesioned animals showed bilateral neuronal loss, retraction, and cavitation in the ventral region of the hippocampus including the CA1 (Fig. 2C). The lesion spared the dorsal hippocampus and adjacent nuclei (i.e., amygdala and thalamus).

nVH lesion alters microglial distribution and morphology in the PFC

We performed immunohistochemical analyses on IBA-1-stained sections to assess microglia. Analysis of PrL and IL regions of mPFC (see Fig. 2B for schematic representation of their anatomical boundaries) revealed clusters of microglia in close proximity one to another in the nVH lesioned rats at P20 (Fig. 3A–D). Microglia within clusters displayed enlarged and rounder cell bodies compared with other parenchymal microglia. The radial orientation of microglial processes towards the center of the clusters could be also identified (Fig. 3B–D). Three-dimensional reconstruction of a cluster captured at high magnification by confocal microscopy showed direct contacts between the processes from neighboring microglia (Fig. 3E). Interestingly, microglial clusters were rarely identified in the nVH rats at P60 (data not shown). Notably, the formation of clusters was not related to programmed developmental cell death

within the PFC as t-test showed no significant difference in activated caspase-3 immunostained cells between P20 sham and lesioned animals ($t = 0.8562$ $df = 4$, $p = 0.4401$) (Fig. 4).

Quantitative microglial density and morphological analyses were performed in IL and PrL regions separately. Two-way ANOVA results from microglial density in IL showed significant main effects of time [$F(1,12) = 61.94$, $p < 0.0001$], lesion [$F(1,12) = 18.63$, $p = 0.0010$] and time x lesion interaction [$F(1,12) = 8.04$, $p = 0.0150$]. Post-hoc analysis from microglial density in IL region revealed a significant increase in P20 lesioned rats compared to sham animals, whereas no change was observed in P60 animals (Fig. 5A–E). Data analysis from distance to neighboring cells in IL brain region also indicated a significant main effect of time [$F(1,12) = 152.47$, $p < 0.0001$], lesion [$F(1,12) = 35.24$, $p < 0.0001$] and lesion x time interaction [$F(1,12) = 23.12$, $p = 0.0004$]. Post-hoc analysis revealed a significant decrease in distance to neighboring cells in P20 lesioned rats compared to sham animals, but no effect was observed in P60 animals (Fig. 5F).

In PrL region, a two-way ANOVA for microglial density showed a significant main effect of time [$F(1,12) = 51.58$, $p < 0.0001$] and lesion [$F(1,12) = 38.39$, $p = 0.0292$] without lesion x time interaction [$F(1,12) = 0.00$, $p = 0.9560$]. Post-hoc test of microglial density revealed a significant increase in both P20 and P60 nVH lesioned rats compared to respective age group sham animals (Fig. 5L). Data analysis for distance to nearest neighboring microglial cell revealed a significant main effect of time [$F(1,12) = 83.44$, $p < 0.0001$] and lesion [$F(1,12) = 38.39$, $p = 0.0292$] but no lesion x time interaction [$F(1,12) = 0.18$, $p = 0.6791$]. Post-hoc also indicated a reduction in the distance to nearest neighboring cell in pre-pubertal as well as post-pubertal lesioned animals compared to respective age-matched sham animals (Fig. 5M). With respect to spacing index the values were comparable between sham-operated and nVH lesion rats in both brain regions and at both ages (Fig. 5G, N).

We next studied microglial morphology to provide additional insights into their altered function. Representative microglial images from IL region of sham-operated and lesioned animals are shown in Fig. 6A–D. Two-way ANOVA results for arborization area in IL region showed significant main effects of time [$F(1,12) = 27.56$, $p = 0.0002$] and lesion [$F(1,12) = 13.56$, $p = 0.0031$] without time x lesion interaction [$F(1,12) = 1.03$, $p = 0.2756$]. Post-hoc analysis revealed a significant decrease in P60 lesioned rats compared to sham animals, whereas no change was observed in P20 animals (Fig. 6E). ANOVA of data on morphological index (soma

area/arborization area) in IL revealed a significant main effect of time [$F(1,12) = 25.88$, $p = 0.0003$] and lesion [$F(1,12) = 17.04$, $p = 0.0014$] without lesion x time interaction [$F(1,12) = 0.24$, $p = 0.6303$]. Post-hoc test also indicated a significant increase in morphological index at both age points in nVH lesioned rats compared to sham animals (Fig. 6G).

Representative microglial images from PrL region of sham-operated and lesioned animals are provided in Fig. 6H–K. Two-way ANOVA of microglial process arborization area in PrL region showed a significant main effect of time [$F(1,12) = 36.55$, $p < 0.0001$] and lesion [$F(1,12) = 5.61$, $p = 0.0355$] but no lesion x time interaction [$F(1,12) = 3.30$, $p = 0.0946$]. Similar to IL, post-hoc analysis revealed a reduction of arborization area in PrL of lesioned rats at P60 (Fig. 6H-L). With regard to the morphological index, ANOVA identified a significant main effect of time [$F(1,12) = 33.10$, $p < 0.0001$] but not lesion [$F(1,12) = 2.77$, $p = 0.1217$] and two-way interaction [$F(1,12) = 2.16$, $p = 0.1675$]. There was also a trend for an increase of soma area/arborization area ratio in P60 lesioned rats compared to age-matched sham animals (Fig. 6N). ANOVA did not reveal any difference in the soma size between sham and lesioned rats at any age point in any brain areas (Fig. 6F, M). Cell body circularity and solidity were also similar between sham-operated and lesioned rats (data not shown). These results indicate that microglia display early alterations associated with reactivity, which persist in terms of reduced arborization area and increased morphological index (Streit et al., 1999) in the PFC of lesioned rats into adulthood.

nVH lesion induces phagocytic activity in microglia during early development

Detailed morphological changes and phagocytic activity in the mPFC were determined by electron microscopic studies and the representative EM images of microglial process profiles are presented in Fig. 7A–F.

For morphological changes, two-way ANOVA analysis of process area revealed a significant main effect of time [$F(1,1680) = 27.88$, $p < 0.0001$] and lesion [$F(1,1680) = 8.92$, $p = 0.0029$] without lesion x time interaction [$F(1,1680) = 0.46$, $p = 0.4998$]. ANOVA analysis of process perimeter showed significant main effects of time [$F(1,1680) = 37.40$, $p < 0.0001$] and lesion [$F(1,1680) = 6.43$, $p = 0.0113$] but without lesion x time interaction [$F(1,1680) = 1.72$, $p = 0.1895$].

Post-hoc tests suggest that nVH lesion induces early-onset morphological changes in terms of increased process area and perimeter in pre-weaning (P20) nVH lesioned animals compared to age-matched sham animals. However, no change was observed in adult animals for both parameters (Fig. 7G and H). Data on process circularity showed a significant main effect of time [$F(1,1680) = 18.34$, $p < 0.0001$] and time x nVH lesion interaction [$F(1,1680) = 7.99$, $p = 0.0048$] but not of lesion [$F(1,1680) = 0.09$, $p = 0.7703$], and data on process solidity revealed a significant main effect of time [$F(1,1680) = 17.19$, $p < 0.0001$] and lesion x time interaction [$F(1,1680) = 9.91$, $p = 0.0017$] but not of lesion [$F(1,1680) = 0.07$, $p = 0.7976$]. Post-hoc analysis also showed a significant increase of both circularity and solidity in adult lesioned animals compared to sham operated animals, whereas no such effect was observed in younger (P20) ones (Fig. 7I and J). The increase of circularity suggests a reduction in the prevalence of morphological specializations, such as those involved in microglial contacts with synapses (Tremblay et al., 2010), while the increase of solidity (i.e. a measure of plasma membrane ruffling) (Ohsawa et al., 2000) indicates a decrease in microglial process motility and surveillance activity.

Changes in phagocytic activity were measured by counting autophagosomes, which contained (cellular inclusions) or not (vacuoles) digested elements, inside of microglial processes. In this manner, the number of cellular inclusions per process showed a significant main effect of lesion [$F(1,1680) = 8.26$, $p = 0.0041$] and time [$F(1,1680) = 10.64$, $p = 0.0011$] without lesion x time interaction [$F(1,1680) = 0.44$, $p = 0.5049$]. Similarly, the number of vacuoles per process showed a significant main effect of time [$F(1,1680) = 20.21$, $p < 0.0001$], lesion [$F(1,1680) = 6.73$, $p = 0.0096$] and lesion x time interaction [$F(1,1680) = 11.43$, $p = 0.0007$]. Post-hoc revealed a significant increase in the number of cellular inclusions (Fig. 7L) and vacuoles (Fig. 7M) in prepubertal lesioned animals compared to sham-operated ones. The combined results indicate increased autophagosomal activity in the microglia from lesioned animals at P20. However, for both vacuoles and cellular inclusions, no significant effect was measured in post-pubertal animals (Fig. 7L, M). ANOVA also revealed a significant main effect of time [$F(1,1680) = 7.95$, $p = 0.0049$] on the number of microglial contacts with synaptic clefts, which are suggestive of synaptic stripping, by which pre- and post-synaptic elements are separated by intervening microglial processes (Fig. 7K). In addition, ANOVA of extracellular digestion, by which cellular elements are degraded extracellularly also revealed a significant main effect of

lesion [F(1,1680) = 10.09, p = 0.0015] and time [F(1,1680) = 5.98, p = 0.0146] in the absence of lesion x time interaction [F(1,1680) = 0.03, p = 0.8571]. Pairwise comparison of data on cellular inclusions and extracellular digestion also indicated a significant increase in pre-weaning nVH lesioned rats compared to the sham animals (Fig. 7N). These values in post-pubertal animals remained comparable between sham and lesioned groups (Fig. 7L, N).

nVH lesion triggers earlier expression of microglial phagocytic genes in PFC

To provide molecular insights into the underlying mechanisms, qRT-PCR was conducted in the PFC of both P20 and P60 sham and nVH lesioned rats (Fig. 7), quantifying mRNA expression levels of genes related to microglial reactivity, pruning function, and antioxidant defense. Fig. 2A provides a schematic representation of the anatomical boundaries of the mPFC tissue sampled for gene expression studies.

ANOVA analysis of Trem2 revealed significant main effects of time [F (1,20) = 63.58, p < 0.0001], lesion [F (1,20) = 4.81, p = 0.0404] and time x lesion interaction [F (1,20) = 6.92, p = 0.0160]. Post-hoc analysis also showed that the expression of Trem2 is significantly decreased in post-pubertal nVH lesioned rats compared to age-matched sham animals whereas no change was observed in pre-weaning animals (Fig. 8A). Similarly, two-way analysis of Cluster of differentiation 45 (Cd45) gene indicated a significant time x lesion interaction [F (1,20) = 4.93, p = 0.0381]. The expression of Cd45 was also significantly increased in younger (P20) lesioned rats compared to age-matched sham animals, while remaining comparable in post-pubertal animals (Fig. 8B). ANOVA of Cx3cr1 only showed a significant main effect of time [F (1,20) = 10.19, p = 0.0046] (Fig. 8C).

Similarly, ANOVA of C1q involved in synaptic pruning showed significant main effects of time [F (1,20) = 142.94, p < 0.0001] and lesion [F (1,20) = 7.10, p = 0.0149]. Post-hoc test further revealed that C1q expression is significantly enhanced in P20 lesioned rats compared to age-matched sham animals. No difference was observed between groups in post-pubertal animals (Fig. 8D). Analysis of C3 gene expression additionally indicated a significant main effect of lesion [F (1,20) = 13.87, p = 0.0014], while post-hoc test showed a significant increase in C3 expression in younger (P20) lesioned rats compared to age-matched sham animals (Fig. 8E).

Analysis of superoxide dismutase 1 (Sod1) gene associated with antioxidant defense showed a significant main effect of time [$F(1,20) = 55.42$, $p < 0.0001$] but not lesion [$F(1,20) = 2.90$, $p = 0.1040$] or time x lesion interaction [$F(1,20) = 0.02$, $p = 0.8765$]. Similarly, ANOVA analysis of superoxide dismutase 1 (Sod2) revealed a significant main effect of time [$F(1,20) = 76.08$ $p < 0.0001$] but not lesion [$F(1,20) = 2.91$, $p = 0.1034$] or time x lesion interaction [$F(1,20) = 3.12$, $p = 0.0928$]. Analysis of Gpx1 expression also showed a significant main effect of lesion only [$F(1,20) = 5.52$, $p = 0.0292$], without significant effects of time [$F(1,20) = 3.29$, $p = 0.0848$] or time x lesion interaction [$F(1,20) = 1.86$, $p = 0.1882$]. Catalase expression showed a significant main effect of lesion [$F(1,20) = 5.80$, $p = 0.0258$] but not time [$F(1,20) = 1.27$, $p = 0.2733$] or time x lesion interaction [$F(1,20) = 2.40$, $p = 0.1373$]. Post-hoc analysis revealed a significant reduction in Gpx1 and Catalase expression in post-pubertal lesioned rats compared to age-matched sham animals, whereas no significant changes were observed in young (P20) animals. These data are presented in Fig. 8F–I.

Neonatal minocycline treatment reverses schizophrenia-related behaviors in post-adolescent nVH lesioned rats

To assess microglial involvement in the schizophrenia-related behaviors in this model, we performed tests of spontaneous locomotor activity, sensorimotor gating, anxiety, and temporal order memory in adult animals exposed to sham or nVH lesion surgery and minocycline treatment.

3.5.1. Spontaneous locomotor activity

Fig. 9A shows the time-course of locomotor activity (beam breaks) for 60 min in a novel environment. A three-way repeated measure ANOVA showed a main effect of lesion [$F(1,20) = 19.28$, $p < 0.001$], minocycline treatment [$F(1,20) = 6.45$, $p = 0.019$] and time [$F(5,100) = 55.54$, $p < 0.0001$]. Two-way ANOVA of total locomotor activity score for 60 min (Fig. 9B) also revealed significant main effects of lesion [$F(1,20) = 19.28$, $p < 0.001$], minocycline treatment [$F(1,20) = 6.45$, $p = 0.019$] and lesion x minocycline interaction [$F(1,20) = 8.15$, $p < 0.01$]. As reported previously in this model, post-hoc test revealed hyperlocomotion of saline

treated nVH lesioned animals compared to saline-treated sham animals ($p < 0.001$). Further analysis showed that while neonatal minocycline treatment had no significant effect in sham animals, it led to a significant attenuation of locomotor activity in nVH lesioned animals ($p < 0.001$ compared to lesion-saline).

Prepulse inhibition

A three-way repeated measure ANOVA on %PPI across all prepulse (PP) intensities showed main effects of lesion [$F(1,80) = 46.67$, $p < 0.001$], minocycline treatment [$F(1,80) = 4.97$, $p = 0.037$] and PPs [$F(4,80) = 79.20$, $p < 0.0001$] (Fig. 9C). Further, a two-way ANOVA of PPI values collapsed over all PPs revealed a significant lesion x minocycline interaction [$F(1,20) = 5.87$, $p = 0.025$] (Fig. 9D). As reported in this model (Bhardwaj et al., 2012), saline-treated nVH-lesioned animals exhibited a significant reduction in PPI, compared to sham-saline animals ($p < 0.001$). Like locomotor behavior, neonatal minocycline treatment had no significant effect in the sham animals, but it led to a partial normalization of PPI deficits in the lesioned animals ($p < 0.01$ compared to lesion saline). The baseline ASR showed no main significant effect of either lesion [$F(1,20) = 0.10$, $p = 0.75$], minocycline treatment [$F(1,20) = 0.80$, $p = 0.38$] or two-way interaction [$F(1,20) = 0.023$, $p = 0.88$] indicating that startle characteristics did not differ between the groups (data not shown).

Elevated plus maze

A two-way ANOVA of the ratio of time spent in the open arms versus the closed arms showed a main effect of lesion [$F(1,20) = 4.78$, $p = 0.04$] and significant lesion x minocycline treatment interaction [$F(1,20) = 6.40$, $p = 0.01$] but no main effect of minocycline treatment [$F(1,20) = 1.63$, $p = 0.21$). A post-hoc analysis on the ratio of the time revealed that the lesion-saline animals spent a significantly more time in the open arm compared to sham-saline animals ($p < 0.001$) indicating a decreased anxiety-like behavior in the NVHL animals. This behavior of lesioned animals was significantly attenuated by minocycline treatment ($p < 0.01$ compared to lesion saline) (Fig. 9E).

Temporal order memory

Neonatal VH lesioned animals were significantly impaired in temporal order memory. Two-way ANOVA on the discrimination ratio indicated a main effect of lesion [$F(1,20) = 15.21$,

$p < 0.001$] and lesion x minocycline treatment interaction [$F(1,20) = 5.02$, $p = 0.03$], but no main effect of minocycline [$F(1,20) = 2.14$, $p = 0.15$]. Post-hoc tests confirmed the memory deficit in saline-treated lesioned rats compared to saline-treated sham animal ($p < 0.01$). Neonatal minocycline administration had no significant effect in sham animals; however, its administration to the nVH lesioned rats significantly improved their temporal order memory performance ($p < 0.05$ compared to lesion saline) (Fig. 9F). No significant effect of lesion or minocycline on total exploration time of the objects was observed (lesion: [$F(1,20) = 0.1781$, $p = 0.6737$]; minocycline: [$F(1,20) = 0.0319$, $p = 0.8566$]; lesion x minocycline: [$F(1,20) = 0.0105$, $p = 0.9982$]) (Fig. 9G).

Gene expression in the mPFC of nVH lesioned animals following neonatal minocycline administration

Lastly, to assess the expression of genes related to microglial function, synaptic pruning and oxidative stress, mPFC tissue from the saline and minocycline treated sham and nVH lesioned animals from the above cohort were processed for qRT-PCR analysis. We selected one gene each from the microglia homeostasis, synaptic pruning and antioxidant defense system that we studied in the first cohort, section 3.4. A two-way ANOVA of microglia homeostasis gene, Cx3cr1 did not show any significant main effect of lesion [$F(1,19) = 0.014$, $p = 0.97$], minocycline treatment [$F(1,19) = 0.56$, $p = 0.46$] or lesion x minocycline treatment interactions [$F(1,19) = 0.02$, $p = 0.88$]. A two-way ANOVA of the synaptic pruning gene, C3 showed a non-significant trend of increase as reported in the first cohort [$F(1,19) = 3.24$, $p = 0.08$]. However, there were no significant effects of minocycline treatment [$F(1,19) = 0.85$, $p = 0.36$] or lesion x minocycline treatment interactions [$F(1,19) = 0.02$, $p = 0.86$]. Analysis of antioxidant defense system gene, catalase also did not show any significant main effect of lesion [$F(1,19) = 1.13$, $p = 0.30$], minocycline treatment [$F(1,19) = 3.09$, $p = 0.09$] or lesion x minocycline treatment interactions [$F(1,19) = 0.14$, $p = 0.70$]. Results are shown in Fig. 10.

Discussion

In the current study, we provide evidence that early-life lesion of the VH triggers microglial alterations and oxidative stress in distant and connected structures such as the mPFC, which may be responsible for the synaptic reorganization and cognitive and behavioral deficits in the nVH lesion rats. Consistent with reports that microglial functions are modulated by neural activity (reviewed in (Tay, Savage, et al., 2017), we believe that microglial impairments in the lesioned animals, beginning at early postnatal development and persisting at least until adolescence, result from the loss of VH inputs within the PFC. Our data showing a protective effect of early minocycline treatment on the behavioral deficits of lesioned rats provides functional support to the molecular and morphological changes observed in the microglia.

To investigate the mechanisms underlying the loss of dendrites and spines in the prefrontal cortical pyramidal neurons following the nVH lesion, we focused on microglia, immune cells which are responsible for the elimination and refinement of synaptic connections and maturation of dendritic spines in healthy developing brain (Hong et al., 2016; Mosser, Baptista, Arnoux, & Audinat, 2017; Tay, Savage, et al., 2017). This process is mediated, among others, through the classical C3/C1q complement pathway (Olincy & Stevens, 2007; Schafer et al., 2012). After phagocytosis, the digestion of synaptic components is regulated by autophagy-related protein 7 (Kim & Cho, 2017). Alteration in synaptic pruning or digestion during prenatal or postnatal brain development leads to synaptic and wiring abnormalities, as well as deficits in social interaction, repetitive behavior, grooming behavior, working and spatial memories in rodents (Kim & Cho, 2017; Lane et al., 2017; Squarzoni et al., 2014; Zhan et al., 2014). The first evidence for microglial homeostatic changes in our lesioned rats comes from the increased number of microglial clusters that we observed in the PFC of nVH rats at P20. Microglial clustering was previously identified in mice after axonal lesion surgery (Dissing-Olesen et al., 2007) and in patients with multiple sclerosis or head injury (Clark, 1974; van Horssen et al., 2012). Microglial clusters have been shown to strongly express human leukocyte antigen DR (HLA-DR), cluster of differentiation 68 (CD68), intercellular adhesion molecule-1 (ICAM1), cluster of differentiation 34 (CD34) and nuclear bromodeoxyuridine (BrdU) signals (Dissing-Olesen et al., 2007; van Horssen et al., 2012), suggesting that microglia within clusters might be proliferative, reactive, or phagocytic in the PFC of lesioned rats, which warrants further investigation. Notably, the

formation of clusters is not due to microglial response to programmed cell death in the PFC as assessed by activated caspase-3 immunostaining.

RT-PCR, and detailed microglial density, distribution, and morphological analyses were performed on confocal images to assess changes in microglial physiological activity in the PFC of lesioned rats. Consistent with the observation in the VH of lesioned rats (Drouin-Ouellet et al., 2011), increased microglial density and morphological index, previously associated with reactive phenotypes (Streit, Walter, & Pennell, 1999), were identified in PFC of lesioned rats at P20, suggesting increased phagocytic and pro-inflammatory activity. In support of a pro-inflammatory activity, we observed increased gene expression of Cd45 in the PFC of lesioned rats at P20. Expressed by microglia and bone-marrow derived monocytes, CD45 plays a key role as a regulator of inflammatory responses, modulating, for instance, the activation and proliferation of peripheral immune cells that include lymphocytes. Whether the infiltration of peripheral monocytes contributes to the increased density of IBA1-positive cells that we measured warrants further investigation. Furthermore, exacerbated phagocytic activity in terms of increased expressions of C1q and C3 was identified from RT-PCR experiments. Recent studies reveal that gene expression of complement component 4A (C4A), a component involved in synapse elimination during postnatal brain development, is elevated in post-mortem brain samples from schizophrenia patients (Sekar et al., 2016). As C4 has been shown to promote microglial phagocytosis through C3 activation, our data suggest that reactive microglia may contribute to the excessive removal of synapses previously observed in our nVH model (Flores, 2005; Ryan et al., 2013).

Ultrastructural analyses were conducted to assess, at a high spatial resolution, changes in microglial reactivity and phagocytic activity in the PFC of lesioned rats. Consistent with the qRT-PCR and fluorescent immunohistochemistry results, microglia were found to have thicker processes, measured by an increased profile area and perimeter at P20. An accumulation of cellular inclusions, vacuoles and extracellular digestion inside/around the microglial processes were also identified in PFC of lesioned rats at P20 from EM analysis, suggesting exacerbated phagocytic activity. Although phagocytic activity becomes less obvious after adolescence, as indicated by a decreased Trem2 gene expression without further increase in the accumulation of cellular inclusions and vacuoles, evidence of microglial reactivity in terms of higher density,

reduced process arborization area, and increased morphological index (Streit et al., 1999) are observed in the PFC of lesioned rats at P60. Differences in microglial processes ultrastructure were also observed at adolescence, particularly increased profile circularity and solidity associated with reduced ruffling and motility, which indicates lasting alterations in microglial phenotype and surveillance function. These data are consistent with the post-mortem and in vivo PET studies demonstrating exacerbated inflammation and microglial reactivity in the brain of adult schizophrenia patients as well as people at ultra-high risk of psychosis (Bloomfield et al., 2016; Laskaris et al., 2016).

Our gene expression analysis also proposes that oxidative stress may be triggered together with microglial reactivity in the PFC of lesioned rats, since lipopolysaccharide-stimulated microglial cells increase their expression of inducible nitric oxide synthase (iNOS) while downregulating the astroglial nuclear factor-erythroid 2-related factor 2 (Nrf2)-inducible antioxidant in culture (Correa, Mallard, Nilsson, & Sandberg, 2011; Kaneko et al., 2012). The expressions of antioxidant Gpx1 and Catalase are downregulated in the PFC of lesioned rats at P60, suggesting redox imbalance. These results are consistent with those of O'Donnell's group showing that increased oxidative response in the PFC is a potential mechanism that may affect cognition in the nVH lesioned animals (Cabungcal et al., 2014).

Considering the early exacerbation of microglial reactivity and phagocytic activity in the PFC of lesioned rats, we then investigated whether modulation of microglial activity by minocycline treatment just after the nVH lesion could prevent the behavioral deficits in post-adolescent rats. Although minocycline does not only target microglia, also affecting astrocytes, endothelial cells, and peripheral immune cells such as monocytes, the tetracycline derivative has been shown to provide anti-inflammatory, anti-apoptotic (i.e. suppression of neurodegeneration) as well as antioxidative effects in animal models and humans with neurodegenerative diseases such as Alzheimer disease, cerebral ischemia, and Parkinson's disease. Recently, minocycline was also shown to normalize microglial phagocytic activity in a mouse model of schizophrenia induced by prenatal challenge with the viral mimic Poly I:C (Mattei et al., 2017). We investigated schizophrenia-relevant behaviors that are influenced by the PFC, and in which deficits have previously been reported in nVH lesioned rats, e.g., novelty-induced locomotor activation, sensorimotor gating, anxiety-like behavior as well as temporal-order memory. Deficits in

temporal-order memory (i.e., memory of objects encountered more recently versus those encountered before) have not been previously described in this model. However, consistent with a role of the PFC-temporal cortex circuits in this behavior (Hannesson, Howland, & Phillips, 2004; Howland et al., 2008), we were not surprised to observe a deficit in the NVHL animals. Compared to the findings from Drouin-Ouellet and colleagues (Drouin-Ouellet et al., 2011), we have shown that minocycline is able to rescue the hyperlocomotion, reduced anxiety-like behavior, deficit in temporal order memory and PPI deficit in the post-adolescent lesioned rats. We also assessed gene expression in the mPFC of adult nVH lesioned and sham animals after neonatal minocycline to see if the behavioral changes may be related to alterations in genes associated with microglial homeostasis, synaptic pruning and anti-oxidant defense system. Although a trend of lesion-induced increase in the expression of C3 gene was noted in this cohort, neonatal minocycline treatment was not found to have any significant effect on the expression of genes studied (C3, Cx3cr1, and catalase). While this appears to be contrary to our expectation, it is possible that other synaptic pruning and microglia homeostasis genes or their protein products during other developmental time-points may be involved in the behavioral rescue by minocycline that we observed. Thus, measuring the expression of the genes during pre-puberty (P15-20) and their protein levels could further provide mechanistic insights in the behavioral rescue as a result of neonatal minocycline treatment. While an extrapolation of findings in rats to human neuropsychiatric disorders is at best speculative, it is interesting to note that many studies describe microglial 'activation' in schizophrenia brains (Laskaris et al., 2016), while clinical studies report reduction of the positive and negative symptoms in schizophrenia patients that received minocycline as an add-on treatment to antipsychotics (Solmi et al., 2017). Accordingly, microglial functions should be closely examined in developmental disorders involving cortical dysconnectivity.

Conclusion

In conclusion, our findings suggest that an early-life disconnection of the VH produces microglial alterations in distant and connected structures such as the PFC. The changes in microglia begin early after the VH lesion and persist through adulthood. While the mechanisms driving microglial changes are unknown, based on previous evidence, we speculate that a loss of

VH inputs to the developing PFC may be one of the signals leading to alterations in microglial physiology. These changes are functionally relevant since suppressing microglial activity after the lesion attenuated the appearance of some behavioral deficits induced by the lesion. Our data provide a potential mechanism by which the development of prefrontal neuronal functions and behaviors are compromised following an early damage to the VH. The findings also have implications for developmental neuropsychiatric disorders such as schizophrenia where a disruption of hippocampal connectivity as well as brain inflammation have been observed.

Acknowledgments and Disclosures

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Conflicts of interest

The authors declare no conflicts of interest.

Figures

Figure 1

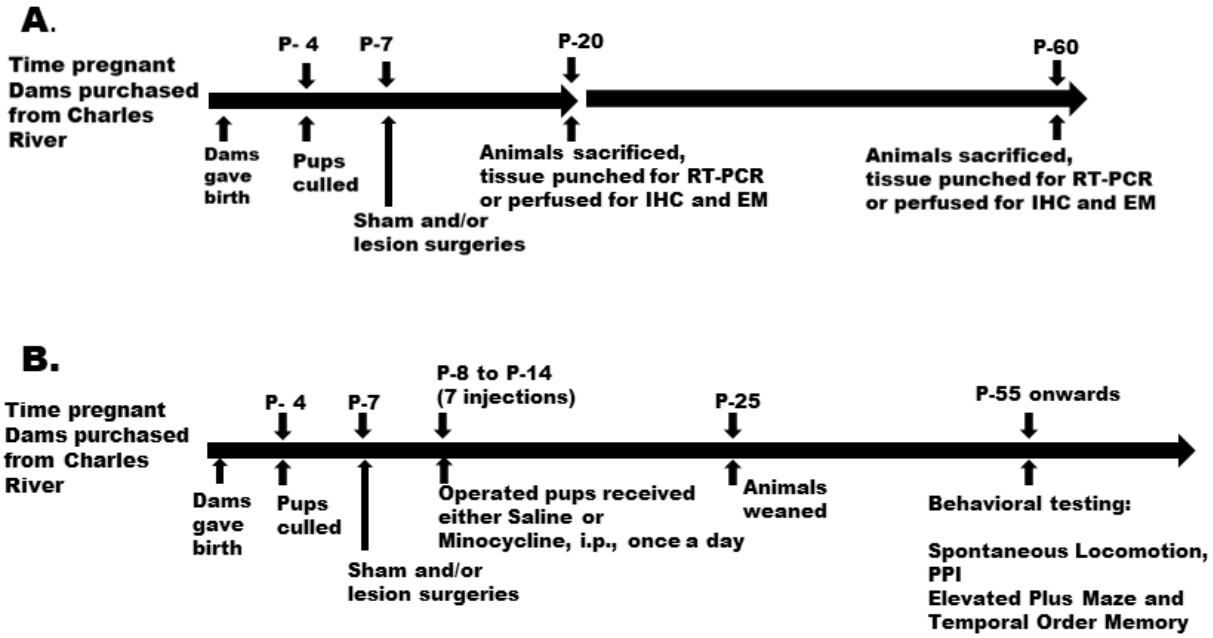


Figure 1. Experimental design. Male pups from Sprague-Dawley rat dams were operated for ventral hippocampal lesioning at P7. (A) For the analysis of microglial distribution and morphology, ultrastructure, as well as related gene expression, different cohorts of animals were sacrificed either by cervical dis-location or aldehyde perfusion at pre-pubertal (P20) and/or post-pubertal (P60) age and their brain were processed as explained in method section. (B) In an-other cohort of sham and lesioned animals, from P8-14, all operated pups were given either saline or minocycline. Animals were weaned at P25 and behavioral testing began at P55 onwards.

Figure 2

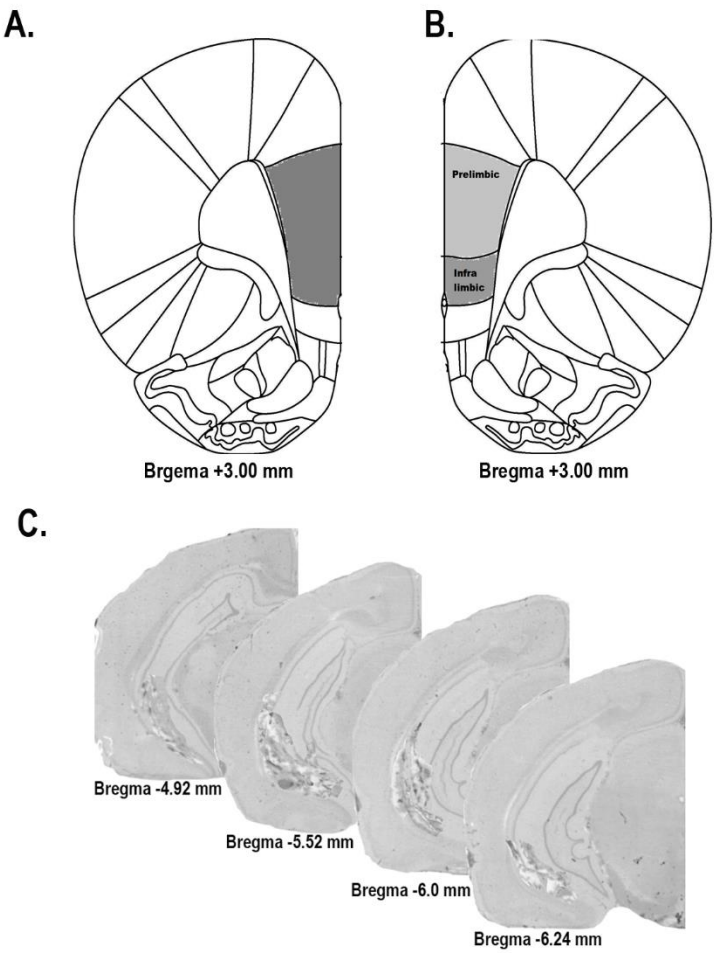


Figure 2. Schematic representation of the rat brain regions used to analyze microglia-related mRNA expression. (A) Specific medial prefrontal cortex brain region sampled for RT-PCR analysis is shown infilled areas. (B) Represents the anatomical boundaries from prelimbic as well as infralimbic brain regions, usedto study microglial morphology and ultrastructure in this study. (C and D) Shows the cresyl violet stained representative photomicrographs of P70 NVHL and sham brain sections, indicating ibotenic acid-induced neuronal loss and cavitation in the rostral to caudal aspects of the ventral half of the hippocampus in lesioned animals.

Figure 3

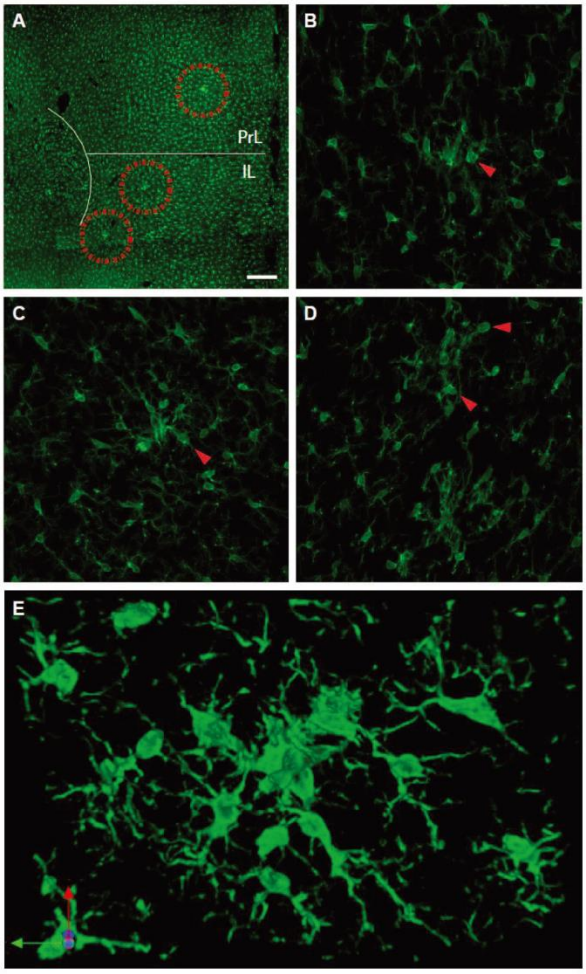


Figure 3. Confocal images showing microglial clustering in prefrontal cortex of lesioned rats at P20. (A) Low magnification (10x) confocal overview of IBA1-immunostaining in the prelimbic (PrL) and infralimbic (IL) regions showing the regular spacing and ramified morphology of microglia. Three representative clusters of microglial cells are circled. (B–D) Higher magnification confocal images showing examples of microglia with enlarged and rounder cell bodies (arrowheads) within the clusters, compared with the elongated and triangular shapes which are observed outside the clusters. (E) 3D reconstruction of a cluster captured with the confocal at high magnification showing direct contacts between the processes from neighboring microglia. Scale bar = 100 μ min (A).

Figure 4

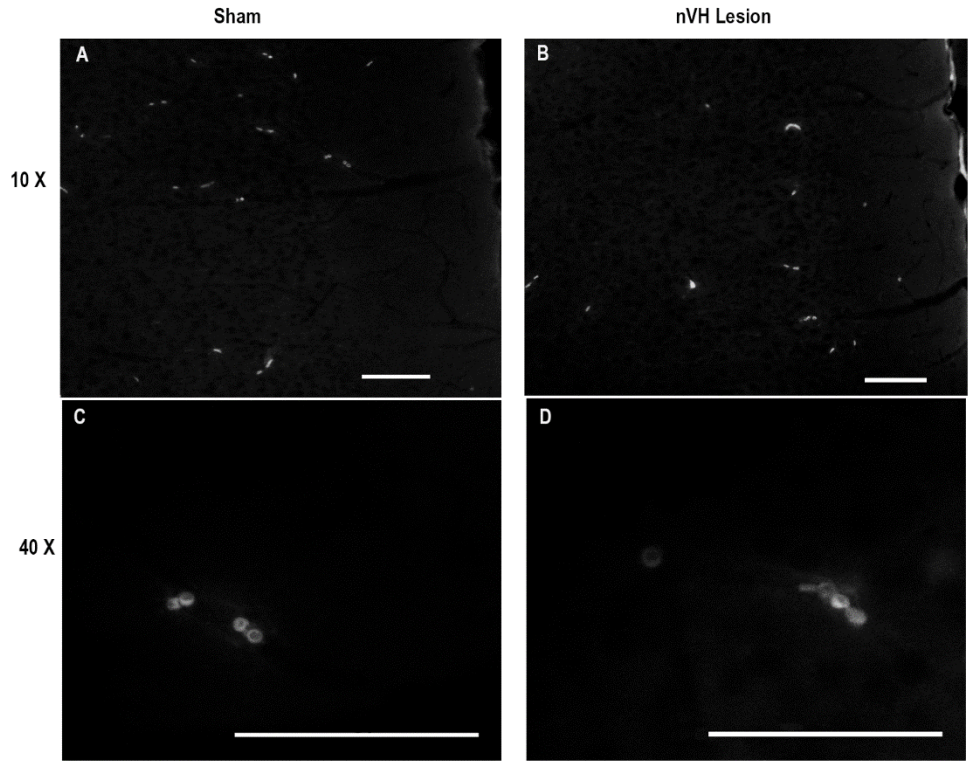
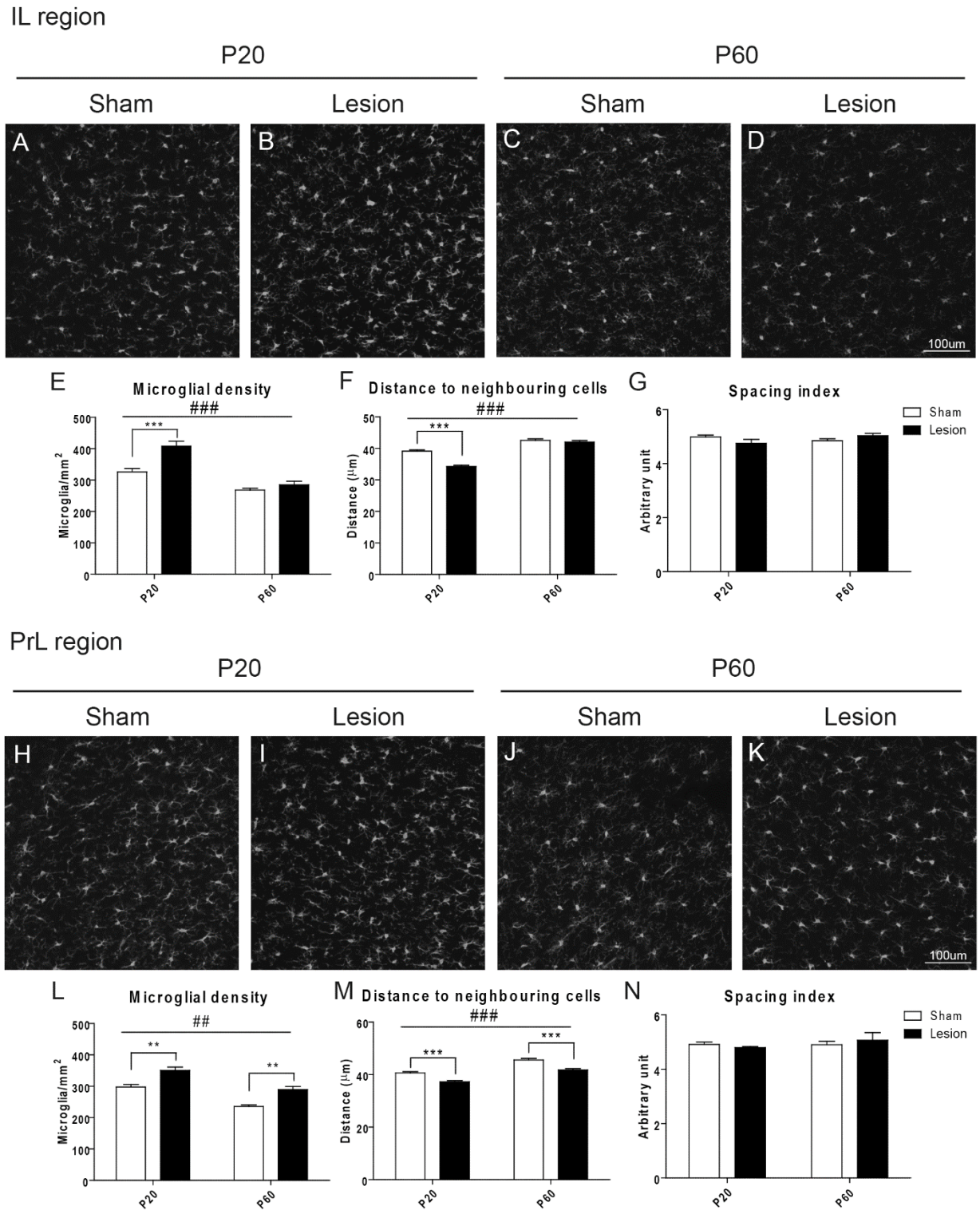


Figure 4. Immunohistochemical staining for cleaved Caspase-3 in the medial prefrontal cortex of sham and neonatal ventral hippocampus lesioned rats at postnatal day20. Representative images of sections from sham animals at 10X and 40X respectively (A and C). Representative images from lesioned rats at 10X and 40 X respectively (B and D). Scale bar: 100μm. (E) shows the quantification of the Caspase-3 positive cells from both sham and NVH lesioned animals at 10X magnification.

Figure 5


IL region
P20
P60
Sham
Lesion
Sham
Lesion
A
B
C
D
100um
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Microglial density
###
***
Microglia/mm 2
F
Distance to neighbouring cells
###
***
Distance (μm)
G
Spacing index
Arbitrary unit
Sham
Lesion
P20
P60
PrL region
P20
P60
Sham
Lesion
Sham
Lesion
H
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Microglia/mm 2
M
Distance to neighbouring cells
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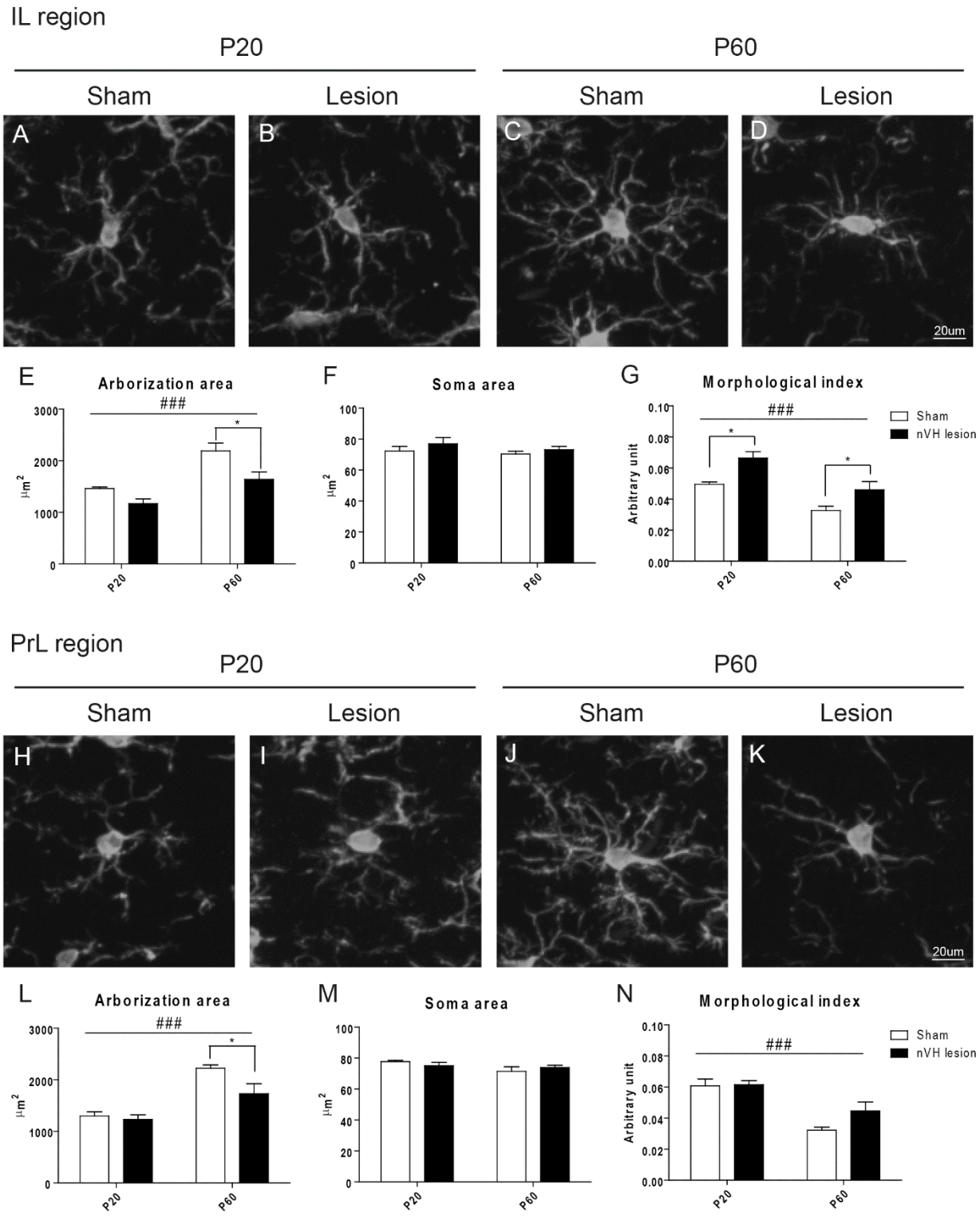
Distance (μm)
N
Spacing index
Arbitrary unit
Sham
Lesion
P20
P60

Figure 5. Effects of NVH lesion on microglial density and distribution in the IL and PrL regions. (A–D) Low magnification (20x) pictures showing the density ofIBA1-stained microglial cells in IL region. (E–G) Microglial density, distance to nearest neighboring cell and spacing index in IL region are shown. (H–K) Low magnification (20x) pictures showing the density of IBA1-stained microglial cells in PrL region. (L–N) Microglial density, distance to nearest neighboring cell and spacing index in PrL region are shown. n = 4 mice in all groups. Scale bar: 100μm**p< 0.01, ***p< 0.001 as compared between sham operated and lesioned animals at same time point, ##p< 0.01, ###p< 0.001 as a significant main effect of time in the parameters.

Figure 6


IL region
P20
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Sham
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F
Soma area
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G
Morphological index
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Arbitrary unit
P20
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Sham
nVH lesion
PrL region
P20
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Sham
Lesion
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Lesion
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Arborization area
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P20
P60
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Soma area
100
80
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20
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µm2
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P60
N
Morphological index
###
0.10
0.08
0.06
0.04
0.02
0.00
Arbitrary unit
P20
P60
Sham
nVH lesion

Figure 6. Effects of NVH lesion on microglial morphology in the IL and PrL regions. (A–D) Magnified pictures showing the morphology of IBA1-stained microglial cells in IL region. (E–G) Arborization area, soma area, and morphological index in IL region are shown. (H–K) Magnified pictures showing the morphology of IBA1-stained microglial cells in PrL region. (L–N) Arborization area, soma area, and morphological index in PrL region are shown. n = 4 mice in all groups. Scale bar: 20μm ***$p < 0.001$ as compared between sham operated and lesioned animals at same time point, ###$p < 0.001$ as a significant main effect of time in the parameters.

Figure 7

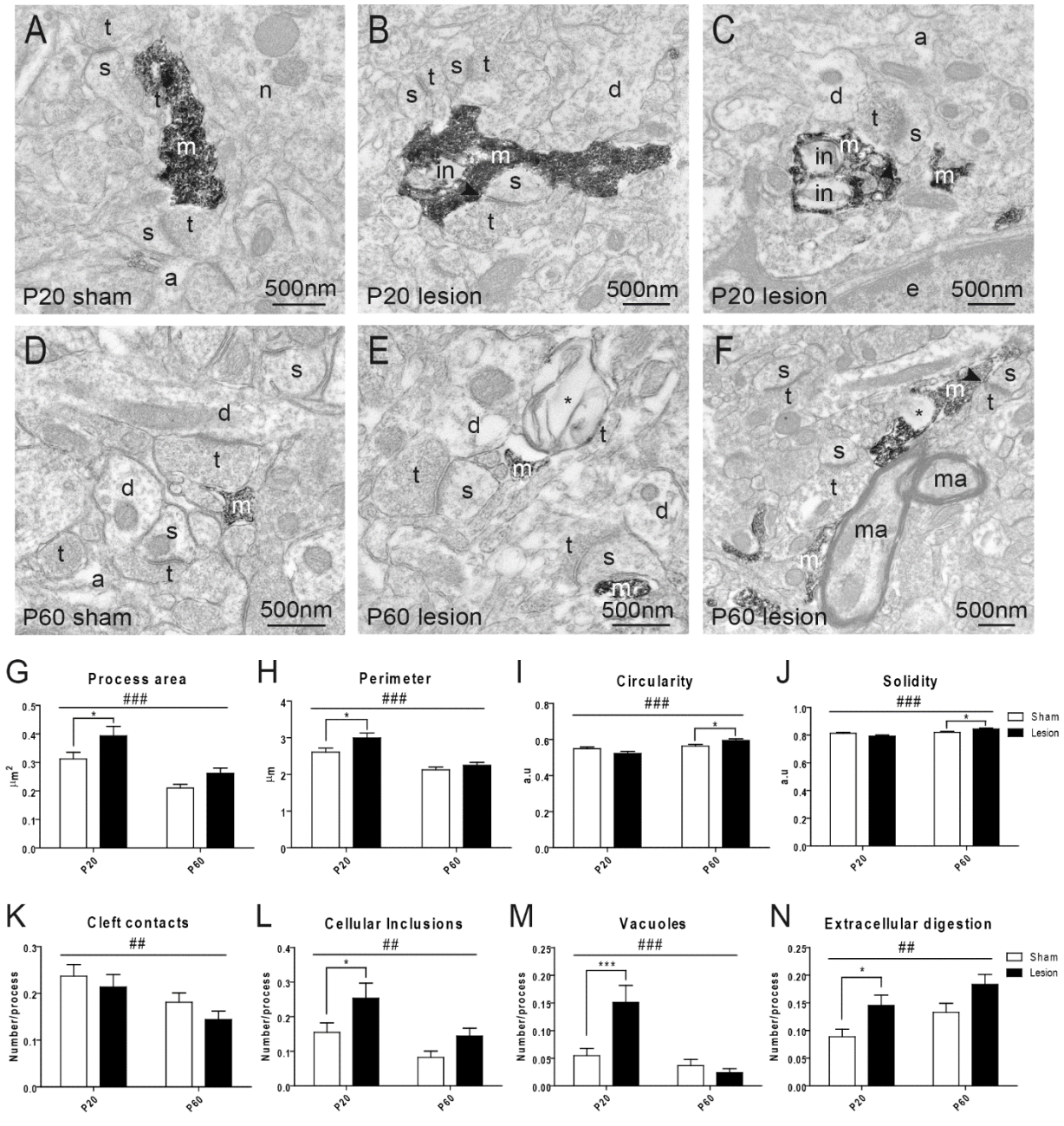

Figure 7. Effects of NVH lesion on microglial ultrastructure in the prefrontal cortical (IL + PrL) region. (A–F) Examples of processes from microglia (m) that show strong immunoreactivity for IBA1. Examples of cellular inclusions (in) are shown in B and C. Extracellular digestion (*) is shown in E and F. (G–N) Quantification of microglial process area,

perimeter, circularity, solidity, and numbers of synaptic cleft contacts, cellular inclusions, vacuoles and extra-cellular digestion. n = 351–458 profiles (IBA1-stained processes) in 4 mice in all groups. Arrowhead = synaptic cleft, s = dendritic spine, t = axon terminal, d = dendrite, a = peri synaptic astrocyte, n = nucleus, ma = myelinated axon.*p< 0.05, ***p< 0.001 as compared between sham operated and lesioned animals at same time point, ##p< 0.01, ###p< 0.001 as a significant main effect of time in the parameters.

Figure 8

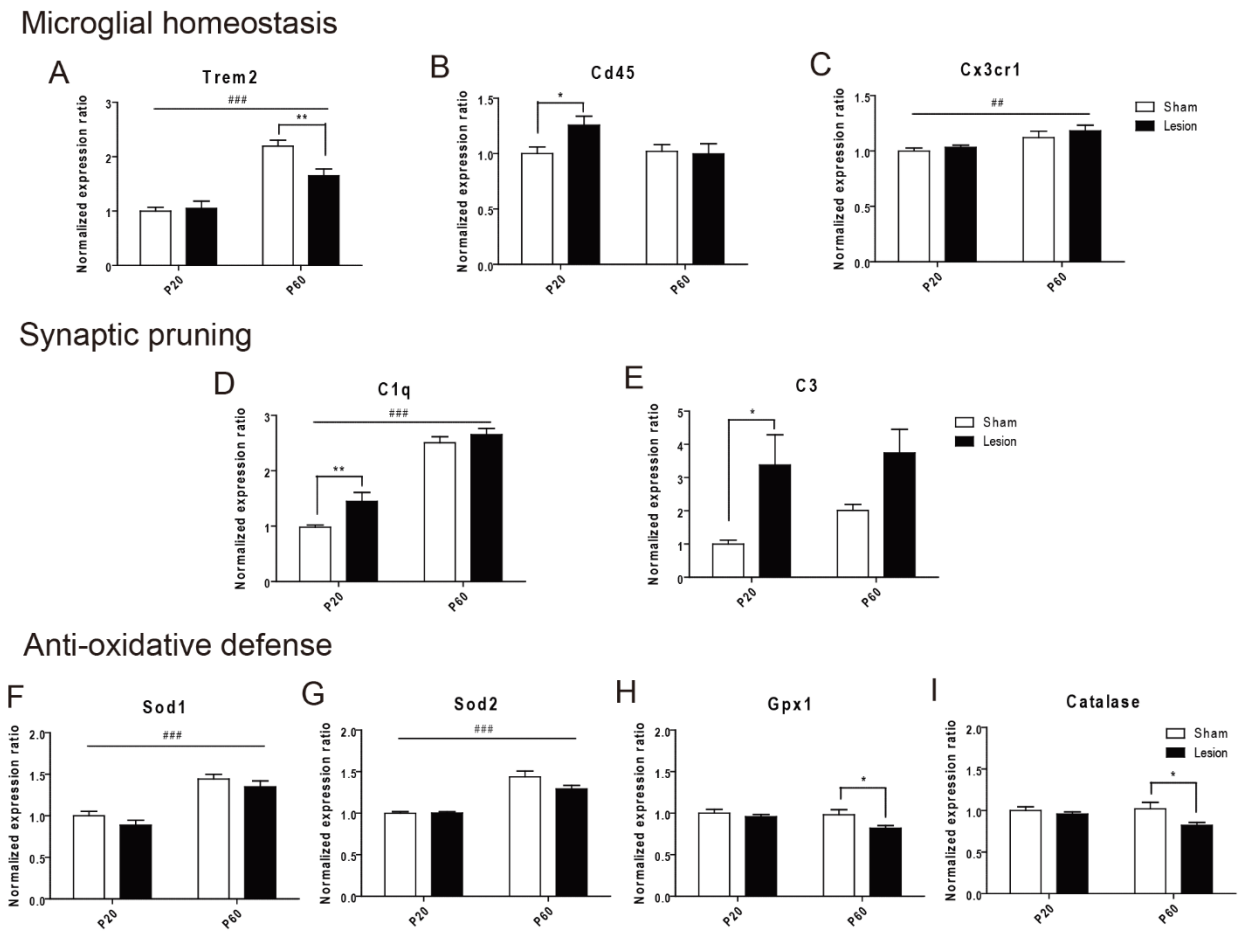

Figure 8. Gene expression studies in the PFC of nVH lesion rats. Genes related to(A–C) microglial homeostasis (Trem2, Cd45,Cx3cr1), (D–E) synaptic pruning function (C1q,C3) and (F–I) antioxidative defense (Sod1,Sod2,Gpx1,Catalase)were quantified by quantitative RT-PCR. *p< 0.05, **p< 0.01 as compared between sham operated and lesioned animals at same time point, ##p< 0.01, ###p< 0.001 as a significant main effect of time in the gene expressions.

Figure 9

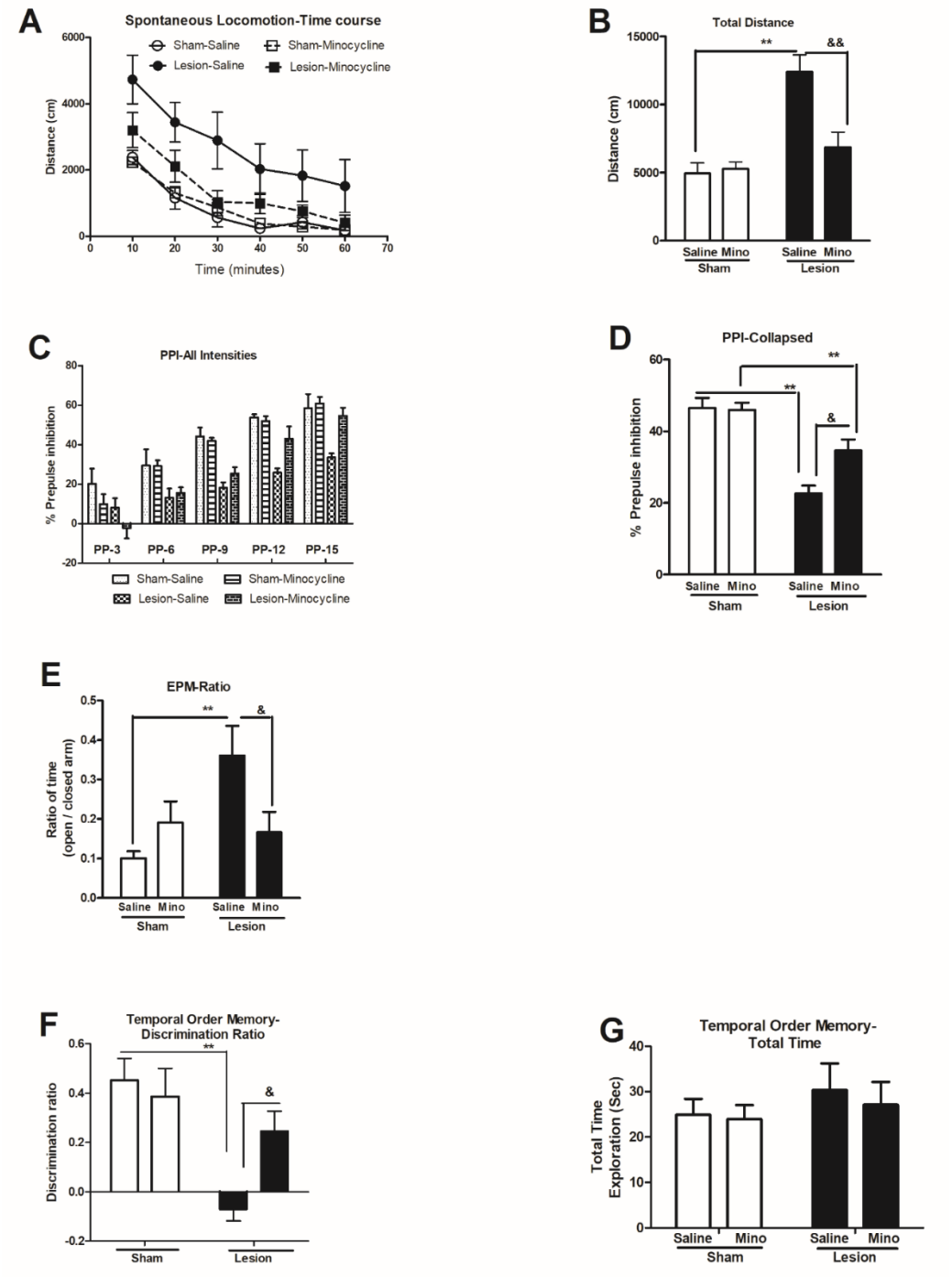

Figure 9. Protective effects of neonatal minocycline administration on behavioral deficits of adolescent NVH lesioned rats. (A–B) Spontaneous hyperlocomotion in NVH lesioned rats is rescued by neonatal minocycline treatment. (A) Time course of locomotor activity over 60 min

presented in 10 min bins. (B) Total distance traveled during the whole 60 min session. A two-way ANOVA revealed a significant lesion x minocycline treatment interaction; Tukey's post hoc test showed that lesion-induced increase in locomotor activity is attenuated by neonatal minocycline administration (&&)(C–D) PPI deficit of acoustic startle response in lesioned rats is rescued by neonatal minocycline treatment. (C) PPI across all prepulse (PP) intensities. (D) Prepulse inhibition collapsed across all PP levels. Two-way ANOVA showed significant Lesion x Mino interaction and post-hoc tests reveal a significantly higher PPI levels in minocycline-treated lesioned rats compared to saline-treated lesioned animals (&). (E) Alterations in anxiety-like behavior (ratio of the time spent in open vs. closed arms) in adolescent NVH lesioned rats is prevented by neonatal minocycline treatment. Two-way ANOVA showed a significant Lesion x Mino interaction and post-hoc test reveals significant reduction of open/closed arm time ratio in minocycline-treated lesioned animals compared to saline-treated lesioned rats (&). (F–G)Deficit in temporal order memory in NVH lesioned rats is attenuated by neo-natal minocycline treatment. (F) Two-way ANOVA of discrimination ratio showed a significant lesion x minocycline interaction. Post-hoc Tukey's showed significant attenuation of deficit by minocycline in lesioned rats (&: $p < 0.05$compared to Lesion-Saline group). (H) No significant difference between sham and lesioned rats in total time of exploration during the choice phase of the temporal order memory test.

Figure 10

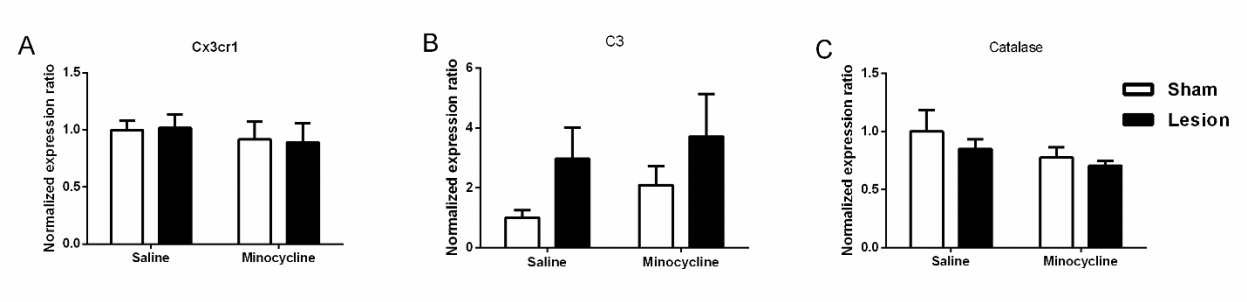

Figure 10. Gene expression study in the adult medial prefrontal cortex of neonatal saline or minocycline treated sham and nVH lesioned rats. Genes related to (A) microglial homeostasis (Cx3cr1), (B) synaptic pruning function (C3) and (C) anti-oxidative defense (Catalase) were quantified by quantitative RT-PCR. Two-way ANOVA failed to show a significant main effect lesion, minocycline treatment or lesion x minocycline treatment interaction.

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CONNECTING STATEMENT TO CHAPTER III

In chapter II, our results reveal increased microglial phagocytic activities, accompanied by early development specific increase in the expression of complement molecules (C1q and C3) induced by NVHL. The microglial alterations persist with a reduction of anti-oxidative defense during adulthood. Further, suppression of microglial reactivity by neonatal minocycline treatment was able to rescue some of the schizophrenia-related behavioral deficits in the adult rats.

These results suggested a dysregulation of neuro-immune function and an overload of oxidative stress within PFC of NVHL animals. Phagocytic microglia are a major source of inflammatory cytokines with in the brain. Pro- and anti-inflammatory cytokines are regulated by microglia (Wang et al., 2015); (Andreou et al., 2017) and a balance between them has been found to be important for neural functions (Spulber et al., 2009; Yamato et al., 2014). And since our second chapter implicated the role of microglia in synaptic pruning; we hypothesized that alterations in the pro- and anti-inflammatory cytokine balance in NVHL animals could be involved in synaptic and behavioral changes. This hypothesis was tested in the third chapter; we conducted the experiments using NVHL rats, measured the expression of pro and anti-inflammatory cytokines, behavioural outcomes and synaptic morphology.

CHAPTER III

Role of prefrontal cortex anti- and pro-inflammatory cytokines in the development of abnormal behaviors induced by disconnection of the ventral hippocampus in neonate rats

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Abstract

Neonatal disconnection of ventral hippocampus (VH) outputs in rats has been reported to lead to post-pubertal behavioral and synaptic changes of relevance to schizophrenia. Increased oxidative and inflammatory load in the prefrontal cortex (PFC) has been suggested to mediate some of the effects of neonatal VH lesion (NVHL). In this study, we hypothesized that developmental imbalance of anti- and pro-inflammatory factors within the PFC might affect synaptic development thus contributing to the adult NVHL-induced behavioral deficits. Ibotenic acid-induced excitotoxic NVHL was performed in postnatal day (PD) 7 male Sprague-Dawley rats and the mRNA levels of select pro- and anti-inflammatory cytokines were measured in the medial PFC (mPFC) at two developmental time points (PD15 and PD60). We observed a development-specific increase of pro-inflammatory cytokine, interleukin (IL)-1β mRNA at PD15, and an overall reduction in the expression and signaling of transforming growth factor beta 1 (TGF-β1), an anti-inflammatory cytokine, at both PD15 and PD60 in the NVHL animals. These cytokine changes were not seen in the somatosensory cortex (SSC) or tissue surrounding the lesion site. Daily administration of systemic recombinant TGF-β1 from PD7-14 prevented the appearance of hyperlocomotion, deficits in prepulse inhibition (PPI) of startle and social interaction (SI) in post-pubertal (PD60) NVHL rats. Neonatal supplementation of TGF-β1 was also able to attenuate dendritic spine loss in the layer 3 mPFC pyramidal neurons of NVHL animals. These results suggest that early damage of the VH has long-lasting inflammatory consequences in distant connected structures, and that TGF-β1 has potential to confer protection against the deleterious effects of developmental hippocampal damage.

Introduction

Disrupted functional connectivity between the hippocampus and the prefrontal cortex (PFC), presumably of developmental origin, is considered a core deficit in schizophrenia and other neurodevelopmental psychiatric disorders (Godsil et al., 2013; Bahner & Meyer-Lindenberg, 2017). In the rodents, monosynaptic glutamatergic afferents from the CA1 and subiculum of the ventral hippocampus (VH) to the medial PFC (mPFC; (Thierry et al., 2000), form a critical pathway mediating the communication between the two structures. This interaction between the VH and the mPFC begins early in life (first postnatal week) in the form of theta-gamma coupled oscillations (Brockmann et al., 2011). A number of studies have attempted to assess whether an early-life disruption of the VH output could be a mechanism underlying later development of abnormal behaviors and neural alterations observed in neurodevelopmental disorders such as schizophrenia. Indeed, several reports show that excitotoxic neonatal VH lesion (NVHL) in rat pups (PD7) leads to adult schizophrenia-related behavioral deficits in sensorimotor gating, social interaction (SI) and cognition (for reviews see (Marcotte et al., 2001; Tseng et al., 2009).

While the VH is connected to several cortico-limbic regions, it is interesting that NVHL animals exhibit numerous cellular and synaptic changes in the PFC at adulthood. For example, reduced dendritic spine density in layer 3 and 5 pyramidal neurons in the mPFC, imbalance in excitatory and inhibitory synaptic transmission (Flores, 2005; Ryan et al., 2013), impaired synaptic plasticity (Bhardwaj et al., 2014) and alterations in parvalbumin (PV) interneurons (Behrens et al., 2007) have all been reported in adult animals following NVHL. However, local mechanisms within the PFC that drive aberrant neural development and behavior in NVHL model are poorly understood. Increase in inflammatory responses and oxidative stress were suggested to play a critical role in the abnormal maturation of PV neurons in NVHL rats (Cabungcal et al., 2014). Consistent with this idea, we recently reported a development- specific effect of the NVHL on mPFC microglial reactivity and expression of genes implicated in oxidative stress and synaptic pruning (Hui et al., 2019). Given that a critical balance of pro- and anti-inflammatory cytokines is important for neural functions (Spulber et al., 2009; Yamato et al., 2014) and alterations in pro- and anti-inflammatory cytokines have been reported in the brain and cerebrospinal fluid (CSF) of schizophrenia patients (Trepanier, Hopperton, Mizrahi, Mechawar, & Bazinet, 2016; A.

K. Wang & Miller, 2018), we hypothesized that alterations in this balance in NVHL animals could be involved in synaptic and behavioral changes.

In the brain, pro- and anti-inflammatory responses are critically regulated by microglia (W. Y. Wang, Tan, Yu, & Tan, 2015; Andreou et al., 2017). Increased expression of pro-inflammatory cytokines like interleukin (IL)-1β, IL-6 and tumor necrosis factor-α (TNF-α) play a detrimental role on the development of the brain (Deverman & Patterson, 2009; W. Y. Wang et al., 2015). Overall the pro-inflammatory cytokines have a detrimental effect on dendritic spine development (Bitzer-Quintero & Gonzalez-Burgos, 2012). IL-1β and IL-6 are immediate early proteins secreted by microglia and have been found to induce oxidative stress and reduce dendritic length (Gilmore et al., 2004). Elevated levels of IL-6 within the brain leads to abnormality in the shape, length and distributing pattern of dendritic spines and also affects the excitatory/inhibitory synaptic neurotransmissions (Wei et al., 2012). TNF-α in conjunction with microglia is a key mediator affecting synaptic remodeling and thereby affecting long term potentiation (LTP) and long term depression (LTD; (Goverman, 2009; Kondo, Kohsaka, & Okabe, 2011). In contrast, anti-inflammatory cytokines belonging to the transforming growth factor beta (TGF-β) family as well as IL-10 and IL-1 receptor antagonist (IL-1RA) suppress inflammation and have been suggested to exert beneficial and neuroprotective actions (Dobolyi et al., 2012; Adzic et al., 2018). TGF-β1, in particular plays a role in neuronal development and synaptic plasticity as well as in neuroprotection and regulation of microglial physiology (Spittau & Krieglstein, 2012; Butovsky et al., 2014). For example, IL-1β induced microglial activation and its associated oxidative stress markers were found to be blocked by TGF-β1 (Basu, Krady, Enterline, & Levison, 2002), and upregulation of canonical TGF-β signaling pathway (via p-Smad-2/3) was found to contribute to quiescent microglia phenotype thereby reducing inflammatory overload with in central nervous system (CNS; (Abutbul et al., 2012). Administration of exogenous TGF-β1 human recombinant protein was able to rescue the LTP and object recognition memory deficits which were caused by inhibiting TGF-β signaling. TGF-β1 has also been implicated in schizophrenia; a gene-set enrichment analysis of genome-wide association analysis data showed one of the pathways being related to TGF-β (Jia, Wang, Meltzer, & Zhao, 2010).

In this study, we assessed pro- and anti-inflammatory cytokines in the mPFC of NVHL rats and asked whether neonatal administration of recombinant TGF-β1 could rescue the behavioral and cellular deficits observed in the lesioned rats at post-pubertal ages.

Materials and Methods

Neonatal Ventral Hippocampal Lesion (NVHL) and TGF-β1 Treatment

This study was carried out in accordance with the guidelines of the Canadian Council of Animal Care. The protocol was approved by the McGill University Animal Care Committee. Twenty pregnant female Sprague-Dawley rats at 15–18 days of gestation were obtained from Charles River Laboratories (QC, Canada). Rat dams were housed individually on a 12 h light-dark cycle with ad libitum food and water in a temperature and humidity-regulated room, where they were allowed to give birth. On PD7, male pups were weighed (15–17 g) and were randomly selected for neonatal ventral hippocampal lesion (NVHL) or sham surgery according to our previously described procedures (Flores et al., 1996; Ryan et al., 2013). Briefly, pups were anesthetized by hypothermia (by covering in crushed ice for 18–20 min) and secured on a modified platform fixed to Kopf stereotaxic apparatus. The "lesion" group received bilateral infusion of 0.3 μl ibotenic acid (Catalog No. 0285; Tocris; 10 μg/μl in 0.1 M phosphate-buffered saline (PBS) over a period of 2 min using a 30-gauge needle connected to an infusion pump, while the "sham" group received the same volume of PBS into the VH (coordinates: AP −3.0 mm from Bregma, ML ±3.5 mm from midline, and DV −5.0 mm from dura). Following this, the skin was sutured using vetbond tissue glue and the pup's ears were tagged. After surgery, pups were placed on a heating pad until full recovery and returned to their respective mothers.

Separate cohorts of sham and lesioned animals were used for measurement of cytokines level (IL-1β, IL-6, TNF-α, IL-10, TGF-β1 and IL-1RA) and TGF-β1 signaling proteins at neonatal (PD15) and young adult (PD60) ages. Another cohort of sham and lesioned animals were randomly divided into two groups, one receiving recombinant TGF-β1 (Catalog No. T7039; Sigma (200 ng/kg; i.p.) or equivalent volume of saline. TGF-β1 was administered 2 h before NVHL surgery and continued for next 7 days (from PD8-PD14). At PD21 the animals were weaned and housed in pairs. At adulthood (PD60), all four groups of animals (Sham-Saline,

Sham-TGF-β1, Lesion-Saline and Lesion-TGF-β1) were subjected to behavioral tests followed by cytokine measurements.

RNA Extraction and Cytokine Gene Expression Using QRT PCR

Animals were sacrificed by decapitation and their brains were removed and sliced into 1 mm thick slices. From one cohort of animals (Sham and lesion) medial PFC (infralimbic and prelimbic cortices) and two control brain regions (Somatosensory cortex (SSC) and the region around VH) were micropunched and the tissues of both hemispheres from each animal were pooled and stored at −80°C until use. Another cohort of animals which were neonatally administered with saline or recombinant TGF-β1 was sacrificed after the behavioral tests. Their brains were extracted and only mPFC micropunched and stored at −80°C until further use. RNA extraction was performed using Trizol reagent (Catalog No. 15596026; ThermoFisher Scientific) with the Purelink RNA minikit (Catalog No.12183018A; ThermoFisher Scientific). Yield and the quality of the RNA was determined using Nanodrop and agarose gel electrophoresis. Two micrograms of RNA was used for the cDNA synthesis using the high capacity cDNA reverse transcription kit (Catalog No. 4368814; Applied Biosystem).

Primers for IL-1β (F-CACCTCTCAAGCAGAGCACAGR-GGGTTCCATGGTGAAGTCAAC), IL-6 (F-GCCCTTCAGGAACAGCTATGA R-TGTCAACAACATCAGTCCCAAGA), TNF-α (F-CCAGGAGAAAGTCAGCCTCCT R-TCATACCAGGGCTTGAGCTCA), TGF-β1 (F-TGGCGTTACCTTGGTAACC R-GGTGTTGAGCCCTTTCCAG), IL-10 (F-AAAGCAAGGCAGTGGAGCAG R-TCAAACTCATTCATGGCCTTGT), IL-1RA (F-GAGACAGGCCCTACCACCAG R-CGGGATGATCAGCCTCTAGTGT) and a housekeeping gene GAPDH (F-AGCCCAGAACATCATCCCTG R-CACCACCTTCTTGATGTCATC) were designed using the NCBI DNA sequence and the primer express software. QRT PCR was performed using SYBR green PCR master mix (Catalog No. A6001; Promega) according to the manufacturer's protocol. The following cycling conditions were used in Applied Biosystem Real time PCR 7,500 machine. Initial denaturation at 95°C for 10 min, followed by 40 cycles with denaturation at 95°C for 15 s, annealing at 60°C for 1 min and elongation at 72°C for 1 min. A standard and a melting curve for all the genes were obtained to check the efficiency of the primers. $2^{-\Delta\Delta CT}$ method was used to calculate the fold changes.

Immunoblotting for TGF-β1 Signaling Proteins

Sham and NVHL animals at PD60 were sacrificed by decapitation and the brains were sliced into 1 mm thick slices. The area corresponding to the prelimbic and infralimbic mPFC was rapidly dissected and homogenized in Tris ethylenediaminetetraacetic acid (EDTA) buffer (containing phenylmethylsulfonyl fluoride (0.2 mM), leupeptin (1 mM), pepstatin (1 mM), sodium orthovanadate (1 mM) and SDS (0.1%)). Western blotting was done as described previously (Bhardwaj et al., 2014). A 15 μg protein was electrophoresed on 4%–20% Tris-glycine gels and blotted to nitrocellulose membranes. The blots were incubated with 1:2,500 dilution of rabbit polyclonal antibody against phospho-Smad-2/3 (Catalog No. 8828S; Cell Signaling) or total-Smad-4 (Catalog No. 49515S; Cell Signaling) followed by anti-rabbit IgG: peroxidase-linked second antibody (Catalog No. 7074; Cell Signaling, 1:2,500). The blots were developed using chemiluminescence detection system (Catalog No. NEL103E001EA; Perkin-Elmer) and exposed to X-ray film. After phospho-Smad-2/3 probing, the blot was stripped and probed for total-Smad-2/3 (Catalog No. 5678S; Cell Signaling) followed by restriping and reprobing for α-tubulin antibody (Catalog No. T5168; Sigma, 1:5,000). Relative optical densities (ROD) of bands were analyzed on image analysis system (MCID-4, Imaging Research).

In order to verify whether exogenously administered TGF-β1 at neonatal ages directly modifies TGF-β signaling in the brain, another set of control animals were injected with either recombinant TGF-β1 (5 μg) or saline intraperitoneally at PD7 and the animals were sacrificed 2 h later. The mPFC was extracted and homogenized to measure the level of Smad-2/3 phosphorylation by Western blotting as described above.

Behavioral Testing

Behavioral testing was performed at PD60; the same sets of animals were used for all behavioral tests except a few animals which had to be excluded due to ill-health. The tests were performed during the light phase of the normal 12 h light-dark cycle (lights on at 8:00 and off at 20:00) starting with the least stressful, in the order presented below. Tests were separated by at least 72 h.

Spontaneous Locomotor Activity

The spontaneous locomotor activity was assessed as described previously (Bhardwaj et al., 2012), using acrylic activity chambers (AccuScan Instruments Inc., Columbus, OH, USA; L × W × H = 40 × 40 × 30 cm) in a dimly lit room. The chambers area was equipped with infrared sensors. Animals were brought from their home environment to the testing room and immediately placed in the activity boxes where their activity was monitored during the next 60 min. Data was collected using the Versamax Software. The total horizontal activity in the whole session (60 min) was used to analyze the locomotor behavior.

Social Interaction (SI)

The three-chamber method as described previously (McKibben, Reynolds, & Jenkins, 2014) was used to measure the sociability of the rodents with a conspecific as well as social discrimination memory of familiar vs. novel conspecific rat. Two wire mesh cages (20 cm × 20 cm × 15 cm) were placed in two chambers on the right and left of the central chamber and the central chamber was devoid of any objects. This method involved three steps; one, habituation where the test animals were placed in the central chamber and allowed to explore all the chambers for 10 min. After this the animals were placed back in their home cage. For the testing procedure, a stranger (S1) or an unfamiliar rat (same strain, sex and age) was placed in any one of the wire mesh cages and the test animals were reintroduced in the central chamber and allowed to explore all the chambers for 5 min. The interaction between the test animal and the stranger (S1) as compared to the empty wire mesh cage was a measure of sociability. For the measure of social novelty preference or social memory, after the first 5 min of interaction, the test animal was placed back in the home cage for 5 min (Retention interval) during which another novel stranger (S2) or unfamiliar rat was introduced in the second wire mesh cage.

After the retention interval, the test animal was reintroduced into the central chamber and was allowed to explore all the chambers for 5 min. The animal behavior was recorded and the interaction with S1 and S2 rat was measured and scored as sociability and social memory respectively. The interaction between the two rats was measured by nose contacts, sniffing within a distance of 1 cm. The videos were scored in a “blinded” manner for the total time spent interacting. This time measured was referred to as exploration time. Sociability and social memory analysis were carried out by calculating the exploration ratio. The sociability

exploration ratio was calculated with the following equation: time spent with stranger 1 (S1)/Total time of interaction (S1 + empty mesh cage) * 100. The following equation was used to measure social memory: time spent with the novel animal (S2)/Total time of interaction (S1+ S2) * 100. Total time of interaction is the time spent by the test animal in interacting with the familiar animal (S1) and the novel animal (S2).

Prepulse Inhibition (PPI)

As described previously (Bhardwaj et al., 2012), we used a commercially available system (SR-LAB; San Diego Instruments, San Diego, CA, USA) equipped with a cylindrical Plexiglas animal enclosure and a small electric fan, which generated a 70-dB background noise and provided ventilation to measure prepulse inhibition (PPI) in sound-attenuating chambers. Sound pressure levels (dB(A) weighting) were measured at the position of the rat's ears. Broadband noise pulses were presented via a speaker positioned directly above the animal. An accelerometer affixed to the animal enclosure frame was used to detect and transduce motion resulting from the animal's response. Noise pulse parameters were controlled using SR-LAB software, which also recorded responses. Animals were acclimated to the enclosure for 5 min before being tested during 37 discrete trials. On the first two trials, the magnitude of the startle response to a 120-dB white noise pulse was measured. These first two startling pulses were presented to habituate the animals to the testing procedure and thus were omitted from the data analysis; all subsequent trials were included in analysis. On the subsequent 35 trials, the startle pulse was either presented alone or 100 ms after the presentation of 30 ms prepulse. Acoustic startle response (ASR) to the pulse was measured following trials with prepulse intensities of 6, 9, 12 and 15 dB above background noise. Prepulses were varied randomly between trials, and each prepulse was presented five times; animals were randomly presented with the startle pulse alone during the other 10 trials. The average inter-trial interval was 15 s (range, 5–30 s). Startle responses were determined automatically by the SR-LAB analysis suite. Startle magnitude was calculated as the average of the startle responses to the pulse-alone trials. PPI was calculated according the formula: %PPI = 100 − (startle response for prepulse + pulse trials/startle response for pulse alone trials) × 100%.

Golgi Cox Staining

After the behavioral testing, few animals (n=3-4) from each group were used for Golgi-Cox staining to assess the morphological changes including dendritic complexity and spine density in mPFC following NVHL and TGF-β1 administration. 48–72 h after the last behavior testing, animals were rapidly decapitated, and the brains were extracted and washed with chilled Milli-Q water. Golgi impregnation was performed according to the specifications of the FD Rapid GolgiStain kit (Catalog No. PK401FD; NeuroTechnologies, INC) with the following optimizations: whole brains were treated for silver impregnation for 12 days, cryoprotected with 30% sucrose solution for 72 h, and sectioned at 200 μm in a vibratome in a 6% sucrose solution. Brain sections were mounted on gelatin-coated slides, lightly pressed and kept in moist container until developed, clarified, and then cover slipped using Permount following FD Rapid GolgiStain kit guidelines. The layer III pyramidal neurons (10 neurons per animal) from the mPFC were traced and a three-dimensional reconstruction of the neurons was carried out using the Neurolucida software (Leica microscope). Mean values of total basilar dendritic length and spine density were calculated. For dendrite arborization pattern, Sholl analysis (number of intersections per each radius 5 μm) was employed as described previously (Baharnoori, Brake, & Srivastava, 2009).

Histological Examination

Following sacrifice and brain extraction, 35 micron coronal sections at the level of the VH were mounted on pre-coated microscope slides and stained with cresyl violet staining solution (0.5%) for verification of the lesion. Only animals with bilateral lesions confined to the VH with no significant damage to the dorsal part or adjacent thalamic nuclei were included in data analyses.

Data Analysis

All reported values are mean ± standard error of the mean (SEM). All data except dendritic morphology were analyzed using Prism (GraphPad, Version 6). Student t-test (two tailed) was used to determine gene and protein expression changes in sham and lesioned animals. Two-way

ANOVAs followed by tukey's post hoc tests were used to determine interactions between lesion status and TGF-β1 treatment. Three-way ANOVA was used to determine interaction between lesion, TGF-β1 treatment, timeline of horizontal activity and prepulse intensities. General Linear Model (repeated measure) in SPSS was used to analyze the interaction of lesion and TGF-β1 treatment on dendritic morphology (Sholl analysis). Greenhouse-Guiser corrected F and df are reported for the general linear model analysis. For all analysis $p < 0.05$ was considered statistically significant.

Results

Lesion Verification

As reported earlier from our lab (Bhardwaj et al., 2012, 2014), NVHL animals showed bilateral neuronal loss, retraction, and cavitation's in the ventral half of the hippocampus including the CA1 (Figure 1) of NVHL animals, but not of sham-operated animals. The lesion spared the dorsal hippocampus and the adjacent nuclei (i.e., amygdala and thalamus).

An Imbalance in the Expression of Pro- and Anti-inflammatory Cytokines in PFC of NVHL Rat

Analysis of the mRNA expression data at PD15 (Figure 2) showed a significant increase in the expression of pro-inflammatory marker IL-1β in mPFC of the NVHL rats ($t = 4.839$, $df = 7$, $p = 0.0019$); however, the expression of the other pro-inflammatory cytokine, IL-6 ($t = 1.350$, $df = 3$, $p = 0.2697$) and TNF-α ($t = 2.672$, $df = 3$, $p = 0.0756$) were not affected. On the contrary, we found a significant reduction in the expression of anti-inflammatory marker TGF-β1 ($t = 5.735$, $df = 2$, $p = 0.0291$) in lesioned animals. The expression of other anti-inflammatory markers, IL-10 ($t = 0.6837$, $df = 2$, $p = 0.5647$) and IL-1RA ($t = 1.828$, $df = 2$, $p = 0.2090$) remained comparable between sham and lesioned animals. At young adulthood (PD60), data analysis did not reveal any significant effect of lesion on the expression of IL-1β ($t = 0.2532$, $df = 4$, $p = 0.8126$), IL-6 ($t = 0.3160$, $df = 4$, $p = 0.7678$), TNF-α ($t = 0.6061$ $df = 4$ $p = 0.5771$), IL-10 (t =

1.295 df = 4, p = 0.2652) and IL-1RA (t = 0.3093, df = 4, p = 0.7725), whereas a significant reduction in TGF-β1 expression (t = 5.222, df = 4, p = 0.0064) in NVHL rats still persisted.

The data analysis from the area around VH showed no significant difference between the sham and lesion animals in the expression of IL-1β (t = 1.948, df = 3, p = 0.1466), IL-6 (t = 1.186, df = 3, p = 0.3212), TGF-β1 (t = 1.580, df = 3, p = 0.2122), IL-1RA (t = 1.024, df = 3, p = 0.3813) at PD15 or at PD60 (IL-1β (t = 0.9057, df = 3, p = 0.4318); IL-6 (t = 0.6960, df = 3, p = 0.5365); TGF-β1 (t = 0.5643, df = 3, p = 0.6120); IL-1RA (t = 2.742, df = 3, p = 0.0712)). The data analysis from PD15 sham and lesioned animals from SSC brain region did not reveal any significant effect on the expression of IL-1β (t = 1.332, df = 3, p = 0.2749), IL-6 (t = 1.015, df = 2, p = 0.4169), TGF-β1 (t = 0.3362, df = 3, p = 0.7589) and IL-1RA (t = 1.403, df = 3, p = 0.2552). Similar to the neonatal (PD15) group, the data analysis from postpubertal (PD60) animal's SSC also did not reveal any significance between sham and lesioned animals; (IL-1β (t = 2.005, df = 3, p = 0.1386), IL-6 (t = 0.9785, df = 3, p = 0.4000), TGF-β1 (t = 1.282, df = 3, p = 0.2900) IL-1RA (t = 0.9438, df = 3, p = 0.4149)). The results are shown in Figure 3.

Decrease in TGF-β Signaling in the mPFC of NVHL Animals

In order to further investigate whether the decrease in mPFC TGF-β1 mRNA expression has functional significance, we assessed TGF-β canonical signaling components, phospho-Smad (p-Smad-2/3), total-Smad (t-Smad-2/3) and Smad-4 from mPFC, using Western blotting in PD60 sham and lesioned rats. Consistent with mRNA levels, data analysis revealed a significant decrease in the normalized phosphorylated Smad-2/3 protein levels in NVHL rats compared to sham rats (t = 4.53, df = 8, p = 0.002). No significant change in the levels of t-Smad-2/3 (t = 1.04, df = 8, p = 0.32) or Smad-4 (t = 0.4509, df = 10, p = 0.6617) was observed. The results are shown in Figure 4.

Systemic Neonatal TGF-β1 Administration Modulates TGF-β Signaling in Brain

In order to assess if exogenous TGF-β1 treatment at neonatal age affects TGF-β signaling in the brain, we gave single high dose (5 μg) of TGF-β1 or saline intraperitoneally at PD7 to control rat

pups. Two hours after the injection, pups were sacrificed, brains were removed and their mPFC was isolated and processed for phospho-Smad-2/3 Western blotting. Student's t-test analyses of protein levels of phospho-Smad-2/3 ROD data (normalized to α-tubulin) showed a significant increase ($t = 3.607$, $df = 6$, $p = 0.011$) in the protein levels of phospho-Smad-2/3 following TGF-β1 administration compared to saline injections (Figure 5B).

The Effect of Neonatal Recombinant TGF-β1 Administration on the Behavior of NVHL Animals at PD60

Locomotor Activity

A three-way repeated measure ANOVA on horizontal activity of all the animals over time showed no significant three-way interaction on lesion × TGF-β1 treatment × timeline of horizontal activity ($F(1,605) = 0.53$, $p = 0.883$; Figure 6A). Further analysis was performed on the total horizontal activity for the whole session (Figure 6B), which showed a sustained increased activity in saline treated lesioned animals. More specifically, two-way ANOVA of the data for the whole session (i.e., 60 min) revealed a significant main effect of lesion ($F(1,55) = 9.96$, $p = 0.002$), TGF-β1 treatment ($F(1,55) = 7.59$, $p = 0.008$) and lesion × TGF-β1 interaction ($F(1,55) = 5.85$, $p = 0.018$). As reported previously in this model, post hoc tukey's test revealed hyper-locomotion in saline-treated NVHL animals compared to saline-treated sham animals ($p = 0.0021$). Further post hoc analysis showed that while neonatal TGF-β1 treatment had no significant effect in sham animals, it led to a significant attenuation of locomotor activity in NVHL animals as compared to lesion-saline animals ($p = 0.0031$).

Social Interaction (SI)

Two-way ANOVA for sociability did not reveal any significant main effect of lesion ($F(1,37) = 0.1343$, $p = 0.7161$), treatment ($F(1,37) = 0.09869$, $p = 0.7552$) and lesion × TGF-β1 treatment interaction ($F(1,37) = 2.218$, $p = 0.1448$); indicating that all groups of animals spend about the same amount of time interacting with S1 animal (Figure 6C). Social memory measured from PD60 sham and NVHL animals showed a significant main effect of TGF-β1 treatment ($F(1,37) = 7.782$, $p = 0.0083$) and lesion × TGF-β1 treatment interaction ($F(1,37) = 6.166$, $p = 0.0177$) but no significant main effect of lesion ($F(1,37) = 3.565$, $p = 0.0669$). Post hoc tukey's test showed a

significant reduction in social memory in the saline treated NVHL animals compared to the Sham-saline group ($p = 0.0249$). This reduction in social memory was significantly reversed after the neonatal administration of TGF-β1 in lesioned animals ($p = 0.0040$) compared to lesion-saline group (Figure 6D).

Prepulse Inhibition (PPI) of Acoustic Startle Response (ASR)

A three-way repeated measure ANOVA on %PPI across all PP intensities (PP6-15) did not show any significant lesion × TGF-β1 treatment × PP intensities interaction ($F(1,96) = 1.10$, $p = 0.35458$; Figure 6E). %PPI data with all PPs collapsed were then analyzed using a two-way ANOVA (Figure 6F). Analysis from %PPI on collapsed prepulse intensities showed a significant main effect of lesion ($F(1,32) = 15.35$, $p = 0.0004$), and lesion × TGF-β1 treatment interaction ($F(1,32) = 5.29$, $p = 0.027$) but no TGF-β1 treatment ($F(1,32) = 0.047$, $p = 0.829$). Tukey's post hoc tests showed that there is a significant deficit in %PPI in lesion-saline animals compared to sham-saline animals ($p = 0.001$). Further post hoc test revealed that neonatal TGF-β1 treatment had no effect on PPI deficit on any group of animals (Figure 6F). Two-way ANOVA on ASR data did not reveal any significant main effect of either lesion ($F(1,31) = 0.2166$, $p = 0.6449$), treatment ($F(1,31) = 1.325$, $p = 0.2585$) or lesion × TGF-β1 treatment interaction ($F(1,31) = 1.085$, $p = 0.30560$; Figure 6G).

Dendritic Complexity and Spine Density

We further examined the dendritic complexity and spine density of adult sham and NVHL animals following the neonatal TGF-β1 treatment. Figure 7A shows the representative photomicrographs of Golgi-stained mPFC pyramidal neurons of saline as well as TGF-β1 administered sham and lesioned animals at adulthood. Two-way analysis on dendritic spine density (number of spines per 10 μm) revealed a significant main effect of lesion ($F(1,10) = 10.28$, $p = 0.009$) and lesion × TGF-β1 treatment interactions ($F(1,10) = 7.07$, $p = 0.02$) but no main effect of TGF-β1 treatment ($F(1,10) = 3.108$, $p = 0.1084$). As reported previously, post hoc tukey's test revealed, that spine density was significantly reduced from layer III pyramidal neurons of saline treated NVHL animals compared to sham-saline animals ($p = 0.002$). Further, data analysis revealed that while neonatal TGF-β1 treatment had no effect in sham animals, it

rescued the spine density loss in lesion-TGF-β1 animals compared to lesion-saline group ($p = 0.010$). Two-way ANOVA on the dendritic length (μm) between sham and lesion animals however, did not show significant main effect of lesion ($F(1,10) = 0.10$, $p = 0.75$), TGF-β1 treatment ($F(1,10) = 1.9$, $p = 0.19$) or lesion × treatment interaction ($F(1,10) = 0.73$, $p = 0.41$). Similarly, the analysis on the dendritic complexity (Sholl analysis), did not show any significant main effect of either lesion ($F(5.8,58) = 1.18$, $p = 0.32$), treatment ($F(5.8,58) = 1.46$, $p = 0.21$), or lesion × treatment × intersections interaction ($F(5.8,58) = 0.65$, $p = 0.68$). Whereas the main effect of dendrite intersections within different group was found to be significant ($F(5.8,58) = 128.87$, $p = 0.00$). Results are shown in Figure 7B.

Effects of Neonatal TGF-β1 Administration on mPFC IL-1 β Levels

Two-way ANOVA from PD15 group of animals following TGF-β1 administration did not reveal any main effect of either lesion ($F(1,20) = 0.7030$, $p = 0.4117$) or TGF-β1 treatment ($F(1,20) = 2.122$, $p = 0.1607$) but a significant lesion × treatment interaction ($F(1,20) = 8.910$, $p = 0.0073$) was observed in IL-1β mRNA expression. Tukey's post hoc test showed a significantly increased IL-1β mRNA expression in lesion-saline group compared to sham-saline animals ($p = 0.0169$). Further analysis showed that while TGF-β1 administration had no effect on sham animals, it led to significantly attenuated IL-1β mRNA expression in lesion-TGF-β1 administered group compared to saline administered—lesioned animals ($p = 0.0244$). Two-way analysis on PD60 group of sham and lesioned animals revealed only a significant main effect of TGF-β1 treatment on IL-1β mRNA expression ($F(1,15) = 16.56$, $p = 0.0010$). No significant main effect of lesion ($F(1,15) = 0.02207$, $p = 0.8839$) or lesion × TGF-β1 treatment interaction ($F(1,15) = 0.1568$, $p = 0.6977$) was observed (Figure 8).

Discussion

In the current study, we observed a reduction in the expression and signaling of TGF-β1 and an increase in the expression of IL-1β mRNA in the mPFC of NVHL animals during neonatal and post-pubertal periods suggesting a persistent increase of inflammatory load in distant connected structures following neonatal lesion of the VH. Notably, the lesion did not produce inflammatory

reaction in other structures not directly connected, e.g., SSC nor in the brain tissues surrounding the lesion site. This suggests a particular vulnerability of the hippocampal-PFC circuit following the lesion possibly due to a loss of the ventral hippocampal excitatory drive to the PFC. Our data provide a mechanistic explanation to the growing evidence suggesting an important role of the developing VH inputs in shaping adult PFC physiology and behavior (Liu & Carter, 2018).

The neonatal VH lesioned rats have been previously shown to display a number of cellular and behavioral changes linked to the PFC (Swerdlow et al., 2001; Chambers & Taylor, 2004). In our recent study, we provided the evidence of increased microglial reactivity, phagocytic activity and inflammation in the PFC of NVHL rats (Hui et al., 2019). Microglia are a major source of early inflammatory genes like IL-1β within the CNS and IL-1β imbalance has been associated with cognitive and other behavioral deficits (C. L. Murray, Obiang, Bannermcan, & Cunningham, 2013; Tsai, 2017). We believe that the increased expression of IL-1β mRNA might be a key factor in regulating the behavioral and synaptic development in lesioned animals during adulthood. We acknowledge that not measuring IL-1β protein levels is a limitation in our study as it could have further strengthened our conclusions; however, taken together, our data suggest a key role of IL-1β in contributing to the inflammatory overload observed within the PFC. Upregulation of canonical TGF-β signaling pathway (via p-Smad-2/3) was found to contribute to quiescent microglia phenotype thereby reducing inflammatory overload within CNS (Abutbul et al., 2012; Kierdorf & Prinz, 2013). IL-1β exerts a suppressive effect on TGF-β1 mediated Smad-2/3 phosphorylation (Benus et al., 2005; Roman-Blas, Stokes, & Jimenez, 2007; Lim et al., 2012), suggesting that the reduction of TGF-β1 in PFC of lesion animals observed in our study may be due to the suppressive effect of elevated IL-1β mRNA on TGF-β signaling. Thus, we hypothesized that neonatal administration of TGF-β1 in NVHL animals could rescue the inflammation-driven schizophrenia- related behavioral and cellular deficits in NVHL animals.

Pro-inflammatory cytokines are tightly regulated by anti-inflammatory cytokines; IL-1β must overcome the anti-inflammatory effects of TGF-β1 to boost pro-inflammatory responses (Coussens & Werb, 2002). Our data shows that neonatal TGF-β1 administration attenuated the increase in IL-1β mRNA in the PFC of lesioned animals. TGF-β1 has been reported to inhibit IL-1β signaling by disrupting the Pellino1/IRAK1 complex by Smad6 (K. C. Choi et al., 2006). Thus, suggesting that neonatal administration of recombinant TGF-β1 could be playing a critical

role in counterbalancing the imbalance of IL-1β-TGF-β1 within PFC in lesion animals. NVHL-induced hyperlocomotion, deficits in PPI and SIs in the post-pubertal animals were also rescued by the administration of TGF-β1at neonatal age. Moreover, neonatal TGF-β1 also prevented the loss of spines observed in the mPFC of lesion animals. Reduced dendritic spine density in the PFC pyramidal neurons has been shown in both human schizophrenia brains as well as in the NVHL animals (Glantz & Lewis, 2000; Ryan et al., 2013) and suggested to alter glutamatergic and GABAergic neurotransmission and cognitive dysfunctions (Lewis & Gonzalez-Burgos, 2008; Selemon, 2013). In vitro and in vivo studies provide evidence towards the role of TGF-β1 regulating synaptogenesis in cortical and thalamic neurons (Diniz et al., 2012; Bialas & Stevens, 2013). TGF-β signaling pathway plays a critical role in neuron specification and its disruption has been identified as one of the factors contributing to neurodevelopmental disorders such as schizophrenia (Yi, Barnes, Hand, Polleux, & Ehlers, 2010; Nakashima et al., 2018). Apart from its role in synaptic growth, TGF-β signaling has been suggested to maintain synaptic homeostasis. Sun's group used a Smad-4 knockout mouse model to show that TGF-β signaling deficits leads to an imbalance in the synaptic homeostasis in the hippocampus resulting in behavioral abnormalities such as hyperactivity and deficits in PPI (Sun et al., 2010). Further, microglia have been reported to work synergistically along with TGF-β1 to regulate microglia mediated synaptic pruning (Bialas & Stevens, 2013). Thus, it is possible that reduced TGF-β1 could be a key player in the synaptic abnormalities observed in the PFC of NVHL animals.

Although our data shows a functional effect of systemically administered recombinant TGF-β1, similar to what (Caraci et al., 2015) observed, it should be pointed out that Kastin's group has reported that circulating TGF-β1 does not cross the blood brain barrier (BBB) in adult rats (Kastin, Akerstrom, & Pan, 2003). However, they also showed that TGF-β1 can cross BBB when experiments were done in serum free condition, suggesting that TGF-β1 is brain- penetrant under certain conditions, at least in the adult animals. Thus, in our experiments it was important to demonstrate that the recombinant TGF-β1 reaches the brain in the neonatal period and the behavioral results that we obtained are indeed due to the effect of recombinant TGF-β1 in the brain. We observed increased phosphorylation of Smad-2/3, a key intracellular signaling partner of TGF-β1, in the TGF-β1 injected animals as compared to the saline injected animals (Figure 5). Accordingly, we believe that due to immaturity of the BBB in neonates, TGF-β1 likely crosses the BBB and exerts its signaling effects resulting in the behavioral rescue that we observed. It

should be pointed out that we do not know the actual concentration of injected TGFβ1 in the brain; however, the activation of Smad-2/3 does suggest a physiological meaningful action of the injected recombinant TGF-β1.

Our data are consistent with recent evidence pointing towards an important role of neuroinflammation in neurodevelopmental disorders such as schizophrenia (Sekar et al., 2016; Fan & Pang, 2017). A meta-analysis of cytokines in the CSF showed similar results, i.e., higher levels of pro- and lower levels of anti-inflammatory cytokines in patients with schizophrenia (A. K. Wang & Miller, 2018). Evidence supporting the role of increased inflammation towards NVHL adult behavioral deficits has been provided in a previous study (Drouin-Ouellet et al., 2011). Interestingly, in our study the inflammatory overload is only observed during early development (around PD15) within the mPFC (a region distant from the lesion site) which is a critical time point of circuit development. In summary, our study shows that TGF-β1 may act as a neuroprotective agent to confer protection against the NVHL-induced behavioral, cellular and molecular deficits and suggests a closer examination of anti-inflammatory growth factors in the pathology of disorders such as schizophrenia.

Acknowledgments and Disclosures

Author Contributions

AJ conceived, designed and performed the experiments and wrote the first draft of the manuscript. SB helped in performing experiments and data analysis and manuscript writing. LS contributed to the development of research idea and implementation, discussion of the results and part of the manuscript writing.

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Figures

Figure 1

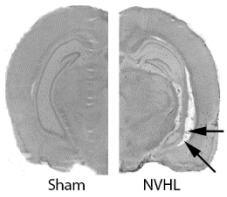

Figure 1. Verification of neonatal ventral hippocampus lesion (NVHL). Cresyl violet stained representative images showing an intact ventral hippocampus in sham animals (left). NVHL brain (right) shows cell loss, cavity and disorganisation in the ventral hippocampus (as shown by arrows).

Figure 2

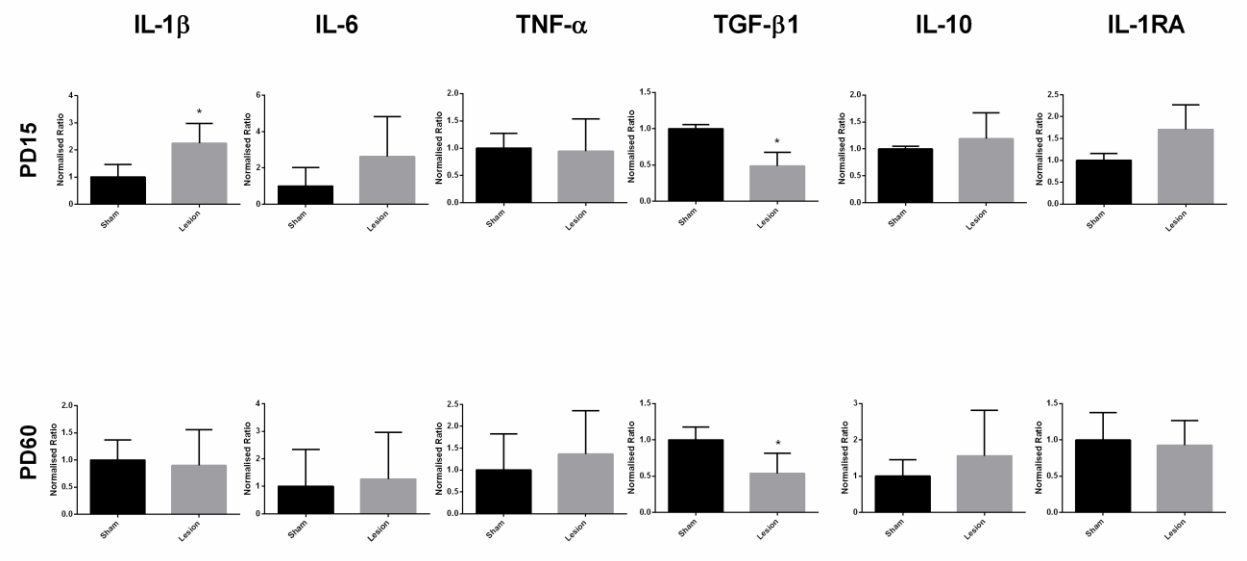

Figure 2. Levels of expression of pro- and anti-inflammatory cytokines from sham and neonatal ventral hippocampus lesion (NVHL) animals from medial prefrontal cortex at PD15 and PD60. Quantification of Q-RTPCR data (Normalised ratio of gene of interest over housekeeping gene); genes of interest quantified include interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), transforming growth factor-β1 (TGF-β1), interleukin-10 (IL-10) and interleukin-1RA (IL-1RA) at two developmental time points PD15 and PD60.

Expression of IL-1β was found to be significantly increased in the NVHL groups as compared to the sham group at PD15 ($p=0.0019$). Decreased expression of TGF-β1 was observed in the NVHL group as compared to sham group both at PD15 ($p=0.0291$) and PD60 ($p =0.0064$). No significant change in the expression of IL-6, TNF-α, IL-10, IL-1RA was observed between sham and lesion group at PD15 and PD60. (n=4-8 per group).

Figure 3

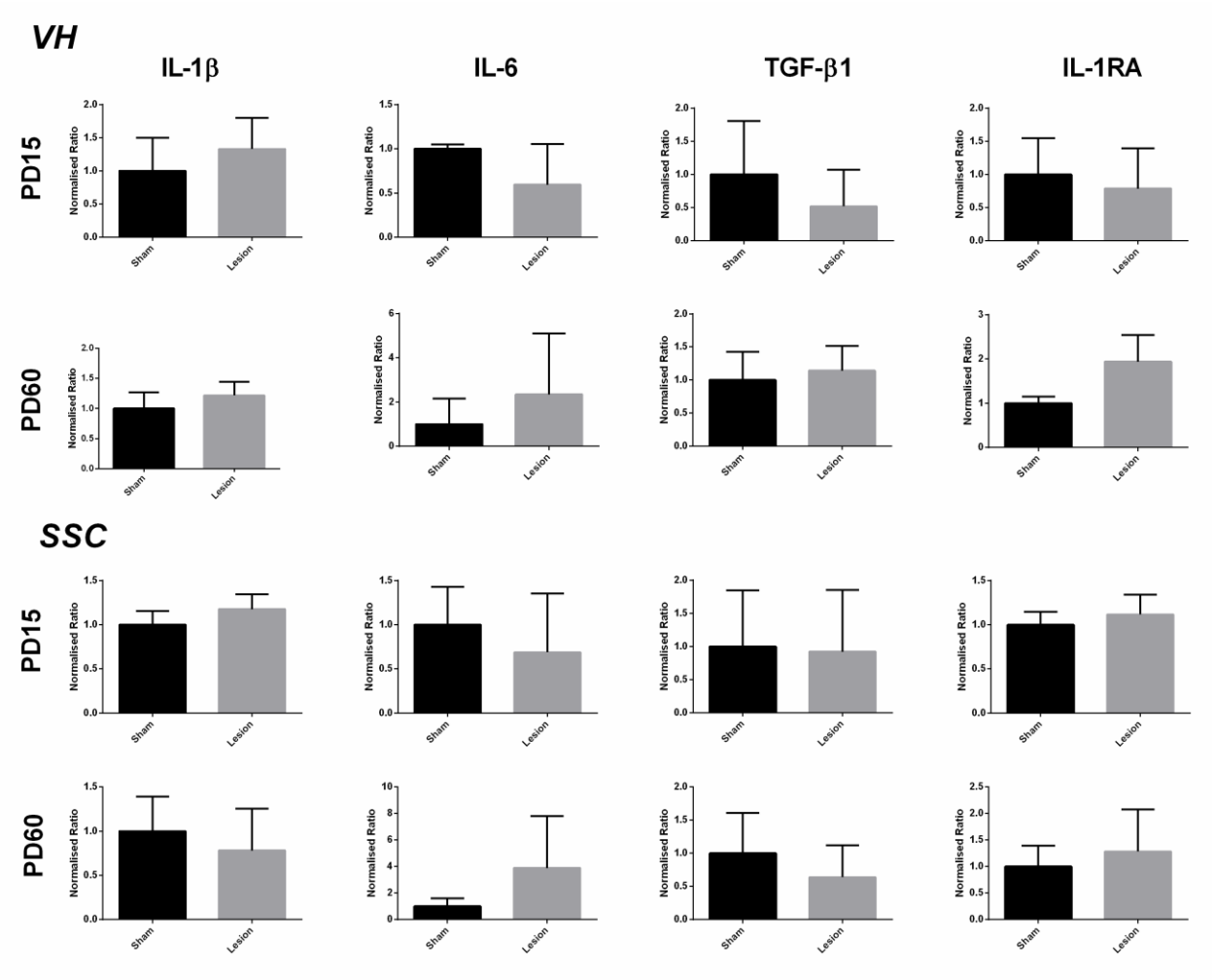

Figure 3. Levels of expression of pro- and anti-inflammatory factors from sham and neonatal ventral hippocampus lesion (NVHL) animals within ventral hippocampus (VH) and somatosensory cortex (SSC) at PD15 and PD60. Quantification of Q-RTPCR data (Normalised ratio of gene of interest over housekeeping gene); genes of interest quantified include interleukin-1β (IL-1β), interleukin-6 (IL-6), transforming growth factor-β1 (TGF-β1) and interleukin-1RA (IL-1RA) at two developmental time points PD15 and PD60. No significant change was observed in the expression of above-mentioned genes between sham and lesion at PD15 and PD60. (n=3-4 per group).

Figure 4

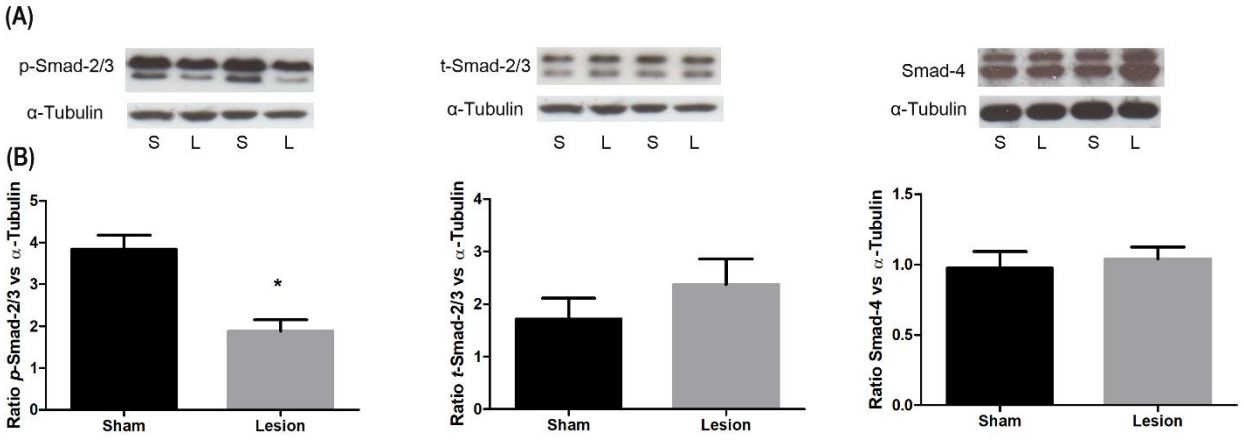

Figure 4. Expression of TGF-β signalling protein from sham and neonatal ventral hippocampus lesion (NVHL) animals from medial prefrontal cortex at PD60. (A). Representative western blots showing the expression of phospho (p)-Smad2/3, (total) t-Smad-2/3, total-Smad-4 and α-tubulin in samples prepared from medial prefrontal cortex tissue of sham (S) and NVHL (L) rats. The blot was developed for p-Smad-2/3, stripped and probed for t-Smad-2/3 and restriped and reprobed for α-tubulin antibody. (B) Quantitation of western blotting data (ROD ratio of p-Smad-2/3, t-Smad-2/3 and Smad-4 over α-tubulin). A significant decrease in p-Smad-2/3 was observed in NVHL rats (p=0.003). No significant change in the normalized levels of t-Smad-2/3 and Smad-4 was observed between sham and lesion group. (n=5-6 per group).

Figure 5

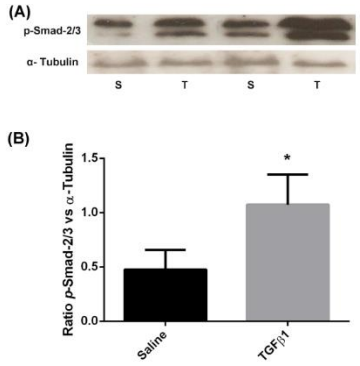

Figure 5. A single acute systemic injection of TGF-β1 regulates its brain intracellular signaling. (A) Shows the representative western blot images showing the increased expression of p-Smad-2/3 in TGF-β1 (T) injected group compared to saline (S) injected group. (B) The quantification of the Western blotting data (ROD ratio of p-Smad2/3 over α-tubulin). A significant increase in p-Smad-2/3 was observed in TGF-β1 injected group as compared to saline injected group ($p=0.011$). ($n=4$ per group).

Figure 6

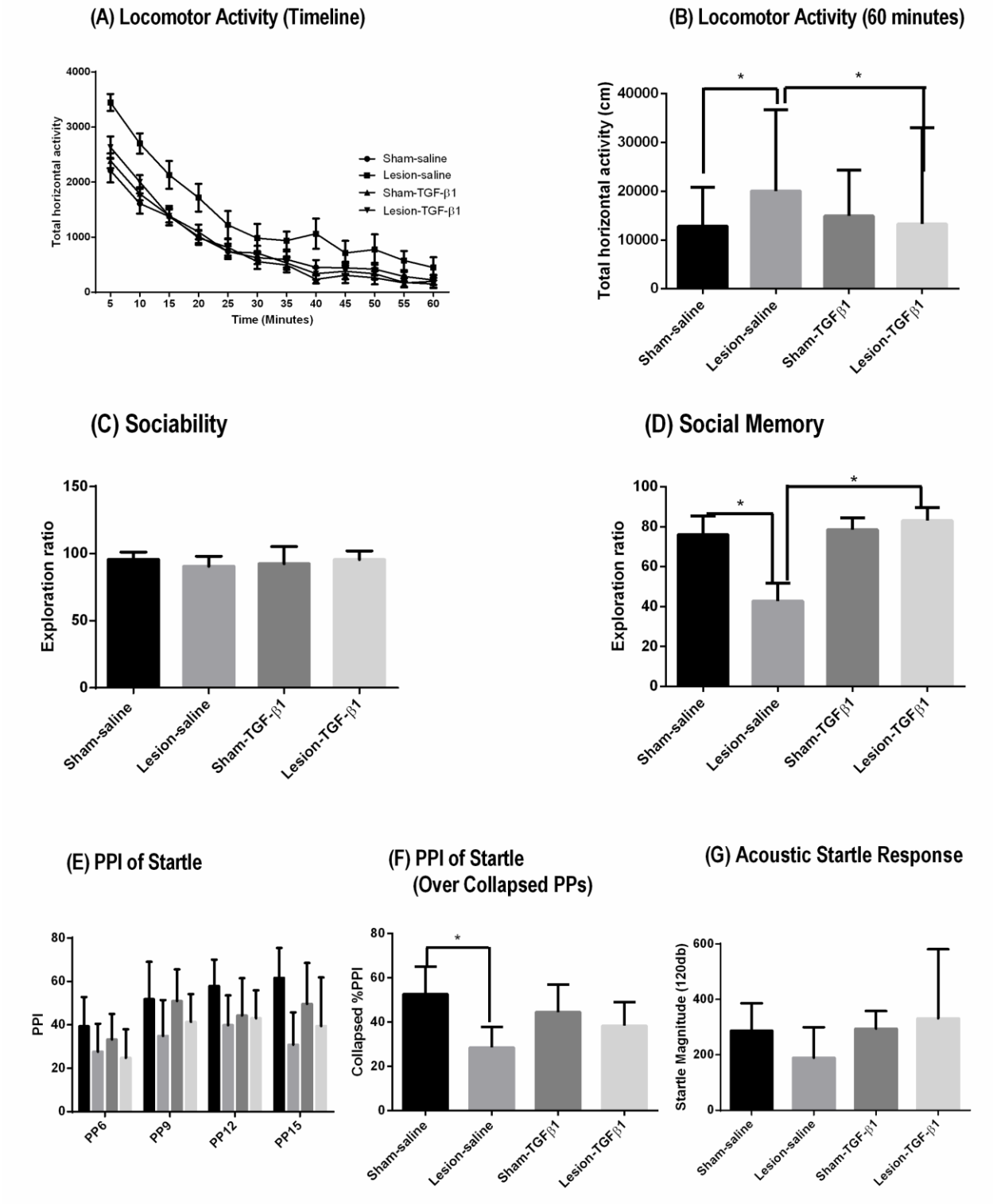
(A) Locomotor Activity (Timeline)
Total horizontal activity
Time (Minutes)
Sham-saline
Lesion-saline
Sham-TGF-β1
Lesion-TGF-β1
(B) Locomotor Activity (60 minutes)
Total horizontal activity (cm)
(C) Sociability
Exploration ratio
(D) Social Memory
Exploration ratio
(E) PPI of Startle
PPI
PP6
PP9
PP12
PP15
(F) PPI of Startle
(Over Collapsed PPs)
Collapsed %PPI
(G) Acoustic Startle Response
Startle Magnitude (120db)

Figure 6. Effect of neonatal TGF-β1 administration on sham and neonatal ventral hippocampus lesion (NVHL) animals at PD60. (A, B) Spontaneous locomotor activity. (A) Time course of locomotor activity as assessed by the horizontal activity in 5 minutes bin. (B) Total horizontal activity during the whole 60-min session. Two-way ANOVA showed a significant lesion x TGF-β1 treatment interaction on the horizontal activity ($p=0.018$). (C, D) Sociability and social memory (C) Indicates similar preferences between sham and NVHL animals for S1 animals. (D) In social memory test, significant lesion x TGF-β1 interactions was observed ($p=0.017$). (E- G) Prepulse inhibition (PPI) of acoustic startle response (E) Prepulse inhibition, as a function of prepulse intensities (PP; 6-15). (F) Prepulse inhibition collapsed across all PPs. A significant 2-way (lesion x TGF-β1) interactions was observed ($p=0.026$). (G) Baseline acoustic startle response was not significantly different between groups. (n=7-18 per group).

Figure 7

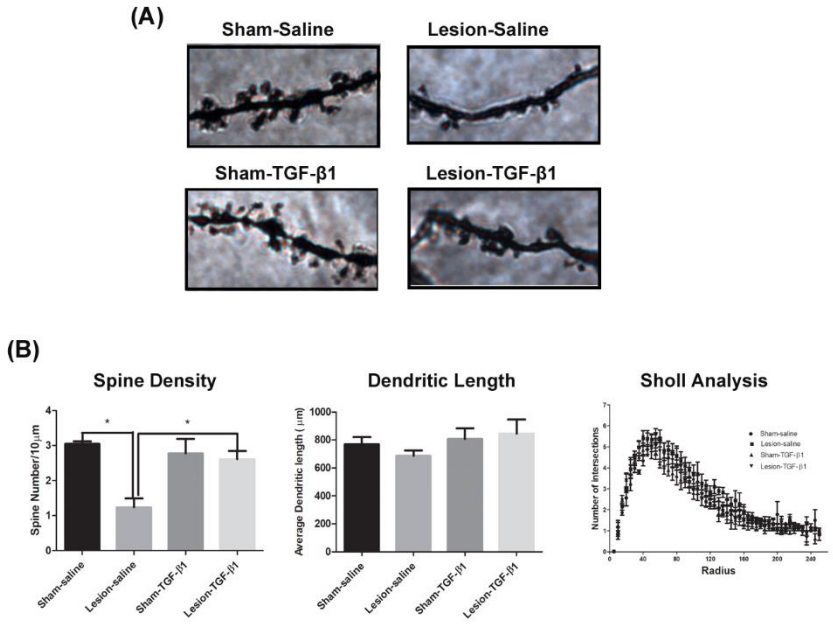

Figure 7. Effect of neonatal TGF-β1 administration on dendritic complexity and spine density in sham and neonatal ventral hippocampus lesion (NVHL) animals within medial prefrontal cortex at PD60. (A) Photomicrograph showing representative golgi-cox impregnated basilar dendrite from pyramidal neuron of layer III medial prefrontal cortex at PD60 in sham-saline, lesion-saline, sham- TGF-β1 and lesion- TGF-β1 groups. (B) Two-way ANOVA showed a significant lesion x TGF-β1 treatment interaction on dendritic spine density (number of spines per 10µm) ($p=0.02$). Total dendritic length (µm) did not change significantly as a result of lesion x TGF-β1 treatment interaction among the four groups ($p=0.41$). Data analysis from dendritic complexity as measured by sholl analysis (Number of dendritic intersections per each sholl radius 5µm) did not reveal any significant lesion x TGF-β1 treatment x dendritic intersections interaction between the four groups. (n=3-4 per group).

Figure 8

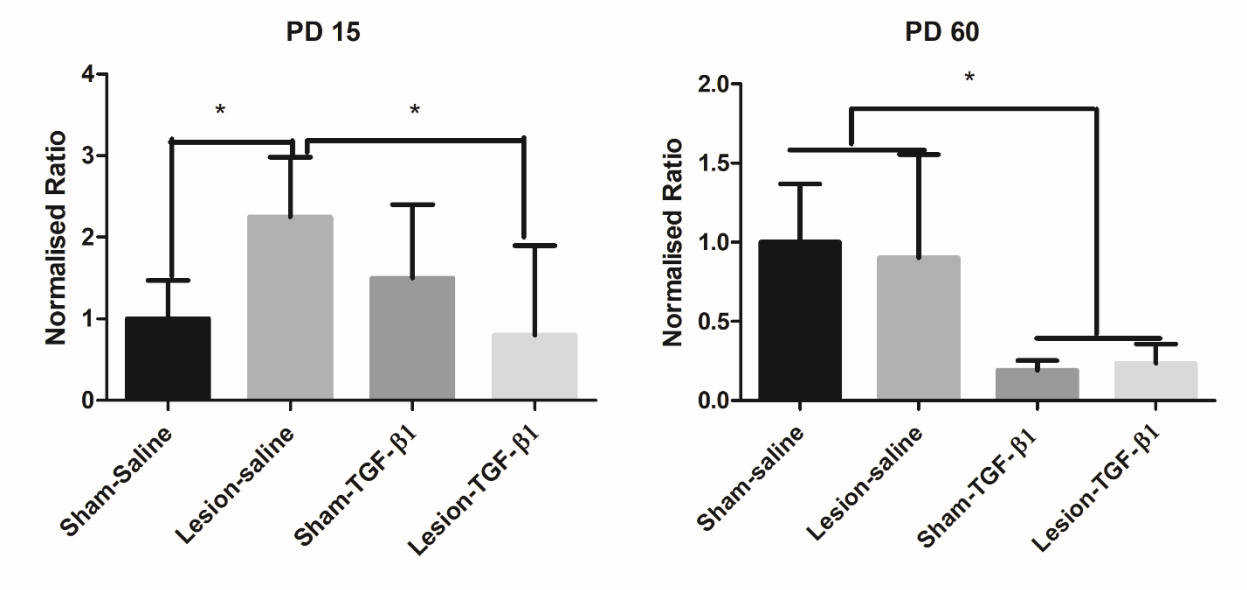

Figure 8. Levels of expression of interleukin-1β (IL-1β) from sham and neonatal ventral hippocampus lesion (NVHL) animals from medial prefrontal cortex at PD15 and PD60 following neonatal TGF-β1 administration. Quantification of Q-RTPCR data (Normalised ratio of IL-1β gene over housekeeping gene) expression at two developmental time points PD15 and PD60. A 2-way ANOVA revealed a significant lesion x TGF-B1 interaction from PD15 group of animals (p=0.007). Post-hoc test showed that IL-1β expression was significantly increased in the lesion-saline group as compared to sham-saline group (p=0.0169) at P15. A significant reduction of IL-1β was observed in the lesion-TGF¬-β1 group as compared to lesion-saline group (p=0.0244). From PD60 group of animals only a significant main effect of TGF-β1 treatment was observed on IL-1β expression (p=0.001). (n=4-8 per group).

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CONNECTING STATEMENT TO CHAPTER IV

In chapter III our results demonstrated a development specific imbalance of pro- and anti-inflammatory factors in the PFC of the NVHL animals. We showed that this imbalance could be ameliorated by neonatal TGF-β1 administration and was associated with the rescue of NVHL-like behavioral and synapse related aberrations. The results presented in Chapter II and III are suggestive of a crucial role of microglia and TGF-β1 in NVHL animals. These factors have been suggested to modulate neuroinflammation within the brain. Therefore, the next logical step would be to decipher the mechanism on how these factors in the PFC of the NVHL animals regulate behavioral and cellular alterations. Since some of the factors resulting in neuroinflammation are regulated by miRNAs and their impact on oxidative stress, we hypothesized a role of miRNAs in modulating neuroinflammation and thereby regulating NVHL-related behavioral and cellular alterations. We tested this hypothesis in Chapter IV by using NVHL rats. Here, we measured the expression of selected miRNAs, oxidative stress and cognitive function. The results presented in Chapter IV are unpublished.

CHAPTER IV

Role of miRNAs and its effect on oxidative stress in neonatal ventral hippocampus lesion induced neuroinflammation.

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Keywords: Ventral hippocampus, mPFC, oxidative stress, miRNA, neuroinflammation, cognition, schizophrenia.

#to be submitted.

Abstract

MicroRNAs (miRNAs) are short non-coding RNAs known to regulate neuroinflammation-related genes and pathways in the central nervous system and have been implicated in schizophrenia. Neuroinflammation has been suggested to cause schizophrenia-related behavioral and cellular deficits in the neonatal ventral hippocampus lesion (NVHL) model in rats. Recent studies from our group and others have provided evidence pointing towards the role of medial prefrontal cortex (mPFC) oxidative stress, microglia function, and pro- and anti-inflammatory factors in the behaviors of the NVHL animals. The mechanism leading to these deficits in the mPFC as a result of NVHL remains largely unknown. Based on the immuno-modulatory role of miRNAs, we hypothesized a possible role of these short non-coding RNAs in contributing to neuroinflammation in the mPFC of the NVHL animals. In order to test this hypothesis, we induced NVHL using ibotenic acid at postnatal day (PD) 7 in male Sprague-Dawley rat pups. Adult animals were used to measure cognitive flexibility, expression of miRNAs and oxidative stress in the mPFC. Consistent with previous studies, we found an impairment in cognitive flexibility and increased oxidative stress in the NVHL animals compared to control animals. We also found an increased expression of miRNAs 134 and 137 in the mPFC of NVHL animals. Our data suggests a role of these miRNAs in regulating PFC-dependent cognitive function in NVHL animals.

Introduction

MicroRNAs (miRNAs) are evolutionarily conserved small noncoding RNAs (~22 nucleotides) expressed in different cells within the central nervous system (CNS) and have been implicated in schizophrenia (Schratt et al., 2006; Schizophrenia Psychiatric Genome-Wide Association Study, 2011). They regulate gene expression by post transcriptional repression and mRNA degradation (Filipowicz et al., 2008). They are reported to be a key modulator of genes related to oxidative stress and inflammation (Ha & Kim, 2014; Xie et al., 2018). For example, inhibition of miR-137 has been reported to reduce oxidative stress by regulating the expression of an anti-oxidative defense enzyme, gluthatione peroxidase (Gpx1) (Li, Li, Wei, & Li, 2016). Other miRNAs, such as miR-103 was reported to modulate oxidative stress by activating a DNA repair polymerase enzyme (Dluzen et al., 2017). MiR-146 expression was shown to be negatively correlated with the expression of oxidative stress indicators in the brain (Xie et al., 2018). Other miRNAs such as the miR-124 has been found to modulate neuro-inflammation by downregulating the production of pro-inflammatory (IL-6 & TNF-α) and upregulating anti-inflammatory (IL-13 & IL-10) cytokines (Hatziapostolou et al., 2011; Ponomarev, Veremeyko, & Barteneva, 2011). Interestingly, miR-21 has been reported to have a dual function of regulating genes related to both oxidative stress and inflammation (Sheedy et al., 2010; van den Bosch, Palsson-Mcdermott, Johnson, & O'Neill, 2014). MiRNAs are also believed to regulate the level of inflammation and oxidative stress within the brain by modulating genes-related to microglia, the resident immune cells of the CNS (Ponomarev, Veremeyko, & Weiner, 2013). For example, miR-124 was shown in promoting microglial quiescence, thereby reducing inflammatory overload within the brain (Ponomarev et al., 2011).

Increased level of oxidative stress and inflammation in the prefrontal cortex (PFC) has been suggested as a possible mechanism contributing to adult schizophrenia-related behavioral and cellular deficits by our group and others using the neonatal ventral hippocampus lesion (NVHL) rats (Joseph, Bhardwaj, & Srivastava, 2018; Hui et al., 2019); Cabungcal et al., 2014). Ventral hippocampus (VH) lesion in neonates was induced by an excitotoxin-ibotenic acid, which resulted in adult behavioral deficits such as amphetamine-induced hyper-locomotion and deficits in prepulse inhibition (PPI) of startle, social memory and cognitive function (Tseng et al., 2009). Altered cognitive flexibility observed in NVHL animals was found to be dependent on PFC

function (Gruber et al., 2010). Besides behavioral deficits, PFC-related cellular deficits such as reduced dendritic spine density in layer 3 and 5 pyramidal neurons, imbalance in excitatory and inhibitory synaptic transmission (Ryan et al., 2013) and impaired synaptic plasticity (Bhardwaj et al., 2004) have also been reported in adult NVHL animals.

Recent study from our group revealed reduction in the expression of genes related to anti-oxidative defense markers such as glutathione peroxidase and catalase in the PFC of lesion animals (Hui et al., 2019). A development specific increase of pro-inflammatory (IL-1β) and decrease of anti-inflammatory (TGF-β1) factor was also observed in the PFC of NVHL animals. We further showed that neonatal supplementation of TGF-β1 could rescue both NVHL-related behavioral and cellular deficits in adulthood (Joseph et al., 2018). This increase in oxidative stress and inflammation in the NVHL animals was suggested to cause alteration in microglial morphology and phagocytic function as observed in our study. Additionally, microglia dysfunction was also found to be associated with increased mRNA levels of complement molecules (C3 and C1q) and suggested to be involved in synaptic pruning in the PFC of NVHL animals (Hui et al., 2019). Interestingly, the expression of synaptic pruning-related complement proteins (C1q & C3) in cortical neurons were found to be regulated by miRNAs such as miR-132 and miR-134 (Xu et al., 2019; Schratt et al., 2006; Benoit & Tenner, 2011). Synaptic pruning-related function of miRNAs have been implicated in the development and maturation of neurons and thereby have been suggested to regulate behaviors such as memory and cognitive functions (J. Gao et al., 2010; Fonken, Gaudet, Gaier, Nelson, & Popovich, 2016). Our data from previous studies suggests a role of neuroinflammation in NVHL-related behavioral and cellular deficits. But the mechanism leading to these deficits as a result of NVHL remains largely unknown. In this study, we hypothesized the role of miRNA and its effects on oxidative stress in modulating neuroinflammation and thereby regulating NVHL-related behavioral and cellular deficits. In this study we examined cognitive flexibility and expression of miRNAs in the mPFC. We also assessed the effect of miRNAs expression on the level of oxidative stress in microglia in adult animals. Consistent with previous studies, our results showed a deficit in cognitive flexibility and increase in oxidative stress in the mPFC of the NVHL animals. We also observed an increase in the expression of select miRNAs (miR-134 and miR-137) in the mPFC of lesioned rats. Together, our data provides a possible mechanism via miRNAs by which the development of PFC could be compromised following early damage to the VH.

Materials and Methods

Neonatal Ventral Hippocampal Lesion (NVHL)

This study was carried out in accordance with the guidelines of the Canadian Council of Animal Care. The protocol was approved by the McGill University Animal Care Committee. Three pregnant female Sprague-Dawley rats at 15–18 days of gestation were obtained from Charles River Laboratories (QC, Canada). Rat dams were housed individually on a 12 h light-dark cycle with ad libitum food and water in a temperature and humidity-regulated room, where they were allowed to give birth. On P7, male pups were weighed (15–17 g) and were randomly selected for neonatal ventral hippocampal lesion (NVHL) or sham surgery according to our previously described procedures (Flores et al., 1996; Ryan et al., 2013). Briefly, pups were anesthetized by hypothermia (by covering in crushed ice for 18–20 min) and secured on a modified platform fixed to Kopf stereotaxic apparatus. The "lesion" group received bilateral infusion of 0.3 µl ibotenic acid (Catalog No. 0285; Tocris; 10 µg/µl in 0.1 M phosphate-buffered saline (PBS) over a period of 2 min using a 30-gauge needle connected to an infusion pump, while the "sham" group received the same volume of PBS into the VH (coordinates: AP −3.0 mm from Bregma, ML ±3.5 mm from midline, and DV −5.0 mm from dura). Following this, the skin was sutured using vetbond tissue glue and the pup's ears were tagged. After surgery, pups were placed on a heating pad until full recovery and returned to their respective mothers.

Behavioral Testing- Attentional set-shifting test

Attentional set-shifting test (ASST) measures PFC dependent cognitive flexibility in rodents. The ASST protocol used in our study has been previously described by (Birrell & Brown, 2000). The adult sham and NVHL animals were food deprived for a total duration of 8 days prior the ASST to reach and maintain 85% of body weight. The ASST was performed in a large Plexiglas box (80 × 40 × 25 cm; L × W × H) containing two choice chambers and one waiting chamber. The choice and waiting chambers were divided by a central partition and the mice had access to the choice chambers by raising the central partition (Figure 1A). The test animals were habituated to the testing box over three days, receiving two sessions of 20 min per day. The test animals were trained to find a buried food reward (1/2 ring of froot loops, Kellogg, Canada), by

digging the bedding in ceramic bowls (10 x 10 cm, D x H) placed in each choice chamber. Once the animals were able to dig efficiently and retrieve the food reward (6 correct responses in a row), they were introduced to the testing phase. The animals were tested on a series of discriminations presented during the following stages of ASST: simple discrimination (SD), compound discrimination (CD), compound discrimination reversal (CDR), intra-dimensional shift (IDS), intra-dimensional reversal (IDR), extra-dimensional shift (EDS), and extra-dimensional reversal (EDR). Different dimensions and combination of stimuli used at different stages is listed in Table 1. Testing began with SD, in which the animals discriminated between the baited (with fruit loop) and unbaited (without fruit loop) bowls along one dimension only (odor). The first four trials of the SD were exploratory trials, in which the animals were allowed to dig the unbaited bowl and correct their choice. For subsequent trials and stages, the test animals were not allowed to correct their response. Following SD test, CD was performed by presenting two dimensions, one was the same dimension used during SD and second dimension was the digging medium (texture- e.g. confetti or cotton flowers). During the compound reversal discrimination stage, the animals must learn that the previously correct stimulus during CD within the rewarded dimension is now incorrect. After criterion was reached for CDR, a new combination of odors and digging mediums were presented at the IDS stage and the animals were required to discriminate according to the already trained dimensions (e.g. Nutmeg or garlic & wool or plastic pearls). Following which the animals were tested on the reversal of IDS stimuli during the IDR stage. Once again, a new pair of stimuli was introduced during the EDS, but now the animals had to shift their attention and respond to the previously irrelevant dimension that was now baited. The final stage of ASST was EDR during which the animals were tested by presenting a reversed order of EDS stimuli.

Total RNA Extraction and miRNA Expression Using QRT-PCR

Following behavioral test, a subgroup of both sham and lesion animals were used to measure the expression of miRNA in the PFC. Total RNA and miRNA were extracted using miRNeasy Micro Kit (Qiagen, Catalog No-217084) and stored at −80 °C. All the samples were checked for genomic DNA contamination and the RNA quality assessed by Nano drop. The complementary DNA of the miRNAs were made using miScript II RT Kit (Qiagen, Catalog No.-218160) and

used for qRT–PCR. We measured the expression of specific miRNAs in our sample by using miScript SYBR Green PCR Kit (Qiagen, Catalog No.-218073) and miScript Primer Assays (Qiagen) for the following miRNAs: miR134 (5'UGUGACUGGUUGACCAGAGGGG), miR137 (5'UUAUUGCUUAAGAAUACGCGUAG), miR124 (5'CGUGUUCACAGCGGACCUUGAU) and miR21 (5'UAGCUUAUCAGACUGAUGUUGA) with ABI PRISM 7900 Real-Time PCR System (Life Technologies). RNU6-6P (Qiagen, MS00033740) was used an internal control. All assays were performed in duplicate and the average cycle threshold (Ct value) determined.

Immunofluorescence and Cell Counting

A separate cohort of adult sham and lesion were anesthetized and perfused trans-cardially with 0.1 m PBS (pH 7.4), followed by 4% paraformaldehyde in 0.1 m PBS. The brains were removed, post-fixed overnight at 4°C, and then transferred to 30% sucrose for 48 hr at 4°C. Coronal sections (35 μm) were collected at levels corresponding to mPFC using a microtome (LeicaVT1200S). For staining, the sections were rinsed in 1xPBS, blocked and incubated in primary antibodies overnight at 4°C. The primary antibodies were used to identify oxidative stress marker, 8-oxo-7, 8-dihydro-20-deoxyguanine (8-Oxo-dG) (Abcam) and microglia marker, IBA-1 (Novus Biologicals). The next day, the sections were further processed and incubated in secondary antibodies (anti-mouse, Alexa Fluor® 488 & anti-goat, Alexa Fluor® 680) and mounted on slides. Distribution of immunoreactive cells were studied by viewing the slides with an Olympus upright fluorescence microscope (BX63) at 20× magnification. Cells immunoreactive for 8-Oxo-dG were counted in the mPFC layer 2/3 using the QuPath software. The cell counts were divided by the area of the corresponding region to determine cell density per square micrometer.

Data analysis

Reported values are mean ± standard error of the mean (SEM). The data were analyzed using Prism (GraphPad, Version 6). Two-tailed unpaired t-test was used to determine cell density and miRNA expression changes. Two-way repeated measure ANOVA followed by Bonferroni's

post-hoc test was used to determine interactions between lesion and stages of ASST. For all analysis $p < 0.05$ was considered statistically significant.

Results

The effect of neonatal VH lesion on adult cognitive behavior.

We examined the effect of neonatal VH lesion on cognition by using ASST during adulthood. Our data analysis revealed a significant main effect of ASST stage ($F(6,90) = 85.47$, $p = 0.0001$), lesion ($F(1,15) = 12.39$, $p = 0.0031$) and stage x lesion interaction ($F(6,90) = 3.29$, $p = 0.005$). Post hoc test showed a significant increase in the number of trials to reach criterion at EDS stage in the lesion animals compared to sham ($p = 0.0001$). No significant difference between the two groups were observed at the other stages: SD ($p = 0.999$), CD ($p = 0.999$), CDR ($p = 0.999$), IDS ($p = 0.189$), IDR ($p = 0.592$), EDR ($p = 0.999$). As expected, further analysis between the different stages revealed that at the EDS stage sham animals required more trials than at the IDS stage ($p=0.0001$). However, during this IDS to EDS attentional shift the lesion animals required far more trials to reach criterion ($p=0.0001$). Overall, our behavioral data revealed an impairment in attentional shift in the lesion group compared to the sham group as shown in Figure 1B.

MiRNA expression in the mPFC of NVHL animals.

Analysis of miRNA data at P60 showed a significant increase in the expression of miR-137 ($t = 4.655$, $df = 7$, $p = 0.002$) and miR-134 ($t = 3.265$, $df = 7$, $p = 0.013$) in the mPFC of NVHL animals compared to sham. The expression of miR-124 ($t = 0.349$, $df = 7$, $p = 0.737$) and miR-21 ($t = 1.975$, $df = 7$, $p = 0.088$) was not found to be significantly different between the sham and lesion animals. The results are shown in Figure 2.

Level of oxidative stress in the mPFC of NVHL animals.

In order to determine the level of oxidative stress in microglia as a result of NVHL, we examined the expression of an oxidative stress marker (8-Oxo-dG) in the microglia cells (IBA1). In the adult mPFC, 8-oxo-dG expressing cells were not found to be co-labeled with microglia cells in both sham and lesion group (Figure 3A). Further, we performed cell counting of 8-oxo-dG

immuno-positive cells in the mPFC and revealed a significant increase in the density of 8-oxo-dG cells in the adult lesion animals compared to the sham animals ($t = 2.745$, $df = 6$, $p = 0.0335$; Figure 3B). Our data shows an increased level of oxidative stress in the adult mPFC as a result of NVHL.

Discussion

In the current study we observed a deficit in cognitive flexibility as a result of neonatal VH lesion. We also found increased expression of miRNAs (miR-137 & miR-134) and oxidative stress in the mPFC of adult lesion animals. Deficits in prefrontal cortical functions, such as working memory and attention, have previously been demonstrated in NVHL rats (Tseng et al., 2008; Lipska, Jaskiw, & Weinberger, 1993). Our observations of a deficit in behavioral flexibility assessed by ASST in NVHL animals are consistent with other groups and further establishes the idea that early developmental disruption in the VH critically affects the development of PFC-dependent cognitive functions later in life (Marquis et al., 2008; Brady, 2009; Placek et al., 2013). ASST measures the ability of animals to learn and discriminate between stimuli within different dimensions. During the initial stages of ASST, the animals learn to discriminate between two stimuli within the same dimension. Whereas during reversal stages, the animals learn to switch their attention from the previously rewarded stimuli within the same dimension. However, during the later stages the animals shift their attention from the previously rewarded dimension to a different dimension that has been now rewarded (Birrell & Brown, 2000). ASST paradigm is considered to be a rodent analogue of Wisconsin Card Sort test (WCST) which is often employed to measure deficits in executive functions in schizophrenia patients (Monchi, Petrides, Petre, Worsley, & Dagher, 2001; Cavallaro et al., 2003). As expected, in our studies we found that NVHL animals were normal in their ability to learn to discriminate between two stimuli within a dimension (odor) during the SD stage. The NVHL animals also showed no learning deficit during the CD, IDS and reversal learning stages, where the animals had to associate reward within the same dimension as SD i.e. odor. Previous studies show that lesion to the orbitofrontal cortex (OFC) was reported to affect reversal learning (Bissonette et al., 2008; Birrell & Brown, 2000). It is thus possible that this brain circuit is apparently not affected by the NVHL. However, we found that the NVHL animals showed

significant deficit in disengaging with previously rewarded dimension and learn to associate and choose the new rewarded dimension. Additionally, the shift from intra-dimension to extra dimension was found to be significantly affected in NVHL animals. This particular behavior in the ASST is believed to be highly dependent on the function of the infralimbic (IL) region of the medial PFC (Mukherjee & Caroni, 2018; Bissonette, Powell, & Roesch, 2013). This observation is consistent with previous reports that show a number of cellular and behavioral deficits related to the IL region of mPFC in NVHL animals (Cabungcal et al., 2014; Marquis, Goulet, & Dore, 2006). Although the issue of homologies between rodent and primate PFC is still being debated (Laubach, Amarante, Swanson, & White, 2018), it is interesting that the deficits in executive functions in schizophrenia are often related to abnormalities in the dorsolateral prefrontal cortex, a region presumably homologous to the medial PFC of rodents.

Previous studies from our group and others using NVHL model have reported evidence on the deleterious effect of increased oxidative stress and inflammation on behavioral and cellular function in the mPFC of NVHL animals (Tendilla-Beltran et al., 2019; Hui et al., 2019); Cabungcal et al., 2014). Both oxidative stress and neuroinflammation were suggested to cause alteration in microglia morphology and function in the mPFC of the NVHL animals (Hui et al., 2019; Joseph et al., 2018). Interestingly, there are reports showing a role of miRNAs in modulating microglial morphology and function (Ponomarev et al., 2013; Woodbury et al., 2015). For example, miRNAs alterations promote the secretion of pro-inflammatory cytokines by microglia and induce oxidative stress (Butovsky et al., 2006). Our previous study found a development specific reduction of anti-oxidative defense markers, glutathione peroxidase 1 (Gpx1) and catalase in the mPFC of NVHL animals (Hui et al., 2019). Our data here found increased miR-137 expression in the mPFC of NVHL animals; interestingly miR-137 has been reported to inhibit the genes related to the expression of Gpx1 enzyme (Matouskova, Hanouskova, & Skalova, 2018). Additionally, miR-200 is known to modulate the genes related to the expression of 8-oxoguanine DNA glycosylase (OGG1) and thereby increase the level of an oxidative stress marker (8-Oxo-dG) (Tinaburri et al., 2018). In our study here, we looked at the expression of an oxidative stress marker, 8-Oxo-dG in microglia cells and found no significant indication of oxidative stress in microglia cells in the mPFC of lesion animals. However, we found an overall increase in the number of cells expressing 8-Oxo-dG in the mPFC of lesion animals. Our data thus suggests an increased level of oxidative stress in other neuronal and non-

neuronal cells, which could be contributing to behavioral and cellular deficits in NVHL animals. Besides oxidative stress, other miRNAs such as miR-124 and miR-21 have been reported to modulate neuroinflammation by downregulating the production of some pro-inflammatory cytokines (Ponomarev et al., 2011; van den Bosch et al., 2014). However, our study on the expression of miR-124 and miR-21 did not reveal any difference between the sham and lesion groups. Since some of the neuroinflammatory alterations observed previously in the NVHL animals are development specific, it is possible that the changes in the expression of miR-124 and miR-21 could be relevant to other developmental time-point. We believe that measuring miRNA expression at other developmental time points should be further investigated. However, our study does suggest a role of at least one of the miRNAs, i.e., miR-137, in the regulation of oxidative stress and neuroinflammation during adulthood as a result of neonatal VH lesion.

Complement mediated synaptic pruning role of microglia has been suggested to be regulated by miRNAs (Xu et al., 2019). The synaptic pruning role of microglia has been found to be mediated by complement proteins (C4) and have also been implicated in schizophrenia (Sekar et al., 2016). In our previous study we observed altered expression of complement genes such as C1q and C3 in the mPFC of NVHL animals (Hui et al., 2019). In our data here we found an increased expression of miR-134 and miR-137 in the lesion animals. Notably, overexpression of both miRNAs (miR-134 & miR-137) have been known to increase synaptic pruning and alter synaptic function in neurons (Schratt et al., 2006; He et al., 2018). Besides synaptic pruning, miRNAs were also reported to regulate schizophrenia-related behaviors such as social memory and cognition in certain animal models of schizophrenia (Lippi et al., 2016).

Our data are also consistent with the evidence pointing towards an important role of miRNAs in neurodevelopmental disorders such as schizophrenia (M. P. Forrest et al., 2018; Calabro et al., 2016). Post mortem analysis of PFC in patients with schizophrenia has shown an upregulation of miR-134, similar to what we observed in our animal model (Santarelli et al., 2011). Notably, among the 108 chromosomal loci identified to be associated with schizophrenia in a Genome wide association study, the miR-137 locus was ranked the second in the list (Schizophrenia Working Group of the Psychiatric Genomics, 2014). Collectively, our study in NVHL animals suggests that the loss of developing VH inputs to PFC and other brain regions alters miRNAs expression affecting neuroinflammation and thereby regulating cellular and cognitive functions

during the maturation of PFC. We suggest miRNAs as a possible mechanism in the prefrontal cortex-related behaviors of NVHL animals, a putative neurodevelopmental animal model of schizophrenia.

Acknowledgments and Disclosures

Author Contributions

AJ conceived, designed and performed the experiments and wrote the first draft of the manuscript. LS contributed to the development of research idea and implementation, discussion of the results and part of the manuscript writing.

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Figures

Figure 1.

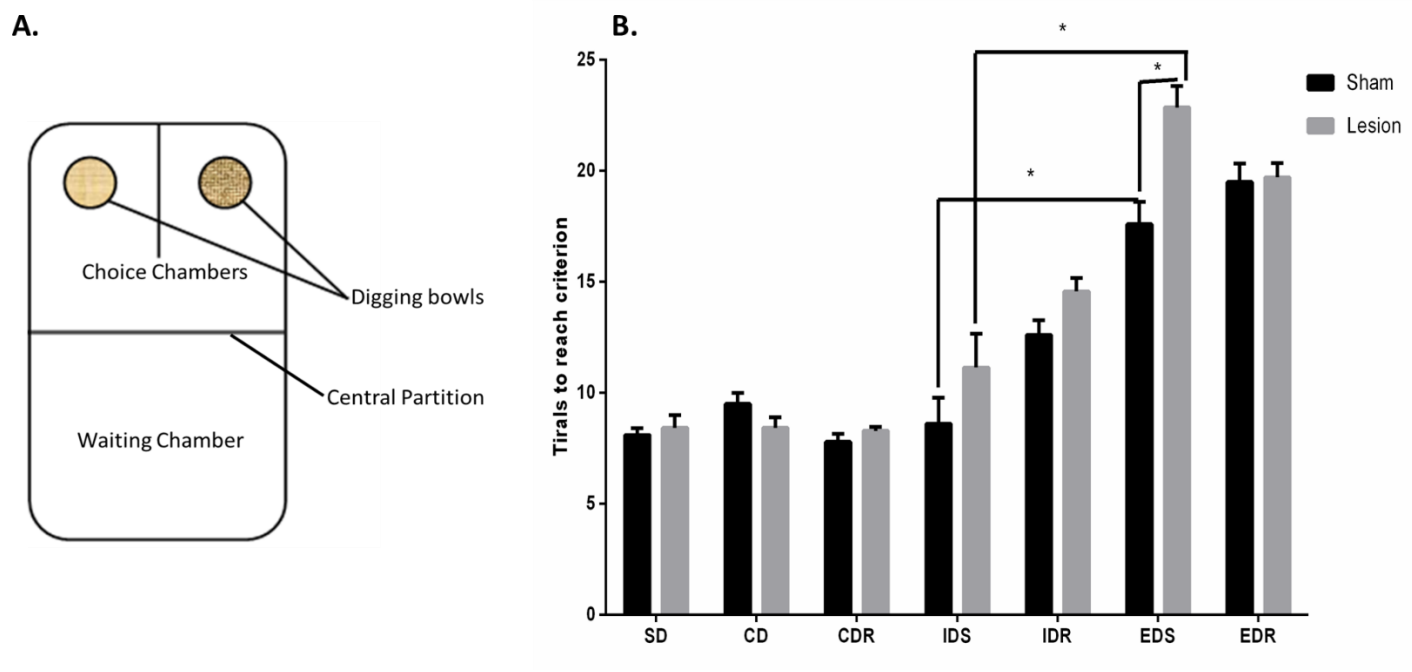

Figure 1. Cognitive behavioural outcome in adult sham and neonatal ventral hippocampus lesion (NVHL) animals during attentional set-shifting test. (A) Schematic representation of the attentional set-shifting testing apparatus. (B) Mean number of trials to reach criterion in adult sham and neonatal ventral hippocampus lesion (NVHL) on each discrimination stage in the attentional set-shifting test. Analysis of the mean number of trials revealed a significant increase in the number of trials required by lesion animals compared to sham animals during the extra-dimensional shift (EDS) (*p=0.0001). No significant difference between the two groups was observed in following discrimination stages: simple discrimination (SD- p =0.999), compound discrimination (CD- p =0.999), compound discrimination reversal (CDR- p =0.999), intra-dimensional shift (IDS- p =0.187), intra-dimensional reversal (IDR- p =0.592) or extra-dimensional reversal (EDR- p =0.999). NVHL group required highest number of trials to reach criterion at EDS stage as compared to other stages. Significant difference between EDS and IDS in sham (*p=0.0001) and lesion group (*p=0.0001) was reported. (n=7-10).

Figure 2.

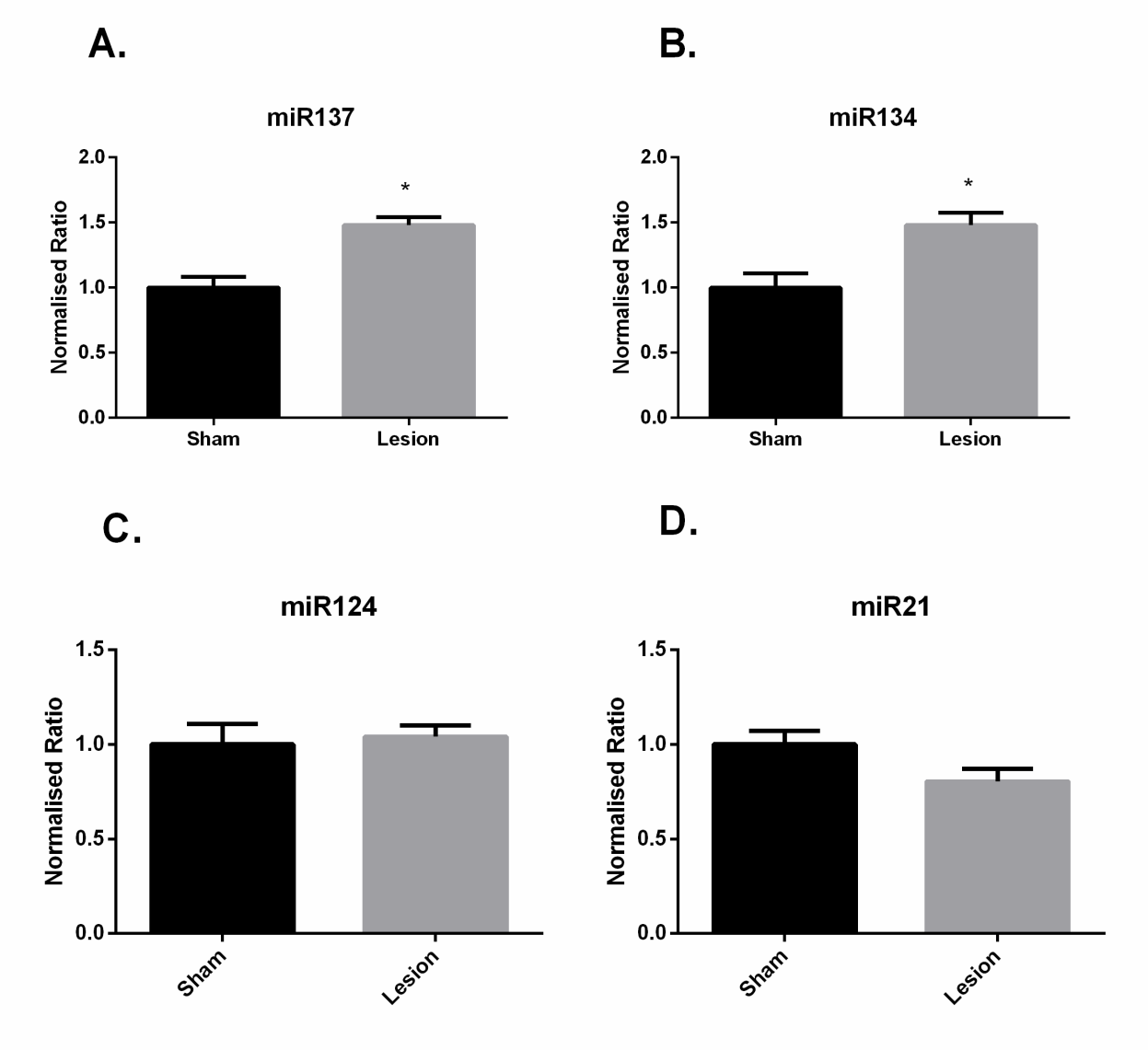

Figure 2. Level of expression of miRNAs from sham and neonatal ventral hippocampus lesion (NVHL) animals in the medial prefrontal cortex at P60. Quantification of Q-RTPCR data (Normalised ratio of miRNAs over control miRNA) expression at P60. (A, B) Unpaired two-tailed t test revealed a significant increase in the expression of miR-137 (*p =0.002) and miR-134 (*p =0.013) in the NVHL group as compared to the sham group. (C, D) No significant effect of NVHL was found on the expression of miR-124 (p =0.737) and miR-21 (p =0.088) between the two groups. (n=4-5).

Figure 3.

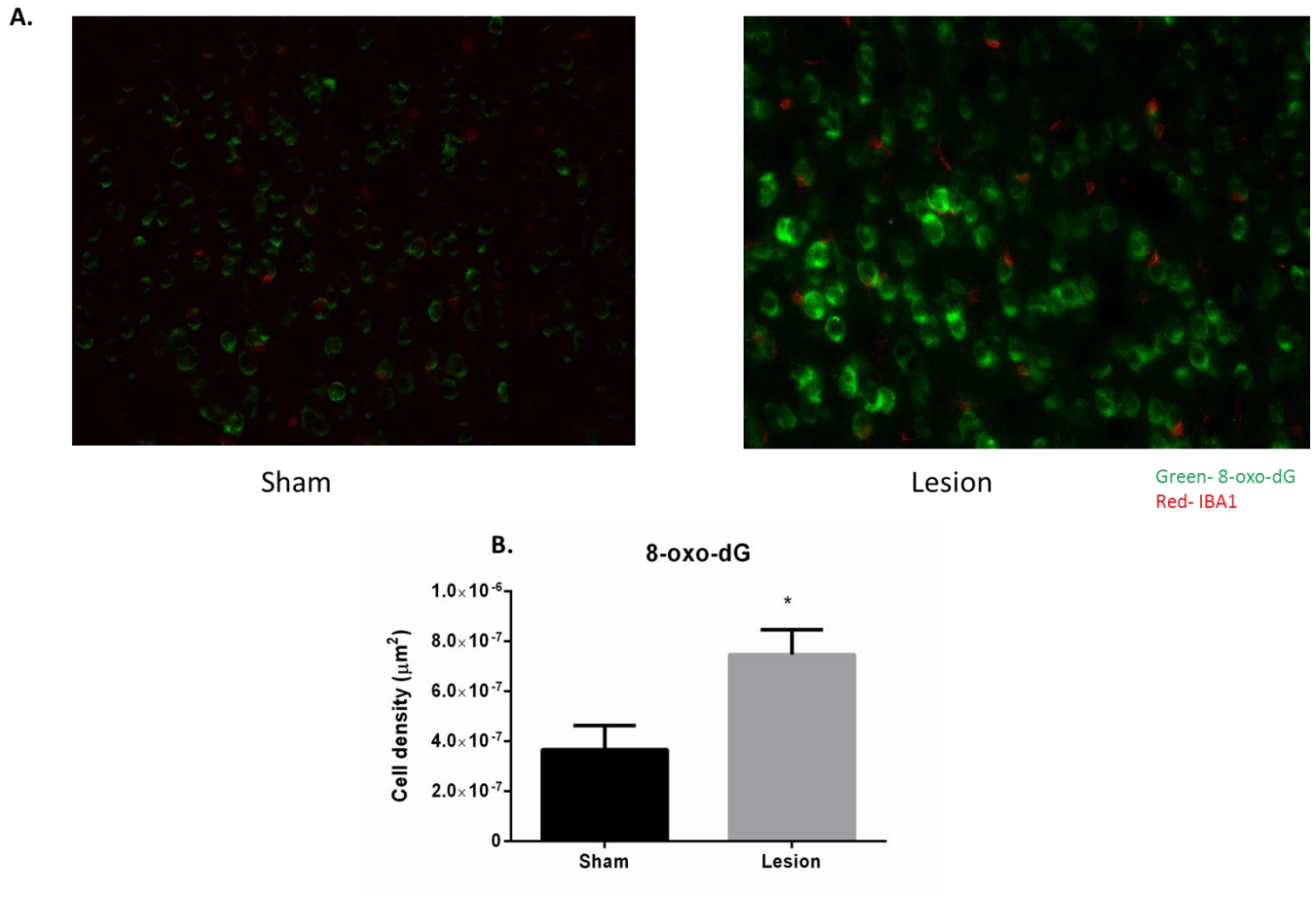

Figure 3. Level of oxidative stress in the mPFC as a result of neonatal ventral hippocampus lesion (NVHL). A. Representative images showing immuno-fluorescent expression of the oxidative stress marker (8-oxo-dG; green) and microglia cells (IBA1; red) in the mPFC of adult sham and lesion rats. The 8-oxo-dG expressing cells were not found to be co-labeled with microglia cells in both sham and lesion group. B. A significant increase of 8-oxo-dG immune-positive cells in the mPFC of NVHL animals compared to sham group (*p-0.0335). (n=4).

Table 1. Different dimensions and combination of stimuli used in the attentional-set shifting test.

ASST stages	**Dimensions**		**Exemplar compound stimuli**	
	Relevant	Irrelevant	Correct	Incorrect
Simple discrimination (SD)	Odor		Basil/bedding	Onion/bedding
Compound discrimination (CD)	Odor	Texture	Basil/Confetti	Onion/Confetti
			Basil/Cotton flowers	Onion/Cotton flowers
Compound discrimination reversal (CDR)	Odor	Texture	Onion/Confetti	Basil/Confetti
			Onion/Cotton flowers	Basil/Cotton flowers
Intra-dimensional shift (IDS)	Odor	Texture Texture	Garlic/Wool	Nutmeg/ Plastic pearls
			Garlic/ Plastic pearls	Nutmeg/Wool
Intra-dimensional reversal (IDR)	Odor	Texture	Nutmeg/Wool	Garlic/ Plastic pearls
			Nutmeg/ Plastic pearls	Garlic/Wool
Extra-dimensional shift (EDS)	Texture	Odor	Gravel/Thyme	Beads/Paprika
			Gravel/Paprika	Beads/Thyme
Extra-dimensional reversal (EDR)	Texture	Odor	Beads/Thyme	Gravel/Paprika
			Beads/Paprika	Gravel/Thyme

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CONNECTING STATEMENT TO CHAPTER V

In the previous chapters using a neurodevelopmental model we showed that the neonatal loss of cells within the VH is implicated in adult behavioral and cellular deficits. In chapter II we revealed a development specific increase in the expression of complement molecules (C1q and C3) and suggested the role of microglia in synaptic pruning in the mPFC of NVHL animals. In chapter III we showed an alteration in the pro- and anti-inflammatory cytokine balance to be associated with reduced spine density in the mPFC of adult NVHL animals. We further provided an evidence in chapter IV of the role of miRNAs as possible mechanistic link in the behavioral and cellular alterations in the PFC of the NVHL animals. Since the NVHL model uses the indiscriminate excitotoxicity of the ibotenic acid to cause a lesion; the specific role of the neonatal VH neuronal populations on adult behavioral and cellular deficit cannot be inferred from this model. Thus, the next question we wanted to address was the contribution of specific cellular populations in the VH of neonatal animals in modulating adult behavioral and cellular deficits. We hypothesized a critical role of neonatal VH excitatory neurons in regulating adult behaviors and spine density. We tested this hypothesis in Chapter V by using a non-lesion viral based method. The results presented in Chapter V are unpublished.

CHAPTER V

Effect of neonatal ablation of ventral hippocampus excitatory neurons on behavioral and cellular changes in adult mice.

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Abstract

Ventral hippocampus (VH) modulates behaviors such as executive control, emotions and goal directed behaviors by connectivity to various cortical and limbic regions (prefrontal cortex (PFC), nucleus accumbens and amygdala). Developmental behavioral disorders are hypothesized to arise from a disruption in VH-PFC/limbic connectivity. A model to test this hypothesis, the neonatal ventral hippocampus lesion (NVHL) model in rats, has revealed that lesion of the VH in pups leads to adult schizophrenia-like behavioral deficit (hyperlocomotion, social memory deficit and cognitive deficits). Since NVHL model uses the indiscriminate excitotoxicity of the ibotenic acid to lesion the VH, the specific role of the neonatal VH neuronal populations on adult behavior cannot be inferred from this model. Thus, we reasoned that neonatal ablation of the VH excitatory neurons may be responsible for adult behavioral and cellular deficits. In order to test this hypothesis, an adeno-associated viral 8 (AAV8) vector carrying diphtheria toxin A (dTA) or a control virus without dTA was microinfused bilaterally in the VH of C57Bl6/J calcium/calmodulin-dependent protein kinase II (CaMKII)-cre mice at neonatal age P12. After 21 days of microinjection, we confirmed the virus expression, injection site and ablation of the excitatory neurons by immunohistochemistry using mCherry and CaMKII antibodies followed by cell counting. Our data showed ablation of a significant number of excitatory neurons within the VH as a result of neonatal dTA (NDT) virus injection compared to the control virus. Another cohort of male NDT virus injected mice were allowed to grow to adulthood when we assessed select behaviors. Our data showed no significant changes in spontaneous locomotor activity in the NDT group of mice compared to the control virus group. However, the NDT-injected group exhibited a deficit in social memory as well as spatial memory retention compared to the control group as assessed by social novelty and Barnes maze tests, respectively. Adult dTA virus-injected group also exhibited altered expression of genes involved in presynaptic function (synaptophysin) and synaptic pruning (complement C4 & C1q) in the mPFC. Taken together, our data show that developmental ablation of excitatory neurons in the VH can lead to abnormalities in social, cognitive functions and synaptic molecules implicated in schizophrenia.

Introduction

Schizophrenia is a neurodevelopmental disorder exhibiting a range of pathological and behavioral impairments (Owen, O'Donovan, Thapar, & Craddock, 2011). Early developmental insults during perinatal period has long term effect on circuit development and behaviors later in life (van Os et al., 2009). Schizophrenia symptoms typically emerge during late adolescence or in early adulthood, although cognitive deficits precede the illness, are progressive and persist throughout the course of the disorder (Sorensen et al., 2006). Schizophrenia patients exhibit abnormality in two key brain regions, the prefrontal cortex (PFC) and hippocampus (HPC) which play a crucial role in regulating cognitive functions (Sakurai et al., 2015; Strange et al., 2014).

Development of hippocampal connectivity with PFC and other regions of the brain (nucleus accumbens and amygdala) have been found to modulate schizophrenia-related behaviors (Volk & Lewis, 2010). The dorsal hippocampus (DH) is predominantly involved in spatial learning and memory whereas the ventral hippocampus (VH) modulates executive control, emotion and goal-directed behaviors (Fanselow & Dong, 2010; Howland et al., 2008; Strange et al., 2014). During early development VH mono or poly-synaptic inputs target one or more projection neurons in the PFC to drive specific adult behavioral outputs including social memory and cognition (O'Neill, Gordon, & Sigurdsson, 2013; Padilla-Coreano et al., 2016). Synchronized theta-gamma oscillations between VH and the PFC respectively are particularly important for working memory (O'Neill et al., 2013; Sigurdsson & Duvarci, 2015).

A role of early developmental abnormality in the hippocampus on adult schizophrenia-like behaviors has previously been studied by ours and other groups using the neonatal ventral hippocampus (NVH)-lesioned rats (Marcotte et al., 2001; Tseng et al., 2009; Joseph et al., 2018; Hui et al., 2019). This model using excitotoxin ibotenic acid to induce VH lesion in neonates showed adult animals exhibiting behavioral deficits such as amphetamine-induced hyperlocomotion and deficits in prepulse inhibition (PPI) of startle, social memory and prefrontal-related cognitive function. The behavioral changes were found to be associated with a number of cellular and molecular changes in the mPFC after NVH lesion. For example, reduced dendritic spine density in layer 3 and 5 pyramidal neurons in the mPFC, imbalance in excitatory and inhibitory synaptic transmission (Joseph et al., 2018; Hui et al., 2019; Ryan et al., 2013) and impaired synaptic plasticity (Bhardwaj et al., 2014) have all been reported in adult

animals following NVHL. A limitation of this model is that the role of specific cell populations within the VH in causing adult behavioral or cellular deficits cannot be easily deciphered.

Like other brain regions, the VH contains excitatory and inhibitory neurons as well as non-neuronal cells such as astrocytes, oligodendrocytes and microglia that have been suggested to play a crucial role in different behaviors (X. Wang, Pinto-Duarte, Sejnowski, & Behrens, 2013; Okuyama, Kitamura, Roy, Itohara, & Tonegawa, 2016; Peng et al., 2019). For example, optogenetic inhibition of CA1 VH excitatory neurons in adult mice has been reported to result in social memory deficits (Okuyama et al., 2016). However, optogenetic activation of the VH excitatory neurons were shown to cause hyperlocomotion and a deficit in short-term memory (Wolff et al., 2018). Further, inhibition of the CA3 glutamatergic neurons has been suggested to play a role in motivational and emotional behaviors (Sweeney & Yang, 2015). Besides the excitatory neurons, GABA inhibitory neurons as well as interneurons (eg., parvalbumin expressing neurons) within the VH have also been suggested to modulate behaviors such as sensorimotor processing and cognition (Nguyen et al., 2014; Caballero, Flores-Barrera, Cass, & Tseng, 2014). Non-neuronal cells such as microglia and astrocytes have also been suggested to regulate VH neuronal excitability and modulate motivation and fear-related behaviors in mice (Peng et al., 2019; Jinno, Fleischer, Eckel, Schmidt, & Kosaka, 2007).

In this study, we assessed the contribution of excitatory neuronal subpopulations within the developing VH in the development of adult behavioral and molecular functions. We used a viral construct with cre-dependent expression of diphtheria toxin subunit A (dTA) to ablate the excitatory neurons in the VH of neonatal C57Bl6/J CaMKII-cre mice (P12). Our results show that animals with excitatory neuronal ablation exhibit deficits in social memory and memory retention during adulthood. We also observed alterations in the mRNA expression of a presynaptic protein (synaptophysin) and complement synaptic protein (C4 & C1q) in the mPFC of adult animals after neonatal ablation of the VH excitatory neurons. Together, these data suggest a crucial role of the developing VH excitatory neurons in cognitive and synaptic maturation of the PFC.

Materials and Methods

Animal Care and use of animals was in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of Facility Animal Care Committee at the Douglas Mental Health University Institute (DMHUI), McGill University. CaMKII-cre adult mice (Male and female) were obtained from Charles River Laboratories (Québec, Canada) and underwent breeding to obtain homozygotes CaMKII-cre line. One homozygous CaMKII-cre male was paired with two homozygous CaMKII-cre females for breeding. Homozygous CaMKII-cre male pups were genotypes after breeding and were used for the experiments except a few animals which had to be excluded due to ill-health. They were housed in a temperature- and humidity-regulated environment at the animal facility of DMHUI. Animals were housed on a 12 h light–dark cycle with ad libitum food and water.

AAV injection- The diphtheria toxin A (dTA) subunit containing viral construct AAV8.flex. dTA.mCherry (University of North Carolina vector core centre) and the control AAV8.flex. mCherry (Canadian neurophotonics platform- University of Laval) were microinjected in the VH of the male neonatal CaMKII-cre mice (P12). The pups were assigned to the dTA virus and control virus groups at random within the same litter. In order to perform the microinjections, the pups were anesthetized with 2%–5% isoflurane and immobilized on a stereotaxic frame (David Kopf Instruments). A volume of 0.25µl of dTA or control virus was injected bilaterally on each side of the VH at a rate of 0.05 µl/min using a 10-µl Hamilton syringe. The coordinates used to target the VH of neonatal mice were AP -3.3, ML ±3.2, DV -4. After surgery, the pups were housed with the dams until. Viral expression after 21 days was analyzed using standard histological analysis.

Immunofluorescence and Cell Counting

A cohort of control and dTA virus injected mice were anesthetized and perfused trans-cardially with 0.1 m PBS (pH 7.4), followed by 4% paraformaldehyde in 0.1 m PBS. The brains were removed, post-fixed overnight at 4°C, and then transferred to 30% sucrose for 48 hr at 4°C. Coronal sections (35 µm) were collected at levels corresponding to ventral hippocampus using a

microtome (LeicaVT1200S). For staining, the sections were rinsed in 1xPBS, blocked and incubated in primary CaMKII alpha monoclonal antibody (Invitrogen) overnight at 4°C. The next day, the sections were further processed and incubated in secondary antibody (anti-mouse, Alexa Fluor® 488) and mounted on slides. Distribution of immunoreactive cells were studied by viewing the slides with an Olympus upright fluorescence microscope (BX63) at 20× magnification. Cells immunoreactive for CaMKII-α were counted in the pyramidal layers of the VH using the QuPath software. The cell counts were divided by the area of the corresponding region to determine cell density per square micrometer.

Behavioral Testing

The following behavioral tests were performed at P60 in AAV-dTA and control virus injected CamKII-cre mice- spontaneous locomotor activity, social interaction and Barnes maze test. The same cohort of animals were used for behavioral tests that were each separated by 72 h. The tests were performed during the light phase of 12 h light-dark cycle (lights on at 8:00H and off at 20:00H) starting with the least stressful, in the order presented below. These behavioral tests were selected due to their relevance to schizophrenia and other neurodevelopmental disorders (Powell et al., 2012).

Spontaneous Locomotor Activity

Spontaneous locomotor activity was assessed in a dimly lit room using acrylic activity chambers (20 × 20 × 29.5 cm, L × W × H) adapted for use with mice (AccuScan Instruments Inc., Columbus, OH, USA) as described previously (Bhardwaj et al., 2012). The chambers were equipped with infrared sensors to monitor locomotor activity. Animals were brought from their home cage to the testing room and immediately placed in the activity boxes where their activity was monitored during the next 120 minutes and data collected using Versamax Software. Total horizontal activity was used to analyze the locomotor behavior.

Social Interaction (SI)

The three-chamber method of Crawley (Crawley, 2004) as described previously (McKibben et al., 2014) was used to measure the sociability of the rodents with a conspecific as well as social discrimination memory of familiar vs. novel conspecific mouse. The testing box (60 x 25 x 25cm, L x W x H) comprised three chambers (a central and two adjoining) with opening to allow movement of mice in all chambers. Two small circular wire mesh cages (10cm x 5cm, DxH) were placed in two chambers on the right and left of the central chamber. The test animals were first habituated to the testing box by placing them in the central chamber and they were allowed to explore all chambers for 10 min. After this, the animals were placed back in their home cage. For the testing procedure, an unfamiliar mouse (same strain, sex and age) (stranger 1, S1) was placed in any one of the wire mesh cage and test animals were reintroduced in the central chamber. The test animal was allowed to explore all chambers for 5 min. The interaction between the test animal and the stranger mouse (S1) as compared to the empty mesh cage was considered as a measure of sociability. To measure social novelty preference or social memory, after the first 5 min of interaction, the test animal was placed back in the home cage for 5 min (Retention interval) during which another novel stranger (S2) or unfamiliar mouse was introduced in the second mesh cage. After the retention interval, the test animal was reintroduced into the central chamber and could again explore all chambers for 5 min.

Throughout the experiment, the interaction of the test mouse with the wire mesh cage or unfamiliar mice were video recorded and scored for sociability and social memory. The social interaction was measured by nose contacts and sniffing within a distance of 1 cm. The videos were scored in a "blinded" manner and the interaction time measured between test animals and mice in the mesh cage (exploration time). Sociability and social memory analysis were carried out by calculating the exploration ratio. The sociability exploration ratio was calculated with the following equation: time spent with stranger 1 (S1)/Total time of interaction (S1 + empty mesh cage) * 100. The following equation was used to measure social memory: time spent with the novel animal (S2)/Total time of interaction (S1+ S2) * 100. Total time of interaction is the time spent by the test animal in interacting with the familiar animal (S1) and the novel animal (S2).

Barnes maze

Barnes maze test was used to measure working memory, reference memory, and cognitive flexibility (O'Leary & Brown, 2012). In this test, the test animal was placed in a dry circular platform (69 cm diameter) with 16 circular holes (4.45 cm diameter) equally spaced around the perimeter. The test animal learned to locate a target hole and enter the escape box (13cm × 29cm × 14cm), located below the surface of the platform. The maze was elevated 50 cm above the floor and covered with curtains. Shapes (triangle, circle, and rectangle) were placed on the curtain around the maze at specific locations and served as spatial visual cues. The location of the different spatial visual cues were consistent throughout the experiment. Two 150-W flood lamps positioned above the maze and a radio was placed below the maze, which were used as aversive stimuli. The behavior was video-recorded using an overhead camera placed on top of the maze. We used a shortened version of the Barnes maze protocol as previously described by (O'Leary & Brown, 2012). The test animals completed five phases of testing including habituation, acquisition training phase, acquisition probe test, reversal training phase, and reversal test. During habituation (Day1), the test mice were placed in the center of the platform and allowed to explore the maze for 3 minutes in the absence of target hole and presence of aversive stimuli. After which the test mice went through the acquisition training phase, which consisted of 5 days (2trials/day). During this phase, the test mice were placed in the center of the platform and both aversive cues were turned on, until the mice entered the target hole. For each training trial the test mice were given 3 minutes to locate the target hole and enter in the escape box. After 3 minutes in case the test mice failed to enter the escape box, they were guided towards the target hole and allowed to enter the escape box. The second trial was performed after 5 minutes (inter-trial interval). After each trial, mice remained in the escape box for 30 s before being returned to their holding cage. During acquisition training phase, the latency to find the escape hole was considered as a measure of learning. Acquisition probe test was performed after the last day of acquisition by placing the test mice in the center of the platform in the presence of aversive cue and with the target escape hole blocked. The mice were given 3 minutes to explore the maze and the time spent around the target hole was measured. Twenty four hours following the probe test, the mice were trained for reversal learning for 5 days (2 trials/day). Reversal training and the probe test were performed similar to the acquisition training, but the location of the target hole was moved 180◦ from its location during acquisition phase. The videos were

scored using a software ANY-maze to measure the latency to enter the target hole and the time spent around the target hole during training and probe testings. For both acquisition and reversal learning phases the average of the two trials were calculated and used to measure learning for each day.

RNA Extraction and Gene Expression Using QRT-PCR

Gene expression study was performed on a subgroup of neonatal dTA virus and control virus injected mice after the behavioral test. Adult mice were sacrificed by decapitation, their brains were extracted and sliced into 1mm slices. The infra-limbic and pre-limbic regions of the mPFC were micro punched and the tissues of both hemispheres from each animal were pooled and stored at −80°C until use. RNA extraction was performed using Trizol reagent (Catalog No. 15596026; Thermo Fisher Scientific) with the Purelink RNA mini kit (Catalog No.12183018A; Thermo Fisher Scientific). Yield and the quality of the RNA was determined using Nanodrop and agarose gel electrophoresis. Two micrograms of RNA were used for the cDNA synthesis using the high capacity cDNA reverse transcription kit (Catalog No. 4368814; Applied Biosystem).

Primers for Transforming growth factor-β1 (TGF-β1) (F-TACCATGCCAACTTCTGTCTGGGA, R-ATGTTGGACAACTGCTCCACCTTG), Synaptophysin (F-TGTGTTTGCCTTCCTCTACTC, R-TCAGTGGCCATCTTCACATC), C1q (F-CAAGGACTG AAGGGCGTGAA, R- CAAGCGTCATTGGGTTCTGC), C3 (F-CACCGCCAAGAATCGCTAC, R-GATCAGGTGTTTCAGCCGC), C4 (F-TCTCACAAACCCCTCGACAT, R-AGCATCCTGGAACACCTGAA) and housekeeping gene GAPDH (F-ATGACATCAAGAAGGTGGTG, R- CATACCAGGAAATGAGCTTG) were designed using the NCBI DNA sequence and the primer express software. QRT-PCR was performed using SYBR green PCR master mix (Catalog No. A6001; Promega) according to the manufacturer's protocol. The following cycling conditions were used in Applied Biosystem Real time PCR 7500 machine. Initial denaturation at 95°C for 10 min, followed by 40 cycles with denaturation at 95°C for 15 s, annealing at 60°C for 1 min and elongation at 72°C for 1 min. A standard and a melting curve for all the genes were obtained to check the efficiency of the primers. $2-\Delta\Delta CT$ method was used to calculate the fold changes.

Data Analysis

All reported values are mean ± standard error of the mean (SEM). All data were analyzed using Prism (GraphPad, Version 6). Two-tailed unpaired t-test was used to determine cell density, behavioural, gene expression changes. Two-way repeated measure ANOVA followed by tukeys post-hoc test was used to determine interactions between virus, timeline of horizontal activity and days of acquisition and reversal training phase. For all analysis $p < 0.05$ was considered statistically significant.

Results

Virus expression and ablation verification.

AAV control and AVV-dTA viruses were microinjected in the VH of neonatal CaMKII-cre mice and their expression within the VH was confirmed by mCherry immune-reactive cells after 21days of microinjection as shown in Figure 1. In order to verify the ablation of excitatory within the VH, we performed cell counting of CaMKII immune-positive neurons. The data show a significant decrease in the number of CaMKII-positive neurons within the VH as a result of neonatal dTA virus injection compared to the control virus ($t = 2.678$, $df = 6$, $p = 0.036$; Figure 2).

The effect of neonatal ablation of excitatory neurons within the VH on adult behaviors.

Locomotor activity- Our data analysis revealed no significant effect of neonatal VH neuronal ablation on spontaneous locomotor activity in adult CaMKII-cre mice compared to mice microinjected with the control virus. A two-way repeated measure ANOVA showed no significant two-way interaction on virus × timeline of horizontal activity ($F(23,368) = 0.914$, $p = 0.5$; Figure 3A). Further analysis on total horizontal activity for the whole session was also found to be not significant between the two groups ($t = 1.011$, $df = 16$, $p = 0.327$; Figure 3B).

Social interaction (SI) – Analysis on sociability data using two-tailed unpaired t-test did not reveal any significant difference between the control and dTA virus injected group ($t = 2.715$, $df = 16$, $p = 0.015$; Figure 3C). However, the ablated adult animals showed a significant reduction in social memory compared to controls ($t = 0.810$, $df = 16$, $p = 0.4$; Figure 3D).

Barnes maze test- We examined Barnes maze test training data and revealed no significant difference in learning between the control and dTA virus injected group. Two-way repeated measure ANOVA on the latency to escape over five days revealed no significant two-way interaction on virus × days during both acquisition ($F(4,56) = 0.994$, $p = 0.4$; Figure 3E) and reversal training phase ($F(4,56) = 1.614$, $p = 0.183$; Figure 3F). Further analysis on the acquisition probe test revealed a significant decrease in short-term memory retention; as shown by the time spent around the escape hole among the neonatal dTA virus injected group compared to control virus injected group ($t = 4.409$, $df = 14$, $p = 0.006$; Figure 3G). Whereas, no significant effect of neonatal ablation of excitatory neurons was observed in the reversal probe test between the two groups ($t = 1.131$, $df = 14$, $p = 0.278$; Figure 3H).

Effects of neonatal ablation of excitatory neurons within the VH on synaptic pruning-related genes in the mPFC.

We examined the mRNA expression data (Figure 4) and showed a significant decrease in the expression of Synaptophysin ($t = 4.290$, $df = 9$, $p = 0.002$; B), C1q ($t = 2.314$, $df = 9$, $p = 0.046$; C) and C4 ($t = 2.869$, $df = 9$, $p = 0.018$; E) in mPFC of neonatal dTA virus injected group as compared to control virus injected group. The expression of other complement and synaptic pruning-related genes including TGF-β1 ($t = 1.296$, $df = 9$, $p = 0.227$; A) and C3 ($t = 0.786$, $df = 8$, $p = 0.4$; D) were not found to be significantly different between the two groups.

Discussion

In the current study, we observed deficits in some aspects of social and cognitive functions as a result of ablation of neonatal VH excitatory neurons. We also found a reduction in the expression of complement and synaptic pruning-related genes in the mPFC of adult mice. Our data points towards a crucial role of the developing VH excitatory neurons in regulating behaviors and synaptic pruning in the mPFC of adult mice.

Previous studies have provided evidence supporting the role of VH excitatory neurons in regulating social memory and cognition in adult mice (Okuyama et al., 2016; Wolff et al., 2018). Our data here found a key role of neonatal VH excitatory neurons in regulating adult social memory function. Our behavioral analyses revealed a deficit in some aspects of cognition in our

model; during Barnes maze test we observed no difference in the learning pattern between the neonatal dTA virus and control virus injected groups. This could be as a result of the mice in our study adopting a serial search strategy to navigate the escape; which is a hippocampus-independent process and thus not affected as a result of ablation of the neonatal VH excitatory neurons (O'Leary & Brown, 2013). However, further analysis of the Barnes maze test data revealed a deficit in short-term memory retention among the mice in the dTA virus injected group; suggesting a crucial role of the neonatal VH excitatory neurons in modulating adult cognitive behaviors.

Previous studies have elucidated the role of neonatal VH in regulating adult behavioral deficits (eg., amphetamine-induced hyper-locomotion, deficit in social memory, PPI and cognition) using NVHL model (Marcotte et al., 2001; Joseph et al., 2018). In this model, the excitotoxicity of ibotenic acid used to lesion the VH results in loss of all types of cells within the developing VH; therefore, limiting our understanding of the specific role of the neonatal VH neuronal populations on adult behaviors. Whereas, in this study by ablating the excitatory neurons we provide a better understanding on the role of neonatal VH excitatory neurons in regulating adult behaviors (social memory and cognition); which were also seen to be altered in NVHL model. Besides adult behavioral deficits, cellular deficits (eg., impaired synaptic plasticity and imbalance in excitatory and inhibitory synaptic transmission) in the mPFC have also been reported in previous studies as a result of NVHL (Bhardwaj et al., 2014; Ryan et al., 2013). A development specific increase in the expression of synaptic pruning-related gene (C1q) was observed in the mPFC of adult NVHL animals (Hui et al., 2019). Whereas, in this study we observed reduced expression of genes involved in presynaptic function (synaptophysin) and synaptic pruning (complement C4 & C1q) in the mPFC of adult mice as a result of neonatal ablation of the VH excitatory neurons. The disparity with respect to the expression of C1q gene between the neonatal VH lesion and neonatal VH excitatory neuronal ablation could be due to a key role of developing VH excitatory neurons in regulating homoeostatic functioning of the complement system in the PFC of adult animals. Altered expression of complement proteins such as C1q and C4 have been identified at immature spines and have been reported to work synergistically with microglia to regulate synaptic pruning within the PFC (Chu et al., 2010; Sekar et al., 2016). Our data suggests a crucial role of neonatal VH excitatory neurons in modulating circuit refinement and synaptic plasticity in the PFC.

A significant proportion of VH excitatory neurons are reported to project to mPFC and other limbic regions (Brockmann et al., 2011). The VH excitatory neurons projecting to mPFC were shown to modulate adult social and cognitive behaviors (Okuyama et al., 2016; Godsil et al., 2013). VH neurons fire at high frequency theta oscillations, these oscillations in the VH act in co-ordination with the gamma oscillations (30–100 Hz) in the PFC to regulate working memory and cognitive function (Thierry et al., 2000; Pastalkova et al., 2008). Inactivation of the VH neurons were shown to cause reduced theta-gamma synchrony between VH and mPFC, thus suggesting a crucial role of VH neurons in regulating mPFC neuronal activity and behaviors (Sigurdsson & Duvarci, 2015; Liu & Carter, 2018; Padilla-Coreano et al., 2016). Further, a synchronized VH-PFC pathway was shown to maintain a balance in excitatory and inhibitory neurotransmission in the PFC and regulate social memory and cognitive function (Phillips, Robinson, & Pozzo-Miller, 2019; Liu & Carter, 2018). Although, we assume in our study that the ablation of neonatal VH excitatory neurons would result in reduced excitatory neurotransmission in the VH, we have not measured it, and this could be one of the limitations of our study. However, overall our behavioural data suggests the role of neonatal VH excitatory neurons in regulating adult cognition and formation of social memories.

Our data are consistent with the evidence pointing towards a key role of developing VH in schizophrenia-related behavioural and cellular deficits (van Os et al., 2009). Evidence supporting the role of neonatal VH towards schizophrenia-related behaviours have been provided previously by us and others (Joseph et al., 2018; Tseng et al., 2009). Interestingly, in this study we have used a viral based ablation method to decipher the role of early developmental VH excitatory neurons in some aspect of cognitive and social function. Our data suggests a crucial role of neonatal VH neuronal activity in regulating VH-PFC connectivity and thereby modulating schizophrenia-related behavioural and cellular deficits. Increased neuronal activation within the HPC has been found to alter HPC-DLPFC functional connectivity during working memory task in the schizophrenia patients as compared to the healthy controls (Meyer-Lindenberg et al., 2005). Additionally, optogenetic activation of the excitatory neurons of the VH in an animal model was found to cause schizophrenia -related cognitive dysfunction (Wolff et al., 2018). Further, hippocampal hyperactivity was also found to be associated with reduced expression of glutamate-metabolizing enzyme; which has been suggested to alter synaptic function and lead to hippocampal volume loss (Grimm et al., 2018; Lander et al., 2019). Reduced hippocampal

volume and hippocampal hyperactivity has been reported in individuals at high-risk of developing schizophrenia (Harrisberger et al., 2016; Schobel et al., 2009). A meta-analysis found both pre- and post-synaptic proteins to be significantly reduced in hippocampal and cortical region of schizophrenia post mortem samples (Osimo, Beck, Reis Marques, & Howes, 2019). Our data from this study point towards similar behavioral and cellular deficits observed as a result of increased hippocampal activity. Future studies examining neonatal VH inhibitory and non-neuronal cells could be carried out to better understand the role of developing VH in shaping mPFC circuit and function. In summary, our study using viral ablation of excitatory neurons further establishes a critical role of neonatal VH excitatory neurons in driving certain adult onset behavioral and cellular abnormalities in the mPFC.

Acknowledgments and Disclosures

Author Contributions

AJ conceived, designed and performed the experiments and wrote the first draft of the manuscript. SB helped in performing experiments and data analysis. LS contributed to the development of research idea and implementation, discussion of the results and part of the manuscript writing.

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Figures

Figure 1.

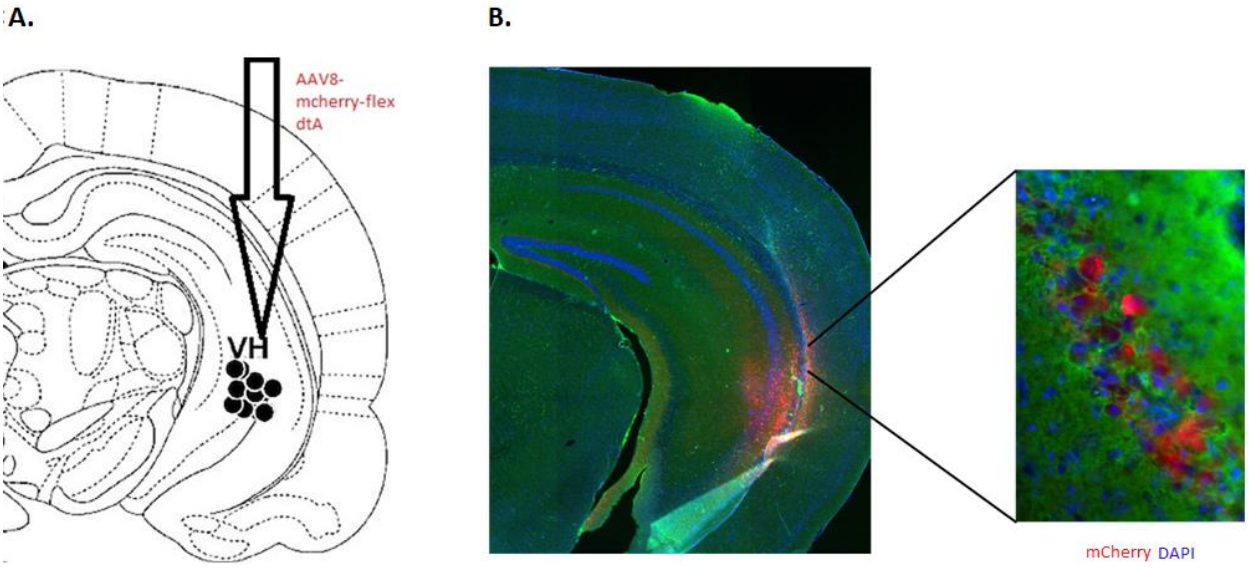

Figure 1. Virus expression in the ventral hippocampus after 21 days. A. Schematic of the ventral hippocampus (VH) and the arrow showing the site of AAV injection in the neonatal mice. B. Representative image showing the expression of the virus within the VH confirmed by mCherry immuno-positive cells.

Figure 2.

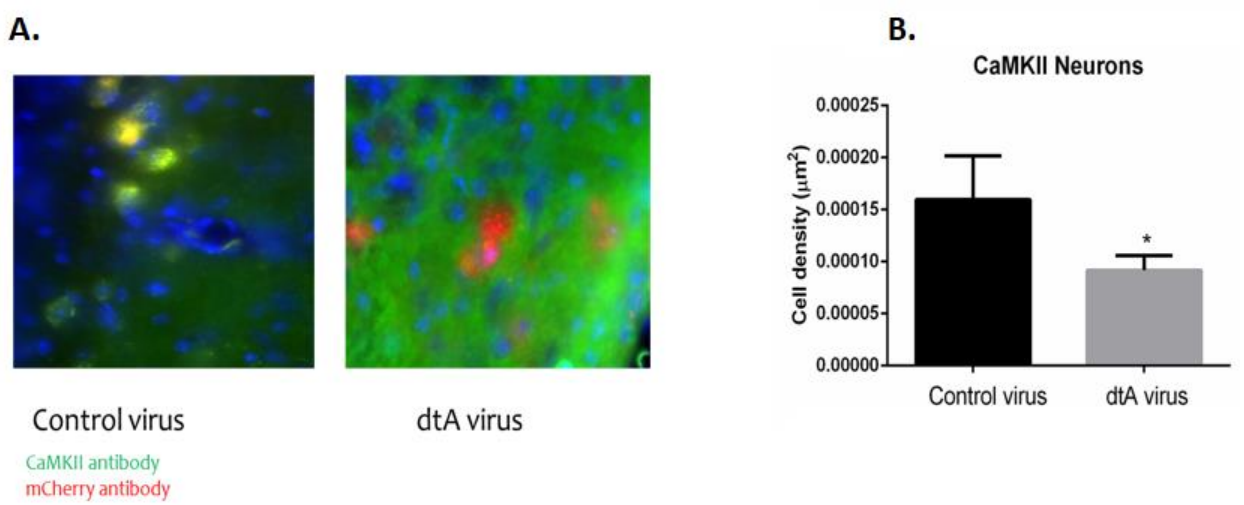

Figure 2. Confirmation of ablation within the ventral hippocampus. A. Control virus expressed mCherry (red) and co-labelled with immuno-fluorescent CaMKII (green) immuno-positive excitatory neurons. dTA virus expressed mCherry with no co-labelling with CaMKII expressing neurons indicating ablation of excitatory neurons. B. A significant reduction of CaMKII-positive neurons within the VH of dTA virus injected group as compared to control virus injected group (*p-0.0366). (n=4 per group).

Figure 3.

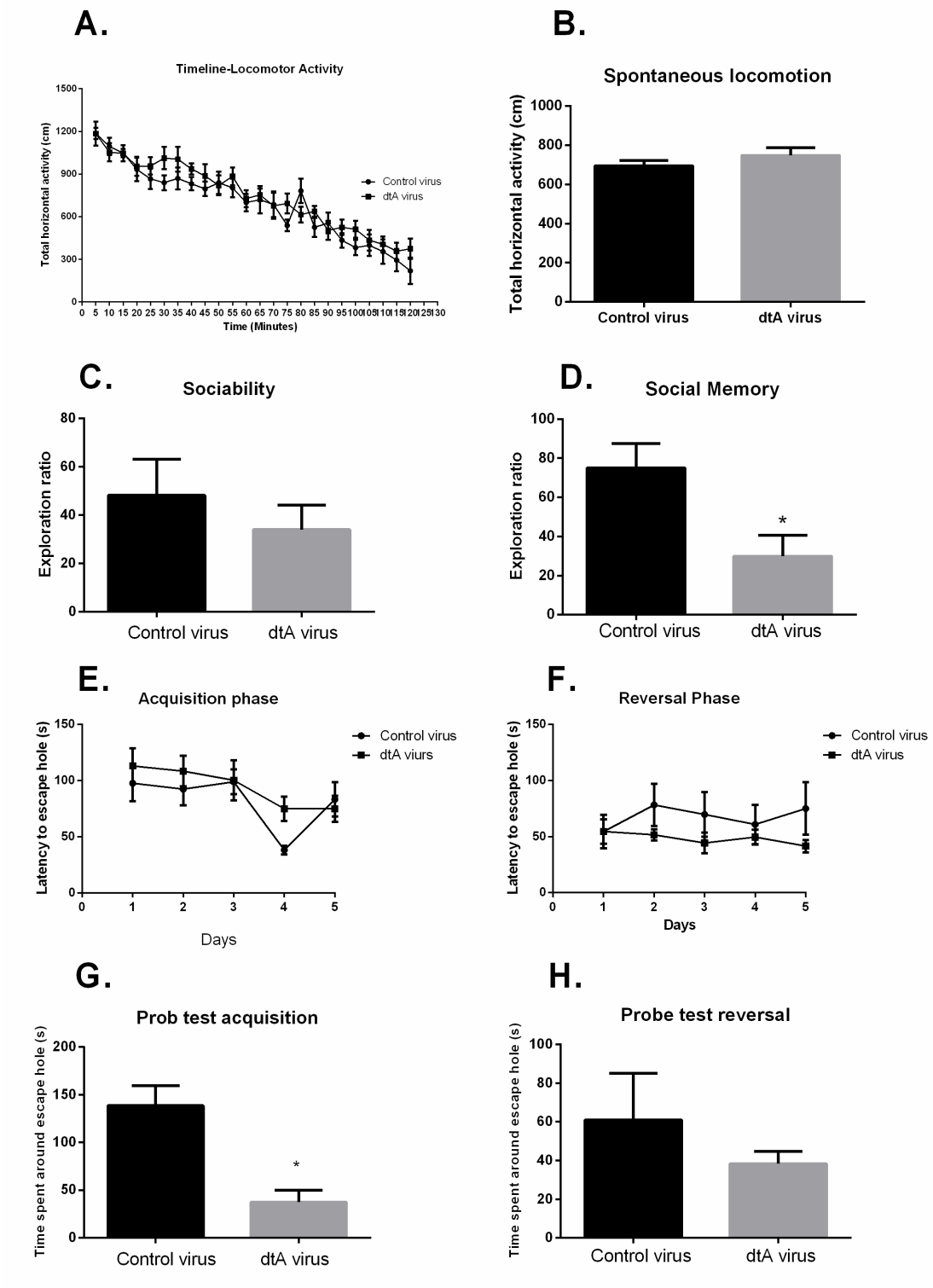
A.
Timeline-Locomotor Activity
Total horizontal activity (cm)
Time (Minutes)
Control virus
dtA virus
B.
Spontaneous locomotion
Total horizontal activity (cm)
Control virus
dtA virus
C.
Sociability
Exploration ratio
Control virus
dtA virus
D.
Social Memory
Exploration ratio
*
Control virus
dtA virus
E.
Acquisition phase
Latency to escape hole (s)
Control virus
dtA viurs
Days
F.
Reversal Phase
Latency to escape hole (s)
Control virus
dtA virus
Days
G.
Prob test acquisition
Time spent around escape hole (s)
*
Control virus
dtA virus
H.
Probe test reversal
Time spent around escape hole (s)
Control virus
dtA virus

Figure 3. Effect of neonatal neuronal virus mediated ablation of excitatory neurons within the VH on adult onset behaviours. A. Two-way repeated measure ANOVA revealed no significant interaction on virus x timeline of horizontal activity between the control and dTA injected group ($p = 0.5$). B. Unpaired two tailed t test revealed no significant difference in spontaneous locomotion (p-0.4873) between the control virus and dTA virus injected group. C. Social interaction test revealed no significant effect of neonatal dTA ablation on sociability (p-0.4296). D. A significant reduction in social memory (*p- 0.0153) was observed among the dTA virus injected group compared to control virus group. E & F. Barnes maze test revealed no significant interaction of virus x days on learning in both the acquisition ($p = 0.4$) and reversal training phase ($p = 0.183$) between the two groups. G. Unpaired two tailed t test revealed a significant reduction in the time spent around the escape hole during the acquisition probe test (*p- 0.0006) among the neonatal dTA virus injected group. H. No significant difference between the two groups was observed in the reversal probe test (p- 0.2769). (n=8-11 per group).

Figure 4.

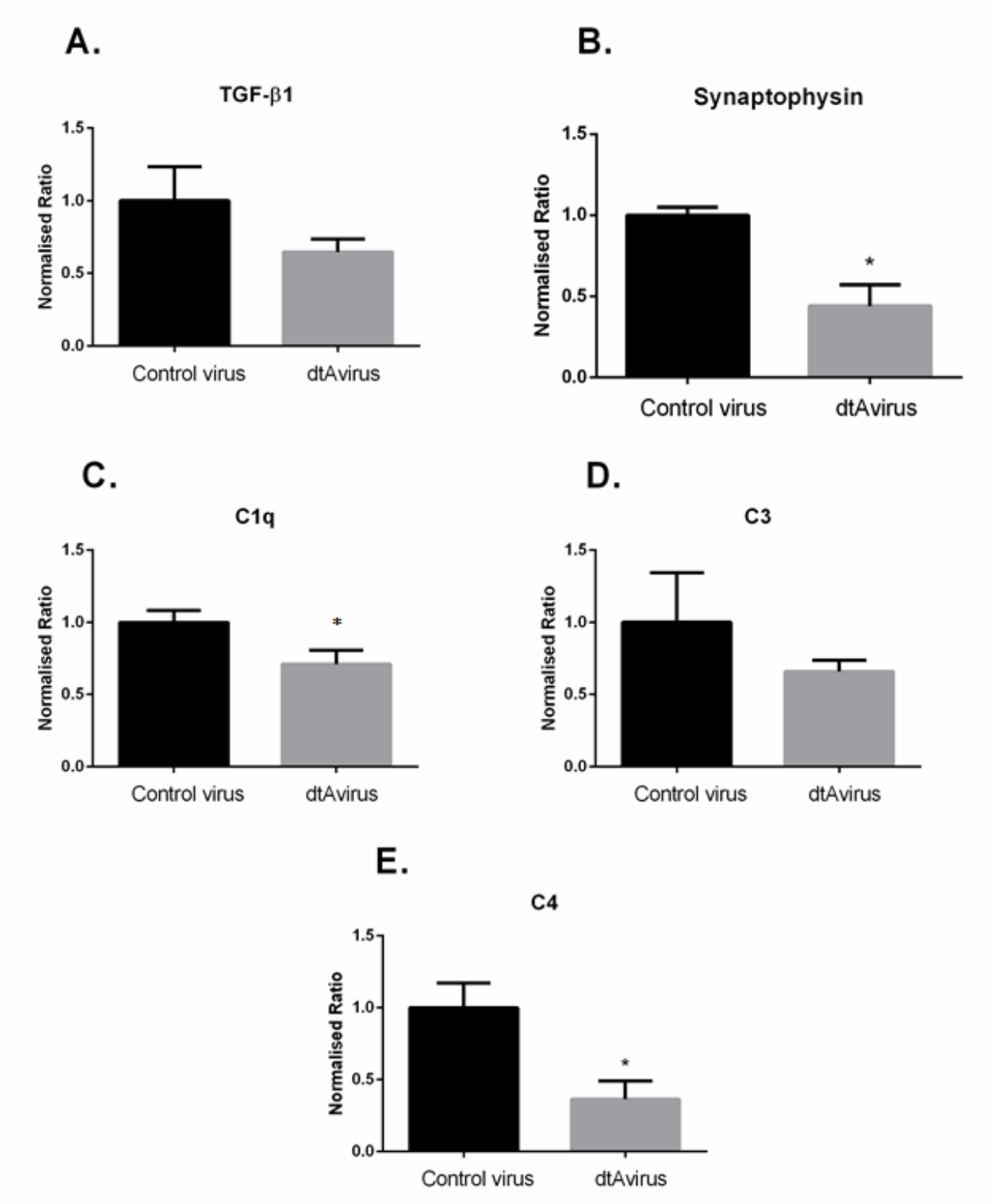
A.
TGF-β1
Normalised Ratio
1.5
1.0
0.5
0.0
Control virus
dtAvirus
B.
Synaptophysin
Normalised Ratio
1.5
1.0
0.5
0.0
*
Control virus
dtAvirus
C.
C1q
Normalised Ratio
1.5
1.0
0.5
0.0
*
Control virus
dtAvirus
D.
C3
Normalised Ratio
1.5
1.0
0.5
0.0
Control virus
dtAvirus
E.
C4
Normalised Ratio
1.5
1.0
0.5
0.0
*
Control virus
dtAvirus

Figure 4. Effect of neonatal neuronal virus mediated ablation of excitatory neurons within the VH on the expression of synaptic pruning-related genes in the mPFC. Quantification of QRT-PCR data (Normalised ratio of gene of interest over housekeeping gene); genes of interest quantified include transforming growth factor-β1 (TGF-β1), Synaptophysin and Complements (C1q, C3 and C4). A. Expression of Synaptophysin was found to be significantly reduced within the mPFC of neonatal dTA virus injected group compared to control virus injected group (**p*- 0.002). C. & E. Reduced expression of C1q (**p* =0.046) and C4 (**p*- 0.0188) was observed in the dTA virus injected group compared to control virus group. A. & D. No significant change in the expression of TGF-β1 (p- 0.227) and C3 (p- 0.4542) (d) was observed between the two groups. (n=4-6 per group).

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CHAPTER VI

GENERAL DISCUSSION

Perinatal period is a critical phase of brain development and any insults during this period has long term effect on circuit development and schizophrenia-related behaviors later in life (Weinberger, 1987; Welham et al., 2009). Development of ventral hippocampus (VH) connectivity with medial prefrontal cortex (mPFC) and other brain regions has been known to regulate schizophrenia-related adult behavioural and cellular deficit (Godsil et al., 2013). Our group and others further established the importance of this early developmental period and showed that neonatal ventral hippocampus lesion (NVHL) causes post-pubertal emergence of a range of schizophrenia- related behavioural deficit such as deficit in sensorimotor gating, memory and cognition (Lipska et al., 1993; Tseng et al., 2009). Besides behaviour, some of the core schizophrenia-related cellular deficits exhibited by NVHL animals were excessive loss of spines and synaptic function in the mPFC (Ryan et al., 2013). Interestingly, excessive spine loss was suggested to cause the schizophrenia-related behavioural deficits. Therefore, in our study we explored the mechanisms involved in synapse elimination and cortical maturation during early developmental period. This would allow for identifying drug targets and intervention before the post-pubertal emergence of the schizophrenia symptoms.

Recent studies provide evidence supporting the role of immune system and complement mediated inflammation in the development of schizophrenia (Sekar et al., 2016; Schizophrenia Working Group of the Psychiatric Genomics, 2014). However, it has been challenging to decipher the exact mechanisms involved in linking neuroinflammation and schizophrenia pathophysiology. In my thesis, we have identified different cellular and molecular components contributing to neuroinflammation and thereby regulating schizophrenia-related behavioural and cellular deficits in the NVHL model. Previous studies, suggested a role of oxidative stress and inflammation in NVHL induced cellular and behavioural deficits (Tendilla-Beltran et al., 2019; Cabungcal et al., 2014). In our study we found reduced expressions of antioxidant markers such as glutathione peroxidase (Gpx1) and catalase in the PFC of adult lesioned rats (Hui et al., 2019). Besides oxidative stress, our data revealed an increase in the expression of a pro- inflammatory IL-1β and a decrease of an anti-inflammatory TGF-β1 mRNA in the mPFC of NVHL animals (Joseph et al., 2018). We also observed an increase in the expression of Trem2 and CD45 genes which further increased the inflammatory overload in the mPFC of NVHL animals (Hui et al., 2019).Consistent with our data, previous studies have suggested a role of oxidative stress and inflammation as a potential mechanism in regulating cognition in the NVHL animals (Cabungcal

et al., 2014; Drouin-Ouellet et al., 2011). Interestingly, the increase of oxidative stress and inflammation in our study has also been shown to be associated with altered microglia morphology and function in NVHL animals. Based on our data and previous studies, it is possible that a development specific immune activation could have primed microglia and altered its morphology and phagocytic function (De Picker, Morrens, Chance, & Boche, 2017; Streit et al., 1999). Notably, altered microglia morphology and function has also been suggested to upregulate the level of pro-inflammatory cytokines and oxidative stress in the CNS (Butovsky et al., 2006).

Pro-inflammatory cytokines are tightly regulated by anti-inflammatory cytokines; IL-1β must overcome the anti-inflammatory effects of TGF-β1 to boost pro-inflammatory responses (Coussens & Werb, 2002). In our study, neonatal administration of recombinant TGF-β1 attenuated adult schizophrenia-related behaviours in NVHL animals. Interestingly, in a recent study TGF-β1 was reported to upregulate the expression of Olfml3, a postnatal microglia-specific gene and thereby regulate microglia function and morphology (Neidert, von Ehr, Zoller, & Spittau, 2018). Further, in our study normalizing microglial phagocytic function by minocycline in neonates was also found to rescue adult schizophrenia-related behaviours in NVHL animals (Hui et al., 2019). The anti- inflammatory role of TGF-β1 has also been known to contribute to quiescent microglia phenotype and thereby suggested to regulate complement-mediated synaptic pruning function of microglia (Bialas & Stevens, 2013; Sekar et al., 2016; Abutbul et al., 2012; Kierdorf & Prinz, 2013). Based on our data and previous studies, it is possible that reduced expression of TGF-β1 in the mPFC of NVHL animals could be contributing to altered microglia morphology and function and thereby regulating synaptic function. Future work calls for closer examination of anti-inflammatory and microglia-specific genes for development of better models to study schizophrenia-related behavioural and cellular deficits.

This thesis provides further evidence on the role of neuroinflammation in neurodevelopmental disorders such as schizophrenia. Consistent with our data, post mortem studies found an increase in the expression of pro-inflammatory and decrease of anti-inflammatory cytokine in the PFC of schizophrenia patients (Pandey, Rizavi, Zhang, & Ren, 2018; Iwamoto & Kato, 2006). Interestingly, a number of studies have described alterations in microglia morphology and

density in schizophrenia brains (Tay, Bechade, et al., 2017; Laskaris et al., 2016). Additionally, an upregulation of oxidative stress has also been identified in schizophrenia post mortem samples (Gawryluk et al., 2011; Yao et al., 2004). While an extrapolation of findings in rats to human neuropsychiatric disorders is at best speculative, it should be pointed out that in our study neonatal supplementation of TGF-β1 reduced the expression of pro-inflammatory cytokine IL-1β thereby rescuing behavioural and cellular deficits in adult NVHL animals. Several clinical trials support the use of anti-inflammatory agents such as inhibitors of COX2, TNF-α, IL-1β and IL-6 as an adjunctive treatment to antipsychotics (Rodrigues-Amorim et al., 2017; Muller et al., 2004). Other agents such as N-acetyl cysteine and minocycline have also been used in clinical studies to reduce the symptoms in schizophrenia patients (Solmi et al., 2017; Lavoie et al., 2008). However, it should be pointed out that the anti-inflammatory therapeutic agents have been found to cause several side effects in schizophrenia patients (Muller et al., 2004); therefore identifying a need for target specific modulators of neuroinflammation with in the brain. This thesis has identified certain modulators of neuroinflammation in the mPFC and suggest further exploration of these mechanisms to develop a more effective way to lower neuroinflammation in the CNS.

MicroRNAs (miRNA) post-transcriptionally regulate gene expression and their abnormal expression has been associated with schizophrenia-related behavioural and cellular deficits (Santarelli et al., 2011). MiRNAs are emerging to be novel and non-invasive biomarkers of complex disorders such as schizophrenia. Evidence supporting the significance of miR-137 as a biomarker of schizophrenia has been provided by a GWAS study which identified miR-137 loci to be highly associated with schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics, 2014). Some of the target genes regulated by miR-137 such as calcium voltage-gated channel subunit alpha1 C (CACNA1C), transcription factor 4 (TCF4) and zinc finger protein 804A (ZNF804A) were reported to increase the susceptibility of schizophrenia (Valles, Martens, De Weerd, Poelmans, & Aschrafi, 2014). These genes in an animal model have been associated with behavioural phenotypes such as cognitive flexibility. Interestingly, in our study we found impairment in cognitive flexibility and increased expression of miR-137 in the mPFC of NVHL animals. In addition to GWAS, post mortem studies also report an upregulation of miR-134 in the PFC of schizophrenia patients (Santarelli et al., 2011). Interestingly, our study also revealed an increased expression of miR-134 in the mPFC of lesion animals. Besides identification of miRNA in the brain tissue, circulating miRNAs have also been detected and measured in

biological fluids and could be used as possible diagnostic and prognostic biomarkers of schizophrenia.

Recent advancement in miRNAs research has pointed towards their role as potential therapeutic targets. MiRNAs have been known to regulate genes related to neuroinflammation and we suggest their role in NVHL induced inflammation and oxidative stress. For example, miR-137 has been reported to inhibit the genes related to the expression of anti-oxidant enzyme thereby protecting the cells from oxidative damage (Matouskova et al., 2018). miRNAs (miR-134 & miR-137) have also been known to modulate genes relevant to synaptic pruning thereby affecting the development of different brain regions implicated in schizophrenia (Fonken et al., 2016); (He et al., 2018). Manipulation of the expression of these miRNAs would allow us to better understand mechanisms involved in the PFC leading to schizophrenia-related behavioural and cellular deficits.

In the series of experiments performed using NVHL model we have further established the role of neuroinflammation in schizophrenia-related behavioural and cellular deficits. A limitation of this model is the inability to decipher the role of specific cell populations within the VH in causing adult behavioral or cellular deficits due to the indiscriminate excitotoxicity of ibotenic acid-induced lesion. In the last chapter of this thesis, we developed a viral based method expressing diphtheria toxin subunit A (dTA) to ablate excitatory neurons in the VH of neonatal C57Bl6/J CaMKII-cre mice. Previous studies in adult mice have reported the role of adult VH excitatory neurons in modulating social and cognitive behaviors (Okuyama et al., 2016; Godsil et al., 2013). Whereas, in this study we particularly targeted early developmental time point and observed a role of neonatal excitatory neurons in schizophrenia- related behavioural and cellular alterations. Interestingly, unlike NVHL model only some aspects of social and cognitive deficits were observed in this model. Besides behaviours, we also observed alteration in the expression of complement and synaptic pruning- related genes that have been suggested to be involved in regulating inflammation and synaptic pruning via microglia. Our data suggests that viral ablation of neonatal excitatory neurons alters VH neuronal activity and affects the development of VH projections to PFC and other limbic brain region. Our viral based model could be considered as a refined neurodevelopmental model of schizophrenia to better understand mechanisms in the mPFC that could be contributing to schizophrenia-related behavioural and cellular deficits.

Concluding Remarks

The work embodied in my thesis provides evidence for a role of neuromodulators during early development of the brain and its implications in neurodevelopmental disorders such as schizophrenia. Our data on the expression of different cellular and molecular components such as those related to inflammatory and oxidative processes suggest a possible explanation on how an early life hippocampal impairment may affect the maturation of distant connected structures during adulthood. In my thesis work, we have also identified a role of neonatal VH excitatory neuronal populations in adult-onset social and cognitive behaviors and have developed an improved viral based model to study schizophrenia-related behavioural and cellular deficits. Our study identified development specific neuroinflammation-related alterations and call further exploration of these mechanism to develop better drug targets. Our study opens the possibility of future research on the role of miRNAs, pro- and anti-inflammatory cytokines as predictors or biomarkers of neurodevelopmental disorders such as schizophrenia.

Future directions

Previous studies from our group and other have identified cellular alteration in both pyramidal and inhibitory neuron in the NVHL animals. Since our data here revealed neuroinflammation-related mRNAs and miRNAs changes from all the mPFC cells; it is imperative to study cell specific expression of different neuromodulators that were found to be altered in our study. Further comparing the cell specific expression in different brain regions and at different developmental time points could determine the specificity of the neuroinflammation-related alterations. In our viral based neonatal dTA ablation model we assumed that the ablation of neonatal VH excitatory neurons may result in reduced excitatory neurotransmission in the VH, we have not measured it, and this could be one of the limitations of our study. Therefore, future experiments could explore the role of VH sub-region-specific ablation and its effect on neuronal activity in modulating schizophrenia-related behavioral and cellular deficits. Additionally, future experiments could also explore the role of VH-PFC monosynaptic projections on schizophrenia-related behaviours. A retrograde virus in the mPFC in combination with a cre-specific dTA expressing virus in the VH would ensure ablation of only those neurons in the VH that are projecting to mPFC. This would improve our understanding on how early developmental insults to the ventral hippocampus may affect prefrontal cortex maturation and behaviours.

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