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DISSERTATION

Effects of deep brain stimulation (DBS) on microglia phenotype in rodent model of psychiatric disorder

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von

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To my beloved family

Content

List of abbreviations

Major depressive disorder (MDD)

Dopamine (DA)

N-methyl-D-aspartic acid (NMDA)

Phencyclidine (PCR)

Interleukin (IL)

Tumor necrosis factor (TNF)

Central nervous system (CNS)

C-Reactive protein (CRP)

Corticotrophin cortisol hormone (CRH)

Adreno-corticotrophin hormone (ACTH)

Indoleamine 2,3 Dioxygenase (IDO)

Positron emission tomography (PET)

Cerebro-spinal fluid (CSF)

Colony-stimulating factor (CSF)

Transforming growth factor (TGF)

Nitric oxide (NO)

Embryonic day (E)

Human leukocyte antigen (HLA)

Polyriboinosinic-polyribocytidylic acid (Poly I:C)

Quinolinic acid (QA)

Inducible nitric oxide synthase (iNOS)

Lipopolysaccharide (LPS)

Deep brain stimulation (DBS)

Food and Drug Administration (FDA)

Treatment-resistant depression (TRD)

Medial forebrain bundle (MFB)

Subcallosal cingulate (SCC)

Nucleus accumbens (Nacc)

Ventromedial prefrontal cortex (vmPFC)

Forced swim test (FST)

Sprague Dawley (SD)

Pre-pulse inhibition (PPI)

Acoustic startle reflex (ASR)

Dorsomedial thalamus (DM)

Flinders Sensitive Line (FSL)

Flinders Resistant Line (FRL)

Gestation day (G)

Postnatal day (P)

Anterior to bregma (AP)

Lateral to the midline (ML)

Ventral to dura (DV)

Phosphate Buffered Saline (PBS)

Caudate putamen (CPu)

Polymerase chain reaction (PCR)

Blood-brain barrier (BBB)

Long-term potentiation (LTP)

Long-term depression (LTD)

Indoleamine 2,3-dioxygenase (IDO)

α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)

1a. Abstract (English)

Background Since inflammation was closely linked to the onset and development of mental disorders, recent studies focus more and more on the contribution of the resident immune cells of the brain the microglia. Evidence from clinical and animal studies has demonstrated a change in the microglial phenotype in both schizophrenia and depressive disorder. Deep brain stimulation (DBS) is a novel treatment for neurological disorders. It has been proved that DBS can effectively ameliorate the symptoms of psychiatric disorders, but the effects of DBS on microglia in schizophrenia and depression are not clear yet.

Methods We were using immunohistochemistry and PCR techniques to investigate the changes of microglia with and without DBS treatment in a rat model of schizophrenia and depressive disorder.

Results In the rat model of schizophrenia, microglial density and soma size in the hippocampus and nucleus accumbens (Nacc) were significantly increased. Deep brain stimulation (DBS) treatment in the medial prefrontal cortex (mPFC) and Nacc effectively attenuated these changes of microglia in both regions. More than that, Nacc-DBS treatment also raised the density of microglia in the mPFC and caudate putamen (CPu) in the schizophrenic rats. In the depressive rat model, the density of microglia in the mPFC was deregulated compared to the control group. However, there was no significant DBS effect detected in the depression model. Furthermore, we confirmed that implantation of electrode can cause an activation of microglia in the targeted region and DBS current reduced microglia activity.

Conclusion The results thus show that there is microglial dysregulation in a rat model of schizophrenia and depression. DBS treatment can normalize microglia density and soma size in both targeted area as well as projection area in schizophrenic rats, but no valuable effect of DBS on microglia can be detected in the rat model of depression.

1b. Abstrakt (German)

Hintergrund Neue Erkenntnisse zeigen das Entzündungsreaktionen im Gehirn einen großen Einfluss auf die Entstehung und die Entwicklung von psychischen Erkrankungen haben. Im speziellen zeigen eine steigende Anzahl von Studien den Einfluss von Mikroglia, als Gehirn spezifische Immunzellen, auf diese Erkrankungen. Sowohl klinische als auch Tier basierte Studien zeigen eine phänotypische Veränderung von Mikroglia in der Schizophrenie als auch depressiven Erkrankungen. Weitere Studien zeigen, dass eine Tiefenhirnstimulation (eng. Deep Brain Stimulation = DBS) die Symptome von psychischen Erkrankungen lindern kann. Jedoch ist der Effekt einer Tiefenhirnstimulation auf Mikroglia noch nicht aufgeklärt.

Methoden Um die phänotypischen Veränderungen in Mikroglia aufzuklären wurden immunhistochemische Färbungen und qualitative RT-PCR von wahlweise eines Rattenmodels für Schizophrenie oder depressive Erkrankungen mit und ohne Tiefenhirnstimulationsbehandlung durchgeführt.

Ergebnisse Im Schizophrenierattenmodel ist sowohl die Dichte als auch die Größe des Zytosomas der Mikrolgia im Hippocampus und Nucleus accumbens (Nacc) gegenüber einer gesunden Ratte signifikant reduziert. Diese Reduktion konnte durch eine Tiefenhirnstimulation im Cortex praefrontalis medialis (eng. medial prefrontal cortex = mPFC) oder im Nacc aufgehoben werden. Darüber hinaus führt eine Tiefenhirnstimulation im Nacc im Schizophrenierattenmodel zu einer Erhöhung der Mikrogliadichte im mPFC und im Caudate Putamen (Cpu). Im Rattenmodel für depressive Erkrankungen reduziert sich die Dichte der Mikroglia nur im mPFC. In diesem Fall konnte die Tiefenhirnstimulationsbehandlung keine signifikante Änderung herbeiführen. Des Weiteren konnten wir zeigen, dass die Implantation der Elektrode für die Tiefenhirnstimulation an sich Mikroglia in der jeweiligen Region inflammatorisch aktiviert. Diese Aktivierung kann durch eine Aktivierung der Tiefenhirnstimulation jedoch wieder reduziert werden.

Fazit Die Ergebnisse zeigen eine Dysregulation der Mikroglia im Rettenmodel der Schizophrenie und der depressiven Erkrankungen. Im Falle des

Schizophrenierattenmodels kann eine Tiefenhirnstimulation die Dichte und die Größe des Zytosomas der Mikroglia sowohl im Implantations- als auch im Projektionsbereich normalisieren. Im Rattenmodel der depressiven Erkrankungen konnte kein Effekt der Tiefenhirnstimulation auf die Mikroglia festgestellt werden.

2 Introduction

2.1 Depression and schizophrenia

2.1.1 Definition and epidemiological features

The most common psychiatric disorder is depression (including major depressive disorder, MDD). Nearly one in five people experience an episode of major depression in their lifetime worldwide. It was the fourth leading cause of disability across all countries in 2001 and will be perhaps the second most disabling disease of all forms of illness in 2020 (World Health Organization 2001). Biology, psychology, and social factors have an effect on the incidence of this disease. The time of onset is normally between the ages of 20 and 30 years, with a peak between 30 and 40 years (Hamilton et al. 2011). Depressive patients lose their interest and the ability to feel pleasure (anhedonia), suffer from anxiety, sadness, irritability or restless feelings. These patients are often unable to communicate, experience serious sleep disturbances, hopelessness and even end their lives by committing suicide. Depression causes not only a tremendous negative impact for the patients themselves, but also for the whole family (Crown et al. 2002). Another serious mental disorder with unclear causes is schizophrenia. Schizophrenia is often characterized by a failure to recognize what is real and attended by abnormal social behavior. "Splitting of mental functions" is also used to describe this kind of disease. Unlike depression, the onset of schizophrenia is mostly sub-acute in young adulthood, peaking around 25 to 27 years and is much rarer in childhood. Males are affected earlier than females (Cascio et al. 2012). According to the World Health Organization statistics, approximately 1% of the world’s population suffers from schizophrenia and the disorder resulted in 20,000 deaths in 2010 (R. Lozano et al. 2012). In 2011, there were more than 24 million patients worldwide (Mas-Expósito et al. 2011). Clinical symptoms of schizophrenia are diverse and complex. They are related to various aspects such as perception, feeling, emotion, and behavior, therefore it is generally described in terms of positive and negative (or deficit) symptoms. The positive symptoms are: unclear or confused thinking, auditory, or visual hallucinations and false beliefs. Positive symptoms generally respond well to medication (American Psychiatric Association and Association 2000). The negative symptoms of schizophrenia are: inability to experience pleasure, deficits of emotion and motivation,

poverty of speech and social difficulties. These patients usually suffer from poor adjustment before the onset of the illness and are less responsive to medication (Smith, Weston, and Lieberman 2010).

2.1.2 Clinical diagnosis and treatment

The diagnosis of psychiatric disorders is based on the patient's behavior reported by relatives and friends, patient's self-reported experiences and a mental status examination. Physicians may request tests for physical conditions that can cause similar symptoms, but there is no specific laboratory test for major depression or schizophrenia. The most common treatment for psychiatric patients is pharmacotherapy. Serotonin uptake inhibitors like Zoloft (sertraline) is primarily used to treat major depression in outpatients, and antipsychotic medication balancing dopamine, glutamate and serotonin, like risperidone, olanzapine, amisulpride and clozapine, are considered to be the first-line treatment for schizophrenia (NICE 2009). Other treatment options also include psychosocial therapy or electroconvulsive therapy. However, although a broad range of treatments has been developed, a significant portion of psychiatric patients remain refractory to multiple modes of therapies (Cleary et al. 2015). Apart from this, the use of antipsychotics can lead to various adverse side effects, which commonly include headaches, weight gain, and extrapyramidal side effects, such as tardive dyskinesia, etc. Therefore, new treatments are urgently needed for those patients.

2.1.3 Etiology

Although physicians and scientists have made great efforts in dealing with this issue, the pathogenesis of schizophrenia and depressive disorder is still uncertain. In recent decades, various hypotheses of the etiology of schizophrenia and depression have been proposed.

Dopaminergic hyperfunction is assumed as a possible underlying cause of schizophrenia: Crow (1984) et al. found an increased number of the dopamine D2 receptors in the basal ganglia and nucleus accumbens in schizophrenic patients, especially related with positive symptoms. Large doses or prolonged use of amphetamine, cocaine and similar drugs can inhibit DA re-uptake and increase levels

of dopamine in the brain, causing schizophrenia-like symptoms in sane people (Curran, Byrappa, and McBride 2004). Another hypothesis of the cause of schizophrenia is glutamate hypofunction. It has been reported that hypofunctioning glutamate receptors could be a contributing factor in the development of schizophrenia (Mechri et al. 2001). Augmentation of NMDA receptor function through D-serine or glycine in schizophrenia patents with negative symptoms showed a good clinical treatment result (Balu and Coyle 2015). Glutamate receptor antagonists, such as phencyclidine (PCR), ketamine or other non-competitive and competitive NMDA receptor antagonists, can induce both positive and negative symptoms in animals (Nishikawa and Ishiwata 2013).

For depression, the monoamine hypothesis states that the main symptoms of depression are due to the imbalance of the three main monoamine neurotransmitters (dopamine, norepinephrine and serotonin) in the brain (Nutt 2008). Low levels of norepinephrine and dopamine following serotonin decrease give rise to a lack of motivation and pleasure, anxiety and a low interest in life. Two meta-analysis in 2009 showed that stressful life events (abuse, discrimination, sleep deprivation, etc.) and genetic susceptibility were probably associated with depression onset and development (Munafo et al. 2009; Risch et al. 2009).

Recent studies focus more and more on the relationship between inflammation and mental disorders. In fact, as early as a hundred years ago there has been evidence supporting this view. Karl A. Menninger, an American psychiatrist, was the first to reveal an association between influenza exposure and psychotic disease after the outbreak of influenza pandemic in 1918 (Meyer 2014).

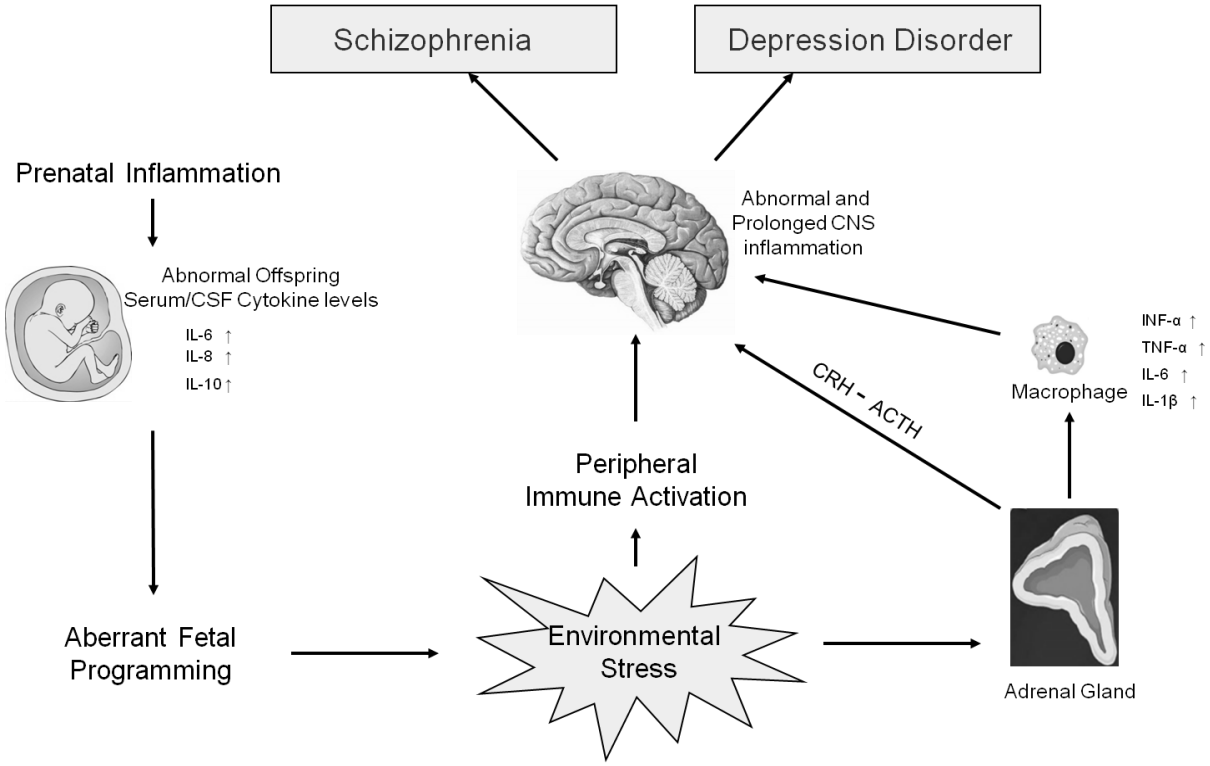


Figure 1. Inflammation hypothesis of psychiatric disorders.

Maternal immune activation increased brain and peripheral cytokine levels in offspring, which caused aberrant brain development and oversensitivity to immune challenge. Subsequent environmental stress may lad to abnormal CNS Inflammation and contribute to the onset of schizophrenia. Meanwhile, long-lasting mental stress in adolescent or adult age can directly induce the release of pro-inflammatory cytokines and alter corticotrophin-releasing hormone (CRH) - adrenocorticotropic hormone (ACTH) signaling, which increases inflammatory cytokines in the CNS and negatively affects brain function. These changes may be involved in the pathology of depressive disorder.

2.2 CNS inflammation and psychiatric disorders

2.2.1 Immune system and CNS function

Although this early neuroimmune hypothesis of psychotic disease sank into oblivion after Dr. Menninger's report, it was reanimated by another research group. Torrey et al. suggested that latent viruses might be involved in the development of psychosis (Torrey, Bartko, and Yolken 2012), this finding has led to a great expansion of this field. Now, various infectious agents and even autoimmune diseases are being considered to play an etiopathological role in mental disorders. Inflammation is a complex response to microbial invasion or tissue damage, which includes several

steps such as increased vascular permeability and blood supply, and the activation and recruitment of immune cells. It is associated with not only infectious, but also non-infectious processes. It has been reported that there was an increase in proinflammatory cytokines such as IL-6, TNF-α and a downregulation of the anti-inflammatory cytokine IL-10 in first-onset psychosis as well as in acute psychotic relapse (Miller et al. 2011). After treatment, proinflammatory cytokines in stable patients are no longer elevated, but several anti-inflammatory cytokines are (Potvin et al. 2008; Suvisaari et al. 2011). Cytokines are small proteins secreted by cells that contribute to both coordination of the host response to infection and several other functions. They exert effects as key regulators of inflammatory processes in the peripheral tissues as well as in the central nervous system (CNS). In addition, they have been shown to be important for proper neural system maturity and function (Carpentier and Palmer 2009; Deverman and Patterson 2009). During fetal brain development, they participate in neurogenesis, gliogenesis, axon pathfinding and the development of microglia. Besides, they also contribute to the process of neuronal cell survival, synapse regulation, brain repair and neural stem cell renewal and differentiation (de Araujo, da Silva, and Dos Santos 2009). One of the most important roles of cytokines is their involvement in neuroplasticity, which is crucial to memory formation and to the stability of neuronal networks by maintaining the balance between nerve cell excitation and inhibition (Boulanger 2009). When the abnormal secretion of cytokines occurs, it can lead to an interruption of this normal neuroplastic function or an imbalance of this regulation, as well as neurogenesis (Romer et al. 2011).

2.2.2 Inflammation evidence link to depressive disorder

Clinical as well as preclinical trials show a connection between depression and inflammation. In several clinical studies, an increase of cytokines in peripheral blood has been observed. A number of meta-analyses (Dowlati et al. 2010; Howren, Lamkin, and Suls 2009; Valkanova, Ebmeier, and Allan 2013) have demonstrated increased levels of CRP, IL-6, IL-1β and TNF- α in the serum or plasma of depressed patients. Common symptoms like reduced appetite, rapid weight loss and reduced interest in the physical and social environment, were reported to be caused by systemic infectious or inflammatory conditions (Dantzer 2009). Furthermore,

significant depressive symptoms are frequently seen in hepatitis C patients as a side effect of cytokine interferon alpha treatment (Navines et al. 2012). Maes et al. also proposed that organic inflammation, like autoimmune disease, cancer, systemic infections, as well as environmental stressors, stressful life events can trigger depression via inflammatory processes (Maes et al. 2009). There are several possible mechanisms with which inflammation can influence CNS functions and cause depressive symptoms. Inflammatory cytokines from peripheral tissue and activated immune cells in the brain can affect the modulation of neurotransmitter metabolism and neurotoxic mechanisms, decrease the neurotrophic support, reduce neurogenesis and trigger the release of stress hormones like corticotrophin cortisol, hormone (CRH) and adreno-corticotrophin hormone (ACTH)(Cattaneo et al. 2015). For instance, cytokines induce the enzyme Indoleamine 2,3 Dioxygenase (IDO) to break down tryptophan (a serotonin precursor) into kynurenine, which can be converted into a neurotoxic substance (quinolinic acid) and harm the neuronal function (Myint and Kim 2014). This cytokine pathway is suggested to be the common mechanism linking the immune status with the increased risk of depression.

2.2.3 Inflammation evidence link to schizophrenia

Using positron emission to mography (PET), two small clinical studies including schizophrenic patients and matched controls, have found evidence of ongoing inflammation in the hippocampus (Janine Doorduin et al. 2009) and in the total gray matter (van Berckel et al. 2008). In the CSF of psychotic patients during an acute episode, elevated levels of IL-1 and glial protein S100B, and an increased number of macrophages and activated lymphocytes has been reported (Nikkila et al. 2001; Rothermundt et al. 2004; Soderlund et al. 2009). In addition, many antipsychotic drugs like perospirone, ziprasidone and quetiapine seem to have a potential anti-inflammatory effect (Bian et al. 2008). A recent meta-analysis showed that nonsteroidal anti-inflammatory drugs in an appropriate dosage can be useful as effective adjuvant drugs in schizophrenia (Sommer et al. 2012). With the progress of related studies, the prenatal period seems highly sensitive to the damaging effects induced by environmental insults such as infections (Meyer, Yee, and Feldon 2007). Mednick et al. (Mednick et al. 1988) have already reported that prenatal maternal exposure to an influenza epidemic in greater Helsinki was closely linked to an

increased incidence of schizophrenia in the offspring. Thereafter, the prenatal infection hypothesis of schizophrenia received a greater significance and reputation. Many studies have pointed out that maternal infections, not only influenza but also prenatal exposure to a number of other viral agents, such as polio, herpes simplex, rubella, or bacterial pathogens causing sinusitis, pneumonia, tonsillitis, as well as protozoan parasites, are associated with a greater risk of schizophrenia (Brown et al. 2004, 2009; Miller et al. 2013; Mortensen et al. 2007).

The fetal immune system normally begins to develop during the second trimester, while findings linking specific infections to schizophrenia have mostly been from first- and second-trimester infections (Brown and Derkits 2010). Several hypotheses have been presented to explain why prenatal exposures increase the risk of schizophrenia. Among them, the most prevailing one suggests that an activated maternal immune system could be the reason for the harmful effects on the developing fetal central nervous system (Kneeland and Fatemi 2013; Watanabe, Someya, and Nawa 2010; Ygberg and Nilsson 2012). Preclinical research indicated that prenatal maternal infections have the ability to alter cytokine levels in the fetal body, not only peripherally but also in brain tissue (Boksa 2010). These inflammatory cytokines can result in CNS insult through different mechanisms: antibodies acting as receptor agonists or antagonists, antigen modulation as well as interaction with the fetal immune system (Diamond et al. 2009).

Moreover, overexpression of cytokines during the periods of neurodevelopment and maturity may alter neural substrate sensitivity and permanently change the “set-point” of the neural immune system, sequentially constitute psychopathology of patients and brain responses to immune challenge. This not only has short-term but also long-lasting consequences for the emergence of postnatal brain dysfunctions, in which pathological processes within relevant cerebral areas occur during early brain development (Meyer, Yee, and Feldon 2007). Overall, cytokines, produced by the mother during infection or autoimmune disorder, could lead to prematurity and abnormal activation of the fetal immune system, which can cause severe brain developmental disturbance and dysfunction, in which cytokines have an important role (Deverman and Patterson 2009).

2.3 Microglia

2.3.1 Origin and properties of microglia

Since inflammation was closely linked to the onset and development of mental disorders, microglia might be involved in the pathological process. Recent findings have shown that microglia are different to macrophages. Precursors leave the yolk sac and enter the neural tube on E8.5-E9.0 (Greter and Merad 2013), after that they gain lineage-specific gene expression and develop into adult microglia. The maturity of microglia is also different from macrophages. A study published in 2013 shows that both colony-stimulating factor 1 (CSF-1) and transcription factor Myb are essential for macrophages but not for microglia (Hashimoto et al. 2013; Schulz et al. 2012; Yona et al. 2013). In contrast, TGFb-1 is crucial for the persistence of microglia in the central nervous system, however, macrophages do not need this cytokine to sustain their vitality (Koeglsperger et al. 2013). In the adult CNS, there is considerable heterogeneity in microglia density in different brain regions, but in general, microglia constitute approximately 10% of the total cells (Kettenmann, Kirchhoff, and Verkhratsky 2013). Microglia can take several morphological forms during their entire viable period. During early developmental stages they show an amoeboid appearance, just like macrophages in the peripheral tissue. In adulthood, they will change their form into a ramified morphology with long and thin processes. These processes of microglia directly appose dendritic spines, presynaptic terminals and perisynaptic astrocytic clefts (Tremblay, Lowery, and Majewska 2010), this good location makes it possible for them to monitor both synaptic function and neuronal firing activity (Kettenmann et al. 2011; Koizumi et al. 2013). Despite this positioning, microglia are highly motile cells. It has been estimated that they can explore the whole brain area in a few hours through extruding and retracting their processes (Nimmerjahn, Kirchhoff, and Helmchen 2005).

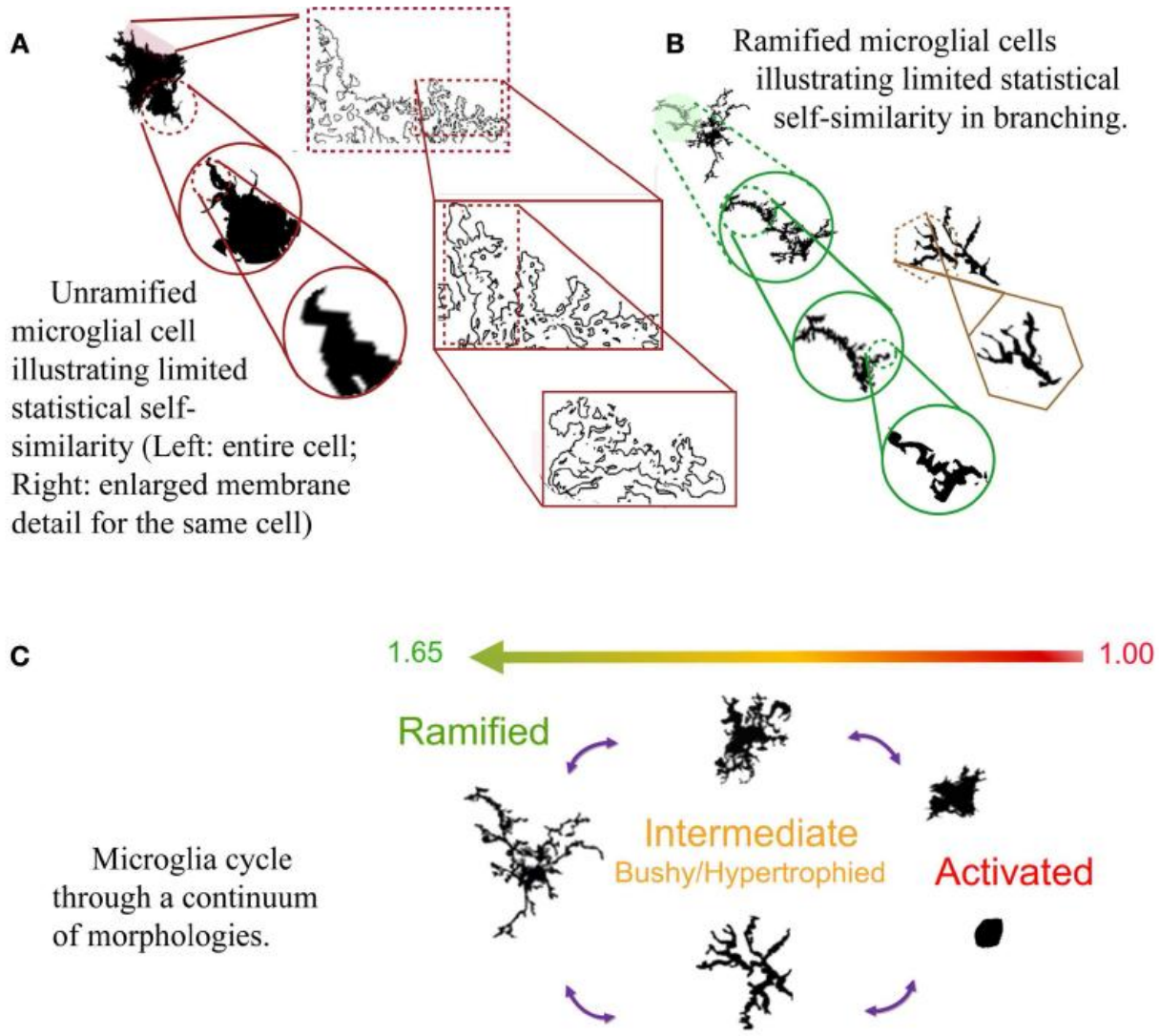


Figure 2. Two typical microglial morphologies

(A) Unramified microglia morphology. (B) Ramified microglia morphology. (C) The cycle of microglia morphology from non-pathological to Pathological condition. Adapted from (Karperien, Ahammer, and Jelinek 2013).

The general view about microglia is that they are the inflammatory mediators in diseases of the central nervous system. It has become the consensus that microglia have the ability to rapidly respond to any injury, infection, or disease in the CNS, they can change the morphology through cytoskeletal rearrangements, secrete specific inflammatory cytokines, and proliferate, even if the area of damage is only as small as one neuron (Davalos et al. 2005). Activated microglia can release various cytokines like IL-6, tumor necrosis factor (TNF)-α, interleukin (IL)-1β and nitric oxide (NO)(Bilbo and Schwarz 2009). This activation of microglial cells is crucial for the

maintenance of the homeostasis of the CNS. Microglia can therefore be seen as a highly programmed system to eliminate any threat and maintain the stability of the central nervous system.

With more and more studies focusing on microglia, as well as the rapid development of advanced technology, we are starting to understand other functions of microglia in the CNS. They not only participate in a variety of mechanisms to regulate and maintain normal nervous system functions, but also play an important role in the development and maturation of the central nervous system during specific time periods. A series of important studies pointed out that microglia have been found to be required for the development of mature synapses during embryogenesis and to regulate the number of functional synapses both *in vivo* and in culture (Ji et al. 2013). More importantly, they also regulate adult neurogenesis (Sierra et al. 2010).

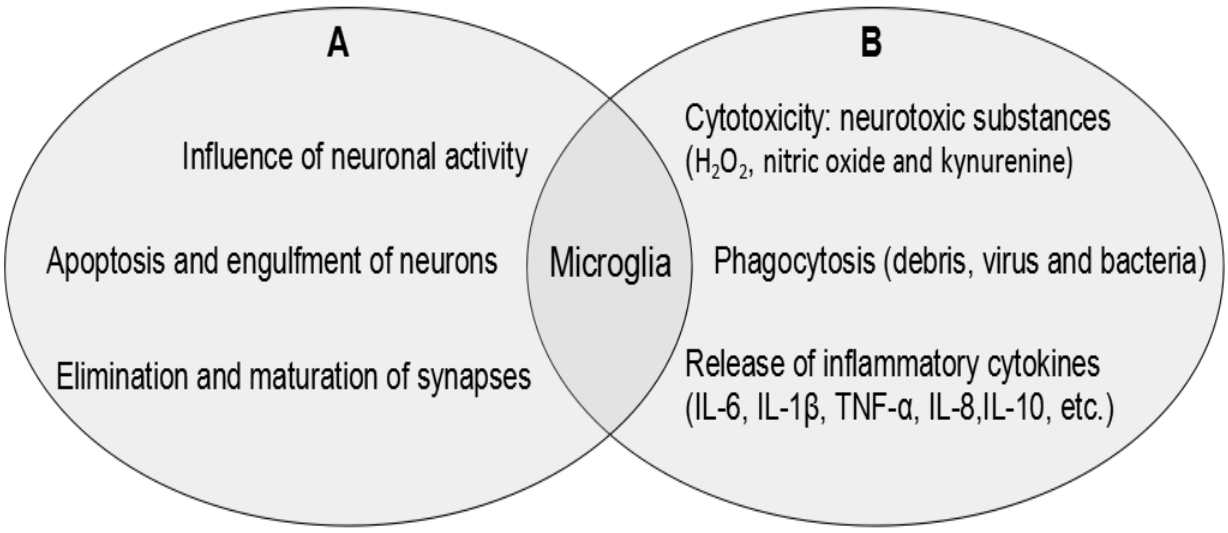


Figure 3. Property of microglia

(A) Functions of microglia under physiological conditions. (B) Functions of microglia under pathological conditions.

2.3.3 Microglial dysregulation in schizophrenia

In 1999, an abnormal activation of microglia was found in a subset of schizophrenic patients by a small post-mortem study (Bayer et al. 1999). More recently, A larger post-mortem study showed a degeneration of HLA+ microglia cells in patients with schizophrenia (Wierzba-Bobrowicz et al. 2004), such as damaged mitochondria, cytoplasm shrinkage, thinning and shortening, or even fragmentation of their processes (Wierzba-Bobrowicz et al. 2005).

Furthermore, by using ultrastructural analysis, degenerated microglia have also been found to show irregular nuclear contours, vacuolated cytoplasms and increased lysosomes (Uranova et al. 2010). Several other post-mortem studies have provided further evidence of microglial activation or degeneration in schizophrenia. One of these findings demonstrated that HLA-DR+ microglia densities were increased in the posterior hippocampus in paranoid schizophrenia, while residual schizophrenia patients only had an increased density of lymphocytes rather than microglia in the same region (Busse et al. 2012). This may indicate that the variation of microglia might be different among different subtypes of schizophrenia.

In pre-clinical trials, researchers use a variety of animal models to study the relationship between microglia and schizophrenia. In a mouse model of schizophrenia, which is based on a cryolesion in the parietal cortex, an increase of microglia density with neurodegeneration in cingulate cortex and hippocampus was induced (Sargin et al. 2009). Maternal polyriboinosinic-polyribocytidylic acid (Poly(I:C)) exposure can lead to offspring presenting with schizophrenia-like behavioral changes. By using this method, researchers found an increased number of microglia and reduced arborization in the striatum and the hippocampus in young adult mice (Ratnayake et al. 2012). Similar density and morphology abnormalities of microglia were also observed in another experimental model of schizophrenia, which is associated with hyperbilirubinemia (Liaury et al. 2012).

2.3.4 Microglial dysregulation in depressive disorder

Clinical evidence of microglial dysregulation in depressive disorders is limited. For instance, Bayer et al. (Bayer et al. 1999) found that only one out of 6 patients with major affective disorders showed that microglia activation (HLA-DR expression

increase) increases in the hippocampus in a post-mortem study. Similarly, in another post-mortem study, Steiner et al. (Steiner et al. 2008) suggested that there is no alteration in microglial density in several brain regions (mediodorsal thalamus, dorsolateral prefrontal cortex, hippocampus, and anterior cingulate cortex) in depressive patients compared to controls. But it is worthwhile to note that up-regulated microglial activity has been observed to be highly related with completed suicide in patients with depression compared to healthy controls (Steiner et al. 2011). Furthermore, compared to the healthy control, they demonstrated an increased density of microglia positive for a neurotoxin, quinolinic acid, and N-methyl-D-aspartate glutamate receptor agonist which is produced and released by activated microglia in suicidal depressed patients.

Animal models have been used to further investigate the link between microglia and mood disorders. Increased microglia activation in the prefrontal cortex has been found in the chronic psychological stress rat model of depression (Pittenger and Duman 2008); anti-inflammatory drugs such as minocycline, which can effectively block microglia activation, is able to reverse microglial abnormalities as well as attendant cognitive dysfunction in stressed animals (Hinwood et al. 2012). The same antidepressant-like effects also have been proved by another research group (Arakawa et al. 2012). In another chronic stress model, characterized by social defeat, an increased number of de-ramified Iba1+ microglia, and the levels of cytokines associated with cytotoxic microglial activation (IL-1β, IL-6, TNF-α, and iNOS) in CD11b+ cells in the medial prefrontal cortex, amygdala, and hippocampus have been reported (Wohleb et al. 2012). Further events showed that activated microglia can cause long lasting abnormal behavioral consequences. Neonatal rats which were exposed to LPS had long-term increased hippocampal microglial activation with anxiety-like behavior in adulthood (Wang et al. 2013)

2.4 Deep brain stimulation (DBS)

2.4.1 Background of DBS

Deep brain stimulation (DBS) is a neurosurgical procedure using stereotactic technology. The surgeon implants an electrode in a specific brain region. With an electrical current at a certain frequency and intensity, DBS can change the activity of related neuro-circuitry and treat CNS disorders. For current applications, DBS

electrical currents are modulated by pulse width, frequency, and amplitude. Superior to the general surgery, these parameters can be further modified postoperatively with an external programmer according to treatment outcome. In clinical studies, investigators can approximate the stimulated effect as frequency determines inhibition or activation, pulse width determines the surface area of stimulated neural tissue, and amplitude determines the strength of the effect. High-frequency stimulation blocks the activity of the neural structure and clinically mimics the effects of a lesion (Breit, Schulz, and Benabid 2004). Target areas of stimulation electrodes implantation suggested to be pathophysiological relevance to the respective disorders. The applied stimulation affects neural function of the DBS-target as well as associated projection brain regions (Lujan, Chaturvedi, and McIntyre 2008). For movement disorders like Parkinson's disease and essential tremor, the FDA has granted permission for the use of DBS treatment and systematic investigations have shown benefit compared with other forms of treatment. Nowadays, DBS has already become a common clinical practice and more than 100,000 patients worldwide have had this surgery performed (A. M. Lozano and Lipsman 2013). The first instance of DBS for psychiatric disorders was in 2003, capsular stimulation effectively ameliorated the core symptoms of patients with severe, long-standing, treatment-refractory obsessive-compulsive disorder (Nuttin et al. 2003). This long-term benefit has been repeatedly reported across various investigative centers. Others also including addiction, anorexia nervosa and Alzheimer's disease (Williams and Okun 2013).

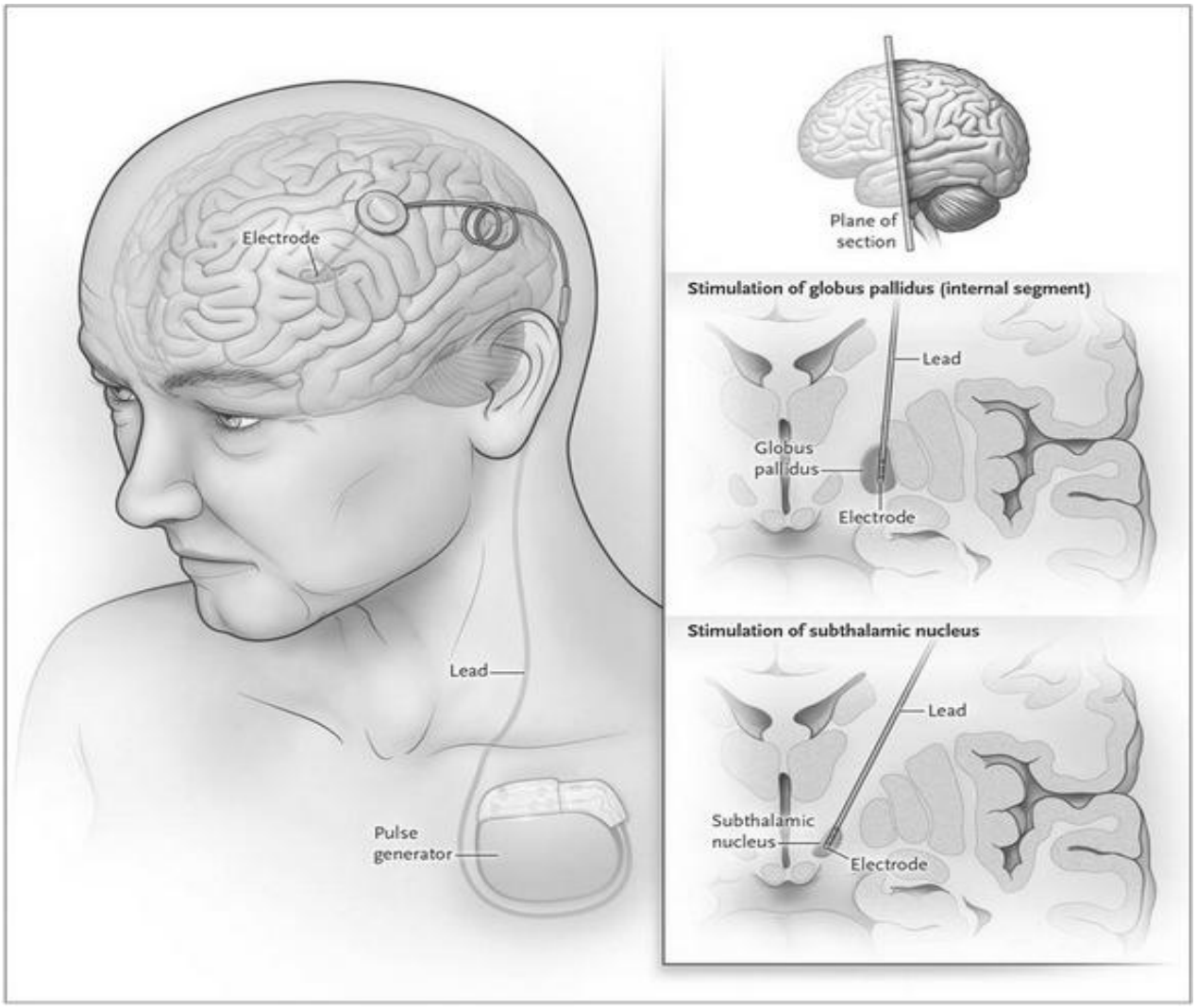


Figure 4. Deep brain stimulation (DBS) system in humans.

Surgeon sends electrical current with a certain frequency and intensity to a precise target brain region of the patient (for example globus pallidus or subthalamic nucleus) through an implanted electrode, which is connected to a pulse generator, in order to change the activity of related neuro-circuitry and treat CNS disorders. Adapted from(Okun 2012)

2.4.2 The application of DBS in depression

Depression is a heterogeneous mental disease. It is characterized by a variety of symptom constellations, arising from a few dysfunctional nodes that are involved in one or several mood networks, such as overactivity of the subcallosal cingulate or the dysregulation of anhedonia and reward-circuitry (Downar et al. 2014). Patients who consistently experience major depressive disorder (MDD) and fail to respond to two adequate treatments of antidepressant medications from different drug classes, are commonly referred to as experiencing treatment-resistant depression (TRD).

Beneficial effects for treatment resistant depression via DBS were found in 2005. Mayberg et al.(Mayberg et al. 2005) were the first to report that the patients' symptoms had been greatly improved after DBS in the subgenual cingulate (Cg25)(Wani et al. 2013). .According to recent studies, DBS for those patients showed a significant improvement in functionality as well as symptomatology, with the benefits lasting at least the first three years. Response and remission rates are normally in the range of 25 to 50% (Pierce and Vassoler 2013).

The DBS target choice in depression may be selected according to the nature of the depressive symptoms, coupled with the possible side-effects and relevant comorbidities (Greenberg et al. 2010). The existing common targets for DBS in depression include the medial forebrain bundle (MFB), the VC/VS, the subcallosal cingulate (SCC) (brain area 25 [BA 25]) as well as the nucleus accumbens (Nacc)(Schlaepfer et al. 2013). These major DBS targets have been studied for treatment-resistant individuals at the clinical stage, and all have demonstrated positive results in small studies. Apart from these regions, the inferior thalamic peduncle (ITP) and lateral habenula are also assumed to be potentially efficacious DBS targets (Sartorius et al. 2010).

Animal models have been used to evaluate the effect and find new targets for DBS treatment of depression. According to Hamani et al., the ventromedial prefrontal cortex (vmPFC) of rodents can represent the Cg25 in humans (Hamani et al. 2010). It has been shown that DBS of the vmPFC can exert symptom-specific anti-depressant effects in the forced swim test (FST) in both the chronic, mild stress rat model of depression and naive Sprague Dawley (SD) rats (Hamani and Nobrega 2012). The FST includes two swimming sessions, performed on consecutive days, with treatment given between sessions. In this test, a decrease in immobility time is the hallmark of an antidepressant-like treatment effect.

2.4.3 The application of DBS in schizophrenia

Pharmacological treatment with antipsychotic medication is the first-line therapy for schizophrenia patients and can effectively reduce the risk of relapse. Other therapies, such as counseling, job training and social rehabilitation are also important in psychotic patient treatment. However, there has not been much progress in developing alternatives. Almost all current antipsychotics have essentially the same

mechanism as drugs which were discovered in the 1950s. Furthermore, about 10-30% of patients have little or no response to treatment and a large proportion of them who received long-term medication suffer from various side-effects (Leucht et al. 2012). As mentioned above, DBS might be an option for the treatment of mental disorders. Unfortunately, there has been no evidence on the effects of DBS in schizophrenia patients from clinical trials, but more than a therapeutic technique, DBS can also serve as an experimental tool in animal studies of schizophrenia. Samuel G. at el (Ewing and Grace 2013) treated MAM rats, a validated rat model of schizophrenia using mitotoxin methylazoxymethanol acetate, by using high frequency stimulation on the ventral hippocampus. The study showed that DBS effectively restored the deficits in the processing of auditory evoked potentials, which has long been associated with schizophrenic patients. As a behavioral output parameter, pre-pulse inhibition (PPI) deficits have been widely used to identify cognitive and functional deficits in schizophrenic animal models (Swerdlow et al. 2008). Disrupted PPI of the acoustic startle reflex (ASR) can well reflect the deficits in sensorimotor gating or early attentional control, which is especially linked with schizophrenic symptoms (Braff, Geyer, and Swerdlow 2001). By performing high-frequency DBS in mPFC and DM, Winter et al. (Klein et al. 2013) proved this treatment can effectively attenuate PPI-deficits in the maternal Poly (I:C) injected rat model of schizophrenia. In another schizophrenia rodent model which induced the schizophrenia-like symptoms by ketamine injection, treatment with medial septum and nucleus accumbens stimulation separately, could also show a significant improvement in the PPI test as well as hyperlocomotion (Ma and Leung 2014). This suggests that DBS in rodent models has a beneficial effect on behavioral measures.

However, due to the inherent differences between species and the limitations of experimental techniques, until now, the mechanisms of DBS effectiveness remain unknown. More efforts have to be made to uncover this mystery in the foreseeable future.

2.5 Aim of the dissertation

1. Are there microglial changes in various brain regions (mPFC, Nacc, CPu and hippocampus) in the rat model of schizophrenia and depressive disorder?
2. Do microglial products (cytokines) change in certain brain regions in a rat model of schizophrenia?
3. How do microglia change after the implantation of electrodes in a targeted brain region?
4. Does DBS treatment affect microglia in local brain regions?
5. How do microglia change in projection areas of the brain in a rat model of schizophrenia and depression after mPFC-DBS treatment?
6. How do microglia change in projection areas of the brain in a rat model of schizophrenia and depression after Nacc-DBS treatment?

3 Materials and methods

3.1 Materials

3.1.1 Devices and equipment

Product	Company
Balances BL610	Sartorius (Göttingen, Germany)
Confocal microscope TSC SPE	Leica Microsystems (Wetzlar, Germany)
Perfusion system WAS02	DITEL (Prague, Czech Republic)
Stereotactic head holder	David Kopf Instruments (Tujunga, USA)
Weighing scales	Sartorius (Göttingen, Germany)
Vibratome VT1000S	Leica Microsystems (Wetzlar, Germany)
Water bath 1008	GFL (Brugwedel, Germany)
Cryostat CM 3050S	Leica Microsystems (Wetzlar, Germany)
pH meter CG840	Schrott (Mainz)
Thermocycler FAST 7500 Real-Time PCR System (qPCR)	Applied Biotsystems (Foster City, USA)
Vortex	Janke & Kunkel, Germany
FACSAria™ III flow cytometer	(BDBiosciences,Erembodegem, Belgium)
Cell culture incubator Heracell	Heraeus Instruments (Hanau, Germany)
Cell strainer	Corning Incorporated (USA)
Spectrophotometer Nanodrop ND-1000	Thermo Scientific (Schwerte, Germany)

Table 1. Devices and equipment

3.1.2 Reagents and chemicals

Reagents/Chemicals	Company
Aqua Poly/mount	Poly sciences Europe (Eppelheim,Germany)

Phosphate Buffered Saline (PBS)	Life Technologies (Rockford, USA)
Sodium chloride (NaCl)	Carl Roth (Karlsruhe, Germany)
Triton X-100	Merck (Darmstadt, Germany)
Donkey serum	Sigma-Aldrich (Munich, Germany)
Polyriboinosinic-polyribocytidylic acid (Poly(I:C))	SIGMA (Germany)
Dental acrylic cement	Heraeus-Kulzer (Hanau, Germany)
Hank's balanced salt solution (HBSS)	Life Technologies (Rockford, USA)
Foetal calf serum	Life Technologies (Rockford, USA)
Dulbecco's Modified Eagle Medium (DMEM)	Life Technologies (Rockford, USA)
Percoll™	GE Healthcare (Fairfield, USA)
CD11b/CD45 microbeads	eBioscience (San Diego, USA)
RNAase mini RNA isolation kit	Qiagen (Hilden, Germany)
Superscript II reverse transcriptase kit	Invitrogen (Karlsruhe, Germany)
Chloral hydrate	Merck (Darmstadt, Germany)

Table 2.Reagents and chemicals

3.1.3 Antibodies for immunohistochemistry

Epitope	Dilution	Provider
Iba1 (ab5076)	1:250 (IHC)	Abcam(Cambridge, UK)
DAPI	1:200	Sigma-Aldrich (Munich, Germany)
NeuN	1:250	Merck Millipore (Massachusetts, USA)
Alexa Fluor®647-conjugated donkey anti-goat IgG	1:200	Dianova (Hamburg, Germnay)

Cy™3-conjugated donkey anti-chicken	1:200	Dianova (Hamburg, Germnay)

Table 3.Antibodies for immunohistochemistry

3.1.4 Buffers

Buffer name	Composition
1X Tris Buffered Saline (TBS)	1.21 g Tris, 8.76 g NaCl, dissolve in 1 L aqua distilled water, pH 7.4
Blocking buffer (TBS+)	0.05%Trition X-100, 5% donkey serum in TBS
1X Phosphate Buffered Saline (PBS)	8 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4, 0.24 g KH2PO4. in 1 L aqua distilled water, pH 7.4
Percoll solution buffer	75% v/v myelin gradient buffer, 22% v/v Percoll, 2.4% v/v 1.5M NaCl
1X FACS buffer	2% v/v FCS in PBS

Table 4.Buffers

3.1.5 DBS system

Components	Company
Lithium thionyl chloride batteries EF651625	EVE Energy Co., Ltd （china）
MOSFET switches REF3125	Texas Instruments （USA）
Dual retriggerable monostable multivibrator 74HC123	Texas Instruments （USA）
Charge pump LM2704	National Semiconductor (USA)
Current sources PSSI2021SAY	NXP (Holland)
MOSFET transistor switch 2N7002	NXP (Holland)
Rodent jackets	Harvard Apparatus Ltd.（USA）

Concentric bipolar platinumeiridium electrodes	Nano biosensors Nazareth （Israel）

Table 5. DBS system

3.1.6 Software

Software	Company
Fiji	NIH (Bethesda, USA)
Leica LAS AF	Leica (Wetzlar, Germany)
Microsoft Office 2007	Microsoft (Berlin, Germany)
Microsoft Windows 7 professional	Microsoft (Berlin, Germany)
GraphPad Prism v5.0	GraphPad Software (La Jolla, USA)

Table 6. Software

3.2 Methods

3.2.1 Animals

Rats from the Flinders Sensitive Line (FSL) and from the control Flinders Resistant Line (FRL) (Karolinska Institutet, Stockholm, Sweden) were used to investigate the effects of DBS on microglia in depressive disorders. A rat model of schizophrenia was established by injecting Poly(I:C) into pregnant rats (Wistar, Harlan Laboratories, Germany), injecting saline instead of Poly(I:C) in the control group. All experimental protocols conformed to the guidelines of the European Communitys Council Directive (86/609/EEC) for care of laboratory animals and were approved by the local ethics committee (Senate of Berlin and Dresden). All the animals were housed in a temperature- and humidity-controlled vivarium with a 12-h light/dark cycle (lights on: 6 a.m-6 p.m.). Food and water were available ad libitum. All efforts were made to minimize animal suffering and to reduce the number of animals used.

3.2.2 Poly(I:C) injections

Rats (Wistar, Harlan Laboratories, Germany) were mated at about three months of age. We define the first day after copulation as day one of pregnancy and on

gestational day 15, all pregnant rats randomly received a single i.v. injection through the tail vein of either Poly(I:C) (4.0 mg/kg, SIGMA, Germany) dissolved in 200 µl 0.9% NaCl, or vehicle (saline)(Klein et al. 2013). After this, all rats were placed in their original cages and left undisturbed until parturition time. At postnatal day (PND) 21, male pups were weaned and housed by sex and litter (Figure 5).

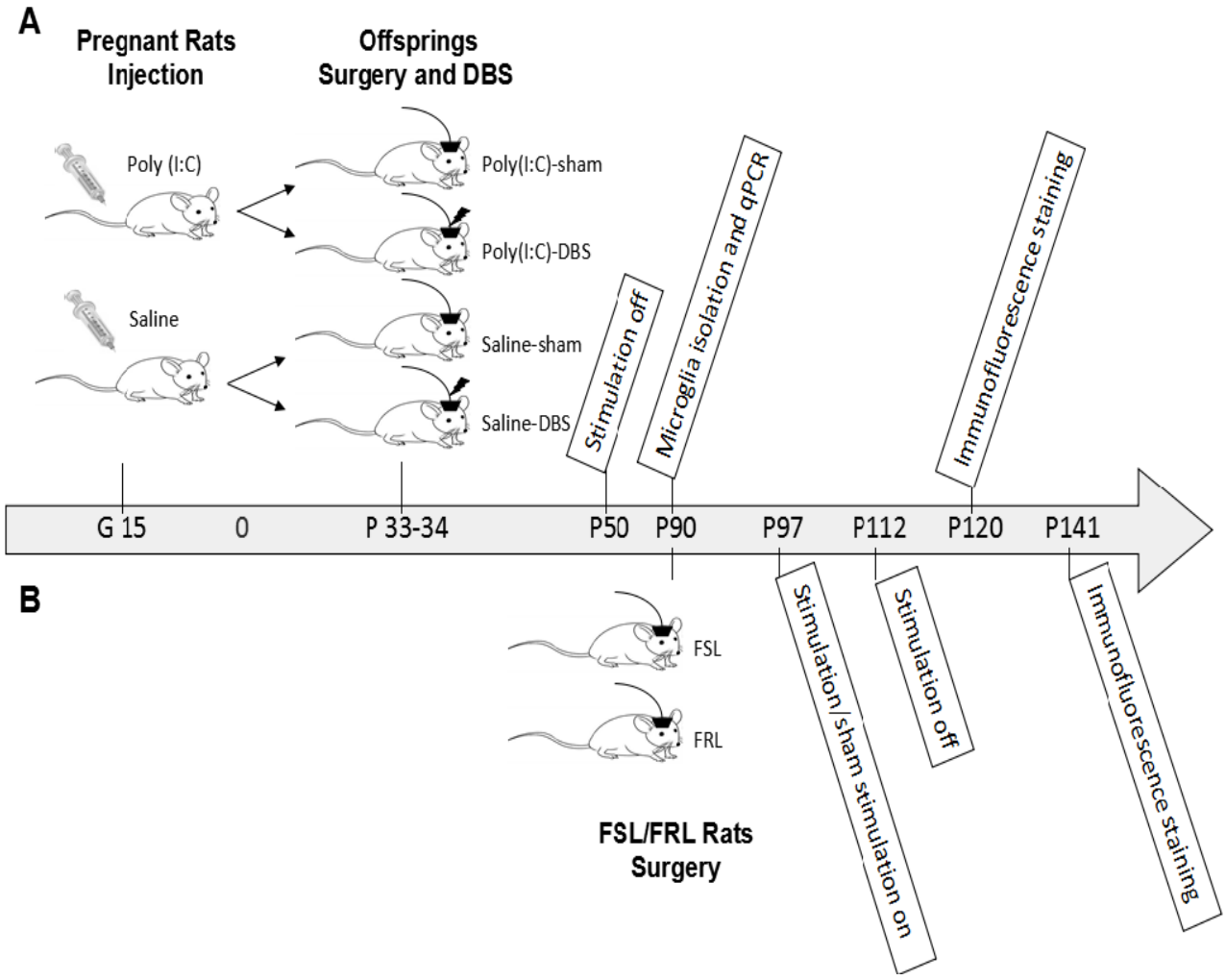


Figure 5. Time line of the experimental protocols.

(A) The investigative procedure of the effects of deep brain stimulation (DBS) on microglia in a rat model of Schizophrenia. G, gestation day; P, Postnatal day; qPCR, quantitative real-time polymerase chain reaction. (B) The investigative procedure of the effects of DBS on microglia in a rat model of depressive disorder. FSL, Flinder Sensitive Line; FRL, Flinder Resistant Line.

3.2.3 Surgery and deep brain stimulation treatment

1) Rat model of schizophrenia: During the PND33 and PND34, the male offspring of the Poly(I:C) group (n=33) and saline group (n=35) were bilaterally and stereotactically implanted with DBS electrodes (concentric bipolar platinum iridium electrodes with connector; Nano biosensors Nazareth, Israel). Procedures were performed as described previously (Djodari-Irani et al. 2011). Pups were anaesthetized through a balanced anesthesia (Medetomidin 0.15 mg/kg, midazolam hydrochloride 2.00 mg/kg, fentanyldihydrogencitrate 0.005 mg/kg). For each operation, the incisor bar was set at 3.3 mm below the interaural line. The skin was cut open to sufficiently expose the skull, and the electrodes were implanted in the medial prefrontal cortex (mPFC) or nucleus accumbens (Nacc) with the following coordinates (according to the atlas of Paxinos and Watson, 1997): mPFC: t3.6 mm anterior to bregma (AP), 0.6 mm lateral to the midline (ML), and -3.4 mm ventral to dura (DV); Nacc: 1.2mm to AP, 1.8 to ML, -8,1mm to DV. Using stainless steel screws and dental acrylic cement (Technovit®, Heraeus-Kulzer, Hanau, Germany), electrodes were fixed to the skull surface and the incision closed with stitches, and the electrodes and stimulator connected (Table 7). The stimulator was carried on a rodent jacket (Harvard Apparatus Ltd.) which includes a dedicated on/off switch so that rats can move freely with the device (Figure 6). 2) Rat model of depressive disorder: rats from FRL (n=12) and FSL (n=12) were also implanted with DBS electrodes in mPFC or Nacc at PND 90. All the processes of anesthesia and surgery were the same as above.

In order to avoid the stimulator being frequently knocked against the top of the cage, we housed all the animals in cages with a large vertical space after surgery. Randomly selected rats from each group had the stimulator switched on (treated group; stimulation parameters: frequency 130Hz; pulse 150µA; duration 90µs), stimulator was switched off in the remaining rats (untreated group). The treatment lasted 15 days to mimic chronic DBS treatment in patients, the stimulator was then switched off in the treated group, leaving all the rats undisturbed until they reached adulthood.

Part	Value	Package	Quantity
Battery	3.6 V	LTC-5PN	1
Switch	-	-	1(B)
Regulator	REF3125	SOT23	1
Inductor	10 µH	0805	1
Capacitor	4.7 µF	0603	3
Capacitor	4.7 µF	0805	3
Charge pump	LM2704	SOT23-5	1
Diode	Schottky	SOD123	1
R1	510 kΩ	0603	1
R2	33 kΩ	0603	1
Dual one-shot	74HC123	TSSOP16	1
Freq cap	1 µF	0603	1
Freq res	18 KΩ	0603	1
Pulsewidth cap	0.01 µF	0603	1
Pulsewidth res	20 KΩ	0603	1
Current source	-	SOD-353	2
Current res	4.53 KΩ	0603	2
Switching	MOSFET	SODT23	3

Table 7. Component list of stimulator

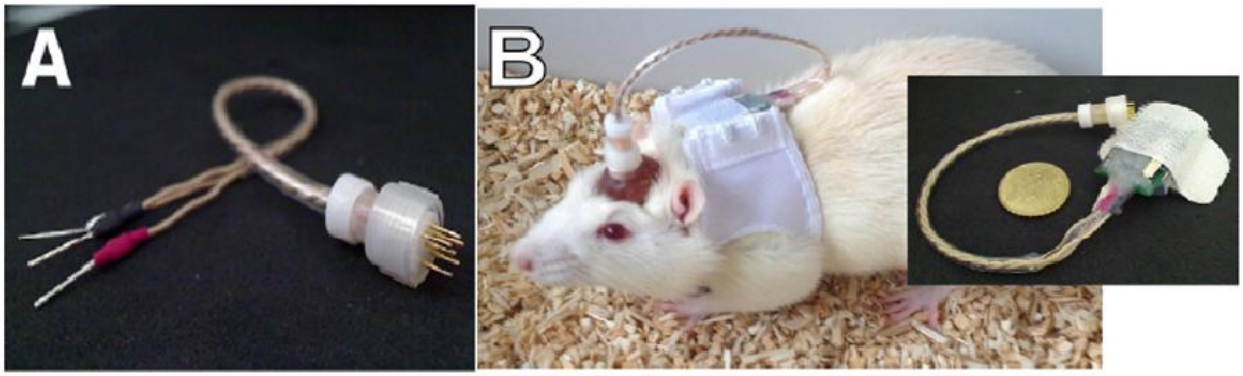


Figure 6. System of deep brain stimulation.

(A) 3 channel 363-SL/3 cable. (B) Electrode connected with a stimulator which was carried on a rodent jacket with secure attachment provided by a hook-and-loop fastener. Adapted from (Ewing et al. 2013).

3.2.4 Isolation of microglia

Rats from each group (Poly(I:C)-sham, n=9; Poly(I:C)-DBS, n=8; saline-sham, n=9; saline-DBS,n=10) were deeply anaesthetised (sodium pentobarbital, 30 mg/kg) and perfused (1% phosphate buffered saline (PBS), Gibco®, Life Technologies). The hippocampus was dissected from the removed brain and dissociated mechanically. Tissue fragments were washed by using 1X Hank's balanced salt solution (HBSS) (Gibco®, Life Technologies) and then centrifuged (2 min at 900g, 4ºC). The tissue was incubated with deoxyribonuclease and trypsin-deoxyribonuclease at room temperature for 2 min. After incubation, the suspension was mixed by repeated up and down pipetting to enhance the enzymatic digestion reaction. Foetal calf serum (10% v/v, FCS, Gibco®, Life Technologies), 1X DMEM solution (Gibco®, Life Technologies) and penicillin-streptomycin-glutamine (1% v/v, PenStrepGlut, Gibco®, Life Technologies) were added to the mixture to terminate the reaction. The mixture was sieved by using a 70µm cell strainer (FALCON, Corning Incorporated). The residue was smashed through a syringe plunger and washed with DMEM complete solution (DMEM/FCS/ PenStrepGlut). The filtrate was centrifuged (10 min at 500g, 4ºC) and resuspended in 25 ml percoll solution buffer (75% v/v myelin gradient buffer; 22% v/v Percoll^{TM}, GE Healthcare; 2.4% v/v 1.5M NaCl). PBS (5mL) was slowly added to the surface of the suspension. The suspension was centrifuged again (20 min at 950g, 4ºC). The myelin and suspension were removed carefully and the pellets were resuspended in 1X FACS buffer (PBS containing 2% v/v FCS). Cell suspensions were incubated with CD11b and CD45 microbeads (1:500, eBioscience) for 20 min and centrifuged for 5 min at 500g. Cell pellets were resuspended in FACS buffer and sieved again through a 70µm cell strainer. CD11b and CD45 positive cells were then collected by FACSAria™ III flow cytometer (BD Bioscience) in FACS tubes.

3.2.5 Total RNA isolation, cDNA synthesis and quantitative PCR detection

Total RNA was isolated using RNeasy® or Rneasy® Plus Micro Kit (QIAGEN) according to the manufacturer's instructions. The concentration of isolated RNA was measured with the nanodrop-1000 spectrophotometer (PeqLab Biotechnologie GmbH). cDNA synthesized by using SuperScript II reverse transcriptase kit (Invitrogen^{TM}, Life Technologies). IL-1β, TNF-α and IL-6 mRNA levels were determined by quantitative real-time polymerase chain reaction (qPCR) using primers

(BioTeZ Berlin-Buch GmbH) (Table 8). Quantitative real-time PCR was carried out in at least duplicate by using SYBR PCR mix (Applied biosystems®, Life Technologies) in a FAST 7500 real-time PCR system according to the manufacturer's instructions. 1ng/µl cDNA per sample was added into a total volume of 19.5µl. H2O was used as negative control and β-actin was used as reference. The thermal cycling protocol involved 43 cycles. Quantification of mRNA was calculated though the comparative Ct ($2^{-\Delta\Delta Ct}$) values.

Gene product	Primer sequences	Annealing Temperature
Rat TNF-α	5'- GATCGGTCCCAACAAGGAGG-3' 5'- CAGCTGCTCCTCCGCTT-3'	60°C
Rat IL-1β	5'- CTGTCTGACCCATGTGAGCTG-3' 5'- AGGGATTTTGTCGTTGCTTGTC-3'	60°C
Rat IL-6	5'- CCACCCACAACAGACCAGTAT-3' 5'- GCATCATCGCTGTTCATACAATCA-3'	60°C
Rat β-actin	5'- CCACCTCACAAGCATCCTTTCT-3' 5'- CCAGAGGCATACAGGGACAA-3'	60°C

Table 8. qPCR primers

3.2.6 Brain section preparation

Animals (maternal Poly(I:C)/saline-injected rats at P120, n=32; FSL/FRL rats at P141, n=24) were decapitated under deep anesthesia (chloral hydrate 50 mg/kg, Merck, Darmstadt, Germany). Brains were removed within seconds, shock frozen and sectioned in the coronal plane at 40mm thickness. Only animals with correct electrode placements were included in the statistical analysis. Sections were stored in -20℃ in phosphate buffered saline (PBS).

3.2.7 Immunofluorescence staining of microglia

The selected brain sections (4-5 slices from each area of each animal) from both the schizophrenia model (Poly(I:C)/saline group) and depression model (FSL/FRL group)

were collected and put into a 12-well plate (3 slices per well). The sections were washed with TBS buffer (PH 7.4, 10min × 3-5 times, room temperature) and incubated in blocking buffer TBS+ (5% donkey serum in TBS) for 1h for permeabilization. The sections were then incubated with primary antibody (goat-anti-Iba1;1:250;Abcam) and chicken primary antibodies against NeuN (1:250, *Chemicon*®, Merck Millipore) in TBS/DKS, 350ml per well, overnight at 4℃. On the second day, after 6 washes with TBS at room temperature，the slices were blocked again in TBS+ buffer for 15min. The blocking buffer was then removed and secondary antibody (Alexa Fluor®647-conjugated donkey anti-goat and Cy™3-conjugated donkey anti-chicken; 1:200; dianova), Dapi (1:200; Sigma-Aldrich) with TBS+ buffer was added into each well (350ml per well). Slices were incubated for 2h at room temperature. After the final 6 washes with TBS, the slices were mounted with Aqua-Poly/Mount mounting medium onto microscope slides and stored at 4℃ in preparation for scanning.

3.2.8 Confocal microscope scanning and image analysis

Brain slices were scanned by using a confocal microscope (Leica, Wetzlar, Germany). 4 to 5 pictures of each brain region (mPFC, CPu, Nacc and hippocampus (dentate gyrus)) per animal were taken with the same parameter setting of the microscope (Objective lens: 10X; Resolution: 1024 ×1024; Laser: Dapi and CY5; Zoom size: 1.5; Z-stack: 8-10μm per step). Tile scanning was used only for the scanning of the hippocampus (6 tiles images). The density and soma size of microglia in each region were measured blind by using Fiji ImageJ software (NIH, Bethesda, Maryland, USA).

3.2.9 Statistical analysis

All data represent at least 3 independent experimental objects. Error bars represent standard error of the mean. Data sets were analyzed using the software GraphPad Prism v5.0 software (La Jolla, CA, USA). Comparisons between two groups were carried out using an unpaired t-test. Multiple groups were tested using one-way ANOVA or two-way ANOVA followed by Bonferroni post-hoc test. Statistical significance was determined using p values < 0.05 (*), $P < 0.01$ (**) and $P < 0.001$ (***).

4 Results

4.1 Microglia dysregulation in the animal model of schizophrenia

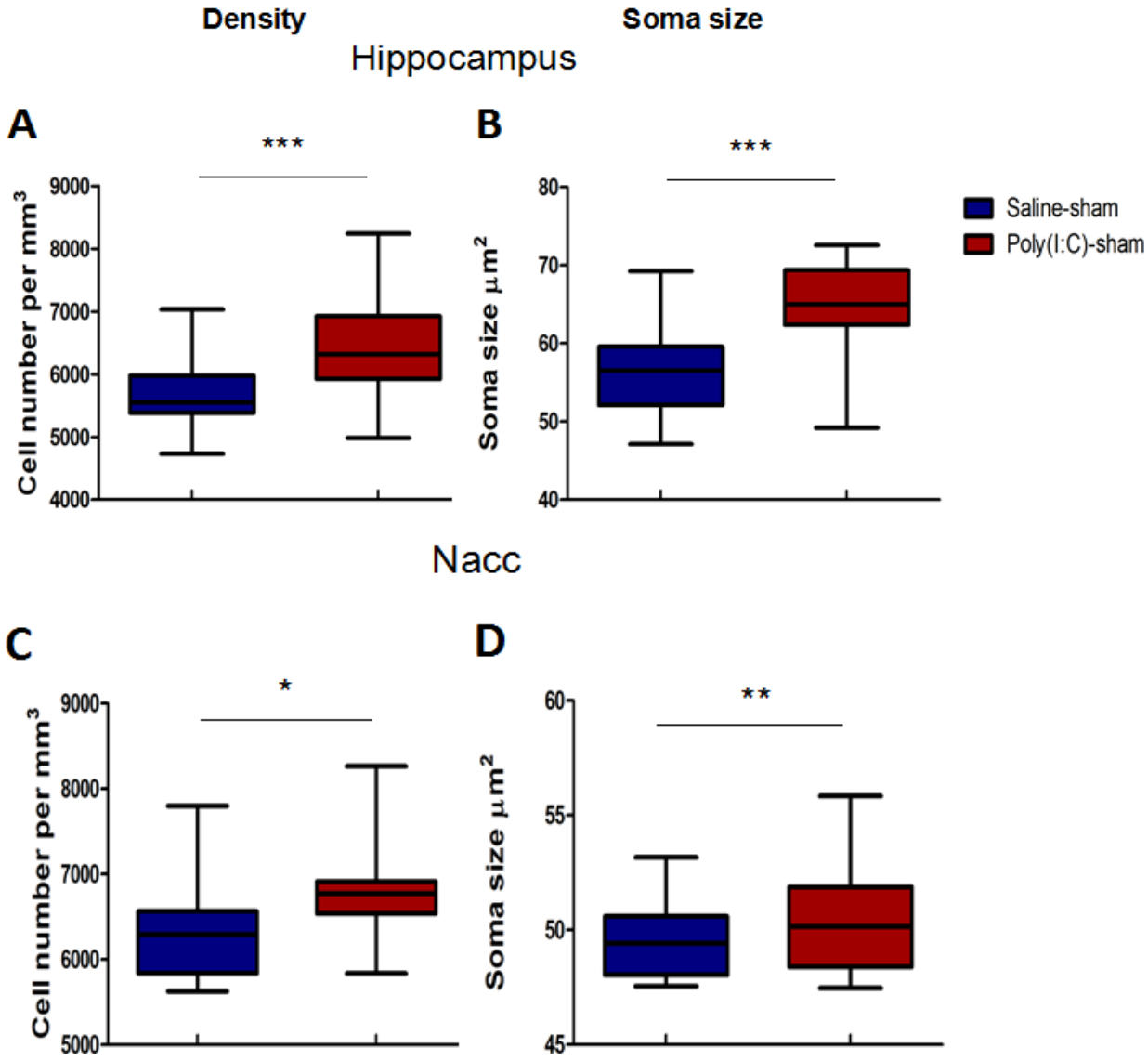
4.1.1 Microglia density and soma size is increased in hippocampus and nucleus accumbens (Nacc) in the Poly(I:C) group

In order to study the density and soma size changes of microglia, we used an immunofluorescence method to mark microglia with the ionized calcium-binding adapter molecule (Iba1), which is an excellent marker for the analysis of microglial morphology, and scanned various brain regions by using an SPE microscope (Leica Microsystems, Wetzlar, Germany). We calculated the cell density and soma size, as an indicator of cell activity, using image processing software (Fiji, powered by MediaWiki). First, for investigating how microglia change in a rat model of schizophrenia, we compared the density and soma size of microglia in the saline-sham group with the Poly(I:C)-sham group in different brain regions. In the hippocampus, both density (fig.7A, saline-sham vs. Poly(I:C)-sham, 5687 ± 95.46 vs. 6495 ± 183.4, unpaired t test $p < 0.0001$) and soma size (Fig.7B, saline-sham vs. Poly(I:C)-sham, 56.57 ± 1.226 vs. 64.71 ± 1.296 unpaired t test $p < 0.0001$) of microglia in the Poly(I:C)-sham group are significantly greater compared to saline-sham group, which indicates that microglia were highly activated in the hippocampus (Fig.8 A2 and B2) in schizophrenic rats. In the Nacc, the same results of maternal Poly(I:C) injection can be observed, the density (Fig.7C, saline-sham vs. Poly(I:C)-sham, 6325 ± 137.1 vs. 6752 ± 107.4 unpaired t test $p < 0.0179$) as well as soma size (fig.7D, saline-sham vs. Poly(I:C)-sham, 50.69 ± 0.3721 vs. 54.11 ± 1.119, unpaired t test $p < 0.0069$) of microglia are significantly increased in Poly(I:C)-sham group (Fig.8 A4 and B4).

4.1.2 The density of microglia is decreased in caudate putamen in the Poly(I:C) group

In another two interesting areas, there were no signs of an increase in microglia density in CPu (Fig.7G and Fig.7H) or in mPFC (Fig.7E and Fig.7F) after maternal Poly(I:C) injection. However, in contrast, as the results show, we observed a decreased microglial density (Fig.8 A8) in the Poly(I:C)-sham group (Fig.7G, saline-

sham vs. Poly(I:C)-sham, 6634 ± 95.99 vs. 5973 ± 116.3 unpaired t test $p < 0.0001$) in CPu, which is also an important area that is closely linked to abnormal neurotransmitter metabolism in schizophrenia disorder (Reinhart et al. 2015).



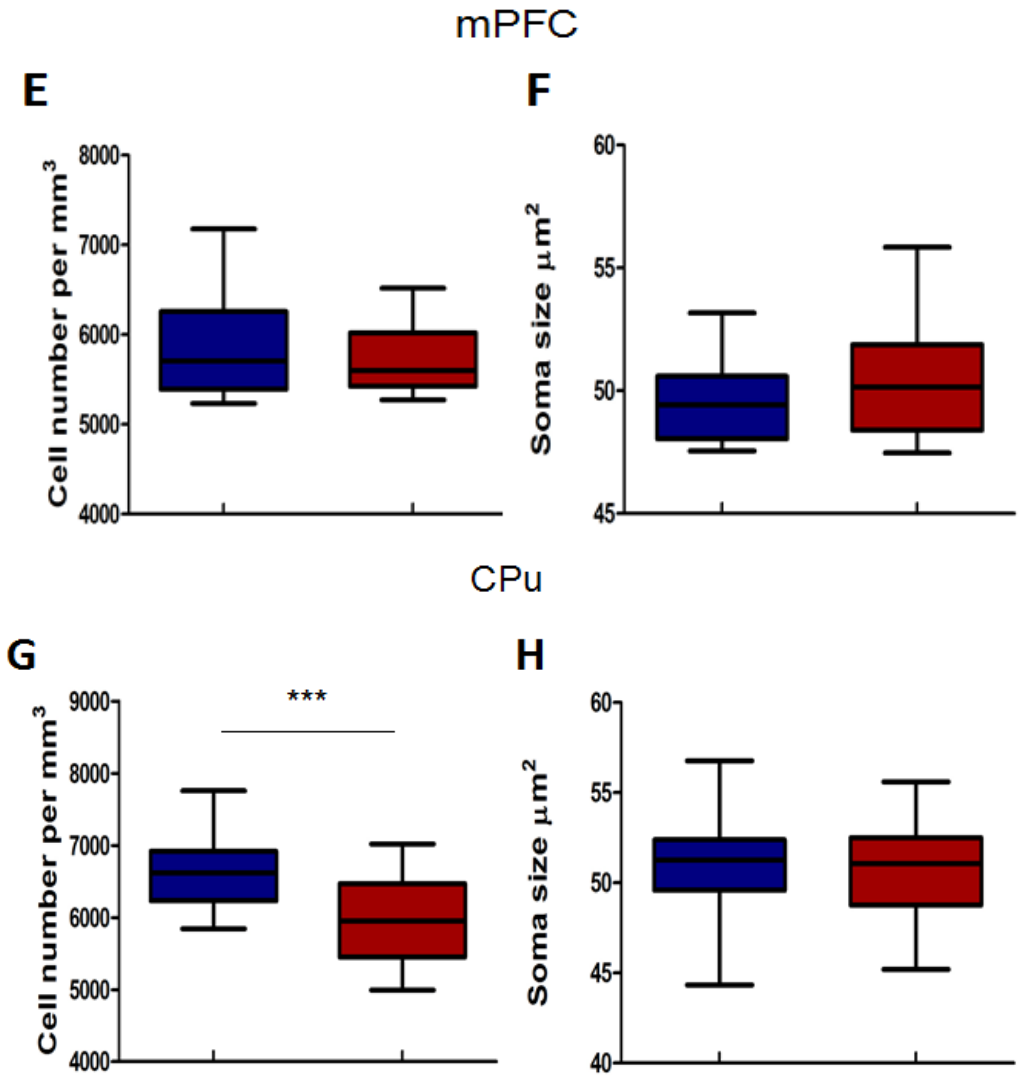


Figure 7. In a rat model of schizophrenia, the density and soma size of microglia are increased in the hippocampus and nuclear accumbens (Nacc), but the density of microglia was decreased in the caudate putamen.

After maternal Poly(I:C) injection, changes in microglial density and soma size can be observed in their offspring by marking microglia through immunofluorescence staining (Iba1). In the hippocampus, both density (A) and soma size (B) of microglia were increased compared to the saline-sham group. In the Nacc, the density (C) as well as the soma size (D) of microglia were also significantly increased in Poly(I:C)-sham group compared to the saline-sham group. There was no microglial changes in mPFC between the Poly(I:C)-sham group and the saline-sham group (E and F). However, microglial density was significantly decreased in CPu in Poly(I:C)-sham group (G). Significance levels are denoted by *$p < 0.05$, ** $p < 0.01$ and ***$p < 0.001$ based on unpaired t test.

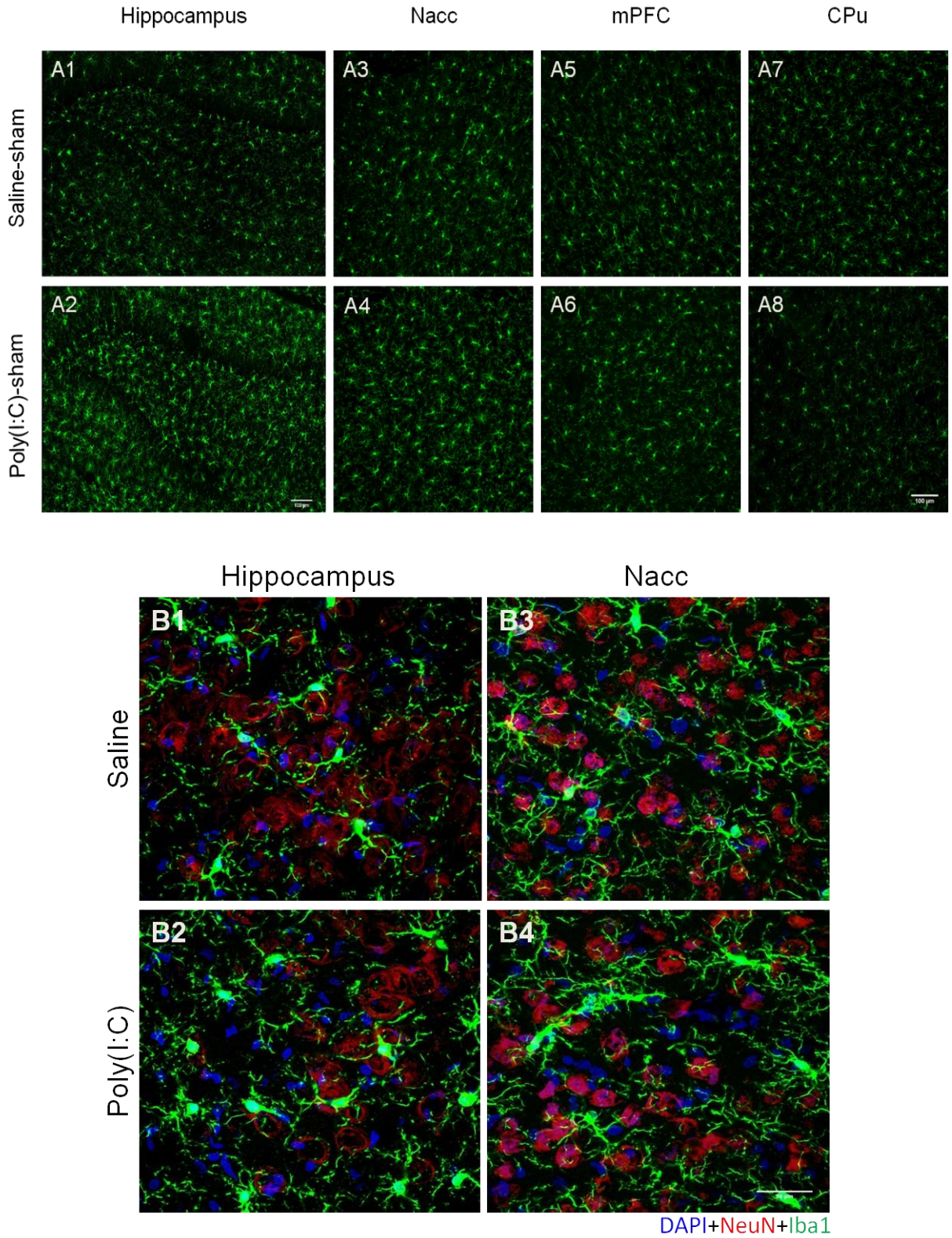


Figure 8. The density and morphology of microglia in various brain regions in the poly(I:C) group and saline group.

(A) Density of microglia in the hippocampus, Nacc, mPFC and CPu in the Poly(I:C) group compared to the saline group. (B) Morphologic changes of microglia in the hippocampus and Nacc in the Poly(I:C) group compared to saline group.

4.2 Effects of DBS on microglia in the schizophrenia model

4.2.1 Electrode implantation per se can lead to an increase in the density and soma size of microglia in local brain region, which were normalized by DBS

Since we detected the changes in microglia in a schizophrenia-like rat model, we have performed high frequency electric deep brain stimulation in mPFC and Nacc to investigate the changes of microglia after DBS treatment.

In mPFC, which is the target area of the implanted electrode, in order to study the effects of DBS electrode implantation per se as well as the high-frequency electric current on microglia in this targeted region, we put the non-implantation group (Electrodes implanted in Nacc without DBS treatment), implantation group (electrodes implanted in mPFC without DBS treatment) and implantation-DBS group (electrodes implanted in mPFC with DBS treatment) together and compared both the density and soma size of microglia with each other by using two-way ANOVA followed by Bonferroni post-hoc test. As expected, we observed a significant microglial morphology change, enlarged cell bodies with highly ramified processes (Fig.10 B3 and B4), in both the saline and the Poly(I:C) implantation group compared to the non-implantation groups (Fig.9A Poly(I:C)-non-implantation (5724±118.1) vs. Poly(I:C)-implantation (6741±154.2) Bonferroni post-hoc $p < 0.001$; saline-non-implantation (5893±169.9) vs. saline-implantation (7197±238.1), Bonferroni post-hoc $p < 0.001$; $F_{2,83} = 21.5$, $p < 0.0001$) (Fig.9B Poly(I:C)-non-implantation (50.62±0.805) vs. Poly(I:C)-implantation (59.75±0.992) Bonferroni post-hoc $p < 0.001$; saline-non-implantation (49.58±0.478) vs. saline-implantation (62.04±0.569), Bonferroni post-hoc $p < 0.001$; $F_{2,83} = 56.8$ $p < 0.0001$). These changes may be due to a local inflammation that was caused by the electrode implantation. The same effect of electrode implantation has also been reported by a recent animal study (Perez-Caballero et al. 2014). In the implantation-DBS group, the electric current in the targeted region showed an effect of reduced microglial density and soma size compared to the implantation group. This would be a hint that chronic DBS treatment can reduce the inflammation response in the local brain region (Fig.9A Poly(I:C)-implantation (6741±154.2) vs. Poly(I:C)-implantation-DBS (6071±135.7), Bonferroni post-hoc $p < 0.01$; saline-implantation (7197±238.1) vs. saline-implantation-DBS (6417±188.4) Bonferroni post-hoc $p < 0.01$; $F_{2,83} = 21.5$, $p < 0.0001$) (Fig.9B Poly(I:C)-implantation (59.75±0.992) vs. Poly(I:C)-implantation-DBS (55.08±1.055),

Bonferroni post-hoc $p < 0.001$; saline-implantation(62.04±0.569) vs. saline-implantation-DBS (56.677±1.294), Bonferroni post-hoc $p < 0.001$; $F_{2,83} = 56.8$ $p < 0.0001$).

To further confirm our findings, we also investigated another implantation target region, the nucleus accumbens. The same trend of microglial density change can be seen after electrode implantation in the saline group, but it is not significantly different (Fig.9C). In the Poly(I:C) group, there have been increased microglia density and soma size in Nacc in naïve schizophrenic rats. This would be an explanation as to why there was no difference in microglial density after implantation. However, the electric current in the local brain region decreased both the microglial density and soma size after treatment compared to the non-implantation group in the Poly(I:C) rats (Fig.9C Poly(I:C)-non-implantation (6751±107.4) vs. Poly(I:C)-implantation-DBS (6091±242.7), $F_{2,84} = 3.8$ Bonferroni post-hoc $p < 0.05$) (Fig.9D Poly(I:C)-non-implantation (54.11±1.119) vs. Poly(I:C)-implantation-DBS (48.97±0.721), $F_{2,79} = 4.6$ Bonferroni post-hoc $p < 0.001$).

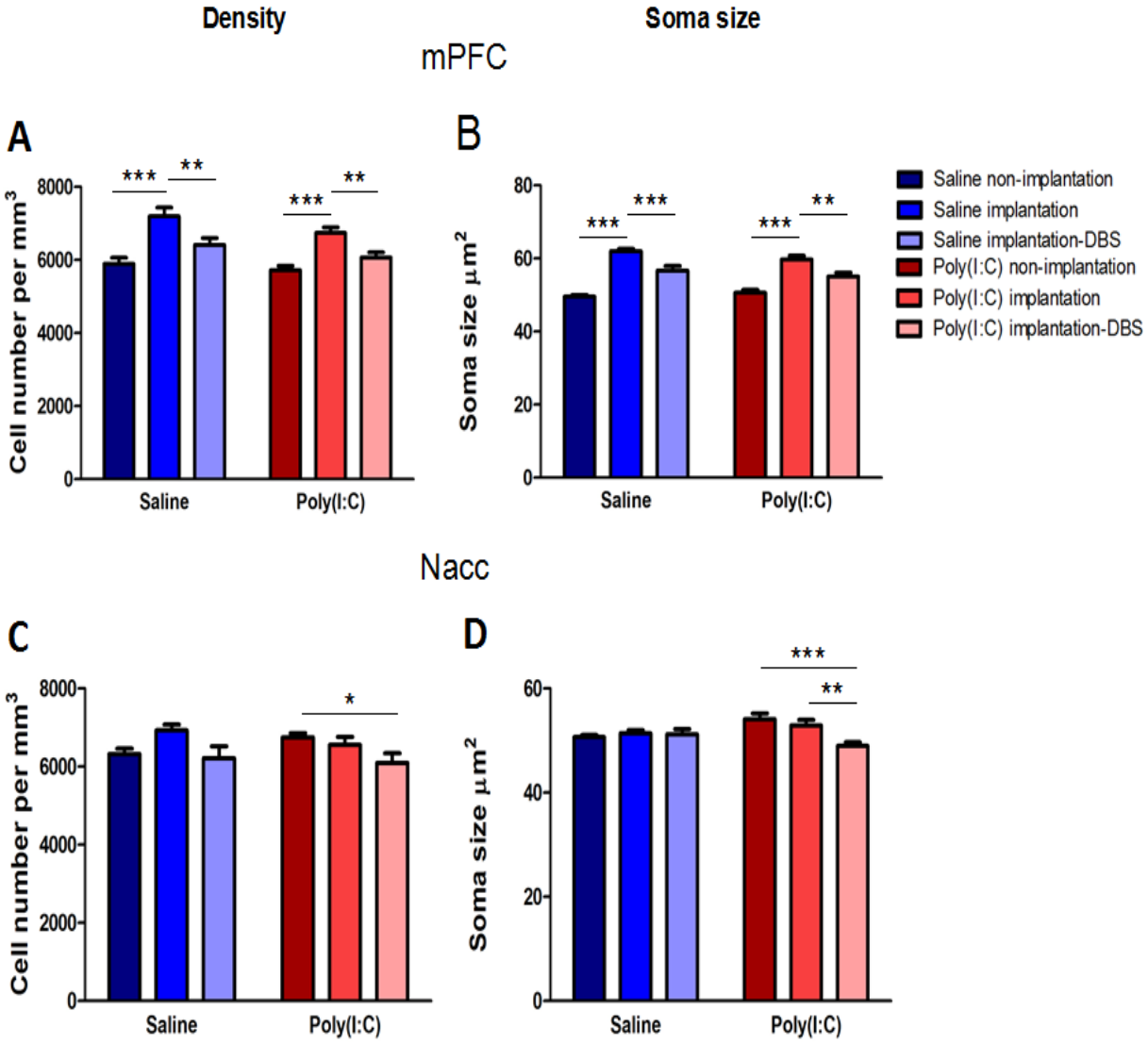


Figure 9. Electrode implantation per se can lead to an increase in the density and soma size of microglia in the local brain region, which were attenuated by DBS.

To study the effects on microglia of electrode implantation per se as well as DBS current in the targeted brain region, we compared the density and soma size of microglia in the non-implantation group, implantation group and implantation-DBS group. In mPFC, microglial density (A) and soma size (B) in the Poly(I:C) and saline groups were significantly increased in the implantation group compared to the non-implantation group. After mPFC-DBS treatment, the local effects of DBS reversed these changes. The density and soma size of microglia were normalized. To confirm our findings, another implantation target region, nucleus accumbens, was also investigated. The same trend of microglial density change can be observed after electrode implantation in saline and Poly(I:C) groups (C), and the DBS current effectively attenuated increased microglial density (C) and soma size (D) in the Poly(I:C) group in the local brain region.

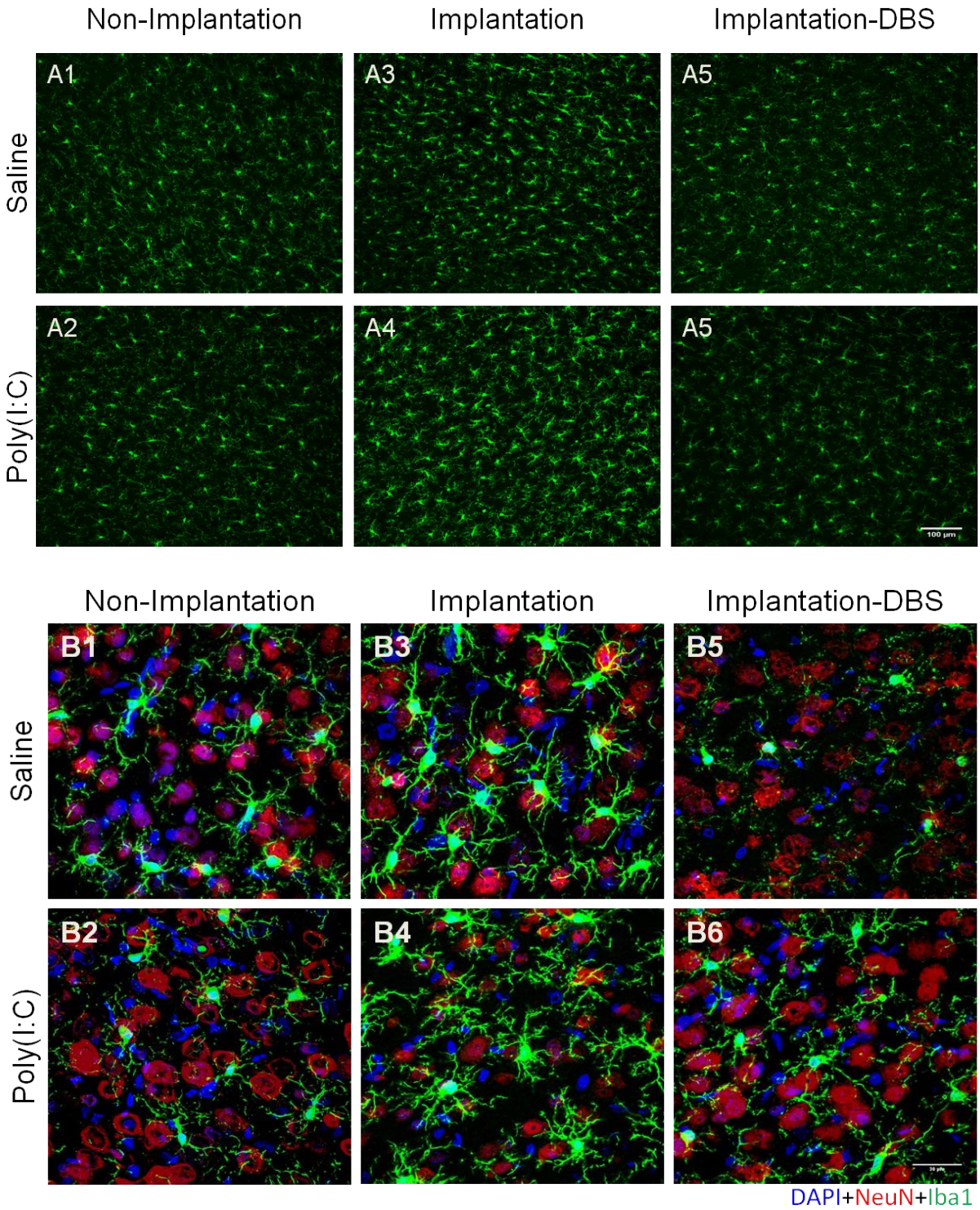


Figure 10. The density and morphologic changes of microglia caused by electrode implantation and DBS current in the targeted brain region (mPFC).

(A) The density change of microglia in the targeted region among Non-implantation, implantation and implantation-DBS group. (B) The morphologic change of microglia due to electrode implantation and DBS current in the targeted region.

4.2.2 mPFC DBS can effectively attenuate microglial changes in the projection area (hippocampus and Nacc)

To study the effects of DBS on microglia, we performed DBS treatment in mPFC. As a brain region which is closely related to schizophrenic behavior, the hippocampus has always been the focus in many earlier studies (Cocchi, Drago, and Serretti 2015). As we expected, increased density (Fig.11A, Poly(I:C)-sham vs. Poly(I:C)-DBS, 6411 ± 295.5 vs. 5566 ± 106.0 unpaired t test $p = 0.0087$) and soma size (Fig.11B, Poly(I:C)-sham vs. Poly(I:C)-DBS, 66.06 ± 1.169 vs. 58.00 ± 0.7171 unpaired t test $p < 0.0001$) of microglia in poly(I:C)-DBS group in hippocampus were normalized by DBS treatment. In another area, where microglia were highly activated in Poly(I:C)-DBS group, nucleus accumbens, we observed the same effects of DBS on microglia, both microglial density and soma size were significantly attenuated by mPFC-DBS treatment (Fig.11C, Poly(I:C)-sham vs. Poly(I:C)-DBS, 6752 ± 107.4 vs. 5714 ± 150.2 unpaired t test $p < 0.0001$; Fig.11D, Poly(I:C)-sham vs. Poly(I:C)-DBS, 54.11 ± 1.119 vs. 50.19 ± 0.3663 unpaired t test $p = 0.0037$). In the projection region caudate putamen (CPu), DBS treatment showed no influence on either density or soma size of microglia. The density of microglia was still lower in the Poly(I:C)-DBS group compared to the saline-DBS group (Fig.11E, saline-DBS vs. Poly(I:C)-DBS, 6212 ± 62.14 vs. 5756 ± 92.94 unpaired t test $p = 0.0003$).

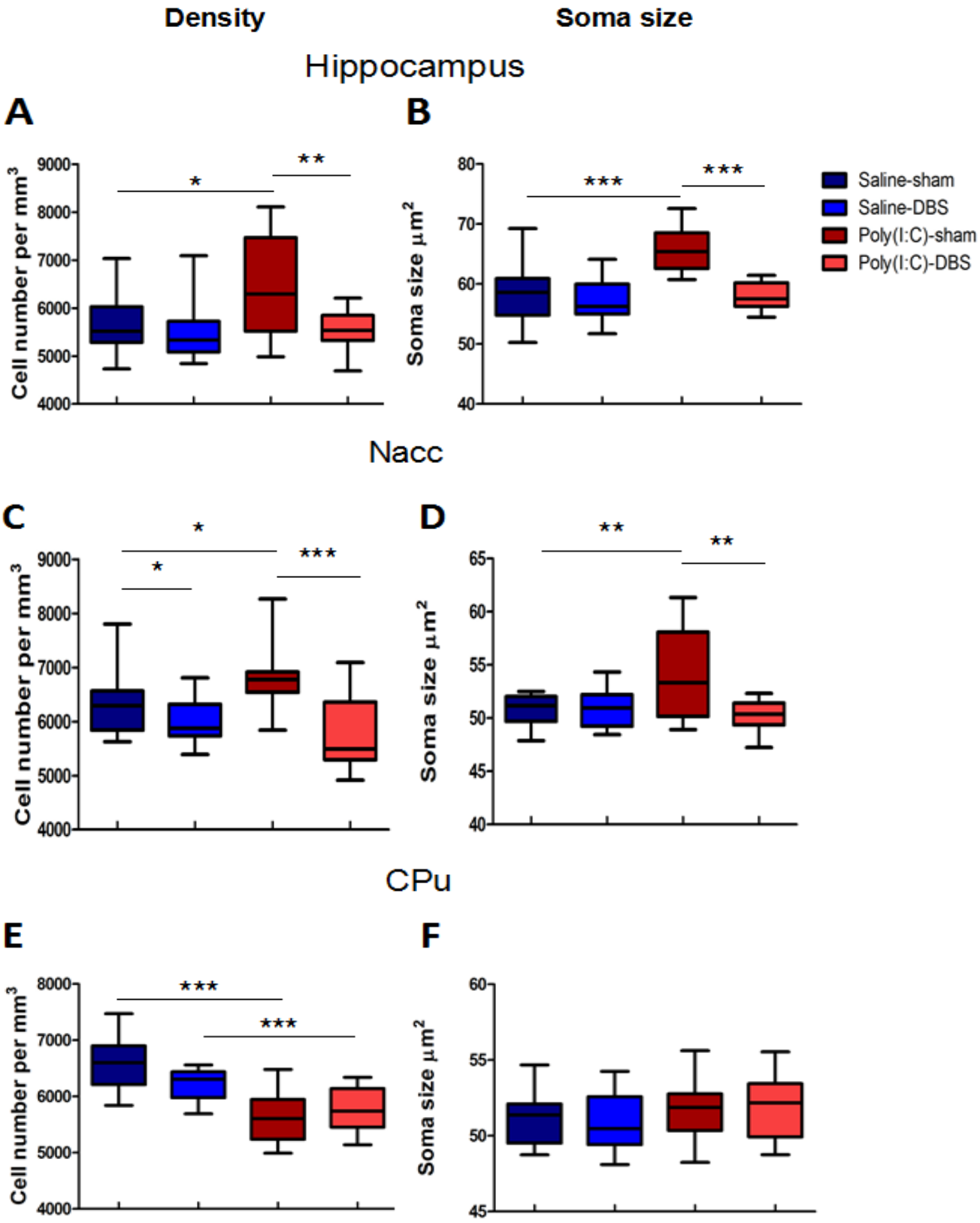


Figure 11. mPFC DBS can effectively attenuate the changes of microglia in the hippocampus as well as the Nacc in the Poly(I:C) group.

Since we have understood how DBS can affect microglia in the local brain region, we performed DBS in mPFC to observe microglia changes in the projection areas. In the hippocampus, DBS treatment effectively attenuated the changes of microglia in the Poly(I:C)-

DBS group compared to Poly(I:C)-sham group (A and B). The same effects on the changes of microglia density and soma size can be seen in Nacc (C and D). There was no significant change in microglia after mPFC-DBS treatment, the density of microglia still lower in Poly(I:C)-DBS group compared to saline-DBS group (E). Significance levels was denoted by *p < 0.05, ** p < 0.01 and ***p < 0.0001 based on unpaired t test.

4.2.3 Nacc DBS attenuates microglial density and soma size in the hippocampus in the Poly(I:C) group

As a further step, we investigated the changes of microglia in another treatment group, where DBS electrodes were targeted in the nucleus accumbens (Nacc). After DBS treatment, similar to the mPFC-DBS treatment group, the density (Fig.12A, Poly(I:C)-sham vs. Poly(I:C)-DBS, 6586 ± 219.9 vs. 5837 ± 191.1 unpaired t test p = 0.0175) of microglia in the hippocampus was significantly reduced in Poly(I:C)-DBS group, while the soma size of microglia was also decreased by DBS treatment, but was not significant compared to the Poly(I:C)-sham group (Fig.12B).

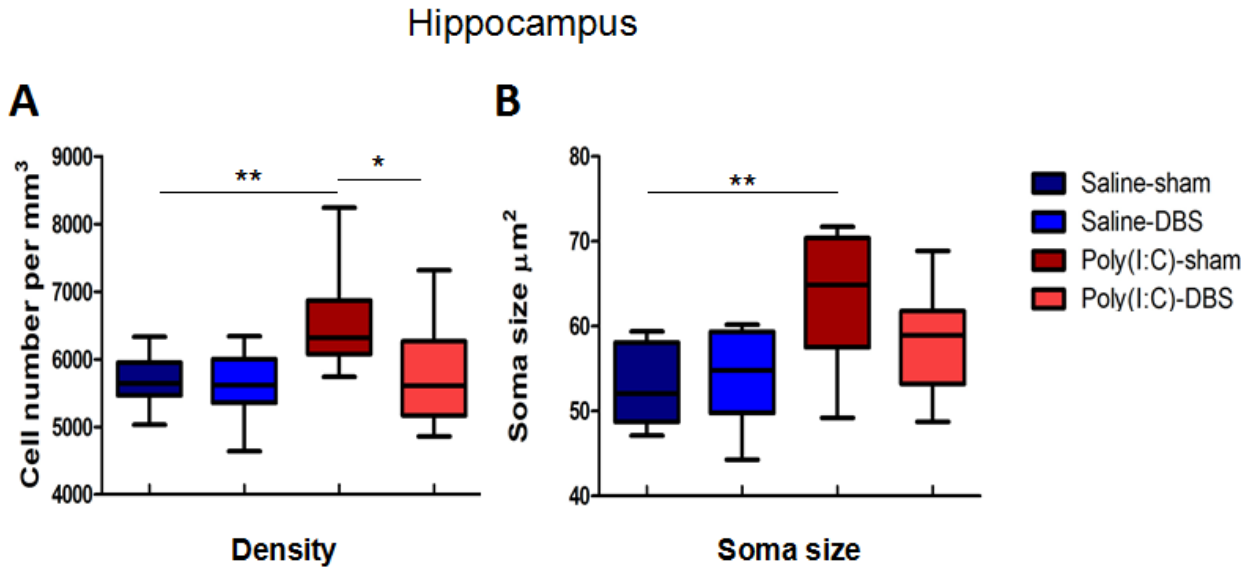


Figure 12. Nacc DBS attenuates the density of microglial in the hippocampus in the Poly(I:C) group.
DBS treatment was performed in Nacc to further study the effects on microglia in the projection areas. The density of microglia in the hippocampus was normalized after treatment (A), while the soma size of microglia was also decreased by DBS, it was however, not significant compared to Poly(I:C)-sham group (B). Significance levels are denoted by *p < 0.05, ** p < 0.01 based on unpaired t test.

4.2.4 Nacc DBS treatment increases the density of microglia in the mPFC and CPu in the Poly(I:C) group.

Interestingly, in the caudate putamen (Cpu), we observed a dramatic increase in the density of microglia in the Poly(I:C)-DBS group compared to the saline-DBS group and Poly(I:C)-sham group after DBS treatment (Fig.13A, saline-DBS vs. Poly(I:C)-DBS, 6428 ± 213.2 vs. 7461 ± 226.0 unpaired t test p = 0.0033; Poly(I:C)-sham vs. Poly(I:C)-DBS, 6404 ± 145.7 unpaired t test P = 0.0007), but there was no difference in the soma size of microglia (Fig.13B) among each of these groups. Taking into account that the microglia density was down-regulated in the Poly(I:C)-sham group, it could be a beneficial effect for schizophrenia treatment. Not coincidentally, we found the same effect of DBS on microglia in mPFC, the density of microglia in Poly(I:C)-DBS group significantly higher than the saline-DBS group (Fig.12C, saline-DBS vs. Poly(I:C)-DBS, 6360 ± 244.6 vs. 7466 ± 259.3 unpaired t test p = 0.0066; Poly(I:C)-sham vs. Poly(I:C)-DBS, 5724 ± 118.1 vs. 7466 ± 259.3 unpaired t test P = 0.0007), again, no difference can be seen in the value of soma size (Fig.13D).

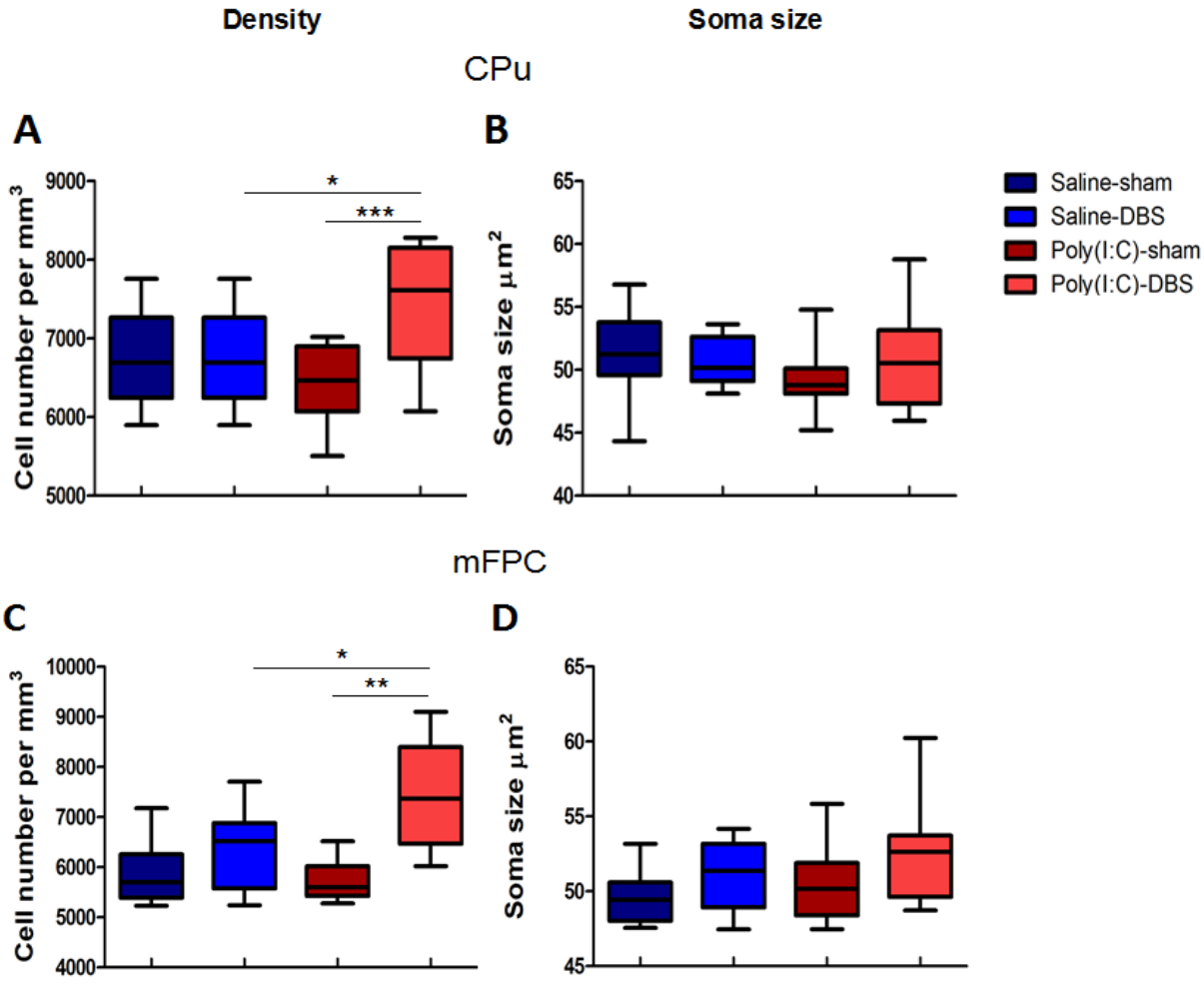


Figure 13. Nacc DBS treatment increases the density of microglia in the mPFC and CPu in the Poly(I:C) group.
DBS treatment in the Nacc caused an unexpected significant increase in microglial density in the CPu (A) and mPFC (C) in the Poly(I:C)-DBS group compare to other groups. No similar effect can be seen on the soma size of microglia in these two areas (B and D).

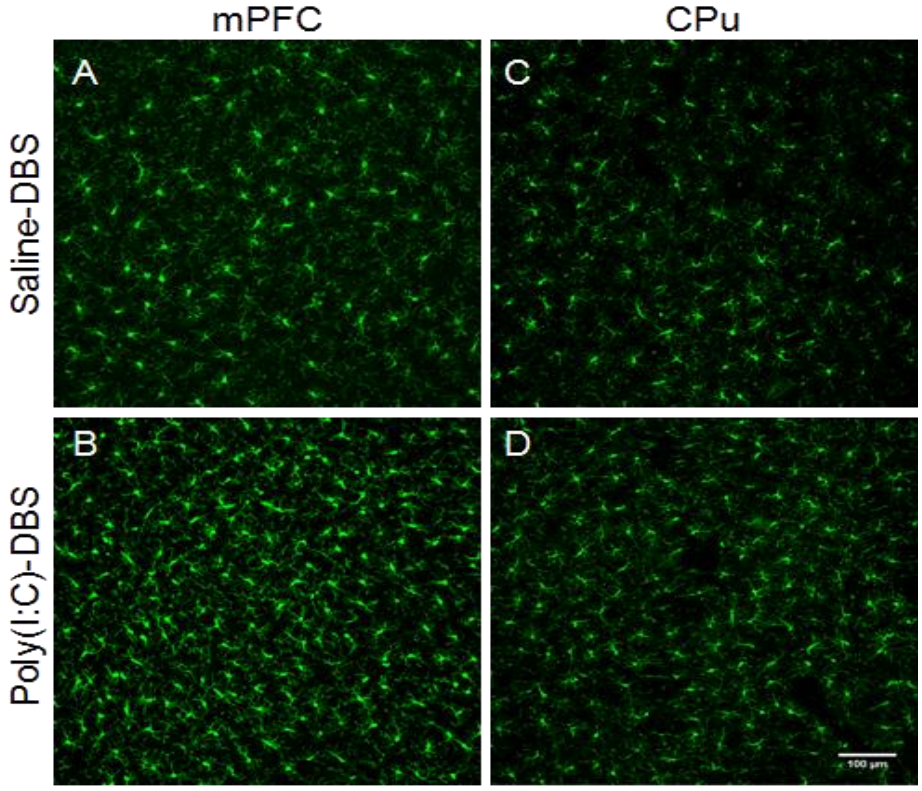


Figure 14. The density of microglia in the mPFC and CPu was increased after Nacc-DBS treatment in the Poly(I:C) group compared to saline group.
After Nacc-DBS treatment, the density of microglia in the mPFC (B) and CPu (D) were significantly increased in the Poly(I:C) group compared to the saline group (A and C).

4.2.5 The levels of cytokine expression in microglia in the hippocampus

Cytokine changes were always involved in the inflammatory response and neurodegeneration process in mental disorders. Since we observed a hyperactivity of microglia in the hippocampus in schizophrenic rats and an attenuation after mPFC-DBS or Nacc-DBS treatment separately, we decided to investigate various cytokine levels expressed by microglia in the hippocampus with and without mPFC-DBS treatment. CD11b+ and CD45+ cells were isolated through FACS, and cytokine production (TNF-α, IL-6, IL-1β) was evaluated by qPCR. According to our findings, even though IL-1β and IL-6 levels showed a trend of increasing in the Poly(I:C)-sham group, there were no significant changes among the groups (Fig. 15).

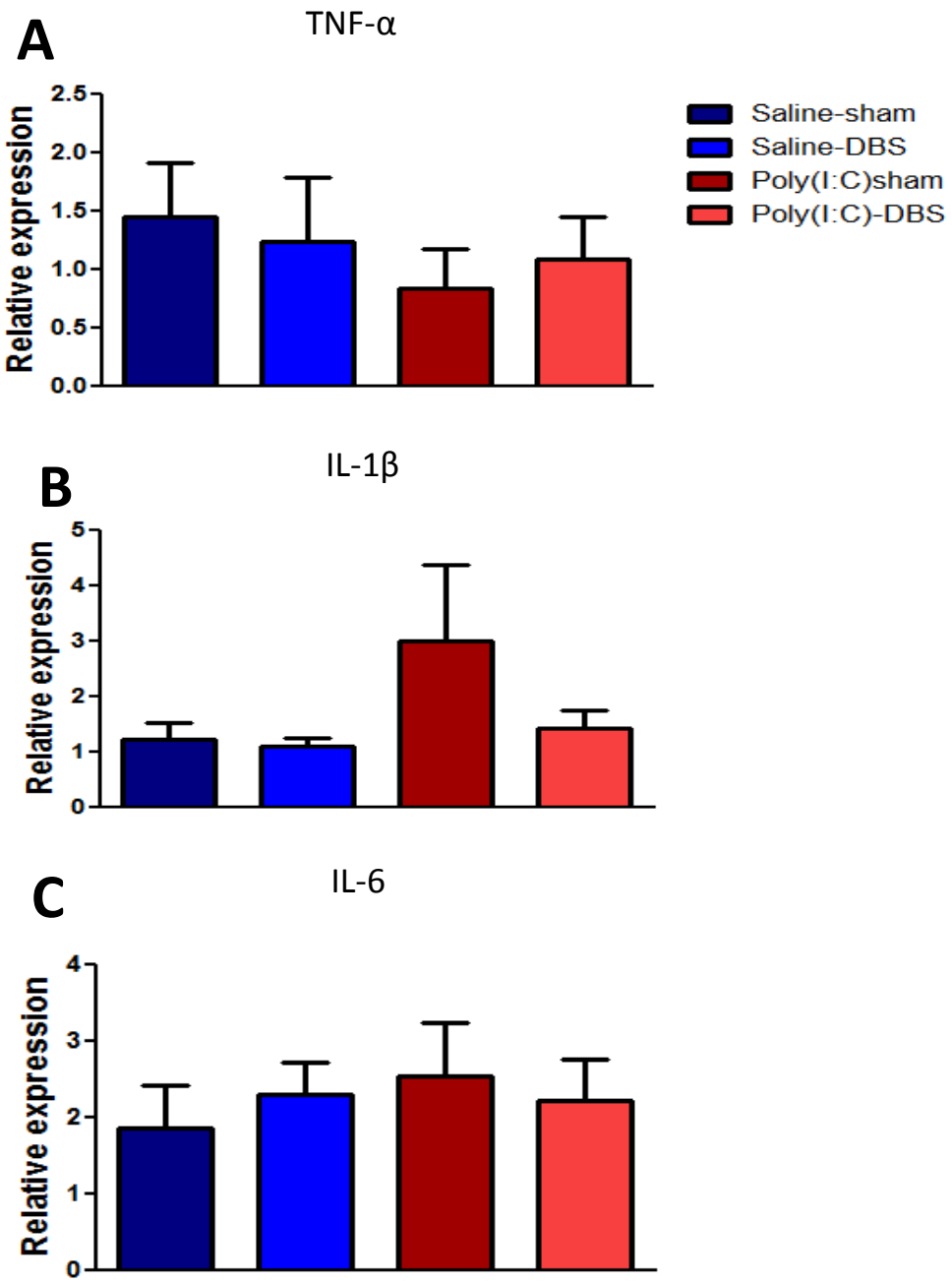


Figure 15. There was no significant change in the level of cytokine expression in microglia in the hippocampus with or without DBS treatment in the Poly(I:C) group or saline group.

The levels of TNF-α (A), IL-1β(B) and IL-6 (C) expression in microglia cells in the Poly(I:C) group showed no significant difference compared to the saline group in the hippocampus. DBS treatment in the mPFC did not influence the expression levels of microglial cytokines in this projection area.

4.3 The density changes of microglia in the depression model

Since inflammation may be involved in the pathology of depressive disorder. We investigated the density of microglia in two different brain regions (mPFC and Nacc)

in FSL rat and in control FRL rat. There was no significant difference in the density of microglia in the Nacc in FSL group compared to FRL group (Fig. 16B). However, in mPFC, which has been repeatedly reported that it is involved in neurotransmitter dysregulation in many depression-related studies, we found the density of microglia was significantly lower in FSL rats than FRL rats (Fig. 16A, FRL vs. FSL, 11590 ± 277.5 vs. 9554 ± 185.5, unpaired t test $p < 0.0001$). This finding implies that the small number of microglial cells or hypofunction of microglia may contribute to the development of depressive disorder.

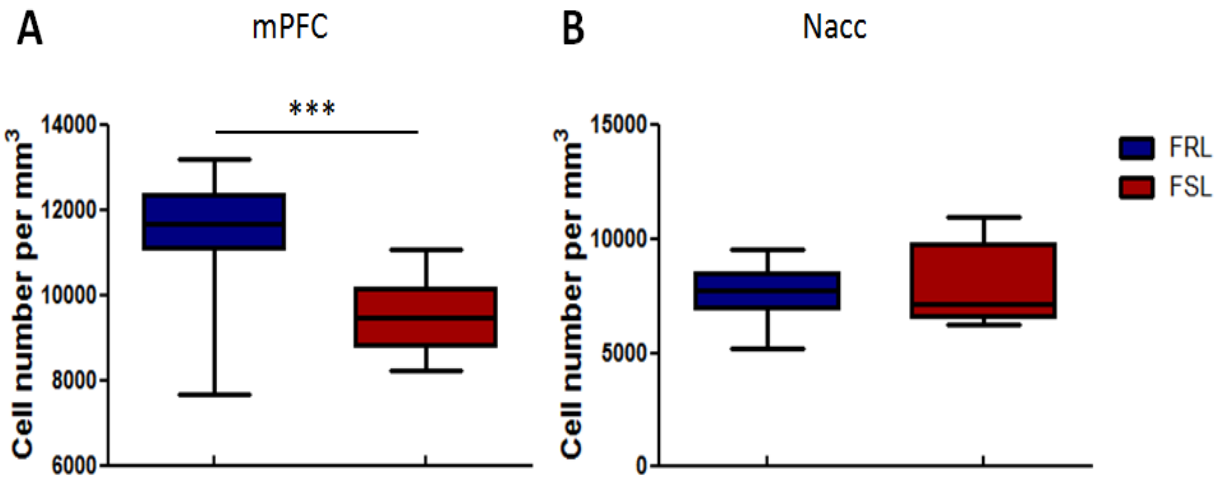


Figure 16. The density of microglia was significantly lower in FSL rats compared to FRL rats in the mPFC and there was no difference between two groups in the Nacc.

Comparing the density of microglia in FSL rats to FRL rats, we observed a significant difference between the two groups in the mPFC (A). No difference in microglia density can be seen in Nacc (B).

4.4 Effects of DBS on microglia in a depression model

4.4.1 Electrode implantation and DBS current did not significantly change the density of microglia in the targeted brain region

DBS was performed in the mPFC in both FSL rats and FRL rats to investigate the effects of DBS on microglia in the rat model of depressive disorder. Two-way ANOVA analysis was used to evaluate the change in microglial density among the non-implantation group, implantation group and implantation + DBS group in two animal lines. Electrode implantation slightly increased the density of microglia in FSL group, but did not reach a significant level (Fig. 17). Unfortunately, in contrast to our above finding in Poly(I:C)/saline-injected rats, there was no significant difference in the

density of microglia after implantation surgery or DBS treatment in the mPFC in either FSL or FRL rats.

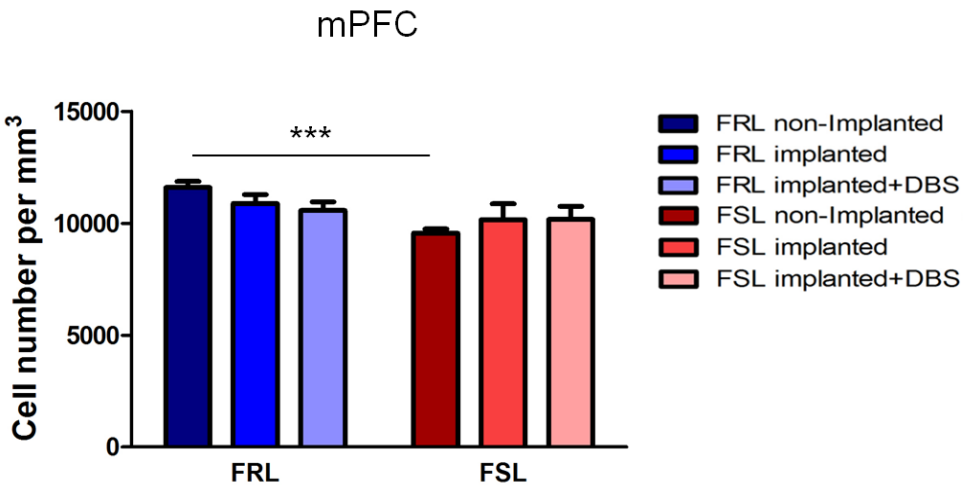


Figure 17. The effects of implantation per se and DBS treatment on the density of microglia in the targeted region (mPFC) in FRL rats and FSL rats.

Electrode implantation per se did not increase the density of microglia in the targeted region compared to the non-implantation group in either FSL or FRL rats. DBS treatment in FSL and FRL rats showed no significant influence on the density of microglia in the targeted region either.

4.4.2 mPFC-DBS treatment did not change the density of microglia in projection area (Nacc) in FSL/FRL rats

DBS treatment was performed in the mPFC in both FSL and FRL rats to study the effect of electric current on microglia in the projection area. In our study, we did not find significant changes in microglia in the Nacc due to the treatment. The density of microglia in FSL and FRL rats remained at the same level after DBS compared to the sham group (Fig. 18)

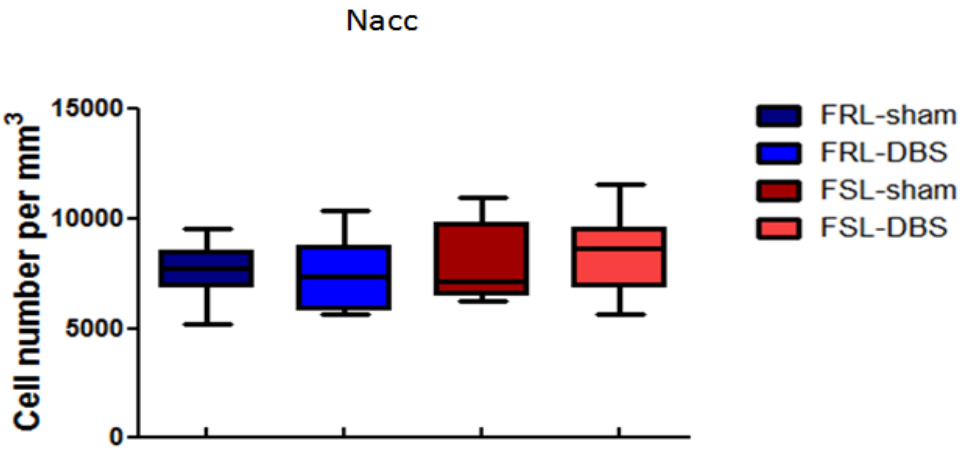


Figure 18. mPFC-DBS treatment did not change the density of microglia in the projection area (Nacc) in FSL/FRL rats.

There was no difference in microglial density in the Nacc after DBS treatment in the mPFC. The density of microglia in FSL and FRL rats remain at the same level after DBS compared to the sham group.

5 Discussion

5.1 Microglial dysregulation in a schizophrenia model

According to the inflammation hypothesis, abnormally activated microglia may be involved in the pathology of schizophrenia. The development of animal models provided an excellent avenue to determine the causality of inflammation and mental disorders. Maternal administration of Polyriboinosinic-Polyribocytidilic Acid (Poly(I:C)) is one of the most popular and widely used methods to establish a prenatal immune challenge model of schizophrenia (Meyer and Feldon 2012; Meyer 2014). In our study, we used this rat model to study the role of microglia in schizophrenia disorder. By using immunofluorescence staining through the labeling of microglia with Iba1, which is restricted to microglial cells, we showed a significant increase in both microglia density and soma size, which represents an upregulation of microglia activity in the hippocampus and Nacc in the schizophrenia group compared to the control group. The density and soma size are the two characteristic features of microglial activation. Kozlowski et al. (Kozlowski and Weimer 2012) provided strong evidence that microglia activation can be quantified *in situ* through morphological analysis, specifically, they also showed that the increase in the soma size was the only parameter that shows an dose-dependent effect of LPS among other morphological changes. Recent research suggested that a maternal infection could

leave microglia permanently in an activated state, and a subsequent immune challenge in later life causes an abnormal response from the primed microglial cells, giving rise to an unbalance between pro- and anti-neurogenetic molecules, followed by neuronal apoptosis and brain damage (Monji et al. 2013). This view is consistent with microglial activation which could be a result of a degenerative process, for instance, cleaning up cellular debris. Alternatively, dysregulated activation of microglial processes may cause neuronal damage and degeneration, like the long discussed role microglial activation has on the pathophysiology of neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease, and dementia related to the human immunodeficiency virus. Moreover, in an open label clinical trial, Miyaoka and colleagues have found a significant improvement in both positive as well as negative symptoms after minocycline, a broad-spectrum tetracycline antibiotic that inhibits the activation of microglia, was added to an antipsychotic in subjects with schizophrenia (Miyaoka et al. 2008). Results from our previous study also confirmed this beneficial effect of minocycline in the Poly(I:C) model of schizophrenia (Mattei et al. 2014).

Outcomes of both human patients and animal models of schizophrenia have shown increased microglial density and activity in the hippocampus. Several clinical studies have found evidence of microglia activation in the hippocampus, temporal and frontal cortex of patients with schizophrenia (J. Doorduin et al. 2009; Radewicz et al. 2000). An increased number of microglial cells with reduced arborization, which is a sign of activation, were also found in the hippocampus of offspring following maternal Poly(I:C) exposure (Juckel et al. 2011). The malfunction of the nucleus accumbens (NAc or Nacc), which is correlated with the regulation of cognitive processing of pleasure, aversion, reward and reinforcement learning, was reported to contribute to the pathological progress of schizophrenia (Goda et al. 2015). Increased density of microglia has been found in both previous (Mattei et al. 2014) and current studies. Unfortunately, to the best of our knowledge, very little is known about the influence of activated microglia in the Nacc in schizophrenia. One supporting piece of evidence from our collaboration group (Winter et al. 2009) demonstrated a significantly reduced 5-HT level in the Nacc in Poly(I:C) model of schizophrenia. Microglial cells appear to function as crucial regulators of serotonin production through pro-inflammatory cytokines in the brain. Hyperfunction of microglia can upregulate the

function of indoleamine 2,3-dioxygenase (IDO), which leads to a decrease of 5-HT level through the Kynurenine pathway (Watkins, Sawa, and Pomper 2014). No sign of microglia activation has been found in the mPFC and CPu in the Poly(I:C) group compared to control group, which is consistent with our previous findings (Mattei et al. 2014). This observation is supported by another group as well. By using the same animal model of schizophrenia, Garay et al (Garay et al. 2013) reported the density of microglia in the mPFC, ventral striatum and cingulated cortex in schizophrenic rats were not different from those of healthy rats.

Brain regions	Maternal Poly (I:C) injected rats					
	Untreated		mPFC-DBS		Nacc-DBS	
	Density	Soma size	Density	Soma size	Density	Soma size
Hipp	+	+	-	-	-	-
Nacc	+	+	-	-	-	-
CPu	-	NC	NC	NC	+	NC
mPFC	NC	NC	-	-	+	NC

Table 9. Changes of microglia in maternal Poly(I:C) injected rats and the effects of DBS treatment on different targets on microglia. "+", increase; "-", decrease; NC, not changed.

5.2 Cytokine changes in the schizophrenia model

Microglia and macrophages share the same marker CD11b. Using flow cytometry, we isolated CD11b+ cells from the hippocampus and analyzed the mRNA levels of cytokines (IL-1β, IL-6, TNF-α). We found no significant change in these cytokines in schizophrenic rats compared to the baseline. However, the resulting outcome suggests, albeit inconsistently, that the IL-1β, IL-6, and TNF-α cytokine networks are activated in schizophrenia. Alterations in the levels of inflammatory cytokines in the peripheral blood, cerebrospinal fluid (CSF) and brain of schizophrenic patients have consistently been reported (Al-Amin, Nasir Uddin, and Mahmud Reza 2013). One of the most important roles of cytokines is that they are involved in neuroplasticity, which is crucial to the memory formation and to the stability of neuronal networks

through keeping nerve cell excitation and inhibition in balance (Boulanger 2009). When the abnormal secretion of cytokines appears, it could, for instance, lead to an interruption in this normal neuroplastic function or an imbalance of this regulation as well as neurogenesis. Considering the characteristics of the maternal Poly(I:C) injection model, which only show schizophrenic-like behavior in adult offspring (Vuillermot et al. 2010), such inconsistency between cell density and cytokine productions could be explained by the variations in age. The animals sacrificed for cytokine testing (PND 90) were much younger than those sacrificed for cell counting (PND 120). Furthermore, our previous study confirmed that the production of TNF-α and IL-1β derived from microglia, in the PND 128, was enhanced in the Poly(I:C) offspring in microglia sorted from the hippocampus (Mattei et al. 2014). Also these rats were older than the ones we used for cytokine measures. There is a possibility that age might have an effect. It is also worth noting that microglia are not the only source of inflammatory cytokines，peripheral cytokines crossing blood-brain barrier (BBB); activation of astrocytes and dendritic cells entering brain tissue under CNS inflammatory conditions can contribute to the upregulation of cytokines as well (Geissmann et al. 2010).

5.3 Microglia dysregulation in a depression model

The view, that microglia play an important role under physiological conditions when immune challenge is absent, raises the possibility that the disturbance of this balance can lead to pathological conditions. In our present study, we showed that the density of microglia in the mPFC was deregulated in FSL rats compared to FRL rats (Table 10). This finding implies that the small number of microglial cells or hypofunction of microglia may contribute to the development of depressive disorder. A series of recent studies has shown that microglia could be an important regulator of persistent synaptic plasticity, as well as learning and memory. Parkhurst et al. (Parkhurst et al. 2013) reported that microglia-depleted mice appeared significantly different in a novel object recognition test, contextual fear conditioning test and rotarod training test compared to control groups. Researchers observed a delay in synaptic pruning in the hippocampus as well as in the visual cortex by using mice lacking the chemokine receptor CX3CR1, which is only expressed by microglia (Tremblay, Lowery, and Majewska 2010). This delay will cause an excess of dendritic spines. Elimination of

the excessive synapses is known as “synaptic pruning”. Unlike the function of microglia during CNS disorders, it is a common physiological cell refining process. The persistent plasticity of synapses has two main functions, one is to maintain existing normal synaptic function and the other one is to change synaptic function actively according to the inputs received from neurons, called activity-triggered synaptic plasticity. According to whether enhancement or inhibition of synaptic efficacy is needed, activity-triggered persistent synaptic plasticity is divided into long-term potentiation (LTP) and long-term depression (LTD), which is commonly considered closely related to learning and memory in the adult CNS. Malfunction of LTP processes could be involved in the onset and development of depressive disorders (Racagni and Popoli 2008). CNS-TGFb-1-deficient mice, in which the number of microglia will largely decrease after E14.5 without neuronal loss, an enhancement of LTD and a reduction of LTP was observed in the hippocampus. This dramatic change of activity-triggered persistent synaptic plasticity finally led to severe motor dysfunction in adulthood and premature death (Butovsky et al. 2014). It has been proved by a recent study that LTP in the mPFC was markedly impaired in a rat model of depression (Burgdorf et al. 2015).

Furthermore, the microglial KARAP/DAP12 (DAP12KI)-deficient mouse model (Roumier et al. 2004) has been shown to have a decreased expression of AMPA receptors and increased synaptic GluN2B NMDA receptor function, which is due to synaptic immaturity. These changes are crucial for the progress of depressive disorder. On the one hand, AMPA receptors are composed of four subunits (GluA1-4), and they are the primary mediators of excitatory synaptic transmission. Reduced GluA1 levels in the prefrontal cortex, hippocampus and amygdale have been found in quite a few rodent stress models of depression (Chandran et al. 2013). Pharmacological studies proved that AMPA potentiators have antidepressant properties and, on the contrary, antidepressants can increase GluA1 and GluA2 expression in the Nacc and PFC (Li et al. 2010). On the other hand, Early findings show that the non-competitive NMDA antagonist (Dizolcipine [MK-801]) and competitive NMDA antagonist (2-amino-7-phosphonoheptanoic acid [AP-7]) can mimic the effects of clinically effective antidepressants in animal models of depression (Trullas and Skolnick 1990). Preclinical trials have also suggested that

chronic therapy with antidepressants induces a reduction both in the expression and function of the NMDAR.

These mechanisms have already been elaborated in many publications which are related to glutamine system dysfunction of depressed patients. All these indicate that deregulation of microglia, which insult synaptic pruning and maturation and result in deficits in both hippocampal-dependent learning and motor learning, can contribute to the development of depression in later life.

Brain region	The density of microglia			
	Untreated		mPFC-DBS	
	FSL	FRL	FSL	FRL
mPFC	-	NC	NC	NC
Nacc	NC	NC	NC	NC

Table 10. The density of microglia in FSL/FRL rats and the effects of mPFC-DBS on microglia in different brain regions. "-", decrease; NC not changed.

5.4 Treatment effects of DBS in schizophrenia and depression

Deep brain stimulation (DBS) is a novel therapy for CNS disorders. Through a surgically implanted electrode, it provides focal electrical neural-network regulation within a brain circuit or circuits of interest. Initially, DBS systems were aimed to address dysfunctional circuits in patients diagnosed with treatment-resistant movement disorders, like Parkinson or essential tremor. Recently, a considerable body of evidence from pre-clinical studies has demonstrated a bright side of DBS as a treatment for mental disorders (Kocabicak et al. 2015; Lujan, Chaturvedi, and McIntyre 2008). Previous studies from our collaboration group also proved that performing high frequency DBS in the mPFC and globus pallidus effectively improved PPI deficits in schizophrenic rats (Klein et al. 2013).

Due to our findings regarding the abnormal activation of microglia in naïve schizophrenia and depression rats, we administered high frequency DBS in the mPFC and Nacc in schizophrenic rats, and DBS in the mPFC in depressed rats, to

observe the effects of DBS on microglia in these models of psychiatric endophenotypes. As we expected, the activity of microglia was upregulated in the implantation group compared to non-implantation group. Similar effects of electrode implantation per se on microglia in targeted brain regions can be observed in both disease models. We propose that the changes of microglia could be caused by a local lesion due to the electrode insertion process. This physiological defense reaction has also been described in a recent study, which reported an obvious insult after intracranial electrode implantation, which is reflected by local glial activation and increased inflammatory mediator levels (Perez-Caballero et al. 2014). Furthermore, this study also proved that the inflammatory response in the PFC gave rise to a transient antidepressant-like effect in the animal model. Increased expression of P11, a key regulator of anti-depressant responses (Warner-Schmidt et al. 2011), and more strongly expressed close to the insertion site, could be responsible for this beneficial effect of the implantation per se in the targeted area. This finding can give a hint to the clinical therapy and a new insight into the pathology of depression.

5.4.1 Effects of DBS on microglia in targeted area

The outcomes of our study showed that chronic high frequency DBS significantly reduced microglial density and soma size in both targeted regions in the Poly(I:C) group and the saline group, while in FSL/FRL rats MG density and soma size was unchanged by DBS. Evidence related to the effects of DBS on microglial changes is very limited. To the best of our knowledge, we are the first who study the changes of microglia in animal models of psychiatric disorders after DBS treatment. However, rather than directly acting on microglia and reducing the density, we assumed that high frequency DBS can inhibit the increase of microglia activity at the very beginning. Mechanical injury of brain tissue through implantation surgery or the pathological process of the CNS disease, whatever the triggering factor, will lead to a migration and proliferation of microglia caused by neuroinflammatory reaction. It has been reported Iba1+ and CD45+ cells are reduced in the damaged cortex hours after transcranial direct current stimulation of the ischemic area in a mouse model of stroke (Peruzzotti-Jametti et al. 2013). Moreover, according to the lesioning hypothesis, DBS at high frequencies can ameliorate pathological hyperactivity at the targeted brain sites. It has been well known that high frequency DBS produces an

overall net inhibition of the stimulated target, and the depolarization block was considered to be the major mechanism of this action (Benazzouz and Hallett 2000).
Other potential mechanisms, such as a reduction in the abnormally increased neuronal firings and firing patterns through blockade of voltage-gated currents as well as GABAergic inhibition, were also demonstrated by recent studies (Chiken and Nambu 2013). So far, direct inhibition of current and suppressed neuronal function and the disruption of information flow between cells caused by DBS in local brain region could give rise to a modulation of the inflammatory reaction, and prevent microglia activation or migration. To some extent, this effect can be understood as a neuroprotective function of DBS within the targeted region, which reduced secondary injury sustained by activated microglia (Doyle, Simon, and Stenzel-Poore 2008).

5.4.2 Effects of DBS on microglia in the projection area

More than just the effect on neuronal function of one specific brain region, DBS has the capacity to deliver its effects to the downstream and upstream areas of stimulated targets within certain neural circuits. Our study has demonstrated the beneficial effects on the changes of microglia after chronic DBS treatment in the rat model of schizophrenia. Both mPFC-DBS and Nacc-DBS significantly attenuated increased microglia density and soma size in the hippocampus, while no significant change in microglia cytokine production can be observed after mPFC-DBS. Combining our findings gained from untreated schizophrenic rats, we confirmed that DBS can influence the projection area and reduce the hyperactivity of microglia, which supports the view that DBS could potentially ameliorate the symptoms of psychiatric patients. An in vitro study demonstrated that the viability of LPS-activated microglia was significantly decreased after electric current stimulation without cytokine production changes (Pelletier et al. 2015). Interestingly, as well as the inhibition of microglial soma size and density, Nacc-DBS also increased the density of microglia in both mPFC and CPu in the Poly(I:C) group.
Compared to the effects of DBS in the local brain region, the underlying mechanisms of how DBS affects projection areas seems much more complex than simple inhibition or excitement. First, according to the pattern of neurotransmitters (glutamatergic and GABAergic neurotransmitters) delivered in basal ganglia-thalamo-cortical-circuitry, DBS treatment in different targeted areas within this circuitry could

cause different outcomes of microglia activity in downstream and upstream regions. For instance, glutamine is closely linked to the activation of microglia (Kaushal and Schlichter 2008). As mentioned above, mPFC-DBS, which produces an overall net inhibition of the stimulated target, inhibits glutamatergic neurotransmission to the Nacc and hippocampus and down-regulates microglial activity. Similarly, blocked signaling transmission due to DBS in Nacc may increase the glutamine upstream (mPFC) through negative feedback and decrease glutamine downstream (hippocampus), which leads to different outcomes of microglia activity in different projection areas. Second, from an anatomical perspective, the mPFC and CPu are close to the Nacc. Electric power of DBS diminishes with increasing distance from the stimulation point. Low intensity current may not be enough to inhibit neural function, on the contrary, it can mimic a mild lesion and cause inflammatory response. It has been proved that an electric field can polarize and accelerate the migration of different types of immune cells (Pelletier et al. 2015). However, concrete evidence related to the long-term outcomes of DBS in animal models of mental disorders is still very scarce. Whether this response results from electric per se or complex cell interactions, or both, will require further investigation.

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Affidavit

“I, Le Dong, certify under penalty of perjury by my own signature that I have submitted the thesis on the topic “Effects of deep brain stimulation (DBS) on microglia phenotype in rodent model of psychiatric disorder”. I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My interest in any publications to this dissertation corresponds to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

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