UNIVERSITY OF CALIFORNIA SAN DIEGO

Increased Secretion of IL-6 in Bipolar Patient iPSC-derived Astrocytes Decreases Neuronal Synaptic Activity

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

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The Thesis of Vipula Racha is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California San Diego

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DEDICATION

For my parents

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LIST OF ABBREVIATIONS

BD	Bipolar Disorder
IL-6	Interleukin-6
IL-1b	Interleukin-1 beta
iPSC	Induced Pluripotent Stem Cell
GPC	Glial Precursor Cell
MEA	Multiple Electrode Array
NPC	Neural Progenitor Cell

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ABSTRACT OF THE THESIS

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Bipolar disorder (BD) is a neuropsychiatric disease characterized by intermittent episodes of mania and depression that detrimentally affects a person's ability to carry out day-to-day tasks. Life expectancy is reduced by 10 to 15 years along with an increased suicide rate and comorbidities such as cardiovascular disease and diabetes. Studies have shown that synaptic plasticity and inflammation play a major role in the pathophysiology of bipolar disorder, but how they contribute is still unknown. Astrocytes, an important glial cell type in the brain, are significant in both synaptic plasticity and inflammation but are currently understudied in bipolar disorder. Therefore, we wanted to know the effect that astrocytes derived from induced pluripotent stem cells (iPSCs) from bipolar patients have on the

synaptic activity of control neurons measured via multiple electrode array during inflammatory conditions. BD astrocytes showed increased secretion of IL-6, an inflammatory cytokine, when activated with IL-1b, a pro-inflammatory cytokine. Both co-culture with BD astrocytes and addition of conditioned media from BD astrocytes decreased action potentials in control neurons, which was rescued by adding an IL-6 blocking antibody. Our results suggest that increased IL-6 secretion in response to inflammatory stimuli in BD astrocytes may lead to altered neuronal activity in BD patients that could contribute to the bipolar phenotype. These results may further elucidate the etiology of BD and help scientists understand the role of astrocytes and inflammatory proteins in BD.

Introduction

Bipolar disorder prevalence and current treatment options

Bipolar disorder is a complex mood disorder that affects 2.3 million people in America, and has a worldwide prevalence rate of 1-3% (Muneer, 2016). Patients who suffer from this disease have cyclic mood states ranging from high energy, manic states to low energy, depressive states (Rybakowski, 2014). During manic episodes, patients can battle with insomnia, lack of impulse control, excessive spending, and other symptoms related to excess energy. In contrast, during depressive episodes, which typically last longer, patients are prone to symptoms such as excessive sleeping, depression, lack of appetite, and anhedonia (Rybakowski, 2014).

Bipolar disorder reduces the quality of life of those affected by it and their families. About 25% of patients with bipolar disorder attempt suicide over the course of the disorder (Hilty et al., 2006). Bipolar disorder has a high comorbidity with other psychiatric disorders, such as substance abuse, attention-deficit hyperactivity disorder, and anxiety disorders (Hilty et al., 2006). Currently, the medicine preferentially used to treat bipolar disorder patients is the mood-stabilizing drug lithium. While lithium is the first drug of choice, it is not effective for 33% of patients (Rybakowski, 2014), has associated side effects, and long-term lithium treatment can interfere with kidney function or lead to permanent kidney damage (Gitlin, 2016). Other therapeutic drugs, such as valproate and antipsychotic medication, are also used to treat bipolar disorder, particularly the manic state, if lithium does not work. But, the side effects of these medicines can further reduce the patient's quality of life (Hilty et al., 2006). Therefore, studying the underlying cellular dysfunction in bipolar disorder could help developing more effective treatment plans for patients.

Heritability in bipolar disorder

Bipolar disorder has been shown to have an 80-85% heritability (Muneer, 2016; McGuffin et al., 2003), suggesting a strong genetic component. The most recent GWAS studies of bipolar disorder found 30 significant genetic loci, 20 of which were new (Gordovez and McMahon, 2020). Studies into the genetics of bipolar disorder consistently highlight three genes: *ANK3*, *CACNA1C*, and *TRANK1*. *DCLK3* has also been associated with bipolar disorder, along with schizophrenia. Bipolar disorder also seems to share common weak risk factors with schizophrenia and major depression (Gordovez and McMahon, 2020). These various risk factors may become additive to present with BD symptoms, which could be better explored using polygenic risk scores (Gordovez and McMahon, 2020). Therefore, even though BD has a high heritability, a biological cause of bipolar disorder has not yet been found, likely due to environmental influences and the polygenic nature of the disorder (Hilty et al., 2006).

Bipolar disorder and inflammation

One underexplored biological factor in bipolar disorder is imbalanced inflammatory signaling, which is associated with bipolar disorder in several ways. (Hamdani et al., 2012; Goldstein et al., 2009; Vonk et al., 2007; Weiner et al., 2011; Cassidy et al., 1999). Bipolar disorder patients have been shown to have increased levels of inflammatory markers and higher concentrations of pro-inflammatory cytokines such as IL-6 and TNFa in their blood (Hamdani et al., 2012; Goldstein et al., 2009). They have been reported to develop organ-specific auto-immunity (Vonk et al., 2007) and to have chronic and mild inflammation (Goldstein et al., 2009). Bipolar disorder also has a high comorbidity with diseases with a

significant inflammatory component, such as cardiovascular disease and diabetes mellitus (Weiner et al., 2011; Cassidy et al., 1999). Studies show that people with bipolar disorder have almost double the risk of the general population of cardiovascular mortality (Weiner et al., 2011). They also have an increased risk of diabetes mellitus, having a prevalence of almost three times the general population (Cassidy et al., 1999).

Inflammatory signaling is a major communication system for the immune system and is mediated by immunocompetent cells, meaning cells that can generate an immune cascade in response to an antigen or foreign particle. These cells, such as T-cells and B-cells, start an immune response by secreting specific cytokines and chemokines, such as IL-1b or TNFa, to signal, or activate, surrounding cells to begin certain inflammatory processes. When this system is imbalanced, e.g. a cell cannot stop secreting IL-1b, it may lead to significant downstream effects, such as increased cell death or autoimmunity (Wagoner, et al., 1999).

The immune response in the central nervous system is known as neuro-inflammation. IL-1b, a pro-inflammatory cytokine, is a primary driver of neuro-inflammation (Sani et al., 2015). As a neuropsychiatric disease, bipolar disorder is associated with the central nervous system, especially the brain. *In vivo* studies have shown increased activation of immune cells in the hippocampi of bipolar patients, which then was associated with increased neuronal damage (Benedetti et al., 2020). Monocytes, a type of immune cell, in the brain showed mRNA overexpression of inflammatory genes in bipolar patients (Benedetti et al., 2020). Also, some treatments of bipolar disorder, such as valproate and anti-psychotic drugs, have immunoregulatory effects, such as reducing cytokine production or regulating microglial activation (Sani et al., 2015). These data suggest that bipolar disorder may have an important neuro-inflammatory component, which has not been fully explored.

Astrocytes' role in neuro-inflammation

Astrocytes are key regulators of neuro-inflammation (Wagoner et al., 1999). These star-shaped glial cells are the most abundant cell type in the human brain, and our understanding of their importance to normal brain function is increasing over time (Allen, 2014). Astrocytes play a primary role in neurogenesis, neuronal survival, and neuroplasticity by generating a multitude of neurotrophic factors (Allen, 2014). They are essential for neuronal development, maturation and signaling processes (Allen, 2014). Astrocytes are also immunocompetent as they are regulators of inflammation. When activated by pro-inflammatory cytokines, such as IL-1b or TNFa, they produce and secrete chemokines and inflammatory cytokines such as IL-6 and IL-8 that can influence neuronal processes, such as neuronal signaling or survival (Wagoner et al., 1999; Campbell et al., 1993).

Astrocytes triggered by inflammatory markers can have either neuroprotective or neurodegenerative pathways triggered. The behavior of astrocytes may depend on the individual inflammatory stimuli and time and duration the astrocyte is activated (Colombo and Farina, 2016). Additionally, astrocytes are heterogeneous cells, so the astrocytic type may also determine whether it has a neuroprotective or neurodegenerative effect. *In vivo*, astrocytes are exposed to a variety of inflammatory stimuli that activate distinct pathways, which may not be reflected *in vitro* (Colombo and Farina, 2016). A specific subtype of reactive astrocytes, known as A1 astrocytes, promote neurotoxicity when induced by microglia (Liddelow et al., 2017). Another subtype of reactive astrocytes, known as A2 astrocytes, promote neuronal survival (Li et al., 2019). These studies show that astrocytes can have a dual effect on neurons during inflammation, and astrocytes' role in inflammation needs to be more explored, especially the contributions of different astrocyte subtypes.

Inflammation and neuronal signaling

Studies have shown that imbalanced inflammatory signaling in the brain and particularly in astrocytes can lead to dysfunctional neuronal signaling. In transgenic mice, overexpression of IL-6, released by astrocytes in response to neuro-inflammatory signals, led to neurologic disease, seizure, neurodegeneration, and an increase of astrocytes as a result of brain trauma (Campbell et al., 1993). IL-6 overexpression was detected in tissue samples from the cerebellum of autistic patients, another neuropsychiatric disease (Wei et al., 2011). When IL-6 was overexpressed in primary mouse cerebellar granule cells, the cells had impaired cellular adhesion and migration and increased formation of excitatory synapses, not inhibitory (Wei et al., 2011). Santos and colleagues plated IL-1b-activated or non-activated primary and hiPSC-derived astrocytes with hiPSC-derived neurons. The neurons co-cultured with activated astrocytes were significantly less active than neurons with non-activated astrocytes (Santos et al., 2017). Inflammatory factors can also support or increase neuronal activity, though it depends on dose, environment, and length of exposure (Barkho et al., 2009; Fujishita et al., 2009; Nelson et al., 2012; Sun et al., 2017). These data show that imbalanced inflammatory signaling could cause altered neuronal signaling, which has also been associated with bipolar disorder.

Dysfunctional neuronal signaling in mood disorders

Altered neuronal signaling has been implicated as a possible phenotype in mood disorders, including bipolar disorder and major depressive disorder (Stern et al., 2018; Stern et al., 2019; Vadodaria et al., 2019b). A meta-analysis of fMRI studies on bipolar disorder showed that there was increased activity in limbic brain regions, such as the hippocampus,

and decreased frontal lobe activity in bipolar patients compared to neurotypical individuals (Chen et al., 2011). Hyperexcitability or increased neuronal firing from bipolar hippocampal neurons was seen in patch clamp recordings when compared to control neurons (Mertens et al., 2015; Stern et al., 2018). These neurons were derived from the fibroblasts (skin cells) or lymphoblasts (blood cells) of human patients with bipolar disorder via induced pluripotent stem cells (iPSCs). However, the direct role of astrocytes has not been evaluated in these studies.

Astrocytes' role in synapse regulation

Astrocytes have a key role in regulating synapses and, during brain development, can connect multiple synapses of different neurons by attaching to the axons of multiple neurons via astrocytic projections (Jones et al., 2012). Through a similar mechanism, they are able to monitor and alter neuronal synapses in the adult brain (Chung et al., 2015). Immature astrocytes secrete thrombospondins (TSPs) which promote synaptogenesis in the CNS, specifically TSP1 and TSP2 (Christopherson et al., 2005). Immune-reactive astrocytes also secrete these proteins, which could result in unwanted synaptogenesis after injury and inflammation (Christopherson et al., 2005). Astrocytes are significant in neurotransmitter reuptake, especially glutamate, which allows them to control synaptic transmission. They express excitatory amino acid transporter 1 (EAAT1) which is required to reuptake glutamate, an excitatory neurotransmitter, from the synaptic cleft (Allen, 2014). This reuptake terminates synaptic transmission between neurons, which controls the strength of excitation signals the post-synaptic neuron gets and determines whether the post-synaptic neuron will fire an action potential.

Astrocytes are also involved in synaptic pruning (Allen, 2014). Synaptic pruning occurs during development when the overall number of synapses reduces to create more efficient pathways of communication. *In vitro* studies in mice show that addition of mouse astrocytes to primary mouse neurons in a co-culture system significantly increased the activity and survival of the neurons (Jones et al., 2012). However, many studies of bipolar disorder, especially those *in vitro*, focus solely on neurons and discount the importance of astrocytes. As astrocytes are key to developing a neural network leading to complex synaptic activity, the role of astrocytes in signaling, and especially signaling irregularities in neurological disorders, needs to be considered.

The synaptic activity or communication between neurons can be detected as electrical signals using multiple electrode array (MEA) technology. MEA allows *in vitro* mesoscale electrophysiology experiments, by recording a neuronal population and giving data about the rate of spontaneous synaptic events, such as action potentials (Obien et al., 2015). One of its advantages is that it enables measurement of the activity of multiple neurons at different sites in the culture simultaneously for long-term recordings (Obien et al., 2015). This technology allows the creation of a co-culture system of neurons and astrocytes, in which we can measure the effect of astrocytes on neuronal activity. This has previously been done using mouse neurons and astrocytes, where the neurons showed a significant increase in growth and number of synapses (Jones et al., 2012). As astrocytes are a potential connection between neuronal inflammation and synaptic activity, the effect of bipolar disorder on astrocytes must be explored, particularly the bipolar astrocytic response to inflammatory stimuli.

Human cellular models for mood disorders

In order to study astrocytes' response to inflammation in bipolar disorder, a human cellular model is desirable because human astrocytes are more complex than mouse astrocytes and have different responses to drugs (Santos et al., 2017). This increased complexity is likely due to enhanced calcium signaling, the method by which astrocytes communicate, and the increased number of astrocytes projections in human astrocytes, allowing a single astrocyte to connect to more neuronal axons than mice (Vasile et al., 2017). Human astrocytes are both larger and morphologically distinct from mouse astrocytes (Han et al., 2013). When human glial progenitor cells, which are precursor cells to astrocytes, were engrafted into a live mouse brain, the astrocytic calcium signaling and learning of the mouse were significantly increased, showing that astrocytes likely have functionally different roles in humans (Han et al., 2013). Human astrocytes also express different genes than mice astrocytes (Santos et al., 2017). There are other limitations with using a mouse model apart from the distinct nature of human astrocytes. It is challenging to generate a transgenic mouse model that accurately portrays the bipolar phenotype, including the cyclic manic and depressive states and given the polygenic nature of the disease (Marchetto et al., 2010).

Human induced pluripotent stem cells (hiPSC) derived cellular models are used to study a variety of disorders often unique to humans, including neurodegenerative (e.g., Alzheimer's disease, multiple sclerosis) and mood (e.g., bipolar disorder, depression) disorders (Marchetto et al., 2010). iPSCs are often generated by cellular reprogramming of skin biopsies from patients and controls, resulting in stem cell-like cells with the potential to become many different cell types of the body (Takahashi et al., 2007). By getting patient-derived cells via iPSC technology, scientists are able to obtain cell types, such as hippocampal

neurons or astrocytes, which would be difficult, if not impossible, to get primary cells from live patients. These cells will also reflect the genome of patients with the disease or disorder of interest (Marchetto et al., 2010). In bipolar disorder, where the genetic cause is unknown and there are many genes of interest, having cells that contain the patient genome is invaluable (Hilty et al., 2006).

Proposal

As astrocytes are essential for both the neuro-inflammatory response and neuronal synaptic activity which are both implicated in bipolar disorder, we explored the effect of bipolar astrocytes on the synaptic activity of healthy control neurons when the astrocytes were activated by the primary neuro-inflammatory driver, IL-1b. We investigated how bipolar astrocytes responded differently to inflammatory stimuli and found that they had increased secretion of IL-6, an inflammatory cytokine, compared to control astrocytes. We also showed that both patient-derived BD astrocytes and IL-1b-activated astrocytes reduced neuronal activity in a co-culture system and that IL-6 is involved in this pathway. From this study, we gained a greater understanding of the role of astrocytes in bipolar disorder and the understudied inflammatory component of bipolar disorder. This information may allow us to discover novel ways of treating and possibly diagnosing patients with bipolar disorder and other neurological disorders where inflammation plays a role.

Materials and Methods

Patient Selection and Reprogramming

This study was performed with 2 cohorts of Bipolar patients and controls that have been collected, and iPSC lines were derived and characterized and reported in previous studies (Mertens et al., 2015 and Stern et al., 2018). Both cohorts are detailed below.

The first cohort of patients with Bipolar disorder, type 1, were participants of a drug response clinical trial at the University of California, San Diego: Veteran's study (VA) and Pharmacogenomics of Bipolar Disorder Study (PGBD). All subjects provided written informed consent and all procedures were approved by local human subjects committees. Subjects were Caucasian males, and patient characteristics have been previously described (Mertens et al., 2015). 3 BD patients were responsive to lithium (Li) treatment, and 3 BD patients were non-responsive to Li treatment. Fibroblasts obtained from sterile skin biopsies were reprogrammed to pluripotent stem cells using the Cyto-Tune Sendai kit (Invitrogen) according to the manufacturer's instructions as previously described (Mertens et al., 2015).

The second cohort of patients with BD, type 1, were participants in genetic studies at Dalhousie University and their clinical characteristics were described previously (Stern et al., 2018). All subjects (4 healthy age-matched controls, 3 BD patients responsive to Li, and 3 BD patients non-responsive to Li) were Caucasian males and provided written informed consent. Epstein–Barr virus (EBV)-immortalized B-lymphocytes from the patients were reprogrammed to pluripotent stem cells using Yamanaka Episomal vector set according to the manufacturer's instructions as previously described (Stern et al., 2018).

The University of California, San Diego (UCSD), the Salk Institute Institutional Review Board and the Embryonic Stem Cell Research Oversight Committee (IRB protocol

#09-0003) approved all procedures. The clinical characteristics of the fibroblast and lymphocyte (lymphoblast) cohorts are described in Tables 1 and 2, respectively.

Generation of hiPSC-Derived Immuno-Responsive Astrocytes

iPSC lines were cultured on Matrigel-coated plates in mTeSR1 medium (STEMCELL Technologies) with daily media changes. Embryoid bodies were prepared from confluent iPSC plates. 1 mg/mL collagenase IV (Gibco) was used to mechanically dissociate the iPSCs for 15 minutes. These cells were then plated on low-adherence plates in mTeSR1 medium with 10 uM ROCK inhibitor (STEMCELL Technologies) and incubated overnight on a

Table 1: Clinical Characteristics of Subjects with Bipolar Disorder from Fibroblast Source

Cell line	Category	Age	Diagnosis	Sex	Ethnicity	Rapid Cycling	Family History	Psychosis	Time in Study (months)	Termination reason	Study
RS14474	R	65	BPI	M	Caucasian	No	No	No	24	Completed study	PGBD
SM11692	R	59	BPI, PTSD	M	Caucasian	No	Yes	No	23	Relapsed-mixed episode	VA
TC11822	R	57	BPI, PTSD, ADHD	M	Caucasian	No	Yes	No	22	Relapsed-depression	VA
RDK9691	NR	54	BPI, PTSD	M	Caucasian	Unknown	Yes	Unknown	3	Failed to respond	VA
WC14934	NR	69	BPI	M	Caucasian	Yes	No	No	4	Failed to respond	VA
149-51	NR	22	BPI, ADHD	M	Caucasian	No	Yes	Yes	5	Failed to respond	PGBD
149-59	C	n/a	n/a	M	Caucasian	n/a	n/a	n/a	n/a	n/a	n/a
149-60	C	n/a	n/a	M	Caucasian	n/a	n/a	n/a	n/a	n/a	n/a
149-61	C	n/a	n/a	M	Caucasian	n/a	n/a	n/a	n/a	n/a	n/a
149-62	C	n/a	n/a	M	Caucasian	n/a	n/a	n/a	n/a	n/a	n/a

shaker at 90 RPM. To undergo glial progenitor cell (GPC) differentiation, the embryoid bodies were cultured in suspension with Astrocyte Medium (AM, ScienCell) supplemented with 500 ng/mL Noggin (R&D Systems) and 10 ng/mL PDGF-AA (Peprotech) for 14 days and then, for 1 more week, supplemented with only PDGF-AA. The embryoid bodies were then dissociated using papain and plated on 10 µg/mL poly-L-ornithine (Sigma) and 5 µg/mL laminin (Invitrogen)-coated plates. They were cultured and expanded on the previously-mentioned coated plates in AM supplemented with 20 ng/mL fibroblast growth factor 2 (FGF2, Joint Protein Central) and 20 ng/mL epidermal growth factor (EGF, Humanzyme).

For differentiation from GPCs to astrocytes, DMEM/F12 Glutamax (Thermo Fisher Scientific) supplemented with N2 (Thermo Fisher Scientific) and B27 without retinoic acid

Table 2: Clinical Characteristics of Subjects with Bipolar Disorder from Lymphoblast Source

Cell ID	Category	Age	Age at onset	Diagnosis	Sex	Ethnicity	Episodes off Li*	Episodes on Li*	Years on Li at sampling	Response score**	SCC code
21868	R	50	31	BPI	M	Caucasian	3M 4D	0	5	9/10	SBP010
35794	R	41	34	BPI	M	Caucasian	5M 1D	0	3	10/10	SBP005
7762	R	34	15	BPI	M	Caucasian	3M 1D	0	4	9/10	SBP007
24339	NR	51	35	BPI	M	Caucasian	1M 1D	1M	2	3/10	SBP001
20026	NR	58	22	BPI	M	Caucasian	3M 5D	2D 1RC	6	1/10	SBP002
14501	NR	40	24	BPI	M	Caucasian	4M 3D	7M	7	0/10	SBP004
37014	C	62	n/a	n/a	M	Caucasian	n/a	n/a	n/a	n/a	SBP011
31616	C	25	n/a	n/a	M	Caucasian	n/a	n/a	n/a	n/a	SBP008
40064	C	51	n/a	n/a	M	Caucasian	n/a	n/a	n/a	n/a	SBP009
7158	C	53	n/a	n/a	M	Caucasian	n/a	n/a	n/a	n/a	SBP012

* M – mania; D – major depression; RC – period of rapid cycling
** (Manchia et al., 2013)

(Thermo Fisher Scientific) and 10% fetal bovine serum (FBS, Biowest) was added to low-confluency GPC plates, using a previously described method (Santos et. al, 2017). Media was changed thrice a week. After 2 weeks of differentiation, the cells were transferred to plastic plates (Corning). Once the cells were 4 weeks old, they were considered ready for the experiment. Cerebellar human fetal primary astrocytes from ScienCell were used as a positive control for human astrocyte markers and were cultured in AM ScienCell medium as described by the manufacturer.

Immunocytochemistry

Astrocytes were fixed on plastic slides using 4% paraformaldehyde solution for 15 minutes at 4 ^{o}C. 10% horse serum and 0.1% Triton X-100 in PBS was used to block antigens and permeabilize the cell membrane for 1 hour and room temperature. The cells were incubated overnight at 4 ^{o}C with primary antibodies in 10% horse serum. The secondary antibodies (1:250, Jackson Laboratories) were incubated for 1 hour at room temperature in 10% horse serum. The primary antibodies used, dilutions, and companies are detailed in Table

Table 3: List of Primary Antibodies Used

Primary Antibody	Species	Dilution	Company
Nestin	mouse	1:500	EMD Millipore
S100b	rabbit	1:1000	Dako
GFAP	mouse monoclonal	1:250	EMD Millipore
NFIA	rabbit	1:250	Novus Bio
CD44	rat	1:100	BD Pharmingen
ALDH1L1	mouse	1:100	EMD Millipore
A2B5	Mouse IgM	1:50	EMD Millipore
Vimentin	goat polyclonal	1:500	EMD Millipore

3. Fluorophore conjugated donkey secondary antibodies were used. DAPI was used to stain the cells for nuclei detection. Immunocytochemistry was performed as previously described (Vadodaria et al., 2019a).

Cytokine array blots

5-week astrocytes (3 control and 4 BD lines) were treated with 10ng/mL recombinant human IL-1b (R&D) or PBS (vehicle) in astrocyte differentiation medium (DMEM/F12 Glutamax supplemented with N2. B27, and 10% FBS) after a PBS wash. 5 hours after treatment, 1 mL of media was collected and flash-frozen in liquid nitrogen to use in the cytokine array blot. Cells were detached as described above and counted to determine that similar cell numbers were present across cell lines and treatment conditions. Cytokine levels were determined by Proteome Profiler Human Cytokine Array from R&D systems using the manufacturer's instructions. FIJI software was used to comparatively quantify the intensity of cytokines (Schindelin et al., 2012).

Astrocyte-Neuron Co-culture and Multiple Electrode Array

For co-culture experiments, control iPSC-derived neural progenitor cells (NPCs) were plated on a 96-well poly-L-ornithine (poly-O, Sigma, 100 μg/mL) and laminin (Invitrogen, 100 μg/mL) coated MEA plate (Axion Biosystems) to undergo neuronal differentiation. After 3 weeks of differentiation, 4-week old astrocytes were plated on top of the neurons in the MEA plate. Cells were fed thrice a week with co-culture media: DMEM/F-12 with Glutamax (Gibco), 2% B-27 without vitamin A (Invitrogen), 1% N-2 (Invitrogen), 0.2 μM Ascorbic Acid (Sigma), 20 ng/mL glial cell-derived neurotrophic factor (GDNF, PeproTech), 20 ng/mL

brain-derived neurotrophic factor (BDNF, PeproTech), and 500 μg/mL cyclic AMP (cAMP, TOCRIS), 1% FBS, 1% penicillin streptomycin (ScienCell).

Neuronal activity was measured on the Maestro MEA system, an AxIS software (Axion Biosystems), three times a week before media changes. Each plate was acclimatized to the MEA Maestro for 10 minutes then recorded for 10 minutes. A bandwidth with a filter for 200 Hz to 3 kHz cutoff frequencies was used. An adaptive threshold of 6 times the standard deviation of the estimated noise on each electrode was used for spike detection. Data analysis for these recordings was done using the Axion Biosystems Neural Metrics Tool. An active electrode was defined as an electrode that recorded at least 5 spikes/minute. Bursts were identified from each individual electrode using an adaptive Poisson surprise algorithm. Network bursts were defined as a minimum of 10 spikes/minutes within a maximum of 100 milliseconds when 25% of the electrodes in the well are classified as active. Only active wells were included in MEA analysis.

Conditioned media experiments

100,000 – 150,000 iGluta neurons (Fujifilm Cellular Dynamics, Inc.) were plated on each well of a 96-well MEA plate (Axion Biosystems) and cultured with iGluta media: 100 mL Brainphys Neuronal Media (STEMCELL Technologies), 2 ml of iCell Neural Supplement B (Fujifilm Cellular Dynamics, Inc.), 1 ml of iCell Nervous System Supplement (Fujifilm Cellular Dynamics, Inc.), 1ml N-2 (Invitrogen), 100µl of 1µg/ml Laminin (Invitrogen), and 1ml penicillin streptomycin (ScienCell) as instructed by the manufacturer. The plate was recorded every other day for 1 week before media changes to measure baseline activity.

In parallel, 4-week old astrocytes (4 BD lines and 2 Control lines) were activated with 10ng/mL IL-1b. After 5 hours of treatment, the cells were washed with PBS and their media was changed to iGluta media with 1% FBS added in order to make conditioned medium. 18 hours later, the astrocyte conditioned medium was removed from the astrocytes and added on top of the neurons cultured for 1 week on the MEA plate. Neuronal activity was measured before addition of conditioned media, immediately after, 3 hours after, 6 hours after, and 12 hours after conditioned media addition.

For IL-6 experiments, conditioned media was collected like above and divided into two treatment groups for each cell line, both activated and non-activated: 50ng/ml IL-6 blocking antibody (R&D Systems) or no addition of factors. 4 BD lines and 2 control lines were used. The treated conditioned media was added on top of iGluta neurons plated on an MEA plate, like above. Neuronal activity was measured before addition of conditioned media, immediately after, 3 hours after, 6 hours after, and 12 hours after conditioned media. After recording, the medium was changed back to non-conditioned media for 4 days to return neurons back to baseline. Then, the neurons were washed with PBS, and aCM from each astrocyte line was added back to the respective well. The MEA plate was immediately recorded, then 20 ng/mL IL-6 (R&D Systems) added to each well and the plate was recorded immediately again.

Statistical Analysis

All results are expressed as mean ± SEM. Unpaired two-tailed t-tests were used to determine statistical significance, with $p < 0.05$ signaling significance.

Results

Generation and validation of iPSC-derived astrocytes

For our experiments, we wanted to use human astrocytes from individuals with bipolar disorder to compare with human astrocytes from healthy individuals that both respond to inflammatory stimuli. Therefore, we generated immune-responsive astrocytes from hiPSCs derived from control and BD individuals using a glial precursor cell (GPC) intermediate, following a previously described method from our lab (Santos et al., 2017). By using a GPC intermediate, we were able to expand, freeze, thaw, and store these proliferating cells. These GPCs could then be efficiently differentiated into astrocytes. This protocol began by generating embryoid bodies (EBs) from confluent iPSCs. These EBs were cultured in the presence of Noggin and PDGF-AA, in order to differentiate into GPCs (Figure 1A). After a period of 3 weeks, the EBs were disassociated and were plated and expanded as GPCs in culture medium with epithelial growth factor (EGF) and fibroblast growth factor 2 (FGF2). Once sufficiently expanded, the medium was switched to astrocyte medium for astrocyte differentiation. We observed morphological changes when cells differentiated from GPCs to mature astrocytes; cells became larger and flatter (Figure 1A). Astrocytes were considered mature when cultured for approximately 4 weeks in astrocyte medium.

To confirm that we had successfully generated iPSC-derived astrocytes from both control and BD lines, we used quantification of immunostaining to confirm cell type. The glial identity of the GPCs was confirmed by expression of cell surface ganglioside antigen A2B5 and nuclear factor-I A (NFIA), a glial transcription factor. For both control and BD lines, over 80% of the GPCs co-expressed both glial markers, showing that the cells were GPCs (Figure 1B). To validate that we successfully differentiated astrocytes from GPCs, we

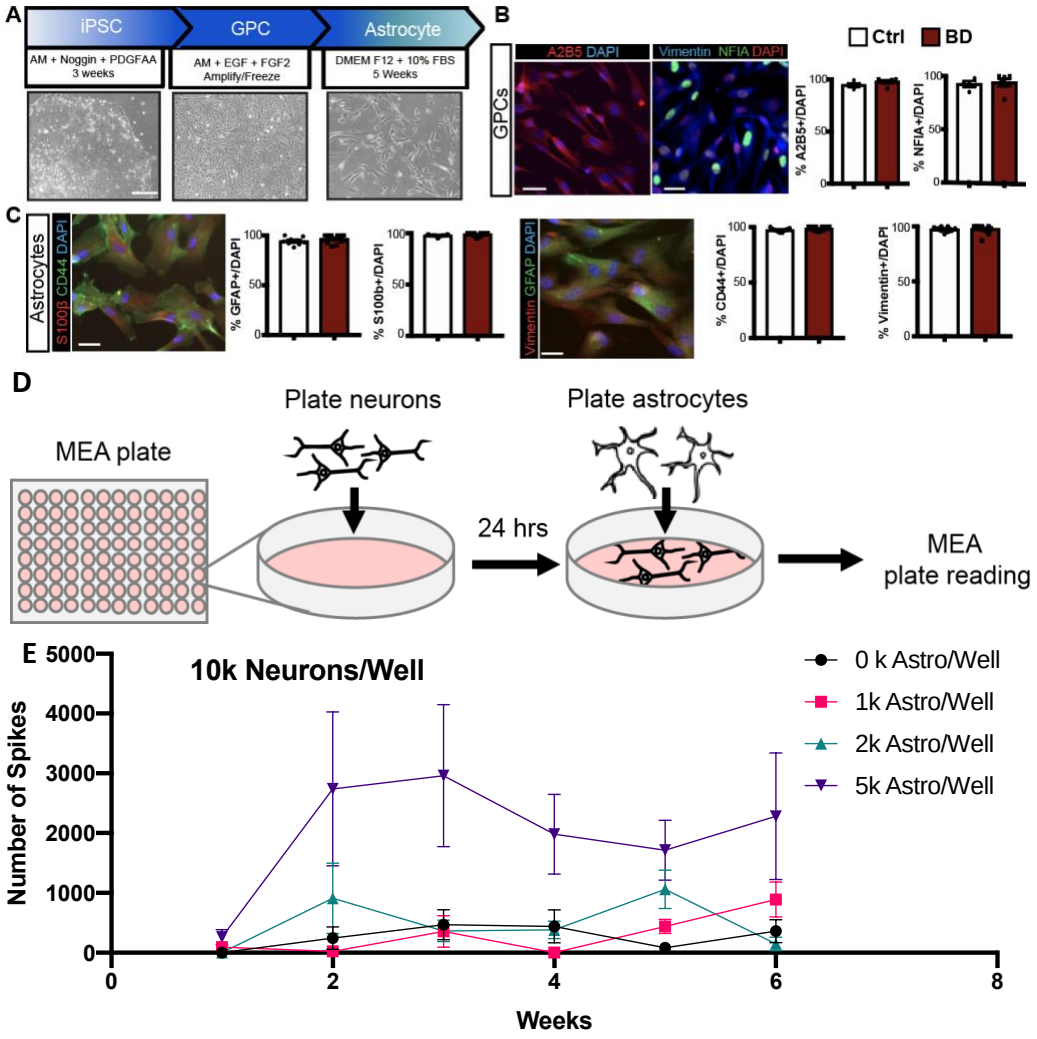

Figure 1: Characterization of iPSC-derived astrocytes and optimization of co-culture model

(A) 8-10 week timeline of astrocyte differentiation from hiPSCs. Bright field images are representative of cell type: fibroblast-derived iPSC, GPCs, and 5-week old astrocytes. Scale bar 300 µm.

(B) Representative images and quantification of GPCs immunostained with early glial markers: A2B5 (red)/DAPI (blue), Vimentin (blue)/NFIA (green)/ DAPI (red). Scale bar 50 µm.

(C) Representative images and quantification of 4-5 week old astrocytes immunostained with astrocytic markers: S100B (red)/ CD44 (green)/ DAPI (blue), Vimentin (red)/ GFAP (green)/ DAPI (blue). Scale bar 50 µm. Cell number quantifications are over DAPI and expressed as mean ± SEM. n = 4 control and 6 BD individuals with experiments in triplicate.

(D) Schematic of co-culture: a monolayer of neurons was plated on an MEA plate and astrocytes were plated on top at least 24 hours later.

(E) 6-week timeline of the number of action potentials (spikes) in co-culture of 10,000 neurons/well with varying concentrations of astrocytes expressed as mean ± SEM. n = 12.

immunostained for the canonical astrocytic markers: glial fibrillary acidic protein (GFAP) and Vimentin (intermediate filament proteins), S100b (a calcium binding protein), and CD44 (a cell surface binding protein). For all cell lines used in the experiment, nearly all astrocytes (90-100%) co-expressed all astrocytic markers (Figure 1C). These data confirmed that we had successfully generated a homogenous population of astrocytes from iPSCs from control and BD individuals.

Optimization and validation of the co-culture system

Astrocytes are well known to regulate synaptic activity of neurons and play major roles in synaptic pruning, transmission, and generation (Allen, 2014; Chung et al., 2015). In order to test the astrocytes' impact on neuronal synaptic activity, we wanted to measure control neurons' synaptic activity in co-culture with iPSC-derived astrocytes using the multiple electrode array (MEA) system. Before determining any differences in our astrocyte lines, we had to first optimize the system to give viable results, especially to determine the most effective ratio of neurons to astrocytes, and validate that it worked as theorized. As a general scheme, we planned to first plate neurons on the MEA plate and then plate astrocytes on the plate at least 24 hours later, to allow the neurons time to attach to the plate (Figure 1D). Plating neurons first allowed direct contact between the electrode and neuron, which the MEA plate requires. If astrocytes and neurons were plated simultaneously, astrocytes would likely attach to the electrodes quicker than neurons and prevent accurate recordings of synaptic activity (Auld et al., 2013).

We tested four different astrocyte concentrations (0 astrocytes/well, 1,000 astrocytes/well, 2,000 astrocytes/well, or 5,000 astrocytes/well) and two methods of plating

neurons (plating 10,000 NPCs and differentiating directly in the MEA plate or plating 30,000 2-week-old neurons sorted by FACS on the MEA plate). One control iPSC-derived neuron line and one control iPSC-derived astrocyte line were used for this experiment. The number of spontaneous electrical events (spikes) per well was used as the metric for synaptic activity. There were six replicate wells per condition, and recordings were taken twice a week for this co-culture experiment.

The data show that the 5,000-astrocyte condition is the most active co-culture active throughout the 6-week experiment when co-cultured with 10,000 NPCs per well (Figure 1E). Addition of astrocytes, on average, increased the activity of neurons, as expected. Results in Figure 1E were from neurons differentiated from NPCs on the MEA plate. Interestingly, the sorted neurons were very inactive compared to the neurons differentiated from NPCs on the MEA plate (data not shown). This may have been due to disruption of previously formed neural networks when dissociating, sorting, and re-plating the neurons. Additionally, when sorting using fluorescence-activated cell sorting (FACS), cell aggregates are broken down to single cells and neurites may have been broken during this process and did not grow back due to loss of synaptic plasticity in mature neurons.

These results showed that the astrocyte-neuron MEA co-culture model is a viable method of measuring the effect of astrocytes on the synaptic activity of neurons. A higher number of astrocytes correlated with higher synaptic activity, with the 5,000 astrocytes per well condition showing the highest level of activity. As the concentration of neurons per well was sufficient to generate high levels of synaptic activity, we aimed to plate approximately 10,000 NPCs and at least 5,000 astrocytes per well in future experiments.

Differential response of BD astrocytes to inflammation

Astrocytes are immunocompetent, meaning they can respond to inflammatory stimulation and produce inflammatory cytokines of their own. They can be activated by pro-inflammatory cytokines, such as TNFa and IL-1b, that are secreted from microglia (Liddelow et al., 2017) and join the neuroinflammatory cascade. The astrocytes we generated were immunoreactive, meaning that they respond to inflammatory stimuli similarly to those *in vivo*. To determine whether BD astrocytes had a differential response to inflammation compared with control astrocytes, we first performed a time course experiment to determine the peak time of cytokine secretion in the culture medium. We collected the media from 5-week old

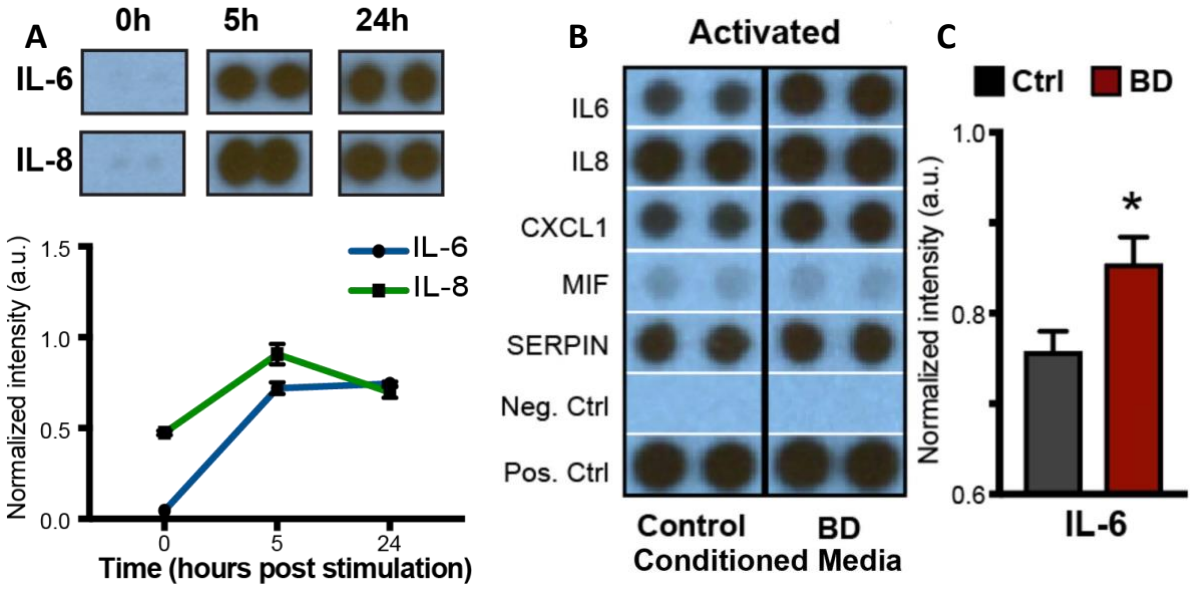

Figure 2: Increased secretion of IL-6 by BD astrocytes
(A) Time course of IL-6 and IL-8 secretion 0 hours, 5 hours, and 24 hours after IL-1b activation. Blot intensity was quantified and plotted over time (arbitrary units, a.u.). IL6 (blue); IL8 (green).
(B) Representative cytokine blots show level of cytokines in the conditioned media of control and BD astrocytes 5 hours after IL-1b activation
(C) Quantification (normalized intensity, a.u.) of IL-6 levels from the cytokine blot array in the medium of control (black bar) and BD (red bar) astrocytes

IL-1b activated control astrocytes 0 (before), 5, and 24 hours after activation (Figure 2A).

Using a cytokine blot array, we stained and quantified the level of IL-6 and IL-8, two key downstream cytokine targets, and observed that the levels peaked at 5 hours and plateaued around 24 hours. Therefore, we examined the cytokine profile of control and BD astrocytes (3 control lines and 4 BD lines) 5 hours after activation to determine which cytokine levels significantly changed (Figure 2B). We observed induction of secretion of IL-6, IL-8, CXCL1, MIF, and SERPIN. Of those activation-induction cytokines, only IL-6, IL-8, and CXCL1 showed a slight increase in BD conditioned medium compared to control conditioned medium, and only IL-6 significantly increased in BD conditioned medium (Figure 2C). This showed that BD astrocytes do have an increased response to IL-1b-mediated inflammation, particularly IL-6. Additionally, in future experiments, all IL-1b activated astrocytes were incubated with IL-1b for a 5-hour period before use (Figure 3A), as that incubation period was shown to be the peak time for cytokine secretion.

Effect of inflammation-activated and BD patient-derived astrocytes on neuronal activity

As inflammation plays a role in synaptic activity, we wanted to determine whether inflammation-activated astrocytes affected neuronal activity differently than non-activated astrocytes. We activated 4-week old astrocytes with IL-1b for 5 hours then plated them on top of 3-week old neurons from a healthy individual in an MEA plate (Figure 3A). Astrocytes were generated from two different iPSC sources, lymphoblasts and fibroblasts, each with 6 BD patients and 4 healthy individuals. 10,000 NPCs and 7,000 astrocytes were plated on each well and each condition per line had 4 replicate wells.

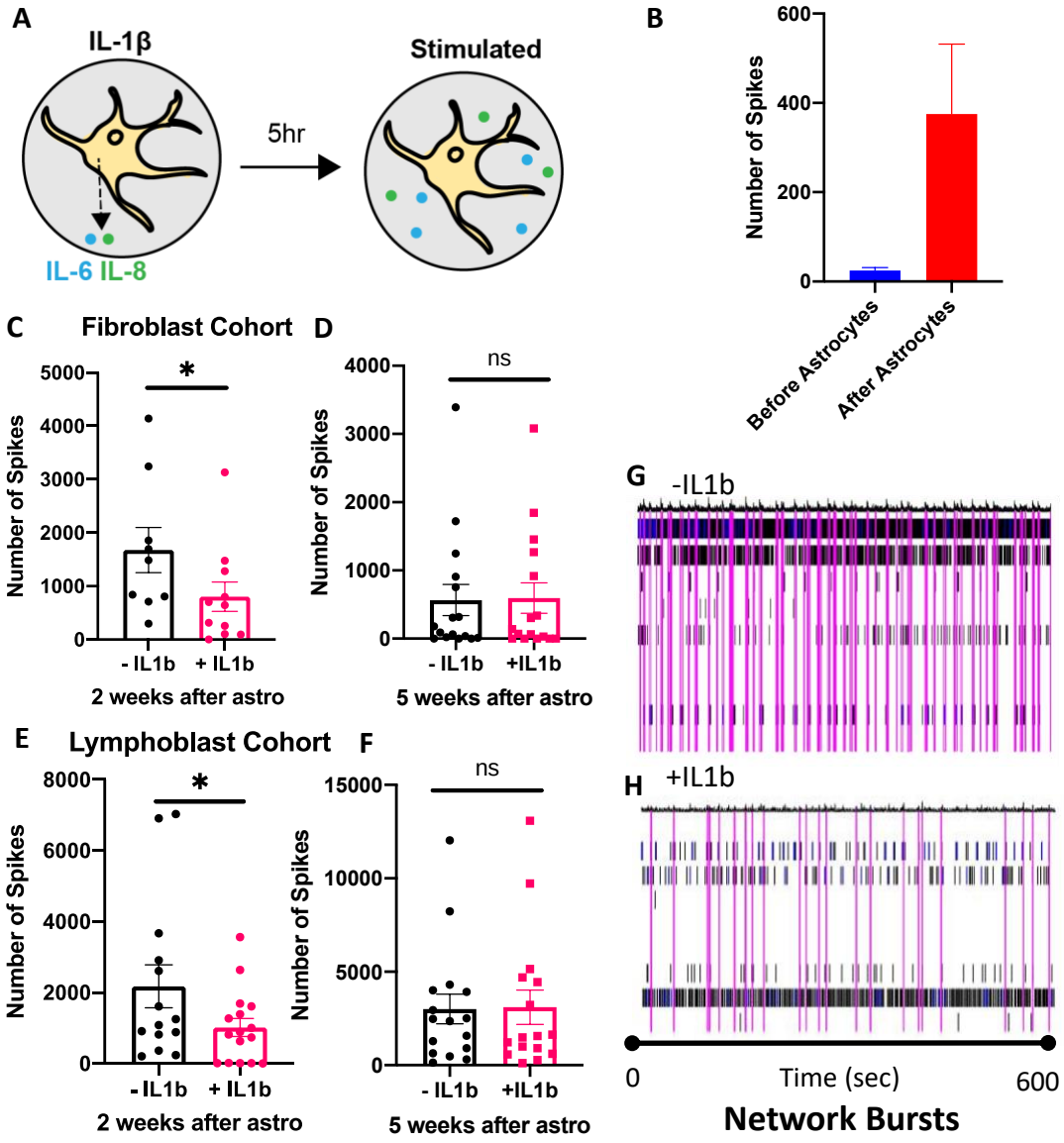

Figure 3: Decreased activity of control neurons when co-cultured with IL-1b-activated astrocytes

(A) Schematic of activation of astrocytes with IL-1b to induce cytokine production

(B) Number of action potentials (spikes) before (blue bar) and 12 hours after astrocytes (red bar) were added to co-culture with neurons.

(C-F) Neurons were co-cultured with astrocytes pre-treated with either IL-1b (pink bar) or vehicle (black bar) from fibroblast derived iPSCs (C, D) or lymphoblast-derived iPSCs (E, F). At 2 weeks (C, E) and 5 weeks (D, F) of co-culture, the neuronal activity was measured. Each dot represents one active well.

(G-H) Representative raster plot showing average spikes (black lines) and network bursts (pink lines) in neurons co-cultured with non-activated (G) and activated (H) astrocytes. Individual electrodes are plotted (y axis) over time (x axis)

Statistical significance was determined using the unpaired two-tailed t-test * $p<0.05$

We performed a time course experiment to determine whether the astrocytes increased neuronal activity, as previously observed (Figure 1E). Recordings were taken before addition of astrocytes and 12 hours after addition of astrocytes, and we observed a significant increase of synaptic activity after astrocytes were added, regardless of astrocyte line (Figure 3B). Additionally, comparing the activity of neurons co-cultured with astrocytes to neurons without astrocytes showed that co-cultured neurons were significantly more active over time (data not shown). The results show the neuronal activity approximately 2 and 5 weeks after astrocytes were added to the neurons (Figures 3 C-F).

When astrocytes were activated with IL-1b, they initially decreased the activity of neurons compared to non-activated astrocytes (Figures 3 C, E). This was apparent in both the lymphoblast and fibroblast cohorts. We observed a reduction in two key parameters of neural activity: number of spikes (Figures 3 C, E) and number of network bursts (Figures 3 G, H). Raster plots showing network burst activity of co-cultures with activated control astrocytes (Figure 3G) were compared to those with non-activated control astrocytes (Figure 3H). This showed that the functional impact of IL-1b mediated inflammation on synaptic activity was negative.

We then tested whether BD astrocytes affected neuronal activity differently than control astrocytes. Interestingly, when co-culturing BD astrocytes with control neurons, we also observed a significant reduction of neuronal activity in both number of spikes and network bursts (Figure 4). Additionally, IL-1b activated BD astrocytes further reduced neuronal activity compared to non-activated BD astrocytes (Figures 4 F-G). Astrocytes from both the fibroblast and lymphoblast cohort showed this BD phenotype (Figures 4 A, C). These

results showed that BD astrocytes also have a negative functional impact on neuronal activity, and this effect can be exacerbated by IL-1b activation.

As these co-cultures were recorded for 6-7 weeks, the longevity of the effect of the

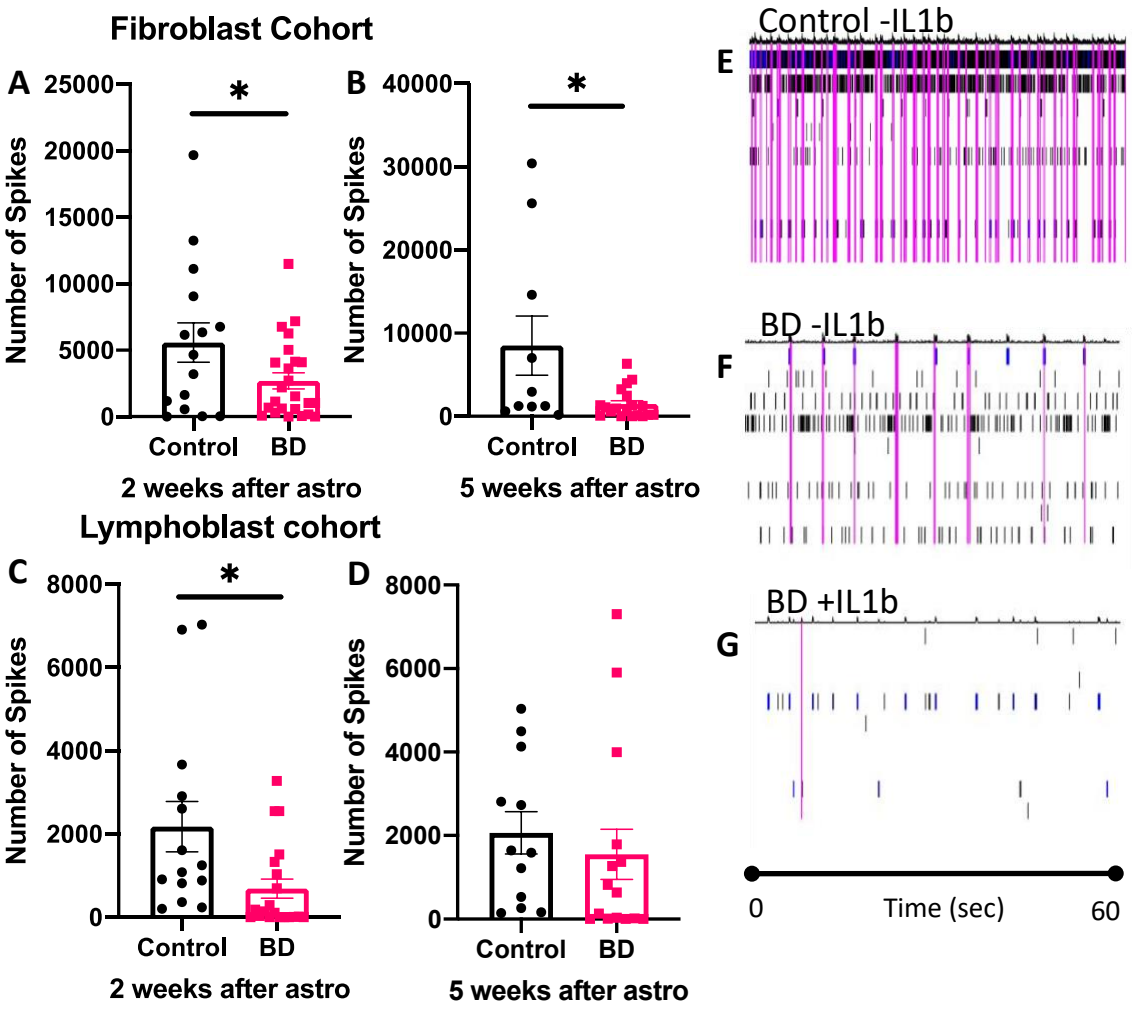

Figure 4: Decreased activity of control neurons when co-cultured with BD patient astrocytes

(A-D) Neurons were co-cultured with either BD (pink bar) or control (black bar) astrocytes from two different iPSC sources. At 2 weeks (A, C) and 5 weeks (B, D) of co-culture, the neuronal activity was measured. Each dot represents one active well. Statistical significance was determined using the unpaired two-tailed t-test *$p<0.05$

(E-G) Representative raster plot showing average spikes (black lines) and network bursts (pink lines) in neurons co-cultured with control (E), BD (F), and activated BD (G) astrocytes. Individual electrodes are plotted (y axis) over time (x axis).

activated or BD astrocytes was recorded. Around 5 weeks, the neurons co-cultured with astrocytes were still active, but there was no reduction of neuronal activity in neurons co-cultured with activated astrocytes compared to controls (Figure 3 D, F). On the other hand, neurons co-cultured with BD astrocytes still showed that reduction in activity 5 weeks after astrocyte addition (Figure 4 B, D). This difference in the longevity of impact may be because changes to activated astrocytes are temporary, and once they stop receiving inflammatory stimuli, they could return to their baseline regulation of neuronal activity. BD astrocytes retained the observed phenotype, which suggests that the decrease of neuronal activity is due to the BD genotype or permanent altered gene expression, not only a temporary response to inflammatory stimuli.

These data show that both inflammation-activated and BD patient-derived astrocytes have a detrimental impact on neuronal function. The longevity of the impact is shorter for activated astrocytes than BD astrocytes. But, the co-culture method used does not differentiate between contact-dependent and contact-independent phenotypes, as the neurons and astrocytes are always in direct contact. Therefore, we next asked whether direct contact was necessary to observe these phenotypes to begin to understand the mechanism by which this phenotype occurs.

Direct contact is not necessary for effect of astrocytes on neuronal activity

To test whether the activated and BD astrocytes could reduce neuronal activity in a contact-independent manner, we used a conditioned media system in which media from one cell type (astrocytes) is transferred to the other cell type (neurons) so that the two cell types do not interact directly. We examined the effect of astrocyte-conditioned media (aCM) from

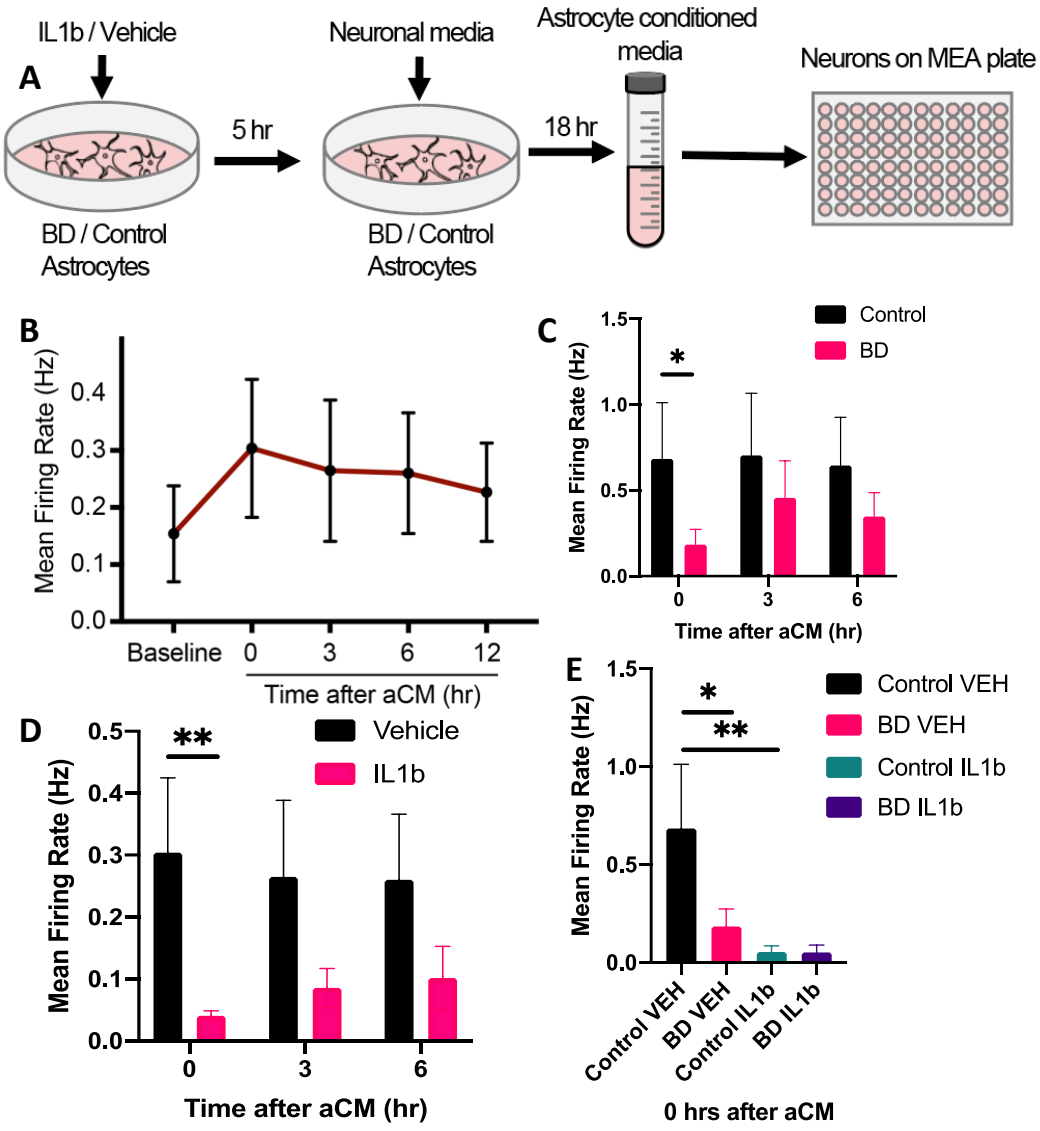

Figure 5: Activated astrocytes or BD astrocytes decreases the activity of neurons in a contact-independent manner

(A) Schematic of conditioned media experiment. Monolayer of control or BD astrocytes are pre-treated for 5 hours with vehicle or IL-1b, then incubated with neuronal media for 18 hours. The astrocyte-conditioned neuronal media is collected and used to culture control neurons on an MEA plate, where activity records were performed.

(B) Time course measurements of activity (spikes per minute) were done from before addition of aCM to 12 hours after addition of aCM

(C) Spikes per minute were recorded on neurons cultured with aCM from control astrocytes (black bar) and BD astrocytes (pink bar) at three timepoints

(D) Spikes per minute were recorded on neurons cultured with aCM from non-activated astrocytes (black bar) and activated astrocytes (pink bar) at three timepoints

Figure 5 (continued):
(E) Spikes per minute were recorded on neurons immediately after culture with aCM from control (black bar), activated control (pink bar), BD (turquoise bar), and activated (purple bar) BD astrocytes. Bars show mean ± SEM. Statistical significance was determined using the unpaired two-tailed t-test *p<0.05, **p<0.01

control or BD lines on the activity of control glutamatergic neurons in culture on an MEA plate (Figure 5A). 2 control astrocyte and 4 BD astrocyte lines from the fibroblast cohort were activated with either IL-1b or vehicle for 5 hours, as previously done in the co-culture model. Commercial glutamatergic neurons (iGluta neurons from CDI) were used for this experiment to obtain a more homogenous population of neurons. As they were mature neurons and not prone to rapid proliferation like NPCs, 100,000 neurons were plated per well.

We performed a time course experiment to measure the activity of the neurons before aCM was added, immediately after (0 hours after), 3 hours after, 6 hours after, and 12 hours after. There was an immediate increase in activity after addition of aCM, which gradually reduced over time (Figure 5B), showing that astrocytes could promote neuronal activity in a contact-independent way, likely through secreted factors. aCM from BD lines significantly reduced neuronal activity compared to aCM from control lines immediately after aCM addition, but this difference reduced at the 3 and 6-hour time points (Figure 5C). Similarly, neurons cultured with aCM from activated astrocytes had significantly decreased activity relative to neurons cultured with aCM from non-activated astrocytes. This difference also became less pronounced over time (Figure 5D). The results from the 0 hours after aCM addition time point corroborate our findings from the co-culture system (Figure 5E), showing that BD and activated astrocytes likely secrete factors that play a role in regulating the decrease of neuronal activity. Our previous cytokine array blot showed that BD astrocyte secretion of IL-6 significantly increased after IL-1b activation (Figure 2B). This suggested

that astrocyte-secreted IL-6 or its downstream targets potentially could regulate the observed decrease of neuronal activity when co-cultured with BD or activated astrocytes. Therefore, we next asked whether IL-6 contributed to or regulated the observed phenotype.

Secreted IL-6 from BD astrocytes regulated neuronal activity

We hypothesized that the observed effects of BD and activated astrocytes may be partly mediated by IL-6. To test this hypothesis, we performed the conditioned media experiment again with aCM from BD astrocytes. Control glutamatergic neurons were either treated with aCM from non-activated BD astrocytes, activated BD astrocytes, or activated BD astrocytes with IL-6 blocking antibody. The results showed that neurons cultured with activated BD astrocyte-conditioned medium had significantly lower activity compared to

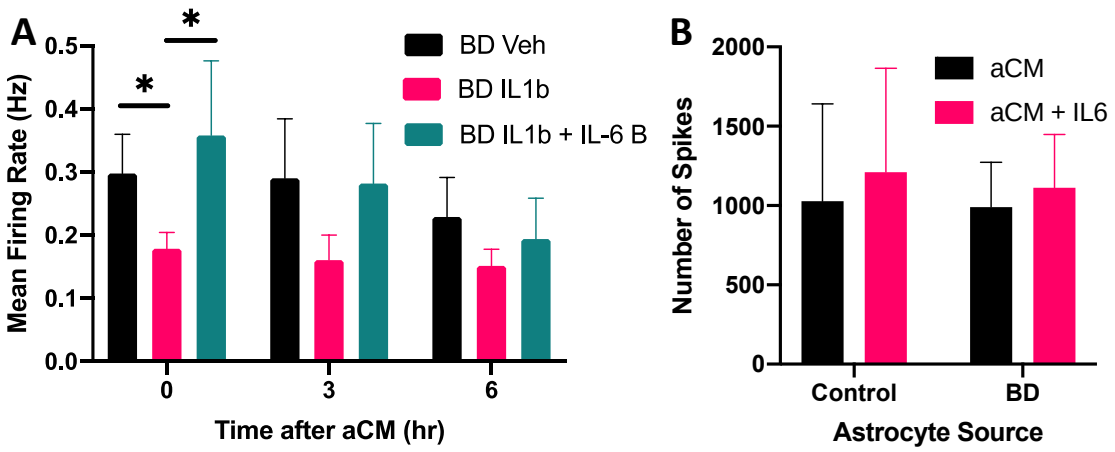

Figure 6: Increased secretion of IL-6 by BD astrocytes may mediate observed decrease of neuronal activity
(A) Time course of neuronal activity of control neurons cultured with aCM from BD (black bar), activated BD (pink bar), and activated BD + IL-6 antibody blocker (turquoise bar) astrocytes at 0, 3, and 6 hours after aCM addition.
(B) Neuronal activity of control neurons immediately after addition of aCM (black bar) or aCM treated with IL-6 (pink bar) from control or BD astrocytes. Bars show mean ± SEM. Statistical significance was determined using the unpaired two-tailed t-test *$p<0.05$

neurons cultured with non-activated BD astrocyte-conditioned medium immediately after addition of aCM (Figure 6A).

Importantly, at the same time point, neurons cultured with activated BD astrocyte-conditioned medium with added IL-6 blocking antibody had significantly higher activity compared to neurons cultured with activated BD astrocyte-conditioned medium, showing a significant rescue effect. To be sure that the IL-6 blocking antibody did not increase neuronal activity in the absence of astrocytes, control neurons were cultured in non-conditioned neuronal media containing the same concentration of IL-6 antibody, and no significant difference in neuronal activity was observed (data not shown). Interestingly, when IL-6 was directly added to aCM from control and BD astrocytes, there was no observed significant difference in neuronal activity, showing that IL-6 alone was not sufficient to decrease neuronal activity (Figure 6B). Taken together, our results showed that IL-6 is a partial mediator in the negative effect of activated BD astrocytes on neuronal activity.

Discussion:

We optimized two systems, co-culture and conditioned media, to measure both the contact-dependent and contact-independent effects of human patient iPSC-derived astrocytes on the synaptic activity of neurons and derived immune responsive astrocytes from healthy and BD iPSCs. Our results show that iPSC-derived astrocytes from control and bipolar patients activated with IL-1b, a pro-inflammatory cytokine and driver of neuro-inflammation, reduced control neuronal activity compared to non-activated astrocytes, measured via MEA. These results were consistent in both the co-culture system, where astrocytes directly contacted the neurons, and in the conditioned media system, where only the astrocytic conditioned media was added to the neurons and no direct neuron-astrocyte contact occurred. These results are supported by Santos and colleagues (2017) who showed that inflammatory competent iPSC-derived astrocytes reduce iPSC-derived neuronal activity when activated with inflammatory stimuli. Overexpression of IL-6, another inflammatory cytokine, in the astrocytes of mice led to neurologic disease and seizures (Campbell et al., 1993). These data come together to support that neuro-inflammation mediated by cytokine activity and astrocytes has adverse effects on neuronal activity in the brain.

Our results also show that astrocytes derived from patients with bipolar disorder decreased the activity of neurons in both co-culture and conditioned media systems compared to control astrocytes. Previous studies have shown a link between mood disorders, such as bipolar disorder, and altered synaptic activity which can be rescued with lithium, a common treatment used for bipolar disorder, suggesting that dysfunctional synaptic activity in the brain may be a phenotype in bipolar patients which could be treated by a common bipolar treatment (Mertens et al., 2015; Stern et al., 2017). However, Mertens and colleagues (2015) showed

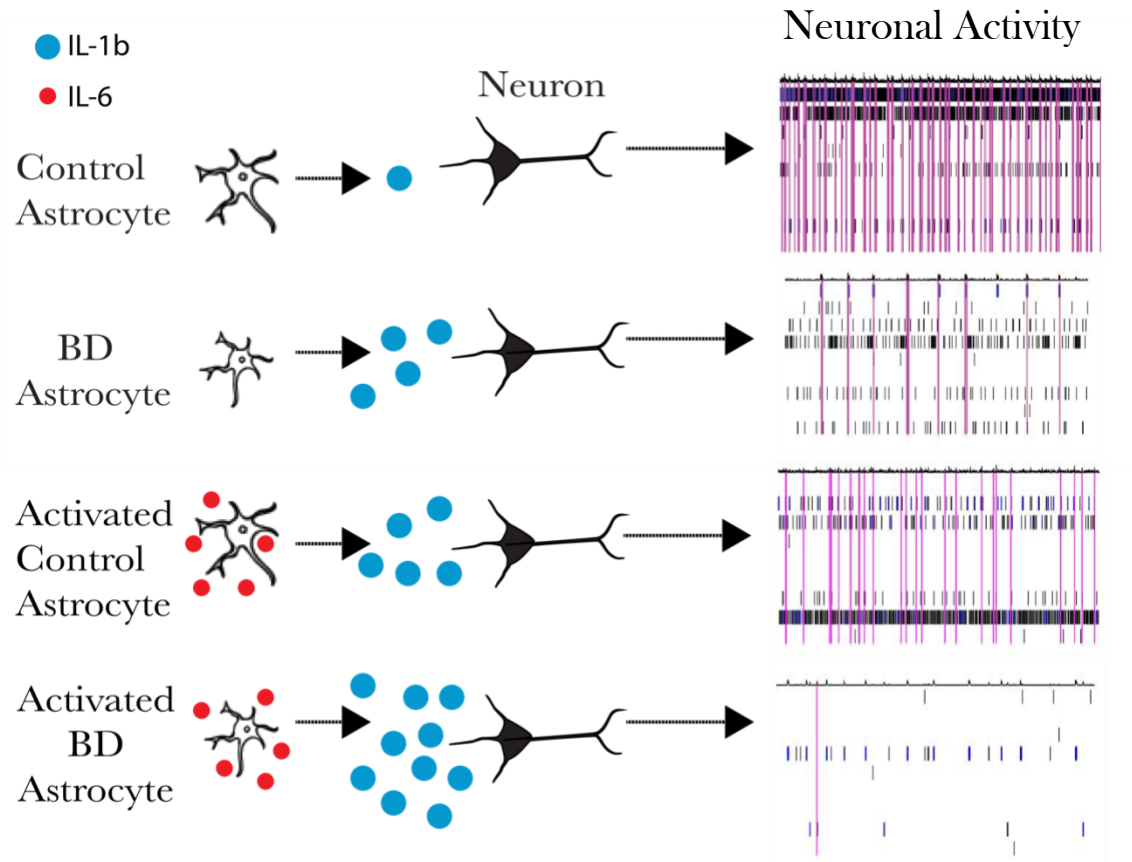

Figure 7: Graphical schematic of mechanism by which BD and activated astrocytes may decrease control neuron activity

hyper-excitability, or increased activity, of iPSC-derived hippocampal neurons from bipolar patients, which contradict our findings that bipolar astrocytes result in decreased neuronal activity. As their experiment focused on only bipolar neurons and did not have astrocytes, this difference in results could be due to differences in interactions between neurons and astrocytes in bipolar disorder.

This raises the question as to whether bipolar patients have hyperactive neurons in the brain, or if this increased activity is mitigated in the presence of astrocytes. Further research studying both neurons and astrocytes together in bipolar disorder, such as with a co-culture of bipolar neurons and bipolar astrocytes, may help elucidate these interactions and how they

may differ from neurotypical individuals. Our results show that bipolar astrocytes directly give rise to a reduction of control neuronal synaptic activity, which may be due to altered neuron-astrocyte interactions in bipolar disorder.

Using conditioned media experiments, we showed that the decrease of neuronal activity by both bipolar and IL-1b-activated astrocytes is mediated through secreted factors, likely cytokines, as only the media was transferred, so there was no direct astrocyte and neuron interaction. As we detected increased IL-6 secretion in IL-1b activated bipolar iPSC-derived astrocytes compared to control astrocytes using a cytokine array blot, we added an IL-6 blocking antibody to the astrocytic conditioned media and observed a partial rescue effect, where the neuronal activity in BD-activated aCM-fed cultures increased to BD-non-activated aCM levels. This showed that IL-6 secreted by astrocytes has a role in regulating neuronal activity. Previous studies support our findings that there are increased levels of IL-6 and IL-1b in bipolar patients (Hamdani et al., 2012). Elevated levels of IL-1b and IL-6 have been found in the blood and cerebrospinal fluid of patients with psychiatric disorders including bipolar disorder, particularly during manic episodes (O'Brien et al., 2006). These findings come together to show that increased IL-6 production in astrocytes from bipolar patients is involved in reducing neuronal activity.

Intriguingly, although IL-6 blocking antibody in aCM rescues the negative effect of IL-1b activation, we found that exogenous application of IL-6 in aCM had no detrimental effect on the neurons. IL-6 is a complex pleiotropic cytokine that has both neuroprotective and neurodegenerative effects. There are two pathways that IL-6 affects cells by: the classical pathway, associated more with neuroprotection, and the trans-pathway, which is associated more with neurodegeneration (Rothaug et al., 2016). IL-6 has been associated with depression

in rats, and IL-6 injections into rat brains led to depressive-like behaviors (Gruol et al., 2015). IL-6 expression has also been linked to altered synaptic activity and transmission in mice (Nelson et al., 2012). IL-6 was shown to have a neuroprotective effect when added exogenously at 1000pg/mL and incubated for 24 hours or in IFN-y-activated astrocyte conditioned medium (Sun et al., 2017; Fujishita et al., 2009). These effects may be dose and context-dependent, as IL-6 was shown to promote neuronal differentiation at 20ng/mL in the absence of retinoic acid (RA), but inhibit it at 50ng/mL in the presence of RA (Barkho et al., 2009). Additionally, the synaptic effects of IL-6 may vary dependent on the length of exposure. Acute exposure led to depression of synaptic activity such as LTP, while chronic exposure using transgenic mice led to enhanced fEPSPs (Nelson et al., 2012). Exogenous application of IL-6 in studies ranges from 0.01 ng/mL – 200 ng/mL, and lower concentrations are associated with physiological conditions and higher concentrations with disease conditions (Gruol et al., 2015).

To explore whether addition of IL-6 to our generated aCM can induce a decrease of neuronal activity, different and higher concentrations of IL-6 and durations of exposure should be tested as the effects are dose-dependent and time-dependent. Also, effects of IL-6 exposure likely should be recorded in neurons that have not been exposed to non-physiological concentrations of IL-6 as the effects decrease over time. If exogenous IL-6 still does not induce a response, it may not be inducing a response alone and may be forming a complex with another secreted molecule in the presence of neurons. In the future, we hope to better understand the mechanism by which IL-6 regulates neuronal activity after future experiments. Additionally, according to the cytokine blot, CXCL1 had a slight but not significant increase in aCM from BD astrocytes compared to aCM from control astrocytes.

This chemokine has been implicated in inflammatory pain pathways and may serve as another potential mediator of neuronal activity, along with IL-6 (Cao et al., 2014).

Due to the results of this study, we believe that differential responses to inflammation in bipolar astrocytes, specifically secretion of IL-6, compared to control astrocytes may lead to altered neuronal activity or astrocyte-neuron interactions that could contribute to the bipolar phenotype (Figure 7).

Our results are robust among our multiple patient-derived astrocyte lines. But, the current sample size of 12 bipolar lines and 8 control lines is rather small, due to the low throughput nature of deriving iPSC-derived cell lines, as the typical variability between cell culture model lines is relatively high. Also, as our astrocytes are iPSC-derived, they are likely more similar in age to astrocytes in fetuses than those in the brains of bipolar patients whose average age of onset is 25, and we do not yet have reliable biomarkers for tracking the developmental age of astrocytes (Rybakowski, 2014). As this experiment was done in vitro, the brain micro-environments and all brain cell types, which play a major role in controlling neuronal activity, were not present in the co-culture system. Despite these limitations, we believe our findings elucidate aspects of the understudied role of astrocytes in neuro-inflammation and bipolar disorder and provide a potential therapeutic target in IL-6, which could help find new and more effective treatments for bipolar disorder. As we have showed increased secretion of inflammatory signals occurs in bipolar disorder, anti-inflammatory agents may be another potential avenue for bipolar treatment, especially as they are currently showing promise for treatment of major depressive disorder, a mood disorder related to bipolar disorder (Benedetti et al., 2020).

In the future, as mentioned earlier, we will compare a co-culture system of bipolar neurons and bipolar astrocytes to a co-culture of control neurons and control astrocytes to examine potential differences in astrocyte-neuron interactions and neuronal activity and examine synaptic puncta densities in more detail in correlation with neuronal activity. Additionally, we will work to further understand how exactly IL-6 is involved in regulating neuronal activity in BD and activated astrocytes and explore CXCL1 as an alternate potential target. We will also attempt to introduce microglial cells to the co-culture model to increase complexity. Microglia, as the resident immune cells in the brain, also play major roles in neuro-inflammation and secrete the IL-1b that activates astrocytes and trigger neuro-inflammation (Hewett, 2012). We also plan to use brain organoid models to obtain the 3-D model structure of brains, as currently our co-culture model is 2-D because it is a monolayer plated on wells. Adding microglia and using an organoid model would also allow us to more closely model the in vivo manifestation of bipolar disorder.

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