

## RAPID COMMUNICATION

# Reduced Neurite Density in Neuronal Cell Cultures Exposed to Serum of Patients with Bipolar Disorder

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## Abstract

**Background:** Increased inflammatory markers and oxidative stress have been reported in serum among patients with bipolar disorder (BD). The aim of this study is to assess whether biochemical changes in the serum of patients induces neurotoxicity in neuronal cell cultures.

**Methods:** We challenged the retinoic acid-differentiated human neuroblastoma SH-SY5Y cells with the serum of BD patients at early and late stages of illness and assessed neurite density and cell viability as neurotoxic endpoints.

**Results:** Decreased neurite density was found in neurons treated with the serum of patients, mostly patients at late stages of illness. Also, neurons challenged with the serum of late-stage patients showed a significant decrease in cell viability.

**Conclusions:** Our findings showed that the serum of patients with bipolar disorder induced a decrease in neurite density and cell viability in neuronal cultures.

**Keywords:** bipolar disorder, neurite density, RA-differentiated SH-SY5Y cells, systemic toxicity

## Introduction

Bipolar disorder (BD) affects about 2% of the world's population, with sub-threshold forms affecting up to a further 2% (Merikangas et al., 2007). The course of BD is highly variable, and a subset of patients seem to present a progressive course

associated with brain changes (Cao et al., 2016) and functional impairment (Rosa et al., 2014). Nonetheless, the molecular foundations for this illness progression are just beginning to be explained. It is known that brain-derived neurotrophic factor

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(BDNF) serum levels were decreased in the late-stage of BD when compared to those at an early stage of the illness (Kauer-Sant'Anna et al., 2009). Moreover, altered cellular resilience was reported in late-stage patients (Pfaffenseller et al., 2014). In addition, patients at a late stage of illness present increased levels of C-C motif ligand 11 (CCL11) and decreased levels of CXCL827 chemokines (Panizzutti et al., 2015). However, what is not known is how these abnormal peripheral blood markers may lead to, or be involved with, brain changes in BD.

In 2011, our group hypothesized that impairments in neuroplasticity of BD patients may be translated into shrinkage of the brain structures by reducing neurites and intercellular connections in the neuronal network (Berk et al., 2011). We also suggested that the aforementioned biochemical changes may play a causal role in this scenario, which has been called the systemic toxicity (Kapczinski et al., 2010). Recently, several studies reported reductions in the volume of the left hippocampi (Cao et al., 2016) and frontal cortices of patients with late-stage BD (Abe et al., 2015). These findings are in line with the pioneering work of Strakowski and colleagues (2002), which reports increased ventricle volumes in multiple-episode patients with BD compared to those who had only one episode. However, the causal role of the abnormal peripheral blood markers on neuronal cells of the patients with late-stage BD has not been investigated yet.

In the present study, we used an *in vitro* approach with the retinoic acid (RA)-differentiated human cell line SH-SY5Y exposed to serum of bipolar patients. The differentiated human neuroblastoma cell line, SH-SY5Y, has been used as an experimental model to assess molecular and biochemical pathways involved in the pathophysiology of brain disorders (Lopes et al., 2010) and for neurotoxicological experiments in developmental and mature neurons (Schonhofen et al., 2015). This model has the advantage of being derived of human cells, displaying neuronal morphology, and neuronal markers (as high neurite density, tyrosine hydroxylase, dopamine transporter) during RA-differentiation (Lopes et al., 2010).

Therefore, the aim of the present study was to assess whether biochemical changes in serum of patients with BD could induce neurotoxicity in neuronal cell cultures.

## Methods

The Ethical Committee of the Hospital de Clínicas de Porto Alegre (HCPA) approved the study (application number: 12-0102). All subjects had signed the informed consent.

## Subjects

We recruited 12 patients with BD from the Bipolar Disorders outpatient clinic of the HCPA. We also selected six healthy controls matched by age and gender from the blood donation center of HCPA. They had no previous history of psychiatric illness as well as no history of psychiatric or neurologic disorders in first-degree relatives. Inclusion criteria were euthymic subjects with BD type 1 according to the DSM-IV and aged between 18 and 60 years. Exclusion criteria were a history of autoimmune diseases or a history of chronic infection/inflammatory disorders, as well as any severe systemic disease or use of immunosuppressive therapy.

## Assessments

Subjects were evaluated through a socio-demographic history form. Axis-I diagnoses and clinical and functioning characteristics were assessed using the Structured Clinical Interview for DSM-IV axis-I Disorders (SCID-I) and Functioning Assessment Short Test

(FAST), respectively, which were administered by trained staff. Current dimensional mood symptoms were assessed with the Hamilton Depression Rating Scale (HDRS; Hamilton, 1960) and the Young Mania Rating Scale (YMRS; Young et al., 1978). Euthymia was defined by the HDRS score < 8 and YMRS score < 5.

The patients were classified in stages I to IV, based on functional impairment, as well as patterns of episode recurrences and severity of clinical features (Kapczinski et al., 2009). Patients were stratified in early-stage (stage I or II) or late-stage (stage III or IV) of BD. Of note, we used the same staging criteria of previous studies from our group (Fries et al., 2013; Pfaffenseller et al., 2014).

## Sample Collection

Four milliliters of blood were collected from each subject by venipuncture into a free-anticoagulant vacuum tube. After withdrawal, the blood was centrifuged at 3000g for 10 minutes and the serum was stored at -80°C until assayed.

## Cell Culture and Treatment

The neuronal differentiation of human neuroblastoma SH-SY5Y cells were performed in accordance with the protocol established by Lopes and colleagues (2010). The neuronal differentiation is induced by reducing the fetal bovine serum (FBS) in the culture medium at 1% plus 10 µM all-trans retinoic acid (Enzo Life Sciences, Inc.) for seven days. At the end of this protocol, the cells acquire the morphological and biochemical characteristics of mature, differentiated neurons. After the neuronal differentiation protocol, the cells were treated with inactivated serum (56°C for 30 min) of controls and bipolar patients (1%) for 24h. The cells treated with FBS were used as the control group for the experiment.

## Cell Viability

Cell viability was evaluated by the quantification of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction to a blue formazan product by cellular dehydrogenases, as previously described (Mosmann, 1983). The cells were seeded in 12-well plates and 24-well plates at a density of 160 000 and 80 000 cells/well, respectively. After treatment, the medium was discarded and MTT (0,5 mg/mL; Sigma-Aldrich) was added to each well and the plate were incubated for 1 h at 37°C. Then, the MTT was discarded, and dimethyl sulfoxide was added to solubilize the formazan crystals. Absorbance was determined at 560 and 630 nm in a SoftMax Pro Microplate Reader (Molecular Devices). Data were expressed as percentage of experimental control group of three independent experiments.

## Immunofluorescence

The immunofluorescence was performed using anti-βIII tubulin antibody (Alexa 488-conjugated; Sigma-Aldrich) and with Nuclear dye Hoechst 33342 (0.25 µg/µL; Sigma-Aldrich). Randomly selected images were captured using an EVOS Flويد Cell Imaging Station (Thermo Fisher Scientific Inc.) and analyzed with Nikon Imaging Software (NIS), the NIS-elements. The neurite density was assessed using the AutoQuant Neurite software (implemented in R program) and expressed as arbitrary units (AU) as described previously (Schonhofen et al., 2015).

## Statistical Analysis

Statistical analyses were performed using the SPSS 18.0 software (SPSS Inc.). The normality of data distribution was assessed using

the Shapiro–Wilk test. Data are expressed as mean  $\pm$  standard deviation (SD) with a t-test and one-way ANOVA used where appropriate ( $n = 6$ ).  $P$  values  $< 0.05$  were considered significant.

## Results

There was no difference in age and gender among patients at early- and late-stage BD and healthy controls. Moreover, illness duration and medication status were not different between patients at early and late stages (Table 1). We performed a RA-differentiation protocol of SH-SY5Y cells and replaced the fetal bovine serum (FBS) with the serum of bipolar patients and control subjects (Figure 1A) and assessed neurite density and cell morphology using immunofluorescence (Figure 1B). We found a reduction in the neurite density of RA-differentiated SH-SY5Y cells treated with the serum of patients with BD compared to healthy controls ( $p = 0.0153$ ). Furthermore, when the effect of the serum of patients at a late stage were compared to the serum of the control group, we also found a significant reduction in neurite density ( $p = 0.0089$ ). There was no difference when the serum of patients at the early stages was compared to healthy controls (Figure 1C). There was no difference between the serums of patients and controls in cell viability analysis. However, higher serum neurotoxicity was found to be attributed to late-stage patients, leading to a significant decrease in cell viability compared to the serum of both early-stage patients ( $p = 0.0290$ ) and healthy control subjects ( $p = 0.0075$ ; Figure 1D).

## Discussion

The present study showed that the serum of patients at a late stage induced a significant reduction of neurite density and a decrease in the cell viability compared to the serum of healthy controls. In addition, the serum of patients at a late stage caused a significant decrease in the cell viability compared to those at an early stage. Besides, we presented a potential new model for the study of illnesses that affect the central nervous system as BD, where it would be possible to evaluate the molecular and biochemical changes featured in BD.

Previously, a study reported the neurotoxic effect of the serum of patients with BD in human endothelial cells by inducing apoptosis (Politi et al., 2008). Moreover, another study showed that the serum of euthymic patients with BD had detrimental effects on peripheral blood mononuclear cells function (Herberth et al., 2011). To our knowledge, however, our work was the first to evaluate the effect of serum of patients at different stages of BD in cellular parameters using human neuronal cells.

Our findings corroborate the hypothesis that patients with BD might have a loss of neuronal connectivity leading to neuroplasticity and cellular resilience impairments (Rajkowska, 2002). Thus, they suggest that the serum of patients with BD, mainly those at the late stage of the illness, may contain chemicals that could be toxic and alter neural cells, as proposed by the systemic toxicity hypothesis (Kapczinski et al., 2010). Specifically, our previous work showed that mood episodes are associated with peripheral changes in inflammation, oxidative stress, and neurotrophin markers (Kapczinski et al., 2010). In this sense, the cumulative damage caused by the recurrent mood episodes may explain why the serum of patients at a late stage is more neurotoxic than of patients at an early stage.

Moreover, these findings add to the notion of neuroprogression. The term neuroprogression has been proposed as the pathological rewiring of the brain that takes place in parallel with the clinical and neurocognitive deterioration in the course of BD (Berk et al., 2011). This hypothesis may explain why some patients with BD have a progressive course associated with a shortening of inter-episodic intervals, functional and cognitive impairment, treatment refractoriness, and suicide attempts (Merikangas et al., 2007; Rosa et al., 2014).

In addition, there was no difference between groups in illness duration (Table 1). This finding corroborates current staging models in BD, where the number of episodes and functioning impairments are more relevant to the definition of stages than length of illness (Kapczinski et al., 2014).

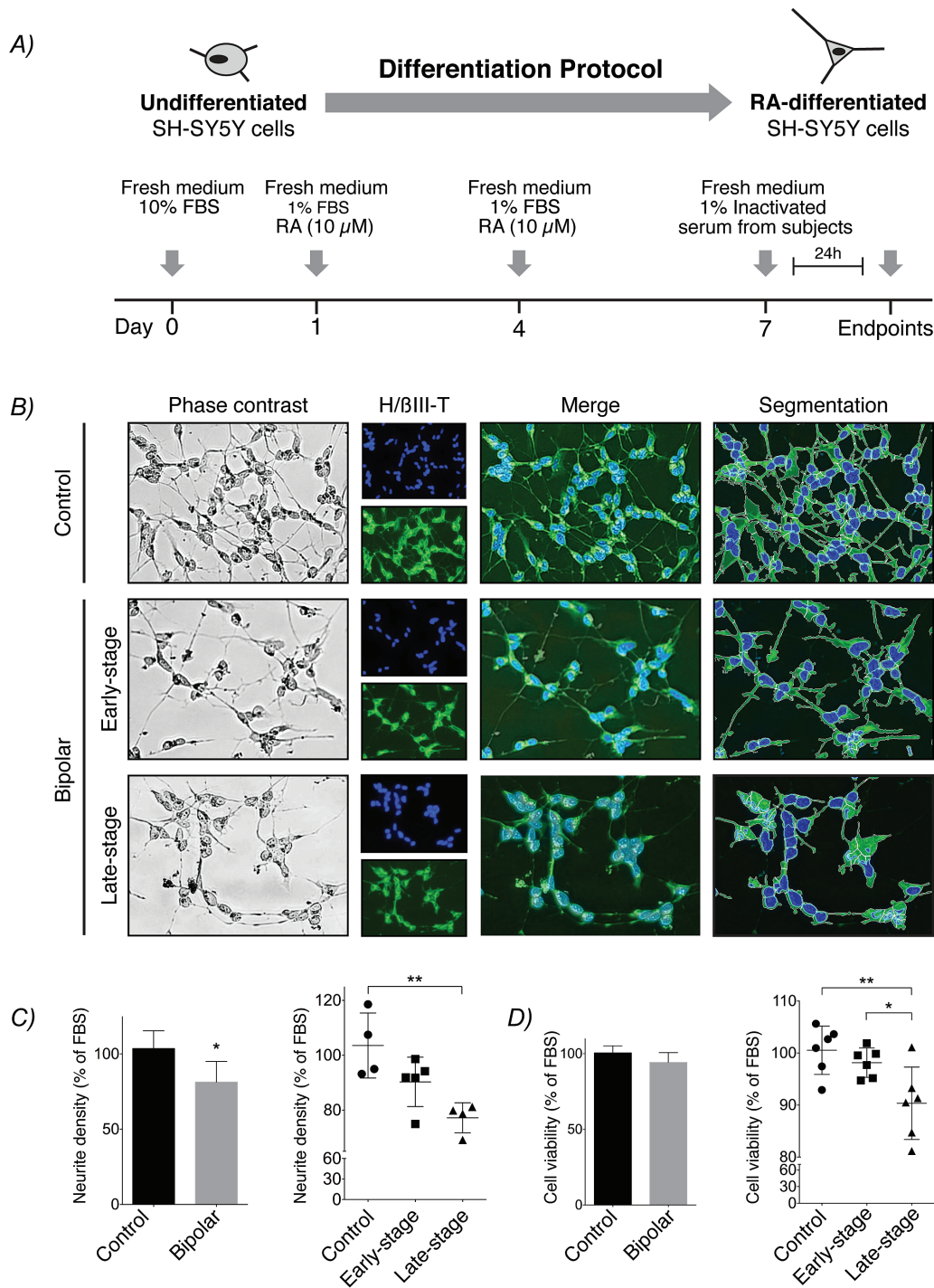
Our study has some limitations. First, there is a question as to what extent neuronal cell line experiments reflect what actually happens *in vivo*. Addressing this issue, a recent study proposed a model wherein transient or persistent disruption of blood-brain barrier integrity is associated with decreased central nervous system protection and increased permeability of proinflammatory and oxidative stress substances from the peripheral blood into the brain in patients with BD (Patel and Frey, 2015). Also, a positron emission tomography scan study reported that there is neuroinflammation in the brain of patients with BD (Haarman et al., 2014). Second, the sample size is small. Third, the patients were on medication, which could potentially change the serum biological markers. However, we observed that 24h treatment with different medications has no detrimental effects in the cell viability in our study (data not shown). Moreover, there was no difference between medication status between patients at the early and late stages (see Table 1). Future research with drug-free patients would be important to evaluate the effect of drugs in this model. Finally, studies with large sample sizes are also needed to replicate our findings.

**Table 1.** Clinical and Demographic Characteristics of Controls and Euthymic Patients at Early vs. Late Stages of Bipolar Disorder

Euthymic patients	Early (n = 6)	Late (n = 6)	Control (n = 6)	$p$
Age (years)	48.2 $\pm$ 4.7	49.0 $\pm$ 5.0	48.8 $\pm$ 5.1	0.904 <sup>a</sup>
Gender (male/female)	2/4	2/4	2/4	1.00 <sup>b</sup>
Duration of illness	24.3 $\pm$ 11.29	21.5 $\pm$ 5.6	n/a	0.595 <sup>c</sup>
Number of episodes	5.67 $\pm$ 3.5	15.83 $\pm$ 7.02	n/a	0.010 <sup>c</sup>
Medications (%)				
Mood stabilizers	83.3%	50%	n/a	0.545 <sup>d</sup>
Antidepressants	16.6%	0%	n/a	1.00 <sup>d</sup>
Atypical antipsychotics	16.6%	33.3%	n/a	1.00 <sup>d</sup>
Typical antipsychotics	0%	16.6%	n/a	1.00 <sup>d</sup>
Benzodiazepines	16.6%	0%	n/a	1.00 <sup>d</sup>

<sup>a</sup>analysis of variance, data expressed as mean  $\pm$  standard deviation; <sup>b</sup>chi-square test; <sup>c</sup> independent-samples t-test, data expressed as mean  $\pm$  standard deviation;

<sup>d</sup>Fisher's Exact Test. n/a = not applicable.



**Figure 1.** Protocol design, neurite density, and cell viability of retinoic acid (RA)-differentiated SH-SY5Y cells challenged with bipolar disorder (BD) serum. (A) RA differentiation protocol of human SH-SY5Y cells. At day 0, the exponentially-growing proliferative SH-SY5Y cells (ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM) / F12 (1:1) medium supplemented with 2 mM glutamine, 100  $\mu\text{g}/\mu\text{L}$  gentamycin, and 0.25 mg/mL amphotericin B and containing 10% fetal bovine serum (FBS) and maintained in a humidified atmosphere with 5%  $\text{CO}_2$  at 37°C. After 24 hours (day 1), the previous medium was removed and a fresh medium containing 1% of FBS and 10  $\mu\text{M}$  of RA (differentiation medium) was added. Three days later (day 4), the differentiation medium was replaced by a fresh one. At day 7, SH-SY5Y cells were ready to perform the experiments of interest. The medium was replaced by fresh medium with 1% of serum of bipolar patients and controls, instead of FBS. The treatment lasted 24 h, when the endpoints were analyzed. (B) Representative phase contrast and fluorescent images of human RA-differentiated SH-SY5Y cells labeled with nuclear dye Hoechst 33342 (H) and anti- $\beta$ III tubulin ( $\beta$ III-T) treated with the serums of bipolar patients and controls. Merge is the combination of phase contrast, H, and  $\beta$ III-T images for analysis in the AutoQuant neurite software. Representative neurite segmentation shows the neurite density per cell body, identified by the AutoQuant neurite software. (C) Neurite density analysis. Comparison between bipolar patients and the control group ( $p = 0.0153$ ) and between late-stage patients and controls ( $p = 0.0089$ ) showed statistical differences. (D) Cell viability analysis. Comparison between bipolar patients and the control group did not show a statistical difference. However, when comparing the late-stage group of patients to the control group ( $p = 0.0075$ ) and late- and early-stage patients ( $p = 0.0290$ ) there was a statistical difference. Fetal bovine serum (FBS) was considered as 100% of cell viability. Data are presented as mean  $\pm$  standard deviation. A t-test and one-way ANOVA were performed when appropriate. \*Significant differences were considered when  $p < 0.05$ . \*\*Significant differences were considered when  $p < 0.01$ .



In summary, we analyzed the effect of exposure to the serum of patients with BD in human cells differentiated into neuron-like cells. Our results showed neurotoxic activity in the serum of BD patients, particularly late-stage patients. In addition, we developed a new experimental model using neuronal RA-differentiated human cell cultures.

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## Statement of Interest

Drs Wollenhaupt-Aguiar, Pfaffenseller, de Saraiva Chagas, Castro, Passos, and Klamt have no declaration of interest. Dr Kapczinski has received grants or research support from AstraZeneca, Eli Lilly, Janssen-Cilag, Servier, NARSAD, and the Stanley Medical Research Institute; has been a member of speakers' boards for AstraZeneca, Eli Lilly, Janssen, and Servier; and has served as a consultant for Servier. Dr Kauer-Sant'Anna is on speaker/advisory boards for, or has received research grants from, NARSAD, Stanley Medical Research Institute, CNPq-Universal, CNPq/INCT-TM, FIPE-HCPA, and Novartis.

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