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Brief Communication

Suberoylanilide hydroxamic acid increases progranulin production in iPSC-derived cortical neurons of frontotemporal dementia patients

Sandra Almeida [a], Fuying Gao [b,c], Giovanni Coppola [b,c], Fen-Biao Gao [a,*]

[a] Department of Neurology, University of Massachusetts Medical School, Worcester, MA, USA
[b] Department of Neurology, Semel Institute for Neuroscience and Human Behavior, David Geffen School of Medicine, University of California, Los Angeles, CA, USA
[c] Department of Psychiatry, Semel Institute for Neuroscience and Human Behavior, David Geffen School of Medicine, University of California, Los Angeles, CA, USA

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ABSTRACT

Mutations in the *granulin* (*GRN*) gene cause frontotemporal dementia (FTD) due to progranulin haploinsufficiency. Compounds that can increase progranulin production and secretion may be considered as potential therapeutic drugs; however, very few of them have been directly tested on human cortical neurons. To this end, we differentiated 9 induced pluripotent stem cell lines derived from a control subject, a sporadic FTD case and an FTD patient with progranulin S116X mutation. Treatment with 1 μM suberoylanilide hydroxamic acid (SAHA), a histone deacetylase inhibitor, increased the production of progranulin in cortical neurons of all subjects at both the mRNA and protein levels without affecting their viability. Microarray analysis revealed that SAHA treatment not only reversed some gene expression changes caused by progranulin haploinsufficiency but also caused massive alterations in the overall transcriptome. Thus, histone deacetylase inhibitors may be considered as therapeutic drugs for *GRN* mutation carriers. However, this class of drugs also causes drastic changes in overall gene expression in human cortical neurons and their side effects and potential impacts on other pathways should be carefully evaluated.

1. Introduction

Frontotemporal dementia (FTD), the second most common form of presenile dementia, is associated with degeneration in the frontal or temporal lobes and deficits in cognition, behavior, and language (Boxer and Miller, 2005). About 40% of FTD cases are familial and the nature of their genetic basis may help in the design of therapeutic approaches. Some forms of FTD can be caused by gain-of-toxic functions in disease genes, such as in the case of FTD linked to chromosome 3 (FTD-3; Skibinski et al., 2005), where mutant CHMP2B impairs multiple cellular pathways including the autophagy pathway (Filimonenko et al., 2007; Lee et al., 2007; Lu et al., 2013), endolysosomal signaling (Urwin et al., 2010; West et al., 2015), and miR-124 mediated regulation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (Gascon et al., 2014). In these cases, reducing the production of toxic mutant proteins may represent a potential therapeutic approach. On the other hand, FTD can also be caused through a loss-of-function mechanism. For instance, haploinsufficiency of progranulin (PGRN; Baker et al., 2006; Cruts et al., 2006) or TANK-binding kinase 1 (TBK1; Freischmidt et al., 2015; Pottier et al., 2015) also causes familial FTD. Thus, molecular interventions that can increase the expression level or activity of these proteins are potential avenues for therapeutic intervention.

The expression levels of intracellular and secreted PGRN are regulated through multiple mechanisms. For instance, microRNA-659, microRNA-29b, and microRNA-107 regulate PGRN expression posttranscriptionally (Jiao et al., 2010; Rademakers et al., 2008; Wang et al., 2010). PGRN production or secretion can be increased by inhibitors of vacuolar adenosine triphosphatase and some clinically used alkalizing drugs (Capell et al., 2011), as wells as by protein disulfide isomerases (Almeida et al., 2011). Extracellular levels of PGRN are also influenced by its rate of uptake through sortilin-mediated endocytosis (Carrasquillo et al., 2010; Hu et al., 2010) and small molecules that block PGRN-sortilin binding increase extracellular PGRN (Lee et al., 2014). Moreover, histone

* Corresponding author at: Department of Neurology, University of Massachusetts Medical School, Worcester, MA 01605, USA. Tel.: 508-856-8504; fax: 508-856-2811.
E-mail address: fen-biao.gao@umassmed.edu (F.-B. Gao).

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deacetylase (HDAC) inhibitors (including suberoylanilide hydroxamic acid, SAHA) have been shown to increase PGRN levels in cultured cell lines (Cenik et al., 2011). However, most of these drugs have not been tested directly on human cortical neurons.

We previously developed an induced pluripotent stem cell (iPSC) model of PGRN deficiency and identified a gene expression signature related to PGRN deficiency in iPSC-derived cortical neurons (Almeida et al., 2012). Here, we tested the effect of SAHA treatment on human cortical neurons differentiated from 9 iPSC lines and analyzed their gene expression profiles. We show that SAHA increases expression levels of *GRN* mRNA as well as intracellular and secreted PGRN in iPSC-derived human cortical neurons. Moreover, some gene expression changes observed in *GRN* mutant cortical neurons were reversed by SAHA treatment, which also caused massive alterations in the overall transcriptome. The implications of these findings are discussed.

2. Materials and methods

2.1. Differentiation of FTD-patient specific iPSCs into postmitotic neurons

The iPSC lines used in this study were generated and extensively characterized previously (Almeida et al., 2012). Neuronal differentiation into cortical neurons follows a 4-stage differentiation process: embryoid bodies (EBs), rosettes, neurospheres, and neurons. Briefly, iPSC colonies were dislodge with accutase (Millipore) and left in suspension to form EBs. At day 5, EBs were carefully broken up and attached to plates to initiate the formation of rosettes. Ten-day-old rosettes were isolated using dispase and grown in suspension as neurospheres. Neurospheres were dissociated after 3 weeks, and the cells were plated on glass coverslips (BD Biosciences) or plates coated with poly-D-lysine (0.1 mg/mL) and laminin (10 μg/m). Neurons were used after 2–4 weeks in culture.

2.2. SAHA treatment and PGRN measurements

Two-week-old neurons were incubated with 1-μM SAHA or dimethyl sulfoxide (DMSO; in fresh culture medium) for 24 hours. The medium was collected and cells were lysed with NP-40 buffer (20-mM Tris, pH 8, 137-mM NaCl, 2-mM EDTA, 1% NP-40) followed by 3 freeze-thaw cycles. To remove cell debris, the culture medium and the cell lysates were centrifuged at 12,000 rpm at 4 °C for 10 minutes. Cell lysate supernatants were assayed for protein concentration with the BioRad reagent assay. PGRN levels in total cell lysates and culture medium were determined with an ELISA kit (Alexis Biochemicals, San Diego, CA, USA) according to the manufacturer's instructions. Data were normalized to protein concentration.

2.3. Cell viability assay

Healthy control, sporadic FTD, and PGRN S116X neurons were incubated with 1-μM SAHA or DMSO for 24 hours and then assessed for cell viability. For the experiments using a stress inducer, PGRN S116X neurons (lines 1 and 14) were further incubated with 10-nM staurosporine (STS) or DMSO for additional 24 hours. Cell viability was determined with the water soluble tetrazolium-1 cell-proliferation assay (Roche Applied Science, Penzberg, Germany), according to the manufacturer's instructions. Values were expressed as the percentage of cells treated with DMSO (control).

2.4. RNA extraction and quantitative polymerase chain reaction

Total RNA was isolated with RNeasy kit (Qiagen) and reverse transcribed to cDNA with TaqMan reverse transcription reagent kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Quantitative polymerase chain reaction (PCR) was performed with SYBR Green Master Mix (Applied Biosystems) and forward and reverse primers (*GRN*-forward—tgcaccaccacctgcttagc; *GRN*-reverse—acggtaaagatgcaggagtgg; *GAPDH*-forward—tgcaccaccacctgcttagc; *GAPDH*-reverse—ggcatggactgtggtcatgag). Ct values for each sample and gene were normalized to *GAPDH*.

2.5. Microarray analysis

Total RNA was extracted from iPSC-derived cortical neurons using standard protocols. At least 3 replicates were run per sample category: sporadic FTD, PGRN S116X mutation carrier and control, and 3 treatment conditions (untreated, DMSO, and SAHA) for a total of 80 arrays. RNA quantity was assessed with Nanodrop (Nanodrop Technologies) and quality with the Agilent Bioanalyzer (Agilent Technologies). As per the manufacturer's protocol, 200 ng of total RNA were amplified, biotinylated, and hybridized to Illumina Human Expression Beadchips V4, querying the expression of approximately 47,000 transcripts. Raw data were analyzed by using Bioconductor packages as previously described (Coppola, 2011). Quality assessment was performed by examining the interarray Pearson correlation and clustering based on the top variant genes was assessed. Three arrays resulted outliers and were excluded from subsequent analyses. Contrast analysis of differential expression was performed by using the LIMMA package (Smyth, 2005). After linear model fitting, a Bayesian estimate of differential expression was calculated, and a false discovery rate was estimated and q-value threshold was set at 0.05, corresponding to a 5% false discovery rate. To identify changes specific to *GRN* mutation carriers (*GRN* signature), we compared PGRN S116X samples to sporadic FTD and control combined. To identify treatment-related effects, we compared SAHA-to DMSO–treated cells. Gene Ontology analysis was performed using DAVID (http://david.abcc.ncifcrf.gov/). Raw and normalized array data will be deposited within the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo/).

2.6. Statistical analysis

Values are expressed as mean ± standard error mean. The significance of differences among multiple groups was determined with a 1-way analysis of variance followed by a Tukey-Kramer post hoc test (GraphPad Prism version 5.04). Differences were considered significant at $p < 0.05$.

3. Results

We previously generated iPSC lines from skin fibroblast cells of a control subject, a sporadic FTD case and an FTD patient with the PGRN S116X mutation (Fig. 1A and B and Almeida et al., 2012). To examine the effect of SAHA on human cortical neurons, we differentiated 3 iPSC lines of each subject into postmitotic cortical neurons as described before (Fig. 1C). By performing quantitative PCR analysis, we confirmed that *GRN* mRNA level in cortical neurons with the PGRN S116X mutation is about 50% of that in control neurons or neurons derived from a sporadic FTD case (Fig. 1D). Treatment with 1-μM SAHA for 24 hours restored *GRN* mRNA level in cortical neurons with the PGRN S116X mutation to that of control neurons (Fig. 1D). As expected, SAHA also increased *GRN* mRNA level in cortical neurons without *GRN* mutations (Fig. 1D). Under these experimental conditions, cell viability of the neurons was not affected as measured by the water soluble tetrazolium-1 assay (Supplementary Fig. 1). At the protein level, SAHA treatment also increased the intracellular PGRN level in cortical neurons