Both a schizophrenia susceptibility gene, *NOS1AP*, and an antipsychotic agent, clozapine, stunt dendrite development in human iPSC-derived neurons

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Abstract

Abnormal dendritic arbor development has been implicated in a number of neurodevelopmental disorders, such as autism and Rett syndrome, as well as the neuropsychiatric disorder schizophrenia. NOS1AP, a protein encoded by a schizophrenia susceptibility gene, is an intracellular factor that alters neuronal morphology and synaptic structures. Brain samples from subjects with schizophrenia have elevated levels of NOS1AP in the DLPFC, a region of the brain associated with cognitive function. Furthermore, we previously reported that NOS1AP negatively regulates dendrite branching in rat hippocampal neurons. For patients with the *NOS1AP* schizophrenia risk factor, the cognitive deficits could be linked to neurodevelopmental defects. To investigate the role that NOS1AP plays in human dendritic arbor development, we used human iPSC technology to generate human neurons. We found that increased protein levels of NOS1AP decrease dendrite branching in human neurons at the developmental time point when both primary and secondary branching actively occurs. Next, we tested whether an antipsychotic drug is able to restore normal dendritogenesis in these human neurons. Preliminary results suggest that the antipsychotic agent clozapine decreases dendrite branching in the developing human neurons, both alone and when in combination with increased NOS1AP protein levels. Our studies show how an *in vitro* model of a schizophrenia vulnerability factor could be used as a platform to screen drugs for preventative therapies. In addition, this *in vitro* model of human dendrite development can be used to determine whether prenatal exposure to drugs that act in the CNS produce potentially lasting adverse effects on fetal neurodevelopment.

Materials and Methods

Human induced pluripotent stem cell (hiPSC) Derivation.
Human foreskin fibroblasts (HFFs) were infected with retroviruses containing cDNA of OCT4, SOX2, KLF4, and c-MYC. These four transcription factors integrated into the fibroblasts' genomes and after 24 days of expression resulted in the reprogramming of the cells into a pluripotent stem cells.

hiPSC differentiation to Neural Stem Cells (NSCs) and neurons.
hiPSCs were grown in medium supplemented with Noggin, a signaling molecule that plays a key role in neural induction. 18 days after neural induction, the medium was supplemented with Fibroblast Growth Factor, a growth factor that elicits neural stem cell proliferation. For differentiation into neurons, NSCs were grown in medium supplemented with Brain Derived Neurotrophic Factor, a growth factor that functions in the maturation of neurons.

Immunocytochemistry (ICC).
ICC was done on the cells to confirm cell type. Cells were fixed in methanol at -20˚ C for 15 min. Cells were permeabilized and blocked in 2% Normal Goat Serum and 0.1% Triton in PBS at room temperature for 1 hr. The cells were incubated with primary antibodies against either stem cell markers, NSC markers, or neuronal markders for 2 hrs, washed with 1xPBS twice, and then incubated with secondary antibodies with attached fluorophores for 1hr.

Western Blot.
12 µgs of protein from cell lysates scraped in TEE were loaded and resolved on a 12% SDS polyacrylamide gel and transferred to Immobilon P membrane (Millipore) in transfer buffer lacking SDS. The blot was probed with a rabbit antibody to NOS1AP, a mouse antibody to GAPDH, and visualized using ECL plus with a secondary antibody coupled to horseradish peroxidase.

Electrophysiology.
Three week old neurons were patch clamped in the whole cell configuration using thick walled glass pipettes with a resistance of 4-6 mega Ohms. To activate voltage gated ion channels, neurons were voltage clamped at -80mV, with a jump to -30 mV, and return to -80 mV. Additional jumps were made at 10 mV increments with the final jump to +30 mV. In current clamp mode, a depolarizing current injection was applied to activate voltage gated sodium channels to generate action potential activity.

Transfection and Cell Imaging.
At days differentiating (DD) 19, human neurons were transfected with pCAG-GFP, pCAG-GFP-NOS1AP-L, or pCAG-GFP-NOS1AP-S using Lipofectamine 2000. Immediately after transfection, DMSO or 3.0 µg/ml clozapine (therapeutic dosage) was added to the medium. 48 hours after transfection, cells were fixed with 4% PFA and stained for GFP and MAP2. For dendrite branching analysis, neurons that were both MAP2- and GFP-positive were imaged at 20x using an Olympus Optical IX50 microscope with a Cooke Sensicam CCD cooled camera, fluorescence imaging system, and ImagePro software. Neurons were blindly traced in NeuronJ (NIH, Bethesda, MD) and NeuronStudio (Mt. Sinai Medical School, NYC, NY). Data were analyzed using MatLab (MathWorks, Natick, MA). Sholl analyses were measured at 6 µm intervals and dendrites were counted if >3 µm.

Acknowledgements

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Background and Significance

- Schizophrenia results from a complex interaction between genetic and environmental factors.
- *NOS1AP* has been identified as a schizophrenia susceptibility gene.
- Postmortem brain samples from patients with schizophrenia have increased protein levels of NOS1AP (Figure 1) in the DLPFC, a brain region associated with cognitive function.
- Antipsychotics are most effective in treating the positive symptoms of schizophrenia, while there is little improvement in the negative or cognitive symptoms.
- What are the functional implications of increased levels of NOS1AP on dendritic arbor development in human neurons?
- Can a currently available antipsychotic agent restore normal dendrite development in human neurons (Figure 2)?

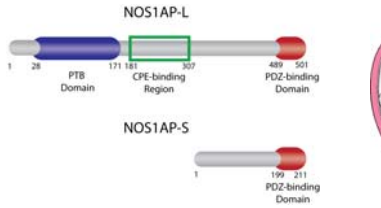

Figure 1. Domains of NOS1AP-Long and NOS1AP-Short. The long isoform of *NOS1AP* produces a protein with a PTB domain, CPE-binding region, and a PDZ-binding domain (NOS1AP-L). The short isoform of *NOS1AP* produces a protein with only the PDZ-binding domain (NOS1AP-S).

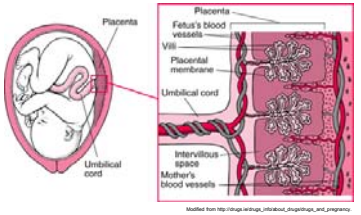

Figure 2. Drugs cross the placenta and reach the fetus. Numerous antipsychotics have demonstrated passage from the maternal to the placental circulation at varying levels. The effects of drugs on fetal development depend on the fetus' stage of development and the level of drug exposure.

Results

Reprogramming of human fibroblasts to human induced pluripotent stem cells

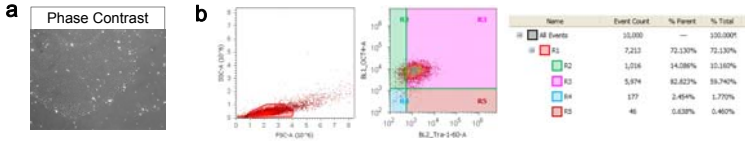

Figure 3. a. Image of a hiPSC colony. 10x. **b.** Flow cytometric analysis of hiPSCs for OCT4 and Tra-1-60.

Generation of human neural stem cells and functional neurons from hiPSCs

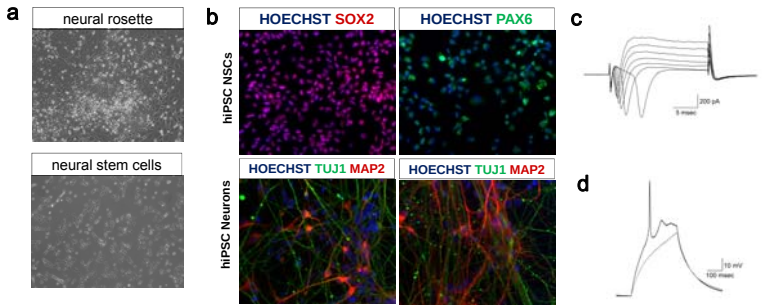

Figure 4. a. Phase contrast images of a human neural rosette and human neural stem cells (NSCs). 10x. **b. Top.** hiPSC derived NSCs express SOX2 (red) and PAX6 (green). Hoechst (blue). 10x. **Bottom.** hiPSC derived DD 14 (left) and DD 28 (right) neurons express TUJ1 (green) and MAP2 (red). HOECHST (blue). 40x. **c.** Human neurons show normal sodium and potassium currents when voltage-clamped. **d.** Human neurons show normal induced action potentials when current-clamped.

NOS1AP protein levels alter during dendritogenesis of differentiating hiPSC-derived neurons

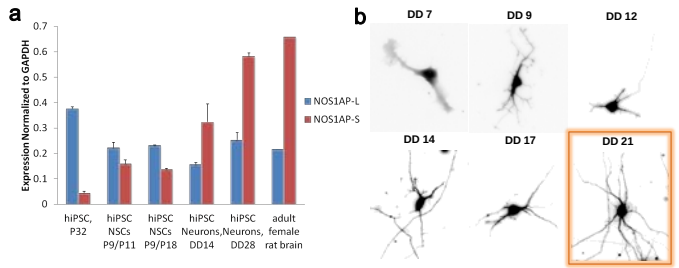

Figure 5. a. Densitometry analysis of cell lysates from various hiPSC-derived cell types via immunoblotting for two isoforms of NOS1AP. **b.** Pattern of neurite growth monitored through transfection of hiPSC-derived NSCs with a vector encoding peGFP. Active primary and secondary branching occurs at DD21 in developing hiPSC-derived neurons.

NOS1AP-L and -S negatively regulate dendrite branching in hiPSC-derived neurons

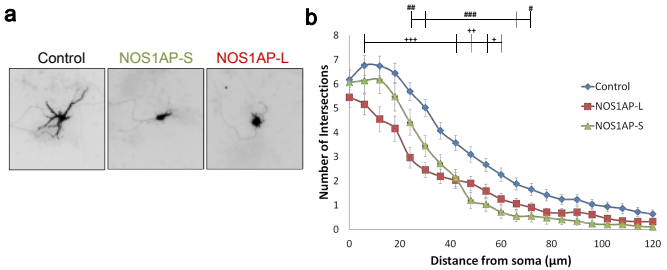

Figure 6. NOS1AP-L and -S expression in hiPSC-derived neurons for 48 h decreases dendrite branching. **a.** GFP images of representative neurons transfected at DD19 with GFP, NOS1AP-S, or NOS1AP-L and then fixed and immunostained for dendrite counting at DIV 21. **b.** Proximal Sholl analysis within the first 120 µm from the soma. # Control vs NOS1AP-S, + Control vs NOS1AP-L, # or + $p < 0.05$, ## or ++ $p < 0.01$, ### or +++ $p < 0.001$. p values were determined by two-way ANOVA followed by Bonferroni multiple-comparisons test. Error bars indicate SEM. n = 52 neurons, Control; n = 31, NOS1AP-L; n= 29, NOS1AP-S.

Treatment with clozapine trends toward a reduction in dendrite branching

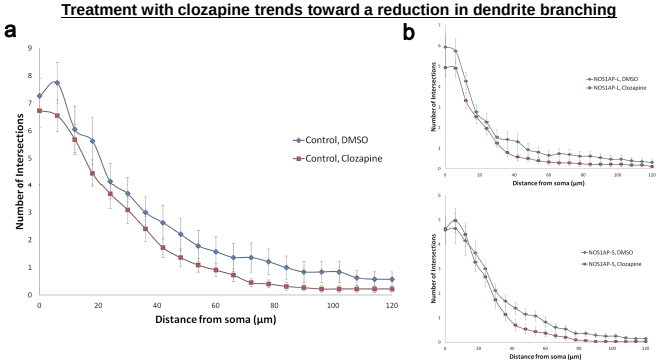

Figure 7. Treatment of hiPSC-derived neurons with clozapine for 48 hours trends toward a reduction in dendrite branching. All figures represent proximal Sholl analysis within the first 120 µm from the soma. **a.** Neurons transfected with GFP were treated with either vehicle or clozapine. **b.** Neurons transfected with either NOS1AP-S or NOS1AP-L were treated with either vehicle or clozapine. Error bars indicate SEM. n = 19 neurons, Control, DMSO; n = 22, Control, Clozapine; n = 28, NOS1AP-S, DMSO; n = 30, NOS1AP-S, Clozapine; n = 26, NOS1AP-L, DMSO; n = 28, NOS1AP-L, Clozapine.

Conclusions

- Symptoms observed in patients with schizophrenia could be due to neurodevelopmental defects that occurred *in utero* caused by increased levels of NOS1AP protein.
- The developed *in vitro* model of a schizophrenia vulnerability factor can be used as a platform to screen drugs for preventative therapies.
- Prenatal exposure to drugs has the potential to be beneficial or detrimental to fetal neurodevelopment.