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Patch-clamp recordings and calcium imaging followed by single-cell PCR reveal the developmental profile of 13 genes in iPSC-derived human neurons☆

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Abstract Molecular genetic studies are typically performed on homogenized biological samples, resulting in contamination from non-neuronal cells. To improve expression profiling of neurons we combined patch recordings with single-cell PCR. Two iPSC lines (healthy subject and 22q11.2 deletion) were differentiated into neurons. Patch electrode recordings were performed on 229 human cells from *Day-13* to *Day-88*, followed by capture and single-cell PCR for 13 genes: *ACTB*, *HPRT*, *vGLUT1*, *βTUBIII*, *COMT*, *DISC1*, *GAD1*, *PAX6*, *DTNBP1*, *ERBB4*, *FOXP1*, *FOXP2*, and *GIRK2*. Neurons derived from both iPSC lines expressed *βTUBIII*, fired action potentials, and experienced spontaneous depolarizations (UP states) ~2 weeks before *vGLUT1*, *GAD1* and *GIRK2* appeared. Multisite calcium imaging revealed that these UP states were not synchronized among hESC-H9-derived neurons. The expression of *FOXP1*, *FOXP2* and *vGLUT1* was lost after 50 days in culture, in contrast to other continuously expressed genes. When gene expression was combined with electrophysiology, two subsets of genes were apparent; those irrelevant to spontaneous depolarizations (including *vGLUT1*, *GIRK2*, *FOXP2 and DISC1*) and those associated with spontaneous depolarizations (*GAD1* and *ERBB4*). The results demonstrate that in the earliest stages of neuron development, it is useful to combine genetic analysis with physiological characterizations, on a cell-to-cell basis.

Abbreviations: AP, action potential; MEFs, mouse embryonic fibroblasts; EB, embryoid bodies; NE, neuroepithelial (rosettes); CC, current clamp; VC, voltage clamp; V_R, resting membrane potential; R_{IN}, input resistance.

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Introduction

Question

An understanding of the molecular genetic basis of psychiatric disorders will be enhanced by studying gene regulation in postmitotic human fetal neurons (Girard et al., 2012; Moore et al., 2011b). However, when geneticists analyze the neuronal transcriptome, the brain tissue contains many non-neuronal cell types, including astrocytes, oligodendrocytes, microglia, blood cells, capillaries, endothelium and ependymal cells (Iwamoto et al., 2005; Mirnics et al., 2006; Mudge et al., 2008; Rollins et al., 2009), causing an obvious contamination and dilution of the neuronal transcriptome. Here we ask if gene analysis could be combined with electrophysiology to eliminate the contamination by non-neuronal cells. We propose a strategy in which cells are patched (patch-clamp), characterized electrophysiologically, stored individually and then analyzed for genes of interest using single-cell PCR. If successful, such a strategy would allow one to compare the gene expression profiles between healthy and affected individuals while focusing only on positively-identified neurons. This would not be the only benefit of patch electrode recordings. Neurons are composed of many subtypes. It is possible that genetic abnormalities in schizophrenia, for example, exist only in one particular neuron subtype (e.g. GABAergic interneurons (Benes et al., 1991)), or at one particular time point of development, and at one particular neuronal maturation stage (e.g. immature neuron (Jaaro-Peled et al., 2009)). Patch-clamp recordings can potentially segregate neurons between neuronal subtypes and maturation stages (McCormick et al., 1985; Moody & Bosma, 2005; Moore et al., 2009), and combined with single-cell PCR (Esumi et al., 2006; Morris et al., 2011; Stahlberg & Bengtsson, 2010; Vullhorst et al., 2009), these important questions can be tackled.

iPSCs

Major ethical and practical obstacles preclude the use of human brains in experiments. Recent advances in stem cell technology may solve this problem (Takahashi et al., 2007). Briefly, a small skin sample is taken from a human subject and skin cells are converted into induced pluripotent stem cells (iPSCs) and later differentiated into neurons (Pedrosa et al., 2011). This method allows researchers to have access to developing human neurons, to perform molecular characterizations of neurons at different maturation stages, and also to perform electrophysiological measurements on living human neurons. Human neurons differentiated from iPSCs harvested from both healthy and diseased human subjects (Brennand & Gage, 2012; Ming et al., 2011; Pedrosa et al., 2011) may bring us closer to understanding mental diseases with strong neurodevelopmental components (Currenti, 2010; Jaaro-Peled et al., 2009; Karlsgodt et al., 2008; Lewis & Levitt, 2002; Rapoport et al., 2005).

22q11.2 deletion

Although there are limitations with iPSC-derived neurons, such a system guarantees that the proteins and potential regulatory mechanisms of humans are encoded in the cellular preparation, which can be used in controlled experimental conditions. In this study we used two iPSC lines. One line was derived from a control subject without mental disease. The other iPSC line was derived from a schizophrenia patient with a deletion on 22q11.2. A patient with 22q11.2del was chosen for this study because 22q11.2del is a distinct genetic abnormality, found in 1% of patients with schizophrenia (Bassett & Chow, 2008). Instead of using a typical molecular genetic approach (tissue homogenization), here we performed patch electrode recordings on individual neurons, characterized their maturation stage physiologically, captured their cell bodies one at a time, and then performed single cell qPCR analysis of mRNA expression.

Materials and methods

Stem cell culture

Three stem cell lines were used in the current study (two iPSC lines and one human embryonic stem cell line (hESC H9)). The two iPSC lines were created at the Albert Einstein College of Medicine using the Yamanaka transcription factors (*c-MYC*, *SOX2*, *Klf4*, and *OCT4*) and both lines have been used in a previous report (Pedrosa et al., 2011). iPSC-01 (normal, passages 8–15) and iPSC-15 (22q11.2 deletion, passages 11–14, in reference called SZ22del15-6) cells were co-cultured with MEFs in ES media consisting of: 80% DMEM/F12, 20% KOSR, 1 mM glutamine, 1× NEAA, 4 ng/ml bFGF, and 7 nl/ml β-mercapto ethanol (Sigma, St. Louis). All cell culture reagents were from Invitrogen (Grand Island, NY) unless otherwise noted. hESC-H9 colonies were obtained from the University of Connecticut Stem Cell Core.

Neuronal differentiation of iPSCs

Stem cell lines were differentiated using a five stage protocol (Fig. 1A) with defined media constituents (Belinsky et al., 2011; Iacovitti et al., 2007). This protocol does not use typical brain region morphogens in Stages 4–5 (besides those present in B27), and so is associated with the default production of forebrain neurons (Zeng et al., 2010). Briefly, stem cell colonies (Stage 1) were disassociated by collagenase, and stem cell aggregates were incubated for four days in ES media without bFGF on Ultralow adherence plates (Costar, Wilkes Barre, PA) (Stage 2). 5 μM dorsomorphin (Chemdea, Ridgewood, NJ) and 5 μM SB431542 (Ascent, Princeton, NJ) were added to the media from *Days 1* to *8*. Stem cell aggregates were then seeded on dishes coated with 1:100 Geltrex and allowed to expand for 4–8 days in NEP-basal medium (Stage 3) until neuroepithelial colonies appeared. NEP-basal medium consisted of DMEM/F12, 1 mg/ml BSA (Sigma), 1× N2, 1× B27 supplements, and 1× penicillin/streptomycin/anti-mycotic. 30 min before selecting the resultant neuroepithelial colonies, 0.66 mg/ml ROCK inhibitor (Y27632, Wako USA, Richmond, VA) was added to the media. Colonies with neuroepithelial morphology were removed by trituration and seeded with 0.66 mg/ml ROCK inhibitor on glass cover slips coated with 1:100 Geltrex. Cells were grown in NEP-basal medium with 20 ng/ml bFGF for 7 days (Stage 4). Cells were then maintained in NEP-basal medium in the presence of