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Efficient derivation of cortical glutamatergic neurons from human pluripotent stem cells: A model system to study neurotoxicity in Alzheimer's disease☆

Tandis Vazin [a,b,c,h], K. Aurelia Ball [d,h], Hui Lu [c,e,h], Hyungju Park [c,e,h], Yasaman Ataeijannati [f,h], Teresa Head-Gordon [a,b,d,g,h], Mu-ming Poo [c,e,h], David V. Schaffer [a,b,c,h,*]

[a] The Department of Chemical and Biomolecular Engineering, USA
[b] The Department of Bioengineering, USA
[c] Helen Wills Neuroscience Institute, USA
[d] Graduate Group in Biophysics, USA
[e] Division of Neurobiology, Department of Molecular and Cell Biology, USA
[f] Department of Molecular and Cell Biology, USA
[g] Department of Chemistry, USA
[h] University of California, Berkeley, CA 94720, USA

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ABSTRACT

Alzheimer's disease (AD) is among the most prevalent forms of dementia affecting the aging population, and pharmacological therapies to date have not been successful in preventing disease progression. Future therapeutic efforts may benefit from the development of models that enable basic investigation of early disease pathology. In particular, disease-relevant models based on human pluripotent stem cells (hPSCs) may be promising approaches to assess the impact of neurotoxic agents in AD on specific neuronal populations and thereby facilitate the development of novel interventions to avert early disease mechanisms. We implemented an efficient paradigm to convert hPSCs into enriched populations of cortical glutamatergic neurons emerging from dorsal forebrain neural progenitors, aided by modulating Sonic hedgehog (Shh) signaling. Since AD is generally known to be toxic to glutamatergic circuits, we exposed glutamatergic neurons derived from hESCs to an oligomeric prefibrillar forms of Aβ known as "globulomers", which have shown strong correlation with the level of cognitive deficits in AD. Administration of such Aβ oligomers yielded signs of the disease, including cell culture age-dependent binding of Aβ and cell death in the glutamatergic populations. Furthermore, consistent with previous findings in postmortem human AD brain Aβ-induced toxicity was selective for glutamatergic rather than GABAeric neurons present in our cultures. This in vitro model of cortical glutamatergic neurons thus offers a system for future mechanistic investigation and therapeutic development for AD pathology using human cell types specifically affected by this disease.

Introduction

AD is a neurodegenerative disorder characterized by an abundance of Aβ peptides generated from amyloid precursor abnormal cleavage by membrane-associated secretases (Murphy and LeVine, 2010). The development of AD pathology precedes cognitive symptoms and diagnosis by many years (Lazarczyk et al., 2012), presenting challenges for studying early disease stages to aid in the discovery of preventive drugs.

Over the past decade numerous transgenic animal models of AD have been generated to aid in understanding mechanisms of the disease in humans (Gotz and Ittner, 2008). However, such animals must express multiple pathological proteins at levels higher than endogenous genes to exhibit AD pathology, highlighting the value of developing complementary models to enable investigations in human cells with gene expression patterns closer to endogenous levels. In particular, in vitro models derived from hPSCs offer strong platforms for basic research and subsequent therapeutic development for early stages of AD.

Tapping into this potentially exciting new class of disease models requires efficient differentiation of hPSCs into neurons affected by AD. In particular, glutamatergic neurons are severely afflicted in the cerebral cortex, and disruption of their circuits is associated with the hallmark memory deficits of AD (Francis et al., 1993; Greenamyre et al., 1988). In contrast, studies of human postmortem AD brain suggest that

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* Corresponding author at: 278 Stanley Hall, University of California, Berkeley, CA 94720, USA.
E-mail address: schaffer@berkeley.edu (D.V. Schaffer).

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GABAergic neurons are spared from death (Rossor et al., 1982). To date, the sensitivity of human cortical glutamatergic neurons to Aβ has not been studied within in vitro models, and such efforts would benefit from renewable sources of glutamatergic neurons derived from hPSCs that could serve as human disease models of AD. We have thus developed an hPSC-based system to examine how Aβ neurotoxicity affects enriched populations of both human cortical glutamatergic and GABAergic neurons.

During development, glutamatergic neurons are generated from the dorsal telencephalon, whereas GABAergic neurons emerge from the ventral telencephalic region (Wilson and Rubenstein, 2000), due in part to Shh-mediated patterning. Here, we show that hESCs and iPSCs differentiated to a dorsal phenotype, with the aid of Shh pathway inhibition and stimulation with FGF-2, primarily give rise to glutamatergic neurons. In contrast, without Shh inhibition, NPCs adapted a ventral phenotype and primarily gave rise a GABAergic fate.

AD is primarily characterized by Aβ plaques; however, amyloid plaque load shows a weak correlation with dementia in AD (Naslund et al., 2000). By comparison, soluble Aβ oligomer levels correlate more closely with AD pathology (Kuo et al., 1996). Thus, a stable, oligomeric Aβ form called "Aβ globulomers" has been prepared and increasingly studied (Barghorn et al., 2005, Gellermann et al., 2008). While human AD brain neuropathology studies show elevated levels of Aβ oligomers surrounding cortical neuronal processes, which may cause synaptic impairment (Viola et al., 2008), the differential neurotoxic effects of these oligomers on different human cortical neuronal populations remain to be elucidated. Such results may enhance our understanding of the contribution of this toxic species to the disease process, aid future elucidation of molecular mechanisms for its actions, and help resolve differences in disease progression between for example familial AD and corresponding animal models (Gotz and Ittner, 2008).

In this study, the derivation of human cortical cultures primarily comprised of glutamatergic neurons enabled investigation of the recently proposed role of Aβ globulomers in AD pathology. Specifically, this intermediate form of Aβ was toxic to human glutamatergic neurons in a cell culture age-dependent manner. Furthermore, the results show that Aβ globulomers exert a selective neurotoxicity for glutamatergic rather than GABAergic neuronal populations.

Materials and methods

Cell culture

The H1 (WiCell) and HSF6 (UC San Francisco) hESC lines, and the MSC and fibroblast derived iPSC lines (a kind gift from George Q. Daley, Children's Hospital Boston, Boston, MA), were cultured on Matrigel-coated cell culture plates (BD) in mTeSR1 maintenance medium (Stem Cell Technologies). NPCs were isolated from the hippocampus of adult female Fischer 344 rats (Palmer et al., 1999) and cultured in medium containing DMEM/F12 (Invitrogen) supplemented with N2 (Invitrogen) and 20 ng/ml FGF-2 (Peprotech). Neuronal differentiation of adult rat NPCs was achieved by withdrawing FGF-2 and adding 1 μM retinoic acid and 5 μM forskolin for 5 days.

Cortical differentiation of human pluripotent stem cells

In adherent conditions, hPSCs were seeded at a density of 5×10^4 cells/cm^2 in growth medium. At 50% confluence, the medium was gradually changed to neural basal medium (Invitrogen) containing N2 and B27 (Invitrogen). SMAD signaling inhibitors LDN193189 (Stemgent, 1 μM) and SB432542 (Tocris Biosciences, 10 μM) were added from day 1 to day 7 of neural induction. Cyclopamine (Calbiochem, 400 ng/ml) and FGF-2 (Peprotech, 10 ng/ml) were added from days 3 to 14 of differentiation. After 12–14 days, cells were mechanically passaged into poly-L-ornithine (Sigma Aldrich) and laminin (Invitrogen, 20 μg/ml) coated plates and allowed to undergo maturation for an additional 3–6 weeks. BDNF (10 ng/ml, Peprotech) was added to cultures one week after initiation of this neuronal maturation. For EB mediated neural differentiation, PSCs were aggregated for 4 days in ultra low-attachment plates (Corning) and then seeded on Matrigel-coated plates. Cyclopamine (5 μM) and FGF-2 (10 ng/ml) were added to the cultures the following day until day 12 of neural induction. At day 14, structures with a rosette-like morphology were mechanically isolated and plated on poly-L-ornithine and laminin coated plates and allowed to undergo neuronal maturation for 4 weeks. BDNF (10 ng/ml) was added to the cultures one week after rosette isolation.

Gene expression analysis by RT-PCR

Complementary DNA was synthesized from 1 μg total RNA, isolated at day 12 of neural induction and day 40 of neuronal maturation, using random primers and MultiScribe Reverse Transcriptase (Applied Biosystems) in a 20 μl reaction according to the manufacturer's recommendations. The PCR analysis was carried out with Taq DNA polymerase (New England Biolabs). Equal amounts of RNA were tested in PCR reactions under the same conditions to verify the absence of amplification of genomic DNA. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was amplified as an internal control in gene expression analysis. Primer sequences (Table 1) were obtained from the PrimerBank website (http://pga.mgh.harvard.edu/primerbank/) and synthesized by Life Technologies.

Immunocytochemistry

Cultures were fixed with 4% paraformaldehyde or 3% paraformaldehyde and 2% glutaraldehyde for 10–15 min. The primary antibodies used were: mouse anti-nestin (1:50; R&D Research), rabbit anti-Pax6 (1:200; Covance), rabbit anti-BF1 (1:100, Abcam), mouse anti-Otx2 (1:50, R&D Systems), mouse anti-MAP2 (1:500, BD Biosciences), rabbit anti-glutamate (1:1500, Sigma Aldrich), rabbit anti-GBA (1:2000, Sigma Aldrich), rabbit anti VGluT1 (1:3000, Synaptic Systems), rabbit anti-TBR1 (1:1000, Millipore), rabbit anti-CUX1 (1:1000, Santa Cruz Biotechnology), rat anti-CTIP2 (1:1000, Abcam), rabbit-anti Aβ (1:500, Millipore), and rabbit anti-cleaved caspase-3 with Alexa Fluor 488 Conjugate (1:100, 1:50, Cell Signaling). For co-staining with antibodies against Aβ and glutamate or GABA, the Aβ primary antibody was conjugated to Alexa 647 dye (Invitrogen). Cultures were (with the exception of cultures stained with labeled caspase-3 and Aβ) incubated with fluorescent-labeled secondary Alexa 594-conjugated anti-rabbit and Alexa 488-conjugated anti-mouse antibodies in PBS containing 1% BSA for 1 h (1:1000, Invitrogen). Cultures were counter-stained with DAPI (Molecular Probes) and imaged using a Zeiss Axio Observer A1 inverted microscope.

Electrophysiology

Whole-cell recording was made from neurons after 50 days of neuronal maturation using a patch clamp amplifier (MultiClamp 700B, Axon Instr.) under infrared differential interference contrast optics. Microelectrodes were made from borosilicate glass capillaries, with a resistance of 4–5 MW. To apply glutamate, air pulses (10 ms duration at 1 Hz) were applied by Picrospritzer III (Parker Hannifin) to a glass pipette with the tip of ~5 μm in diameter. Generally, EPSPs induced by puffed glutamate were recorded at −70 mV in current-clamp mode. For recording action potentials, cells were held at −70 mV in voltage-clamp mode. The intracellular solution for whole-cell recording of EPSPs and action potentials contained (in mM) 140 potassium gluconate, 5 KCl, 10 HEPES, 0.2 EGTA, 2 $MgCl_2$, 4 MgATP, 0.3 Na_2GTP and 10 Na_2-phosphocreatine, pH 7.2 (adjusted with KOH).

For recording spontaneous EPSCs (sEPSCs), cells were pre-treated with the extracellular bath solution containing 50 μM picrotoxin to

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