SCREENING IN IPSC DERIVED NEURONS FOR MODIFIERS OF ALZHEIMER'S DISEASE RELATED PHENOTYPES

charles river

Shushant Jain [1], Malika BsiBsi [1], Dirk Duinsberg [1], Niki Heuvelmans [1], David F. Fischer [2], Jeroen De Groot [1]
[1] Charles River, Leiden, The Netherlands
[2] Charles River, Chesterford Research Park, United Kingdom

1 ABSTRACT

Alzheimer's Disease (AD) is a complex disorder with increasing prevalence and socio-economic burden. Clinically characterized by severe cognitive decline with major deficits in memory, speech and movement.
The majority of therapeutic strategies for AD were aimed at reducing Aβ production or enhancing Aβ or TAU clearance as these two proteins make up the amyloid plaques and neurofibrillary tangles commonly found upon pathological analysis of brains from AD patients. Continued lack of clinical validation of these targets by candidate drugs has necessitated alternative options for therapeutic intervention. Using a multi-parametric high content phenotypic readouts with neurons derived from human differentiated iPSCs with familial AD mutations, we aim to optimize a platform for CRISPR based rescue screens for the various phenotypes associated with the mutations, such as endo-lysosomal transport, synaptic dysfunction and neuronal toxicity. The multiple-phenotypic rescue approach will enable identification novel key pathways and/or targets which could serve has drug candidates for the treatment of AD

2 METHODS

hiPSCs culture: hiPSCs lines derived from healthy subjects were procured from the Regenerative Medicine Program and AD hiPSCs were procured from Coriell. Presence of the familial mutations were confirmed by Sanger sequencing and droplet digital PCR.
All cells were tested for viability, sterility, cell line identity, karyotype and expression of pluripotency markers prior to the creation of master and working cell banks. hiPSCs were cultured on matrigel as described by StemCell Technologies (Maintenance of Human Pluripotent Stem Cells in mTeSRTM1 manual) using mTeSR1 as the cell culture media and gentle dissociation agent for passaging. If spontaneous differentiation was observed, ReLeaSer (StemCell Technologies) was used to maintain an undifferentiated culture.
Generation of NGN2 inducible stable iPSCs: hiPSCs from healthy controls and AD donors were transduced with lentivirus encoding NGN2 and placed under antibiotic selection at pre-determined concentrations for 10 days to generate a polyclonal stable pool of hiPSCs inducibly expressing NGN2
Differentiation of NGN2-stable iPSCs to cortical neurons: hiPSCs were re-plated as single cells and expression of NGN2 was induced by the addition of doxycycline to differentiate the iPSCs to neuronal precursors (NPC). 4 days after single seeding and induction of NGN2, NPCs, were trypsinized and re-plated in to PLO/matrigel coated 96 well plates at a density of 30,000 cells/cm^2. 2 days post seeding of the NPCs, cultures were treated with a pre-determined optimal concentration of Ara-C to prevent the emergence of astrocytes.

2 METHODS (CONT)

CRISPR knockout of target gene: NPCs were nucleofected (Amaxa) with the high fidelity Cas9 Nuclease (IDT) and a pool of guide RNAs (gRNAs; Synthego) to generate the knockout of the target gene. Knockout of the target gene was confirmed by sanger sequencing and ICE analysis (Synthego) and was estimated to be 98%.
Immunocytochemistry (ICC): hiPSC-derived neurons were fixed using methanol free 4% paraformaldehyde in PBS pH 7.4 for 10 min at room temperature. Samples were permeabilised for 10 mins with PBS containing 0.1% Triton X-100. Cells were washed in PBS, three times for 5 min. Cells were blocked in PBST containing 10% fetal bovine serum (FBS) for 1hr at RT. After washing 3 x times with PBST, all primary antibodies were incubated with the cells O/N at 4°C in blocking buffer. After washing 3 x times with PBST, all secondary were incubated with the cells for 1hr at RT, washed 3 times with PBST and counterstained with DAPI.
High content imaging and image analysis: Images were acquired using an InCell 6000 (GE Healthcare) using a plan-apochromat 40x objective and the appropriate excitation and emission lasers for the fluorophores
Image quality control: only well-segmented cells were included. Apoptotic and out-of-focus cells were excluded. Cells touching the border of the image were removed to avoid analysis of artificially cropped cells. Custom image analysis algorithms were developed in InCell Developer (V6.3.2) software to quantify multiple phenotypic features such as neurite length, vesicle size, shape, area and distribution
Statistical analysis: All statistical analysis was performed in GraphPrism.

3 RESULTS

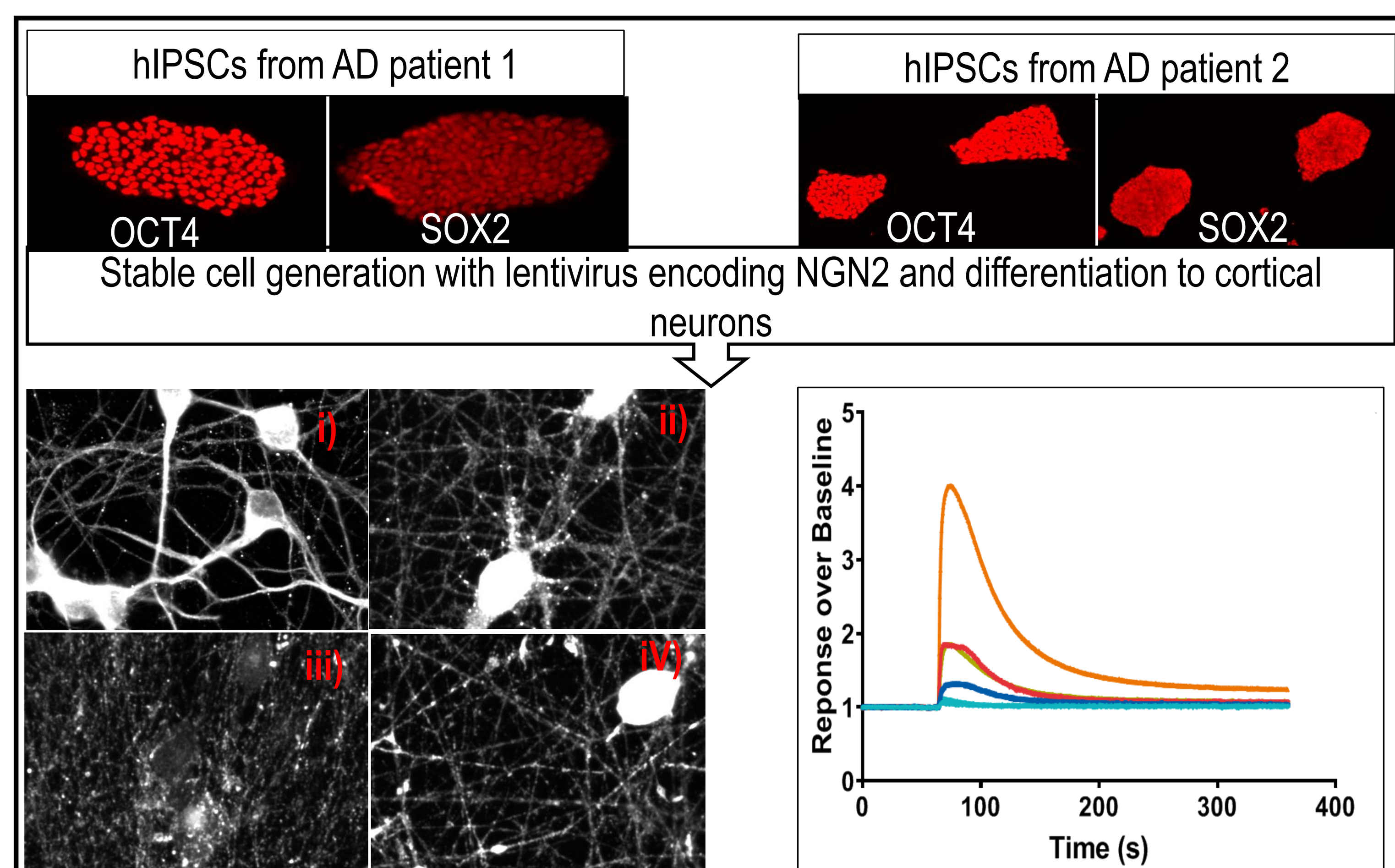

Figure 1: Quality control of patient derived iPSCs and differentiation to cortical neurons via overexpression of NGN2
iPSCs were cultured as described above and stained for pluripotency markers OCT4 and SOX2, NANOG and TRA-160 (data not shown). Polyclonal stable iPSCs, inducibly expressing NGN2, were generated and subsequently differentiated for a total of 28 days. Differentiated neurons were stained for the cortical markers Bassoon (i), Homer1 (ii), Synaptophysin (iii) and Synapsin (iv). Calcium flux experiments were also performed to confirm neurons have active synapses

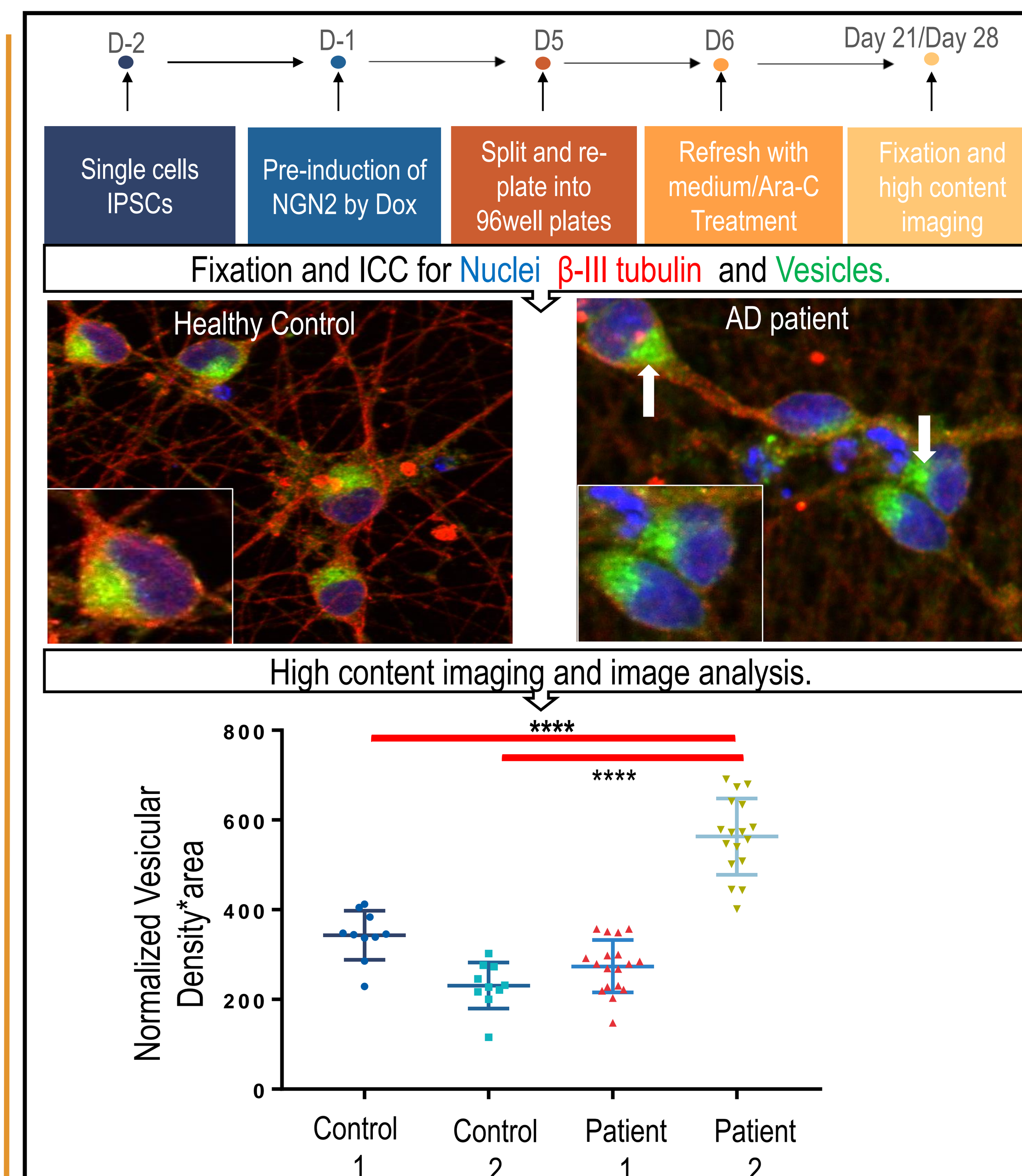

Figure 2: Quantification of vesicle size and area in neurons differentiated from control and AD patient iPSCs
iPSCs were differentiated for a total of 28 days. Differentiated neurons were stained for β-III-tubulin and a specific vesicular marker. Fixed cells were imaged and image analysis performed to extract a number features related to vesicular number, area, density and distribution within the soma and neuritic structures.
There was a significant accumulation of vesicles were observed in neurons from AD patients compared to neurons from healthy iPSCs (indicated by white arrows). A Mann Whitney test was performed to determine significance (**** $P < 0.0001$)

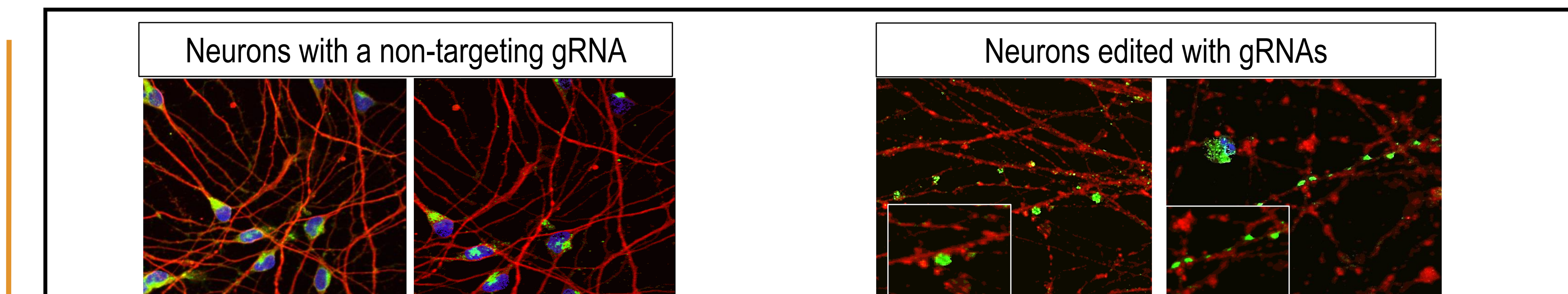

Figure 3: CRISPR knockout of a target gene leads to stalling and accumulation of enlarged vesicles in neurons
NPCs were nucleofected with either a non-targeting gRNA (left) or a pool gRNAs targeting a specific gene (right). NPCs were differentiated to neurons and stained for Nuclei, β-III tubulin and Vesicles. Knockout of the target gene caused axonal and pre-synaptic accumulation of vesicles similar to that observed in AD brains (Krstic & Knuesel, Nat Rev Neurol, 2013).

4 CONCLUSION

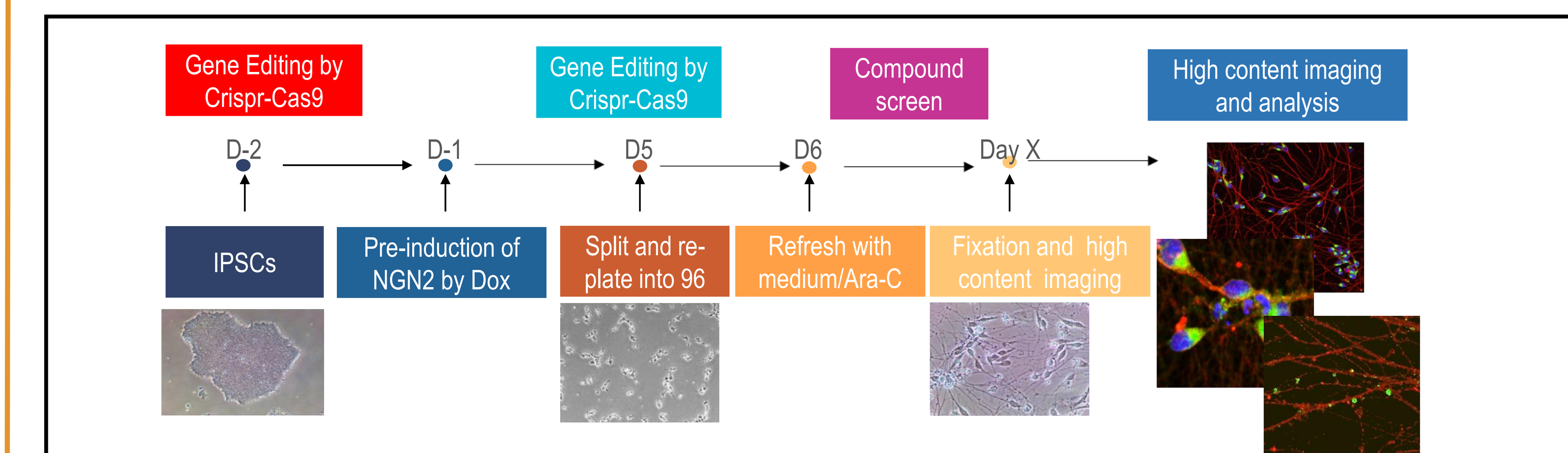

Figure 4: Scalable protocol for the generation of mature cortical neurons from human iPSCs which can modified by CRISPR for target identification or validation