"Identifying Clinically Relevant Compounds and their Molecular Targets Modulating the Neuronal Excitability in ARSACS Patients"

Dr. Mohan Babu
University of Regina

The iPSC line from ARSACS patients harboring the above-mentioned mutations and the corresponding isogenic controls were differentiated into MNs following previously established protocol. Upon completion of the protocol, iPSC-derived MNs were verified by neuronal (NFL^+) and motor neuron ($HB9^+$) markers. Furthermore, the efficiency of iPSC differentiation into MNs was assessed on the control iPSC line by counting the proportion of $NFL^+/HB9^+$ cells. Imaging the intracellular calcium wave is a key technique for measuring neuronal activity. The most commonly used method is to utilize genetic (GCaMP) or chemical (Fluo-4 AM) based calcium indicators and imaging calcium waves on a confocal microscope. However, calcium imaging in a high-throughput manner requires a specialized imaging platform or significant modification on a confocal microscope to facilitate live cell imaging in 96- or 384-well plates. Herein, we used a Ca^{2+} binding fluorescent probe (Fluo4-AM) and a multi-well plate reader to measure the intracellular calcium flux as a proxy to access MN activity in a medium throughput fashion. To access the hypo- or hyperexcitability of ARSACS MNs, iPSCs from patients and isogenic controls were seeded into 96 well plates in triplicate and allowed to differentiate. After completion of the differentiation protocol, the basal Ca^{2+} flux of the control and patient lines was measured using a GloMax Discover Microplate reader. Both the ARSACS iPSC-derived MNs lines showed altered intracellular calcium flux in comparison to the isogenic gene-corrected control, indicating the defective channels, mitochondrial and endoplasmic reticulum function and downregulation of calcium buffering proteins which are reported in cerebellar ataxia. A pilot study was performed to establish the experimental conditions to optimize drug screening assay and uncover the compounds modulating neuronal excitability. Briefly, ARSACS MNs carrying the *SACS* mutation and control were treated with randomly selected compounds from the FDA-approved compound library, in 96-well plates for 24 h, in triplicate, at 3µM concentration to balance potency and off-target effects. The intracellular calcium was monitored using the GloMax Discover Microplate reader as mentioned above and calcium flux was expressed as relative fluorescence units (RFU) compared to the DMSO-treated control. For the compound(s) to be considered hits, we set cutoffs (+/- 30%) based on a previous publication. So, the compound(s) that resulted in increased or decreased intracellular calcium flux greater than or equal to the set cutoff after 6 or 24 hrs treatment were considered a potential hit. In the primary screening, we tested 20% of the total compounds from the library thus far and identified a few potential hits that modulated the ARSACS neuronal excitability. These hits were then reconfirmed by a secondary screen using the same methodology. To discern compounds that induce changes in neuronal excitability due to toxicity, we assessed the relative efficacy and toxicity of the identified compounds at different concentrations and also determined the half-maximal inhibitory concentration (IC_{50}) based on alterations in calcium flux after 6 and 24 hours of treatment using a CellTiterGlo assay, which measures cellular ATP levels. This approach helped distinguish whether the observed alteration in neuronal activity is primarily due to cytotoxicity rather than a genuine impact on cell viability.

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