Functional alteration in gating behavior of IP3R channel mediating Calcium Signaling as common biomarker in Autism Spectrum Disorder

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Abstract

Background: Current clinical measures used in diagnosing Autism Spectrum Disorder (ASD) have been adapted to screen core characteristics that range widely in severity. However, even at its most refined, ASD remains a group of developmental disorders that is defined by behavioral qualities, not by its pathogenesis. Studies on the genetic architecture of ASD and related neurological disorders have brought light to the central role of calcium channelopathies and its effects on calcium signaling pathways rationally implicated in diverse aspects of ASD pathogenesis.

Objective: We propose that depressed function in IP3R mediated calcium release channels in the ER may be a reliable diagnostic biological marker for monogenic and 'sporadic' forms of ASD.

Methods: Functional components of calcium signaling were dissected in ASD and a super-resolution STORM system was used to obtain molecular-resolution optical patch clamp analysis on monogenic ASD models. Fibroblasts derived from skin biopsies of healthy, unaffected individuals, and patients with rare monogenic forms or 'sporadic' autism were cultured and monitored for agonist-evoked calcium signals using a high throughput FLIPR assay. Human induced pluripotent stem cells (hiPSCs) were generated from primary fibroblasts using the Thermo-Fisher Sendai virus protocol. hiPSCs were differentiated into neuronal progenitors, and measured with UV-activated caged iP3.

Results: Local IP3-mediated calcium signaling was decreased in fibroblasts derived from patients with monogenic forms of ASD when compared to those derived from healthy, control patients. Likewise, IP3-mediated calcium signaling was repeatedly decreased in fibroblasts derived from patients with monogenic or 'sporadic' forms of ASD when compared to healthy, control patients. iPSC- derived neuronal precursors from patient fibroblasts also share this signaling defect.

Conclusion: Our results strongly implicate deregulated calcium signaling in the pathogenesis of ASD and supports IP3-mediated calcium signaling as a diagnostic biological marker for ASD. Furthermore, a high-throughput FLIPR assay may be used as a highly reproducible diagnostic that is able to capture differences in IP3-mediated calcium signaling.

Introduction

Autism Spectrum Disorders (ASD) is a group of neurodevelopmental disorders characterized by impaired social communication and interaction, and repetitive and stereotyped behaviors. Genomic sequencing technology have improved our understanding of ASD, and have pointed towards calcium ion channel variants as a possible mechanism of disease. Calcium signaling is involved in many cellular functions that span multiple physiologic systems. In most cells, intracellular calcium is released by inositol trisphosphate (IP3) receptor calcium ion channels on the endoplasmic reticulum, a major calcium store. This release is spatially and temporally coordinated to elicit specific functions including neuronal excitability, neurotransmitter release, cell secretion, gene expression, and apoptosis.

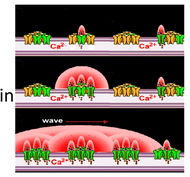


Figure 1. Single channel gating is altered in ASD. Ca^{2+} ion signaling is initiated by IP3 from G-protein coupled membrane receptor, first at a single channel. It is propagated by Ca^{2+} within a cluster, then spreads in waves throughout a cell by a positive feedback mechanism that involves diffusion of neighboring Ca^{2+}

Methods and Materials

FLIPR Ca^{2+} imaging

Skin fibroblasts were seeded in 96-well plates and loaded with 2 µM of Fluo-8 AM. Cell were stimulated with 100 µM ATP or 1 µM ionomycin in Ca^{2+}-free HBSS in triplicates.

Single-cell Ca^{2+} imaging

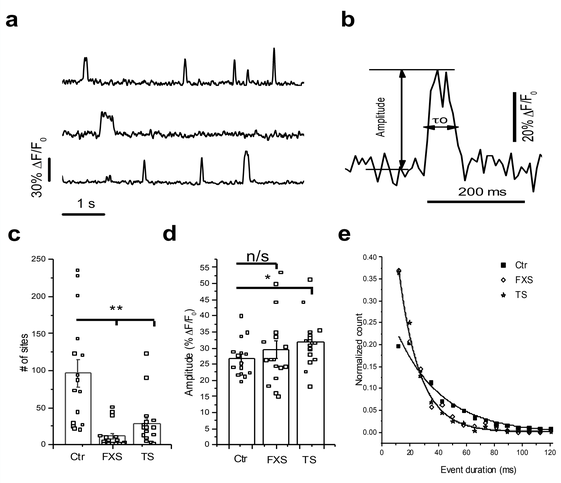
Cells seeded in glass-bottomed dishes were loaded with 4 µM Fluo-8 AM and 1 µM caged i-IP_3 (ci-IP_3) for 45 mins. $[Ca^{2+}]_i$ changes were imaged with a 40x oil objective at 30 frames sec^{-1}. A single flash of UV light was used to uncage i-IP_3. For local Ca^{2+} signals, cells were loaded with Ca^{2+} indicator Cal520, c-iIP_3 and 10 µM EGTA-AM for an hour. $[Ca^{2+}]_i$ signals were imaged using an Apo TIRF 100x (NA=1.49) oil objective at 129 frames sec^{-1}.

Human Induced Pluripotent Stem Cells

Human induced pluripotent stem cells (hiPSCs) were generated from the fibroblasts using the Thermo-Fisher Sendai virus protocol. For the differentiation, hiPSCs form EBs in suspension culture for the first 7 days and then are plated and develop into colonies containing rosette, neuroepithelial cells. At day 16, neural progenitors can be observed in the edge and the rosette-containing colonies are detached and grown in suspension to form neuroepithelial sphere.

Results

Figure 2. Local IP_3-mediated Ca^{2+} signaling is decreased in FXS and TS



a. "Optical Patch Clamp" traces of individual events. These reflect stochastic single molecule conformational transitions that "gate" the intracellular calcium channel

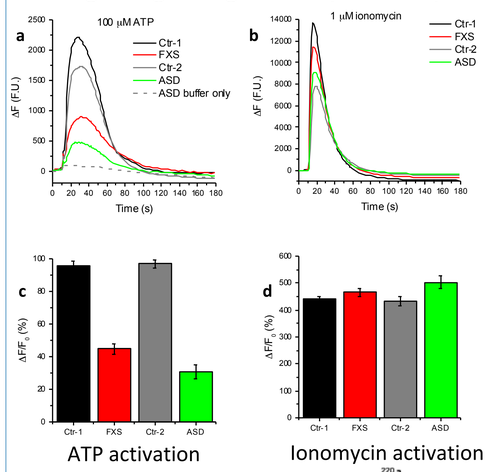
b. Magnified single Ca^{2+} event showing peak amplitude and event duration (t_0) at half-maximal amplitude.

c. Total numbers of Ca^{2+} release sites following photorelease of i-IP_3. Flicker opening of channels contributes to undercount.

d. Unaltered mean amplitudes of all events following the photolysis at each site.

e. Distributions of event durations (at half maximal amplitude) derived from all events identified in FXS (open diamonds), TS (stars) and control cells (black squares). Time constants t_0 is 15 ms for both FXS and TS (a brief "flicker" opening) compared to 32 ms for opening of control.

Figure 3. High-throughput FLIPR screen to monitor IP_3-mediated Ca^{2+} signaling changes in response to purinergic activation



a. Traces of ATP-induced Ca^{2+} events in zero Ca^{2+} solution.

b. Traces of ionomycin-induced Ca^{2+} events in zero Ca^{2+} solution.

c. Percent change of maximum Ca^{2+} release relative to basal measurement in ATP induced Ca^{2+} signaling.

d. Percent change of maximum Ca^{2+} release relative to basal signal in IM induced Ca^{2+} signaling.

e. Normalized values of ATP responses to IM responses.

Figure 4. IP_3-mediated Ca^{2+} response is significantly depressed across monogenic and sporadic forms of ASD

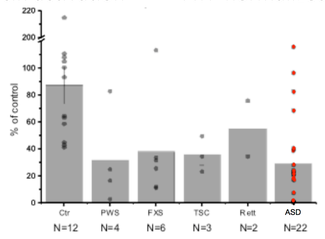


Figure 5. Step-by-step illustration of iPSC differentiation.

a. Differentiation of human iPSC to GABA interneurons involves 4 stages, including embryonic body (EB) formation, induction of neuroepithelial cells (NE), patterning of MGE progenitors and differentiating to GABA neurons.

b. Under a defined system, hiPSCs were differentiated into neurons.

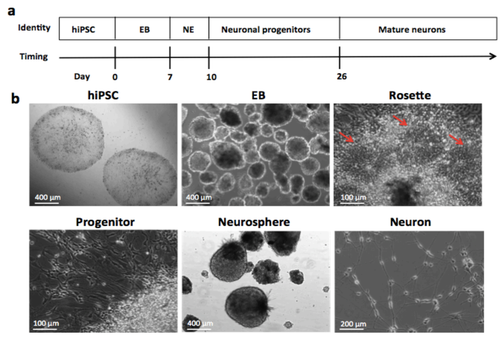
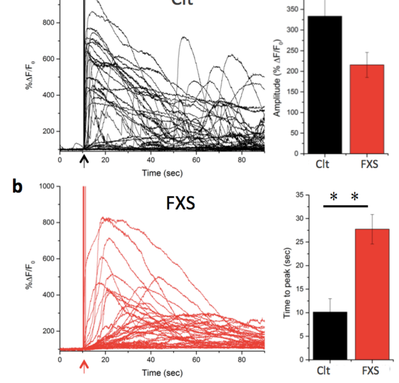


Figure 6. IP_3-mediated Ca^{2+} signaling is decreased in neuronal progenitors from a FXS patient, similar to fibroblasts.

a - b. Superimposed traces of single-cell Ca^{2+} response to uncaging of ci-IP_3 in control **(a)** and FXS **(b)** progenitors. Arrow indicates time of the UV flash.

c. Mean amplitudes and latencies to peak of Ca^{2+} fluorescence signals in FXS progenitors (red) and matched controls (black).



Discussion

Currently, ASD is diagnosed using subjective, clinical, behavioral assessments that delay diagnosis until at least 2 years. Our project suggests that intracellular calcium signaling is a likely ASD biomarker that can be detected using *in vitro* high throughput assay measurements. An ROC curve evaluates parameters to separate affected from unaffected individuals for diagnostic purposes . The area under the curve (AUC) suggests that our assay is quite robust in discriminating between syndromic or sporadic ASD samples and controls.

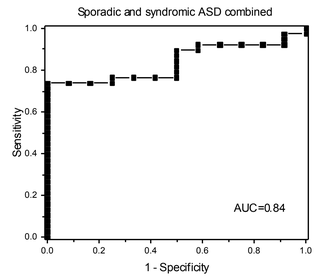


Figure 7. ROC Curve shows 73% sensitivity and 92% specificity of high throughput assay in discriminating ASD samples from control samples

Conclusions

- ASD has no defined biomarkers for diagnostics or novel drug discovery
- In rare forms of monogenic ASD syndromes, a molecular defect in IP_3 channel gating is resolved showing all forms have a short flicker open time.
- A high-throughput screen was developed to capture this defect in the monogenic ASD and typical, sporadic ASD samples.
- iPSC- derived neuronal precursors from patient fibroblasts share this signaling defect.
- Therefore, IP_3R signaling appears to be at a node in a signaling pathway at which many forms of ASD are unified into a shared defective output.
- ROC curves can distinguish, with high sensitivity and specificity, between syndromic and sporadic ASD samples, which signal similarly in this assay, and neurotypical controls.
- This biomarker may come to be useful as an adjunct diagnostic and potentially in a screen for novel therapeutics for ASD.