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Intracellular calcium dysregulation in autism spectrum disorder: An analysis of converging organelle signaling pathways☆

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ABSTRACT

Autism spectrum disorder (ASD) is a group of complex, neurological disorders that affect early cognitive, social, and verbal development. Our understanding of ASD has vastly improved with advances in genomic sequencing technology and genetic models that have identified > 800 loci with variants that increase susceptibility to ASD. Although these findings have confirmed its high heritability, the underlying mechanisms by which these genes produce the ASD phenotypes have not been defined. Current efforts have begun to "functionalize" many of these variants and envisage how these susceptibility factors converge at key biochemical and biophysical pathways. In this review, we discuss recent work on *intracellular calcium signaling in ASD*, including our own work, which begins to suggest it as a compelling candidate mechanism in the pathophysiology of autism and a potential therapeutic target. We consider how known variants in the calcium signaling genomic architecture of ASD may exert their deleterious effects along pathways particularly involving organelle dysfunction including the endoplasmic reticulum (ER), a major calcium store, and the mitochondria, a major calcium ion buffer, and theorize how many of these pathways intersect.

1. Autism and autism spectrum disorders

Autism spectrum disorder (ASD) is a group of neurodevelopmental disorders characterized by core deficits in social interaction, verbal and non-verbal communication, and repetitive interests and behaviors [1]. Over the past few decades, the increase in awareness for autism and the development of more sensitive screening tools have resulted in a steady rise in diagnoses worldwide [2]. However, these reasons alone are insufficient to explain the epidemic rise in its incidence. Notably, this rise is also reflected by a significant increase in societal and economic consequences, which include direct costs, such as special education and medical care as well as indirect costs, including loss of productivity, previously approximated at $268 billion per year in the USA in 2015, and estimated to increase to $461 billion per year by 2025 [3].

Today, ASD is estimated to affect 1 in 59 children, varying by gender and racial and ethnic groups [4]. The clinical diagnosis for autism is typically based on behavioral observations and assessments such as the Autism Diagnostic Interview – Revised (ADI-R) and the Autism Diagnostic Observation Schedule (ADOS), which may only be administered beginning at 2 years of age [5–7]. Although early diagnosis and intervention have shown improvements in long-term clinical outcomes [8,9], the average age of diagnosis is 4 years old [4]. This delay is exacerbated because autism is defined solely by behavioral qualities that are age-dependent, creating a lengthy process that the families experience known as a "diagnostic odyssey". Despite major progress in the genetic and neurobiological underpinnings of this disorder, its pathogenesis remains unclear. Currently, autism is understood as a highly heritable disorder, caused by a combination of multiple genetic and significant, yet poorly defined environmental factors [10,11]. Likewise, ASD is widely heterogeneous in its phenotype as it is often comorbid with symptoms including gastrointestinal and immune system abnormalities, mitochondrial dysfunction [12,13], and neuropsychiatric disorders, such as developmental delay, epilepsy, and attention deficit hyperactivity disorder (ADHD). This recognition begins to change the perception of autism from being a disorder affecting only the central nervous system, to a systemic disorder affecting

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multiple physiological systems [14].

Advances in DNA sequencing technology and bioinformatics have improved our ability to identify risk variants associated with complex disorders [15]. However, one of the greatest challenges that remains is to interpret how these factors converge along molecular pathways to produce the disparate abnormalities we see in autism. In this review, we focus on early genomic and more recent biophysical evidence that point towards organellar and intracellular calcium signaling as a target pathway in the pathophysiology of autism. Calcium signaling is involved in many essential cellular functions that span multiple organelles, tissues, and physiological systems [16,17]. Given its ubiquitous role, it is predictable that a deregulation along any point in the calcium signaling pathway may be capable of disrupting neurological function, which has already been demonstrated by a number of 'calciumopathies' – a diverse group of diseases caused by a disruption of calcium homeostasis [18–20] (this issue of BBA). This mechanistic perspective holds the potential to unify several theories of ASD pathogenesis including those on abnormalities in mitochondrial function, neurotransmitter signaling, excitation/inhibition imbalance and synaptic plasticity, and explain several of its non-neuronal co-morbidities.

We begin this review by discussing the current knowledge of calcium channel variants in the genetic architecture of autism, and follow our discussion by extrapolating 'hubs' in which these variants converge in the endoplasmic reticulum (ER) and mitochondria — two major organelles involved in calcium signaling. Intracellular organelle disease is a rapidly emerging area of medicine [21–23], and is beginning to advance our understanding of common, complex polygenic disorders that have a shared organellar pathophysiology [24]. Several classical monogenetic prototypes are well recognized to produce a distinctive spectrum of diseases of the mitochondria (the mitochondrial encephalomyopathies) [24], the lysosomes (the lysosomal storage diseases) [25] and the peroxisomes (the peroxisome biogenesis disorders) [26,27]. Unlike other organelles, the ER lacks a well-recognized spectrum of genetic diseases (Parys, this issue), though rare ER ryanodine receptor mutants are known to cause myopathies [28], and initial reports are emerging for rare ataxia syndromes caused by mutations of the ER inositol 1,4,5-trisphosphate receptors (IP_3R) [29,30] (for a review, see Bezprozvanny, 2011 [31]). This nascent field has promising leads in defining the mechanisms underlying complex polygenic syndromes, as the ER is known to play a central role in a host of cellular responses to environmental stressors [32,33] and hence is a mechanism by which environmental cues may impact phenotypes. Furthermore, the *function* of its calcium channels have also been shown to be altered in polygenic diseases, such as Alzheimer's disease [34–36]. Organelles are present in essentially all cells of the body, but notably, organellar diseases overwhelmingly affect the central nervous system (CNS). Organelle function is typically studied in human fibroblasts, and samples derived from skin biopsies are already in routine clinical use for the functional diagnosis of mitochondrial, peroxisomal and lysosomal neurological disease [37–39]. Since IP_3R function in signaling has been extensively studied in fibroblasts [40–42], functional diagnostics for the ER neurological diseases are rendered feasible and will be discussed in this review.

2. The genomics of calcium 'channelopathies' in autism spectrum disorder

The high heritability of ASD strongly suggests that genes and the biochemical pathways they subserve are involved in producing the autism phenotype [43]. Historically, the heritability (h^2) of ASD was established by twin studies in which monozygotic twins had a concordance rate of 80–90% for autism, while dizygotic twins had a concordance rate of ~10%. Although the concordance rate for dizygotic twins is much lower than that of the former, it is 10–20 times the risk of the general population (1%) [44]. This heritability calculation demonstrates room for an environmental component of variance that must be responsible for occasional phenotypical differences among discordant identical twins that share *all* genes in common. However, the exact nature of much of the autism heritability remains undefined, as in the studies discussed below, only a small portion (15–20%) of the genetic component of ASD is captured as a specific gene mutation [45,46]. Insights into the nature of the genetic component of ASD have also been furthered by the study of rare, monogenic syndromes that have a high comorbidity with ASD, and their respective murine genetic models [47]. Such disorders, including fragile X (mutation in *FMR1* on chromosome Xq27), tuberous sclerosis (mutations in *TSC1* or *TSC2* on chromosomes 9q34 and 16p13, respectively), and Rett syndromes (mutation in *MECP2* on chromosome Xq28), are caused by a single-gene mutation either inherited or arising *de novo*. While these monogenic disorders provide useful genetic models and insights into ASD, they each account for < 1% of all ASD cases [48,49]. Recently, much of this work has been extensively reviewed [50,51].

Other genetic studies have additionally showed mutations in ion channel genes in subjects with autism, leading to the suggestion that this disorder reflects a 'channelopathy' [52]; a topic we have extensively reviewed [53–55]. The case for this disease mechanism was greatly strengthened by the discovery of Timothy syndrome (TS) and studies of its mutated calcium ion channel [56]. TS is a rare, dominant monogenic syndrome that produces well-defined "long QT arrhythmia" (LQT) and a highly-penetrant ASD phenotype *via de novo* mutations of the *CACNA1C* gene (on chromosome 12p13), which encodes the major alpha subunit of a voltage-activated calcium channel that is expressed in heart and neurons. Our robust understanding of the multiple ion channel mutations contributing to LQT pathogenesis [57], and the bridge provided by TS, bettered our understanding of how that same channel's biophysical lesion might lead to "an LQT-like" neuronal dysfunction in ASD [54]. This recognition opened a gateway for understanding calcium signaling dysregulation as a mechanism of the ASD phenotype [55,58–66].

More recently, copy number variants (CNVs) have been implicated as an important new class of genetic mutations involved in the pathogenesis of ASD [67,68]. CNVs are segments of DNA that have been duplicated or deleted, spanning up to millions of bases (Mb) in length, and are now recognized to add significant structural variability to the human genome [69]. These variants are typically recognized at lengths of > 1-kilobases (kb), but are now reliably detected at lengths of a few dozen base pairs with more sensitive high-depth resequencing techniques. Early whole genome surveys based upon microarray technology previously suggested that there was an increased frequency of CNVs in ASD. However, more sensitive resequencing techniques reveal that both ASD subjects and neurotypical controls share the same frequency (~19%) though CNVs in ASD are ten-fold longer (10.9 kb *vs* 1.2 kb) [70] and similarly ten-fold more likely of damaging an exon. This mirrors the findings of *de novo* point mutations [71,72], which occur at a similar rate in ASD subjects and neurotypical controls, but which are recognized to occur at a position more frequently severely disrupting of gene function in ASD. The *de novo* point mutations [73] are recognized to increase with paternal age, a significant contributor to ASD risk. However, the mechanism by which CNVs arise must be different, as they do not share this paternal-age effect [74]. Several resequencing studies have identified exon-disrupting CNVs of the calcium channel accessory subunit genes [75,76]; and *CACNA2D3* is one of the genes most frequently interrupted by recurring CNVs in ASD. Together these results suggest that there is not an increased mutation risk in ASD, but that if the mutations occur at critical genes, they cause the disorder. Therefore, the nature of these targeted genes should help define ASD pathogenesis.

The discovery of rare variants or mutations associated with ASD has dramatically shifted our understanding of its genetic landscape. Advances in sequencing technology, accompanied by dramatic decreases in its costs, permitted genome-wide association studies (GWAS) that are based upon locus resequencing, instead of merely common

Table 1
Calcium channel subunit genes carrying risk variants implicated in ASD.

Protein	Description	Normal function	Disease association
CACNA1C	Voltage-regulated L-type calcium channel, alpha 1C subunit	Regulates entry of Ca^{2+} into excitable cells: muscle contraction, hormone/ neurotransmitter release, gene expression, cell cycle	Timothy Syndrome, ASD, psychiatric diseases
CACNA1D	Voltage-regulated calcium channel, alpha 1D subunit	High-voltage activated, long lasting Ca^{2+} activity	Sinoatrial node dysfunction and deafness, ASD, psychiatric diseases
CACNA1E	Voltage-regulated R-type calcium channel, alpha 1E subunit	High-voltage activated rapidly inactivated	ASD, psychiatric diseases
CACNA1F	Voltage-regulated L-type calcium channel, alpha 1F subunit	Regulates entry of Ca^{2+} into excitable cells: muscle contraction, hormone/ neurotransmitter release, gene expression, cell cycle	ASD and X-linked congenital stationary night blindness
CACNA1G	Voltage-regulated T-type calcium channel, alpha 1G subunit	Regulates entry of Ca^{2+} into excitable cells: muscle contraction, hormone/ neurotransmitter release, gene expression, cell cycle	ASD; intellectual disability; juvenile myoclonic epilepsy
CACNA1H	Voltage-regulated T-type calcium channel, alpha 1H subunit	Regulates neuronal and cardiac pacemaker activity	Familial autism; childhood absence epilepsy
CACNA1I	Voltage-regulated T-type calcium channel, alpha 1I subunit	Characterized by a slower activation and inactivation compared with other T-channels	Possibly implicated in ASD
CACNA2D3	Voltage-regulated calcium channel, alpha 2/delta 3 subunit	Accessory calcium channel subunit; regulates entry of Ca^{2+} into excitable cells	ASD
CACNA2D4	Voltage-regulated calcium channel, alpha 2/delta 4 subunit	Accessory calcium channel subunit; regulates entry of Ca^{2+} into excitable cells	Gene deletion along with CACNA1C leads to ASD
CACNB2	Accessory calcium channel beta-2 subunit	Contributes to the function of Ca^{2+} channels. Modulates voltage dependence of activation and inactivation, and controls trafficking of the Ca^{2+} channel family	ASD, psychiatric diseases

single nucleotide polymorphisms (SNPs). Compared with earlier studies with common variant SNPs, strong genome-wide associations, for the first time, could be reproducibly detected [43], underlining new and more significant associations with calcium signaling proteins, now emerging as predominant in the architecture of ASD. These large, unbiased genome-wide studies have implicated > 800 genes in ASD and account for about half of the ASD liability even though not a single gene accounts for > 1–2% of total autism cases. The pathways impacted by these variants, however, are not random, but are largely clustered into three major functional groups: those modifying chromatin structure, those altering transcription factors or those impacting calcium signaling [71,73,77–80] (Collation in public domain: http://www.mindspec.org/autdb.html). Together these findings suggest that ASD predominantly follows a polygenic pattern of inheritance caused by several weakly penetrant genetic variants – either arising *de novo* or inherited from parents – and that many act in combination in key cellular calcium signaling pathways that incrementally enhance the susceptibility of ASD [10,11,81]. Among them are a large number of variants in genes encoding specific calcium channel subunits (Table 1) and key calcium signaling proteins with which they interact. Most prominent and reproducible among the calcium channel variants discovered in ASD are those in the loci encoding alpha subunits of the voltage-activated calcium channel genes, *CACNA1C*, *CACNA1D*, *CACNA1H*, *CACNA1G*, *CACNA11*, *CACNA1E*, those of their accessory subunits, *CACNB2*, *CACNA2D3*, *CACNA2D2* and *CACNA2D4*, as well as the *GRIN2B* locus, encoding the calcium-permeable *N*-methyl-D-aspartate (NMDA) receptor ion channel subunit found at excitatory synapses throughout the brain. Several ASD-associated variants that should also be noted are those found in loci encoding channels that closely interact functionally with these channels, which include those in the voltage-gated sodium channel alpha subunits *SCN2A*, *SCN1A*, and *SCN8A* and the 15q12-located *GABRB3* $GABA_A$ receptor gene encoding a ligand-gated chloride ion channel found at inhibitory synapses throughout the brain. It is important to note that many of the same channel loci contribute to the genetic architecture of schizophrenia and autism [82,83].

It is clear that the genetic architecture of autism is highly varied, including *de novo* and inherited mutations in genes that are highly or weakly penetrant, as well as inherited and *de novo* duplication or deletion CNVs of a range of sizes found in sporadic or recurrent chromosomal regions [84]. The mechanism and frequency of mutations in ASD do not seem to be altered, rather the genes they have *hit* appear relevant in the pathogenesis of autism. Interestingly, loci encoding calcium signaling proteins appear to be reproducibly targeted by all of the classes of mutations in ASD.

3. Calcium signaling pathways

The significant impact predicted from dysregulated calcium signaling can be appreciated by recognizing its diverse roles in cell function. Unlike other ions that all participate in the electrical activity of cell membranes, calcium signaling plays the additional role of coupling electrical activity to biochemical, metabolic, transcriptional and translational processes. The calcium ion is an ancient second messenger that coordinates this wide range of cellular processes through its intracellular spatial and temporal gradients [85]. These gradients are established and maintained by a rich array of calcium pumps, exchangers, voltage-activated and ligand-gated ion channels, and a diverse set of families of calcium-binding proteins, that together permit tight regulation of cytosolic and organellar calcium concentrations and downstream signals. Further, these are explicitly required for higher cognitive functions such as synaptic plasticity [86], memory [87], neuron excitability [88,89], neurotransmitter release [90,91], axon growth [92], and long-term changes in gene expression in neurons [93].

In neurons, calcium channels are particularly important for the initiation and propagation of action potentials. Once an action potential arrives at the axon's presynaptic terminus, local voltage-gated calcium channels are opened to permit an influx of calcium ions, amplified by the release of intracellular calcium stores. Together, these actions trigger the fusion of intracellular synaptic vesicles containing neurotransmitters with the plasma membrane of the presynaptic cell and allow the release of neurotransmitters into the synaptic junction with the postsynaptic cell.

These events are tightly regulated by intracellular calcium homeostasis. At rest, cytosolic calcium concentration is maintained at ~100 nM, a value 10,000-fold lower than the extracellular calcium concentration. Upon stimulation, calcium ions are mobilized from intracellular stores, such as the ER, or from the extracellular environment, to increase cytosolic calcium concentrations. The mitochondrion tightly regulates these changes by rapidly sequestering calcium ions into the mitochondrial matrix through mitochondrial calcium uniporter (mCU) channels [94]. Likewise, calcium ions are more slowly taken up by the ER calcium ATPase pump (SERCA) or cleared out of the cell through calcium ATPase (PMCA) pumps that line the plasma membrane [95].

Vitamin D, specifically 1α,25-dihydroxyvitamin D_3 (D_3), plays a major role in whole-body calcium homeostasis *via* its nuclear receptor and the regulation of gene expression. In addition to its role in

regulating gut absorption and bone mobilization, it also plays a recognized role in cell proliferation, differentiation and modulation of voltage-gated calcium channels and PMCA expression [96]. The mitochondria ultimately produce active D_3, and notably, D_3 insufficiency occurring in mid-gestation to infancy has been reproducibly recognized as a significant risk factor in ASD [97,98] and a potential therapeutic target [99].

4. Inositol trisphosphate (IP_3)-mediated calcium signaling

IP_3Rs are a family of calcium-permeable ion channels ubiquitously expressed and predominantly found in the ER membrane of nearly all known cells (for an extensive review, see Foskett et al. 2007 [100]). Functional IP_3Rs are tetramers, with subunits consisting of a large cytosolic N-terminus, six transmembrane domains containing the ion channel pore, and a short cytosolic C-terminus [100,101]. In mammals, the IP_3R family is comprised of three separate gene products (IP_3Rs types 1–3) and a number of splice variants [100]. At the protein level, IP_3R isoforms are 60–80% homologous, and their functional domains are similar. However, each isotype has a different affinity for IP_3 and calcium, and are differentially modulated by ATP, cAMP and protein kinases. Similarly, different forms of IP_3Rs exhibit distinct and overlapping expression patterns with most cell types expressing more than one isoform, which provides a large degree of spatial and temporal plasticity in IP_3-generated calcium signaling.

IP_3-mediated calcium signal transduction is typically initiated by stimulation of cell surface receptors linked to the activation of phospholipase C isoforms β or γ (PLC- β/γ) [102]. PLC-β is activated following stimulation of G-protein coupled receptors linked to the heterotrimeric Gq family proteins (GPCRs) [16], whereas PLC-γ is turned on by phosphorylation in response to tyrosine kinase-linked cell surface receptor stimulation [103]. Activation of PLC leads to the generation of IP_3 and diacylglycerol (DAG) following hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2). IP_3 liberated from the inner leaflet of the plasmalemma diffuses into the cytoplasm and binds to IP_3Rs located in the ER membrane. The IP_3R forms a calcium-permeable channel in the membrane of the ER, and its opening allows the release into the cytosol of calcium sequestered within the ER lumen [104,105]. Opening of the IP_3R channel requires binding of IP_3 together with calcium ions to receptor sites on the cytosolic face.

Gating of IP_3R by calcium is biphasic, such that small elevations of cytosolic calcium induce channel opening, whereas larger elevations cause inactivation [100,106]. The positive feedback by calcium underlies the process known as calcium-induced calcium release (CICR), whereby calcium is released in a regenerative manner that originates as a single channel fundamental event, known as a blip [107], and may remain restricted to a cluster of IP_3Rs, producing local calcium signals known as calcium puffs [108], or may propagate throughout the cell as a saltatory wave involving the recruitment of multiple puff sites by successive cycles of calcium diffusion and CICR. Thus, IP_3-mediated calcium signaling represents a hierarchy of calcium events of differing magnitudes [109,110], and the spatial patterning and distribution of IP_3Rs is critical to ensure high specificity of cellular responses.

In neurons, IP_3R-mediated calcium release is involved in synaptic plasticity and memory [87,111], neuronal excitability [88,89], neurotransmitter release [90,91], axon growth [92] and long-term changes in gene expression [90], highlighting the central integrating position and crucial role played by IP_3Rs [112]. The IP_3R is a key signaling hub in the canonical metabotropic glutamate receptor (mGluR) pathway [87,102], where IP_3-induced calcium response in neurons propagates along the dendrite as a wave and is distinct from action potential-induced calcium fluctuations in both temporal and spatial aspects. Unlike brief spike-evoked calcium signals that occur throughout the cell, IP_3-mediated events start at a local proximal dendrite and then spread to the soma as a calcium wave. This was first observed in hippocampal neurons [113,114]. In hippocampal CA1 slices, brief pre-treatment with group 1 mGluR agonists has been shown to facilitate the induction of long-term potentiation (LTP) that manifests in an enhanced magnitude and stability of LTP [115]. However, stronger activation of the group 1 mGluRs induces long-term depression (LTD) [116,117], a form of synaptic plasticity and a candidate mechanism for the cellular basis of motor learning [87]. Application of a group 1 mGluR agonist acutely reversibly depressed excitatory postsynaptic currents (EPSCs) in rat brain slices. Subsequently, IP_3-mediated calcium waves were observed and characterized in other brain regions – cortical pyramidal neurons and midbrain dopamine neurons, suggesting a ubiquitous role of IP_3 signaling throughout the brain [118,119].

In the mammalian brain, different isoforms of IP_3Rs have distinct expression patterns that vary with brain region and developmental state. IP_3R type 1, encoded by *ITPR1* on chromosome 3p26, is predominantly expressed in neurons, especially in Purkinje cells in the cerebellum. IP_3R type 3, encoded by *ITPR3* on chromosome 6p21, demonstrates predominantly a neuronal pattern of expression that does not overlap with the IP_3R1. For instance, Purkinje cells in the cerebellum are highly enriched in IP_3R1, but have low or undetectable levels of IP_3R3. By contrast, granule cells of the cerebellum and many regions of the medulla display moderately high levels of IP_3R3, whereas IP_3R1 is virtually undetectable in these regions. IP_3R1 expression levels are the highest in cerebellar Purkinje neurons, where IP_3-mediated calcium signaling is necessary for induction of LTD [85]. Interestingly, cerebellar dysfunction has been repeatedly implicated in the pathogenesis of ASD [120].

In these cerebellar Purkinje cells that are especially enriched in IP_3Rs, repetitive parallel fiber stimulation triggers mGluR activation and subsequent IP_3-mediated calcium release from the ER [121,122]. Because both IP_3 and calcium are required for the initial IP_3R activation, some cytoplasmic calcium is necessary. If the concentration of IP_3 is high, a low basal calcium concentration is sufficient, though, an additional source of calcium is required at lower IP_3 concentrations. This dual requirement for the two messengers is met by two inputs to the Purkinje neurons: the climbing fiber input strongly depolarizes Purkinje cells to generate a calcium signal *via* plasmalemmal channels, whereas parallel fiber inputs activate the mGluRs to produce IP_3. Thus maximal activation of calcium release *via* IP_3Rs depends on the timing of co-activation and serves as a coincidence detector for these two types of inputs [123]. Consistent with the role of IP_3R1 in cerebellar LTD, LTD is completely abolished in mice with a genetic deletion of *Itpr1* [87].

In the cortex, different subtypes of IP_3Rs are expressed in almost exclusive fashion in different cell types. ITPR1 is predominantly expressed in neurons across cortical layers and regions, while ITPR2 predominates in microglia and astrocytes (Collation in public domain: http://hgwdev-max.soe.ucsc.edu/tsneViewer/dev3/?ds=autism10x). IP_3R activation in pyramidal neurons is a key signaling hub downstream of mGluRs [87,102], where it leads to a brief hyperpolarization followed by a more prolonged depolarization [89,124]. The initial outward current results from the opening of small conductance calcium-activated K^+ channels [125,126]. This current is proportional to the calcium signal amplitude [89] and can be triggered directly by intracellular uncaging of IP_3 [89,124]. As a result, IP_3-evoked calcium release transiently hyperpolarizes the cell and briefly depresses neuronal excitability, leading to a reduction in firing frequency [89]. Suppressed IP_3-mediated calcium release from the internal stores diminishes the inhibitory K^+ conductance, and produces neuronal hyperexcitability, consistent with observations following mGluR stimulation of ASD-model neurons [127,128]. The trafficking of calcium-impermeable AMPA receptors to the synapse *via* COPII vesicles also depends upon ER calcium release stimulated by mGluRs and downstream of IP_3R activation, and this seems correlated with removal of synaptic high-conductance calcium-permeable AMPA receptors, another process that contributes to LTD [129].

A complex array of downstream signaling events [130] arising from

mGluR activation has been previously reported in FXS [127,131]. In particular, a popular theory largely based upon studies in the mouse model postulates that absence of FMRP over-activates the mGluR-mediated signaling pathway, and thus leads to features associated with FXS [132], including augmented LTD and smaller, elongated dendrites suggestive of a less mature phenotype [133,134]. Moreover, multiple studies from different groups in *Fmr1* knockout mice with pharmacological or genetic reduction of mGluR function showed correction of many of these abnormalities, further demonstrating that mGluRs play a significant role in the pathophysiology of FXS, at least in mice [135–137]. Despite the fact that overactivation of these receptors has long been implicated in the pathophysiology of FXS, surprisingly little is known about the resulting calcium release upon activation of these receptors. Extrapolating the signaling pathway predicted by this model would suggest that the calcium signaling is enhanced in response to postsynaptic activation of mGluR5 in neurons from *Fmr1* KO mice, consistent with previous reports on other molecular aspects of over-activated mGluR signaling in the FXS mouse model [137,138]. This prediction that downstream calcium release from ER IP_3Rs would be increased in FXS is also consistent with preliminary observations we have made in *Fmr1* KO murine neurons and astrocytes (Fig. 1) in response to extracellular activation with group1 mGluR agonists.

A dynamic balance between neuronal excitation and inhibition (E/I) is critical for proper brain development and function. An imbalance is a well-recognized aspect of the neurological abnormalities in autism [139,140]. Extensive evidence indicates that inhibitory GABAergic transmission plays an important role in regulating excitation-inhibition balance [141], and a loss of synaptic $GABA_A$ receptors is often correlated with this imbalance *via* their reduced synaptic efficacy [142–144]. In a recent study, Bannai et al. discovered that IP_3-mediated release of calcium from the ER, induced by activation of mGluRs, promotes $GABA_AR$ clustering and enhanced GABAergic transmission [145]. This pathway was found to be necessary for reversal of $GABA_AR$ dispersal of these receptors caused by an NMDA receptor-dependent calcium influx. Sustained tonic low-level calcium signals from the ER driven by mGluR activation serves to cluster $GABA_AR$ *via* PKC phosphorylation on S327 and S343 of the receptors gamma2 subunit, while phasic sustained calcium influx *via* the NMDAR channel activates calcineurin and dispurses the receptors without effect on those two phosphorylation sites. Hence constitutively diminished IP_3-mediated calcium release, as observed in ASD, may cause altered inhibitory synaptic transmission, excitation-inhibition imbalance, and may be an important factor in disease onset.

In attempting to “functionalize” the “calcium signaling” genomic architecture of ASD discussed above, we had previously revealed a change in IP_3R channel gating. We demonstrated depressed IP_3R-mediated calcium signaling in skin-derived fibroblasts of rare, monogenic forms of ASD – fragile X (FXS) and tuberous sclerosis type 1 and type 2 (TSC). These disorders are not associated with any IP_3R mutations. Using caged IP_3 release and optical patch clamp recording [107], we documented a shared functional state change in channel gating. We found that there was no change in channel conductance or latency in opening, but noted that channels from both FXS and TSC cells shared a common “flicker” kinetic component of opening, in which the elementary event duration follows a single exponential in all the ASD model cells, with a dramatically reduced t½ (15 msec) when compared with controls (32 msec). In addition, we found no change in the intracellular calcium ion store content, and no difference in the abundance of IP_3Rs at the protein level [146]. The phenotype of this discrete molecular change could be captured in single-cell studies and *via* a high-throughput fluorescence plate reader (FLIPR) screen using ATP to activate plasma membrane P2Y receptors [146] (Fig. 2). In still unpublished studies, we also found that control and FXS fibroblasts that were induced into pluripotent stem cells (iPSC) and differentiated into neural progenitor cells (nPCs) retained their signaling-deficient phenotype (Fig. 2b). Provocatively, skin fibroblasts from murine *Fmr1* KO strains do not replicate the findings in the human cells (Fig. 2c), suggesting important differences in the human and murine disorder. This is a possible concern in view of the recurrent failures of drug discovery based upon this model. An explanation for this discrepancy may be elucidated by differences between murine and human proteomes. Recently, a massive mouse genome mapping study demonstrated that the expression pattern of many mouse genes showed considerable divergences from their human orthologs [147]. Likewise, it is worthwhile to note that while FMRP plays the same role of sequestering and silencing translation of multiple mRNAs in both man and mouse, an imperfectly overlapping set of messages are silenced in the two species (De Rubeis et al. 2014, Fig. 1a [71]). Although *Fmr1* KO mouse is believed to be a good model of FXS, it is not obvious that all aspects of the phenotype should correlate.

In a similar study, we extended our high-throughput FLIPR screen to additional forms of monogenic syndromic ASD, which included Rett syndrome and Prader-Willi syndrome, as well as typical sporadic non-syndromic ASD subjects enrolled in the Center for Autism Research and Translation (CART) at UC Irvine for genome sequencing and high-depth phenotyping. Using skin-derived fibroblasts collected from these subjects, we saw a similar decrease in this specific ATP-induced calcium signal when compared with paired controls. Importantly, we again found that there was no difference in intracellular calcium stores [148]. In preliminary unpublished studies, we have begun to compare high-throughput FLIPR screen data of fibroblast cells from individual patients with other rare inborn errors that cause neurological disorders. The fibroblast cells derived from patients with two lysosomal storage diseases, mucopolysaccharidosis type 3B (MPS 3B) and Niemann-Pick type C1 disease (NPC1) signaled similarly to those derived from neurotypical controls, while, surprisingly, cells from the patient with phenylketonuria (PKU) signaled within the range of our ASD patients.

To assess the performance of our high-throughput calcium-signaling assay as a diagnostic, we generated a “receiver operating characteristic” (ROC) curve (Fig. 3). The ROC curve is a standard metric widely used to evaluate diagnostic parameters that discriminate diseased cases from normal cases [149]. The ROC curve expresses the accuracy of a test in terms of *sensitivity*, the ability to capture affected individuals, and *specificity*, the ability to exclude the unaffected individuals. In this study, we differentiated our study subjects as “affected” or “unaffected” using their results from the ADOS-2 assessment – a test classically described as the ‘gold standard’ for ASD diagnoses – then correlated their results to those from the high throughput calcium signaling assay. At every possible calcium signaling value, the sensitivity and the specificity are calculated from a ratio of people who are disease-positive (true positive) or disease negative (false positive). Sensitivity and specificity are inversely related, thus as the signaling ‘threshold’ is increased, the sensitivity and the number of ASD subjects captured also increases. At the same time, however, the number of unaffected controls captured also increases, thereby decreasing the specificity. The area under the ROC curves (AUC) serves as a proxy for a test's accuracy and utility. The 0.85 AUC metric achieved by our ROC test suggests that this calcium signaling assay may be a reliable diagnostic test, though additional replication studies with larger cohort sizes would be required.

5. Other sources of calcium signaling abnormalities in ASD

The evidence discussed in the previous sections posits that the abnormalities in IP_3-mediated calcium signaling contributes to the neurological and behavioral phenotypes seen in autism. However, calcium transduction to the remainder of the cell involves numerous highly interconnected pathways that may be also influenced by abnormalities originating from other sources. Such abnormalities have been published on ryanodine receptors (RyRs) and plasma membrane calcium ATPases (PMCAs), though the pathogenetic mechanism by which they perturb signaling have not been defined. Using this rationale, it is also important to consider the mitochondria as a source of calcium

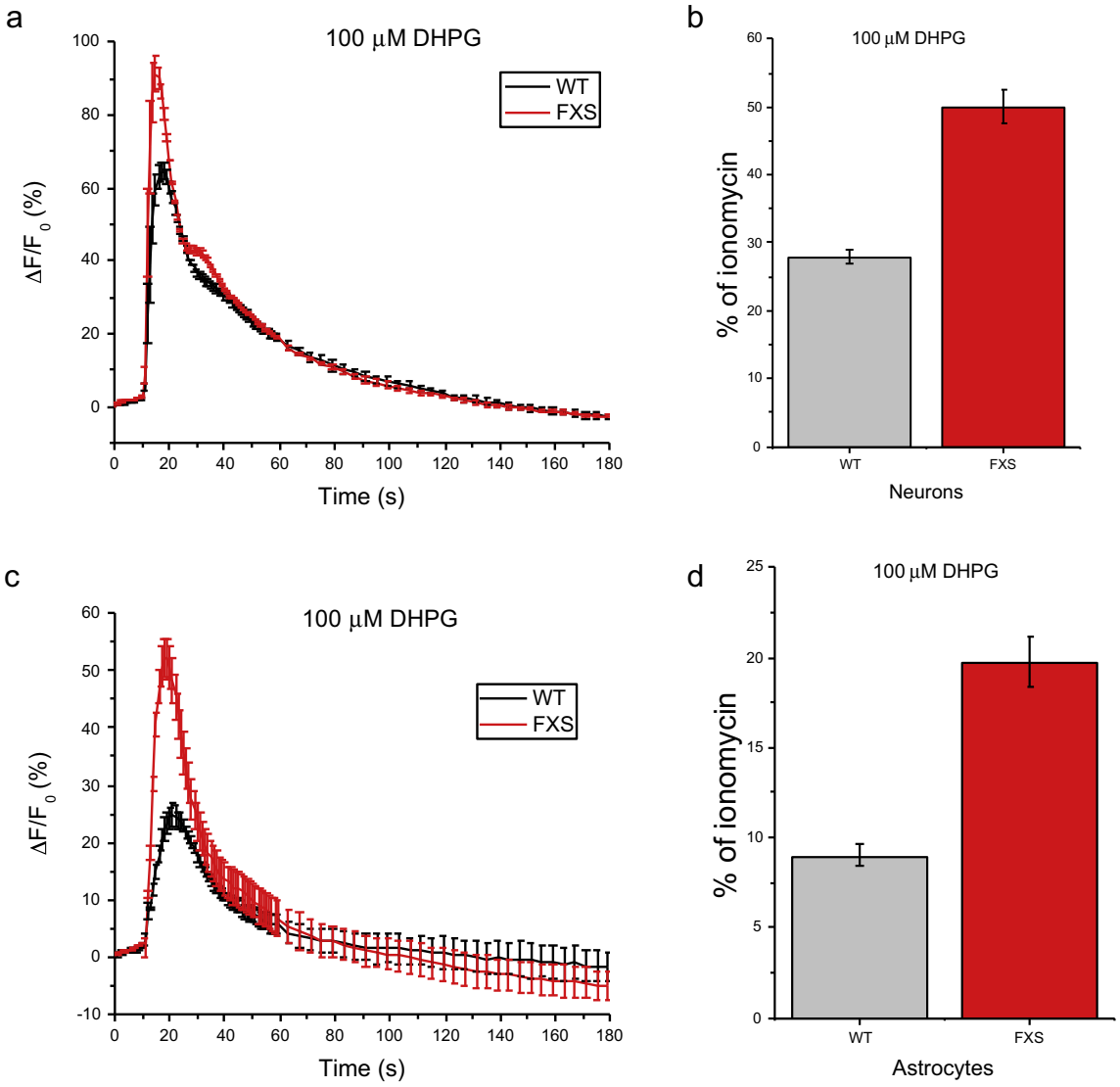

Fig. 1. Representative Ca^{2+} responses to extracellular application of purinergic agonists in absence of extracellular Ca^{2+} in neuron- or astrocyte-enriched cultures from WT or FXS P0 mice.

(a) Averaged FLIPR traces from three independent wells showing changes in Fluo-8 fluorescence over the basal fluorescence ($\Delta F/F_0$) in response to extracellular application of 100 μM DHPG to neuron-enriched cultures. Lines represent mean values ± s.e.m. (b) Peak amplitudes (ΔF) of Ca^{2+} responses to 100 μM DHPG normalized to basal fluorescence (F_0) before stimulation are expressed as a percentage of the mean ionomycin response in neurons from WT mice (grey) or FXS (red). Bar graphs show mean of triplicate measurements after subtracting the artifactual signal resulting from addition of vehicle alone. Data are presented as mean ± s.e.m. (c) Averaged FLIPR traces showing changes in Fluo-8 fluorescence over the basal fluorescence ($\Delta F/F_0$) in response to extracellular application of 100 μM DHPG to astrocyte-enriched cultures. (d) Peak amplitudes (ΔF) of Ca^{2+} responses to 100 μM DHPG normalized to the basal fluorescence (F_0) before stimulation expressed as a percentage of the mean ionomycin response in astrocytes from WT mice (grey) or FXS (red). Bar graphs show mean of triplicate measurements after subtracting the artifactual signal resulting from addition of vehicle alone. Data are presented as mean ± s.e.m.

dysregulation.

Ryanodine receptors are a family of large, homotetrameric calcium channels located in the sarcoplasmic/endoplasmic reticulum membrane that release calcium from intracellular stores. Mutations in genes encoding RyRs have become well recognized as a cause of cardiac myopathies [150,151], however very limited suggestions of a role in ASD have also been shown. In a study following the contributions of known CNVs to ASD, a maternally inherited duplication at 1q43, which encompasses the RyR2 gene, was identified as likely pathogenic in a small group of Lebanese autistic children [152] and in a separate study, RyR2 was identified among 20 missense *de novo* variants found in sporadic cases of childhood onset schizophrenia [153]. The RyR3 gene, found in the critical 15q11–13 region associated with ASD, and the subtype most abundant in brain, when knocked out in mice impaired social interactions [154] but the gene had been found *not* to be associated with ASD in a large Japanese study [155].

Similarly, plasma membrane calcium ATPases have also been highlighted as another source of deregulated calcium signaling in autism. PMCAs are members of a large family of P-type ion pumps that have known roles in global and local calcium homeostasis. Independent genome-wide scans have previously identified a link between the chromosome region 3p25 and autism, prompting gene candidate studies of ATP2B2, a plasma membrane calcium transporting ATPase2 (PMCA2) highly expressed in the central nervous system. In a study conducted by Hu et al., ATP2B2 was found to be one of only 43 genes differentially expressed in ASD samples vs controls [156]. In a separate study, Carayol et al. found significant association between several single nucleotide polymorphisms (SNPs) in ATP2B2 and ASD in males [157] and evidence supporting the association of SNPs in AT2B2 and autism was again found in two additional similar family association

studies [158,158]. More recently, this locus controlling cytoplasmic calcium levels has proven to be one of the most reproducible associations with ASD in resequencing studies, although thus far no biophysical studies have offered evidence of a functional change [77].

A high co-occurrence of mitochondrial diseases and autism has been well documented over the past two decades, suggesting a pathogenetic link between the two disorders. Although the exact mechanism by which the mitochondria produces the autism phenotype is still under intensive study, its role in calcium signaling is becoming a focus. In the following sections, we discuss biochemical and genetic evidence that link deregulation in the mitochondria to autism, and build on our previous evidence in IP_3-mediated calcium deregulation to propose possible points of intersection between the ER and mitochondria that may affect calcium homeostasis in ASD.

6. Mitochondrial dysfunction in ASD

Early suggestions of a link between mitochondrial dysfunction and ASD first emerged more than two decades ago when studies began to detect biochemical correlates of mildly deficient mitochondrial function in patients diagnosed with autism [160–162]. Biological markers including an elevated lactate/pyruvate ratio or elevated alanine– an indication of chronic mild lactic acidosis, or carnitine deficiency– an indication of loss of this mitochondrial carrier molecule through chronic buffering of organic acids, were directly observed in a retrospective case-control study of biochemical markers of mitochondrial energy deficiency present in a general ASD population. In this study [163,164], the signature of significantly reduced plasma carnitine levels, moderate hyperammonemia, and chronic lactic acidosis indicated by elevated plasma alanine and an elevated plasma lactate/pyruvate

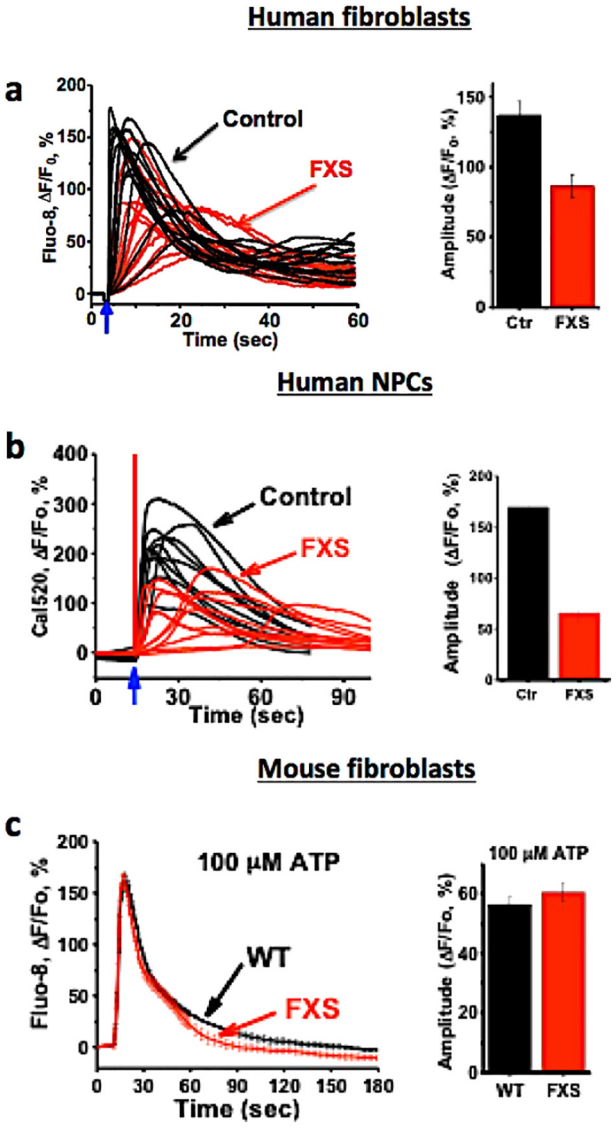

Fig. 2. IP_3 –induced intracellular Ca^{2+} signals are decreased in human fibroblasts and neural progenitor cells with FXS, but not in mouse fibroblasts with FXS.

a. Human skin fibroblasts (FXS, GM09497 and control, GM02912, Coriell Cell Repository) were loaded with Ca^{2+} sensitive indicator Fluo-8 (Kd = 390 nM) and ci-IP_3 (a membrane permeable caged IP_3 which can be photoreleased by UV flash). Left – representative traces show Fluo-8 fluorescence changes (indicative of intracellular Ca^{2+} changes) in individual FXS (red) and control (black) cells, in response to direct IP_3 receptor stimulation. Traces were normalized to baseline averaged for 10 s before stimulation. Blue arrow indicates time of the UV flash. Right – bar graphs indicate average maximum amplitude of Ca^{2+} rises in control cells (black bar) and in FXS cells (red bar). Data are from 13 control and 14 FXS cells, $P < 0.01$ (two sample T-test). (modified from Schmunk et al., 2015, Fig. 3, B [146])

b. Neural progenitor cells, reprogrammed from FXS (GM05185) and control (GM03440) fibroblasts, were loaded with Ca^{2+} sensitive indicator Cal520 (Kd = 320 nM and ci-IP_3). Left – representative traces show Cal520 fluorescence changes in individual FXS (red) and control (black) cells. Traces were normalized to baseline averaged for 10 s before stimulation. Right – bar graphs indicate average peak amplitude of Ca^{2+} rises in control cells (black bar) and in FXS cells (red bar). N = 30 control and 24 FXS cells, $P < 0.001$ (two sample T-test). FXS, Fragile X (YM & JJG unpublished)

c. Average Ca^{2+} responses to extracellular application of P2Y receptor agonist ATP in absence of extracellular Ca^{2+} in skin fibroblasts from wild type and FXS mice. Left – averaged FLIPR traces from three independent wells showing changes in Fluo-8 fluorescence over the basal fluorescence ($\Delta F/F_0$) in response to extracellular application of 100 μM ATP to fibroblast cultures. Lines represent mean values ± s.e.m. Right – average peak amplitudes of Ca^{2+} responses to 100 μM ATP normalized to the basal fluorescence before stimulation in fibroblasts from WT mice (black bar) or FXS mice (red bar).(modified from GS thesis and unpublished GS & JJG).

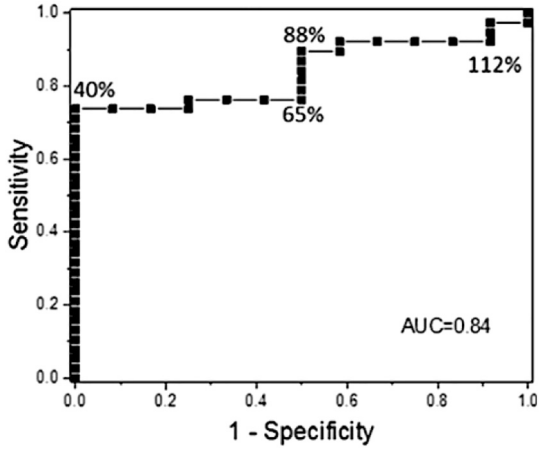

Fig. 3. Receiver operating characteristic (ROC) curve for ATP-evoked Ca^{2+} signaling in sporadic and syndromic ASD. ROC curve results for syndromic ASD cell lines (N = 15), sporadic ASD subjects (N = 23), and unaffected neurotypical controls (N = 12). Sensitivity (the true-positive rate) was plotted against (1-specificity) (the false-positive rate) for each value of Ca^{2+} signaling response normalized to a reference control cell line. Numbers in % reflect Ca^{2+} signaling cutoff values (presented as % of the reference cell line) to illustrate how different threshold values influence specificity and sensitivity of the ROC curve. The ROC curve shows the assay is able to achieve 73% sensitivity and 100% specificity in discriminating ASD samples from neurotypical control samples using a cutoff value of 40% of the reference cell line (modified from Schmunk et al., 2017 [148]).

ratio was observed among a large cohort of ASD patients (n = 100). Subsequently in a separate large population-based study analyzing mitochondrial function in autistic children in Portugal [165], elevated plasma lactate levels were found in 20% of ASD patients, and 7% of patients had a definite diagnosis of a mitochondrial respiratory chain disorder. Significantly, no mtDNA abnormalities were found in this group. Further epidemiological evaluation of the Portuguese cohort revealed functional mitochondrial disorders to be the second most common autism-associated disorder, accounting for 5% of cases [166].

The discovery of different mutations in the mtDNA of patients with a variety of systemic or tissue-specific energy deficiency syndromes established that mtDNA mutations can cause multiple diseases [164,167,168]. Since the mtDNA only contributes to mitochondrial components, finding a disease-associated mutation within the mitochondrial genome would make the strongest argument for mitochondrial dysfunction as a direct primary defect *causing* ASD, though only a few isolated case reports have been published. One study suggested this may be due to technical limitations by demonstrating challenges in identifying heteroplasmic mtDNA mutations in typical nonsyndromic ASD [169]. All mtDNA is only inherited via the egg—hundreds of copies, and none are passed via the sperm. Here, mothers of four patients with autism had detectable levels of a pathogenic "MELAS mutant" (A3243G mtDNA), which is known to cause MELAS (myopathy, encephalopathy, lactic acidosis, strokes) syndrome at high heteroplasmy levels. However, the mtDNA mutation was only detectable in two out of four affected sons, even after mtDNA had been tested from five different tissues [169]. Heteroplasmy arises when a new mutation occurs in a strand of mtDNA, creating populations of wild-type (WT) and mutant chromosomal copies. With time, both the mutant and original WT copy replicate and create a different heteroplasmic mix of the two types of mtDNA in different tissues at each generation. Early on after a *de novo* event, the low heteroplasmy level may cause no phenotype since mixed heteroplasmic mitochondrial populations can support cell survival even when the mutation, like the one described in this study, can cause a very severe functional defect. Several publications have reported mtDNA mutations in atypical ASD patients having seizures as a prominent component of their syndrome [170,171], but more recent comprehensive resequencing studies have shown mixed results. Two studies have found an enrichment for heteroplasmy at conserved, predicted-pathogenic sites in the mtDNA in sibs with ASD compared with neurotypical sibs [172,173], one finding a reduced mtDNA abundance in cells from patients with ASD [173], and another study finding that the very common mtDNA variants that define ancestral haplogroups contribute to ASD risk [174]. However, a large study found no evidence to suggest a major role for mtDNA variation associated with ASD susceptibility [175].

Mitochondrial functions are controlled by both the mitochondrial genome (mtDNA) and over 1000 genes scattered throughout the nuclear chromosomal DNA (nDNA). So while there is only limited evidence that mtDNA variants contribute to ASD risk, there is substantial evidence that chromosomal nDNA variants that impact mitochondrial function *do*. Rare chromosomal rearrangements that alter gene dosage have the well-established ability to link complex phenotypes to specific chromosomal loci. Both visible chromosomal lesions as well as the smaller copy number variants (CNVs) that are below karyotype resolution but still span millions of base pairs, are found in 10–20% of ASD cases and are important contributors to its genomic architecture [67]. As discussed above, *de novo* CNVs represent a major component of the genomic risk to ASD [73]. The most common CNV in ASD is at 16p11, and, counter intuitively, a remarkably similar ASD risk is conferred by deletions or duplications of that 600 kb region [176], a feature of gene dosage shared by several CNV loci associated with ASD.

The most common visible chromosomal rearrangement seen in autism, and the second most common CNV, is an inverted duplication of the imprinted domain on chromosome 15q11-q13 [177]. The extra chromosomal material is maternally derived and typically carried as an extra "marker" chromosome [178]. Filipek and coworkers reported two unrelated children with autism and a maternal 15q inverted duplication marker chromosome, noting biochemical signs of mild mitochondrial dysfunction including elevated serum lactate, pyruvate, ammonia and alanine, elevated urinary lactic acid, a secondary carnitine deficiency, and a biopsy-proven functional partial deficiency of mitochondrial respiratory complex III [179]. The specific dosage-sensitive gene(s) causing the 15q syndrome, however, remains controversial. Evidence of individuals with the full phenotype, but with only small deletions covering just the *CHRNA7* locus that encodes the calcium-permeable nicotinic alpha 7 receptor channel subunit, strongly suggests that this gene was the major contributor to the phenotype [180]. But more recently, while this locus was required for the full phenotype in mouse models, the *OTUD7A* gene, encoding a protease that specifically cleaves ubiquitin linkages, was shown to be responsible for the majority of the behavioral and dendritic spine defects [181]. A similar pattern of biochemical signs of modest mitochondrial energy-deficiency to those of the 15q syndrome are found in another syndromic form of ASD caused by mutations in *MECP2* (located at Xq28) that produce Rett syndrome in heterozygous females but are embryonic lethal in hemizygous males. The female Rett syndrome patients had a modest lactic acidosis and hyperammonemia [182] and the mouse model had functional defects in mitochondrial respiratory complex III [183].

The number of mitochondria in each cell type depends on its energy demand. For instance, the brain has one of the highest demands, consuming about 20% of the total body energy, though it accounts for only 2% of the total body mass. Mitochondrial-produced ATP supports many neuronal functions including synaptogenesis, release of neurotransmitter and synaptic plasticity, intracellular ion homeostasis and action potential generation (for an extensive review, refer to Pei and Wallace, 2018 [184]). Proper mitochondrial function is thus crucial for tissues with high-energy demands as they become especially vulnerable when they are pushed beyond the threshold for normal physiological functioning. As such, the organelle's dysfunction most frequently manifests as an abnormality in brain function, although its dysfunction is present in all cells throughout the body.

In addition to the ion channel genes that confer a risk of ASD discussed above, there is increasing evidence that support the role of mitochondrial carriers and calcium pumps in autism [77,185–187]. SNPs in *SLC25A12*, encoding a brain-specific isoform of the mitochondrial calcium-regulated aspartate/glutamate carrier, were previously associated with ASD in a study involving 197 families [188]. However, this remains a controversial finding on meta-analysis [186,189,190]. In a different study, the product of the *SLC25A12* gene showed significantly increased transport activity in post-mortem brain tissue of six autistic patients when compared with tissue collected from matched controls. This was found to be associated with elevated cytosolic calcium levels in the ASD tissue [191]. However when controlling for the calcium levels, transport activity was identical in isolated mitochondria from ASD and unaffected individuals. Since no mutations or polymorphisms at the locus were associated with the ASD cases [191], these results suggest that the critical link to this altered mitochondrial metabolism in ASD was *caused by* a change in calcium homeostasis. More recently *ATP2B2*, discussed above, encoding a plasma membrane calcium ion pump, has proven to be one of the most reproducible associations with ASD in resequencing studies, although thus far no biophysical studies have offered evidence of a functional change [77].

The analysis thus far suggests that cytosolic calcium signaling, mitochondrial function, and autism have many domains of intersection from clinical, genetic and biochemical/biophysical functional studies. Below, we examine evidence largely from other groups that suggest these findings may be synthesized into a unified underlying mechanism.

7. Mitochondria and ER interactions in calcium homeostasis and metabolism

The mitochondria's role in sequestering large amounts of calcium was first discovered > 50 years ago [192]. Subsequent studies have since been able to identify the mechanism by which calcium transport occurs. Calcium is passively transported across the relatively permeable outer mitochondrial membrane through the VDAC pores, then the inner mitochondrial membrane, largely by the mitochondrial calcium uniporter (MCU), that is electrogenically-driven by the mitochondrial electrochemical potential gradient. This occurs only during periods of high cytosolic calcium ion concentrations (previously reviewed extensively by Marchi et al. 2018 [193]). The primary recognized role of this calcium uptake is to regulate mitochondrial metabolic function according to the cell's needs through an impact on three key dehydrogenases in the tricarboxylic acid (TCA) cycle [194]. As the intramitochondrial calcium ion concentration increases, the TCA cycle is also upregulated, modestly increasing the mitochondrial potential and increasing ATP generation [195–197].

At rest, cytosolic calcium concentrations are strictly maintained between 100 and 300 nM, and, therefore, intracellular ion concentrations during normal physiological cell function will not reach levels that appear necessary for initiating mitochondrial calcium uptake. Thus, it was not clear how mitochondria might participate in physiological calcium signals.

This paradox was solved upon recognizing that mitochondria are located in close proximity to IP_3R channels with close “synapse-like” contacts that were first observed by electron microscopy in 1950s [198], but have been subsequently confirmed by FRET, immunochemistry and mass spectrometry (for review see Rizzuto et al. 2012 [199]; Csordás et al. 2018 [200]). Higher concentrations of calcium ions are reached in these areas compared with bulk cytoplasm [201]. In mammalian cells 5–20% of mitochondrial membranes are estimated to be in these contacts connected by a number of protein tethers that create microdomains that facilitate rapid calcium ion transfer [199]. These domains have come to be known as “mitochondria-associated membranes” (MAMs) (see Fig. 4).

MAMs are regions of tight interactions between the ER and mitochondria, and some of the MAM proteins are related to ER tubule formation and mitochondrial fission and fusion [202]. One of the MAM proteins, mitofusin 2, encoded by *MFN2* on chromosome 1p36, when silenced or ablated disrupts ER morphology and loosens ER-mitochondria interactions, consequently reducing the efficiency of calcium translocation to mitochondria. A reduction of MFN2 decreased calcium uptake by mitochondria, overexpression of MFN2 triggers apoptosis [203] and dominant and recessive mutations in the gene produce a Charcot-Marie-Tooth axonal neuropathy [204]. MAM mitochondria-ER contact sites do not contain RyRs but are enriched with IP_3Rs physically linked with VDAC – the high conductance voltage-dependent channel in outer mitochondrial membrane, through the molecular chaperone, glucose-related protein 75 (GRP75) [205,206]. GRP75 couples the IP_3R to the VDAC to facilitate calcium transfer from the ER lumen to the mitochondrial matrix. Mutations in GRP75 cause mitochondrial disease phenotypes including a dominant sideroblastic anemia, the recessive Even-Plus multiple congenital anomaly syndrome, and heterozygous mutations have been observed overrepresented in patients with Parkinson's disease [202,207].

Patch-clamp studies on isolated mitochondria have now identified MCU, the main route for calcium ion uptake into mitochondria through inner membrane, as a highly calcium-selective ion channel. Recently the structure of MCU (encoded by the *MCU* gene on chromosome 10q22), and the nature of its associated regulatory peptides were defined [208,209]. Two regulatory peptides, MICU1 and MICU2, encoded by genes on chromosome 10q22 and 13q12, appear to work as a regulatory gate sensing calcium ions in cytoplasm, inhibiting MCU opening when cytosolic calcium is low, near resting levels, and promoting opening when calcium is elevated [210]. Therefore, when calcium in the cytoplasm is at resting levels (about 200 nM), it is not transported into the mitochondria through MCU. MICU2 seems to play the role of reducing the affinity of the channel for calcium such that conditions sufficient for calcium uptake are only met by mitochondria very close to the mouth of the IP_3R calcium release site; more distant mitochondria are unable to participate. This phenomenon would help shape the distribution of the cytoplasmic calcium signal that can be “decoded” to carry out, in a highly specific fashion, a number of cellular functions [211]. There are also thought to be other routes for calcium to enter the mitochondrial matrix since the mitochondria of MCU-deficient knockout mice were found to have diminished, but not zero, matrix calcium [212]. Those mice have a markedly impaired ability to perform strenuous work, thus supporting the idea that prompt increases in matrix

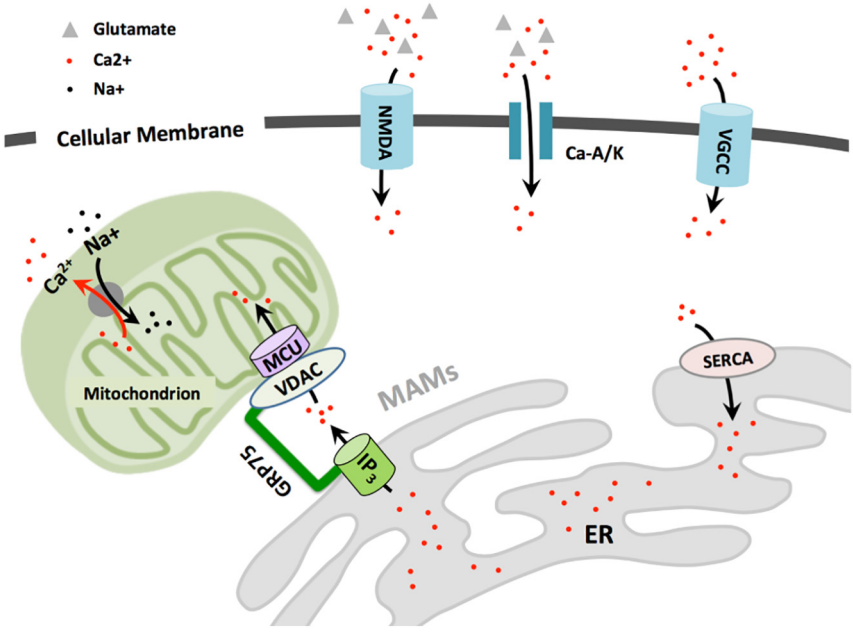

Fig. 4. Schematic of Ca^{2+} signaling in neuronal cells. Ca^{2+} permeates the plasma membrane through NMDA and Ca^{2+}-permeable AMPA channels (Ca-A/K) activated by glutamate, and voltage gated Ca^{2+} channels (VGCC). Once in the cytoplasm, Ca^{2+} quickly accumulates into mitochondria largely through mitochondrial Ca^{2+} uniporters (MCU) and is slowly sequestered into the ER by SERCA pumps. ER is the major Ca^{2+} depot in the cells. Ca^{2+} is released from the ER in response to IP_3 receptor or ryanodine receptor activation. In areas of close co-localization of ER and mitochondria (MAMs), IP_3 receptors (IP_3) directly interact with VDAC1 (VDAC) on the outer mitochondrial membrane assisted by the molecular chaperone glucose-related protein 75 (GRP75). This flux plays a critical role in maintaining mitochondrial energy production and viability. Ca^{2+} leaves the mitochondria largely by Na^+/Ca^{2+} exchanger.

calcium *via* the uniporter are necessary under conditions of increased ATP demand (for review, see Marchi and Pinton 2014 [213]; De Stefani et al. 2015 [214]).

Constitutive IP_3R calcium "feeding" of the mitochondria *via* MAM regions is essential for maintaining sufficient mitochondrial calcium for producing adequate reducing equivalents of NADH to support normal levels of oxidative phosphorylation and hence the suppression of autophagy [215]. Calcium signals transmitted in this fashion enhance mitochondrial basal oxygen consumption and electron transport flux and enhance lactate production of the cells [215]. Blockers of MCU, such as Ru360, had similar effects to genetic knock-out of the three IP_3R loci. The KO phenotype could be rescued with a transfected WT, but not channel-defective (T2591A) or calcium-impermeant (D2550A) IP_3R3, and was similarly eliminated with the IP_3R blocker xestospongin B (XeB). Transfection of the ryanodine receptor RyR2 was without effect. This fairly conclusively defines the essential calcium-signaling pathway to mitochondrial energy metabolism as being calcium release from the IP_3R through MCU into the mitochondrial matrix [215]. This pathway does not impact mTOR, but rather involves pyruvate dehydrogenase kinase and AMPK phosphorylation. Interestingly, these essential highly localized stochastic calcium signals required to maintain optimal mitochondrial function can be observed as quantal events with TIRF microscopy and "optical patch clamp" techniques [216]. In a salts solution and the absence of nutrients and growth factors these IP_3R elementary channel openings occur with an unchanged amplitude, but at a ten-fold reduced frequency [215], a finding of net decreased calcium flux reminiscent of that seen in our studies of fibroblasts from ASD subjects. *This observation perhaps provides the critical link between the clinical findings of mitochondrial dysfunction, lactic acidosis, an absence of mtDNA mutations, and IP_3R flicker openings of the channel we have observed in ASD.*

With strong neuronal stimulation, a substantial load of calcium fills the cytosol by entering across the plasma membrane through ligand- and voltage- gated calcium channels. As cytosolic calcium is tightly regulated, this load is cleared by a comparatively rapid uptake by mitochondria and more gradual transfer into the ER lumen or back across the plasma membrane by calcium ATPases [199]. The rapidly sequestered mitochondrial calcium is then slowly released through the mitochondrial calcium exchangers back into the cytosol, thus maintaining a persistently elevated level of calcium for a prolonged period. In the presynaptic terminals this elevated calcium level enhances the release of neurotransmitter from synaptic vesicles [86,217]. Several studies have demonstrated that preventing calcium uptake by mitochondria abrogates the short-term increase in post-stimulus release of neurotransmitter [218–221]. This mechanism is implicated in regulating synaptic transmission [221,222], converting the strength of an input stimulus into the duration of neurotransmitter release [220] and participating in various forms of synaptic plasticity [223,224], such as facilitation, and post-tetanic potentiation. All are attributed to the effects of a residual elevation in presynaptic calcium, acting on one or more molecular targets that appear to be distinct from the secretory trigger responsible for fast exocytosis and phasic release of transmitter in response to single action potentials [225]. However, with excess neuro-stimulation, occurring in pathologic conditions such as ischemia, brain trauma or prolonged seizures, and hence over-accumulation of calcium, mitochondrial function can become disrupted, typically resulting in increased reactive oxygen species (ROS) production [226,227] and the opening of the mPTP pore. This promotes mitochondrial swelling and the release of cytochrome C and other pro-apoptotic peptides [195], and ultimately apoptosis.

Free radicals (FR) and ROS are generated in the cell as byproducts of cellular metabolism. Mitochondria and the electron transport chain (ETC) of their inner membrane are considered to be the major source of both in most mammalian cells [197,228]. At physiological conditions "redox homeostasis", a balance between FR/ROS generation and scavenging, is tightly regulated by cellular antioxidant systems. Either too high or too low concentrations can cause deleterious effects [197,229]. Deficiencies of mitochondrial function, especially the abnormalities of ETC complexes, can cause imbalance in FR/ROS production thus disrupting homeostasis and leading to mitochondrial and cellular damage.

At high concentrations, FR/ROS is a real danger posed to living organisms since they can damage cellular elements. This includes oxidative damage of lipids in cellular membranes, proteins and DNA. It is important to note that this environment is particularly dangerous for mitochondrial DNA since it does not have the protective histones or elaborate repair mechanisms of nuclear DNA, so is much more prone to oxidative damage and hence to *de novo* mutations. A modest amount of FR/ROS, however, does play an important physiological role that can even mediate cellular processes involved in protection from oxidative damage and reestablishing "redox homeostasis". They also can serve as signaling molecules for different physiological functions, including initiating the coordinated activation of mitochondrial fission or autophagy [229,230].

Raturi et al. highlighted the emerging idea that calcium signaling and the redox state of the ER are correlated, especially at the MAMs [231]. ER calcium release and uptake mechanisms are directly modulated by redox-sensitive chaperones and oxidoreductases. In addition, a close interconnection exists between calcium and ROS at the ER-mitochondrial junction. Using an H_2O_2 sensor, Booth et al. demonstrated that H_2O_2 is transferred from the mitochondrial matrix to the ER–mitochondrial sites [232]. These H_2O_2 transients are able to modulate ER calcium release, since blocking of H_2O_2 release eliminated normal calcium oscillations [205,232].

Accordingly, the MAMs functions appear to play a critical role in unifying into a single mechanism many of the diverse observations in ASD that impact ROS, energy-deficiency, calcium signals, IP_3Rs and mitochondrial dysfunction.

8. Concluding remarks

The findings discussed in this review indicate that IP_3R-mediated signaling is affected in a majority of cases of ASD, pointing to the ER as a functional "hub" where the different cellular signaling pathways hinted at in the genomic architecture of ASD converge to contribute to its pathogenesis. This defect was first demonstrated at the molecular level in our previous work where multiple samples of three distinct monogenic Mendelian genetic disorders, each highly comorbid with ASD, all shared the same distinct change in optical patch clamp records—a flicker channel opening distinctly different in kinetics from that observed in the neurotypical cells [146]. This change in channel gating is caused by a still-undefined post-translational event, as there are normal calcium stores, a normal abundance of the IP_3R proteins, and they do not contain mutations on their encoding genes. This change may be a reflection of a protein phosphorylation or other covalent modification [233], an altered clustering of IP_3R proteins with themselves or other signaling proteins in the complex [101], or a different distribution of mobile non-signaling *vs* static calcium-signaling IP_3R sites [107]. This molecular calcium-signaling defect could also be captured in a high-throughput FLIPR screen by activating P2Y purinergic receptors in cells from several additional monogenic syndromes *co*-morbid with ASD as well as typical polygenic sporadic ASD, allowing them to be resolved from those of neurotypicals [148].

In addition to its role in calcium homeostasis, the ER serves as a key integrator of environmental stressors with metabolism and gene expression as it mediates a host of broadly ranging cell stress responses such as the heat shock and unfolded protein responses [234,235]. This battery of well-defined ER stress responses may well be the window through which environmental components, long-sought as contributing to ASD risk statistics, achieve their effect. It thus holds the potential to reveal relevant environmental, prenatal or physiological stressors contributing to the disorder, and perhaps its "epidemic" increase. Because

of the ubiquitous nature of IP_3R signaling and its diverse roles in almost all cells of the body, deficits in IP_3-mediated calcium signaling may not be limited to neurological correlates of ASD, but may also explain other characteristic ASD-associated heterogeneous symptoms that cannot be readily traced to neural circuits, such as those of the gastrointestinal tract [236,237] and immune system [238,239].

As we have discussed in this review, clinical signs of cellular stress in ASD have frequently been attributed to defective mitochondrial function, which is well documented in patients with ASD. In view of the emerging role recognized to be played by MAMs in biophysical studies, and in the absence of mtDNA mutations, but still with multiple ASD-associated chromosomal mutations in genes encoding proteins interacting with mitochondrial function, it is reasonable to consider the possibility that mitochondria play a *secondary* role in the disorder – potentially downstream of MAM interaction with an upstream IP_3R dysfunction. This is further supported by highly reproduced clinical findings of biochemical signatures of mitochondrial energy deficiency and enhanced ROS production in ASD. The emerging role of ER-mitochondrial interactions in the control of cell metabolism has laid a foundation for beginning to formulate this model to unify the clinical findings in ASD with those resolved at the cellular and molecular level.

Together, this perspective also opens the prospect that a skin biopsy sample could become a functional cellular diagnostic and surrogate clinical trial outcome end-point measure, much as long has been the case for neurogenetic encephalopathies caused by defects in mitochondria, lysosomes and peroxisomes [37–39,240]. Using this cell-based assay, novel or repurposed candidate drugs may be used to evaluate their efficacy on a subject's cells prior to their enrollment in a clinical trial as a measure to feasibly improve subject homogeniety and hence the prospects for successful trials and novel medications to help children with autism and their families. Likewise, this assay may be used as a diagnostic test that could help shorten the median age to diagnosis of ASD, which currently stands at 4–5 years old. This delay, classically involving a “diagnostic odyssey” to several specialists, delays diagnosis well after many critical developmental stages. Early diagnosis minimally ends the odyssey and would allow early intervention with behavioral therapy, known to be most effective at early time points.

Although human skin fibroblasts have advantages as a diagnostic sample and as a model cell system to study ASD, the central pathology of ASD lies in neuronal dysfunction. To truly understand the disease pathogenesis these studies need to be extended to examine IP_3/calcium signaling in neurons. Recent advances in stem cell biology and somatic cell reprogramming now allow the generation of patient-derived induced pluripotent stem cells (iPSCs) to be differentiated into neurons, glia and other cell types *in vitro* [241]. We have begun to use neuronal and glial cells derived from fibroblasts from a selected set of monogenic and sporadic ASD subjects and matched, neurotypical controls. Ultimately, this will allow a determination of how calcium signaling is altered in the central nervous system cells from ASD subjects, how these alterations relate to the corresponding deficits in the fibroblasts from which the iPSC cells are derived, and provide a means to explore consequences for neuronal excitability. Given the ubiquity of the ER IP_3 calcium signaling pathway across all cells of the body, and decades of experience with other organellar diseases, it is reasonable to expect that the deficits observed in fibroblasts from ASD subjects will be reflected in neuronal function; however, that remains to be experimentally determined, and will be a critical achievement. The correlation between calcium signaling deficits in neurons *vs*. fibroblasts among different subjects should reveal the extent to which neuronal deficits can be inferred from measurements in fibroblasts, and whether similar correlations hold true across different monogenic and sporadic cases of ASD. If such correlation exists, it would greatly support the view that calcium signaling deficiencies may play a causative role in ASD pathogenesis and provide an enormously powerful tool to dissect the underlying mechanisms.

Conflict of interest

None of the authors have a conflict of interest.

Transparency document

The Transparency document associated with this article can be found, in online version.

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