Review

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John Jay Gargus *

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Article

Genetic Dissection of Energy-Deficiency in Autism Spectrum Disorder

John Jay Gargus

Professor, Medical genetics and genomics-Pediatrics, Physiology&Biophysics, Founding Director Center for Autism Research and Translation, University of California, Irvine, Founding Scientist & Chief Scientific Officer, NeuroQure, 15375 Baranca Parkway, Suite E-104, Irvine, CA 92618; jjgargus@neuroqure,com

Summary

ASD reflects a mismatch between recently evolved brain regions and their high metabolic demands, driven by genomic evolution impacts on lipid signaling and mitochondrial vulnerability.

Abstract

An important new consideration of Autism Spectrum Disorder (ASD) is the bioenergetic mechanisms underlying the recent rapid evolutionary expansion of the human brain, and the fundamental risks this poses for mitochondrial dysfunction and calcium signaling abnormalities, and their potential role in ASD with insights from the BTBR mouse model of ASD. The rapid brain expansion in Homo sapiens, particularly in the parietal lobe, has led to increased energy demands, making the brain vulnerable to metabolic disruptions seen in ASD. Mitochondrial dysfunction in ASD is characterized by impaired oxidative phosphorylation, elevated lactate and alanine levels, carnitine deficiency, abnormal reactive oxygen species, and altered calcium homeostasis. These dysfunctions are primarily functional rather than due to mitochondrial DNA mutations. Calcium signaling plays a crucial role in neuronal ATP production, with disruptions in ITPR-mediated ER calcium release observed in ASD patient-derived cells. This impaired signaling affects the ER-mitochondrial calcium axis, leading to mitochondrial energy deficiency, particularly in high-energy regions of the developing brain. The BTBR mouse model, with a unique Itpr3 gene mutation, exhibits core autism-like behaviors and metabolic syndromes, providing valuable insights into ASD pathophysiology. Various interventions have been tested in BTBR mice, as in ASD, but none have directly targeted the Itpr3 mutation or its calcium signaling pathway. This review highlights the need for further research into metabolic resilience and calcium signaling as potential diagnostic and therapeutic targets for ASD.

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The Rapidly Expanded Homo Sapiens Brain and ASD Vulnerability

The adult human brain accounts for only 2% of body mass, but requires >20% of basal metabolic energy, a value that approaches 60% in the neonate. Apparently these energetic demands restricted early hominid brain volume to ~500cc for millions of years [1,2,3], but early genus Homo brain volume slowly began to expand over the past 2Myr, and then about 60-70kyr ago it underwent a rapid expansion to ~1500cc the current size of the modern Homo sapiens brain[1]. This was accompanied by a wide assortment of HAR (human accelerated region) and human-specific genetic changes that affect PUFA (polyunsaturated fatty acid) metabolism, such as the FADS (tight linkage block of Fatty Acid Desaturase genes clustered at chromosome 11q12), transport (e.g. MFSD2A) and lipid-mediated signaling (e.g. phosphoinositides, endocannabinoids), all involved in shaping

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neuronal membranes with an influence on synaptic plasticity by regulation of calcium signaling, and all vulnerable to disruption in neurodevelopmental disorders [4].

Most of the Homo sapiens' unique brain expansion occurred within the parietal lobe, particularly at the precuneus on the medial surface of the parietal lobe just rostral to the cuneus of the occipital lobe and behind the paracentral lobule, dorsal to the splenium of the corpus callosum. This network has high synaptic densities and maintains large dendritic arbors that rely heavily on aerobic metabolism. Importantly the precuneus is *functionally* connected to many regions across multiple lobes and serves as an integrative default mode network (DMN) hub which carries out visuo-spatial imagery from the first-person perspective, involved in self-awareness, social cognition, and memory, all uniquely human cognitive capacities of special interest in ASD, as these phenotypes allude to features of ASD and this region shows both functional and structural abnormalities in ASD, including reduced connectivity and altered energy metabolism[5,6,7,8].

In ASD, mild mitochondrial dysfunction is a common finding, evidenced by impaired oxidative phosphorylation, elevated lactate and alanine levels, carnitine deficiency, abnormal reactive oxygen species (ROS) and altered calcium homeostasis, but much less frequently mitochondrial DNA mutations [9,10,11,12,13,14]. Recently a large meta-analysis demonstrated that altered brain pH and lactate levels are commonly observed in many animal models of neuropsychiatric disorders and ASD, providing further evidence supporting the hypothesis that altered brain pH and lactate levels are not mere artifacts, but are rather involved in the underlying disease pathophysiology [15].

In ASD these are primarily functional mitochondrial defects [10], and they are particularly relevant in the context of energy-hungry brain regions like the precuneus and its cortical "social brain" network, areas amongst the brain structures displaying the highest resting metabolic rates [5] and vulnerablities in ASD.

Mitochondria play a critical role in fueling the brain's highly oxidative-dependent energy needs. The mitochondria's oxidative metabolism of glucose, the human brain's normal energy source, provides 16 times the ATP of anaerobic metabolism. A few minutes of anoxia causes a rapid shutdown of the brain, leading to coma and death [16]. Even brief fasting illustrates the human brain's high sensitivity to its extraordinary energy demands. At the onset of fasting, blood glucose is quickly consumed, and the liver begins to release glucose from glycogen stores to temporarily support the brain while other tissues switch to oxidizing long-chain fatty acids. However, these fats cannot cross the BBB, so again a special process is developed to support the brain when glycogen's glucose is gone. Ketone bodies, acetoacetate and β-hydroxybutyrate, which can travel on monocarboxylate transporters across the BBB, are produced for the brain by the liver to be oxidized by the mitochondria to achieve a high ATP yield [17]. While other primate brains utilize ketone bodies to a limited extent, they still largely relying on glucose. The evolution of the modern human brain not only greatly enhanced the capability of ketone body production, but also generated large fat supplies in the fetus, unique among the primates [18], to assure a secure supply of lipids for newborn brain growth and its energy supply.

Autism Spectrum Disorder (ASD) may arise in part from a fundamental mismatch between the unique structural and functional expansion of recently evolved human brain regions—particularly association cortices involved in complex cognition—and the exceptionally high metabolic demands these regions impose. Evolutionary genomic changes, including human-specific regulatory sequences and gene networks, have reshaped pathways crucial for lipid signaling, such as the synthesis, transport, and metabolism of polyunsaturated fatty acids that are essential for neuronal membrane integrity and synaptic function [19]. Simultaneously, these genomic shifts have introduced vulnerabilities in mitochondrial biology, affecting processes like calcium handling, oxidative phosphorylation, and reactive oxygen species detoxification. The combined effect is that newly evolved cortical circuits may operate close to metabolic limits, rendering them particularly susceptible to mitochondrial dysfunction and impaired lipid-mediated signaling, which together contribute to neurodevelopmental disruption and the diverse phenotypic spectrum observed in ASD.

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Neurons have huge, fluctuating ATP demands because of variable rates of synaptic transmission and required restorative ion pumping to reestablish transmembrane ionic gradients of Na^+, K^+ and Ca^{2+}. The rate of mitochondrial ATP production is homeostatically regulated to match ATP consumption in response to a signal that energy demand has increased, and that signal is overwhelmingly cytosolic calcium sensed as it is sequestered by the ER [20]. Additionally, prolonged ER calcium release can deplete ER stores, triggering store-operated calcium entry (SOCE) to replenish calcium levels and sustain mitochondrial energetics [21]. The drop in luminal ER Ca^{2+} concentration is sensed by the stromal interaction molecule (STIM) proteins, especially STIM1. When ER calcium levels fall, STIM1 undergoes a conformational change and migrates to areas of the ER membrane close to the plasma membrane (ER-PM junctions). STIM1 physically interacts with Orai1, a calcium channel in the plasma membrane, triggering it to open, resulting in store-operated calcium entry (SOCE) — an influx of extracellular Ca^{2+} into the cytosol. SOCE replenishes ER calcium levels by allowing newly entered Ca^{2+} to be pumped back into the ER via SERCA pumps [22].

Besides serving as the major intracellular calcium store, the ER's other major job is protein folding and post-translational modifications, both highly ATP-dependent and involving chaperones like BiP (GRP78), calnexin, and calreticulin. When cellular ATP levels fall, these endoplasmic reticulum (ER) energy-intensive processes of protein folding and quality control deteriorate due to reduced SERCA (Sarcoplasmic/Endoplasmic Reticulum Ca^{2+}-ATPase) pump activity. Physiologically the pump actively transports Ca^{2+} ions from the cytosol to sequester them in the lumen of the ER, and its failure produces the drop in ER luminal Ca^{2+} that impairs proper protein folding. As a result, unfolded and misfolded proteins accumulate within the ER lumen, triggering "ER stress". This stress, alternatively referred to as the unfolded protein response (UPR) is orchestrated by three principal ER stress sensors: IRE1, PERK, and ATF6. IRE1 (inositol-requiring enzyme 1) possesses both kinase and endoribonuclease activity; upon activation, it catalyzes the unconventional splicing of XBP1 mRNA, removing a 26-nucleotide intron, thereby producing a potent transcription factor (XBP1s) that induces genes involved in protein folding, ER-associated degradation (ERAD), and lipid synthesis to expand ER capacity [23]. PERK (protein kinase RNA-like ER kinase) phosphorylates the α-subunit of eukaryotic initiation factor 2 (eIF2α), globally attenuating cap-dependent protein translation to reduce the influx of new proteins into the stressed ER. However, this phosphorylation paradoxically enhances translation of specific mRNAs, such as ATF4, a transcription factor that activates genes related to amino acid metabolism, antioxidant responses, and apoptosis under prolonged stress [24]. ATF6 (activating transcription factor 6), meanwhile, is translocated from the ER to the Golgi upon ER stress, where it is cleaved by site-1 and site-2 proteases. The liberated cytosolic fragment (ATF6-N) migrates to the nucleus to upregulate expression of ER chaperones (e.g., BiP/GRP78, GRP94) and ERAD components, thereby promoting restoration of ER proteostasis [25]. Together, these pathways maintain cellular homeostasis but can trigger apoptosis if ER stress remains unresolved.

This physiological homeostatic regulation, mediated by the ER calcium reservoir, upon "stress" stimulation, releases calcium uniquely through its inositol 1,4,5-trisphosphate receptors (ITPRs) which are physically coupled to the mitochondrial voltage-dependent anion channel (VDAC) via the molecular chaperone GRP75 (glucose-regulated protein 75) , forming an exclusive molecular bridge to MCU (Mitochondrial Calcium Uniporter) in the inner mitochondrial membrane; the high local calcium concentrations at MAMs overcoming MCU's low Ca^{2+} affinity. Once inside mitochondria, Ca^{2+} activates three key dehydrogenases of the TCA cycle: pyruvate dehydrogenase (PDH), isocitrate dehydrogenase (IDH), α-ketoglutarate dehydrogenase (α-KGDH). This boosts the cycle's NADH and $FADH_2$ production and hence their donation of electrons into the inner mitochondrial membrane electron transport chain (ETC) enhancing ATP synthesis by oxidative phosphorylation. Hence, Ca^{2+} couples neuronal firing into a bioenergetic homeostatic loop with a Ca^{2+} -sensing arm during sequestration by the ER and an effector arm of Ca^{2+}efflux through the ER's ITPR coupled to the VDAC of the mitochondrion and the Ca^{2+}-sensitive rate of mitochondrial ATP production [26].

Recent studies have demonstrated the effector arm of this critical homeostatic loop is functionally altered in ASD. In fibroblasts and iPSC-derived GABAergic interneuron precursors

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from ASD patients, it has been demonstrated that ITPR calcium release from the ER is impaired [27,28,29,30]. Optical patch-clamp single-channel recordings reveal a molecular abnormality of ITPR gating, resulting in brief flicker openings of otherwise normal ITPR channels. These single-channel kinetic abnormalities are similar to those found in genetic seizure, migraine and arrhythmia channelopathy syndromes [31,32], suggesting ASD is an organellar calcium channelopathy. This flicker opening is best revealed as a low flux and a slow, low calcium wave signaling signature in response to P2Y receptor-induced calcium release from the ER [28].

Our group initiated the fibroblast calcium transport studies when the Autism Sequencing Consortium, and others carrying out ASD re-sequencing studies, began being able to identify clusters of significant risk genes for ASD [33,34,35,36]. Along with clusters of transcription factors and clusters of chromatin proteins, the largest, and biophysically most tractable, cluster were synaptic calcium signaling proteins. In functionalizing this genetic clue, with plans to produce iPSC neurons for complete biophysical characterization, studies of components of calcium flux in the primary skin cells themselves were undertaken and surprisingly revealed a shared defect in intracellular calcium store release in several independent skin samples of Fragile X, Tuberous Sclerosis 1 and Tuberous Sclerosis 2, all monogenic syndromes highly co-morbid with ASD [28]. The ryanodine receptor release channel was normal, but the component released via the ITPRs was dramatically reduced. Optical patch clamp was used to resolve single channel kinetics of these ITPRs and it revealed a very short open time in the monogenic mutants, with all other properties unchanged from neurotypical controls [28]. This flicker opening suggested that the channel open state was very unstable, rapidly collapsing and producing a greatly reduced calcium store efflux. This could be captured in whole cell imaging studies via P2Y receptor activation on a Molecular Devices FLIPR automated fluorescent imaging plate reader [29]. This high-throughput method was used to study skin samples from the monogenic ASD subjects, but also subjects with typical polygenic non-syndromic ASD documented with research-grade ADOS testing in the ASD range. Area under the curve (AUC) separation on receiver operator curves (ROC) between ASD and neurotypical controls was 84%, comparable to that of the ADOS itself [29].

Since ITPR gating is essential for coupling calcium signaling dynamics to mitochondrial energetics through the molecular bridge between the ER ITPRs and mitochondrial VDAC

pores [37], this defective gating disrupts the homeostatic energy balance axis [30]. Consequently, this results in mitochondrial energy deficiency, a vulnerability particularly pronounced in the developing brain with high energetic needs. This mismatch between the evolutionary development of a complex, energy-demanding brain and its bioenergetic control could contribute to ASD susceptibility, particularly under non-optimal metabolic conditions [30]. The recent evolutionary achievement of an enlarged social brain may come with a biological cost in the form of ASD vulnerability under stressors, potentially reflecting its recent divergence leading to modern *Homo sapiens*, evolution that has not yet stabilized [38, 39].

Mitochondrial energy deficiency caused by this unstable calcium gating and flux may disrupt homeostatic energy balance that may impair developmental network maturation and contribute to social brain deficits in ASD (39). Convergent neuroimaging techniques highlight this same energy-deficiency phenotype in functional brain imaging studies of ASD. fMRI studies show decreased metabolic energy consumption, seen as reduced blood flow, and diminished functional connectivity, found within the default mode network (DMN), especially in expanded areas such as the precuneus, medial prefrontal cortex, and facial recognition area of the fusiform gyrus [40, 41]. EEG data reveal altered alpha and gamma oscillations, reflecting impaired synchronization and insufficient energy availability [42,43,44]. Most specifically, near-infrared spectroscopy (NIRS) studies show blunted mitochondrial cytochrome oxidase responses in the temporal and frontal lobes during language and social interaction tasks [45,46]. These findings suggest that ASD vulnerability and the energy-deficiency phenotype in ASD reflect compromised bioenergetic function in the recently evolved association cortices of the “social brain” that themselves were driven by the rapidly evolving

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chromosomal HARs, and these findings suggest these chromosomal and brain regions are also those most disrupted in ASD.

A Mini-Evolutionary Case Study, the BTBR Mouse: From Abnormal Taste Preferences to Calcium Signaling-Based ASD

An evocative mini-evolutionary tale in mice tells a potent parallel story of ASD energy deficiency hiding in plain sight. The BTBR mouse is widely recognized as one of the most valuable models of typical nonsyndromic autism. This inbred strain was largely developed by sib matings in the mid-20th century at Columbia and was primarily maintained due to its coat color—short for Black and Tan (a^t/a^t) and being heterozygous for the BRachyury T allele (T/+) resulting in a shortened tail, hence BTBR [47]. Through early crosses establishing the strain in the 1950's, they inserted the "tf" tufted allele as a marker for the tightly linked lethal T allele, which became fixed in the colony long before its molecular identity was discovered [48]. Mary Lyon isolated the unique tufted (tf) locus as a spontaneous recessive mutation that caused characteristic waves of hair loss to spread from snout to tail. It was mapped tightly linked to the brachyury (T) complex on chromosome 17 [49], and the tufted pattern of hair loss (tf/tf) was subsequently used as a visible marker to identify carriers of the complex lethal T/T locus [48,50].

The mouse T gene has many known mutant alleles, both spontaneous variants and lab-engineered mutations. Classic spontaneous alleles include the original mutation discovered in 1927, which causes truncated tails and missing sacral vertebrae in T/+ heterozygotes, and is embryonic lethal when homozygous (T/T). The recessive t alleles are defined by their interaction with T to cause tail-lessness in double heterozygotes (T/t) [48]. This complex locus has been intensively characterized over the years and had originally been maintained in the BTBR strain as a segregating locus, but it had been lost and was wild-type (T+/T+) sometime prior to the acquisition of the current strain by the Jackson Laboratory from the McArdle Laboratory in 1994, and therefore, the only studies on the BTBR strain are (a^t/a^t T+/T+ tf/tf) [47,48,50].

BTBR mice were originally studied as models for phenylketonuria (PKU) and metabolic syndromes, showing insulin resistance, hyperinsulinemia, impaired glucose tolerance, pancreatic beta-cell dysfunction, and obesity/type 2 diabetes susceptibility [51]. They were also characterized with abnormal immune responses, being noted to have significantly higher amounts of serum IgG and IgE, of IgG anti-brain antibodies (Abs), and of IgG and IgE deposited in the brain, elevated expression of cytokines, especially IL-33, IL-18, and IL-1β in the brain, and an increased proportion of MHC class II-expressing microglia compared to B6 mice. BTBR mice are also significantly more susceptible to listeriosis than B6 or BALB/c mice, and the Th2-like immune profile of the BTBR mice and their constitutive neuroinflammation suggests an autoimmune-like profile. The heterozygous F1 mice had intermediate levels of Abs and cytokines [52,53,54].

Another phenotype for which the strain was studied was altered gut permeability, which was associated with microbial dysbiosis and immune dysregulation [55]. They have compromised intestinal barrier function, evidenced by increased translocation of FITC-dextran across the intestinal epithelium into the bloodstream, and this is accompanied by reduced expression of tight junction proteins like occludin and zonulin-1, indicating impaired barrier integrity. They suffer a dysbiosis with the gut microbiota composition in BTBR mice differing significantly from that of control strains, with notable alterations in bacterial genera such as *Bacteroides*, *Parabacteroides*, and *Sutterella*. These changes are associated with behavioral abnormalities and immune responses. Further, the gut sustains immune activation with the BTBR mice displaying elevated levels of pro-inflammatory cytokines, including TNF-α and IL-6, in colon tissues. This suggests an ongoing inflammatory response that may contribute to both GI and behavioral symptoms [55].

Compromised sensory systems were also recognized in the BTBR mice. They demonstrated distinct sensory processing anomalies such as altered nociceptive thresholds, with behavioral assessments indicating that BTBR mice have heightened sensitivity to painful stimuli, suggesting altered nociceptive processing [56].

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By the early 21st century, the Monell Chemical Senses Center in Philadelphia was developing murine screens for food taste preferences [57]. Here the BTBR strain stood out and it was intensively studied because it had an unusually high taste preference for calcium solutions. On detailed characterization, it was also found to have markedly abnormal preference scores for exemplars of the classic taste qualities of sweet, umami, and bitter, and comparably large abnormalities in preferences for the less well-accepted taste qualities of carbohydrate, calcium, and pyrophosphate. Smaller abnormalities were present in the taste responses to sour and salty taste compounds and to two monovalent chlorides (KCl and NH_4Cl) but not to $ZnCl_2$ or the irritant capsaicin. They further tried to isolate the taste preference locus but found that the same mutation is responsible for the BTBR strain's bad hair and its odd taste preferences. This caused them to focus on the "tf" (tufted) allele that had arisen spontaneously and was introduced into the BTBR strain during its early history soon after 1956, all of which suggested that the signal transduction cascade in type 2 taste receptor cells was impacted by the "tf" allele itself [58].

Also at the turn of the 21st century, as a major component of ASD was coming to be recognized as genetic, several systematic screens of mouse strains for autism-relevant behaviors were undertaken, and here a strong connection between the BTBR inbred mouse strain and ASD phenotypes were discovered, and this became the phenotype for which the strain was known [50,59,60,61]. These early findings reported that among numerous strains tested, BTBR T+^tf^/J mice displayed strikingly low sociability—much less social interaction than typical strains—along with high levels of repetitive self-grooming. Studies explicitly framed these strain differences as "autism-like" behavioral variations.

Following up on this lead, the Crawley lab at the NIMH published a detailed behavioral characterization of BTBR T+^tf^/J mice, confirming that BTBR exhibits all three core diagnostic features of ASD: abnormal reciprocal social interactions, impaired communicative behaviors, and repetitive/stereotyped behaviors [62,63,64]. They showed, for example, that BTBR mice engage in very little social sniffing or play compared to standard C57BL/6J mice, emit fewer social vocalizations, and display repetitive grooming at high frequency. They proposed BTBR as a model of idiopathic autism, given the breadth of its behavioral phenotype. Soon after, the Jackson Laboratory and other groups embraced BTBR as the ASD model, noting the strain's unique traits (e.g., JAX describes BTBR as exhibiting "several symptoms of autism…including reduced social interactions, unusual vocalizations, and low exploratory behavior" and a 100% incidence of corpus callosum agenesis)[65,66]. Notably, the "T" gene itself is not mutated in Jackson Labs BTBR (the strain carries the wild-type T allele, hence "T+"), so the connection to the T locus was historical—the BTBR name traces back to a brachyury mutation in its lineage. Nevertheless, the identification of BTBR's autism-like behavior raised the question of whether genetic factors in the T region (on Chr17) or elsewhere in BTBR's genome underlie its ASD-like phenotype.

It is particularly noteworthy that while eventually the behavioral phenotype of BTBR helped define it as a robust nonsyndromic ASD model with all three key behavioral features, all of its other previously-discovered phenotypes, discussed above, were never discussed as a core part of its ASD syndrome [67,68]. However, even a passing observation of the common complaints of families in an ASD clinic will call to mind the BTBR's evocative tactile sensory issues, its unusual food sensitivity and GI and immune complaints, not to mention the typical chronic lactic acidosis found in many patients (and now in all of the 109 models of ASD from Japan [15]. While many physicians caring for patients with ASD tackle these problems, and many do consider them part of the child's syndrome and therapy, none have yet been incorporated into the formal definition of the ASD syndrome [69].

In the meantime, the unusual taste preferences of BTBR drew further attention at Monell, and they carried out detailed recombination mapping of the BTBR T+^tf^ locus [70]. Now, in the post-genome era, they ultimately identified the *Itpr3* gene to carry the causal mutation [70]. The JAX catalog [50], by 2014, fully describes their sequencing of the mutation: "sequencing reveals a 12 bp deletion in Exon 23 (Chr17: 27238069, Build 38.1), which codes for amino acids 983–986 of the ITPR3 type 3 IP_3 receptor channel [70], as the mutation arose early in the history of the BTBR strain (in or

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soon after 1956). This mutation is not found in 18 other strains." The JAX catalog now reports six alleles of *Itpr3* in seven different genetic backgrounds; most are constitutive or conditional knockouts, and it notes that mice homozygous for a knockout allele are viable, fertile, and exhibit no apparent abnormalities in pancreatic and salivary secretion. However, only one mutation in this gene (that specific unique spontaneous "tf" allele in BTBR) results in the distinctive, yet poorly understood unusual hair and the now well-understood, inseparably-linked low sweet preference, as well as a full suite of model ASD phenotypes. While other alleles have been generated, there is no published report of an effort to recapitulate the now-known "*Itpr3*^tf^" allele [50]. The region encompassing amino acids 983–986 deleted by the "tf " mutation lies within the regulatory domain, which is crucial for modulating channel activity through interactions with various proteins and post-translational modifications. The deletion leads to a significant reduction of functional ITPR3 protein in certain tissues, such as taste receptor cells, and results in impaired taste perception. However, the mutation does not completely abolish ITPR3 function, suggesting a hypomorphic effect rather than a complete loss of function. Further, this partially functional protein may confer a gain-of-function aspect to the mutation via its potential to alter the receptor's critical regulatory mechanisms, potentially leading to aberrant calcium signaling.

With the specific identification of the mutation, the JAX catalog soon updated its BTBR designation to BTBR T+ *Itpr3*^tf^/J, and it remains the prime strain recognized for autism-like phenotypes. Behaviorally, a large number of groups and studies continue to expand on the range of phenotypes by which BTBR mice display robust autism-like behaviors [71,72,73]: they have profoundly impaired social interactions (failing to engage in normal amicable sniffing, social play, or mating behaviors with partner mice), deficits in social communication (for instance, BTBR pups emit fewer ultrasonic distress calls, and adults have atypical vocalization patterns), and repetitive behaviors (such as excessive self-grooming and a unique vertical jumping or "inchworming" stereotypy). These behavioral traits in BTBR are so pronounced that they fulfill the mouse analogs of autism's diagnostic triad. In fact, BTBR is often described as an idiopathic autism model with "face validity" for core autism symptoms [50,72,74].

Beyond behavior, BTBR mice also exhibit neuroanatomical and neurodevelopmental abnormalities reminiscent of findings in some people with ASD. Most strikingly, BTBR has a 100% penetrant absence of the corpus callosum, the major tract connecting the brain's hemispheres. This agenesis of the corpus callosum is accompanied by a markedly reduced hippocampal commissure and reduced cortical thickness in BTBR brains [65,66]. Histological studies have documented modest and selective alterations in glia, neurons, and synapses in BTBR forebrain, along with reduced neurogenesis in the adult hippocampus. Of all markers examined, the most distinctive changes were seen in the neurodevelopmental proteins NG2, PSA-NCAM, NeuroD, and DCX, consistent with aberrant development of the nervous system in BTBR mice, and novel substrates to link callosal abnormalities and autistic behaviors [66].

MRI and histological studies have further found that BTBR brains differ in white matter and subcortical structure volumes: for example, reduced size of the corpus callosum and internal capsule, changes in the cerebral peduncle, and volume differences in regions like the hippocampus, striatum, and globus pallidus [67]. Such changes align with neurodevelopmental connectivity anomalies hypothesized in autism. BTBR also shows altered cortical organization — one study noted disruptions in the formation of functional cortical areas and early neurogenesis in BTBR, paralleling neurodevelopmental patterning deficits [73].

At the cellular and molecular level, BTBR mice have been found to have neuroimmune and synaptic differences relevant to ASD. For instance, BTBR has an elevated baseline of activated microglia and astroglial markers in certain brain regions, and it mounts an exaggerated neuroinflammatory response compared to control mice. Induced systemic inflammation leads to significantly more microglial activation in BTBR than in B6 mice [52]. This hyper-reactivity of the immune cells in the brain may mirror the increased neuroinflammation observed in some individuals with autism.

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On the synaptic side, unbiased transcriptomic and proteomic profiling of BTBR brain tissue has revealed alterations in expression of many autism-relevant genes and proteins, characterized by multiple genetic and epigenetic aberrations [75]. While pathway analyses point to disruption of several inter- and intracellular signaling pathways, many genes and proteins involved in the development and maintenance of proper connectivity within the brain were also affected. Notably, BTBR brains show dysregulated levels of BDNF (Brain-Derived Neurotrophic Factor) and SHANK3 (a synaptic scaffolding protein), among other molecules, compared to typical mice. Both BDNF and SHANK3 are critical for synaptic development and have been implicated in human ASD, so their alteration in BTBR mice provides a mechanistic clue linking BTBR strain genetics to ASD-like neural phenotypes [75]. Functionally, BTBR neurons and oligodendrocytes may develop differently — for example, studies have noted precocious myelination patterns in the BTBR brain (i.e., early myelin deposition in frontal brain regions) and reduced neuronal plasticity, which could underlie some behavioral rigidities [76].

Strikingly, while the Monell group has made use of the identification of the "tf" locus to recognize that there is a role for the *Itpr3* receptor in the signal transduction cascade in type 2 taste receptor cells through G-protein coupled receptors (GPCR) and lipid signaling molecules [70], this mechanistic analysis has not yet been reflected in the ASD studies, which continue to use increasingly broad genomic and proteomic approaches to ferret out some mechanistic connection in BTBR to the ASD phenotype. Despite the widespread use of BTBR in ASD research, no published study has yet attempted to rescue or isolate the *Itpr3*^tf^ allele using modern CRISPR/Cas genome editing. No targeted correction or complementation cross has been reported to dissect whether Itpr3 is necessary or sufficient for the ASD-like traits in this strain [71,73].

This gap is both a cautionary tale and an opportunity: as we increasingly use animal models to infer molecular mechanisms, we must attend closely to their full genomic context. And in the case of BTBR, we may have been using, unknowingly, the conspicuously best available in vivo model of ITPR-linked neurodevelopmental dysfunction all along. BTBR poses a unique model for translational biology in ASD. The BTBR T+ Itpr3^tf^/J strain therefore represents a natural in vivo model of altered ITPR3 function. Unlike total knockouts (0% function) or knockout heterozygotes (50% function), this hypomorphic allele has apparently some specific unique gain-of-function characteristic such that uniquely this homozygote mimics the subtle, partial ITPR-signaling deficits seen in ASD patient cells [28,29,30]. It offers a rare chance to link molecular-level dysfunction with emergent behavioral phenotypes in a widely accepted animal model.

Moreover, because BTBR also demonstrates immune, sensory, GI, and metabolic alterations—domains increasingly implicated in children's syndromes with ASD pathophysiology—it could enable a systems-level dissection of how ER ITPR calcium signaling intersects with broader physiological domains of the "*full* ASD syndrome".

The simplest interpretation of the aggregate data is that the *Itpr3^tf^* allele is etiologic and diagnostic of the BTBR strain, rather than simply an innocuous linked- marker to its autism-relevant behaviors. But to date, no published experimental evidence has been attempted to link Itpr3 hypomorphic function to BTBR's neurobehavioral phenotype, and no engineered line isolates the *Itpr3^tf^* allele or rescues it to test causality. So there is a major knowledge gap explicitly testing what seems to be the clear contribution of impaired ER calcium release via Itpr3 to ASD-like behavior in BTBR, as has been explicitly observed in ADOS-confirmed children with ASD. This most parsimonious mechanistic hypothesis appears to be one surprisingly not yet studied. *Itpr3^tf^* is not yet considered a major ASD gene in BTBR; it is usually never mentioned in a publication and those that do at most mention an effect on taste; it remains an incidental, historical marker for this strain, a strain best felt to typify typical polygenic nonsyndromic ASD, unlike major gene mutations such as those in *Shank3* or *Fmr1*. Studies routinely compare the BTBR homozygote to a WT line, without studying the heterozygote phenotype. There is no widely available "BTBR T+" strain without *Itpr3^tf^* for direct comparison. The tf allele is essentially part of the BTBR package — you can't currently order a "tf-negative" BTBR from a standard supplier [50,74].

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Despite ignoring the *Itpr3* mutation as a target, over the past 20 years many interventions have been tested on BTBR mice to reverse or ameliorate its ASD-like behaviors. These interventions span pharmacological, dietary, microbiome, epigenetic, and environmental strategies.

Pharmacological interventions include: mGluR5 antagonists such as MPEP, which reduce repetitive behaviors [77], and selective negative allosteric modulators like GRN-529, which reduce both repetitive behaviors and social deficits [78]. Dopamine D2 receptor modulation has been recommended, based on findings of increased D2R density in the striatum of BTBR mice. Agents like risperidone and aripiprazole are suggested to normalize stereotypic behavior [79]. GABAergic interventions such as nanoformulated bumetanide, an NKCC1 ion cotransport inhibitor. When injected into the medial prefrontal cortex (mPFC), it reduces hyperexcitability and improves social interaction [80]. Even epigenetic and environmental approaches have been included, such as HDAC inhibition with sodium phenylbutyrate, which reduces repetitive self-grooming and rescues social and cognitive deficits [81] and environmental enrichment and neurostimulation, both shown to improve sociability and reduce repetitive behaviors in BTBR [82]. Furthermore, dietary interventions have also been explored, such as PUFA supplementation (omega-3 fatty acids), which had minimal impact [83], and ketogenic diet, which by contrast, has shown reproducible benefits in reducing repetitive grooming, anxiety, and improving vocal communication—especially when applied in juvenile BTBR mice [84,85]. More recently the microbiome has been an area of growing interest, finding that a butyrate-producing probiotic improves intestinal barrier function and reduces BTBR behavioral abnormalities [86].

The key point is that all of these therapeutic manipulations target behavioral phenotypes but none target Itpr3 directly. Most interventions act on synaptic plasticity, excitation-inhibition balance, GABAergic function, or global epigenetic modulation. The unique mechanistic implications of the *Itpr3^tf^* allele—and the BTBR strain's apparent ITPR dysfunction in taste bud signaling—have been left largely unexplored in therapeutic studies to date---except in taste perception [69].

One mechanism by which the ITPR potentially contributes to ASD is via its control over cell death and survival decisions. This occurs through regulation of ER calcium release and, consequently, autophagy, apoptosis, and impacts on cellular proliferation and migration. These processes are critical not just in neurodevelopment, but also in cancer, where IP_3R3 has recently emerged as a key regulator [87,88,89].

Final Synthesis

Putting all this together, a powerful idea emerges. One that says this: Autism reflects a mismatch between the evolutionary demands of our modern brains and the metabolic systems that support them. The strongly supportive observations are:

- The evolutionary importance of, and potentially critical historical ecological niche permitting, a newly-evolving energy-hungry enlarged human "social brain", conveying the uniquely human set of "theory of mind" capabilities underlying language and culture, but that are specifically challenging in ASD.
- The critical role of ITPR channel gating in calcium signals that homeostatically control mitochondrial oxidative metabolic energy production, and the molecular ITPR gating defect that compromises calcium signaling and energetics, that is observed in patients with an ADOS-confirmed diagnosis of ASD.
- The findings that the unique BTBR mouse model of ASD has cryptically harbored a unique missense mutation of that same mechanism, the *Itpr3* gene, that *causes* a syndrome not merely of taste dysfunction and unusual hair, but that includes *all* conventional ASD behavioral abnormalities as well as an additional broad, recognizably-patient-related set of physiological abnormalities, of the gut, immune cells and sensory systems, *all* related to its ability to model a *typical ASD syndrome*, and already mechanistically-linked to signaling via the ITPR.

This synthesis implies that our social, symbol-using enlarged energy-hungry brains gave us language, art, weapons, and culture, allowing us to "read each others minds" and thereby form

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conquering tribes larger than families and "emerge from Africa" to cover the entire globe [90,91], but they also created a huge energetic burden. If something disrupts the delicate balance of energy supply during brain development, whether due to mitochondrial issues, calcium signaling problems, or environmental stressors, it may cause these newly-evolved energy-demanding networks to fail and tip the scale toward developmental conditions like autism. This theory does not suggest that evolution "caused" autism. Instead, it offers a new framework for understanding why certain children may be more vulnerable, especially when their developing brains do not receive the necessary energy support they need. It also opens new paths for intervention. If ITPR signaling and mitochondrial energy metabolism play a role in ASD, it might allow specific targeted diagnostics and therapeutics to be developed. Treatments that support mitochondrial function or improve calcium signaling could be beneficial for some individuals. With new specific targets comes new interventions. Research in these areas is still evolving, but early findings are promising.

A Hopeful Future

ASD is complex and multifaceted. No single explanation will account for every individual's experience. But by looking back to our evolutionary past and connecting it to the molecular, cellular, and systems realities of the brain, we may better understand the biological roots of autism, and how to support those on the spectrum better today.

Understanding ASD through the lens of energy metabolism opens the door to novel diagnostics and interventions. One company at the forefront of this approach is NeuroQure (Neuroqure.com), which offers the *ASD Insight@* test. *ASD Insight* is a first-of-its-kind offering that analyzes calcium signaling abnormalities in patient-derived cells as early as two days after birth through a discarded foreskin or minimally invasive skin sample [92]. By measuring how calcium flows through IP3R channels that are found in all cells of the body and play a critical decisive step in transducing a host of cellular signals into action, the test helps identify mitochondrial and cellular signaling patterns that may contribute to the development of ASD symptoms. This cellular molecular biophysical analysis represents a significant leap toward more biologically informed autism diagnostic solutions and treatments that support metabolic resilience in the brain.

It is important to note that when it comes to ASD, early detection is crucial. It allows for tailored interventions during critical developmental windows, enhancing communication, social skills, and overall quality of life for children with ASD [93, 94,95]. By identifying potential challenges before behavioral symptoms emerge, families and healthcare providers can proactively plan and implement strategies that support optimal developmental outcomes.

In summary, both the evolutionary role of mitochondrial energetics and synaptic signaling lipids in the emergence of the culminating worldwide, large hyper-prosocial brain that led to the development of modern human-specific, culture-inducing prosocial skills, and the variant or mis-expression of many of these late-evolving skills in the phenotype of autism, as well as the same battery of deficits being recognized in the best mouse model of ASD, which by happenstance has a unique mutation and a unique defect in function of of the Itpr3 receptor channel, makes a compelling rationale for how an assay of this ITPR function, even in skin cells from a newborn child, could provide a window into vulnerability to ASD. This is above and beyond the empirical finding that assay of this ITPR molecular dysfunction provides a 75-85% AUC ability to distinguish ASD [29]. Perhaps by turning our focus to this well-understood mechanism, rather than broadcast agnostic multi-omics hunts, we may, first, be able to hasten diagnosis, and therefore supportive therapy, but then perhaps next an era of precision guided therapeutics to a known structure.

The structure of the inositol 1,4,5-trisphosphate receptor type 3 (IP_3R3), encoded by the ITPR3 gene, has been elucidated through advanced structural biology techniques. Notably, a high-resolution cryo-electron microscopy (cryo-EM) structure of the human ITPR3 was recently published, providing detailed insights into its architecture and functional mechanisms [96]. These structural studies demonstrate the structural heterogeneity of ITPRs in the presence of IP_3, ATP, and Ca^{2+}, and correlating these structures with their plausible functional states should allow definition of

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conformational changes at different gating states that underpin ITPR activation and gating. These structures should prove to be foundational for dissecting the function of the "tf" mutation and potential therapeutics targeted at the functionally implicated target for ASD therapy, as has been done for other ion channels underlying other channelopathies [97,98,99,100,101].

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