Metabolic and Behavioral Features of Acute Hyperpurinergia and the Connection to Autism Spectrum Disorder

Zarazuela Zolkipli-Cunningham

University of California San Diego School of Medicine

Jane C. Naviaux

University of California San Diego School of Medicine

Tomohiro Nakayama

University of California San Diego School of Medicine

Charlotte M. Hirsch

University of California Irvine

Jonathan M. Monk

University of California San Diego School of Medicine

Kefeng Li

University of California San Diego School of Medicine

Lin Wang

University of California San Diego School of Medicine

Thuy P. Le

University of California San Diego School of Medicine

Simone Meinardi

University of California Irvine

Donald Blake

University of California Irvine

Robert K. Naviaux (✉ mnaviaux@health.ucsd.edu)

University of California San Diego School of Medicine <https://orcid.org/0000-0001-5832-2297>

Research

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Abstract

Background. Since 2012, studies in mice, rats, and humans have suggested that abnormalities in purinergic signaling may be a final common pathway for many genetic and environmental causes of autism spectrum disorder (ASD). The current study in mice was conducted to characterize the bioenergetic, metabolomic, breathomic, and behavioral features of acute hyperpurinergia triggered by systemic injection of the purinergic agonist and danger signal, extracellular ATP (eATP).

Methods. Responses were studied in C57BL/6J mice with ASD-like behaviors in the maternal immune activation (MIA) model and controls. Basal metabolic rates, respiratory exchange ratios ($RER = VCO_2/VO_2$), and locomotor activity were measured in CLAMS cages. Plasma metabolomics measured 401 metabolites. Breathomics measured 98 volatile organic compounds.

Results. A 0.5 $\mu\text{mol/g}$ dose of ATP dropped whole body oxygen consumption by $74\% \pm 6\%$ (mean $\pm$ SEM, 5,303 to 1,382 ml/kg/hr, $p < 0.0001$) and rectal temperature by $6.2^\circ \pm 0.3^\circ\text{C}$ ($p < 0.0001$) in 30 minutes. Over 200 metabolites from 37 different biochemical pathways were changed. MIA animals were hypersensitive to the metabolic and behavioral responses triggered by eATP and poly(IC). Breathomic and metabolomic analysis revealed changes in folate-methylation-1-carbon, purines, pyrimidines, acyl-carnitines, glycolysis, aromatic and branch-chain amino acids, Krebs cycle, glutathione, urea cycle, phospholipids, sphingolipids, eicosanoids, cholesterol, bile acids, vitamins, and microbiome metabolism similar to children with ASD.

Limitations. The responses to ATP were studied in only a single genetic strain of mice (C57BL/6J). Although similar to metabolic results reported in FVB mice and a small clinical trial in children with ASD, the generalizability of these results to larger studies in humans is unknown. The chronic effects of repeated postnatal ATP exposures were not tested.

Conclusions. Acute hyperpurinergia produced metabolic and behavioral changes in mice that were similar to those found in children with ASD. These behaviors and metabolic changes were associated with mitochondrial functional changes that were profound but reversible.

Introduction

Over the past decade our group has tested a new unifying hypothesis for the origin and treatment of autism spectrum disorder (ASD) in animal models [1-3] and a small human clinical trial [4]. This new hypothesis proposes that the behavioral symptoms and neurobiology of ASD are the result of a new kind of metabolic syndrome that arises from abnormalities in purinergic signaling. Purinergic signaling was first described by Geoffrey Burnstock in 1970 [5], and refers to the action of purines like ATP, ADP, AMP, and adenosine, and some pyrimidines like UTP and UDP-glucose, when they bind to specific cellular receptors [6]. Interestingly, ATP or its metabolites have also been found to be co-neurotransmitters and neuromodulators at every immunologic [7, 8] and neural synapse that has been studied to date [9]. Persistent abnormalities in purinergic signaling can be traced to a new functional state of the cell and its neighbors. This new functional state is maintained by mitochondrial-nuclear crosstalk and is called the cell danger response (CDR) [10]. Because this new metabolic syndrome traces to the evolutionarily conserved cell danger response, the new conceptual

framework for the origin and treatment of ASD can be called the CDR hypothesis. Experiments have shown that the CDR is not limited to disturbances in ATP metabolism. The CDR is a highly coordinated, multisystem response that involves hundreds of metabolites and over 30 different metabolic pathways. Once deployed, the metabolic changes of the CDR propagate and cause changes in gene expression, cytokines, methylation, neuroendocrine and autonomic circuits, the microbiome, the immune system, and behavior. Despite the multisystem nature of the CDR, a picture is emerging that shows disturbances in purinergic signaling are at or near the root of the tree of all the changes that occur when a cell is threatened by genetic or environmental danger.

All stressed cells release ATP in proportion to the degree of environmental threat through stress- and redox-gated pannexin/P2X7 and other channels in the plasma membrane, and by vesicular export [11, 12]. The stress can come in the form of positive threats like infection, poisoning, genotoxicity, physical or psychological trauma. Stress can also come from negative threats like deficiencies in oxygen, water, calories, vitamins, nutrients, or even gravity [13]. Each of these stresses can lead to an increase in ATP release. Extracellular ATP then serves as a pro-inflammatory molecule and damage associated molecular pattern (DAMP) [14, 15] that is an effector of the CDR. The release of ATP to the extracellular space has the effect of decreasing intracellular energy reserves, prompting further adaptive changes in mitochondria, metabolism, and gene expression. The words used to describe these adaptive changes in response to changing environmental conditions have varied according to the experimental system being studied. When studying memory in neurons, the adaptive changes have been called long-term potentiation [16]. When studying other cell types and organisms, this highly conserved adaptive response has been called allostasis [17], hormesis [18], and adaptive homeostasis [19]. Each term is used to describe a different aspect of the same biology. The molecular aspects of the CDR include the integrated stress response (ISR) [20]. The systemic features of the CDR are coordinated by remote sensing and signaling (RSS) mechanisms that involve ATP and other metabolites like glutamate that bind to receptors, regulate, or are conducted by solute carriers (SLCs) and ABC transporters [21]. Earlier work on the connection between the immune response and cellular damage and danger by Polly Matzinger and Ephraim Fuchs was published in the 1990s [22, 23]. The systemic, or whole-body CDR starts with the cellular ISR that is coordinated by mitochondria [20, 24, 25]. More recent work has now tied the CDR to healing, aging, early life stress events that can change the trajectory of child development [26], and the cellular response to environmental pollution [27-29].

Mitochondria not only coordinate the CDR by producing 90% of the ATP in the cell, regulating cellular redox, energy metabolism, and epigenetics [30, 31], but also play a pivotal role in both the response to [29], and regeneration after injury [32]. Purinergic signaling abnormalities have also been found by gene ontology analysis of transcriptomic data from post-mortem brains of children with ASD [33]. The purinergic signaling abnormalities found in that study were correlated with ASD behaviors and with a coordinated set of abnormalities in mitochondrial oxidative phosphorylation, protein and lipid synthesis. Other metabolic markers of the CDR, like changes in tryptophan, methionine, folate, and glutathione metabolism have been described in several cohorts of children with ASD also trace to a new state of mitochondrial function [34-36].

The first genetic evidence that abnormalities in purinergic signaling might be involved in the pathogenesis of ASD came in 1969 [37]. This was the report of a child with ASD, high uric acid from increased purine metabolism, and a mutation in phosphoribosyl pyrophosphate synthase (PRPPS) that eliminated feedback

control and led to superactivity of this rate-limiting enzyme in *de novo* purine synthesis [38]. Over the next 50 years, several other examples of genetic disorders of purine and pyrimidine metabolism that cause ASD have been reported [39]. Point mutations in mitochondrial DNA have also been shown to cause ASD [40]. Based on these genetic leads, we tested the antipurinergic drug suramin to treat the Fragile X genetic model of autism in mice. That study found the top metabolic pathway that changed in association with the correction of ASD-like behaviors was purines [1].

Aims of the study

In the current mouse study, we used a combination of bioenergetic, breathomic, metabolomic, and behavioral analysis for two aims: 1) to test the effect of ATP injection in typically developing control animals as an experimental model of hyperpurinergia, and 2) to test the effects of postnatal ATP or poly(IC) injection in animals with pre-existing ASD-like behaviors in the maternal immune activation (MIA) model [41-43].

Materials And Methods

Animals and Husbandry

All studies were conducted in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) under UCSD IACUC-approved animal subjects protocol number S06135. C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained on *ad libitum* Harlan Teklad 8604 mouse chow and water. Animals were housed at an ambient temperature of 22-24°C and humidity of 40-50%, in a controlled access vivarium with a 12h light-dark cycle; lights on at 7 am and off at 7 pm. The thermoneutral zone for adult mice about 25-30 grams in weight is 29-31°C [44]. Below this temperature, additional calories must be burned to maintain body temperature. The human biological age equivalent for the C57BL/6J strain of laboratory mouse (*Mus musculus*) was estimated from the following equation: 12 years for the 1st month, 6 years for the 2nd month, 3 years for months 3-6, and 2.5 years for each month thereafter [2, 45]. Therefore, an 8-month old mouse would be about 35 years old ($= 12 + 6 + 3 \times 4 + 2.5 \times 2 = 35$ years) on a human timeline.

Reagents

ATP, ADP, GTP, adenosine (Ado), cAMP, cGMP, UTP, CTP, TTP, and poly(IC) were purchased from Sigma. Sterile saline was made from endotoxin-free, nuclease free H₂O and used as the solvent for all solutions.

Nucleotide administration

All nucleotide and drug challenge experiments were conducted between 9:00 am and 1 pm. Mice were given intraperitoneal (IP) doses of nucleotides or saline at an equal volume of 20 μ l/g. Intravenous doses were administered by lateral tail vein injection in maximum volumes of 5 μ l/g. Doses up to 0.5 μ moles/g were given as a 25 mM solution. Pilot dose-response experiments revealed that higher doses (>0.5 μ moles/g, as 50-100 mM solutions) of ATP IP resulted in non-linear phenotypic dose responses as they approached saturation. To initiate the maternal immune activation (MIA) model, pregnant dams received an IP injection of poly(IC) (Sigma-Aldrich Cat# P9582;) (0.17 A260 U/g; 2 mg/kg IP) on gestational day E12.5 and E17.5. Control dams

received normal saline (0.15 M NaCl; 5 µl/g IP) on E12.5 and E17.5. Pregnant dams were provided with nesting material and left undisturbed until offspring were weaned at 3-4 weeks of age. The postnatal challenge dose of poly(IC) (0.17 A260 U/g; 2 mg/kg IP) was tested in 8-9-month-old animals. Daily temperature was recorded for 14 days, then weekly for 4 weeks after poly(IC) administration.

Metabolomics

Blood was collected by submandibular vein lancet [46] into lithium-heparin BD microtainers (Cat# 365971, Becton-Dickinson) and inverted 10 times. Plasma was separated by centrifugation at 1500 g for 5 min in an Eppendorf microfuge and frozen at -80°C until use. Blood draws were performed between the hours of 9 am and 1 pm. Targeted, broad-spectrum, metabolomic analysis of 613 metabolites from 63 biochemical pathways was performed by LC-MS/MS as described [47] with minor modifications. A total of 401 of the 613 targeted metabolites were measurable in the plasma of both males and females. L-cysteine was not measured independently because of oxidation to its disulfide cystine that occurred in whole plasma with storage.

Breathomics

Breathomic analysis of natural gases and volatile organic compounds in mice was performed as described with minor modifications [48]. Six 10-month old C57BL/6J male mice were used in this study. Three mice received intraperitoneal injections with physiologic saline, and 3 were injected with 0.5 µmol/g ATP (0.6 ml IP of a 25 mM solution in 30-gram animals). Samples of exhaled mouse breath were collected from a 10 L glass bulb containing a mouse fitted with three sampling ports. One of the sampling ports was connected to a 1.9 L electropolished stainless steel canister via a Swagelok Nupro bellows valve using stainless steel flex tubing. Prior to sampling, the canisters were baked at 150°C for 24 hours, flushed with ultra-high purity helium, and vacuumed to 10⁻² mm Hg. Upon injection, each mouse was immediately placed in the sampling vessel and breath samples were collected for 20 seconds at 1, 5 and 10-minute intervals into three separate 1.9 L canisters for each mouse. For purposes of this analysis, the results for all 3 time points were pooled and averaged. Room air samples were collected before and after the study for background air analysis. The samples were analyzed for CO/CO₂ and CH₄ in addition to a list of about 100 volatile organic compounds (VOCs) using the 6-column-detector gas chromatography system in the Rowland/Blake laboratory at UC Irvine. The CO/CO₂ measurements were carried out using a Carbosphere 80/100 packed column with a flame ionization detector (FID) for CO and a thermal conductivity detector (TCD) for CO₂. CH₄ determination was performed on a separate GC system consisting of a packed column terminating at an FID. Analysis on the multi-column system began with cryogenic pre-concentration of sample at 400 mm Hg, followed by injection into 6 separate columns, contained in pairs in a combination of 3 ovens (Hewlett-Packard 6890 series GC system). Temperature ramp programs were set at -60°C to 220°C for 2 ovens and -20°C to 200°C for the third. This GC system utilized several detectors including FID, electron capture (ECD), and mass selective detectors (MSD). The combination of columns and detectors includes DB-1/FID (Agilent), PLOT+DB-1/FID (Agilent), Restek1701/ECD (Restek), DB-5+Restek1701/ECD (Agilent, Restek) and DB-5ms/MSD (Agilent), many of which quantify the same gases. The redundant gases for each column were plotted against one another, to ensure a linear trend. This elucidated chemical differences of any co-eluting peaks by comparison of different

column results. Exhaled hydrogen sulfide (H₂S) or methanethiol (monomethylsulfide) was not quantified in this analysis.

Chemokines and Cytokines

The plasma cytokine response to IP ATP (0.5 μmol/g) or saline was examined at baseline, 30-minutes and 4-hours after injection in 6-month old female C57BL/6J mice. Heparinized plasma was diluted 2-fold in saline and analyzed at the UCSD CTF-C Core Lab using the 7-plex MSD Multispot Assay system (Meso Scale Diagnostics, LLC, Gaithersburg, MD, USA) according to manufacturer instructions. The 7 cytokines examined and the lower limit of detection (LLOD in pg/ml) were: IL10 (11), CXCL1 (3.3), IL12p70 (35), IL1b (0.75), TNFa (0.85), IL6 (4.5), and IFNg (0.38).

Corticosterone Assays

Four-month old C57BL/6 females (N = 7 per time point) were treated with 0.5 μg/g ATP IP or saline at time 0. Blood samples were collected before, 30-minutes after, and 4-hours after treatment. Plasma concentrations of corticosterone were measured by ¹²⁵I double antibody radioimmunoassay using 10 μl of plasma diluted 1:200 with assay buffer (MP Biomedicals LLC, Orangeburg, NY). The intraassay coefficient of variation (CV) was 4% with an interassay CV of 7%.

Temperature Measurements

A BAT-12 Microprobe digital thermometer and RET-3 mouse rectal probe (Physitemp Instruments, Clifton, New Jersey) were used to obtain a temperature precision of +/-0.1°C, taking care to minimize stress-induced hyperthermia, as previously described [3].

Basal Metabolic Rate

Food intake, oxygen consumption and locomotor activity were measured using the Comprehensive Lab Animal Monitoring System (CLAMS, Columbus) in 13-minute cycles over a 3-hour period after nucleotide injection. Each mouse was housed in individual CLAMS cages with ad libitum access to Teklad 8604 standard mouse chow and water. Oxygen consumption (VO₂) and carbon dioxide production rates (VCO₂) were used to calculate the respiratory exchange ratio (RER = VCO₂/ VO₂).

Behavior

We developed a purinergic behavioral response scale (PBRs) to quantify the magnitude of the behavioral response to purines and pyrimidines. The PBRs was a 3-point (0-absent, 1-present, and 2-severe) severity scale that assessed 6 characteristics: 1) open field avoidance, 2) decreased exploratory behavior or locomotor activity, stillness, 3) imbalance/ataxia in a cage rim walk test, 4) piloerection, 5) rapid shallow breathing/panting, 6) shivering/rigors. The PBRs range was 0 (normal) to 12.

Statistical Analysis

Results are reported as mean $\pm$ SD unless otherwise noted. Peak area under the curve (AUC) data from metabolomics were $\log_2$ transformed, scaled by control standard deviations, and the resulting z-scores analyzed by univariate non-parametric analysis by Mann-Whitney U test, and parametric analysis of ≥ 3 groups by ANOVA. Cytokine data were $\log_2$ transformed using the generalized log transformation formula $\text{glog}_2 = \log_2(y+1)$ to accommodate fractional concentrations between 0 and 1 pg/ml. Simple Linear Regression was used to compare dose-dependent rates of temperature decline. Linear Mixed Models analysis was used for longitudinal time and temperature data. Metabolomic data were analyzed by multivariate partial least squares discriminant analysis (PLSDA). Post hoc correction for multiple hypothesis testing after ANOVA was done by Fisher's least significant difference method in MetaboAnalyst [49, 50]. The false discovery rate (FDR) method of Benjamini and Hochberg [51] was used for all other analyses. Bayesian false discovery rates were estimated using the Storey q value [52]. Metabolites with variable importance in projection (VIP) scores were determined by PLSDA. Significant metabolites were grouped into pathways and their VIP scores summed to determine the rank-ordered significance of each biochemical pathway. Random forest analysis [53] was used to rank metabolites for their ability to distinguish the different treatment groups using mean decrease in accuracy (MDA) scores. *k*-nearest neighbor (*k*-NN) clustering was used to identify metabolite groups that contributed in different ways to the experimental groups [54]. *k*-NN clusters were ranked by the sum of the VIP scores ≥ 1.0 and by positive MDA scores. Dendrograms were Euclidean using Ward clustering. Pearson and Spearman correlation and logistic multiple regression was used to identify metabolites most associated with acute ATP injection. Results were organized into biochemical pathways and visualized in Cytoscape version 3.4.0. Statistical methods were implemented in Stata (Stata/SE12.1, StataCorp, College Station, TX), Prism (Prism 8, GraphPad Software, La Jolla, CA), Python, or R.

Results

Study Overview

The overall design of this study is illustrated in Figure 1. Six different experimental techniques were used to characterize the response to acute ATP injection.

Metabolomics

Overview at 30 minutes

A total of 202 (50%) of 401 metabolites measured were significantly changed by ATP injection in 30 minutes (Table S1-S5, Figure S1-S12). The rank order of metabolites that were most changed is illustrated in Figure 2A. These metabolites belonged to 37 different biochemical pathways (Figure 2BC, Table S3) and showed FDRs < 0.05 , VIP ≥ 0.9 , and t-test p values < 0.05 (Table S2). There was a generalized decrease in plasma amino acids. Methionine levels in particular were strongly decreased, dropping by 43% (Table S2). All amino acids—both essential and non-essential—were affected (Figure S3). The average decrease across the 19 amino acids measured was -5.0 ± 2.6 (Z-score $\pm$ SD; Table S2). Fifteen ceramides and a class of phospholipids enriched in lysosomal and exosomal membranes, bis(monoacylglycero)phosphates (BMP), were also decreased (Table S3, Figure S6). Phosphatidylinositol (PI) lipids were decreased. The polar head groups of the major phospholipids were increased. This included choline, phosphorylcholine, ethanolamine and myoinositol.

Myoinositol, which is a phospholipid head group derived by PI lipid activation to inositol phosphates for calcium signaling and subsequent processing by phosphatases, was sharply increased by ATP injection. Other head groups like phosphorylcholine and ethanolamine were also increased. Phosphatidylethanolamine (PE) lipids were decreased, while their precursors phosphatidylserine (PS) lipids were increased. Several arachidonate-derived eicosanoids, including 5-HETE and 13S-HODE, and the endocannabinoid anandamide, were also increased (Figure S4, Table S2). Dopamine was strongly increased 30 minutes after ATP injection with a Z-score of +5.5 (Figure 2A, Table S2). Other striking effects of acute ATP injection included an increase in lactate, glycerol-3-phosphate, and pyrimidines like orotic acid, thymidine, and thymine. As expected, purine metabolites were strongly increased (Figure S2, Figure 2A-C, Figure S9). These included xanthosine, allantoin, inosine, hypoxanthine, xanthine, and uric acid. Allantoin is disproportionately increased compared to uric acid in the mouse because mice have an intact uricase gene, while primates do not [55]. Adenosine triphosphate (ATP) was rapidly metabolized and was not detectable in any of the plasma samples (Table S1; detection limit = 100 nM in heparinized plasma, data not shown).

The top 2 of 10 *k*-NN clusters at 30 minutes after ATP injection were responsible for 79% of the metabolic impact and contained 134 metabolites with VIP scores ≥ 1.0 , from 36 different metabolic pathways. Seventy-one (71) metabolites were increased with a mean Z-score of $+3.2 \pm 1.8$ (mean $\pm$ SD; cluster #2, Table S5, Figure S4) and belonged to a superpathway that included purines but was comprised of 26 different biochemical pathways. Sixty-three (63) metabolites were decreased with a mean Z-score of -3.4 ± 2.0 , including 19 of 19 measured amino acids, (cluster #1, Table S6, Figure S5) and belonged to a second superpathway comprised of an overlapping set of 26 different biochemical pathways (Tables S2-S5).

Overview at 4 hours

A total of 54 (13%) of 401 metabolites measured were changed at 4 hours after ATP injection (Figure 2D-F, Tables S1, S6-S9, Figure S1-S12). After 4 hours of recovery, one of 8 animals had recovered sufficiently to be indistinguishable from controls by multivariate analysis, while most others had not recovered completely. This is seen graphically by the overlap of sample points and the 95% confidence limits shown in the 2D PLSDA plots (Figure 2G; blue and pink circles), and in the dendrograms that show a clean statistical separation of ATP- and saline-treated animals at 30 minutes, but an intermixed response after 4 hours (Figure 2H). During subacute recovery measured 4 hours after eATP injection, phospholipids and sphingolipids, were decreased. Several bile acids like glycocholic and taurocholic acid remained decreased. Increased turnover of phospholipids was evidenced by an increase in the phospholipid head groups phosphorylcholine, ethanolamine, and myoinositol. Several markers of cellular oxidation were increased 4 hours after ATP injection. These included increased oxidized glutathione (GS-SG) and cystine (CysS-SCys), and increased markers of carnosine metabolism such as 1-methylhistidine and histidine. Changes in nitrogen metabolism were marked by increases in agmatine, homoarginine, and urea (Figure S4).

The top 3 of 10 *k*-NN clusters at 4 hours after ATP injection were responsible for 88% of the metabolic impact and contained 45 metabolites with VIP scores ≥ 1.5 that belonged to 27 different metabolic pathways (Table S9-S11). Thirty-two (32) metabolites in the top *k*-NN cluster were increased with a mean Z-score of $+2.2 \pm 0.7$ (mean $\pm$ SD; cluster #3, Table S10) and belonged to a superpathway comprised of 19 different biochemical

pathways. The second two superpathways contained 13 metabolites that were decreased with a Z-score range of -2.0 to -2.7 and were made up of 7 biochemical pathways (clusters #7 and #8, Table S9, S11).

A heat map of the 30 most increased or decreased metabolites is shown in Figure 2I. The proportional effects of purinergic signaling on all the biochemical pathways measured at 30 minutes and 4 hours after injection are illustrated in the Cytoscape map in Figure S1. Quantitative changes in purines, amino acid, methylation, sulfur, polyamine, and nitrogen metabolism are illustrated in Figure S9. Principal components analysis showed that metabolomics explained 81.2% and 74.9% of the phenotypic variance in animals at 30 minutes and 4 hours after ATP injection, respectively (Figure S10).

eATP and the microbiome

Eleven products of microbiome metabolism increased 30 minutes after eATP injection (Figure S7). These included an increase in the cysteine precursor O-acetylserine, the leucine precursor isopropylmalic acid, the carnitine metabolite trimethylamine-oxide (TMAO), the histamine metabolite imidazoleacetic acid, two phenylketones from microbial tyrosine metabolism, 4-hydroxyphenyllactic acid and 4-hydroxyphenylpyruvic acid, and the aryl hydrocarbon receptor-binding immunomodulatory molecule and tryptophan metabolite indoxyl-3-sulfate [56]. The mean Z-score for increased microbiome metabolites in the plasma was $+3.5 \pm 1.5$ (Table S2). Only two microbiome metabolites, butyrylcarnitine ($Z = -5.7$) and vitamin K2 (menaquinone; $Z = -1.2$) were decreased 30 minutes after ATP injection. Unsubstituted purine, thought to be a marker of purine-rich food intake [57], was decreased ($Z = -5.2$) 30 min after ATP. The significance of this is not yet understood. No microbiome metabolites were abnormal 4 hours after ATP injection, although glycocholic, taurocholic, and taurodeoxycholic bile acids remained low ($Z = -1.5 \pm 0.2$; Table S6).

eATP and plasma vitamin concentrations

A broad range of vitamins were acutely changed in the plasma 30 minutes after ATP injection (Figure S8). Thiamine (B1), niacin (B3), pyridoxic acid (B6), and choline were increased by a mean Z-score of $+3.4 \pm 1.6$ (Table S2). In contrast, the plasma intermediates and effectors of 1-carbon metabolism were decreased. These included serine, glycine, and trimethyl-glycine (betaine), with a mean Z-score of -4.4 ± 1.8 (Table S2). Vitamin D3 (cholecalciferol) was also decreased (Z-score = -1.5), although active 1,25-dihydroxy Vitamin D3 was unchanged (Table S2). Thiamine (B1), niacin (B3), and pyridoxic acid (B6) remained increased in the plasma 4 hours after eATP injection, with a mean Z-score of $+1.8 \pm 1.1$ (Table S6, Figure S8). Other vitamins and cofactors that were increased at 4 hours included 5-methyl tetrahydrofolic acid (mTHF), dimethylglycine, flavin adenine dinucleotide (FAD; B2), and L-carnitine with a mean Z-score of $+1.7 \pm 0.9$. No vitamins were decreased 4 hours after ATP injection (Table S6 and S9).

Breathomics

Overview

Accurate measurements of exhaled gases requires normalization for minute volumes using the rate of CO₂ production [48]. We found that ATP injection stimulated the release of volatile organic molecules ranging from 1 to 5 carbons in length in the first 10 minutes (Figure 3A-H). These included the three different 1-carbon

species: carbon monoxide (CO), methanol, and methane. One 2-carbon, sulfur-containing volatile was increased by ATP injection: dimethylsulfide. The remaining volatiles that were produced by acute hyperpurinergia included acetaldehyde (C2), acetone (C3), butyraldehyde (C4), and isoprene (C5; Figure 3A-H).

Chemokines and Cytokines

Cytokines were measured at baseline, 30-minutes and 4-hours after ATP or saline injection to permit comparison of metabolomic and cytokine data at these time points. The chemokine CXCL1, also known as KC and GROa, was increased 2.8 times compared to saline injections (526 ± 118 pg/ml vs 188 ± 75 ; $p < 0.0002$). CXCL1 binds the G-protein coupled receptor CXCR2 and facilitates the arrest of rolling neutrophils and monocytes at sites of inflammation [58]. The anti-inflammatory interleukin, IL10 was also increased (95 ± 56 vs 36 ± 12 ; $p < 0.04$) (Figure 3I-K). ATP was known to stimulate IL10 secretion from microglial cells in culture [59], but had not been studied in animals. By 4 hours, each of these had returned to baseline levels. IL6 trended toward being increased at 30-minutes but animal-to-animal variability in the saline controls limited a stronger statistical conclusion without a larger sample size (Figure 3 K). IL1b, TNFa, IFNg, and IL12p40 were measured but unchanged at 30 minutes and 4 hours after IP ATP injection (data not shown).

eATP Effects on Corticosterone Release

Previous studies have shown that adrenal corticoid synthesis and release are directly stimulated by purinergic signaling at the adrenal cortex, independent of ACTH [60]. We found that plasma corticosterone peaked 30 minutes after injection of ATP, then trended below baseline levels by 4 hours (Figure 3L). This pattern of response was consistent with acute stimulation of corticosterone release, followed by feedback inhibition of hypothalamic corticotropin releasing hormone (CRH) and ACTH. CRH and ACTH levels were not measured in this study.

eATP Effects on Body Temperature

We tested several nucleotides for their hypometabolic effects at the high dose of $0.5 \mu\text{mol/g}$ IP in both males and females (Figure 4AB, Figure S10). All adenine-containing purines (adenosine, AMP, ADP, and ATP) produced a decrease in rectal temperature with a nadir that was reached 30-60 minutes after injection and recovery by 120 minutes. This effect lasted longer when the dose was administered intravenously instead of IP (Figure 4C). The behavioral changes caused by ATP also lasted longer when given IV (Figure 4D). ADP was most potent at these high doses of $0.5 \mu\text{mol/g}$ IP in both males and females (Figure 4AB). We next evaluated the gender-specific potency of each purine under non-saturating doses of 0 to $0.20 \mu\text{mol/g}$ measured at 15 minutes to reflect the initial phase of the metabolic response. Under these conditions we found that females were about 70% more sensitive to the hypothermic effects of ATP, i.e., had a more rapid decrease in temperature (Figure 4E, Table 1), while males were more than twice (108%) as sensitive to ADP (Figure 4F, Table 1). AMP and adenosine were equally potent in both males and females (Figure 4GH). We also examined the metabolic effects of several other purines and pyrimidines and cyclic nucleotides at equimolar doses of $0.5 \mu\text{mol/g}$ IP compared to saline and ATP (Figure S11). In males, only cAMP showed a hypometabolic effect similar to ATP. In females, both cAMP and GTP showed some activity, but both were less potent than ATP.

eATP Effects on Behavior

The behavioral effects of ATP injection were stereotyped and dose dependent. The purinergic behavioral response scale scored the change in 6 behavioral characteristics: open field avoidance, decreased exploratory behavior or locomotor activity, rapid shallow breathing or panting, shivering or rigors, piloerection, and imbalance or ataxia. The onset of behavioral changes after a high dose (0.5 $\mu\text{mol/g}$) of ATP IP occurred within 1-2 minutes, peaked at 30 minutes, and resolved by 60 minutes of IP injection (Figure 4I). Normal movements and self-grooming behavior gradually reappeared after 45 - 60 minutes, but the abnormal behavioral features were prolonged after an intravenous (IV) dose of ATP (Figure 4D). When non-saturating, low doses of ATP were given IP (0.025 - 0.20 $\mu\text{moles/g}$; Figure 4J), and outcomes were measured in the linear initial phase 15 minutes after injection, significant gender differences were observed in the response to extracellular ATP (eATP injection). Males were $37\% \pm 3\%$ more sensitive to the behavioral effects produced by eATP than females (male behavioral response slope $b = 37.1 \pm 1$; female $b = 27.4 \pm 0.7$; $p < 0.000.1$; Figure 4J, Table 1).

eATP Effects on Whole Body Metabolism

The effects of eATP injection on whole body metabolism and oxygen consumption were quantified in Comprehensive Lab Animal Monitoring System (CLAMS cages, Figure 4K-N) using indirect calorimetry. By 26 minutes after a dose of 0.5 $\mu\text{mol/g}$ ATP, whole body oxygen consumption (VO_2) dropped by $74\% \pm 6\%$ (5,303 to 1,382 ml/kg/hr, $p < 0.0001$; Figure 4K) and the rate of CO_2 production (VCO_2) dropped by $76\% \pm 18\%$ (4323 to 1034 ml/kg/hr, $p < 0.0001$; Figure 4L). The respiratory exchange ratio ($\text{RER} = \text{VO}_2/\text{VCO}_2$) shifted from 0.84 ± 0.08 , reflecting a balanced usage of fat and carbohydrate to nearly complete dependence on fatty acids with an $\text{RER} = 0.70 \pm 0.062$, $p < 0.006$; Figure 4M, Table 1). Locomotor activity declined in both saline and ATP injected animals when placed in the wire-bottomed CLAMS cages for analysis, but the ATP-injected animals were nearly motionless between 26-52 minutes (Figure 4N, Table 1).

eATP Activates a Latent Metabolic Memory Response in the MIA Model of ASD

Acute temperature response

In the MIA model, pregnant female mice are exposed to a simulated viral infection by injection with the toll-like receptor 3 (TLR3) agonist poly(IC). This produces offspring with neurodevelopmental abnormalities associated with both autism spectrum disorders [61] and schizophrenia [62]. We administered ATP or saline to adult MIA offspring of poly(IC)-treated females and wild-type control offspring from saline-treated dams. We used a lower dose of 0.05 $\mu\text{moles/g}$ in females compared to 0.2 $\mu\text{mol/g}$ in males because of the increased sensitivity of females to the hypothermic effects of ATP. All animals were 8-9 months of age. This is the human biological age equivalent of 35-38 years of age (see Materials and Methods). When the male MIA animals were given 0.2 $\mu\text{mol/g}$ ATP, they had a $3.6 \pm 0.3^\circ\text{C}$ mean decrease in temperature (Figure 5A). MIA females had a $2.5 \pm 0.3^\circ\text{C}$ mean reduction in temperature following the 0.05 $\mu\text{mol/g}$ dose of ATP (Figure 5B). Control males and females showed a similar short-term hypothermic response to ATP injection (Figure 5AB, red squares).

Subacute temperature response

We next recorded the body temperatures in MIA animals over 5 days after a single injection of 0.2 μ moles/g ATP (Figure 5CD). Although both MIA and control groups had a similar acute response to ATP injection in the first hour, their subacute response over the next 5 days differed. ATP injection produced a significant rise in basal body temperature for days 1-3 after injection only in the MIA mice (gold triangles; $0.7 \pm 0.1^\circ$ in males, $p < 0.0001$; $0.6 \pm 0.1^\circ$ in females, $p < 0.003$), and not the unprimed wild-type controls, or the MIA animals treated with saline (Figure 5CD, purple vs gold triangles). The core body temperature of the MIA mice then returned to baseline by 5 days after ATP injection.

Month-long temperature response in MIA mice after poly(IC) challenge

We next followed the basal body temperature in 8-month old MIA males and controls for 28 days after a postnatal dose of poly(IC) or saline (Figure 5E). This experiment unmasked a triphasic temperature response to poly(IC) in both the MIA and control animals: 1) An initial increase in temperature on day 1 after poly(IC) (red and gold squares), 2) a decrease in temperature on days 2-4 to below the pre-challenge baseline, 3) a return to baseline in control animals by 5 days (red squares), or a rebound increase of 0.8°C that was sustained between 6 to 14 days (36.3 ± 0.5 vs 35.5 ± 0.2 ; $p < 0.0001$) in the MIA animals challenged with poly(IC), with a gradual return to baseline only after 28 days (gold squares). Poly(IC) injection in both the MIA and control mice produced a similar magnitude of hypothermia on days 2 to 4. Note that the MIA animals with ASD-like behaviors maintained a 0.5°C lower body temperature than saline-treated control animals before the challenge even at 8 months of age (Figure 5E, purple vs black circles to the left of the y-axis; Day 0 control temperature = $36.3 \pm 0.3^\circ\text{C}$ vs poly(IC) = 35.8 ± 0.4 ; 0.5°C difference, $p < 0.03$).

Discussion

Our studies showed that a relatively simple stimulus with a ubiquitous, but strictly compartmentalized metabolite, ATP, had a profound, multisystem effect on metabolism and behavior. Half of all metabolites measured (>200) were changed 30 minutes after ATP injection. Extracellular ATP is rapidly metabolized by cell surface ectonucleotidases CD73 and CD39 [63, 64] and other purine metabolic enzymes that include adenosine deaminase and purine nucleoside phosphorylase [65], AMP deaminase, IMP dehydrogenase, GMP synthase, and guanine deaminase [39]. Metabolism of ATP leads to the production of ADP, AMP, and adenosine, which can each bind different purinergic receptors, transporters, and other proteins, and lead to hypometabolism and can cause hypothermia by several different mechanisms [66]. Other purine metabolites like inosine, xanthosine, and xanthine are also produced. Xanthine is a source of superoxide and hydrogen peroxide through the action of the enzyme xanthine oxidase. Stress-induced increases in xanthine have recently been shown to cause anxiety-associated behaviors like the open field avoidance observed after ATP injection in this study. The mechanism was shown to be binding of xanthine to the adenosine A1 purinergic receptor in the amygdala [67]. The xanthine-anxiety effect is the complex outcome of mitochondrial fragmentation, and increased purine release from CD4+ T-cells under conditions of environmental stress. All the behavioral and the majority of metabolic effects of ATP injection reversed spontaneously within a few hours in the unprimed animals. However, there was an increased magnitude and duration of the response to postnatal eATP stimulation, or by CDR activation with poly(IC) in maternal immune activation (MIA) animals with ASD-like behaviors.

The MIA model has been a classical laboratory model for ASD and schizophrenia for over 10 years [61, 68-70]. The MIA model is typically created by exposing pregnant females to a simulated viral infection using the double strand RNA poly(IC). This results in a 24-hour flu-like syndrome with mild fever and decreased appetite that resolves spontaneously. However, the offspring born after an MIA pregnancy have ASD-like features, activated brain microglia, and abnormalities in synapse structure for life [71]. Detailed systems analysis of the MIA model has shown that it recapitulates many of the behavioral [68], metabolic [4], immune, microbiome [72], and brain synaptic features [3, 73] of children with ASD. In the past, it was shown that toll-like receptor 3 (TLR3) signaling triggered by poly(IC) was important to induce IL6 and IL17, which in turn played key roles in placental and brain inflammatory signaling that preceded the onset of ASD-like behaviors [74]. In humans, significant immunologic challenge and/or inflammation leading to fever during pregnancy, similar to that produced by poly(IC) in mice, is also known to increase the risk of autism [75, 76] and schizophrenia [77]. Repeated exposures to febrile triggers during the second trimester are particularly harmful [76].

Recent work with the MIA model has shown that poly(IC) *per se* is not necessary. A final common path appears to be the activation of purinergic signaling and the cell danger response (CDR) [1-4]. Horváth, et al. showed that injection of ATP itself into pregnant females was sufficient to produce the post-natal cerebellar Purkinje cell drop out and life-long autism-like behaviors that were indistinguishable from the classical MIA model [73]. Extracellular ATP and its metabolite ADP are both classical purinergic agonists and classical damage-associated molecular patterns (DAMPs) that are released from cells in response to nearly every physical, microbial, inflammatory, chemical, or metabolic stress studied to date [11, 14, 15, 78]. Other recent work has shown that mitochondria play a critical role in cellular adaptive response by serving as the substrate for metabolic memory [79]. Mitochondria are crucial in regulating innate immunity and cellular defense [80]. Our results show that gestational immune activation can produce a durable metabolic memory that alters innate immune responses in offspring well into adulthood, and perhaps for life. This raises the possibility that chronic reductions in basal body temperature that are seen clinically in some children with autism [81] might be a marker of a primed and persistent state of the cell danger response (CDR) that occurred as a result of past exposures to infectious, inflammatory, or other environmental stress that occurred during pregnancy or early child development.

Hyperpurinergic hypometabolism and the biological clocks of development and aging

Several hypometabolic states are known in biology. These include hibernation, and less familiar states known as torpor, estivation, diapause, dauer, and caloric restriction [82]. In each case, the cause of the hypometabolic phenotype involves a combination of environmental factors that operate on both genes and metabolism. These hypometabolic states appear to have evolved as a concerted multisystem mechanism to protect the organism from environmental danger. For example, in addition to producing hypometabolism [83], adenine nucleotides like AMP have been shown to protect animals from lethal radiation [84]. Hypometabolism creates a side-effect that disconnects and slows the internal biological clock of the individual compared to the external clock of time experienced by other individuals. In this way, biological time is relative to individualized physiology and is controlled by local organismal and tissue factors. In the case of dauer, the normal 2-week lifespan of the worm *Caenorhabditis elegans*, is extended for up to 4 months under conditions of environmental stress [85]. Development slows to nearly a stop during this time, as the rates of many biological reactions are slowed. The animal does not eat during dauer, so the chance of consuming

something toxic from the environment is eliminated. When the conditions that created the environmental danger are removed, the worm reenters its normal life cycle as if no time had elapsed, even though 2-4 months of external time may have passed. At this point, the internal biological clock of development re-entrains with the external clock of the sun and seasons. The animals that emerge from dauer undergo rapid catch-up development to achieve reproductive maturity in a few days and eventually die at the usual developmental age in 1-2 weeks. Caloric restriction in vertebrates produces a similar slowing of the biological clock associated with aging [27, 86].

It is tempting to speculate that biological aging can be compartmentalized, such that different organ systems age at different rates in a way controlled by the compartmentalization of local stress and the associated release of ATP and other metabokines. For example, if cells in the brain experience stress, they would be expected to release ATP since all stressed cells release ATP and ADP [87, 88]. Since the brain controls whole body metabolism through autonomic and neuroendocrine communication with all the organs, the function of many organ systems like the gut and immune system is affected by brain function. If the stress is transient, for example after the neuromuscular activity associated with exercise, then the associated purinergic signaling would contribute to a beneficial CNS adaptive response. If on the other hand, the stress and associated purinergic signaling were repetitive or chronic, they would activate a more persistent cell danger response, promote microglial activation, and lead to a slowing and alteration of developmental progress in children.

Compartmentalized danger signaling and metabolic slowing in a child could have a protective effect during neurodevelopment. The normal critical windows of readiness for child neurodevelopment might be prolonged because the internal biological clock of the brain has slowed, while the development of organ systems outside the brain might be less affected, or unaffected entirely because they are protected from brain eATP by the blood brain barrier. After the metabolic danger signals that have maintained the compartmentalized purinergic and metabokine signaling have been removed or restored to normal, the internal clock of a child's brain development can restart at a new speed. Experience with malnourished children and inborn errors of metabolism has shown that once a missing metabolic factor or calories are provided, or a metabolic or socioeconomic block removed [89, 90], catch-up development with symptom improvements can be rapid until a new steady state of child development is reached. The outcome of therapy is improved if it is instituted before age 5-10 [91, 92], but symptom improvement can also occur in adults affected by a genetic or neurometabolic block [93]. If this hypothesis is true for even a small fraction of children and adults with ASD, it means that new approaches to therapy, including antipurinergic therapies [4, 94], might fundamentally reset, restart, or improve brain developmental outcomes by normalizing the developmental clock of the brain, allowing it to resynchronize with the developmental clocks of other impacted organ systems like the gut, the microbiome, and immune systems.

The heterogeneous pattern of metabolic recovery after ATP exposure

The recovery from acute hyperpurinergia measured by metabolomics at 4 hours after ATP injection was characterized by greater inter-individual heterogeneity than the acute response at 30 minutes. Individual animals returned to the pre-injection ground state of metabolism in a less-stereotyped, more-individualized way, and by using biochemical pathways that were not simply the reversal of the acute changes. This

probabilistic exit from the cell danger response over time mirrored the non-deterministic cell-cycle re-entry of bacterial persister cells [95], and the transient arousals characterized by periodic rewarming and cooling of hibernating mammals throughout winter [96] until springtime has been firmly reestablished after a long period of harsh environmental conditions.

Plasma amino acids

One of the most striking metabolic effects of ATP injection was the generalized decrease in all measured plasma amino acids within 30 minutes of the hyperpurinergic stimulus. The average decrease across 19 of 19 measured amino acids was -5.0 ± 2.6 standard deviations compared to saline controls. The pool of amino acids in the plasma is exchanged rapidly with tissues and is highly regulated [97]. It is currently unknown into which tissues the amino acids were transported in response to ATP signaling. If the uptake is selective into muscle, the resulting plasma depletion could trigger an amino acid depletion response in another tissue like liver. Such compartmentalization of the amino acid depletion response could lead to the accumulation of uncharged tRNAs and the activation of transcription factors like GCN2. GCN2 initiates a program of metabolic changes associated with the amino acid depletion response that stimulates autophagy and decreases inflammation and mammalian target of rapamycin complex 1 (mTORC1) signaling. Because protein synthesis imposes a significant energy cost on the cell, the amino acid response preserves ATP and GTP, and contributes to the hypometabolic response. GCN2 activation protects the cell from environmental danger [98, 99], and contributes to longevity [100], but at the cost of inhibited healing [27]. The role of extracellular ATP signaling in the GCN2-on/mTORC1-off mediated amino acid depletion response has not yet been investigated.

Vitamins

Vitamins are essential cofactors for enzymes that catalyze thousands of metabolic reactions in the cell. Without an adequate concentration of vitamins in cells, many metabolic reactions will slow or stop. Therefore, a concerted mechanism for rapidly changing the blood concentration of vitamins in response to environmental threat would have the effect of rapidly changing the flux of metabolites through all the biochemical pathways controlled by vitamin availability. Mitochondria are especially rich in enzymes requiring the B vitamins. Thiamine (B1), niacin (B3), pyridoxic acid (B6), FAD (B2), methyl-tetrahydrofolic acid (mTHF, B9), and carnitine were each increased in the plasma after ATP injection. In contrast, the vitamin D precursor cholecalciferol (vitamin D3) was decreased by ATP injection. The mobilization of vitamins and conditionally-essential nutrients like carnitine during stress may deplete intracellular pools in some organs in exchange for making them available in the plasma for other tissue types. For example, if a significant amount of FAD and niacin comes into the plasma from the liver and this is sustained for more than a few hours, then mitochondrial fatty acid oxidation, which requires FAD and NAD⁺ from niacin as cofactors, might be decreased and lead to abnormalities in plasma acyl-carnitines. We did not measure plasma metabolites for more than 4 hours after ATP injection. However, abnormalities in fatty acid oxidation and acyl-carnitine profiles in children with ASD are well known [101]. The effect of purinergic signaling on the redistribution of intracellular vitamin pools in different organs like the liver or muscle, and on excretion in the urine has not yet been investigated.

Microbiome

Another surprising effect of ATP injection was the dramatic increase in metabolites known to be derived predominantly or entirely from microbial metabolism in the gut. Thirteen of 20 measured microbiome metabolites were changed and 11 of these were increased 30 minutes after ATP injection. We hypothesize that systemic exposure to ATP and ADP may increase intestinal permeability by regulating zonulin located in the tight junctions between intestinal epithelial cells and lead to the phenomenon known as a leaky gut [102]. One caveat to this interpretation is that the purinergic stimulus in this study was given by intraperitoneal injection, which transiently exposes the serosal surface of the intestines to higher concentrations of eATP before it is absorbed into the blood. This may amplify the effect of eATP on tight junctions. On the other hand, it has been shown that functional changes in mitochondria directly change the microbiome [103] and intestinal epithelial cell and mucosal function at the host-microbe interface [104]. The inhibition of mitochondrial function by systemic eATP shown in this study might therefore be a direct cause of intestinal epithelial cell functional changes that lead to chronic changes in the microbial ecology of the gut. The differential effects of IP vs IV ATP injection on microbiome metabolites and more direct measures of leaky gut have not yet been studied.

Volatile Organic Compounds in the Breath

Breathomics analysis showed that ATP injection stimulated an increase in several small volatile organic compounds (VOCs) from 1 to 5 carbons in length. The increase in one-carbon redox series carbon monoxide (CO), methanol, and methane supports the concept of a relative block in protein, lipid, nucleic acid, and polysaccharide (macromolecular polymer) synthesis. The CDR-mediated shift toward oxidizing conditions minimizes the chances that an invading microbe can usurp cellular resources for synthesizing its own polymers, and leads naturally to the accumulation of small molecular weight intermediates and monomers [10]. In addition, CO is produced naturally under stress conditions by heme oxygenase, which leads in turn to an increase in the biliary secretion of glutathione and bile acids [105]. This has been traced to the inhibitory effect of CO on the enzyme cystathionine b-synthase (CBS) and to the inhibition of H₂S synthesis by this enzyme [106]. This complex set of interactions may help explain the observation of persistent decrease in plasma bile acids measured 4 hours after ATP stimulation. Since the availability of microbiome-modified bile acids is necessary for binding to and regulating the anti-inflammatory effects of the farnesoid X receptors (FXRs) [107], decreased bile acids can further contribute to the pro-inflammatory state of a persistently activated CDR. The decrease in biosynthetic enzymes and plasma bile acid concentration was corrected in the Fragile X mouse model by treatment with the antipurinergic drug suramin [1]. We also found the 5-carbon isoprene to be elevated after ATP injection. Isoprene is a building block of cholesterol, steroid hormones, bile acids, and the electron carrier CoQ10. Other studies have measured isoprene in the breath of children and found it to be increased with steroid hormone synthesis in puberty [108], and in adults after exercise and even with the normal orthostatic stress of standing up after sitting or reclining [109].

The Triphasic Response

MIA animals showed a triphasic temperature response to the TLR3 activator and double strand RNA, poly(IC), that differed from the response in typically developing controls. There was an acute increase in temperature in

the first day, followed by a hypothermic response from days 2-4, then a hyperthermic rebound that occurred at 5 days and lasted a month. This rebound to above the pre-challenge baseline temperature did not occur in control animals. It is well known in children with ASD that some autism-related behaviors improve from the period immediately preceding a recognized fever, and for the next few days throughout the period of fever. Once the fever remits, the symptoms of ASD return [110]. We did not test the ASD-related behaviors like social approach in the MIA animals in the hours and days following postnatal challenge with poly(IC). Future studies will be needed to quantify the ASD-like behaviors during each phase of the triphasic temperature response to generic innate immune triggers like ATP and poly(IC) to test the similarity to the human ASD fever response.

Limitations

Only one strain of mouse, the C57Bl/6J strain, was used in this study. Although this is the classic laboratory mouse strain used in the MIA model, other genetic backgrounds such as the FVB mouse used in our previous studies of Fragile X syndrome [1], could theoretically show different metabolic responses to ATP injection. However, the specific genetic differences between mouse strains, and even specific mutations leading to ASD, appear not to have a significant effect on purinergic signaling associated with ASD-like behaviors. Several groups have now shown that treatment with the antipurinergic drug suramin was able to correct all the behavioral abnormalities, and most of the metabolic abnormalities, in the MIA model in C57Bl/6J mice, the Fragile X model in FVB mice [1-3], a rat model of ASD caused by prenatal exposure to valproic acid [111], and in a small clinical trial in children with ASD [4]. Metabolomic analysis after correction of the ASD-associated behaviors showed that the top metabolic pathway changed by treatment in all of these studies was purines [1, 2, 4]. ATP injection in rats is also known to cause hypothermia and pro-inflammatory effects in the brain [112] but metabolomics have not yet been performed. Intravenous infusions of ATP have been reported in patients with advanced cancer [113]. However, none of the published studies in humans have reported body temperature or metabolomic responses before and after the IV infusion. Although a small human clinical trial of antipurinergic therapy in children with ASD supports the CDR hypothesis and the crucial importance of purinergic signaling [4], the generalizability of the current mouse studies to larger studies in children with ASD is unknown.

Conclusions

The cell danger response (CDR) is an evolutionarily conserved, multi-system response to environmental stress. The CDR redirects cellular energy resources and metabolic building blocks away from housekeeping functions to new functions for purposes of cellular defense. When this happens during pregnancy or persistently in early childhood, neurodevelopmental disorders can result. Our metabolomic results showed that many aspects of the acute CDR were reproduced experimentally in mice by the systemic injection of the classical purinergic agonist ATP. Acute hyperpurinergia produced a dramatic decrease in whole body oxygen consumption, metabolic rate and temperature, and produced major shifts in mitochondrial metabolism documented by changes in amino acid, fatty acid, nucleotide, phospholipid, bile acid, redox, microbiome, and energy metabolism. If the inflammation associated with P2X7 signaling can be avoided [114], these studies raise the possibility that the development of new pro-purinergic hypometabolic drugs (PPDs) may be able to produce a transient state of cellular hypometabolism and organ cooling that could have utility in the fields of transplant biology, military and motor vehicle trauma, kidney, heart, liver, and brain surgery, and space medicine. On the

other hand, the development of new antipurinergic drugs (APDs) with suramin-like actions might prove therapeutic in clinical conditions characterized by persistent activation of the cell danger response such as autism [1, 2, 4], myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) [115], Gulf War illness [116], post-traumatic stress disorder (PTSD), major depressive disorder (MDD) and bipolar disorder (BD) [117], amyotrophic lateral sclerosis (ALS) [118], and several other developmental, neurodegenerative, and age-related disorders [10, 27].

ATP injection also produced acute behavioral changes that have been previously associated with anxiety in mouse models of ASD [119], and in response to environmental stress [67]. Abnormal behaviors included open field avoidance and a decrease in exploratory behaviors. We also noted transient ataxia. These behaviors and neurological signs have similarities to children with ASD. We found exaggerated and prolonged metabolic responses to danger signals like poly(IC) and extracellular ATP (eATP) in MIA animals with pre-existing ASD-like behaviors compared to typically developing controls. MIA animals were hypersensitive to these generic cell danger signals. In addition, we found that males were more sensitive to the *behavioral* effects of systemic eATP. Females were more sensitive to the *metabolic* effects of eATP reflected by increased changes in body temperature and metabolic rates. It is a common experimental observation to see fewer ASD-like behaviors in female mice compared to their male littermates in the MIA mouse model [3]. The greater sensitivity of females to the metabolic effects of ATP signaling may contribute to the 3 to 1 increased risk of females compared to males for another complex disorder, myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) [120]. The increased behavioral response to ATP signaling and decreased metabolic response in males may contribute to the 4 to 1 increased risk of males compared to females for the development of ASD. The hypersensitivity to extracellular ATP or ADP and poly(IC) in animals with ASD-like behaviors in this study may be relevant for the persistent activation of the CDR by environmental stresses of many kinds known to change child development, including early life stress [26] and environmental pollution [29]. In children with ASD, the hypersensitivity to purinergic agonists and to other innate immune triggers like microbial infections that trigger ATP release, would mean that relatively small increases in extracellular ATP or ADP can produce dramatic behavioral and metabolic changes that can disrupt development and oppose therapeutic efforts.

List Of Abbreviations

Ado: adenosine. APT: antipurinergic therapy. APD: antipurinergic drugs. CDR: cell danger response. MIA: maternal immune activation. TLR3: toll-like receptor 3, PPD: Propurinergic drugs.

Declarations

Competing Interests

The authors declare that they have no competing interests.

Declarations

1. Ethics approval and consent to participate—not applicable
2. Consent for publication—not applicable

3. Availability of data—All metabolomics data is included in Supplemental Table S1

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Author Contributions

ZZ helped design and conducted the experiments, analyzed the data, and wrote the paper. TN helped design, conducted experiments, and edited the manuscript. LW contributed to experimental design and coordination. TPL contributed to biochemical characterizations. JCN contributed to the behavioral studies and conducted the metabolomics. KL performed the metabolomics. CMH, SM, and DB performed the breathomics studies. JMM contributed to the statistical analysis and data visualization. RKN directed the study, designed the experiments, analyzed the data, and wrote the paper.

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Tables

Table 1
Metabolic and behavioral features of acute hyperpurinergia

Phenotype	Parameter (26–30 min post- infusion)	Saline Response (mean)	SD	ATP Response (mean)	SD	Change	Animals Per Group	p value
Metabolic ¹	Oxygen consumption (VO ₂ ml/kg/hr)	5302	710	1382	325	-74%	6	0.0001
	CO ₂ production (VCO ₂ ml/kg/hr)	4324	647	1034	226	-76%	6	0.0001
	RER (VCO ₂ /VO ₂) ²	0.84	0.082	0.70	0.062	-0.14	6	0.006
	Radiated heat (cal/hour)	650	84	180	40	-72%	6	0.0001
	Locomotor activity (xyz photobeam breaks)	742	442	27	16	-96%	6	0.006
Core Temp	Males (°C) ³	37.9	0.38	34.3	0.99	-3.6°C	6	0.0005
	Females (°C) ³	38.4	0.13	32.2	0.26	-6.2°C	6	0.0001
	Male dose response (β = °C/ μ mol/g ATP) ⁴	0	0	-16.5	1.0	n/a	6	0.0005 ⁴
	Female dose response (β = °C/ μ mol/g ATP) ⁴	0	0	-27.8	3.4	n/a	6	—

¹ATP Dose = 0.5 μ mol/g IP C57BL/6J females, 28 weeks old in CLAMS cages. ²Maximum change in RER was not reached for 52 minutes. ³Rectal temperature. 20-week old C57BL/6J. Cage temperature = 22.4°C, ATP dose = 0.5 μ mol/g IP. Males reached maximum hypothermia at 30 minutes, and females reached maximum at 45 minutes after injection. ⁴Linear regression analysis comparing the slopes (β) for males and females: F(1,53) = 13.6, p < 0.0005. ⁵Purinergic Behavioral Response Scale (PBRs). ⁶Linear regression analysis scored at 15 minutes, comparing the slopes (β) for males and females: F(1,52) = 63.85, p < 0.0001.

Phenotype	Parameter (26–30 min post- infusion)	Saline Response (mean)	SD	ATP Response (mean)	SD	Change	Animals Per Group	p value
Behavior ⁵	Males, maximal response (after 0.5 μ mol/g)	0	0	9.7	0.48	n/a	10	0.0001
	Females, max response (after 0.5 μ mol/g)	0	0	10.7	0.48	n/a	10	0.0001
	Male dose response (β = PBRs/ μ mol/g ATP) ⁶	0	0	37.1	1.0	n/a	6	0.0001 ⁶
	Female dose response (β = PBRs/ μ mol/g ATP) ⁶	0	0	27.4	0.7	n/a	6	—
¹ ATP Dose = 0.5 μ mol/g IP C57BL/6J females, 28 weeks old in CLAMS cages. ² Maximum change in RER was not reached for 52 minutes. ³ Rectal temperature. 20-week old C57BL/6J. Cage temperature = 22.4°C, ATP dose = 0.5 μ mol/g IP. Males reached maximum hypothermia at 30 minutes, and females reached maximum at 45 minutes after injection. ⁴ Linear regression analysis comparing the slopes (β) for males and females: F(1,53) = 13.6, p < 0.0005. ⁵ Purinergetic Behavioral Response Scale (PBRs). ⁶ Linear regression analysis scored at 15 minutes, comparing the slopes (β) for males and females: F(1,52) = 63.85, p < 0.0001.								

Figures

Figure 1

Figure 1

Study Overview. Abbreviations: GC—gas chromatography, FID—flame ionization detection, ECD—electron capture detection, MSD—mass selective detection, LC—high performance liquid chromatography, MS/MS—triple quadrupole mass spectrometry.

Figure 2AB. 30 minutes

Figure 2

Metabolomic Analysis of Acute Hyperpurinergia. A. Ranking of metabolites changed 30 minutes after ATP injection by partial least squares discriminant analysis (PLSDA). B. Bubble impact plot of pathways most changed 30 minutes after ATP injection, C. Venn diagram of pathways increased, decreased, or contained increased and decreased metabolites after 30 minutes, D. Ranking of metabolites changed 4 hours after ATP injection by partial least squares discriminant analysis (PLSDA), E. Bubble impact plot of pathways most changed 4 hours after ATP injection, F. Venn diagram of pathways increased, decreased, or contained increased and decreased metabolites after 4 hours, G. Two-dimensional separation of the metabolomes by multivariate PLSDA components after saline and 0.5 and 4 hours post ATP injection, H. Dendrogram showing sharp separation of the metabolome at 30 minutes and the heterogeneous and incomplete return to baseline by 4 hours after ATP injection, I. Heatmap of the top 30 most-changed metabolites 30 minutes and 4 hours after ATP injection. ATP dose = 0.5 $\mu\text{mol/g}$ body weight, n = 7-8 C57BL/6J males per group, age = 12-13 weeks. Abbreviations: VIP—variable importance in projection.

Figure 3A-H. Breathomics

Figure 3

Breathomics, Chemokines, Cytokines, and Corticosterone Response to Acute Hyperpurinergia. Breathomics captured and analyzed exhaled breath at 1-10 minutes after ATP injection (A-H; $n = 3$ C57BL/6J males per group, 3 samples per animal), A. Carbon monoxide, B. Methanol, C. Methane, D. Dimethylsulfide, E. Acetaldehyde, F. Acetone, G. Butyraldehyde, H. Isoprene. Plasma chemokine and cytokine analysis 30 min and 4 hours after ATP injection (I-K; $n = 6-7$ C57BL/6J males per group), I. CXCL1/KC/GRO J. IL10, K. IL6. Plasma corticosterone levels 30 minutes and 4 hours after ATP injection (ATP dose = $0.5 \mu\text{mol/g}$ body weight, $n = 7$ C57BL/6J females per group), L. Corticosterone. Abbreviations: CXCL1—chemokine (C-X-C motif) ligand 1, KC—keratinocyte-derived chemokine, GRO—growth related oncogene alpha, IL10—interleukin 10, IL6—interleukin 6.

Figure 4AB. Temperature

Figure 4

Body Temperature, Bioenergetic, and Behavioral Responses to Acute Hyperpurinergia. A. Male C57BL/6J mice (nucleotide dose = 0.5 $\mu\text{mol/g}$ body weight, $n = 6$ per group, 5-6 months old), B. Female C57BL/6J mice ($n = 6$ per group, 5-6 months old). Intravenous (IV) vs intraperitoneal (IP) dosing (C and D, ATP dose = 0.5 $\mu\text{mol/g}$, $n = 6$ females per group, 5 months old), C. Body temperature response, D. Behavioral response. Gender-specific differences (E-H, temperatures measured at 15 minutes post-injection with 0-0.2 $\mu\text{mol/g}$ ATP, $n = 6$ -8 mice/group) E. Females were more sensitive to the hypothermic effects of ATP, F. Males were more sensitive to the hypothermic effects of ADP, G. Males and females were equally sensitive to the hypothermic effects of AMP, H. Males and females were equally sensitive to the hypothermic effects of adenosine. Behavioral responses (I and J), I. The behavioral response to high-dose ATP was the same in males and females (dose = 0.5 $\mu\text{mol/g}$, $n = 10$ per group), J. Dose-response curves at non-saturating ATP doses revealed that males were more sensitive to the behavioral effects of hyperpurinergia (PBRs scores measured at 15 minutes post-ATP, $n = 6$ per group, 5-6 months old). CLAMS cage analysis of bioenergetics (K-N, ATP dose = 0.5 $\mu\text{mol/g}$, $n = 6$ per group, 28-week old C57BL/6J females), K. The basal metabolic rate measured as the rate of oxygen utilization (VO_2) was decreased by 74% after ATP injection, L. The rate of CO_2 production was decreased by 76% after ATP injection, M. The respiratory exchange ratio (RER) dropped from 0.84 to 0.70 after ATP injection compared to saline, N. ATP injection decreased exploratory activity as measured by light beam breaks compared to saline. Abbreviations: PBRs—purinergic behavioral response scale, SAL—saline, CLAMS—comprehensive laboratory animal monitoring system. RER—respiratory exchange ratio = VCO_2/VO_2 .

Figure 5A-D.

Figure 5

Latent Memory Response to ATP and Poly(IC) in the MIA Mouse Model of ASD. The acute 1-hour response to postnatal challenge with ATP (A and B, $n = 6$ per group) A. Acute response in males, B. Acute response in females. The subacute 5-day response to postnatal challenge with ATP (C and D, $n = 6$ per group, 8-9 months old) C. Five-day response to ATP in males, D. Five-day response to ATP in females, E. The triphasic 1-month response to postnatal challenge with poly(IC) (dose = 2 mg/kg, $n = 6$ males per group, 8-9 months old). Abbreviations: MIA—maternal immune activation mouse model with ASD-like behaviors, Poly(IC)—poly inosinic:cytosinic acid double strand RNA.

Supplementary Files

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