An integrative genomics path towards discovery of environmental contributors to autism

Valerie Hu, Ph.D.
Professor of Biochemistry and Molecular Medicine
The George Washington University
School of Medicine and Health Sciences

Autism Research Institute
Webinar
Jan. 13, 2020
Objectives

- Describe how our integrative genomics studies on autism spectrum disorder (ASD) led to our investigation of endocrine disrupting compounds (EDCs) as potential environmental risk factors for autism.

- Present preliminary findings on the impact of a specific EDC, the herbicide atrazine, on gene expression in a neuronal cell model.

- Describe current studies on epigenetic changes (DNA methylation) in human sperm in association with environmental exposures to long-lived EDCs.

- Discuss how epigenomic alterations caused by EDC exposures may contribute to elevated risk for ASD.
What is autism?

Autism spectrum disorder (ASD) refers to neurodevelopmental disorders that are characterized by core deficits in:

- Reciprocal social interaction/communication
- Restricted, repetitive stereotyped behavior
- Language development and usage

ASD: Classic autism --- PDD-NOS --- Asperger’s

Autism is a significant public health problem worldwide

Prevalence: 1 in 59* individuals; male:female ratio > 4:1

Estimated lifetime costs for raising a child with autism (2017): $2.4 million (US)/individual

*CDC, 2018
What Causes Autism?

Goals:
• To identify genes, biological pathways, and functions for targeted therapies
• To identify diagnostic biomarkers of ASD
• To develop a “systems level” understanding of the pathobiology of ASD

Problem: Heterogeneity

Experimental strategy: Reduce clinical heterogeneity by using scores on a commonly used diagnostic test to subgroup individuals according to severity of behavioral symptoms

Intrinsic and extrinsic environmental factors (e.g., hormones, pesticides)

Genetics (Hardware)
- Mutations
- Copy number variants
- Chromosomal abnormalities

Epigenetics (Software)
- DNA methylation
- MicroRNA expression
- Histone modification
- Chromatin remodeling

Gene expression profile: level of gene activity

ASD phenotypes: brain circuitry, behaviors and symptoms

An integrated genomics approach to autism spectrum disorders
A hierarchical view of the multiple factors that cause or affect risk for autism. In this view, the components of each level can influence those shown below.
Underlying hypothesis:

Autism is a “systems” disorder with severe neurological manifestations, and therefore can be studied using easily accessible tissues, such as blood, which may reflect molecular and functional abnormalities in the brain and reveal biomarkers for diagnosis.

Experimental strategy: “Divide and Conquer”
Reduce heterogeneity by using scores on a commonly used diagnostic test (ADI-R) to subgroup individuals according to severity of behavioral (clinical) symptoms
Autism diagnostic interview-revised ("gold standard" behavioral diagnostic)

Detailed questionnaire (> 100 items) that probes subject’s functioning across multiple behaviors:

• Spoken language (chat, echolalia, pronoun reversal)
• Nonverbal communication (gestures, head nod)
• Social functioning (interactive play, offer comfort)
• Repetitive, stereotyped behaviors (rocking, flapping, lining things up)
• Restricted interests (perserverance on topic, objects)
• Savant skills (visuospatial skills, memory, reading, music, art, math)
K-means cluster analyses of ADI-R scores shows 4 distinguishable clinical phenotypes of ASD individuals

Rows: Individuals (>1900)
Columns: 123 ADIR “items”
Severity scores: 0-3
Bright red: 3 (most severe)
Black: 0 (normal)
Gray: no data

Principal components analysis:
Unsupervised

PCA clusters = ASD subtypes

Gene expression profiling of lymphoblasts
(~40,000 transcripts tested per sample on a single slide)

Questions asked:
- What genes are differentially expressed between autistic individuals vs. controls?
- Can subtypes of ASD be distinguished by gene expression profiling?
- What do differences in gene expression profiles tell us about the respective biology of the ASD subtypes?
- Can these differences be used to discriminate between autistic individuals and controls (that is, can they be used for diagnosis)?
Gene expression profiles can distinguish the 3 ASD subgroups from each other and controls both qualitatively and quantitatively.

Gene expression heatmap
Columns = Individuals
Rows = Genes
Red = Higher gene activity
Green = Lower activity

Principal components analysis
Unsupervised clustering of individuals based on genes in heatmap above:
Each point represents an individual characterized by gene expression profile.
Overlapping as well as unique genes are associated with each subgroup of ASD (compared to controls).

Analyses of genes using Ingenuity Pathway Analysis software reveals associated functions:

- **Language**: 4,160 genes < 5% FDR
- **Cell death (large # genes)**
- **Painful response to normal sensory stimuli**
- **Muscle rigidity**
- **Heightened sensitivity to painful stimuli**
- **Epilepsy**
- **Circadian rhythm**

**Overlap of L and M**
- Cell death
- Inflammation
- Embryonic development
- Synaptic transmission
- Neuronal migration
- Memory/learning
- Muscle tone
- Head size

**Mild**: 502 genes < 5% FDR

**Savants**: 127 genes < 5% FDR

Novel long noncoding transcripts, many are responsive to male hormones (regulatory?)

Hu et al., Autism Res. 2(2):78-97, 2009
Circadian rhythm “clock” genes in severe ASD group affect functions and disorders associated with ASD
Functional analysis of circadian “clock” genes

Associated functions and disorders

• Sleep-wake cycle  
• Memory  
• Learning  
• Cell proliferation  
• Steroid biosynthesis  
• Digestive disorders  
• Inflammation  
• Muscle dysfunction  
• Neuron toxicity

Novel target genes for subtype-specific treatment

• **AA-NAT**: deficiency leads to reduced melatonin biosynthesis  
  ⇒ melatonin supplements?  
• **DPYD**: deficiency leads to reduced beta-alanine and predisposes to epilepsy, mental and motor retardation, and ASD  
  ⇒ beta-alanine supplements or anticonvulsant medications as novel and/or targeted therapies

•  ⇒ **Personalized medicine**
An integrated genomics approach to autism spectrum disorders
A hierarchical view of the multiple factors that cause or affect risk for autism. In this view, the components of each level can influence those shown below.

Subtype-dependent genetic differences
Hu et al., PLoS ONE, 6(4):e19067, 2011

Subtype-dependent gene expression

Do epigenetic mechanisms contribute to gene dysregulation in autism?

An epigenetic mechanism for disrupting genes
Normal
DNA --
Chemical mark

Disease gene -
Does not involve change in sequence

Do epigenetic mechanisms contribute to gene dysregulation in autism?
Identification of methylation differences contributing to autism

Large-scale microarray analyses of cells from discordant twins and siblings

- Differentially methylated genes
- Differentially expressed genes

Overlapping genes

Functionally significant candidate genes

Confirm methylation and expression in lymphoblastoid cells

Evaluate changes in protein level in brain tissues

Methylated DNA precipitated using methyl-cytosine binding proteins; Analyzed on CpG island microarrays

TIGR 40K cDNA microarrays

Hu et al., 2006
Overlap between differentially methylated and expressed ASD candidate genes

Network analysis of candidate genes reveals functions relevant to ASD

RORA and BCL2 selected for further validation & analyses.

Nguyen et al., FASEB J. 24(8):3036-51, 2010
Why is RORA relevant to autism?

Lessons learned from a *Rora*-deficient mouse model (*staggerer*)

- Rora protects brain against oxidative stress and inflammation.
- Rora regulates circadian rhythm.
- Loss of *Rora* leads to developmental defects in cerebellum and loss of Purkinje cells.
- *Rora*-deficient mice are sexually dimorphic with respect to production of cerebellar neurosteroids and Purkinje cell survival during aging, with male mice being more severely affected.
- *Rora* deficiency results in ataxia and hypotonia as well as perseverative behavior and deficits in spatial and discrimination learning.

Implications for ASD

- Neuroinflammation and oxidative stress have been detected in the postmortem autistic brain.
- Circadian rhythm genes are implicated in autism.
- Purkinje cell deficiency is the earliest noticed and most consistent neuroanatomical abnormality in ASD.
- Males are more affected by ASD than females.
Questions regarding RORA’s involvement in autism

Regulators?  →  RORA  →  Targets?

• Given the sexually dimorphic characteristics of Rora-deficient mice, is RORA differentially regulated by male and female sex hormones?

• Is RORA deficiency evident in brain tissues from individuals with ASD?

• RORA is a nuclear hormone receptor which acts as a transcriptional regulator: Which genes are transcriptional targets of RORA, and how do they relate to autism?
RORA expression is oppositely regulated by male and female hormones

Sarachana et al., PLoS ONE, 6(2): e17116, 2011
RORA and aromatase are both reduced in autistic brain

So what?

Aromatase is strongly correlated with RORA.

22 controls; 12 autistic; 40-50 neurons/subject
Error bar = SE; * p-value<0.05

Relevance of decrease in aromatase

- The autistic male to female ratio is at least 4:1
- “Extreme male brain theory”; Baron-Cohen et al. (2005)

Elevated fetal testosterone → Increased risk for autism

However, there was no molecular explanation for higher testosterone levels or for the significant male bias in ASD

Decreased aromatase ⇒ higher testosterone
Model explaining increased testosterone levels and why males may be more susceptible to ASD than females.
RORA is a “master regulator” of other autism-relevant genes.

2544 potential target genes (>400 are autism risk genes)
(highly enriched for autism candidate genes and functions: neurogenesis, synaptic transmission and plasticity, axonogenesis, cognition, learning, memory)

Validated RORA targets (also reduced in ASD brain)

- A2BP1 (RBFOX1)
- CYP19A1 (aromatase)
- HSD17B10
- ITPR1
- NLGN1
- NTRK2 (TRKB)

Neurological functions/disorders:
- synaptic transmission, neuronal excitation, ataxia, developmental delay
- neurogenesis, synaptic plasticity, protects against oxidative stress, social cognition
- mitochondrial integrity, X-linked mental retardation, language impairment
- synaptogenesis, synaptic plasticity, dendritic contact, long-term depression
- adhesion, synaptic remodeling, neuritogenesis, repetitive behavior, spatial memory
- axon guidance, synaptogenesis, synaptic plasticity, mood disorder, learning

Any mechanism that disrupts RORA expression, including environmental factors, may increase risk for ASD.

Genome-wide target analysis (ChIP-on-chip)

ChIP-qPCR analyses and siRNA knockdown/qPCR

Sarachana & Hu, Molecular Autism 4:14, 2013
Linking RORA deficiency to environmental factors

The impact of sex hormones on *RORA* expression suggests that *RORA* may also be dysregulated by endocrine disrupting chemicals.

Endocrine disrupting chemicals (EDCs) are compounds that either mimic endogenous hormones or antagonize their actions, metabolism, or transport, thus interfering with normal hormonal activity and homeostasis.

Is *RORA* a target for gene-environment interactions involving EDCs that may increase risk for ASD?
Examples of EDCs

- **Atrazine** – herbicides
- **Bisphenol A (BPA)** – plastics, dental sealants, paper receipts
- **DDT** (long-lived breakdown product: **DDE**) - pesticides
- **Phthalates** – soft toys, flooring material, cosmetics, air fresheners
- **Polychlorinated biphenyls (PCBs)** – coolants, lubricants
- **Polybrominated diphenyl ethers (PBDEs)** – flame retardants, textiles
- **Valproic acid** – drug for epilepsy, bipolar disorder, major depression

Major concerns regarding EDC exposures:
- Effects of cumulative exposures from persistent organic pollutants (POPs), e.g., **DDE**, PCBs and PBDEs
- Epigenetic changes, particularly in germline (sperm and egg) cells, that may be propagated transgenerationally
Atrazine

• Common herbicide
• EPA: a 90-day average of 37.5 ppb (175 nM) is currently accepted as “safe” in community water systems
• Easily absorbed by GI tract, lungs, or skin
• Literature reports the effects of atrazine on sexual differentiation in wildlife
The United States congenital birth defect rates by month of conception versus atrazine concentrations in surface water.

“Low-dose” amounts of atrazine have a bidirectional effect on the expression of RORA in neuronal cell cultures after 2 hours.
Results of gene expression profiling on Affymetrix HTArrays
Overlap of differentially expressed genes induced by 0.1nM and 10nM atrazine and transcriptional targets of RORA

Pathways affected:
- Axon guidance
- Glutamate receptor signaling
- Chemokine, notch, and ephrin receptor signaling

Neuronal functions:
- Development, growth, morphology, and guidance of neurons
- Neuritogenesis
- Synaptic development and transmission

RORA target genes:
- Migration and cell death of granule cells in cerebellum
- Formation of brain
- Seizure disorder
- Mental retardation
- Movement disorders

Kocher and Hu, unpublished data
Dysregulation of *RORA* expression by EDCs, such as atrazine, is a potential mechanism for gene x environment interactions that may increase risk for ASD by inducing a “domino effect” leading to the deregulation of transcriptional targets of RORA as well as many other genes that may contribute to the neuropathology of ASD.

- While our studies so far have focused on immediate effects of EDCs, what are the long-term effects of EDC exposures, especially *in vivo*?
- How might the impact of environmental agents be transmitted across generations?
The study: Female rats were injected with atrazine on days 8-14 of gestation (F0). Male and female offspring from different litters of exposed F0 females were bred through 3 generations (F1, F2, F3) without any further exposure to atrazine.

What was examined: - Various pathologies and phenotypes (testis disease, mammary tumors in males and females, early onset of puberty, body weight); - DNA methylation in sperm from male offspring in F1, F2, and F3 generations

Results: F1 – no disease but lower body weight
          F2 – increased frequency of testis disease and mammary tumors in males and females, early onset of puberty in males, leaner females
          F3 – increased frequency of testis disease, early puberty in females, motor hyperactivity and lean phenotype in both males and females

DNA methylation in sperm is increasingly altered in F1 to F2 to F3 generations
(My analysis of this data shows significant enrichment in ASD genes in F2 and F3.)
Impact of endocrine disruptors on the human sperm methylome

Specific aims:
• Investigate DNA methylation in association with exposure to high and low levels of p,p’-DDE, a long-lived (persistent) breakdown product of DDT pesticide
• Determine pathways and functions affected by differentially methylated genes (DMGs)
• Determine if DMGs are enriched in autism risk genes

Samples:
• Semen obtained from young adult men in the Faroe Islands (Denmark) whose natural diet of pilot whale meat and blubber exposes them to high levels of endocrine disruptors (e.g., p,p’DDE)

Experimental approach: Whole genome bisulfite sequencing (WGBS) of sperm DNA

Bisulfite conversion

Percent methylation calculated and differentially methylated regions (DMRs) identified
Differential methylation patterns clearly separate the low exposure group from the high exposure group.

Methylation data were adjusted for covariates including age, BMI, sperm motility, time in storage, smoking status, and batch effects.

Majority of DMRs are in protein coding regions

Maggio et al., 2019; unpublished data (Master’s thesis)
Differentially methylated genes from both discovery (32) and validation (20) sample sets are significantly enriched in ASD risk genes in SFARI Gene

Functional analyses of DMGs also show significant associations with development of central nervous system, synaptic transmission, hormone metabolism, social behavior, language and movement disorders, autism and intellectual disability.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSMD1</td>
<td>CUB and sushi domain-containing protein 1</td>
</tr>
<tr>
<td>HDAC4</td>
<td>Histone deacetylase 4</td>
</tr>
<tr>
<td>RBFOX1</td>
<td>RNA binding protein fox-1 homolog 1</td>
</tr>
<tr>
<td>CHRM3</td>
<td>Muscarinic acetylcholine receptor M3</td>
</tr>
<tr>
<td>JARID2</td>
<td>Protein Jumonji</td>
</tr>
<tr>
<td>KHDRBS2</td>
<td>KH domain-containing, RNA-binding, signal</td>
</tr>
<tr>
<td></td>
<td>transduction-associated protein 2</td>
</tr>
<tr>
<td>TM4SF20</td>
<td>Transmembrane 4 L6 family member 20</td>
</tr>
<tr>
<td>EBF3</td>
<td>Transcription factor COE3</td>
</tr>
<tr>
<td>GRM5</td>
<td>Metabotropic glutamate receptor 5</td>
</tr>
<tr>
<td>CDH9</td>
<td>Cadherin-9</td>
</tr>
<tr>
<td>NRXN2</td>
<td>Neurexin-2</td>
</tr>
<tr>
<td>TSHZ3</td>
<td>Teashirt homolog 3</td>
</tr>
<tr>
<td>SYT1</td>
<td>Synaptotagmin-1</td>
</tr>
<tr>
<td>CTNNB1</td>
<td>Catenin beta-1</td>
</tr>
</tbody>
</table>

Maggio et al., 2019; unpublished data
Differential methylation of SNORD115 imprinted region on chromosome 15 shows genome-wide significance after multiple testing correction

Maggio et al., 2019; unpublished data

**SNORD115 block**
DMRs, this study
DMRs associated with ASD

**SNORD115 block**
*Single DMR block with genome-wide significance across 52 samples combined

¶Same region was found to be significantly differentially methylated in sperm from fathers with ASD children relative to fathers of unaffected children (Feinberg et al., 2015).

This suggests potential for environmentally induced ASD-associated epigenetic alterations that may be transmitted transgenerationally through germline cells.
An integrative genomics approach to autism spectrum disorders
A hierarchical view of the multiple factors that cause or affect risk for autism. In this view, the components of each level can influence those shown below.
Summary

- Neurodevelopmental disorders, such as ASD, have complex etiologies, involving genetic susceptibilities in combination with environmental risk factors (triggers).
- Environmental chemicals, such as atrazine, may increase risk for ASD in part through dysregulation of RORA (a master regulator of genes associated with pathobiology of ASD) as well as many other genes involved in neurodevelopmental processes.
- Human exposures to EDCs can also be associated with DNA methylation changes in sperm of exposed men that impact genes involved in neurodevelopment and autism.
- Epigenetic changes that occur in germline cells may be partially responsible for the heritability or transmission of a disorder across generations following initial environmental exposures.
- Thus, it is critically important to understand not only the genetic components of ASD, but also how environmental contributors (including social and psychological stressors) interact with genes, primarily through epigenetic mechanisms, to impact health and human development.
Acknowledgements

Support:
- NIEHS, NIMH, Autism Speaks, Simons Foundation, The Catherine B. McCormick Genomics Center (GWU)

Minyi Xu, M.S.
Sex hormone effects on RORA

AnhThu Nguyen
Global methylation analysis

Mara Steinberg
ASD phenotyping

Kyung Soon Kim, M.S.
Gene expression

Kristen Kocher, M.S.
Impact of EDCs on RORA

Angela Maggio, M.S.
DDE/Sperm methylation

Henry Shu
DDE effects on cells

Jessica Debski, M.S.
DDE/miRNA in sperm

Tewarit Srarachana, Ph.D.
Gene and miRNA expression; Regulation of RORA and target gene analyses

Collaborators
- John Quackenbush, Ph.D.: Dana Farber Cancer Inst.
- Ray-Chang Wu, Ph.D.: Biochem/Molec. Med., GWU
- Norman Lee, Ph.D.: Pharm/Physiol, GWU
- Yinglei Lai, Ph.D.: Dept. of Statistics, GWU
- Gerd Pfeifer, Tibor Rauch: City of Hope, CA
- Husseini Manji, Rulun Zhou, Guang Chen: NIMH
- Anjene Addington, Ph.D.: NIMH
- Zohreh Talebizadeh: Children’s Mercy Hospital, MO
- Melissa Perry, GWU, SPHHS
- Pal Weihe, Faroese Hospital System, Faroe Islands
- Philippe Grandjean, Harvard SPH, U. So. Denmark
- Janine LaSalle, UC Davis
- Ben Laufer, UC Davis

Support: NIEHS, NIMH, Autism Speaks, Simons Foundation, The Catherine B. McCormick Genomics Center (GWU)
Selected references relevant to presented material

Future studies should address:

• How exposures at critical periods of early development (prenatal and perinatal) cause neurodevelopmental changes that manifest at a later time period
• How the effects of environmental exposures are transmitted across generations
• Differential susceptibility of males and females to specific environmental exposures
• How exposure to chemical mixtures (in a real-world scenario) may elicit different effects than exposure to a single chemical
• How chemical exposures and social or psychological stressors may interact to increase (or decrease) the impact on human development and health