

Examining environmental contributors to autism spectrum disorder

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Collaborative for Health and the Environment

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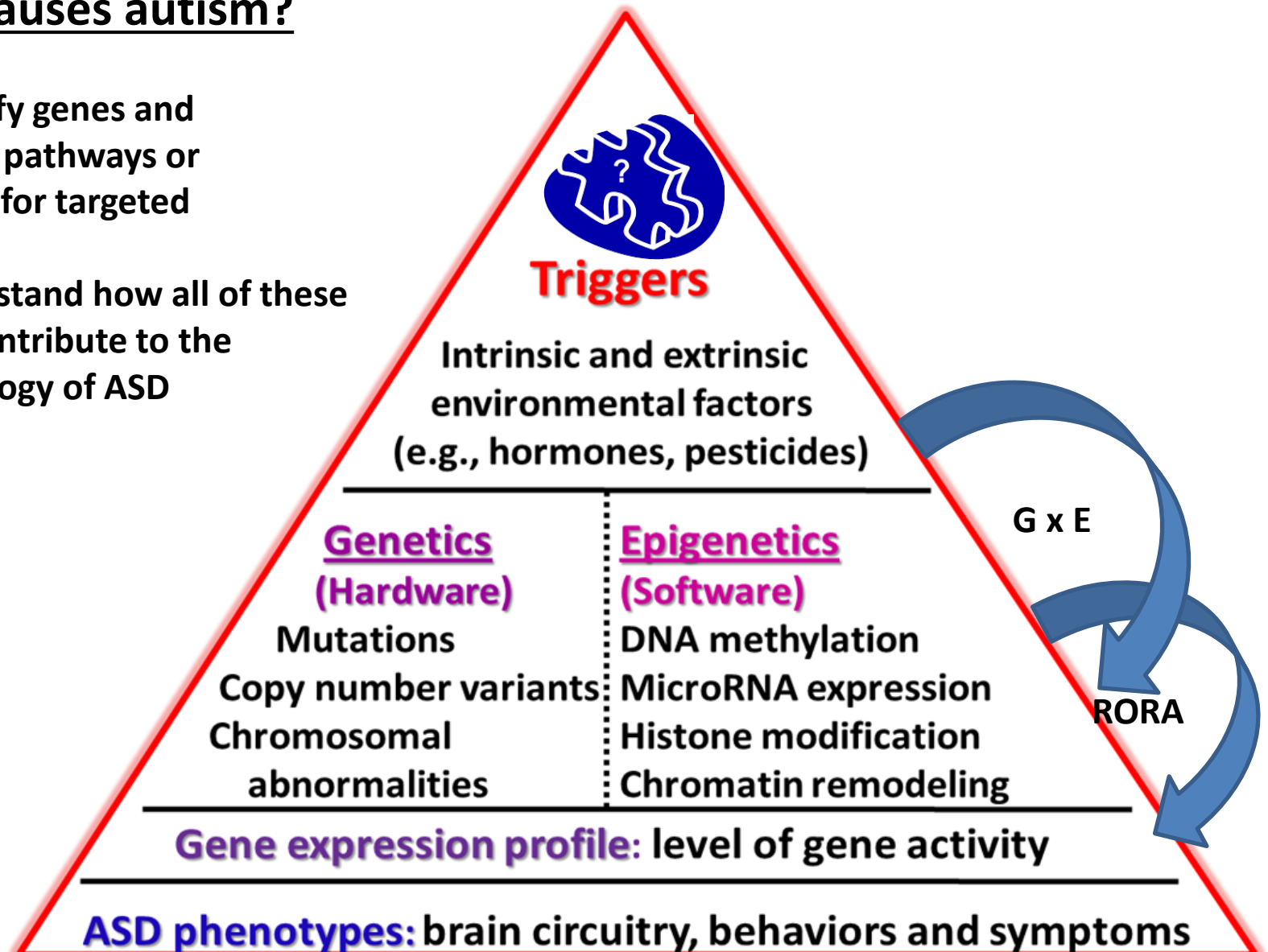
Objectives

- Describe how our integrative genomics studies on autism spectrum disorder (ASD) led to our investigation of endocrine disrupting compounds as potential risk factors for autism
- Present findings on the impact of a specific EDC, atrazine, on gene expression in a neuronal cell model
- Discuss how alterations caused by environmental exposures may be transmitted across generations

What causes autism?

Goals:

- To identify genes and biological pathways or functions for targeted therapies
- To understand how all of these factors contribute to the pathobiology of ASD



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.

Why the interest in *RORA*?

Rora-deficient mice reveal that:

- Rora protects brain against oxidative stress and inflammation.
- Rora regulates circadian rhythm (sleep-wake cycle).
- Loss of *Rora* leads to defects in cerebellum & loss of Purkinje cells.
- Male mice are more severely impacted by Rora deficiency.
- *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.
- Sleep problems are often associated with autism.
- Purkinje cell deficiency is the earliest noticed and most consistent brain abnormality in ASD.
- Sex bias: Males are more affected by ASD than females.

New findings from our lab linking *RORA* to ASD

- *RORA* expression is reduced in both peripheral cells and brain tissues from a subset of individuals with ASD. [Hu et al., Autism Research, 2009; Nguyen et al., FASEB J., 2010]
- Increased methylation at the *RORA* promoter was associated with decreased expression in lymphoblastoid cells from siblings with ASD but not from controls. [Nguyen et al., FASEB J., 2010]
- *RORA* is oppositely regulated by male and female sex hormones in a manner suggesting potential involvement in the sex bias in ASD. [Sarachana et al., PLoS ONE, 2011]
- Reduced expression of *RORA/Rora* coupled with its higher correlation with expression levels of its target genes in the male brain (of both mice and humans) suggests that *RORA* deficiency may have a greater impact on males than on females. [Hu et al., Molecular Autism, 2015]

RORA is a “master regulator” of many autism risk genes

An additional 500 more transcriptional targets were confirmed by RNAseq in stable shRORA-KD cells



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

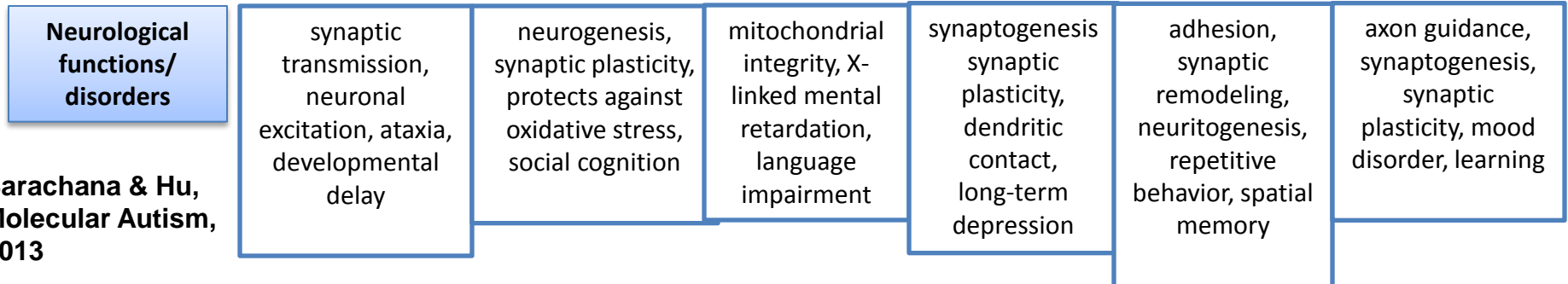
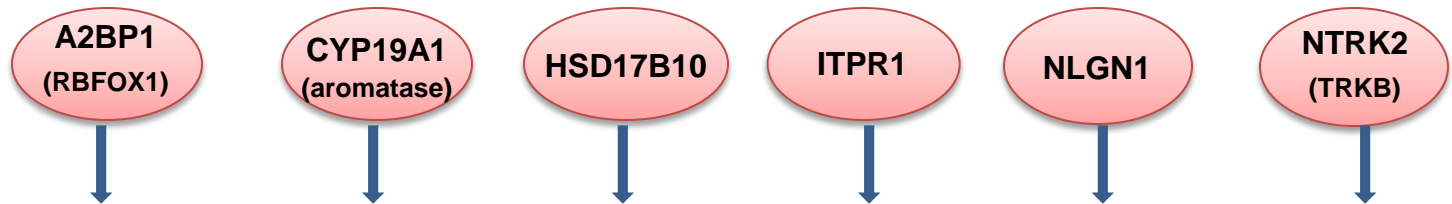
Genome-wide target analysis (ChIP-chip)

2544 potential target genes

(highly enriched for autism candidate genes and functions: neurogenesis, synaptic transmission and plasticity, axonogenesis, cognition, learning, memory)

Functional knockdown and ChIP-qPCR analyses

Validated RORA targets (also reduced in ASD brain)



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
- 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., PCBs and PBDEs
- Epigenetic changes, particularly in germline (sperm and egg) cells, that may be propagated transgenerationally

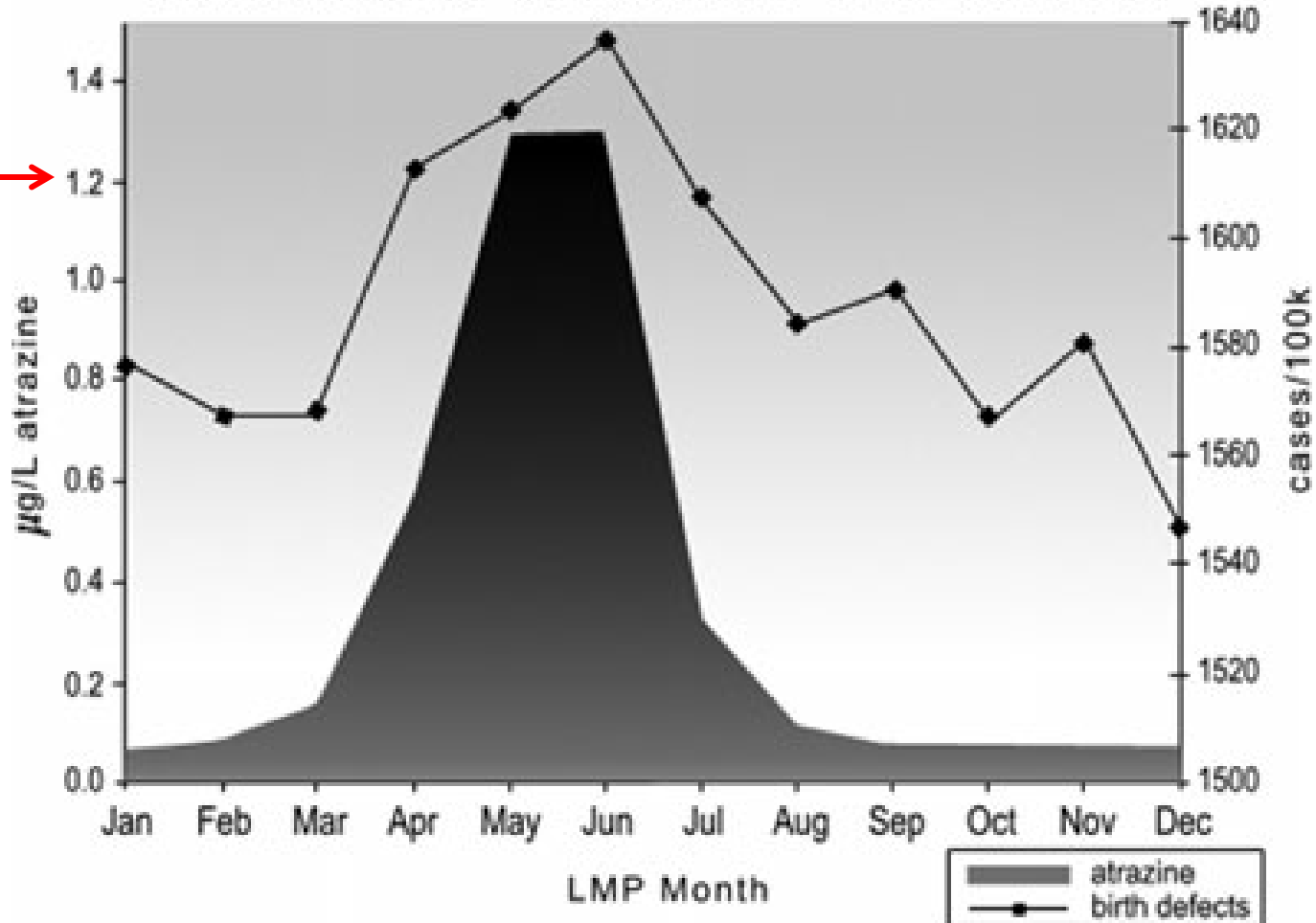
Atrazine

- 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 gut, lungs, or skin
- Studies report the effects of atrazine on sexual differentiation in wildlife

Atrazine Herbicides	
Aatrex	Keystone
Atrazine	Laddok S-12
Basis Gold	Lariat
Bicep	Liberty ATZ
Breakfree ATZ	Lumax
Breakfree ATZ Lite	Marksman
Bullet	Rifle Plus
Degree Extra	Shotgun
Field Master	Stalwart Xtra
Ful time	Sterling Plus
G Max Lite	Tremor AT Lite
Guardsman	Volley ATZ
Harness Xtra	
Harness Xtra 5.6	(as of 2010)

Birth Defects vs Atrazine 1996-2002 US

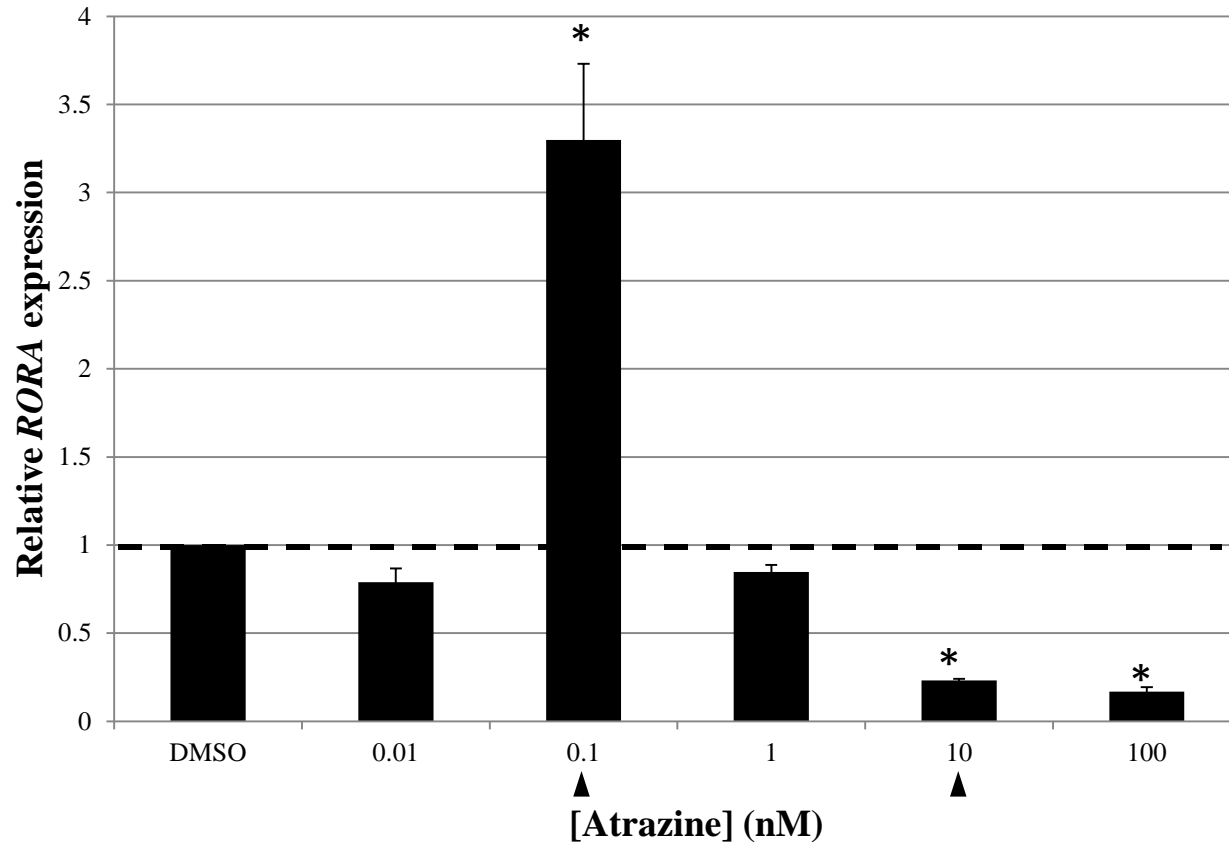
5.5 nM →
[175 nM
is "safe"
by EPA]



The United States congenital birth defect rates by month of conception versus atrazine concentrations.

Winchester, Huskins, and Ying. Agrichemicals in surface water and birth defects in the United States. *Acta Paediatrica*, 2009

“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

0.1 nM ATR

10 nM ATR

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

Summary

Our exploratory studies suggest that dysregulation of *RORA* expression by EDCs, such as atrazine, is a potential mechanism for gene-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?

Atrazine induced epigenetic transgenerational inheritance of disease, lean phenotype and sperm epimutation pathology biomarkers

Margaux McBirney, Stephanie E. King, Michelle Pappalardo, Elizabeth Houser, Margaret Unkefer, Eric Nilsson, Ingrid Sadler-Riggelman, Daniel Beck, Paul Winchester, Michael K. Skinner* PLOS ONE | September 20, 2017

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 (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

Results of DNA methylation (epigenetic) analyses

[DMR = differentially methylated regions]

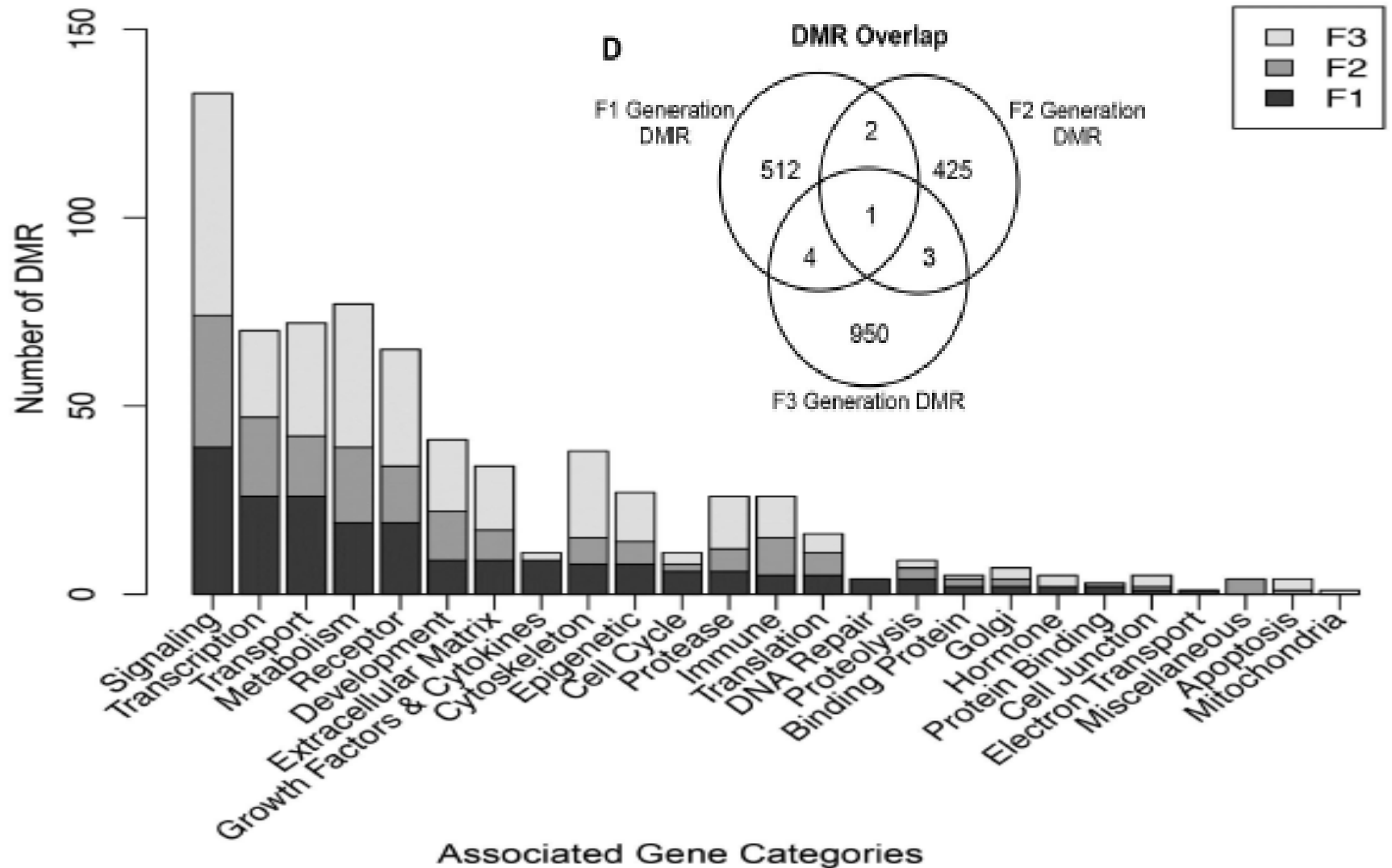


Fig 8. DMR associated gene categories. F1, F2 and F3 generation functional gene categories versus number of DMR per category. Insert color code for F1, F2 and F3 generation presented.

Take-home messages

- Neurodevelopmental disorders, such as ASD, have complex etiologies, involving genetic susceptibilities in combination with environmental risk factors (triggers).
- Atrazine, an EDC, may increase risk for ASD through dysregulation of *RORA* (a master regulator of genes associated with pathobiology of ASD) as well as many other genes involved in neurodevelopmental processes.
- Atrazine has also been shown in a rat model to induce epigenetic changes in sperm that are manifested through several generations through F3, following the initial exposure of the pregnant F0 dam.
- Epigenetic changes that impact germline cells may be partially responsible for the heritability or transmission of a disorder across generations following initial EDC exposure.

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

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ASD phenotyping



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Gene expression



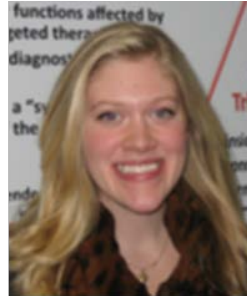
Tewarit Sarachana, Ph.D.
Gene and miRNA expression;
Regulation of *RORA* and target
gene analyses



AnhThu Nguyen
Global methylation
analysis



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



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

Collaborators

- John Quackenbush, Ph.D.: Formerly of TIGR
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