

Lead Investigator: Benjamin C. Nephew, PhD

Autism spectrum disorders (ASD) are pervasive neurodevelopmental disorders characterized by deficits in communication and social interactions, as well as the presence of stereotypic behaviors¹. ASD may affect as many as 1 in 45 children in the USA³, but the global prevalence is still under-recognized⁴. Numerous gene mutations have been identified in patients with ASD, but no direct link has so far been uncovered except for a small percentage of cases associated with Tuberous Sclerosis, Fragile X syndrome, Rett syndrome and PTEN deficiency⁵. As a result, even though there are a number of genetically-based mice with phenotypes resembling autism⁶, they do not adequately reflect ASD and there is an urgent need for appropriate animal “models” of ASD⁷. A number of perinatal allergic, genetic, environmental, immune and infectious factors may increase the risk of or contribute to the pathogenesis of ASD⁸, especially the regression that appears in about 50% of children with ASD in about 3 years of life. These could act through activation of a unique tissue immune cell, the mast cell (MC)^{9, 10}. In addition to histamine, stimulated MCs secrete other vasoactive and pro-inflammatory mediators such as the preformed kinins and proteases, as well as the *de novo* synthesized leukotrienes, prostaglandins, chemokines (CCXL8, CCL2), cytokines (IL-4, IL-6, IL-1, TNF) and vascular endothelial growth factor (VEGF)⁹. MCs are not only considered critical for the development of allergic reactions⁹, but also for immunity¹¹ and inflammation¹². In fact, many studies have reported that allergic diseases in preschoolers are strongly associated with psychological and behavioral problems¹³. We had proposed that MC-derived mediators could disrupt the blood brain barrier (BBB) and cause “allergy of the brain”¹⁴, thus contributing to the pathogenesis of ASD¹⁵. Advanced functional imaging techniques such as fMRI and diffusor tensor imaging (DTI), which is ideal for investigating neuronal connectivity, have documented broad neuroanatomical changes in rodent models of ASD^{16, 17} that may be related to inflammatory mediated developmental changes.

Gestational infection and exposure to air pollution are linked to increased risk of ASD

Increasing evidence suggests that there is immune dysregulation in ASD¹⁸. The maternal immune activation (MIA) rodent model involves maternal infection that leads substantial increases in circulating IL-6 levels that cross the placenta at midgestation and induce the release of fetal stress hormones resulting in fetal injury. Viral poly(I:C) injection in pregnant mothers, one MIA method, dramatically raises IL-6 in the placenta and brains of mice and also induces autistic-like behavior, and a single IL-6 injection can mimick these behavioral changes¹⁹. One interesting possibility, now supported by numerous epidemiology and animal studies is that traffic-related pollution (TRAP) can also contribute to the development of ASD^{20, 21, 22}. In addition to the epidemiology on TRAP, there is also corroborating evidence that shows that ambient ultrafine particles, which are found in TRAP can travel up the olfactory nerve and enter the brain²³.

Specific Aim 1: Determine the effects of maternal infection and inhalant particulate matter pollution on inflammation, neuroanatomy, and autism-like behavior in male rat offspring.

General Procedures

We will use male rats because ASD is more common in males, rat dams are excellent behavioral models for perinatal manipulations, and rat brains are larger for improved magnetic resonance imaging and diffusion tensor imaging (MRI/DTI) imaging. We propose to have the following two groups: (1) saline and no pollution (Control) and (2) MIA and airborne particulate matter (PM) pollution exposure (Stress). Both the MIA and PM exposures represent environmental stressors that may be involved in ASD etiology through immune mediated effects on the brain. This study will involve 20 mated females (Kingston, NY, n = 10 dams per group) and 2 male offspring (weaned at day 21) from each litter to avoid litter effects (n = 20 male offspring per group). All rats will be kept on a 14:10 hr light-dark cycle in virus-free sections of a modern animal facility at the Cummings School and are allowed *ad libitum* access to food and water.

Male rat offspring will undergo open field, social approach, social interaction, social recognition, and hole board behavioral testing at 25-30 days. These tests have successfully been used in the past to show ASD-like social, anxiety, and cognitive behavior in rodents following viral stimulation of the mothers during gestation, mentioned earlier. Males subjected to the behavioral tests will be bled, then euthanized on day 31 between 08:00-10:00 to collect blood samples to assess basal levels of CRH, IFN- γ , IL-6, IL-8, TNF, and VEGF. The euthanized animals will then be transported 10 miles to UMass medical center for MRI and DTI imaging at the Center for Comparative NeuroImaging (CCNI) fMRI facility, where Dr. Nephew has an active collaboration. Littermates will be euthanized at the same time to sample the brain to assess whole brain levels of CRH, IFN- γ , IL-6, IL-8, TNF, and VEGF.

The MIA model

Pregnant rats will be injected in the tail vein with 4 mg/kg poly(I:C) freshly dissolved in 0.9% sterile PBS on gestation day 15²⁴. Control dams will be injected with the same volume of PBS.

Whole body inhalant TRAP exposure

Confirmed pregnant dams and their eventual litters will be housed within the environmental chambers (CH Technologies, USA) shown below (Fig.1) for the duration of gestation and lactation. The environmental exposure will involve whole body inhalant exposure to PM particles collected from the Harvard Ambient Particle Concentrator using methods adapted from an established protocol²⁵. Dams to be injected by poly (I:C) on day 15 of gestation will be exposed to re-aerosolized particles for 5 hours a day/ 3 days a week for the duration of gestation and lactation (6 weeks). Dams will be kept with their pups in the environmental chambers throughout lactation, and control animals will undergo the identical procedures with filtered air exposure.



Fig. 1 Environmental Chambers

Behavioral testing

Open field and social approach, interaction, and recognition will be performed in a progressive, integrated test as described previously with the addition of a novel vs. familiar social recognition test following the social interaction. Anxiety and repetition behaviors will be assessed throughout the social tests. Cognitive testing will be done the day before or after social testing and assess performance on a plus maze as previously described².

Cytokine and peptide measurements: Cytokines (IFN- γ , IL-6, TNF) as well as neurotensin and CRH in the serum and brains will be measured by commercially available ELISA kits, and quantitative PCR, as described previously in mice^{26, 27}.

MRI and DTI imaging: Male offspring brains will be analyzed with MRI and DTI using established methods^{17, 28} at the Center for Comparative Neuroimaging at UMass Medical Center, where Dr. Nephew has an ongoing collaboration with CCNI director and Associate Provost Dr. Jean King to make detailed group comparisons in neuroanatomy and structural connectivity and identify potential neural mechanisms for behavioral effects of MIA and PM stress.

Statistical analysis: All data from the two groups will be compared using the Mann-Whitney U test within each group as it is not known if they follow a normal distribution. Pearson correlations will be used to test for associations between behaviors, biomarkers and neuroanatomy. Analysis of MRI and DTI data will use established methods and be conducted at the CCNI.

Expected outcomes: We expect that the stress group will exhibit impaired cognition and social behavior, increased anxiety and repetitive behaviors, increased peripheral CRH and inflammatory factors, and altered neuroanatomy from the MRI DTI scans. It is expected that the imaging data will reveal differences in both neuroanatomical volumes and structural connectivity (white matter track morphology) which will strongly suggest changes in functional neural connectivity which can be explored in future studies which will incorporate targeted neural investigations of inflammatory and behaviorally relevant gene and protein expression and resting state functional connectivity and stimulus based functional MRI. The data as whole will establish the value of the combined MIA and PM environmental model of ASD and provide a strong foundation for additional grant applications.

Timeline

The animal work will take 4-5 months, assays and data analysis will require an additional 5 months, and the final 2 months will allow for unexpected delays and/or be devoted to grant preparation for the fall 2017 NIH grant deadline. We already have plans to apply for an R21 in the fall of 2016.

Innovation and significance

Our result will indicate how stress and environmental exposure may contribute to the pathogenesis of ASD through inflammatory mechanisms especially for the 50% of children with ASD that regress for no apparent reason at age about 3 years old. As a result, child and adolescent outpatient mental health services in the USA have increased considerably²⁹. Moreover, the annual economic burden for ASD was recently estimated at \$268 billion for 2015 and is projected to reach \$416 billion in 2025³⁰.

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