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Prenatal and early life diesel exhaust exposure disrupts cortical lamina organization: Evidence for a reelin-related pathogenic pathway induced by interleukin-6

Yu-Chi Chang^a, Ray Daza^{b,1}, Robert Hevner^{b,c,1}, Lucio G. Costa^{a,d}, and Toby B. Cole^{a,e,*}

^aDepartment of Environmental and Occupational Health Sciences, University of Washington, Seattle, WA, USA

^bCenter for Integrative Brain Research, Seattle Children's Research Institute, Seattle, WA, USA

^cDepartment of Neurological Surgery, University of Washington, Seattle, WA, USA

^dDepartment of Medicine and Surgery, University of Parma, Parma, Italy

^eCenter on Human Development and Disability, University of Washington, Seattle, WA, USA

Abstract

Several epidemiological studies have shown associations between developmental exposure to traffic-related air pollution and increased risk for autism spectrum disorders (ASD), a spectrum of neurodevelopmental disorders with increasing prevalence rate in the United States. Though animal studies have provided support for these associations, little is known regarding possible underlying mechanisms. In a previous study we found that exposure of C57BL/6J mice of both sexes to environmentally relevant levels (250–300 $\mu\text{g}/\text{m}^3$) of diesel exhaust (DE) from embryonic day 0 to postnatal day 21 (E0 to PND21) caused significant changes in all three characteristic behavioral domains of ASD in the offspring. In the present study we investigated a potential mechanistic pathway that may be of relevance for ASD-like changes associated with developmental DE exposure. Using the same DE exposure protocol (250–300 $\mu\text{g}/\text{m}^3$ DE from E0 to PND21) several molecular markers were examined in the brains of male and female mice at PND3, 21, and 60. Exposure to DE as above increased levels of interleukin-6 (IL-6) in placenta and in neonatal brain. The JAK2/STAT3 pathway, a target for IL-6, was activated by STAT3 phosphorylation, and the expression of DNA methyltransferase 1 (DNMT1), a STAT3 target gene, was increased in DE-exposed neonatal brain. DNMT1 has been reported to down-regulate expression of reelin (RELN), an extracellular matrix glycoprotein important in regulating the processes of neuronal migration. RELN is considered an important modulator for ASD, since there are several polymorphisms in this gene linked to the disease, and since lower levels of RELN have been reported in brains of

*Corresponding author at: Department of Environmental and Occupational Health Sciences, University of Washington, Box 357234, 1959 NE Pacific St., Seattle, WA, USA. ycc2@uw.edu (Y.-C. Chang), ray.daza@seattlechildrens.org, rdaza@ucsd.edu (R. Daza), rhevner@uw.edu, rhevner@ucsd.edu (R. Hevner), lgcosta@uw.edu (L.G. Costa), tobycole@uw.edu (T.B. Cole).

¹Present affiliation: Dept of Pathology, University of California at San Diego, La Jolla, CA, USA.

Conflict of interest

All authors declare that they have no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://doi.org/10.1016/j.bbi.2019.01.013>.

ASD patients. We observed decreased RELN expression in brains of the DE-exposed mice at PND3. Since disorganized patches in the prefrontal cortex have been reported in ASD patients and disrupted cortical organization has been found in RELN-deficient mice, we also assessed cortical organization, by labeling cells expressing the lamina-specific-markers RELN and calretinin. In DE-exposed mice we found increased cell density in deeper cortex (lamina layers VI–IV) for cells expressing either RELN or calretinin. These findings demonstrate that developmental DE exposure is associated with subtle disorganization of the cerebral cortex at PND60, and suggest a pathway involving IL-6, STAT3, and DNMT1 leading to down regulation of RELN expression that could be contributing to this long-lasting disruption in cortical laminar organization.

Keywords

Autism spectrum disorder; Air pollution; Diesel exhaust; Neuroinflammation; Reelin; Cortical lamina organization; Mice

1. Introduction

The escalating prevalence of autism spectrum disorder (ASD) in recent years has prompted research in understanding the role played by environmental risk factors in the etiology of the disease. The most recent prevalence rate for ASD in the United States has been reported to be 1 in 59, indicating a more than ten-fold increase in the past 40 years (Baio et al., 2018). Although increasing prevalence may be attributed to broadening of diagnostic definition, the contribution of environmental risk factors cannot be discounted. An epidemiological study conducted in California estimated that 26.4% of the increased autism prevalence can be attributed to change in diagnostic practices between 1992 and 2005 (King and Bearman, 2009). However, in support of the significance of environmental contributions to autism etiology, an epidemiological study looking at ASD association in monozygotic and dizygotic twins concluded that environmental components have an even larger effect than genetic components in predicting ASD outcome (Hallmayer et al., 2011).

Of all the environmental risk factors associated with ASD, air pollution exposure is the most ubiquitous, affecting a large number of individuals, especially in densely populated areas in Central and North America and in South and East Asia (Calderón-Garcidueñas et al., 2015; Pandis et al., 2016; van Donkelaar et al., 2014). High exposure levels of particulate matter ($PM_{2.5} > 100 \mu g/m^3$) over extended periods have been commonly experienced by populations living in these areas (Brook et al., 2010). Developmental exposure to traffic-related air pollution (TRAP) has been associated with increased ASD risk in many recent epidemiological studies conducted in North America and Europe (Becerra et al., 2013; Suades-González et al., 2015; Talbott et al., 2015; Volk et al., 2011; Volk et al., 2013), as well as in Asia (Jung et al., 2013). Two epidemiological studies conducted as part of the Nurses' Health Study II Cohort indicated that exposure to TRAP during the third trimester resulted in the strongest association of ASD outcome comparing to earlier trimesters (Raz et al., 2015; Volk et al., 2013), while another study found robust association with early-life exposure (Raz et al., 2018). Converging evidence from animal studies support the hypothesis

that TRAP may represent an important contributor to ASD etiology (reviewed in Costa et al., 2017).

PM, a component of diesel exhaust (DE), is known for its ability to cross cellular membranes and cause oxidative damage. We have previously reported that acute DE exposure in adult mice causes neuroinflammation and oxidative stress (Cole et al., 2016), as well as microglia activation both in vivo and in vitro (Cole et al., 2016; Roqué et al., 2016). Developmental DE exposure in rodents has been shown to increase levels of pro-inflammatory cytokines in placenta, fetal brain and fetal lung (Auten et al., 2012; Bolton et al., 2012; Li et al., 2018; Weldy et al., 2013). Elevated levels of interleukin-6 (IL-6) and of interleukin-17 α (IL-17 α) have been shown to be sufficient in eliciting ASD-like behavior in offspring from maternal immune activated (MIA) dams, subjected to lipopolysaccharide or poly (I:C) treatment to mimic bacterial or viral infection during pregnancy (Choi et al., 2016; Samuelsson et al., 2006; Shin Yim et al., 2017; Smith et al., 2007). Binding of IL-6 to its cognate receptor activates the kinase pathway involving Janus kinase 2 (JAK2) and Signal Transducer and Activator of Transcription 3 (STAT3) (Chang et al., 2005; Erta et al., 2012; Hsiao and Patterson, 2011; Tsukada et al., 2015). Activated STAT3 forms homodimers that translocate into the nucleus, where they bind to DNA and act as transcription factors (Hsiao and Patterson, 2011; Parker-Athill and Tan, 2010; Tsukada et al., 2015). The expression of DNA methyltransferase 1 (DNMT1) has been shown to be modulated by STAT3 (MuhChyi et al., 2013; Zhang et al., 2005). DNMT1 is abundantly expressed in both developing and adult mammalian brains (Inano et al., 2000; Robertson et al., 1999; Veldic et al., 2004), and is responsible for both de novo methylation and maintenance of DNA methylation patterns. DNMT1 has been shown to bind directly to, and to exert epigenetic alterations at the reelin (RELN) promoter region (Kundakovic et al., 2009). Indeed, treatment with a DNMT inhibitor results in a dose-dependent increase of reelin expression in an in vitro model (Kundakovic et al., 2007). In brains of individuals with other neurodevelopmental disorders (e.g., schizophrenia or bipolar disorder), over-expression of DNMT1 and increased binding of DNMT1 to the RELN promoter have been observed (Dong et al., 2015). A methylation sequencing study conducted with post-mortem temporocortical tissue samples revealed that ASD patients display significantly heavier methylation in the 5' region of the RELN gene promoter, while controls exhibiting more extensive methylation at the 3' promoter region. (Lintas et al., 2016).

RELN is a secreted extracellular protein that has been known to modulate neuronal migration and dendrite formation during CNS development. In adult mice, RELN has been shown to play a role in synapse formation (D'Arcangelo, 2014; Levenson et al., 2008; Michetti et al., 2014). Several lines of evidence suggest the importance of RELN in the pathogenesis of ASD. First, decreased RELN levels have been reported in brains of ASD patients (D'Arcangelo, 2014). Second, mice haploid-deficient in RELN have been shown to exhibit some ASD-related behavioral phenotypes (Michetti et al., 2014). Third, cortical disorganization has been reported in both ASD patients and in RELN-deficient mice (Boyle et al., 2011; Stoner et al., 2014). Fourth, differences in DNA methylation patterns within the RELN promoter were reported in ASD patients (Grayson et al., 2006). Given RELN's involvement in organizing cortical structural architecture (Fukumitsu et al., 2006; Boyle et al., 2011), we decided to investigate cortical laminar organization in the somatosensory

cortex using the cortical layer-specific markers RELN and calretinin. In the adult cortex, RELN is expressed mostly by Cajal–Retzius cells in cortical layer I and also by some GABAergic interneurons in layers II through VI (Impagnatiello et al., 1998; Pesold et al., 1998). Calretinin is a calcium-binding signaling protein, expressed in a subpopulation of GABAergic interneurons in layers II/III and IV (Gonchar, 2008). A recent study showed that activation of neurons in the S1DZ region of the somatosensory cortex leads to ASD-like behavioral changes in mice (Shin Yim et al., 2017), supporting the idea that structural changes in the somatosensory cortex could be involved in ASD-like behaviors.

We have reported previously that developmental DE exposure causes deficits in the three hallmark categories of ASD behavior, i.e., social interaction in the reciprocal interaction and social preference tests, social olfactory and vocal communication, and repetitive behavior (Chang et al. 2018). While other studies have also reported subsets of ASD-like behavior changes, such as repetitive/ impulsive behavior and social deficits upon developmental exposure to air-pollution or PM (Allen et al., 2016; Church et al., 2018; Li et al., 2018; Thirtamara et al., 2013), the environmentally relevant level of DE exposure (250–300 $\mu\text{g}/\text{m}^3$) used in our study appeared to produce a more robust behavioral phenotype (Chang et al. 2018). Given that developmental DE exposure has been shown to cause ASD-like behavioral changes, and an increase in neuroinflammation (Bolton et al., 2017, 2012), we investigated a potential mechanism that could lead from neuroinflammation to cortical disorganization as seen in ASD. In the present study, we demonstrate that developmental DE exposure results in disorganization of cortical lamina in the somatosensory cortex, and present evidence for a potential mechanistic pathway involving IL-6, STAT3, DNMT1 and dysregulation of RELN expression that could explain these ASD-related effects of DE exposure.

2. Methods

2.1. Animals and exposure

Two-month-old male and female C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and housed in the University of Washington Northlake Diesel Exposure Facility under specific pathogen free conditions on a 12-h light/dark cycle in an Allentown caging system (Allenton, NJ, USA) supplied with filtered air, and free access to water and food. The overall study design is shown in Fig. 1. Following one week of acclimation, each male was paired with two females for timed mating. Evidence of a vaginal plug served as confirmation of successful mating, and mated females were considered to be at embryonic day (E)0 upon identification of vaginal plug, and were randomly selected to be individually housed in cages supplied with either diluted DE or filtered air (FA) from E0 to postnatal day 21 (PND21). DE exposure was carried out for six h/day and five days/week at the level of 250–300 $\mu\text{g}/\text{m}^3$ of $\text{PM}_{2.5}$ concentration, corresponding to a time-weighted hourly average of 35.71–44.64 $\mu\text{g}/\text{m}^3$. DE was generated on site from a Yanmar YDG5500 diesel generator fueled with standard highway-grade number two diesel fuel obtained from local fuel distributors and operated under load. Generated DE then passed through a two-step dilution system with dynamic control of fine particulate matter ($\text{PM}_{2.5}$), maintaining constant exposure level at 250–300 $\mu\text{g}/\text{m}^3$. Chemical composition and particle size characterization of the DE have been previously described in detail (Fox et al., 2014; Gould

et al., 2008). During the exposure period, mice in both groups (DE or FA) were housed in the same room under identical conditions, subjected to the same noise level and light cycle. Mice were weaned at PND21 and group-housed (5 mice per cage) with same-sex, same treatment mice for the duration of the experiment. All animal experiments were consistent with the National Research Council Guide for the Care and Use of Laboratory Animals, as adopted by the National Institutes of Health, and were approved by the University of Washington Institutional Animal Care and Use Committee.

2.2. Tissue collection

For all measures, the litter was used as the statistical unit, i.e., one male and one female mouse was used from each litter for each end-point. Five pregnant dams from each exposure group were euthanized with CO₂ at E16.5. Placenta and matching fetal tails were snap-frozen in liquid nitrogen and stored at -80°C for cytokine evaluation by ELISA and sex genotyping, respectively. At PND3, PND21, and PND60, pups born to DE- and FA-exposed dams were euthanized by CO₂ narcosis followed by cervical dislocation. At PND3 whole brain samples were rapidly dissected and snap-frozen in liquid nitrogen, then stored at -80°C for quantitative real-time PCR (qRT-PCR) and Western-blot analysis. At PND21 and PND60, the cerebral cortex was rapidly micro dissected and snap-frozen in liquid nitrogen then stored at -80°C. Additionally, five animals/group/sex were euthanized by CO₂ narcosis followed by cervical dislocation at PND60, and transcardially perfused with 10 ml of phosphate buffered saline (PBS) followed by 10 ml of 4% paraformaldehyde. Brains were then carefully removed from the skull, placed into 4% paraformaldehyde at 4°C overnight for additional fixing, then cryoprotected in 30% sucrose at 4°C until the brains sank to the bottom of the tube. After cryoprotection, brains were hemisected and embedded in Tissue-Tek* CRYO-OCT cutting compound (Fisher Scientific, Pittsburgh, PA) with midline facing the bottom of the standard size Cryomold® (25 × 20 × 5 mm, Sakura Finetek, Netherlands) to ensure a consistent sectioning angle. The embedded brains were stored at -80°C for later immunohistochemical analysis.

2.3. Quantitative Real-time qPCR

Levels of mRNA of IL6, DNMT1, DNMT3a, DNMT3b, DNMT3l, and RELN were measured by qRT-PCR after normalization to the house-keeping gene GAPDH (encoding glyceraldehyde-3-phosphate dehydrogenase) in whole brain samples from DE- or FA-exposed PND3 pups. RELN mRNA levels were also measured in cortical samples from PND21 and PND60 mice. RNA was extracted by blending frozen brain samples in TRIzol reagent (Thermo Fisher Scientific, Rockford, IL) with a tissue homogenizer followed by chloroform extraction and washing with 70% ethanol according to standard procedures. RNA was further purified with the Gene JET RNA purification kit (Thermo Fisher Scientific Inc., Rockford, IL) according to the RNA clean-up protocol provided in the kit. Quality and concentration of RNA isolates were confirmed by Nano Drop (Thermo Fisher Scientific Inc., Rockford, IL) measurements (260/280 ratio > 1.8, 260/230 ratio between 2.0 and 2.2). Reverse transcription was done using the iScript[™] cDNA Synthesis kit (Biorad; Hercules, CA) with 1 µg of RNA per 20 µl reaction. The iTaq[™] Universal SYBR® Green One-Step Kit (Biorad; Hercules, CA) was used for signal detection during real-time quantitative PCR on a Bio-Rad CFX384 Real-Time PCR Detection System (Biorad; Hercules, CA) with IL6,

DNMT1, DNMT3a, DNMT3b, DNMT3L, RELN, and GAPDH primers adapted from the primer data base (Wang et al., 2012), as shown in Supplemental Table 1. Relative mRNA expression of target genes was normalized to the housekeeping gene GAPDH, relative expression (dCq) was calculated according to Haimes and Kelley (2010), and expression in DE exposed animals was compared with control animals of the same sex. GAPDH expression was not affected by DE treatment.

2.4. Measurement of IL-6 levels by ELISA

IL-6 protein levels were measured in E16.5 placentas using the MDS V-PLEX Plus ELISA kit (MesoScale Discovery, Gaithersburg, MD, USA). Briefly, tissue was quickly homogenized and sonicated on ice in Cell Lysis buffer (10 mM HEPES; 150 mM NaCl; 1 mM CaCl₂; 0.5 mM MgCl₂; 10 µg/ml leupeptin; 10 µg/ml aprotinin; 1 mM PMSF; 50 mM NaF) supplemented with complete mini protease inhibitor cocktail tablets (Sigma-Aldrich; St. Louis, MO) according to the manufacturer's directions. Total protein content was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL), and protein concentrations in all samples were adjusted to 10 µg/µl with cell lysis buffer. 25 µl of homogenate of equal protein concentration/well was loaded and run in triplicate according to the manufacturer's instructions. The electro-chemiluminescent signal was visualized using SECTOR S 600 (MesoScale Discovery, Gaithersburg, MD, USA).

2.5. Measurement of phosphorylated STAT3 (Tyr705) and total STAT3 by Western blot

In brief, frozen brain tissue was homogenized on ice in RIPA buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate) supplemented with complete mini protease inhibitor cocktail tablets and PhosSTOP phosphatase inhibitor cocktail tablets (both from Sigma- Aldrich; St. Louis, MO) using a glass Potter-Elvehjem homogenizer. The protein content of each sample was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL). 35 µg of protein were loaded into pre-cast gels (10% Bis-Tris gels) and electrotransferred onto a polyvinylidene difluoride (PVDF) immunoblot membrane (0.45 µm). After transfer, membranes were cut to isolate the 86 kDa bands for STAT3 and p-STAT3 from the 43 kDa bands for β- actin. The membranes were blocked with 5% (w/v) non-fat milk for oneh. The top portion of the membranes were incubated with a rabbit anti-Phospho-Stat3 (Tyr705) antibody (1:500; Catalog No. 9145; Cell Signaling Technology, Danvers, MA) and horseradish peroxidase-con jugated anti-rabbit secondary antibody (1:1000; Catalog No. 7074; Cell Signaling Technology, Danvers, MA). The bottom portion of the membranes were incubated with a mouse anti-β-actin antibody (1:1000; Catalog No. 554022; BD Pharmingen, San Jose, CA) and a horseradish peroxidase-conjugated anti-mouse polyclonal secondary antibody (1:1000; Catalog No. 554002; BD Biosciences, San Jose, CA). The membranes were developed with a chemiluminescent substrate (ECL kit from Thermo Scientific, Waltham, MA). The top portion of the membranes were stripped with Restore™ Western Blot Stripping Buffer and re-blocked with 5% non-fat milk before incubating with a rabbit anti- Stat3 antibody (1:2000; Catalog No. 12640; Cell Signaling Technology, Danvers, MA) followed by a horseradish peroxidase-conjugated antirabbit secondary antibody (1:1000; Catalog No. 7074; Cell Signaling Technology, Danvers, MA), then were developed as described above. Band intensity was measured by densitometry

using ImageJ (provided by the National Institutes of Health), and the intensity of the bands was normalized to β -actin content.

2.6. Immunohistochemical analysis of cortical lamina organization

Brains from PND60 mice embedded in optimum cutting temperature (OCT) compound (Sakura Finetek USA, Torrance, CA) were cut sagittally at 10–12 μ m starting 2000 μ m away from midline, and the somatosensory cortex region was sampled at 200 μ m intervals for five serial sets. The sections were direct-mounted on glass slides and air dried before being stored at -80°C . Immunohistochemistry was performed as previously described (Englund et al., 2005). The following primary antibodies were utilized at the indicated dilutions: mouse anti-RELN (1:1000; EMD Millipore, MAB5364); rabbit anti-Calretinin (1:2000; Swant, CR 7697); Alexa Fluor 488-conjugated or 568-conjugated goat anti-mouse or rabbit IgG (Thermo Fisher Scientific, 1:600). The nuclear counterstain DAPI (4',6-diamidino-2-phenylindole) (Sigma, St. Louis, MO) was used to label DNA following the manufacturer's instructions, after incubation with primary and secondary antibodies. Brains from five animals in each experimental group were processed and analyzed, and 3–5 sections/brain were collected. Digital immunofluorescence images were obtained on a Zeiss Axio Imager Z1. In each image, the cortical depth (distance between ventricle and pia mater), was divided into 10 evenly-spaced bins, with bin 1 positioned nearest to the pia mater. Fluorescent-labeled cells were counted in each of the 10 bins and the area of each bin was measured using Adobe Photoshop, with the researcher blinded to the experimental groups. Cell density in each bin was calculated by dividing the total area of the bin by the total number of cells.

2.7. Statistical analyses

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc.; San Diego, CA, USA). Differences in IL-6 levels in placenta and STAT3-phosphorylation in PND3 brains between DE- and FA-exposed groups were determined by Student's t-test. mRNA levels of genes of interest (IL-6, DNMTs, reelin) were analyzed by two-way ANOVA followed by Bonferroni post-hoc comparison to test the effect of exposure and potential sex differences. For immunohistochemistry, the two-tailed Student's t-test was used to assess differences in cell density between FA and DE brains of the same sex in each bin.

3. Results

3.1. Developmental DE exposure increases IL-6 expression

Elevated levels of the pro-inflammatory cytokine IL-6 were found in neonatal (PND3) pup brains and in placentas (E16.5) from mice exposed to DE. Neonatal brains from DE-exposed PND3 pups of both sexes showed significantly increased levels of IL-6 mRNA compared to FA-exposed mice of the same sex and age (Fig. 2A), when normalized to the housekeeping gene GAPDH (Two-way ANOVA; main effect of DE exposure $F(1, 16) = 13.48$, $p = 0.0021$; Bonferroni posttest adjusted p -value: Male $p = 0.037$, Female $p = 0.041$; no significant effect of sex or DE exposure X sex interaction were found). There were no differences in GAPDH expression between DE- and FA-exposed animals (not shown). In E16.5 placenta, IL-6 protein levels measured by ELISA were significantly increased by DE exposure (Fig. 2B) [Two-tailed T-test with Welch's correction; $T(14.9) = 2.54$, $p = 0.023$].

3.2. Developmental DE exposure activates STAT3 through phosphorylation at Tyr705

Total STAT3 and phospho-STAT3 (Tyr705) were measured by Western blot analysis in whole brain lysates from PND3 pups to assess the extent of STAT3 activation. After normalizing to β -actin, phospho-STAT3 (Tyr705) immuno-blotting revealed increased band intensity for phospho-STAT3 in DE-exposed mice (male and female) compared to FA controls ($n = 10$, Two-tailed T-test with Welch's correction; $T(16.26) = 2.285$, $p = 0.036$) (Fig. 3A), while no differences were found in total STAT3 levels (Fig. 3B). This finding indicates increased STAT3 activation by phosphorylation at residue Tyr705 at PND3 during the early postnatal period of developmental DE-exposure.

3.3. Developmental DE exposure is associated with increased DNMT1 expression

To assess the expression of DNMTs [known to be modulated by STAT3 (Huang et al., 2016; Zhang et al., 2005)], qPCR analysis was conducted with RNA samples isolated from PND3 whole brains. Relative expression of the target genes DNMT1, DNMT3a, DNMT3b, DNMT3l were normalized to the housekeeping gene GAPDH. Expression of DNMT1 increased significantly in brains of DE-exposed mice (Two-way ANOVA; main effect of DE exposure $F(1, 12) = 11.91$, $p = 0.0048$; Bonferroni posttest adjusted p-value: Male $p = 0.0052$; No significant differences due to sex ($p = 0.4659$) or DE exposure X sex interaction ($p = 0.0824$) were found; Fig. 4A). Other DNMTs, i.e. DNMT3a, DNMT 3b, and DNMT 3l, showed no significant changes in expression due to DE exposure (Fig. 4B–D). There were no differences in GAPDH expression between DE- and FA-exposed animals (not shown).

3.4. Developmental DE exposure is associated with decreased RELN expression in brain at PND3

Since DNMT1 has been shown to down-regulate RELN expression (Kundakovic et al., 2009; Noh et al., 2005), we hypothesized that the increased DNMT1 expression in DE-exposed mice may decrease levels of RELN mRNA. At PND3, a significant decrease in RELN mRNA levels was found in whole brain samples of DE-exposed mice compared to same-sex control mice (Two-way ANOVA; main effect of DE exposure $F(1,15) = 28.66$, $p < 0.0001$; Bonferroni posttest adjusted p-value: Male $p = 0.0007$, Female $p = 0.0168$; no statistically significant interaction was found between DE exposure X sex; however, there were significant differences in RELN expression between FA-control male and female mice at PND3 [$F(1,15) = 16.54$, $p = 0.0010$; Fig. 5A)]. No significant differences in RELN expression due to DE exposure were found in cerebral cortex of mice at PND21 (Fig. 5B) or PND60 (Fig. 5C).

3.5. Developmental DE exposure is associated with disorganization of cortical lamina

RELN is known to be a critical player in guiding the process of neuronal migration during cortical development (D'Arcangelo, 2014; Franco et al., 2011; Jossin and Cooper, 2011; Reiner et al., 2015), and disrupted cortical organization has been reported in RELN deficient "reeler" mice (Boyle et al., 2011). Immunohistochemical analysis was performed with the lamina-specific markers RELN and calretinin to examine cortical organization in PND60 brains of mice exposed to either DE or FA during development. Significant differences were seen in the cortical distribution of both RELN- and calretinin-positive cells. The

distribution of RELN-positive cells revealed ectopic clusters of cells in deeper layers of the cortex in both DE-exposed males [clusters located in bins 4, 6, 7 and 8; Two-tailed T-test with Welch's correction; bin 4: $T(7.88) = 2.97$, $p = 0.036$; bin 6: $T(7.64) = 2.89$, $p = 0.028$; bin 7: $T(7.49) = 2.53$, $p = 0.024$; bin 8: $T(7.21) = 2.37$, $p < 0.001$; Fig. 6C] and females [clusters located in bins 5, 6, 7, and 9; Two-tailed T-test with Welch's correction; bin 5: $T(7.79) = 2.76$, $p = 0.008$; bin 6: $T(7.85) = 2.83$, $p = 0.039$; bin 7: $T(7.67) = 2.53$, $p = 0.024$; bin 9: $T(7.21) = 2.63$, $p = 0.033$; Fig. 6D], with the cell clusters located outside of the marker's normal destined distribution, as seen in FA control animals of the same sex (e.g., for males, compare Fig. 6A and B). When comparing cell density in all sampled areas (all 10 bins combined) no significant difference in RELN+ cell density was found between DE- and FA-exposed mice, indicating that the average density of RELN+ cells across all cortical layers was not affected by DE exposure.

The distribution of calretinin-positive cells in DE-exposed mice also showed higher cell density clusters in deeper layers of cortex in both DE-exposed males [clusters located in bins 5 and 7; Two-tailed T-test with Welch's correction; bin 5: $T(7.61) = 2.44$, $p = 0.042$; bin 7: $T(7.73) = 1.54$, $p = 0.023$; Fig. 7C] and females [clusters located in bins 7 and 9; Two-tailed T-test with Welch's correction; bin 7: $T(6.33) = 3.10$, $p = 0.020$; bin 9: $T(7.99) = 3.211$, $p = 0.012$; Fig. 7D], as compared to FA control animals of the same sex. Again, when comparing cell density across all sampled areas (all 10 bins combined) no significant difference in calretinin+ cell density was found between DE- and FA-exposed mice. Thus, immunohistochemistry analysis revealed statistically-significant cortical disorganization in DE- exposed males and females, with both markers presenting clusters of cells in deeper layers within the somatosensory cortex, suggesting neurons were under-migrated during corticogenesis in the DE-exposed mice.

4. Discussion

Positive associations between developmental TRAP exposure and increased risk for ASD have been reported in several epidemiological studies (Becerra et al., 2013; Suades-González et al., 2015; Talbott et al., 2015; Volk et al., 2011; Volk et al., 2013). Various animal studies have also reported ASD-like behavioral changes due to air-pollution exposure during development (Church et al., 2018; Li et al., 2018; Thirtamara et al., 2013; Chang et al., 2018). We have previously found that developmental exposure to DE (from E0 to PND21) causes behavioral changes in all three characteristic domains of ASD, i.e. an increase in repetitive behavior, disrupted verbal and olfactory communication, and social behavior deficits (Chang et al., 2018). However, possible mechanisms underlying the developmental effects of air pollution and their potential roles in ASD are still unknown. Exposure to ambient ultra-fine particles (UFP) has been reported to induce inflammation and microglial activation leading to ventriculomegaly and excitatory/inhibitory imbalance (Allen et al., 2016). Prenatal DE exposure has been shown to cause toll-like receptor 4-dependent microglial activation, as well as astrocyte activation in mice (Bolton et al., 2017; Li et al., 2018). We hypothesized a possible pathway that may lead from neuroinflammation to cortical laminar disorganization, a morphological alteration observed in brains of autistic patients as well as in the MIA mouse model of autism (Choi et al., 2016; Stoner et al., 2014). Findings from this MIA study lend support to our hypothesized pathway to cortical

disorganization (Fig. 8), a pathway that involves elevated levels of the cytokine IL-6, phosphorylation of STAT3, and up regulation of DNMT1, leading ultimately to down regulation of RELN expression and altered cortical organization during development.

In the present study, we indeed demonstrate effects of developmental DE exposure on the cortical organization of the somatosensory cortex, and provide evidence suggesting that the above pathway may be involved in producing these effects. As expected, we observed an increase of IL-6 protein levels in E16.5 placenta (Fig. 2B), and of IL-6 mRNA expression in PND3 neonatal whole brain (Fig. 2A). Other studies have also reported increased levels of IL-6 in placenta and fetal brain caused by developmental DE exposure in mice (Auten et al., 2012; Bolton et al., 2012; Fujimoto et al., 2005), although the latter studies utilized higher concentration of DE particles. A recent study reported significant up-regulation of IL-17 α in serum and in mononuclear cells isolated from placenta after induction of inflammatory response with Poly (I:C) injection (a MIA model) (Choi et al., 2016). The same study also showed that the increase in IL-17 α was IL-6 dependent, as IL-6 is a key factor for TH17 cell differentiation (Choi et al., 2016). We attempted measuring protein levels of IL-17 α in E16.5 placenta samples by ELISA, but the results were inconclusive, as most samples were under the detection limit (data not shown). Since IL-17 α is produced by a small population of differentiated TH17 cells, in future studies measuring expression levels of the IL-17 α receptor subunit A (IL-17Ra) may be a more robust and reliable approach in characterizing the inflammatory response associated with ASD-like behavioral phenotypes.

In agreement with our hypothesis, we found that developmental DE exposure induced STAT3 activation as evidenced by the increased levels of phosphorylation at residue tyrosine 705, a phosphorylation site known to be activated by JAK2 (Chang et al., 2005), while levels of total STAT3 were unaffected (Fig. 3A and B). We also found up regulation of DNMT1 (Fig. 4A) in the brains of the DE-exposed offspring, which is known to be modulated by STAT3 (MuhChyi et al., 2013; Zhang et al., 2005). Increased expression of DNMT1 has been reported in lungs of C57BL/6 mice exposed to concentrated ambient PM_{2.5} at a level of 5.5×10^5 particle/cm² (Soberanes et al., 2012). In mice prenatally exposed to 73.61 μ g/m³ of ambient PM_{2.5} for 5 h/day, increased expression of DNMT1, DNMT3a, and DNMT3b were detected in adult heart samples (Tanwar et al., 2017). The finding of an increased expression of DNMT1 suggested potential alterations of DNA methylation in DE-exposed neonatal pups. In particular, DNMT1 has been shown to modulate the expression of RELN, an important neurodevelopmental signaling molecule, by directly binding and modifying DNA methylation status at the RELN promoter region (Kundakovic et al., 2007; MuhChyi et al., 2013; Zhang et al., 2005). As expected, RELN mRNA levels in brains of PND3 pups were significantly decreased upon developmental DE exposure (Fig. 5A). Since epigenetic modifications often result in long-lasting effects on gene expression, we also measured RELN mRNA levels at two later ages (PND21 and PND60) but found no significant difference in RELN expression due to DE exposure (Fig. 5B and C). These results are not surprising given the importance of early developmental events in shaping the organization of brain structure. The decreased levels of RELN at PND3, but not at PND21 or PND60, are consistent with an early impact of DE exposure on RELN-related developmental effects. Considering the fact that developmental DE exposure was associated with disrupted cortical laminar organization in the somatosensory cortex at PND60, long after the DE exposure

concluded, it is likely that the early developmental changes in RELN expression were important for altering the cortical organization at PND60. Decreased RELN expression (D'Arcangelo, 2014) and differential DNA methylation patterns within the RELN promoter (Grayson et al., 2006) have both been found in ASD patients compared to control individuals. It is important to note that the current study has not demonstrated causal relationships between these events and the effects on cortical organization, and it is possible that other events or pathways are equally or even more important. It is reasonable to suggest that disrupted RELN expression during crucial developmental stages, as seen in the current study, could result in long-lasting laminar disorganization, considering the important role of RELN in modulating the process of neuronal migration.

To investigate whether developmental DE exposure may induce structural abnormalities in the cerebral cortex as observed in ASD (Stoner et al., 2014), we carried out an immunohistochemical analysis of cortical laminar organization in the somatosensory cortex of PND60 mice. Specifically, we determined the distribution of cells expressing cortical lamina-specific markers RELN and calretinin in different cortical layers. Structural abnormalities in the SIDZ region of the somatosensory cortex have been found to play an important role in modulating autism-like behavior (Shin Yim et al., 2017). In cerebral cortex of control mice, RELN is mostly localized in layer I and more sparsely in layers II through VI (Impagnatiello et al., 1998; Pesold et al., 1998). We found a larger proportion of RELN positive cells concentrated within layers IV and V in brains of both male and female mice developmentally exposed to DE, while the total number of RELN+ cells in FA- or DE-exposed animals was not statistically different. In contrast, calretinin is typically expressed in GABAergic interneurons localizing in layers II/III and IV (Gonchar, 2008), and this was confirmed by our findings in cerebral cortex of FA-exposed mice (Fig. 7). Developmental DE exposure caused changes in the distribution of calretinin-positive cells, which were localized in layers V and VI at significantly higher cell density compared to control mice of the same sex (Fig. 7). Although the level of cortical disorganization found in DE-exposed mice is mild compared to findings in mouse models of ASD (Boyle et al., 2011; Choi et al., 2016), our results parallel reports of small patches of disorganization found in prefrontal cortex of adolescent ASD patients (Stoner et al., 2014). Considering that disruption of developmental events elicited by a pervasive environmental toxicant such as air pollution would have long-lasting effects in organizational structure of the brain, the public health implication suggested by our finding is rather striking. Two possible scenarios can be considered that could result in dis-localized RELN+ and calretinin+ cells: disruption in neuronal migration, and/ or disruption in the fate of neuronal differentiation. Further histological assessment with markers involving neurogenesis and differentiation (e.g. PAX6, Tbr2, and Tbr1) (Englund et al., 2005; Mihalas and Hevner, 2017) would be helpful to distinguish between the two possibilities.

In the current study and in our previous study examining ASD-related behaviors, sex differences were observed in many, but not all, of the effects of DE exposure, with males generally being more sensitive. For example, the effects of DE exposure on DNMT1 and RELN in the brains of PND3 pups were greater in males than in females (Figs. 4A and 5A). This is interesting given the strong male bias in autism spectrum disorder (ASD), where prevalence rate have been reported to be much higher in male patients than in female

patients (Richards et al., 2015; Loomes et al., 2017). While no direct mechanistic explanation has been attributed to the sex bias in ASD, recent studies suggest that differences in behavioral phenotype between males and females may contribute to this bias (Werling and Geschwind, 2013; Richards et al., 2015; Loomes et al., 2017). However, no sex differences were seen with DE exposure in IL-6 mRNA levels or STAT3 phosphorylation levels at PND3, and there were no statistically significant sex differences in cortical laminar organization at PND60 (Figs. 2A, 3A, 6, 77). Nevertheless, it is intriguing that when sex differences were detected, it was generally the males that were more sensitive to the effects of DE exposure.

5. Conclusion

The association between air pollution and increased ASD risk has been reported by many epidemiological studies and by a number of animal studies, yet mechanistic studies supporting these findings are just starting to emerge. We have found that developmental exposure to DE is associated with increased neuroinflammation, activation of the JAK2/STAT3 pathway as detected by STAT3 phosphorylation, decreased expression of RELN, and long-term changes in cortical lamina organization. The biochemical and histological changes observed in developmentally DE exposed mice parallel findings in both ASD patients and ASD-like animal models. Future studies are much warranted not only to gain further understanding in how gene-environment interaction modulates ASD, to improve protection for vulnerable populations, and for exploring other potential mechanisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ASD	autism spectrum disorder
DE	diesel exhaust
DNMT	DNA methyltransferase
ELISA	enzyme-linked immunosorbent assay

FA	filtered air
GABA	gamma-aminobutyric acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
E0	embryonic day 0
IL17a	interleukin 17a
IL6	interleukin 6
JAK2	Janus kinase 2
MIA	maternal immune activation
PAX6	paired box 6
PM	particulate matter
PND	postnatal day
Poly (I:C)	polyinosinic-polycytidylic acid
qRT-PCR	real-time quantitative reverse transcription polymerase chain reaction
RELN	reelin
STAT3	signal transducer and activator of transcription 3
Tbr2	T-Box, Brain 1
TH17	T helper 17 cell
TRAP	traffic related air pollution
UFP	ultrafine particles

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Experimental design

Fig. 1.

General experimental design. Exposure started at the beginning of pregnancy upon confirmation of copulation plug [Embryonic day 0 (E0)] and ended at weaning [postnatal day 21 (PND21)]. Exposure to diesel exhaust (DE) was conducted for 6 h/day, 5 days/week at the level of 250–300 $\mu\text{g}/\text{m}^3$ $\text{PM}_{2.5}$ concentration. At E16.5, a subset of pregnant dams exposed to either DE or FA were sacrificed, and placenta were collected for evaluation of inflammatory cytokine IL-6 levels by ELISA. At PND3, brains were collected for qPCR and Western blot assays. At PND21 cortical samples were collected for qPCR, and at PND60 cortical samples were collected for qPCR and perfusion-fixed cryo-protected brains were collected for immunohistochemistry.

Fig. 2.

Developmental DE exposure increases IL-6 expression. (A) Interleukin 6 (IL-6) mRNA levels in whole brain lysate from PND3 pups exposed to filtered air (FA) or diesel exhaust (DE) from E0 to PND3. After normalizing to the house keeping gene GAPDH, significantly increased IL-6 expression levels, calculated as the relative expression (dCq) and plotted as mean ($\pm$ SE), were found in DE-exposed males and females compared to FA-exposed controls of the same sex. (* $p < 0.05$; two-way ANOVA with Bonferroni post-test; $n = 5$). (B) IL-6 protein levels in E16.5 placenta were measured by enzyme-linked immunosorbent assay (ELISA). Increased IL-6 protein levels, plotted as mean ($\pm$ SE), were found in E16.5 placenta from dams exposed to DE compared to control dams (* $p < 0.05$; two tailed t-test with Welch's correction; $n = 10$). FA, filtered-air; DE, diesel exhaust; M, male; F, female.

Fig. 3.

STAT3 phosphorylation in brains from PND3 mice. STAT3 phosphorylation at residue tyrosine 705 (Tyr705) was measured by Western blot in whole brain lysate from PND3 mice. Increased levels of phosphorylated STAT3 (Tyr705) were found in DE-exposed mice [mixed sex, (* $p < 0.05$; two tailed t-test with Welch's correction; $n = 10$)] compared to FA-exposed controls (A). There were no differences in total STAT3 protein levels (T-STAT) associated with DE exposure (B). Both phosphorylated STAT3 (Tyr705) and total STAT3 levels were normalized to the loading control, beta-actin. Results represent the mean ($\pm$ SE) of 5 pups from different litters for each experimental group. (C) Representative Western blot image. FA, filtered-air; DE, diesel exhaust; M, male; F, female.

Fig. 4.

mRNA expression of DNMTs in brains of PND3 mice. mRNA Levels of DNMTs (1, 3a, 3b, 3l) were measured in brains of male and female PND3 mice exposed to FA or DE.

Expression of DNMT1 increased in DE-exposed mice compared to FA-exposed control mice, particularly in males (A). Expression levels of other DNMTs (3a, 3b, 3l) did not show statistically significant differences between treatments (B, C, D). Relative mRNA expression of DNMTs were normalized to the house keeping gene GAPDH and calculated as the relative expression (dCq). Results represent the mean ($\pm$ SE) of five pups from different litters for each experimental group (** $p < 0.01$, two-way ANOVA with Bonferroni post-test). FA, filtered-air; DE, diesel exhaust; M, male; F, female.

Fig. 5.

Developmental DE exposure decreases RELN expression in brain. Reelin (RELN) mRNA levels in brain were measured at three developmental time points (PND3, PND21, and PND60). (A) At PND3, expression of RELN in whole brain samples was significantly lower in DE-exposed mice compared to FA control mice of the same sex. (B) RELN expression in the cerebral cortex of PND21 mice showed no significant change due to DE exposure in either male or female mice. (C) RELN expression in cerebral cortex of PND60 mice also showed no significant change due to DE exposure in either male or female mice. RELN expression levels were normalized to the house keeping gene GAPDH and calculated as the relative expression (dCq). Results represent the mean ($\pm$ SE) of 4–5 pups from different litters for each experimental group (* $p < 0.05$, *** $p < 0.001$; two-way ANOVA with Bonferroni post-test). FA, filtered-air; DE, diesel exhaust; M, male; F, female.

Fig. 6.

RELN positive cells in somatosensory cortex of PND60 mice. In cerebral cortex of control mice, reelin (RELN) is mostly localized in layer I and more sparsely in layers II through VI (Impagnatiello et al., 1998; Pesold et al., 1998). (A, B) Representative image of RELN+ cells (green) with nuclei counterstain DAPI (blue) from FA- and DE-exposed male mice. Distribution of RELN-positive cells revealed ectopic clusters in deeper layers of cortex; in males, increased RELN+ cell density was found in bins 4, 6, 7, and 8 (C), and in females, RELN+ cell density increased in bins 5, 6, 7, and 9 as a consequence of DE-exposure (D). No significant difference was found in the total number of RELN+ cells per sampled area due to DE exposure. Results represent the mean ($\pm$ SE) of 5 mice from different litters for each experimental group; 3–5 sections/mouse were examined (* p < 0.05, ** p < 0.01, *** p < 0.001; unpaired t-test with Welch's correction). FA, filtered-air; DE, diesel exhaust; M, male; F, female; reelin, RLN. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 7.

Calretinin positive cells in somatosensory cortex of PND60 mice. In cerebral cortex of control mice, calretinin is typically expressed in GABAergic interneurons localizing in layers II/III and IV (Gonchar, 2008). (A, B) Representative image of calretinin+ cells (red) with nuclei counterstain DAPI (blue) from FA- and DE- exposed males. Distribution of calretinin+ cells revealed ectopic clusters in deeper layers of cortex; in males, increased calretinin+ cell density was found with DE exposure in bins 5 and 7 (C) and in females, calretinin+ cells formed ectopic clusters in bins 7 and 9 due to DE exposure (D). No significant difference was found in number of calretinin+ cells per sampled area due to DE exposure. Results represent the mean ($\pm$ SE) of 5 mice from different litters for each experimental group; 3–5 sections/mouse were examined (* $p < 0.05$; unpaired t-test with Welch's correction). FA, filtered-air; DE, diesel exhaust; M, male; F, female; calretinin, CR. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 8.

Proposed mechanism for developmental DE exposure-induced cortical disruption.

Developmental exposure to DE is associated with neuroinflammation, evidenced by elevated levels of IL-6. This in turn leads to activation of the JAK2/STAT3 pathway leading to STAT3 phosphorylation; activated STAT3 acts as a transcription factor and up-regulates DNMT1 expression, which modulates reelin (RELN) expression via DNA methylation. Known for its critical role in guiding the process of neuronal migration during development, decreased expression of RELN during critical developmental periods would lead to altered cortical structure as observed in ASD (Stoner et al., 2014). Of note is that with the same period and level of developmental DE exposure we previously found significant alterations in all three characteristic behavioral domains of ASD (communication, repetitive behavior, social interactions) (Chang et al., 2018).