Common Genetic Variants and Risk of Brain Injury After Preterm Birth

WHAT’S KNOWN ON THIS SUBJECT: Preterm birth is strongly associated with alterations in brain development and long-term neurocognitive impairment that are not fully explained by environmental factors.

WHAT THIS STUDY ADDS: Common genetic variation in genes associated with schizophrenia and lipid metabolism modulates the risk for preterm brain injury; known susceptibilities to neurologic disease in later life may be exposed by the stress of preterm birth.

BACKGROUND: The role of heritable factors in determining the common neurologic deficits seen after preterm birth is unknown, but the characteristic phenotype of neurocognitive, neuroanatomical, and growth abnormalities allows principled selection of candidate genes to test the hypothesis that common genetic variation modulates the risk for brain injury.

METHODS: We collected an MRI-linked genomic DNA library from 83 preterm infants and genotyped tag single nucleotide polymorphisms in 13 relevant candidate genes. We used tract-based spatial statistics and deformation-based morphometry to examine the risks conferred by carriage of particular alleles at tag single nucleotide polymorphisms in a restricted number of genes and related these to the preterm cerebral endophenotype.

RESULTS: Carriage of the minor allele at rs2518824 in the armadillo repeat gene deleted in velocardiofacial syndrome (ARVCF) gene, which has been linked to neuronal migration and schizophrenia, and rs174576 in the fatty acid desaturase 2 gene, which encodes a rate-limiting enzyme for endogenous long chain polyunsaturated fatty acid synthesis and has been linked to intelligence, was associated with white matter abnormality measured in vivo using diffusion tensor imaging (P = .0009 and P = .0019, respectively).

CONCLUSIONS: These results suggest that genetic variants modulate white matter injury after preterm birth, and known susceptibilities to neurologic status in later life may be exposed by the stress of premature exposure to the extrauterine environment. Pediatrics 2014;133:e1655–e1663
Preterm delivery affects 10% of births worldwide and is associated with neurodevelopmental impairment.\textsuperscript{1,2} Adverse outcome is strongly associated with a phenotype that commonly combines: cognitive dysfunction and special educational needs; reduced early somatic and brain growth; and cerebral white matter injury.\textsuperscript{3–6} This is only partly explained by known environmental stresses such as ischemia, inflammation, and altered nutrition.\textsuperscript{7} However, neuroanatomic and neurocognitive functions are strongly heritable\textsuperscript{8–11} and the characteristic abnormalities seen in preterm infants allow principled choices of candidate genes that might modulate the risk for adverse neurodevelopmental outcome after preterm birth.

The role of prematurity in cerebral development and damage has been much investigated by using MRI and diffusion tensor imaging (DTI), which provide sensitive measures of abnormal morphology and microstructure that correlate with neurodevelopmental outcome.\textsuperscript{5,12–16} Computational techniques are available that define a cerebral endophenotype that is closely related to later neurocognitive outcome. These include tract-based spatial statistics (TBSS), a method for group-wise analysis of multiple fractional anisotropy (FA) images derived from DTI, and deformation-based morphometry, which quantitates local brain growth.\textsuperscript{17,18} These methods allow powerful unbiased group-wise studies of genetic risk.\textsuperscript{19–21}

Based on studies of the imaging and clinical phenotype we hypothesized that the development of preterm cerebral injury could be related to genes involved in white matter development or human cognition and behavior. We used TBSS and deformation-based morphometry to examine the risks conferred by carriage of particular alleles at single nucleotide polymorphisms (SNPs)\textsuperscript{22} located within a small number of candidate genes (Supplemental Table 1).

These genes were selected because of previous functional evidence related to the preterm cerebral endophenotype including cognitive/behavioral dysfunction, neuronal/glial development, and endogenous long-chain polyunsaturated fatty acid production, which is essential for neural development. We now report 2 genetic variants that modulate white matter injury after preterm birth.

**METHODS**

The study was conducted according to the principles of the Declaration of Helsinki, and ethical approval was obtained from the UK National Research Ethics Service. Written parental informed consent was obtained.

The cohort consisted of preterm neonates who received care at Queen Charlotte’s and Chelsea Hospital between January 2005 and October 2008, underwent DTI and MR imaging in the neonatal period, and whose parents consented to the collection of DNA. Infants were not eligible if they had a chromosomal abnormality, congenital malformation, or congenital infection.

**Genotyping**

The DNA OG-575 kit was used for sampling of saliva at term equivalent age (DNAGenotek, Kanata, Canada). Genotyping was performed by using the Sequenom iPLEX genotyping assay and MALDI-TOF mass spectrometry (Sequenom Inc, San Diego, CA). To maximize the genetic information obtained from studying the candidate genes, an efficient set of SNPs for genotyping were identified by using the Tagger algorithm\textsuperscript{22} as implemented in the Haplovew software.\textsuperscript{25} Tag SNP sequences were extracted from the HapMap genome browser release\textsuperscript{28} and included if the minor allele frequency (MAF) exceeded 5% using population descriptor CEU (Utah residents with Northern and Western European ancestry from the CEPH collection). Exclusion criteria: <85% genotyping success per SNP, and/or statistically significant deviation from Hardy-Weinberg equilibrium ($P < .001$).

**Imaging**

MR imaging was performed on a Philips 3-Tesla system (Philips Medical Systems, Netherlands) using an 8-channel phased array head coil. Single-shot echo-planar imaging DTI was acquired in the transverse plane in 15 non-collinear directions using the following parameters: repetition time (TR): 8000 ms; echo time (TE): 49 ms; slice thickness: 2 mm; field of view: 224 mm; matrix: 128 × 128 (voxel size: 1.75 × 1.75 × 2 mm$^3$); b value: 750 s/mm$^2$; SENSE factor: 2. T1-weighted 3D MPRAGE were acquired with parameters: TR = 17 ms, TE = 4.6 ms, inversion delay = 1500 ms, flip angle = 13°; acquisition plane = sagittal, voxel size = 0.82 × 1.05 × 1.6 mm, FOV = 210 × 167 mm and acquired matrix = 256 × 163. T2-weighted fast spin-echo: TR = 8700 ms, TE = 160 ms, flip angle = 90°; acquisition plane = axial, voxel size = 1.15 × 1.18 × 2 mm, FOV = 220 mm, and acquired matrix = 192 × 186.

All examinations were supervised by a pediatrician experienced in MR procedures. Infants were sedated with oral chloral hydrate (25–50 mg/kg) before scanning, and pulse oximetry, temperature, and electrocardiography data were monitored throughout. Ear protection was used for each infant, comprising earplugs molded from a silicone-based putty (ColteneWhaledent, Mahwah, NJ) placed in the external ear and neonatal earmuffs (MiniMuffs, Natus Medical Inc, San Carlos, CA).

**Data Analysis**

**DTI**

DTI analysis was performed by using FMRIB’s Diffusion Toolbox (v2.0) as implemented in FMRIB’s Software Library (FSL v4.1.5; www.fmrib.ox.ac.uk/fsl).\textsuperscript{24} Each infant’s diffusion-weighted image DWI was registered to their
respective non–diffusion-weighted \((b = 0)\) image and corrected for differences in spatial distortion owing to eddy currents. Images were brain extracted using Brain Extraction Tool (BET v2.1), diffusion tensors calculated voxelwise, and FA maps generated. TBSS\(^\text{18}\) was performed by using a modified pipeline specifically optimized for neonatal DTI analysis.\(^\text{19}\) The aligned data were used to create a mean FA map and a mean FA skeleton that represents the center of all white matter tracts common to the group. The FA skeleton was thresholded at \(FA \geq 0.15\) before each infant's aligned FA data were projected onto it. TBSS was used to assess the relationship between FA and genotype. For each SNP, infants were grouped according to presence of the minor allele, which enabled a 2-group comparison of minor homozygote or heterozygote versus major allele homozygote. SNP data were excluded if MAF in the study population was <5%. The FA values of the 2 groups of infants were compared by using voxel-wise cross-subject linear regression, with gestational age at birth and age at scan entered in the model, because these are known to influence FA during early development. All images were subject to family-wise error correction for multiple comparisons after threshold-free cluster enhancement.\(^\text{25}\)

Control of type I error for multiple SNP testing was carried out using the q-value algorithm, selecting the bootstrap method to calculate the Benjamini and Hochberg FDR of 5%.\(^\text{26,27}\)

**Morphometric Analysis**

Volumetric analyses were performed in 2 stages by using published methods.\(^\text{28,29}\)

First, the MR image of each subject was co-aligned with a spatiotemporal atlas of the anatomy of the neonatal brain closest to the age of the subject.\(^\text{28,29}\)

The correspondence obtained by this registration allowed the average estimates of structural segmentations to be used as priors in a segmentation method, which estimated the same structures in the native space of the subject.\(^\text{28}\) The resulting segmentation of each structure is represented as a “soft” density map with the value at each voxel ranging from 0 to 1 indicating the proportion of the voxel occupied by each structure. By summing these values over all voxels, an estimate of the volume for each structure in each image was calculated.

Second, a spatiotemporal atlas was constructed from 142 T2-weighted images acquired between 29 and 44 weeks' postmenstrual age (PMA)\(^\text{31,32}\) and is publicly available at www.brain-development.org. At each specific age in the range, the corresponding atlas volume is an estimate of the average of the anatomicies of subjects close in age. By iteratively applying kernel regression to the affine transformations between each subject’s image and the average, an unbiased estimate of the average shape is obtained. The transformations between each acquired image and the final atlas were used to propagate structural segmentations from each subject’s native space. In this way, as well as obtaining a spatiotemporal atlas of the MR images, further spatiotemporal atlases of white matter, grey matter, cerebrospinal fluid, subcortical grey matter, brainstem, and cerebellum were generated. Automatic thalamic segmentations were acquired using published methods.\(^\text{33}\)

Clinical, demographic, and nutritional data were tested for normality by using Shapiro-Wilk test and are presented as medians and ranges if the data did not conform to a normal distribution. Data were analyzed by using independent sample \(t\) test for continuous variables if there was equality of variance between groups (Levene’s test) and \(X^2\) test for proportions. Statistical analysis was performed by using SPSS v19.0 (SPSS Inc, Chicago, IL).

**RESULTS**

We collected an MRI-linked genomic DNA library from 83 preterm infants who had a median PMA at birth of 28.\(^\text{15}\) weeks (range, 23.\(^\text{2}\) to 39.\(^\text{6}\) weeks) and median birth weight of 1052 g (range, 550–2140 g). MR images were acquired at median PMA 40.\(^\text{1}\) weeks (range, 29.\(^\text{1}\) to 47.\(^\text{6}\) weeks). Forty-five participants were male (54%), and 38 (45%) were white British (see complete ethnic distribution in Supplemental Table 2). Fourteen (17%) had bronchopulmonary dysplasia defined as a need for respiratory support and/or supplemental oxygen at 36 weeks PMA.

**Common Genetic Variants and White Matter Microstructure**

Thirty-five tag SNPs across 13 genes were tested (Supplemental Table 1). After correction for multiple testing we found that 2 tag SNPs were associated with significant alterations in FA in white matter: rs2518824 in the ARVC\(^\text{2}\) gene \((P = .0009; \beta\) coefficient \(-0.029, 95\%\) confidence interval \([-0.039 to -0.019]\) and rs174576 in the fatty acid desaturase 2 \((FADS2)\) gene \((P = .0019; \beta\) coefficient \(-0.034, 95\%\) CI, \(-0.047 to -0.021)\). Carriage of the minor allele in no other tag SNP was significantly associated with FA in white matter after correction for multiple tests (Fig 1 and Supplemental Table 1). There was no significant difference in the prevalence of bronchopulmonary dysplasia between infants who had and those who did not have the minor allele for either of the significant SNPs.

After correcting for degree of prematurity at birth and PMA at scan, carriage of the minor allele \((G)\) at rs2518824 in the \(ARVC\) gene was associated with reduced FA in white matter for the 80 individuals who had genotype data for this SNP and DTI \((P = .0009)\). The minor allele frequency was 66% \((\text{minor homozygote/heterozygote})\). Additional SNPs rs1110477 in ARVC\(^2\) and rs9532377 in the catechol-O-methyl transferase \((\text{COMT})\) gene, both at locus
22q11.2, were nominally associated with change in FA, but these relationships were not significant after the omnibus correction threshold was applied ($P = .0282$ and $P = .0413$, respectively; for linkage disequilibrium plots see Supplemental Fig 1). Carriage of the minor allele (A) at rs174576 in $FADS2$ was associated with reduced FA in specific white matter tracts for the 73 individuals who had genotype data for this SNP and DTI ($P = .0019$), and none of the remaining tag SNPs in the $FADS2$ gene were associated with FA changes after correction for multiple tests. The MAF was 57%.

Infants who carried the minor allele (G) at rs2518824 in the $ARVCF$ gene had reduced FA in the corpus callosum, the superior corona radiata, the fornix, and the centrum semiovale (Fig 2). Infants who carried the minor allele (A) at rs174576 in $FADS2$ had reduced FA in white matter within the posterior corona radiata (Fig 3). The mean PMA at birth of the group that carried the minor allele was 29.28 weeks, which was older than that of the group without the minor allele (28 weeks) ($P = .032$).

Because of a possible interaction between polymorphisms in $FADS2$ and breast milk exposure on IQ in childhood,$^{34,35}$ we investigated whether the 2 groups differed in early nutritional exposures. There was no difference in the proportion of infants who were exclusively breastfed at term equivalent age between the 2 groups (60% vs 55%, $P = .812$), and there was no significant difference in parenteral nutrition use between those who had the minor allele and those who did not (4 vs 5.5 days, $P = .132$).

TBSS is designed to identify supra-threshold voxels, so when a regional effect is detected without due cause, the possibility that it underlies a more global effect should be considered. Therefore we assessed the spatial distribution of difference in FA at the significance threshold $P < .005$ for rs2518824 and rs174576 (Figs 2 and 3). No other tag SNP was associated with voxel-wise differences that were significant at this threshold (Fig 1 and Supplemental Table 1).

**DISCUSSION**

Based on studies of the characteristic preterm phenotype, we predicted a number of genes that could modulate the risks for cerebral abnormalities, and found that SNPs linked to genetic regions associated with schizophrenia,$^{36,37}$ and linking lipid metabolism to intelligence,$^{34,35}$ predicted changes in the cerebral endophenotype.

Common genetic variation at chromosome 22q11.2 is consistently associated with neuropsychiatric disorders.$^{37-40}$ Within this cytogenetic band we focused on $ARVCF$ and $COMT$ for a number of reasons: haplotypic associations spanning these genes have been associated
with schizophrenia; overexpression of the region in mice leads to alterations in incentive learning and working memory; common variants in \textit{ARVCF} are associated with an intermediate MRI phenotype that includes altered FA in patients who have schizophrenia; and SNPs in \textit{COMT} are associated with white matter changes in preterm-born adults.

The association between a tag SNP in \textit{ARVCF} and reduced FA in white matter after preterm birth could be explained by: common variation at \textit{ARVCF} influencing white matter microstructure, which has been reported in adult-onset schizophrenia; the SNP being in strong linkage disequilibrium (LD) with non-genotyped SNPs that span \textit{COMT} (Supplementary Fig 1A); or a regulatory role on gene expression. Two SNPs in \textit{COMT} (rs1110477 and rs9332377) approached significance and were in intermediate LD with rs2518824, which could account for the reduced significance of these associations.

\textit{ARVCF} is a member of the catenin family and it modulates neural cell-cell adhesion.
and migration. It is richly expressed in the human ganglionic eminence and in neurons that migrate from the ganglionic eminence to the intermediate zone, the amygdaloid complex, and the thalamus during fetal life. We and others have reported that volume and neuronal loss in the dorsomedial nucleus of the thalamus is associated with preterm birth, and these data focus attention on perturbed neuronal migration as a possible mechanism for abnormal thalamic development, which contributes to the encephalopathy of prematurity.

The FADS2 gene, located on chromosome 11q12.2, encodes \( \delta-6 \) desaturase, a rate-limiting enzyme on the pathway of endogenous docosahexaenoic acid and arachidonic acid production. These long-chain polyunsaturated fatty acids (LC-PUFAs) accumulate in the brain in abundance from the third trimester to 18 months postpartum and are essential for neurogenesis, neurotransmission, and protection from oxidative stress.

The candidacy of FADS2 as a risk modulator for preterm brain injury is raised because FADS2 gene variants have functional effects on LC-PUFA availability, with minor allele carriage at common SNPs including rs174576 being associated with altered levels of arachidonic acid and docosahexaenoic acid in phospholipid, serum, and breast milk, and FADS2 variants may interact with early dietary exposures to influence childhood IQ.

We found that minor allele carriage at rs174576 is associated with lower FA in white matter, after controlling for non-genetic confounders. rs174576 is associated with LC-PUFA phenotypes and is in strong LD with other functional SNPs at the FADS2 locus (Supplemental Fig 1B). The effect size of SNPs in the FADS2 locus is large, with approximately one-third of the variability of PUFA and LC-PUFA levels in human tissues attributable to genotype.

These effects were detected through the use of TBSS, which provides a novel and powerful method for detecting group-wise differences in white matter microstructure predictive of later neurodevelopmental outcome, overcoming the diagnostic imprecision of neurodevelopmental assessment in early childhood and allowing significantly smaller sample sizes. Modeling and neonatal clinical studies have shown that clinically significant changes in FA can be detected in groups as small as 10 patients per group. The quantitative abnormalities described here show similarity to alterations seen in older preterm children and adolescents, suggesting that the influence of prematurity and genetic factors on neural systems that underpin neurodevelopmental function is operative before the time of normal birth.

We used the International HapMap Project population descriptor CEU (Utah residents with Northern and Western European ancestry) to define haplotype frequency, because this descriptor represents the largest proportion of the study population. Our study group was diverse, and although it is possible that ethnicity is a confounding factor in the analysis, we did not adjust for it for the following reasons: firstly, self-declared maternal ethnicity is an imprecise surrogate for neonatal ethnicity; secondly, both parents did not always share the same ethnicity; and thirdly, we adjusted the TBSS model only for factors that are known to influence FA. In future studies it may be possible to investigate the effect
of ethnicity on FA by studying very large populations, which would allow for adjustment for genetic ethnicity, or by restricting analyses to ethnically homogeneous groups.

This study provides proof of concept of genetic pathways that warrant investigation in large-scale association studies, including inflammation, hypoxia signaling, and myelination pathways. The detected effects were specific to white matter, despite brain volume being highly heritable and the common association between white matter damage and reduced brain volumes.66,67 This raises the possibility that although volumetric and white matter changes are associated in the preterm phenotype, genetic vulnerability may be separable. It is also possible that techniques that provide a more highly resolved quantification of structure, but which are not yet validated for use in the developing brain, may be sensitive to genetic difference.

CONCLUSIONS

These results suggest that genetic vulnerabilities to cognitive and neuropsychiatric abnormalities are revealed by the environmental stress of preterm delivery. This is consistent with recent data showing that preterm infants have higher rates of neuropsychiatric disease in adult life,58 as well as the observation that schizophrenia- and autism-associated genes are overexpressed in developing mouse cortical subplate,69 a region of specific vulnerability during preterm life.70 Future research could focus on perturbations in neuronal migration and lipid metabolism in the causal pathway to preterm brain injury.

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Dr Boardman conceptualized and designed the study, made substantial contributions to data acquisition (participant recruitment and image acquisition) and analysis and interpretation of data (identification of candidate genes and tag SNP information and statistical analysis of image and genotype data), and drafted the manuscript; Dr Walley conceptualized and designed the study, made substantial contributions to analysis and interpretation of genetic data (identification of tag SNP information, design of Sequenom assays, and performance of SNSpol and FDR analyses), and revised the manuscript for important intellectual content; Dr Ball made substantial contributions to analysis and interpretation of image data (tract-based spatial statistics) and revised the manuscript for important intellectual content; Dr Takousis made substantial contributions to analysis and interpretation of genetic data (design of Sequenom assays, genotyping experiments, QA tests, collation of data output, and generation of summary statistics) and revised the manuscript for important intellectual content; Dr Krishnan made substantial contributions to the acquisition and interpretation of image data (tract-based spatial statistics) and revised the manuscript for important intellectual content; Mrs Hughes-Carre made substantial contributions to the acquisition of data (genetic material) and revised the manuscript for important intellectual content; Dr Aljabar made substantial contributions to data analysis and interpretation (structural magnetic resonance image data) and revised the manuscript for important intellectual content; Dr Serag made substantial contributions to image data analysis and interpretation (structural magnetic resonance image data) and revised the manuscript for important intellectual content; Mrs King made substantial contributions to the acquisition and interpretation of nutritional data and revised the manuscript for important intellectual content; Drs Merchant and Srinivasan made substantial contributions to acquisition of image data and revised the manuscript for important intellectual content; Professor Froguel, Professor Hajnal, and Professor Rueckert made substantial contributions to conception and design and revised the manuscript for important intellectual content; Professor Counsell made substantial contributions to conception and design of the study and acquisition, analysis, and interpretation of image data and revised the manuscript for important intellectual content; Professor Edwards conceptualized and designed the study, made substantial contributions to analysis and interpretation of data, and drafted the manuscript; and all authors approved the final manuscript as submitted.

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