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Epigenetic and genetic variation at the *IGF2/H19* imprinting control region on 11p15.5 is associated with cerebellum weight

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Abbreviations: IGF2, insulin-like growth factor 2; CTCF, CCCTC-binding factor; DMR, differentially methylated region; ICR, imprinting control region; RT-PCR, real-time PCR; mRNA, messenger RNA; SNP, single nucleotide polymorphism

IGF2 is a paternally expressed imprinted gene with an important role in development and brain function. Allele-specific expression of *IGF2* is regulated by DNA methylation at three differentially methylated regions (DMRs) spanning the *IGF2/H19* domain on human 11p15.5. We have comprehensively assessed DNA methylation and genotype across the three DMRs and the *H19* promoter using tissue from a unique collection of well-characterized and neuropathologically-dissected post-mortem human cerebellum samples (n = 106) and frontal cortex samples (n = 51). We show that DNA methylation, particularly in the vicinity of a key CTCF-binding site (CTCF3) in the imprinting control region (ICR) upstream of *H19*, is strongly correlated with cerebellum weight. DNA methylation at CTCF3 uniquely explains ~25% of the variance in cerebellum weight. In addition, we report that genetic variation in this ICR is strongly associated with cerebellum weight in a parental-origin specific manner, with maternally-inherited alleles associated with a 16% increase in cerebellum weight compared with paternally-inherited alleles. Given the link between structural brain abnormalities and neuropsychiatric disease, an understanding of the epigenetic and parent-of-origin specific genetic factors associated with brain morphology provides important clues about the etiology of disorders such as schizophrenia and autism.

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Introduction

Allele-specific gene expression is known to occur at many autosomal loci, primarily mediated by cis-acting sequence variation.[1] A smaller number of loci are subject to genomic imprinting, an important epigenetic phenomenon regulating the monoallelic expression of genes in a parent-of-origin specific manner. There are approximately 50 known classically imprinted genes in the human genome,[2] and their allele-specific expression is critical for normal embryonic growth and development.[3] A converging body of evidence highlights the particular importance of genomic imprinting in neurodevelopment and brain function,[4] with a recent study in mouse documenting widespread parental-origin effects on gene expression across the brain.[5] Early findings from mouse chimeras demonstrated that maternally- and paternally-inherited genomic information has a differential effect on brain growth,[6] and recent research emphasizes the role of imprinted genes in behavioral and cognitive functions.[7-9] It has been hypothesized that the dysregulation of genomic imprinting during neurodevelopment could adversely affect normal neurobiological functioning and mediate risk of neuropsychiatric disorders in later life.[7,10]

The *IGF2/H19* locus, located on human chromosome 11p15.5, is one of the best-characterized imprinted regions. Work in mice and humans shows that these two neighboring genes are reciprocally imprinted in most somatic cells—the *IGF2* gene is silenced on the maternal allele, whereas the *H19* gene is silenced on the paternal allele—with allele-specific expression primarily regulated by allele-specific DNA methylation at the *H19* promoter and three well-defined differentially methylated regions (DMRs) [11] (**Fig. 1**). Of particular importance is the *IGF2/H19* imprinting control region (ICR) DMR, which contains seven binding sites for the methylation-sensitive, zinc-finger protein CCCTC-binding factor (CTCF). CTCF binding at these sites is thought to facilitate the assembly of a chromatin insulator that blocks interaction between the *IGF2* promoter and enhancers 3' of the *H19* gene. CTCF binds to the unmethylated maternal allele, silencing *IGF2* while allowing expression of *H19*. On the paternal allele, CTCF binding (and insulator assembly) is blocked by DNA methylation, facilitating *IGF2* expression.[11] Furthermore, mouse models show that a specific 54 bp methylated region in the paternal *Igf2 DMR2* is necessary for *Igf2* expression,[12] while methylation of the paternal *H19* promoter

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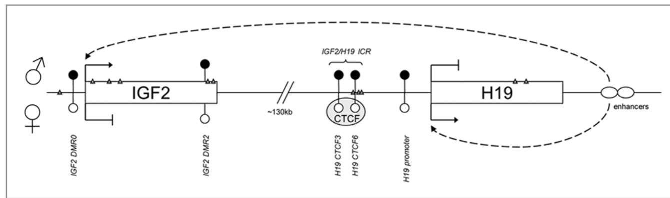

Figure 1. Schematic map of the human *IGF2/H19* locus on chromosome 11p15.5, with paternal allele (♂) on the top and maternal allele (♀) on the bottom. DMRs are represented by circles: filled circles indicate a typically methylated allele and empty circles a typically unmethylated allele. In the current study, DNA methylation was assessed across amplicons spanning *IGF2 DMR0, IGF2 DMR2,* the 3rd and 6th CTCF binding sites of the *IGF2/H19* ICR *DMR* (*H19 CTCF3*, *H19 CTCF6*), and the *H19 promoter*. SNPs genotyped are shown as gray triangles (from left to right rs3842773, rs3741211, rs3213221, rs3213223, rs680, rs3168310, rs10732516, rs2071094, rs2107425, rs2839701, rs217727).

acts to repress expression of the *H19* gene.[11] IGF2 has been shown to be a major driver of prenatal growth in mouse, with placenta-specific *Igf2* transcripts controlling the growth of the placenta and the supply of maternal nutrients to the developing fetus.[13] IGF2 also acts as part of the IGF signaling pathway to regulate the postnatal growth of somatic tissues, including the brain.[14]

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Despite being a classically imprinted region, there is evidence of considerable epigenetic heterogeneity at the human *IGF2/H19* locus; DNA methylation in the region is a normally distributed quantitative trait, which can be influenced by both genetic[15] and early-life nutritional factors.[16] For example, periconceptional folic acid supplements are associated with hypermethylation of *IGF2*,[17] and individuals conceived (or in early gestation) during the Dutch hunger winter famine 1944–1945 show alterations in *IGF2* methylation.[16] Of note, while the developmental timing of exposure appears to be important—only exposure to famine during the periconceptional period is associated with altered *IGF2* DNA methylation—the changes appear to be stable and maintained into adulthood. Such persistent epigenetic alterations offer a mechanistic link between adult health and environmental conditions during development.[18] Intriguingly, periconceptional exposure to famine is also associated with structural brain abnormalities and an increased risk of neuropsychiatric disease.[19-22] Numerous studies have linked brain morphometry to a range of neuropsychiatric disorders including schizophrenia, bipolar disorder, major depressive disorder and autism spectrum disorders, and a detailed meta-analysis concludes that brain weight is significantly reduced in schizophrenia.[23] Together these findings support the hypothesis that environmental influences on imprinted genes such as *IGF2* could result in abnormal fetal growth and neurodevelopment, potentially leading to increased risk of neurodevelopmental disorders and schizophrenia.[24]

IGF2 is the most abundantly expressed IGF in the adult rat central nervous system (CNS),[25] and directly controls brain growth and development. For example, IGF2 in cerebrospinal fluid (CSF) has an age-dependent effect on the proliferation of neural stem cells and adult brain cells.[26] Studies in mouse show that during the early postnatal period *Igf2* is monoallelically expressed in the cerebellum,[27] helping to regulate the proliferation of granule cell precursors which ultimately determine its final size and shape.[28] In addition to its role in neurodevelopment, IGF2 has been implicated in a number of important neurocognitive functions in the brain including memory consolidation and enhancement in rats.[9] Previously, we reported preliminary evidence of an association between DNA methylation at *IGF2 DMR2* and brain weight in adult males[29] and hypothesized that this could explain the epidemiological findings of increased schizophrenia and brain abnormalities in famine-exposed individuals.

In this study we comprehensively assessed DNA methylation, genetic variation and gene expression across the extended 11p15.5 *IGF2/H19* region using tissue from a unique collection of well-characterized and neuropathologically-dissected post-mortem human cerebellum and frontal cortex brain samples. Our data provide strong evidence that epigenetic and parent-of-origin genetic variation in this region mediate between-individual differences in brain morphology.

Results

Cross-tissue patterns of IGF2/H19 DNA methylation and gene expression. We quantified DNA methylation across four amplicons spanning two known *IGF2/H19* DMRs and the *H19* promoter in reference samples obtained from six different tissues: frontal cortex [Brodmann's area 9 (BA9)], superior temporal gyrus, (BA22), visual cortex, (BA17), cerebellum, whole blood and saliva. Very similar levels of DNA methylation across all amplicons were observed in each tissue (**Table S1 and Fig. S1**), with the *H19 CTCF3* region being the most stable across tissues (mean DNA methylation = 40%, SD < 0.01).

IGF2 and *H19* expression was compared across cerebellum, frontal cortex, liver, placenta, skeletal muscle and spleen using qRT-PCR. Unlike DNA methylation, gene expression was found to be highly tissue-specific; both transcripts were detected in the cerebellum and frontal cortex, but at lower levels compared with the peripheral tissues tested (**Fig. S2**). Of note, higher levels of both *IGF2* (39X) and *H19* (12X) mRNA were observed in cerebellum compared with frontal cortex. No significant correlation between DNA methylation and steady-state mRNA was observed for either gene in either brain region indicating that *IGF2/H19* DNA methylation patterns may be disassociated from gene expression in adult brain tissue. Alternatively, other DNA modifications such as 5-hydroxymethylation, which is known to be relatively prevalent in the cerebellum,[30] may be influencing transcription at this locus.

Cerebellar DNA methylation and total brain weight. We quantified cerebellum DNA methylation across five amplicons spanning the three known *IGF2/H19* DMRs and *H19 promoter* (**Figs. 1 and S3**). Guided by our previous preliminary data using samples from the Stanley Medical Research Institute (SMRI),[29] we first examined the association between cerebellum DNA methylation at *IGF2 DMR2* and total brain weight in males in an extended combined sample set, with the addition of samples from the London Brain Bank for Neurodegenerative Diseases (LBBND). Again, DNA methylation was positively correlated

with total brain weight at multiple individual CpG sites, with increased significance compared with our previous analyses (**Fig S4**). This observation is consistent with experimental data demonstrating that a specific 54 bp methylated region in the mouse *Igf2 DMR2*, homologous to the region assessed by our human *IGF2* DMR2 assay is necessary for high-level transcription of the paternal *Igf2* allele; deletion of this methylated region results in reduced *Igf2* expression and fetal growth retardation.[12] Our analysis of cerebellum DNA methylation and total brain weight showed that two other amplicons were significantly correlated with total brain weight. *IGF2 DMR0* methylation was negatively correlated (rho = -0.22 p = 0.042) while *H19 promoter* methylation was positively correlated (rho = 0.25 p = 0.017) (**Table 1**). In both cases, however, the correlations with total brain weight did not remain significant after controlling for gender and age (**Table 1**).

Table 1. Correlations between DNA methylation and total brain weight

	Correlation with total brain weight	Partial correlation with total brain weight	
		Adjusted for age	Adjusted for gender
IGF2 DMR0	-0.22*	-0.23*	-0.18
IGF2 DMR2	0.15	NA	NA
H19 CTCF3	-0.10	NA	NA
H19 CTCF6	0.04	NA	NA
H19 promoter	0.25*	0.11	0.14

*p < 0.05; **p < 0.01; ***p < 0.001; NA, not applicable.

Cerebellar DNA methylation and cerebellum weight. Cerebellar DNA methylation across the *H19 CTCF3* amplicon was strongly and robustly correlated with cerebellum weight (rho = -0.61, p = 1.55e-05) (**Table 2 and Fig. 2**). This correlation remained highly significant after we adjusted for age, sex and total brain weight minus cerebellum weight (i.e., net brain weight) (**Table 2**), and sex-stratified analyses showed that it was present in both males and females separately (**Table S2**). To complement the correlation analysis we ran a multiple regression for cerebellum weight including *H19 CTCF3* methylation, age, gender and net brain weight as independent variables. The overall regression model was highly significant (adjusted R-squared = 0.50, F = 11.22, p = 4.62e-06), explaining approximately 50% of the variance in cerebellum weight, with *H19 CTCF3* methylation (unstandardized β = -76.38, SE = 17.1, standardized β = -0.53, p = 7.27e-05) and net brain weight (i.e., total brain weight minus cerebellum weight) (unstandardized β = 0.05, SE = 0.02, standardized β = 0.36, p = 0.0085) being predictive. To determine if *H19 CTCF3* methylation explained unique variance in cerebellum weight, we conducted a hierarchical linear regression with the significant variables from the original regression model: net brain weight on the first step and *H19 CTCF3* methylation on the second step. As expected net brain weight was significantly associated with cerebellum weight, explaining 30% of the variance (ΔR-squared = 0.30, Δf = 17.19, p = 0.0001). Strikingly, *H19 CTCF3* methylation, added on the second step, uniquely explained an additional 25% of the variance in cerebellum weight (ΔR-squared = 0.25, Δf = 21.041, p = 4.57e-05). To ensure that data from our DNA methylation assays were not mediated or confounded by single nucleotide polymorphisms (SNPs), representative samples spanning the full range of average methylation levels were sequenced across the region assessed in our methylation assay and found to be genetically identical. DNA methylation was not significantly correlated with cerebellar weight at any of the other amplicons.

Frontal cortex DNA methylation and brain/cerebellum weight. To assess the tissue-specificity of the observed association between *IGF2/H19* DNA methylation and cerebellum weight we repeated the analysis using DNA extracted from frontal cortex tissue from the LBBND cohort. We used Spearman's rank correlation tests to assess the relationship between frontal cortex DNA methylation and (1) total brain weight (2) net brain weight and (3) cerebellum weight. Although the average levels of DNA methylation was similar between the frontal cortex and cerebellum samples, between-individual variation was not significantly correlated across the tissues and we found no association between frontal cortex DNA methylation and any of the brain weight measures. These results indicate that the relationship between *H19 CTCF3* methylation and cerebellum weight is specific to DNA from the cerebellum itself.

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Cerebellar DNA methylation and genotype. In addition to quantifying DNA methylation, we genotyped 11 polymorphic SNPs (**Fig. 1**) spanning the *IGF2/H19* imprinting domain selected from previously published genetic associations with morphological and physiological phenotypes (**Fig. S5**).[15,31-33] Several SNPs proximal to the *IGF2* gene were associated with DNA methylation at *IGF2 DMR0*, both when assessed individually (**Fig. S6**) and as part of multi-marker haplotypes (**Table S3**). This is consistent with the findings of previous work in reference 15 and 34, including a recent genome-wide study by Zhang and colleagues using cerebellum samples from the SMRI,[34] indicating that DNA methylation at *IGF2* is influenced by genotype.

Genotype and brain/cerebellum weight. Standard genetic association analyses showed that none of the SNPs tested were directly associated with total brain or cerebellum weight. Imprinted regions, however, are known to harbor genetic variants that influence phenotype in a parental-origin-specific manner, with opposite effects associated with maternally- and paternally-inherited alleles.[35] Due to the lack of parental DNA samples, we were unable to directly ascertain the parental-origin of alleles for the SNPs genotyped in this study. Using an allele-specific bisulfite PCR assay however, we could accurately infer the parental-origin of rs2107425, located within the *H19 CTCF6* amplicon, by discriminating between methylated (paternal) and unmethylated (maternal) alleles. rs2107425 is located in a block of strong linkage disequilibrium upstream of the *H19* region (**Fig. S5**), and is in complete phase with several other SNPs, including rs2071094 and rs10732516 genotyped in this study, for which we were thus also able to infer parental origin. Taking inferred parent-of-origin of these SNPs into account, samples were split into three groups: wild-type homozygotes at rs2107425 (CC) (n = 41), and heterozygotes (CT) with a paternally inherited (n = 33) or maternally

Table 2. Correlations between DNA methylation and cerebellum weight

	Correlation with cerebellum weight	Partial correlation with cerebellum weight		
		Adjusted for age	Adjusted for gender	Adjusted for net brain weight
IGF2 DMR0	-0.27	NA	NA	NA
IGF2 DMR2	-0.15	NA	NA	NA
H19 CTCF3	-0.61***	-0.65***	-0.63***	-0.59***
H19 CTCF6	-0.15	NA	NA	NA
H19 promoter	0.07	NA	NA	NA

*p < 0.05; **p < 0.01; ***p < 0.001; NA, not applicable.

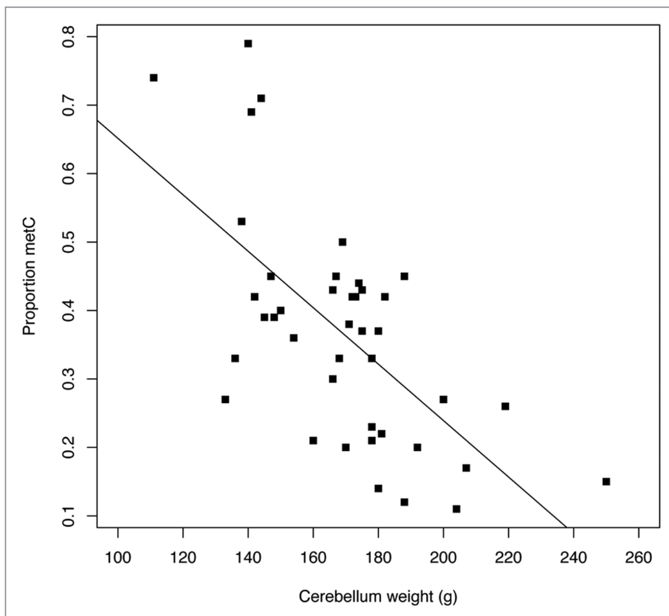

Figure 2. Epigenetic variation at the *IGF2/H19* ICR is associated with cerebellum weight in post-mortem brain samples. DNA methylation (metC) at *H19 CTCF3* is significantly associated with cerebellum weight (r = -0.61, p = 1.55e-05).

inherited (n = 23) T allele. Although there was no significant difference in total brain weight between the genotype groups (F = 1.89, p = 0.16, n = 96), a highly significant difference was found with cerebellum weight in the LBBND sample. A post-hoc Tukey test showed that the two heterozygous groups were driving this difference (p = 0.01); paternally-inherited T alleles were associated with a significantly lower cerebellum weight (157.8 g ± 4.1 g) than a maternally-inherited T allele (187.3 g ± 10.1 g) (t = -3.11, p = 0.005) (**Fig. 3**). Further analysis of the LBBND cohort revealed that a paternally-inherited T allele was associated with a significantly higher total brain weight (1317.6 g ± 117 g) than a maternally-inherited T allele (1503.4 g ± 195 g) (t = -2.99, p = 0.007), but no significant difference was observed in relation to net brain weight, indicating that the effect was specifically driven by the cerebellum. The allele-specific genotype of each sample was subsequently verified by repeating our allele-specific bisulfite-PCR assay on frontal cortex DNA from the same group of individual donors with the same result.

Discussion

Our data support a role for epigenetic and parental-origin-specific genetic variation at the *IGF2/H19* ICR in mediating growth and development of the cerebellum. The current study represents the first detailed investigation of *IGF2/H19* DNA methylation and expression in human brain tissue. Our data indicate that both *IGF2* and *H19* are expressed at detectable levels in the brain, albeit at much lower levels compared with peripheral tissues such as liver and placenta. We show that DNA methylation in cerebellar DNA, in the vicinity of a CTCF-binding site (CTCF3) upstream of *H19*, is strongly correlated with cerebellum weight. Hierarchical regression analysis showed that ~25% of the variance in cerebellum weight is uniquely explained by DNA methylation at *H19 CTCF3*. This effect appears to be tissue-specific, with no significant correlation between DNA methylation and cerebellum weight observed using frontal cortex DNA from the same samples. We also report that genetic variation at this ICR is strongly associated with cerebellum weight in a parental-origin specific manner, with maternally-inherited alleles associated with a 16% increase in cerebellum weight compared with paternally-inherited alleles.

Previous studies have shown that imprinted regions can harbor genetic variants that influence phenotype in a parental-origin-specific manner, with opposite effects associated with maternally- and paternally-inherited alleles.[35] Of note, polymorphisms at human 11p15.5 have been shown to have a parent-of-origin specific association with placental and fetal growth[36,37] and type-2 diabetes.[38] The parent-of-origin specific effect on cerebellum weight observed in our analyses resembles the "bipolar dominance" pattern of imprinting previously reported for postnatal growth at imprinted regions in mice[39] and with birth weight in humans,[36] whereby heterozygous individuals stratified by paternally- and maternally-inherited alleles have opposite phenotypes, with homozygous individuals showing intermediate levels.

Current evidence suggests that alterations in DNA methylation at the *IGF2/H19* ICR affects the binding of CTCF, regulating the developmental expression of *IGF2* and *H19* by mediating promoter interactions with enhancer elements located downstream of *H19*.[11] The large between-tissue differences in gene expression we observe contrast with the cross-tissue stability of *IGF2/H19* DNA methylation levels, an observation recently reported for multiple imprinting DMRs by Woodfine and colleagues.[40] The observed somatic methylation patterns potentially reflect

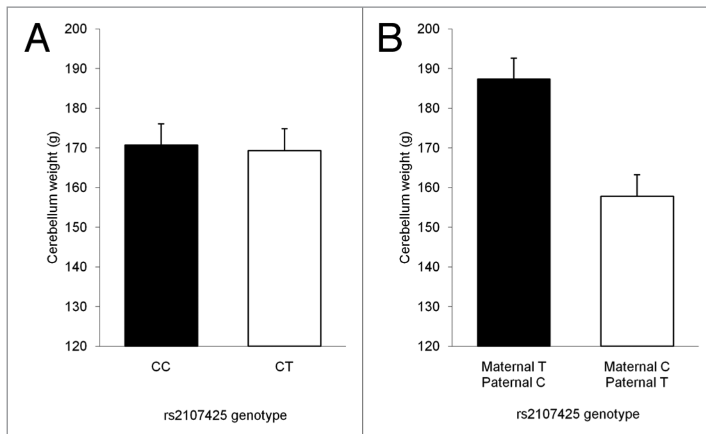

Figure 3. Alleles of rs2107425 at *H19 CTCF6* show a parental-origin-specific association with cerebellum weight. (A) No difference in cerebellum weight is observed between CC homozygotes and CT heterozygotes not stratified by parental origin ($t = 0.184$, $p = 0.855$). (B) CT heterozygotes stratified by the parental-origin of alleles are significantly different ($t = -3.11$, $p = 0.005$), with a paternally-inherited T allele being associated with a 16% lower cerebellum weight than a maternally inherited T allele. Bars represent mean ± SEM.

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epigenetic changes occurring at an early developmental stage, and it is possible that other functional mechanisms influence absolute levels of transcription at the *IGF2/H19* locus in adult tissue. Indeed, it has been shown that although CTCF consistently binds to unmethylated ICR elements, this is not always associated with predictable gene expression in the brain.[41] In addition, emerging evidence suggests that CTCF binding at these same sites may mediate long-range inter-chromosomal interactions in mice.[42,43] We therefore hypothesize that DNA methylation and genetic variation at the *IGF2/H19* ICR may regulate growth and development of the brain via more complex mechanisms of epigenetic regulation, which include both local and long-range chromosomal interactions. Given the evidence that the epigenetic regulation of *IGF2/H19* transcription is tissue-specific during early human development[44] and a recent study in mouse reporting heterogeneity in *Igf2* allelic expression across brain regions,[5] it is not surprising that our analysis of cerebellar DNA methylation revealed a strong correlation specifically with cerebellum weight and not with net brain weight. Our subsequent analysis of DNA methylation in the frontal cortex provides additional evidence that the association between *H19 CTCF3* methylation and cerebellum weight is cerebellum-specific.

Data from a recent analysis of developmental gene expression shows that *IGF2* expression in the human cerebellum increases between ~150–500 d post-conception (**Fig. S7**).[45] The timing of this upregulation of IGF2 coincides a period during which the cerebellum is observed to undergo a 4-fold increase (384%) in volume in a study of normative fetal brain growth.[46] The human cerebellum has a protracted postnatal developmental period,[47,48] which could leave it particularly vulnerable to environmentally-induced epigenetic changes during pre- and peri-natal development, such as to famine which has been previously shown to alter imprinting patterns at this genomic region as discussed in the introduction.[17] Indeed, studies in mice have already shown that early undernutrition is associated with both decreased cerebellum weight and alterations to the IGF system within the cerebellum.[49] Research on the human cerebellum has focused mainly on its role in motor control and sensory-motor integration. However, considerable recent evidence indicates that it also plays a critical role in cognition and emotion.[50] Paleoneurological work shows that the cerebellum evolved reciprocally with the cerebral hemispheres to coincide with emerging cognitive behaviors.[51,52] Its role in higher cognitive function is supported by in vivo neuroimaging studies which show that the cerebellum is activated in a wide-range of cognitive tasks (reviewed in ref. 53). Given that cerebellar abnormalities are among the most consistently reported structural findings in autism and attention deficit hyperactivity disorder,[47] and progressive loss of cerebellar volume has been reported in childhood-onset schizophrenia,[54] our data have important implications for the etiological study of neuropsychiatric disorders. These findings also suggest a possible mechanism linking prenatal exposure to famine and an increased risk of disorders such as schizophrenia.[55]

The biological effects of IGF2 are mediated by several IGF receptors and binding proteins. Work in rats shows that the IGF type 1 receptor is particularly highly expressed in the developing cerebellum, midbrain and olfactory bulbs.[25] The IGF type 2 receptor (IGF2R) has an almost exclusive affinity for IGF2 and is also expressed in many brain regions including the lower brainstem in the rat.[56] In biological fluids IGFs bind to six distinct binding proteins (IGFBP1–6). In the cerebrospinal fluid IGFBP6 has the highest affinity for IGF2 (10–100 times higher than any other IGFBPs).[57] Interestingly, transgenic mice overexpressing IGFBP6 in the CNS show a 25% to 35% reduction in cerebellar size and weight specifically.[58]

This study has a number of limitations. Although we were careful to control for variables such as age, sex and postmortem delay, it is plausible that our findings are biased by confounding or unmeasured variables. Our sample includes brains from two large independent brain-banks, but replication in additional samples would be optimal to further confirm these data. Furthermore, the bisulfite modification method used in this study does not discriminate between methylated cytosines and hydroxymethylated cytosines,[59] so any future studies of *IGF2/H19* methylation should utilize the newly developed techniques that allow detection of hydroxymethylation. This may be particularly relevant because hydroxymethylation appears to play a complex role in both the activation and repression of gene expression[60] and is relatively enriched in the Purkinje cells of the cerebellum.[30] Finally, we were unable to directly examine allele-specific patterns of DNA methylation or gene expression, and although our allele-specific bisulfite-PCR assay highlights a strong parental-origin effect for genotypic variation, we could not obtain parental DNA samples from our post-mortem brain samples. Future work will expand these analyses to other brain regions and explore other imprinted genes that are known to be functionally important in the brain.

To conclude, we report that epigenetic and genetic variation in the *IGF2/H19* region is associated with cerebellum weight. Given the link between structural brain abnormalities and neuropsychiatric disease, an understanding of the factors influencing brain morphology provides important clues about the etiology of disorders such as schizophrenia and autism.

Materials and Methods

Samples. Cerebellum samples (n = 60) were obtained from the SMRI, and cerebellum (n = 46) and frontal cortex (n = 51) samples from the LBBND. Subjects were approached in life for written consent for brain banking, and all tissue donations are consented, collected and stored following legal and ethical guidelines. All samples were dissected by a trained neuropathologist, snap-frozen and stored at -80°C. Accurate measures of total brain weight were obtained for both sample sets at autopsy, with specific cerebellum weight data also obtained for samples from the LBBND (**Tables S4 and S5**). DNA was isolated from each sample using a standard phenol-chloroform extraction method, and tested for degradation and purity prior to analysis.

DNA methylation analysis across IGF2/H19 DMRs. Genomic DNA (0.5 μg), extracted from neuropathologically-dissected brain samples, was treated with sodium bisulfite using the EZ 96-DNA methylation kit (Zymo Research, CA) following the manufacturer's standard protocol. Fully methylated and unmethylated samples were included throughout the experimental procedure as assay controls.

IGF2 DMR2 primers were obtained from the Sequenom online Standard EpiPanel (www.epidesigner.com). Primers for all other DMRs were designed using Sequenom's EpiDesigner software (www.epidesigner.com) and tested for specificity and sensitivity. Bisulfite-PCR amplification was performed in duplicate using Hot Star *Taq* DNA polymerase (Qiagen, UK) and optimized cycling conditions. **Table S6** summarizes the experimental conditions used for each bisulfite-PCR assay. Following bisulfite-PCR amplification, DNA methylation was quantitatively assessed at individual CpG sites using the Sequenom EpiTYPER system (Sequenom Inc., CA) as described previously in reference 61. This technique employs base specific cleavage followed by MALDI-TOF mass spectrometry (MS) in which the mass ratio of the cleaved products provides highly quantitative methylation estimates for CpG sites within a target region.

Prior to analysis, stringent data quality control and filtering was performed to remove potentially unreliable measurements. All data from CpG fragments identified by EpiTYPER as having low mass or high mass (outside the MS analytical window), silent peak overlap (two overlapping peaks, one with no CpGs) or overlap (two overlapping peaks, both with CpGs) were discarded. Next, CpG fragments potentially confounded by a SNP or CpG fragments with a measurement success rate below 80% were discarded. Finally, DNA samples with less than 60% complete measurement success rate per amplicon were discarded. CpG sites included in the final analyses are shown in **Table S7**.

All statistical analyses were conducted within the R statistical environment (www.r-project.org). Correlation analysis was used to assess the relationship between cerebellar DNA methylation and (a) total brain weight and (b) cerebellum weight. First, average DNA methylation at each of the five amplicons was screened to identify those amplicons that showed a significant correlation with total brain or cerebellum weight (Spearman's rank correlation, $\alpha = 0.05$). For those amplicons that were significantly correlated, we assessed the robustness of the correlation using partial correlations to control for age and gender, as adult brain weight is known to decrease with age and differs between the sexes.[62] To identify any interactions with gender correlation analyses were repeated stratified by sex.

We also performed bivariate correlations to assess whether additional variables (autopsy time, sample set and net brain weight) were correlated with cerebellum weight. We adjusted for any significant variables in partial correlations between cerebellar DNA methylation and cerebellum weight. In addition we ran a multiple regression for cerebellum weight with cerebellar DNA methylation, age, sex, together with any other factor significantly correlated with cerebellum weight, as independent variables. Finally we employed a hierarchical linear regression to determine the unique variance in cerebellum weight contributed by DNA methylation.

Based on the results of the initial IGF2/H19 methylation analysis we repeated the above analyses using frontal cortex tissue from an overlapping set of LBBND donors to assess the tissue-specificity of any significant differences.

Genotype analysis. Eleven SNPs spanning the *IGF2/H19* region were selected for genotype analysis based on previously published associations with physiological- and growth-related phenotypes.[15,31,63,64] The location of these SNPs is shown in **Figure 1**. Eight of the selected SNPs (rs3842773, rs3741211, rs3213221, rs3213223, rs680, rs3168310, rs2839701, rs217727) were genotyped using a multiplex SNaPshot assay (Applied Biosystems, CA) following the manufacturer's standard protocol. For details of the genotyping assays and PCR conditions see **Table S8**. SNaPshot products were analyzed on the 3130 x l DNA Analyzer (Applied Biosystems, CA) and automated genotype analysis of the data was performed with GeneMapper 4.0 (Applied Biosystems, CA).

The remaining 3 SNPs (rs10732516, rs2071094, rs2107425) were genotyped by direct genomic DNA sequencing using BigDye Terminator chemistry (Applied Biosystems, CA). Primers were designed to cover the region assessed in the *H19 CTCF6* DNA methylation assay. For details of the genotyping assay and PCR conditions see **Table S8**. Products were analyzed on the 3,130 x l DNA Analyzer (Applied Biosystems, CA) and alleles called using Sequencher (GeneCodes, MI) software.

Haploview 4.2 (www.broadinstitute.org/haploview) was used to test whether the genotyped SNPs were in Hardy-Weinberg equilibrium (HWE): all 11 SNPs were found to be in HWE. Lewontin's D' and the linkage disequilibrium (LD) coefficient r^2 were all calculated using Haploview to measure the LD between all pairs of biallelic loci. HWE p values, minor allele frequencies and LD between the SNPs are given in **Table S9**.

ANOVAs were performed to individually test each SNP for an association with total brain weight, cerebellum weight and mean

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percentage cerebellar DNA methylation at each of the DMRs. To further interrogate the association between genotype and methylation we used UNPHASED 3.1.4 (www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased) to test haplotypes in the *IGF2* and *H19* genes for association with mean cerebellum DNA methylation values (**Table S3**).

Parent-of-origin genotype analysis. We used a second, allele-specific, *H19 CTCF6* methylation assay to infer the parent-of-origin of 3 SNPs: rs2107425, rs2071094 and rs10732516. Experimental procedures were performed as detailed above. The presence of a SNP (rs2107425) at position 22 of the reverse primer (calculated from the 3' terminus) results in complete biased amplification of the bisulfite-treated DNA with samples classified as fully methylated (SNP allele on unmethylated maternal allele), ~50% methylated (no SNP alleles), or unmethylated (SNP on methylated paternal allele) as reported by Tost and colleagues (**Fig. S8**).[65] The genotype of rs2107425 was confirmed via direct genomic sequencing across the *H19 CTCF6* region (as described above). These analyses revealed that rs2071094 and rs10732516 are in complete phase with rs2107425, thus sharing inferred parent-of-origin. ANOVAs were used to test for a significant difference between each of the genotype groups (split by parental-origin) for (1) total brain weight and (2) cerebellum weight.

Gene expression analysis. RNA was isolated from the LBBND brain samples using the Trizol extraction method and purified using an RNeasy Mini Kit with DNase I digestion (Qiagen, UK), according to manufacturer's instructions. RNA was tested for degradation and purity using an Agilent 2100 Bioanalyzer and RNA 6000 Nano kit (Agilent Technologies, UK). Using the Precision Nanoscript Reverse Transcription Kit (Primer Design Ltd., UK), according to manufacturer's instructions, 1 μg RNA from each sample was reverse transcribed to cDNA. The geNORM housekeeping selection kit (Primer Design Ltd., UK) was used to assay 12 housekeeping genes in a representative subset of the samples (n = 12). Using NormFinder software, the two most stable genes for normalization were found to be splicing factor 3a, subunit1 (*SF3A1*) and ubiquitin-C (*UBC*) for cerebellum RNA, and β-actin (*ACTB*) and *UBC* for the frontal cortex RNA. Interestingly, β2-microglobulin (β*2M*) was identified as the least stable reference gene in the cerebellum samples from individuals with high and low brain weight, which concurs with recent work reporting a correlation between β2M expression and brain weight in a set of cerebellum samples from the SMRI.[66] Optimized assays for *SF3A1*, *UBC*, *ACTB*, *IGF2* and *H19* were obtained from Primer Design (Southampton, UK).

qPCR was performed using the ABI PRISM 7900HT (Applied Biosystems, UK). Human biobank cDNA samples (liver, placenta, skeletal muscle, spleen and testes) were included in all experimental procedures to allow between-tissue comparisons of gene expression. Mean duplicate Ct values were calculated for each sample and then used to calculate relative expression using the ΔCt method.[67] In brief, the normalization index was calculated as the mean of the two housekeeping genes. ΔCt was then calculated by subtracting the normalization index from the mean target Ct, and then the relative expression value was calculated using the equation $2^{-\Delta Ct}$.

Spearman's rank correlation tests ($\alpha = 0.05$) were used to assess the relationship between relative expression values and DNA methylation within the same tissue. For the three SNPs for which we had inferred parental origin (rs2107425, rs2071094 and rs10732516), ANOVAs were used to test for a significant difference between genotype group (split by parental-origin) and relative expression.

We used the ΔΔCt method to assess gene expression across the Biobank cDNA samples from different tissues and brain tissues.[67] The expression value for each tissue (relative to frontal cortex) was calculated using the equation $2^{-\Delta\Delta Ct}$.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Ethical Statement

The HTA license number for the LBBND is 12,293. This work is covered by ethical approval from the local NHS ethical review board (REC reference 10/H0808/114).

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Note

Supplemental material can be found at:
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