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Haploinsufficiency of Interferon Regulatory Factor 6 Alters Brain Morphology in the Mouse

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Abstract

Orofacial clefts are among the commonest birth defects. Among many genetic contributors to orofacial clefting, Interferon Regulatory Factor 6 (*IRF6*) is unique since mutations in this gene cause Van der Woude (VWS), the most common clefting syndrome. Furthermore, variants in *IRF6* contribute to increased risk for non-syndromic cleft lip and/or palate (NSCL/P). Our previous work shows that individuals with either VWS or NSCL/P may have cerebral anomalies (larger anterior, smaller posterior regions), and a smaller cerebellum. The objective of this study was to test the hypothesis that disrupting *Irf6* in the mouse will result in quantitative brain changes similar to those reported for humans with VWS and NSCL/P. Male mice heterozygous for Irf6 ($Irf6^{gt1/+}$; n = 9) and wild type ($Irf6^{+/+}$; n = 6) mice at comparable age underwent a 4.7T MRI scan to obtain quantitative measures of cortical and subcortical brain structures. There was no difference in total brain volume between groups. However, the frontal cortex was enlarged in the $Irf6^{gt1/+}$ mice compared to that of wild types (p = 0.028) while the posterior cortex did not differ. In addition, the volume of the cerebellum of $Irf6^{gt1/+}$ mice was decreased (p = 0.004). Mice that were heterozygous for *Irf6* showed a similar pattern of brain anomalies previously reported in humans with VWS and NSCL/P. These structural differences were present in the absence of overt oral clefts. These results support a role for *IRF6* in brain morphometry and provide evidence for a potential genetic link to abnormal brain development in orofacial clefting.

Keywords

IRF6; oral clefts; brain development; magnetic resonance imaging; mouse model

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CONFLICT OF INTEREST STATEMENT
The authors have no conflicts of interest to declare.

INTRODUCTION

Orofacial clefts are a disturbance of craniofacial development. This malformation is thought to result from a combination of genetic and environmental factors affecting cell migration, proliferation, adhesion, differentiation, and/or apoptosis [Burdi, 2006; Knight et al., 2006]. Collectively, orofacial clefting affects 1 in 700 live births making it one of the most common birth defects worldwide [Canfield et al., 2006]. Clefts can occur as part of a defined syndrome or in an isolated manner. Isolated clefts are commonly termed non-syndromic clefts of the lip and/or palate (NSCL/P) and comprise roughly 70% of all oral clefts [Jones, 1988].

In addition to the facial cleft, many individuals affected with NSCL/P manifest functional problems, including speech abnormalities [Timmons et al., 2001], behavioral abnormalities [Hunt et al., 2005], and cognitive deficits [Jocelyn et al., 1996]. The cognitive deficits are specifically language-based and often manifest in language-based learning difficulties. In fact, the rate of learning disabilities in this population is nearly ten times greater than the general population [Richman et al., 1988].

The cause of speech, behavioral, and cognitive deficits in NSCL/P has been debated. The traditional view is that these deficits are secondary issues, due to other factors related to the cleft such as chronic otitis media, exposure to anesthesia during operations, or even the psychological stress of living with a facial cleft [Jocelyn et al., 1996; Millard and Richman, 2001; Riski, 2006]. But more recently, it has been suggested that deficits in speech, cognition, and behavior are a direct result of abnormal brain development and are therefore primary neurodevelopmental issues that present in parallel with the cleft. Initial support for this theory was provided by the observation that the development of the face and the brain are intimately linked [Kjaer, 1995]. Our laboratory has focused on brain structure and function in children and adults with NSCL/P hypothesizing that the factors responsible for disruption in the development of craniofacial structures may also adversely affect the development of the brain [Rutter et al., 2004]. In a study evaluating brain structure using Magnetic Resonance Imaging (MRI), men with NSCL/P were compared to matched controls, and a unique pattern of brain morphology was found. The two groups did not differ in overall brain size, however there was a shift in tissue distribution in which the anterior cerebrum (in particular, the gray matter) was enlarged while the posterior cerebrum was abnormally small (in particular, the white matter). In addition, there was a significant deficit in cerebellar volume [Nopoulos et al., 2000; 2002].

Of the many known genetic factors involved in cleft lip and palate (CLP), Interferon Regulatory Factor 6 (*IRF6*) is unique because DNA variants in this gene contribute to syndromic and non-syndromic forms of orofacial clefting [Kondo et al., 2002; Zucchero et al., 2004]. Loss-of-function mutations in *IRF6* cause Van der Woude syndrome, the most common syndromic form of CLP, comprising lower lip fistulas in addition to the cleft [Kondo et al., 2002]. Furthermore, a common single nucleotide polymorphism in *MCS9.7*, an enhancer element near *IRF6*, contributes to the risk of NSCL/P [Rahimov et al., 2008]. The pattern of brain structure changes in men with VWS was very similar to the pattern seen in the men with NSCL/P; compared to matched controls, the VWS subjects had significant

enlargement of the anterior regions of the cerebrum (in particular gray matter), and a volumetric reduction of the cerebellum [Nopoulos et al., 2007]. This pattern of structural brain changes was observed in a subset of patients with VWS who had no cleft, but lip pits only. This suggests that the abnormal brain morphology seen in VWS is a pleiotropic manifestation and maybe present even in the absence of a cleft.

In an effort to better understand the relationship between *Irf6* and the brain changes associated with orofacial clefting, we assessed quantitatively the brain phenotype in *Irf6* mutant mice. Mice homozygous for the null allele die perinatally with limb, craniofacial and epidermal anomalies [Ingraham et al., 2006; Richardson et al., 2006]. However, mice heterozygous for *Irf6* survive to adulthood, superficially appear normal, and lack an orofacial cleft and lip pits [Ingraham et al., 2006]. Therefore, the heterozygous mouse constitutes a unique approach to assess potential alteration in brain structure in the context of genetic alteration in Irf6 *without* a clefting phenotype. Furthermore, the mice present with the advantage of being free of the environmental influences (chronic otitis media, exposure to anesthesia, or psychological stress) encountered in humans with clefts.

We evaluated total brain and regional brain structures of the *Irf6* heterozygous mouse compared to wild type controls using volumetric MRI in order to test the hypothesis that disrupting *Irf6* in the mouse will result in quantitative brain changes similar to those reported for humans with VWS and NSCL/P.

MATERIALS AND METHODS

A total of nine male mice heterozygous for the Irf6^{gt1} allele were compared to six wild type mice (*Irf6*$^{+/+}$). All mice were cared for according to the Animal Care and Use Review Form (ACURF) at the University of Iowa, Iowa City, IA. Mice were housed in an environment under light-, temperature-, and humidity-controlled conditions. Food and water were available *ad libidum*. In order to avoid variability in brain structure due to age or sex, a sample of age-matched male mice was used. All mice were 25 weeks old, which corresponds to fully mature animals. The *Irf6*^{gt1} allele was previously described and is maintained in a C57BL/6J background [Ingraham et al., 2006].

Magnetic resonance imaging (MRI) was performed on a 4.7 Tesla Varian small-bore scanner. All acquisitions utilized a 25 mm diameter transmit/receive coil for high-resolution imaging. Mice were anesthetized with isoflurane (3% induction, 1.5% maintenance) and transferred to the scanner for imaging. After a series of three localizer scans (each about five seconds long), a set of T2-weighted fast spin-echo images was acquired in the axial plane. The protocol parameters were TR/TE = 2100/60 ms, echo train length of eight, 0.5 mm thick contiguous slices with in-plane resolution of 0.16 mm × 0.16 mm over a 256 × 256 matrix using 12 signal averages. The total time for the entire protocol was about 40 minutes.

All MRI data were processed using BRAINS software developed locally at the University of Iowa [Magnotta et al., 2002]. The mouse brain atlas used for segmentation purposes was the mouse Biomedical Informatics Research Network (mBIRN) atlas, which was constructed

using T2-weighted magnetic resonance microscopy (MRM) from 11 WT C57BL/6J mice at the University of California, Los Angeles [MacKenzie-Graham et al., 2006].

For our pipeline process we employed a directed acyclic pipeline architecture using Nipype [Gorgolewski et al., 2011], a Python-based wrapping library for neuroimaging applications. The mBRIN atlas was registered to the input T1 file using b-spline warping within BRAINSFit [Johnson et al., 2007], a mutual information driven application developed under the ITK framework. The atlas was then resampled using BRAINSResample to match the voxel lattice of the T1 image, thereby allowing one-to-one correspondence between atlas and image. Finally, we computed the volume measurements for each desired region of our atlas as the sum of voxels within a given label times the volume of a voxel (Fig. 1).

The atlas defined 43 regions of interest. The regions were then grouped into the following areas: amygdala, hypothalamus, pituitary, thalamus, total brain volume, basal ganglia, brainstem, cerebrospinal fluid (CSF), cerebellum, hippocampus, white matter tracts, anterior cortex, and posterior cortex. The anterior cortex was further subdivided into the Olfactory and Frontal Cortices. The posterior cortex was similarly subdivided into the posterior, entorhinal, and perirhinal cortices. Volumes were reported in mm^3.

All analyses were performed using Statistical Package for Social Science (SPSS), version 19.0 for Windows (SPSS Inc., Chicago Ill). Due to the small sample size, non-parametric analysis using the rank of all measures was utilized in order to minimize any effects of outliers. Analysis of variance (ANOVA) was used to evaluate total brain volume across groups. The remainder of the brain regions was compared across the two groups using Analysis of Covariance (ANCOVA), controlling for total brain volume.

RESULTS

Table I shows the results of the analysis comparing *Irf6*$^{gt1/+}$ and wild type mice on general brain measures. Both groups had a similar total brain and white matter volume. However, the *Irf6*$^{gt1/+}$ mice showed a statistically significant increase in cortical volume, ($F = 9.80$, $p = 0.008$). In contrast to the cortical enlargement, the cerebellum in the *Irf6*$^{gt1/+}$ mice displayed markedly smaller volumes compared to wild type mice, ($F = 12.43$, $p = 0.004$). Significant differences were also found in the amygdala indicating it was enlarged in the *Irf6*$^{gt1/+}$ mice ($F = 6.04$, $p = 0.030$).

Regional analysis of the cortex in the *Irf6*$^{gt1/+}$ is also found in Table I. When the cortex was further divided into an anterior and posterior portion, the overall cortical enlargement was accounted for by greater volumes of the anterior cortex ($F = 6.21$, $p = 0.028$). Posterior cortex volumes were not significantly different from those of wild type mice. When the anterior cortex was further subdivided, the frontal region ($F = 9.13$, $p = 0.010$) was significantly enlarged. The olfactory cortex was also enlarged, but this finding was not statistically significant.

DISCUSSION

These findings largely mirror those from human studies on the brains of adult males with NSCL/P and VWS. Although the *Irf6*$^{gt1/+}$ mice are without orofacial clefts or lip pits (the clinical hallmarks of VWS), both the mice and humans with NSCL/P and VWS showed an almost identical pattern of brain structure abnormalities, including a subset of adult patients with VWS and lip pits only (no cleft). Specifically, an increase in volume in the anterior portions of the cerebrum (cortical gray matter in particular) and a reduction in overall cerebellum volume were common to humans and mice (amygdala volume was not measured in the adult humans). These results support a potential link between *Irf6* and the abnormal brain phenotype observed in human orofacial clefting.

Although preliminary, these results lend support to our working hypothesis that the structural brain changes observed in NSCL/P are not simply the secondary consequences of hearing loss, psychosocial factors, or anesthesia. Rather, they suggest that at least some of the genetic factors that underlie NSCL/P risk may also result in the characteristic pattern of brain morphology that is typically observed in this population. Structural factors during early embryogenesis may link this observed pattern of brain dysmorphology, characterized by disproportionate increases in forebrain regions, to the failure of fusion between adjacent facial prominences. Several studies have suggested that the size and position of the developing frontonasal mass exerts an influence on the position and orientation of immediately adjacent tissue components of the nascent face responsible for forming the upper lip and palate, components required to approximate and contact one another in a precise spatiotemporal sequence to ensure the structural integrity of the midface [Diewert et al., 1993; Jiang et al., 2006 Weinberg et al., 2009; Parsons et al., 2011]. An improved characterization of the complex network of molecular signals coordinating the forebrain and face during development will be essential to understanding how such structural integration is achieved [Creuzet et al., 2004; Marcucio et al., 2005].

Based on our prior work, the pattern of brain abnormalities is not identical across all cleft types [van der Plaas et al., 2010; DeVolder et al., 2013]. In particular, individuals with cleft palate only show a different pattern in which the cerebellum is not reduced in volume but instead shows a different distribution of cerebellar tissue [DeVolder et al., 2013]. This is especially interesting when considering that variation in *IRF6* is associated with an increased risk for cleft lip only or combined cleft lip and palate and not with an increased risk of cleft palate only [Rahimov et al., 2008]. Thus our finding of reduced cerebellar volume in the heterozygous mouse more closely resembles the pattern found in the cleft types most strongly associated with alterations in *IRF6* (cleft lip only and combined cleft lip/palate).

We chose to work on the *Irf6*$^{gt1/+}$ mouse because it is the best model for the evaluation of the role of *IRF6* in human disease. We recognize that this mutant murine strain does not have a cleft phenotype, but a number of practical limitations required us to work with this model. As mentioned earlier, although mice homozygous for the null allele have clefts, they die perinatally [Ingraham et al., 2006; Richardson et al., 2006]. Embryological studies of these mice are not possible due to technical limitations. In addition, many mice that are

heterozygous for mutations in other cleft-related genes (*Tp63*, *Tfap2a*, *Tbx1*, etc.) similarly lack an overt orofacial cleft [Lindsay et al., 2001; Mills et al., 1999; Zhang et al., 1996]. Even in human studies, the subset of individuals with VWS and no cleft (only lip pits) exhibited the same abnormal brain morphology as those with NSCL/P [Nopoulos et al., 2007]. The fact that our mouse model (which lacked a clefting phenotype) showed a similar pattern of brain structure abnormalities provides even further evidence that mutations in *IRF6* alone (even in the absence of an orofacial cleft) are sufficient to cause aberrant neurodevelopment.

The biological mechanism by which disruptions in *Irf6* translate into altered brain phenotypes is unknown. However, in a recent study, a mouse *Irf6* enhancer (MCS9.7) was fused to the *B-galactosidase* gene and showed strong expression in the hindbrain and some expression in the anterior pole of the forebrain early in development [Fakhouri et al., 2012]. Additional work is needed to connect the observed phenotypic changes to altered gene expression and to pinpoint the timing and sequencing of events leading to abnormal brain morphogenesis. A recent paper found no significant change in mandibular morphometry in Irf6 adult het mice using micro-CT [Boell et al., 2013], although mandibular hypoplasia was observed in knockout embryos [Ingraham et al., 2006]. This suggests that development of the brain is more sensitive to *Irf6* dosage than development of the mandible. Similarly, landmark-based morphometric analyses would be an excellent follow-up study and provide additional information on how the brain is developing in response to alterations in *Irf6*. However, this type of analysis would require a larger sample size than the current study. Hopefully the findings presented here can provide a basis for landmark selection in future morphometric studies in this mouse model.

The current finding of abnormal brain morphology in $Irf6^{gt1/+}$ mice parallels the abnormal brain morphology of adult human NSCL/P and VWS subjects. Taken together, our data provide strong evidence that abnormalities in *IRF6* are directly related to changes in brain structure in NSCL/P and VWS subjects. Though the underlying functional mechanism is still unclear, it appears that IRF6 may provide a genetic link between abnormal craniofacial and abnormal neurodevelopment common to both VWS and NSCL/P.

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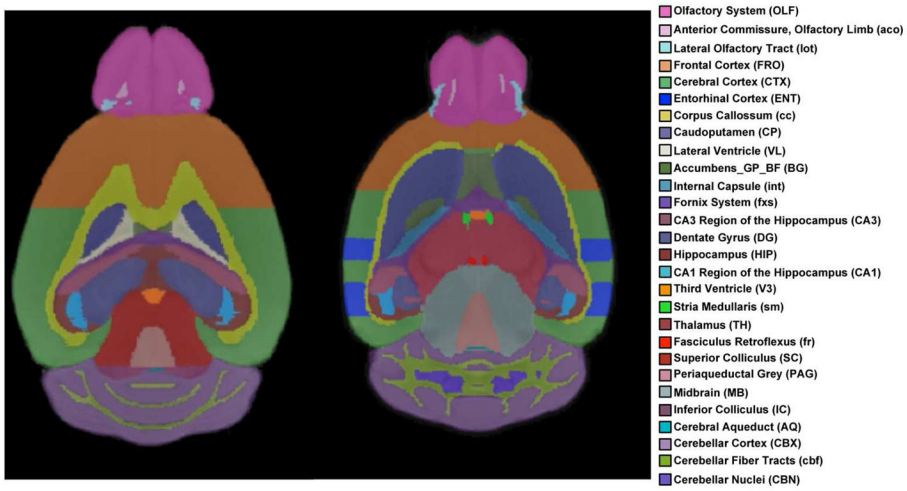

Figure 1. Volumetric Labels of mBIRN Atlas use for Segmentation of Mouse Brain

Horizontal sections of mouse brains showing the volumetric labels of the mouse Biomedical Informatics Research Network (mBIRN) Atlas. The image on the left shows a more dorsal section passing through the hippocampus; the image on the right shows a more ventral section passing through the thalamus.

Table I

Results of general brain measures and regional cortex measures, between group comparison.

	Irf6$^{+/+}$ (Wild type, n = 6)		*Irf6*$^{gt1/+}$ (n = 9)			
	Volume (mm^3)	**S.D.**	**Volume (mm^3)**	**S.D.**	**F***	**P***
Total Brain	457.89	17.18	465.47	9.81	0.12#	0.737#
Cortex	171.58	8.87	178.97	4.44	9.80	0.008
Anterior	69.22	7.33	77.23	5.65	6.21	0.028
Olfactory	27.27	4.84	32.43	3.65	4.44	0.056
Frontal	41.96	2.90	44.80	2.38	9.13	0.010
Posterior	102.35	4.00	101.74	3.23	0.31	0.585
Cortex	93.90	3.61	93.68	3.04	0.14	0.712
Entorhinal	5.05	0.60	4.83	0.37	0.25	0.626
Perirhinal	3.40	0.29	3.23	0.24	2.08	0.174
White Matter	124.59	6.98	126.63	4.15	0.02	0.888
CSF	3.07	0.35	3.08	0.31	0.12	0.731
Cerebellum	60.52	5.06	56.17	3.34	12.43	0.004
Amygdala	1.03	0.08	1.12	0.05	6.04	0.030
Basal Ganglia	35.07	1.75	35.88	1.33	1.97	0.186
Hippocampus	26.35	1.32	26.39	2.05	0.27	0.618
Hypothalamus	12.50	1.29	13.68	0.79	4.41	0.057
Pituitary	2.06	0.38	2.23	0.37	0.39	0.542
Thalamus	21.12	0.79	21.32	1.62	0.12	0.739

#ANOVA based on ranked variables

*ANCOVA based on ranked variables, controlling for Total Brain volume