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Neurochemical changes in the pericalcarine cortex in congenital blindness attributable to bilateral anophthalmia

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Coullon GS, Emir UE, Fine I, Watkins KE, Bridge H. Neurochemical changes in the pericalcarine cortex in congenital blindness attributable to bilateral anophthalmia. *J Neurophysiol* 114: 1725–1733, 2015. First published July 15, 2015; doi:10.1152/jn.00567.2015.—Congenital blindness leads to large-scale functional and structural reorganization in the occipital cortex, but relatively little is known about the neurochemical changes underlying this cross-modal plasticity. To investigate the effect of complete and early visual deafferentation on the concentration of metabolites in the pericalcarine cortex, ^{1}H magnetic resonance spectroscopy was performed in 14 sighted subjects and 5 subjects with bilateral anophthalmia, a condition in which both eyes fail to develop. In the pericalcarine cortex, where primary visual cortex is normally located, the proportion of gray matter was significantly greater, and levels of choline, glutamate, glutamine, *myo*-inositol, and total creatine were elevated in anophthalmic relative to sighted subjects. Anophthalmia had no effect on the structure or neurochemistry of a sensorimotor cortex control region. More gray matter, combined with high levels of choline and *myo*-inositol, resembles the profile of the cortex at birth and suggests that the lack of visual input from the eyes might have delayed or arrested the maturation of this cortical region. High levels of choline and glutamate/glutamine are consistent with enhanced excitatory circuits in the anophthalmic occipital cortex, which could reflect a shift toward enhanced plasticity or sensitivity that could in turn mediate or unmask cross-modal responses. Finally, it is possible that the change in function of the occipital cortex results in biochemical profiles that resemble those of auditory, language, or somatosensory cortex.

anophthalmia; blindness; choline; magnetic resonance spectroscopy; visual cortex

IT IS WELL ESTABLISHED that the loss of a sense early in life results in brain areas normally associated with that sense being recruited by the remaining modalities, termed cross-modal plasticity (Bavelier and Neville 2002; Merabet and Pascual-Leone 2010). In blind individuals, reorganization of the occipital cortex for auditory and tactile processing has been demonstrated in a diverse range of neuroimaging and behavioral studies (for a review, see Voss 2013) and is accompanied by structural (Bridge et al. 2009; Shimony et al. 2006) and metabolic (De Volder et al. 1997) changes. However, whereas structure and function have been extensively characterized, relatively little is known about neurochemical changes in the occipital cortex of blind humans.

Many animal models of congenital blindness investigating neurochemistry have used dark rearing rather than binocular enucleation. Investigations of dark rearing suggest that reorganization is driven by changes to the inhibitory and excitatory pathways that underlie the development of the occipital cortex. For example, occipital cortex excitability is thought to increase following visual deprivation (Movshon and van Sluyters 1981; although see Zheng et al. 2014). Any increase in excitability may be due to an upregulation of cholinergic pathways. Dark-reared kittens show increased choline acetyltransferase (a cholinergic marker) in primary and extrastriate visual areas (Fosse et al. 1989). There is also an attenuation of the inhibitory GABAergic circuits in dark-reared kittens (Fosse et al. 1989) and rats (Benevento et al. 1995). Thus early visual deprivation attributable to dark rearing seems to result in attenuated inhibitory circuits and strengthened cholinergic transmission; these alterations may increase cortical excitability and have been shown to extend the critical period (Lee and Nedivi 2002). The extent to which dark-reared animals demonstrate cross-modal plasticity in the occipital cortex, however, has not yet been determined, so a direct comparison is difficult to make.

In the human brain, neurochemicals can be quantified within a localized region of tissue using ^{1}H magnetic resonance spectroscopy (MRS). Application of this method has allowed investigations of neurochemical differences in the occipital lobe of people who are blind (Bernabeu et al. 2009; Boucard et al. 2007; Weaver et al. 2013). An early study by Boucard and colleagues (2007) found no difference in any measured metabolites when comparing patients with degenerative visual disorders (acquired glaucoma and age-related macular degeneration) with sighted subjects. In contrast, again investigating subjects with predominantly acquired blindness, Bernabeu et al. (2009) found an increase in concentrations of *myo*-inositol (*myo*-Ins), a marker of glial cell activity, in primary visual cortex (V1). The only study to investigate neurochemical changes attributable to early blindness in the human brain found increased levels of *myo*-Ins but also choline and creatine (Cr)/phosphocreatine (PCr) [total creatine (tCr)] and decreased levels of γ-amino-butyric acid (GABA) (although this difference was not significant following correction for multiple comparisons) (Weaver et al. 2013). No significant changes were found in glutamate (Glu) or *N*-acetylaspartate (NAA). The inconsistencies among these previous reports most likely reflect the variability in age at blindness onset and extent of vision loss.

Here, we used a combination of MRS and structural imaging to examine neurochemical effects of blindness attributable to anophthalmia. In bilateral anophthalmia, both eyes fail to develop, and thus there is no pre- or postnatal stimulation of

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the visual system. Given that anophthalmia is not associated with any other neurological impairment in these individuals, they are an ideal population to study the effects of sensory deprivation on healthy brain tissue. In this cohort, unlike previous cohorts with early blindness, neurochemical changes could be thought to reflect differences in cortical maturation attributable to the lack of stimulation along the visual pathway during prenatal development. It is not yet clear whether anophthalmia results in a unique phenomenology, or can be thought of as an extreme form of early blindness.

Using this unique population, we aimed to compare previously described structural differences (Bridge et al. 2009) with a novel investigation of neurochemical changes to further our understanding of the considerable functional changes evident in these subjects (Coullon et al. 2015; Watkins et al. 2012, 2013).

MATERIALS AND METHODS

Participants

Five subjects with bilateral anophthalmia participated in the study (mean age 31 yr, range 25–38 yr, 1 female). All had previously participated in imaging studies (Bridge et al. 2009) and are referred to as *cases 1*, *2*, *3*, *5*, and *6* to match this previous work. The data presented here were acquired 7–8 yr after the first study. *Case 4* from that study was not scanned again. *Case 1* has a mutation in the gene *OTX2*; none of the cases have any neurological history beyond the cause of their blindness (see Bridge et al. 2009 for more details). Fourteen sighted controls with normal or corrected-to-normal vision also participated (mean age 25.6 yr, range 19–30 yr, 8 females). This study was granted ethical approval by the Central University Research Ethics Committee at the University of Oxford, and all participants gave informed consent before participation.

MR Imaging Acquisition

All scans were acquired using a Siemens Trio 3-Tesla whole-body MRI scanner and a 32-channel coil at the Oxford Centre for Clinical Magnetic Resonance Research (OCMR, University of Oxford). A high-resolution T1-weighted MPRAGE image was acquired for accurate MRS voxel placement and subsequent structural analyses (repetition time = 2,040 ms, echo time = 4.7 ms, flip angle = 8°, 192 transverse slices, 1-mm isotropic voxels).

^{1}H MRS was performed using two voxels (each 2 × 2 × 2 cm). The first was placed over the occipital midline of each subject, centered on the calcarine sulcus (V1 in sighted controls). The voxel was positioned medially and was large enough to cover as much of the V1 region as possible while avoiding signal contamination from fat and lipids of the skull (Fig. 1*A*). In 2-mm Montreal Neurological Institute standard space, the mean central coordinates (and SD) across control participants were $x = 1.0$ (1.5), $y = -82.3$ (3.2), and $z = 9.7$ (4.0), and anophthalmic participants were $x = 0.7$ (1.7), $y = -79.8$ (3.7), and $z = 13.4$ (11.5). The second was a control voxel placed midline over the paracentral lobule (Fig. 1*B*, referred to here as sensorimotor cortex). The mean central coordinates (and SD) for this voxel in control subjects were $x = 0.4$ (1.0), $y = -28.7$ (5.8), and $z = 54.3$ (2.8). In anophthalmic participants, the mean central coordinates were $x = 0$ (2.0), $y = -29.6$ (2.8), and $z = 55.8$ (1.5). There were no significant differences in voxel position between the groups in any dimension or in either voxel.

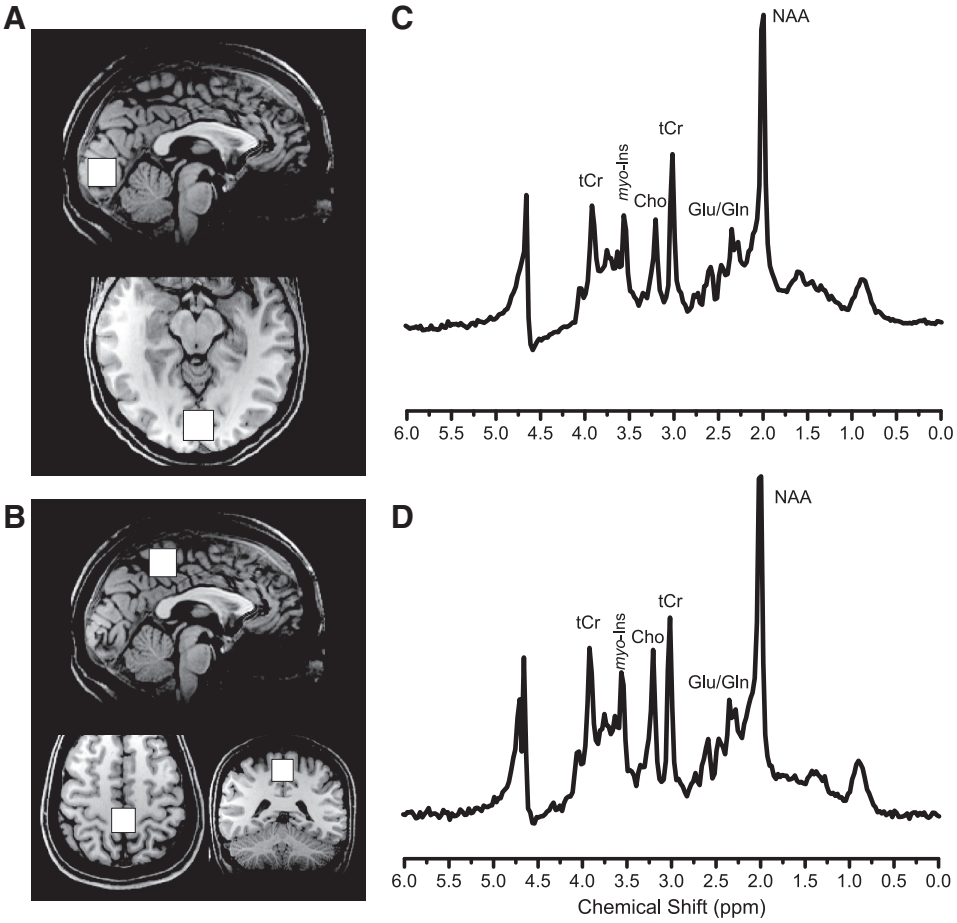

Fig. 1. Example location of a V1 voxel (*A*) and sensorimotor voxel (*B*) in a sighted subject, as well as the raw spectra acquired from these V1 (*C*) and sensorimotor (*D*) voxels. The main peaks for total creatine (tCr), *myo*-inositol (*myo*-Ins), total choline (tCho), glutamate (Glu)/glutamine (Gln), and total *N*-acetylaspartate (tNAA) are labeled.

B0 shimming was achieved using a GRESHIM. Spectra were measured with a semi-adiabatic localization by adiabatic selective refocusing (semi-LASER) sequence (echo time = 28 ms; repetition time = 4 s; 64 averages) with variable power radio frequency pulses with optimized relaxation delays (VAPOR), water suppression, and outer volume saturation (Deelchand et al. 2015; Oz and Tkac 2011). Unsuppressed water spectra acquired from the same volume of interest were used to remove residual eddy current effects and to reconstruct the phased array spectra (Natt et al. 2005). Data were acquired in single-shot mode, i.e., each single acquisition was saved separately. Single-shot spectra were frequency and phase corrected before averaging over 64 scans.

Data Analysis

MRS metabolites were quantified using LC model (Provencher 1993) (version 6.3-1B). The model spectra of alanine, aspartate, ascorbate/vitamin C, glycerophosphocholine (GPC), phosphocholine (PC), Cr, PCr, GABA, glucose, glutamine (Gln), Glu, glutathione, lactate, *myo*-Ins, NAA, *N*-acetylaspartylglutamate (NAAG), phosphoethanolamine, *scyllo*-inositol, and taurine were generated based on previously reported chemical shifts and coupling constants (Govindaraju et al. 2000) by using GAMMA/PyGAMMA simulation library of versatile simulation, pulses, and analysis (VESPA) for carrying out the density matrix formalism. Simulations were performed with the same radio frequency pulses and sequence timings as that on the 3T system in use. Eight LC model-simulated macromolecule resonances were included in the analysis at the following positions: 0.91, 1.21, 1.43, 1.67, 1.95, 2.08, 2.25, and 3.00 parts per million (ppm) (Schaller et al. 2013). Resonances were assigned according to their known ^{1}H chemical shift along the spectrum (*x*-axis, in ppm); total NAA (tNAA), Glu/Gln, tCr, total choline (tCho), and *myo*-Ins (Fig. 1, *C* and *D*). If the pairwise inverse correlation between two metabolites was consistently high (correlation coefficient < -0.3) in a given region, their sum was reported, such as NAA + NAAG (tNAA), Cr + PCr (tCr), GPC + PC (tCho), and Glu/Gln. Absolute neurochemical concentrations were extracted from an average of 64 water-suppressed and drift-corrected runs.

The T_2 relaxation of tissue water content (80 ms; Deelchand et al. 2015) was taken into account in the LC model fitting. Signal loss attributable to T_2 relaxation of metabolites was assumed to be negligible because metabolites have long T_2 relaxation times compared with our echo time of 28 ms (Deelchand et al. 2015).

Brain tissue segmentation. Structural images were brain extracted and tissue-type segmented using the FMRIB Software Library Brain Extraction Tool (Smith 2002) and FMRIB Automated Segmentation Tool (Zhang et al. 2001). The percentage of gray matter, white matter, and cerebrospinal fluid (CSF) within the MRS voxel was calculated from the resulting images and used to correct metabolite concentrations for CSF fraction (F_{CSF}) by multiplying measured metabolites by [1/(1 − CSF)]. This approach assumed no metabolites in CSF, as demonstrated with proton spectroscopy on clinical field strength scanners (Lynch et al. 1993).

Correction for water content. To determine whether the differences in metabolite concentrations were due to differences in the voxel tissue content, a second analysis calculated metabolite concentrations relative to an unsuppressed water spectrum acquired from the same voxel, assuming a water content of 72% for white matter, 82% for gray matter, and 100% for CSF (Gelman et al. 2001; Randall 1938). The water signal was then corrected individually for each participant using the following equation: water correction = $[F_{GM} \times GM_{water}$ (0.819) + $F_{WM} \times WM_{water}$ (0.72)] + $[F_{CSF} \times CSF_{water}$ (1)], where GM is gray matter and WM is white matter.

Statistical analysis. Statistical analyses were performed using SPSS software (SPSS Version 20 for Mac). First, a repeated-measures ANOVA was performed to compare neurochemical concentrations in the two voxel locations across the two groups. Homogeneity of variance was assessed using Mauchly's test of sphericity; if significant, the Greenhouse-Geisser correction and adjusted degrees of freedom were reported. Second, post hoc tests compared group neurochemical concentrations for each voxel location. Because of differences in numbers between the two groups and the small size of anophthalmia group, nonparametric Mann-Whitney *U*-tests were performed. Results were considered statistically significant if $P < 0.05$ after Bonferroni correction for the number of comparisons made. Mann-Whitney tests were also used to compare the proportion of gray and white matter in the voxel across the two groups.

The size of the sample studied is by necessity very small because congenital bilateral anophthalmia that is isolated (i.e., there are no other systemic effects) is very rare. The small sample size obviously affects the power of our study to detect true positive differences. Furthermore, the findings that we report remain significant with a false-positive rate of 5% corrected for the multiple comparisons made across the number of neurochemicals measured, and this rate would be the same if the sample were larger. We believe that greater sensitivity is achieved in this small sample because of the homogeneous cause of blindness and the complete lack of stimulation of the visual pathway by light or endogenous activity. Even so, there remains a large amount of interindividual variability that we assume reflects differences in other uncontrolled aspects of early experience (i.e., the level of environmental enrichment). There are no obvious explanations (based on our knowledge of the participants' experience) that can explain this variability; there were no consistent differences among the anophthalmia subjects across the various neurochemicals studied.

RESULTS

All sighted controls showed normal T1-weighted structural images. Consistent with a previous report (Bridge et al. 2009), the anophthalmic occipital cortex appeared structurally normal.

Several Metabolites Are Increased in the Occipital Cortex of Anophthalmic Participants

Concentrations of six previously studied (Weaver et al. 2013) neurochemicals of interest, tCr, *myo*-Ins, tCho, Glu/Gln, tNAA, and GABA, within the V1 and sensorimotor voxels were compared between the sighted and anophthalmia groups. Figure 2 shows the mean neurochemical concentrations for the anophthalmia (●) and sighted control (○) groups. The concentrations in both the V1 and sensorimotor voxels are shown.

A repeated-measures ANOVA (neurochemical concentrations × voxel location × group) highlighted significant differences in neurochemical concentrations between the two voxel locations [main effect of voxel location $F(1,16) = 79.2$, $P < 0.001$; interaction voxel location × neurochemical concentrations $F(1.9,29.7) = 35.6$, $P < 0.001$]. This was expected, as neurochemical levels are known to vary across cortical regions (Pouwels and Frahm 1998). Importantly, the ANOVA revealed that differences in neurochemical concentrations between the two groups were dependent on voxel location [interaction voxel location × neurochemical concentration × group, $F(1.9,29.7) = 7.6$, $P < 0.001$]. Mann-Whitney *U*-tests were then performed to further investigate these neurochemical differences in V1 and sensorimotor cortex. In the V1 voxel, the anophthalmia group showed significantly higher concentrations in tCr ($P < 0.001$) and tCho ($P < 0.001$) levels. Concentrations of *myo*-Ins ($P < 0.005$) as well as Glu/Gln ($P < 0.005$) were also significantly higher in the anophthalmia group. All of these differences survive Bonferroni correction for six comparisons. Neither tNAA nor GABA levels differed

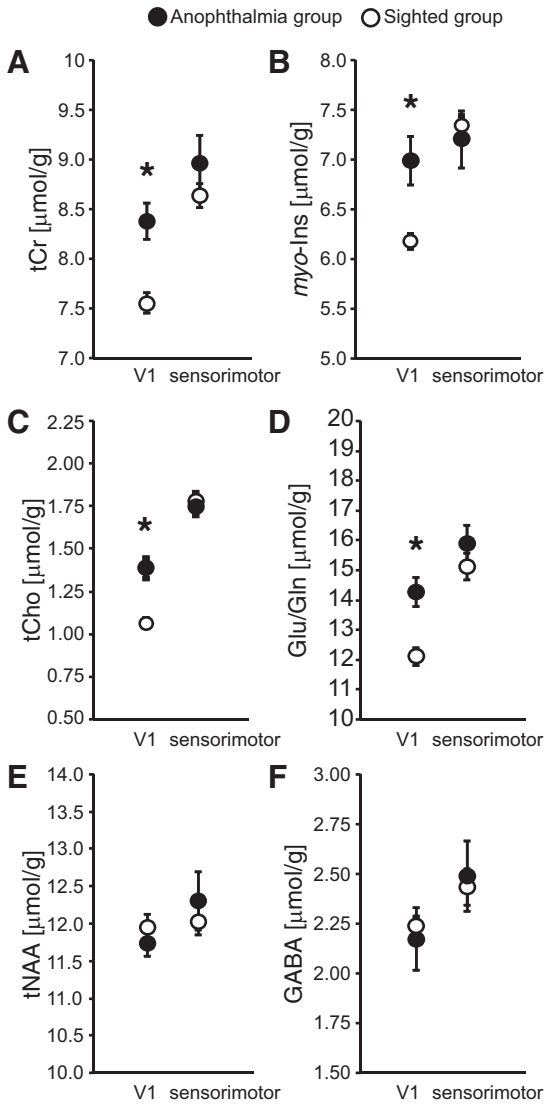

Fig. 2. Group mean concentrations in the 2 magnetic resonance spectroscopy voxels for the 6 metabolites of interest: tCr (*A*), *myo*-Ins (*B*), tCho (*C*), Glu/Gln (*D*), tNAA (*E*), and γ-amino-butyric acid (GABA) (*F*). Error bars represent the standard error of the mean. *Significant difference (after Bonferroni correction) between groups in that voxel location. Concentrations are relative to H_2O.

between the anophthalmia and sighted groups. In the sensorimotor voxel, no differences were found between the two groups for any of the six metabolites of interest.

To illustrate the relative neurochemical concentrations in the V1 voxel for anophthalmia subjects compared with the sighted controls, individual anophthalmia data points were normalized to the V1 control mean and presented in Fig. 3 as *z*-scores. In the case of both tCr and tCho, the five anophthalmic subjects had concentrations that exceeded the mean + 1 SD (at least) of the sighted control mean.

To ensure that differences in metabolite concentrations between the sighted and anophthalmia groups were not due to spectral quality, Cramer-Rao bands (% SD), signal-to-noise ratios, and line width (in Hz) were assessed with Mann-Whitney *U*-tests. Cramer-Rao bands did not differ between the two groups for any of the six neurochemicals in either voxel, tCr (V1 $P = 1.00$, sensorimotor $P = 0.82$), *myo*-Ins (V1 $P = 0.62$, sensorimotor $P = 0.89$), tCho (V1 $P = 0.07$, sensorimotor $P = 0.96$), Glu+Gln (V1 $P = 0.26$, sensorimotor $P = 0.89$), tNAA (V1 $P = 0.75$, sensorimotor $P = 0.82$), and GABA (V1 $P = 0.19$, sensorimotor $P = 1.00$). Neither spectral signal to noise nor line width differed between groups in either the V1 voxel (signal-to-noise ratio $P = 0.16$; line width $P = 0.96$) or sensorimotor voxel (signal-to-noise ratio $P = 0.26$, line width $P = 0.75$).

To ensure that differences in sex and age of the control group did not affect results, two analyses were performed on the control group. First, for each of the six metabolites of interest, age was correlated with metabolite concentration. There were no significant or trend correlations for any of the metabolites, suggesting that, within the limited range used, age does not measurably affect the MRS concentrations of the metabolites studied here. Second, the control group was divided into male and female subgroups, and *t*-tests were used to determine whether there were sex differences. Although there were no differences between male and females participants for tCr, *myo*-Ins, Glu+Gln, tNAA, or GABA, there was a trend level difference for tCho, with males showing higher concentration than females ($t = 2.1$; degrees of freedom = 12; $P = 0.054$). To ensure that this difference did not affect the main results, the analysis for tCho was repeated using only the males in the control group. tCho was still significantly greater in the anophthalmic population ($P < 0.001$). Furthermore, the female anophthalmic participant did not have extreme values for any metabolite. Thus differences in age and sex between the control and anophthalmic group are unlikely to have caused the group differences reported here.

Tissue Content Analysis

Segmentation of the brain tissue within the V1 voxel indicated that there was a significantly greater proportion of gray matter in the anophthalmia group compared with the sighted control group [control mean = 0.49 (SE 0.01); anophthalmia mean = 0.55 (SE 0.02); $U = 10$, $P = 0.01$], consistent with the increase in cortical thickness previously shown (Bridge et al. 2009). The increase in gray matter was accompanied by a

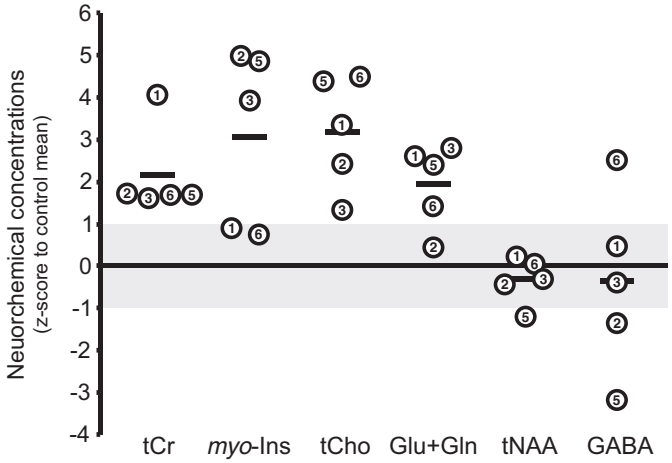

Fig. 3. Neurochemical concentrations in V1 for the anophthalmic participants. For display purposes, *z*-scores for each anophthalmic participant (relative to sighted controls) for the 6 neurochemicals of interest are presented here. The black lines indicate the anophthalmia group mean. The gray band indicates 1 SD above and below the control mean.

decrease in white matter, as seen in Fig. 4, and increase in CSF. In contrast, there was no difference in the tissue composition in the sensorimotor voxel [control mean = 0.53 (SE 0.01); anophthalmia mean = 0.47 (SE 0.03); $P = 0.07$] (Fig. 4).

Because some metabolites have different concentrations in gray and white matter, we performed three additional analyses to investigate the consequences of differing tissue composition on metabolite concentrations.

First, because water content differs across gray and white matter, metabolite concentrations were normalized to the water content of the voxel. Taking water content into account did not change the statistical significance of the group differences for any metabolite concentrations.

Second, we examined the effect of the gray matter fraction on the V1 metabolite concentrations. The relationship between metabolite levels in the V1 voxel and the gray matter fraction in the V1 voxel are plotted in Fig. 5. The lack of any statistically significant relationship between metabolite concentration and gray matter fraction in the control group suggests that the tissue content alone is unlikely to explain the results. Furthermore, as can be seen in Fig. 5, for four of the five anophthalmic subjects, there was a control subject with a similar gray matter fraction. In each case, the concentrations of tCho and tCr were higher in anophthalmic subjects even when comparing these control subjects with similar gray matter fractions. A similar pattern was observed for Glu/Gln (Fig. 5*D*) and *myo*-Ins (Fig. 5*B*) but to a slighter lesser extent.

Finally, in addition to the group difference in gray matter fraction within the V1 voxel, the CSF fraction was higher in the anophthalmia group. To demonstrate that differences in V1 metabolite concentrations between the two groups do not solely depend on correction for CSF content and are visible in the spectra before CSF correction, the mean raw spectrum for each group, normalized to the water peak, was computed. Figure 6 shows the mean spectrum for the anophthalmia group (black line) and the control group (gray line) before any correction for CSF. The level of peaks of interest for each group is indicated by the arrows. tNAA is noticeably reduced in the anophthalmia group compared with the control group, a finding not evident after correction for CSF. Because the previous study of Weaver et al. (2013), who corrected for proportion of CSF, did not find a reduction in NAA in the early blind group, it is likely that this reduction reflects the increased CSF proportion in the voxel for the anophthalmia group. Increased peaks for tCho, tCr, and *myo*-Ins are also evident even in the raw data.

DISCUSSION

In vivo neurochemical and structural assessment of the pericalcarine cortex (V1) in anophthalmic individuals revealed significantly higher levels of tCho, *myo*-Ins, tCr, and Glu/Gln and a higher proportion of gray matter compared with sighted controls.

Visual Deprivation Results in Elevated Choline and myo-Ins Levels

Choline is found in cell membranes and is generally considered a marker of membrane, or structural, integrity. tCho represents a combination of two choline-containing compounds, PC and GPC. Choline levels in the V1 voxel were lower in both groups compared with the sensorimotor voxel; regional variability in gray matter choline levels have been noted in the past, particularly between occipital and parietal cortices (Pouwels and Frahm 1998).

Consistent with reports from early blind individuals (Weaver et al. 2013), choline levels were robustly elevated in the pericalcarine cortex (V1) but not in the sensorimotor cortex of anophthalmic subjects. This robust difference across groups was somewhat surprising, given that, in adulthood, increased choline is generally found in disease states as a marker of membrane breakdown, possibly attributable to demyelination.

One potential explanation for the elevated choline is that it reflects altered cholinergic pathway activity, consistent with previous reports from blind animals (Dehay et al. 1996; Fosse et al. 1989; Wu et al. 2013). Although acetylcholine is a very small component of the choline peak, it may provide a marker of cholingeric tone (Satlin et al. 1997; Wang et al. 2008) because phospholipids play a key role in providing choline for acetylcholine synthesis (Beloueche-Babari et al. 2010; Boulanger et al. 2000; Cantley 2002; MacKay et al. 1996). The regulation and expression of cholinergic pathways depends on whether there is visual input (Gu 2003). Thus these findings

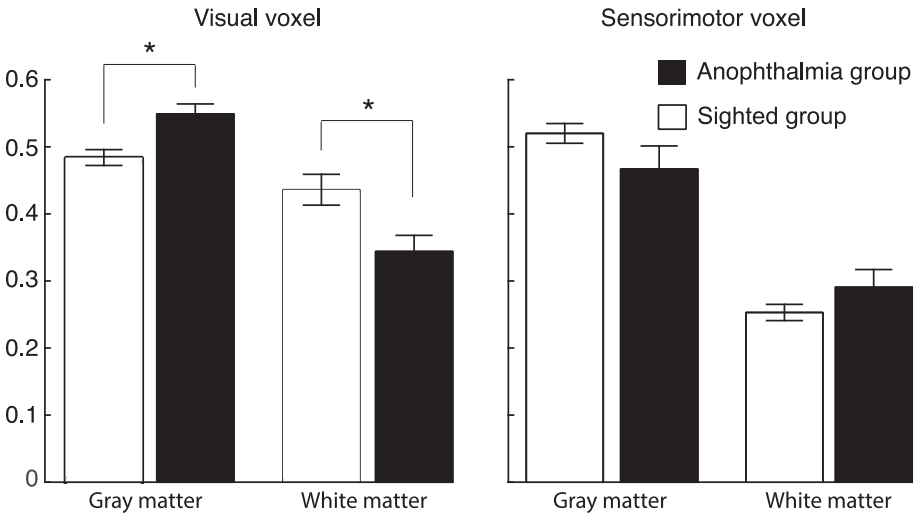

Fig. 4. The gray matter and white matter fractions for the V1 (*A*) and sensorimotor (*B*) voxels are shown for the anophthalmic and control participants. *$P < 0.05$.

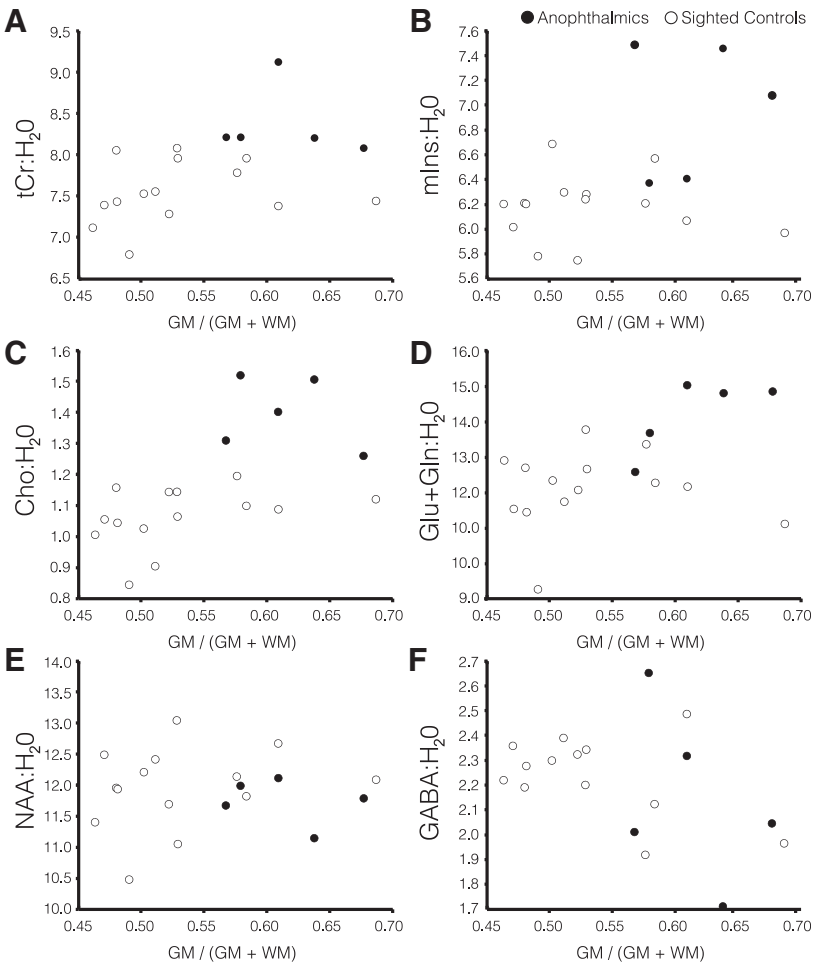

Fig. 5. Metabolite concentrations in V1 voxel plotted against gray matter (GM) fraction for the sighted (○) and anophthalmia (●) subjects. For all metabolites, there was a lack of significant correlation between gray matter fraction and metabolite levels in sighted control subjects. WM, white matter.

might reflect an upregulation of activity in pericalcarine cholinergic circuits.

A second nonexclusive explanation is that some of the higher choline in the anophthalmic pericalcarine (V1) cortex could be explained by an increase in the number of cells in anophthalmic subjects, which would be consistent with the increased gray matter proportion that we find in the same region in these subjects (although it is not yet demonstrated that increased cell number is the cause of the increased cortical thickness found using structural MRI; Bridge et al. 2009). Furthermore, volumetric changes as a result of dark rearing or early enucleation in animal models seem to be confined to layer IV (Bengoetxea et al. 2013; Dehay et al. 1996). In any case, the increase in tCho concentration cannot be explained by higher cortical gray matter alone because, even when comparing subjects with similar values of gray matter, tCho concentrations were higher in the anophthalmic participants (Fig. 5*C*).

Consistent with previous studies of both early blind (Weaver et al. 2013) and late blind (Bernabeu et al. 2009) individuals, *myo*-Ins levels in V1 were significantly higher in the anophthalmia group compared with sighted controls. *Myo*-Ins (the most common biological stereoisomer of inositol) is produced within glial cells and astrocytes; increased levels of the neurochemical are usually viewed as indicative of increased glial proliferation or increased glial size (Soares and Law 2009). Although traditional dark rearing reduces the astrocyte population (Argandoña et al. 2003; Muller 1990), astrocyte density is actually enhanced in dark-reared animals given access to both an enriched environment and voluntary exercise (Argandoña et al. 2009).

Elevated levels of both choline and *myo*-Ins, such as those observed in anophthalmic V1 are also characteristic of immature cortex. Concentrations of both neurochemicals, as measured by MRS, are high at birth; choline levels briefly continue to increase and typically peak at 3 mo. Levels of both neurochemicals gradually decrease following the onset of cortical maturation and pruning (Bluml et al. 2013), thus suggesting that increased levels of these two neurochemicals in adulthood (and in the absence of disease) could be a marker of delayed cortical development. However, although dark rearing delays the onset of the critical period (Lee and Nedivi 2002), it is not clear that it can be delayed indefinitely or that having a cortex that remains undifferentiated or is nonspecialist in terms of function would be beneficial. Moreover, it might be expected that the cross-modal responses that are observed in early blind and anophthalmic subjects would signal the end of the critical period.

Visual Deprivation Results in Elevated tCr Levels

Cr, which is measured in MRS as an approximately equal proportion of Cr and PCr, is linked to ATP reserves in the brain (Miller 1991). In line with previous MRS studies of blind macaques (Wu et al. 2013) and early blind humans (Weaver et

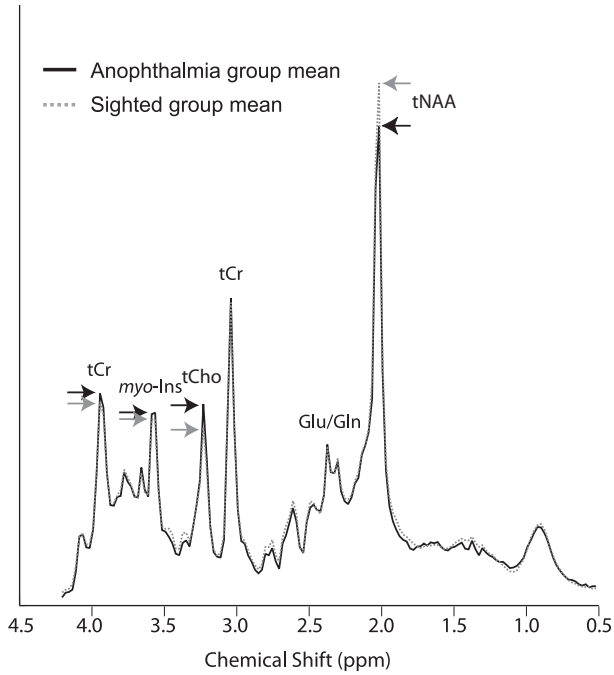

Fig. 6. Overlay of the raw mean spectra acquired in V1 for the anophthalmia group (black) and control group (gray). The tNAA peak is higher in the control group, likely reflecting the increased proportion of neuronal tissue in the voxel relative to the anophthalmia group. The peaks for tCr, *myo*-Ins, and tCho are higher for the anophthalmia group, indicating that differences, although small, are visible at the individual level in the spectra even before correction for cerebrospinal fluid (CSF) content. The increase in Glu/Gln is presumably too small to be detected in the absence of CSF correction.

al. 2013), tCr levels in V1 were significantly higher in anophthalmia than sighted controls. As a potential marker of energetic metabolism, increased levels of tCr in anophthalmia and early blindness are consistent with evidence of upregulated metabolic processing in the occipital cortex of early blind subjects at rest and during auditory and tactile tasks (De Volder et al. 1997; Uhl et al. 1993; Veraart et al. 1990; Wanet-Defalque et al. 1988). Combined with increased levels of choline and *myo*-Ins, this result may suggest that there are increases in cell activity, glial cell number, and/or metabolism in the pericalcarine cortex of anophthalmia.

Elevated Glu/Gln Levels in Anophthalmia

In contrast to previous MRS studies with early-onset (Weaver et al. 2013) and late-onset (Bernabeu et al. 2009) blindness, we found that levels of Glu and Gln in the occipital cortex were significantly increased in anophthalmia compared with sighted controls. Quantifying these metabolites at 3T can be technically challenging, and, in the study of Weaver et al. (2013), the measurements were reported to be extremely unreliable. Similarly, the lack of difference in Bernabeu et al. (2009) could either be methodological or attributable to the individuals having acquired blindness.

Our finding is consistent with animal models showing that glutamatergic pathways are affected by visual deprivation, as well as a recent MRS study showing increased Glu/Gln levels in neonatally blind macaques (Wu et al. 2013). In animal models, critical periods of development under conditions of deprivation can be extended, and plasticity in adulthood can be encouraged via glutamatergic pathways (Bear and Singer 1986; McCoy et al. 2009); enhancement of excitatory cholinergic circuits have been observed in dark-reared cats (Fosse et al. 1989). According to these models, the increased Glu and choline we observe in anophthalmic V1 could be indicative of increased activity in excitatory pathways. As the balance of excitatory and inhibitory circuits is thought to be important for synaptic plasticity, it is possible that increased occipital cortex excitability in anophthalmia and early blindness allows for large-scale reorganization of the region and cross-modal plasticity (Bavelier and Hirshorn 2010).

Large Variability in GABA Levels Across Anophthalmic Individuals

Given the animal literature (Benevento et al. 1995; Fosse et al. 1989) and results suggestive of reduced GABA in early blind individuals (Weaver et al. 2013), it seems possible that the lack of a significant GABA effect in the present study may be due to a combination of small subject numbers and the inherent difficulty in extracting reliable GABA concentrations. Indeed, as depicted in Fig. 3, we found that there was considerable variability in GABA levels across the five anophthalmic participants.

It is not clear whether this difference in findings can be attributed to methodological differences in how MRS GABA was measured. Weaver et al. (2013) used a relatively standard MEGA-PRESS sequence; however, their rest-retest reliability was low. Although the sequence that we used is less commonly used than MEGA-PRESS, it has been shown to produce spectra of consistent quality and enable reliable quantification of at least 13 metabolites, including GABA (Deelchand et al. 2015). Moreover, the reliability of the sequence was confirmed in the study of Deelchand et al. (2015) by replicating the spectra with different MRI scanners at different scan sites (including the scanner used in this experiment).

Voxel Tissue Content

Increased cortical thickness has been previously shown in the pericalcarine cortex in both the anophthalmic participants studied here (Bridge et al. 2009) and early blind (Jiang et al. 2009; Voss and Zatorre 2012) subjects. Presumably as a result of this difference in cortical thickness, the proportion of gray matter within the V1 voxel was significantly larger in the anophthalmic compared with the sighted group.

Because the relative concentrations of many metabolites are known to differ in gray and white matter (Wang and Li 1998) and others are still being debated in the literature, the differing proportions of gray and white matter within the MRS voxel are problematic. We therefore took multiple steps to determine whether the increased concentrations of the four neurochemicals were simply a result of increased gray matter fraction. Correcting by the relative amount of water in the tissue did not change the findings. Furthermore, the metabolite concentrations for anophthalmic participants tended to be significantly higher than those in control subjects who had comparable proportions of gray matter.

Comparison with Early Blind Populations

Anophthalmic participants are distinct from other early blind populations in that they have never received any visual stimulation either from light or endogenous activity through retinal

waves. It is not yet clear whether anophthalmia results in a unique phenomenology or can be thought of as an extreme form of early blindness. In support of the latter hypothesis, the increased thickness of the pericalcarine cortex observed in early blind individuals (Anurova et al. 2014) appears to be even more extreme in the anophthalmic population (Bridge et al. 2009). Similarly, the lateral geniculate nucleus is more atrophied in anophthalmic than in early blind individuals (Cecchetti et al. 2015). In our study, we found a greater elevation of tCr and tCho in anophthalmic individuals compared with the elevation found in early blind individuals by Weaver et al. (2013). These results suggest that the lack of retinal waves and/or residual light exposure results in even more dramatic effects on occipital cortex development than found in congenital blindness.

Interestingly, despite our approach taken to study a small population with a homogenous cause of blindness and the same experience with light, there remains considerable interindividual variability in neurochemical differences that we assume are due to the extreme visual deprivation. Presumably adding further variance in causes of blindness, age of onset, and experience with light perception (even in utero) would make it even more difficult to find consistent differences in the biochemical architecture of the occipital cortex.

Link Between Altered Neurochemistry and Cross-Modal Plasticity

What is the relationship between these changes in neurochemistry and the cross-modal plasticity that has been demonstrated within occipital cortex both for early blind (Amedi et al. 2007; Anurova et al. 2014; Bedny et al. 2011; Collignon et al. 2011; Lewis et al. 2010; Pietrini et al. 2004) and these anophthalmic individuals (Coullon et al. 2015; Watkins et al. 2012, 2013)? We have suggested that the neurochemical profile is similar to the immature visual cortex, which potentially provides a longer period in which the cross-modal plasticity can occur. Alternatively, the difference may reflect the unmasking and strengthening of existing connections. The occipital cortex has been shown to receive multi-modal input (Innocenti et al. 1988), and it may be that these existing connections are unmasked in the absence of visual input. Finally, it is also the case that different cortical regions also have different neurochemical profiles. Indeed, Fig. 2 illustrates the considerable differences in neurochemical concentration between the sensorimotor voxel and V1. In all the metabolites showing a difference between anophthalmic and control subjects, we found that the concentrations within the blind subjects were more similar to those measured in the sensorimotor voxel. Thus an alternative possibility is that the changes that we see reflect a respecification of cortex as a result of blindness that incorporates neurochemistry as well as a functional role.

Conclusions

The neurochemical changes that we describe suggest that lack of eyes and stimulation along the visual pathway in anophthalmia has affected the development of excitatory circuits of the occipital cortex as well as more gross aspects of cortical development (e.g., thickness). Thus the absence of visual input has not triggered the normal pattern of maturation of the pericalcarine region. Future work will be needed to determine the relationship between these neurochemical and structural differences and the substantial functional reorganization found in pericalcarine cortex as a result of complete blindness.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: G.S.L.C., K.E.W., and H.B. conception and design of research; G.S.L.C. performed experiments; G.S.L.C. and U.E.E. analyzed data; G.S.L.C., U.E.E., I.F., K.E.W., and H.B. interpreted results of experiments; G.S.L.C., U.E.E., and H.B. prepared figures; G.S.L.C. drafted manuscript; G.S.L.C., U.E.E., I.F., K.E.W., and H.B. edited and revised manuscript; G.S.L.C., U.E.E., I.F., K.E.W., and H.B. approved final version of manuscript.

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