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Effects of Bilateral Enucleation on the Size of Visual and Nonvisual Areas of the Brain

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Alterations in the activity of one sensory system can affect the development of cortical and subcortical structures in all sensory systems. In this study, we characterize the changes that occur in visual and nonvisual areas of the brain following bilateral enucleation in short-tailed opossums. We demonstrate that bilateral enucleation early in development can significantly decrease brain size. This change is driven primarily by a decrease in the size of the thalamus, midbrain, and hindbrain, rather than a decrease in the size of the cortical hemispheres. We also found a significant decrease in the size of the lateral geniculate nucleus in bilaterally enucleated animals. Although the overall size of the neocortex was the same, the percentage of neocortex devoted to visual areas V1 (primary visual area) and caudotemporal area were significantly smaller in bilaterally enucleated opossums and the percentage of neocortex devoted to the primary somatosensory area (S1) was significantly larger, although S1 did not change in size to the same extent as V1. Our data suggest that during development the relative activity patterns between sensory systems, which are driven by activity from unique sets of sensory receptor arrays, play a major role in determining the relative size and organization of cortical and subcortical areas.

Keywords: cross-modal plasticity, marsupial, *Monodelphis domestica*, neocortex, V1

Introduction

The neocortex is a highly dynamic structure that is capable of assuming a unique organization and pattern of connectivity to match the physical demands and fluctuations that occur naturally in the environments in which an animal develops. We have known for some time that altering neural activity generated at the sensory receptor array, which is the first biological interface between the environment and brain, can change the functional organization and connectivity of the thalamic and cortical structures associated with the altered modality. For example, Wiesel and Hubel established that visual experience is necessary for normal development within primary visual cortex (V1, see Table 1 for abbreviations) in cats and monkeys (Wiesel and Hubel 1963, 1974; Hubel et al. 1977). Since then, numerous studies have examined the roles of sensory-driven activity and spontaneously generated activity in the normal development of cortical and subcortical visual structures (for review, see Feller and Scanziani 2005; Huberman 2007), and it is clear that both types of activity are important for normal visual development (e.g., Rakic 1981; Shatz 1990; Gordon and Stryker 1996; Huberman et al. 2006; Li et al. 2006; Smith and Trachtenberg 2007).

The role of early experience has been examined in other sensory systems as well. In the somatosensory system, multiple studies in mice have demonstrated that removal of vibrissae follicles early in development leads to the absence of the corresponding barrels in the barrel field in primary somatosensory area (S1) (Van der Loos and Woolsey 1973; Rice and Van der Loos 1977) and that mice with supernumerary vibrissae develop extra barrels in S1 that correspond to the location of the additional vibrissae (Van der Loos and Dorfl 1978; Van der Loos et al. 1984, 1986). In the auditory system, Zhang et al. (2002) have shown that alterations in the auditory environment, which occur early in development, result in a reorganization of the primary auditory area (A1) with a disordered topography and a degradation of frequency–response selectivity. Taken together, these studies demonstrate that alterations in the patterns of activity from sensory receptor arrays, which in natural environments reflect alterations in the patterns of physical stimuli present, can dramatically influence the development of functional organization and connectivity of cortical fields (e.g., see Pallas 2001; O'Leary and Nakagawa 2002; Majewska and Sur 2006; Krubitzer 2007 for review).

Although our understanding of the extent to which cortical and subcortical structures can be reorganized following alterations in sensory-driven activity has increased over the past three decades, most studies have focused on the types of changes that occur within a sensory system following changes in the sensory receptor array and/or the activity that it generates. However, there is also evidence that alterations in the activity of one sensory system can affect the overall development of cortical and subcortical structures in the other sensory systems. This phenomenon, in which a change in one sensory system alters the development of the remaining sensory systems, is called cross-modal plasticity.

Several studies have shown that the loss of one sensory modality can alter the functional organization and connectivity of cortical and subcortical structures associated with both the lost and spared sensory modalities. For example, Rauschecker and Korte (1993) demonstrated that early bilateral lid suture in cats resulted in a respecification of higher order visual areas. Specifically, the majority of neurons in the anterior ectosylvian visual area responded to auditory and tactile stimulation rather than visual stimulation. This change has been attributed to an expansion of neighboring auditory and somatosensory fields at the expense of extrastriate visual areas (for review, see Rauschecker and Henning 2001). This type of cross-modal plasticity has even been observed in primary sensory areas. For example, in hamsters, mice, rats, and opossums, bilateral enucleation early in development altered the modality of

response of neurons in V1 in that they responded to auditory and tactile stimulation (Izraeli et al. 2002; Kahn and Krubitzer 2002a; Chabot et al. 2007; Piche et al. 2007). Together, these results are similar to what has been shown in congenitally blind humans where regions of the neocortex normally activated by visual stimulation become responsive to auditory and tactile stimulation (for review, see Bavelier and Neville 2002). These alterations in functional organization are likely due to changes in connectivity that occur along the entire neuroaxis (Negyessy et al. 2000; Hunt et al. 2005; Karlen et al. 2006; Chabot et al. 2007).

Work in bilaterally enucleated opossums is more complete than in other animal models in that the functional organization across most of sensory neocortex has been determined and the alterations in cortical and subcortical connectivity have been described (Fig. 1). As in the studies described above, studies in the short-tailed opossum have demonstrated that following the removal of visual input early in development via bilateral enucleation the neurons in V1 and extrastriate cortex respond to auditory, somatosensory, or auditory and somatosensory stimulation (Kahn and Krubitzer 2002a; Karlen et al. 2006). However, unlike previous studies, we also observed cross-modal changes in the functional organization of the primary sensory areas associated with the remaining, intact sensory systems. For example, in some opossums, many neurons in S1, which normally respond only to somatosensory stimulation, responded to auditory stimulation or to more than one sensory modality. These changes in the functional organization of V1

Table 1
Neuroanatomical abbreviations

Abbreviation	Neuroanatomical area
A1	Auditory area
AD	Anterodorsal nucleus
AV	Anteroventral nucleus
CT	Caudotemporal area
ER	Entorhinal cortex
FM	Frontal myelinated area
LD	Laterodorsal nucleus
LG	Lateral geniculate nucleus
LGd	Dorsal lateral geniculate nucleus
LGv	Ventral lateral geniculate nucleus
LP	Lateral posterior nucleus
MG	Medial geniculate nucleus
MM	Multimodal cortex
OB	Olfactory bulb
OT	Optic tract
PYR	Pyriform cortex
S1	Primary somatosensory area
S2	Secondary somatosensory area
TMH	Thalamus, midbrain, and hindbrain
V1	Primary visual area
V2	Secondary visual area
VL	Ventrolateral nucleus
VP	Ventral posterior nucleus

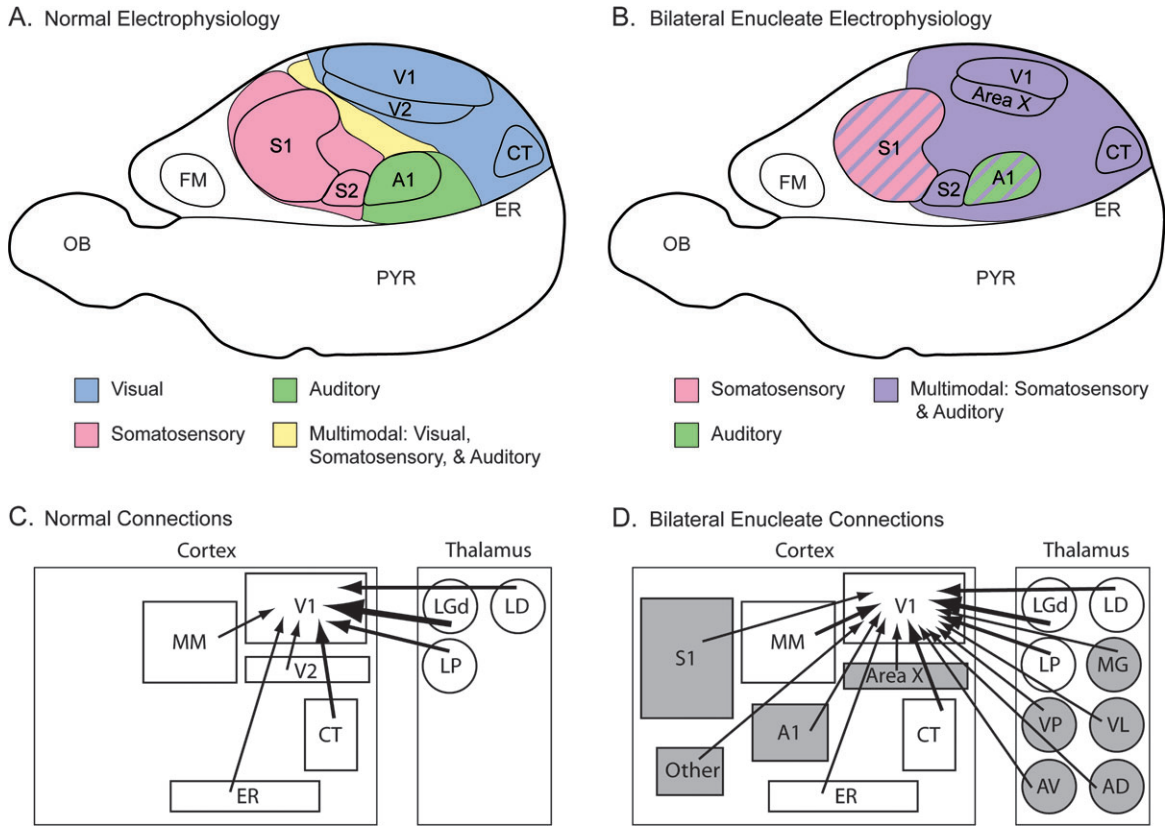

Figure 1. In short-tailed opossums, the functional organization (*A* and *B*) and connections of V1 (*C* and *D*) in normal (*A* and *C*) and bilaterally enucleated (*B* and *D*) animals. In bilaterally enucleated opossums, all the cortex that would normally be devoted to visual processing contains neurons responsive to somatic, auditory, or both somatic and auditory stimulation (compare *A* [blue] and *B* [purple]). Further, many neurons in S1 and A1, which normally respond only to one modality, respond to a different type of modality or to more than one sensory modality (purple stripes). These changes in the functional organization of V1 and other primary cortical areas were accompanied by alterations in cortical and subcortical connections such that V1 received inputs from S1, A1, and somatosensory and auditory nuclei of the thalamus (compare *C* and *D*). Thus, the loss of one sensory modality had a profound affect not only on the regions of the brain associated with that modality but also with regions of the brain, including primary cortical areas, associated with the spared sensory systems. Based on Kahn and Krubitzer (2002a) and Karlen et al. (2006). Abbreviations are defined in Table 1.

and other primary cortical areas were accompanied by alterations in cortical and subcortical connections such that V1 received inputs from S1, A1, and somatosensory and auditory nuclei of the thalamus (Karlen et al. 2006). Thus, the loss of one sensory modality had a profound affect not only on the regions of the brain associated with that modality but also with regions of the brain, including primary cortical areas, associated with the spared sensory systems.

In the present study, we further characterize the alterations that occur in visual and nonvisual areas of the brain following early bilateral enucleations by examining subcortical and cortical architecture. Specifically, we quantify the size of the dorsal and ventral divisions of the lateral geniculate nucleus (LG) in the thalamus and the size of the primary sensory areas V1, S1, and A1 in the neocortex. We hypothesized that the size of brain structures associated with the lost sensory system would decrease while the size of those structures associated with the remaining sensory systems would increase.

Materials and Methods

All procedures were approved by the Animal Use and Care Administrative Advisory Committee of the University of California, Davis, and conform to National Institutes of Health guidelines. Twenty-two normal short-tailed opossums (*Monodelphis domestica*) and 8 bilaterally enucleated opossums were examined in these studies. All animals ranged in age from 6 to 32 months, with no significant difference between the ages of the animals in the 2 groups.

Enucleation Surgery

As in previous studies (Kahn and Krubitzer 2002a; Karlen et al. 2006), animals were enucleated on postnatal day 4 (P4) to ensure survival of the pups. At this age, the ganglion cell axons have just entered the optic tract (OT) (Dunn et al. 2001), and thalamocortical afferents have not yet arrived at the cortex, which occurs between P5 and P15 (Molnar et al. 1998). This stage is roughly equivalent to embryonic day 14 (E14) in the rat and E13 in the mouse (Molnar et al. 1998). Adult females were anesthetized with Alfaxan (20 mg/kg) administered intramuscularly to keep them immobilized, and the individual young were anesthetized by hypothermia. Body temperature, heart rate, and respiration were continuously monitored throughout the surgery. Once the pups were anesthetized, their eyes were manually excised under microscopic guidance. The skin surrounding the eyes was replaced over the exposed eye socket and secured with surgical glue. Pups remained with the mother for 4 weeks, and were then hand-reared until postnatal week seven. The animals were then housed individually. Although the eyelids did form, they did not open, and postmortem dissection revealed a membrane covering the eye sockets.

Measuring Brain Size

Once animals reached maturity (after 5 months of age), each animal was euthanized with an overdose of sodium pentobarbital (Beuthanasia, 250 mg/kg intraperitoneally), and perfused transcardially with 0.9% NaCl in 0.1 M phosphate buffer (PB; pH 7.4), then with 4% paraformaldehyde (PA) in PB, followed by 4% PA + 10% sucrose in PB. Each brain was blocked behind the cerebellum, and everything anterior to the level of the spinal cord was analyzed. The brains were photographed (RT Spot camera, Diagnostic Instruments, Inc.), weighed on a laboratory scale (A-250, Denver Instrument Company), and the volume of each brain was measured.

As previously described (Karlen and Krubitzer 2006), volume measurements were taken using fluid displacement (Scherle 1970; Weibel 1979; Mayhew et al. 1990; Howard and Reed 2005), which relies on the Archimedean principle of fluid displacement and is comparable to the Cavalieri method for measuring volume (Mayhew et al. 1990). Specifically, a beaker of 0.9% normal saline was placed on a laboratory scale, and a plastic mesh basket was suspended below the surface of the saline but above the bottom of the beaker by a laboratory stand using a fine thread. The apparatus was tared, and the brain was placed in the basket. The weight in milligrams not borne by the thread, which is equal to the volume of the brain in cubic millimeters, was recorded, and the procedure was repeated 5 times. An average of 5 measurements were used as the final volume to increase the accuracy of the measurement.

Following weight and volume measurements for the whole brain, the cortical hemispheres were removed from the remaining thalamus, midbrain, and hindbrain (TMH) using a procedure that has been performed in a variety of animals, including mice, rats, star-nosed moles, and opossums (Welker and Woolsey 1974; Welker 1976; Catania and Kaas 1995; Huffman et al. 1999). Briefly, the anterior commissure was cut, and the cortex was gently peeled away from the thalamus, breaking the axons in the corona radiata adjacent to the thalamus. The hippocampus was not separated from the cortex and was included in the cortical measurement. The weight and volume of each hemisphere and the remaining TMH block were taken as described above. All tissues were stored in 4% PA until histological processing began.

Histological processing

The TMH block and the cortical hemispheres were cryoprotected by immersion in 30% sucrose overnight before sectioning. The TMH block was frozen and sectioned coronally on a freezing microtome at a thickness of 40 μm. Sections were collected in PB. Alternate sections were stained for Nissl or myelin (Gallyas 1979). Adjacent sections processed for Nissl and myelin were used to determine the borders of thalamic nuclei for 6 normal opossums and 6 bilaterally enucleated opossums.

Cortical hemispheres were manually flattened and sectioned tangentially to the pial surface on a freezing microtome at 20 μm. Sections were collected in PB. Alternate sections were stained for myelin for 17 normal opossums and 7 bilaterally enucleated opossums. Because darkly myelinated regions are coextensive with functional boundaries (Huffman et al. 1999; Catania et al. 2000; Frost et al. 2000; Kahn et al. 2000), cortical fields were defined based on myelin density. Cytoarchitectonic boundaries were not examined in this study because the brains where cut tangentially, and Nissl stains are not helpful for identifying cortical field boundaries in this plane of section. However, myelin density boundaries have previously been shown to coincide with the cell density boundaries of layers IV and VI in V1, and both myelin and cell density boundaries have been directly correlated with the functional boundaries of V1 (Kahn and Krubitzer 2002a).

Quantification of the LG

In all, 6 normal opossum cases and 6 bilaterally enucleated opossum cases were coded with randomly generated numbers. A camera lucida (Axioskop, Zeiss) was used to draw the borders of the LG at a fixed magnification. The experimenter was blind to condition when defining the borders of the nuclei. Borders of the LG were based on pairs of adjacent Nissl- and myelin-stained sections that contained the nuclei of interest. We chose to examine the dorsal lateral geniculate nucleus (LGd) because it is one of the major targets of the retina and thus was most likely to have alterations in size following bilateral enucleation. We examined ventral lateral geniculate nucleus (LGv) because it also receives retinal input. Both of these nuclei have clearly defined boundaries. Other targets of the retina in *M. domestica* include the superior colliculus, lateral posterior nucleus, pretectal complex, and nucleus of the OT (Kahn and Krubitzer 2002b). Because the boundaries of these nuclei are less well defined than the boundaries of the LGd and LGv, quantification of these nuclei and, in particular, of the superior colliculus would have been more subjective than identifying boundaries in the LG. In addition, it would have been difficult to separate the sources of variability in those measurements (particularly the variability attributed to measurement error) because of the subjectivity of the boundaries. Thus, for this study, we focused on only quantifying the boundaries of the LG.

The volume of the LGd and LGv were calculated using the Cavalieri method (Gundersen and Jensen 1987; Howard and Reed 2005) as determined by the formula:

$$V=(T \cdot A_1)+(T \cdot A_2)+\cdots+(T \cdot A_m)=T \cdot \sum_{i=1}^{m} A_i,$$

where V is the total volume of the nucleus, T is the thickness of the section, and A_i is the cross-sectional area of the object seen on the ith slice. Because adjacent Nissl and myelin sections were used to determine nuclei borders, we set T = 80 μm. We did not adjust the calculated volume for tissue shrinkage or section compression because we were primarily interested in the difference between groups, and the brains from both groups underwent the same histological procedures. We assumed that changes due to shrinkage or section compression would affect both groups equally. Finally, comparisons between groups were based solely on volume measurements; we did not count the number of cells in each nucleus.

Quantification of Cortical Field Size

To quantify cortical field sizes, myelin borders were drawn for every section containing cortical fields of interest using a Jena microfiche reader. Sections were quantified if the boundaries of one or more cortical areas could be identified. All sections that met this criterion were reconstructed for 17 normal opossum cases and 7 bilaterally enucleated opossum cases. Because no single section accurately represents all the cortical field borders, boundaries from all the reconstructed sections were compiled to make one composite reconstruction to represent each hemisphere (as described in Karlen and Krubitzer 2006). The composite reconstruction was scanned into a computer, and area measurements were acquired using the NIH Image 1.62 program (Rasband 1997–2008). This program has been used to measure cortical areas in previous studies (Huffman et al. 1999; Wahlsten et al. 2003; Airey et al. 2005; Hunt et al. 2006; Karlen and Krubitzer 2006).

Statistical Analysis

Descriptive statistics of each measurement are presented. When appropriate, means plus or minus the standard deviation are given. Differences between normal and bilaterally enucleated opossums were evaluated using independent samples t-tests. Levene's test for equality of variances between groups was used to determine whether there was a significant difference in the variance of each of the measurements described below. Based on this, equal variances were assumed for all t-tests unless otherwise noted.

Results

We examined the effect of early bilateral enucleation on the size of subcortical and cortical areas in the short-tailed opossum. We compared overall brain size using weight and volume measurements, the volume of the dorsal and ventral divisions of the LG, and the relative size of primary sensory cortical fields (defined as cortical field area divided by total area of the dorsolateral neocortex) between normal and bilaterally enucleated opossums. We used architectonic boundaries to define subcortical nuclei and cortical fields because they have been previously related to functional boundaries in short-tailed opossums (Huffman et al. 1999; Kahn et al. 2000), and they can be easily identified, reliably quantified, and consistently compared across animals.

Quantification of Body Size and Brain Size

Body size was measured in normal and bilaterally enucleated adult animals using a laboratory scale. All the animals were weighed prior to being euthanized. Weights ranged from 60 to 143 g. There was no significant difference between the 2 groups in the body weights of the animals.

Brain size was determined by 2 measurements, weight and volume (Fig. 2). We found a significant difference between normal and bilaterally enucleated opossums in the weight of the brain [$t(28)$ = 3.377, P = 0.002]. The average brain weight was 828 ± 68 mg for normal animals and 729 ± 81 mg for bilaterally enucleated animals. Once the left and right hemispheres were separated from the TMH, each of the resulting

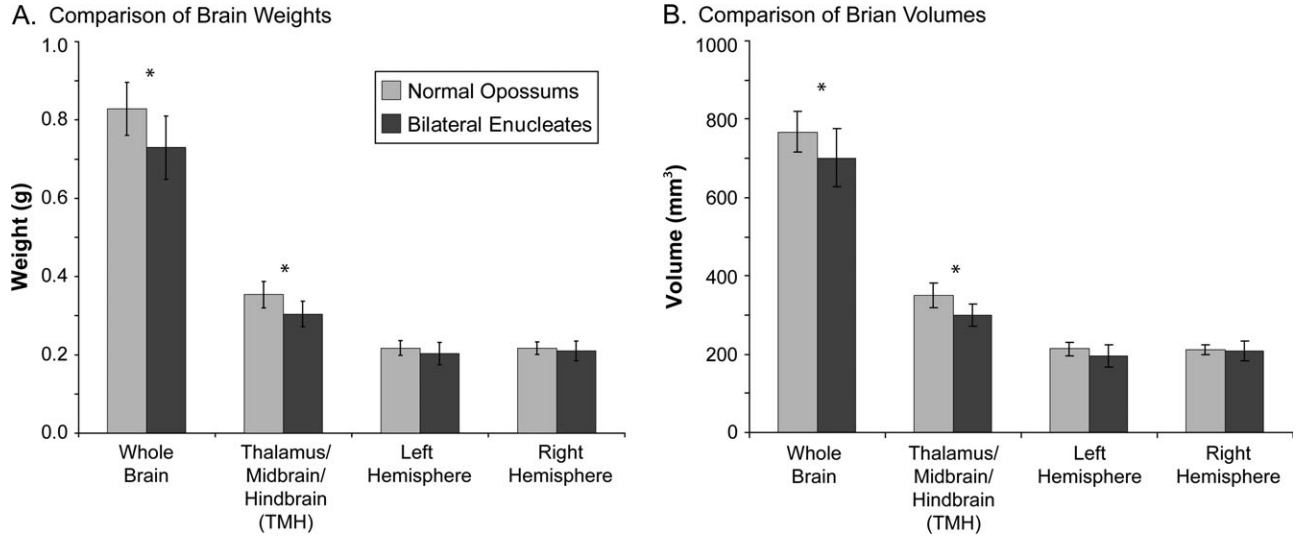

Figure 2. Brain size was determined by 2 measurements, weight (*A*) and volume (*B*). In (*A*), we found a significant difference between normal and bilaterally enucleated opossums in the weight of the brain [$t(28) = 3.377$, $P = 0.002$]. When the left and right hemispheres were separated from the TMH and the volume of each section was measured, there was a significant difference in the weight of the TMH between normal and bilaterally enucleated opossums [$t(28) = 3.617$, $P = 0.001$]; however, there was no significant difference in the size of the cortical hemispheres. In (*B*), we found corresponding significant differences in the volume of the brain [$t(26) = 2.642$, $P = 0.014$] and the TMH [$t(28) = 3.909$, $P = 0.001$]. There was no significant difference in the volume of the cortical hemispheres. Both measurements indicate that the decrease in brain size was driven solely by a decrease in the size of the TMH because there was no change in the size of the cortical hemispheres.

blocks was then weighed independently. For normal animals, the TMH weighed 354 ± 34 mg, the left hemisphere weighed 217 ± 18 mg, and the right hemisphere weighed 217 ± 16 mg. For bilaterally enucleated animals, the TMH weighed 304 ± 32 mg, the left hemisphere weighed 203 ± 28 mg, and the right hemisphere weighed 209 ± 25 mg. There was a significant difference between normal and bilaterally enucleated opossums in the weight of the TMH [$t(28) = 3.617$, $P = 0.001$]; however, there was no significant difference in the size of the cortical hemispheres (Fig. 2*A*). This indicates that the decrease in brain weight was driven by a decrease in the size of the TMH, with no change in the size of the cortical hemispheres.

As with brain weights, we found a significant decrease in the volume of the brain in bilaterally enucleated opossums [$t(26) = 2.642$, $P = 0.014$]. The average brain volume was 768 ± 53 mm^3 for normal animals and 702 ± 75 mm^3 for bilaterally enucleated animals. There was a significant difference between normal and bilaterally enucleated opossums in the volume of the TMH [$t(28) = 3.909$, $P = 0.001$] but no difference in the size of the cortical hemispheres (Fig. 2*B*). In normal animals, the volume of the TMH was 349 ± 32 mm^3, the volume of the left hemisphere was 213 ± 18 mm^3, and the volume of the right hemisphere was 212 ± 13 mm^3. In bilaterally enucleated animals, the volume of the TMH was 299 ± 29 mm^3, the volume of the left hemisphere was 195 ± 28 mm^3, and the volume of the right hemisphere was 208 ± 25 mm^3. These results indicate that, as with brain weight, the decrease in brain volume was driven by a decrease in the size of the TMH, with no change in the cortical hemispheres.

Thalamic Cytoarchitecture and Quantification of the LG

The architectonic appearance of the major sensory nuclei of the thalamus, including the LGd, the ventral posterior nucleus (VP), and the medial geniculate nucleus (MG), has been described for both normal and bilaterally enucleated opossums (Kahn and Krubitzer 2002b; Karlen et al. 2006). Examples of a normal case (Fig. 3*A*) and bilateral enucleate case (Fig. 3*B*) are shown. Both the LGd and LGv are darkly stained and densely packed with cells, as determined by Nissl staining, and are moderately myelinated (Fig. 3). Borders for each reconstruction were drawn using adjacent Nissl- and myelin-stained sections. Reconstructions of the LGd and LGv were made for all sections containing these nuclei by experimenters blind to condition.

The progression of the LGd and LGv from caudal to rostral can be seen in Figure 4, which shows the borders from every other section taken from 2 normal opossum cases and 2 bilaterally enucleated opossum cases. Although the general shape and location of the LGd and LGv are similar in normal and enucleated opossums, the medial-to-lateral width of these nuclei was reduced throughout the series of sections in bilaterally enucleated animals. Further, there were fewer sections through the thalamus in which the LGd and LGv were present in bilaterally enucleated animals as compared with normal animals, which is consistent with a decrease in the overall size of the thalamus. Finally, there was a conspicuous lack of an OT in the bilaterally enucleated animals.

The Cavalieri method was used to quantify the volume of the LGd and LGv in normal and bilaterally enucleated opossums. The LGd was 0.404 ± 0.065 mm^3 in normal animals and 0.217 ± 0.020 mm^3 in bilaterally enucleated animals (Fig. 5). Levene's test for equality of variances determined that equal variances could not be assumed between the 2 groups ($F_{1,22} = 13.449$, $P = 0.001$). Using an independent samples *t*-test with equal variances not assumed, we found that the volume of the LGd was significantly smaller in bilaterally enucleated opossums (Fig. 5; $t[13.127] = 9.557$, $P < 0.001$). The LGv was 0.103 ± 0.016 mm^3 in normal animals and 0.064 ± 0.006 mm^3 in bilaterally enucleated animals. Levene's test for equality of variances determined that equal variances could not be assumed between the 2 groups ($F_{1,22} = 13.650$, $P = 0.001$). Using an independent samples *t*-test with equal variances not assumed, we found that the volumes of the LGv were significantly smaller in bilaterally enucleated opossums, as compared with normal animals (Fig. 5; $t[14.202] = 7.775$, $P < 0.001$).

Cortical Myeloarchitecture and Quantification of Cortical Areas

Cortical boundaries were determined using flattened sections that were stained for myelin (Fig. 6). There was no significant difference between normal and bilaterally enucleated opossums in the area of the flattened cortical sheet, as measured using the NIH Image program. The area of the cortical sheet was 52.3 ± 10.0 mm^2 for normal animals and 50.3 ± 6.5 mm^2 for bilaterally enucleated animals. Further, we found no difference between the left and right hemispheres within each group for any of the measurements described below, indicating that the 2 hemispheres were symmetrical in both normal and bilaterally enucleated opossums, as previously described for normal animals (Karlen and Krubitzer 2006).

The cortical sheet was divided into 2 regions: the dorsolateral neocortex, defined as cortex medial to the rhinal sulcus, but not including the medial wall or orbital cortex, and the pyriform cortex (PYR)/olfactory bulb (OB), defined as cortex lateral to the rhinal sulcus (Fig. 6). To control for any changes that may have occurred during histological processing, the area of each region was standardized as a percent of the cortical sheet (defined as area of the region divided by total area of the cortical sheet including dorsolateral neocortex and PYR). There was no significant difference between normal and bilaterally enucleated opossums in the percent of the cortical sheet occupied by the neocortex or the PYR/OB. The percent of the cortical sheet occupied by the neocortex was 44.3 ± 2.8% in normal animals and 43.6 ± 2.0% in bilaterally enucleated animals; the percent occupied by the PYR/OB was 55.7 ± 2.8% in normal animals and 56.4 ± 2.0% in bilaterally enucleated animals.

In both normal and bilaterally enucleated opossums, the primary sensory areas, including V1, S1, and A1, are easily defined by their darkly myelinated appearance (Fig. 6). The major difference between normal and bilaterally enucleated opossums is that bilaterally enucleated opossums have a darkly myelinated region just lateral to V1, termed area X (Rakic 1988; Rakic et al. 1991) or default extrastriate cortex (Dehay et al. 1989, 1996a, 1996b). As described previously in opossums (Kahn and Krubitzer 2002a; Karlen et al. 2006), area X appears cytoarchitectonically and myeloarchitectonically distinct from both V1 and the secondary visual area (V2) in normal animals, and it is similar in location and appearance to area X described in enucleated primates. Obviously, area X in opossums is not homologous to area X in primates; the terminology is meant only to reflect the emergence of a novel field that is similar in location and appearance to area X in primates.

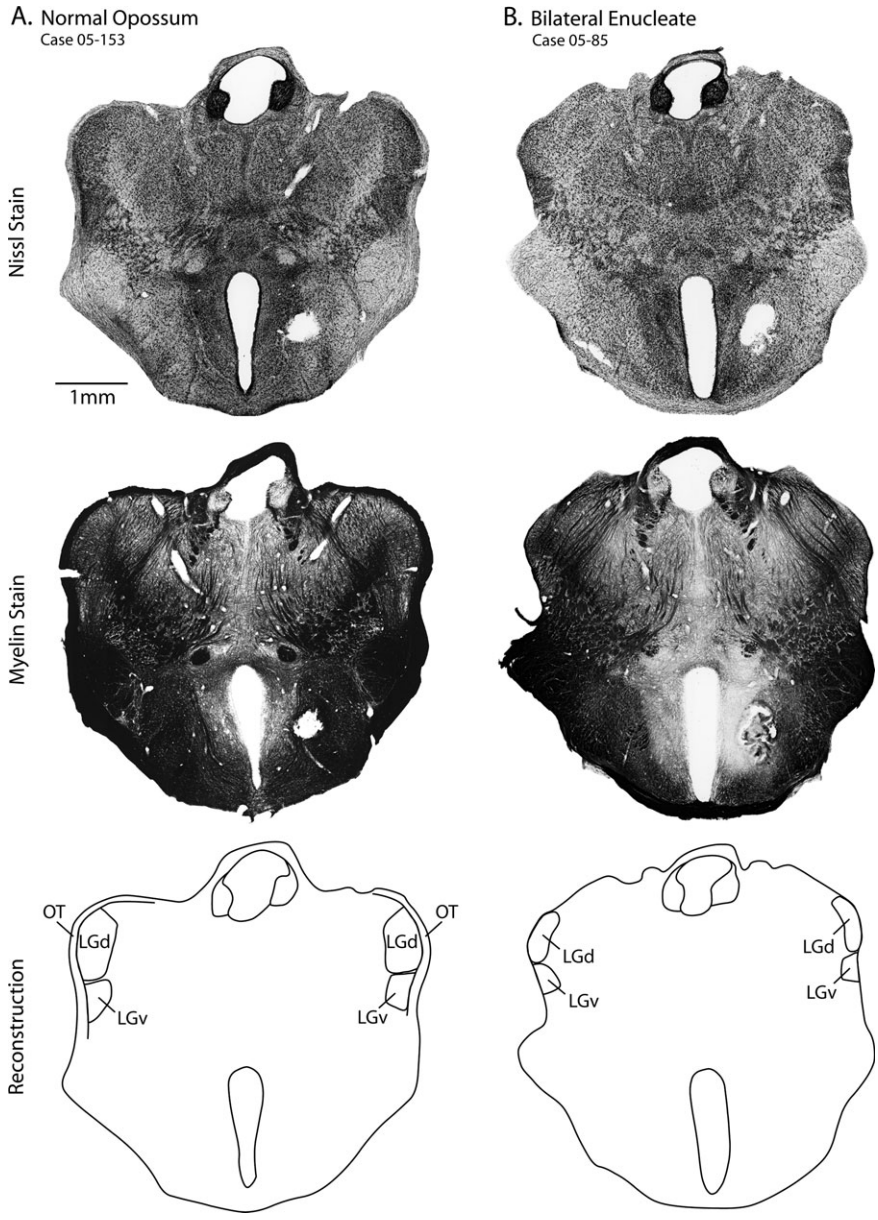

Figure 3. Thalamic nuclei were identified using Nissl (top row) and myelin stains (middle row). Examples of a normal case (*A*) and a bilateral enucleate case (*B*) are shown. Both the LGd and LGv are darkly stained by Nissl, densely packed with cells, and moderately myelinated. There was a conspicuous lack of an OT in the bilaterally enucleated animals. Borders for each reconstruction (bottom row) were drawn using adjacent Nissl- and myelin-stained sections. Reconstructions of the LGd and LGv were made for all sections containing these nuclei by experimenters blind to condition. Dorsal is up, scale bar = 1 mm; abbreviations are defined in Table 1.

A comparison between normal and bilaterally enucleated opossums of the percent of the cortical sheet devoted to each cortical field size is shown in Figure 7. The relative size of each area is as follows: V1 occupied 11.4 ± 1.9% of the neocortex in normal opossums and 5.5 ± 0.9% in bilaterally enucleated opossums, caudotemporal area (CT) occupied 3.2 ± 0.4% of the neocortex in normal opossums and 2.8 ± 0.4% in bilateral enucleates, S1 occupied 18.8 ± 1.8% of the neocortex in normal opossums and 21.2 ± 2.0% in bilateral enucleates, secondary somatosensory area (S2) occupied 2.3 ± 0.3% of the neocortex in normal opossums and 2.3 ± 0.3% in bilateral enucleates, A1 occupied 5.2 ± 0.9% of the neocortex in normal opossums and 5.3 ± 0.6% in bilateral enucleates, and finally, frontal myelinated area (FM) occupied 4.8 ± 0.6% of the neocortex in normal opossums and 4.4 ± 0.5% in bilateral enucleates.

For V1, Levene's test for equality of variances determined that equal variances could not be assumed between the 2 groups ($F_{1,40} = 6.375$, $P = 0.016$). Using an independent samples *t*-test with equal variances not assumed, we found that the relative size of V1 was significantly smaller in bilaterally enucleated opossums, as compared with normal animals [$t(39.156) = 13.478$, $P < 0.001$]. Similarly, the CT, another cortical field involved in visual processing in normal animals, was significantly smaller in bilaterally enucleated opossums as compared with the normal animals [$t(40) = 2.475$, $P = 0.018$]. Conversely, S1 was significantly larger in bilaterally enucleated opossums, as compared with the normal animals [$t(40) = -3.936$,

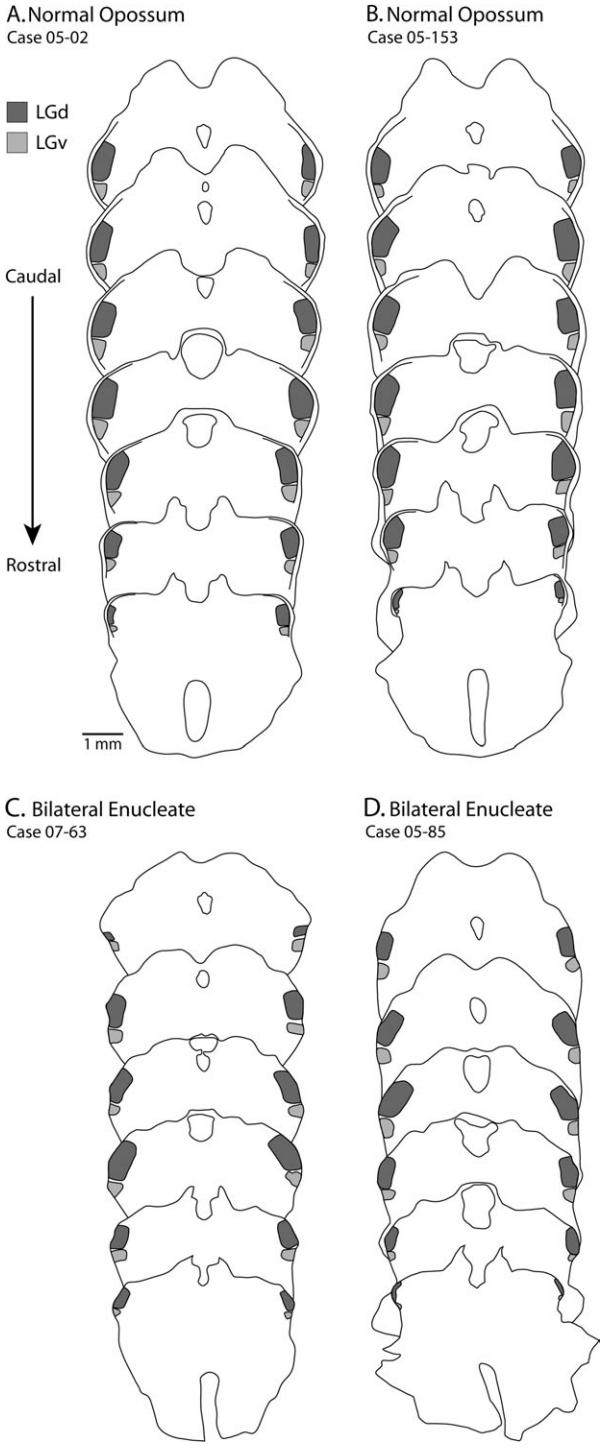

Figure 4. The progression of the LGd (dark gray) and LGv (light gray) from caudal (top) to rostral (bottom). Examples of two normal cases (*A* and *B*) and 2 bilaterally enucleated cases (*C* and *D*) are shown. Although the general shape and location of the LGd and LGv are similar in normal and enucleated opossums, the medial-to-lateral width of these nuclei was reduced throughout the series of sections in bilaterally enucleated animals, and there were fewer sections through the thalamus in which the LGd and LGv were present in bilaterally enucleated animals as compared with normal animals. There was also a conspicuous lack of an OT in the bilaterally enucleated animals. Although reconstructions were made for all sections containing these nuclei, only every other reconstruction is illustrated here. Experimenters were blind to condition when reconstructing the borders. Dorsal is up, scale bar = 1 mm; abbreviations are defined in Table 1.

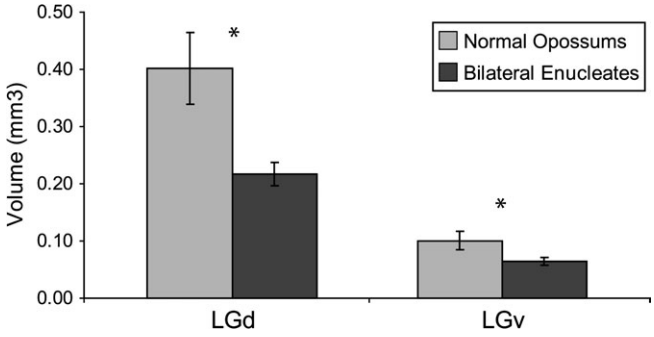

Figure 5. The Cavalieri method was used to quantify the volume of the LGd and LGv in normal (light gray) and bilaterally enucleated (dark gray) opossums. Experimenters were blind to condition when measuring the size of the nuclei. The volume of the LGd was significantly smaller in bilaterally enucleated opossums [$t(13.127) = 9.557$, $P < 0.001$]. There was also a significant decrease in the volume of the LGv in bilaterally enucleated opossums [$t(14.202) = 7.775$, $P < 0.001$]. Abbreviations are defined in Table 1.

$P < 0.001$]. There was no difference between groups in the relative size of S2, A1, or FM. Thus, we found that although there was no change in the overall size of the neocortex, the percentage of the neocortex devoted to the visual areas V1 and CT was significantly smaller in bilaterally enucleated opossums, and the percentage of the neocortex devoted to the somatosensory area S1 was significantly larger in bilaterally enucleated opossums, as compared with normal adults.

Discussion

In the present study, we demonstrate that bilateral enucleation early in development decreases the size of the brain, as measured by both weight and volume. This decrease is not caused by a change in the size of the cortical hemispheres but rather by a reduction in the size of the TMH. When we examined the visual pathway more specifically, we found a decrease in the size of the LGd and LGv in the thalamus. Although the overall size of the cortex is the same, the relative size of cortical fields changed in bilaterally enucleated opossums. Specifically, as compared with normal adults, the percentage of the neocortex devoted to the visual areas V1 and CT is significantly smaller in bilaterally enucleated opossums, whereas the percentage of the neocortex devoted to the somatosensory area S1 is significantly larger. Based on these results, as well as previous studies of functional organization and connectivity, it appears that early sensory loss not only alters the organization, connectivity, and size of structures associated with the deprived sensory system, but also cortical areas devoted to other sensory modalities as well.

Gross Anatomical Changes Following Bilateral Enucleation

One of the most striking findings in this study was a 14% reduction in the size of the TMH (both in weight and volume; Fig. 2), which is in stark contrast to the cortex that showed no change in size between normal and bilaterally enucleated animals. This decrease in the size of the TMH seemed to drive the reduction in total brain size and was most likely due to changes to several subcortical nuclei, such as the LGd and LGv, as well as the loss of the OT. These differences may be due to the differing rates at which the thalamus and the cortex

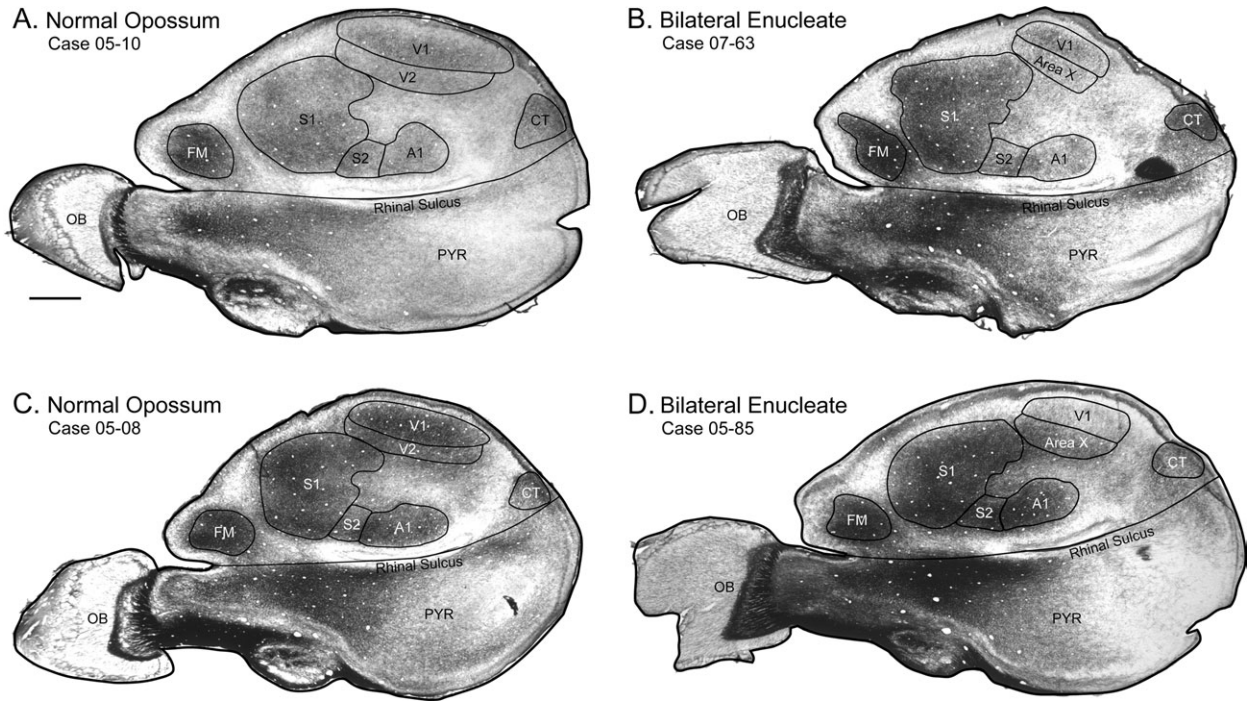

Figure 6. Digital images showing myeloarchitecture in flattened cortical sections of normal (*A* and *C*) and bilaterally enucleated (*B* and *D*) opossums. Although only one section from each case is shown, all cortical boundaries cannot be appreciated in a single section, and the entire series of sections was used to create a comprehensive reconstruction to determine the boundaries of cortical fields (as described in Karlen and Krubitzer 2006). In normal animals, primary sensory areas (V1, S1, and A1) are all darkly myelinated. As has been reported previously (Kahn and Krubitzer 2002a), the myeloarchitectonic appearance of most cortical areas is similar in normal and bilaterally enucleated animals. However, V1 is markedly smaller, and there is a darkly myelinated region adjacent to V1 called area X in bilateral enucleates (*B* and *D*). Dorsal/medial is up, rostral is left; scale bar = 1 mm; abbreviations are defined in Table 1.

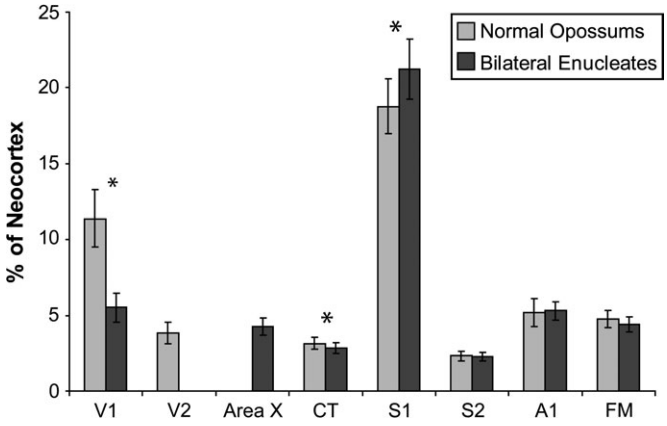

Figure 7. Comparisons in the percent of the cortical sheet devoted to each cortical field between normal (light gray) and bilaterally enucleated (dark gray) opossums. We found the relative size of V1 to be significantly smaller in bilaterally enucleated opossums [$t(39.156) = 13.478$, $P < 0.001$], and the size of CT to be significantly smaller in bilaterally enucleated opossums [$t(40) = 2.475$, $P = 0.018$], as compared with normal animals. Conversely, S1 was significantly larger in bilaterally enucleated opossums than in normal animals [$t(40) = -3.936$, $P < 0.001$]. There was no difference in the relative sizes of A1, S2, or FM between groups. Abbreviations are defined in Table 1.

develop. Specifically, at P4 (the age when our enucleations were performed), the ganglion cell axons from the retina have just entered the OT (Dunn et al. 2001), thalamocortical axons have started to grow out of the dorsal thalamus and arrive at the cortex by P5, and the thalamic nuclei begin to differentiate at P5 (Molnar et al. 1998). In the cortex, the first cells of the cortical plate are just beginning to arrive at P4, the cortical layers form between P4 and P20 (Molnar et al. 1998), and cortical development continues through P45 (Saunders et al. 1989). Thus, plasticity may be greater in neural regions that develop later and continue to develop for longer periods of time than those that develop earlier. Another possibility is that structures that receive direct input from the primary afferents, such as the LGd and LGv, are more dramatically affected by the loss of inputs and their activity, then are second order structures, like the neocortex.

Interestingly, structural alterations at subcortical levels have also been noted with early monocular enucleations in monkeys (e.g., Rakic and Riley 1983). These authors demonstrate that the early removal of one eye results in an increase in the size

and number of axons in the remaining optic nerve. This demonstration of remarkable subcortical plasticity within the visual system suggests that neurons in one structure can influence the size of another structure that shares synaptic territory.

Subcortical Changes Following Bilateral Enucleation

When we examined the thalamus in more detail, we observed that both the LGd and LGv were significantly smaller in bilaterally enucleated opossums (LGd decreased 46% and the LGv decreased 37%; Fig. 5). Although the reduction in size of the LGd may, in part, account for alterations in the size of V1 (described below), the reduction in the size of the LGv is unlikely to contribute to the changes observed in V1. These results are consistent with previous studies which demonstrate that bilateral enucleation in early postnatal rats causes a reduction in the size of the LGd and the LGv in the anteroposterior axis, as well as a decrease of 35–45% in the cross-sectional area of the LGd and 21–24% in the cross-sectional area of the LGv (Tsang 1937). In mice, the LGd loses more than half its volume following early postnatal bilateral enucleation. This decrease is due to a reduction in the size of neurons as well as in the number of neurons and glial cells in the LGd (Heumann and Rabinowicz 1980). In cats bilaterally enucleated on the day of birth, the size of the LG decreases by 53%, as compared with normal controls (Berman 1991). Lastly, in primates enucleated prenatally, there is a loss of lamination in the LG (Dehay et al. 1996a), and the volume of the LG decreases by 43–68% (Williams et al. 1987; Rakic 1988; Rakic et al. 1991; Dehay et al. 1996a). Further, there is a relationship between the age of enucleation and the reduction in LG size such that the earlier an animal is enucleated, the greater the LG is reduced (Rakic et al. 1991; Dehay et al. 1996a). In primates, this decrease in the size of the LG can be attributed in part to a decrease in the number of cells (Williams et al. 1987; Rakic et al. 1991). In the current study, we did not count the number of cells, so it is unclear whether the changes we observed were due to a decrease in cell size or in cell number. Taken together, these studies in a variety of species demonstrate that the loss of primary sensory afferents early in development can lead to a significant decrease in the size of the LG. The magnitude of the size decrease differs between studies and across species and is likely related to the developmental stage at which the retinal inputs are lost (Chabot et al. 2007).

Despite the severe atrophy of the LG, it is remarkable that it is present at all given the complete lack of retinal input, especially in species in which this loss occurred very early (e.g., primates, opossums). This retention of the LG is due, in part, to the genetic constraints imposed on the developing thalamus that predetermine the formation of structures regardless of input (Lim and Golden 2007). In fact, although this discussion deals mainly with activity-dependent contributions to nervous system construction, the role of genes is also paramount, and recent studies clearly demonstrate the important role they play in the formation of cortical fields (e.g., Sur and Rubenstein 2005; O'Leary et al. 2007 for review) and thalamocortical interactions (e.g., Uziel et al. 2006; Harada et al. 2007 for review).

The retention of the LG is also likely due to the invasion of inputs from other modalities, which would lead to a sensory respecification of this nucleus. Evidence for the latter comes from studies in congenitally blind mice and mice bilaterally enucleated at birth. In these animals, ascending somatosensory projections from the dorsal column nuclei innervate the LGd (Asanuma and Stanfield 1990). Further, in bilaterally enucleated hamsters (Izraeli et al. 2002), anophthalmic mice (Chabot et al. 2008), and naturally blind animals, such as the blind mole rat (Doron and Wollberg 1994), the inferior colliculus provides a major source of input to the LGd. Interestingly, the topographic relationship of the connections from the LG to the neocortex in bilaterally enucleated animals is roughly normal, as has been demonstrated in ferrets and tree shrews, although in tree shrews the laminar organization of LG is disrupted (Brunso-Bechtold and Casagrande 1981; Brunso-Bechtold et al. 1983; Guillery et al. 1985). Thus, following early sensory loss, subcortical nuclei like the LG can be commandeered for cross-modal processing by the remaining, intact sensory modalities, and these changes in subcortical structures are relayed to the neocortex (Negyessy et al. 2000; Hunt et al. 2005).

Cortical Field Changes Following Bilateral Enucleation

Although we saw no change in the overall size of the cortex in bilaterally enucleated opossums, the relative size of V1 decreased by 51%, as compared with normal animals (Fig. 7). This reduction in the size of V1 has been described qualitatively in short-tailed opossums (Kahn and Krubitzer 2002a; Karlen et al. 2006) and has been previously demonstrated in primates that were bilaterally enucleated prenatally (Rakic 1988; Dehay et al. 1989, 1991; Rakic et al. 1991). As with the LG, in primates there is a relationship between the age of enucleation and the reduction in the size of V1 such that the earlier an animal is enucleated, the more the V1 is reduced in size (Dehay et al. 1991, 1996a; Rakic et al. 1991). Furthermore, in primates this decrease in V1 is accompanied by an increase in the size of extrastriate visual fields (Dehay et al. 1996a), where extrastriate fields were defined by subtracting the size of V1 from the total occipital-temporal cortex size. Dehay and colleagues found that there was a linear relationship between the age of enucleation and size of extrastriate visual cortex such that the younger an animal was at the time of enucleation the larger extrastriate visual cortex became. There was no correlation between age of enucleation and occipital-temporal cortex size. Unlike primates, our opossums did not show a corresponding increase in extrastriate areas following enucleation. In fact, in bilaterally enucleated animals, we saw a 10% decrease in the size of the CT (Fig. 7), an area that is proposed to be associated with visual processing (Huffman et al. 1999). This finding differs from previous studies in primates because the extrastriate visual area CT in opossums decreases in size while extrastriate cortex in primates appeared to increase in size (Dehay et al. 1996a).

An interesting observation was the inclusion of a new architectonic zone (area X) at the rostrolateral border of V1. In bilaterally enucleated primates, a new architectonic zone in a similar location was also observed and termed area X (also called hybrid cortex, Dehay et al. 1991; Rakic et al. 1991) or default extrastriate cortex (Dehay et al. 1996a, 1996b). We use the area X terminology to demonstrate similarity, not homology. To our mind, this instance of what appears to be a novel architectonic zone that emerges after bilateral enucleation in 2 very distantly related species is extremely interesting. Given that the common ancestor of primates and marsupials existed about 180 million years ago (Murphy et al. 2004), the similarity of this region in terms of location and architectonic appearance

in both primates and marsupials suggests that there are highly conserved developmental mechanisms involved in cortical field emergence that impose considerable constraints on the development and evolution of cortical fields.

It is tempting to consider area X a new, although nonhomologous, cortical field in these species, which was first suggested by Rakic (Rakic et al. 1991). Nevertheless, given the available data, there are several equally plausible interpretations regarding the status of area X. First, as noted above, it is possible that area X is a new cortical field. Support for this proposition comes from its unique architectonic appearance (Rakic 1988; Dehay et al. 1989; Rakic et al. 1991; Kahn and Krubitzer 2002a) and from previous electrophysiological recordings of this region in bilaterally enucleated opossums (Kahn and Krubitzer 2002a). Functionally, area X is a multimodal area in which neurons respond to somatosensory stimulation of the head, neck, and face and to auditory stimulation. Second, it is possible that area X is a reorganized portion of area 18 (Dehay et al. 1996a). In bilaterally enucleated opossums, area X is approximately the same size as area 18 (Fig. 7) and is in a similar location to area 18. However, its architectonic appearance is markedly different than area 18 in both primates and opossums (Rakic 1988; Dehay et al. 1989, 1996b; Rakic et al. 1991; Kahn and Krubitzer 2002a). Finally, it is possible that area X is an expansion of multimodal cortex, which is supported by the electrophysiological recording data (Kahn and Krubitzer 2002a). Although all three of these hypotheses are viable, to determine which may be true, it will be necessary to examine the connections of area X as well as the early molecular and anatomical development of this area in a variety of animals that have undergone early bilateral enucleation.

We found no change in the size of A1 in bilaterally enucleated opossums, which is consistent with other enucleation studies in which only higher order auditory areas have been found to change following bilateral enucleation (for review, see Rauschecker and Henning 2001). Conversely, we did find an expansion of S1 in bilaterally enucleated opossums, which suggests that in opossums, somatosensory cortex, rather than extrastriate cortex or auditory areas, enlarges at the expense of V1 and extrastriate area CT. This change in somatosensory cortex following enucleation has not been described in primates, but comparisons between previous studies in primates and the current study in opossums are problematic. In primates, a restricted area of visual cortex was measured, as opposed to the entire cortical sheet in opossums, and the size of cortical fields relative to the overall size of the cortical sheet was not considered in primates. Further, S1 has not been measured in studies of bilateral enucleated primates. Thus, it is not known whether nonvisual cortical fields expand following early bilateral enucleation in primates. Finally, previous studies, in which the functional organization of much of the cortical hemisphere was ascertained, demonstrate that multimodal cortex (MM) appears to expand in size in bilaterally enucleated opossums (Kahn and Krubitzer 2002a), and this expansion may account for some of the reallocation of cortical space observed in the present study. MM is located caudal to S1, medial to A1, and rostrolateral to V1. Because all the boundaries of MM could not be accurately and consistently drawn across cases, we did not include this region of cortex in our measurements.

There is evidence that cross-modal changes occur in nonvisual cortical areas in other mammals as well. For example, in rats bilaterally enucleated on the day of birth, the functional border of S1 is shifted posteriorly, as compared with normal adults (Toldi et al. 1990). Also, the receptive fields of neurons within individual barrels were altered, such that in some barrels, receptive fields were significantly enlarged, and in other barrels, the angular sensitivities of the neurons were better defined (Toldi et al. 1994). In bilaterally enucleated mice, the overall size of the barrel field in each hemisphere increases significantly, as well as the size of individual barrels (Bronchti et al. 1992; Rauschecker et al. 1992). Although this increase in barrel size was restricted to a subset of whiskers, there was no indication that the remaining barrels decreased in size. This suggests that there was an increase in the overall size of S1, although S1 was not explicitly measured in these studies, nor was it determined what effect this increase in barrel cortex had on the size of other cortical areas.

The types of cross-modal changes observed in experimental animals are also observed in naturally blind animals, such as the blind mole rat. Blind mole rats live in a dark, subterranean environment. They rely little upon their visual system and more extensively on their auditory system for navigation as well as for communication with conspecifics (for review, see Bronchti et al. 2002). In addition, there is evidence that S1 has expanded by about 20% in blind mole rats, as compared with sighted rats (Necker et al. 1992). Further, the remnants of their visual system have been commandeered for processing auditory stimuli. For example, the size of the LGd is significantly smaller in blind mole rats, as compared with seeing rodents, due to a reduction in the number of retinofugal axons and the subsequent degeneration of the projections (Bronchti et al. 1991; Rehkamper et al. 1994). The LG receives input from the inferior colliculus (Bronchti et al. 1989), which in turn projects to "visual" cortex. Consequently, neurons in this visual cortex respond to auditory stimulation (Bronchti et al. 1989; Heil et al. 1991). Thus, in the blind mole rat, there is a decrease in the size of "visual" areas, such as LG and V1, an expansion of the remaining sensory systems, and the regions of the brain that in other mammals would normally process visual stimuli are coopted by the remaining sensory systems.

Although cross-modal plasticity is usually studied experimentally, evidence from naturally blind animals, like the blind mole rat, and other mammals with extreme specializations indicate that cross-modal interactions occur naturally during the development of the nervous system. These interactions between sensory systems are important for allowing the cortical phenotype, generated from any given genotype, to match environmental demands and fluctuations. Thus, the relative patterns of activity between sensory systems, resulting from variable, complex, and often dynamic environments, play an important role in optimizing fundamental characteristics of cortical organization including cortical field modality, sensory domain allocation, patterns of connectivity, and cortical field size.

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Notes

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