Development of Connections in the Human Visual System During Fetal Mid-Gestation: A DiI-Tracing Study

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Abstract. Animal studies have shown that connections between the retina, lateral geniculate nucleus (LGN), and visual cortex begin to develop prenatally. To study the development of these connections in humans, regions of fixed brain from fetuses of 20–22 gestational weeks (GW) were injected with the fluorescent tracer DiI. Placement of DiI in the optic nerve or tract labeled retinogeniculate projections. In the LGN, these projections were already segregated into eye-specific layers by 20 GW. Retinogeniculate segregation thus preceded cellular lamination of the LGN, which did not commence until 22 GW. Thalamocortical axons, labeled from DiI injections into the optic radiations, densely innervated the subplate, but did not significantly innervate the cortical plate. This pattern was consistent with observations of a “waiting period” in animals, when thalamocortical axons synapse in the subplate for days or weeks before entering the cortical plate. Cortical efferent neurons (labeled retrogradely from the optic radiations) were located in the subplate and deep layers of the cortical plate. In summary, human visual connections are partially formed by mid-gestation, and undergo further refinement during and after this period. The program for prenatal development of visual pathways appears remarkably similar between humans and other primates.

Key Words: Corticothalamic; DiI; Lateral Geniculate Nucleus; Retina; Striate Cortex; Subplate; Thalamocortical.

INTRODUCTION

Although tremendous progress has been made in mapping brain connections and their development in animals, our knowledge of pathways in the human brain has remained imprecise (1). A more detailed knowledge will be essential for recognizing abnormal connections, which may contribute significantly to the pathogenesis of some neurological and psychiatric diseases (e.g. mental retardation, cerebral palsy, epilepsy, and schizophrenia). Furthermore, by comparing the development of axon pathways in normal and pathological brains, it should be possible to learn when and how connections become faulty in disease conditions and so gain insights into their pathogenesis. Recently, the development of axon pathways has been studied in a few areas of the human brain—for example, the arcuate nucleus of the medulla (2) and the hippocampal formation (3)—but many important connections, including those between the thalamus and cortex, have not yet been explored.

In the present study, the development of human visual connections was traced during the period around mid-gestation using the fluorescent tracer DiI. This lipophilic molecule diffuses both anterogradely and retrogradely through the axolemma. The visual system was selected for study for several reasons: First, the visual system affords an opportunity to examine connections between the thalamus and the isocortex (neocortex), which may develop according to the same general principles throughout their extent (4, 5). Second, there is a rich context of previous studies on the development of visual pathways in animals, including primates (6–11), providing a basis for comparative analysis. Third, previous studies have described the histological development of the human lateral geniculate nucleus (LGN) (4, 12–17) and visual cortex (4, 11, 18, 19) in detail. Fourth, the anatomical components of the visual system (i.e. the optic nerves and tracts, lateral geniculate nuclei, and calcarine cortex) are easily identified in fetal brains so that DiI injections can be placed accurately for experiments.

Classic studies using axon impregnation have suggested that retinal fibers innervate the LGN remarkably early in the 40-week human gestation. (Gestational age is defined as the time elapsed since the first day of the last menstrual period [20].) Fibers in the optic tract reach the LGN anlage by about 7 gestational weeks (GW) (12, 13). Synapses between optic fibers and LGN cells are formed by about 13–14 GW (17). At such early fetal ages, the LGN anlage appears as a homogeneous collection of cell bodies. Later, the LGN neurons aggregate intro 6 cellular layers, which begin to be evident at about 22 GW (4, 12, 14, 15). Each cellular layer ultimately receives innervation from only 1 eye. The segregation of retinogeniculate afferents into eye-specific layers has not been studied in humans, but in other mammals the retinal afferents from each eye initially overlap within the LGN and then redistribute by an activity-dependent (tetrodotoxin-sensitive) process (21–23). Furthermore, afferent segregation precedes LGN cellular lamination in all mammals that have been studied thus far (22, 23). In cats, for example, retinal afferents segregate 2 weeks before cellular layers become visible in the LGN (22). One goal of the present...
study was to determine if human retinogeniculate afferents segregate prior to 22 GW when LGN cellular layers first appear.

Previous histological studies also suggest that connections from the LGN to the cortex begin to form prior to mid-gestation in humans. Electron microscopy has shown that axons of presumed thalamic origin make synapses in the subplate (a transient cellular and synaptic layer beneath the developing cortical plate) by about 11–13 GW, and possibly as early as 8.5 weeks (4, 11, 24). However, synapses do not form within the cortical plate (the anlage of the mature cortex) until about 23–25 GW (11, 24). Thus, thalamocortical axons seem to “wait” in the subplate for several weeks before innervating the cortical plate. Indeed, axon tracing studies in cats (25) and monkeys (7, 11) have shown that thalamocortical fibers wait in the subplate for some weeks before growing radially into the cortical plate. Histochemistry for acetylcholinesterase, a marker of fetal thalamocortical axons, suggests that humans also have a waiting period (11), although this has never been documented by axon tracing. Thus, a second goal of the present study was to determine if human thalamocortical fibers end in the subplate at mid-gestation, which would be consistent with the existence of a waiting period in humans.

The development of cortical efferent connections has been studied very little in humans. In other mammals the first projections out of the cortex arise from neurons located in the subplate (5, 26). Generally, the corticothalamic projections develop concurrently with the thalamocortical projections, so that cortical axons reach the LGN at about the same time as LGN axons reach the cortex (7, 9, 25, 27). In mature animals, layer 6 is the major source of corticobulbar and corticospinal projections (5), although this distinction may not hold during development when different connections may form transiently (28). The third goal of the present study was to determine which layers give rise to cortical efferent projections in humans.

In sum, the major objectives of this study were 1) to test the hypothesis that visual pathways in humans develop according to the same principles as in other mammals; 2) to better characterize the stage of connectional development in human fetal brains so that the effects of intrauterine insults might be better understood; and 3) to provide further information about normal connections in humans that could be used for comparison with pathological specimens.

MATERIALS AND METHODS

Four fetal brains were used for DiI tracing (ages 20–22 GW). The brains were removed from therapeutically aborted fetuses examined in autopsies at the Brigham and Women’s Hospital (Boston, MA). The protocol was approved by the Human Protection Committee. The brains were removed within 4–6 hours after death and fixed by immersion in at least a 10-fold excess volume of cold (−4°C) 0.1 M phosphate buffer (pH 7.2) containing 4% paraformaldehyde fixative and 4% sucrose. The brains were normal as determined by visual inspection and histological examination.

After fixation for 2–7 days, the brains were dissected into blocks for DiI injections. Each block of tissue was used for a separate experiment. Tissue blocks contained the following regions: 1) for optic tract injections: the LGN and ipsilateral optic tract; 2) for optic nerve injection: the LGN, ipsilateral optic tract, optic chiasm, and both optic nerves; 3) for optic radiations injection: the occipital lobe, removed by a coronal cut through the hemisphere at the temporo-occipital junction. Small crystals (200–400 μm) of DiI (Molecular Probes, Eugene, OR) were implanted into the optic tract (n = 4), optic nerve (n = 2), or optic radiations (n = 2) using a 30 gauge needle. Each block was injected with DiI in only 1 location.

Blocks were incubated in fixative at room temperature for 17–45 weeks to allow for diffusion of the DiI tracer. The blocks were then cryoprotected, sectioned coronally on a freezing sliding microtome, counterstained with DAPI (a fluorescent DNA-binding molecule) (Sigma, St. Louis, MO), and examined by fluorescence microscopy as described previously (3). Slides or negatives of fluorescence micrographs were scanned digitally and assembled into montages using Photoshop (Adobe).

For histological examination, hematoxylin and eosin-stained paraffin sections through the LGN of normal fetal brains were also examined and photographed. These slides were from the collection of fetal autopsy cases at the Brigham and Women’s Hospital.

RESULTS

Emergence of LGN Cell Layers

Previous studies have found that the LGN first forms as a homogeneous collection of cells and subsequently develops its characteristic 6-layered structure over the period from 22–25 GW (4, 12, 14, 15). The 2 magnocellular layers become evident first (15) at the medial edge of the LGN (Fig. 1A). By 25–27 GW, all 6 cellular layers are visible (Fig. 1B). The LGN cell layers are initially oriented parallel to the dorsoventral axis (Fig. 1B), but due to rotation and differential growth of the thalamus during development, become oriented almost parallel to the mediolateral axis by the time of birth (4, 14, 15).

Retinogeniculate Projections

The projection of retinal axons into the LGN was studied after DiI injection into the optic nerve to label axons originating from 1 eye, or into the optic tract to label the entire complement of axons from both eyes. At 20 GW, DiI placement into 1 optic nerve labeled large bundles of axons that entered the LGN at an oblique angle and then terminated in alternating fields of dense and sparse innervation, suggestive of eye-specific laminar territories (Fig. 2A). The impression that these fields were related
Histological development of the human LGN (coronal sections, H&E). A: At 22 GW, faint lamination is visible at the medial edge of the nucleus, heralding the emergence of magnocellular layers 1 and 2. The cell-dense structure (asterisk) is the caudal (temporal) ganglionic eminence. B: At 27 GW, all 6 LGN layers are now visible. The layers at this age are oriented parallel to the dorsoventral axis, but later in development become more closely aligned with the mediolateral axis due to growth and rotation of the dorsal thalamus (4, 14, 15). Axes: dorsal (D), lateral (L), medial (M), and ventral (V). Scale bar, 1 mm for both panels.

to the future cellular layers of the LGN was supported by their dorsoventral orientation, the same orientation as for early cellular layers (Fig. 1B). Furthermore, DiI labeling delineated 6 distinct zones identified as likely precursors of the 6 cellular layers of the LGN (Fig. 2A). DAPI staining of the same section showed no evidence of cellular lamination (Fig. 2B), indicating that retinogeniculate afferent segregation preceded cellular lamination. DiI placement in the optic tract at 20 GW labeled fiber bundles that terminated throughout a diffuse territory within the LGN and showed no evidence of alternating fields (Fig. 2C). There was likewise no evidence of cellular lamination by DAPI staining (Fig. 2D). Thus, the segregation of retinogeniculate afferents was observed only when afferent fibers were labeled from 1 eye, but not from both eyes (i.e. segregation was eye-specific).

At 22 GW DiI placement in the optic tract labeled terminal fields that were not strictly homogeneous, but
Fig. 2. DiI transport to the fetal LGN from injections into the optic nerve or optic tract (coronal sections). DiI fluorescence is shown in the upper panels (A, C, E), and DAPI fluorescence (which labels cell nuclei) of the same sections in the lower panels (B, D, F). A: DiI transport from the left optic nerve to the right LGN, 20 GW. Prospective layers 1, 4 and 6 were brightly labeled in accordance with the mature pattern of LGN innervation from the contralateral eye in primates (7). Labeled fiber bundles entered the LGN obliquely (arrowheads) before segregating into laminar territories. Afferent segregation appeared incomplete, especially in the ventral portion of the nucleus. Dorsal (D), ventral (V), medial (M), and lateral (L) axes are indicated. B: DAPI fluorescence confirmed that cellular lamination had not yet developed. C: DiI transport from the left optic tract to the left LGN, 20 GW (same brain as in [A]). The LGN was diffusely labeled. D: DAPI fluorescence showed absence of cellular lamination. E: DiI transport from the right optic nerve to the right LGN, 22 GW. Labeling was concentrated in cellular layers, demonstrated by DAPI fluorescence in the same section (F). Scale bar, 1 mm for all panels.

were related to the emerging cellular layers of the LGN. Stripes of intense DiI labeling (Fig. 2E) were located in each of the cell-dense LGN layers (Fig. 2F). Faint DiI labeling was evident in thinner stripes, corresponding to the interlaminar zones. This result indicated that retinogeniculate innervation was densest within cellular layers, even at the earliest stages of their formation. The stripes of faint labeling were not related to eye-specific segregation, but to concentration of afferents in the cell-dense laminar compartments. Optic nerve innervation of the LGN was not studied at this age, due to a lack of specimens with adequately preserved optic nerves and chiasm.

Finally, no retrogradely labeled neurons were observed in the LGN after DiI injection into either the optic nerve or the optic tract at 20 or 22 GW. This result was consistent with the unidirectional flow of visual projections from the retina to the LGN, but not vice versa.

Geniculocortical and Cortical Efferent Projections

Connections between the LGN and calcarine (visual) cortex were studied by injecting DiI into the optic radiations. DiI was placed in the intermediate zone (developing white matter) of the medial occipital lobe, within 2–3 cm of the presumptive striate cortex. This site was chosen because at mid-gestation the distance between the LGN and calcarine cortex is already many centimeters and thus too long for efficient DiI labeling.

DiI injection into the optic radiations labeled radial glia (not shown), as well as corticothalamic and cortical efferent axon systems (Fig. 3). Radial glia were identified
Fig. 3. Dil transport to the fetal occipital cortex from an injection in the optic radiations (coronal sections). A: Dil labeling was most intense in the subplate, a relatively cell-sparse zone directly beneath the cortical plate. Retrogradely labeled neurons (arrows) were present in the cortical plate and in the subplate where they were obscured by intense fiber labeling. B: DAPI fluorescence of the same section shown in (A). The normal histology of the mid-gestational cortex is demonstrated. C: Double exposure for Dil and DAPI fluorescence showed that labeled fibers were concentrated in the subplate, directly beneath the cortical plate. Arrows indicate retrogradely labeled neurons in the cortical plate. D: Higher magnification of Dil fluorescence in the cortical plate. Retrogradely labeled neurons (arrows) had round or pyramidal cell bodies with thick ascending dendrites that ended in an apical tuft. E: DAPI fluorescence of the same section shown in (D). The cortical plate has 3 distinct layers corresponding to prospective layers 5 and 6 and, superficially, a zone of dense cortical plate (dcp) containing newly migrated cells (4). F: Double exposure for Dil and DAPI fluorescence. Retrogradely labeled neurons were located mainly in prospective layers 5 and 6 and in the subplate (obscured due to intense fiber labeling). Retrogradely labeled cortical plate neurons gave rise to apical dendrites ending in tufts in the deep half of the marginal zone. Abbreviations: cp, cortical plate; iz, intermediate zone; mz, marginal zone; sp, subplate; svz, subventricular zone; vz, ventricular zone. Scale bar, 1 mm for A–C and 400 μm for D–F.
Fig. 4. Comparison of visual system development in the macaque monkey (upper line) and human (lower line). In humans, the conventional 40-week gestational period includes 12 days after the last menstrual period but prior to fertilization (20). Horizontal arrows indicate events that may occur prior to the indicated age due to gaps between ages that have been studied. Horizontal lines above the monkey timeline indicate the periods when cells destined for each cortical layer undergo their last cell division, i.e. cell birthdates (8). See text for other references. Abbreviations: CP, cortical plate; LGN, lateral geniculate nucleus; SP, subplate.

Comparison of Human and Monkey Visual System Development

To compare the development of visual pathways in humans and monkeys, a timeline was constructed indicating when developmental events occur relative to the entire gestational period from conception to birth (Fig. 4). The results showed a remarkable degree of similarity between these species. For example, LGN lamination begins at approximately the same relative time in gestation, and the thalamocortical waiting periods cover overlapping time...
segments (11). This comparison supports the idea that events in visual development that are not amenable to experimental study in humans, such as the dates of last mitotic division (“birthdates”) of cells in different layers and the tempo of cell migration from the ventricular zone to the cortex, are probably also conserved between monkeys and humans.

**DISCUSSION**

In the present study, 5 specific issues in human brain development were addressed: 1) Do pathways in the human visual system develop according to the same general plan as in other mammals, especially primates? 2) Does segregation of retinogeniculate afferents precede cellular lamination in the LGN? 3) With regard to cortical development, is the subplate relatively wide in humans (and other primates) as compared with other mammalian groups (11)? 4) Is there a waiting period for thalamocortical axons in humans? 5) Which cell layers give rise to cortical efferent projections in humans? In addition, the present study helps to establish a normal baseline of visual system development at mid-gestation, which may be used for evaluation of fetal brains with potentially abnormal connections.

The present results support the overall hypothesis that visual connections in humans develop similarly as in other mammals. DiI injection into 1 optic nerve at 20 GW clearly demonstrated that afferent segregation (Fig. 2A) occurred prior to cellular lamination (Fig. 2B) in humans, as it does in all other mammals studied thus far (22, 23). Likewise, DiI injection into the optic radiations showed that the great majority of thalamocortical fibers terminated in the subplate during mid-gestation (Fig. 3A–C), as is the case in fetal monkeys at mid-gestation (6, 7, 11). The subplate is also the primary target of thalamocortical afferents in nonprimate mammals (e.g. cats [25]). The origin of cortical efferent fibers from neurons in developing layer 5, layer 6, and the subplate (Fig. 3D–F) is also consistent with findings in other mammals (5, 26). Finally, the sequence and tempo of fetal visual development are very similar between humans and monkeys (Fig. 4).

The results of thalamocortical axon labeling reinforce previous evidence that the subplate is expanded in humans and other primates, as compared with nonprimate mammals. Kostovic and Rakic (11) used histochemistry for acetylcholinesterase, a marker of thalamocortical axons, to study human fetal cortex and found that the acetylcholinesterase-rich zone (presumed subplate) in visual cortex was equal in width to the overlying cortical plate. In the present study, the DiI results likewise indicated that the zone beneath the cortical plate that contained thalamic afferents—the defining feature of the subplate (11)—was approximately equal in width to the overlying cortical plate (Fig. 3A–C). In contrast, the subplate in nonprimate mammals is markedly thinner than the cortical plate (5, 11, 25). The expansion of the subplate in primates may be important for organizing complex cortical afferents, not only from the thalamus, but also from ipsilateral and contralateral areas of cortex (11).

The thalamocortical labeling results were also consistent with the hypothesis that humans have a prolonged waiting period, but since only the period from 20–22 GW was studied, the full duration of the waiting period could not be determined. Studies using electron microscopy alone, DiI studies at more ages will be necessary to accurately delineate the waiting period.

The neurons that give rise to cortical efferent pathways were localized to prospective layers 5 and 6, and the subplate (Fig. 3D–F). In other mammals, the cortical efferent pathways originate from these same layers, each of which projects to distinct targets. Subplate neurons pioneer the first axonal pathway out of the cortex and terminate in the vicinity of the thalamus (5, 26). Clasca et al (28) have presented evidence, however, that collaterals of layer 5 axons grow into the thalamus before axons from the subplate or layer 5, and that the layer 5 collaterals subsequently retract. In mature animals, layer 5 axons provide the major cortical projection to the brainstem and spinal cord (5, 28) and layer 6 axons provide the major projection to the thalamus (5, 27). Further studies will be necessary to resolve the roles of projections from each layer during development.

The apical dendrites of retrogradely labeled cortical neurons formed elaborate tufts within the deep half of the marginal zone (Fig. 3D–F). Interestingly, this sublayer is also rich in synapses (11, 24) and cytochrome oxidase (19), a marker of overall neuronal functional activity (29). Together, these observations suggest that the apical dendrites of cortical efferent neurons participate in neuronal circuits that are synaptically and electrically active during fetal life. Likely sources of axonal input to this layer include Cajal-Retzius neurons or, possibly, monoaminergic afferents from outside the cortex (4, 11). The significance of these circuits is unknown.

One limitation of the present study is that few brains were studied (n = 4). Although it would clearly be valuable to have more cases and study additional ages, tissues suitable for DiI tracing are difficult to obtain. The DiI method works well only with brain regions that have
been removed without disruption and fixed in paraformaldehyde after a short postmortem interval.

Do Abnormal Connections Contribute to Neuropsychiatric Illnesses?

Given the complexity of axonal pathways in the normal human brain, it seems likely that some neurological and psychiatric diseases may be caused in part by abnormal connections. Thus, one goal of axon tracing studies is to characterize the normal pathways as a basis for comparison of pathological cases. In addition to the visual pathways described here, normal connections have also been traced in the human fetal hippocampus (3), brainstem (2), and intracortical circuits (31). In future studies, diseased brains should be examined with axon labeling methods to test for abnormal connections. One such study has recently been published in which DiI tracing revealed abnormal cortical connections in cases of nodular heterotopia with epilepsy (31).

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REFERENCES


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