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Formation of eye-specific retinogeniculate projections occurs prior to the innervation of the dorsal lateral geniculate nucleus by cholinergic fibers

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Abstract

We compared the developmental periods in the mouse when projections from the two eyes become segregated in the dorsal lateral geniculate nucleus with the time when this nucleus becomes innervated by cholinergic fibers from the brainstem. Changes in labeling patterns of different tracers injected into each eye revealed that segregation of retinogeniculate inputs commences at postnatal day five (P5) and is largely complete by P8. Immunocytochemical staining showed that cholinergic neurons are present in the parabrachial region of the brain stem on the day of birth. However, cholinergic fibers are not evident in the geniculate until P5, and these are sparse at this age, increasing in density to form well-defined clusters by P12. These results indicate that segregation of eye-specific projections during normal development is unlikely to be regulated by cholinergic inputs from the brainstem.

Keywords

mouse; development; visual system; brainstem

INTRODUCTION

The dorsal lateral geniculate (dlgn) of the thalamus provides the main conduit by which visual information from the two eyes is conveyed to the primary visual cortex (Jones, 2006). However, most synapses in the dlgn emanate from non-retinal sources (Guillery, 1969). A prominent, non-retinal structure that innervates the dlgn and contributes the major cholinergic input to the thalamus is the parabrachial region of the brain stem (Steriade *et al.*, 1988; Smith *et al.* 1988). There is considerable evidence to implicate the cholinergic neurons of the parabrachial region in the expression of rapid eye movement activity during sleep (Kodama and Honda, 1996; Meney *et al.*, 1998; Carden *et al.*, 2000) and modulation of dlgn visual responses during arousal and attention (Uhlrich *et al.*, 1995; Ozaki and Kaplan, 2006).

Our interest in the cholinergic projection stems from the possibility that it might have a role in the development and plasticity of binocular retinal inputs to the dlgn. It was demonstrated first

by Rakic in the fetal monkey (Rakic, 1976) and, subsequently, in many other species that early in development the projections from the two eyes overlap in the dlgn before segregating into eye-specific domains, and several lines of evidence indicate that neuronal activity has a key role in this process (Chapman, 2000; Huberman *et al.*, 2003). In particular, several recent papers have relied on $\beta 2^{-/-}$ mice, which lack the $\beta 2$ subunit of the nicotinic acetylcholine receptor, to assess the role of retinal activity on the state of retinogeniculate projections (Rossi *et al.*, 2001; Grubb *et al.*, 2003; Grubb and Thompson, 2004a; Grubb and Thompson, 2004b; Torborg *et al.*, 2004; Torborg *et al.*, 2005). In these animals, normal patterns of retinal activity are perturbed, as is the normal segregation of left- and right-eye projections to the dlgn. Thus, the results of studies with $\beta 2^{-/-}$ mice have been interpreted as supporting the role of retinal activity in the formation of segregated retinogeniculate projections. Because nicotinic acetylcholine receptors are expressed by dlgn neurons (Zoli *et al.*, 1998; Sherman and Guillery, 2002), a potential drawback of this work is that the abnormalities noted in the segregation of ocular projections in the mutant mice might be caused by a lack of $\beta 2$ nicotinic acetylcholine receptor subunit centrally, rather than perturbed patterns of retinal activity.

OBJECTIVES

Our objective was to compare the developmental periods when eye-specific retino-dlgn projections are established with the formation of the cholinergic projection to the dlgn from the brainstem parabrachial region. If cholinergic inputs are present when eye-specific inputs are being established, one could conclude that this non-retinal input might have a role in the segregation process. By contrast, if the parabrachial-dlgn pathway forms later, after the establishment of eye-specific projections, this would rule out a role for cholinergic involvement in the segregation process. The development of cholinergic projections from the parabrachial region to the dlgn has been studied in cats (Carden *et al.*, 2000) but not mice, a species that is gaining considerable acceptance as a model for studies of visual development and plasticity. Here we have used retinal injections of dual tracers to quantitatively assess the period of binocular overlap and segregation in wild-type mice and assessed the formation of the parabrachial-dlgn projections in the same species using anterograde tracing and immunocytochemical methods. The results provide the first documentation of the developing parabrachial cholinergic pathway to the dlgn in mice.

METHODS

Animals

The development of the parabrachial cholinergic input to the dlgn was examined in C57BL/6 mice ranging in age from 1 day after birth (P1) to adulthood. Mouse pups were obtained from a UC Davis colony with breeding pairs from Charles River Laboratories. All protocols were approved by the UC Davis Institutional Animal Care and Use Committee (IACUC), and were in compliance with NIH guidelines.

Immunocytochemistry

Choline acetyl transferase (ChAT)-positive cell bodies in the brainstem and fibers in the dlgn were examined at seven postnatal dates, P1 ($n = 4$), P3 ($n = 5$), P5 ($n = 3$), P8 ($n = 4$), P12 ($n = 3$), P20 ($n = 3$) and in adults ($n = 3$). Mice were euthanized with Eutha-6 (0.1 ml of pentobarbital sodium; Western Medical Supply) and brains were fixed by perfusion using 4% paraformaldehyde in a 0.1 M phosphate buffer in the adults and by immersion perfusion in all other animals. The brains were removed from the skull, fixed overnight and sliced in 50 μ M coronal sections on a vibratome (Leica Microsystems). Adjacent sections containing the parabrachial region (pbr) or dlgn were either stained for Nissl substance using a standard protocol (Clerici and Coleman, 1990) or processed for ChAT immunocytochemistry. Tissue

sections processed for ChAT immunocytochemistry were incubated in 10% normal donkey serum, 0.02% BSA and 0.3% Triton-X100 in 0.1 M phosphate buffered saline (EM Science) for 1 hour to block non-specific staining. To label cholinergic cells, sections were incubated in a 1:50 dilution of an antibody for ChAT (Chemicon) for 72 hours at 4°C. A fluorescent secondary antibody, Alexa Fluor 594 (Molecular Probes) at a 1:500 dilution, was used to visualize ChAT staining. Incubation for 1 hour in DAPI (Molecular Probes) at a 1:500 dilution was used to stain cell bodies to better delimit the boundaries of the dlgn. Tissue was mounted using Vectashield mounting medium (Vector Laboratories) in preparation for imaging.

Tracing eye-specific segregation

Retinal projections were traced by making intraocular injections of cholera toxin- β (CTB) conjugated to fluorescent dyes (Molecular Probes). Mice at P0-P8 were anesthetized by immersion in ice water, whereas older animals (P12-adult) received an injection of 35 mg kg⁻¹ ketamine and 21 mg kg⁻¹ xylazine in physiological saline. Intraocular injections of 1.5 μ l of CTB conjugated to either Alexa dye 488 or Alexa dye 594 in 0.5% saline were made into the far temporal region of the eye with a micropipette. The eyes were treated with ophthalmic antibiotic and the animals placed on a warm surface until full movement was regained. Mice were allowed to survive for 24-48 hours at which time they were euthanized with Eutha-6 and perfused transcardially with 4% paraformaldehyde.

Imaging and analysis

An Olympus FV500 series confocal microscope equipped with a 405 diode, argon and krypton lasers was used to obtain images of the pbr and dlgn processed for ChAT. Confocal images were taken in the xyz axes with a total z-depth of 20 μ m with a 0.31 μ m z-step with a resolution of 1024 $\times$ 1024. Images were captured using 20 $\times$ or 40 $\times$ oil-immersion objectives, collapsed into a single layer using the Olympus Fluoview software, and the cholinergic fibers were traced using Adobe Photoshop. Images of the tissue from animals treated with CTB were captured on a Nikon Eclipse E600 upright microscope equipped with an ORCA-ER C4742-90 CCD camera (Hamamatsu Photonics) using a 10 $\times$ objective. Images were pseudocolored using the Wasabi software (Hamamatsu) and loaded to Adobe Photoshop and Illustrator for construction of montage. Parameters of >30% threshold for white and <30% threshold for black (Huberman *et al.* 2003) were used to analyze the tissue with the Image J program (NIH). Multiplying the threshold images of both projections and determining the number of pixels common to both projections provided a measure of binocular overlap. The number of pixels was selected automatically and measured using the Image J program. Four sections were used for analysis from the middle portion of the dlgn per animal.

RESULTS

Eye-specific segregation

At P1 the projections from the two eyes showed extensive overlap. Fig. 1 provides representative sections from the rostral, medial, and caudal extent of an animal at this age, where the label that stems from the contralateral eye is green and from the ipsilateral eye is red. The contralateral and ipsilateral labeling patterns have been superimposed in the images on the right, and the degree of overlap is signified by yellow, a blend of green and red. The contralateral projection at P1 was found throughout the dlgn whereas the ipsilateral projection is more restricted, being confined largely to the dorsolateral part of the nucleus.

Fig. 2 shows single sections from the dlgn showing the degree of binocular overlap in individual animals at five developmental ages (P1, P5, P8, P12 and P20) and adult animal taken from the core of the binocular region. Note that by P5 the ipsilateral projection occupies a much smaller portion of the dlgn compared to P1, but the degree of overlap is still substantial, as shown by

the yellow coloring. By P8 the retinal projections were largely segregated, indicated by the reduction in the yellow area from the earlier age. Fine refinements in the retinal projections occur at P12, and by P20 the segregation of left- and right-eye retinogeniculate inputs was indistinguishable from the adult state.

To confirm the qualitative impression of the state of the retinogeniculate pathway in the developing mouse, we quantified the extent of the contralateral and ipsilateral label in the dlgn at different ages as well as the degree of binocular overlap (Fig. 3). At P1 the contralateral and ipsilateral projections occupied 57% (s.e.m. = 2.8) and 35% (s.e.m. = 1.5) of the area of the dlgn, respectively, with 15% (s.e.m. = 1.5) of the dlgn containing overlapped projections. No appreciable change was noted at P3 when the contralateral and ipsilateral projections occupy 54% (s.e.m. = 4.8) and 29% (s.e.m. = 3.7), respectively, with 19% (s.e.m. = 3.1) of the dlgn containing overlapping projections. A significant change ($P < 0.01$, t -test) was seen by P5 as the ipsilateral projection decreased to occupy 10% (s.e.m. = 2.23) of the dlgn, whereas the contralateral projection occupied 43% (s.e.m. = 3.3). At this age the area of overlap decreased significantly to 2% (s.e.m. = 0.7) of the dlgn area ($P < 0.01$, t -test). At P8 the ipsilateral projection remained virtually unchanged, occupying 9% (s.e.m. = 0.88) of the dlgn, whereas the contralateral projection increased to 42% (s.e.m. = 1.6), with 0.9% (s.e.m. = 0.45) of the area of the dlgn containing overlap between the projections. At P12 and P20 the ipsilateral projection occupied 8%, of the dlgn, which is comparable to the adult value of 5%. From P12 to the adult, the contralateral projection occupied ~40% of the dlgn area and the degree of binocular overlap was negligible. These quantitative data confirm the qualitative impression summarized in the previous two Figs.

Cholinergic innervation of the dlgn

To identify the presence of cholinergic fibers in the dlgn, serial sections were processed for ChAT immunocytochemistry and adjacent sections stained with cresyl violet. Representative tracings of ChAT-positive fibers and accompanying cresyl violet-stained sections are shown in Fig. 4. The presence of cholinergic fibers in the dlgn was observed first at P5. At this age only a few ChAT-positive profiles were observed, which appeared to be distributed throughout the dlgn. The incidence of cholinergic fibers increased by P8, and by P12 the first sign of cholinergic clusters was apparent. The presence of cholinergic clusters has been used to identify glomeruli and these might indicate the first synapses between cholinergic fibers from the pbr and neurons in the dlgn (Erisir *et al.*, 1997). The density of ChAT-positive fibers in the dlgn increased markedly in the next few days so that by P20 individual fibers could not be resolved.

We also assessed the development of ChAT-positive soma in the pbr, the source of cholinergic fibers to the dlgn. ChAT-positive somas were present by P1 and at all ages studied. Fig. 5 shows representative sections of the pbr from animals of different ages that were matched to represent the same area of the brainstem. We did not attempt to quantify the size of the ChAT-positive cell population, but our impression is that this did not fluctuate appreciably during the developmental period studied.

CONCLUSIONS

- Retinal axons were observed to separate into eye-specific regions in the dlgn beginning at P5, and the separation of left- and right-eye inputs was nearly complete by P8, with subtle refinements occurring at later stages of development.
- ChAT-positive fibers were present first in the dlgn at P5 and cholinergic clusters were seen first at P12.
- ChAT-positive somas were present in the parabrachial region at birth and remained throughout development and adulthood.

- These findings rule out a role for the cholinergic parabrachial projection in the formation of eye-specific retinal-dlgn inputs.

Discussion

Several studies have relied on $\beta 2^{-/-}$ mice to demonstrate that retinal waves play an important role in the formation of eye-specific, retinal-dlgn projections (Rossi *et al.*, 2001; Grubb *et al.*, 2003; Grubb and Thompson, 2004a; Grubb and Thompson, 2004b; Torborg *et al.*, 2005). However, in mature animals, it is known that nicotinic acetylcholine receptors are expressed by dlgn neurons that receive a cholinergic input from the parabrachial region of the brainstem (Zoli *et al.*, 1998; Sherman and Guillery, 2002). Thus, a potential concern when using this animal model to study the role of retinal activity in the formation of retinofugal projections is that the effects observed might be caused by events at the dlgn rather than (or in addition to) the lack of retinal waves. Indeed, a substantial literature has implicated cholinergic modulation in the development and plasticity of sensory systems (Sachdev *et al.*, 1998; Suga and Ma, 2003; Prakash *et al.*, 2004; Kilgard, 2003), including the visual cortex (Kirkwood *et al.*, 1999; Gu, 2003). To date, only one study dealing with the formation of eye-specific projections has attempted to rule out the possibility that blockade of $\beta 2$ nicotinic receptors in the dlgn is a contributing factor to this developmental event. Penn *et al.* (Penn *et al.*, 1998) made a single injection of epibatidine into the lateral ventricle of three ferrets (ranging in age from P0 to P9), which did not prevent the formation of eye-specific projections. The effects of central drug injections are problematic to interpret because it is difficult to establish key factors such as the extent of drug diffusion and the effectiveness of the drug at the intended receptor. For this reason, the question of whether a cholinergic projection from the midbrain to the dlgn has a role in the formation of eye-specific projections remains open.

As an initial step, one approach to resolving this issue is to determine whether or not a cholinergic input to the dlgn is present during the developmental period when eye-specific retinogeniculate inputs are being formed. The results of the present study indicate that eye-specific projections in the mouse are established during a well-delimited temporal period. On the basis of dual-labeling patterns of different tracers injected into each eye, we find that this process commences at P5 and is largely completed by P8. These observations agree with previous studies (Rossi *et al.*, 2001; Muir-Robinson *et al.*, 2002; Jaubert-Miazza *et al.*, 2005). During this period, we found a small number of cholinergic fibers in the dlgn at P5, with the first clusters of such labeled fibers evident by P12 (Fig. 3). At the same time, cholinergic cell bodies were evident in the parabrachial region of the brainstem on the day of birth (Fig. 5). Collectively, these observations indicate that cholinergic cells are present relatively early in development but that the axons of these neurons do not innervate the dlgn until after the segregation of left- and right-eye projection to the dlgn is largely completed. Assuming that the pattern of labeling of cholinergic fibers accurately depicts cholinergic input to the developing dlgn, our results indicate that modulation of neuronal activity in the geniculate by parabrachial neurons does not play a role in the formation of eye-specific projections.

Several years ago, Chapman (Chapman, 2000) reported that normal retinal activity is required to maintain eye-specific segregation after the segregated state has been established earlier in development. To our knowledge, the mechanism for this intriguing observation has not been addressed. Cholinergic modulation of plasticity-related events has been demonstrated in several sensory systems (Sachdev *et al.*, 1998; Kirkwood *et al.*, 1999; Suga and Ma, 2003; Prakash *et al.*, 2004) and, for the most part, these studies have been concerned with cortical events. Because our results indicate that cholinergic projections to the mouse dlgn become apparent soon after eye-specific projections are formed, it is now of interest to determine whether this input from the brainstem to the dlgn is required to maintain the segregation of left- and right-eye inputs.

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Fig. 1. The distribution of projections from the contralateral and ipsilateral eyes to the dlgn at P1 Retino-dlgn projections were traced using CTB conjugated to a red and green label in the right eye and left eye, respectively, with overlap between the contralateral and ipsilateral projections apparent as yellow. Eye-specific segregation was not seen in any segment of the dlgn at P1. Samples were 150 μ m apart in coronal sections and represented rostral, medial and caudal segments of the dlgn. Scale bar, 150 μ m.

Fig. 2. Development of eye-specific regions in the dlgn

Retino-dlgn projections were traced using CTB conjugated to a red and green label in the right eye and left eye, respectively, with overlap between the contralateral and ipsilateral projections apparent as yellow. The contralateral projection (green) occupies the majority of the dlgn in early stages of postnatal development and is absent in a dorsal portion of the nucleus at later stages. Ipsilateral projections are found throughout the dorsal portion of the dlgn in early stages of development and form a concise boundary in later stages of development. The formation of eye-specific regions in the dlgn occurs during the first postnatal week in mice (P1-P8), with the greatest change occurring between P1 and P5. After the initial formation of eye-specific

regions, refinement continues during the second postnatal week and reaches adult-like levels by the end of the second week (P12). Scale bar, 150 μm .

Fig. 3. Area of the dlgn occupied by the contralateral, ipsilateral and both (overlap) eyes

Values plotted are mean and sem from four sections from each animal. At P1 there is a substantial overlap between the ipsilateral and contralateral projections. A significant decrease in the area occupied by the ipsilateral and overlapping projections was seen at P5, when eye specific regions begin to form. After P5 the eye-specific regions are refined and reach adult-like levels by P12. (* $P < 0.01$, t -test)

Fig. 4. The presence of cholinergic fibers in the dlgn during development

A developmental series of Nissl-stained sections show the dlgn with a square indicating the portion used to trace the ChAT-positive fibers present at each point during development. The column on the right represents the tracing of ChAT-positive fibers that appeared first at P5 and reached adult-like levels by P20. The presence of cholinergic clusters was seen first at P12 and might indicate synapses between fibers from the pbr and dlgn neurons. ChAT staining was captured on a confocal microscope and represents a z-axis depth of 20 μm . Scale bars: 200 μm , cresyl-violet sections; 50 μm , fiber-tracing sections.

Fig. 5. ChAT staining in the pbr of the brainstem

ChAT-positive somas are present in the parabrachial region at P1 and are present throughout all developmental stages and adulthood. A counter stain for DAPI was used to identify the area being photographed. The pictures represent a segment of the pbr captured with a confocal microscope and represent a z-axis of 20 μm . Scale bar, 50 μm .