

ERK signaling is required for eye-specific retino-geniculate segregation

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Summary

In the mammalian visual system, retinal ganglion cell (RGC) projections from each eye, initially intermixed within the dorsal-lateral geniculate nucleus (dLGN), become segregated during the early stages of development, occupying distinct eye-specific layers. Electrical activity has been suggested to play a role in this process; however, the cellular mechanisms underlying eye-specific segregation are not yet defined. It is known that electrical activity is among the strongest activators of the extracellular signal-regulated kinase (ERK) pathway. Moreover, the ERK pathway is involved in the plasticity of neural connections during development. We examine the role of ERK in the segregation of retinal afferents into eye-specific layers in the dLGN. The

activation of this signaling cascade was selectively blocked along the retino-thalamic circuitry by specific inhibitors, and the distribution of RGC fibers in the dLGN was studied. Our results demonstrate that the blockade of ERK signaling prevents eye-specific segregation in the dLGN, providing evidence that ERK pathway is required for the proper development of retino-geniculate connections. Of particular interest is the finding that ERK mediates this process both at the retinal and geniculate level.

Supplemental data available online

Key words: Visual system development, Retinal ganglion cell, Retino-geniculate segregation, ERK pathway

Introduction

The precise neural circuits of the mammalian brain develop gradually through a process of remodeling of initially diffuse axonal projections. In the visual system, during early development, RGC axons from the two eyes are intermixed within the dLGN; then the adult pattern of segregated zones gradually emerges, as axons remodel their branches and become restricted into eye-specific territories (Hahm et al., 1999; Linden et al., 1981; Sretavan and Shatz, 1986).

Previous findings have indicated that the formation of eye-specific domains in dLGN occurs through interocular competition (Lund et al., 1973; Rakic, 1981) and it has been suggested that this process is driven by neural activity (Shatz and Stryker, 1988), particularly by a correlated pattern of spontaneous retinal activity (retinal waves) present in the developing retina (Feller et al., 1996; Galli and Maffei, 1988; Wong et al., 1995). Indeed, selective blockade of retinal activity in both eyes prevents formation of the eye-specific layers in the dLGN, whereas blocking activity in one eye results in an expansion of the territory occupied by the active untreated eye at the expense of axons from the treated eye (Grubb et al., 2003; Huberman et al., 2002; Penn et al., 1998; Rossi et al., 2001). Conversely, if one eye is made more active, its layers within the dLGN expand (Stellwagen and Shatz, 2002). However, the role of retinal activity in retino-geniculate development remains a debated issue (Sengpiel and Kind, 2002). Some findings favor the hypothesis that correlated

retinal activity plays an instructive rather than a permissive role in the development of dLGN (Stellwagen and Shatz, 2002), whereas others suggest that the presence, but not the spatiotemporal pattern, of retinal activity is required (Huberman et al., 2003). In addition, Cook et al. (Cook et al., 1999) have reported that binocular blockade of neural activity does not prevent segregation of retinal axons but just delays it, implying that specific cues might be involved in this process (Huberman et al., 2002; Huberman et al., 2003; Muir-Robinson et al., 2002).

Some molecular systems have been suggested to play a role in the remodeling of retino-geniculate circuitry (Corriveau et al., 1998; Menna et al., 2003; Pham et al., 2001; Ravary et al., 2003; Upton et al., 1999); however, the intracellular cascades required in shaping these connections have remained unclear. Extracellular signal-regulated kinases (ERKs) are signal-transducing enzymes that have been shown to participate in a diverse array of cellular programs (Grewal et al., 1999). They are activated by phosphorylation on threonine and tyrosine residues and, once activated, are able to phosphorylate other downstream kinases and transcription factors (Davis, 1993; Pizzorusso et al., 2000; Seger and Krebs, 1995). Recently, it has become evident that the ERK pathway can play multiple roles in the activity-dependent regulation of neuronal functions. It is involved in cellular models of synaptic plasticity, such as LTP and LTD (English and Sweatt, 1997; Kawasaki et al., 1999; Martin et al., 1997), and in addition, behavioral studies have revealed a requirement of this pathway in learning and long-term memory (Atkins et al., 1998;

Mazzucchelli et al., 2002). Recent findings have highlighted the importance of ERKs in visual cortical plasticity (Di Cristo et al., 2001), and in morphological changes in dendritic or axonal structure (Kim et al., 2004; Markus et al., 2002; Vaillant et al., 2002; Wu et al., 2001).

In the present study, we examined the role of the ERK pathway in the development of eye-specific domains in dLGN, focusing specifically on the localization of its action along the retino-thalamic circuitry. We blocked ERK phosphorylation in the retina or dLGN during the period in which eye-specific segregation normally occurs and then analyzed the distribution of optic projections in the dLGN. We demonstrate that the active form of ERK is crucial for the refinement of retinogeniculate connections, as its inhibition in dLGN blocks the eye-specific segregation completely. Furthermore, our results provide evidence that, for a correct retino-thalamic pattern formation, ERK signaling is required on both geniculate neurons and retinal ganglion cells. This is demonstrated by the finding that a separate blockade of ERK activation in retinal or geniculate-cortical neurons partially arrests the segregation process, whereas a concurrent blockade at these sites arrests it completely.

Materials and methods

Animal treatment

Newborn Long-Evans rats were used in this study, in accordance with the Italian Ministry of Public Health guidelines for care and use of laboratory animals.

Stock solutions of U0126 or PD98059 (25 mM; Promega and Calbiochem, respectively) were prepared in 100% DMSO. For lower concentrations we used dilutions in saline. Fluorescent latex microspheres (Lumafuor Corp.), 50-200 nm in diameter, were incubated overnight at 4°C in a 1:5 mix of microspheres to U0126, PD98059 or DMSO+saline (vehicle). Beads were then centrifuged and resuspended in saline (Riddle et al., 1997; Riddle et al., 1995).

The intraventricular injections were performed under ether anesthesia by inserting into the left lateral ventricle a 30 gauge needle connected to a Hamilton syringe. 1 µl of latex beads coated with 500 µM U0126 or 1 mM PD98059 or vehicle was injected. The following coordinates, which vary according to the age of the animal, were used: 3 mm lateral and 1.5-0.2 mm posterior to bregma until age P6, or 1 mm anterior to bregma at P8; 2-3 mm deep from the pial surface. The location of injections was confirmed by the presence of fluorescent latex microspheres throughout the lateral ventricle.

Intraocular injections of fluorescent latex beads coated with 1 mM U0126 were performed using a glass micropipette, with a tip diameter of 30 µm, connected to a microinjector. The micropipette was inserted at the ora serrata, and the drug was slowly released into the vitreous. Intraocular injections of epibatidine (1 mM in DMSO, Sigma) were carried out in the same manner.

For visual cortex injections, the skull was opened on the left side to expose much of the length of area 17. Microspheres (1 µl) coated with 250 µM U0126 were injected at eight different sites using a glass micropipette. Retrograde transport and LGN labeling were obtained 48 hours later. To confirm the presence of beads within dLGN projection neurons, 48 hours after injection of beads, we also labeled the geniculate-cortical neurons by injecting the retrograde tracer FluoroGold (2%; Molecular Probes) into the visual cortex; three days later animals were sacrificed.

Immunohistochemistry

Animals were perfused with ice-cold 4% paraformaldehyde in 0.1 M TBS and 1 mM sodium orthovanadate (pH 7.4). Brains were then

removed and cryoprotected in 30% sucrose overnight. Coronal sections of the brain, 50 µm thick, were cut on a freezing microtome. After a blocking step, tissue sections were incubated with pERK (1:1000, Sigma), pAkt (1:50, Promega) or pCaMKII (1:500, Promega) antibody and then exposed to the appropriate biotinylated secondary antibody (1:200, Vector). Antibody binding was visualized by the ABC kit (Vector) and nickel-enhanced diaminobenzidine (DAB) reaction. Images were acquired with a Zeiss HR Axiocam videocamera connected to a Zeiss Axiophot microscope and digitalized by Axiovision software.

pERK immunoreactivity in the dLGN was also detected by incubating sections with Oregon-green 488 goat anti-mouse (1:400, Molecular Probes) or biotinylated horse anti-mouse IgG (1:200, Vector), followed by extravidin-Cy3 (1:300 Sigma). Images were then acquired using an Olympus confocal microscope (Fluoview).

For double-labeling experiments, the neuronal-specific nuclear protein, NeuN (1:500, Chemicon), was added after the end of pERK immunohistochemistry, and then pERK and NeuN immunoreactivities were, respectively, detected with Alexa Fluor 488 and Alexa Fluor 568 secondary antibodies (1:400, Molecular Probes). The sections were observed by using an Olympus confocal microscope.

pERK immunostaining in the retina was performed either on whole-mount or coronal retinal sections. For both treatments, animals were perfused with ice-cold 4% paraformaldehyde in 0.1 M TBS and 1 mM sodium orthovanadate (pH 7.4). Whole-mount retinas were dissected, whereas coronal retinal sections (14 µm thick) were cut using a cryostat and then processed for immunostaining. Retinas were permeabilized in 0.3% Triton X-100 and incubated in anti-pERK (1:200 for wholemounts, 1:1000 for retinal sections), then exposed to the Oregon-green 488 goat anti-mouse antibody (1:200 for wholemounts, 1:400 for sectioned retinas). Background staining was observed in control retinas in which the primary antibody was omitted.

Western blotting

Treated or control retinas were pooled in samples containing two equivalent retinas each and analyzed by immunoblotting. Proteins were extracted with lysis buffer (1% Triton X-100; 10% Glycerol; 20 mM Tris; 150 mM NaCl; 1 mM EDTA, 0.5 mM Na₂VO₄, 10 µg/ml Leupeptin, 10 µg/ml Aprotinin, 1 mM PMSF, 0.5% Na Deoxycholate, 0.1% SDS, 50 mM NaF, 1 mM Na₂MoO₄, 5 mM Na₄P₂O₇) and the total concentration of the samples was assessed with a protein assay kit (BioRad) using a bovine serum albumine (BSA)-based standard curve. Proteins (30 µg) were boiled with sample buffer, loaded on a 12% SDS-PAGE gel and then electrotransferred to nitrocellulose. Blots were blocked with BSA (4%, Sigma) and Tween-20 (0.2%) in TBS for 2 hours, and then incubated with anti-pERK antibody (1:1000, Sigma) overnight with shaking at 4°C. After washing, blots were incubated with HRP-conjugated secondary antibody (0.3 µg/ml, BioRad) in blocking solution for 2 hours, developed by the ECL chemiluminescence system (Amersham) and captured on autoradiographic films (Amersham). Filters were subsequently stripped with stripping buffer (Chemicon) for 30 minutes at 50°C, reblocked, and then reprobed with anti-ERK antibody (1:1000, Sigma) using the same immunoblotting procedure as described above. Films were then digitalized with a camera and densitometric analysis of the bands was performed with MCID-M4 software. pERK levels in treated and control retinas were normalized to ERK by measuring the optical density (OD) of the pERK band and dividing it by the OD of the ERK band, on the same filter. Finally, data from each sample were normalized with respect to controls run on the same gel, and results were presented as a percentage of control values. The mean value for the control eyes was set at 100% (Cotrufo et al., 2003). Statistical significance was determined using Student's *t*-test.

Analysis of pERK-expressing neurons in dLGN

Five animals were used to analyze what type of neurons express pERK

in dLGN. Geniculo-cortical neurons were retrogradely labeled by application of FluoroGold (2%) in the visual cortex; three days later animals were sacrificed. Brain coronal sections were obtained by using a freezing microtome, collected through the dLGN and immunostained. pERK immunoreactivity in the dLGN was detected by incubating sections with appropriate biotinylated secondary antibody (1:200 Vector) followed by extravidin-Cy3 (1:300 Sigma) in 0.1% Triton X-100. Images were then acquired at a 60× zoom 3 with an Olympus confocal microscope. For each animal we selected five sections through the middle of the dLGN, and for each section six different (60×60 μm) randomly spaced fields were acquired. Then, pERK, FluoroGold and pERK-FluoroGold labeled neurons were counted using MetaMorph software.

Analysis of cell density in the retina

Retinas were dissected, fixed with 2.5% glutaraldehyde and then with formaline-ethanol solution (1:9). The whole-mount retinas were then stained with 0.1% cresyl violet and visualized with a Zeiss light microscope. For cell density analysis, two groups of retinas were used: monocular U0126-treated and monocular vehicle-treated. Injections were performed every 48 hours from P2 to P8, and the retinas were analyzed at P9. For each retina we evaluated in blind the density of living cells in the ganglion cell layer (GCL). Specifically, living cells were counted at 100× magnification by using a Zeiss computerized microscope (Stereo Investigator software, Microbrightfield) in an average of 25 fields (80×80 μm). For all measures, fields were equally spaced throughout the retina.

Retino-geniculate axons labeling

All animals received an intravitreal injection of Cholera Toxin B subunit (CTB) conjugated with Alexa Fluor 488 (10 μg/μl, Molecular Probes) in the left eye and CTB-Alexa Fluor 594 in the right eye at least 24 hours before perfusion. All rats were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were dissected after perfusion and cryoprotected in 30% sucrose. Coronal sections, 50 μm thick, were cut on a freezing microtome, collected in a serial order through the entire thalamus and then visualized using an Olympus confocal microscope.

Analysis of retino-geniculate axon projections

Images were collected with an Olympus Optical confocal microscope using UPLAPO 10× lens with numerical aperture (NA) 0.4. Settings for laser intensity, gain, offset and pinhole size were optimized initially and held constant through the study. For each animal, the entire serial order of coronal sections of the dLGN was acquired, and for each section, confocal series of a step size of 2 μm were obtained throughout the whole section thickness (50 μm). These confocal series were then averaged and visualized on a single focal plane. Background was measured as the level of autofluorescence of unstained tissue and was subtracted from the signal fluorescence. Then, the collected images of the dLGN, corresponding to contralateral or ipsilateral projections, were imported to the image analysis system MCID-M4 and used to analyze the areas occupied by the ipsilateral and the contralateral RGC projections. All image analyses were done blind. For each animal we analyzed the five largest sections through the middle of the dLGN, where the two eye-specific domains appear better segregated (Menna et al., 2003; Rossi et al., 2001; Stellwagen and Shatz, 2002). The dLGN boundary and the profile of ipsilateral and contralateral dLGN projections were drawn on the computer screen excluding the ventral LGN and extrageniculate optic tract. Great care was exercised to identify the extension of contralateral projection terminals in the gap area. We defined the line at which the majority of axon terminals stopped at the border of the gap. The relative areas occupied by the ipsilateral and contralateral projections were calculated by dividing the average of the five ipsilateral or contralateral areas by the average of the five total dLGN areas (Rossi et al., 2001). To determine the extent of

overlap between ipsilateral and contralateral projections to the same dLGN, the ipsilateral and contralateral areas were measured, then their sum was subtracted from the total dLGN area and expressed as a percentage of it (Rossi et al., 2001; Stellwagen and Shatz, 2002). The statistical significance of data was evaluated by Student's *t*-test.

Results

Development of retino-geniculate projections

To identify the stage of development at which retinal afferents segregate in the rat dLGN, we analyzed the distribution of retino-geniculate projections at different ages after birth. We used intravitreal injections of cholera toxin B subunit (CTB) conjugated to Alexa Fluor 594 (red label) or Alexa Fluor 488 (green label) to visualize the retinal fibers simultaneously from both eyes in coronal sections of the dLGN. At P4, retinal fibers from the two eyes are still largely intermixed. The projections from the contralateral eye occupy the entire geniculate nucleus (100%; Fig. 1A,I), whereas those coming from the ipsilateral eye are diffusely distributed [19.6±0.8% (mean±s.d.); Fig. 1B,J], overlapping throughout the dLGN (Fig. 1C,K). This area of overlap can be better appreciated if observed at high magnification. In fact, as reported in Fig. 1D, the intermingled pattern of fibers from the two eyes is clearly evident. By P9, the process of segregation is almost accomplished as retinal fibers are segregated into eye-specific regions – the contralateral (92.6±0.5%; Fig. 1E,I) and the ipsilateral zone (9.6±0.7%; Fig. 1F,J) – with only 2.2±0.5% of overlap area (Fig. 1G,K). High magnification of the border between the two projections shows a clear segregation of fibers from the two eyes (Fig. 1H). Note that, in the contralateral projection of P4 rats, a lower density of fibers is visible just in the area where they will leave a gap and the ipsilateral projection will segregate.

ERK activation in dLGN is developmentally regulated

As the activity of ERK requires its phosphorylation (pERK), we first examined whether pERK in the dLGN is regulated during the development of retino-thalamic connections. Analysis by immunohistochemistry at different postnatal stages revealed that pERK staining is present at birth (Fig. 1L), rises during the first postnatal week with a peak by P5 (Fig. 1M) and then declines after P8 (Fig. 1N). At P11 low levels of pERK are observed (Fig. 1O). We co-labeled sections for pERK and NeuN, a specific neuronal marker. As shown in Fig. 1P, almost all pERK-positive cells (green) are immunoreactive for NeuN (red), indicating that pERK expression is restricted to neurons. This regulation of pERK, over the temporal window during which the segregation occurs, suggests that it could be involved in the formation of eye-specific domains.

The inhibition of ERK signaling in dLGN blocks the segregation of retino-geniculate afferents

To assess the role of the ERK pathway in the segregation of retinal afferents within the dLGN we blocked its signaling by using a potent MEK-specific inhibitor, U0126 (Favata et al., 1998). Initially, we tested the capacity of U0126 to block ERK activation in our *in vivo* model. P5 rats, which present high levels of pERK in the dLGN (see Fig. 1M), were injected in one of the lateral ventricles with U0126 (see Materials and

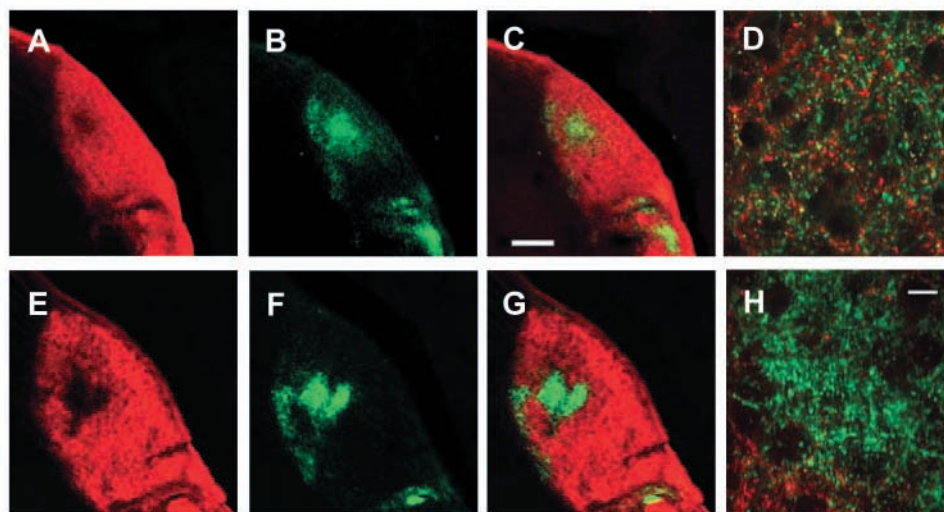
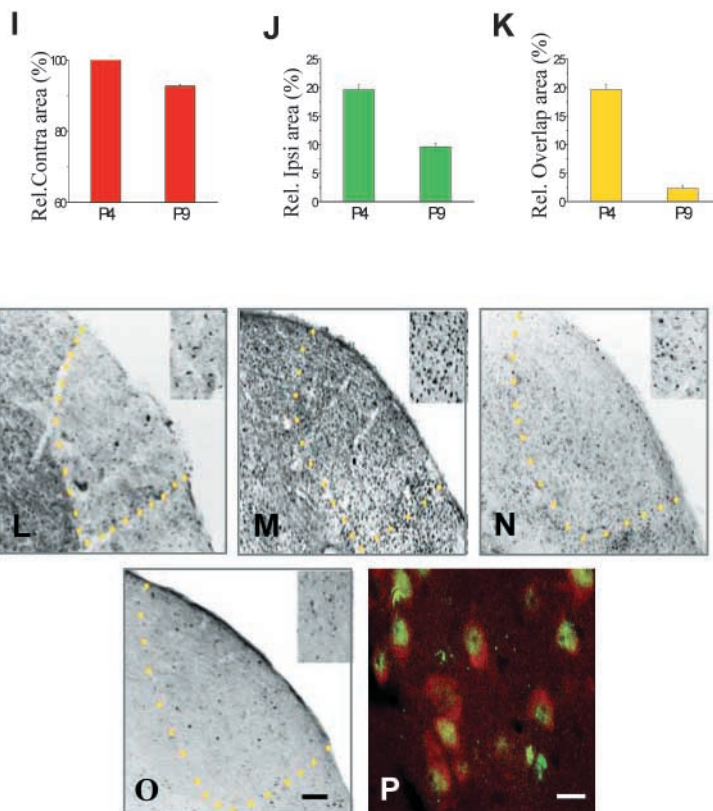


Fig. 1. Development of retino-geniculate connectivity. Representative coronal sections through dLGN of P4 (A-D, $n=3$) and P9 (E-H, $n=3$) rats. Fibers from the ipsilateral projecting eye are labeled with CTB-Alexa Fluor 488 (green), whereas the projections from contralateral eye are labeled with CTB-Alexa Fluor 594 (red). (I,J,K) Quantification of relative contralateral (I), ipsilateral (J) and overlap (K) areas of P4 ($n=3$) and P9 ($n=3$) rats. The areas occupied by ipsilateral and contralateral projections are significantly different between P4 and P9 groups (Student's *t*-test, $P<0.05$ for contralateral and ipsilateral areas). Note that the overlap area decreases from 20% in P4 to 2% in P9 dLGN (K). At P4, high magnification of the area of overlap shows that fibers from both eyes are largely intermixed (D), whereas by P9, high magnification of the border between the two projections shows that these fibers terminate in distinct regions (H). (L-O) pERK expression in the dLGN is developmentally regulated. pERK immunohistochemistry in the dLGN at different postnatal stages reveals an increasing expression of pERK from P0 (L, $n=3$) to P5 (M, $n=4$). Note the reduced staining at P8 (N, $n=3$) and P11 (O, $n=3$). pERK-positive cells (green) co-express the neuronal marker NeuN (red), (P5 rats, $n=4$, P). Error bars depict the s.d. Scale bars: 200 μm for A-C,E-G; 10 μm for D,H; 100 μm for L-O; 50 μm for the insets; 25 μm for P.



methods). After one hour, animals were perfused and pERK staining in the dLGN was examined. Intracerebroventricular (ICV) injection is a well-known technique for drug delivery into the dLGN and our experiment demonstrated that 1 μl of U0126 at a dose of 500 μM was able to drastically reduce pERK expression in dLGN (Fig. 2A,B).

As a control for any non-specific effect of this drug, we examined whether other signaling pathways were altered by pharmacological manipulation with U0126. We focused on CaMKII and Akt, known to be involved in synaptic plasticity (Taha et al., 2002; Wang et al., 2003), and investigated the expression of their phosphorylated forms. We found that ICV

injection of U0126 does not block pCaMKII (Fig. 2D,E) or pAkt (Fig. 2F,G) expression in the dLGN. This result, in keeping with other works (Davies et al., 2000; Favata et al., 1998), indicates that U0126 inhibits specifically ERK signaling and has no effects on the activity of other kinases. Therefore, we injected U0126 into the lateral ventricle of newborn rats and examined the projections from the two eyes.

The drug was injected along with fluorescent latex microspheres to provide prolonged release. Latex microspheres are able to bind different substances by passive absorption and to release them gradually (Penn et al., 1998; Riddle et al., 1997). Indeed, we noted that this method of drug delivery inhibits

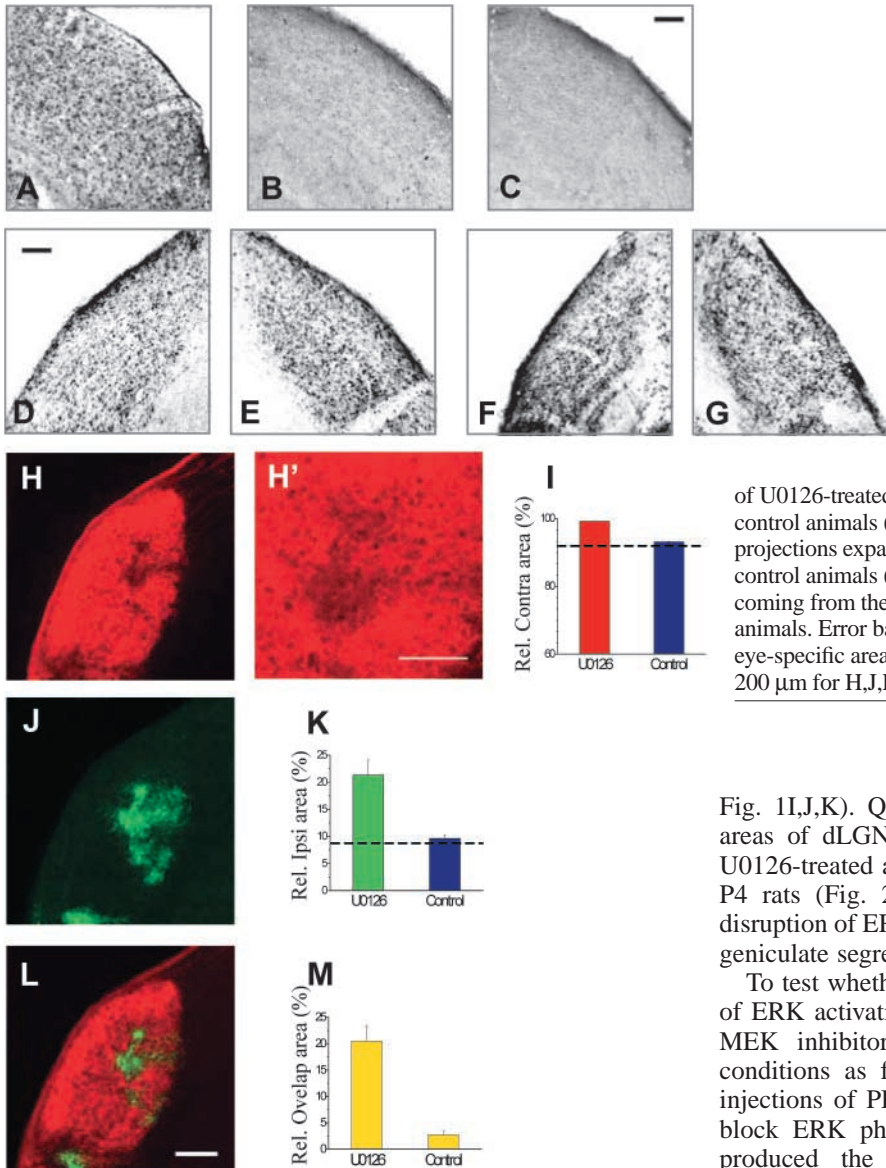


Fig. 2. Effects of the inhibitory action of U0126 and PD98059 in retino-geniculate connections. Intracerebroventricular (ICV) injection of U0126 (500 μ M, P5 rats $n=4$, B) or PD98059 (1 mM, $n=4$, C) drastically reduces ERK activation in the dLGN with respect to the saline injection ($n=3$, A). (D-G) U0126 does not affect pCaMKII or pAkt expression. ICV injection of saline (D) or 1 μ l of 500 μ M U0126 (E) does not block CaMKII phosphorylation in the dLGN. Also pAkt levels in the dLGN remain unchanged after ICV injection of saline (F) or U0126 (G, P4 rats, $n=5$ for each treatment). (H-M) Distribution of retinal projections in the dLGN of P9 rats that received beads-U0126 or beads-vehicle ICV injections. (H,H') Projections from the contralateral eye expand filling the entire dLGN. (I) The relative contralateral area of U0126-treated animals ($n=6$) is significantly larger than that of control animals ($n=5$; Student's t -test, $P<0.05$). (J,K) Ipsilateral projections expand occupying an area twice greater than that of control animals (Student's t -test, $P<0.05$). (L,M) The projections coming from the two eyes largely overlap in the U0126-treated animals. Error bars depict the s.d. Dashed lines indicate the relative eye-specific areas in normal P9 rats. Scale bars: 100 μ m for A-G; 200 μ m for H,J,L; 100 μ m for H'.

pERK expression in the dLGN for 24 hours. pERK expression then recovers gradually until 48 hours after injection (see Fig. S1 at <http://dev.biologists.org/supplemental/>). Hence, beads-U0126 were injected into the left lateral ventricle of newborn rats every 48 hours from P2 to P8 and the projections from the two eyes were examined at P9. Animals injected with fluorescent latex beads coated with the vehicle (DMSO+saline) were used as controls.

We found that blockade of ERK activation in dLGN completely arrests the process of eye-specific segregation. Indeed, in the dLGN ipsilateral to the U0126-injected side, RGC axons coming from the two eyes were largely overlapped (20.5 \pm 2.8%; Fig. 2L,M). The axons from the contralateral eye were present throughout the entire dLGN, occupying 99.2 \pm 0.8% of the total area (Fig. 2H,H',I), and the ipsilateral projection expanded (21.3 \pm 2.8%) occupying an area twice greater than that ipsilaterally innervated in control animals (Fig. 2J,K). Control animals presented exactly the same distribution of fibers as normal P9 rats (Fig. 2I,K,M and see

Fig. 1I,J,K). Quantitative analysis revealed that the relative areas of dLGN occupied by ipsi- or contralateral fibers in U0126-treated animals were comparable to those observed in P4 rats (Fig. 2I,K,M and Fig. 1I,J,K), demonstrating that disruption of ERK signaling results in the total arrest of retino-geniculate segregation.

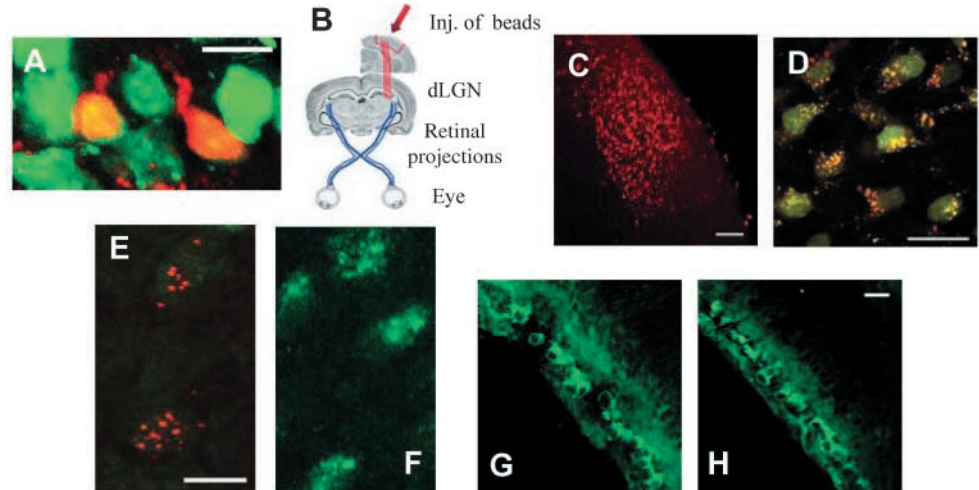
To test whether this result was specifically due to blockage of ERK activation we repeated the experiment using another MEK inhibitor, PD98059. Under the same experimental conditions as for U0126 treatment, we observed that ICV injections of PD98059 at a dose of 1 mM (the dose able to block ERK phosphorylation in the dLGN, see Fig. 2A,C) produced the same effect: abolishment of eye-specific segregation. The relative contralateral projection area (98.2 \pm 0.6%) and ipsilateral projection area (17.9 \pm 2.3%) were not statistically different from the corresponding areas in the U0126-treated animals ($n=6$, Student's t -test, $P>0.05$ for both ipsi and contra projections).

Geniculo-cortical blockade of pERK causes an expansion of retinal projections in the dLGN

As the ICV delivery of U0126 provides blockade of ERK activation at the geniculate level, it was difficult to distinguish whether the effect on eye-specific segregation was due to the blockade of pERK in geniculate neurons, or in RGC fibers, or in other afferent inputs to the dLGN. To assess the site of action of pERK we first investigated whether pERK in the dLGN is expressed by all neurons or only by dLGN projection neurons. We used five P5 rats for this study and for each of them we counted an average of 496.8 \pm 44.5 cells stained with pERK in the dLGN (see Materials and methods for details). Our analysis showed that all of these cells co-localized with dLGN projection neurons labeled with FluoroGold (Fig. 3A). Hence, during the first postnatal week, pERK in the dLGN is expressed

Fig. 3. Specific blockade of pERK in LGN neurons. (A) All pERK-positive cells (red labeling) in the dLGN of P5 rats ($n=5$) co-localize with dLGN projection neurons labeled with the retrograde tracer FluoroGold (green labeling). (B) Schematic representation of visual cortex injections of fluorescent latex beads in newborn rats. (C) A large number of retrogradely labeled neurons are evident in the dLGN after injections of red fluorescent latex beads in the primary visual cortex ($n=4$). (D) The red beads are present throughout the cytoplasm of geniculo-cortical neurons labeled by Fluorogold ($n=3$). (E) Cortical injections of beads-U0126 produce a prolonged blockade of ERK

activation in the dLGN ipsilateral to the injected side. Forty-eight hours after injection, no detectable pERK staining (green) is observed in LGN neurons with red beads inside the cytoplasm (E, $n=7$). (F) Unilateral cortical injections of beads-U0126 do not affect pERK staining in the dLGN contralateral to the injected cortex; indeed, no red beads are observed in this side of the brain ($n=7$). (G,H) Injections of beads-U0126 in the left side of visual cortex do not affect pERK expression in the eye. Coronal sections from the contralateral eye, which sends the major afferents to the LGN, are shown. pERK immunostaining in the RGCs of treated animals (G, $n=7$) is not different from that of controls (H, $n=3$). Scale bars: 10 μm for A,E,F; 100 μm for C; 25 μm for D; 20 μm for G,H.



only by projection neurons. The rate between neurons labeled with FluoroGold and those labeled with FluoroGold-pERK revealed that only half of the projection neurons express pERK (Fig. 3A).

Next, pERK was blocked in the projection neurons of dLGN. To deliver U0126 into the dLGN neurons we took advantage of the capacity of the beads to be retrogradely transported (Katz et al., 1984; Riddle et al., 1995). In fact, if injected into the primary visual cortex (Fig. 3B), the microspheres are taken up by nerve terminals and retrogradely transported into the dLGN ipsilateral to the injected cortex. We observed that, 48 hours after injection, almost all the neurons were labeled (Fig. 3C). At high magnification fluorescent beads were visible into the cytoplasm of the geniculo-cortical neurons retrogradely labeled by FluoroGold (Fig. 3D). Moreover, we noted that pERK expression in the projection neurons of dLGN was still inhibited at 48 hours after injection of microspheres treated with U0126 at a dose of 250 μM (Fig. 3E). Note, this treatment did not alter pERK staining in the dLGN contralateral to the injected cortex (Fig. 3F), or in the retina (Fig. 3G,H), confirming its specific localized action. Therefore, red microspheres treated with U0126 or vehicle (for control animals) were injected into the left primary visual cortex every 48 hours from P2 to P8. Because of the presence of red fluorescent beads in the dLGN, we could label eye projections using only green-CTB. To analyze the fraction of dLGN area occupied by ipsilateral and contralateral projections, at age P8 all the animals, both U0126 treated and controls, were divided in two groups: one group was injected with green-CTB in the left eye to visualize the ipsilateral projections and the other group was injected in the right eye for visualizing the contralateral fibers. When the distribution of retinal inputs was analyzed at P9, we observed that they were not completely segregated. The projections from both eyes occupied significantly larger territories in U0126-treated animals with respect to controls (contra, $95.5 \pm 0.7\%$; ipsi, $14.6 \pm 2.4\%$; Fig. 4A-D). The overlap between the ipsilateral and contralateral

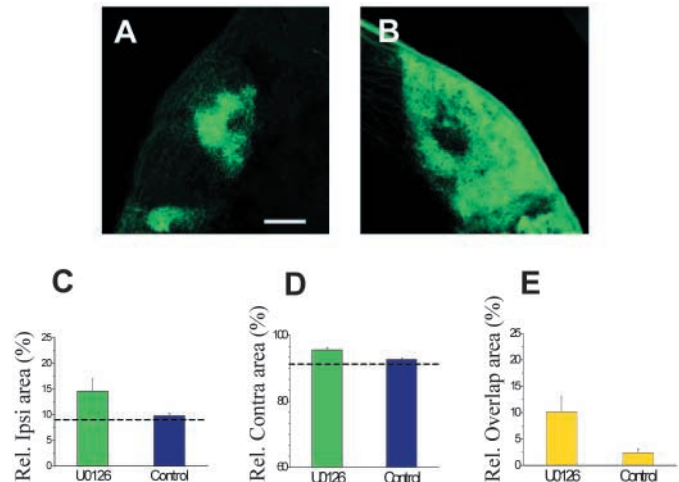


Fig. 4. dLGN projection neurons contribute to the development of retino-geniculate connectivity. (A-E) Coronal sections of dLGN in P9 rats ($n=18$) that received injections of retrogradely transported beads-U0126 in the primary visual cortex, and quantification of relative contralateral, ipsilateral and overlap areas in the dLGN. Both ipsilateral (A) and contralateral (B) projections are diffusely distributed in the dLGN. The relative ipsilateral (C) and contralateral (D) areas of U0126-treated animals ($n=18$) are statistically greater than those of control animals ($n=10$), (Student's *t*-test, $P < 0.05$ respectively). (E) The relative overlap area of U0126-treated animals is larger than that of control animals. Error bars depict the s.d. Dashed lines indicate the relative eye-specific areas in normal P9 rats. Scale bar: 200 μm .

areas was evident ($10.1 \pm 2.8\%$), even if at a lower extent when compared to the overlap produced by the ICV U0126 administration (Fig. 4E, and see Fig. 2M). Retinal axons of control animals were segregated as in normal P9 rats (Fig. 4C,D,E and see Fig. 1I,J,K). This result shows that ERK

signaling in the projection neurons of dLGN is crucial during eye-specific segregation.

Binocular retinal blockade of ERK activation causes an expansion of the optic projections in the dLGN, whereas monocular blockade causes a retraction of them

As the blockade of pERK in geniculo-cortical neurons affects only partially the eye-specific segregation, ERK signaling at other sites is likely to be involved in this process. We therefore analyzed whether pERK in the RGCs plays a role in retinal fiber segregation. First, we checked the dose of U0126 able to block pERK expression in the retina: two groups of P5 rats were binocularly injected with saline or U0126 and were perfused 1 hour later. The eyes and the brain were then dissected and processed for pERK immunostaining in the retina and dLGN. We observed that 1 μ l of U0126 at a dose of 1 mM strongly reduced pERK in the retina (Fig. 5A,B) but it did not alter pERK expression in the dLGN (data not shown). We performed these controls also by injecting binocularly U0126-coated beads and noted that at 48 hours after injection, pERK expression in the retina was reduced by $52\pm 19\%$ with respect to control animals (Fig. 5D). In the meantime, no changes were observed in pERK levels in dLGN (Fig. 5E,F), indicating that this treatment blocks pERK only at the retinal level. Next, binocular retinal injections of fluorescent latex beads coated with U0126 or vehicle (for control animals) were performed, every 48 hours from P2 to P8. At P9, analysis of retinal axon distribution revealed a partial segregation of retinal afferents from both eyes in their specific domains. There was an expansion of the territory occupied by the contralateral ($96.6\pm 1.7\%$; Fig. 5G,J) and ipsilateral ($15.1\pm 4.3\%$; Fig. 5H,K) projections, resulting in an overlap in $11.7\pm 3\%$ of the dLGN (Fig. 5I,L), thus a loss of refinement of retino-geniculate projections.

We then investigated what might be the effect of pERK blockade in only one eye, a model that imposes an interocular imbalance in pERK levels. Intravitreal injections of U0126 were performed using the same dose and schedule as for binocular injections. At P9, after monocular retinal pERK blockade, we observed that projections from the treated eye were retracted from their territories in the dLGN, whereas projections from the untreated eye were expanded, invading territories normally occupied by the other eye (Fig. 6A,B and Table 1). No alteration of the eye-specific pattern was observed in animals monocularly injected with vehicle (Fig. 6D,E). The opposing effects of bilateral and monocular blockade indicate that pERK is required for interocular competition during the eye-specific segregation. Moreover, the contrasting results of these two experiments indicate that the retraction of retino-geniculate projections obtained after monocular treatment was not due to mechanical damage in the retina, or to other non-specific effects. In fact, we found that the cell density in the ganglion cell layer (GCL) of treated retinas was not statistically different from that of controls (Student's *t*-test, $P > 0.05$). The number of living cells per field was 69.3 ± 6.1 for U0126-treated retinas ($n=5$) and 66.1 ± 2.5 for vehicle-treated retinas ($n=4$) (Fig. 6C,F). Taken together, our results indicate that pERK in the RGCs is required for the normal segregation of retino-geniculate projections.

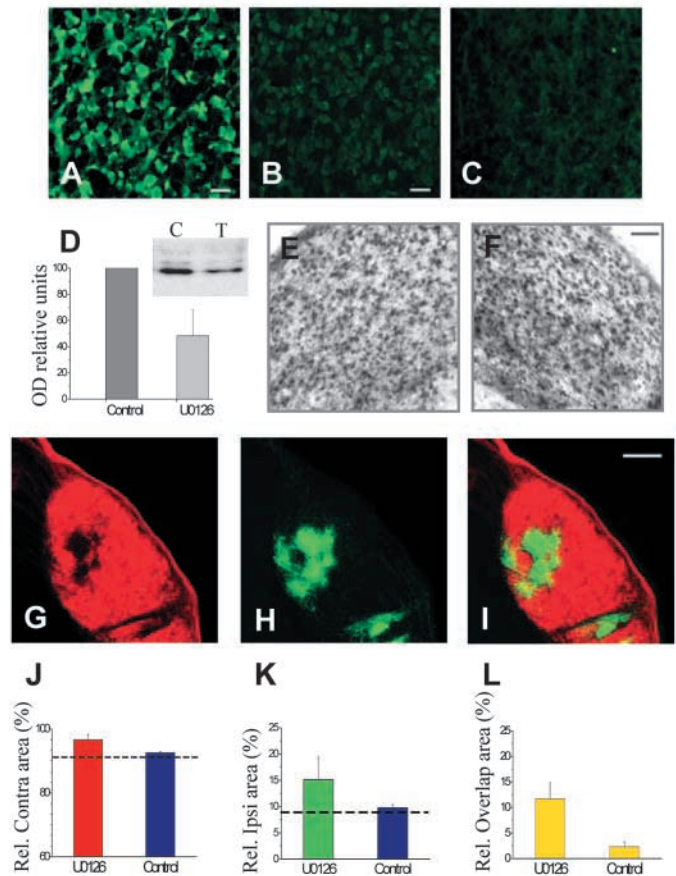


Fig. 5. Effects of binocular retinal injections of U0126. pERK staining in P5 rats is evident in all cells of the GCL injected with saline (A, $n=3$) but is strongly decreased after 60 minutes of U0126 treatment (B, $n=4$). (C) Control retinas with no primary antibody show background fluorescence. (D) Western blot analysis reveals that, at 48 hours after intravitreal injections of beads-U0126 (1 mM), pERK levels in the retina are decreased by $52\pm 19\%$ with respect to controls injected with beads-vehicle, Student's *t*-test, $P < 0.05$ (C, control; T, U0126-treated). Each column is an average of six different samples. Each sample pools together two retinas. (E,F) In P5 rats, no differences in pERK levels within the dLGN are observed 48 hours after binocular injections of beads-vehicle (E, $n=6$) or beads-U0126 (F, $n=6$). (G-L) Eye-specific segregation in P9 rats that received binocular intravitreal injections of beads-U0126. (G,H) Both contralateral and ipsilateral projections are diffusely distributed in the dLGN. (J,K) The relative contralateral and ipsilateral areas of U0126-treated animals ($n=7$) are significantly larger than those of control animals ($n=6$), (Student's *t*-test, $P < 0.05$, respectively). (L,L) The relative overlap area of U0126-treated animals is larger than that of control animals. Error bars depict the s.d. Dashed lines indicate the relative eye-specific areas in normal P9 rats. Scale bars: 25 μ m for A,B,C; 50 μ m for E,F; 200 μ m for G,H,I.

Concurrent blockade of pERK in the retina and dLGN neurons abolishes retino-geniculate segregation

We have seen that eye-specific segregation is only partially affected by blocking pERK in RGCs, or in the projection neurons of the dLGN. We wondered whether the total arrest of segregation obtained by ICV delivery of U0126 could be ascribed to pERK blockade both on RGCs and geniculate

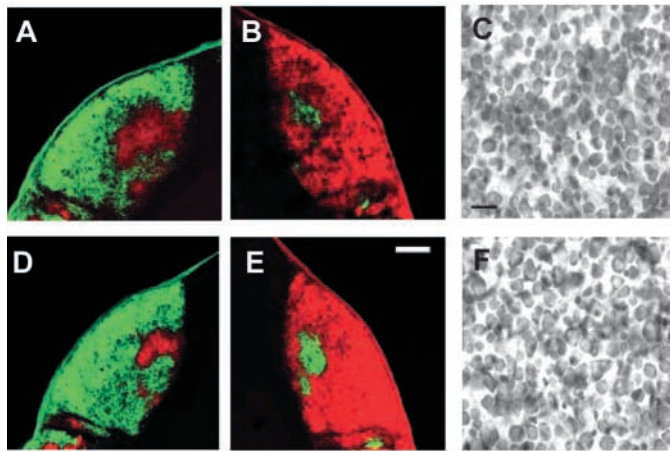


Fig. 6. Monocular retinal blockade of ERK activation. (A,B,D,E) Representative images of both sides of the dLGN of rats that received monocular injections of beads-U0126 (A,B; $n=7$) or vehicle (D,E; $n=7$). Projections from the treated eye are labeled with CTB-Alexa Fluor 488 (green) and those from untreated eye with CTB-Alexa Fluor 594 (red). Note that in the U0126-treated animals both contralateral (A) and ipsilateral (B) fibers from the treated eye (green) occupy smaller territories than the corresponding projections from the untreated eye (red, A,B). Conversely, in control rats (D,E), fibers from both eyes occupy similar areas in the dLGN. (C,F) Cresyl violet staining of cells in the GCL. Living cell density in the retinas of rats monocularly injected with U0126 (C; 69.3 ± 6.1 cells per field, $n=5$) is not statistically different (Student's *t*-test, $P > 0.05$) from that of vehicle-treated retinas (F; 66.1 ± 2.5 cells per field, $n=4$). Scale bars: 200 μm for A,B,D,E; 20 μm for C,F.

neurons. In support of this possibility was the observation that ICV injections of U0126 blocked pERK in dLGN and retina (see Fig. S1 at <http://dev.biologists.org/supplemental/>). To elucidate this issue, we inhibited ERK activation by injecting beads-U0126 into both eyes and, at the same time, into the primary visual cortex, every 48 hours from P2 to P8, as described in the previous experiments. At P9, we observed that in the dLGN ipsilateral to the injected cortex, projections from both eyes largely overlapped each other ($18.5 \pm 1.3\%$; Fig. 7E). The contralateral fibers filled all the dLGN ($99.3 \pm 0.7\%$; Fig. 7A,C) and the ipsilateral area was largely expanded ($19.2 \pm 2.8\%$; Fig. 7B,D) giving rise to a total blockade of segregation. The distribution of retino-geniculate projections

Table 1. Eye-specific segregation after monocular pERK blockade

		Treated eye	Untreated eye
U0126-treated rats	Contralateral	84.35 ± 2.5	95.97 ± 3.6
	Ipsilateral	5.80 ± 3.7	18.30 ± 2.5
Control rats	Contralateral	90.39 ± 1.1	90.24 ± 1.3
	Ipsilateral	11.17 ± 2.6	11.22 ± 1.4

In rats that received monocular pERK blockade the ipsilateral and contralateral projections from the treated eye retract from their specific territories in the dLGN, whereas the projections from the untreated eye expand. In control animals that received monocular injections of vehicle, fibers from both eyes are normally segregated. Ipsilateral and contralateral projection areas of treated animals ($n=7$) are significantly different from those of controls ($n=7$; Student's *t*-test, $P < 0.05$ for treated and untreated eye).

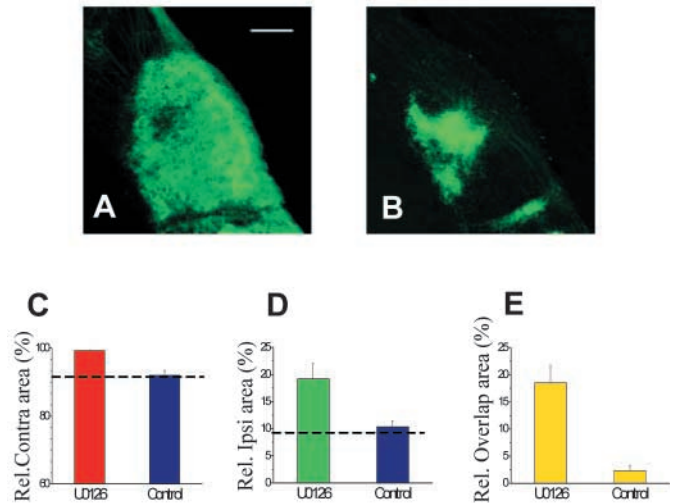


Fig. 7. Effects of concurrent pERK blockade in both retinas and geniculate neurons. In treated animals ($n=10$) the contralateral fibers fill nearly the entire nucleus (A,C), whereas those coming from the ipsilateral eye are largely diffused in the dLGN (B,D), overlapping through 18.5% of the total area (E). This distribution of fibers is significantly different from that of control animals ($n=6$; Student's *t*-test, $P < 0.05$ for contralateral and ipsilateral). Error bars depict the s.d. Dashed lines indicate the relative eye-specific areas in normal P9 rats. Scale bar: 200 μm .

showed a strong similarity with that obtained after pERK inhibition in the dLGN by ICV injections of U0126 (see Fig. 2), suggesting that the ERK pathway acts both on geniculate neurons and on RGCs to form the correct retino-thalamic pattern.

Retinal activity regulates ERK phosphorylation in the retina and dLGN

Different findings have shown that blocking spontaneous retinal activity can prevent the formation of eye-specific retino-geniculate connections (Penn et al., 1998; Rossi et al., 2001; Huberman et al., 2002; Grubb et al., 2003). To investigate whether any relationship between activity and pERK exists during this process, we performed intraocular injections of epibatidine, a desensitizing agent for nicotinic receptors that is able to block retinal activity (Feller et al., 1996). Previous studies performed in ferrets (Penn et al., 1998) or mice (Rossi et al., 2001) have reported that repeated binocular injections of epibatidine during the first postnatal week block the formation of eye-specific layers in the dLGN. We observed the same effect in rats. Indeed, binocular injections of 1 mM epibatidine, every 48 hours from P3 to P7, resulted in the complete blockade of eye-specific layer formation: projections from the contralateral eye occupied the entire dLGN and those from the ipsilateral eye expanded extensively (Fig. 8A,B). Thus, P5 rats were binocularly injected with epibatidine (1 mM) or vehicle for controls. After 48 hours, animals were perfused, and retinas and brains immunostained for pERK expression in the GCL and dLGN. As shown in Fig. 8, we observed that the blockade of retinal activity drastically reduced pERK levels in the retina and dLGN (Fig. 8C,E) with respect to controls (Fig. 8D,F). This result indicates that retinal activity drives ERK activation in both these sites.

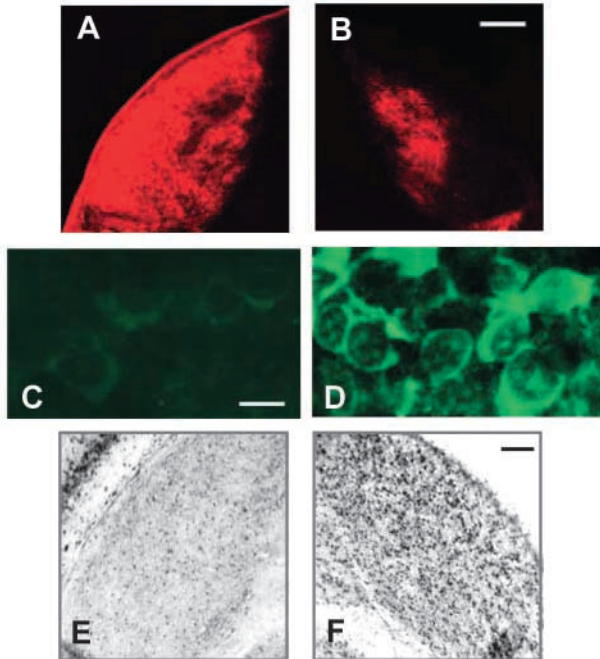


Fig. 8. Effects of binocular retinal injections of epibatidine. (A,B) Representative coronal sections of dLGN in P9 rats that received binocular injections of epibatidine ($n=3$). The retinal fibers of only one eye labeled with CTB-Alexa Fluor 594 (red) are shown, as the other eye projection is identical. The contralateral fibers fill nearly the entire nucleus (A) and the ipsilateral projections are expanded considerably (B). (C,D,E,F) Forty-eight hours after binocular injection of epibatidine, the expression of pERK in retina and dLGN of P7 rats (C,E, $n=4$) is strongly decreased compared with controls injected with vehicle (D,F, $n=3$).

Discussion

Role of ERK pathway in the development of retino-geniculate neurons

We have explored a role for the ERK pathway in the remodeling of visual system connectivity in rats. Our results show that ERK signaling is crucial for the eye-specific segregation of retinal fibers, and that it mediates this process both at the retinal and the geniculate level.

The inhibition of ERK activation in the dLGN through ICV delivery of U0126 or PD98059 results in a complete arrest of the eye-specific segregation process. The RGC axons from the two eyes remain extensively overlapped in their target region, showing a strong similarity with the intermingled pattern of fibers observed in normal P4 rats.

The effect obtained after ICV injections of U0126 potentially originates from the inhibition of pERK on geniculate neurons, or on afferent inputs into the dLGN, or both. To distinguish between these possibilities, ERK activation was separately blocked only on RGCs of both eyes or on dLGN projection neurons. For each treatment, our data demonstrated that fibers from both eyes were only partially segregated within the dLGN. The partial segregation was specific in both experiments and was not due to a partial inhibitory action of U0126. Indeed, the concentration of U0126 in the retina and in the projection neurons of the dLGN was

effective, as we have clearly demonstrated a strong reduction of pERK staining at both these sites.

Together, our results demonstrate that for the correct development of retino-thalamic circuitry, activation of ERK only on RGCs or on geniculate projection neurons is not sufficient. Importantly, we observed that pERK in the dLGN is expressed only by the dLGN projection neurons (Fig. 3A); hence, it is likely that the complete disruption of the segregation process observed after ICV-U0126 treatment is caused by a blockade of ERK activation, both on RGC and dLGN projection cells. One might argue that the effect of ICV treatment could also involve other afferent inputs to the dLGN. However, the concurrent inhibition of pERK on geniculocortical relay neurons and RGCs faithfully replicated the results found after ICV-U0126 treatment, making it unlikely that there was any consistent contribution of pERK present in other afferent inputs, except RGCs. In summary, these results provide strong evidence that eye-specific segregation cannot develop without the relative contribution of pERK on both RGCs and dLGN projection neurons. In view of this, our findings highlight the role of LGN projection neurons in the formation of eye-specific domains in the dLGN. It has been previously reported that thalamic NMDA receptors do not contribute to the eye-specific segregation in the ferret dLGN (Smetters et al., 1994). However, although the activity of postsynaptic cells, at least the NMDA-mediated activity, does not seem to be required, our study indicates that the postsynaptic cell per se is involved in the segregation of eye-specific layers in the dLGN.

Retinal activity and ERK signaling: a possible link during eye-specific segregation

A large body of work indicates that spontaneous retinal activity, present in the developing retina (Feller et al., 1996; Galli and Maffei, 1988; Wong et al., 1995), shapes the retino-geniculate connectivity during early development. Our results show that the ERK cascade, both at the retinal and geniculate level, plays a key role in the segregation of retinal projections and that ERK activation is affected by retinal activity during this process. It should be noted that the alterations of retino-geniculate pattern observed in our experiments after pERK blockade, are very similar to the alterations due to electrical activity blockade. Indeed, pERK blockade in dLGN by ICV U0126 treatment (Fig. 2) and electrical activity blockade in dLGN by intracranial infusion of tetrodotoxin (TTX) (Shatz and Stryker, 1988) result in a complete arrest of retinal fiber segregation. In addition, the monocular blockade of pERK (see Table 1 and Fig. 6), or of retinal activity (Penn et al., 1998), produces a retraction of projections of the treated eye and an expansion of the territory occupied by projections from untreated eye. It is unlikely that U0126 affected neuronal activity and thus altered segregation through a non-specific mechanism. Indeed, *in vivo* studies performed in our laboratory have demonstrated that, in animals treated with U0126, the electrophysiological properties of neurons are not affected (Di Cristo et al., 2001); this is in agreement with several *in vitro* studies (English and Sweatt, 1997; Impey et al., 1998). Hence, on the basis of these results, and on the fact that retinal activity drives ERK phosphorylation in the retina and dLGN, we suggest that retinal activity signals via ERK to regulate retinal fiber segregation in the RGCs and in the dLGN

projection neurons. The proposed model is consistent with the findings that binocular blockade of all retinal activity prevents segregation (Huberman et al., 2002; Penn et al., 1998), but that only simultaneous blockade of pERK in the retina and the dLGN yields the same result.

The formation of eye-specific projection patterns in the dLGN occurs, in different species, during the first postnatal week. Thus far, this process was considered to reflect eye-specific segregation, whereas recently it has been proposed that eye-specific segregation can be dissociated from eye-specific layer formation (Huberman et al., 2002; Muir-Robinson et al., 2002). These studies suggest that spontaneous retinal activity in the first postnatal week is crucial in establishing eye-specific layers; hence, the first postnatal week may constitute the crucial period for eye-specific layer formation. However, it is not yet known what might be the potential determinants of this crucial period. Because patterns of retinal activity seem to be insufficient to instruct the development of retino-geniculate connectivity (Huberman et al., 2003), an appealing hypothesis is that the formation of eye-specific layers might be determined both by the level of endogenous retinal activity (Stellwagen and Shatz, 2002) and by the transient expression and recognition of molecular markers in the retina and/or dLGN (Eglen et al., 2003; Huberman et al., 2002; Huberman et al., 2003; Muir-Robinson et al., 2002). In this context, pERK might influence the crucial period for layer formation as it has two interesting properties that make it a likely candidate as molecular determinant of the crucial period. First, it is developmentally regulated, as its pattern of expression correlates with the timing for layer formation, and second, pERK expression is modified by retinal activity. We can speculate that during the crucial period electrical activity may work hand in hand with ERK signaling to activate transcription factors, and in this way, to regulate function and spatiotemporal distribution of specific molecular cues.

Neurotrophins as potential activators of ERK

Growth factors such as neurotrophins (NTs) signal via ERKs during many different neuronal functions, including cell differentiation (Barnabe-Heider and Miller, 2003), survival (Han and Holtzman, 2000; Watson et al., 2001), axon growth (Atwal et al., 2000) and dendrite formation (Miller and Kaplan, 2003; Vaillant et al., 2002). These factors can also mediate the synaptic plasticity of neural connections via the ERK pathway (Patterson et al., 2001). Although there is a wide range of cellular processes in which NTs are known to be involved, as yet no literature has reported any role for NTs, except for BDNF, in the formation of eye-specific pattern. Recently, we showed that endogenous anterogradely transported BDNF is required for the proper development of retino-geniculate connectivity (Menna et al., 2003). In that study, the binocular blockade of BDNF caused a retraction of retinal projections rather than an expansion, as we have obtained after binocular pERK blockade. Moreover, BDNF seems to act independently of retinal activity. These results may suggest that for the formation of eye-specific domains BDNF signaling does not converge into ERK; the action of BDNF may proceed in parallel to that of the ERK pathway. However, a second scenario may exist: BDNF action might be mediated by ERK. If so, the different alterations in retino-geniculate connectivity observed in these two studies (Menna et al., 2003) (this paper)

might be explained by the different levels of ERK activation, depending on the BDNF and electrical activity pathways, respectively. Indeed, it is noted that the level of ERK activity is a crucial component during plastic processes. For example, it has been reported that either blockade or overexpression of ERK signaling reduces LTP levels or enhances dendritic growth (English and Sweatt, 1997; Kim et al., 2004; Komiyama et al., 2002). Considering these observations, we may suppose that, during the formation of a correct retino-thalamic pattern ERK signaling may be induced not only by electrical activity but also by neurotrophins, which together act in concert to finely regulate the eye-specific segregation.

ERK targets

If the MEK/ERK pathway is crucial in the remodeling of visual system connectivity, what are the relevant ERK substrates? CREB (cAMP response element binding protein) has been shown to be one of the targets of ERK signaling during processes of neuronal plasticity such as learning and memory (Impey et al., 1999), as well as during developmental visual cortical plasticity (Cancedda et al., 2003). Moreover, Stryker and colleagues (Pham et al., 2001) have shown that the CRE/CREB pathway contributes to the refinement of retino-geniculate projections. Considering these findings and ours reported here, we favor the hypothesis that the ERK pathway controls eye-specific segregation through CREB. However, ERK signaling does not always converge with CREB. For example, there is a positive coupling between the ERK cascade and CREB phosphorylation during LTP (Davis et al., 2000; Impey et al., 1998), but not during LTD (Thiels et al., 2002). Other potential targets of pERK at the cytoplasmic level, such as cell adhesion molecules, cytoskeletal elements or synaptic proteins (Bailey et al., 1997; Matsubara et al., 1996; Suzuki et al., 1995), may also be involved in the propagation of ERK signaling during retino-thalamic development.

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