

# Distinct Roles for Spontaneous and Visual Activity in Remodeling of the Retinogeniculate Synapse

Bryan M. Hooks<sup>1,2</sup> and Chinei Chen<sup>1,2,\*</sup>

<sup>1</sup> Department of Neurology  
Neurobiology Program  
Children's Hospital, Boston  
Harvard Medical School  
300 Longwood Avenue  
Boston, Massachusetts 02115  
<sup>2</sup> Program in Neuroscience  
Harvard Medical School  
220 Longwood Avenue  
Boston, Massachusetts 02115

## Summary

Sensory experience and spontaneous activity play important roles in development of sensory circuits; however, their relative contributions are unclear. Here, we test the role of different forms of activity on remodeling of the mouse retinogeniculate synapse. We found that the bulk of maturation occurs without patterned sensory activity over 4 days spanning eye opening. During this early developmental period, blockade of spontaneous retinal activity by tetrodotoxin, but not visual deprivation, retarded synaptic strengthening and inhibited pruning of excess retinal afferents. Later in development, synaptic remodeling becomes sensitive to changes in visually evoked activity, but only if there has been previous visual experience. Synaptic strengthening and pruning were disrupted by visual deprivation following 1 week of vision, but not by chronic deprivation from birth. Thus, spontaneous activity is necessary to drive the bulk of synaptic refinement around the time of eye opening, while sensory experience is important for the subsequent maintenance of connections.

## Introduction

In the visual system, patterned sensory experience is thought to be necessary for the precise functional refinement of topographic maps at the synaptic and cellular level (Kandel and Jessell, 2000). Indeed, vision appears to play a role in synaptic rearrangements at every level of the sensory system, from aiding in the refinement of retinal ganglion cell (RGC) arbors into ON and OFF substrata (Tian and Copenhagen, 2003) to the refinement of ocular dominance columns in primary visual cortex (Wiesel and Hubel, 1963). During critical periods of development, visual deprivation can strongly alter excitatory connections in visual cortex (Maffei et al., 2004). Deficits produced by manipulations of sensory activity support the conclusion that vision is key in synapse establishment, maintenance, and plasticity.

In contrast to visual activity, spontaneous activity plays an earlier, more general role in development, including neuronal cell fate determination and neurotrans-

mitter specification (Borodinsky et al., 2004) and regulation of axon guidance cues in the developing spinal cord (Hanson and Landmesser, 2004). In the lateral geniculate nucleus (LGN), the subcortical region to which RGCs project, gross anatomical refinement of axon arbors is deficient in the absence of activity (Hahn et al., 1991; Shatz and Stryker, 1988; Sretavan et al., 1988). Proper layer formation thus requires a combination of molecular guidance cues, such as Eph receptors and ephrins, and spontaneous activity (Pfeiffenberger et al., 2005). Spontaneous activity, which sweeps across the retina in waves of increased RGC firing, provides the activity required for the coarse eye-specific layering that separates input from the two eyes in the LGN (Galli and Maffei, 1988; Meister et al., 1991; Penn et al., 1998; Demas et al., 2006). The bulk of eye-specific layer formation is completed around p8 in mouse (Godement et al., 1984; Muir-Robinson et al., 2002), a week before eye opening.

Long after gross anatomical refinement is complete, further remodeling or refinement at the retinogeniculate synapse continues for up to 3 weeks after eye opening. Pruning, the elimination of many initial retinal inputs onto a given geniculate cell until only one or a few afferents remain, and strengthening, the process by which each remaining retinal fiber more strongly activates its LGN target, characterize the developmental maturation of this sensory connection (Chen and Regehr, 2000; Joubert-Miaza et al., 2005). At a corresponding developmental stage following eye opening, the visual receptive field is sharpened (Blakemore and Vital-Durand, 1986; Daniels et al., 1978; Tavazoie and Reid, 2000). Because synaptic remodeling occurs around the time of eye opening (p12–p14 in mouse), patterned sensory input is widely assumed to drive refinement (Gandhi et al., 2005). However, this assumption has not been truly tested because earlier studies did not determine relative contributions of sensory activity, spontaneous activity, and activity-independent mechanisms in the establishment and maintenance of these connections.

Here, we test the relative contributions of visual activity and spontaneous activity in the developmental remodeling of the mouse retinogeniculate synapse. In this synapse, presynaptic cells can be monitored independently from corticothalamic inputs, and changes in synaptic connectivity are large and quantifiable. For a variety of RGC activity manipulations, we monitor a number of parameters of synaptic remodeling, including synaptic strength, AMPA receptor-mediated currents (AMPAR)/NMDA receptor-mediated currents (NMDAR) ratio, NMDAR current decay time course, and the average number of retinal inputs that innervate a given relay neuron. Using these parameters, we find that spontaneous activity drives the elimination of the majority of excessive afferents and strengthening of the remaining inputs over a 4 day period spanning eye opening. Dark rearing during the same period does not delay maturation, suggesting that vision plays a limited role at this stage. Furthermore, chronic deprivation of visual activity from birth results in changes in the AMPAR/NMDAR

\*Correspondence: chinei.chen@childrens.harvard.edu

ratio and NMDAR current decay timecourse, but not in strengthening or pruning even 2 weeks after eye opening. In contrast, normal visual experience followed by deprivation during a later period of development (after p20) is capable of inducing synaptic weakening and recruitment of additional retinal fibers. These findings demonstrate that spontaneous activity drives the bulk of retinogeniculate refinement, while previous visual experience is required for later synaptic plasticity.

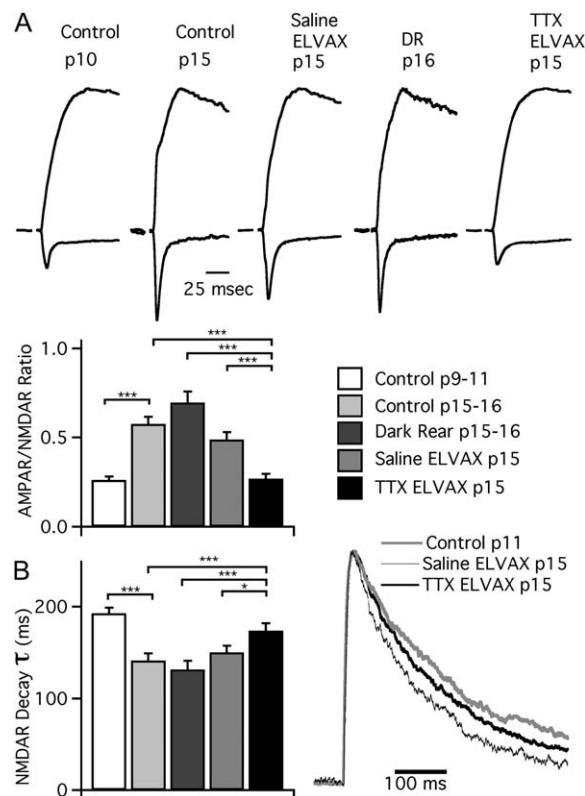
## Results

### Short-Term Activity Blockade but Not Sensory Deprivation Disrupts Synaptic Remodeling

Because a large number of retinal fibers are eliminated during the few days around the time of eye opening (Chen and Regehr, 2000), we tested the hypothesis that visual activity drives the maturation of synaptic properties by using short-term activity manipulations from ages p9 to p16. We compared the effects of two conditions of activity manipulation in C57BL/6J mice. First, we blocked visually evoked activity by dark rearing (DR) mice from p11. To ensure equivalent amounts of visual experience in each of the control animals, we physically opened their eyes at p12. Second, we inhibited retinal action potentials with tetrodotoxin (TTX), which blocks both visual and spontaneous activity. We achieved continuous blockade of retinal activity for 3–4 days by surgically implanting pieces of slow release polymer, ELVAX, containing TTX in the vitreous humor of p11 animals. The effectiveness of block was assessed by an ophthalmological exam of the direct and consensual pupillary light reflex (Prusky and Ramoa, 1999; see Experimental Procedures). We assessed the effect of all manipulations on synapse maturation at p15–p16. By altering the voltage-clamp holding potential, two subtypes of ionotropic glutamatergic current were differentiated: fast, transient inward currents at −70 mV (AMPAR) and more slowly decaying outward currents at +40 mV (NMDAR) that required postsynaptic depolarization to open and were thus blocked at −70 mV.

Toxin blockade of retinal activity had striking effects that more physiological manipulations of visual input (DR) did not. TTX treatment altered the ratio of the peak AMPAR/NMDAR EPSC amplitudes at the retinogeniculate synapse over the developmental period between p11–p15. Figure 1A (top) compares the normalized maximal AMPAR and NMDAR currents recorded from different ages in development. In p9–p11 mice, NMDAR currents dominate (A/N ratio = 0.26), while in older animals (p15–p16), the AMPAR component significantly increases (average A/N ratio = 0.58,  $p < 0.001$ ). Saline ELVAX mice and DR mice display an AMPAR/NMDAR ratio comparable to unmanipulated controls. In contrast, the AMPAR/NMDAR ratio remains small in p15 TTX ELVAX-treated mice, similar to p9–p11 mice and significantly reduced compared to control, saline ELVAX, and DR mice. A summary of the mean ratio values for the developmental period p9–p16 shows that blockade of presynaptic activity retards the normal developmental changes in the ratio (Figure 1A, bottom).

Changes in the level of spontaneous activity also had a strong influence on the normal developmental changes in NMDAR current time course, the slowly de-



**Figure 1. Spontaneous Activity Blockade but Not Visual Deprivation Disrupts Normal Maturation**

(A) (Top) Maximal current traces at −70 mV (inward) and +40 mV (outward) show AMPAR/NMDAR current ratio changes over development from p10 (far left) to p15–p16 in control, DR, and saline ELVAX-treated animals, but not in TTX ELVAX (far right). Traces are normalized to peak NMDAR current. (Bottom) Average AMPAR/NMDAR current ratio in mice from p9 to p16 under various conditions of activity manipulation.

(B) (Right) Representative NMDAR current traces in p11 animals and p15 animals treated with both saline and TTX ELVAX. (Left) Decay  $\tau$  values (ms) are significantly slower in TTX-treated animals than in other conditions at p15.

\* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ . n = 27–37. Control (p9–p11), 36 cells from 11 animals; control (p15–p16), 30 from 8; DR, 27 from 10; Saline ELVAX, 36 from 10; TTX ELVAX, 37 from 8.

caying outward current in Figure 1B. Previous studies show that the NMDAR decay time course accelerates over development in an activity-dependent manner (Carmignoto and Vicini, 1992; Hestrin, 1992). Changes in NR2 subunit composition from slowly decaying isoforms (NR2B) to more rapid ones (NR2A) underlie this change (Monyer et al., 1994). Consistent with these studies, relay cells from young animals (p9–p11) showed a slow decay ( $192.9 \pm 6.0$  ms). This time course was significantly faster in p15–p16 control mice ( $141.5 \pm 7.8$  ms;  $p < 0.001$ ). Visual deprivation did not prevent this acceleration of NMDAR decay time course. However, under complete blockade of activity in p15 TTX-treated mice, NMDAR decay ( $173.7 \pm 8.0$  ms) was significantly slower than unmanipulated controls, DR mice, and saline ELVAX controls. This is consistent with activity blockade preventing the developmental switch from slowly decaying NMDA receptor subunits (NR2B) to the more rapidly decaying subunit (NR2A) expressed in adults.

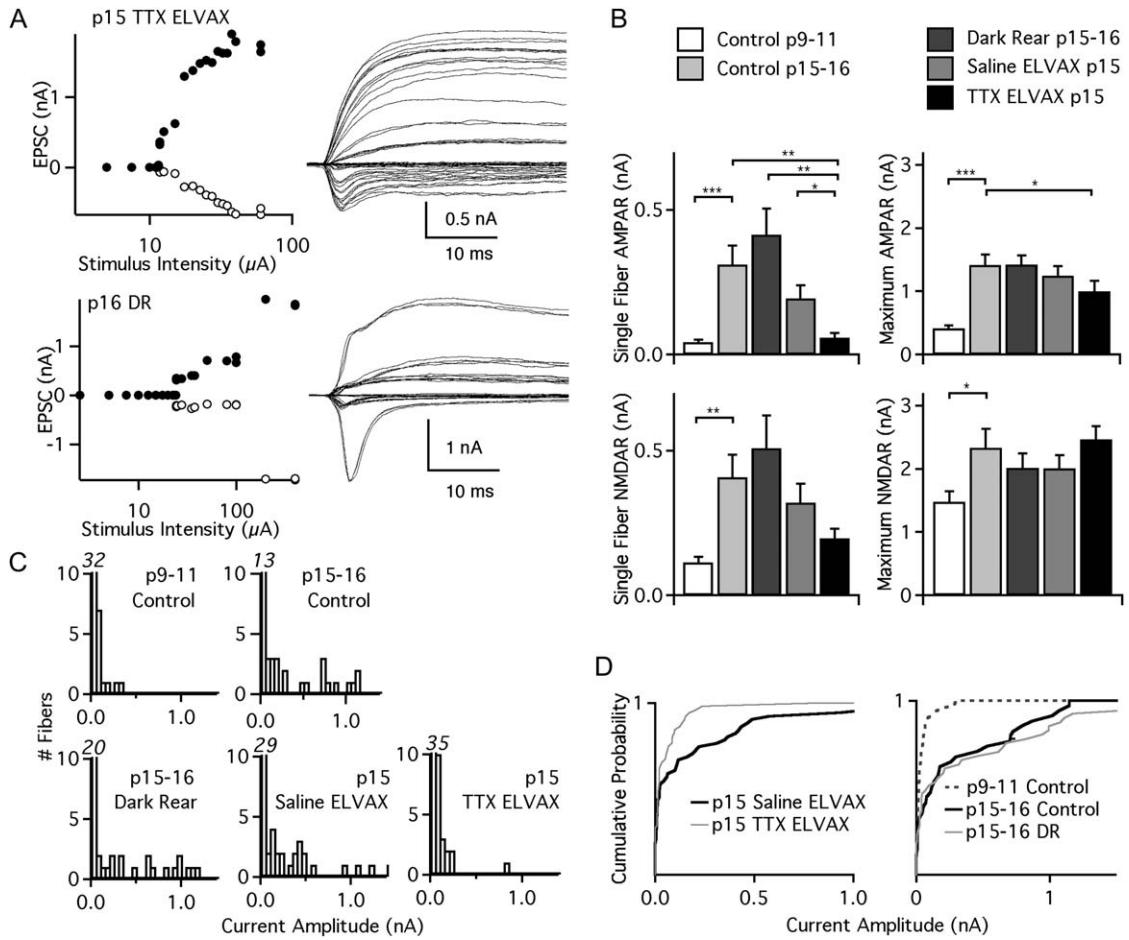


Figure 2. Spontaneous Activity Blockade but Not Dark Rearing Blocks Maturation of Single-Fiber Strengthening

(A) Representative recordings from activity-manipulated animals. (Top) Cell from a p15 TTX ELVAX-treated mouse with graph (top left) of peak EPSC amplitude (nA) versus stimulus intensity for both AMPAR- (white circles) and NMDAR-mediated (black circles) components of synaptic current. (Top right) Superimposed EPSCs recorded from the same relay neuron at  $-70$  mV (inward currents) or  $+40$  mV (outward currents) while increasing the stimulus intensity. (Bottom) Similar presentation for an LGN neuron from p16 DR mouse.

(B) Summary of EPSC amplitudes for AMPAR (top) and NMDAR (bottom) currents elicited in response to single-fiber stimulation (left) or maximal stimulation (right, note change in vertical scale).  $n = 35-53$  per condition (single fiber) and  $n = 17-30$  (maximal current). We found a trend toward smaller currents for saline ELVAX when compared to control. However, these differences were not statistically different.

(C) Histograms of single-fiber AMPAR current amplitude in 50 pA bins for p9 to p16 mice. Numbers above first bin indicate total points less than 50 pA (off scale). (Top) Control mice and (bottom) various conditions of activity manipulation are shown.

(D) Single-fiber AMPAR currents in cumulative probability histograms. (Left) Saline and TTX ELVAX compared at p15. (Right) Control and DR animals at p15-p16 compared to younger controls (p9-p11).

\* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ .

### Spontaneous but Not Visual Activity Drives Synaptic Strengthening and Pruning at Eye Opening

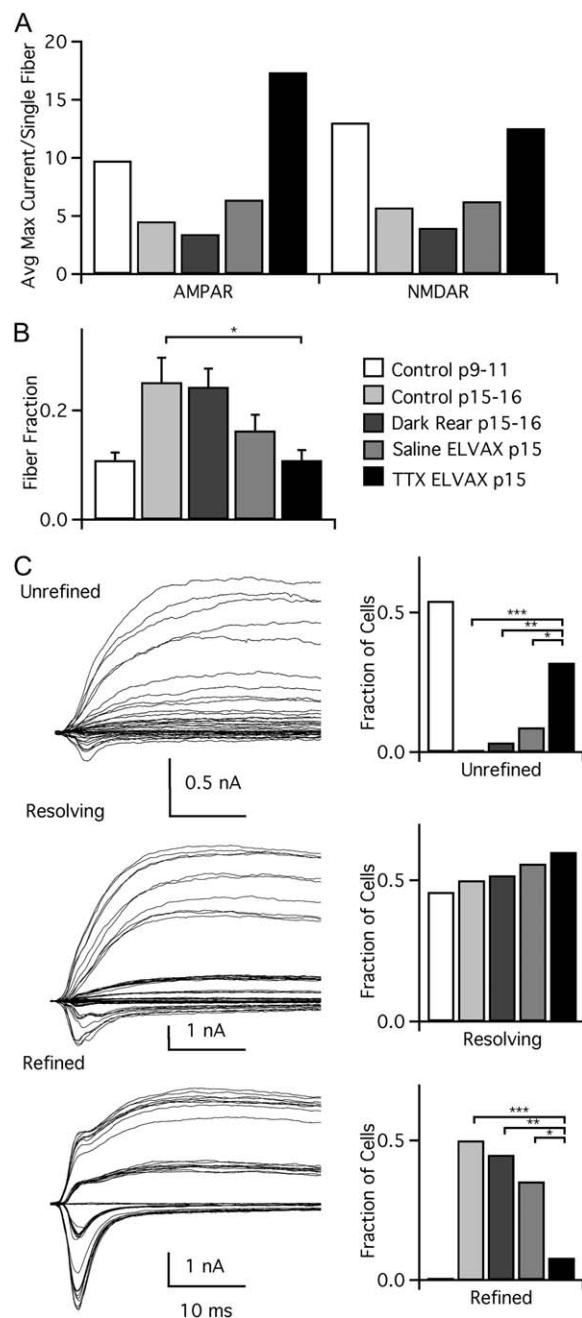
Figure 2A shows a representative example of how we examine synaptic refinement by determining single-fiber and maximal current for both populations of glutamate receptors (AMPAR and NMDAR). The optic tract stimulus intensity is increased in small, incremental steps until a minimal response emerges from noise. We define this minimal response as the single-fiber current amplitude. Stimulus intensity is then increased in regular increments to record the maximum response (see Experimental Procedures). The peaks of the rapid inward currents (AMPAR,  $-70$  mV) and slower decaying outward currents (NMDAR,  $+40$  mV) are plotted against stimulus intensity in Figure 2A, left. Overlaid current responses from LGN cells obtained from a representative p15 TTX-treated mouse (Figure 2A, top)

and a p16 DR mouse (bottom) are displayed on the right.

Figure 2B summarizes the effects of activity manipulations on single-fiber (minimal stimulation) and maximal currents. During the period spanning eye opening (p9-p16), average single-fiber currents strengthen significantly, and average maximal currents increase as well in control animals. Reduction in visual activity (DR) alone did not alter single-fiber current strength or maximum current compared to control in p15-p16 animals. Similarly, average maximum currents also increased in TTX-treated animals compared to p9-p11. But, in TTX blockade, single-fiber currents did not increase to the degree seen in control, DR, or saline-treated controls. TTX treatment left animals with significantly smaller single-fiber AMPAR currents, about 1/4 that of control and saline ELVAX mice.

At young ages, a large number of fibers remain small, resulting in a skewed distribution of single-fiber current strength. To better illustrate the effect of TTX, we examined the distribution of single-fiber AMPAR currents, shown in Figure 2C as amplitude histograms. Virtually all inputs are less than 100 pA until p15. With age, 30%–40% of the inputs become larger, ranging from 100 to more than 2000 pA. This maturation is not altered in DR mice or in mice treated with saline ELVAX. However, the larger inputs are conspicuously absent in p15–p16 mice treated with TTX ELVAX, and there are significantly fewer fibers that strengthen to greater than 100 pA (15% [8/53] of all inputs in TTX versus 54% [19/35] in control, 48% [20/42] in DR, and 40% [21/52] in saline;  $p < 0.003$  by Fisher's exact test). This difference is emphasized when comparing the cumulative probability histogram of peak single-fiber AMPAR current between TTX ELVAX and saline ELVAX (Figure 2D, left). The immature pattern of this distribution is emphasized by comparison of control mice at p9–p11 to p15–p16 with DR mice sharing the control pattern (Figure 2D, right). Notably, a similar analysis of single-fiber NMDAR current amplitudes revealed no significant effect on the amplitude distributions with TTX treatment or DR (data not shown).

Did this effect of activity blockade on synaptic strengthening also disrupt synaptic pruning? We estimated the remaining afferents for each cell using three methods. We could not simply count the number of steps in current amplitude as previous studies (O'Brien et al., 1978; Jackson and Parks, 1982) have done because less refined cells have a large number of weak afferents, and it is difficult to distinguish small differences due to trial-to-trial variation from independent connections. We determined a rough estimate of the number of remaining fibers from the ratio of average maximal and single-fiber currents (Figure 3A), as in our previous study (Chen and Regehr, 2000). TTX ELVAX-treated animals have a large number of connections (~13, estimated using AMPAR currents; ~17 using NMDAR currents) compared to control p15–p16 (~5, AMPAR; ~6, NMDAR), and are more similar to young control animals (~10, AMPAR; ~13, NMDAR). However, this count is not amenable to statistical analysis. Thus, we also computed a fiber fraction: the amount of synaptic current contributed to each cell by a single fiber was measured as a fraction of the maximal current for each cell. This normalized for the variability in the total current magnitude between cells and allowed averaging between all cells. The average fiber fraction for each condition is plotted in Figure 3B, with each single afferent representing  $25.1\% \pm 4.4\%$  of the synaptic input to control p15–p16 animals. Similar fractions exist for both DR ( $24.3\% \pm 3.3\%$ ) and saline ELVAX ( $16.3\% \pm 2.9\%$ ) conditions. However, in TTX treatment, each fiber's contribution to the total current remains about as low as in p11 control animals, representing only  $10.9\% \pm 1.8\%$  of the synaptic input ( $p = 0.025$  versus control,  $p < 0.002$  for combined control and saline ELVAX versus TTX ELVAX). We thus estimate that about four retinal afferents remain connected to each LGN cell in control animals at p15–p16, while about ten afferents remain in TTX ELVAX, similar to that present at p9–p11. Note that both methods are likely to underestimate the total



**Figure 3. Synaptic Pruning Is Deficient in TTX-Treated but Not Visually Deprived Animals**

(A) Fibers remaining computed by ratio of average maximal current to average single-fiber current, calculated independently for AMPAR and NMDAR currents.

(B) Fiber fraction for p9–p16 animals. Each single fiber divided by the maximal current for the same relay neuron estimates the fraction of the cell's total current contributed by that fiber, averaged for all cells treated to the same *in vivo* activity manipulation.  $n = 50$ –82.

(C) (Top) Example traces illustrate three categories of refinement. Unrefined p9 relay neuron from control mouse. (Middle) Resolving p16 relay neuron from dark-reared mouse. (Bottom) Refined p16 relay neuron from dark-reared mouse. (Right) Fraction of cells for a given activity manipulation in each state of refinement.

Cells evaluated in control p9–p11,  $n = 24$ ; control p15–p16,  $n = 30$ ; DR,  $n = 29$ ; saline ELVAX,  $n = 34$ ; TTX ELVAX,  $n = 25$ . \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ .

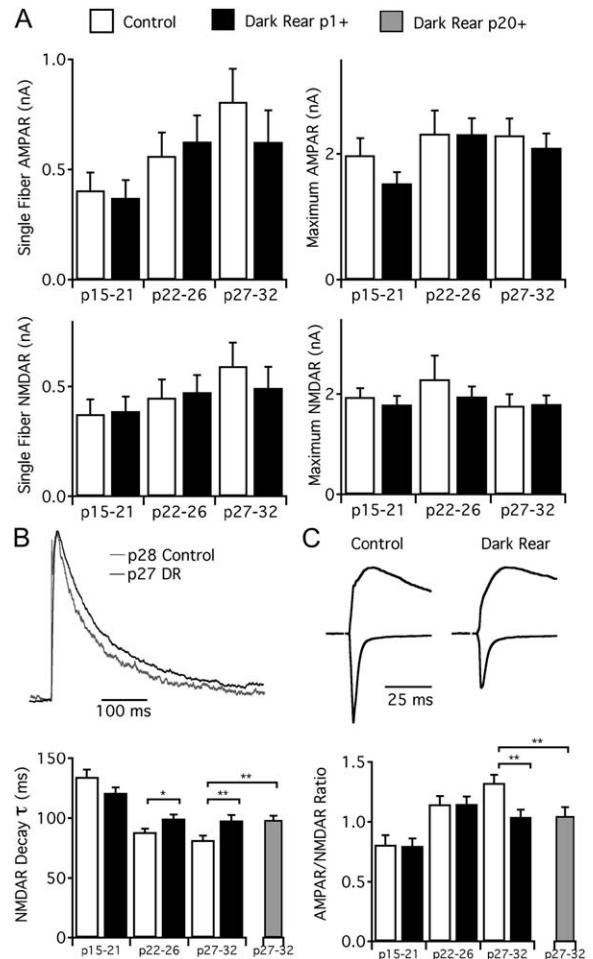
number of fibers since afferents outside the plane of the slice are removed, not all fibers are stimulated, and inputs to the same cell are not all equivalent. Thus, our estimated number of afferents should be taken as relative for comparison to other conditions and not as an absolute measurement.

We further assessed whether the estimates of afferent number obtained using numerical estimates were consistent with the range of responses observed from single relay neurons. An examination of synaptic responses to stimulation between minimal to maximal current revealed that response amplitudes increased in a stepwise manner, indicating the sequential recruitment of distinct presynaptic fibers. At p15–p16, a great deal of heterogeneity exists among synaptic responses of different relay neurons. In some cells, clear, distinct steps made counting the number of fibers straightforward. However, electrical noise, trial-to-trial variation, and small fiber amplitude prohibited distinguishing distinct steps in other cells. Thus, to roughly assess the degree of refinement for a given condition of activity manipulation, we sorted cells (blind with respect to condition) into three categories. *Refined* cells are defined as those cells in which there are from one to three clear, distinct steps in current amplitude. *Resolving* neurons show between four and six steps in current amplitude, with a subset of the steps being clear and distinct. *Unrefined* cells have little or no clear distinction between steps, with a continuous rise in current amplitude with increasing stimulus intensity, which we interpret as a large number of afferents, each with small synaptic strength. Figure 3C shows a significant decrease in the number of refined cells in TTX-treated mice only. Short-term dark rearing, as expected, did not disrupt synaptic pruning and maturation. Conversely, TTX blockade increased the number of unrefined cells. This gauge of synaptic refinement was thus consistent with the other methods described above and, taken together, support the hypothesis that spontaneous activity is sufficient to mediate changes in retinogeniculate connectivity.

#### Chronic Dark Rearing Results in Only Modest Changes in Synapse Maturation

Because previous studies had shown sensory deprivation-induced plasticity from retina to cortex in the visual system, our short-term studies may have missed effects of dark rearing by depriving the animals too briefly. Therefore, we visually deprived mice from near birth (p1 or earlier) into young adulthood (up to p32), evaluating synaptic strengthening and pruning over a wider range of ages. Comparison of the three age groups between p15–p32 revealed that the average single-fiber strength for both AMPAR and NMDAR was not significantly different between control and DR mice (Figure 4A, left). Furthermore, maximal current amplitudes were not different between control and chronically dark-reared animals (Figure 4A, right).

Other aspects of synapse maturation were more strongly influenced by chronic dark rearing. Figure 4B shows that the normal developmental acceleration of NMDAR decay  $\tau$  was slowed in long-term dark-reared animals compared to age-matched controls after p22. The differences between dark reared and control are not present at earlier ages, consistent with our short-



**Figure 4. Small Effect of Chronic Visual Deprivation on Synaptic Maturation**

(A) Summary of EPSC amplitudes of AMPAR and NMDAR currents elicited in response to single-fiber stimulation (left) and maximal stimulation (right) for animals in age groups from p15 to p32. n = 23–68.

(B) (Top) Representative normalized NMDAR current decay traces. (Bottom) NMDAR current decay  $\tau$  is summarized for all age groups. NMDAR decay  $\tau$  values (ms) for late DR are added to the right of the graph for comparison. Late dark rearing alone is sufficient to achieve the same deficit as chronic dark rearing. n = 27–46.

(C) (Top) Representative traces from mature mice in control (p28) and chronic dark rearing (p27). Traces are normalized to peak NMDAR current. (Bottom) Average AMPAR/NMDAR current ratio in mice from p15 to p32 for control and DR. Ages p27–p32 are appended for late DR. n = 29–46.

\*p < 0.05, \*\*p < 0.01, and \*\*\*p < 0.001. Control (p15–p21), 45 cells from 14 animals; control (p22–p26), 33 from 11; control (p27–p32), 35 from 13. Dark reared (p15–p21), 46 cells from 12; DR (p22–p26), 37 from 11; DR (p27–p32), 29 from 12. Late dark rearing (p27–p32), 36 cells from 12.

term results, suggesting that either sustained reduction in visual experience retards the normal developmental acceleration or that absence of visual experience at this later point in development determines the NMDAR time course. It is worth noting, though, that the developmental endpoint reached is similar: both control ( $81.6 \pm 3.7$  ms) and chronic dark-reared ( $98.1 \pm 4.4$  ms) animals have NMDAR currents that decay much faster than young p9–p11 animals ( $192.9 \pm 6$  ms). Furthermore, chronic dark rearing resulted in reduced

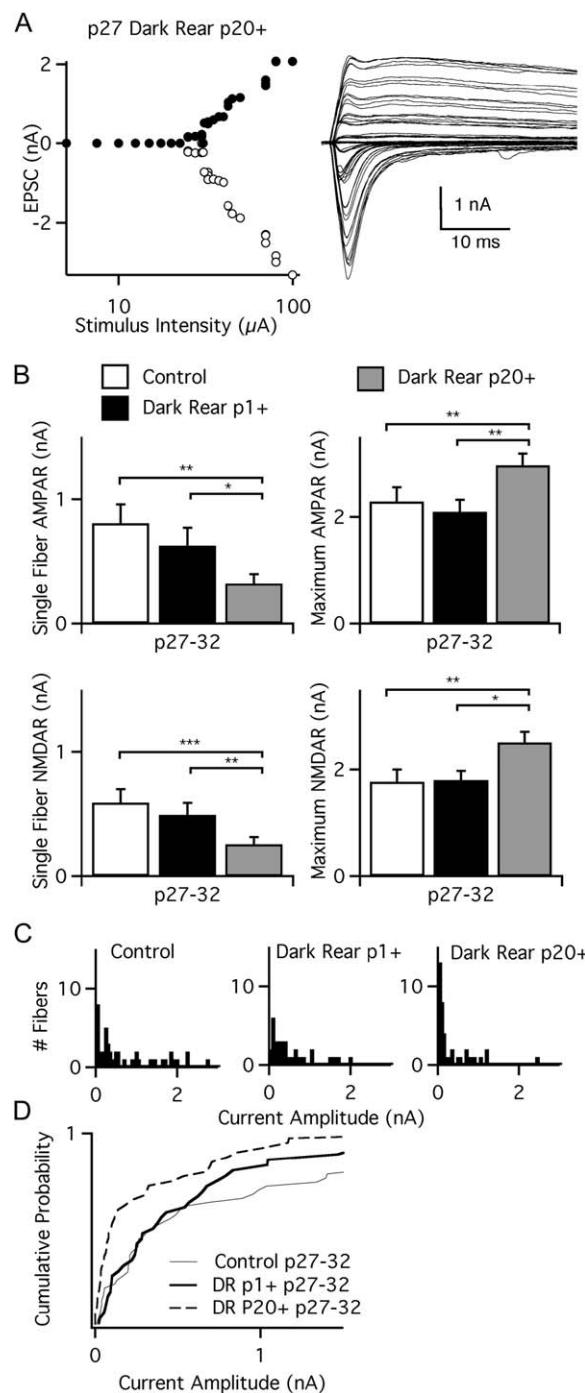
AMPA/NMDAR current ratio at p27–p32 (Figure 4C). Again, however, the developmental endpoint reached in DR ( $1.0 \pm 0.1$ ) is more similar to control ( $1.3 \pm 0.1$ ) than young p9–p11 animals ( $0.3 \pm 0.0$ ). Since chronic dark rearing results in deficits in NMDAR current decay and AMPA/NMDAR ratio but not in current amplitudes and afferent elimination, we conclude that different features of synaptic maturation can be independently regulated by distinct properties of visual experience.

### Deprivation following Visual Experience Disrupts Synaptic Remodeling

The deficits in NMDAR current time course and AMPA/NMDAR ratio in chronic dark-reared mice become apparent late in development, raising the possibility that sustained visual deprivation is necessary to alter normal synapse maturation. Alternatively, this synaptic deficit could be due to visual deprivation during a distinct sensitive period in later development, similar to the cortical critical period for ocular dominance plasticity (Hensch, 2005). To distinguish between these possibilities, we initiated dark rearing relatively late in development to determine whether similar synaptic changes would occur in older mice during a shorter period of visual manipulation. We deprived mice from p20, and sacrificed animals at p27–p32 after 7–12 days of DR.

Indeed, a short period of visual deprivation after p20 (late DR) is sufficient to cause the same deficiency in AMPA/NMDAR ratio (Figure 4C) and NMDAR current decay (Figure 4B) that we see with chronic dark rearing. Evoked synaptic currents from late DR animals showed similar characteristics to their chronically deprived counterparts, with significant changes relative to control adult animals.

Remarkably, although we had not found changes in single-fiber current amplitudes during chronic DR manipulations, both AMPAR and NMDAR single-fiber currents were significantly reduced by late DR (Figure 5B). Furthermore, maximal AMPAR and NMDAR currents were both increased by this short period of late dark rearing. These results strongly suggested that the number of afferents connected to relay neurons increased following late visual deprivation after p20. To quantify this, we assessed synaptic connectivity using similar methods as used for assessing our short-term manipulations. First, amplitude histograms and cumulative probability histograms of AMPAR-mediated single-fiber currents (Figures 5C and 5D) demonstrate a reduction in the average strength of retinogeniculate connections after late DR. Second, we estimated the number of fibers present at p27–p32: this indicated an increase in afferent number following late visual deprivation. The number was approximately five for control animals p15–p16 (Figure 3A) and three at p27–p32 (Figure 6A). In late DR animals, our estimate of the remaining afferents from the ratio of average maximal and single-fiber currents is greater than ten afferents. This is consistent with our fiber fraction calculations (Figure 6B) that show a significant change following late visual deprivation. Each fiber represents only  $13.4\% \pm 2.4\%$  of the synaptic current to a given LGN relay neuron, similar to the  $10.9\% \pm 1.4\%$  in p9–p11 animals and significantly less than that in mature control and chronically DR animals. Both these estimates are



**Figure 5.** Late Dark Rearing After p20 Disrupts Normal Developmental Synaptic Remodeling

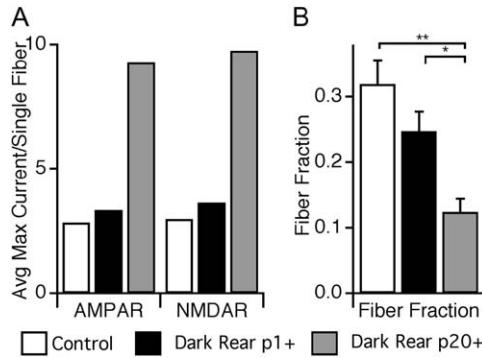
(A) Representative response to incremental increase in stimulus intensity for a p27 mouse dark-reared from p20 until sacrifice. Recordings displayed as in Figure 2A.

(B) Summary of EPSC amplitudes of AMPAR and NMDAR currents elicited in response to single-fiber stimulation (left) and maximal stimulation (right) for animals dark reared from age p20, n = 28–48. Data for control and chronic DR are those from Figure 4A.

(C) Histograms of single-fiber AMPAR current amplitude in 50 pA bins for p27 to p32 mice.

(D) Single-fiber AMPAR currents in cumulative probability histograms.

\*p < 0.05, \*\*p < 0.01, and \*\*\*p < 0.001.



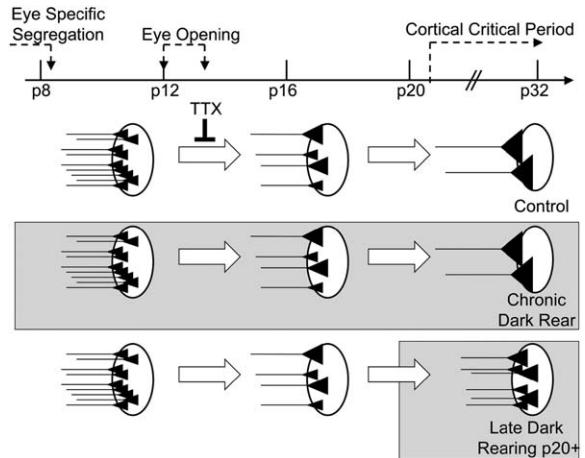
**Figure 6. Late Dark Rearing After p20 Disrupts Synaptic Pruning**  
Measures of synaptic pruning for p27–p32 mice. (A) Afferents remaining computed by ratio of average maximal current to average single-fiber current, computed independently for AMPAR and NMDAR currents. (B) Fiber fraction for chronic and late visual manipulations, computed as in Figure 3.  $n = 60$ –80. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ .

consistent with an increase in the number of connected retinal afferents per LGN cell following late DR.

## Discussion

Our studies reveal two developmental periods where spontaneous activity (p9–p16) and visually evoked activity (after p20) drive specific aspects of synaptic remodeling (see Figure 7). The prevailing theories of sensory system development invoke the importance of sensory experience in the refinement of sensory circuits (Foeller and Feldman, 2004; Katz and Shatz, 1996). These ideas arise from studies that demonstrate changes in synaptic function associated with sensory manipulations (Aizenman et al., 2003; Hensch et al., 1998; Takahashi et al., 2003; Zhou et al., 2003). Here we show that in the context of the full developmental range of synapse maturation between p9 and p32, sensory activity governs only the late stage of synaptic remodeling. Our experimental system allowed us to differentiate between the contributions of spontaneous and visually evoked activity, thus demonstrating the relative importance of the two by dissecting the different aspects of activity in developmental strengthening and pruning of synapses. We find that the majority of synapse elimination after p9 is driven by spontaneous activity alone, while visual experience plays a later role in the maintenance and plasticity of synaptic connections.

At another CNS synapse that remodels during development, the climbing fiber to Purkinje cell synapse of the cerebellum (Crepel et al., 1976; Mariani and Changeux, 1981), two phases of synaptic refinement have also been proposed (Hashimoto and Kano, 2003, 2005). The majority of synapse elimination occurs between p3 and p6, with a later phase (p10–p14) that correlates with the onset of multivesicular release. Previous studies of climbing fibers demonstrate that disruption of many postsynaptic signaling pathways can alter the later phase of pruning when only a few fibers remain (Hashimoto and Kano, 2005); however, it is not known what mechanisms affect the earlier phase of synapse elimination, when approximately ten climbing fibers contact a single Purkinje cell (Sugihara, 2005). At the retinogeni-



**Figure 7. Summary of Roles of Activity in Retinogeniculate Developmental Maturation**

A diagram of the phases of retinal afferent maturation and pruning. In early stages, each LGN relay cell is innervated by many RGC axons. Between p11 and p16, the majority of these excess connections are eliminated, and those that remain are strengthened. Visual deprivation does not seem to retard this process, but synaptic elimination is blocked in the absence of spontaneous retinal activity. At older ages (after p20), late visual deprivation but not chronic dark rearing results in recruitment of additional afferents and a less mature synaptic phenotype.

culate synapse, because the activity pattern of RGCs in response to both spontaneous activity and sensory experience is well characterized, we can assess the functional significance of each phenomena. By contrast, such a distinction would not be possible at the climbing fiber to Purkinje cell synapse, as little is known about the in vivo stimuli that excite climbing fibers.

## A Major Early Pruning Step Depends on Spontaneous Activity

Our evidence supports the hypothesis that presynaptic spontaneous activity alone is sufficient to drive the bulk of retinogeniculate synapse maturation, even in the absence of sensory experience. During ages around eye opening (p9–p16), this major step in functional development involves the strengthening of a subset of presynaptic retinal afferents, the elimination of a majority of retinal afferents, and a change in the kinetics of NMDAR current time course. As spontaneous retinal wave activity persists until p15 (Demas et al., 2003), waves are a major source of action potential activity in ganglion cells during this developmental period and are thus a likely candidate for the in vivo activity that drives functional synaptic remodeling. The earlier anatomical segregation of retinal afferents into eye specific zones has been shown to rely on this retinal wave activity (Penn et al., 1998; Torborg et al., 2005; Demas et al., 2006). It is possible that the morphological correlate to the continued functional maturation we observe occurs by finer axon arbor rearrangement within each eye-specific layer. Note, however, in our preparation, the contribution of spontaneous RGC action potentials and spontaneous mEPSCs is not differentiated from that of retinal waves. Thus, it is still unclear which specific features of spontaneous retinal activity (such as spike frequency

or burst pattern), if any, play a role in functional synapse maturation.

We observe an increase in both AMPAR and NMDAR components of synaptic current during synaptic strengthening during the earlier phase of synaptic remodeling (p9–p16). Previous studies have demonstrated that synaptic strengthening proceeds by the activity-dependent trafficking of AMPA receptors (Gomperts et al., 2000; Luscher et al., 1999; Malinow and Malenka, 2002; Takahashi et al., 2003). Observed increases in NMDAR-mediated current may occur through a separate mechanism that does not rely on visual or spontaneous retinal activity. Our findings support this model by showing that strengthening of single-fiber AMPAR currents is disrupted by blockade of spontaneous activity, while the effect on single-fiber NMDAR currents is not as strong. At early ages, we show that *in vivo* spontaneous activity is sufficient for driving AMPAR into developing retinogeniculate synapses. Retinal waves persist to p15–p16, regardless of whether animals have visual experience (Demas et al., 2003). Thus, we hypothesize that spontaneous retinal activity may contribute to the majority of RGC firing, while the onset of vision contributes a smaller share and thus produces no deficit in animals DR from p11–p16. The period during which spontaneous activity is dominant, then, is closed by the cessation of retinal waves and not the onset of visual experience. Consistent with this hypothesis, the AMPAR/NMDAR ratio is altered by deprivation in older animals since, at the later developmental period, retinal waves are not present and visually evoked activity may dominate. This does not explain why AMPAR/NMDAR ratios are normal in chronically deprived animals from p15–p26, suggesting other processes vary over development, including the plasticity of relay neurons.

One interesting observation about the distribution of our single-fiber AMPAR amplitudes is that a subset (30%–40%) of retinal afferents to the developing LGN strengthen a great deal during p15–p16. It seems that not only is spontaneous activity a prerequisite for strengthening of individual fibers but that, in the complete absence of activity, excess synapses are not eliminated. The fact that only a subset of fibers strengthens is consistent with competition-based (Hebbian) mechanisms of synapse strengthening selecting a subset of afferents with coincident depolarization instead of all fibers strengthening uniformly (Kasthuri and Lichtman, 2003; Katz and Shatz, 1996). However, looking in more detail at the current amplitude distributions of single fibers that remain weak (<100 pA), we see no clear evidence for synaptic weakening, though it is unclear whether this does not occur at the retinogeniculate synapse, or if resolution of small fiber currents is limited by noise in our technique.

Our results in LGN differ from recent work that suggests that eye opening in mice causes rapid synaptic changes in the superficial superior colliculus (SC). At short intervals after eye opening, Lu and Constantine-Paton report rapid pruning within 24 hr after eye opening which is blocked by eyelid closure (Lu and Constantine-Paton, 2004). In contrast, our short-term dark-rearing data show that reduction of visual experience does not delay synaptic pruning in the LGN. However, at older ages, both LGN (our data) and SC (Lu and Constan-

tine-Paton, 2004) show a large degree of synaptic pruning in chronically visually deprived animals a week after eye opening. That pruning occurs with a normal time course in deprived LGN but not SC could be accounted for by cell type-specific differences of the pre- and post-synaptic cells. Such specificity is shown in cortex, where the rules governing the response to visual deprivation are highly cell pair specific (Maffei et al., 2004). Thus, it appears that the rules governing synaptic maturation may also be cell type specific for RGC connections.

### Chronic Activity Manipulations Alter Subtle Aspects of Synaptic Remodeling

Chronic manipulation of sensory experience (DR) had a surprisingly mild phenotype compared to our original hypothesis that visual activity was required for the functional strengthening and pruning of retinogeniculate afferents. Neither the average single-fiber strength nor maximal currents were affected by even the most prolonged periods of deprivation. Other synaptic properties, such as the AMPAR/NMDAR current ratio and NMDAR decay  $\tau$  become significantly altered after almost 3 weeks of chronic dark rearing. These synaptic changes are not secondary to a cumulative loss of visual experience because they can be reproduced by late DR for a shorter period after p20. Instead, our data suggest that visual experience during the fourth postnatal week plays a role in establishment or maintenance of these certain synaptic properties. On the other hand, it is worth noting that neither chronic nor short-term deprivation results in developmental stasis or a return to a completely immature state, but rather a mild deficit compared to the fully mature state. Thus, we find that different features of synaptic remodeling are separable and regulated by distinct properties of activity.

It is important to note that our technique may miss certain aspects of synaptic reorganization following visual manipulations. Since we quantify the changes at the synaptic level, information about the spatial organization of afferents is lost. Furthermore, we cannot distinguish whether relay cells retain ON or OFF RGC afferents or remain responsive to both. Consistent with our chronic DR experiments, visual deprivation does not disrupt normal orientation and direction selectivity of LGN cells in cats, though orientation selectivity is reduced in cortex, where monocular deprivation has more dramatic effects (Cynader et al., 1976; Hubel and Wiesel, 1970; Zhou et al., 1995). Thus, we cannot assess changes in the sensory map topography or receptive field shape due to additional afferents, or determine whether remaining ganglion cell afferents are from adjacent cells or disparate locations in the retina.

### Visual Deprivation following Sensory Experience Activates Synaptic Plasticity in Mature Animals

While chronic visual manipulations did not cause dramatic changes at the retinogeniculate synapse, we found that more significant disruptions to normal synaptic connectivity result from late dark rearing following a week of normal visual experience. Not only are the single-fiber current amplitudes of both AMPAR and NMDAR currents reduced by this manipulation, but total currents are enhanced, which suggests a greater number of RGCs connected to each LGN relay neuron.

Our rough estimates of number of retinogeniculate afferents (Figure 6A) indicate a doubling of inputs (from approximately three to four inputs to seven to ten inputs) after 7 days of visual deprivation from p20. The source of the additional retinal inputs recruited is unclear. We cannot be certain whether the additional afferents are adjacent to existing contacts, nor can we distinguish whether these afferents represent new contacts or the re-establishment of connections to previously connected RGC axons.

Our findings from late dark rearing experiments are consistent with the idea that visual experience is needed to maintain appropriate sensory maps as previously proposed (Crair et al., 1998; Carrasco et al., 2005). There is, however, a striking difference between our findings in the LGN and those of previous reports in other areas of the visual system. In the cat visual cortex or the hamster superior colliculus, normal development of sensory maps and receptive fields, respectively, progressed in the absence of visual experience, but with prolonged deprivation these maps or receptive fields subsequently deteriorated. In contrast, at the retinogeniculate synapse, synaptic remodeling in chronically dark-reared mice is indistinguishable from control. Instead, our results demonstrate that previous experience dictates the response to late sensory deprivation. With previous visual experience prior to the cortical critical period, the connectivity of the retinogeniculate synapse becomes sensitive to changes in visually evoked stimuli. This response is quite distinct from the synaptic response to continued deprivation during the cortical critical period when mice are dark reared from birth.

Several questions regarding the role of previous visual experience in response to deprivation remain unanswered. Future studies that delineate how much sensory experience is sufficient to enable plasticity in response to deprivation and how rapidly plasticity is induced will provide insight into the cellular and molecular mechanisms that are involved. Our experiments around the time of eye opening show that 4 days are sufficient for the elimination of a large number of excess connections. However, recruitment of a similar number of additional fibers in response to late DR may require a longer period of time. Finally, it is not clear whether the synaptic plasticity that we observe is transient or persistent. Subdivision of the late dark-rearing data into two sets (p27–p29 and p30–p32) reveals no trend in single-fiber currents and remaining fiber numbers, suggesting that the deprivation-induced changes may be persistent.

One possible model that could explain the distinct effects of chronic and late sensory deprivation relies on the fact that dark rearing from p20 coincides with the murine cortical critical period for ocular dominance plasticity, around p23–p35 (Hensch, 2005). Developing animals exposed to normal visual experience before this age may acquire the ability to express functional synaptic plasticity in response to a change in sensory input. Notably, there is recent evidence that supports the idea that previous visual experience is necessary for normal gene regulation (Majdan and Shatz, 2006). In contrast, animals that are chronically dark reared are not capable of similar plasticity because a specific activity-dependent program has not been activated. Since triggering of cortical plasticity relies upon inhibitory cir-

cuit maturation (Hensch et al., 1998), it will be interesting to test whether changes in inhibitory circuits under activity manipulation could cause changes in retinal afferent connectivity.

Another possible model for these somewhat paradoxical findings is that chronic dark rearing activates homeostatic or compensatory mechanisms that, while not observed in the synaptic characteristics we measured, nonetheless render these animals less sensitive to prolongation of dark rearing past p20. The specific mechanisms that render normal animals receptive to synaptic plasticity following p20 while chronically dark-reared animals are resistant to synaptic changes are not clear. Nevertheless, we conclude that the age during *in vivo* synapse maturation at which an activity modification occurs plays an important role in determining the system's response.

In summary, we have shown two distinct periods of synaptic development at the mouse retinogeniculate connection and identified the different roles played by spontaneous activity and visual experience in synapse remodeling and maintenance. These studies have also revealed that synaptic plasticity at the later period in development is dependent on the previous history of visual experience at the retinogeniculate synapse. Identification of signaling molecules that are differentially regulated during one or both of these developmental periods will enable the future exploration of the cellular mechanisms underlying these activity-dependent changes.

## Experimental Procedures

### LGN Slice Preparation

All experimental procedures were performed in compliance with animal protocols approved by the IACUC at Children's Hospital, Boston. C57BL6/J mice (aged p9–p32) were anesthetized using the inhalant isoflourane and decapitated. The brain was then rapidly removed and placed in a 4°C choline-based cutting solution containing (in mM): NaCl 78.3, NaHCO<sub>3</sub> 23, glucose 23, choline chloride 33.8, KCl 2.3, NaH<sub>2</sub>PO<sub>3</sub> 1.1, MgCl<sub>2</sub> 6.4, and CaCl<sub>2</sub> 0.45. LGN slices (250 μm) were cut as previously described using a sapphire blade (DDK) on a vibratome (Leica VT1000S) (after Chen and Regehr, 2000; Turner and Salt, 1998). Slices were allowed to recover at 31°C for 20–25 min in the choline cutting solution and for 20–35 min in isotonic saline solution (in mM: NaCl 125, NaHCO<sub>3</sub> 25, glucose 25, KCl 2.5, NaH<sub>2</sub>PO<sub>3</sub> 1.25, MgCl<sub>2</sub> 1, and CaCl<sub>2</sub> 2). Oxygenation (95% O<sub>2</sub>/5% CO<sub>2</sub>) was continuously supplied during cutting and recovery.

### Electrophysiology

Whole-cell voltage-clamp recordings of thalamic relay neurons from the contralateral monocular region of the dorsal LGN were performed as previously described (Chen and Regehr, 2000). Recording glass electrodes of 1.2–2.2 MΩ resistance were filled with an internal solution of (in mM) CsF 35, CsCl 100, EGTA 10, HEPES 10, and pH 7.32 (with CsOH). D600 (0.1 mM; methoxyverapamil hydrochloride; Tocris, MO) was added to block voltage-gated calcium channels. Slices were continuously perfused with isotonic saline and the GABA<sub>A</sub> receptor antagonist, 20 μM bicuculline (Tocris, MO), to inactivate local inhibitory circuits. Saline-filled glass pipettes were placed up to 1 mm away in the optic tract. Single-fiber determination was performed as previously described (Chen and Regehr, 2000). Single-fiber measurements included a second input from a given cell if it was recruited during incremental increase in stimulus intensity (0.25 μA) and clearly resolvable (five times greater in amplitude) from the first input. Maximal current response was obtained by increasing the stimulus intensity until the current response stopped rising. The stimulating electrodes were moved to determine which location would give the largest postsynaptic response; this was the location used for recordings. NMDAR current time course was

determined with a single exponential fit to the current evoked by maximal stimulation of the optic tract while holding the relay neuron at +40 mV for 500 ms following stimulation.

#### Dark Rearing

Control animals for dark-reared experiments were raised in microisolator cages in standard animal facility conditions under a 12 hr light/12 hr dark cycle. Dark-reared pups and their mothers were placed in a light-tight container in which luminance was measured at <0.02 lux by photometer (Light Probe Meter, Extech Instruments). Experimental dark-reared animals were transferred to the lab for slice preparation in an opaque box (as were control animals in blind experiments) to minimize light exposure prior to sacrifice. For short-term manipulations of visual activity, litters of C57BL6/J mouse pups were split from p11 (or earlier) to permit rearing of littermates in dark or control conditions, a 12 hr light/dark cycle, and short-term dark rearing was begun at p11. The eyes of control-reared littermates were opened at p12 with an incision between the eyelids to ensure equivalent amounts of visual experience for the control group. After 3–4 days of visual experience (or deprivation), retinogeniculate brain slices of p15–p16 mice were obtained and response to optic tract stimulation quantified. For long-term experiments, mice were reared in complete darkness from p1 or earlier to avoid concerns that visual stimuli presented through closed eyelids (Akerman et al., 2002) would affect results.

#### TTX ELVAX Insertions

ELVAX (DuPont) ethylene-vinyl acetate copolymer containing TTX or saline was synthesized from beads as previously described (after Prusky and Ramoa, 1999). Phosphate-buffered saline (PBS) was used in control ELVAX. The resulting polymer was cut into 175  $\mu$ m disks on a cryostat. ELVAX was inserted into the vitreous humor of anesthetized (avertin) p11 mice (Cook et al., 1999). A small incision was made in the sclera just below and parallel to the limbus on the ventral surface of the left eye. Three small pieces of ELVAX polymer (~250  $\mu$ m length and width) were placed in the vitreous humor using fine forceps after rinsing briefly in 95% EtOH and then PBS to remove surface TTX and to minimize initial burst of drug release. The eye incision was sealed using gelfoam and the eye covered with antibiotic (Neomycin, Polymyxin B, Bacitracin; CVS Pharmacy, Boston, MA) to minimize infection. Recordings were made from the monocular region of the right LGN corresponding to the affected contralateral eye.

The effectiveness of TTX-ELVAX insertion was assessed by ophthalmological exam. Animals with damaged or infected eyes were rejected. Only animals that exhibited block of both the direct pupillary light response in the injected eye as well as the consensual light response in the uninjected eye were used for electrophysiological recordings. Pupil size was measured with a reticule on a dissecting microscope, and only animals with pupil diameters greater than 0.8 mm were used for experiments. ELVAX insertion was typically effective for 3–4 days.

#### Evaluation of Synaptic Pruning

We attempted to quantify the degree of synaptic pruning of excess retinogeniculate afferents to dLGN relay cells using three different measures that gave estimates of number of connected fibers, with the understanding that the number is not exact and likely an underestimate due to the limitations inherent to the slice preparation. First, we estimated the number of afferents to each LGN cell recorded based on the size of the single-fiber current as a percent of the total current for that cell. This allowed us to gather estimates for each fiber from each cell, making differences in fiber number statistically testable. Furthermore, by expressing the fiber fraction as percent, this allowed for normalization of input size and made inclusion of silent synapses possible (division by zero would occur if the maximal current were divided by single fiber for each cell). The number of fibers estimated using this method is in agreement with coarse estimates based on the stimulus-response profile of each cell outlined below.

In a second method by which we investigated afferent refinement, synaptic responses were recorded from LGN principal neurons at both +40 mV and –70 mV while varying the stimulus intensity from minimal stimulation (required to excite a single fiber) to maximal stimulation (to excite the bulk of the optic tract). Stimulation was increased in increments of 2.5  $\mu$ A between 0  $\mu$ A and 40  $\mu$ A (with larger

increments at higher current intensities), except in cases where smaller steps were used to verify that a large jump in current was due to the recruitment of a single fiber. When overlaid, these traces indicated the number of afferents to the postsynaptic cell: clusters of postsynaptic response (current amplitude) were interpreted as the separate fibers recruited during stimulation. This stimulus-response profile allowed a visualization of the rough number of afferents to the cell. Cells were categorized (blind with respect to condition) into refined, resolving, and unrefined as described above. Third, we simply took the ratio of the average maximal current to the average single-fiber current for each age group and visual manipulation condition.

#### Statistical Analysis

Mean values of AMPA/NMDA current ratios, NMDAR current decay time course  $\tau$ , and fiber fraction were compared using Student's *t* test. Normality of current amplitude distributions was tested by comparison to a theoretical normal distribution using a Kolmogorov-Smirnov test. This test was then used to evaluate differences in non-normal distributions. Values for animals p16 and younger were typically not normally distributed (see Figure 2C). Fisher's exact test was used to examine differences between treatment conditions where cells were divided into categories (such as for evaluation of synaptic pruning, Figure 3C).

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