

Silent Synapses during Development of Thalamocortical Inputs

John T.R. Isaac,*[§] Michael C. Crair,[†]
Roger A. Nicoll,^{†‡} and Robert C. Malenka*^{††}

*Department of Psychiatry

[†]Department of Physiology

[‡]Department of Molecular and Cellular Pharmacology
University of California
San Francisco, California 94143

Summary

During development, activity-dependent mechanisms are thought to contribute to the refinement of topographical projections from the thalamus to the cortex. Because activity-dependent increases in synaptic strength may contribute to the stabilization of synaptic connections, we have explored the mechanisms of long-term potentiation (LTP) at thalamocortical synapses in rat somatosensory (barrel) cortex. During early postnatal development (postnatal days 2–5), we find that a significant proportion of thalamocortical synapses are functionally silent and that these are converted to functional synapses during LTP. Silent synapses disappear by postnatal day 8–9, the exact time at which the susceptibility of these synapses to LTP is lost. These findings suggest that the activity-dependent conversion of silent to functional synapses due to correlated pre- and postsynaptic activity may contribute to the early development and refinement of thalamocortical inputs to cortex.

Introduction

A fundamental property of thalamocortical inputs to primary sensory areas of cortex is their organization into well-defined topographic maps of the peripheral sensory environment. In the visual cortex, the final pattern of thalamic inputs is not entirely genetically predetermined but rather is refined over the course of early development via an activity-dependent process during which thalamic axons are thought to compete for their postsynaptic cortical targets. The most compelling evidence in support of this view comes from studies in which the normal pattern of connectivity is disrupted either by reducing normal neuronal activity pharmacologically or by disrupting this activity using sensory perturbations. This modification of thalamocortical afferent topography by perturbations in neuronal activity appears to be most robust during a defined period in early development, so-called critical periods (Goodman and Shatz, 1993; Singer, 1995; Katz and Shatz, 1996).

An active area of theoretical and experimental research has been the elucidation of the cellular and molecular mechanisms by which neuronal activity controls the precise final patterning of thalamocortical afferents.

A prominent hypothesis for which there is some theoretical and experimental support has been that mechanisms analogous to long-term potentiation (LTP) and long-term depression (LTD) play an important role in activity-dependent development (Bear et al., 1987; Singer, 1995; Katz and Shatz, 1996). To address this question, in previous work, Crair and Malenka (1995) examined the occurrence of LTP at thalamocortical synapses in a slice preparation (Agmon and Connors, 1991), which contained the afferent connections from the ventrobasal nucleus of the thalamus to layer IV cells in somatosensory (barrel) cortex. In slices prepared from early postnatal rats (P3–P7), robust, NMDA receptor-dependent LTP could be elicited reliably. However, beginning at P8, LTP was essentially impossible to induce even though the induction protocol provided all of the ingredients that are thought to be required to trigger LTP (Crair and Malenka, 1995). This critical period for LTP induction closely matches the critical period during which thalamocortical afferent topography is sensitive to sensory perturbations. Thus, this finding provided strong correlative evidence in support of a role for LTP in the development of thalamocortical circuitry.

In the present work, we have explored possible mechanisms underlying LTP at thalamocortical synapses in rat barrel cortex. In particular, we have examined whether functionally silent synapses may exist and whether their conversion to functional synapses may contribute to LTP. Recently, we and others presented evidence that in hippocampal CA1 pyramidal cells, synapses exist that appear to contain functional NMDA receptors but no functional AMPA receptors (Isaac et al., 1995; Liao et al., 1995; Durand et al., 1996). Thus, when the cell is near its resting membrane potential, these synapses are functionally silent even when transmitter release occurs. Following activation of NMDA receptors during standard LTP induction protocols, these silent synapses could be converted to functional ones. Such a mechanism can explain many features of NMDA receptor-dependent LTP in the hippocampus and is particularly attractive as a developmental mechanism by which activity can induce functional synapse formation (Liao et al., 1995; Liao and Malinow, 1996; Durand et al., 1996). We find that silent synapses indeed exist at thalamocortical inputs during early postnatal development and that they can be converted to functional synapses by an LTP induction protocol. However, silent synapses disappear by approximately postnatal day 8, the exact time at which it becomes difficult to generate LTP.

Results

In an initial series of experiments, we used minimal stimulation techniques (Isaac et al., 1996) to examine the quantal changes that contribute to the generation of LTP at thalamocortical synapses. By using small stimulation strengths, it is possible to record small excitatory postsynaptic currents (EPSCs) that are due to the activation

[§]Present address: Department of Anatomy, University of Bristol School of Medical Sciences, Bristol BS8 1TD, United Kingdom.

of a single synapse or a small number of synapses and to distinguish these from synaptic failures. From such data, one can calculate the failure rate and the so-called potency (Stevens and Wang, 1994), which is defined as the average size of the synaptic response when release of transmitter occurs. (If a single synapse is being studied, potency will be the same as the quantal size.) This type of analysis has been performed by a number of investigators studying LTP in the CA1 region of the hippocampus. Results have varied, but it is generally agreed that when LTP is accompanied by a decrease in failure rate but no change in potency, these results are most readily explained by postulating that LTP was caused solely by an increase in the probability of transmitter release (Stevens and Wang, 1994; Bolshakov and Siegelbaum, 1995). When a change in potency has been observed, however, a change in probability of release alone is unlikely to account for LTP since the increase in potency is large and any change in failure rate is small (Isaac et al., 1996). In these circumstances, it is necessary to postulate a change in quantal size and often in addition, a change in the number of functional synaptic contacts (Isaac et al., 1996).

Figure 1 shows an example of a minimal stimulation experiment performed in a thalamocortical slice prepared from a neonatal animal (P5). The initial failure rate of the synaptic responses was 0.40, and the potency was 10.8 pA. LTP was induced simply by depolarizing the cell to -10 mV while maintaining the baseline stimulation rate. This protocol prevents any possible frequency-dependent changes in axon excitability while providing the essential requirements for eliciting NMDA receptor-dependent LTP. Upon returning the cell to -70 mV, it is apparent that robust LTP (167%) had been elicited (Figures 1A and 1B) and that this was accompanied by a significant change in potency (to 16.1 pA; Figure 1B₃) and a decrease in failure rate (to 0.30). A histogram of the EPSCs before and after LTP (Figure 1C) indicates that LTP caused a significant proportion of the small events to disappear and be replaced by larger events.

A summary of eight such experiments from P2–7 slices is shown in Figure 2. As found previously (Crair and Malenka, 1995), the maximal LTP was not achieved immediately following the induction protocol but took 10–20 min to develop and stabilize (Figure 2). Detailed analysis of each experiment revealed that potency increased significantly in 7 of the 8 cells (to $142 \pm 5\%$ of control, $n = 8$; Figure 2B₁). However, in only one cell could a change in potency completely account for the increase in synaptic strength during LTP. The success rate (i.e., $1 - \text{failure rate}$) also changed in 7 of 8 cells but to a smaller degree than the change in potency (to $129 \pm 8\%$ of control, $n = 8$; Figure 2B₂). Again, however, in only one cell could this change in success rate completely account for LTP. Thus, in 75% of the cells, changes in both potency and success rate were observed.

How should these results be interpreted? The classic interpretation of a change in the failure rate is that the probability of transmitter release has been modified and, thus, one possibility is that LTP was caused entirely by an increase in the probability of release. Such a

mechanism would require that the observed increase in potency was due to an increased likelihood of each stimulus causing simultaneous release from multiple synapses. However, if we assume that even though we used minimal stimulation techniques, a large number of synapses were activated during the baseline, the change in release probability that would be required to account for the change in potency would have caused a larger decrease in failure rate than was observed. Therefore, as was the case in a previous study of LTP in hippocampal CA1 pyramidal cells (Isaac et al., 1996), it is necessary to propose that LTP at thalamocortical synapses involves changes in quantal amplitude together with an additional mechanism causing an increase in either the probability of release or the number of functional synaptic contacts. Indeed, it has recently been suggested that at two different classes of inputs in the hippocampus, some proportion of synapses may be functionally silent and that LTP may be caused, at least in part, by the conversion of these silent synapses to functional ones (Isaac et al., 1995; Liao et al., 1995; Tong et al., 1996). Thus, a reasonable hypothesis is that the observed change in potency at thalamocortical synapses following LTP may be due, in part, to similar mechanisms.

To determine directly whether an LTP induction protocol could convert a silent synapse to a functional one, we used a stimulation strength that elicited no EPSCs at -70 mV. As shown in Figure 3, we then performed a standard LTP induction protocol by simply depolarizing the cell to 0 mV for 50 stimuli. In five of seven cells tested from P2–5 slices, when the cell was returned to -70 mV, fast EPSCs now appeared. Interestingly, in all five cells, there was some variable (1–7 min) delay before the appearance of the EPSCs. It is tempting to speculate that this variable delay in the conversion of silent to functional synapses following the LTP induction protocol may contribute to the slow growth of LTP of evoked EPSCs that has been observed (Crair and Malenka, 1995).

What mechanism might account for the silent synapses and their conversion to functional ones during LTP? In the CA1 region of the hippocampus, it has been suggested that some proportion of synapses may be silent because they lack functional AMPA receptors while still retaining NMDA receptors (Isaac et al., 1995; Liao et al., 1995). To test whether similar synapses exist at thalamocortical inputs, we again performed experiments in which the stimulus strength was initially turned down to the point at which no synaptic responses were observed while holding the cell at -70 mV (Figures 4A and 4B₁), except that in these experiments, the pipette solution contained 10 mM BAPTA to prevent the induction of LTP (Crair and Malenka, 1995). Depolarizing the cell to $+50$ mV revealed a mixture of failures and synaptic responses with the time course and shape of NMDA receptor-mediated events (Figure 4B₂). This appearance of EPSCs cannot be attributed to some drift in the stimulation parameters since, when the cell was returned to -70 mV, again no EPSCs were observed (Figure 4B₃). Furthermore, application of D-APV completely blocked the events observed at $+50$ mV (Figures 4A and 4B₄), confirming that they were mediated by NMDA receptors.

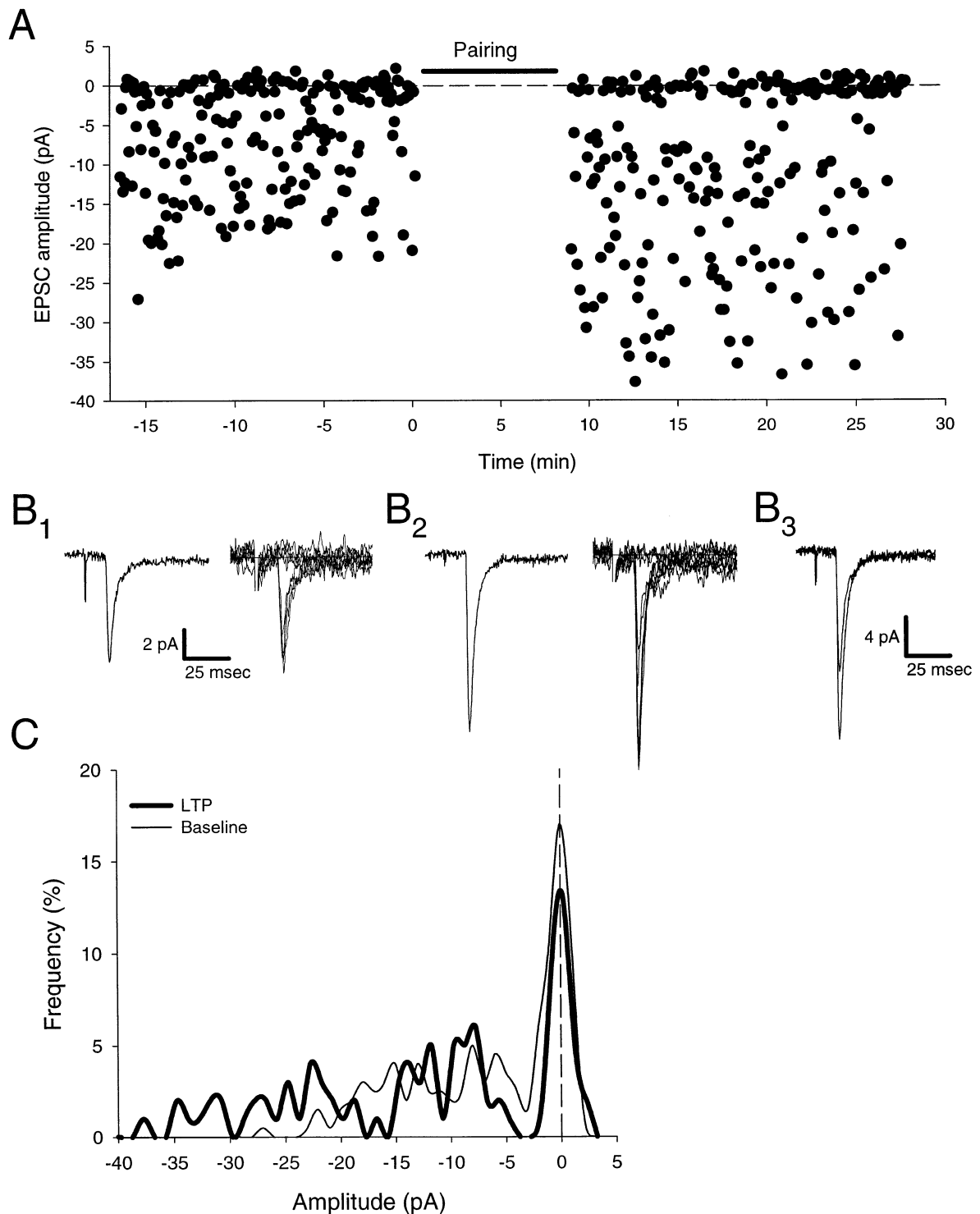


Figure 1. An Example of LTP at Thalamocortical Synapses Using Minimal Stimulation and Perforated Patch-Clamp Recording (P5 Animal)

(A) Individual response amplitudes monitored during the time course of an experiment. Time shown is relative to the start of pairing.

(B) Traces from experiment in (A).

(B₁, left) Average of 100 responses during baseline.

(B₁, right) Ten superimposed consecutive responses from baseline.

(B₂, left) Average of 100 responses during LTP (5 min after the end of pairing).

(B₂, right) Ten superimposed consecutive responses during LTP.

(B₃) Superimposed averages of successes only during baseline (smaller trace) and LTP (larger trace; same epochs as used for averages in [B₁] and [B₂]).

(C) Amplitude histograms (bin width = 1 pA) of all data from baseline (thin line) and during LTP (thick line).

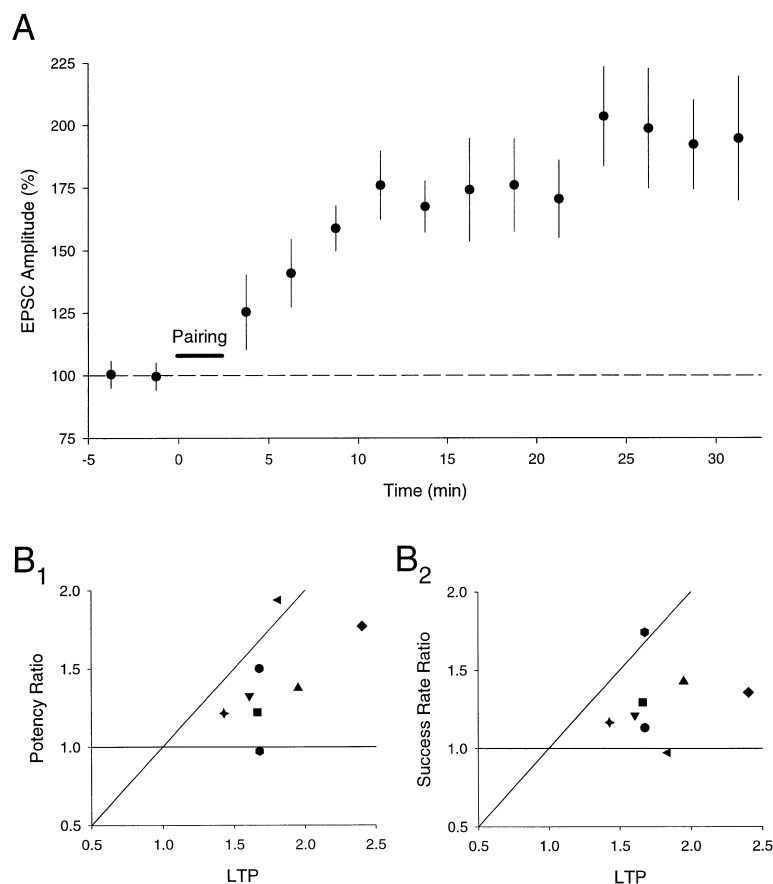


Figure 2. LTP at Thalamocortical Synapses in P2-7 Animals Is Associated with an Increase in Potency and Success Rate

(A) Summary graph of LTP ($n = 10$; 8 were minimal stimulation experiments, and 2 examined larger evoked EPSCs). Time shown is relative to the start of pairing.

(B) Potency and success rate analysis for the eight cells in which minimal stimulation was used (each experiment is coded with a symbol that is consistent for both graphs).

(B₁) Potency ratio (LTP/baseline) plotted as a function of LTP magnitude. The horizontal line represents the expected change in potency for an increase in q only at a single release site. The diagonal line represents the change in potency for an increase in q only.

(B₂) Success rate ratio (LTP/baseline) plotted as a function of LTP magnitude. The horizontal line represents the expected change for an increase in q only. The diagonal line represents the change for an increase in Pr alone at a single site.

Similar results from a total of six cells (P2-5 slices) are summarized in Figure 4C. In an additional seven cells, no responses were observed when the cells were depolarized to positive potentials, presumably because no synapses were being activated.

As a second test for the existence of silent synapses that contain NMDA receptors but lack functional AMPA receptors, we used minimal stimulation techniques and compared the failure rates at hyperpolarized (-70 mV) and depolarized ($+40$ mV) holding potentials. At silent synapses expressing functional NMDA receptors only, EPSCs will be detected at depolarized potentials but will appear as failures at hyperpolarized potentials because of the voltage-dependent blockade of NMDA receptors. Thus, if silent synapses exist, depolarizing the cell should cause an apparent decrease in the failure rate (Liao et al., 1995). One dramatic example of such an experiment is shown in Figure 5. At -70 mV, the failure rate was 0.32, and the EPSCs (Figure 5B₁) were fast with no discernible slow component. Upon depolarization of the cell to $+40$ mV, the failure rate decreased dramatically (to 0.04; Figure 5C), and the EPSCs now exhibited a prominent slow tail (Figure 5B₂), which was completely blocked by D-APV (25 μ M) (Figure 5B₃). Importantly, the failure rate returned to close to its control value measured at -70 mV when the D-APV was applied.

Thus far, we have presented evidence that some proportion of thalamocortical synapses in neonatal slices

of barrel cortex are functionally silent and that these can be converted to functional synapses by an LTP induction protocol. Given that the ability to generate LTP at these synapses appears to disappear at \sim P8-9, an important question is whether silent synapses are still present at this time. To estimate the proportion of silent synapses at different ages, we again used minimal stimulation techniques to compare the failure rate at hyperpolarized versus depolarized membrane potentials. As illustrated in Figure 5, if some of the apparent failures at hyperpolarized potentials are due to transmitter release occurring at synapses with NMDA receptors but no functional AMPA receptors, the failure rate will decrease when the cell is depolarized. Figure 6A shows the failure rates for populations of cells recorded from slices of different ages. At P2-3 and P4-5, there was a clear decrease in failure rate in 13 of 14 cells when they were depolarized (P2-3 failure rate at -70 mV = $0.44 \pm .07$, at $+40$ mV = $0.29 \pm .09$, $n = 6$; P4-5 failure rate at -70 mV = $0.52 \pm .04$, at $+40$ mV = $0.31 \pm .06$, $n = 8$). Importantly, application of D-APV (25 μ M) at the depolarized membrane potentials returned the failure rate to values close to those observed at -70 mV. This indicates that the decrease in failure rate at depolarized potentials was indeed due to activation of NMDA receptors. A significant decrease in the magnitude of the difference in failure rates at the two membrane potentials first became apparent at P6-7 (failure rate at -70 mV = $0.45 \pm .05$; at $+40$ mV = $0.36 \pm .06$; $n = 11$). This

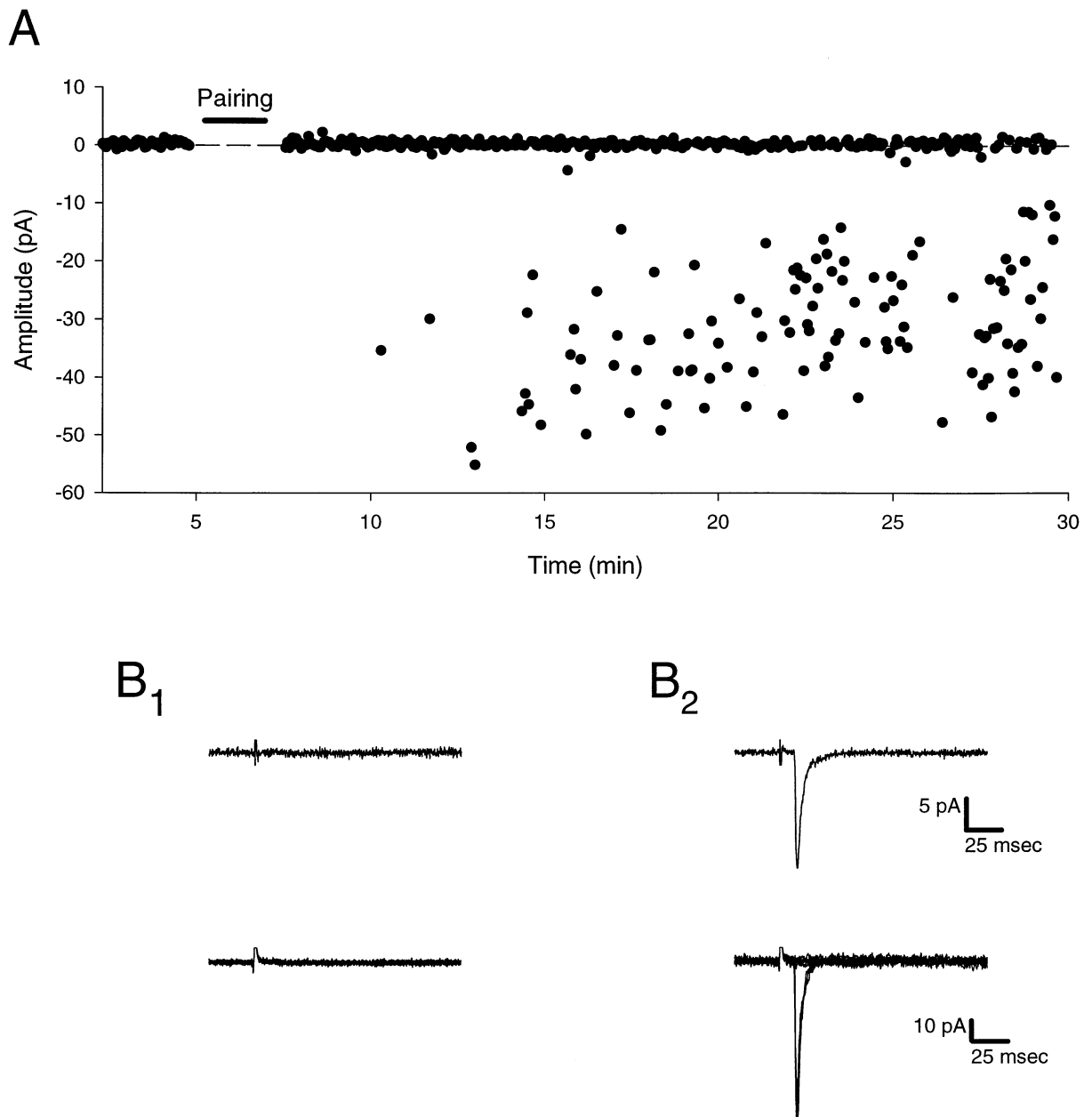


Figure 3. Induction of LTP at Silent Synapses (P4 Animal; All Data from One Experiment)

(A) Response amplitudes during the time course of an experiment.

(B₁, top) Average of all 50 baseline responses before pairing.

(B₁, bottom) Ten superimposed consecutive responses during the baseline.

(B₂, top) Average of 50 responses 15 min after the end of pairing.

(B₂, bottom) Ten superimposed consecutive responses from 15 min after the end of pairing.

observation is consistent with the hypothesis that the proportion of silent synapses decreases as the thalamocortical circuit develops. In strong support of this hypothesis, in P8–9 slices, only one of nine cells exhibited a significant decrease in failure rate when depolarized (Figure 6A₁), and the mean failure rate actually increased slightly (failure rate at -70 mV = $0.50 \pm .08$; at $+40$ mV = $0.52 \pm .09$; $n = 9$).

Figure 6B shows a summary of these data plotted as the difference in success rate at the two membrane

potentials, a measure which is proportional to the percentage of silent synapses being stimulated when compared to the total population of activated synapses. Assuming that the average probability of release at synapses containing only NMDA receptors and those containing both NMDA and AMPA receptors is similar, it is also possible to estimate directly the percentage of synapses that are silent at hyperpolarized membrane potentials simply by comparing the failure rates at the two potentials (Liao et al., 1995; Liao and Malinow, 1996).

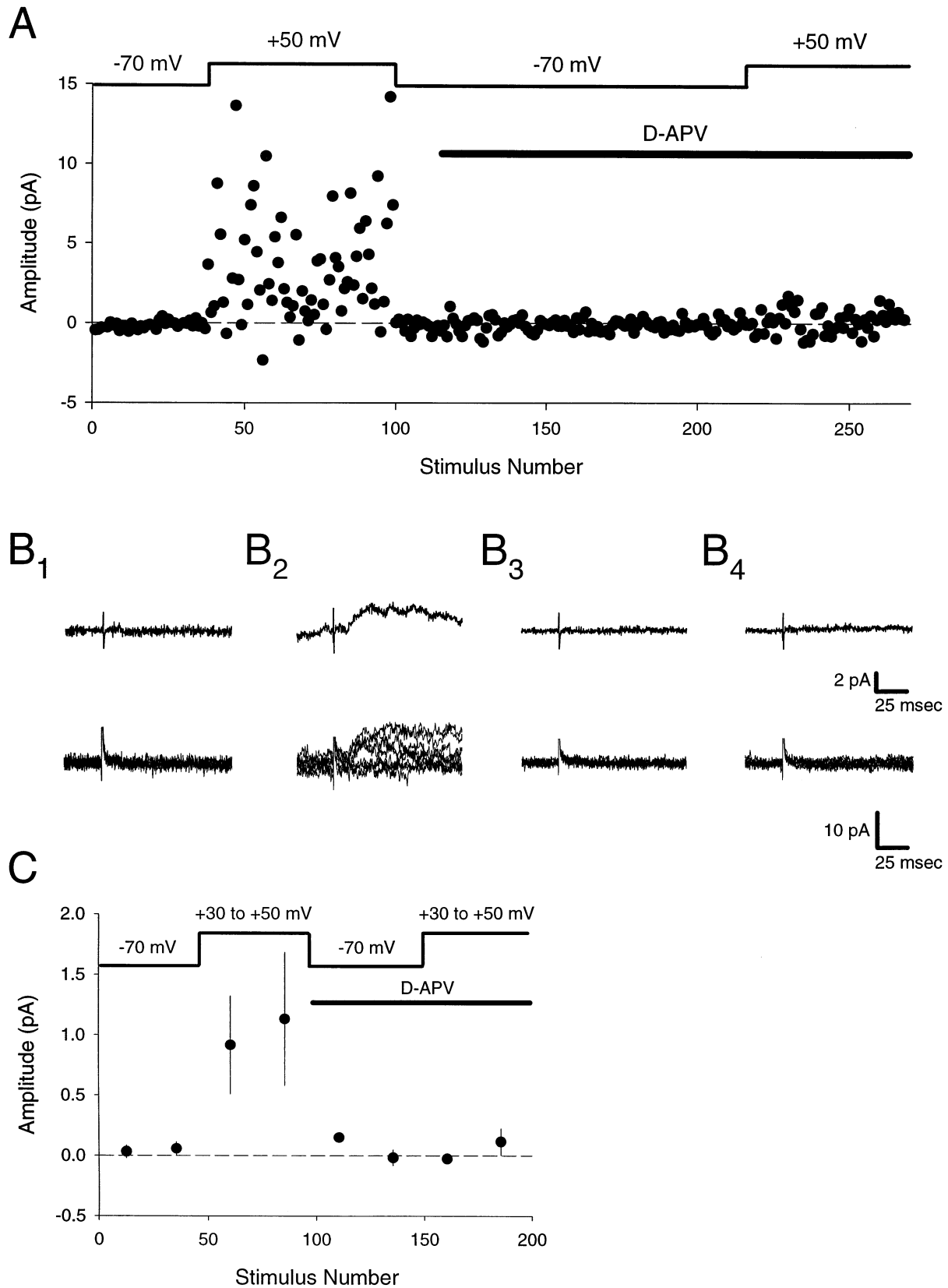


Figure 4. An Experiment Demonstrating the Existence of Silent Synapses When No Synaptic Response Was Detected at a Holding Potential of -70 mV (P5 Animal; [A] and [B] from One Experiment)

(A) Individual response amplitudes during the time course of an experiment (membrane potential is indicated at the top).

Performing this calculation (see Liao et al., 1995) yields the following estimates for the proportion of silent synapses: P2–3, 34%; P4–5, 43%; P6–7, 21%; and P8–9, 0%. Thus, these analyses strongly suggest that by P8–9, silent synapses at thalamocortical inputs have essentially disappeared.

Discussion

A major question in neurobiology is how the precise patterns of neuronal connections in the mammalian central nervous system are formed during development. Based primarily on work in the visual system, it is thought that while activity-independent processes are used to establish the gross topology of connections, the refinement of these connections in a manner that allows appropriate processing of sensory information strongly depends on neural activity (Goodman and Shatz, 1993; Singer, 1995; Katz and Shatz, 1996). The mechanisms by which activity modifies neural circuitry have been the subject of much interest. Theoretical work has pointed out that correlation-based rules of synaptic modifications (“neurons that fire together wire together”) can explain many features of the development of the mammalian visual system (Bienenstock et al., 1982; Miller et al., 1989; Miller, 1996). Since Hebbian synaptic modifications exhibit many of the characteristics required by theoretical models of neural circuit development, a prominent hypothesis has been that NMDA receptor-dependent LTP, which exhibits Hebbian properties, may play a fundamentally important role in the development of the visual system and its modification by sensory experience (see Bear et al., 1987; Fox and Daw, 1993; Goodman and Shatz, 1993; Singer, 1995; Katz and Shatz, 1996).

In support of this hypothesis, it has been found that NMDA receptor-dependent LTP can indeed be generated in both young visual cortex (Artola and Singer, 1987; Komatsu et al., 1988; Kimura et al., 1989; Tsumoto, 1992; Bear et al., 1992) and at developing retinogeniculate synapses (Mooney et al., 1993). The ability to generate LTP in visual cortex shows an age-dependent decline that roughly parallels the critical period (Perkins and Teyler, 1988; Komatsu et al., 1988; Kato et al., 1991; Kirkwood et al., 1995) and importantly, the susceptibility to LTP can be prolonged by dark rearing in a manner analogous to the prolongation of the critical period by this behavioral manipulation (Kirkwood et al., 1996). Work in simpler systems has also provided evidence that the susceptibility to LTP correlates with the activity-dependent refinement of afferent arborizations (Schmidt, 1990).

The present study was motivated by the hypothesis that, as suggested for the development of the visual system, LTP may also play an important role in the development of the precise topographic organization of thalamic afferents to layer IV cells in rodent barrel cortex. One major advantage of this system is the ability to obtain slices in which the entire longitudinal course of thalamic afferents to cortex can be retained (Agmon and Connors, 1991). This preparation therefore allows one to stimulate in thalamus, record monosynaptic responses in layer IV, and examine in detail the properties of LTP at the exact connections that are modified by sensory experience. In previous work (Crair and Malenka, 1995), we found that the ability to generate LTP at thalamocortical synapses in barrel cortex closely paralleled the critical period for the experience-dependent reorganization of thalamocortical afferents, a process that depends on postsynaptic activity (Schlaggar et al., 1993). However, the importance of activity in the normal development of the barrel fields has been questioned since disruption of postnatal activity appeared to have no effect on the normal barrel pattern when assessed using histochemical methods (Chiaia et al., 1992; Henderson et al., 1992; Schlaggar et al., 1993). Recently, this apparent contradiction has been resolved with the demonstration that blockade of glutamate receptors in barrel cortex during the critical period significantly disrupts the topographic refinement of thalamocortical afferents without changing the apparent gross morphology of barrels (Fox et al., 1996). Thus, as in visual cortex, postsynaptic activity in barrel cortex appears to be critical for the fine tuning of appropriate neuronal connections.

In an initial set of experiments, we used minimal stimulation techniques to help determine the exact synaptic modifications that might contribute to LTP. In seven of the eight cells examined, a change in the probability of release alone could not account for LTP, and it was necessary to postulate that a change in quantal size and/or the number of functional synapses had occurred. Because one mechanism by which synapse number can be increased involves the conversion of functionally silent synapses that only express NMDA receptors to synapses that contain both AMPA and NMDA receptors (Isaac et al., 1995; Liao et al., 1995; Durand et al., 1996; Wu et al. 1996), we performed experiments that allowed us to look for the existence of such synapses. In slices taken from young P2–7 animals, we obtained clear evidence that a significant proportion of synapses do not contain functional AMPA receptors and that such synapses can be converted to AMPA-containing ones by a standard LTP induction protocol. However, by P6–7, the proportion of silent synapses has decreased, and by

(B₁, top) Average of 40 responses at a membrane potential of -70 mV.

(B₁, bottom) Eight superimposed consecutive responses at a membrane potential of -70 mV.

(B₂, top) Average of 40 responses at a membrane potential of $+50$ mV.

(B₂, bottom) Eight superimposed consecutive responses at a membrane potential of $+50$ mV.

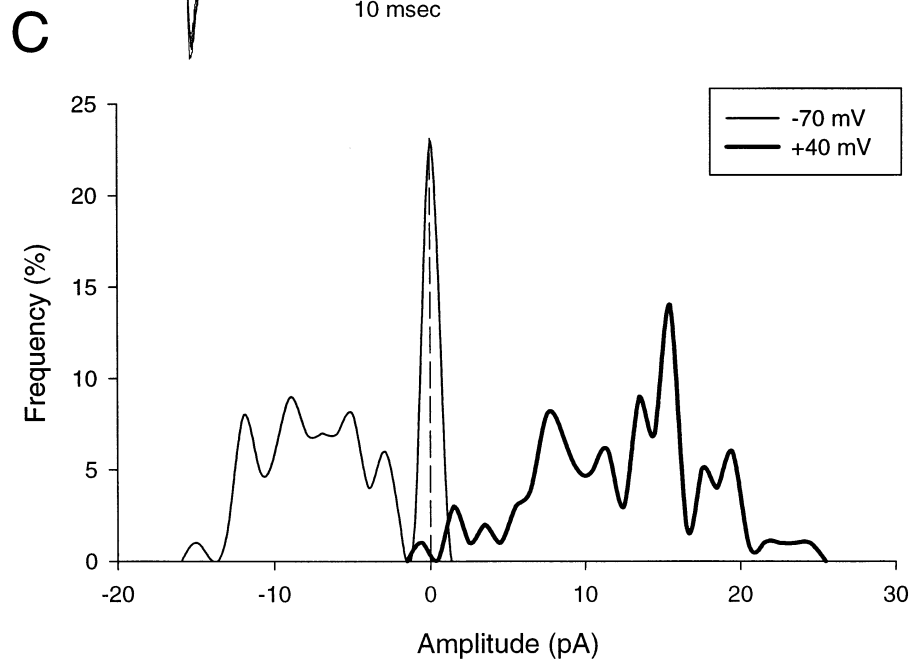
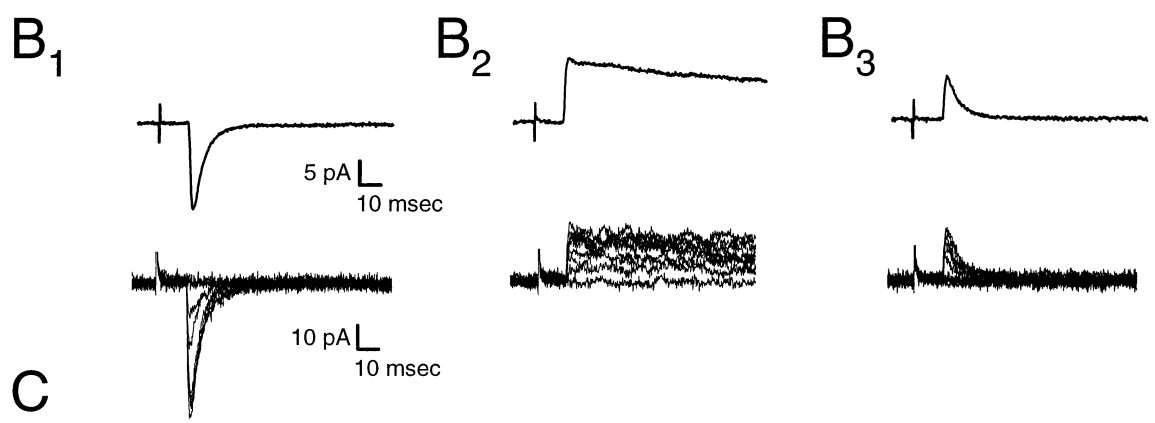
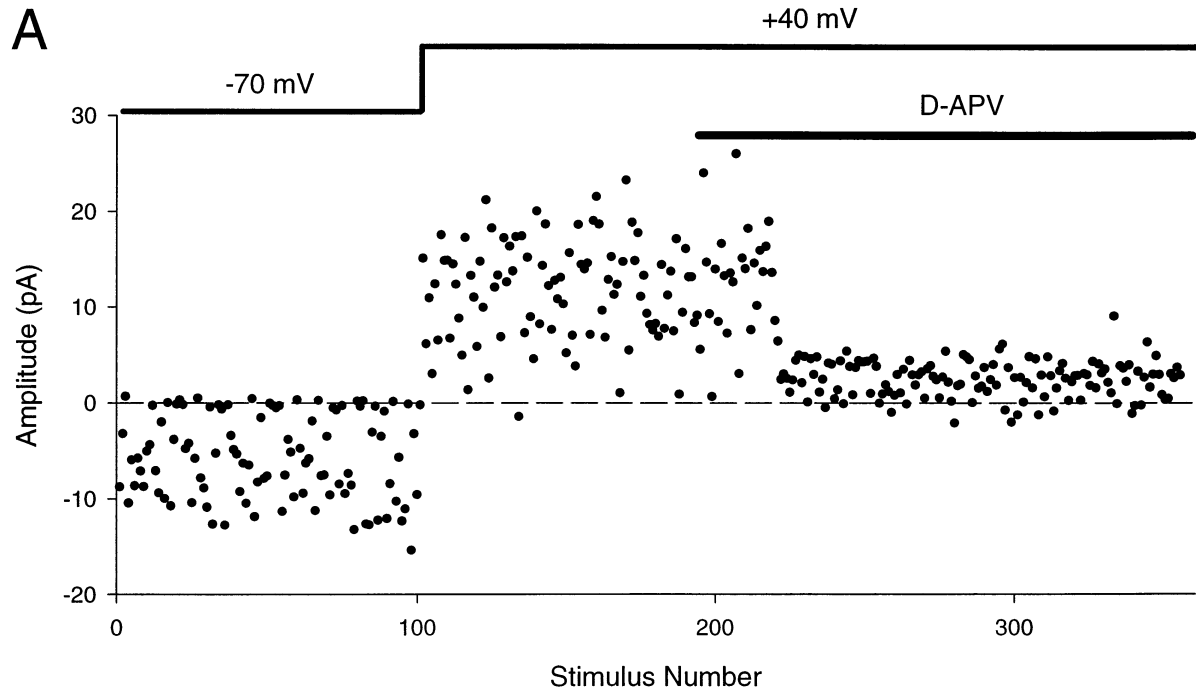
(B₃, top) Average of 40 responses at a membrane potential of -70 mV following collection of responses at $+50$ mV.

(B₃, bottom) Eight superimposed consecutive responses at a membrane potential of -70 mV following collection of responses at $+50$ mV.

(B₄, top) Average of 40 responses at a membrane potential of $+50$ mV in the presence of $25 \mu\text{M}$ D-APV.

(B₄, bottom) Eight superimposed consecutive responses at a membrane potential of $+50$ mV in the presence of $25 \mu\text{M}$ D-APV.

(C) Summary graph from 6 similar cells (P2–5 animals; data were collected at $+30$ – $+50$ mV in the presence of D-APV in 2 of 6 cells).



P8–9, silent synapses are no longer detectable. This disappearance of silent synapses is consistent with and indeed may account for the large decrease in the relative contribution of NMDA receptor–mediated synaptic currents that occurs over the same period (Crair and Malenka, 1995).

There clearly is also a tight temporal correspondence between the disappearance of silent synapses and the loss of the ability to generate LTP (Crair and Malenka, 1995). A likely scenario to explain this finding is that LTP occurs at thalamocortical connections during normal development and involves the conversion of silent synapses to functional ones. This would suggest that initially following synaptogenesis, many synapses contain only NMDA receptors and do not efficiently transmit information unless the cell is depolarized. With adequate depolarization, presumably supplied by correlated activity from other synapses on the same cell, these NMDA receptor–only synapses could be converted to functional ones. Silent synapses may therefore provide an important substrate by which experience (i.e., activity) can result in the actual addition of functional elements to neural circuitry rather than simply changing the gain of existing synaptic contacts. A developmental decrease in silent synapses has also been observed in the hippocampus (Liao and Malinow, 1996; Durand et al., 1996) and the frog optic tectum (Wu et al., 1996), suggesting that such mechanisms may be generally important for developmental modifications of neural circuitry.

An important assumption underlying our experiments is that we are indeed recording thalamocortical synaptic responses. Because we are stimulating in the thalamus, several millimeters from the cortex, it is conceivable that we are consistently antidromically stimulating the recurrent collaterals of layer VI corticothalamic afferents, which make monosynaptic contacts on layer IV cells. We think that this is unlikely for two reasons. First, the cell bodies of thalamocortical neurons in VB should have a lower activation threshold than the terminals of corticothalamic axons. Furthermore, in mature animals, corticothalamic axons have smaller diameters than thalamocortical afferents (Jones and Powell, 1969; Ferster and Lindstrom, 1985). These differences in activation thresholds make it unlikely that in our experiments, all of which (except two cells) involved a minimal stimulation protocol, we had any significant contribution from collaterals of corticothalamic axons. Second, recording the cortical responses to VB stimulation in neonatal animals (P0–14) using voltage-sensitive dyes revealed robust monosynaptic signals in layer IV and very weak signals in layers V–VI (Crair et al., 1993, Soc. Neurosci. abstract).

Thus, there was minimal antidromic activation of layer V–VI cells.

A second technical issue is whether developmental changes in layer IV cells' structure or basic electrophysiological properties might have caused the apparent disappearance of silent synapses. This is unlikely since the major changes accompanying development would make it more likely to observe silent synapses in older cells rather than less likely. Specifically, an increase in the size of the dendritic arbor would increase the spatial filtering of fast AMPA receptor–mediated currents generated distally on dendrites and thereby decrease their measured amplitude. The decrease in input resistance with development (Crair and Malenka, 1995) would also make it more difficult to record AMPA receptor–mediated EPSCs. Formally, it is also possible that silent synapses have not been converted to functional ones during development but rather constitute a very small fraction of the sampled synapses at P8–9. For example, there is a dramatic increase in the arborization of thalamocortical afferents early in development (Catalano et al., 1996). If all of the new terminations form functional synapses containing both AMPA and NMDA receptors, it is conceivable that silent synapses are still present but went undetected in our analysis. Such a scenario, however, requires that the ability to convert silent to functional synapses is not utilized during development.

Our data strongly suggest that there is a significant developmental decrease in the proportion of synapses that are functionally silent and that this decrease parallels both the critical period and the susceptibility to LTP. Recently, however, it has been suggested that in the hippocampus, the silent synapses at which only NMDA receptor–mediated EPSCs are observed may not be due to the lack of functional AMPA receptors but instead may be attributed to the spillover of glutamate from a presynaptic bouton onto an adjacent postsynaptic site that is located on a cell that is not directly contacted by the releasing bouton (Kullmann et al., 1996). Assuming that the concentration of glutamate at the spillover synapse was much lower than in the synaptic cleft at which vesicle exocytosis occurred, it was proposed that because of the differential affinity of NMDA receptors and AMPA receptors for glutamate (Pateneau and Mayer, 1990), NMDA receptors but not AMPA receptors would be activated by this glutamate spillover. If such a scenario explains all of the present data, and therefore all thalamocortical synapses actually express both NMDA and AMPA receptors, it is necessary to postulate that the observed change in silent synapses during development is due to dramatic developmental changes

Figure 5. An Example of an Experiment Demonstrating the Existence of Silent Synapses Using Analysis of the Difference in Failure Rates between a Holding Potential of -70 mV and $+40$ mV (P4 Animal; All Data from One Experiment)

(A) Individual response amplitudes during the time course of an experiment (membrane potential is indicated at the top).

(B₁, top) Average of 100 responses at a membrane potential of -70 mV.

(B₁, bottom) Eight superimposed consecutive responses at a membrane potential of -70 mV.

(B₂, top) Average of 100 responses at a membrane potential of $+40$ mV.

(B₂, bottom) Eight superimposed consecutive responses at a membrane potential of $+40$ mV.

(B₃, top) Average of 100 responses at a membrane potential of $+40$ mV in the presence of $25 \mu\text{M}$ D-APV.

(B₃, bottom) Eight superimposed consecutive responses at a membrane potential of $+40$ mV in the presence of $25 \mu\text{M}$ D-APV.

(C) Amplitude histogram (bin width = 1.0 pA) of all responses at -70 mV (thin line) and all responses at $+40$ mV before the addition of D-APV (thick line).

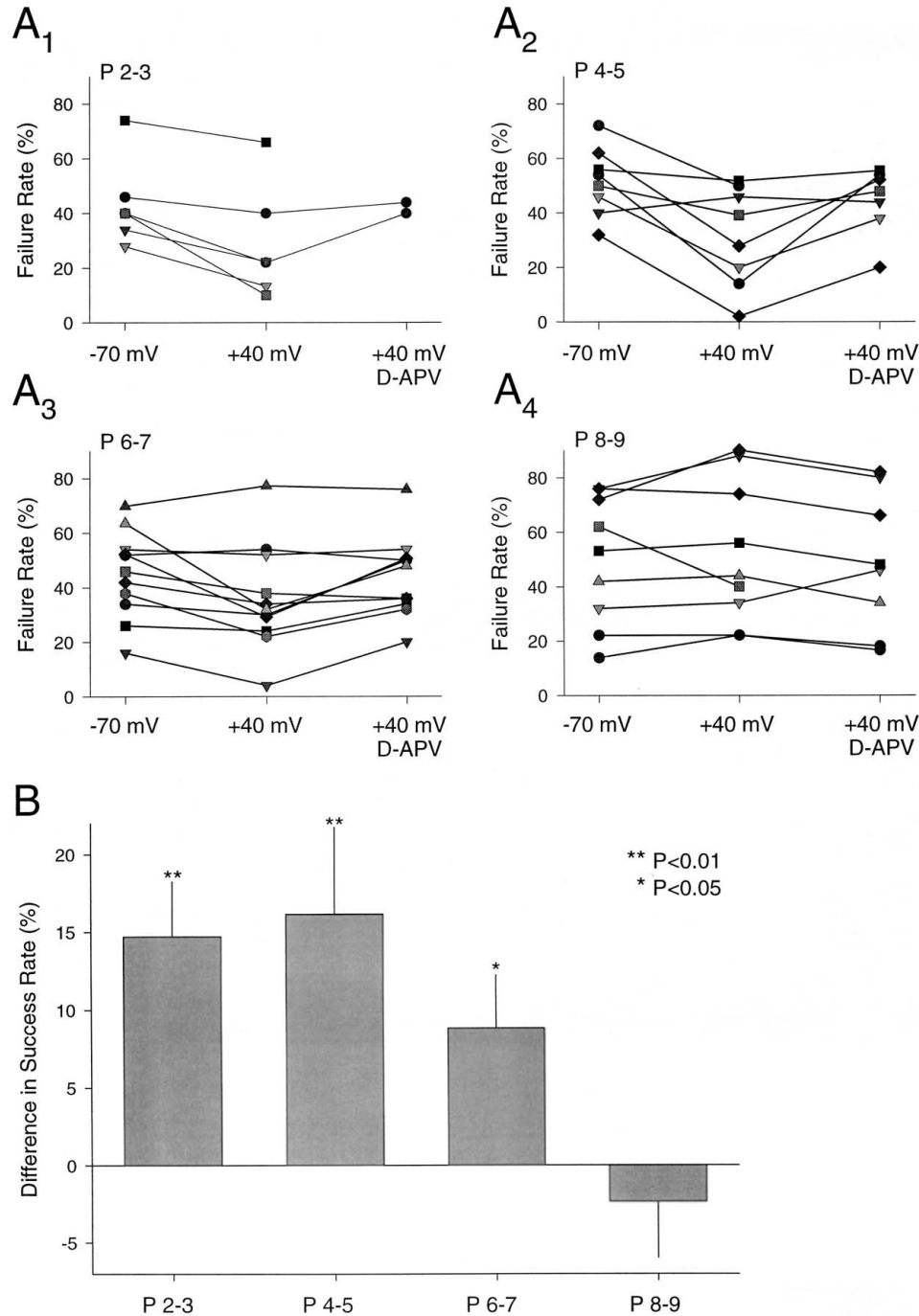


Figure 6. Silent Synapses Exhibit a Distinct Developmental Profile

(A) Graphs showing failure rates from individual experiments at holding potentials of -70 mV, $+40$ mV, and $+40$ mV in the presence of 25 μ M D-APV.

(A₁) Experiments from 2- and 3-day-old animals ($n = 6$).

(A₂) Experiments from 4- and 5-day-old animals ($n = 8$).

(A₃) Experiments from 6- and 7-day-old animals ($n = 11$).

(A₄) Experiments from 8- and 9-day-old animals ($n = 9$).

(B) Success rate ($1 - \text{failure rate}$) difference (success rate at $+40$ mV $-$ success rate at -70 mV) plotted as a function of age from all experiments shown in (A).

in glutamate uptake mechanisms such that by P8-9, glutamate no longer can spillover from one synapse onto another. Furthermore, to explain the LTP data (Figures 1-3), it is necessary to propose that during our

control recording period, we were stimulating presynaptic boutons that did not release neurotransmitter and that LTP, via some retrograde messenger, switched on these boutons so that they now functioned normally.

Thus, according to this scenario, there still must be a developmental decrease in the proportion of silent thalamocortical synapses, but they are presynaptically, not postsynaptically, silent. Indeed, such a "switching on" of silent presynaptic release sites may occur at the crayfish neuromuscular junction (Wojtowicz et al., 1994) and at the mossy fiber synapse in the CA3 region of the hippocampus (Tong et al., 1996).

We have provided direct evidence that functionally silent synapses exist in neocortex and that at thalamocortical inputs, they disappear early in development. It remains unclear whether all newly formed synapses initially contain only NMDA receptors or whether during synaptogenesis, there is a random intermixing of silent and functional connections. In either case, the conversion of silent to functional synapses via an LTP-like mechanism may be an important mechanism by which correlated presynaptic afferent activity can result in the formation of functionally relevant connections (Liao et al., 1995; Durand et al., 1996; Wu et al., 1996) and thereby help set up the appropriate topographic representation of sensory experience in the cortex.

Experimental Procedures

Slices (500 μ m thick) were prepared from pups of timed-pregnant Long-Evans rats (P0 was defined as the first 24 hr after birth) as previously described (Agmon and Connors, 1991; Crair and Malenka, 1995). Briefly, the brain was removed and placed in ice-cold extracellular solution before paracoronaral slices were prepared at an angle between 40° and 50° relative to the midline. Slices were allowed to recover for 2–4 hr before being placed in the recording chamber where they were submerged beneath a continuously superfusing solution at room temperature (23°–25°C) saturated with 95% O₂, 5% CO₂. The standard extracellular solution contained 119 mM NaCl, 2.5 mM KCl, 2.5 mM CaCl₂, 1.3 mM MgSO₄, 1.0 mM NaH₂PO₄, 26.2 mM NaH₂CO₃, 11 mM glucose, and 0.1 mM picrotoxin. Picrotoxin was not used in the experiments shown in Figures 1 and 2.

Whole-cell voltage-clamp recordings using 3–6 M Ω electrodes were made from cells in layer IV of the somatosensory cortex. The somatosensory cortex was identified by visualizing the barrels under oblique transillumination and evoking short latency field EPSPs and whole-cell EPSCs by stimulation in the ventrobasal nucleus of the thalamus (Agmon and Connors, 1991; Crair and Malenka, 1995). The whole-cell recording solution was: 117 mM cesium gluconate, 20 mM HEPES, 0.2 mM EGTA, 5 mM TEA-Cl, 3.7 mM NaCl, 4 mM Mg-ATP, and 0.3 mM GTP (pH 7.2 with CsOH, 275 mOsm). In experiments designed to detect the existence of silent synapses, 10 mM BAPTA was included in the whole-cell solution and replaced 10 mM of cesium gluconate. In two LTP experiments, perforated patch-clamp recordings were used (Isaac et al., 1996). In 25 cells, biocytin (0.4%) was included in the pipette solution. A random selection of 10 of these cells was processed, and all were found to be in layer IV when the tissue was Nissl stained. Cells were held at –70 mV during recordings unless otherwise indicated. Input resistance (1–5 G Ω) and series resistance (5–40 M Ω) were monitored continuously during recordings, as previously described (Isaac et al., 1995). A concentric bipolar stimulating electrode placed in the ventrobasal nucleus of the thalamus was used to evoke monosynaptic thalamocortical responses in layer IV cells. Initial stimulus intensity was kept low so that no synaptic responses were elicited. It was then increased slowly until the lowest intensity that elicited a mixture of responses and failures was found. Responses were accepted as monosynaptic if they exhibited short and constant latency that did not change with increasing stimulus intensity. EPSCs were evoked at a stimulus frequency of 0.2–0.33 Hz. For a given experiment, once stimulation was commenced, it was maintained at that frequency without interruption for the duration of the experiment. LTP was

induced by depolarizing the cell to –10 mV for 50 consecutive stimuli without alteration of stimulus frequency. In the two perforated patch-clamp experiments, cells were paired in the same manner but for 100 stimuli.

Data were collected using an Axopatch 1-D amplifier, filtered on-line at 2 kHz, digitized at 5 kHz, and analyzed on-line as previously described (Mulkey and Malenka, 1992). EPSC amplitudes were estimated by measuring the average of a 1–2 ms window at the peak of the EPSC relative to the same window taken immediately before the stimulus artifact. EPSC amplitude histograms were constructed by plotting the frequency histogram of all events with a smoothed line (spline) in SigmaPlot. Failure rates were estimated using the methods of Liao et al. (1995). Briefly, the number of responses with an amplitude >0 pA were determined, and this was doubled to provide the number of failures. Any offset of the failures peak from zero was estimated by averaging between 30 and 100 visually identified failures. In a subset of cells, failure rates were also determined visually and compared to the estimate provided by the method above. Potency (Stevens and Wang, 1994) was defined as the mean amplitude of the EPSC (calculated by averaging all of the trials together) divided by the success rate (1 – failure rate). In the experiments designed to detect silent synapses by estimating the difference in failure rates at –70 mV and +40 mV, EPSC amplitudes were estimated using the same window for both holding potentials. In the figures, for clarity of display, the stimulus artifact of the averaged waveforms was subtracted using an average of the same number of visually identified failures. Data are expressed as mean \pm SEM. For statistical tests of significance, a two-tailed paired Student's *t*-test was used.

Acknowledgments

R. C. M. is a member of the Center for Neurobiology and Psychiatry. R. A. N. is a member of the Keck Center for Integrative Neuroscience and the Silvio Conte Center for Neuroscience Research. This work was supported by grants from N.I.H. (to R. C. M. and R. A. N.) and a McKnight Investigator Award (to R. C. M.). J. T. R. I. was supported by a Wellcome Prize Travelling Research Fellowship. M. C. C. was supported by an N.R.S.A. from N.I.H.

Received December 26, 1996; revised January 24, 1997.

References

- Agmon, A., and Connors, B.W. (1991). Thalamocortical responses of mouse somatosensory (Barrel) cortex in vitro. *Neuroscience* 41, 365–379.
- Artola A., Singer, W. (1987). Long-term potentiation and NMDA receptors in rat visual cortex. *Nature* 330, 649–652.
- Bear, M.F., Cooper, L.N., and Ebner, F.F. (1987). A physiological basis for a theory of synaptic modification. *Science* 237, 42–48.
- Bear, M.F., Press W.A., Connors, B.W. (1992). Long-term potentiation in slices of kitten visual cortex and the effects of NMDA receptor blockade. *J. Neurophysiol.* 67, 1–11.
- Bienenstock, E.L., Cooper, L.N., Munro, P.W. (1982). Theory for the development of neuron selectivity: orientation specificity and binocular interaction in visual cortex. *J. Neurosci.* 2, 32–48.
- Bolshakov, V.Y., and Siegelbaum, S.A. (1995). Regulation of hippocampal transmitter release during development and long-term potentiation. *Science* 269, 1730–1734.
- Catalano, S.M., Robertson, R.T., and Killackey, H.P. (1996). Individual axon morphology and thalamocortical topography in developing rat somatosensory cortex. *J. Comp. Neurol.* 366, 36–53.
- Chiaia, N.L., Fish, S.E., Bauer W.R., Bennett-Clarke, C.A., and Rhodes, R.W. (1992). Postnatal blockade of cortical activity by tetrodotoxin does not disrupt the formation of vibrissa-related patterns in the rats somatosensory cortex. *Dev. Brain Res.* 66, 244–250.
- Crair, M.C., and Malenka, R.C. (1995). A critical period for long-term potentiation at thalamocortical synapses. *Nature* 375, 325–328.
- Durand, G.M., Kovalchuk, Y., and Konnerth, A. (1996). Long-term

- potentiation and functional synapse induction in developing hippocampus. *Nature* 381, 71–75.
- Ferster, D., and Lindstrom, S. (1985). Augmenting responses evoked in area 17 of the cat by intracortical axon collaterals of corticogeniculate cells. *J. Physiol.* 367, 217–232.
- Fox, F., and Daw, N.W. (1993). Do NMDA receptors have a critical function in visual cortical plasticity? *Trends Neurosci.* 16, 116–122.
- Fox, K., Schlaggar, B.L., Glazewski, S., and O'Leary, D. (1996). Glutamate receptor blockade at cortical synapses disrupts development of thalamocortical and columnar organization in somatosensory cortex. *Proc. Natl. Acad. Sci. USA* 93, 5584–5589.
- Goodman, C., and Shatz, C. (1993). Developmental mechanisms that generate precise patterns of neuronal connectivity. *Cell/Neuron Rev. Suppl.* 10, 77–98.
- Henderson, T.A., Woolsey, T.A., and Jacquin, M.F. (1992). Infraorbital nerve blockade from birth does not disrupt central trigeminal pattern formation in the rat. *Dev. Brain Res.* 66, 146–152.
- Isaac, J.T.R., Nicoll, R.A., Malenka, R.C. (1995). Evidence for silent synapses: implications for the expression of LTP. *Neuron* 15, 427–434.
- Isaac, J.T.R., Hjelmstad, G.O., Nicoll, R., and Malenka, R.C. (1996). Long-term potentiation at single fiber inputs to hippocampal CA1 pyramidal cells. *Proc. Natl. Acad. Sci. USA* 93, 8710–8715.
- Jones, E.G., and Powell, T.P.S. (1969). An electron microscopic study of the mode of termination of cortico-thalamic fibres within the sensory relay nuclei of the thalamus. *Proc. R. Soc. Lond. [Biol.]* 172, 173–185.
- Kato N., Artola A., Singer W. (1991). Developmental changes in the susceptibility to long-term potentiation of neurones in rat visual cortex slice. *Dev. Brain Res.* 60, 43–50.
- Katz, L.C., and Shatz, C.J. (1996). Synaptic activity and the construction of cortical circuits. *Science* 274, 1133–1138.
- Kimura F., Nishigori A., Shirokawa T., Tsumoto T. (1989). Long-term potentiation and N-methyl-D-aspartate receptors in the visual cortex of young rats. *J. Physiol. (Lond.)* 414:125–144.
- Kirkwood, A., Lee, H., and Bear, M. (1995). Co-regulation of long-term potentiation and experience-dependent synaptic plasticity in visual cortex by age and experience. *Nature* 375, 328–331.
- Kirkwood, A., Rioult, M., and Bear, M. (1996). Experience-dependent modification of synaptic plasticity in visual cortex. *Nature* 381, 526–528.
- Komatsu, Y., Fujii, K., Maeda, J., Sakaguchi, H., Toyama, K. (1988). Long-term potentiation of synaptic transmission in kitten visual cortex. *J. Neurophysiol.* 59, 124–141.
- Kullmann, D.M., Erdemli, G., and Asztely, F. (1996). LTP of AMPA and NMDA receptor-mediated signals-evidence for presynaptic expression and extrasynaptic glutamate spill-over. *Neuron* 17, 461–474.
- Liao, D., and Malinow, R. (1996). Deficiency in induction but not expression of LTP in hippocampal slices from young rats. *Learning Mem.* 3, 138–149.
- Liao, D., Hessler, N.A., and Malinow, R. (1995). Activation of postsynaptically silent synapses during pairing-induced LTP in CA1 region of hippocampal slice. *Nature* 375, 400–404.
- Miller, K.D. (1996). Synaptic economics: competition and cooperation in synaptic plasticity. *Neuron* 17, 371–374.
- Miller, K.D., Keller, J.B., and Stryker, M.P. (1989). Ocular dominance column development: analysis and simulation. *Science* 245, 605–615.
- Mooney, R., Madison, D., and Shatz, C. (1993). Enhancement of transmission at the developing retinogeniculate synapse. *Neuron* 10, 815–825.
- Mulkey, R.M., and Malenka, R.C. (1992). Mechanisms underlying induction of homosynaptic long-term depression in area CA1 of hippocampus. *Neuron* 9, 967–975.
- Pateneau, D.K., and Mayer, M.L. (1990). Structure-activity relationships for amino acid transmitter candidates acting at N-methyl-D-aspartate and quisqualate receptors. *J. Neurosci.* 10, 2385–2399.
- Perkins, A.T., and Teyler, T.J. (1988). A critical period for long-term potentiation in the developing rat visual cortex. *Brain Res.* 439, 222–229.
- Schlaggar, B., Fox, K., and O'Leary, D. (1993). Postsynaptic control of plasticity in developing somatosensory cortex. *Nature* 364, 623–626.
- Schmidt, J.T. (1990). Long-term potentiation and activity-dependent retinotopic sharpening in the regenerating retinotectal projection of goldfish: common sensitive period and sensitivity to NMDA blockers. *J. Neurosci.* 10, 233–246.
- Singer, W. (1995). Development and plasticity of cortical processing architectures. *Science* 270, 758–764.
- Stevens, C.F., and Wang, Y. (1994). Changes in reliability of synaptic function as a mechanism for plasticity. *Nature* 371, 704–707.
- Tong, G., Malenka, R.C., and Nicoll, R.A. (1996). Long-term potentiation in cultures of single hippocampal granule cells: a presynaptic form of plasticity. *Neuron* 16, 1147–1157.
- Tsumoto, T. (1992). Long-term potentiation and long-term depression in the neocortex. *Prog. Neurobiol.* 39, 209–328.
- Wojtowicz, J.M., Marin, L., and Atwood, H.L. (1994). Activity-induced changes in synaptic release sites at the crayfish neuromuscular junction. *J. Neurosci.* 14, 3688–3703.
- Wu, G.-Y., Malinow, R., and Cline, H.T. (1996). Maturation of a central glutamatergic synapse. *Science* 274, 972–976.