Presynaptic Efficacy Directs Normalization of Synaptic Strength in Layer 2/3 Rat Neocortex After Paired Activity

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INTRODUCTION

Paired bursts of pre- and postsynaptic action potentials (APs) are believed to be a physiological mechanism of plasticity at many central synapses (e.g., Markram and Tsodyks 1996; Paulsen and Sejnowski 2000). Paired recordings from hippocampal cultures and cortical slices suggest that the direction of synaptic plasticity that paired activity produces is dependent on the order of the presynaptic and postsynaptic spikes (Bi and Poo 1998; Markram et al. 1997). Pairing presynaptic spikes shortly before postsynaptic spikes produces long-term potentiation (LTP), whereas pairing postsynaptic spikes before presynaptic spikes produces long-term depression (LTD), with less temporal spike constraint (Bi and Poo 1998; Feldman 2000; Markram et al. 1997). The initial strength of the synapse may also dictate whether a synapse potentiates, with weaker synapses potentiating preferentially over stronger ones (Bi and Poo 1998). The relative timing of the presynaptic and postsynaptic spikes could be reflected by both the amplitude and kinetics of calcium transients in spines, with larger, more transient calcium signals producing LTP and smaller, longer-lasting ones producing LTD (Cormier et al. 2001; Hansel et al. 1997; Ismailov et al. 2004; Koester and Sakmann 1998; Yang et al. 1999).

Pairing presynaptic before postsynaptic spikes has been shown to induce both LTP and LTD in individual layer 2/3 pyramidal neurons of young rodent cortex (Ismailov et al. 2004; Zhou et al. 2005). These studies both used extracellular stimulation and involved the simultaneous stimulation of multiple synapses, so were unable to address properties of individual inputs. In layer 5 cortical pyramids pairing postsynaptic APs with excitatory postsynaptic potentials (EPSPs) produced LTP at proximal synapses but LTD at distal synapses (Sjostrom and Hauser 2006). This effect was attributed to dendritic action potential backpropagation as dendritic depolarization converted LTD to LTP at the more distal synapses (Sjostrom and Hauser 2006). The diverse effects of paired activity on individual EPSPs raises the question of whether initial properties of a connection are important in determining the type of plasticity exhibited by the individual synaptic connections. A proportion of layer 2/3 connections from 3-wk-old cortex were shown to conform to a simple binomial release model when subjected to a quantal analysis (Hardingham et al. 2006), enabling one to look at initial quantal parameters and changes in both pre- and postsynaptic quantal parameters after plasticity.

Here we show that in layer 2/3 pyramidal connections paired activity produces equal proportions of cells showing LTP and LTD. We find that those connections that exhibit LTP are of smaller mean amplitude than connections that show LTD. Quantal analysis reveals that plasticity is mostly presynaptic and those connections that potentiate are weaker presynaptically than those that depress. Pairing thus acts to normalize the synapses’ presynaptic strength. Moreover, distal synapses potentiate by a larger magnitude than proximal ones and do so by additional postsynaptic mechanisms.

METHODS

Slice preparation and intracellular recording

All recordings were made from brain slices taken from 19- to 27-day-old Sprague-Dawley rats. Animals were killed by cervical dislocation and parasagittal slices of visual cortex (400 μm thick) were prepared by conventional methods (Hardingham and Larkman 1998). Slices were maintained at 23°C in artificial cerebrospinal fluid (ACSF) containing (in mM): 119 NaCl, 3.5 KCl, 1 NaHPO₄, 2.5 CaCl₂, 1 MgSO₄, 26 NaHCO₃, and 10 glucose, bubbled with 95% O₂ -5% CO₂.
Whole cell voltage recordings were made from the somata of adjacent pairs of pyramidal neurons within layer 2/3 (predominantly layer 2) of visual cortex using an AxoProbe 1A amplifier (AxonInstruments), selected by near-infrared differential interference contrast (DIC) video microscopy (Dodd and Ziegglansberger 1990) using a Zeiss Axioskop upright microscope equipped with a ×40 water-immersion objective at 23 or 35°C. Recording pipettes contained (in mM): 110 potassium gluconate, 10 KCl, 2 MgCl₂, 2 Na₂ATP, 10 EGTA, 2 CaCl₂, and 10 Hepes, adjusted to pH 7.3 and 290 mOsml and were of resistance 2–5 MΩ. Before seal formation, neurons were selected as being pyramidal by the presence of a prominent apical dendrite. Subsequently, the neuron could be identified as a pyramid by its asymmetric spikes with faster rise phases than decay phases, typical of pyramidal neurons in this layer (Mason et al. 1991; McCormick et al. 1985). Series resistance measurements were measured by bridge balance settings and were between 20 and 40 MΩ. Series resistances and pipette capacitances were compensated during a recording and recordings were rejected if resistance changed by >20%. Resting membrane potentials were −69 ± 2 mV and input resistances were in the range 100–250 MΩ.

Single or paired APs at a 50-ms interval were elicited (by current injection) in one neuron and on-line spike-triggered averaging was used to detect any resultant EPSP in the other neuron. If no EPSP could be detected, the pair of cells was tested for a connection in the other direction. Once a synaptic connection had been identified, single APs were induced in the presynaptic cell at 0.1 Hz by injection of short (5- to 10-ms) pulses of depolarizing current. Postsynaptic responses were amplified, low-pass filtered at 2 kHz, digitized at 5 kHz using a Cambridge Electronic Design CED 1401 A/D board and recorded on a PC for analysis off-line. Postsynaptic neurons were held at membrane potentials more negative than −70 mV to ensure that EPSPs were dominated by α-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid (AMPA)–receptor-mediated currents but holding current was rarely necessary. Slices were continually perfused with ACSF during recording.

After control periods of recording (normally 100 trials), a paired AP protocol was applied to the connection (Supplemental Fig. S1).¹ Postsynaptic APs were timed so that they fired 5 ms after presynaptic APs. Trains of 20 paired APs were evoked in the cells at 20 Hz. Ten trains, fired at 0.5 Hz, made up a group of paired action potentials (200 paired APs). Three of these groups (at one/minute) were induced in the cells (600 paired APs in all) and connections were recorded again thereafter at 0.1 Hz until recording instability occurred. Mean amplitudes of 60 min of postpairing data were normalized to control periods of recording to give amplitude changes.

**Measurement of EPSP amplitude**

For each EPSP recorded the peak amplitude from each spike-triggered sweep was measured off-line using a computer routine that compared the average voltage during a 0.4- to 2-ms period of baseline potential with the average voltage during a period of the same duration at the EPSP peak. For each EPSP, the measurement windows were determined from the average EPSP waveform. Measurements of noise were obtained using the same time windows used to measure the EPSP, but implemented in an area of baseline remote from the EPSP. At least three separate noise measurements were taken for each EPSP, from nonoverlapping parts of the baseline, to calculate the mean noise SD. This noise SD was subtracted from the EPSP SD using the equation

\[
\text{EPSP SD}^2 = (\text{SD of combined EPSP + noise})^2 - (\text{noise SD})^2
\]

To verify that the postsynaptic changes in EPSP amplitude we observed in these experiments were changes in AMPA currents we sought to verify that the EPSPs we were recording at −70 mV were exclusively AMPA mediated. In 50 μM 2-amino-5-phosphonovaleric acid (APV), EPSPs were on average 1.06 ± 0.04 times their control value (n = 5). Therefore N-methyl-D-aspartate (NMDA) receptors do not appear to contribute significantly to EPSPs recorded in these cells at −70 mV.

Paired-pulse ratios (PPRs) were measured at a 50-ms interval and second EPSPs were measured in the same way as the first EPSP in a pair of stimulations. PPRs were defined as being second EPSP/first EPSP.

**Selection of EPSP data**

Only EPSP recordings remaining stable for ≥100 consecutive trials of the control period of recording were included in the final data set. Stable periods of data were defined as those where the mean and SD, taken over successive epochs of 50 trials, remained close to their values for the first epoch. The SD was required to remain within 30% of its initial value, whereas the mean amplitude was required to remain within 3 SDs of the first epoch (= ±0.5 SD). A study in the hippocampus suggested that there could be significant drifts in quantal size over time (Larkman et al. 1997), which were sometimes associated with inverse changes in release probability. With no net effect on the mean amplitude (unpublished observations). This possibility is minimized by imposing stability criteria on the SD as well as on the mean amplitude because for a binomial process, changes in release probability only minimally affect the SD, whereas changes in quantal size have a much greater effect on the SD.

**Extracting quantal parameters**

Histograms of amplitude-frequency distributions of EPSPs from stable periods of data often (28 from 50 recordings) contained regularly spaced peaks, indicative of a quantal release of neurotransmitter at the synapses. It was previously shown that neocortical synapses appear to operate with similar release probabilities, which are target derived (Koester and Johnston 2003), and so can be approximated with a simple binomial model (Hardingham et al. 2006). Therefore the working hypothesis was that the EPSP amplitudes were drawn from a simple binomial distribution characterized by the number of release sites N, release probability P, and quantal size Q (Larkman et al. 1997). Experimental noise was represented as a Gaussian with SD σₑ. We incorporated an offset S to allow for the fact that the mean amplitude of failures may differ slightly from zero, arising from extracellular field effects (Stricker et al. 1996). Finally, we included a parameter σₒ representing quantal variance, which could be Type 1 or flat (Type 1 and Type 2 combined), whichever was the better fit.

Models were fitted to stable experimental data samples from control and postpairing periods of recording of ≥100 trials (range 100–1,650 trials, mean 166 ± 32 trials) using the method of maximum likelihood (Press et al. 1993). The noise σₑ was obtained by fitting a single Gaussian to a noise distribution measured from the postsynaptic neuron. For a given number of release sites N, the continuously variable parameters (Pᵣ, Q, σₒ, S) were then fitted to the data so as to maximize the likelihood (LN) of the model fit. The optimal N was defined to be that with the highest LN. Starting from n = 1, N was increased until it was either fourfold larger than the N value with the highest LN or far encountered, or 20, whichever occurred first.

Locating a global maximum in a multidimensional parameter space is a nontrivial matter. It was performed with the FMINSEARCH algorithm from MATLAB’s Optimization Toolbox. To guard against being misled by a local maximum, every fit was repeated with ten different randomly chosen starting positions in the parameter space. During development, the performance of this algorithm was validated against a simulated annealing algorithm (Press et al. 1993) implemented in C++, with three different cooling regimes (JC Read, unpublished data).

¹ The online version of this article contains supplemental data.
Adequacy of fitted model

To test whether the proposed fit was acceptable as a model of the experimental data, seven goodness-of-fit statistics were considered: the Kolmogorov–Smirnov \( D \) statistic (Press et al. 1993), the sum of the squared differences between the model and data cumulative distributions, and the \( \chi^2 \) statistic for five different bin sizes. The power of the \( \chi^2 \) statistic depends strongly on the bin size used. With too few bins, the test is too coarse to catch local deviations of the data from the model predictions. Conversely, if too many bins are used, the number of data points falling in any one bin is small and subject to large sampling fluctuations, so the statistic again tolerates poor fits. The optimal number of bins depends on the data set. By using a range of different bin numbers (20, 30, 50, 75, and 100) for each data set, we ensured that each data set would be exposed to a rigorous test. Distributions of these statistics under the null hypothesis, that the experimental data had actually been drawn from the fitted model, were obtained by Monte Carlo simulation (implemented in MATLAB on a PC). In all 5,000 sets of simulated data, each the same size as the experimental data set, were generated from the fitted model and the seven goodness-of-fit statistics were calculated for each simulated data set. For each statistic, we calculated what proportion \((f)\) of simulated data sets yielded higher values of the statistic (indicating worse fits) than the experimental data. A value of \( f > 5\% \) means that the null hypothesis cannot be rejected at the 5\% level on the basis of the statistic. Finally, we applied an additional test, using the proportion of events that failed to evoke a simulated EPSP, \( p_{\text{fail}}\). The Monte Carlo distribution of failure rates could then be compared with the \( p_{\text{fail}}\) observed experimentally. The failure rate test is a two-tailed test, so the null hypothesis is accepted at the 5\% level provided that the experimental \( p_{\text{fail}}\) lies between the 2.5 and 97.5\% quantiles of the Monte Carlo distribution. The quantal model describing each period of experimental data was rejected if any of our tests provided evidence to reject the null hypothesis at the 5\% level. For 22 out of 50 of our recordings we were unable to obtain a satisfactory binomial model, similar to the proportion reported by Koester and Johnston (2005) to conform to a binomial model. This was either as a result of the fitting algorithm being unable to compute an optimal solution or because the model failed on one or more of the rigorous statistical tests. Correlations between experimental parameters were tested using linear regressions.

RESULTS

Paired activity depresses strong connections and potentiates weak connections

The purpose of the investigation was to study the effect of paired pre- and postsynaptic activity on connections between layer 2/3 cortical pyramids. The pairing protocol consisted of 600 paired action potentials in bursts of 20 (details of protocol in METHODS), similar to that used by Markram and Tsodyks (1996). From a total of 78 connections recorded before and after paired activity, 50 were judged to be sufficiently stable during the control period and had sufficient data recorded postpairing be included in the final data set. From these 50 connections, 16 showed long-lasting potentiation in mean amplitude after pairing (threshold of a 20\% increase in EPSP amplitude, mean increase of 109 ± 20\%; Fig. 1A, amplitude all \( P < 0.001 \) compared with baseline), 20 connections showed LTD (threshold of 20\% decrease in amplitude, mean decrease 33 ± 2\%; Fig. 1A, amplitude all \( P < 0.001 \) compared with baseline), whereas 14 showed no change in mean amplitude [no change (nc), Fig. 1A]. Therefore a protocol designed to produce LTP if recorded extracellularly produced a heterogeneous response in individual cells.

To determine whether similar heterogeneous responses to the pairing protocol would also be seen at more physiological temperatures we also carried out 11 further experiments at 35°C using the same pairing protocol and found the occurrence of LTP and LTD to be very similar, with three occasions yielding LTP, four no change in amplitude, and four LTD (data not shown). Therefore individual cortical cells produce heterogeneous responses to an LTP protocol independent of temperature.

We investigated whether various basic properties of the connections could predict the direction of plasticity observed in recordings. The first observation we made was that connections of smaller mean amplitude were more likely to potentiate, whereas those of greater mean amplitude were more likely to depress (Fig. 1B). This therefore meant the variance of the population amplitude distribution decreased significantly after pairing [Fig. 1C, \( P < 0.05 \) using Levene’s test (Levene 1960)]. Consistent with this finding, we found a negative correlation between initial EPSP amplitude and the normalized change in EPSP amplitude after pairing (\( r = 0.42, P < 0.01, \) Fig. 1D). We next looked at the relationship between the connection failure rate and change in amplitude after pairing. We found a strong positive correlation between failure rate of a connection and the increase in EPSP amplitude after pairing (\( r = 0.68, P < 0.001, \) Fig. 1E).

We also looked at various other measures of initial presynaptic strength at these synapses and how these parameters were related to changes in EPSP size. Paired-pulse ratio (PPR; EPSP/EPSP) is often used as a gauge of presynaptic release probability (\( P_r\)), with high values referring to low release probabilities (Bender et al. 2006; Markram and Tsodyks 1996; Volgushev et al. 1997). We found a weaker positive correlation between initial PPR at a 50-ms interpulse interval and change in mean amplitude after pairing (\( r = 0.37, P < 0.05; \) Fig. 2A).

The skew of an amplitude distribution can also give an indication of the release probability of a connection (by comparing mean and median values), with high values of skew referring to low release probabilities (Ledermann 1980). There was a positive correlation between initial skew of amplitude distribution and change in mean amplitude after pairing (\( r = 0.39, P < 0.01; \) data not shown).

Together, these results suggest presynaptic strength determines the direction and magnitude of plasticity that a connection exhibits in response to paired pre- and postsynaptic neuronal firing. After paired activity, weak connections become stronger and strong connections become weaker.

Quantal analysis reveals \( P_r \) is the critical determinant of plasticity

It was previously shown that connections at this synapse and developmental stage can be described with a simple binomial model (Hardingham et al. 2006). A quantal analysis of the recordings was carried out to look in more detail at the contribution of initial individual synaptic parameters (\( N \) and \( P_r\)) to the presynaptic regulation of plasticity and also how the parameters changed after potentiation or depression. Twenty-eight of the 50 connections (just over half the overall data set) had simple binomial fits successfully applied to control periods of recording and postpairing periods of recording. These 28 included nine connections that showed LTP, ten that showed
LTD, and nine that showed no change in mean amplitude. Values of quantal size ($Q$), release probability ($Pr$), and number of release sites ($N$) were assigned to each connection pre- and postpairing. Two further validations of the quantal peaks in histograms recorded from this synapse are their continued existence at a lower Mg$^{2+}$/H$^+$ ratio and release probability $[Q(1 Ca^{2+}/2 Mg^{2+})] = 0.93 \pm 0.07$[$Q(2.5 Ca^{2+}/1 Mg^{2+})], n = 8$ (Hardingham et al. 2006) (Fig. 4) and their presence in histograms of the response to a second stimulation 50 ms after the first stimulation, again at a lower release probability ($Q$ response to the second stimulation $= 0.87 \pm 0.04$). The mean quantal variance of those connections that were fitted successfully ($29\%$) was lower than that of connections that could not be fitted ($45 \pm 4\%$), suggesting that low quantal variance was an important property for binomial fitting. These levels of quantal variance are in close alignment to those found in similar age recordings in the hippocampus (Jonas et al. 1993) where quantal variance of successfully fit data sets was $22\%$ and there was also a similar proportion of successfully fitted data to the present study (roughly $50\%$) (Jonas et al. 1993).

A graph of release probability plotted against skewness of amplitude distribution in the present study was linear and passed through the y-axis (zero skew) at a $P_r$ of 0.57 ($r = 0.67, P < 0.001$; data not shown). Those connections that did not show significant changes in mean amplitude after pairing showed little change in quantal parameters (Supplemental Fig. S4). As a result, histograms of EPSP amplitudes both before and after the pairing protocol had similar peak spacings and histogram shape (Supplemental Fig. S4).

We found another strong negative correlation between initial $P_r$ and change in mean amplitude after pairing ($r = 0.66, P < 0.001$, Fig. 2B), consistent with the observations of Fig. 1. There was no correlation between initial $Q$ value for the connection and change in mean amplitude ($r = 0.28$, NS, Fig. 2C), nor number of release sites of the connection and change in mean amplitude (not shown, average $N$ for the EPSPs was $2.5 \pm 0.2$, range 1–6). This mean $N$ value is in close proximity to the number of anatomical contacts that were identified between layer 2/3 pyramids in rat cortex of comparable age ($2.8 \pm 0.7$, range 1–4; Feldmeyer et al. 2006). Because the variance in $N$ for the population of connections is relatively small, failure rate can be used as a
nonderived measure of presynaptic strength [for a simple binomial process, failure rate = \( (1 - P_r)\)]. Change in release probability after pairing was also negatively correlated with initial release probability \( (r = 0.69, P < 0.001, \text{Fig. 2D}) \). As well as determining whether connections potentiate, initial values of \( P_r \) also predict by how much \( P_r \) can increase.

As stated earlier, connections were split into three groups: those that exhibited LTP after pairing; those that showed LTD; and those that did not change in mean amplitude (nc). Series resistances were not different between the three groups of cells (not shown), one possible explanation of why some cells potentiated whereas others depressed. We were interested to investigate whether there were differences between mean synaptic parameters of these three groups of connections. In control periods of recordings, cases of LTD had lower \( P_r \) values \((0.26 \pm 0.03)\) than connections showing no change in mean amplitude \((0.47 \pm 0.04)\), which in turn had lower \( P_r \) values than cases of LTD \((0.69 \pm 0.06, P \text{ values } all < 0.05, \text{Fig. 2E})\). Consistent with this, initial PPRs of connections that potentiated were also significantly greater than connections that depressed \((P < 0.05, \text{Supplemental Fig. S5A})\). Changes in PPR after pairing were also negatively correlated with changes in mean amplitude after pairing \((r = 0.41, P < 0.01, \text{Supplemental Fig. S5B})\). However, changes in mean amplitude after pairing were better correlated with either changes in release probability \((r = 0.89, P < 0.001, \text{Supplemental Fig. S5C})\) or connection failure rate \((r = 0.73, P < 0.001, \text{Supplemental Fig. S5D})\) and equally well by skew \((r = 0.41, P < 0.01, \text{not shown})\) as by PPR. This is consistent with the correlations shown in Figs. 1E and 2, A and B; that is to say, connection failure rate or derived release probability appear to be more accurate indicators of presynaptic strength than PPR or skew. The variance of PPR values for all 50 connections was also lower after pairing (Levene’s test, \( P < 0.05; \text{data not shown} \)). When release probabilities of the three populations of connections were compared after pairing, they showed no differences \((all P \text{ values } > 0.05, \text{Fig. 2E})\). There were differences in initial connection failure rates and skewness of amplitude distributions between cells exhibiting LTP and LTD, although again there were no significant differences postpairing (Fig. 2F and Supplemental Fig. S5E). Variance of the failure rate diminished in a similar manner to the connection mean amplitude after pairing (Levene’s test, \( P < 0.01 \)). We also compared both the somatic pre- and postsynaptic action potential width of these three groups because it has been reported that a broader
postsynaptic action potential favors LTD (Zhou et al. 2005). There were no differences between the groups of cells. Connections that showed LTD had total mean spike widths of 3.8 ± 0.2 ms presynaptic neuron and 3.8 ± 0.2 ms postsynaptic neuron. Connections that showed LTD had mean spike widths of 3.9 ± 0.2 ms presynaptic and 4.0 ± 0.2 ms postsynaptic.

These data strongly state that the release probability of a connection dictates the direction of the plasticity that paired activity produces. Paired activity thus results in homogenization of amplitude and presynaptic strength within a population of connections.

Potentiation occurs predominantly by presynaptic mechanisms

The 16 connections that showed LTP were examined to look at changes in EPSP properties in more detail and also changes in quantal parameters after LTP. Potentiations were stable during the hour of recording after LTP induction (little short-term plasticity, Fig. 3A). Potentiations varied greatly in magnitude, from 32 to 319%, with a mean of 109 ± 20% of population mean. Connections that potentiated exhibited smaller mean amplitude and greater transmission failures than other connections (mean amplitude 176 ± 39 μV in control periods for LTP connections compared with an average of 484 ± 69 μV for all connections (P < 0.001) and failure rate of 0.54 ± 0.04 compared with average of 0.30 ± 0.06 for all connections; Fig. 2E). Mean amplitudes of inputs that potentiated increased to 314 ± 58 μV after pairing compared with the population mean of 410 ± 45 μV after pairing (P > 0.05, NS). Potencies of connections (a measure of postsynaptic strength: mean amplitude divided by success rate of transmission; i.e., an EPSP of 200 μV with a failure rate of 0.5 would be of potency 400 μV) went from 378 ± 72 to 456 ± 85 μV (P < 0.05), an increase of 32 ± 13% compared with a much larger 109 ± 20% increase in mean amplitude. Failure rates declined significantly after potentiation [from 0.54 ± 0.04 in control periods to 0.31 ± 0.04 after pairing (Fig. 2F)]. These results all largely suggest that increases in presynaptic function are responsible for the potentiation. Failure rates of potentiating connections postpairing became comparable to other connections [0.26 ± 0.04, NS (Fig. 2F)]. Skew values and PPRs of potentiating EPSPs also decreased after pairing (Supplemental Fig. S5). These data all suggest a large presynaptic component to potentiation at these synapses.

Normalized CV²/amplitude plots can give an indication of the locus of changes in synaptic efficacy. Gradients steeper than unity are considered predominantly presynaptic, whereas those less steep than unity are considered predominantly postsynaptic (Malinow and Tsien 1990). Trajectories depend on initial and final values of Pr as well as postsynaptic contributions to potentiation. Normalized 1/CV² plots of LTP data most often had trajectories steeper than the unity line,
again indicating predominantly presynaptic potentiations, although there was substantial heterogeneity between individual lines (Fig. 3B), suggesting the locus varied between individual connections. The mean normalized increase in 1/CV² (to 3.18 ± 0.41) was greater than the mean increase in amplitude (2.09 ± 0.20), implying a predominant presynaptic locus for the LTP. Three connections had a CV² trajectory less than the unity line, suggesting significant postsynaptic increases. Quantal analysis techniques were corroborative with these observations; the other potentiations were largely presynaptic. The mean locus of the potentiation was 82±8% presynaptic (m change, Fig. 3C) and 18±8% postsynaptic (Q change, Fig. 3C). An example of a largely presynaptic LTP is shown in Fig. 3D and an example of a mixed pre-/postsynaptic potentiation in Supplementary Fig. S6A. The predominant presynaptic change after LTP referred to a mean increase in quantal output (m = N × P_r) of 91 ± 19%, going from initial values of 0.47 ± 0.05 to postpairing 0.85 ± 0.09 (Fig. 3C). This was largely by an increase in P_r (0.26 ± 0.04 to 0.44 ± 0.05, Fig. 2E). Only one of the connections had a best-fit value of N that increased after pairing. This could also be satisfactorily explained by a simple binomial model with an increase in P_r (with N constrained). There is strong evidence against the existence of silent synapses at this age (Rumpel et al. 2004) and our data are consistent with this. It would therefore seem that the increase in m seen after LTP was brought about by an increase in P_r (Fig. 3C). As stated earlier, P_r values of LTP connections were lower than P_r values of other connections in control periods (P < 0.05) but not after pairing (Fig. 2E, NS).

Additional postsynaptic changes occur at the more distal synapses

We were interested in why there was a 10-fold range in the magnitudes of potentiation. As already stated, a number of connections exhibited postsynaptic changes in addition to the ubiquitous presynaptic changes. We found a correlation between magnitude of potentiation and size of postsynaptic change (r = 0.68, P < 0.05, Fig. 4A) in addition to the almost expected correlation between magnitude of potentiation and presynaptic change (r = 0.67, P < 0.05; data not shown). We were interested why a certain minority of connections increased by postsynaptic modifications as well as presynaptic changes, so we looked at various properties of EPSPs showing these postsynaptic changes and established how they differed from other connections.

Rise time (10–90% EPSP amplitude rise time) is a measure of the distance of the synapses from the soma (Magee and Cook 2000). EPSPs from distal synapses have slower rise times at the soma than proximal ones and decay by a greater amount from their initial amplitudes in neocortical pyramidal neurons (Williams and Stuart 2002). We found that EPSP rise time was not correlated with initial EPSP amplitude, release probability, or quantal size of connection (Supplemental Fig. S6A).
S7). EPSP rise time was also not correlated with series resistance of the recording (not shown).

Average rise times of connections that potentiated were not different from those that depressed or those that did not change in mean amplitude, and did not significantly change during the course of the experiments (Fig. 4B). However, when only connections that showed potentiation were considered, we found a correlation between EPSP rise time and magnitude of potentiation \((r = 0.52, P < 0.05, \text{Fig. 4C})\). We were concerned that the rise time/degree of potentiation correlation might be attributable to the inclusion of EGTA in the electrode filling solution, causing increased calcium buffering at proximal synapses and reducing the potentiation at these synapses. Therefore we repeated a set of experiments without EGTA or \(\text{Ca}^{2+}\) in the electrode solution (ef) and found the same rise time/potentiation magnitude correlation (rise time to potentiation) was still present \((r = 0.97, P < 0.05, \text{Fig. 4C})\). Combining the zero EGTA/zero \(\text{Ca}^{2+}\) efs with the EGTA/\(\text{Ca}^{2+}\) efs data improved the rise time/potentiation magnitude correlation \((r = 0.57, P < 0.01; \text{data not shown})\), suggesting that the electrode solution did not have an effect on the physiology. There was also a similar probability of occurrence of the various forms of plasticity with zero EGTA/zero \(\text{Ca}^{2+}\) efs (12 experiments: four LTP, four LTD, and four no change in amplitude). The lack of effect of EGTA was perhaps to be expected because neurons contain a large amount of buffered calcium; thus a more physiological electrode filling solution would indeed contain both calcium and calcium buffer. Many experimenters investigating synaptic transmission and plasticity used EGTA intracellularly when using whole cell techniques (Choi et al. 2003; Kullmann and Nicoll 1992; Schubert et al. 2003) and observed both pre- and postsynaptic changes in LTP (Kullmann and Nicoll 1992). Effects of EGTA on synaptic release were previously documented for layer 5 cells (without any \(\text{Ca}^{2+}\) in the electrode; Ohana and Sakmann 1998), but in our hands control experiments with no pairing protocol produced little change in mean amplitude after 1 h of recording (only 1% \pm 12% depression on average, \(n = 10; \text{data not shown})\).

We also found a correlation between initial EPSP rise time and postsynaptic change after LTP \((r = 0.75, P < 0.01, \text{Fig. 4D})\), although there was no correlation between EPSP rise time and presynaptic change \((r = 0.01, \text{NS, Fig. 4E})\). Therefore it seems that distal synapses are able to potentiate by a greater magnitude, by additional postsynaptic mechanisms. This may explain the lack of correlation between rise time/initial amplitude and rise time/initial \(Q\) for recorded EPSPs (Supplemental Fig. 7, NS) because distal synapses can show greater potentiation than proximal ones and thus compensate for their more remote location. To confirm these observations, EPSPs were split in half, into the most proximal inputs and the more distal inputs. The two groups were compared for amplitude changes after LTP, presynaptic changes, and postsynaptic changes. The magnitude of the LTP and postsynaptic component of LTP were larger for distal inputs than proximal inputs (both \(P\) values <0.05), whereas the presynaptic component was comparable (\(P \geq 0.05, \text{NS, Supplemental Fig. 8})\). When all connections were considered (including nonpotentiating connections), we found a positive correlation between EPSP rise time and postsynaptic change after pairing \((r = 0.42, P < 0.05; \text{data not shown})\) and a negative correlation between initial \(Q\) value and postsynaptic change \((r = 0.43, P < 0.05; \text{data not shown})\).

**LTD also occurs predominantly by a reduction in quantal release**

Twenty connections \((40\%)\) showed a 33 \pm 2\% reduction in mean amplitude 1 h after the pairing protocol (Fig. 5A). Connections that showed LTD had larger initial EPSP amplitudes than average \([790 \pm 139 \mu V \text{ compared with the population average of } 484 \pm 69 \mu V \text{ (} P < 0.05\)]\), which depressed to 512 \pm 94 \mu V after pairing, compared with the population average of 410 \pm 45 \mu V (NS). They also showed fewer transmission failures \([\text{failure rates were } 0.15 \pm 0.04 \text{ in the control period compared with a population average of } 0.30 \pm 0.06 (P < 0.05)\), after pairing failure rates increased to 0.25 \pm 0.06 postpairing compared with a population average of 0.26 \pm 0.04 (NS, Fig. 2E).

There is evidence for both presynaptic (Torii et al. 1997) and postsynaptic (Eder et al. 2002) components to neocortical LTD. Normalized CV\(^2\)/amplitude plots for individual experiments in the present study mostly had trajectories steeper than the unity line, suggesting a similar predominantly presynaptic mechanism to the LTD, but again there was considerable heterogeneity between individual plots with a number of lines above the diagonal (Fig. 5B). The mean normalized reduction in 1/CV\(^2\) was to 45 \pm 6\% the control value compared with a reduction in amplitude of 33 \pm 2\%. Therefore there seems to be a mixture of both pre- and postsynaptic depression at the 2/3 synapse. Potencies of connections that showed LTD went from 854 \pm 130 to 608 \pm 88 \mu V \text{(} P < 0.001\), again consistent with an appreciable postsynaptic component to the depression.

When a quantal analysis was performed on these connections that showed LTD, the reduction in mean amplitude was found to be largely by a reduction in \(m\) (found on 80\% of occasions, \(P < 0.01, \text{Fig. 5C})\), but depression in \(Q\) was also commonly observed (60\% of occasions). There were comparable reductions in individual synaptic parameters \(N, P, \text{ and } Q\) but none was significant on its own (Fig. 5C). Changes in \(m\) after LTD could again in all cases be adequately described by reductions in release probability, with \(N\) held constant, but could not always be described by changes in \(N\) (with \(P\) held constant). These results are again consistent with the observations of Rumpel et al. (2004). An example of a largely presynaptic LTD is given in Fig. 5D; an example of a largely postsynaptic LTD is given in Supplementary Fig. 6B.

Relationships between EPSP rise time and magnitude or locus of depression did not exist as for the LTP data (not shown) and thus it was unclear why some depressions were presynaptic and others postsynaptic.

**Discussion**

The main findings of this study are that at 3-wk-old layer 2/3 cortical synapses, the release probability of a connection determines whether the synapse will potentiate or depress in response to paired neuronal activity. Potentiation occurs by predominantly presynaptic mechanisms, whereas additional postsynaptic mechanisms operate for more distal synapses.

**Normalization of synaptic strength after paired activity**

We show that in layer 2/3 neurons paired activity is acting to reset the presynaptic strength to intermediate values (to \(P_r\) values of about 0.5). Potentiation occurs predominantly by
increases in $P_r$ and there appears to be an upper limit to attainable values of $P_r$. This is consistent with the observation that only weak, relatively undeveloped connections are capable of potentiation. However, the reasons that the strength of layer 2/3 synapses normalize in response to paired activity are unclear. Between the ages of 2 and 4 wk there has been shown to be a switch from paired-pulse depression to paired-pulse facilitation in the cortex, which may indicate a net reduction in release probability over this period (Reyes and Sakmann 1999). For this to happen there would need to be greater levels of depression than potentiation. Activity-dependent scaling has been previously shown to occur postsynaptically in neocortical neurons (Turrigiano et al. 1998); here we show a means by which scaling occurs presynaptically.

Results from other studies have also suggested a normalization of connection strength after pairing. Connections of smaller mean amplitude were shown to potentiate preferentially in hippocampal cultures (Bi and Poo 1998). In a previous report where both LTP and LTD were observed in response to the same induction protocol (in layer 2/3 rat visual cortex), initial paired-pulse ratio (PPR), a measure of presynaptic strength, was shown to predict the direction of the plasticity in individual cells (Volgushev et al. 1997, 2000), whereas changes in PPR after pairing were negatively correlated with initial PPR values. Similar heterogeneous directions of plasticity from paired neuronal firing were reported at adult corticostriatal synapses (Akopian et al. 2000). In the present study, a quantal analysis of layer 2/3 cortical connections shows that the initial $P_r$ of a connection determines more accurately whether the connection potentiates or depresses than PPR ($r = 0.65$, $P < 0.001$ for $P_r$ compared with $r = 0.37$, $P < 0.05$ for PPR). We also found negative correlations between initial PPR and change in PPR after pairing ($r = 0.82$, $P < 0.001$).

A similar negative correlation between initial $P_r$ and change in $P_r$ after LTP to this study was previously found in the hippocampus (Larkman et al. 1992). High initial values of $P_r$ may reflect previous saturation of LTP at the connections and thus only subsequent depression of the connection is possible (Akopian et al. 2000). Conversely, low values of $P_r$ may reflect previous history of LTD so there is therefore a larger scope to potentiate (Akopian et al. 2000).

Other factors have been implicated in determining the direction of plasticity that layer 2/3 neurons exhibit in response to paired activity. Postsynaptic action potential width was proposed to be a determining factor of direction of plasticity in the entorhinal cortex (Zhou et al. 2005) and differing kinetics of postsynaptic calcium transient are proposed to be a determinant in the immature visual cortex (Ismailov et al. 2004). Our data show postsynaptic action potential width is not a critical determinant in the 3-wk-old visual cortex. We cannot rule out the possibility that postsynaptic calcium transients in our postsynaptic neurons varied in cells showing different responses to paired activity, although this seems unlikely because the major determinant of plasticity direction is presynaptic strength. Different levels of calcium elevation in spines were previously shown to cause opposite directions of plastic-

FIG. 5. Depression also involves predominantly presynaptic changes. A: in a subset of recordings (20 from 50), paired APs produced a long-lasting reduction in EPSP mean amplitude. reductions in amplitude were $33 \pm 2\%$. Averaged raw data show an EPSP that depressed in the control period of recording and postpairing. B: normalized 1/CV2/mean amplitude plots showed considerable heterogeneity between connections, but the mean gradient was again steeper than unity (open symbols), suggesting predominantly presynaptic mechanisms. C: mean changes in amplitude, $m$ ($N \times P_r$), $N$, $P_r$, and $Q$ after LTP. Only changes in amplitude ($P < 0.001$) and $m$ ($P < 0.01$) after LTD were significant. D: example of a largely presynaptic LTD with simple binomial model fits in control periods (left) and depressed recordings (right).
The locus of synaptic plasticity after paired activity

The locus of long-term potentiation at central synapses is still a much debated topic. Locus may depend on the synapse being studied, the stage of development recorded at, the timescale of the measurements, the composition of the intracellular and extracellular solutions being used, and the induction protocol used (for recent reviews see Malenka and Bear 2004; Malinow 2003). In the neocortex, LTP is predominantly presynaptic, although postsynaptic modifications have been reported (Hardingham and Fox 2006; Markram and Tsodyks 1996; Volgushev et al. 1997, 2000). Potentiation is again believed to be presynaptic at the mossy fiber synapse in the hippocampus (Weisskopf and Nicoll 1995) but many reports were made of postsynaptically expressed LTP at the hippocampal Schaffer Collateral or at least a mixed pre-/postsynaptic locus (Malenka and Bear 2004; Malinow 2003; Nicoll 2003). LTP in response to a burst of stimulations at cortical synapses was previously shown to be a redistribution of synaptic efficacy over the burst rather than a net increase in synaptic efficacy, consistent with a largely presynaptic locus (Markram and Tsodyks 1996). The fidelity of the postsynaptic response to bursts is more preserved in hippocampal LTP, consistent with a more postsynaptic locus of expression (Buonomano 1999; Selig et al. 1999). Many previous cortical plasticity studies were carried out at room temperature and results are consistent with more physiological temperatures in many respects, including both synaptic properties and plasticity outcomes (e.g., Ismailov et al. 2004; Volgushev et al. 1997; Zhou et al. 2005). The present study confirmed a predominant presynaptic mechanism for both LTP and LTD at the cortical layer 2/3 synapse.

Because postsynaptic calcium transients were previously shown to be responsible for both LTP and LTD (Bender et al. 2006), this would indicate the involvement of a retrograde messenger to produce both reductions and increases in presynaptic function. Nitric oxide was strongly implicated in LTP in supra-granular layers of cortex (Hardingham and Fox 2006; Haul et al. 1999; Nowicky and Bindman 1993). Endocannabinoids were implicated in postsynaptic cortical LTD in layer 2/3 cortex (Bender et al. 2006; Sjostrom et al. 2003, 2004). Paired activity could produce both messengers simultaneously. Presynaptic targets of nitric oxide (e.g., guanyl cyclase) could become desensitized after recent LTP inducing activity. The direction of synaptic plasticity could depend on the relative size of responses to the two retrograde signals. If the release probability is low, the potentiating response of nitric oxide could dominate, whereas if release probability is high the response to nitric oxide may be saturated and the depressing response of endocannabinoids could dominate. In this way the differing responses of neurons to the same protocol could be explained.

In the present study, whereas presynaptic modifications were ubiquitous at the synapses studied, postsynaptic modifications were restricted to distal synapses. Distal synapse amplitude was shown to be larger at the synaptic terminal than at more proximal sites, both in cortex and hippocampus, whereas EPSPs are of more similar size at the soma regardless of synapse location (Andrasfalvy and Magee 2001; Magee and Cook 2000; Smith et al. 2003; Williams and Stuart 2002). These results are consistent with the lack of correlations between EPSP rise time (location) and somatic amplitude or $Q$ (postsynaptic efficacy) in the present study (Supplemental Fig. S7). It was proposed that distal synapses may be initially larger in amplitude as the result of a greater number of AMPA receptors at the synapses (Andrasfalvy and Magee 2001; Smith et al. 2003). Distance-dependent scaling of this form could be explained by distal synapses being preferentially potentiated, or potentiated by a greater magnitude than proximal ones. Results in this study suggest that increased potentiation of distal synapses does indeed occur and that it does so by additional postsynaptic modifications to the ubiquitous presynaptic changes.

This is appreciably different from that reported at the same neurons at two distances along the apical dendrite where potentiation was shown to be smaller at distal synapses (Froemke et al. 2005). However, one must take into account the fact that a large number of the excitatory synaptic inputs to these layer 2/3 neurons (85–90%) are on the basal and apical oblique dendrites (Larkman 1991), which were not investigated by Froemke et al. Modeling studies indicate that the range of rise times for EPSPs that we recorded are within those predicted for basal and apical oblique inputs (Trevelyan and Jack 2002; Trevelyan, unpublished observations). The longer rise time EPSPs would be located on the terminal ends of the dendrites. Because their amplitude at the soma is similar to the more proximal input EPSPs, they must be generated by synaptic conductances at least as large as the more proximal EPSPs. Because the local input impedance is greater in the terminal dendritic region, the local voltage excursion will be much greater than that for more proximal inputs (for theoretical examples see Jack et al. 1975; Redman 1973). This predicted larger local voltage excursion might be more likely to cause greater calcium entry, particularly through voltage-dependent calcium channels. The size of postsynaptic calcium transients caused by bursts of action potentials along the basal dendrites in layer 5 neurons from 3- to 4-wk-old rats has now been shown to be larger distally than more proximally (Kappa and Stuart 2006). Whether these larger distal calcium transients also occur in layer 2/3 and whether they can recruit additional postsynaptic modifications remain to be seen.

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REFERENCES


