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21. Cosmid DNAs were labeled and hybridized in pairs to G1 interphase cell nuclei from fibroblast cell cultures as described (3, 6, 22). One site was labeled with Texas red (biotinylated probe detected with avidin–Texas red), and one site was labeled with fluorescein isothiocyanate (FITC) [digoxigenin-labeled probe detected with sheep antidigoxigenin and FITC-conjugated donkey anti-sheep immunoglobulin G (IgG) antibodies]. The two fluorochromes were viewed simultaneously through a double bandpass filter (Chromatechnology). Photographic slides of randomly selected nuclei were made for each tested pair (15-s exposures, ≥10 nuclei per field, Scotch 3M 640T film). Slides were projected at ~10⁴× magnification onto a digitizing board with a nominal resolution of 40 lines per millimeter (Summagraphics, Seymour, CT). The coordinates of paired red and green fluorescent spots were identified and were entered through the digitizing board into a computer for further analysis. With this approach, ~5000 measurements can be accumulated per person·day.
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Dependence of Cortical Plasticity on Correlated Activity of Single Neurons and on Behavioral Context

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It has not been possible to analyze the cellular mechanisms underlying learning in behaving mammals because of the difficulties in recording intracellularly from awake animals. Therefore, in the present study of neuronal plasticity in behaving monkeys, the net effect of a single neuron on another neuron (the "functional connection") was evaluated by cross-correlating the times of firing of the two neurons. When two neurons were induced to fire together within a short time window, the functional connection between them was potentiated, and when simultaneous firing was prevented, the connection was depressed. These modifications were strongly dependent on the behavioral context of the stimuli that induced them. The results indicate that changes in the temporal contingency between neurons are often necessary, but not sufficient, for cortical plasticity in the adult monkey: behavioral relevance is required.

Learning processes are usually assumed to be mediated by lasting changes in synaptic efficacies, a phenomenon known as "synaptic plasticity." However, the underlying mechanisms are only partially understood

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(1). Many computational models of associative learning have adopted Hebb's postulate (2) and suggested that experience-dependent synaptic changes depend mainly on the contingency [that is, the correlation (3)] between the firing times of two interacting neurons (4, 5). Although much evidence supports the necessity of correlated activity for synaptic plasticity in vivo in the brain of mammals (5, 6), that assumption has not been tested at the level of two single cells. Furthermore, little is known about other factors that may affect this

requirement for synaptic plasticity (7). Evidence from psychological studies suggests a crucial role for behavioral factors in neural plasticity: Thorndike argued that a connection is significantly modified only if its activation is associated with outcomes important to the animal's behavior (8). Because of methodological and technical difficulties, the role of behavioral context has been often overlooked in the study of the synaptic mechanisms underlying learning in mammals (9).

To overcome such difficulties, we used a different approach: instead of studying the efficacy of single synapses, we examined the correlation between the firing times of pairs of neurons. The correlation manifests the net effect of the whole synaptic substrate through which the two neurons interact, including both direct and indirect connections; it represents the "functional connection" (10) between the two neurons. Lasting changes of functional connections ("functional plasticity") represent lasting changes of cortical functioning as mediated by synaptic plasticity. Our methodological approach was to examine whether and how "general behavioral factors," such as attention, motivation, and reinforcement, affect functional plasticity. These factors were not differentiated in this study and henceforth are included in the term "behavior."

Neuronal activity was recorded from the auditory cortices of two adult monkeys (11). In each session, the extracellular activities of two to ten single neurons were recorded simultaneously. One hundred and fifty-eight pairs of neurons that exhibited positive or negative correlation in their activity were selected for this study. The dependence of functional plasticity on the contingency between the activities of the two neurons (henceforth referred to as "contingency") and on behavior was tested by combined cellular conditioning and behavioral paradigms (Fig. 1). The activity of one neuron in each pair (the "CS neuron") was regarded as the conditioned stimulus (CS), and the activity of the other neuron (the "CR neuron") as the conditioned response (CR) (12). An auditory stimulus capable of eliciting or suppressing activity in the CR neuron was used as the unconditioned stimulus (US). The US served both for pairing the activities of the two neurons and for guiding the monkey's behavior during the performance of an auditory discrimination task.

The combined paradigm yielded three combinations. (i) "Conditioning associated with behavior" occurred when the monkey performed the task, and the connections (direct, indirect, or both) between the neurons were conditioned. Conditioning was applied by pairing the US with the CS neuron activity (the auditory stimulus was

delivered 2 to 4 ms after the CS neuron fired a spike), thereby modifying the contingency between the activities of the two neurons (13). During task performance, the monkey had to attend to the US in order to get a reward (Fig. 1); at each trial, a train of auditory stimuli (the US) was delivered. After a variable interval (0.8 to 2.2 s), the nature of the stimuli was changed from either a band-pass noise to a pure tone or from a lower frequency to a higher frequency tone (Fig. 1). To receive a drop of juice, the monkey had to release a touch switch within 0.5 s. The duration of each conditioning session varied from 70 to 850 s. (ii) "Conditioning without behavior" occurred when trains of the US were delivered in the same manner as during task performance but the monkey was not rewarded and did not perform the task. (iii) "Pseudoconditioning" occurred when the monkey performed the task but the contingency between the activities of the two neurons was not affected; the occurrence of the US was not correlated with the activity of the CS neuron. The US was delivered at random times, with an average rate similar to the average rate measured in the conditioning paradigm.

Functional connections were evaluated by cross-correlation histograms (CCHs) (14, 15). Firing patterns of single neurons were examined by autocorrelation histograms (ACHs) (14, 15). Cross-correlation histograms between all conditioned neurons and their ACHs were computed and presented on-line by a fast microcomputer (Intel 310). We evaluated functional plasticity quantitatively by estimating the strength of the connections before and after the conditioning periods (16). During both periods no stimuli were delivered. We estimated the strength by calculating the gain of the connection from the area under the peak (or trough) of the CCH, excluding the expected value (17). The gain equals the average number of spikes that were added to the spike train of the CR
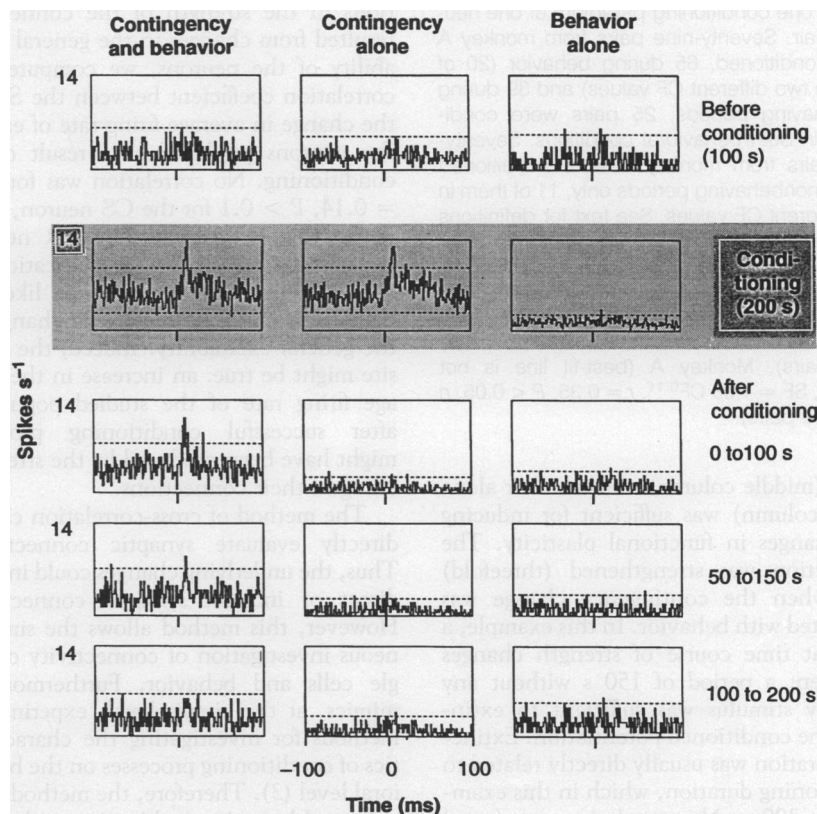
neuron after each spike of the CS neuron.

Figure 2 illustrates the potentiation of functional connection induced by conditioning associated with behavior. The lower graph shows the gain of the connection as a function of time during two conditioning processes. The weak connection was potentiated during the first conditioning period (indicated by the left horizontal filled bar) and remained strong after conditioning was stopped. The potentiation was extinguished during the next 12 to 13 min of "spontaneous" activity. After the con-

nection had returned to its original strength, a second conditioning process was applied, and similar results were obtained. The changes in functional plasticity are illustrated by the CCHs (upper row) that were calculated before (left) and after the second conditioning period.
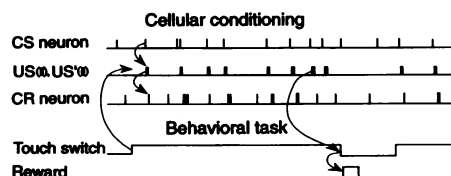
Conditioning associated with behavior always yielded stronger modifications of the connection between a given neuronal pair than pseudoconditioning or conditioning without behavior. Figure 3 depicts one pair for which neither a contingency change
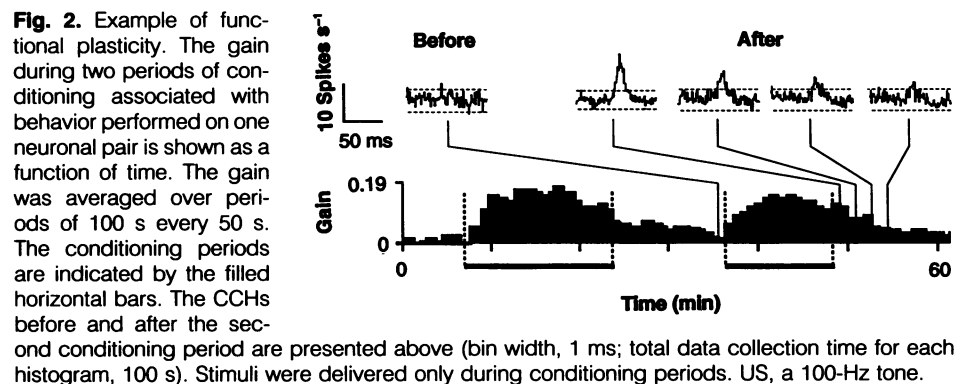
Fig. 2. Example of functional plasticity. The gain during two periods of conditioning associated with behavior performed on one neuronal pair is shown as a function of time. The gain was averaged over periods of 100 s every 50 s. The conditioning periods are indicated by the filled horizontal bars. The CCHs before and after the second conditioning period are presented above (bin width, 1 ms; total data collection time for each histogram, 100 s). Stimuli were delivered only during conditioning periods. US, a 100-Hz tone.
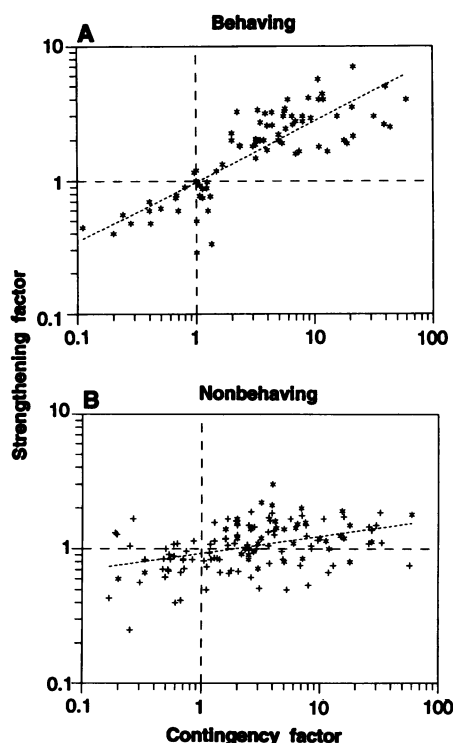

Fig. 1. Experimental paradigms. **(Top)** Cellular conditioning paradigm. Each thin vertical line represents a single spike. During conditioning, the US (filled bars) was delivered immediately after the CS neuron fired a spike. The CR neuron usually responded to each US with one or more spikes. Curved arrows illustrate the causal sequence. As a result, the contingency between the activities of the two neurons increases. The empty bars denote the US', an auditory stimulus different from the US but usually still effective in activating the CR neuron. **(Bottom)** The behavioral task (see text).

Fig. 3. Requirement of both behavior and contingency for the induction of functional plasticity on a single neuronal pair. **(Upper row)** Preconditioning CCHs. **(Second row)** Conditioning CCHs. Only during this period were stimuli delivered. **(Third through fifth rows)** Postconditioning CCHs describing three successive, partially overlapping periods of 100 s each (timings are indicated on the right of each row). **(Left column)** Contingency and behavior ("conditioning associated with behavior"). **(Middle column)** Contingency alone ("conditioning without behavior"). **(Right column)** Behavior alone ("pseudoconditioning"). Bin width, 1 ms. US, a wide-band ("white") noise.

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Fig. 4. Effect of behavior and contingency on functional plasticity. Data gathered from two monkeys: (∗) monkey A, (+) monkey B (nonbehaving only). Each dot represents average values for one conditioning paradigm of one neuronal pair. Seventy-nine pairs from monkey A were conditioned, 65 during behavior (20 of them in two different CF values) and 39 during nonbehaving periods; 25 pairs were conditioned in both behavioral conditions. Seventy-nine pairs from monkey B were conditioned during nonbehaving periods only, 11 of them in two different CF values. See text for definitions of factors (CF and SF). **(A)** Conditionings associated with behavior. Best-fit; SF = 0.98 CF$^{0.45}$, $r = 0.81$, $P < 0.0001$, $n = 85$ (65 pairs). **(B)** Conditionings without behavior. Both monkeys, SF = 0.93 CF$^{0.13}$, $r = 0.41$, $P < 0.01$, $n = 129$ (118 pairs). Monkey A (best-fit line is not shown), SF = 1.03 CF$^{0.11}$, $r = 0.35$, $P < 0.05$, $n = 39$ (39 pairs).

alone (middle column) nor behavior alone (right column) was sufficient for inducing any changes in functional plasticity. The connection was strengthened (threefold) only when the contingency change was associated with behavior. In this example, a different time course of strength changes was seen: a period of 150 s without any auditory stimulus was sufficient to extinguish the conditioned potentiation. Extinction duration was usually directly related to conditioning duration, which in this example was 200 s. No correlation was found between the extinction duration and the amount of strengthening.

To assess quantitatively the relative contributions of contingency and behavior to the observed changes in functional connections, two factors were defined: The strengthening factor (SF), defined as the gain immediately after conditioning divided by the gain before conditioning; and the contingency factor (CF), defined as the area under the peak (or peaks) during conditioning (the "induced gain") divided by the gain before conditioning. Figure 4 describes the dependence of SF on the CF in the behaving condition and in the nonbehaving condition. The combined effect of contingency and behavior is evident: When conditioning paradigms were carried out during behavior (Fig. 4A), a clear dependence of strengthening on contingency was seen. When the contingency was increased by more than 50% (CF > 1.5) the connections were potentiated, and when it was decreased by more than 50% the connections were depressed. The regression line suggests that, as a first approximation, the SF is about equal to the square root of the CF. On the other hand, when behavior was not involved (Fig. 4B), similar changes in the contingency yielded much smaller (although statistically significant) changes in the strength of the connections.

Functional plasticity was frequently accompanied by an increase in the average firing rate of the neurons (average about 20%). To test the possibility that modifications in the strength of the connections resulted from changes in the general excitability of the neurons, we computed the correlation coefficient between the SF and the change in average firing rate of each of the neurons produced as a result of the conditioning. No correlation was found ($r = 0.14$, $P > 0.1$ for the CS neuron, and $r = 0.19$, $P > 0.05$ for the CR neuron, two-tailed t test). Thus, modifications in connectivity strength were not likely to have been produced merely by changes in the general excitability. Indeed, the opposite might be true: an increase in the average firing rate of the studied population after successful conditioning processes might have been produced by the strengthening of their connections.

The method of cross-correlation cannot directly evaluate synaptic connectivity. Thus, the underlying changes could involve direct or indirect synaptic connections. However, this method allows the simultaneous investigation of connectivity of single cells and behavior. Furthermore, it mimics, at the circuit level, experimental methods for investigating the characteristics of conditioning processes on the behavioral level (3). Therefore, the method demonstrated here, in combination with other methods (6, 9), may bridge the gap between cellular and behavioral levels. Indeed, these results might provide an explanation for certain findings obtained by these previously applied methods.

The results demonstrate that lasting modifications of functional connections can be induced in the auditory cortex of the adult behaving monkey without the use of intracranial stimulation. Initially, each neuronal pair had some constant, steady-state level of contingency. The connections were strengthened after an increase in the contingency, were depressed when the contingency decreased, and generally were not changed when the contingency was not changed (18). In general, these results support the "generalized Hebb-Stent rule" (2, 4, 5), but they also suggest a refinement to this rule: a change in synaptic efficacy is not determined by the absolute level of the contingency but rather depends on the deviation from its steady-state level. Thus, a positive contingency, which is smaller than the steady-state level, will cause a decrease, not an increase, in the efficacy. Furthermore, the modifications were much weaker when the stimuli that evoked them carried no behavioral relevance, which suggests a crucial role for behavioral factors in modulating the Hebbian-like mechanisms (19). We conclude that the mechanisms of learning that underlie neuronal plasticity in the cortex of adult monkeys obey the essential features of both the Hebb-Stent rule and Thorndike's Law of Effect (20).

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17. Three types of "strength evaluators" were used in this study: gain, contribution, and synchronous gain (15). There was no significant difference between the results obtained with these three evaluators. The gain, a simple and straightforward evaluator, is used in this paper.

18. There are few examples in which connections were depressed when contingency was either not changed or slightly increased (Fig. 4), which suggests the existence of an additional, non-Hebbian, mechanism. For example, input activa-

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Control by Asparagine Residues of Calcium Permeability and Magnesium Blockade in the NMDA Receptor

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The N-methyl-D-aspartate (NMDA) receptor forms a cation-selective channel with a high calcium permeability and sensitivity to channel block by extracellular magnesium. These properties, which are believed to be important for the induction of long-term changes in synaptic strength, are imparted by asparagine residues in a putative channel-forming segment of the protein, transmembrane 2 (TM2). In the NR1 subunit, replacement of this asparagine by a glutamine residue decreases calcium permeability of the channel and slightly reduces magnesium block. The same substitution in NR2 subunits strongly reduces magnesium block and increases the magnesium permeability but barely affects calcium permeability. These asparagines are in a position homologous to the site in the TM2 region (Q/R site) of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors that is occupied by either glutamine (Q) or arginine (R) and that controls divalent cation permeability of the AMPA receptor channel. Hence AMPA and NMDA receptor channels contain common structural motifs in their TM2 segments that are responsible for some of their ion selectivity and conductance properties.

Excitatory synaptic transmission in the vertebrate central nervous system is mediated by glutamate, which activates glutamate receptor ion channels. These receptors are divided into two functional subtypes: the AMPA receptors mediate fast synaptic currents and NMDA receptors mediate currents that are slower and longer lasting. NMDA receptors mediate an inflow of Ca^{2+} into the postsynaptic cell, which is controlled by extracellular Mg^{2+} in a voltage-dependent manner (1). The NMDA receptor subunits NR1 (2) and NR2 (3) are distantly related to each other and contain, in their transmembrane 2 (TM2) segment, an asparagine at a position homologous to the Q/R site (4) of the AMPA receptor subunits (Fig. 1A). The amino acid occupying the Q/R site of AMPA receptor subunits is controlled by RNA editing (5)

and determines conductance rectification and the divalent cation permeability of the ion channel (6). Arginine in this site imparts a much lower Ca^{2+} and Mg^{2+} permeability to the AMPA receptor channel than the glutamine residue (6). Engineering an asparagine into this critical position of AMPA receptor subunits imparts to the altered channel a high permeability for Ca^{2+} compared to Mg^{2+} (4). We therefore investigated the effect of replacing the asparagine (7) in the position homologous to the Q/R site of AMPA receptor subunits on the Ca^{2+} and Mg^{2+} permeability of recombinantly expressed NMDA receptors.

Whole-cell currents were activated by fast application of L-glutamate to transfected 293 cells expressing NMDA receptor channels (7). Voltage ramps were applied before and during agonist application to compare changes in reversal potentials when the extracellular solution was changed from high Na^+ (140 mM) to high Ca^{2+} (110 mM) or high Mg^{2+} (110 mM). The difference in the reversal potential between the wild-type NR1-NR2A and the mutant NR1(N598Q)-NR2A channel after a switch from high Na^+ to high Ca^{2+}

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