the single-substitution mutants exhibited little or no transferase activity under the conditions we used (Table 1); however, unlike the double mutants, it retained reactivity with neutralizing mAb 1B7. Only small amounts of enzymatic activity could be detected when the amount of 4-1 protein in the assay was increased (Fig. 2A); repeated determinations indicated that the specific ADP-ribosyltransferase activity of the S1 analog was reduced by a factor of approximately 5000. Measurement of the NAD glycohydrolase activity associated with the single-residue substitution mutants (Fig. 2B) revealed a pattern similar to that obtained from evaluation of ADP-ribosyltransferase activity. The S1 analog 4-1 exhibited little or no detectable glycohydrolase activity, indicating a reduction in the magnitude of this activity by a factor of 50 to 100.

The results obtained here substantiate our previous conclusion (*18*): the region of S1 homology (residues 8 to 15) with cholera and *E. coli* toxins contains amino acids that are important for enzyme activity. The magnitude of the effect exerted by substitution of Arg^9 on both transferase and glycohydrolase activities suggests that this residue plays an essential role in the enzymatic mechanisms of the S1 subunit. We recently introduced the $Arg^9 \rightarrow Lys$ mutation into full-length recombinant S1 and found that transferase activity was reduced by a factor of approximately 1000. This observation indicates that the substitution at residue 9 is alone sufficient to attain the striking loss in enzyme activity and that the coincidental replacement of the two amino-terminal aspartate residues in the mature S1 sequence with the Met-Val dipeptide that occurs in S1/6A is not required to achieve this reduction.

Inactivation of enzymatic and toxic activity through single amino acid substitutions effected by site-directed mutagenesis has been achieved for both *Pseudomonas* exotoxin A and diphtheria toxin (DTX) (*25*). In addition, Porro *et al.* (*26*) have shown that an enzymatically inactive mutant form (CRM197) of DTX elicits a protective response. These studies support the utility of "genetic toxoiding" and have prompted similar efforts with respect to PTX. Black *et al.* (*27*) have recently described a strain of *B. pertussis* that produces a mutant form of PTX with reduced ADP-ribosyltransferase activity. The strain showed little or no evidence of PTX-mediated biological activity (for example, lymphocytosis or histamine sensitization) when injected into mice, thereby supporting the prediction that suppression of S1-associated enzyme activity will result in a corresponding reduction in toxic effects. However, the mutant strain also had diminished immunoprotective capability when used in the intracerebral challenge model and this diminished capability was interpreted to reflect an immunopotentiating effect of the ADP-ribosyltransferase activity. It will be of interest to evaluate the effects of the 4-1 mutation that retains reactivity with a known protective mAb on the toxic and immunogenic properties of the holotoxin.

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Pharmacological Induction of Use-Dependent Receptive Field Modifications in the Visual Cortex

JOACHIM M. GREUEL,* HEIKO J. LUHMANN,† WOLF SINGER

Lasting modifications of the receptive fields of neurons in the visual cortex can be induced by pairing visual stimuli with iontophoretic application of the neuromodulators acetylcholine and noradrenaline or the excitatory amino acids *N*-methyl-D-aspartate (NMDA) and L-glutamate. The modifications are obtained in less than 1 hour and persist for more than 40 minutes. Thus, acetylcholine and norepinephrine have a permissive role in use-dependent neuronal plasticity. These results support the notion of a postsynaptic threshold for neuronal malleability that differs from that of sodium-dependent action potentials.

DURING EARLY POSTNATAL DEVELopment, receptive fields of visual cortex neurons are susceptible to experience-dependent long-term modifications (*1*). Activation of cortical neurons by retinal input alone is insufficient to trigger these changes. There appears to be a threshold for induction of use-dependent modifications (*2, 3*) that is reached only if retinal activity is coincident with additional nonretinal gating signals (*4*). Proprioceptive affer-

Max Planck Institute for Brain Research, Deutschordenstrasse 46, 6000 Frankfurt 71, Federal Republic of Germany.

*Present address: Troponwerke, Department of Neurobiology, P.O. Box 801060, D-5000 Köln 80, Federal Republic of Germany.
†Present address: Stanford University Medical Center, Department of Neurology, C 338, Stanford, CA 94305.

ents from extraocular muscles, nonspecific thalamic projections, and the noradrenergic and cholinergic pathways have been identified as sources of these gating signals (*5*). Inactivation of these systems prevents experience-dependent modifications without abolishing neuronal responses to light. Blockade of the NMDA receptor with the antagonist D,L-amino-5-phosphono-valeric acid (AP5) has a similar effect (*6*). Thus, the threshold for induction of use-dependent modifications may be related to voltage-dependent mechanisms that have a high activation threshold such as the NMDA receptor–mediated Ca^{2+} conductance and the high-threshold Ca^{2+} channels. Manipulations that abolish a phenomenon are valuable for screening but raise problems of specificity (*7*). Therefore, in this study we sought evidence that acetylcholine (ACh) and norepinephrine (NE) facilitate use-dependent and long-lasting modifications of cortical responses, and that strong activation of postsynaptic neurons is crucial to plastic modifications of response properties.

In anesthetized, paralyzed kittens, retinal stimulation alone activates cortical neurons but does not induce lasting changes of cortical receptive fields (*8*), probably because the required gating signals of nonretinal origin are reduced by anesthesia (*9*). This preparation is thus suitable for testing whether local application of ACh and NE and of activators of the NMDA mechanism facilitates long-term modifications in response to visual stimulation. Therefore, NE and ACh or the excitatory amino acids (EAAs) L-glutamate (GLU) and NMDA, or both, were applied to cortical neurons by microiontophoresis, and the neurons were simultaneously monitored electrophysiologically and activated by light stimuli to the retina.

Thirty-four 4- to 6-week-old kittens and four adult cats were prepared for single cell recording (*10*). Once a neuron was isolated its receptive field properties were analyzed with hand-held stimuli, and the responses were quantified by computing histograms to standardized stimuli. In control experiments we assessed the spontaneous variability of responses of 31 cells by repeated measurements for up to 60 min. In these cells the spontaneous variations of response amplitudes did not exceed ±40% of the mean. In agreement with previous investigations (*11*) we never observed spontaneous changes in ocular dominance, orientation preference, or direction selectivity.

For induction of long-term modifications we applied the drugs in synchrony with the light-induced cell discharge ("conditioning") (*12*). To induce changes in ocular dominance we conditioned light responses from the eye that was initially less effective in driving the cortical cell. For changes in orientation or direction preference we stimulated the more effective eye but conditioned responses to stimuli that had an orientation and direction of motion different from those preferred by the neuron. These suboptimal stimuli were adjusted to elicit a clear-cut response even in the absence of drug application.

The neuromodulators (ACh and NE) and EAAs were first applied together to maximize the chance of obtaining use-dependent modifications. In later experiments the neuromodulators and the EAAs were applied separately. Ejection currents were adjusted to produce a clear enhancement of the light-evoked response. At the end of the conditioning period the response properties of the cells were reassessed in the absence of pharmacological stimulation. A change in response was accepted as significant and causally related to conditioning when (i) the modification was selective for responses to particular stimulus configurations and hence could not be attributed to global changes of excitability, (ii) the change in response amplitude exceeded ±80% of the mean baseline value determined prior to conditioning, and (iii) the modification outlasted conditioning by at least 5 min. Of 48 cells selected for conditioning, 26 cells (54%) fulfilled these criteria. Typically, the changes in response properties consisted of an enhancement of the responses to the conditioned

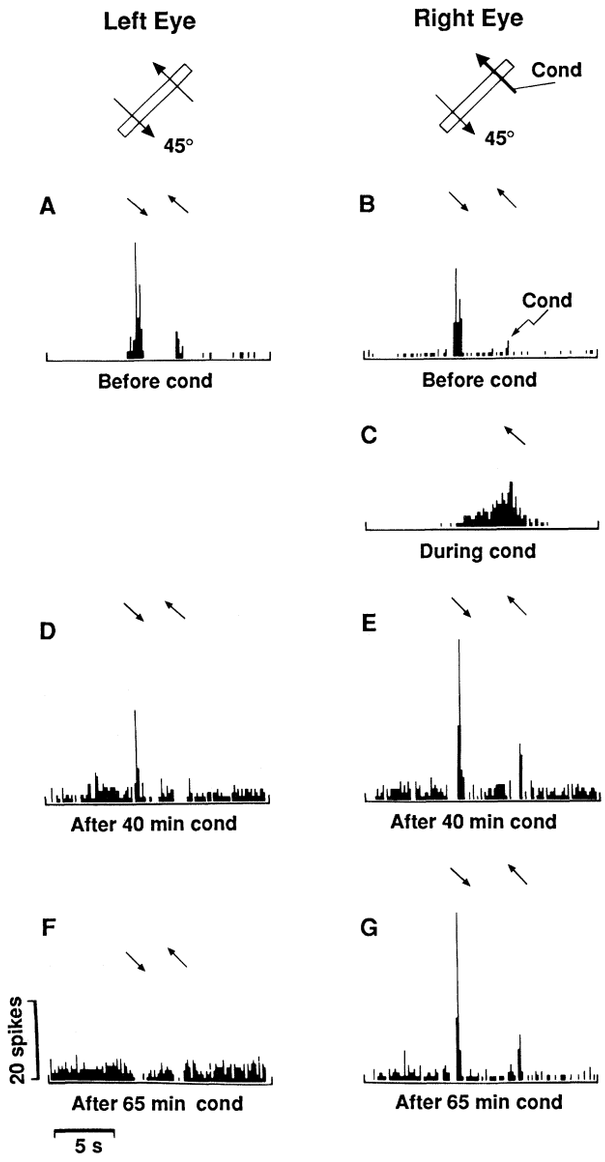

Fig. 1. Modifications of ocular dominance in a direction-selective complex cell due to the reduction of nonconditioned responses. The cell was located at a depth of 1360 μm, preferred a downward-moving oblique (45°) light bar, and responded well to stimulation of both eyes prior to conditioning [compare response histograms (**A**) and (**B**)]. The schemes above the histograms indicate the orientations of the applied visual stimuli and the arrows indicate their direction of movement. The arrows above the histograms indicate the time and the direction of the stimulus passage over the receptive field. The response that was subsequently conditioned was elicited through the right eye by an upward-moving, optimally oriented light stimulus [thick arrow in the scheme above (B)], and is indicated by "Cond" in histogram (B). For conditioning, the stimulus was presented every 20 s, whereby a shutter occluded the stimulus while it moved in the preferred direction. For pharmacological stimulation, ACh (+34 nA), NE (+30 nA), NMDA (−2 nA), and GLU (−20 nA) were applied simultaneously. The onset and the duration of the iontophoretic currents were adjusted so that the drug effects were essentially confined to the visually evoked response. In this case the onset of the iontophoretic pulse preceded the visual response by 5 s and the pulse lasted 6 s. Histogram (**C**) shows the response during conditioning. (**D**) and (**E**) show responses to light stimulation alone after 40 min of conditioning and 5 min after the last conditioning cycle. (**F**) and (**G**) show responses recorded 5 min after the end of an additional conditioning period of 25 min. All histograms were compiled from responses to 10 stimulus presentations.

stimuli or a reduction of the responses to nonconditioned stimuli or both ($n = 20$). Occasionally, decreases of the conditioned responses were also observed ($n = 6$).

A representative example for modification of the ocular dominance of a binocular neuron is illustrated in Fig. 1. In this case ACh, NE, NMDA, and GLU were applied in conjunction with the response to a light stimulus that was presented to the right eye only. After a conditioning period of 40 min, the response to the conditioned stimulus showed a small enhancement (Fig. 1E), whereas the response to the same stimulus when presented to the left eye was markedly reduced (Fig. 1D). This differential effect excludes the possibility that the reduction of the nonconditioned response resulted from a nonspecific reduction in excitability. Conditioning was then continued for another 25 min. The responses elicited through the conditioned right eye showed no further enhancement (Fig. 1G). However, despite a slight increase of resting activity, the excitatory responses from the left eye were completely abolished and replaced by episodes of reduced firing (Fig. 1F).

An example of the modification of orientation preference is illustrated in Fig. 2 for a cell that before conditioning preferred horizontally oriented light stimuli. In this case NMDA and GLU were applied in conjunction with the response to an obliquely oriented, downward-moving light bar. After 30 min of conditioning, the response to the previously preferred horizontal stimulus was strongly reduced (Fig. 2C) and the response to the conditioned stimulus showed a marked increase. This enhancement was selective for the conditioned direction of movement (Fig. 2D). Thus, conditioning had altered the cell's orientation and direction selectivity. These modifications persisted throughout the 15-min control period, during which no further stimuli had been applied (Fig. 2, E and F).

In the 26 cells that showed response modifications, these modifications persisted throughout the entire postconditioning control period that lasted up to 40 min (*13*). All drug combinations were effective in promoting lasting changes in response properties (Table 1). The combined application of the neuromodulators and EAAs appeared to be more effective than application of the neuromodulators and the EAAs alone, but this trend was not significant ($P > 0.05$, χ^2). Simple cells appeared to be more susceptible to modifications than complex cells; changes that met our criteria occurred in 19 of 27 simple cells (70%), but only in 7 of 21 complex cells (33%). Modifications of orientation preference were observed in 15 of 25 neurons (60%), modifications of ocular dominance in 9 out of 19 cells (47%), and modifications of direction selectivity in only 12 of 32 cells (37.5%).

To test whether the observed response modifications were due to the contiguity of visual and pharmacological stimulation, we examined the effects of visual ($n = 18$) and pharmacological stimulation ($n = 16$) alone. The visual or pharmacological stimuli were applied repeatedly once every 20 s for 30 min, but we never observed any consistent response modification. We also applied visual and pharmacological stimuli in alternation at intervals of 8 to 12 s with the visual ($n = 7$) or the pharmacological stimulus ($n = 7$), preceding, respectively. This separation proved sufficient to abolish direct effects of the drugs on the light-evoked

Table 1. Effectiveness of the applied agents in promoting modifications of response properties. Most attempts to condition cells with neuromodulators were made with ACh ($n = 17$) alone because it was more often excitatory than NE (*24*). ACh promoted a persistent change in response properties in eight cases and was ineffective in nine cells. In the remaining two experiments, ACh and NE were applied together. In most cells conditioned with EAAs, we ejected NMDA and GLU simultaneously ($n = 10$), because with NMDA alone the margin between facilitatory and cytotoxic doses was very narrow. Combined application allowed the usage of lower doses of NMDA and produced persistent response modifications in six of ten neurons. In the remaining three cells, NMDA was used alone. A response modification occurred in one cell. The row "Neuromodulators and EAAs" contains data from all experiments where at least one neuromodulator and one EAA were used together for conditioning. The following combinations were applied: ACh, NE, NMDA, and GLU (nine cells, changes in six neurons); ACh, NMDA, and GLU (two cells, change in one neuron); ACh, NE, and NMDA (three cells, changes in two neurons); and ACh and GLU (two cells, change in one neuron).

Agent	Modification of response properties	No change of response properties
Neuromodulators	9 (47%)	10 (53%)
EAAs	7 (54%)	6 (46%)
Neuromodulators and EAAs	10 (62%)	6 (38%)

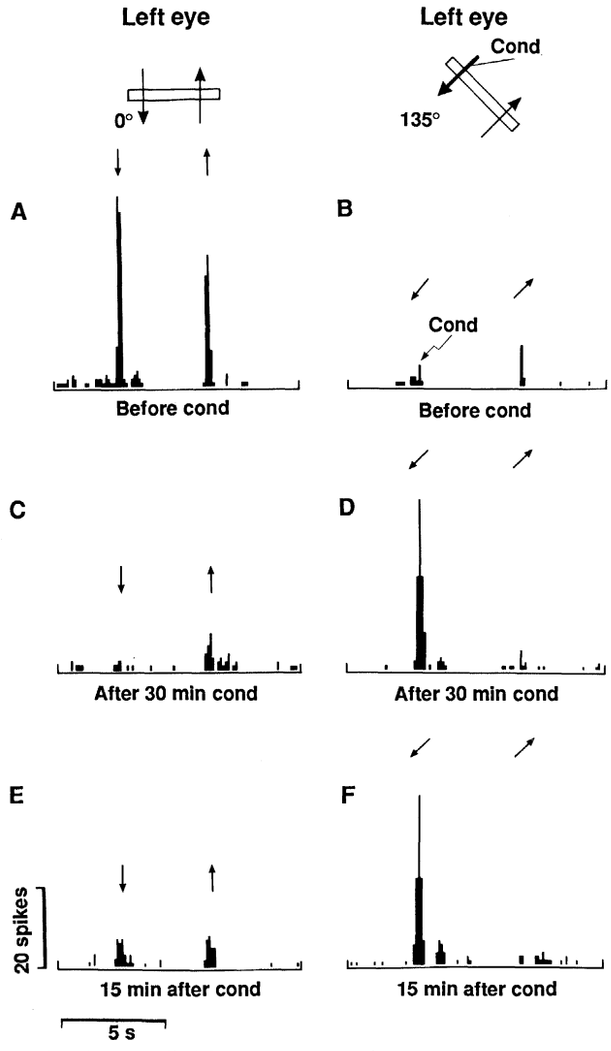

Fig. 2. Modification of the orientation preference of a simple cell as a result of enhancement of the conditioned and suppression of the nonconditioned responses. This cell was located at a depth of 500 μm in the supragranular layers. It was only excitable from the left eye and had a clear preference for horizontally oriented light stimuli [compare (A) with (B)] and a weak preference for downward movement (A). For conditioning, NMDA (−10 nA) and GLU (−20 nA) were applied while the cell was stimulated with an obliquely oriented (45° off the optimal, horizontal orientation), downward-moving light bar. The conditioned stimulus and the respective response are marked with "Cond." The current pulse started 4 s before the onset of the light response and lasted 5 s. This procedure led to an enhancement of the light-evoked response (not shown). (**A**) and (**B**) control responses before conditioning; (**C**) to (**F**) responses recorded 5 min (C and D) and 15 min (E and F) after the end of conditioning that had been applied for 30 min. For other conventions see Fig. 1.

responses. This "pseudo conditioning" procedure also failed to induce modifications of response properties.

To test whether response plasticity was related to the developmental stage of the animals, we applied the same conditioning procedure to four adult cats. We studied a total of 21 cells, 9 with simple and 12 with complex receptive fields. We conditioned the light responses either with combined application of the neuromodulators and EAAs (n = 12) or with the EAAs (n = 5) or ACh (n = 4) alone. Only two neurons, one complex and one simple cell, changed their response properties during conditioning (*14*).

Our results show that long-lasting modifications of receptive-field properties of striate cortex neurons can be induced in less than 1 hour in anesthetized and paralyzed cats. These modifications resemble the vision-dependent changes that occur in alert animals, both with respect to the nature of the changes and the age-dependent decline of malleability (*1*). This suggests that our conditioning procedure activated the same adaptive mechanisms as does natural experience. Thus, our results confirm that the neuromodulators ACh and NE and the ligands of the NMDA receptor promote experience-dependent, long-term modifications of neuron response properties in the visual cortex (*15*). The neuromodulators and EAAs were more effective when applied in combination, suggesting synergy between the actions of the two classes of neurotransmitters. This agrees with recent evidence obtained in the cat somatosensory cortex (*16*). The pseudoconditioning experiments further indicate that the applied drugs must be present during light-induced activity in order to be effective. Since visual stimulation alone induced action potentials but did not alter the receptive fields in a long-lasting way, the process leading to use-dependent modifications apparently has a threshold that differs from that of Na^+-dependent action potentials (*2, 3*). In analogy to results from in vitro studies on use-dependent synaptic plasticity, this threshold could be related to the activation threshold of NMDA- or voltage-dependent Ca^{2+} conductances (*17*). In fact, all four drugs could facilitate response modifications by increasing the probability that retinal signals reach the activation threshold of Ca^{2+} conductances (*18*). This interpretation is compatible with the recently published result that response modifications similar to those described here can be obtained when retinal signals are paired with direct activation of visual cortex neurons by positive currents and potassium extrusion from the recording pipette (*19*). However, synergistic interactions between retinal and nonretinal signals could also occur at the level of the numerous second messengers that are triggered by NMDA, GLU, ACh, and NE.

In the majority of the successfully conditioned neurons (77%), the response modifications occurred as predicted by the Hebb (*20*) and Stent (*21*) rules for synapse modification and as expected from experience-dependent changes in receptive fields (*22*). In general, inputs that were activated in synchrony with the facilitated responses of the postsynaptic cell were strengthened and nonactivated inputs were weakened. However, in six cells the activated inputs were weakened. This could reflect an anti-Hebbian mechanism at the recorded cell (*23*) but could also result from Hebbian modifications if these comprised polysynaptic pathways and changed the balance between excitatory and inhibitory influences.

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10. Anesthesia was induced with sodium hexobarbital (5 mg per kilogram of body weight, intraperitoneally) and xylazine hydrochloride (10 mg per kilogram of body weight) (5%) intramuscularly. Anesthesia was maintained during recording either with nitrous oxide (70% N_2O, 30% O_2) and intravenous infusion of pentobarbital (1.8 mg per kilogram of body weight per hour, n = 14) or with nitrous oxide and 0.2 to 0.4% halothane (n = 18). Paralysis was assured in both cases with intravenous infusion of 0.7 mg per kilogram of body weight per hour of hexacarbacholinbromide. Single unit activity was recorded with potassium citrate (1.5M)–filled micropipettes. For further details see (*3*).
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12. Because of procedural similarities with classical conditioning the term "conditioning" is now also used by neuroscientists [F. Morell, T. J. Hoeppner, L. de Toledo-Morell, *Exp. Neurol.* **80**, 111 (1983); Y. Frégnac, S. Thorpe, D. Shulz, E. Bienenstock, *Soc. Neurosci. Abstr.* **10**, 1078 (1984)]. Classical behavioral and cellular conditioning involve repeated pairing of two independent stimuli leading to a change in the respective behavioral or cellular response to the conditioned stimulus. Typically, combined visual and iontophoretic stimuli were repeated at intervals of 20 s for at least 30 min, amounting to a minimum of approximately 100 pairings. The drugs were applied through a seven-barreled micropipette that was glued to the recording electrode. The latter protruded from the phoresis barrels by 50 μm to minimize current effects on the soma of the recorded neuron and to allow for a more even distribution of drugs throughout the cortex in which the cell's processes ramify. One of the phoresis barrels was filled with saline and compensated the applied retention and ejection currents so that the net current flow between the iontophoresis barrels and the tissue was zero. This barrel could also be switched to a noncompensated phoresis mode to control for direct current effects. The drug concentrations, pHs, and the ejection and retention currents were, respectively, ACh (2M, pH 4.5), +20 to +50 nA (ejection) and −8 to −10 nA (retention); NE (0.2M, pH 3.8), +20 to +70 nA (ejection) and −8 to −10 nA (retention); NMDA (50 mM, pH 8), −2 to −18 nA (ejection) and +12 nA (retention); GLU (0.1M, pH 8.5), −5 to −20 nA (ejection) and +10 nA (retention). For drug ejection, currents were applied for 3 to 6 s.
13. The postconditioning control periods always lasted for as long as we could record from the neurons. Of the 26 cells modified, 9 were followed for 15 min, 2 for 30 min, and 1 for 40 min. For the remaining cells the control period lasted between 5 and 15 min.
14. In the complex cell, which was conditioned with ACh, NE, NMDA, and GLU, the response to the conditioned stimulus was enhanced and the response to the optimal stimulus was reduced after conditioning. In the simple cell, which was conditioned with ACh only, we observed a selective decrease of the conditioned response, which recovered completely 10 min after the end of the conditioning.
15. We consider it unlikely that the response modifications were due to the iontophoresis currents rather than to the applied drugs for the following reasons. (i) Because of the compensation circuit, the net current flow between the phoresis barrels and the brain tissue was zero (see T. W. Stone, *Microiontophoresis and Pressure Ejection* (Wiley, New York, 1985), vol. 8, pp. 27–28. (ii) Application of noncompensated currents to the saline-filled control barrel had no effect on cell activity. (iii) Currents of opposite polarity were used for the ejection of ACh-NE and NMDA-GLU, respectively, but both regimes were effective, and when applied together appeared to have synergistic rather than antagonistic effects.
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