Blockade of Cholinergic Receptors in Rat Barrel Cortex Prevents Long-Term Changes in the Evoked Potential During Sensory Preconditioning

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Maalouf, M., A. A. Miasnikov, and R. W. Dykes. Blockade of cholinergic receptors in rat barrel cortex prevents long-term changes in the evoked potential during sensory preconditioning. J. Neurophysiol. 80: 529–545, 1998. We offer evidence that acetylcholine (ACh) is involved in the emergence of functional neuronal plasticity induced by whisker pairing. Evoked potentials were recorded within the barrel cortex of awake, adult rats before, during, and after one of five paradigms. In the pairing procedure, each of 50 deflections of a whisker (S1) was followed 150 ms later by the deflection of a second whisker (S2). The explicitly unpaired control procedure differed by the lack of contiguity and contingency between the stimulation of S1 and S2. In the three remaining groups, pairing was performed 30 min after an intraperitoneal injection of either 0.5 ml of saline (150 mM NaCl), 100 mg/kg of atropine methyl nitrate (0.5 ml of AMN in saline), or 100 mg/kg of atropine sulfate (0.5 ml of ATS in saline). Changes in responsiveness to S1 were compared with, and adjusted by, changes in responsiveness to stimulation of S2. Changes in potentials evoked by S1 were interpreted as a change in neuronal excitability occurring when the first innocuous stimulus systematically predicted the appearance of the second innocuous stimulus. When whisker pairing was performed alone or in the presence of either saline or AMN (a blocker of muscarinic cholinoreceptors that does not cross the blood-brain barrier, BBB), responses to S1 increased, whereas, in the presence of ATS (blocker of muscarinic cholinoreceptors that does cross the BBB) or following the explicitly unpaired control, they decreased. The effects of saline, AMN, and ATS on the evoked potential without vibrissae pairing were opposite to those observed when these substances were injected and pairing occurred. Analysis of the behavioral state of the animal showed that the changes observed in the evoked potential could not be attributed to changes in behavioral state. The changes in responsiveness to S1 induced by whisker pairing were independent of neuronal excitability, did not occur in the absence of contingency and contiguity between S1 and S2, were blocked by the muscarinic receptor antagonist ATS, but not by blockade of muscarinic modulation of normal synaptic transmission. Thus activation of muscarinic cholinoreceptors within the CNS were a necessary condition for this form of neuronal plasticity.

INTRODUCTION

As a result of increased longevity, the number of people affected by Alzheimer’s disease (AD) has been increasing steadily, provoking massive efforts to understand its neurobiological basis and to find effective treatments for this disease. AD is characterized by a slowly progressing deterioration of higher brain functions, including learning and memory. Early postmortem studies of patients with AD revealed that the nucleus basalis of Meynert (NBM), an important structure for learning and memory, displays characteristic histopathologic lesions associated with extensive cell loss.

The NBM is the major source of cortical acetylcholine (ACh) (Johnston et al. 1981; Mesulam 1989). Several lines of evidence support an important role for ACh in learning and, more generally, in neuronal plasticity: the levels of ACh increase in various regions of the cerebral cortex (CCx) during learning (Butt et al. 1997); ACh can modulate neuronal excitability and enhance the responsiveness of cortical neurons to afferent stimuli for periods of time that outlast its presence (Kotlyar and Ovcharenko 1978; Metherate et al. 1987, 1988a,b); changes in the firing of suspected NBM cholinergic neurons has been reported during learning (Pirch et al. 1991; Richardson and DeLong 1991; Wilson and Rolls 1990a,b); cholinergic antagonists or lesions of the NBM prevent or, at least, delay the acquisition of various learned tasks (Butt and Hodge 1995; Jacobs and Juliano 1995); the success of cholinergic pharmacotherapies (the best developed approach being the use of AChE inhibitors) in treating cognitive deficits has been very limited (see Enz et al. 1993; Giacobini 1993); and the cholinergic hypothesis of learning and memory dysfunction in AD does not take into account the lesions reported in various other regions equally important for learning and memory, most importantly the CCx, a major source of afferents to the NBM and, at the same time, its main target (Esiri 1989; Hof and Morrissoson 1994).
The aim of the present study is to examine the nature of the interaction between ACh and neuronal plasticity in the somatosensory CCx and thereby its significance for learning and memory. We describe experiments involving whisker pairing, an associative learning paradigm consisting of repeatedly pairing two mystacial vibrissae that is believed to induce plastic changes within the vibrissae representation of the primary somatosensory “barrel” cortex (Maalouf et al. 1998). Evoked potentials were collected from several barrels simultaneously before, during, and after awake, but restrained, adult rats were subjected to whisker pairing in the absence of or after intraperitoneal injection of either saline, the muscarinic antagonist atropine sulfate (ATS) or atropine methyl nitrate (AMN), an analogue of ATS that does not cross the blood-brain barrier. Our results suggest that systemic administration of ATS prevents the emergence of synaptic plasticity within barrel cortex during whisker pairing.

METHODS

Surgical preparation of animal to be studied awake

All procedures were approved by the local committee on animal care; they respected the standards established by the Canadian Council of Animal Care. Adult male Sprague-Dawley rats (350–550 g; 471.3 ± 57.4 g, mean ± SD), housed individually with food and water ad libitum, were anesthetized with intraperitoneal injections of 35 mg/kg pentobarbital sodium (65 mg/ml in an aqueous propylene glycol base with 2% benzyl alcohol; Somnotol, MTC Pharmaceuticals, Cambridge, Ontario, Canada). Supplemented were added as required to maintain areflexia until the end of the surgery. An antibiotic was administered systemically (enrofloxacin 50 mg/ml in N-butyl alcohol and water, 0.05 ml im; Baytril, Chemargo, Etobicoke, Ontario, Canada) and the eyes of the animal were covered with baby oil (Johnson and Johnson). After the injection of a local anesthetic (0.3 ml of lidocaine hydrochloride 20 mg/ml; Xylocaine, Astra Pharmaceuticals, Mississauga, Ontario, Canada), a central portion of the scalp was excised and the temporal muscles reflected. Two teflon-insulated stainless-steel wires were placed over the cervical muscles to monitor the electromyogram (EMG). Three stainless steel screws to which teflon-insulated stainless steel wires were soldered were inserted in the skull. The first two were used to record the electro-oculogram (EOG; +5 to +6 mm anterior to the bregma, at the right edge of the skull) and electroencephalogram (EEG; over the right parietal lobe: 2.5 mm posterior to the bregma and 2 mm lateral to the midline) respectively. The third one (over the left orbital sinus: +5 to +6 mm anterior to the bregma and 2 mm lateral to the midline) served as a reference. Eight additional stainless steel screws were inserted posteriorly to lambda and on the lateral sides of the skull to help anchor the attachments.

A craniotomy was performed over the left hemisphere from +3 to −3 mm, anteroposterior (AP) and 2 to 5 mm mediolateral (ML) to the midline, and a small portion of the dura was removed. Two arrays of 4, 25 μm-diameter, formvar-insulated nichrome wires (A-M Systems., Everett, WA) were inserted successively into the left hemisphere between −2 and −3.5, AP, and 3 and 4.5, ML, with vertical and horizontal angles of 35–45°. The tips of the electrodes (1–2 MΩ impedance at 1 kHz) were separated by ≤0.5 mm. During the penetration, multunit activity from all four electrodes (connected together) was monitored continuously. The insertion of the electrode assembly was terminated when neurons responding to the deflection of one or several vibrissae were encountered. The array was then stabilized with dental acrylic cement. After insertion of the second four-electrode assembly, the exposed brain was covered with low-melting-point wax and more dental acrylic was used to cover the exposed skull and attachments. Two parallel stainless steel tubes secured by two shorter tubes were imbedded in the dental acrylic. This rectangular arrangement of tubes was fixed to the recording apparatus where it served to stabilize the head and restrain the animal during the experiment (Maalouf et al. 1998).

Electrophysiology and experimental protocols

Animals were allowed 72 h to recover from surgery before being placed in the recording apparatus for one to four practice sessions of ±1–2 h to adapt them to restraint. Before the first experiment, multunit activity from each electrode was briefly recorded (filter set at 300–3,000 Hz) to identify the principal whisker that drove the neurons sampled by that electrode. Evoked potentials were filtered (band-pass filter set at 1.6–70 Hz) and amplified at 10,000–30,000 with a Neurofax electroencephalograph (5–15 μV/ mm Nihon Kodhen, Tokyo). Filtering at band-pass of 1–100 Hz and an amplification of 5,000 (AM 502 & TM 504. Tektronix, Beaverton, OR) was used for the processing of the EEG, EMG, and EOG signals. All signals were digitized with a CED 1401 plus laboratory computer interface (Cambridge Electronic Design, Cambridge, UK) at a resolution of 2,000 samples/s (sp/s) for evoked potentials and at 500 sps for the EEG, EMG, and EOG.

At the beginning of the experiment, two whiskers were selected. The one stimulated first during pairing (S1) was always rostral to the other (S2). In addition, S2 had to be the principal whisker of one of the barrels that we could record from. Each stimulus consisted of a discrete, 5-ms–long, 3-mm horizontal deflection of the whisker. The stimulating device driving each of the whiskers consisted of a miniature speaker with one end a light-weight tube of dried grass glued to the membrane. The stimulated whisker was inserted into other end of the tube and stayed there for the duration of the experiment. S1 and S2 were each stimulated separately during 20 trials to establish baseline responses (Fig. 1, A and B). After that, the animal was injected intraperitoneally, while still in the recording box, with 0.5 ml of either 150 mM NaCl (saline), the solution of ATS (100 mg/kg; Research Biochemicals International, Natick, MA) in saline, or the solution of AMN (100 mg/kg; Sigma, Saint-Louis, MO) in saline. Thirty minutes later, a new baseline was established by stimulating first S1, then S2, during 20 trials each (Fig. 1, C and D). Whisker pairing then was performed 50 times, the interstimulus interval between S1 and S2 being 150 ms (Fig. 1E). After that, 20 deflections of S2 followed by 20 deflections of S2 were presented again (Fig. 1, G and H). This final set of independent stimuli for S1 and S2 allowed evaluation of changes in the responses of each whisker that might have been brought about by their pairing. The intertrial interval varied constantly in all sections of the protocol between four preset values (5, 7, 9, or 11 s) to minimize a possible conditioning to time (Delacour and Houcine 1987).

In six preliminary experiments involving whisker pairing alone as well as in the explicitly unpaired controls, recording of the first baseline for each whisker was omitted and the animals did not receive any injections. In unpaired controls, the interstimulus intervals were 2.5, 3.5, 4.5, or 5.5 s (half the intertrial intervals used during in the pairing procedures) and each whisker could be stimulated up to five times in a row (Fig. 1F).

Cortical responses to whisker stimulation

To calculate the averaged response over several presentations, we selected and combined only evoked potentials with similar
shapes. The pattern that occurred most frequently was chosen for analysis; evoked potentials contaminated by artifacts related to movements of the animal were eliminated. On average, 53.7 ± 2.1% (mean ± SE) of the recorded potentials were used. There were no statistically significant differences among the various treatment groups in terms of number of evoked potentials chosen for analysis (1-way analysis of variance (ANOVA) and Kruskal-Wallis ANOVA on ranks; P < 0.05) nor was there any correlation (linear regression analysis P > 0.05) between the values obtained for each group and the percentage of potentials eliminated.

Evoked potentials from up to seven different electrodes could be recorded while simultaneously monitoring the EEG, EMG, and EOG. Deflection of a single whisker could elicit evoked potentials in several barrels. Our observations suggested that the more distant was the whisker from the whisker giving the largest response (principal).

FIG. 1. Experimental paradigms. A and B: initial measures of responsiveness; whisker stimulation consisted of 10 or 20 single, 5-ms-long horizontal deflections of the whisker S1 (A) followed by the same number of deflections of whisker S2 (B). Stimuli were separated by intervals of 5, 7, 9, or 11 s arranged in pseudorandom sequences (C and D) drug tests; whisker stimulation in patterns identical to those shown on A and B but performed 30 min after the intraperitoneal injection of either saline, atropine sulfate (ATS), or atropine methyl nitrate (AMN). E: pairing; during pairing, stimulation of S1 preceded the stimulation of S2 by 150 ms but pairings still occurred after a pseudorandom interval of 5, 7, 9, or 11 s. F: explicitly unpaired controls; concomitant stimulation of S1 and S2. This paradigm differed from pairing by the lack of a structured temporal relationship between stimulation of S1 and S2. In these experiments, the stimulations of one whisker or the other occurred every 2.5, 3.5, 4.5, or 5.5 s, but deflection of the same whisker could not occur more than 5 times in a row. G and H: final measure of responsiveness; repeated whisker stimulation, identical to the one shown in A and B used to obtain a measure of the effects of pairing.
TABLE 1. Percentages of evoked potentials used in each treatment group

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<tr>
<th>Group</th>
<th>Evoked Potentials, %</th>
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<tr>
<td>Pairing</td>
<td>52.3 ± 14.3</td>
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<tr>
<td>Pairing + saline</td>
<td>53.8 ± 13.3</td>
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<tr>
<td>Pairing + AMN</td>
<td>50.2 ± 8.3</td>
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<tr>
<td>Pairing + ATS</td>
<td>61.6 ± 20.4</td>
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<tr>
<td>Control</td>
<td>50.4 ± 6.8</td>
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Values are means ± SD. Averaging across all groups, the percentage of evoked potentials used was 53.7 ± 2.1 (Mean ± SEM) of all the recordings. The treatment groups did not differ statistically from each other based on the one-way analysis of variance (P = 0.51) and Kruskal-Wallis analysis of variance on ranks (P = 0.85).

TABLE 2. Number, time schedule, and design of experiments performed in each animal

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Thirty-four experiments were conducted in nine different animals. The number of experiments per animal ranged from one to nine, the average being 3.8 ± 2.3 (Mean ± SD). * C, control; P, pairing; S, saline; ATS, atropine sulfate; AMN, atropine methyl nitrate.
between the values in each bin of the spectral distribution histogram (see Norusis 1990). The EOG was used as an indicator of rapid eye movement (REM) sleep.

Data analysis
To quantify the effects of the various experimental protocols, two different approaches were used. In the first one, the percentage of change in the amplitude of the first component of evoked potentials was calculated for S1 and S2. Then the measures obtained for S2 were subtracted from those of S1

$$\Delta A = 100(\frac{R^{S1}_2 - R^{S1}_1}{R^{S1}_1}) - 100(\frac{R^{S2}_2 - R^{S2}_1}{R^{S2}_1})$$

where $R^{S1}_1$ and $R^{S2}_1$ were the amplitude of responses to stimulation of whiskers S1 and S2, respectively, before ($R^1$) and after ($R^2$) the pairing. The adjusted percentages of change in the responses to S1

![Fig. 2. Changes attributable to pairing in the responses recorded from barrel for which S2 is the principal whisker. Five waveforms before (A and B), during (C), and after (D and E) pairing are illustrated. Amplitude of the 1st component (at the moment in time when the absolute minimum occurred) of the potentials evoked by stimulation of S1 decreased by 5.7% after pairing and by 27.1% for S2. Subtracting the change in responses to stimulation of S2 from those of S1 yielded a 21.4% increase in the relative size of the responses to S1 (see also Fig. 4A). ▼, time of whisker deflection.](http://jn.physiology.org/)

![Fig. 3. Explicitly unpaired control procedure. After repeated, unpaired whisker deflections (▼), the responses to stimulation of S1 recorded in the barrel for which S2 is the principal whisker, decreased by 1.5%. However, after adjustment for the 10.1% increase seen in the responses to S2, responses to stimulation of S1 relative to the changes in those of S2, decreased by 11.6% (see also Fig. 4B). Five traces are shown for each experimental condition.](http://jn.physiology.org/)
approach was based on evidence in the literature suggesting that this conditioning paradigm changes the relationship between responses to S1 and S2. As well, these relative measures minimized any generalized change in excitability that affected all areas of somatosensory cortex.

The effects of saline or drug administration without pairing were evaluated by comparing the amplitude of the first component of the evoked potentials measured 30 min after the drug injection with the first components collected before the injection with t-test and Mann-Whitney rank-sum test ($P < 0.05$).

Using a cluster analysis, recordings having markedly different evoked potential profiles, when compared with the overall sample, also were excluded. These criteria, albeit stringent, excluding ~50% of the potentials, prevented the analysis of waveforms contaminated by artifacts and spontaneous fluctuations of the EEG.

![Figure 5](https://example.com/fig5.png)

**FIG. 5.** Effects of pairing after the intraperitoneal injection of saline. These responses were recorded from a barrel for which S2 is the principal whisker. Responses to stimulation of S1 decreased by 7.7% and responses to stimulation of S2 decreased by 21.4%. Adjusted difference represents a 13.7% increase (see also Fig. 7A). Saline was injected 30 min before the 1st recording of responses to S1 shown on the graph. \( \triangledown \), moments of stimulation; only 5 sample traces are shown for each experimental situation. (Table 1). The identical approach was used for all treatment groups and both one-way ANOVA ($P = 0.51$) and Kruskal-Wallis ANOVA on ranks ($P = 0.85$) revealed no statistically significant difference in our exclusion procedures among groups.

**Histology**

At the end of the last experiment, an electric current (10 \( \mu \)A for 15 s) was passed through three or four of the electrodes to mark the recording sites. The animal then was anesthetized deeply with an intraperitoneal dose of pentobarbital sodium. The thoracic cage was opened, and the animal was perfused through the aorta with
500 ml of phosphate-buffered saline (pH 7.3) followed by 500 ml of phosphate-buffered 4–10% solution of paraformaldehyde. The brain was removed from the skull and placed in 0.8 M sucrose for ≥48 h. Frontal sections were cut with a cryotome at a thickness of 50 μm and mounted on chrom-alum coated slides for staining with cresyl violet.

RESULTS

The results were obtained from 34 experiments performed on nine rats. The number of experiments per animal ranged from one to nine, the average being 3.8 ± 2.3 (mean ± SD; Table 2). The explicitly unpaired control procedure was applied in 6 of the 34 cases. The remaining ones involved whisker pairing either without any injection or after the intraperitoneal administration of saline, AMN or ATS (Table 3).

Three animals were subjected to more than one type of protocol to verify whether the effects of different treatments could be reproduced in the same animal. The first animal was exposed to two sessions of explicitly unpaired whisker stimulation followed by pairing and then a control session. The second animal was subjected to three control sessions followed by three pairing experiments with injections of saline. The third one received nine pairing sessions, the first five and the seventh in the presence of ATS and the remaining ones with the injection of saline. In total, 18 experiments were performed in rats that were subjected to more than one type of treatment. However, a rat was never subjected to more than one treatment per day. The group where pairing was performed after injection of AMN was the only one where rats were only exposed to a single procedure.

FIG. 6. Effects of saline injection without pairing. Responses to stimulation of S1 recorded from a barrel for which S2 is the principal whisker after intraperitoneal injection of saline increased by 6.8%. The responses to stimulation of S2 increased by 37.3%. The difference was a 30.5% decrease (see also Fig. 7B), without a dramatic change in the shape of the potential. ▼, moments of stimulation; only 5 sample traces are shown for each experimental condition.

FIG. 7. Saline injection with (A) and without (B) pairing. First 2 columns: average, normalized response to stimulation of S1 and S2 before and after pairing. Difference for S1 (ΔS1) and S2 (ΔS2) were negative, and the net effect (ΔS1 − ΔS2) was +13.7%, whereas without pairing the net effect was −30.5%. Pattern of changes in general reproduces the one, shown in Fig. 4, A and B, indicating that injection of a neutral agent does not affect the results of pairing. A and B: Site M91.4.
Different whisker combinations were used, however, S1 always was rostral to S2. On average, S1 and S2 were separated by 1.5 ± 0.6 columns and 1.0 ± 0.6 rows. In Euclidian coordinates, the distance between them was 1.9 ± 0.6 whiskers.

**Effects of whisker pairing**

In six experiments, whisker pairing was performed during 50 trials where the stimulation of S1 preceded the stimulation of S2 by 150 ms.

*ADJUSTED PERCENTAGES OF CHANGE IN THE RESPONSES TO S1.* On average, the responses to S1 and S2 decreased by 5.9 ± 7.4 and 16.1 ± 8.9% (mean ± SE), respectively, after pairing. Figures 2 and 4A illustrate the situation where responses to S2 decline more than those to S1 (4 of the 6 cases). In one of the remaining cases, responses to S1 dropped more than S2, whereas in the other, responses to stimulation of S1 and S2 both climbed, however, the increase was larger for responses to S1.

Because the change in the responses to S2 was taken as a reference, it was subtracted from the change in the responses to S1, and the residual change was used to estimate the effect of the experimental manipulation. These differences were averaged across all experiments within a group. The result was positive, suggesting that whisker pairing produced an increase in the amplitude of S1 with respect to S2 (10.3 ± 4.9%). This was also true after injection of either saline (5 experiments showing a 14.5 ± 6.4% average increase; Figs. 5 and 6A; Fig. 7 shows responses with and without pairing) or AMN (5 experiments showing a 14.3 ± 6.1% average increase; Table 4 and Figs. 8 and 10A; see also Fig. 9). With the t-test ($P < 0.05$), the means of the three groups did not differ from each other, but each was significantly higher than the decrease (6 experiments; −7.2 ± 5.0%) observed in the explicitly unpaired controls (Figs. 3 and 4B). The results of the Mann-Whitney rank-sum test confirmed this outcome; pairing alone, pairing after saline injections and pairing after AMN injections were significantly different from the explicitly unpaired controls. The results are summarized in Fig. 14 and Tables 4 and 5.

**TABLE 3.** *Number of experiments performed in each treatment group and the number of rats they were obtained from*

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Experiments</th>
<th>Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recordings from S2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pairing</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Pairing + saline</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Pairing + AMN</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Pairing + ATS</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Recordings from S1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pairing</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Recordings from barrels serving unstimulated whiskers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pairing</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Control</td>
<td>13</td>
<td>2</td>
</tr>
</tbody>
</table>

AMN, atropine methyl nitrate; ATS, atropine sulfate.

**FIG. 8.** Responses observed in the barrel for which S2 is the principal whisker before (A and B) and after (D and F) pairing (C) in the presence of an intraperitoneal injection of AMN 30 min before the pairing. Responses to stimulation of S1 increased by 9.3%, whereas those to S2 decreased by 8.1%, leading to a corrected increase of 17.4% in the responses to stimulation of S1 (see also Fig. 10A). ▼, moments of stimulation; 5 traces are shown for each panel.

When available, data collected from barrels neighboring the barrel for which S2 was the principal whisker (i.e., S1 or other, unstimulated whiskers) showed the same tendency seen in barrels related to S2; pairing increased responses to stimulation of S1 relative to S2, whereas control procedure provoked their decrease (Table 6). However, these differences were smaller and were statistically significant only when the data from all the experiments in the barrels for which S1 was the principal whisker and other unstimulated whiskers were combined.

**DIFFERENCE OF RATIOS ($\Delta R$).** The ratio of the response to S1 over the response to S2 ($R_{S1}/R_{S2}$) increased after
whisker pairing alone (0.14 ± 0.05; mean ± SE) or after the intraperitoneal injection of saline (0.12 ± 0.06) or AMN (0.10 ± 0.04), whereas the ratio decreased after the explicitly unpaired control procedure (−0.05 ± 0.04). The differences between the pairing and control groups were statistically significant with both the t-test and Mann-Whitney rank-sum test (P < 0.05; Tables 4 and 5).

Applying the preceding analyses to data collected from barrels for which S1 is the principal whisker or other, unstimulated whiskers yielded results similar to those obtained for the barrels for which S2 was the principal whisker. The \((R_1):(R_2)\) ratio increased after pairing and decreased after the explicitly unpaired control (Table 6), however, the pairing and control groups differed significantly from one another only when the data collected from all of these off-focus regions was combined, again suggesting that the effects of pairing were smaller in the barrels for which S2 was not the principal.

**Effects of saline, AMN and ATS on the responses to whisker stimulation before pairing**

Observations of the waveforms recorded during the experiments suggested that the injected drugs alone affected the amplitude of the evoked potential. This was most evident with ATS. To evaluate these effects, evoked potentials were recorded 30 min after the injection of saline (Figs. 5 and 7B), AMN (Figs. 9 and 10B) or ATS (Figs. 12 and 13B; see also Fig. 11), and the amplitudes of the first component were normalized relative to the baseline established at the beginning of the experiment. In all three groups, the potentials generated by stimulation of both S1 and S2 were greater after the injections but only ATS caused a significant increase. However, the changes attrib-
TABLE 4. Summary of the results from recordings in the cortex for which S2 is the principal whisker for each experimental condition

| Procedure   | To S1 | To S2 | To S2 Adjusted for S1 | Percentage Change | Ratio of S1 Over S2 | Ratio of S1 Over S2
|--------------|-------|-------|-----------------------|-------------------|-------------------|-------------------|
| Pairing      | $-5.9 \pm 7.4$ | $-16.1 \pm 8.9$ | $10.3 \pm 5.0$ | Before: $0.87 \pm 0.11$ | After: $1.01 \pm 0.14$ | Difference: $0.14 \pm 0.05$
| Pairing + saline | $6.3 \pm 8.7$ | $-8.2 \pm 10.9$ | $14.5 \pm 6.4$ | $0.73 \pm 0.06$ | $0.85 \pm 0.05$ | $0.12 \pm 0.06$
| Pairing + AMN | $20.7 \pm 12.6$ | $6.4 \pm 7.9$ | $14.3 \pm 6.1$ | $0.83 \pm 0.04$ | $0.93 \pm 0.05$ | $0.10 \pm 0.04$
| Pairing + ATS | $3.8 \pm 8.5$ | $12.9 \pm 9.1$ | $-9.1 \pm 8.2$ | $0.74 \pm 0.06$ | $0.70 \pm 0.10$ | $-0.04 \pm 0.08$
| Control      | $-5.1 \pm 8.2$ | $2.1 \pm 4.2$ | $-7.2 \pm 5.0$ | $0.71 \pm 0.05$ | $0.66 \pm 0.07$ | $-0.05 \pm 0.04$

Values are means ± SE.

TABLE 5. Statistical evaluation of the significant differences between the various experimental conditions

<table>
<thead>
<tr>
<th>Procedure</th>
<th>$t$</th>
<th>$P$</th>
<th>$t$</th>
<th>$P$</th>
<th>$t$</th>
<th>$P$</th>
<th>$t$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pairing</td>
<td>0.52</td>
<td>0.61</td>
<td>0.57</td>
<td>0.58</td>
<td>1.91</td>
<td>0.07</td>
<td>2.49</td>
<td>0.03*</td>
</tr>
<tr>
<td>Pairing + saline</td>
<td>0.27</td>
<td>0.80</td>
<td>0.02</td>
<td>0.98</td>
<td>2.16</td>
<td>0.04*</td>
<td>2.69</td>
<td>0.03*</td>
</tr>
<tr>
<td>Pairing + AMN</td>
<td>0.23</td>
<td>0.83</td>
<td>1.63</td>
<td>0.12</td>
<td>2.18</td>
<td>0.04*</td>
<td>2.75</td>
<td>0.02*</td>
</tr>
<tr>
<td>Pairing + ATS</td>
<td>2.18</td>
<td>0.04*</td>
<td>1.51</td>
<td>0.15</td>
<td>3.15</td>
<td>0.01*</td>
<td>0.14</td>
<td>0.89</td>
</tr>
<tr>
<td>Control</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.16</td>
<td>0.88</td>
</tr>
</tbody>
</table>

Evaluation applied to the adjusted percentages of change in the responses to S1 ($t$-test, 1st line; Mann-Whitney rank-sum test where available, 2nd line) and to the difference of ($R_{s1}$):($R_{s2}$) ratios ($t$-test, 3rd line) in the barrels for which S2 is the principal whisker. * $P < 0.05$.
plotted in 100-dimensional space, >90% of the trials were shown to have similar frequency distributions; <10% differed by 1 or 2 orders of magnitude. When the distances were ordered and plotted on a semilogarithmic scale of distance versus rank-order, they formed a smooth, gradually increasing progression until near the end when there was a sharp increase to much larger values. The minority of trials during the experiment that contributed to these larger values were interpreted as cases where the animal had been in a different behavioral state.

Figure 15 shows the analysis from one experiment. The distances on the left are small relative to one another suggesting that they arose from cases where the power spectra were nearly identical. In the long midportion of the graph, the distances were approximately of the same length and thus formed a group having similar characteristics. By contrast, the rapidly rising arm on the right side of the figure represents distances that are 1–2 orders of magnitude larger than the majority. Based on this analysis, we chose to exclude all trials where the power spectra differed from the majority by a factor of ≈10. Because we know that during the majority of the data collection period the animals were alert but resting, the minority of cases when the animal was in some other state (perhaps excessively active) should not seriously alter our conclusions, and exclude the possibility that our results could be attributable to an effect of changes in behavioral state.

**Histology**

Recording sites (n = 61) were identified in frontal sections of eight animals. These were located in or near layer IV; 34% were actually in layer IV and almost equal numbers were obtained from layers III (23%) and V (20%). Fewer data were obtained from layers II (11%) and VI (12%). This laminar distribution is close to the one reported by Maalouf et al. (1998) with single-unit recordings, suggesting that our data are derived from a similar neuronal populations at similar depths to the neurons studied by single-unit methods.

**Discussion**

Our experimental procedure offers several advantages: 1) whisker pairing, a behaviorally relevant sensory conditioning paradigm (see Carvell and Simmons 1996; Hutson and Mastroton 1986; Kossut 1992), is combined with electrophysiological recordings, histological identification of the laminar distribution of recording sites and the use of pharmacological agents that allowed us to distinguish the effects of a muscarinic antagonist on the CNS from those on the peripheral nervous system; 2) evoked potentials reflect a summation of membrane currents from a large population of neurons many being below the threshold necessary to generate action potentials and therefore providing information that cannot be not be revealed by recordings of action potentials (see Martin 1991); 3) the behavioral state of the animal, as revealed by the EEG, EMG, and EOG, can be correlated with the probability of emergence of plasticity (see Cruikshank and Weinberger 1996).

The data described in this paper confirm with a different method, the conclusion of previous single-unit studies by Maalouf et al. (1998) that whisker pairing increases neuronal responsiveness to stimulation of S1 relative to S2 in the barrel for which S2 is the principal whisker. In addition, we report that similar changes in nearby barrels were smaller, implying a localization of the plastic changes to the cortex for which S2 is the principal whisker, and that the cortical plasticity emerging after whisker pairing depends on the action of ACh on muscarinic receptors in the CNS.

**Cortical responses to whisker stimulation**

The deflection of a single whisker provokes a neuronal response in the barrel cortex of awaken or lightly anaesthetized rats consisting of three phases: an initial, prompt excitation, followed by inhibition, and then a longer lasting excitation (Ebner and Armstrong-James 1990). In more deeply anaesthetized animals, the third component does not appear, and the response is frequently limited to a single excitatory phase, although the inhibitory phase has been recorded intracellularly (Carvell and Simons 1988). Similar patterns were observed recently with imaging techniques that rely on voltage-sensitive dyes (Kleinfeld and Delaney 1996).

The origins of the late components (≈500 ms) of the evoked potential remain relatively unknown. Several proposed mechanisms hypothesize a role for the multiple reciprocal connections that sensory areas form with various corti-
Effects of whisker pairing on cortical responses to whisker stimulation

Our recordings in the cortex for which S2 is the principal whisker clearly show that whisker pairing, either alone or after systemic injection of saline or AMN, enhanced the difference in responses to stimulation of S1 relative to S2 whereas the explicitly unpaired control procedure decreased it. After pairing (Fig. 4), reactivity to the second signal in the pair declined (see also Diamond and Weinberger 1989; Lennartz and Weinberger 1992; Rescorla 1988; Simons 1983, 1985). This change can be interpreted as a neural representation of the fact that the second stimulus would follow the first one; the sensitivity to the second (harmless) stimulus was adjusted accordingly. On the other hand, when the first signal did not predict the appearance of the second

FIG. 11. Effects of pairing after the intraperitoneal injection of ATS. ATS was applied 30 min before pairing of S1 and S2. Waveforms of potentials evoked by S1 and S2 before are shown in A and B, respectively. Two whiskers were paired 50 times (C). After pairing, the amplitude of the responses evoked by S1 decreased by 6.5% and the responses to S2 increased by 10.4% (D and E, respectively). Average, corrected response (by subtracting changes in S2 from those in S1) was a 16.9% decrease (see also Fig. 13A). ▼, moments of stimulation; 5 sample traces are shown for each panel.

FIG. 12. Changes in the responses recorded from a barrel for which S2 is the principal whisker before (A and B) and after (C and D) intraperitoneal injection of ATS. Amplitude of the 1st component of the potentials evoked by stimulation of S1 and S2 increased by 18.6 and 25.0%, respectively. Corrected response to stimulation of S1 was a 6.4% decrease (see also Fig. 13B). ▼, moments of stimulation; 5 sample traces are shown for each panel.
could be related to the degree of arousal caused by the procedure itself.

These results confirm previous findings by Maalouf et al. (1998), who recorded single-unit activity in the barrel cortex of awake rats and found that responses to stimulation of S1, when averaged across several neurons, were enhanced by whisker pairing and were reduced after unpaired presentations of stimuli to S1 and S2. Siucinska and Kossut (1996) reached similar conclusions about classical conditioning in barrel cortex in the barrel cortex of mice from a study involving desoxyglucose; classical conditioning caused an enlargement of the metabolically active region serving as the conditioned stimulus (CS). Bakin and Weinberger (1990) described similar changes in neuronal responses from the auditory cortex of guinea pigs undergoing classical conditioning with a tone serving as a CS.

Maalouf et al. (1998) suggested that the plasticity induced in barrel cortex by whisker pairing obeys the covariance rule originally described by Hebb (1949). The covariance rule can be divided into two parts: when presynaptic activation is synchronized repeatedly with increased levels of postsynaptic activation, the synapses linking the two elements are potentiated and when there is no correlation between pre- and postsynaptic activities, synaptic efficacy is reduced (see Cruikshank and Weinberger 1996). The results described in this study consolidate the hypothesis of Maalouf et al. (1998). During pairing, the interstimulus interval was 150 ms so the stimulus to S2 was presented when the activity of the recorded neurons was still clearly enhanced by the previous stimulation of S1 (Fig. 2C). This was not the case in the explicitly unpaired controls.

The conditions set by the rules of covariance were met in several regions of barrel cortex during pairing since deactivations of S1 and S2 both activated neurons in several barrels. Responses to stimulation of S1 were enhanced significantly in Fig. 13. ATS injection with (A) and without (B) pairing. First two columns: average normalized response to stimulation of S1 and S2 before and after treatment. Net difference after pairing was −16.9%, whereas without pairing that difference was −6.4%. Pattern of changes in responses to both whiskers during pairing (top) in general reproduces the one shown in Fig. 4B when the stimuli were applied randomly. A and B: Site M94.1.

TABLE 7. Effects of saline, AMN and ATS on the responses to whisker stimulation recorded from the barrels for which S2 is the principal whisker

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Number of experiments</th>
<th>Percentage of change in the responses to S1</th>
<th>Percentage of change in the responses to S2</th>
<th>Adjusted change in the responses to S1</th>
<th>Before</th>
<th>After</th>
<th>Difference of ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>5</td>
<td>11.2 ± 6.3</td>
<td>22.7 ± 9.8</td>
<td>−11.6 ± 10.9</td>
<td>0.78 ± 0.05</td>
<td>0.73 ± 0.06</td>
<td>−0.05 ± 0.08</td>
</tr>
<tr>
<td>AMN</td>
<td>5</td>
<td>7.8 ± 16.5</td>
<td>25.2 ± 17.8</td>
<td>−17.4 ± 11.1</td>
<td>0.94 ± 0.03</td>
<td>0.83 ± 0.04</td>
<td>−0.11 ± 0.07</td>
</tr>
<tr>
<td>ATS</td>
<td>12</td>
<td>68.1 ± 21.8</td>
<td>51.2 ± 16.0</td>
<td>16.9 ± 13.1</td>
<td>0.66 ± 0.04</td>
<td>0.74 ± 0.06</td>
<td>0.08 ± 0.06</td>
</tr>
</tbody>
</table>

Values are means ± SE.
the muscarinic antagonist ATS but not AMN, an analogue that does not cross the blood-brain barrier. In spite of the greater variability in the responses observed after ATS injection, it was evident that the cortex no longer adjusted to the predictive value of S1 followed by S2. When the blocker of muscarinic cholinoreceptors could not cross the blood-brain barrier, the responsiveness to the second (harmless) stimulus was still capable of being adjusted, offering support of the idea that ACh plays a role in cortical neuronal plasticity (Dykes 1997).

In the absence of pairing, the effects of ATS on responses to stimulation of S1 relative to S2 were not significantly different from those of a control condition (saline injection) and from AMN. However, because these changes in the opposite direction were significant, it suggests that cholinergic mechanisms are not the only mechanism of plasticity in sensory cortex. Based on these data, we suggest that ACh is necessary for the plastic changes induced by associative learning at the cortical level and that this function is independent from its influence on normal synaptic transmission. However, even though these results confirm a conclusion derived from the previous work on the importance of ACh for learning (see INTRODUCTION), they do not solve the controversy of whether ACh affects learning and memory directly or through an increase in attention that might have been provoked by the paired stimuli. This question has been raised by several studies that showed that enhanced cortical cholinergic release was correlated with sensory stimulation or increased attentional processing (Inglis and Fibiger 1995; Sarter and Bruno 1997) as well as with learning (Butt et al. 1997).

Our data are not incompatible with any of these views, and we propose that ACh is involved in both attentional processing of sensory stimuli and in the mechanisms of plasticity underlying learning (see Dykes 1991, 1997). First of all, ACh has been shown, on the one hand, to take part in the generation of sensory-evoked potentials, particularly the late components (Meador 1995; Sannita 1995) and, on the other hand, to be necessary for working memory in the prefrontal cortex (Granon and Poucet 1995) and the hippocampal formation (Myers et al. 1996). Hasselmo and his colleague (Hasselmo 1995; Hasselmo and Bower 1993) have suggested that ACh shifts hippocampal circuits toward a state that is appropriate for acquiring new inputs and prevents stored information from interfering with learning by selectively depressing transmission in intrinsic fibers while not affecting responses evoked by afferent fiber stimulation. Interestingly, Inglis and Fibiger (1995) found that ACh re-
lease in the prefrontal cortex and the hippocampus is increased during sensory stimulation, and Butt et al. (1997) showed a further increase during tactile learning.

ACh most probably accomplishes these functions through its interactions with postsynaptic muscarinic receptors. It can decrease neuronal adaptation and lengthen the duration of action potentials in pyramidal cells by decreasing several potassium (K⁺) outward currents, including the voltage-dependent nonactivating Iₐ, the fast-inactivating Iₐk, and the calcium (Ca²⁺)-activated K⁺ current responsible for slow afterhyperpolarization (Bernardo and Prince 1982; Madison et al. 1987; McCormick 1993). Moreover, it activates a voltage-dependent cation nonselective inward current (Haj-Dahmane and Andrade 1996) and has been shown to reduce inhibition by enhancing K⁺ outward currents in GABAergic interneurons (see McCormick 1993). In contrast on the presynaptic side, ACh decreases the amplitude of synaptic potentials, presumably by activating inhibitory receptors on glutamatergic presynaptic terminals (Hasselmo 1995; Hasselmo and Barkai 1995; Hasselmo and Bower 1993). This would explain why cortical responses to whisker deflection are enhanced in the presence of atropine, whereas those evoked by stimulation of the hindpaw a few milliseconds after electrical stimulation of the basal forebrain are reduced (Verdier et al. 1995).

These same mechanisms that decrease neuronal adaptation and increase action potential duration also can facilitate long-term potentiation (LTP), a form of plasticity believed by many to be activated by learning and to underlie memory storage. Because action potentials last longer under the influence of ACh, the probability and duration of opening of N-methyl-D-aspartate (NMDA) channels and probably voltage-dependent Ca²⁺ channels are increased, leading in turn to the entry of larger amounts of Ca²⁺ into the postsynaptic cell (Brocher et al. 1992; Segal 1992). This rise in cytoplasmic Ca²⁺ also may be achieved by muscarinic potentiation of phosphoinositide turnover, resulting in the synthesis of inositol triphosphate, which subsequently enhances the release of Ca²⁺ from intracellular stores (Jones 1993). The rise in intracellular Ca²⁺ concentration ultimately would lead to the activation of the cascade of second-messenger events leading to synaptic plasticity. Consistent with this model are the findings of Verdier et al. (1995), who demonstrated that pairing electrical activation of the basal forebrain with hindpaw stimulation in anesthetized rats leads to a long-term enhancement (over an hour) of cortical responsiveness to the tactile stimulus and that this enhancement requires the activation of NMDA receptors. In addition, the data presented in this paper indicate that the muscarinic antagonist atropine is sufficient to block an NMDA-dependent form of cortical plasticity (Maalouf et al. 1998).

**Tenable hypotheses**

Thus ACh might facilitate the emergence of plasticity in the CCx at two levels: by enhancement of working memory via extension of a period of time during which external stimuli provoke their neural representation within the cerebral cortex and by directly affecting plasticity through the facilitation of the opening of postsynaptic NMDA channels and increasing the intracellular levels of Ca²⁺ that promote induction of LTP. Because a cholinergic deficit would prevent the facilitation of these two processes, it might be at these levels that the absence of ACh has an important role in production of cognitive impairments that characterize Alzheimer’s disease. Treatment directed to correction of cholinergic malfunction, however, should take into account the transient temporal needs for ACh as well as the fact that degeneration also occurs in other neuromodulatory systems involved in learning and memory.

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