Role of the Basal Forebrain Cholinergic Projection in Somatosensory Cortical Plasticity

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Sachdev, Robert N. S., Shao-Ming Lu, Ron G. Wiley, and Ford F. Ebner. Role of the basal forebrain cholinergic projection in somatosensory cortical plasticity. J. Neurophysiol. 79: 3216–3228, 1998. Trimming all but two whiskers in adult rats produces a predictable change in cortical cell-evoked responses characterized by increased responsiveness to the two intact whiskers and decreased responsiveness to the trimmed whiskers. This type of synaptic plasticity in rat somatic sensory cortex, called “whisker-pairing plasticity,” first appears in cells above and below the layer IV barrels. These are also the cortical layers that receive the densest cholinergic inputs from the nucleus basalis. The present study assesses whether the cholinergic inputs to cortex have a role in regulating whisker-pairing plasticity. To do this, cholinergic basal forebrain fibers were eliminated using an immunotoxin specific for these fibers. A monoclonal antibody to the low-affinity nerve growth factor receptor 192 IgG, conjugated to the cytotoxin saporin, was injected into cortex to eliminate cholinergic fibers in the barrel field. The immunotoxin reduces acetylcholine esterase (AChE)-positive fibers in S1 cortex by >90% by 3 wk after injection. Sham-depleted animals in which either saporin alone or saporin unconjugated to 192 IgG is injected into the cortex produces no decrease in AChE-positive fibers in cortex. Sham-depleted animals show the expected plasticity in barrel column neurons. In contrast, no plasticity develops in the ACh-depleted, 7-day whisker-paired animals. These results support the conclusion that the basal forebrain cholinergic projection to cortex is an important facilitator of synaptic plasticity in mature cortex.

INTRODUCTION

To test the hypothesis that cholinergic inputs to cortex influence plasticity in the mature sensory neocortex, we designed experiments to eliminate the cholinergic inputs before inducing activity-dependent plasticity. The paradigm used the anatomically parceled whisker representation in the somatic sensory (S-I) cortex of rats that is characterized by clusters of neurons in layer IV, known as barrels, one for each large contralateral facial whisker (Woolsey and Van der Loos 1970). Neurons in each barrel column respond at the highest magnitude and shortest latency to deflections of a principal whisker, which constitutes the center receptive field (CRF) for barrel neurons (Armstrong-James and Fox 1987). The same neurons respond with fewer spikes and at a latency >10 ms to 2–10 additional whiskers, which together constitute the surround receptive field (SRF).

A simple method for modifying the responses of neurons in barrel field cortex of adult rats is to trim some, but not all, whiskers close to the rat’s face. Whiskers that have been trimmed are less likely to be stimulated during movements of the vibrissal pad (Vincent 1912; Welker 1964), and consequently a bias in activity levels is created in the whisker-to-barrel-cortex pathway. Activity arising from the trimmed whiskers is reduced, whereas activity from the intact whiskers continues unabated. The whisker trimming results in predictable and reproducible changes in cortical cell response properties. The resulting changes in cortical cell responses constitute a robust example of plasticity in the mature somatosensory cortex called “whisker-pairing plasticity” (Armstrong-James et al. 1994; Diamond et al. 1993).

In the present experiments, the D2 whisker was always left intact along with one adjacent D-row whisker (either D1 or D3), and recording was always from cells anatomically localized to the D2 barrel column in cortex. There are two main effects of pairing whiskers for >3 days. One effect is that cells in the D2 barrel column increase their response to test stimuli applied to the principal D2 whisker. The other is that the intact or “paired” D-row surround whisker responses are elevated. This enhancement of paired whisker evoked responses is followed by a decrease in response stimulation of all the trimmed surround whiskers. The difference in response to the two adjacent spared D-row whiskers creates a bias (i.e., changes the normal 1:1 ratio of the intact-to-trimmed surround D-row whisker response; this creates the “S/D-row bias”) that is greatest after 1–2 wk but persists for months. Diamond, Huang, and Ebner (1994) established that the earliest S/D-row bias occurs in layers II/III and V before any changes in the layer IV cells. This result suggests that whisker pairing induces intracortical synaptic modifications before it modifies activity in the cortical cells that receive the majority of the specific thalamic inputs.

Results from a number of previous studies suggest that acetylcholine (ACh) plays a role in modifying cortical receptive fields. For example, plasticity induced by sensory preconditioning of whiskers is prevented by the microiontophoresis of atropine, a muscarinic antagonist, in barrel cortex (Delacour et al. 1990). ACh iontophoresis enhances the response level of cortical neurons to somatic stimulation and increases their receptive field size (Bassant et al. 1990; Dykes and Lamour 1988; Metherate et al. 1988a,b). ACh released by direct stimulation of the nucleus basalis (NB) produces long-lasting facilitation of cortical cell responses (Tremblay et al. 1990a). Finally, NB stimulation enhances the responses evoked by whisker stimulation in some, but not all, cortical neurons (Howard and Simons 1994).

The vast majority of cholinergic fibers to the somatosensory...
sory cortex arise from the NB (Eckenstein et al. 1988; McKinney et al. 1983; Mesulam et al. 1983). Fibers immunoreactive for the ACh synthetic enzyme, choline acetyltransferase (ChAT) or stained for the ACh degradative enzyme, acetylcholine esterase (AChE) can be detected in all layers of the rat parietal cortex, but the innervation to laminae I–III and V is particularly dense (Houser et al. 1985; Kristy 1979a, b; Lysakowski et al. 1989; Umbriaco et al. 1994). Given that cholinergic fibers are dense in the supragranular layers, where the earliest changes are induced by whisker pairing, a reasonable hypothesis to test is that the destruction of the basal forebrain cholinergic projection fibers to the somatosensory cortex would diminish or eliminate the intracortical components of whisker pairing plasticity. To test this hypothesis, 192 IgG conjugated to saporin, an “immunotoxin” specific for cholinergic basal forebrain neurons, was injected directly into the cortex before whisker pairing and recording. All brains were examined for the extent of the cholinergic depletion. A preliminary account of this work has been published (Sachdev et al. 1995).

METHODS

Experimental subjects were adult male Long-Evans rats, 2-mo old, weighing 275–325 g at the initiation of the experiments. Animals were housed with one to two litter mates.

Experimental groups

The experiments were designed to determine the effect of ACh depletion on cortical receptive field properties in barrel field cortex. To quantify the depletion, a few sections containing the barrelfield were taken from every animal and stained for AChE fibers. Subsequently the number of AChE fibers remaining in the barrelfield were counted. In one experimental group (n = 5), the immunotoxin was injected into the left cortex (Fig. 1A) and 21 days after the injection receptive fields were characterized (depletion alone). In a second experimental group (n = 6 animals), the immunotoxin was injected into the left cortex, and 14 days later whiskers were trimmed on the right side of the face for 7 days (depletion with whisker pairing; Fig. 1B). At the end of the 7 days of whisker pairing, during which the animals could use only their paired whiskers instead of their normal contingent of ≈25 whiskers, the responses of D2 barrel column cells were analyzed under urethan anesthesia (Fig. 1C).

The control groups consisted of sham-depleted animals. The sham depletion was produced by injection of saporin unconjugated to 192 IgG antibody before whisker pairing (saporin + IgG) (n = 2) or by injection of saporin alone (n = 2). Finally, comparable experimental and control groups of injected animals (n = 6) were used for anatomic analysis to characterize the effect of the toxin and extent of the depletion in coronal sections. All animals were examined 21 days after the injection procedure.

ACh depletion

The barrel cortex was depleted of its cholinergic inputs by injection of 192 IgG conjugated to saporin. The 192 IgG, a monoclonal antibody to the low-affinity nerve growth factor receptor, binds an extracellular epitope of the receptor on the basal forebrain cholinergic fibers in cortex and is internalized. When the conjugate is internalized by cells that have the low-affinity receptor on their terminals, it leads to cell death. In the present study, 62 ng of the immunotoxin in phosphate buffer was injected directly into the cortex ≈2 mm medial to the barrel field. Previous studies have delivered this dose into the ventricle where it results in the death of the cholinergic neurons in the NB, the medial septal nucleus, and diagonal band (Heckers et al. 1994; Wiley et al. 1991). To visualize where the toxin was delivered into cortex, a minute amount of Chicago sky blue was dissolved in the vehicle.

Animals were anesthetized with pentobarbital sodium (Nembutal, 50 mg/kg) and placed in a stereotaxic frame. A small 1.5 × 1.5 mm opening was made in the skull, 1.0 mm lateral to and 1.0 mm caudal to Bregma. A 31-gauge shallow-beveled hypo-tube (Small Parts) connected via polyethylene tubing (A-M Systems) to a Hamilton syringe was used to deliver the toxin. The delivery system first was filled with mineral oil, then the toxin was drawn into the delivery tube with negative pressure. The hypo-tube was pushed through the dura and ≈1.5 mm below the surface of cortex. One microliter of the toxin was injected slowly during 15–20 min,
and the delivery tube was left in place for 10 min after the injection. Once the tube was removed, the wound edge was sutured, the animal was placed under a heat lamp and allowed to recover before being placed back with one other animal.

**Sham depletion**

Using the methods described in the preceding text sham depletions were made by injecting either the cytotoxin, 7.5 ng of saporin (1.5 mg/ml, molecular weight 55K), mixed with an equal volume of 192 IgG in vehicle, or saporin alone (7.5 ng, 1 μl).

**AChE fiber counting**

To document the amount of depletion, we used a method similar to that reported by Stichel and Singer (1987). They counted the number of fibers crossing a grid in the ocular of a microscope; we counted the number of fibers crossing a grid on a video screen. Sections cut coronally or tangential to the cortical surface were placed on a Leitz microscope stage (Leica, Aristoplan) and captured at ×40 on a video screen. Using a BioQuant Measurement-OS/2 software, a grid that subtends 180 μm across and 130 μm from top to bottom (subdivided into 10 × 10 μm squares) is laid over the image. Fibers crossing the grid-lines could be selected manually and subsequently counted using the BioQuant system.

Statistical analysis of the number of fibers in the barrel and septa were performed using counts from two D-row barrels in each animal. The χ² test was used to assess the significance of the difference in the number of fibers in the barrel and septa of sham-depleted animals. The χ² test also was used to assess the difference in the number of fibers in the sham- and ACh-depleted animals. In depleted cases, no distinction could be made between barrel and septa because no AChE staining pattern remained so the mean number of fibers in the barrelfield of the ACh- and sham-depleted animals was compared.

**Whisker trimming**

The experiments required an examination of cortical cell responses in the D2 barrel column following either a bias in activity created by trimming one but not the other D-row whisker next to D2 or no whisker trimming. Whiskers on the left side of the face remained intact in all cases, whereas on the right side of the face all but two whiskers were clipped to the level of the fur for periods of 7 days (whisker pairing). In these experiments, the principal D2 whisker was always left intact and paired with one in-row surround whisker, either D1 or D3. Either whisker—D1 or D3—left intact is called the “D-paired” whisker. The in-row surround whisker which is cut is called the “D-cut” whisker.

These animals constituted the whisker-paired group. For the duration of whisker pairing the whiskers were cut every 2 days. On the day of the experiment, all whiskers were trimmed to the same length, ~3–5 mm from the skin so that they could be stimulated in an equivalent way.

**Preparation for physiology**

Rats were anesthetized with urethan (1.5 g/kg ip, 30% wt/vol in distilled water). Body temperature was maintained at 37°C, with the aid of a feedback regulated heating pad. An opening was made in the skull to expose the postero medial barrel cortex in S1 on the left side. A small incision was made in the dura through which the electrode entered the cortex. To find the D2 barrel column, penetrations were made into the layer four barrels until cells were found that responded to D2 whisker stimulation with a latency of <10 ms. During recording, the depth of anesthesia was maintained at a constant level by supplementing the animal with one-tenth of the original dose when necessary, as determined by the rate of spindling and burst discharges. Animals in this study were maintained at around two to three bursts per second as monitored by listening to the audio monitor and by measuring the burst frequency on a Gould digital storage oscilloscope.

**Whisker stimulation**

Individual whiskers were deflected repeatedly by a wire glued to one end of a piezoelectric wafer that was controlled by a digital stimulator. Using a surgical microscope, the wire was positioned just below a whisker without touching it. The whisker was deflected 200 μm upward, with a rise and fall time of 0.5 ms and a duration of 3 ms. Fifty stimuli were presented at 1 Hz to whisker D2 and to each of its immediate surround whisker neighbors (D1, D3, C2, and E2) to generate post-stimulus time and latency histograms.

**Recording and data analysis**

Carbon fiber microelectrodes (Armstrong-James and Millar 1979) were used to record action potentials. Electrodes were advanced through the cortex using a stepping hydraulic microdrive (Kopf Instruments) at an angle perpendicular to the pial surface, so that the cortical laminae of the same column would be encountered sequentially. D2 whisker stimulation was required to give the signature best response with a latency <10 ms (in the barrel) during each penetration.

Spontaneously active units were isolated by the use of a time-amplitude window discriminator (Bak Electronics). The accepted action potential wave-forms were displayed on a digital storage oscilloscope ( Nicolet ), permitting examination of each accepted action potential for the duration of the stimulation. A Cambridge Electronics Design 1401 plus processor and BMES 486 computer were used to generate on-line peristimulus time histograms (PSTHs), raster plots, and latency histograms at 1 ms resolution. Data were collected 50 ms before onset of stimulus and for 100 ms after the offset of the stimulus. The period of data collection before the stimulus was used to estimate spontaneous activity. All raw data on timing of action potentials and delivery of stimuli was stored on a hard disk for offline analysis.

The magnitude of response to whisker stimulation was first examined in latency and PSTHs. During the recording session, the principal whisker ( always D2 for this study ) was determined by the quality of each neuron’s response to whisker stimulation. Stimulation of a barrel neuron’s principal whisker elicits a short-latency (5–10 ms) response and produces a larger number of spikes than the number evoked by stimulation of any other whisker. Latency histograms were constructed by taking the first spike occurring after 3 ms poststimulus during each of the trials. The bin containing the most spikes defined the modal latency. For purposes of obtaining a mean modal latency for each whisker, units that did not respond to the stimulation of a whisker were excluded from the mean modal latency calculation.

PSTHs at 1-ms bin resolution also were constructed for each unit. For all animals in an experimental group, PSTHs to D2 stimulation were summed to graph the average PSTH across all cells in a barrel column. Average spontaneous activity was subtracted from each poststimulus time bin.

Nonparametric statistical analysis of data were carried out by applying the Mann Whitney U test (MWU) for independent samples and Wilcoxon matched-pairs signed-rank test (WMPSR) for related samples.

**Identification of recording sites**

We required that all cells included in this study be located within the cortical D2 barrel column. At the time of recording, all we
ACh DEPENDENT PLASTICITY IN BARREL CORTEX

Phosphate buffer solution. Once the brains sank, the cortical mantle was peeled off and flattened between glass slides. Frozen tangential sections were cut at 30 μm on a sliding microtome and processed for CO activity (Wong-Riley and Welt 1980) and AChE histochemistry. CO-stained sections were used to identify barrels and to determine the location of each track, whereas the AChE sections were used to assay the degree of the depletion. Sections were left in the CO staining solution (60–90 mg cytochrome-c/100 ml of 0.1 M phosphate buffer, 30 mg 3,3'-diaminobenzidine, and 4% sucrose) until barrels were delineated clearly in tangential sections. The time necessary to obtain this staining varied from 6 to 16 h and depended on the quality of perfusion and fixation and the time of sectioning relative to the perfusion.

AChE histochemistry

The procedures used to localize AChE-positive fibers were developed by Koelle (1955), modified by Jacobowitz and Creed (1983), and described earlier in detail in Clinton and Ebner (1987). Free floating sections from both the depleted hemisphere and the control hemisphere were run through the staining procedures at the same time. Sections were preincubated for 30 min at 38°C in 24% sodium sulfate and 1.25 μM tetraisopropylpyrophosphoramide (iso-OMPA), to suppress pseudocholinesterase staining. The sections then were transferred into the incubation solution, which contained (in mM) 4 acetylthiocholine iodide as a substrate, 2 copper sulfate, and 80 magnesium chloride plus 0.94 μM iso-OMPA in 24% sodium sulfate (pH 6.0). After 2 h of incubation, the tissue was washed at room temperature in 20% then 10% solutions of sodium sulfate for 5 min and 1 min, respectively. While the sections were in a water rinse for 1 or 2 min, the substrate binding solution consisting of 4% ammonium sulfide in phosphate buffer (pH 6.0) was prepared. Sections were developed in this solution for several minutes, then rinsed in distilled water, and the stain was fixed in 10% formalin solution. Sections were left overnight in fix at 4°C, mounted on subbed slides, dried, and toned in 0.2% gold chloride. After another water rinse, tissue was placed in a 5% sodium thiosulfate solution for 5 min, rinsed, dehydrated in an alcohol series, cleared in Hemo-D, and coverslipped.

FIG. 2. Injection site in a cytochrome c-oxidase (CO)-stained tangential section. This photomicrograph illustrates the site of IgG 192-saporin injection, which is visible as a sphere of light staining. This necrotic zone surrounds the injection site. Arrowhead points to an electrolytic lesion made in the final recording track in the D2 barrel of this animal. Calibration bar 1 mm.

knew was that stimulating the D2 whisker produced the shortest latency responses (<10 ms in the barrel) and the largest response magnitude of any whiskers tested. Recording sites were marked by passing a DC current of 1 μA for 1 s (electrode tip negative) at the bottom of every second track during a recording session. On the last track of each recording session, three lesions were made, one in the barrel and one above and one below it (1–2 μA for 3 s at each site). The small spherical lesion produced by these methods was visible in histological cytochrome c-oxidase (CO)-stained sections, facilitating reconstruction of the recording sites (Fig. 2). The lesions were used both for establishing the location of the electrode in the barrel, and for confirming the depth of the electrode in the barrel column.

Histology

On termination of the experiment, rats were perfused transcardially with 75–125 ml of heparinized 0.1 M phosphate buffer, followed by either 4% buffered paraformaldehyde or by a buffered periodate-lysine–paraformaldehyde (2.5%) fixative (McLean and Nakane 1974). Subsequently, the brains were removed, postfixed overnight at 4°C, and placed in a 20% sucrose, 10% glycerol phosphate buffer solution. Once the brains sank, the cortical mantle was peeled off and flattened between glass slides. Frozen tangential sections were cut at 30 μm on a sliding microtome and processed for CO activity (Wong-Riley and Welt 1980) and AChE histochemistry. CO-stained sections were used to identify barrels and to determine the location of each track, whereas the AChE sections were used to assay the degree of the depletion. Sections were left in the CO staining solution (60–90 mg cytochrome-c/100 ml of 0.1 M phosphate buffer, 30 mg 3,3'-diaminobenzidine, and 4% sucrose) until barrels were delineated clearly in tangential sections. The time necessary to obtain this staining varied from 6 to 16 h and depended on the quality of perfusion and fixation and the time of sectioning relative to the perfusion.

RESULTS

The key requirement of these experiments is the near-total depletion of cholinergic projection fibers in barrel field cortex without compromising the cholinergic innervation of other parts of the brain. We therefore begin the results with a description of the depletion before describing the physiological effects of destroying the cholinergic projections.

Site of injection

At the site of injection of either the 192 IgG conjugated to saporin or saporin alone or saporin unconjugated with 192 IgG in vehicle, a variable amount of necrosis is apparent in the CO-stained tangential sections that was medial to barrel field cortex (Fig. 2). The area of necrosis is apparent as a lightly stained region extending ~1 mm around the injection site. The damage around the injection site does not extend into the barrel field.

Normal AChE staining pattern

In normal animals, the AChE staining pattern in the somatosensory cortex is similar to that described earlier by other investigators (Eckenstein et al. 1988; Kristt 1979a,b).
In coronal sections, layers I and layer V are densely stained, layers II–III are less densely stained, and layer IV is lightly stained (Fig. 3A, Table 1). The pattern of layer IV staining results in part from the orientation of the fibers; in layer IV AChE fibers are oriented predominantly vertically toward the pial surface, whereas in the other layers, more fibers run obliquely or parallel to the pial surface (Fig. 3A).

In adjacent tangential sections CO and AChE delineate the barrels (Fig. 4, A and B). In the CO stain, the barrels stain more darkly than the septa (Fig. 4A). The adjacent AChE-stained sections (Fig. 4B) form a complementary image where the septa are more densely stained than the barrels. The difference in AChE staining density between barrels and septa can be appreciated by an examination of the sections at higher power (Fig. 5, A–C). Fiber counts confirm that the septa have 30% more fibers than do the barrels (Tables 2–4). There is some variability between animals, but the number of fiber crossings is remarkably similar from barrel to barrel in a single animal (Table 3). In every animal, the number of fibers in the septa is always greater than the number in the barrels.

**AChE staining pattern in the depleted brain**

Two to 3 wk after the injection of 192 IgG linked to saporin, the barrel field cortex is depleted of AChE-positive fibers (Fig. 3B). In coronal sections, the depletion extends through all laminae of the cortex. Except for the scattered darkly stained cortical cell bodies and their associated processes, very few fibers remain on the depleted side. The lateral border of the depleted zone varies from animal to animal, but in all cases included in the physiological analysis, the entire barrel field cortex was depleted. In tangential sections, the pattern of AChE fibers is no longer evident after depletion (Fig. 4D). The dense CO staining in the barrels remains (Fig. 4C). The toxin injections depleted the hemispheres examined for histology by an average of 93% (P < 0.0001, χ² test) in the number of AChE-stained fibers in the barrel-field compared with controls (Table 3). In the animals used for physiology, the decrease in AChE-stained fibers averaged 91% (Table 4). In the sham-depleted animals, there is no decrease in the AChE-stained fibers. In all cases a few, lightly stained cortical cell bodies remained AChE positive (Figs. 3B and 6A).

**SRF plasticity in D2 barrel column of ACh-depleted animals**

In the ACh-depleted cases without whisker trimming, the D2 barrel responses are not biased toward surround whisker (Fig. 7A): that is, no significant difference exists between the responses evoked by D1 or D3 whisker stimulation (WMPsR, P = 0.1).

Whisker pairing for 7 days usually produces a sign shift toward the intact whiskers where the intact whisker evokes a 100% better response than does the cut whisker, and where 70% of the neurons respond better to the paired whisker as opposed to the cut whisker. In the ACh-depleted, 7-day whisker-paired cases, there is also no significant bias (WMPsR, P = 0.4) toward the D-paired whisker (Fig. 7B).

**SRF plasticity in D2 barrel column of sham-depleted animals**

As expected, the sham-depleted animals show the D-paired/D-cut bias after 7 days of whisker pairing (Arm-

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**TABLE 1. AChE fiber crossings in coronal sections**

<table>
<thead>
<tr>
<th>Layer (µm)</th>
<th>Animals</th>
<th></th>
<th></th>
<th>Average*</th>
</tr>
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<tbody>
<tr>
<td>Layer I (40–90 µm)</td>
<td>344</td>
<td>243</td>
<td>386</td>
<td>324 ± 42</td>
</tr>
<tr>
<td>Layer II, III (200–350 µm)</td>
<td>692</td>
<td>617</td>
<td>641</td>
<td>650 ± 22</td>
</tr>
<tr>
<td>Layer IV (500–650 µm)</td>
<td>462</td>
<td>442</td>
<td>505</td>
<td>470 ± 19</td>
</tr>
<tr>
<td>Layer V (850–1,000 µm)</td>
<td>762</td>
<td>722</td>
<td>686</td>
<td>723 ± 22</td>
</tr>
</tbody>
</table>

Fiber counts were made at ×40 (magnification) on a 180 × 130 µm² area that was subdivided into 10-µm squares, making a grid across the section. Fibers and cortical cell bodies crossing this grid are counted. For layer one, an area of 50 × 180 µm² is examined. AChE, acetylcholine esterase. * Means ± SE.
FIG. 4. CO- and AChE-stained adjacent tangential sections. A: tangential section stained for CO from the right (control) side displaying the densely stained barrels and the lightly stained septa. B: adjacent tangential section stained for AChE. Fibers are sparser in barrels and denser in the septa. C: tangential CO-stained section from a depleted hemisphere appears normal. D: adjacent tangential section stained for AChE shows no sign of the barrel-septal pattern after the cholinergic basal forebrain fibers have been destroyed. Calibration bar, 1 mm.

strong-James et al. 1994). As shown in Fig. 7C, the whisker paired with D2 gives a 100% larger response than the D-cut whisker (WMPSR, P < 0.001). The mean modal latency for the paired whiskers was 18.0 ± 1.3 (SE) ms and for the cut whiskers was 22.0 ± 2.5 ms.

D2 whisker plasticity after whisker pairing

By definition, the D2 whisker produces the highest magnitude response in D2 barrel column cells. Whisker pairing increases the ability of the D2 whisker to drive the D2 barrel cells. D2 stimulation also evokes significantly greater number of spikes in animals that are ACh depleted and whisker paired than in animals that are just ACh depleted (MWU, P < 0.001). This result indicates that the principal whisker still can be potentiated by whisker pairing after ACh depletion. There are no significant changes in the mean modal latencies to D2 whisker stimulation: in the saporin sham-depleted whisker-paired animals, the D2 mean modal latency is 8.9 ± 0.5 ms; in the ACh-depleted animals, a mean modal latency of 8.7 ± 0.7 ms is obtained, whereas in the ACh-depleted whisker-paired animals the latency is 9.2 ± 0.3 ms.

Depth analysis

In Fig. 8, data obtained from the whisker-paired animals are plotted against depth from pial surface. At all depths, in the saporin/192 IgG sham-depleted animals (Fig. 8A), the response is substantially and uniformly larger when the paired whisker is stimulated than when the cut whisker is stimulated. In the ACh-depleted animal (Fig. 8B), the effect of stimulating the cut whisker is not consistently different from the effect of stimulating the paired whisker.

Summed PSTH

The PSTH of the D2 whisker response for each cell is summed, corrected for number of cells to make a direct comparison of the PSTHs between conditions. Figure 9 shows the summed PSTHs for the D2 whisker. Note that the decrease in the overall magnitude of the response for the ACh-depleted nonwhisker-paired animals is noticeable throughout the PSTH (compare Fig. 9, A with B) but is especially evident in the first 20 ms poststimulus.

Short-latency, intermediate, and long-latency responses

To further examine the timing characteristics of the responses, the PSTHs generated by each whisker were broken down into four latency epochs: 3–10, 10–20, 20–50, and 50–100 ms poststimulus (Fig. 9, insets). The whisker-paired ACh-depleted animals have significantly more spikes in the 10- to 20-ms bin than the depleted but unpaired animals (WMPSR, P < 0.001).

Individual case data

In some cases, the investigator is blind to the history of the animal: the sham-depleted (saporin alone) and ACh-depleted litter-mate (case 51 and 52, respectively) are prepared on the same day and are housed together during the whisker pairing period. The toxin injection in case 52 produced only a partial depletion of the barrel-field (Fig. 6B). In this case, the number of fibers increase progressively from the medial injection site toward the barrel cortex. The reduction in AChE fibers ranges from 90% at the injection site to 60% in the lateral edges of the barrel field. The summary physiology data from a single penetration through the D2 barrel column of these animals with different degrees of depletion are shown in Fig. 10. The expected D-paired/D-cut bias is present in the sham-depleted case 51. There was no bias toward the D-paired whisker in ACh-depleted cases. A trend toward a bias is present in the incompletely depleted case 52.
TABLE 2.  \textit{AChE} fiber crossings in tangential sections of normal animals

<table>
<thead>
<tr>
<th></th>
<th>Right</th>
<th></th>
<th>Left</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Barrel</td>
<td>Septa</td>
<td>Barrel</td>
</tr>
<tr>
<td>Normal 1</td>
<td>418</td>
<td>614</td>
<td>421</td>
</tr>
<tr>
<td>Normal 2</td>
<td>417</td>
<td>621</td>
<td>451</td>
</tr>
</tbody>
</table>

Fiber counts were made at \( \times 40 \) (magnification) on a \( 180 \times 130 \mu m^2 \) area that was subdivided into 10-\( \mu m \) squares, making a grid across the section. Fibers and cortical cell bodies crossing this grid are counted.

\textit{Distribution of penetrations}

In normal animals, the position of a cell within the D2 barrel with respect to its surrounding barrels affects the magnitude (and the latency) of responses elicited by each of the surround whiskers. If a cell is located in barrel D2 closer to the E2 than the C2 barrel there is a predictably larger response to stimulation of the E2 whisker.

Examination of the distribution of the penetrations within the D2 barrel provides a good estimate of whether the recording sites are skewed exclusively toward one or another side of the barrel, and especially whether the recording sites were inadvertently biased toward the D-paired or the D-cut barrel. The distribution of penetrations in the D2 barrel are displayed in a schematic format in Fig. 11 (A–C). Sections containing the electrolytic lesions were magnified, the lesions were transferred onto the schematic, and the remaining tracks not associated with an electrolytic lesion were interpolated. The recording sites for the sham-depleted animals (Fig. 11A), the ACh-depleted alone animals (Fig. 11B), and the ACh depleted with whisker pairing animals (Fig. 11C) are distributed throughout the barrel and are not biased toward any particular barrel.

\textit{Spontaneous discharge}

All units recorded in both ACh-depleted animals and sham-depleted animals had some spontaneous activity. There is no significant difference in the spontaneous discharge in any of the conditions reported here. In sham-depleted animals, the spontaneous discharge is \( 0.78 \pm 0.04 \) (SE) Hz, and in ACh-depleted animals, it is \( 0.85 \pm 0.06 \) Hz.

TABLE 3.  \textit{AChE} fiber crossings in tangential sections of injected animals

<table>
<thead>
<tr>
<th>Animal</th>
<th>Control Side (Right)</th>
<th>Injected Side (Left)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Barrel</td>
<td>Septa</td>
</tr>
<tr>
<td>Depleted 1</td>
<td>336</td>
<td>413</td>
</tr>
<tr>
<td></td>
<td>339</td>
<td>540</td>
</tr>
<tr>
<td>Depleted 2</td>
<td>543</td>
<td>749</td>
</tr>
<tr>
<td></td>
<td>521</td>
<td>724</td>
</tr>
<tr>
<td>Depleted 3</td>
<td>319</td>
<td>520</td>
</tr>
<tr>
<td></td>
<td>312</td>
<td>577</td>
</tr>
<tr>
<td>Mean*</td>
<td>395 ± 43.6</td>
<td>585.5 ± 52.6</td>
</tr>
</tbody>
</table>

* Mean ± SE.

\textbf{FIG. 5.} \textit{AChE}-stained fibers in the normal barrel field. \( A \): same section shown in Fig. 4B at higher magnification. Calibration bar, 200 \( \mu m \). \( B \): \textit{AChE}-stained fibers in barrel of the section in Fig. 4B at 4 times the magnification in \( A \). \( C \): \textit{AChE}-stained fibers in septa of section in Fig. 4B. All sections are cut at 30 \( \mu m \). At higher magnification, the differential density of \textit{AChE}-stained fibers is remains apparent. Fiber crossings over the grid within the barrel and septa shown here are were 406 and 568, respectively. On the depleted side, 72 crossings were recorded. Calibration bar for \( B \) and \( C \), 200 \( \mu m \).
TABLE 4. Physiology cases: AChE fiber crossings in tangential sections

<table>
<thead>
<tr>
<th>Control Side (Right)</th>
<th>Depleted Side (Left)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Barrel</td>
</tr>
<tr>
<td>Sham depleted</td>
<td></td>
</tr>
<tr>
<td>Saporin/IgG 192 or</td>
<td>466 ± 33</td>
</tr>
<tr>
<td>Saporin: Cases 37, 38, 57, 59</td>
<td></td>
</tr>
<tr>
<td>ACh depleted</td>
<td></td>
</tr>
<tr>
<td>Whisker paired:</td>
<td>361 ± 33</td>
</tr>
<tr>
<td>Not whisker paired:</td>
<td></td>
</tr>
<tr>
<td>Cases 20–23, 26</td>
<td></td>
</tr>
<tr>
<td>Cases 27, 34, 67, 68, 69</td>
<td>352 ± 24</td>
</tr>
</tbody>
</table>

Note that the AChE-stained sections containing the barrel field representation for cases 23, 59, 68, and 69 were not available. Note also that on the depleted side no AChE pattern remains so no attempt was made to count in just the barrel or the septa.

DISCUSSION

There are two main findings of this study: first, cholinergic fibers can be depleted from the barrel cortex by intracortical injections of IgG 192 conjugated to saporin, and second, the destruction of the basal forebrain cholinergic projections to the cortex prevents SRF response plasticity. A striking feature of the latter finding is that the CRF responses still can be enhanced. Given the density of AChE-stained and ChAT immunoreactive fibers in superficial and deep laminae, we had expected that cholinergic depletion would prevent plasticity in a depth specific manner. Instead, the data suggest that after ACh depletion, the SRF whisker pairing effect is abolished at all depths.

The abolition of SRF plasticity by ACh depletion is consistent with the results of earlier studies that examined the role of cholinergic mechanisms in cortical function and plasticity. Ocular dominance plasticity requires the activation of ACh muscarinic (M1) receptors in the kitten striate cortex (Gu and Singer 1993). Similarly, atropine iontophoresis prevents whisker related associative plasticity in the adult rat barrel cortex (Delacour et al. 1990). Another line of evidence for a cholinergic role in barrel cortex function is demonstrated by 2-deoxyglucose uptake studies in barrel cortex. ACh depletion of the barrel cortex by the injection of an excitotoxin into the basal forebrain reduces the area of evoked cortical 2-deoxyglucose uptake produced by stimulating whiskers and reduces the intensity of 2-deoxyglucose uptake within the area of activation (Jacobs et al. 1991, 1994; Juliano et al. 1991). The decrease in the dimension of 2-deoxyglucose uptake area suggests that the cortical domain around the barrel that is active in the normal animal is no longer active in the depleted animal. Our results are also consistent with the notion that the septal and the superficial cortical layers which facilitate interbarrel relays (Armstrong-James et al. 1991) are not as easily modified after ACh depletion.

Mechanisms underlying ACh function in the barrel cortex

Two questions raised by the results of this study are: how does ACh regulate cortical plasticity and why does ACh depletion have a predominant effect on SRF plasticity?

The role of ACh in plasticity is linked to its role in modifying receptive fields (Dykes 1997). In vivo, in the rat somatosensory cortex, iontophoresis of ACh excites neurons in all layers but the effect is greatest in layers II, III, and V (Bassant et al. 1990; Lamour et al. 1988). In the cat somatosensory cortex, ACh iontophoresis enhances the responsiveness of cortical neurons to somatic stimulation and increases the size of cortical receptive fields (Metherate et al. 1988a,b). ACh released by stimulation of nucleus basalis produces long-lasting facilitation in the cat somatosensory cortex (Tremblay et al. 1990a,b). In the rat barrel cortex nucleus basalis stimulation enhances the whisker evoked response of some, but not all, neurons (Howard and Simons 1994). These same authors demonstrated that some neurons respond to particular whiskers only after NB stimulation.

FIG. 6. AChE-stained fibers in depleted and partially depleted barrel fields. A: in a case where the depletion is >90%, very few fibers can be seen. A few intensely stained AChE-positive cell bodies are seen easily and would be counted in our counting system. B: in a case where the barrel cortex is only partially depleted, more fibers can be seen. This photomicrograph is from case 52 (physiological data are presented in Fig. 11) in which only partial depletion was achieved. In the barrel field, the AChE fiber count was 313, whereas near the injection the count was only 38. Calibration bar, 100 µm.
There are at least two mechanisms that could underlie the cholinergic role in modifying barrel cortex receptive fields.

Several lines of evidence suggest that ACh could have a direct effect on postsynaptic cortical neurons. It is known that the application of muscarinic agonists increases cortical neuron excitability, reduces afterhyperpolarization, reduces...
spike accommodation—all by a reduction of potassium conductances (see McCormick 1992 for a review). A long-lasting, slow depolarization also is observed in the presence of muscarinic agonists (McCormick and Prince 1986). Consequently, the direct effect of ACh in the cortex is to increase cortical excitability, and this might be one mechanism whereby ACh has an effect on cortical plasticity. According to this mechanism, removing the basal forebrain cholinergic inputs to cortex reduces cortical excitability, making it harder to initiate changes in synaptic strength. On the other hand, ACh depletion does not suppress spontaneous activity.

An alternate mechanism also is supported by the available data. In addition to any direct postsynaptic effects of ACh, evidence from various studies suggests that ACh can modulate glutamatergic transmission. Immuno-electron microscopy with antibodies to ChAT suggests that in the visual cortex, cholinergic and glutamatergic terminals are in position to reciprocally modulate each other’s release (Aoki and Kabak 1992). Similar techniques suggest that muscarinic receptors (m1 and m2) in the cortex are present at asymmetric, presumably excitatory amino acid synapses on spines. In addition, m2 receptors have been localized presynaptically on presumptive glutamatergic terminals (Mrzljak et al. 1993). Intracellular recording techniques and modeling indicate that ACh activation of muscarinic receptors can suppress selectively synaptic transmission at some olfactory cortex synapses (Hasselmo and Bower 1993). In the hippocampus, ACh potentiates the entry of calcium induced by activation of N-methyl-D-aspartate receptors (Auerbach and Segal 1994). Taken together these data suggest a second mechanism whereby ACh may influence whisker pairing plasticity, namely by increasing the efficacy of glutamatergic transmission. Either one or both of these mechanisms would be consistent with the results obtained in this study.

One caveat in comparing experiments designed to show cholinergic effects on cortex is that not all of the basal forebrain projecting cells are cholinergic. A recent double labeling study in rat suggests that roughly one-third of all basal forebrain projecting cells are cholinergic, one-third are GABAergic, and one-third use an unidentified neurotransmitter other than ACh or GABA (Gritti et al. 1997). This result suggests that the effects of stimulating or lesioning the basal forebrain region may be quite different from applying specific cholinergic agonists and antagonists within cortex. In addition, results using the “suicide transport” technique for eliminating cholinergic fibers may not be directly comparable with results derived from cholinergic depletion produced by basal forebrain lesions.

The principal mechanism for the generation of receptive fields is reflected in the anatomy. The shorter latency (<10 ms) thalamocortical inputs constitute a signature CRF for the principal whisker. The shaping of the SRF on the other hand is not as clear cut. Both the cortical and thalamic inputs have a role in the shaping of the SRF. For this study, what is important is that regions of the barrel column thought to facilitate corticocortical inputs—the septa, the superficial and deep layers of cortex—all receive a dense cholinergic input whereas the barrels themselves receive a less dense cholinergic input. Thus the removal of the basal forebrain cholinergic inputs would be predicted to have a greater im-

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**FIG. 10.** Individual penetrations in the D2 barrel column. Data from individual penetrations in individual animals are displayed here: case 51 (6 units) is a sham-depleted animal; case 52 (6 units) is an incompletely ACh-depleted animal (see Fig. 5); case 26 (8 units) is an ACh-depleted animal. Bars over the histogram are the standard error for each condition. Ordinate is the mean magnitude of response for cells within the D2 cortical barrel column to 50 stimuli applied to the D2 whisker or 1 of its surround whiskers: D1, D3. Abscissa has the whisker names, where D1 or D3 data have been collapsed into D-cut and D-paired depending on whether these whiskers were paired with D2. D2 barrel responses were collected as described in methods. Number of cells recorded in each penetration in each animal are denoted by N = 6 or 8.
Our procedure produces >90%, unilateral decreases in AChE staining on the injected side over a large portion of the cortical mantle. The importance of obtaining complete depletions has been underscored by recent investigations in which the performance of tasks requiring rats to use their whiskers was correlated with the percentage of depletion (Jacobs and Juliano 1995). Rats with a smaller percentage of AChE fiber reduction return to criterion performance quicker than do rats with larger depletions (Jacobs and Juliano 1995), suggesting that rats can compensate for some loss of cholinergic innervation. The same might be true in the whisker pairing paradigm, where partial ACh depletions have a proportional effect on the whisker pairing bias. It is somewhat more difficult to show graded effects with our paradigm, because partial depletions vary in their extent, as does the position of the electrode within the barrel in any single case.

**AChE STAINING AS A MARKER FOR CHOLINERGIC FUNCTION.** To date, the correspondence among AChE staining, ChAT immunocytochemistry, and the measurement of enzyme activity has suggested that AChE staining is a good marker for cholinergic function: where AChE activity is high or AChE staining is dense, ChAT activity is high and ChAT immunoreactivity is dense (Guela and Mesulam 1996). Furthermore, a loss in ChAT immunoreactivity is usually associated with a loss in AChE fiber/cell body staining (Arendt et al. 1988; Guela and Mesulam 1996; also see Jacobs et al. 1991; but see Webster et al. 1991). In addition, depletions appear just as successful when the immunocytochemistry for vesicular acetylcholine transporter is done in conjunction with AChE histochemistry. One slight difference is that with immunocytochemistry fewer fibers appear stained, and the delineation between the barrel and septa is not as robust as it is with AChE staining (unpublished observations).

### SHAM DEPLETIONS

Sham depletions were made by injections of saporin alone or 192 IgG unconjugated with saporin. The rationale for the use of saporin mixed with 192 IgG is clear: the ACh depletion in the experimental animals occurs because the 192 IgG and saporin are conjugated. The rationale for use of saporin alone as a control is that it is the cytotoxic element in the immunotoxin.

When the immunotoxin IgG 192 linked to saporin is injected into the cortex, most of it is presumably taken up by the targeted terminals, killing the nucleus basalis neurons, and some small quantity is taken up by nonspecific endocytosis. Consequently, the sham-depleted animals showed no targeted cholinergic fiber loss beyond the zone of cell death.

### NORMAL WHISKER PAIRING PLASTICITY

Here we have replicated the “whisker pairing” findings of Diamond, Armstrong-James, and Ebner (Armstrong-James et al. 1994; Diamond et al. 1993). When the D2 whisker is paired with an adjacent D-row whisker for 7 days, a significant change in the corticocortical interactions, and less of an impact on the thalamocortical inputs. The net effect could explain the exclusive impact of cholinergic depletion on the SRF.

**Methodological considerations**

**ACH depletion.** One finding of this study is that single intracortical (in contrast to intraventricular) injections of 192 IgG conjugated to saporin produce long-term, robust (>90%) depletions of cholinergic fibers in the cortex. The intraventricular delivery method has proven effective in killing cholinergic basal forebrain neurons bilaterally (Wiley and Lappi 1994; Wiley et al. 1991). In this study, we were interested in depleting the basal forebrain cholinergic input to the barrel field in only one hemisphere not in both hemispheres.

**FIG. 11.** Recording sites in the D2 barrel of sham-depleted whisker paired (A), ACh depleted (B), and ACh depleted whisker paired (C) conditions. Each number surrounded represents a case. The filled circles associated with each case represent the approximate location of the electrode penetrations. The shaded area associated with a number represents data gathered within the barrel of a particular case. Note that though the distribution of penetrations from condition to condition varies, the penetrations are not skewed toward either the D1 or the D3 barrel. The number of cells recorded from each animal are in A: cases 59 (20 units), 57 (25), 37 (13), and 38 (16); B: cases 67 (18 units), 69 (24), 68 (10), 34 (16) and 27 (8); and C: cases 23 (20 units), 26 (12), 22 (16), 21 (11), and 20 (16).
the response properties of neurons in the D2 barrel column is produced such that the response to the intact whiskers is potentiated. In this study, we obtain results similar to those obtained earlier: the sham-depleted animals develop a significant bias toward the paired whisker response in that the D-paired response is twice that of the D-cut whisker.

The overall response magnitudes in the saporin sham-depleted animals, however, are lower than those obtained in uninjected animals. One reason could be that in this study, an initial surgery is performed on the rats, and a toxin delivery cannula is passed through the dura; this results in local damage to the cortex medial to the barrel field. Even in the control sham-depleted cases, a highly effective toxin (saporin) is injected into the cortex (Wiley and Lappi 1994).

These procedures would by themselves be expected to have some detrimental effect on cortical function. These factors, in addition to the usual sources of variation, like cell selection during recording, result in a lower D2 barrel-column response after whisker pairing when compared with that obtained in the earlier study on normal adult rats. Nevertheless, the main finding of whisker pairing bias—the condition where the paired whisker gives a significantly greater response than the cut whisker—plus the D2 whisker potentiation remain robust and significant, even after saporin injection into the brain.

OTHER EVIDENCE FOR A CHOLINERGIC ROLE IN CORTICAL PLASTICITY. During the early postnatal period, the ocular dominance columns in kitten cortex are easily modifiable by visual experience; closing one eye during the “critical period” biases the neurons in the visual cortex to respond almost exclusively to stimulation of the eye that remains open (Wiesel and Hubel 1963). Kasamatsu and Pettigrew demonstrated that such a shift could be prevented in some cases if, before eye closure, norepinephrine (NE) was depleted from the cortex by infusion of 6-hydroxydopamine (Kasamatsu and Pettigrew 1976, 1979). This finding was challenged subsequently on methodological grounds: 6-hydroxydopamine, the agent used to deplete NE, was mini-pumped continuously during the experiment up to and including during recording and under these conditions may have directly blocked muscarinic receptors in addition to depleting NE. In 1986, Bear and Singer (1986) demonstrated that the depletion of both ACh and NE was sufficient to consistently prevent the ocular dominance shift in kitten cortex. Subsequently, Gu and Singer (1993) demonstrated that blockade of the muscarinic m1 receptor alone was sufficient to prevent ocular dominance shifts, and Brocher, Artola, and Singer (1992) demonstrated that the presence of cholinergic muscarinic agonists increases the probability of obtaining long-term potentiation. The data presented in this paper suggest that in the adult rat ACh depletion alone is sufficient to prevent intracortical plasticity for an extended period of time.

ACh IN SOMATOSENSORY CORTEX. It is widely accepted that the predominant cholinergic projection to the somatosensory cortex, arises from the nucleus basalis in the basal forebrain (Eckenstein et al. 1988; Lysakowski et al. 1989; McKinney et al. 1983; Umbro et al. 1994) and project to all layers (Krisst 1979a; Lysakowski et al. 1989). Our AChE staining results are in agreement with these previous studies; we see dense innervation of laminae I–III and V, and in layer IV, we see that the AChE staining pattern is relatively sparse within the barrels compared with the higher density of fibers in septa. The present results confirm that the number of AChE fibers in the barrels is less dense than in the septa, but the barrels do have two-thirds the number of fibers found in the septa, suggesting that the depletion should have some effect on layer IV barrel neurons.

Further experiments are required to determine whether a longer period of whisker pairing might produce the bias associated with whisker pairing even in the ACh-depleted animals. If so, what we have described is a delay, not a permanent impairment. Our preliminary experiments suggest that even in the 30-day whisker-paired animal, no whisker pairing bias is induced (data not shown).

In conclusion, this study provides further support for the importance of cholinergic mechanisms in cortical plasticity. When whisker-pairing–induced plasticity is expected to be at its maximum, no significant difference develops between the active and inactive surround whisker in ACh-depleted cortex. The surprising presence of D2 whisker potentiation even in the ACh-depleted animals indicates that ACh is not necessary for all types of activity-dependent cortical plasticity. SRF whisker-pairing plasticity requires synaptic modification in cortical neurons that interconnect the barrels and intracortical plasticity appears to be the component most dependent on ACh. CRF plasticity depends on mechanisms within the barrel and can occur without ACh facilitation.

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REFERENCES


Armstrong-James, M., Callahan, C. C., and Friedman, M. A. Thalamocortical processing of vibrissal information in the rat. I. Intracortical origins of surround but not centre-receptive fields of layer IV neurons in the rat S1 barrel field cortex. J. Comp. Neurol. 303: 193–210, 1991.


DYKES, R. W. Mechanisms controlling neuronal plasticity in somatosensory forebrain cholinergic system.


