The Basal Forebrain Cholinergic System Is Involved In Rapid Nerve Growth Factor (NGF)-Induced Plasticity In the Barrel Cortex of Adult Rats

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Abstract: We have previously reported that topical application of NGF to the barrel cortex of an adult rat rapidly augmented a whisker functional representation (WFR) by increasing its area and height within minutes after NGF application. In addition, we found that TrkA, the high-affinity NGF-receptor, was only found on fibers projecting into the barrel cortex. Here we use a combination of techniques including chronic intrinsic signal optical imaging, neuronal fiber tracking and immunohistological techniques, to test the hypothesis that NGF-induced rapid cortical plasticity is mediated by the cortical projections of the Basal Forebrain Cholinergic System (BFCS). Our studies localize the source of the cells in the BFCS that project to a single WFR, and also demonstrate that TrkA-immunoreactive fibers in the cortex are also cholinergic and likely arise from the BFCS. In addition, by selectively lesioning the BFCS cortical fibers with the immunotoxin 192 IgG-saporin, we show that NGF-induced WFR-cortical plasticity is eliminated. These results, taken together with our previously reported imaging results that demonstrated that agonists of the cholinergic system (particularly nicotine) showed transient NGF-like augmentations of a WFR, implicate the BFCS cortical projections as necessary for NGF’s rapid plasticity in the adult rat somatosensory cortex.

Introduction:
Previously we reported that neurotrophins, such as NGF and Brain-Derived Neurotrophic Factor (BDNF), can rapidly induce large-scale plasticity in the adult somatosensory cortex (Prakash et al. 1996a, b). For example, within minutes after topical application to the cortex, NGF caused a transient, large-scale expansion in the area of a WFR coupled with an increase in the height of the WFR. The current study attempts to describe potential underlying mechanisms that mediate this NGF-plasticity. One possibility may be that NGF is acting directly on cortical neurons to induce such plasticity. However, most evidence indicates that in the adult rat, NGF receptors are not expressed by cortical neurons but rather in neurons that project to the cortex (Merlio et al. 1992) and on projection fibers to the somatosensory cortex (Holtzman et al. 1995; Prakash et al. 1996b). This evidence thus suggests that NGF-induced adult cortical plasticity should involve neuronal projections into the cortex that are presynaptically responsive to NGF. Here we present evidence that indicates that the BFCS is the source of these cortical projection fibers and thus implicates the BFCS in the NGF-induced cortical plasticity.

The basal forebrain (BF) in the rat, known as the magnocellular basal forebrain nuclei, includes cells located in the nucleus basalis of Meynert and the septal nucleus of the diagonal band of Broca (Fibiger 1982; Sofroniew et al. 1982; Wenk et al. 1980). Cholinergic cells within this system project to the entire rat cortex (Lehmann et al. 1980) in a loose topographically organized manner (Jimenez-Capdeville et al. 1997; Lamour et al. 1982) and constitute the only known cholinergic projection to the adult cortex (Mesulam et al. 1983). After release, acetylcholine (ACh) can influence target cells in the cortex through two broad classes of cholinergic receptors: muscarinic and nicotinic receptors.

It has been demonstrated that NGF is synthesized in the cortex by the target cells of BF projections in the cortex, and that it binds to receptors located on the BF nerve terminals and is retrogradely transported to cell bodies of BF neurons, where it regulates their survival and function as predicted by the neurotrophic hypothesis (Cuello et al. 1992; Hefti et al. 1989). While extensive evidence exists for the neurotrophic hypothesis in the developing brain, evidence also exists to support the notion that NGF continues to play a maintenance function for the BFCS after the brain matures. Exogenous application of NGF can rescue axotomized BF cholinergic neurons, reverse the decline in cortical function and restore memory (Dekker et al. 1992; Gage et al. 1988; Jacobs et al. 1994; Koliatsos et al. 1990; Kromer 1987; Will and Hefti 1985; Williams et al. 1986). More direct experimental proof for the role of endogenous cortical NGF in the maintenance of the BFCS (Minger and Davies 1992a, b) was obtained when local injections of anti-NGF antibodies were delivered into the cortex of the adult rat (Gutierrez et al. 1997). These rats demonstrated a lack of ACh release in the cortex, a disruption of connectivity between cortex
and BF, and a disruption of learning and memory. The importance of endogenous cortical NGF was also demonstrated by the use of mutant mice (Chen et al. 1997). Heterozygous mutant mice for the NGF gene showing a reduced level of both NGF-mRNA and protein within the cortex had significant learning and memory problems that were accompanied by a loss and shrinkage of BF cells. Infusion of NGF in these adult mutants abolished the memory deficits and corrected the deficits in both area and projection density of BF cells to the cortex.

It is clear that NGF plays a role in maintaining the BF cholinergic projections to the cortex. Two key findings have also suggested the possibility that NGF can also induce immediate release of ACh. Knipper and collaborators obtained in vitro evidence for NGF-induced rapid release of ACh in the cortex (Knipper et al. 1994a; Knipper et al. 1994b). The authors demonstrated such effects in synaptosomes prepared from the rat hippocampus that included BF projection fibers. They showed enhanced ACh release from these synaptosomes starting within 1 minute after NGF application. Anti-NGF antibodies blocked the NGF-induced rapid release of ACh. The rapid induction of ACh release cannot be readily explained by NGF’s known role of regulating the survival and function of BF cholinergic system, as this role requires a longer window of time (hours to days) for these effects. In addition, the rapid release of ACh supports the existence of NGF receptors on the terminals BF projection fibers. Rapid induction of ACh release by NGF was also demonstrated in synaptosomes prepared from the visual cortex of rats, and further demonstrated that such induction of ACh release involves the activation of the high-affinity TrkA receptor and the p75 low affinity neurotrophin receptor (p75) (Sala et al. 1998). Further, we obtained in vivo evidence for NGF’s ability to rapidly increase (within minutes after application) the height and areal extent of whisker evoked activity in the somatosensory cortex (Prakash et al. 1996b). Our previous histological results suggested that NGF’s site of action is not directly on the neurons within the somatosensory cortex but rather on projections originating outside it. Given that BFCS projection neurons are the only type of neurons with both projections to the cortex and NGF receptors (Holtzman et al. 1995), our findings (Prakash et al. 1996b) suggested an in vivo link for NGF’s rapid in vitro effects that may be mediated by the BFCS.

One possible mechanism for NGF’s induced plasticity is that under behavioral conditions when cortical representations need to be rapidly augmented by increasing cortical excitability and by recruiting more neurons, cortical neurons release NGF in an activity dependent manner in addition to the basal release of NGF needed for the survival and maintenance of the BF. The released NGF binds to its receptors (TrkA and p75) located on the BFCS projection fibers in the cortex. The binding of NGF then enhances the release of ACh from BFCS projections within the cortex. The release of ACh, in turn, further enhances cortical activity through ACh receptors, and potentially glutamate release enhancement by ACh. These actions, in turn, lead to an increase in the excitability of neurons within a WFR and the recruitment of additional neurons to the WFR, leading an areal expansion and increased height of the WFR as observed with the imaging results (Prakash et al. 1996b). Elsewhere we reported, in support of this hypothesis, that activation of muscarinic or nicotinic receptors in the somatosensory cortex results in a WFR plasticity that is similar to the NGF-induced plasticity (Penschuck et al. 2002). Here we present data that tested three other critical aspects of our hypothesis: 1) we demonstrate that projections from BFCS reach the level of a single WFR in barrel cortex; 2) we demonstrate that TrkA-immunoreactive fibers present in the somatosensory cortex are also cholinergic fibers that likely arise from the BFCS, and 3) we show that specifically lesioning the BFCS fibers in the barrel cortex results in a loss of the ability of NGF to rapidly augment a WFR. Some of these data have been published previously in abstract form (Prakash et al. 2000a).

Methods:

Imaging. We have followed a procedure described in (Prakash et al. 1996b) and is described briefly here. Adult male Sprague-Dawley rats (300-550 g) were initially anesthetized with
pentobarbital (IP; 50 mg/kg). Throughout the rest of the experiment pentobarbital was injected as needed to maintain the rat in a consistent anesthetic state such that the respiration rate was $1.1 \pm 0.2$ breaths per second, rectal temperature was $37.0 \pm 0.5$ °C, ear color was pinkish-pale, and the corneal reflex in response to a saline drop was weak to mild. 0.1 mL of lidocaine was injected under the scalp and 5 minutes later a single incision was made with a scalpel roughly along the midline of the scalp. Fascia and muscle overlying the parietal cortex were cleared and the bone was cleaned and dried. Next, the skull overlying the somatosensory cortex was removed. However, unlike the previous protocol (Prakash et al. 1996b) in which the dura and arachnoid mater were removed (durectomy), in this set of experiments the dura remained in place but small slits or holes were made in it (durotomy) using a 30-gage needle. This change in protocol was instituted to decrease the probability that rats would have significant brain herniation. A petroleum jelly well was then built around the partially exposed cortex, filled with artificial cerebrospinal fluid (ACSF; 7.250 g NaCl, 0.370 g KCl, 0.170 g KH$_2$PO$_4$, 0.277 g CaCl$_2$, 0.290 g MgSO$_4$, 2.160 g NaHCO$_3$, and 1.800 g glucose (all per 1 L H$_2$O), pH 7.30, 37°C), and sealed with a coverslip.

A 12-bit slow-scan CCD camera (Photometrics, Tucson, AZ) imaged a 6.8 mm x 5.1 mm (192x144 pixels; 35.5 µm pixel width) region of the cortex that was illuminated with a stabilized 100 watt tungsten-halogen light source (Zeiss Instruments) passed through an optical filter (630 nm bandpass 30 nm; Omega Optics, Brattleboro, VT). To reduce potential surface artifacts induced by pulsations of the brain and vessels, the camera was focused 300 µm below the surface of the cortex. The light reflectance was measured for each pixel (9 consecutive frames, 500 ms each) before, during, and after stimulation of the contralateral whisker(s). Whisker stimulation was given with a computer-controlled mechanical stimulator (Bakin Systems II, Irvine, CA) at 5 Hz for 1 s. The inter-stimulus interval between trials was 16 s. Data files were created by the summation of 64 trials, and thus had duration of about 20 minutes. The right C2 whisker was deflected 2° rostro-caudally (0.5 mm at 1.5 cm from the snout) with an average velocity of 30°/s. Eight imaging data files were collected per rat. The first four data files served as baseline reference. Additionally as an internal control to determine the affects of change the ACSF in the petroleum jelly well, the ACSF was replaced between data files 2 and 3. No significant difference in the measured parameters was ever noted after this ACSF change. After the fourth data file, the ACSF was replaced with recombinant human NGF dissolved in ACSF (15 µg in 500 µL).

**Quantitative Analysis of a Whisker Functional Representation.** Analysis methods have been described in detail elsewhere (Chen-Bee et al. 1996; Masino and Frostig 1996; Masino et al. 1993; Prakash et al. 1996b; Prakash et al. 2000b). Briefly, data files were created from the summation of 64 imaging trials. Ratio values for a data file were computer-calculated for each pixel by dividing the reflectance values of the images 0.5-1.5 s post-stimulation by the images 1.0-0.0 s before stimulation. Before analysis, a gaussian filter (half-width 5) was applied to the raw ratio values to filter out high-frequency noise. The peak location of a WFR is defined as the pixel with the most negative ratio value, and this ratio value minus the median ratio value is the peak height of a WFR, which indicates the maximal magnitude of evoked activity. The area at half-height of a WFR is the area bounded by the threshold that contains pixels 50% of the peak height value.

**Relationship Between Imaging and Underlying Neuronal Activity.** We focused our analysis on reflectance changes of 630 nm light occurring between 0.5 and 1.5 seconds post-stimulus onset, an epoch of activity predominantly consisting of localized oxygen consumption in the capillary bed serving activated neurons (Ances et al. 2001; Frostig et al. 1990; Kim et al. 2000; Malonek et al. 1997; Malonek and Grinvald 1996; Thompson et al. 2003), as the area of imaged activity generated from this epoch has been previously found to be well-correlated with the
underlying area of evoked neuronal activity (Brett-Green et al. 2001; Frostig et al. 1990; Masino 2003; Polley et al. 1999). We also have repeatedly found a strong correspondence between the spatial distribution of intrinsic signals and the underlying single unit responses such that: a) the location of the strongest single unit response for each rat coincided with the location of that rat’s strongest intrinsic signal response; b) evoked single unit responses were present at all locations containing evoked intrinsic signal responses; and c) both responses decayed similarly with increasing distance from the location of peak activity (Brett-Green et al. 2001; Masino 2003; Polley et al. 1999). Furthermore, post-imaging single unit recordings demonstrated that plasticity of a WFR (both in its areal extent and height) was identical to the one obtained with intrinsic signal optical imaging (Polley et al. 1999). Thus, for both the normal functional organization of cortex and its plasticity, intrinsic signal optical imaging that is based on localized oxygen consumption has been shown to be a reliable indicator of underlying physiological neuronal activity patterns and their plasticity.

DiI-Retrograde Labeling of Basal Forebrain Cells Projecting to Barrel Cortex. 15 male Sprague-Dawley adult rats (300-600g) were imaged and the exact location of the peak of activity for the C2 WFR was determined using stereotactic methods. The location of bregma was defined as the origin and C2 WFR location was measured in relation to the origin using its peak activity as obtained by imaging through the thinned skull (~150 µm thickness). The average location of C2 was useful in other experiments, such as the 192-IgG-saporin injections described below, and in localizing the C2-barrel in coronal sections. A small burr hole was drilled through the cortex over the peak activity of the WFR. ~1 µL of Fast-DiI oil (Molecular Probes, Eugene, OR) dissolved in dimethylsulfoxide was then pressure injected (PicoSpritzer II, General Valve, Brookshire, TX) through a glass pipette that was lowered 500 µm below and perpendicular to the pial surface. Fast-DiI is a fluorescent molecule that travels along lipid membranes, thus staining axons (anterogradely and retrogradely) and cell bodies (predominantly retrogradely). After waiting 3 minutes, the pipette was slowly retracted and the burr hole was sealed with sterile bone wax. The scalp incision was closed with wound clips and prophylactic antibiotics were administered (Panalog on the eyes, Terramycin over the wound, and Ampicillin subcutaneously in the back). Rats were survived for 6-10 days and then given an overdose of Nembutal (100mg). When completely areflexive, rats were perfused transcardially with 0.1 M PBS at 4° C (~150 mL) followed by fixation with a solution containing 4% paraformaldehyde and 0.25% gluteraldehyde in 0.1 M Sorensen’s buffer (~150mL). The brain was removed and post-fixed in the same solution overnight, followed by immersion for 2-3 days in 30% sucrose dissolved in 0.1 M PB. Upon sinking, the brain was cut coronally on a cryostat at –20°C each section was thaw-mounted serially directly onto gelatin-coated slides. As DiI is prone to dissolution after sectioning, sections were viewed either dry or with 0.1 M PBS and a coverslip. The locations of DiI-positive cell bodies throughout the basal forebrain were noted and photographed using a Rhodamine filter on a fluorescent microscope (Nikon).

Techniques to detect cholinergic neurons and fibers. Cholinergic neurons in the nervous system produce the neurotransmitter acetylcholine by the enzyme Choline Acetyl Transferase (ChAT). ACh is then transported into synaptic vesicles by the Vesicular Acetylcholine Transporter (VACHT). After acetylcholine is released at the synapse, it is rapidly degraded by AcetylCholine Esterase (AChE). Based on these three enzymes, different histochemical methods have been developed to visualize cholinergic neurons. AChE visualization techniques are the oldest and best-characterized staining techniques for cholinergic neurons and fibers. These techniques are the most sensitive visualization techniques but are not specific enough because norepinephrinergic and dopaminergic neurons may also stain positive for AChE (Albanese and
Butcher 1979; Henderson 1989; Tago et al. 1986). Immunohistochemical techniques for staining neurons and fibers for either ChAT of VACHT have proven to be more specific for cholinergic neurons, but are generally less sensitive at elucidating cholinergic fibers than AChE staining (Armstrong et al. 1983; Eckenstein and Sofroniew 1983; Holler et al. 1996; Mizukawa et al. 1986; Satoh et al. 1983; Weihe et al. 1996). Therefore, depending on whether we required sensitivity or specificity, all three staining methods were chosen for different experiments described below.

Co-localizing TrkA and ChAT. We required maximal specificity to demonstrate that cholinergic fibers were also expressing TrkA. Therefore ChAT was chosen for these experiments. Initial studies were conducted to optimize the single staining protocols for TrkA and ChAT, and in few rats double staining with VACHT and TrkA was also performed. A rat was given an overdose of pentobarbital and then perfused transcardially with 4°C phosphate buffered saline (0.1 M PBS, ~150 mL) and fixed with 4% paraformaldehyde (~150 mL). The brain was removed and placed in the same fixative overnight, then transferred to 30% sucrose in 0.1M PB until it sank (~3 days). The brains were frozen (-20°C) and sectioned to 35µm slices. For double staining, 20-35 µm slices were incubated simultaneously with goat anti-ChAT antibody (Chemicon Int. Temecula, CA) and rabbit anti-TrkA antibody (Holtzman et al. 1995) in 5% donkey serum overnight at 4°C. Following rinse in 0.1 M Tris buffered saline (TBS), sections were incubated for 1 hour room temperature in darkness in Cy2-conjugated donkey anti-rabbit antibody and Cy3-conjugated donkey anti-goat antibody (both from Jackson ImmunoResearch Labs). Four controls were used that had identical treatment as the other slices except for the omission of one of the following antibodies: anti-ChAT, anti-TrkA, anti-rabbit, or anti-goat. After rinse in 0.1 M TB, sections were mounted on gelatin-coated slides and dried for about 15 minutes. Sections were treated with Prolong mounting media (Molecular Probes) and coverslipped and stored in the dark at 4°C. 1-2 days later sections were visualized and imaged under a fluorescent microscope (Nikon or Olympus) with a FITC (for Cy2) or rhodamine (for Cy3) filter.

Cholinergic Depletion of a Cortical Whisker Functional Representation. 192 IgG-saporin is a cytotoxin that was developed by R.G. Wiley and colleagues and has been demonstrated to be extremely effective and specific at eliminating cholinergic basal forebrain cells (Book et al. 1994, 1992; Sachdev et al. 1998; Wenk 1997; Wiley 1992; Wiley et al. 1991) (Baskerville et al. 1997; Zhu and Waite 1998). 192 IgG-saporin works by binding to p75 receptors on cholinergic fibers, where it is internalized and transported back to the nucleus. Saporin then binds to ribosomes and irreversibly blocks protein synthesis, which leads to cell death. One effective method of depleting the barrel cortex of cholinergic inputs is by ventricular injection of 192 IgG-saporin (Baskerville et al. 1997; Zhu and Waite 1998); reviewed by (Schliebs et al. 1996; Wiley 1996). However, even more precise, localized depletions of cholinergic inputs are possible by injection of a small amount of 192 IgG-saporin directly into the cortex (Bucci et al. 1998; Sachdev et al. 1998). Data obtained from such localized injections is not confounded by interpretations related to possible secondary effects from the complete cholinergic depletion of a hemisphere(s) and parts of the cerebellum, as obtained with intracerebroventricular injections (Wiley et al. 1991). To test the hypothesis that cholinergic fibers are involved in NGF’s induced plasticity of a WFR, we locally depleted cholinergic fibers within the C2-WFR, using a similar protocol as Sachdev and collaborators who were also using 192 IgG-saporin to test cortical plasticity in the adult barrel cortex (Sachdev et al. 1998). These authors reported that local injection of 192 IgG-
saporin into the barrel cortex results in a loss of the normal cortical plasticity that occurs between two-whiskers when all the other whiskers are trimmed from a rat’s snout (Sachdev et al. 1998).

**Combining Chronic Imaging with Cholinergic Depletion** 30 rats were used in this study, 20 experimental and 10 controls. Of the 20 experimental rats, 8 were successful for all 3 phases described below. Additionally 3 of the remaining 12 experimental rats were successful in the first 2 phases but had only negligible AChE fiber depletion (>75% remaining) in the C2-barrel column, as evident in phase 3. For the control rats, 6 of 10 were successful for all 3 phases.

**Experimental design:**

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**Phase I—Localization of WFRs and injection.** The right C2 WFR was imaged through a thinned skull in pentobarbital anesthetized adult male Sprague-Dawley rats (300-550 g). To avoid direct damage to the C2 WFR, a burr hole in the skull was drilled between 1.5-2.0 mm just medial of the peak of activity of the C2 WFR. 80 ng of 192 IgG-saporin (Chemicon) dissolved in 2 µL of 0.1 M PBS with 0.05% sodium azide (or only 2 µL of PBS and 0.05% sodium azide for controls) was pressure-injected (Picospritzer II, General Valve) through a glass pipette 1500 µm below the pial surface. Similar to (Sachdev et al. 1998), this depth allowed for good penetration and diffusion of the toxin throughout the cortical tissue. Also similar to (Sachdev et al. 1998), the distance of 1.5-2.0 mm from the C2 WFR was found to be close to the maximal distance for inducing an effective lesion in C2 in this dosage range. This distance also prevented direct damage to the C2 WFR from the pipette. Additional care was taken to avoid traumatizing major cortical vasculature with the pipette. Once injected, the pipette was slowly retracted and the burr hole was sealed with sterile bone wax. The scalp incision was closed with wound clips and prophylactic antibiotics were administered (Panalog on the eyes, Terramycin over the wound, and Ampicillin subcutaneously). Rats recovered from surgery overnight in a cage resting on a heated blanket, and then were returned to individual cages in the vivarium for 21 days with free access to food and water on a 12-hour light, 12-hour dark cycle.

**Phase II—NGF-effects on depleted and control cortex.** Similar to (Sachdev et al. 1998), 21 days was chosen as the interval between injection and second imaging of the C2 WFR, this interval allowed for a full recovery from the surgery. 21 days after the first imaging session/injection, rats were briefly observed for gross behavioral changes and then weighed. All
rats gained between 5-100 g over the 21 days and no gross behavioral changes were observed. Rats were then anesthetized with intraperitoneal pentobarbital and local lidocaine and placed in the stereotactic apparatus. The remaining wound clips were removed and the incision site re-opened. The “cortical window” in the skull that from the previous imaging session was frequently revascularized and thus required cleaning and cauterizing. Once cleaned, the cortical window was resected using a dental drill and forceps. To allow access of NGF into the cortical parenchyma, in all rats 3-4 small slits (~100 x 1500 µm each; figure 8) were made in the dura and arachnoid mater using a 30-gauge needle. The slits were made in locations that surrounded and touched the borders of the previously identified location of the C2 WFR, and that avoided tearing the dural vessels. A petroleum jelly well was then built on the bone surrounding the cortical window and filled with ACSF and coverslipped. The imaging protocol as described above and previously (Prakash et al. 1996b) was then used to assess the C2 WFR before and after application of NGF.

**Phase III—Quantifying the cholinergic depletion.** At the end of the imaging session, rats were perfused transcardially with 4 °C 0.1 M PBS (~150 mL) followed by fixation with a solution of 4% paraformaldehyde, 0.5% gluteraldehyde, and 0.2% picric acid in 0.1 M Sorensen’s buffer (~150 mL). The brain was removed and placed in 30% sucrose in 0.1 M PB until it sank. (Approximately half the brains were prepared for tangential cutting: the cortex was dissected from the rest of the brain and flattened to 2.0 mm between two slides and then the slides and brain were placed in 30% sucrose). The brains were then frozen to −20°C and serial sections were cut coronally or tangentially at 40 µm thickness on a cryostat (Zeiss). Alternating sections were stained for cytochrome oxidase (CO (Wong-Riley 1979)) to visual barrels and layer IV. The other alternating sections were stained for acetylcholine esterase (AChE), using Tago’s modifications (Tago et al. 1986). Additionally, slices were obtained from several rats and stained for recombinant human NGF-immunoreactivity to ascertain the penetration of NGF through dural tears. This protocol was similar to as previously described (Prakash et al. 1996b). Sections were mounted onto gelatin-coated slides and allowed to dry, then covered with mounting media and coverslipped. Sections were analyzed and imaged under a microscope (Olympus) and the location of the left and right C2 barrels and their relations to the vascular patterns was ascertained from the CO sections. The vascular patterns from the adjacent AChE section were then compared to CO section vascular patterns and used to determine the location of the C2-barrels in that section. 20x or 40x magnification photomicrographs were then taken in each C2-barrel and in layer I/II directly above the barrel. Photomicrographs were digitized and in PhotoShop 5.0 (Adobe, San-Jose, CA) a 100 µm line was then drawn through the barrel in layer IV, and above the barrel in layer I/II. The line was drawn perpendicular to the pial surface for coronal sections or horizontal for tangential sections. “Fiber crossings” were calculated by adding up the number of intersections the two 100 µm lines made with AChE-positive fibers. AChE fiber depletion was defined as (1-[(Total fiber crossings on injected side) / (Total fiber crossings on control side)]) x 100%. Although there were no obvious differences across the different lamina, we quantified the fiber depletion in layers IV and layer I/II as these layers are presumably the primary loci relevant to NGF’s effects. However there were differences in the fiber depletion in the C2-barrel across different rats. This was most likely from the 1.5-2.0 mm variability in injection site distance from the C2-barrel, which was introduced in order to avoid damaging major blood vessels (see Phase I above). However, this variability across rats proved to be advantageous because it allowed for a correlation of NGF’s induced plasticity with the fiber depletion ratio (figure 4).
Results:

Regional topography of basal forebrain projection to a WFR. To determine whether the general pattern of BFCS projection cells to the somatosensory cortex holds even for a single WFR, we injected Fast-DiI, a retrograde tracer, into the cortical C2-whisker functional representation that resulted in transport of tracer to several areas of the brain, such as the thalamus, zona incerta, and basal forebrain, in similar patterns as we reported in mice (Prakash et al. 2000b). Previous studies have suggested that the somatosensory cortex of the rat receives basal forebrain projections predominantly from the nucleus basalis of Meynert (nBM; which consists of the ventral part of the globus pallidus (vGP), the substantia innominata (S.I.), some cells in the internal capsule, and a few cells scattered in other nuclei (Bigl et al. 1982; McKinney et al. 1983). Our primary interest in this study was to determine the precise regions of the basal forebrain that were labeled. Firstly, to verify the identities of the basal forebrain nuclei that project to the adult rat barrel cortex. Secondly, to determine whether an individual WFR had a more specific basal forebrain projection locus than reported for the rest of the somatosensory cortex (Bigl et al. 1982; Kiss and Patel 1992; McKinney et al. 1983). Consistent with these previous reports that examined the innervation of the entire somatosensory cortex, we found that the C2-WFR was innervated by a scattered group of large cells throughout the nBM (Figure 2). Other cells were found scattered throughout the basal forebrain, but never in as high a concentration as the nBM. Overall, our results suggest that the pattern of projections to a single WFR is not more localized than the general pattern of BF cells that project to the sensory cortex. These results were helpful for the interpretation of the intracortical 192 IgG-Saporin injections described below.

Cytochemistry of Basal Forebrain Projection Cells and TrkA cortical fibers. The basal forebrain contains at least two major classes of neurons that project fibers to the cortex: parvalbumin-immunoreactive (-IR; GABAergic) and ChAT-IR (cholinergic). The cholinergic fibers are the best candidates for also expressing TrkA, because previous work demonstrated that most of the abundant ChAT-IR cell bodies in the basal forebrain are also p75-IR/TrkA-IR (Sobreviela et al. 1994), whereas GABAergic cells in the basal forebrain are p75-IR negative (Kiss et al. 1993). While there is no question that cholinergic basal forebrain cells also express TrkA, there was no direct evidence that cholinergic projection fibers in the cortex co-express ChAT and TrkA.

To assess whether TrkA-IR fibers observed in the cortex are also cholinergic, co-localization studies with TrkA-IR and ChAT-IR were performed. We found that TrkA fibers were less abundant than ChAT fibers (about 1-to-4 ratio, respectively), but that all TrkA-IR fibers in the barrel cortex were also ChAT-IR (Figure 1). Furthermore, the TrkA fibers were sparsely distributed, but present in all cortical layers (I-VI). To further verify this finding, double labeling studies with TrkA-IR and the vesicular acetylcholine transporter (VACHT)-IR were performed (not shown). These experiments demonstrated similar types of staining patterns as the ones presented in figure 1. In summary, we found TrkA-IR only on fibers in the adult rat barrel cortex, and all these fibers also were cholinergic (either ChAT-IR or VACHT-IR).

Necessity of the BFCS for the expression of NGF’s rapid plasticity. Confirming our observation that TrkA expression is found exclusively in projection fibers into barrel cortex and that all TrkA-IR fibers are cholinergic, we next analyzed the results of depletion of the BFCS fibers in the barrel cortex. This depletion was accomplished by injecting 192 IgG-saporin into the cortex near the C2-WFR and then re-imaging of the same C2-WFR three weeks later. Because of our histological studies using Fast-DiI, we knew that the pattern of BF projection cells to a single WFR is similar to the general patterns of BF projection cells to the somatosensory cortex. Thus, imaging-based localized injections of 192 IgG-saporin to the somatosensory cortex near a single WFR would result in confined, yet representative and thus interpretable, pattern of lesions in the
BF. In these rats the depletion was between 35% and 95%. A control group had saline injections into the cortex near the C2-WFR, which had no detectable AChE fiber depletion. Additionally, a third group of rats had injection of 192 IgG-saporin that was subsequently found to have minimal AChE fiber depletion at the C2-WFR (between 0 and 25%). Both the control group (n=6) and the group with an AChE fiber depletion between 0 and 25% (n=3; figure 4) showed increases in both the area at half-height and the peak height after NGF application that were not statistically significantly different from each other (p>0.05, t-test, n=9). We thus pooled the control group and the group with AChE fiber depletion between 0 and 25% group together as the “non-depleted group” (n=9).

To determine if depleting the cortex of the BFCS affected a WFR 21 days after 192 IgG-saporin injection, we compared the area and strength of the C2-WFRs before application of NGF in the depleted and non-depleted groups. There was no difference between the C2-WFR of depleted and non-depleted groups for either the area at half-height (1.38 ± 0.16 mm$^2$ vs. 1.25 ± 0.13 mm$^2$, p>0.05, t-test, n=17) or the peak height (2.64x10$^{-4}$ ± 0.25x10$^{-4}$ vs. 2.39x10$^{-4}$ ± 0.46x10$^{-4}$, p>0.05, t-test n=17). Therefore injection of 192 IgG-saporin locally into the cortex did not have a significant effect on a WFR when compared across rats (figure 6, also compare gray bars in figure 7).

To determine whether topical application of NGF induced WFR plasticity in a BFCS depleted cortex, we examined the area and strength of the C2-WFR before and after NGF application. In the depleted group, the C2-WFR did not change significantly after topical application of NGF, for either area at half-height (1.38 ± 0.16 mm$^2$ vs. 1.33 ± 0.13 mm$^2$ (mean ± SEM), p>0.05, paired t-test, n=8) or the peak height (2.64x10$^{-4}$ ± 0.25x10$^{-4}$ vs. 2.61x10$^{-4}$ ± 0.30x10$^{-4}$, p>0.05, paired t-test, n=8). In contrast, in the non-depleted group both the area at half-height (1.25 ± 0.13 mm$^2$ vs. 1.59 ± 0.14 mm$^2$, p<0.05, paired t-test, n=9) and the peak height (2.39x10$^{-4}$ ± 0.46x10$^{-4}$ vs. 3.31x10$^{-4}$ ± 0.55x10$^{-4}$, p=0.01, paired t-test, n=9) of the C2-WFR increased significantly after topical application of NGF (see figure 3 ‘augmentation’ for the depiction of this specific plasticity).

To determine that the NGF-induced plasticity of the C2-WFR in the non-depleted group was significantly different from the lack of change in the C2-WFR induced by NGF in the depleted group, we also compared changes in the area at half-height and peak height between depleted and non-depleted rats (figures 5, 6). The change in the area at half-height of the C2-WFR after NGF-application was significantly larger in non-depleted versus depleted rats (36.7% ± 11.2% vs. -0.4% ± 7.5%, p<0.05, t-test, n=17). Likewise, the change in the peak height of the C2-WFR after NGF-application was significantly larger in non-depleted versus depleted rats (37.6% ± 10.6% vs. 0.5% ± 8.4%, p<0.05, t-test, n=17). In summary, if the cortex was depleted of more than 35% of BFCS fibers within the C2-WFR, then topical application of NGF to the cortex did not induce the rapid plasticity of the C2-WFR observed in normal, non-depleted cortex.

Lastly, we examined the time course of the NGF-induced plasticity of the C2-WFR in the non-depleted rats. For the depleted rats, topical application of NGF did not result in any significant changes in either the area at half-height or the peak-height at any time-bin measured (up to 100 minutes post-application, one-way repeated-measure ANOVA, n=8; figure 7). For the non-depleted rats, both the area at half-height and the peak height of the WFR were significantly different from pre-application baseline at the time-bin of 26-50 minutes in the presence of NGF (one-way repeated-measure ANOVA, post-hoc Dunnett’s test, p<0.05, n=9; figure 7). After this time bin, the values remained elevated, but were not statistically different from baseline.

In general, the timecourse of NGF’s plasticity of a WFR appears to be somewhat slower in rats with durotomies reported here, versus rats with durectomies previously reported (Prakash et al. 1996b). The slower timecourse of the NGF-induced plasticity of a WFR may be related to a reduced amount of NGF reaching layer 1 through the durotomies compared to durectomies,
Detection of exogenous NGF penetration to the cortical tissue. To determine how well NGF-penetrated the cortex through the dural slits and holes (durotomies), NGF-IR was assessed in 5 rats (figure 8). We found that NGF-IR was detected in layer 1 (40-80 µm), and thus NGF-IR was detected at a depth that was about one-third the depth of preparations previously reported with durectomies (Prakash et al. 1996b). Additionally, NGF-IR was generally localized in the cortex to the site directly under the slits in the dura. It is likely that NGF spread beyond the dural slits, but low-level NGF-IR throughout the pial layer all over the brain obscured our ability to quantify immunoreactivity at depths of less than ~25 µm below the pial surface.

Discussion

Summary of Results. Several experiments were reported here which tested the hypothesis that the BFCS is involved in mediating NGF’s rapid cortical plasticity. First, we found that the projections of the BFCS can target a single WFR in the cortex and are mostly located in the nBM. Second, we found that TrkA-IR fibers are colocalized with ChAT-IR fibers in the adult rat barrel cortex that likely arise from the BFCS and therefore that by binding to TrkA, NGF has the potential to modulate acetylcholine release from the BFCS. Finally, we found that depletion of more than 35% of the AChE fibers that innervate the C2-WFR prevented the rapid NGF-induced plasticity of the C2-WFR after topical application of NGF to the cortex. Overall, these experiments implicate the BFCS as necessary for NGF-induced cortical plasticity.

Cytochemistry of Basal Forebrain Projection Cells and TrkA cortical fibers. The basal forebrain contains at least two major projection neurons that have fibers in the cortex: parvalbumin-immunoreactive (-IR; GABAergic) and ChAT-IR (cholinergic). The cholinergic fibers are the best candidates for also being the TrkA-IR fibers we observed in the barrel cortex, because previous work has shown that most of the abundant ChAT-IR cell bodies in the basal forebrain are also p75-IR/TrkA-IR (Sobreviela et al. 1994). While there is no question that BFCS cells also express TrkA, there is still no direct evidence that their projection fibers in the cortex co-express ChAT and TrkA. This lack of direct evidence may be related to a low sensitivity of TrkA antibodies (Sobreviela et al. 1994), and even some ChAT antibodies at detecting antigen in the relatively thin cholinergic fibers. Nevertheless, as we were able to detect TrkA fibers in the cortex, co-localization studies with TrkA and ChAT (or VACHt) were then performed. We found that while TrkA-IR fibers were less abundant than ChAT fibers, they were also ChAT-IR (or VACHt) (Figure 1). Because TrkA-IR fibers were found to be ChAT-IR (or VACHt-IR), this provided direct anatomical evidence that NGF can act on cholinergic fibers in the barrel cortex. It is interesting that a recent study on the role of NGF in plasticity of the developing visual cortex, led to the same conclusions (Rossi et al. 2002). In this study, cholinergic afferents to the visual cortex were destroyed by application of the excitotoxic drug quisqualic acid to the BF. Afterwards, using Western blot, TrkA and p75 expression was dramatically but not completely reduced in the cortex, suggesting that in the developing rat these receptors are located on predominantly cholinergic terminals. Taken together, these results generalize the anatomical connections between NGF and the BFCS to both the developing and the adult cortex.

NGF/Basal Forebrain Cholinergic System Functional Interactions. One way to test whether the BFCS is implicated in NGF’s plasticity of a WFR is to directly lesion the BFCS with a specific toxin, such as 192 IgG-saporin, to detect whether NGF can still augment a WFR in the lesioned rat. An alternative way is to pharmacologically block the cholinergic receptors located on the cortical BFCS projections by topical application of a general cholinergic antagonist and
detect whether NGF can still induce rapid plasticity of a WFR in such a pharmacologically blocked cortex. Unfortunately, there is no single general antagonist for both muscarinic and nicotinic cholinergic receptors. Therefore, to perform properly such an experiment at least two general antagonists are needed prior to the application of NGF. This is a complicated experiment because the time courses of the antagonists actions following topical application to the cortex is different, as we previously demonstrated for the topical application of muscarinic and nicotinic receptor agonists (Penschuck et al. 2002). Thus a general cholinergic blockade of the cortex is challenging and therefore the interpretation of the effect of NGF application is questionable under such conditions (Penschuck et al. 1999).

Using the 192 IgG-saporin lesion method described above, we found that in rats with depletion of more than 35% of the AChE fibers that innervate the C2-WFR, topical NGF application did not induce WFR plasticity, thus supporting the hypothesis that the BFCS is necessary for the mediation of NGF–induced rapid plasticity of a WFR. Additional support for functional interactions between NGF and the BFCS has been suggested from research on the developing visual cortex of the rat. Pesavento and colleagues have demonstrated in the developing rat visual cortex that NGF modulation of LTP is mediated by the cholinergic system (Pesavento et al. 2000). In addition, NGF-induced increase of ACh and glutamate release from cortical synaptosomes was strongly impaired after elimination of the cholinergic afferents to the visual cortex (Rossi et al. 2002). Taken together, these results generalize the functional interactions between NGF and BFCS as part of an underlying system important for cortical plasticity in both the developing and the adult cortex. This conclusion, however, seem at odds with a recent study that used transplants of NGF-secreting fibroblasts to restore a reduction stimulus-evoked activity in barrel cortex of the adult rat (Rahimi and Juliano 2001). These authors demonstrate that NGF secreted from the grafted fibroblasts could restore a reduction of evoked-activity in a barrel cortex depleted from cholinergic projections from the BFCS. The restoration of stimulus-evoked activity happened in spite of the fact that the cortex was depleted of more than 80% of AChE. These observations led the authors to conclude that NGF can directly affect cortical activity without the presence of the cholinergic system. The explanation for the apparent difference in conclusions may relate to many technical differences on how the experiments were performed and analyzed. Moreover, there are major differences in what was studied in each experiment. Rahimi and Juliano studied the ability of chronic NGF delivery to restore normal cortical activity in a cortex with more than 80% AChE depletion, while we demonstrated the lack of ability of acute NGF application to induce transient plasticity in a cortex devoid of more than 35% of its AChE fibers. Finally, a recent study using a novel antibody to TrkA reported the existence of TrkA-IR on ChAT-IR neurons in layers II-III and layers V-VI in the rat visual cortex (Tropea et al. 2002). If these finding could be replicated in the somatosensory cortex of the adult rat, and the specificity of this novel antibody further verified, it may help in clarifying the differences between ours and Rahimi and Juliano’s studies. In particular if there are TrkA-IR and ChAT-IR neurons in somatosensory cortex in different cortical layers, there may be different layer-dependent effects. In Rahimi and Juliano’s study the fibroblast grafts were placed in the deep layers of the cortex (1.1 mm depth), while our findings where occurring as NGF was penetrating layer I (see below).

There are at least two other potential ways to complement our studies to obtain a better insight for the role of the BFCS system in NGF’s ability to induce rapid plasticity of a WFR. The first method would be to stimulate the BFCS and observe whether WFR’s plasticity is identical to NGF’s induced plasticity. However, it is currently not possible to specifically stimulate only the BFCS within the BF. Nevertheless, stimulation of the BF (which includes cholinergic, GABAergic and other components (Dykes 1997)) in the rat typically induces enhancements of neuronal activity (Jimenez-Capdeville et al. 1997; Metherate and Ashe 1991, 1993). While it remains to be shown, such enhancements at the neuronal level could, in principle, underlie the NGF-induced plasticity of a WFR. The second way to test whether activation of the BFCS is
implicated in NGF’s-induced plasticity of a WFR is to pharmacologically increase acetylcholine receptor activity. Such experiments are reported elsewhere (Penschuck et al. 2002), and used a protocol similar to the protocol reported here, but instead of NGF, cholinergic agonists such as carbachol or nicotine were topically applied to the durotomized cortex. In these experiments, both nicotine and carbachol caused a transient increase in the area of WFR similarly to NGF. However, NGF and nicotine had identical time-courses for this increase, while carbachol had a slower time-course. In addition, nicotine and NGF both augmented a WFR, by increasing the height of the WFR in addition to the expansion of area, whereas carbachol only expanded the area of a WFR without the increase in height (compare ‘augmentation’ to ‘expansion’ in figure 3 for the depiction of both cases). Overall, these results are consistent with the hypothesis that activation of acetylcholine receptors could explain WFR plasticity. They also suggest that NGF’s rapid plasticity could be mediated predominantly through nicotinic receptors.

Is NGF Rapidly Inducing Plasticity by Activating Nicotinic Receptors in Cortical Layer I?
Previously, we showed that at the time point when the NGF-induced plasticity of a WFR was maximal, NGF penetrated layer I of the somatosensory cortex (Prakash et al. 1996b). In the current study, the more limited penetration NGF further suggests that topical application of NGF can affect evoked cortical activity by inducing acetylcholine release from BFCS projection axons within layer I. Assuming that the sensitivity for the detection of the NGF using immunohistological techniques is optimal, the correlation between the penetration and plasticity could be interpreted in the following way. NGF binding to BFCS projections within layer I causes enhancement of ACh release. Indeed layer I contains the highest laminar densities of ACh axons and varicosities originating from the BFCS (Mechawar et al. 2000) thus providing a rich substrate for NGF action. The target of the cholinergic fibers in layer I could be the apical dendrites of pyramidal and bipolar cells from layer II to V that extend to layer I (reviewed by Nieuwenhuys 1994), or direct activation of neurons located within layer I.

One clue to the type of cholinergic target of NGF is its time course. NGF’s plasticity time course and its characteristics (augmentation) seem identical to nicotine’s (Penschuck et al. 2002). These findings suggest that NGF’s-induced rapid plasticity acts predominantly through activation of nicotinic cortical receptors within layer I. This suggestion is supported by recent findings that demonstrate that while nicotinic receptors can be found in all layers of the adult rat somatosensory cortex they are heavily concentrated in layer I (Levy and Aoki 2002). Indeed, recent evidence demonstrates directly that ACh affects neuronal activity in layer I through nicotinic receptors by showing that almost all of layer I interneurons can be excited by ACh nicotinic agonists. Furthermore, by activating selectively the nicotinic receptors of layer I interneurons, it was demonstrated that the predominant role of these interneurons is to inhibit layer II, III non-pyramidal cells, most likely GABAergic interneurons, and thus probably cause a disinhibition of cortical networks (Christophe et al. 2002). These authors further demonstrate that some of layer I interneurons have indeed direct axonal projections to cortical layers II and III. Indirect activation of layers II and III from layer I has also been demonstrated. Stimulating layer I has been found to induce LTP in layers II/III of the cortex indirectly via the cholinergic processes that also reach layer I (Hess and Donoghue 1999). Taken together, cholinergic activation of layer I by NGF can cause large-scale cortical plasticity in deeper cortical layers through direct and indirect mechanisms that could act in concert and seem to be the result of activation of layer I nicotinic receptors.

Modulatory Versus Trophic Effects of NGF. The presence of NGF in the adult cortex is thought to serve long-term processes, such as survival and maintenance of neurons, as an extension of its role during cortical development. These processes occur after NGF binds to TrkA and/or p75 (reviewed in (Bothwell 1995; Kaplan and Miller 1997)). NGF is rapidly internalized upon binding to p75 (Kiss et al. 1993) and/or TrkA (Grimes et al. 1997; Zapf-Colby
and Olefsky 1998) into a vesicle that is transported back to the cell body where its trophic actions occur via second messenger cascades within the cell body and nucleus (recently reviewed in Sofroniew et al. 2001). However, initially upon binding, and throughout the transport process, within this vesicle the NGF/receptor complex actively induces second messenger pathways. Moreover, recently evidence has accumulated to suggest that the signals induced by NGF may play other roles within the cortex that result in more immediate effects such as increasing synaptic efficacy. For example, in slices of rat visual cortex NGF induced a rapid increase in the height of impulse-evoked excitatory postsynaptic currents through AMPA and NMDA receptors, within 5-15 minutes after NGF application linking NGF action to fast modulation via glutamate release (Carmignoto et al. 1997). The authors speculate that the NGF action is mediated presynaptically through severed cholinergic nerve projections in the slice. In addition, adult cortical cell cultures showed rapid (within 10 minutes) activation of calcium-dependent potassium channels by NGF (Holm et al. 1997). Taken together, these results illustrate that NGF is capable of inducing plasticity of adult neurons on several levels. First, initially upon binding to TrkA, NGF induces acute changes in cortical activity through changes in synaptic efficacy; such changes may continue to be induced while the NGF/receptor complex is transmitted back to the cell body. Once the complex reaches the cell body there are also induced changes in protein expression. Hence in adult rat brain, NGF can rapidly augment evoked sensory activity by modulating activity within the BFCS axons, as well as maintaining the survival of the BFCS cells.

**NGF-induced Plasticity: A Proposed Model for Cortical Modulation of a Diffuse Modulatory Projection System.** The cortex is generally portrayed as a passive recipient of modulatory systems projections including cholinergic, dopaminergic, noradrenergic, serotonergic and histaminergic diffuse systems. Our findings implicate the cortical projections of the BFCS in the plasticity induced by NGF and therefore show that under certain conditions, the sensory cortex is not just a passive recipient of the cholinergic projections but an active participant in modulation of their activity, a modulation resulting in a large-scale plasticity of a WFR. Therefore by enhancing release of NGF the cortex can transiently augment sensory representations like WFRs by recruiting additional neurons to respond to a stimulus, resulting in the expansion of the area of a representation, and by enhancing the strength of their responses, resulting in the increased height of the representation. It will be interesting to find out whether such cortical modulation of a diffuse modulatory system is unique to the cholinergic system or whether the cortex can also similarly modulate other diffuse modulatory systems. An interesting prediction of such a model is that the cortical outcome of NGF-induced plasticity should be different than the outcome expected from directly enhancing BF activity (i.e., a direct BF stimulation experiment). In the case of NGF-induced plasticity the cortex modulates only on the BFCS projection fibers within the cortex. In contrast, directly stimulating the BF enhances unselectively the activity of all the different systems that reside within the BF (including the BFCS) and therefore would result in a different cortical activation pattern compared to the selective enhancement of the cholinergic projections inside the cortex itself.

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**Figures:**

**Figure 1:** *TrkA-IR and ChAT-IR co-localize in Nucleus Basalis of Meynert cells and in cortical fibers.* Thin sections of a rat brain were double stained with specific antibodies to ChAT and TrkA. **Top,** layer I of the barrel cortex. ChAT-IR fibers are present near the pial surface of the cortex (upper right). Of these fibers, a few are also immunopositive for TrkA (center panel), as shown by the overlaid image (yellow; see arrowheads). **Middle,** TrkA-IR fibers found in layer V of the barrel cortex. While ChAT and TrkA immunoreactivities are localized to individual fibers (arrowheads), their localized distribution within the fiber is not necessarily overlapping/restricted to the same area. **Bottom,** Immunostaining of a region of the substantia innominata in adult rat nBM (coronal section) shows large ChAT-IR cells that are also immunopositive to TrkA. These images are typical of all rats examined, and similar results were obtained using an antibody to vesicular acetylcholine transporter.
Figure 2: Retrograde labeling studies and cholinergic fiber depletion quantification. Bottom, Dil injection into the supragranular layers of the C2-whisker functional representation yields retrogradely labeled cells predominantly in the ipsilateral nucleus basalis of Meynert (nBM). Coronal section located –1.4 mm posterior from Bregma; right side is schematized, left side is stained for AChE (Brain atlas picture: adapted from (Paxinos and Watson 1986)). The location of the Dil injection site within the C2-WFR supragranular layers is depicted as a red spot under the red arrowhead (shown in a color coronal section stained for CO located -3.0 mm posterior from Bregma). Red dots grossly schematize that the ventral portion of the globus pallidus (GP) had the highest density of retrogradely labeled cells, however similar cells were seen just ventral in the substantia innominata (S.I.) as well as a few scattered cells nearby in the internal capsule (IC). This cell cluster between GP, IC, and S.I. is also known as the nBM. Inset, a photomicrograph of a Dil-labeled cell body (diameter 20 µm) in the nBM following Dil injection. Middle, composite images of coronal sections stained for AChE for control cortex (left) and 192 IgG-saporin injected cortex (right) from one rat at 10X magnification showing the cortical layers I-VI. Note that typically AChE staining revealed fibers 5 times as abundant as ChAT-IR fibers and 15 times as abundant as TrkA-IR fibers (compare to figures 1 and 4). These slices were adjacent slices the CO-stained slices (see bottom of figure) which enabled the depiction of the location of the C2 barrel (green outline). Top 20X magnification of the same slices from the middle panels. Each yellow dot overlies a fiber crossing (dots enlarged for visibility in this figure) through 100 µm lines drawn in layer I and layer IV above and within the C2-barrel. Right side shows the AChE-fiber depleted cortex after local injection of 192 IgG-saporin. In this case the AChE fiber depletion was 80%.
Figure 3: Depiction of potential plasticity of a WFR. A WFR can change in area and/or height. The central 3D-rendered image is a typical pattern of activity for a normal WFR, with green depicting baseline activity and the red peak depicting the maximal activity. A WFR can change after manipulations that induce cortical plasticity. For example, the upper right image depicts an increase in height and area of a WFR, which we term “augmentation,” such plasticity was observed after NGF application (Prakash et al. 1996a, b) and nicotine application (Penschuck et al. 2002). Other types of plasticity have also been observed in WFRs, such as an “attenuation” after BDNF application (Prakash et al. 1996a, b) and an “expansion” after carbachol application (Penschuck et al. 2002).
Figure 4: Depleting the cortex of more than 35% of AChE fibers prevents NGF-induced augmentation. **Top,** Three weeks after cortical IgG-saporin injection and immediately after the imaging session, AChE fiber depletion was calculated by counting fiber crossings (see figure 2). This graph depicts the average number of fiber crossings in layer 1 and 4 for the uninjected control hemisphere and the IgG-saporin injected hemisphere. The white diamonds depict the 6 rats with more than 35% AChE fiber depletion, the black diamonds depict the 3 rats that had 0-25% depletion. **Bottom,** This graph depicts that in cortex with AChE fiber depletion of less than 25%, NGF application augmented a WFR (dark diamonds), whereas in cortex with AChE fiber depletion greater than 35%, NGF application did not augment a WFR (white diamonds).
Figure 5: NGF-induces significant plasticity of a WFR in non-depleted cortex (black triangles), but not in AChE depleted cortex (white dots). Small symbols represent changes from individual rats while large symbols depict the averages and standard error of the mean for the two groups. These two groups were statistically different from each other both for area at half-height and for peak height (see text for details).
**Figure 6**: NGF-induces significant plasticity of a WFR in normal cortex (black symbols), but not in AChE depleted cortex (white symbols). These graphs depict the actual values, not percent normalized (as in figure 5), for the peak height and area at half-height values in individual rats before and after NGF in AChE depleted and non-depleted cortex. Dark lines depict the average trend for each graph.
**Figure 7**: Time-course of NGF’s plasticity in AChE depleted vs. non-depleted cortex. The gray bars denote the average area at half-height (top graphs) or peak height (bottom graphs) of a WFR before NGF application. The white and black bars are the averages at successive time bins in the continual presence of NGF. In cortex with depleted AChE fibers (left graphs, white bars) NGF did not increase either the area at half height or the peak height even after 100 minutes of application. In contrast, in non-depleted cortex (right graphs, black bars), NGF significantly increased the area at half-height and peak height ~30 minutes after its topical application to the cortex. Error bars denote SEM, * denotes p<0.05 vs. pre-application time bin.
**Figure 8:** NGF penetrates the cortex through holes or slits in the dura and arachnoid mater (durotomy). **Left,** NGF-IR (red arrows denote obvious penetration areas) is seen as green in this fluorescent photomicrograph of the first 60 µm tangential section from the pial surface in the barrel cortex. The black and white inset is an image of the whole cortex during the imaging session. The thin black ovals are the sites of the three dural slits; a fourth small hole was also made in this rat at the red arrow. NGF-IR through that hole is shown here. The dark black circle approximates the area at half-height border of the C2-whisker functional representation. The white arrowhead points to a reference surface vein in both the inset and the photomicrograph. **Right,** NGF-IR was detected under a dural slit as green in these two fluorescent photomicrographs of one 40 µm coronal section in the barrel cortex of a different rat. The right image shows the mediolateral extent of visible NGF-IR under a dural slit (between red arrows). The left image is a higher power image taken from the region indicated by the area outlined in white, and demonstrates that in this rat NGF-IR was detectable about 50 µm below the pial surface. Control slices not shown here had staining patterns similar to the regions outside the red arrows.

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