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31. Cell preparations were done in serum-free medium. We have found that a tiny number of contaminating dendritic cells is sufficient to immunize rather than induce tolerance in a certain proportion of female mice. Thus, unless care is taken to rigorously remove these cells, many populations of red cells with ammonium chloride buffer and of T cells with antibodies to Thy1.2 (J1j.10), CD4 (RL172.4), and CD8 (3.155) and guinea pig complement. After passage over two sequential Sephadex G10 columns (Pharmacia), the cells were centrifuged over discontinuous Percoll gradients containing 60% and 70% layers. Cells at the 60 to 70% interface were used for injection. B cells prepared this way were 96.7 to 99.7% B220<sup>+</sup>, <1% CD4<sup>+</sup> or CD8<sup>+</sup>, <1% NK1.1<sup>+</sup>, and were considered free of macrophages or dendritic cells by virtue of their inability, when given 3000 R irradiation, to stimulate allogeneic mixed-lymphocyte reactions or the proliferation of T cell clones (Fig. 3) (14). For LPS-stimulated blasts, purified resting B cells were cultured (4 × 10<sup>6</sup> cells per 2-ml well) for 72 hours in complete medium (CM) containing LPS (50 mg/ml) (*Escherichia coli* O55:B5, Difco, Detroit, MI) and washed four times in phosphate-buffered saline (PBS) before further use. To purify dendritic

cells, we layered spleen suspensions onto plastic dishes (2 spleens per dish; Falcon, Lincoln Park, NJ), in 7 ml of Eagle's Hanks amino acid medium (EHAA) and 1% normal mouse serum (NMS). After 90 min at 37°C, nonadherent cells were removed, the plates were rinsed, and the medium was replaced. After another 8 hours at 37°C, nonadherent cells were collected and centrifuged over a 50% Percoll gradient. Buoyant cells were recovered, washed in EHAA and 1% NMS, and resuspended in PBS for injection. By fluorescence analysis, cells were 83% 33D1<sup>+</sup> (a dendritic cell-specific marker).

32. For proliferation, 10,000 Rachel cells were stimulated in 0.2-ml cultures with graded numbers of stimulators for 48 hours. <sup>3</sup>H-thymidine (5 μCi/ml) was added for the last 12 hours. For killing, 4 × 10<sup>6</sup> responder spleen cells were cultured for 5 days against 2 × 10<sup>6</sup> irradiated (3000 R) stimulator spleen cells in 2 ml of EHAA medium containing 10% fetal calf serum, 50 mM 2-mercaptoethanol, and antibiotics (CM). They were then harvested and tested for cytotoxic activity by the JAM Test, an assay for CTL activity [P. Matzinger, *J. Immunol. Methods* 145, 185 (1991)].

33. We thank S. Guerder for the Rachel T cell clone; A. Bendelac, R. Germain, R. Palacios, W. Paul, R. Schwartz, and A. Singer for their helpful suggestions; and C. Chen for expert technical assistance. This work is dedicated to the memory of Daniel Galos Fuchs.

8 June 1992; accepted 19 August 1992

## TECHNICAL COMMENTS

### Perceptual Correlates of Massive Cortical Reorganization

T. P. Pons *et al.* (1) found cortical maps to be capable of an unexpectedly large degree of reorganization. After long-term (12 years) deafferentation of one limb in adult primates, the cortical area of the brain corresponding to the limb became responsive to stimuli applied to the lower face region. This finding extended the previously recognized maximum area of cortical reorganization in adult primates from a mediolateral distance of 1 to 2 mm to about 10 to 14 mm. Because cells that originally received information from the arm can later receive input from the face, we wondered whether stimuli applied to the face would be mislocalized to the arm. To explore this, we have studied (2, 3) localization of touch sensations in two human patients after amputation of one upper limb and in one patient after amputation of one digit.

We applied light touch (using a cotton swab) or deep pressure to different points on the normal body surface. Stimulation of points even remote from the amputation line evoked precisely localized referred sensations in the phantom limb. We could plot "reference fields," small regions of skin surface that evoked referred

sensations in specific parts of the phantom limb (for example, the digits). Our main experimental findings may be summarized as follows:

(i) Points (reference fields) were not randomly distributed. There were two clusters, one on the same side of the face as the phantom limb and one around the line of amputation. Furthermore, there was a precise one-to-one correspondence between these points and those on the phantom limb (3). (ii) Sensations were referred most often to the hand, especially to the digits with an overrepresentation of the thumb and "pinkie." This may reflect the high cortical magnification of these areas. (iii) The referred sensations were modality-specific; for example, a drop of warm water trickling down the face was felt as "warm water trickling down" in the phantom hand. (iv) Reference fields were somatotopically organized. We suggest that this is a direct consequence of the remapping observed by physiologists (1). (v) There was a vivid persistence or short-term "memory" of complex sensations; when we gripped and released the finger adjacent to an amputated finger the patient felt the phantom finger being "gripped,"

and this sensation persisted for 7 or 8 seconds in the phantom. (vi) Reorganization was relatively rapid. In one patient, our study was carried out 4 weeks after limb amputation rather than 12 years.

That patients "refer" paresthesiae to a "phantom limb" is in itself not new. We have attempted to systematically relate such findings to studies of animal physiology (1, 4). For example, we suggest that the reason we found two clusters of reference fields exhibiting topography—one on the face and one near the amputation line—is because the hand area in Penfield's homunculus (in the somatosensory cortex) is flanked on one side by the face and on the other side by areas around the line of amputation (for example the upper arm and shoulder). We would therefore expect sensory input from both these regions to "invade" the cortical hand area and provide a basis for referred sensations.

The very existence of phantom limbs might be partially explained by our hypothesis. If tactile and proprioceptive input from surrounding tissue "takes over" the brain areas corresponding to the amputated limb, spontaneous discharges arising from neurons innervating these tissues would be misinterpreted as arising from the missing limb. This hypothesis is different from, although not incompatible with, the idea that phantom limbs result from the persistence of a "neurosignature" in a diffuse neuronal pool (5). Our observation that the changes can occur as early as 4 weeks after amputation is especially interesting since it suggests that the reorganization is a result of the unmasking of "silent" synapses rather than of anatomical changes such as "sprouting." Perhaps, even in normal adults, input from the face projects simultaneously to both face and hand areas in the cortex or thalamus (and input from the hand to both hand and face areas). The unwanted input to the hand area, however, may be subject to tonic inhibition (for example, through an inhibitory interneuron) by the "correct" axon carrying a signal from the hand. When an arm is amputated, this occult input is unmasked through disinhibition. It remains to be seen whether this unmasking is permanent or whether the patients eventually begin to "ignore" the referred sensations.

Whatever the ultimate interpretation may be, however, our findings suggest that the adult mammalian brain has a latent capacity for much more rapid functional reorganization over a much greater area than previously thought, a capacity that could conceivably be exploited for therapeutic purposes.

V. S. Ramachandran  
D. Rogers-Ramachandran  
M. Stewart  
Brain and Perception Laboratory,

Department of Psychology,  
University of California, San Diego,  
La Jolla, CA 92093-0109

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27 May 1992; accepted 1 September 1992

**Response:** The preliminary observations of Ramachandran *et al.* are remarkable for they not only suggest that areas of the brain that undergo reorganization after peripheral or central damage are capable of mediating tactile perception, but also that central mechanisms alone can be responsible for sensations in phantom limbs.

It has often been suggested that incidental stimulation of neuromas immediately adjacent to an amputated region is responsible for phantom sensations in a missing limb. Generally, representations of adjacent body parts are located in adjacent regions of cortex. Ramachandran *et al.* have taken advantage of the fact that the cortical representation of the face is not adjacent to represen-

tations of adjacent body parts, but is instead adjacent to the representation of the upper limb. They were thus able to dissociate the effects of stimulating neuromas, which are located at the end of an amputation, from those of stimulating the adjacent cortical body representation. Stimulation of neuromas was clearly not necessary for the perception of phantom sensations, but activation of cortex that had reorganized to respond to inputs from the face was apparently sufficient for such perception.

The mechanism by which such reorganization of the brain takes place is not yet clear. With regard to the rapidity of the phantom limb being activated by touching the face, I agree with Ramachandran *et al.* that sprouting of new inputs seems unlikely, but an unmasking of preexisting inputs also seems unlikely because anatomical studies have repeatedly demonstrated that areas of cortex representing the hand do not receive connections from regions of the brain representing the face. Whatever mechanism is ultimately found to be responsible for the reorganization, Ramachandran *et al.* have made an enormous contribution by showing that such reorganized cortex is capable of processing sensory inputs so that they result in tactile perception.

Tim P. Pons  
Laboratory of Neuropsychology  
National Institute of Mental Health  
Bethesda, MD 20895

21 July 1992; accepted 1 September 1992

## Structural Similarity Between Transforming Growth Factor- $\beta$ 2 and Nerve Growth Factor

The crystallographic determination of transforming growth factor- $\beta$ 2 (TGF- $\beta$ ) reported by S. Daopin *et al.* (1) offers an exciting insight into the structures adopted by this superfamily. However, the fold may not be as unusual as first anticipated. I have analyzed the major details of this topology and have found them to be remarkably consistent with the structure of nerve growth factor (NGF) that was published last year by N. Q. McDonald *et al.* (2).

The most obvious global similarities between the two structures are, first, the long looping antiparallel strands that lead to the unusual absence of a traditional hydrophobic core and, second, their homodimeric form. In addition, the strict conservation of nine cysteines per monomer reported for the TGF- $\beta$  family is mirrored by an equally important set of six cysteines in NGF. All six cysteines from NGF appear to have equivalent sequential and structural locations in TGF- $\beta$  and form their disulfide bonds with equivalent partners (Fig. 1).

A major structural difference between these proteins is the absence, in NGF, of three helices and three disulfide-forming cysteine residues. These differences are particularly significant, as they are interdependent. The extra intrasubunit disulfide bond in TGF- $\beta$  forms a bridge between helix  $\alpha$ 1 and strand  $\beta$ 1 and seems to be vital for amino-terminal stabilization (Fig. 1). As this helix is absent in NGF, the extra disulfide is not required. The remaining cysteine, which forms an intersubunit disulfide in TGF- $\beta$ , is absent in NGF and will probably reflect the different packing arrangements adopted by each homodimer.

The absence of all three helices in NGF would appear to be of only peripheral importance to the core topology, as they are all located in, or near, loop regions. Similar insertions and deletions have been observed in other topologies with low sequence identities, such as plastocyanin (3) and azurin (4), where they also have little effect on the overall fold.