Quantitative Aspects of Corticocortical Connections: A Tracer Study in the Mouse

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This study provides neuroanatomical data relevant to models and simulations of the propagation of activity over the cortex. We administered small injections of the anterograde tracer biotinylated dextran amine to various regions of the mouse cortex (1 per animal). Two-dimensional reconstructions of the cortical surface were made, showing the distribution, size, and density of the terminal fields. Within the injected hemisphere, the largest part of the terminal field always surrounded the injection site and extended over neighboring areas. On average, axons from injection sites of \( \leq 0.1 \text{ mm}^2 \) (containing several thousand neurons) diverged onto a region about 180 times larger than the injection site. The density of stained fibers in distant terminal fields could reach about 25 \( \text{m/ mm}^2 \). More than half of the total terminal field from an individual injection site consisted of weak projections with densities of 3 or 4 \( \text{m/mm}^2 \). The number of main axons entering an individual distant terminal field ranged between 14 and about 890. By indirect arguments we estimate that the density of stained fibers close to the injection site is 3–6 times that in the most densely labeled distant terminal fields. In addition to symmetric projections to the opposite hemisphere, nonhomotopic callosal projections were found.

**Keywords:** anterograde, cortex, dextran amine, divergence, fiber density

**Introduction**

One of the most outstanding properties of the cerebral cortex is its high degree of connectivity within itself. The great majority of the synapses in the cortex are synapses between cortical neurons (Cragg 1967; Braithberg 1974; 1978a). Broadly speaking, there are 2 kinds of connections: local connections, which knit the cortical neurons together into one huge continuous network, and distant connections, which connect distant places directly to each other in a rather selective way (e.g., Jones and others 1978). All types of neurons seem to participate in the local connections via axon collaterals ramifying within the gray matter. Distant connections are made almost exclusively by pyramidal cells (in which we include the spiny stellate cells) either via the white matter or via long fibers within the gray matter. (For occasional reports on distant nonpyramidal connections, see Peters and others 1990; McDonald and Burghalter 1993.) We will see that—at least in the mouse—the 2 systems of local and distant connections are not neatly separable.

The present study seeks to contribute to the understanding of the global connectivity of the cortex. Considerable research has already been made in this field, in days gone by, with the degeneration methods or with “physiological neuronography” (e.g., Bailey and others 1950) and, more recently, with the development of tracer methods. We therefore have a fairly good idea as to how the cortical areas are connected to each other. In the present study we shall not deal with the connectivity between particular areas but rather address the modelers who are concerned with the mechanisms of propagation of activity over the cortex (e.g., Abeles 1991; Köttter and Sommer 2000; Gerstein and Kirkland 2001; Hehl and others 2001; Knoblauch and Palm 2002a, 2002b; Mehrling and others 2003; Robinson and others 2003; Izhikevich and others 2004). Therefore we focus as far as possible on quantitative aspects.

With the aid of small injections of an anterograde tracer, we seek to answer the following questions. 1) How large a part of the cortex can be reached by the neurons located in a small cortical “column”? (See Size of Terminal Fields in the Injected Hemisphere.) 2) What is the density of fibers in a distant terminal field? (See Fiber Densities.) 3) What proportion of the neurons in such a column projects to an individual distant projection site? (See Numbers of Neurons Projecting to a Distant Spot and Number of Neurons at the Injection Site.)

We used the anterograde tracer biotinylated dextran amine (BDA; Veenman and others 1992), and our experimental animal is the mouse. We chose this animal for 2 reasons. First, its small brain makes it easy to investigate the whole cortex for each tracer injection, and second, the results can be combined with other quantitative data gained in previous studies (summarized in Braitenberg and Schütz 1991).

Preliminary results have been published in Schütz and Liewald (2001) and in Chaimow and Schütz (2004). A similar study using a retrograde tracer was made previously in our laboratory (Greilich 1984; Palm 1986).

**Materials and Methods**

**Experimental Animals and Tracer Injections**

Adult white female laboratory mice (21 animals, at least 4 months old) were used for this study. Each animal received one iontophoretic injection of 10% BDA (BDA-10.000; Molecular Probes, Mo Bi Tec, Göttingen, Germany) in 0.01 M phosphate buffer, each in a different position of the neocortex, such that samples from anterior, posterior, dorsal, and lateral regions were obtained. The exact position of the injection was later determined on the histological sections and localized with reference to the map of Caviness (1975). The injections were placed approximately at middle depth of the cortex that, depending on the particular area, corresponds to lower layer III to upper layer V.

For the injection procedure and the histology, we largely followed the protocols by Veenman and others (1992) and Dolleman-Van der Weel and others (1994). (The deviations described below were probably not crucial for the success of the procedure.) The injections were performed under deep anesthesia, using a combination of ketamine hydrochloride and Rompun (xylazin hydrochlorid), intraperitoneally. The glass electrodes had outer tip-diameters of 20–30 \( \mu \text{m} \). The tracer...
was applied in pulses of 5-μA positive current (7 s on, 7 s off) for 15 min. After a survival time of 7 days, the animals received a lethal intraperitoneal injection of 33% chloral hydrate and were first perfused with 0.9% NaCl, containing 0.05% heparin, and then with the fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4).

**Histology**

The brain was removed from the skull the next day and kept overnight in a 30% solution of sucrose in phosphate buffer on a rotator in the refrigerator. The brain was then cut with a freezing microtome into coronal sections of 50-μm thickness. Alternating sections were collected for BDA and Nissl stain in phosphate-buffered saline (PBS; the phosphate buffer was 0.01 M). In some cases where a second tracer (rhodamine-conjugated dextran amine) had been injected (not evaluated in this study), 3 alternating series were collected.

The Nissl stain was carried out with cresyl violet on mounted sections. The sections for the BDA stain were kept in PBS in the refrigerator for up to 4 days. They were then rinsed several times in cold PBS (3 × 10 min) and kept for 1 h in PBS containing 0.5% Triton X-100. For this and all the following steps, the sections were stored in the refrigerator on the rotator. BDA was visualized using the avidin-biotin complex method (ABC-kit PK-4000 Vectastain, Linaris, Wertheim-Bettingen, Germany) and diaminobenzidine (DAB). The sections were kept overnight in the ABC solution in darkness. They were then rinsed several times (2 × 10 min) in PBS and for 10 min in 0.1 M phosphate buffer. The DAB reaction was carried out according to the instructions of the substrate kit for peroxidase (Vector, SK-4100, Linaris, Wertheim-Bettingen, Germany) for about 15 min. The sections were then rinsed several times in phosphate buffer, mounted, air-dried, dehydrated, and covered in Despex.

**Shrinkage**

We did not make systematic measurements on shrinkage of the tissue. However, to relate our measurements to data gained on different material, a short reflection on the shrinkage of the material used in the present study is in order.

There was no difference in size between adjacent BDA- and Nissl-stained sections. The shrinkage of Nissl-stained frozen sections was determined in an earlier article (Schüz and Palm 1989). There, we found a linear shrinkage of the sections to 86% in the 2 dimensions parallel to the plane of section. However, in contrast to our assumption in the previous study, this is not true of the third dimension, which a later study (Schüz A, unpublished data) showed to suffer a linear shrinkage to only 95%. The linear shrinkage factors relevant to the present study are therefore 0.88 in the 2 dimensions of the plane of section and 0.95 in the direction perpendicular to it. The volume shrinkage is then 0.74.

**Two-Dimensional Surface Representations of the Terminal Fields**

Two-dimensional maps of the cortical surface were reconstructed from 10 brains as follows: in a first step, the outlines of every second BDA-stained coronal section were drawn under a table projector at magnification for less densely stained terminal fields. These were then inserted on the drawings with the aid of the landmarks. In a second step, the sections were scrutinized under the microscope at higher magnification for less densely stained terminal fields. These were then inserted on the drawings with the aid of the landmarks. In a third step, the sections were scrutinized under the microscope at higher magnification for less densely stained terminal fields. The volume shrinkage factor relevant to the present study is 0.74.

**Areal Measurements**

On 8 of these maps (Figs. 4 and 5), the size of the terminal fields within the injected hemisphere and the total area of the neocortex of one hemisphere (but also including the cingulate cortex and the presubiculum) were measured. The measurements were carried out with a digital planimeter.

**The Problem of Retrograde Staining**

BDA is mainly, but not exclusively, an anterograde tracer. Individual retrogradely stained neurons are sometimes found in the sections, particularly in the vicinity of the injection site (e.g., Fig. 1). Some of them have a beautiful Golgi-like appearance, with extensive staining of axon collaterals. In most cases, these collaterals could be distinguished from anterogradely stained fibers emanating from the injection site, and so they did not affect the assessment of direct anterograde staining.

However, we cannot exclude that some of these neurons had been stained via an axon collateral and that the tracer then moved on into the distant axonal tree. Such a retrograde/antierograde staining of a neuron located outside the region of uptake (via cell bodies and dendrites) may occasionally simulate an increased divergence if its distant axonal tree does not coincide with those of neurons located within the region of uptake. To minimize this problem, we took the following precautions: 1) the maps on Figures 4 and 5 were made from preparations containing only a few retrogradely stained neurons, 2) we excluded from the maps terminal fields consisting of very few fibers, and 3) The borders of weak projection zones were cut off in Figures 4 and 5 at a corresponding threshold.

**Size of the Region of Uptake**

The size of the injection, that is, the size of the region within which were located the cell bodies of the neurons that have taken up the tracer (via the cell bodies or the dendrites), was defined as the width of the band of descending axons (see Fig. 1).

**Quantification of Fibers in the Terminal Fields**

This was first performed in a semiquantitative fashion. The result is represented in Figures 4 and 5. The regions of highest density (category 1) were visible with the naked eye and are drawn black on the figures. Dense hatching (category 2) represents the regions that were visible under the low-power table projector. Those regions indicated by wide hatching (category 3) contained fibers that could only be recognized under the microscope. The arrowheads in Figures 1 and 3 indicate borders between these 3 categories. These categories (strong, middle, and weak) reflect the amount of stained fibers under a given surface area of cortex, in that dense hatching can either stand for a high density of fibers in one layer or a lower density over several layers. In some cases (Fig. 5 b), it seemed appropriate to make 4 categories, where the 2 middle ones can be considered as subdivisions of category 2 in the other cases.

In 2 of the brains, we made a more precise quantitative assessment of the terminal fields by actually measuring the fiber densities in them. Fiber densities can be measured as length densities. Length density is the total length of a filiform component in a given volume divided by that volume.

We used standard stereological methods to obtain length density estimates (e.g., Hennig 1963a, 1963b, summarized in Weibel 1979; Österby and Gundersen 1988; Mayhew 1991; Russ and Dehoff 2000) and adapted them to our specific task. Circular test lines were superimposed onto the microscopic image (Fig. 2), and their intersections with the stained axons were counted. Length density estimates can be obtained from the number of intersections by the following formula:

\[ L_v = \frac{2N}{\ell t} \]  

(1)

where \( L_v \) is a length density estimate, \( N \) the number of intersections, \( \ell \) the length of the test line, and \( t \) the thickness of the section, in this instance 50 μm. A derivation of this formula can be found in the Appendix. The circular shape of the test lines guarantees that the sampling does not have any directional preference in the plane of section. For the above formula to hold, it had to be assumed that axonal orientations in any plane parallel to the cortical surface, that is,
perpendicular to our vertical sections, are uniformly distributed. (For assumption-free approaches, see Cruiz-Orive and Howard 1990; Gokhale 1990; Larsen and others 1996; Mouton and others 2002.)

The measurements were made with the aid of a computerized system (Eutectic Neuron Tracing System), connected to a microscope, using an objective lens \( \times 40/1 \). By focusing through the section, we could ensure that all intersections were recorded, regardless of the depth within the section. Four symmetrically arranged circular test line segments, each a quarter of a circle, were placed onto the densest spot of a region of termination (Fig. 2). Using the above formula, an estimate for the mean density was calculated.

**Results**

Figure 3 shows a coronal section close to the site of injection in the right hemisphere. Axon collaterals radiate from the injection site within the gray matter, and distant terminal fields can be seen in both hemispheres and in the thalamus (Th). The axons are completely filled up to—and probably also within—their terminal fields.

**Figure 1.** Coronal section, showing the fiber stain close to an injection site. Numbers and arrowheads on the surface of the cortex illustrate the 3 different categories of fiber density used in this study. Long arrows indicate the width of the region of descending axons. The width of this region was taken as the diameter of the region of uptake (Table 1). A limited number of retrogradely stained neurons (upper left) can occur beyond this region. Bar: 100 \( \mu m \).

**Figure 2.** Micrograph showing BDA-stained fibers in a terminal field and the circular test lines superimposed onto them.

**Figure 3.** Example for the appearance of the fiber stain on a coronal section through the telencephalon of the mouse, close to an injection site. Apart from the large terminal field close to the injection site in the right hemisphere, a distant terminal field can be seen in both hemispheres, as well as a terminal field in the thalamus (Th). The numbers and arrowheads on the surface of the cortex indicate the 2 different categories of fiber density that can be recognized at low magnification. Magnification of figure is \( \times 7.5 \).

**Results**

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**Figure 4.** Two-dimensional surface maps of the cortex (neocortex and part of the allocortex) of 6 mice. They are arranged according to the macroscopic location of the injection: central region of the dorsolateral convexity (a–c), lateral (d), anterior (e), posterior (f). Right and left maps correspond to right and left hemispheres, respectively, rostral is at the top. The straight vertical line in each map corresponds to the border between dorsal and medial cortex, the elongated gray field medially corresponds to the corpus callosum (cc), the elongated gray field laterally is the olfactory tract (ot). The pyriform and entorhinal cortex are located laterally of the dotted line (the dotted line marks the dorsal border of the dark layer II of these allocortical areas, except for the most rostral part where it marks the location of the rhinal fissure). The medial piece caudally of the corpus callosum is the transition zone to the subiculum and hippocampus, both of which are not included on the maps. The white spot within the black field indicates the region of uptake. In (a) and (b), the injection was placed into the left hemisphere, in the other cases into the right hemisphere. The black region indicates the region of highest fiber density, and the regions of dense and wide hatching correspond to middle and low fiber densities, respectively.
Figures 4 and 5 show patterns of anterograde staining on maps of the flattened cortex as described in Methods. For each animal, both hemispheres are shown. In 6 animals, the injection was administered into the right, and in 2 animals into the left hemisphere. The black regions correspond to the highest density of staining, dense and wide hatching to progressively lower densities. The white spot in each figure shows the location of the injection site. Its size indicates the extent of the region of uptake as defined in Methods.

Injected Hemisphere
In spite of the large variation in projection patterns, some properties are common to the various injections: a large field of stained fibers around the injection and patches or bands at more distant places, numbering between 1 and 8 in the injected hemisphere. In the large field around the injection site, the overall density of stained fibers decreases toward the periphery, although denser patches sometimes appear within regions of lower density. The core of highest density (black on Figs. 4 and 5) extends about 500 μm (sometimes more) from the injection site and is due to the bulk of axon collaterals of the neurons affected by the injection. The adjacent hatched regions are fed by fibers running horizontally over long distances and by other fibers that take a bow-shaped course through layer VI, some of them scratching the white matter.

In distant terminal fields, individual axons can ramify such that they contribute fibers to several spots located closely to each other. This is, for example, the case in the 3 densely hatched lateral spots in the right hemisphere in Figure 5a.

Comparison with the Caviness Map
It is, of course desirable to have some idea of the cortical areas involved in Figures 4 and 5. Figure 6a shows the classical map in which Caviness (1975) has adapted the scheme of Brodmann (1909) to the mouse neocortex. On this map, we have marked the locations of the injection sites of the brains in Figures 4 and 5 (Fig. 6b). The localization was made by identifying the cortical areas on the Nissl sections according to the cytoarchitectonic criteria used by Caviness (1975). The shape of the map differs from that of the neocortex in our maps by its larger rostrocaudal extent. This is due to the different treatment of the frontal and occipital pole regions: flattening by distortion in the Caviness map, cutting the tip of the poles, and keeping the rest metrically correct as far as possible in our maps. By means of the landmarks indicated in Figure 6b, it is possible to localize approximately some of the terminal fields.

Some observations shall be mentioned. 1) Although the dark field around the injection site can be restricted to the corresponding cortical area, the large terminal field in continuity with it always extends over adjacent areas. 2) There is a striking similarity in the projection patterns in Figure 4b,c. This is obviously due to the fact that these injections were located in the same cytoarchitectonic area. Both injection sites project to a rostromedial region (area 6/8) and laterally to an elongated field comprising the border between area 35 and the adjacent entorhinal cortex and to a shorter elongated field of higher density, which according to the cytoarchitectonic criteria described by Caviness is at least partly contained in area 14. Both injections are located just caudally to the barrel field. 3) The 3 lateral spots in the right hemisphere in Figure 5a are located in different areas: areas 40 and 14. 4) No fiber stain could be found in the pyriform cortex, and in the entorhinal cortex fiber stain was restricted to its lateral border (e.g., Fig. 4b,c). Fiber stain was occasionally found in area 27, the presubiculum (medially posteriorly in Fig. 4f). 5) The small hatched field close to the olfactory tract in the right hemisphere of Figure 5b is located in the orbital region of the frontal pole (area 11).

Size of Terminal Fields in the Injected Hemisphere
Measurements on the size of the terminal fields in the injected hemisphere were made on the maps shown in Figures 4 and 5.
The field in continuity with the injection site was always larger than the sum of the remaining terminal fields (Table 1). In some cases, nearly all projections merged into one large field (Fig. 4e and 5b).

Table 1 shows the results of the measurements (not corrected for shrinkage). In column 1, the sum of the areas of all terminal fields in the injected hemisphere is reported for each of the 8 brains. Columns 2 and 3 show separate values for the areal size of the large terminal field in continuity with the injection site and the sum of the distant terminal fields. Columns 4-6 show separate values for the sums of the strong (i.e., black), medium, and weakly stained regions. The table also shows the approximate diameter of the region of uptake, as well as the cytoarchitectonic area in which the injection site was located.

The total area of the terminal fields ranges between about 7 and 22 mm², with an average of 12 mm². The difference in size can largely be explained by the inevitable variation in the strength of the injections and the corresponding variation in the size of the regions of uptake, with their diameters ranging between 200 and 400 µm (200 µm is the diameter of the dendritic extension of a large pyramidal cell in the mouse). The region of uptake ranged between about 0.03 and 0.1 mm² in area.

As can be seen from Table 1, in all injections the main part of the terminal field consisted of weak projections (widely hatched regions in Figs. 4 and 5). If we exclude these, the size of the terminal fields (medium plus strong) ranges between 2.5 and 8.8 mm², with an average of 4.7 mm².

The average size of the surface area of the entire neocortex of one hemisphere was 64 mm². Thus, the neurons from a middle-sized column of about 300 µm in diameter can reach about 7% of the total neocortex by projections of high plus

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Table 1

<table>
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<th>Brain number</th>
<th>Total terminal field area</th>
<th>Large field around injection</th>
<th>Areal sum of distant spots</th>
<th>Total area of projections</th>
<th>Region of uptake (µm)</th>
<th>Cortical area</th>
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Note: All areal measurements in square millimeters. All measurements refer to the injected hemisphere.

*In this case, 4 different degrees of density were distinguished. "Middle" contains the middle 2 degrees of density.

Cytoarchitectonic area of the injection site, according to Caviness (1975).
middle strength, and even about 19% if the weak projections are included.

If we translate the measurements of Table 1 into degrees of divergence by dividing the area of the terminal fields by the area of the region of uptake, we get an average factor of 73 (with individual values between 45 and 122) for the strong plus middle projections and an average factor of 182 (with individual values between 122 and 298) if we include the weak connections. (The large range in the individual values may be partly due to the fact that the values for the size of the region of uptake are approximations only.)

**Fiber Densities**

In 2 of the brains (Fig. 5) we quantified the densities of stained fibers in distant projections. The contralateral projection in Figure 5b was one of the densest projections in our material (see micrograph in Fig. 7), whereas the rostral spot in the ipsilateral hemisphere was one of the weakest (see Fig. 8).

![Figure 7. Micrograph of the terminal field contralateral to the injection site in Figure 5b, as an illustration of a terminal field of high density.](https://academic.oup.com/cercor/article-abstract/16/10/1474/353970)

![Figure 8. Micrograph of the terminal field of low density measured in the ipsilateral hemisphere in Figure 5b.](https://academic.oup.com/cercor/article-abstract/16/10/1474/353970)

We therefore took these 2 spots as instances of an upper and a lower limit. The densities of the 2 projections measured in Figure 5a were in between.

The results are shown in Figure 5 and Table 2. For comparison with other data (see Discussion), we express these densities in fiber length per cubic millimeters. Table 2 shows the raw data. The data in Figure 5 indicate the maximum densities measured in each spot because the maximum value may be relevant for the question of how strongly distant places in the cortex can influence each other functionally. These maximum values range between 4.6 and 21 m/mm³. The higher value may be an underestimate by some meters per cubic millimeters because in dense regions some intersections with the test line may escape the observer. Thus, the maximum density in the contralateral spot in Figure 5b may be rounded up to 25 m/mm³.

**Numbers of Neurons Projecting to a Distant Spot**

We also counted the number of axons entering a terminal field from below. Assuming that each of these axons comes from a different pyramidal cell, this number is a measure of the number of neurons projecting from the injection site to that terminal field.

The results of these counts are also reported in Figures 5a, b. In Figure 5b, the number of axons entering a terminal field ranged between 14 for the weak spot to about 890 fibers running toward the dense and middle dense region of the callosal spot. The number for the contralateral spot in Figure 5a was about 230 and for one of the ipsilateral spots 540.

**Number of Neurons at the Injection Site**

We also made an estimate of the number of neurons that had taken up the tracer in the brains in Figure 5.

To a first approximation, one can calculate the number of neurons within the region of uptake. This calculation is made on the basis of the diameter of the region of uptake given in Table 1 and the number of neurons under 1 mm² of cortical surface area, which is about 10⁵ in the mouse (Rockel and others 1980; Schüz and Palm 1989).

However, not all the neurons within the region of uptake defined by the bundles of stained descending axons have...
necessarily taken up the tracer. In the core region, all neurons appeared to have taken up the tracer because the density of stained cell bodies there was as high as the known cell density in the mouse (Schüz and Palm 1989). Toward the periphery, the density of stained cells decreases relatively sharply. According to our estimates, an equivalent cylinder, containing as many neurons as stained in the core region and its halo of lesser staining, has a diameter about 60 μm lesser than the region of uptake defined by the stained descending axons.

With this reduced region of uptake, we end up with a number of about 9074 stained neurons in the brain in Figure 5b. Because we are concerned with the pyramidal cells that project over larger distances, we subtract from this number 15% nonpyramidal cells (Peters and others 1985; Braak H and Braak E 1986), which leads to an estimate of about 7700 pyramidal cells.

The injection in Figure 5a was smaller, and some of the cortical gray substance was mechanically destroyed by the injection. The number of stained pyramidal cells was about 2900 in this case.

**Contralateral Projections**

In all cases (with the exception of the brain in Fig. 4f) fiber stain could also be found in the contralateral hemisphere in a position symmetrical to the place of injection.

In some of the injections, additional stain could be found in other (i.e., nonhomotopic) positions on the contralateral side. When such projections were present in the contralateral hemisphere, terminal fields were always found in the corresponding position in the injected hemisphere. This raised the question as to whether these nonhomotopic callosal projections come directly from the injection site or rather are mediated by neurons stained retrogradely through one of their axonal branches and reaching the opposite side through another.

In some cases, marked by an asterisk in Figures 4 and 5, the latter possibility could not be excluded entirely because in these cases some retrogradely stained neurons were found on the side of the injection. Nor can we fully exclude that some of the weakly hatched regions around the homotopic contralateral spots are slightly enlarged due to fibers coming from retrogradely stained neurons in the vicinity of the injection site.

However, in other cases it was clear that nonhomotopic terminal fields were fed directly from the injection site. For example, in the brain in Figure 5a, some 230 fibers entered the contralateral terminal field symmetrical to the injection site, whereas at least twice as many crossed the corpus callosum. The rest of the stained fibers bypassed the homotopic site to reach the terminal field located more laterally.

Occasionally, an individual axon could be seen to ramify in the other hemisphere to reach 2 closely located contralateral terminal fields (e.g., brain in Fig. 4f) or to supply both the symmetrical contralateral field and its weakly stained surroundings (e.g., brain in Fig. 4b).

**Discussion**

**Methods**

**Completeness of the Fiber Stain**

Wherever axons run in the plane of sectioning, they could be followed in their entirety. They showed rich ramifications upon reentering the cortex. This makes it probable that the tracer filled the axonal trees completely.

**Aberrant Staining**

When employing anterograde tracers one should be aware of the possibility of a certain amount of aberrant staining: transneuronal staining, retrograde staining, or staining of fibers passing the injection site. This raises the question of the extent to which the pictures in Figures 4 and 5 are partly the result of such aberrant staining.

We scrutinized our whole collection of BDA tracer preparations, which included deep cortical and subcortical injections, but found no evidence for transneuronal staining. However, evidence could be found for staining of fibers passing an injection site in both retrograde and anterograde directions. In our material, this phenomenon seemed to have been restricted to fibers injured by the electrode. The existence of a limited number of Golgi-like retrogradely stained neurons has already been mentioned, a phenomenon that may be activity dependent (Jiang and others 1993).

Both the fiber stain due to retrogradely stained neurons and the staining of injured fibers of passage are, however, responsible for a small percentage of stained fibers only. We can exclude that these artifacts had any effects on the size of the middle and densely stained terminal fields. Nor do we believe that they increased the size of the weak projection fields more than slightly because we had taken the precautions described in The Problem of Retrograde Staining.

**Depth of the Injection**

The location of the tip of the electrode varied between layers III/IV and V. This had some influence on the width of the region of uptake in the various layers, but in each case, neurons in all layers had taken up the tracer, as could be gathered from the distribution of darkly stained cell bodies.

**Contralateral Projections**

The existence of nonhomotopic callosal projections in rodents has been debated. For a review see Innocenti (1986). The presence of such projections is evident in our material.

The complete lack of contralateral fiber stain in Figure 4f requires some explanation. This map shows an injection into area 17, the main part of which is known to be “acallosal” (also in the mouse; Yorke and Caviness 1975). In addition, as can be seen on the figure, this brain had an exceptionally small corpus callosum, possibly lacking some of the normal callosal fiber components.

**Local versus Distant Connections**

The axonal tree of a typical pyramidal cell is divided into local axon collaterals radiating in all directions within the gray matter and a main axon running through the white matter to a selected distant location. In the small brain of the mouse, this neat distinction into gray matter axon collaterals and white matter main axons can only be made with respect to the callosal and subcortical projections. In the large terminal field continuous with the injection site, the 2 systems cannot be separated because the main axons do not enter the white matter and can therefore often not be distinguished from long axon collaterals. Even in more distant projections within the same hemisphere, the main axons tend to be reluctant to enter the white matter.
In the following discussion, we shall therefore use the terms "local connections" for those fibers constituting the black field around the injection site in Figures 4 and 5 and "distant connections" for all those constituting the hatched regions. The term "distant spots" refers exclusively to the spots of terminal fields disconnected from the large field in continuity with the injection site.

Quantitative Aspects
Let us assume that a nest of neurons has been activated somewhere in the cortex, for example due to thalamic input. The activity will be relatively strong in the immediate vicinity of the activated neurons because all of them contribute axon collaterals to the local field. A particular distant spot will, however, only be reached by some of the activated neurons because only some of the pyramidal cells at a given place will project to the same distant spot. Most pyramidal cells probably have only one main axon projecting to a distant spot in the cortex. This assumption is supported by double-labeling experiments that show that callosally and intrahemispherically projecting neurons are largely separate populations (Andersen and others 1982; Schwartz and Goldman-Rakic 1982; however, for evidence of ramifying main axons in subcortically projecting pyramidal cells see Cowan and Wilson 1994; Deschénes and others 1994; Levesque and others 1996).

What then are the chances that the activity at a given place A transmitted to a particular distant spot B can activate neurons in B? We leave the final answer to the neurophysiologist or the theoretician modeling cortical activity but will deal with this question below as far as possible from an anatomical point of view.

Percentage of Pyramidal Cells Projecting from A to B
The percentage of pyramidal cells that project from a given injection site to some distant spot may be taken as an indication of the relative strength of distant connections. In Figure 5b the number of pyramidal cells projecting to the contralateral spot (ignoring the weak halo) is about 890. This is 12% of the about 7700 pyramidal cells that have taken up the tracer. In the case of Figure 5a, 540 out of 2900, that is, 19% of the stained pyramidal cells, project to the distant ipsilateral spot investigated. This means that 1/8 to 1/5 of the neurons from a given injection site can contribute fibers to a distant spot. Let us now investigate how this translates in fiber densities.

Density of Stained Fibers in Distant Spots
The maximum density of stained fibers measured in a distant spot in Figure 5b was about 25 m/mm³. This is very little compared with the total axonal length in 1 mm³, which is about 4 km in the mouse (Braitenberg and Schütz 1991; for comparable values in the cat see Foh and others 1973), that is, only about 0.6%. (If we take into account a volume shrinkage to 74% in the BDA preparations [see Methods], the maximum density reduces to 19 m/mm³, corresponding to 0.5% of the total axonal population.) In view of the overall arrangement of synapses along axons (Braitenberg 1978b; Amir and others 1993; Hellwig and others 1994; Houzel and others 1994; Anderson and others 2002), we take fiber density as a direct indication of synaptic density. Functionally speaking, this implies that of the 8000 postsynaptic sites on a dendritic tree of a pyramidal cell in the mouse (Braitenberg and Schütz 1991), about 48 synapses can be activated from a distant site of activation (in the event of a strong projection from that site).

Densities of Stained Fibers Close to the Injection Site
How do these values compare with the situation in the local terminal field in the vicinity of the injection site? This question cannot be answered by direct measurement because the density of stained fibers in the black regions in Figures 4 and 5 is too high to be measured by stereological methods.

However, the density of fibers in the black region can be estimated as follows. In former work we came to the conclusion that individual pyramidal cells in the mouse have a total axonal length between 20 and 40 mm within the gray matter, with an average closer to the upper than to the lower value (see Chapter 17 in Braitenberg and Schütz 1991). This enables us to calculate the total length of stained axons in the brains in Figure 5, where we have an estimate of the number of pyramidal cells that have taken up the tracer. In the case of Figure 5b, the estimated number of labeled pyramidal cells was 7700. Taking 35 mm as an average axonal length per neuron, we end up with a total length of stained fibers of 270 m. (In view of the uncertainties inherent in this calculation, we ignore the fact that some of these 7700 pyramidal cells will be output neurons to subcortical structures and therefore contribute less fibers to the cortical gray matter than the average pyramidal cell.)

We can now estimate how much of these 270 m is contained in the batched subregions of both hemispheres. The remainder must then be contained in the black region. For this estimate, we must assign a fiber density and a volume to each of the hatched subregions. For the middle and widely hatched regions we used average values from Table 2 (12 m/mm³ and 3 m/mm³, respectively) and for the densely hatched regions we used the maximum value of the densest spot in Table 2 (21 m/mm³) instead of the average value (17 m/mm³) because—as already mentioned—the measurements in high-density regions tend to be underestimated by a few meters per cubic millimeter. To assign a volume to each subregion, we took the areal size of the region and the cortical depth within which fibers could be found in a given subregion. This depth varied between 100 and 600 μm in the various subregions.

For the brain in Figure 5b, we ended up with a total value of 81 m in all the hatched regions together. This leaves 270 - 81 = 189 m for the black region close to the injection site, that is, 70% of the total length of stained axons. The volume of the black region is 1.34 mm³ (the cortical depth at the injection site was 0.6 mm). The fiber density is then 189 m/1.34 mm³ = 141 m/mm³. This is 5.6 times higher than the maximum density of 25 m/mm³ in a distant field and corresponds to 3.5% of the total axonal density of 4 km/mm³.

We now perform the same kind of estimate with the brain in Figure 5a. There, about 2900 pyramidal cells had taken up the tracer, making a total fiber length of 2900 × 0.035 = 102 m. In the hatched regions, we end up with a sum of 46 m. This leaves 102 - 46 = 56 m for the dark region around the injection site (including the disconnected black spot). Thus, 55% of the total length of stained fibers is located within the dark field. The volume of the black region is 1.47 mm³ (the cortical thickness is 1 mm here) and the fiber density is then 56 m/1.47 mm³ = 38 m/mm³. This is 2.7 times more than the highest distant fiber density measured in this brain (see Table 2).
Axonal Length per Neuron

The above calculations on fiber density can, in turn, also be used to provide a lower bound for the possible average axonal length per neuron within the range of 20--40 mm mentioned above. If we had made the above calculation with an axonal length of 23 mm instead of 35 mm, we would have ended up with a fiber density in the local field of 14 m/mm\(^3\) in the brain in Figure 5a, that is, equal to that in the densest distant field in that brain. Because the stain is always strongest close to the injection site, an average axonal length of 23 mm is definitely too low. (For comparison with axonal length of individual neurons in the cat see Tettoni and others 1998; Binzegger and others 2005.)

If we go to the upper extreme of the range given above, we get an estimate of the upper bound of the percentage of stained axons in the black region. With an average axonal length per neuron of 40 mm, we get a total length of stained axons of 308 m in the brain in Figure 5b and 116 m in the brain in Figure 5a. The percentage of stained pyramidal cell axons in the black region is then 74\% and 60\%, respectively.

To Summarize These Considerations on Fiber Densities

The density of stained fibers in the near field surrounding the injection is always higher—usually much higher—than in a distant spot. However, strong distant projections can reach densities that are one-sixth or even one-third of the local density. High density of fiber stain in the close vicinity of an injection site as compared with more distant patches has also been found in other species (Malach and others 1993; Bosking and others 1997).

The finding that 55--70\% of the stained axons were located in the near field provides an answer to the longstanding question of the relative importance of local versus distant projections of the axonal tree of a pyramidal cell. The data suggest that the local axonal tree is at least as large and probably somewhat larger than the parts projecting to the hatched regions.

Global Aspects of Connectivity

The high degree of divergence of fibers from a small column supports the view of the cortex as a large mixing machine (Braitenberg 1986). The axons originating from a piece of cortex 0.1 mm\(^2\) in size or smaller were distributed in a region measuring about 70 times that area, and even about 180 times that area, if weak projections are included. Injections of comparable size in the cat show an even larger degree of divergence (Henry and others 1990). (For further comparison with larger species, see Burkhalter and Charles 1990; Amir and others 1993; Levitt and others 1993; Lund and others 1993; Pucak and others 1996; Porter 1997; Barone and others 2000; Levitt and Lund 2002; Kondo and others 2003.)

The low density of projections in more than half of the total terminal field from an injection site needs some discussion. One wonders what contribution they can make to the firing of neurons at a distant place. However, weak projections make...
sense in connection with the theory of cell assemblies (Hebb 1949), the theory of synfire chains (Abeles 1982), and other similar ones that rely on cooperative effects in large numbers of neurons. It should also be mentioned that under certain conditions the membrane potential of cortical neurons can be poised just below threshold for firing (Metherate and Ash 1993; Cowan and Wilson 1994; Stern and others 1997; Timofeev and others 2001; Destexhe and others 2003; Légier and others 2005). In this case, weak connections could have a decisive effect.

The large size of the terminal field in continuity with the injection site, extending over neighboring areas, suggests that most of the connectivity between areas is devoted to closely located areas. This seems to be true for small and large brains alike because it is in accordance with tracer studies in cats and monkeys (summarized in Young and others 1995) and with the distribution of fiber lengths in the cortical white matter in humans (Schüz and Braithenberg 2002).

Notes

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Appendix

Here we will give a derivation of the length density formula (formula (1) in Methods) based on our specific task. We want to estimate length density, that is, the total length of a filament structure in a given volume divided by that volume. The number of intersections between line segments with random position and orientation and a superimposed test line is proportional to the length density.

In our microscopic image, we can see only the 2-dimensional projection of these line segments. The first thing we therefore need to know is how the projected line length is related to the actual line length in space. As mentioned in Methods, we assume that all axonal directions in a plane parallel to the cortical surface are uniformly distributed. To our knowledge, this is a reasonable assumption.

The projected lengths \( p \) are shortened relative to the real lengths \( r \) by the factor \( \cos \alpha \), where \( \alpha \) is the angle between the fiber and the plane of section:

\[
P = \frac{r \cos \alpha}{r} = \cos \alpha.
\]

(2)

Given a uniform distribution of 3-dimensional orientations, we are more likely to find small values for \( \alpha \) than large ones (see Fig. 9). The probability density for finding the angle \( \alpha \) is equal to its cosine:

\[
P(\alpha) = \cos \alpha, \quad \alpha \in \left[0, \frac{\pi}{2}\right].
\]

(3)

The expected shortening is the integral of \( p/r \) over all possible angles \( \alpha \) weighted with \( P(\alpha) \):

\[
\left\langle \frac{p}{r} \right\rangle = \int_0^{\pi/2} \frac{\cos \alpha}{r} P(\alpha) = \int_0^{\pi/2} \cos^2 \alpha d\alpha = \frac{\pi}{4}.
\]

(4)

With this result, we can now reduce our original problem to the estimation of the length density in 2 dimensions.

Estimation of length density in 2 dimensions is a classic problem in stereology (Buffon 1777; Smith and Guttmann 1953). The main idea is that the number of intersections between a linear structure such as the projected axons in our case and a test line in a given area depends on how much total length is distributed in that area. The formula for length density in 2 dimensions is

\[
L_A = \frac{\pi N}{2T}.
\]

(5)

\( N \) is the number of intersections and \( L \) is the length of the test line. This formula assumes isotropic and uniform random sampling. One easy way of obtaining isotropy is by using circular test lines. We will now give an intuitive explanation of the above formula. Imagine a set of parallel lines of length \( b \) that is crossed by a perpendicular test line of length \( l_{\text{length}} \) (Fig. 10a). In this case, the number of intersections \( N \) with that test line tells us exactly how much line length \( L \) is contained in the shaded area \( A \).

\[
L_A = \frac{N_b}{l_{\text{length}}}, \quad N = \frac{N_b}{l_{\text{length}}}, \quad \frac{\pi N}{2T} = \frac{N_b}{l_{\text{length}}}
\]

(6)

However, we require a formula that works for arbitrary orientations. This is achieved by replacing the straight test line by a circular one (Fig. 10b). The number of intersections remains identical, whereas the length of the line is now \( \pi/2 \) times longer. This leads to the following formula:

\[
L_A = \frac{N}{l_{\text{length}}} = \frac{N}{l/(\pi/2)} = \frac{\pi N}{2T}.
\]

(7)

Let us now combine formulae (4) and (7). Formula (7) gives us the total length of axonal segments per area, ignoring their shortening due to their projection onto the plane of section. According to formula (4), the average shortening factor is \( \pi/4 \). If we incorporate the section thickness \( t = 50 \, \mu m \), we arrive at formula (1) of Methods:

\[
L_A = \frac{N_b}{l_{\text{length}}} = \frac{\pi N}{2T} \frac{1}{T} = \frac{2}{150} \frac{N}{50 \, \mu m}.
\]

(8)

References


1848 Corticocortical Connections · Schüz and others


