IV. Mapping Experimental Results

A. Neuroanatomical Methods

Generally speaking, neuroanatomical methods can be divided into those using either normal or experimental material. Basically, *normal methods* simply describe the structure of the intact brain. This can be at the level of gross dissection and at the level of tissue sections stained in a variety of ways and examined with the light and electron microscopes. In contrast, *experimental methods* are considerably more powerful for determining the organization of axonal pathways and rely on the production of lesions or on the injection of tracers that move along axons (and dendrites). More specifically, neuroanatomy deals with the gross structure of the nervous system, with the location of its various cell types and the pathways that interconnect them (architectonics), with the shape of individual cells (morphology), with the disposition of intracellular features at the light (fine structure) and electron (ultrastructure) microscopic levels, with the structural organization of neural circuits, and with the cellular localization of various molecules (histochemistry).

The following discussion is meant to provide an overview of strategies for obtaining and mapping neuroanatomical information. An historical approach has been adopted because most of the techniques developed over the years can still be used to advantage, and the results presented in computer graphics format. A guiding principle is that every method has limitations and advantages, so that a variety of approaches is required to clarify any problem in structural neuroscience. Thus, a clear appreciation of these limitations and advantages is necessary for the

critical evaluation of results presented in the literature, and for the intelligent design of experimental analyses.

The early history of graphical brain mapping can be traced easily to the magnificent drawings published by Andreas Vesalius in 1543 (see text fig. 2 in Swanson 1992a). They illustrate not only the gross anatomy of the human brain in various stages of dissection, but at the same time present a series of horizontal sections. These figures remain instructive today, and serve as an inspiration for comparable 3-D computer graphics models. Finer dissections of the brain to reveal the disposition of major fiber tracts and cell masses were pioneered by Varolius (1591) and Vieussens (1685), and again this approach is still used today, mainly for teaching purposes. In fact, careful gross dissection remains the best way for those starting to use neuroanatomical methods to gain an appreciation of the overall structure of the brain—it provides an invaluable orientation.

More resolution was obtained with the perfection of serial tissue section analysis by Stilling (see Schiller 1969) in the first half of the 19th century, particularly because the sections could be examined under the microscope. Although the latter was invented in the 17th century, it was essentially useless for examining animal tissue sections until the refinement of lenses corrected for chromatic and spherical aberrations, beginning in the 1820s (Hughes 1959). Thus, Stilling was able to define and map in a series of huge, exceptionally beautiful drawings many of the cranial nerve and other brainstem and spinal cord nuclei based on the faint outline of distinctive groups of individual neuronal cell bodies, in unstained sections. Advances in microscope technology also led quickly to the revolutionary distinction between neuronal cell body, dendrites, and axon by a number of workers between 1833 and 1865 (see Shepherd 1991;

Jacobson 1993). The latter marks the transition from gross anatomical to fine structural analyses of the nervous system.

The more or less exponential development of selective histological stains for application to sections of nervous tissue also led to greatly increased resolution of fine structure. The first such stain was carmine, introduced by Gerlach in 1858, although more selective stains were then developed for cell bodies (Nissl 1894), myelinated axons or more correctly myelin sheaths (Weigert 1882), and axons themselves (the reduced silver methods of Cajal 1891, and Bielschowsky 1892).

The latter methods quickly led to the development of a new branch of neuroanatomy—*architectonics*. *Cytoarchitectonics* deals with how neurons form distinctive groups based on their appearance in Nissl-stained material, whereas *myeloarchitectonics* deals with the general spatial distribution of myelinated fiber tracts; because axons are found everywhere in the brain, no special field of 'axotectonics' has developed. Another variant, *chemoarchitectonics*, is based on the histochemical detection of specific molecules, and began in earnest with the application of Koelle's acetylcholinesterase method to the rat brain (Koelle 1934). This was followed by the development and application of the histofluorescence method for biogenic amines by the Swedish school (Falck et al. 1962), and later by the development in the 1970s of immunohistochemical and *in situ* hybridization methods that can be used to detect almost any molecule from amino acids to mRNA. The major conceptual problems with histochemical methods in general are proving specificity of the reaction product, and determining the limits of sensitivity (how is no labeling—negative evidence—interpreted?).

Architectonic methods are based on normal rather than experimentally manipulated nervous tissue. Nevertheless, their use is responsible in large part for our current understanding of the

basic parts of the nervous system and their spatial relationships to one another. However, it is now clear that architectonics must be combined with experimental methods for determining the organization of neuronal connections (discussed below). When used to inform each other, these two approaches constitute the most powerful way at this time to illuminate the basic structural organization of the nervous system.

Another group of techniques is used to characterize the morphology of individual neurons, that is, the detailed shape of their cell body, dendrites, and axon. Although Deiters (1865) executed the tour de force of the dissection approach with the very tedious dissociation of individual neurons and glial cells from a number of CNS regions, Golgi's introduction of the silver chromate method in 1873 revolutionized this field, and led several decades later to Cajal's formulation of the neuron doctrine and the concepts of functional polarity and the synapse (Cajal 1995). As is well known, for reasons that are still unclear the Golgi method impregnates more or less completely, and apparently randomly, all parts (except myelinated segments of axons) of a small fraction of the neurons in a tissue block. This includes the cell body, the dendrites and dendritic spines, and the axon and its collaterals, varicosities, and boutons. Thanks to this method, the morphology of most neuronal cell types is known in exquisite detail.

Interestingly, the validity of the Golgi method was confirmed with the discovery of an independent method by Ehrlich (1886), based on supravital staining with methylene blue. Today, the morphology of electrophysiologically characterized neurons can be determined by injecting a variety of markers directly into the cell with a micropipette and allowing time for the marker to fill the cell before histological processing.

Before turning to experimental neuroanatomical methods, it is important to note that the introduction of electronmicroscopy in the 1950s opened the world of ultrastructure to

neuroanatomy, and allowed the first glimpse of synaptic structure (Peters et al. 1991). It is a general truism that synapses cannot be identified unequivocally with the light microscope because its theoretical resolution is on the order of 500 nm, whereas most synaptic clefts are on the order of 20-30 nm wide. The resolving power of the electron microscope in about 0.5 nm. One major limitation of electronmicroscopy is that by necessity very small volumes of tissue are sampled.

Cajal's hypothesis that neurons are functionally polarized (dendrites and cell body receive information and the axon transmits information) provided an unprecedented need to clarify the structural and functional organization of brain circuitry. Unfortunately, most such information provided by the Golgi method has been concerned with local interrelationships between neurons because it is difficult to trace individual axons long distances through serial sections. In addition, dissection methods and serial section analysis of normal brains are grossly inadequate because most fiber tracts in the CNS are complex and heterogeneous, and because defined cell groups typically contain multiple neuronal phenotypes. Clearly, another approach was required.

Waller's experiments in 1850 sowed the seeds for this new approach. He interrupted certain motor cranial nerves in the rabbit, and observed that the segment of nerve distal to the interruption degenerated whereas the brainstem neurons of origin and proximal segment of the nerve were seemingly unaffected. This led him to the fundamental suggestion that the cell body is the trophic or nutritive center of the neuron, and that the axon's vitality depends upon it. This implies that the direction of axonal pathways in the brain can be inferred by the location of fiber degeneration relative to the cutting of a tract or the destruction of a cell group giving rise to a projection. During the 1890s, Weigert was the first to use this approach with great success by applying his myelin stain to the CNS of animals with experimental lesions, and to postmortem

human brains that had suffered localized pathological lesions during life. However, it was often difficult to detect the loss of relatively small numbers of myelinated axons within the vast fields of such fibers so typical of many CNS regions.

A more powerful approach was described by Marchi and Algeri (1885), who developed a method for staining degenerating myelinated fibers selectively, and leaving intact myelin unstained. Thus, degenerating fibers were the only dark profiles observed against light background staining. As helpful as these methods were, they did not stain unmyelinated axons, and even more importantly the unmyelinated terminal ramifications. It was not until 1952 that Nauta and Ryan developed a reduced silver method for staining selectively degenerating axons themselves. This method, based on the suppression of normal axon staining, and its later variants (such as the Fink-Heimer technique), sparked a virtually complete reexamination of central neuronal pathways during the next 20 years, primarily because it was so much more sensitive than the older techniques, and thus revealed many more, unsuspected, pathways. Of equal importance, it was and still is applied at the ultrastructural level to reveal synaptic relationships in neuronal circuits.

These experimental methods rely on the phenomenon of *anterograde axonal degeneration* (Wallerian degeneration, fig. 5A). Another phenomenon, *retrograde cell body degeneration*, was first observed by Gudden (1879) after evulsing certain motor cranial nerves as they exit the brainstem, in young animals. When the animals were killed several weeks later, he observed that the nerve's cell bodies of origin had degenerated. This of course suggested that the cellular origin as well as the termination sites of particular fiber tracts could be determined experimentally by cutting axons, and incidentally, that the survival of neurons may depend on the retrograde delivery of trophic factors from terminals to the cell body. However, it soon became apparent

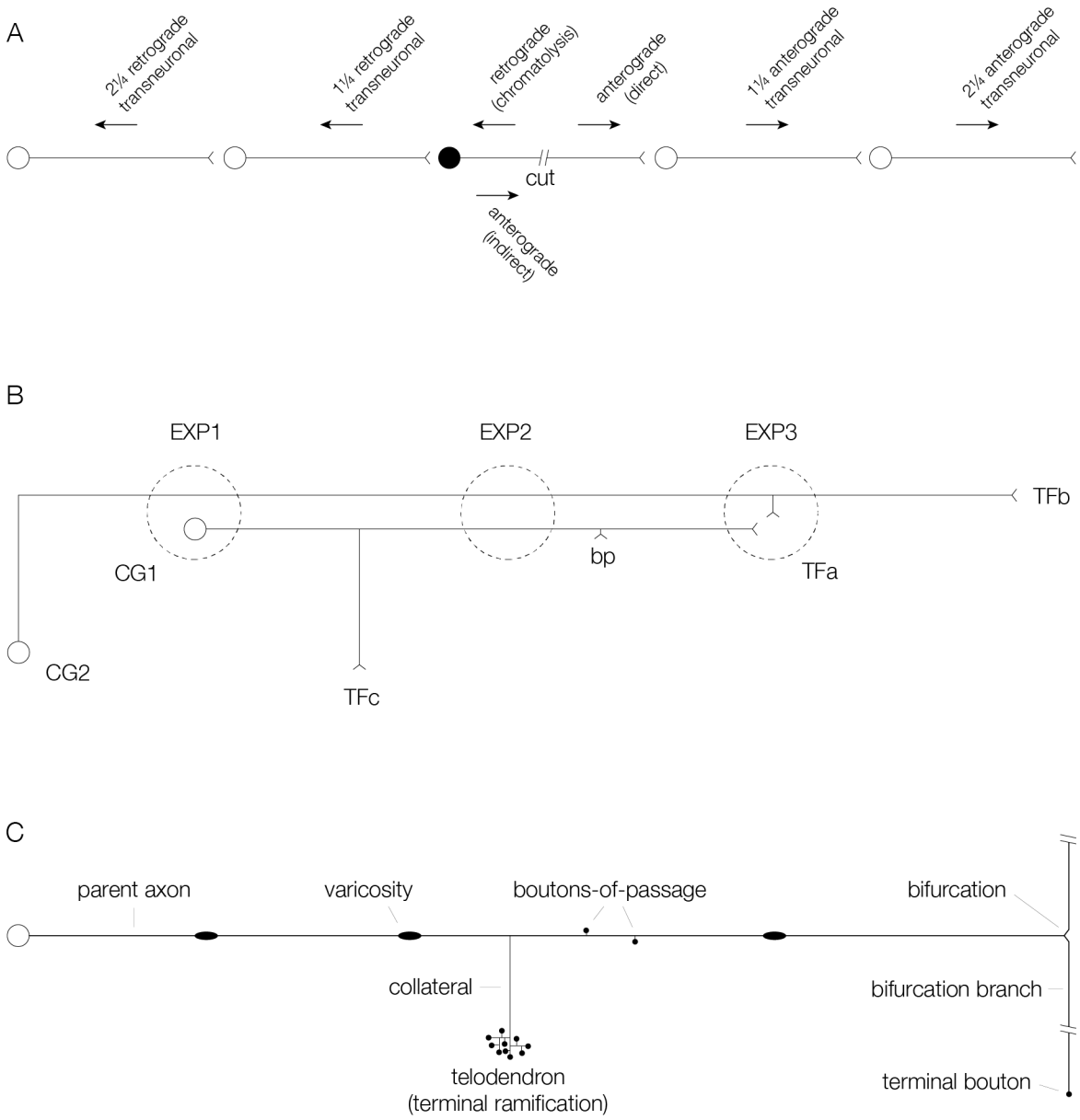

Fig. 5. A. Possible types of degeneration produced by cutting the axon of a neuron (dark circle in the middle of the chain). See text for details. B. A hypothetical brain circuit that is subjected to various experimental procedures (at EXP1, 2, and 3). Two cell groups (CG1, 2) are shown, along with three terminal fields (TFa, b, and c). A terminal bouton-of-passage is also indicated. See text for details. C. Vocabulary of features used to describe axon morphology first defined with the Golgi method, and now appropriate for the PHAL and other methods.

that this method often failed to produce obvious retrograde degeneration in older animals, and it now seems clear that it only works if the parent axon is severed before 'sustaining' collaterals are encountered.

The introduction of the Nissl stain (1894), which relies on basic aniline dyes that interact with nucleic acids (and in particular stacks of rough endoplasmic reticulum in the perikaryon and basal dendrites), much more clearly demonstrated retrograde cell body degeneration, which became known as *chromatolysis*. With severe chromatolysis, the proximal axon may also undergo anterograde degeneration, beginning near the initial segment (indirect Wallerian degeneration). Under favorable circumstances (few sustaining collaterals), anterograde and retrograde degeneration extend beyond the injured neuron itself, leading to *primary*, and even *secondary*, *anterograde and retrograde transneuronal degeneration* (Cowan 1970).

Although much of what we know about the organization of fiber systems in the human CNS is based on degeneration methods (applied postmortem to brains that had suffered pathological insult or surgical intervention), there is one major limitation to their interpretation, in addition to a lack of sensitivity and selectivity compared to modern techniques (see below). That limitation is the possible involvement of *fibers-of-passage*, which will be considered in some detail because it is also a critical factor in the interpretation of current axonal transport tracing methods. The basic concern is illustrated in fig. 5B. Assume that one would like to determine the projections of cell group 1 (CG1). It is thus lesioned (EXP1) and anterograde degeneration is traced to terminal fields a, b, and c (TFa, b, and c). However, it is incorrect to assume that cell group 1 projects to terminal field b. The pathway to terminal field b instead arises in cell group 1, and merely passes through the lesion involving cell group 1: fibers-of-passage have been damaged, leading to a false conclusion (false positive results). In contrast, it is safe to conclude that the fiber tract as a whole projects to terminal fields a and b if it is interrupted at EXP2.

Now assume that one would like to determine the location of neurons projecting to terminal field a. It is lesioned (EXP3) and the entire brain is examined for signs of chromatolysis. It may

be found in cell group 2, but not in cell group 1 (false negative results) because the latter generates proximal sustaining collaterals to terminal field c. The same results would be expected following a lesion of the fiber tract itself (EXP2), rather than the terminal field. Because clear signs of degeneration have different time courses in different parts of the neuron, and in different types of neuron; because actual neural circuits are often much more complex than the simple example given here; and because degeneration methods rely on the destruction of neural tissue (often the very tissue of interest), it is easy to see why there was great interest in developing more powerful approaches.

Such approaches were developed in the 1970s, and were based on fundamental advances in cell biology, rather than on pathological changes. Specifically, they rely on the incorporation into neurons of molecules injected into the extracellular fluid, and their subsequent anterograde and/or retrograde intraaxonal transport—in physiologically intact, rather than lesioned, tissue. Furthermore, they proved to be much more sensitive, and in some cases much more selective, than the degeneration methods.

The first anterograde intraaxonal tracing method was based on the incorporation of injected radiolabeled amino acids into proteins and their subsequent fast axonal transport along the axon and its collaterals to the various terminal fields, where they are concentrated. The basic approach was worked out by Lasek and associates (Lasek et al. 1968) in dorsal root ganglia and applied to the brain by Cowan and associates (Cowan et al. 1972). Because protein synthesis occurs in the cell body (and dendrites), but not in axons, the injected label is transported in detectable quantities *only in the anterograde direction*, and most importantly, *is not incorporated by fibers passing through the injection site* (free labeled amino acids are washed out of tissue during histological processing). Thus, the results of autoradiographic experiments are very

straightforward to interpret, and the method proved considerably more sensitive than those based on degeneration.

However, the autoradiographic method has one major disadvantage: the distribution of labeled axons and terminals must be inferred from a pattern of reduced silver grains in photographic emulsion overlying tissue sections. Thus, whereas labeling at the end of a pathway almost certainly represents a terminal field, it is not possible to infer the existence of synaptic varicosities or boutons *along* a pathway at the light microscopic level. The autoradiographic method is specific (anterograde only) and sensitive, but lacks resolution at the light microscopic level. In Fig. 5B, an injection of cell group 1 labels only terminal fields c and a (and more lightly the axons in between, because label accumulates in terminals), but scattered synaptic varicosities or boutons-of-passage along the axons (bp in Fig. 5B, also see Fig. 5C) cannot be distinguished from the axons themselves. For example, it would not be possible to identify terminals along cerebellar parallel fibers, or along the medial forebrain bundle in the hypothalamus.

The resolution problem was solved in 1984 by Gerfen and Sawchenko, who identified another exclusively anterograde tracer, *Phaseolus vulgaris*-leucoagglutinin (PHAL). For unknown reasons this lectin is incorporated only by dendrites (and possibly cell bodies) under proper injection conditions, and then transported down the axon. Thus, it has the advantages of the autoradiographic method, but in addition has the great advantage of being demonstrated immunohistochemically. With the PHAL method axons are labeled with the clarity of a Golgi impregnation, and the cells of origin are unequivocally labeled in the injection site as well. All of the morphological features associated with axons can be observed in detail (Fig. 5C, also see Risold et al. 1994).

A number of other sensitive anterograde tracers have been developed, but they are also transported in the retrograde direction (for example, cholera toxin subunits or wheat germ agglutinin, WGA, conjugated to horseradish peroxidase, HRP, Wan et al. 1982; and DiI and DiO, Honig and Hume 1989), and it would appear that they can, at least to some extent, be incorporated by damaged or intact fibers-of-passage. Nevertheless, one or another are especially useful in examining multiple pathways in the same brain (in combination with PHAL).

Allusion to retrograde axonal transport of tracers was just made, and this phenomenon has been used to great advantage in pathway characterization. This approach was pioneered by Kristensson and colleagues, who initially injected either HRP or the fluorescent dye evans blue into tongue muscles, and observed their retrograde transport to the brainstem neurons that generate the hypoglossal nerve (see Kristensson et al. 1971).

This approach revolutionized the identification of neurons giving rise to particular terminal fields, central fiber tracts, and peripheral nerves because it was not based on the unreliable production of chromatolysis, and because it has proven extremely sensitive. Kuypers and his colleagues extended this approach by injecting multiple fluorescent dyes with distinct emission characteristics (and subcellular localization) into different terminal fields in the same animal (see Keizer et al. 1983). With this method it is possible not only to determine whether neurons in a particular region innervate multiple terminal fields via axon collaterals, it is possible also to map the spatial distribution and relationships of neurons with different patterns of collateralization. As one simple example, if terminal field a in Fig. 5B is injected with one dye, and terminal field c with another, cell group 1 will contain doubly labeled neurons, whereas cell group 2 will contain singly labeled neurons.

Currently available retrograde tracers have the potential to be taken up by fibers-of-passage, to a greater or lesser extent—which may be an advantage or disadvantage depending on experimental goals. Fluoro-gold (Schmued and Fallon 1986) is a particularly useful tracer because (a) it can be delivered in various ways (iontophoresis, crystals, solution), (b) it can be detected either with direct fluorescence or with immunohistochemistry, (c) it is relatively poorly incorporated by fibers-of-passage, and (d) anterograde transport usually is not observed. Other useful retrograde tracers include HRP itself (Mesulam 1982), cholera toxin subunit B (Luppi et al. 1990), WGA conjugated to HRP (Wan et al. 1982), and various fluorescent dextrans (see Nance and Burns 1990). These tracers are also transported anterogradely, and the fluoro-gold and cholera toxin-based methods are particularly useful because they provide extensive labeling of dendrites in retrogradely filled neurons.

The modern characterization of macrocircuiry structural organization utilizes a combination of anterograde and retrograde axonal tracing, chemoarchitectonics, and cytoarchitectonics. For example, the projections of a cell group can be determined reliably with the PHAL method, and subsets of these projections arising from different neuronal phenotypes within the cell group can be determined with multiple retrograde tracer methods. Then, in combination with immunohistochemistry and in situ hybridization, axonal tracer methods can be used to characterize neurotransmitter and neurotransmitter receptor characteristics of the various cell types (see Sawchenko and Swanson 1981; Kelly and Watts 1996). Finally, this information should be correlated with cytoarchitecture.

The last step is very important because other investigators can identify the boundaries and internal features of well-characterized cell groups without having to repeat the experimental work. Then, it is important to determine the organization of inputs to the cell group. The most

straightforward approach is to place an appropriate retrograde tracer confined within the borders of the cell group, and then apply anterograde tracing/histochemical methods to labeled cell groups. Finally, the synaptology of the circuitry must be determined with the electron microscope. One particularly convenient approach to this general problem is to begin with a combined anterograde/retrograde tracer injection into a particular cell group (see Risold and Swanson 1995a).

This general philosophy guided construction of the atlas maps presented here. While they are based on cytoarchitecture, the borders are informed wherever possible by experimental results. In other words, where experimental work clearly defines a cell group, cytoarchitectonic features that can be used to identify the group are used. Such an approach is very different from that used originally, when cytoarchitecture was based strictly on an observer's interpretation of features in a Nissl-stained section, which often varied considerably between observers. A major reason for using the Nissl stain as a standard is that it is extremely simple to perform on a wide variety of histological material (Simmons and Swanson 1993). And when it cannot be used (for example, on in situ hybridization sections treated with RNAse), adjacent untreated Nissl series should always be prepared for reference purposes.

Additional information about topics outlined here may be obtained in the following particularly useful sources. The best critical analysis of early neuroanatomical methods is found in Chapter 2 of Cajal (1995), and Nauta and Ebbesson (1970) contains a valuable evaluation of methods commonly in use before the 1970s revolution. Detailed, practical information about most current techniques may be found in *Neuroscience Protocols* and its successor, *Brain Research Protocols* (Elsevier).

B. Transferring Data to Atlas Levels

The traditional standard for presenting neuroanatomical data graphically, as opposed to photographically, involves preparing a tracing directly from the histological section containing data. This provides maximum accuracy, and can be done by tracing a standard photograph or a videophoto, or by projecting and thus magnifying the section (or a chrome of the section), for example with a camera lucida or projector, and then drawing the image. Quite often with experimental tracing methods, cytoarchitectonic boundaries must be established from adjacent Nissl-stained sections, which necessarily adds a certain amount of error, though usually small.

It is readily apparent from the literature that there is still a wide range of accuracy in describing and illustrating neuroanatomical results with respect to established structural features of the CNS. Until the late 1980s, neuroanatomical drawings were typically executed with a pencil and published as pen and ink drawings. However, it is now considerably easier and more accurate to prepare such drawings with computer graphics applications, whose files can be used directly by commercial publishers.

Another approach involves transferring neuroanatomical data to a standard series of drawings, for example, an atlas. The great advantage of this strategy is that it simplifies the comparison of results from different experiments or histological methods, particularly when databases are used. The major disadvantage is that the results are less accurate because they are not mapped onto the sections from which they are derived. Results plotted onto standard series are particularly useful for comparing general patterns, and formulating experiments to determine precise relationships. The only accurate way to establish spatial relationships between two staining patterns is to carry out both staining methods on the same tissue section.

Bearing these considerations in mind, it becomes important to *minimize error* as much as possible when transferring data from histological sections to a standard atlas. There are three major sources of error in transferring neuroanatomical data to a standard atlas: (a) differences in plane of section, (b) differences in scale, and (c) nonlinear warping of each section during mounting onto glass slides. These geometric factors will now be considered further.

Geometric Considerations. No matter how carefully brains are mounted on a chuck for sectioning with a microtome, they are always cut in a somewhat different plane of section. This mechanical error can be minimized by cutting the mounting plane of the brain as close as possible to the plane of the atlas. A simple device for accomplishing this is shown in fig. 6.

As a general principle, the error in transferring information from a histological section to a standard drawing is proportional to the magnitude of the difference in plane of section. Consider the brain as a solid object in a three-dimensional Cartesian coordinate (stereotaxic) space. Because the midline of a transverse section (as in the present atlas) can be aligned simply and accurately along a vertical line ($x = 0$), there are two angles that differ (two degrees of freedom) between experimental and standard planes of section. One measures the difference between 'atlas transverse' and 'section transverse', which is commonly referred to as a 'dorsoventral difference'; and the other angle measures the asymmetry between right and left sides, which is commonly referred to as a 'mediolateral difference' and is illustrated in fig. 4 (4° for the atlas).

The consequences of differences in plane of section for any one dimension are illustrated in fig. 7. Note first (left side) that the experimental or test section (T_n) only intersects a particular atlas or standard section (S2, for example) at one point—the only point where there is complete correspondence between the two sections. Moving farther from this point, features represented in the standard and experimental sections display progressively greater differences. As extreme

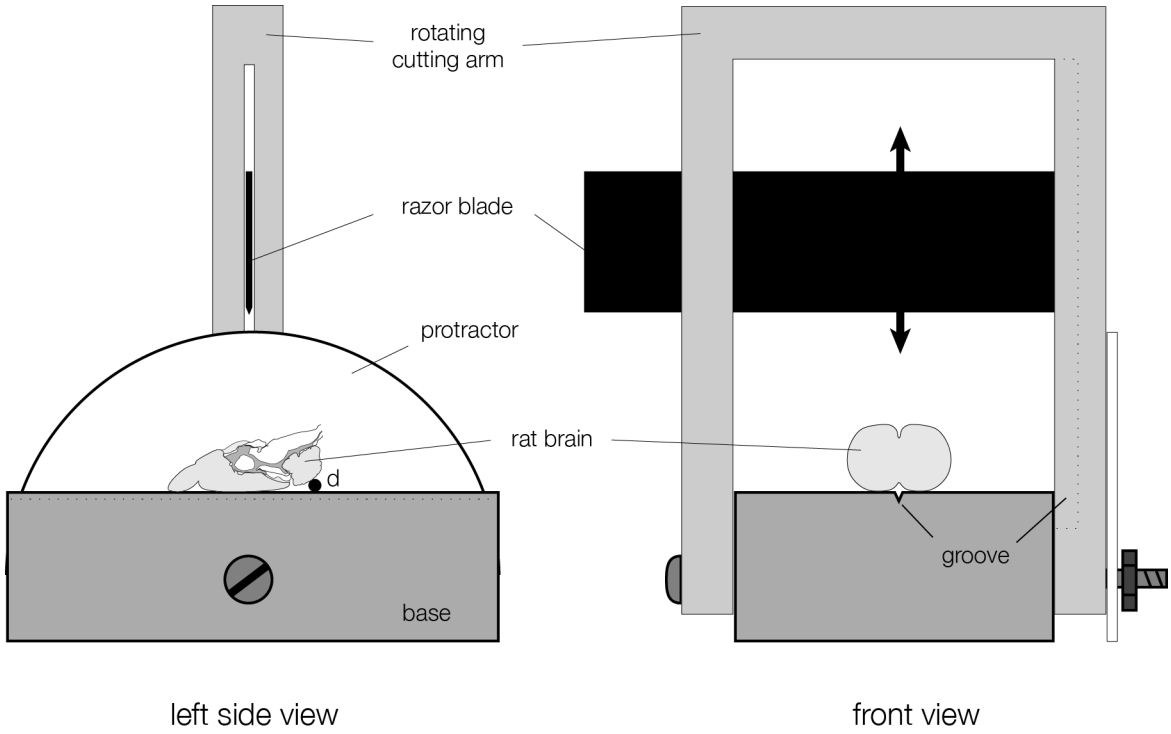

Fig. 6. A simple device made out of wood and a plastic protractor for blocking a rat brain in the approximate plane of the atlas. The dowel under the cerebellum at d is from a Q-tip. It is most convenient to attach the brain to a glass microscope slide with a drop of cyanoacrylate glue. The way brains are mounted in our laboratory, an angle of 1° to the right or caudal (when viewed from the left side) approximates the atlas plane of section. In addition it is necessary to rotate the longitudinal axis of the brain 4° from the longitudinal axis of the cutting device (see fig. 4), indicated by a groove in the front view. If available, it is more convenient, and perhaps more accurate, to use a stereotaxic holder with mounted blade for blocking the brain.

examples, labeling in structure y maps onto structure c in standard drawing S2, whereas labeling in structure b maps onto structure m in the same standard drawing. Clearly, this type of error is greater for smaller structures, and smaller for larger structures.

One way to reduce this type of error is to map data from the experimental section onto appropriate parts of a series of adjacent standard sections. In the example here (fig. 7, right side) this would involve mapping the top third of the experimental section onto atlas section S1, the middle third onto S2, and the bottom third onto S3. Nevertheless, it is obvious that there will always be some error unless the experimental and standard sections are in the same orientation. In principle, this type of error could be virtually eliminated if a 3-D computer graphics model of

the atlas could be sectioned in the same plane as the experimental brain. Assuming no distortions in the experimental sections, data could be mapped directly onto atlas cross sections. However, to be useful for comparisons with other data, the 3-D model with mapped results would then have to be resliced in the standard plane.

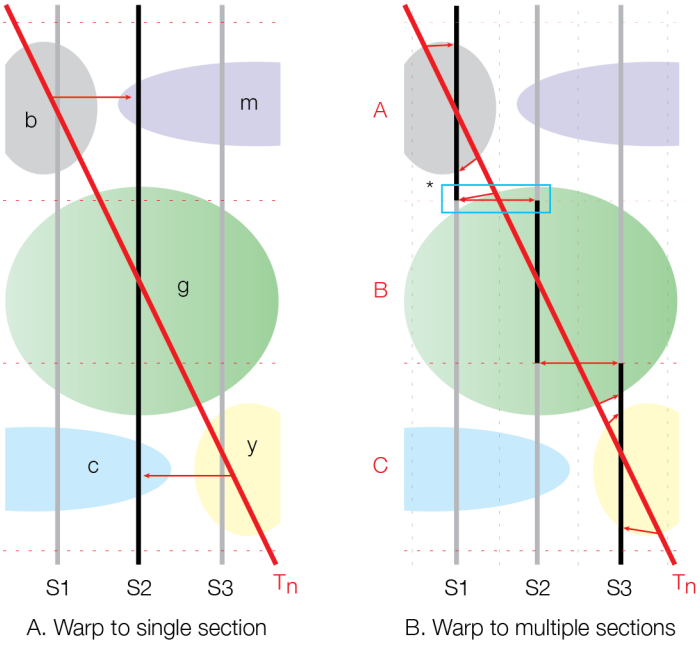

Fig. 7. Left. This figure illustrates the types of errors that can be made when data in a histological section from an experimental or test brain (Tn, in red) is mapped onto a standard atlas drawing (S2, in black). Because no two brains are cut in exactly the same plane of section, there is only one point (in structure g) where the experimental and atlas sections correspond exactly (that is, where they intersect). Moving away from this point in either direction increases the error in transferring data to the atlas level (magnitude indicated by horizontal red arrows). For example, labeled axons in structure b of the experimental section would be mapped onto structure m in the atlas. Right. The types of error just described can be reduced by mapping data systematically from the experimental section onto segments (A-C) of three adjacent atlas levels (S1-3), although serious distortions of the data still occur (approximately horizontal red arrows, especially in blue rectangle). For simplicity of illustration, the problem has been reduced from three dimensions to one.

There are two kinds of distortion that must be dealt with. To a first order of approximation, one is *linear* and has to do with differences in scale. For example, because the atlas brain was embedded in celloidin, it is smaller than brains that have been frozen, sectioned, and treated histochemically. A great deal of this error may be corrected by simple linear scaling, as described for constructing the stereotaxic coordinates for this atlas from the physical coordinates (section IIIB). Therefore, after determining the correct plane of section, linear scaling should be performed on a representation of the experimental section.

The other type of distortion is *nonlinear* and, unfortunately, may be substantial and quite different for every histological

section. These distortions arise whenever a tissue section is mounted on a glass slide for viewing under the microscope, and of course are much more severe in sections from unembedded tissue (such as most frozen sections). The most intractable distortions are due to bubbles, folds, or tears, but because the sections are so thin and fragile, they are also warped in unpredictable ways as they settle onto the glass.

There are a number of warping applications available, which can be used to transform photos or drawings of experimental sections to the shape of standard sections based on a variety of topological algorithms, but it is important to realize that the accuracy of such transformations must be validated, that in general this accuracy is proportional to the number of corresponding fiducial points in the test and standard image, and that images with as much fine detail as the present atlas drawings require a very large number of fiducial points for acceptable warping.

Fiducial points (or a series of such points defining a line) are also useful in determining plane of section relative to the atlas. In general, they should be chosen in the region being mapped. For example, if data is contained in a section through the caudal thalamus, it is important not to choose fiducials in the cerebral hemispheres because in histological mounting their actual position relative to the thalamus can move significantly—they are not attached to the interbrain (see, for example, Atlas Level 32). The best fiducials are the most unequivocal to identify—good examples include the rostral or caudal face of the anterior commissure in the midline, or the rostral face of the posterior commissure in the midline.

Mapping data onto atlas levels. This section deals with transferring neuroanatomical data to atlas levels when this cannot be accomplished quantitatively with scaling and warping algorithms because plane of section and distortion errors are too large to correct accurately. Good examples would include the mapping of PHAL labeled axons from an injection site, or the mapping of

immunohistochemically labeled axons and cell bodies, both of which may involve large areas of the brain.

The basic strategy is to map results structure by structure in the atlas, after determining what plane of section the experimental brain is cut relative to the atlas. For example, the top half of the section may be mapped onto one atlas level, and the bottom half onto the adjacent atlas level (see fig. 7). Bearing this in mind, one structure in the brain is chosen (say nucleus X), and the distribution of label in that structure is determined by examining all sections through it. Unless the observer has a thorough knowledge of nucleus X structure, the distribution of label should be drawn with a camera lucida or from photos, and the boundaries of the nucleus determined as accurately as possible based on Nissl staining. Let us say that labeling is restricted to the caudal half of part b of nucleus X, and that here it is more dense ventrally than dorsally. Next, one examines nucleus X as depicted in the atlas. The 3-D shape of the nucleus is determined by examining it from rostral to caudal in the atlas levels, and then label is drawn in the caudal half of part b, with a greater density ventrally. After nucleus X has been mapped, an adjacent structure is chosen, and the same procedure repeated, and so on until the entire brain has been examined and mapped.

Needless to say, this is an incredibly time consuming procedure, required because there is so much structural information in a series of histochemically stained sections of the brain. Just recall the detail associated with one Golgi impregnated neuron. The time when this procedure can be automated satisfactorily appears remote, although much less so for individual, relatively simple, well-defined cell groups.

It bears repeating that this approach is qualitative rather than quantitative, but has the advantage that general patterns emerging from different brains may be compared readily. And

because the atlas is space filling (that is, every point falls within a named structure—except for very narrow regions between adjacent structures, which can be named as such), the mapper must decide the location of all labeling in the experimental tissue sections. This forces the mapper to consider the distribution of label and the normal structure of the brain in detail. The insightful mapper will sometimes justify changing boundaries in the current version of the atlas, based on new experimental results.

Computer Graphics Methods. A working knowledge of a good computer graphics drawing application (such as the latest version of Adobe *Illustrator* or Macromedia *Freehand*) is essential for modern rendering and display of structural information. These applications are based on *vector graphics*, which are mathematical descriptions of the drawings that have a relatively small file size and can be scaled virtually infinitely without loss of detail. *Pixel-based applications* are appropriate for the manipulation of digital information (scanned photographs or videophotos), but produce crude drawings and very large file sizes.

There are many advantages of vector graphics over pen and ink drawings. On a technical level, line thickness is uniform, and any width and color may be chosen easily; very smooth lines (e.g., for outlines of major structural features in the atlas) can be drawn with Bezier curves, whereas more irregular lines (e.g., axons) can be drawn with a freehand tool; and labeling is easy with a wide range of fonts and sizes. In addition, corrections are trivially easy to make compared to the erasures needed for pen and ink; the drawings may be scaled readily and accurately; and the results may be printed on a common laserjet printer, or at higher resolution (e.g., 2400 dpi) on an Immagesetter.

At a more conceptual level, computer graphics offers the advantage of placing different features of a drawing into separate layers, which are equivalent to a perfectly aligned, unlimited

stack of transparent overlays. This dramatically increases the usefulness of drawings because any combination of layers can be chosen to display and/or print. For example, the template for an atlas level can be 'dissected' so that cell groups are on the bottom layer, fiber tracts are on the next, the basic outlines of the brain and ventricles are next, and abbreviations are on the top. Carrying this approach one step further, the results of a particular PHAL experiment might be placed in a layer over the atlas template (or abbreviation layer), the results of another PHAL experiment placed over the first (preferably in another color), and so on (back cover).

Finally, experience has shown that the time invested in creating good computer graphics drawings pays rich dividends because they (or parts of them copied and pasted) often form the starting point for other drawings, which do not have to be created from scratch. This cloning and modification of existing files is a major advantage of computer graphics over traditional drawing methods.

C. Neuroanatomical Databases

The use of data layers superimposed over atlas templates mentioned in the preceding section lends itself naturally to the concept of a graphical neuroanatomy database. That is, the layers themselves form items in a database, as long as they are aligned correctly over the corresponding atlas level template. Simple queries might take the form of "what is the distribution of PHAL-labeled axons in Level 26 after an injection in the lateral part of the medial preoptic nucleus?; what is the distribution of somatostatin-immunoreactivity in Level 26?; or what is the distribution of AMPA receptor subunit X in Level 26?". The results of the search would display

three layers over atlas Level 26, assuming the relevant information had been entered into the database!

A more sophisticated database structure would allow queries about individual parts of an atlas level, for example, "what is the distribution of somatostatin immunoreactivity in the nucleus reuniens?". Searches within atlas levels and data layers requires spatial indexing between the two. That is, corresponding x, y, and z coordinates need to be assigned to each point in the atlas drawings and data overlays, and the borders of various structures need to be defined in terms of these coordinates.

There are many computer science research issues that must be solved before useful graphical neuroanatomy databases are available and searchable through a Web browser (Dashti et al. 1996). In addition to developing the query structures just mentioned, it is clear that graphical information from different atlases will need to be compared, along with such information from different developmental stages of the same species, and from different species altogether. How to index or correlate such information from different species, ages, and sexes presents a major challenge and time investment.

Furthermore, there will not be a monolithic 'neuroanatomy database'; instead, such information will be distributed in a number of databases, which may use different software, hardware, and conventions. Resolving this heterogeneity in a seamless way for users is also a major challenge for the future. Ultimately, one would like to construct graphical brain models from information in databases, augment and customize these models, and share them in a collaborative environment.

D. 3-D Computer Graphics Models

The computer graphics applications discussed thus far may be either pixel or vector based, and correspondingly, 3-D computer graphics may be either *voxel* (volume element) or *vector* based. Computerized tomography (for example, MRI and CAT scans) is voxel based and has the great advantage that the brain is treated as a space-filling solid, with all of the voxels properly aligned with one another. In addition, a great deal of sophisticated software has been developed for manipulating voxel based models, which can be obtained from living subjects. These advantages are countered by very low resolution: for example, MRIs typically consist of 256x256x512 voxels. A state of the art MRI image of the rat brain is illustrated in fig. 8.

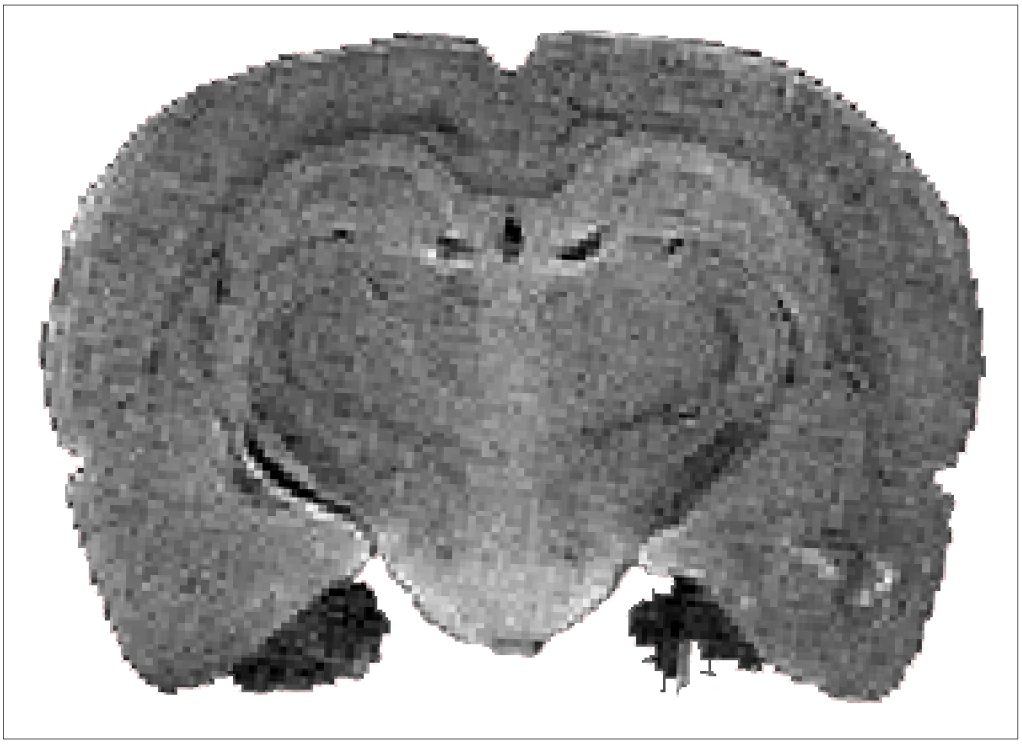

Fig. 8. A high resolution MRI image of the adult rat brain, at about Atlas Level 32. The voxels are 100 μm3, and data for the whole brain took about 24 hours to collect. The male Sprague-Dawley rat weighed 315 g (as did the animal used for the atlas brain). The data set was obtained by Russell Jacobs and Eric Ahrens (Caltech).

Two major problems with voxel based models—low resolution and large file size—are avoided with vector based models. Here, a series of cross sections (the atlas levels) are used, and the surface of individual objects (such as the outlines of a particular cell group in consecutive levels) are "skinned", much like the construction of a ship's hull or an airplane's fuselage. Instead of a space filling model, there is a model based on mathematically defined surfaces, which yield smaller files and infinite scalability. These are important features that minimize rendering times and allow placement of fine details, such as axons and cell bodies, in a gross model of the brain.

There are three obvious uses at the moment for vector based 3-D models of the brain—they can be resectioned in any plane, they can serve as templates for 3-D neuroanatomical databases, and they can display spatially accurate representations of circuits for mathematical modeling of function.

One major problem associated with reconstructing 3-D models from cross sections is that of accurate alignment of the sections in space. While a number of approaches have been taken, none are as yet satisfactory (Toga and Banerjee 1993).

Alignment of the present atlas was accomplished by comparisons with photographic outlines of the brain surface from dorsal, ventral, and midsagittal views, and by "best fit" alignment of a level with the levels rostral and caudal to it. Alignment is not an issue in CT scans, where data are collected as a space filling solid.

The atlas drawings and files presented here can and are being used to construct 3-D computer graphics models, although they need to be modified because they were not designed specifically for such models. For example, to reduce file size some structures in the atlas are not delimited by a closed line; thus, part of the border of some cell groups is formed by adjacent structures such as

other cell groups or a fiber tract. In addition, there are not enough cross sections to model accurately a number of highly irregular (e.g., the ventricular system), or very small (e.g., the trochlear nucleus), structures. Thus, for 3-D modeling, the atlas drawings need to be modified, and in some instances more levels need to be added. Fortunately, serial sections of the atlas brain are available and have been photographed.

Software and hardware for 3-D modeling are developing rapidly, and continue to reduce the great amounts of time required for final rendering of even rather simple 3-D models (much simpler than the complete rat brain). Two applications that are particularly useful for creating brain models from cross sections include Macromedia *Extreme 3D* (introductory) and Bentley *Microstation* (industry standard with built-in database).