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A stereotaxic method for small animals using experimentally determined reference profiles

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In bats conventional stereotaxic methods do not yield sufficient positional accuracy to allow reliable recordings and tracer injections in subnuclei of the auditory system. In a newly developed stereotaxic system experimentally measured patterns of skull profile lines are used to define the animal's brain position with an accuracy of ± 100 μm. By combining the neurophysiological stereotaxic procedure with a standardization of the neuroanatomical processing of the brains, the location of recordings, stimulations or injections can be readily transformed into brain atlas coordinates. This facilitates the compilation and comparison of data within and among animals. The system is not restricted to use in bats and can be readily adapted to other experimental animals.

Introduction

In an attempt to improve the topological accuracy of neurophysiological recordings and focal injections of tracers in small brain areas, a new, reliable stereotaxic technique applicable to small animals was developed.

Since Horsley and Clarke introduced the first systematic stereotaxic device in 1908, a large variety of methods has been developed (for review see e.g. Bures et al., 1976; Bock et al, 1979; De Valois and Pease, 1973). The available stereotaxic methods use coordinates with distinct corporal reference points (e.g. the outer ear canals, the orbital and the position of the upper jaw) and/or skeletal reference points (e.g. bregma and lambda), and then relate their positions in order to position the skull of the experimental animal in a defined coordinate system. The coordinates of the target area are transfered from the brain atlas into coordinates of the stereotaxic device. This procedure is straightforward for systems using rectangular coordinates (e.g. Horsley and Clarke, 1908), whereas in systems with cylindrical or polar coordinates more complicated coordinate transformations are necessary. De-

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spite their complication, the latter systems are more versatile and facilitate the approach to many target areas through the same trepanation hole.

The accuracy of all stereotaxic procedures is limited by the variance of individual skull sizes and the spatial reproducibility of skull landmarks. The choice of landmarks on the skull and the adaptability of the procedure to account for individual deviations of the skull finally determine the reliability of spatial information from a brain atlas for use in neurophysiological experiments. In auditory physiology additional problems arise from the use of earbars for positioning of the animal, as the acoustical transfer function of hollow earbars is complex, notably at high frequencies (e.g. ultrasound in bats). They are virtually unusable for complex auditory stimuli.

The stereotaxic procedure presented in this article circumvents most of the above mentioned problems in 4 ways: (1) earbars are not needed for positioning; (2) contiguous profile lines of the skull are used instead of single points for spatial reference; (3) differences in individual skull sizes are compensated and (4) a specially adapted histological procedure allows continuous verification of both the stereotaxic procedure and the correspondence of the atlas brain series to the in vivo brain position. The method additionally allows the topographical reconstruction of neurophysiological and neuroanatomical data on a common "standard brain" and improves the reliability of interindividual comparisons of data.

Material and Methods

The stereotaxic system consists of 3 major building blocks:

Animal holder combined with a motorized micromanipulator system for the accurate measurement of the actual skull position and for the positioning of electrodes.

Embedding chamber and processing procedure for serial sectioning in standard coordinates.

Computer programs for the processing of neural data in a standard atlas data base (which will not be described in this article).

Animal holder and micromanipulator arrangement

The arrangement of the animal holder and micromanipulator is depicted in Fig. 1 (upper part). The electrode holder (E) can be displaced in 3 orthogonal fixed directions indicated as x, y and z, with a translational resolution of 1 μm in any direction. This is achieved by high resolution micrometer spindles (D_x, D_y and D_z) two of which are driven by stepping motors (M, 1000 steps per revolution). The electrode can be advanced in the z-direction either manually (D_z) or by a piezo stepper (PS, Burgleigh "Inchworm" system). The two stepping motors and the piezo stepper can be controlled by a laboratory computer. The electrode is always advanced vertically (z-direction) in order to avoid translational errors inherent in tiltable micromanipulators. The lateral deviations are negligible due to high precision linear ball bearings in the manipulator tracks.

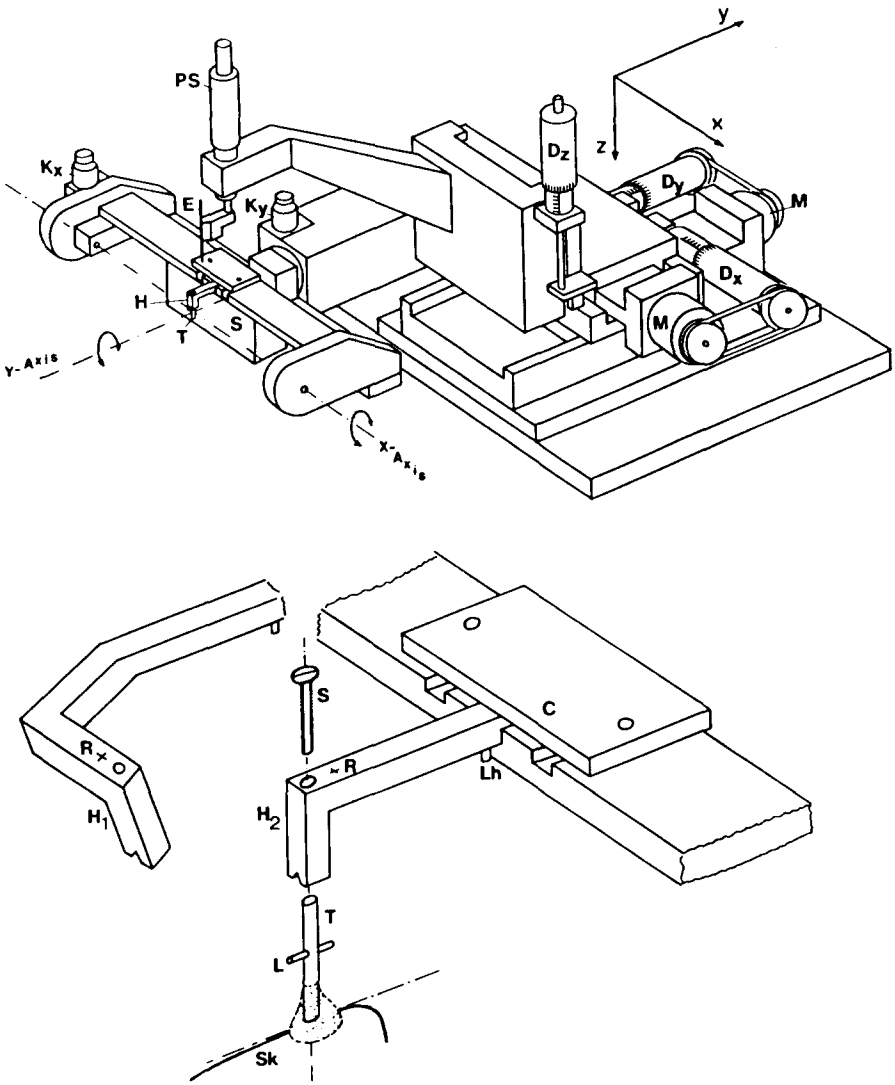


Fig. 1. Upper: schematic drawing of the stereotaxic device. x, y, z indicate the directions for the translational movements of the electrode, X, Y mark the axes around which the animal holder can be turned. Abbreviations: D_x, D_y, D_z micrometer drives along the corresponding directions; E, electrode or probe; H, head holder; K_x, K_y turning knobs for rotation of the animal holder around the corresponding axes; M, stepping motors; PS, piezoelectric stepping device; S, holder for animal restrained in "sandwich"; T, tube to be cemented on animal's skull. Lower: enlarged view of the head holder. The head-holding bar (H_2, for caudal fixation) with the holding tube (T) and the screw (S) in exploded view. Lh adjustment pin, R reference mark, L cross cannula, C clamp, SK skull, H_1 alternative headholder for frontal fixation.

In order to enhance the versatility of the stereotaxic method and to be able to access targets within the brain from different angles, the animal holder was constructed in such a way that it can be tilted around two axes. The animal holder

arrangement is shown in the left part of the upper drawing of Fig. 1. A flat horizontal flange carries at its lower side a small box (S) in which the animal is held in a sandwich-like foam cube tailored to its body outlines to restrain the animal. Only the head is protruding at the front. Above the animal's head the device for immobilizing the skull is mounted to the top side of the flange (H,T). Details of the head holder are depicted in the lower part of the figure. It consists of a holding bar (H_1 or H_2) with a square shaft, a small tube (T) with a fastening screw (S) and a clamp (C). The holding bar fits tightly into a slot milled into the top side of the flange and its longitudinal position is defined by the contact of the pin (Lh) with the flange. The head holder is kept in place by the clamp (C). The small tube (T) fits exactly into the bore through the front end of the head holder and is fastened to it with a screw (S) (S, H_2 and T are shown in exploided view). The outer diameter of the tube, as used in bats, is 2 mm and its length lies between 8 and 12 mm. A cross-cannula (L) defines unequivocally the vertical position and the axial direction of the tube, when fixed to the head holder. The small tube is the only part that is fixed to the skull (SK) of the experimental animal as described later and the different adjustment elements (L, Lh and slots) provide an exactly reproducible fixation of the head in semichronic preparations in which the animal is taken out of the equipment between experimental sessions.

The flange including the animal box (S) and the head holding device can be turned around an axis (*X*-axis in the figure) which allows a rostrocaudal tilt of the animal's head. The bearings of the *X*-axis reside themselves on a carrier bar that can be turned around an axis parallel to the long axis of the animal (*Y*-axis in the figure) and thus the left to right tilt can be adjusted. The angular resolution for both axes is better than 0.04° and adjusted manually with the knobs K_x and K_y. Both imaginary axes run through the animal's skull.

Preparation of the animal

The animals are surgically prepared under halothane anesthesia. The skull area overlying the target brain structure and the area along the rostrocaudal median line are freed from tissue. Depending on the brain area from which recordings are to be made the holding tube (Fig. 1, T) is either cemented on the front or the back of the animal's skull. In the former case a tilted head holder (H_1 in Fig. 1, lower) is used, which by its special form, does not obstruct access to the posterior half of the skull; a straight holder (H_1) is used when the tube is cemented to the back of the skull, in order to allow free access to the front part of the brain, e.g. in the case of cortical structures.

At this stage of the preparation only two rules have to be adhered to for the fixation of the tube:

(1) Its orientation must provide an approximately natural head position, when the animal is fixed in the device.

(2) The cross-cannula of the tube must be accurately aligned with the long axis of the skull, which can be easily done optically under the binocular.

All other corrections to reach a standard position or any other desired orientation of the head relative to the coordinate system of the micromanipulator can be done

later in the stereotaxic holder. In bats the tube is cemented to the skull with cyanoacrylic glue and dental cement, but any other fixation method, e.g. by means of screws in larger animals, is appropriate.

Profile scanning procedure

After the bat's head has been fixed to the stereotaxic device the actual position of the skull relative to the electrode coordinate system (x, y, z) and relative to the turning axes of the animal holder (X, Y) is experimentally determined. Skull coordinates are measured at the tip of a metal probe installed in the electrode holder and referred to a reference mark (R, Fig. 1) on the head fixing bar, in order to make it possible later to determine coordinates independent of the probe or electrode used. First, the alignment of the long axis of the skull with the y-direction of the system is verified at two distant points on the median ridge of the skull (e.g. arrows in Fig. 2B1). A maximal lateral deviation of 150 μm over a 5000 μm rostrocaudal distance is considered negligible for the profile procedure. Then the curvatures of integral parts of the skull surface are measured. In rhinolophid bats the skull profile lines $\pm$ 500 μm parasagittal to the ridge of the skull (Fig. 2B2, arrows) proved to give reliable information of the skull's orientation as a whole if combined with the coordinates of the nose plateau (N, Fig. 2B1). The z-coordinates along the profile lines are measured in steps of 250 μm along the y-direction from the most rostral to the most caudal position of the exposed skull area. The path of the probe in the x- and y- directions is controlled by a DEC LSI 11/23-computer, which automatically adjusts the stepping motors of the micromanipulator drives. At each x, y-coordinate pair the z-drive (Burgleigh Piezo stepper "Inchworm") advances freely and stops automatically when the probe tip makes electrical contact with the skull surface. The z-coordinate at probe contact is read and the probe is withdrawn approximately 1 mm before the next coordinate pair is chosen and the measurement cycle is repeated. The coordinates along the scanning path are stored and graphically represented. The same scanning procedure is used to determine the coordinates of the nose plateau (N in Fig. 2B) and also to evaluate the horizontal left-to-right symmetry of the skull's position. The skin over the nose plateau does not have to be removed and its average thickness is taken into account during the fitting procedure. In principle any scanning path suitable for a positional characterization of the skull can be chosen.

Fitting procedure

The experimentally obtained partial profiles of the skull ($\pm$ 500 μm lateral to the median line) and the nose plateau are compared with the profile line of a "standard skull" and graphically brought into best coincidence with it. The standard profile line of the skull used for the fitting procedure has been averaged for 12 specimens (Fig. 2A, lower). The interrupted lines indicate the maximum individual deviations and the extremes cover the range of skull sizes encountered in adult individuals of different sex, age and body weight. The dotted line above the nose plateau takes into account the thickness of the overlying skin. The included axes define the brain atlas coordinate system and their exact definition will be described later.

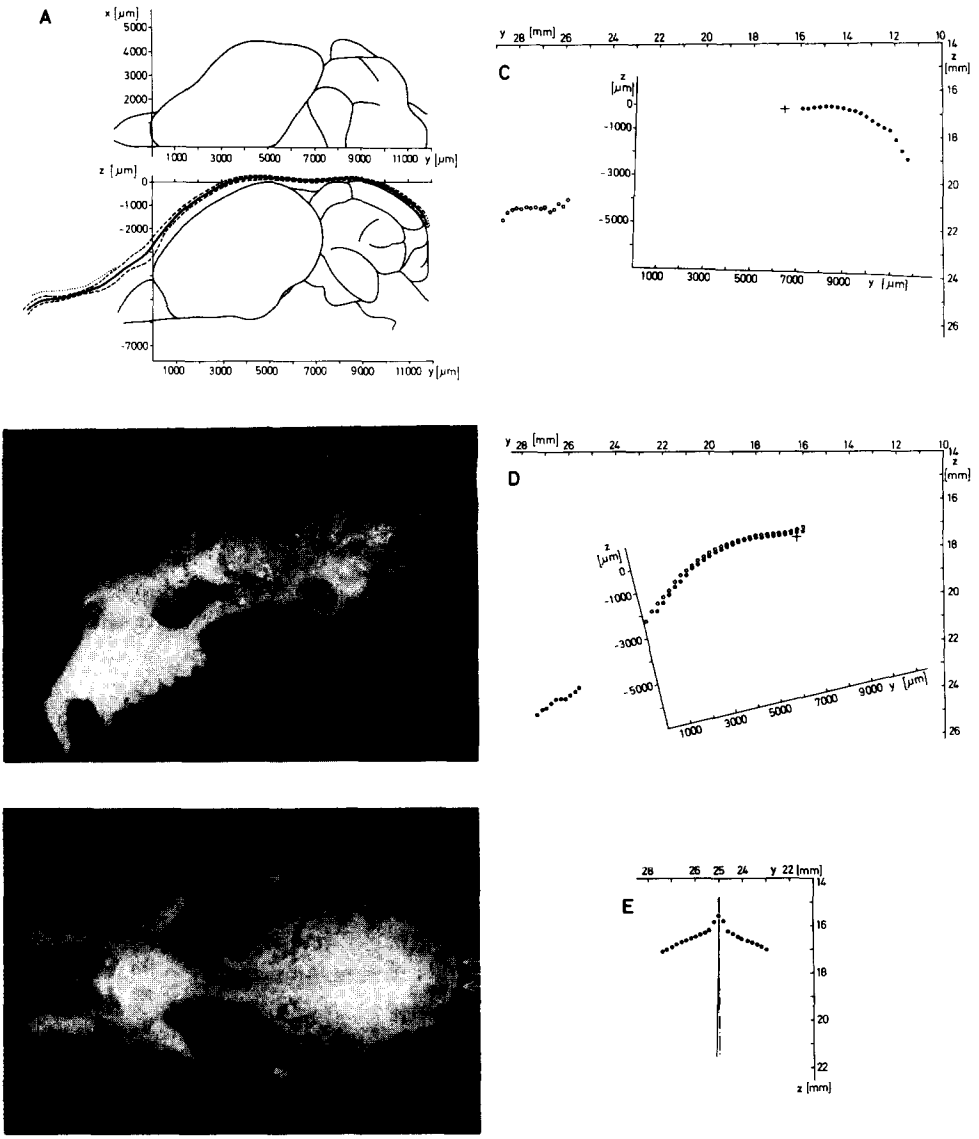


Fig. 2. Skull and brain of the bat *Rhinolophus rouxi*. A: in vivo position of the brain in relation to the parasagittal profile lines ± 500 µm lateral to the median line of the skull (lower) and dorsal view of the brain (upper). The mean profile line was evaluated from 12 skulls and the stippled lines indicate the maximum and minimum individual sizes. The coordinate system gives the coordinates of the brain atlas. The dotted line at the nose profile takes into account the skin overlaying the skull. B1 and B2: lateral and dorsal views of the skull with indication where the profile lines are taken (arrows in B2, stippled marks indicate skull median line). N and arrows in B1 indicate where profile and nose have been measured in C. C and D: experimentally determined parasagittal profile lines in an inferior colliculus and auditory cortex experiment, respectively, represented in the coordinate system of the electrode carrier system (outer axes). The brain atlas coordinate system has been inserted using the best fit of these curves to the mean skull profile line (A). Crosses indicate the position of the turning point of the animal holder in the sagittal plane. E: experimentally determined skull profile line in frontal plane represented in the electrode carrier coordinate system. Only a slight tilt bringing the solid line (symmetry axis of skull) into coincidence with the broken line (vertical direction) is necessary to establish full left to right symmetry.

Graphs 2C and 2D show partial profile lines as obtained in acute experiments for recordings from caudal or rostral parts of the brain, respectively. The outer grid represents the coordinate system of the electrode carrier. When the best fit with the standard skull profile is achieved, which is done by graphical superposition, the standard atlas coordinate axes can be transferred to the drawing of the actual skull position (inner axes). Thus the link between the position of the animal's brain (atlas coordinate system) relative to the electrode reference system is established. Lateral tilt of the animal's skull is evaluated from the left-to-right frontal profile line (Fig. 2E), which is symmetrical relative to the median line in the standard skull position. As the coordinates of the turning axes of the animal holder are mechanically defined and consequently known relative to the brain coordinate system (crosses in Fig. 2C,D,E) any coordinate transformation by tilting or translation can be monitored. The use of the profile lines instead of single reference points proved to be very reliable, and in the rare cases of extreme skull deviations the exact fit can be reached by similarity transformation.

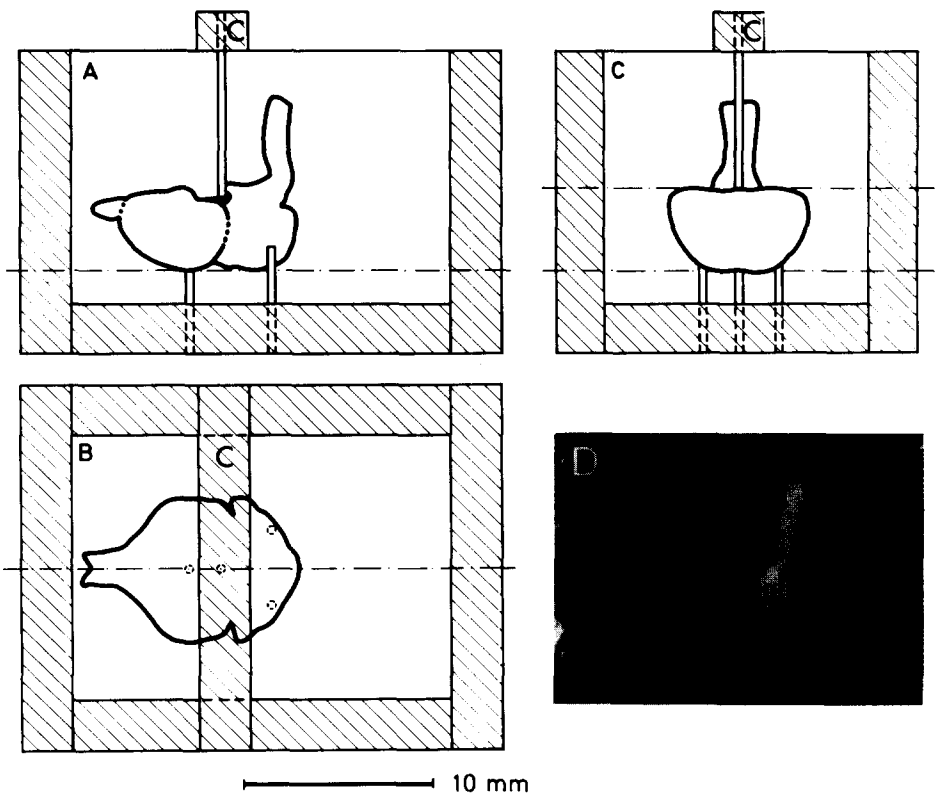
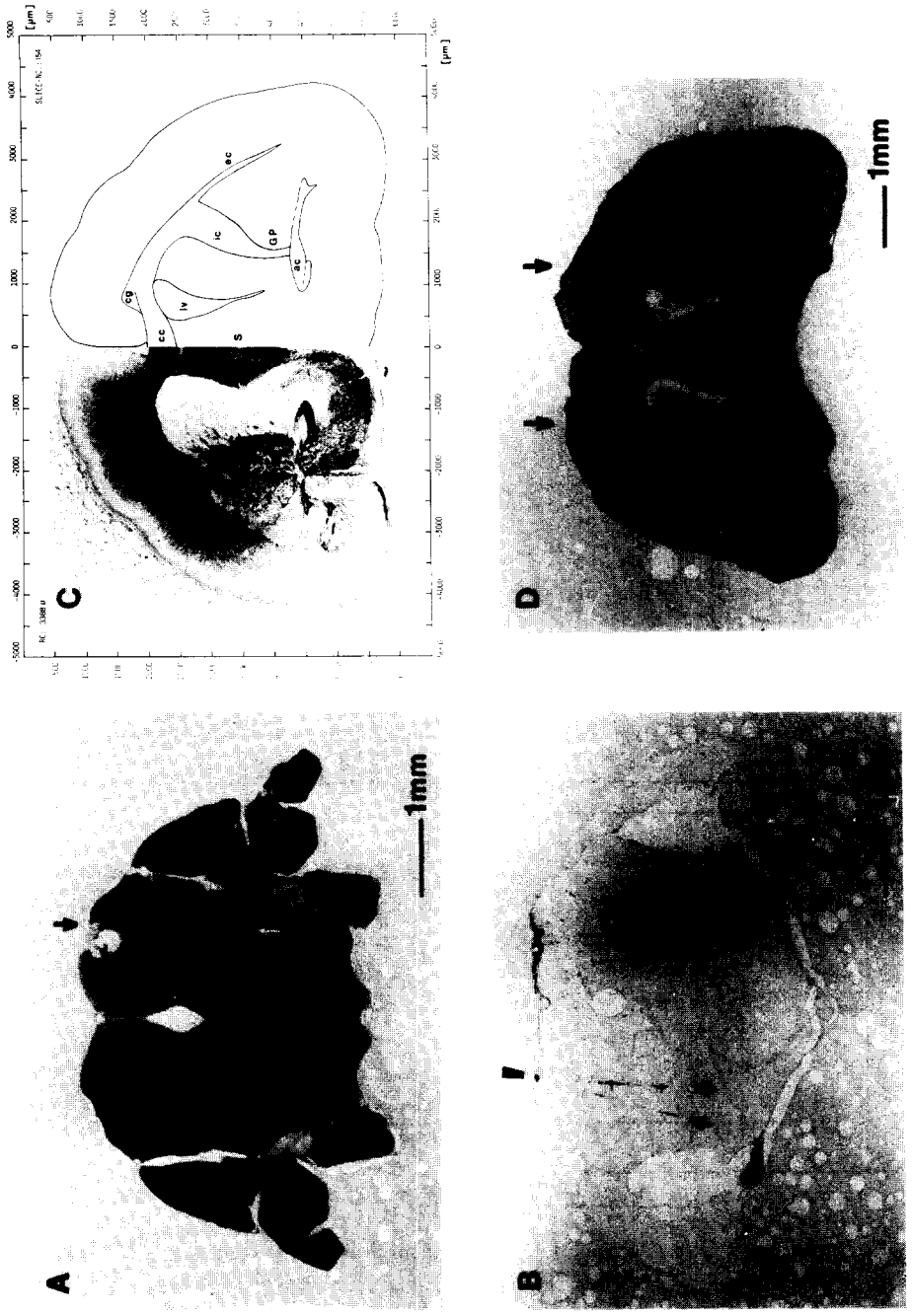


Fig. 3. A, B, C: cubic chamber for embedding of the brain in standard coordinates. The broken lines indicate the guidelines for orienting the fixed brain prior to embedding. Sides are removable to facilitate block removal. D: sagittal view of a gelatine block with embedded brain.


A
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The entire procedure starting with the fixation of the animal in the holder, including the measurement of the profile lines and the determination of the actual skull/brain position by the graphical fitting maximally lasts 1–1.5 h. This investment of time is by and large compensated by the reliability of the stereotaxic information.

Brain–skull fitting and definition of the standard coordinate system

In order to define a common coordinate system interfacing between the stereotaxic procedure and the histological processing of the brain, the in vivo position of the brain relative to the outer profile lines of the skull was determined by scanning the sagittal and parasagittal profile lines also inside the roof of the skull. Since the brain of *Rhinolophus rouxi* fills the cranium completely, the dorsal maxima of photographed parasagittal profiles of undistorted fixed brains of medium size fitted well in these inner profile lines and resulted in a "mean brain position in vivo" as depicted in the graph of Fig. 2A.

The "standard coordinate axes" have been defined as drawn in Fig. 2A: the frontal plane ($y = 0$) is perpendicular to the plane which connects the dorsal extremes of the parasagittal brain profile ($z = 0$) and perpendicular to the sagittal plane ($x = 0$) of the brain. The zero plane of the y-axis was arbitrarily defined as the rostral beginning of the forebrain hemispheres as this level is easily recognized in frontal sections. The plane defined by $z = 0$ was chosen for convenience of the histological processing of the brains, as consistently slicing the brain in the same standardized plane can only be achieved if reliable "landmarks" of the fixed brain can be used during the embedding procedure.

Sectioning of brains in standard coordinates

Reproducible sectioning in standard coordinates is achieved by a standardized embedding procedure in a perspex chamber (Fig. 3) adapted from a design by Andres and von Düring (1981). The chamber has a cubic volume just large enough to accommodate a rhinolophid brain. The walls, two of which are removable, are exactly oriented at right angles and define the possible sectioning planes. The brain is positioned on 3 retractable pins in such a way that its long axis corresponds to the median line of the chamber and the line connecting the dorsal surfaces of the

Fig. 4. Frontal sections of bat brains, A: section through midbrain with electric lesioning tract (1 μA current, electrode retracted at 20 μm/s) crossing the inferior colliculus and parts of the lateral lemniscus (black and white arrows) counterstained for cytochrome oxidase. B: lesioning point at end of penetration (2 μAmin, lower arrows) and lesioning tracts (current application as in A) as visualized in an unstained TMB-processed section. The right side of the section shows TMBreaction product near an HRP-injection site. C: fibre-stained atlas section (left) with corresponding outlines (right) constituting the computer data base (section through auditory cortex). D: frontal section at the same rostrocaudal level as atlas section in C. The section contains two symmetrical HRP injections, is processed after the DAB-procedure and counterstained for cytochrome oxidase. In the cytochrome oxidase stain the fibers appear white, the neuropil in a shaded brown. Abbreviations: ac, anterior commissure; cc, corpus callosum; cg, cingulum; ec, capsula externa; GP, globus pallidus; ic, capsula interna; lv, left ventricle; S, septal region. All examples show the high reliability of the procedure as demonstrated by the coincidence of the standard penetration plane with the sectioning plane of the atlas series.

cerebrum and cerebellum is parallel to the base of the chamber. A fourth pin descending from the cross-bar on top (C) to the ventral side of the brain holds it in this position. For later reference and documentation photographs from all 3 spatial planes can be taken with a Wild photo-macroscope before the brain is embedded by filling the chamber with a mixture of egg yolk or gelatine and glutaraldehyde. After 5–10 min for stabilization the block (Fig. 3D) can be removed and used to make either frontal, horizontal or sagittal serial sections in the planes of the standard coordinate system.

We routinely use the rapid frozen section method, as the tissue quality can be preserved by good fixation and adequate cryoprotection, and practically no shrinkage (max. 10%) occurs. In addition, frozen sections are necessary for many histological procedures.

This embedding procedure yields serial sections of high symmetry (e.g. Fig. 4A,B,D) and sections of different brains can be directly superimposed on sections of the brain atlas (Radtke-Schuller, unpublished). Thus the atlas is not only used to determine the coordinates of target brain structures for neurophysiological recordings and tracer applications, but also constitutes the standard basis on which all neurophysiological and anatomical data of *Rhinolophus rouxi* can be pooled. The reliability of the integral method has been tested with electrolytic lesions: lesioning tracts at predetermined coordinates (x, y) over 2000–4000 μm along the z-axis of the standard coordinate system were visible in full length within 1–2 section of 48 μm thickness at the predicted rostrocaudal position (Fig. 4A,B). As a further demonstration of accuracy, Fig. 4D represents a section containing two HRP-injections (DAB procedure/cytochrome oxidase counterstain) at the same rostrocaudal coordinate. The centers of the two injections are displayed in one single section of 48 μm proving that the neurophysiological as well as the anatomical procedures had a high precision. Comparing section 4D with the corresponding atlas section 4C it is evident that many details (e.g. capsula internal (ic), anterior commissure (ac), septal region (S)) show almost perfect coincidence.

Discussion

Conventional stereotaxic methods as originally introduced by Horsley and Clark (1908), are based on stereotyped fixation of the animal's skull in a rigid frame. The basal plane of the coordinate system is defined by plugs in the external meatuses of the animal's two ears and by clamps on the inferior orbital rim (except in rodents). The position can be further verified by using well-defined landmarks on the skull. The accuracy of the skull position in the stereotaxic frame and hence the position of the brain is dependent on the spatial reproducibility of the fixation points and the landmarks of the skull.

Additional problems in positioning the animal's head arise in auditory research. Especially in small animals great care must be taken not to damage the temporal bones when positioning ear bars. In addition, acoustical stimulation through hollow ear bars presents acoustical transmission problems when high, especially ultrasonic, frequencies are to be used.

We chose an experimental method to evaluate the stereotaxic coordinates of the animal's skull after fixation of the skull with a head holding bar, thus completely avoiding the use of ear bars. The experimental determination of skull position in the reference coordinate system after fixation is based on continuous profile lines of the skull and not on singular landmarks. This constitutes a significant advantage over conventional methods, since the continuous profile lines show characteristic patterns which are largely independent of local deviations of individual skulls. In addition, the pattern of the parasagittal profile lines gives information on whether the individual skull size deviates considerably from the mean size used in the reference system and whether it is necessary to compensate for the deviation by a similarity transformation. In our experiments, in about 90% of the cases the deviations of skull size from the mean was far below 10%. In principle, any other superficial skull pattern (lines or planes) can be used if it characterizes the position and orientation of the skull of the species in use well.

Tiltable electrode carriers create translational deviations of the electrode tip dependent on the advancement of the electrode which are difficult to assess. This problem has been avoided in our procedure by inclining the animal's skull around two horizontally oriented axes instead of tilting the micromanipulator. Thus the accurate position of brain structures within the device oriented system can be determined computationally or graphically by simple rotational transformations and oblique penetrations are easily handled. The stereotaxic system thus combines the particular advantages of rectangular coordinate (electrode carrier) and polar coordinate (animal holder) stereotaxic principles.

The closely adapted "stereotaxic" anatomical procedure which yields accurate sectioning in the standard planes is an indispensable part of the stereotaxic method. Due to the reproducibility of the histological sectioning plane virtually every serial section of an individual brain can be brought into clear coincidence with the standard brain series (atlas). Our experience has shown that even if the histological processing leads to some distortions of the serial sections, it is still possible to relocate the sections within the atlas series.

Pooling of data from different penetrations in the same animal is highly reliable due to the calibration of the electrode tip to the reference point, and spatial accuracy is typically far better than 100 μm. Pooling of data from different individuals is inherently less reliable and the accuracy of absolute positions within the brain as derived from the comparison of lesioning tracts in different animals is better than 200 μm in all 3 dimensions and typically around 100 μm.

A major advantage of the described stereotaxic procedure is that each experiment can be used for updating and optimizing the extant information on skull-to-brain relationships and on individual deviations.

The method is verified and controlled in every experiment by a lesioning tract in the standard z-direction. We routinely find the lesioning path over a depth of 3000 μm within one or two 48 μm thick sections (angular deviation below 1.8°) demonstrating the power of the combined procedure.

A beneficial consequence of the matching of the sectioning plane to the lesioning plane is that even minute lesioning tracts can be visualized if they lie within one

section and that therefore only very low electrolytic currents (less than 2 μA) which only damage minute areas of tissue (about 50 μm diameter) are needed. These low currents can be applied through any kind of electrode (e.g. glass pipettes or metal electrodes) and also during running experiments. Oblique lesioning tracts of this size would appear as single lesioning points in different sections and would be buried in normally occurring tissue artifacts.

The method has been successfully applied in bats for research in several brain areas including the auditory cortex, the medial geniculate body, the inferior colliculus and the nucleus of the lateral lemniscus. In all cases we were able to hit reliably substructures of these nuclei with an accuracy of at least 100–200 μm. The most rigorous test of the method was the chronic implantation of metal electrodes into the cochlear aqueduct through the cerebellum for the recording of cochlear microphonics. In 70% of the cases we were able to hit the 120 μm wide opening at a depth of 5000 μm without any visual control.

Up to now the method has been applied in three bat species, *Rhinolophus rouxi*, *Pteronotus p. parnellii* and *Noctilio albiventris* with similar results. The method should generally be adaptable to other small mammals as the skull information which is finally used for the definition of the stereotaxic position can be freely defined by the experimental needs.

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