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Flattened Cortical Maps of Cerebral Function in the Rat: A Region-of-Interest Approach to Data Sampling, Analysis and Display

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Abstract

We describe a method for the measurement, analysis and display of cerebral cortical data obtained from coronal brain sections of the adult rat. In this method, regions-of-interest (ROI) are selected in the cortical mantle in a semiautomated fashion using a radial grid overlay, spaced in 15 degree intervals from the midline. ROI measurements of intensity are mapped on a flattened two-dimensional surface. Topographic maps of statistical significance at each ROI allow for the rapid viewing of group differences. Cortical z-scores are displayed with the boundaries of brain regions defined according to a standard atlas of the rat brain. This method and accompanying software implementation (Matlab, Labview) allow for compact data display in a variety of autoradiographic and histologic studies of the structure and function of the rat brain.

Keywords

cerebral cortex; rats; brain mapping; topographic maps; software

INTRODUCTION

Rodents represent one of the primary animal models for studying brain function. Typically experimental results are interpreted in relation to one of the standard brain atlases [8,12]. One means for data display is to present functional data on individual brain sections alongside co-registered structural images obtained by histological staining or structural imaging (CT, MRI). While this approach is useful for summarizing local changes, the method of presentation is not

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well suited for mapping activity across the extent of the cortex. Furthermore, most commercially available imaging software packages are not well suited for cortical mapping, and do not provide a means for identification of brain regions according to a brain atlas reference. The current report describes a region-of-interest (ROI) approach to the sampling, analysis, and display of brain imaging data on the flattened map of the rat cerebral cortex. Our method is described in relation to autoradiographic images, though in principle, it would be applicable to any method whose endpoint measurement is that of optical density, fluorescence or colorimetric intensity.

METHODS AND RESULTS

Figure 1 depicts an overview of the methods for generating cortical maps, while figure 2 demonstrates an example of these methods using autoradiographic images of cerebral perfusion in the rat.

Acquisition of brain sections

The interslice distance determined during sectioning provides the spacing of the coronal sections of each brain. The optimal number of slices is best dictated by the experimental paradigm and the spatial resolution of the imaging method. We find for many applications that coronal slices with an interslice distance of 300 microns adequately sample the rat brain. Typically, the cortex of the adult rat brain can then be reconstructed from 52 digitized coronal slices between, for instance, bregma +6.0 mm and -9.3 mm. Identification of an internal cerebral landmark within each brain allows for the coregistration of each animal's set of coronal slices across all animals, such that the same total number of slices, as well as the same number of slices anterior to the landmark is preserved between animals. Such a landmark should be easily recognizable with the naked eye within the imaged sections, and should be relatively invariable across animals. In general, choosing a landmark near the primary region of interest is advisable as this choice creates the greatest local stability [10,13], with bregma being the best reference for the whole brain [11]. We have chosen a position at Bregma -0.3 mm as defined in the 2005 rat atlas [8] as our landmark, specifically as it relates to fusion of the anterior commissures across the midline.

ROI Selection

The ROI selection of cortical regions is applied to digitized autoradiographs of brain sections and proceeds with a software interface developed in Matlab (Matlab 7.1.0.21, release 14, The Mathworks). Regions are sampled on the 8-bit digitized images using two radial hemigrid overlays, with rays spaced in 15° intervals from the midline (Fig. 3), resulting in an average distance between ROIs along the cortical rim of 1.60 $\pm$ 0.39 mm, sufficient to resolve the major cortical regions. Overlay of this template on each digitized brain slice image allows for measurement of the optical density at locations in the cortical mantle in a manner invariant between animals. The center of each hemigrid is placed respectively in the left and the right hemisphere such that the vertical axis runs along the sagittal sulcus and the horizontal axis equally bisects the top and the bottom halves of the brain. Separation or rotation of one or both of the hemispheres due to artifact incurred during freezing or mounting of the specimen can be accommodated by translocation or rotation of the hemigrid.

ROI density measurement

For the analysis of the cortex, the optical density in each slice is measured for 10 locations within each hemisphere at locations in the cortex near the intersections of the hemigrid with the cortical rim. After the hemigrids have been positioned by the user, the program analyzes the image to automatically determine the boundary between the brain and its background. A binary "mask image" is generated based on a threshold gray level in the original image, with

the brain slice changed to white and the background to black [7]. Dilation/erosion and erosion/dilation operations are applied to remove pixels of white in the black background and pixels of black in the white brain rendering the contour of the white brain less “pixelized”. The structuring element in the dilation and erosion operations is a disk with radius equal to 5 pixels (equivalent to 100 microns). These operations are performed only on the mask image that is used to define the brain/background boundary, and do not affect the image itself onto which the ROIs are subsequently placed.

A search is then conducted along each ray of the hemigrids in the binary mask image, from edge to center, to locate the border between the background and the brain. When the fraction of white pixels in the search region (a square of dimension 358 microns, equal to 1.75% of the image height for a 1024×1024 pixel image of 20 microns/pixel) becomes greater than a set percentage (60%), the edge of the brain is assumed to be detected. The program then places an ROI along the ray on the original image at a position that is one ROI-width further in (358 microns center to center) from the edge detected on the binary image. The ROIs are also squares with a width/height that is fixed to 1.75% of the image height (358 microns). This placement avoids any artifacts that may impinge on the cortical surface (e.g. freeze-thaw artifact in the outer cortical rim that may occur when an embedding compound is applied to the frozen brain). As long as the conditions for digitizing the images are kept constant, the ROIs are all of the same size in all images and all brains. Thus, the placement of the ROIs is very reproducible and only affected by the selection of the hemigrad discussed earlier. Users wishing larger or smaller ROIs are permitted to redefine the default ROI size within the software user interface. Ten locations undergo ROI sampling (from 15 to 150 degrees off the vertical). Adding more locations, i.e. at 165 degrees and 180 degrees of the radial hemigrad, is not done because in many slices these locations would be outside of the cortex. After all 20 ROIs have been placed, the user is able to manually reposition the ROIs that are not located satisfactorily (manual positioning of all ROIs is also available as a default option). Simultaneous with each cortical ROI selection is the automatic selection of an ROI on the background, in close proximity to the ROI on the coronal slice, and along the same radial grid line. The subtraction of the optical density or intensity of each brain ROI from the corresponding background ROI allows for correction of inhomogeneities in the background that may arise from the film development or the illumination of the slice during digitization. In the Matlab program, a final comment field allows the user to make annotations regarding data points to discard or replace.

The Matlab ROI selection program allows for export of the optical density data of each ROI and associated background in ASCII format, alongside a code for the experimental group, rat number, and slice number (derived from the file name of each slice). Data deletions, substitutions or corrections can be made in this file, based on annotations made during the ROI selection process. Also saved are image files documenting the locations of the ROIs as selected on each slice. Time commitment for data selection is estimated at less than 1 hour per brain.

Generation of parametric cortical maps

ROI analysis and topographic mapping is performed on the ASCII file using a custom software program written in LabVIEW (National Instruments). Required inputs are (a) the ROI data file, (b) a file describing for each animal the slice containing the coregistration landmark (e.g. bregma -0.3 mm), and (c) a table identifying for each bregma level a number identifying the subset of the possible 10 ROIs to be analyzed in each hemisphere. This number ranges from 6 to 10 and takes account of the fact that at some bregma levels the largest angles in the hemigrad may lie outside the cortex. To allow for the examination of hemispheric asymmetry, the user is prompted to define whether individual ROI values are averaged for each animal across the hemispheres, the p-value for determining statistical significance between the experimental groups, whether the statistical analysis uses one- or two-tailed t-tests, and the interslice spacing.

The user is also prompted to define the minimum number of animals in a group needed to proceed with the data analysis at a single brain location in the case of missing data. This flexibility allows analysis to proceed in the face of missing slices or data discarded because of artifact at an individual brain location.

For every brain, the global mean and standard deviation (SD) is calculated for the ROI densities at all locations in all slices. A z-score transformation [2] is performed on the data to produce patterns of “normalized” regional changes for each animal. This transformation eliminates variations in mean distribution between subjects and experimental groups created by global effects and systematic experimental error, although it prevents detection of true global differences in tracer levels that could be present between experimental groups. Alternatively, the non-normalized data set could be provided for statistical analysis. Group differences in optical density at each ROI location are assessed on the z-scores using t-tests. In addition, mapping of the patterns of differences between groups is performed on the z-scores.

The program provides for display and for export in ASCII format:

- a. The slice coregistration table depicting coregistration at each anterior-posterior bregma coordinate for each brain,
- b. The optical density and z-score matrices for all bregma positions and all locations in all slices. Data can be displayed for individual subjects, individual experimental groups or their differences. Cells in each matrix are associated with specific labels identifying specific brain regions. Region identifiers are derived from the overlay of the radial grid on the digitized images of the coronal rat brain sections from a standardized atlas [8].
- c. The significance matrices of p-values for the results of t-tests performed between groups at each ROI for the results of (b). Region identifiers for each cell of the matrix are used to interpret cells with statistically significant differences.
- d. An anatomic display of the optical density measures or their z-score transformed values on the two-dimensional, flattened cortex. Maps are displayed on a color scale for each animal, each group of animals or group differences. This process is further described below.

The cortical ROIs are displayed as intensity values along a straight line, with an inter-ROI spacing determined by the ROI's circumferential distance along the cortical rim sampled with a sampling interval of 200 μm . The placement of the ROIs is standardized and obtained by placing the 15 degree radial grid on the digitized images of coronal slices in the rat brain atlas [8], and measuring the distance between ROI locations relative to the atlas' standard 5 mm calibration length. This process is facilitated by the absence of gyri and sulci of the rat's brain (lissencephaly). For ROIs sampled at anterior-posterior positions not represented in the atlas, circumferential distances are determined by linear interpolation between the measurements on the preceding and following slices of the atlas. An example of a single line of data points can be seen in the final panel of figure 2. Subsequent slices are then displayed as additional intensity values along a line, whose distance from the prior line is the interslice distance (300 μm). Hence, the z-score or z-score difference data is relocated into a two-dimensional anatomical matrix with cells every 300 μm in the anterior-posterior dimension and every 200 μm in the lateral dimension. Empty cells between the experimental z-score values are filled in using an iterative process of successive linear interpolations. In these interpolations, the value of each interpolated cell is determined by the average of its eight neighboring cells. Interpolation distances for any one of the eight cells varies between 300–1000 μm (1–5 empty cells). Data cells containing empiric z-score measurements are not altered and kept constant throughout the iterative interpolation. After the entire interpolated image is calculated, it is compared to

the preceding interpolated image. The pixel value with the largest relative difference between the current image and the previous image is sought and compared to a convergence threshold (set to 10^{-5}). If convergence is not reached, a new interpolated image is recalculated using the preceding interpolated values. The two-dimensional array of the average z-score values is represented graphically using a color scale on the two-dimensional surface map. The borders between cortical regions, as defined and measured using the hemigrid overlays on the coronal sections of the rat brain atlas [8], are drawn on the cortical maps to allow for a visual correlation between patterns in the data obtained and regional anatomy.

It is worthy of note that in the analysis of data processed with our method, the statistical interpretation of the data is not based on the qualitative anatomic images generated by the interpolation process but rather on the z-score comparisons between measured ROIs as shown in figure 2. The images just helps convey the study results in an intuitive graphical fashion.

DISCUSSION

We describe a method for selection, display and analysis of data derived from histologic sections of the rat brain. Matlab and Labview implementations of the described methods are available through the publisher's website. The method for cortical mapping has been successfully applied in paradigms eliciting motor and somatosensory cortical activation in response to a motor task [4], as well of limbic activation in an auditory conditioned fear paradigm [5]. In a paradigm in which rats were exposed to a 1 kHz/8 kHz alternating auditory stimulus, the described method mapped significant changes in cerebral blood flow to the caudal portions of auditory cortex [3], an area which has been shown by others using microelectrode tonotopic mapping techniques to represent sound frequencies in the lower four octaves (from 0.5 to 8 kHz) [1].

Advantages of the ROI method include the fact that the user, even while taking advantage of the autoplacement routine, always retains the ability to redirect placement of an ROI away from regional tissue artifacts. Furthermore, the ROI method is free of the artifacts introduced by slice registration or warping, as may appear in the case of a voxel-based analysis of the three-dimensionally reconstructed brain such as statistical parametric mapping (SPM) [6]. Region-of-interest analyses that require the user to interactively place the ROIs within a selected brain section can be time intensive and potentially influenced by the subjective nature of placement of the ROI. We have tried to minimize the impact of this limitation with a semi-automated approach to ROI selection on a predefined template. Decreasing the angular spacing of the radial grids and increasing the numbers of ROIs selected within the cortex could be used to detect very localized changes in the cortical images -- a modification our method and software could accommodate.

Coregistration of the rat brains in a data set based on the internal landmark from a single slice carries certain limitations. An error of ± 150 microns is inherent in the assignment of one slice to a specific bregma level given an interslice distance of 300 microns. Furthermore, it is known that some interindividual variation exists in the location of any single landmark, particularly when this landmark is based on a strain or age of rats different from that used in the construction of the standard rat brain atlas [9]. In addition, rat atlases are derived from single animals and hence, themselves show some variability in the definition of specific landmarks (e.g. the initial anterior fusion of the corpus callosum across the midline differs by 680 microns between the 1998 and 2005 Paxinos rat atlases). It would be preferable to determine landmarks and anatomic distances within the cortex based on an atlas that uses statistical sampling of animals of different strains, genders, and ages. However, such atlases are currently not available for the rat brain. For our maps, we have chosen bregma (AP -0.3 mm) as the landmark for alignment as this landmark has been reported to show the greatest

individual stability for whole brain, resulting in a mean absolute error for predicting the position of brain structures of 370 – 490 microns) [11].

Additional sources of error in transferring neuroanatomical data to a standard atlas include differences in the plane of section and nonlinear warping of the section during freezing and mounting onto glass slides. The rat atlas on which our method is based depicts coronal sections perpendicular to the longitudinal axis when animals are placed in a flat skull position. Hence, it is important that coronal section used for the flattened maps be obtained in a similar manner. Though the method of obtaining ROI data using the radial hemigrids addresses issues of distortions that are linear or rotational, it does not address nonlinear distortions. A number of methods are available for transforming images of experimental sections to the shape of standard sections based on a variety of topological algorithms. As noted by Swanson the accuracy of such transformations must be validated and frequently will require the definition of multiple fiducial points for acceptable warping to proceed. However, currently no single approach has proved satisfactory, partly because there are no rigid, internal fiducial structures in the brain [12].

In the display and interpretation of the cortical data, particularly in the designation of borders of individual regions some degree of uncertainty remains. This is both at the level of the reference atlas (compare for instance, the extent of frontal association cortex as defined in the Paxinos and Watson atlas [8] and the Swanson atlas [12]), as well as at the level of ROIs whose placement along any of the rays of the radial grid may in fact be located in a transition zone between two regions. Where possible, we have tried to minimize this uncertainty by including in the nomenclature cortical regions designated as “transition zone”. Furthermore, for the generation of the anatomic maps, interpolation of data between fixed data points carries an inherent limitation of minimizing or exaggerating the full extent of a measured change. Such potential errors could be reduced if more brain slices were sampled at narrower interslice distances and ROIs were sampled within each slice according to a template grid of greater resolution. Our program selects ROIs in the cortex at a fixed distance from the brain’s surface. Consideration could also be given in the future of adopting our software to allow for multiple ROI selections through each of several cortical layers along each ray of the radial grid.

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Figure 1.
Overview of the method for generating cortical maps.

Figure 2.

Method for generating cortical maps applied to an example of the brain imaging of cerebral perfusion. After intravenous injection of the perfusion tracer ($[^{14}\text{C}]$ -iodoantipyrine), brains are sectioned, and autoradiographic images of the distribution of cerebral blood flow related tracer distribution are obtained, followed by region of interest selection in the cerebral cortex using a radial grid. Optical density z-scores are then displayed for each ROI in all slices as a data two dimensional array (left hand table). Also shown is the corresponding region identifier array in which ROIs are identified according to abbreviations in the rat atlas [8] (right hand table). In both cases, ROIs 2 to 8 have been omitted for clarity. Annotation with an asterisk indicates if significant group differences exist at the specified location. Z-scores are also shown as color-coded maps of the flattened cortex. In these maps cortical regions of a single slice are represented on a single line, with values between data points generated using a standard linear interpolation. White dots represent the points at which data was collected. Anatomic boundaries of the cortical areas as defined in a rat atlas [8] are superimposed on the cortical map.

Figure 3.

Method for determining placement of the center of the radial hemigrid: The user examines the midline of the brain and defines a central axis and its degree of tilt by selecting with the cursor of the computer's mouse a top and a bottom point (1–3) within the sagittal plane. The user then defines the lateral shift of that axis (4). The shifted axis, parallel to the central axis, should be aligned with the most medial point of the cerebral cortex of each hemisphere, i.e. closest to the midline. Often this lateral position will fall in the midsagittal plane, but not always. The lateral shift is helpful in accommodating the separation of the cerebral hemispheres that can arise as a result of freezing artifact. The user then selects the top-most border and the bottom-most border of the slice orthogonal to the central axis (5). The hemigrid's center is placed halfway between these two lines along the shifted axis (6–7).