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Protocol

Volumetric measurement of the hippocampus, the anterior cingulate cortex, and the retrosplenial granular cortex of the rat using structural MRI

O.T. Wolf[c,e,f,*], V. Dyakin[a], C. Vadasz[a,d], M.J. de Leon[b,c], B.S. McEwen[e], K. Bulloch[e]

[a]*Laboratory of Neurobehavioral Genetics, Nathan S. Kline Institute for Psychiatric Research, Orangeburg, NY 10962, USA*
[b]*Nathan S. Kline Institute for Psychiatric Research, Orangeburg, NY 10962, USA*
[c]*Center for Brain Health, Neuroimaging Laboratory, NYU School of Medicine, New York, NY 10016, USA*
[d]*Department of Psychiatry, NYU School of Medicine, New York, NY 10016, USA*
[e]*Laboratory of Neuroendocrinology, Rockefeller University, New York, NY 10021, USA*
[f]*Department of Experimental Psychology II, Institute of Experimental Psychology, University of Duesseldorf, Geb 23.02, Ebene 01, Room 43, Universitaetsstrasse 1, 40225 Duesseldorf, Germany*

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Abstract

MRI imaging of the rodent brain is a rapidly growing field in the neurosciences. Relatively limited information is available for regional volume determination. The present paper describes a reliable method for the assessment of the hippocampus, the anterior cingulate cortex, the retrosplenial granular cortex and the ventricles in rats. MRI scans were acquired using a 7 T magnet. The anatomical sampling method was found to be highly reliable with an intra-rater reliability of greater than 0.93. The current protocol should facilitate future in vivo neuroimaging research using animal models of neurodegenerative diseases.

Theme: Disorders of the nervous system

Topic: Neurotoxicity

Keywords: Magnetic resonance imaging (MRI); Volumetric measurement; Hippocampus; Cingulate cortex; Retrosplenial granular cortex; Ventricles; In vivo

1. Type of research

The protocol described is suitable for the volumetric in vivo assessment of the following brain structures in the rat brain: (a) hippocampus, (b) anterior cingulate cortex, (c) retrosplenial granular cortex, (d) ventricles.

Currently, few papers are available describing a method for the in vivo assessment of these brain regions in the rat. Previous reports described a protocol for the in vivo assessment of the hippocampus in tree shrews [9] or in rats [5,6]. However, the two rat papers do not provide reliability data and in one case [6] use an image orientation (sagittal), which in our opinion is not ideal for the analysis of this region. In addition due to space limitations, the two descriptions [5,6] are rather short and therefore difficult to replicate. Last but not least, no method paper to date describes the assessment of the anterior cingulate cortex and the retrosplenial granular cortex. Based on our experience with the analysis of human MRI scans [1–4], the following protocol was developed.

2. Time required

2.1. MRI acquisition

Total time (shimming, scout image acquisition and actual acquisition) in the magnet is approximately 120 min.

*Corresponding author. Tel.: +49-211-811-1799; fax: +49-211-811-2019.
E-mail address: oliver.wolf@uni-duesseldorf.de (O.T. Wolf).

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Acquisition of the 28 coronal image sections used for the volumetric analysis took about 70 min. This time can be reduced if only a portion of the brain is imaged.

2.2. Manual volumetric analysis of the MRI images

A person familiar with basic rat neuroanatomy can be trained to use the software and to reliably determine the anatomic parameters used for the manual volumetric assessment within weeks to months. For the analysis itself, the trained rater needs between 90 and 120 min to manually outline the described regions of interest (ROIs) as well as the headsize (intracranial vault) measure for one subject.

3. Materials

3.1. Animals

Seven 9-week-old adult male Sprague–Dawley rats (weight: 351±15.0 g, mean±S.E.) were selected for this experiment. Four rats reaching stage four seizures induced by kainic acid and three controls (placebo vehicle), as describe elsewhere [12] were used to determine the parameters of this method. All animals were housed in accordance with the Animal Welfare Act [8], given free access to food (rat chow) and water, and kept on a 12-h light, 12-h dark cycle.

3.2. Animal preparation for the MRI scanning

Animals were first lightly anesthetized with 3% isoflurane in 75% NO_2+25% O_2, followed by an i.p. injection of 20 mg/kg diazepam (Elkins-Sinn, Cherry Hill, NJ, USA). For maintenance of anesthesia during experiment, isoflurane was reduced to 1.5% with slight correction for body weight. Temperature was monitored throughout the scan using a rectal probe. Body temperature was controlled through a warm water containing line system, with automatic temperature monitoring. The animal's head was fixed in a head holder (David Kopf Instruments, Tujunga, CA, USA), and limb movements were controlled by wrapping the animal with a flexible textile, which allowed breathing but prevented limb movements.

3.3. MRI system

MRI scans were obtained at the MRI facility of the Nathan S. Kline Institute for Psychiatric Research (Orangeburg, NY, USA). A 7.0 Tesla 40-cm horizontal bore MR system (Magnex Scientific, Abingdon, UK), driven by a Surrey Medical Imaging Systems (SMIS, Guilford, UK) spectrometer was used. The system is equipped with a rodent imaging gradient set (10 cm i.d., 1000 mT/m, 200 ms rise time). A quadrature bird-cage transmit/receive radio-frequency (RF) coil (Morris Instruments Inc., Canada) was employed.

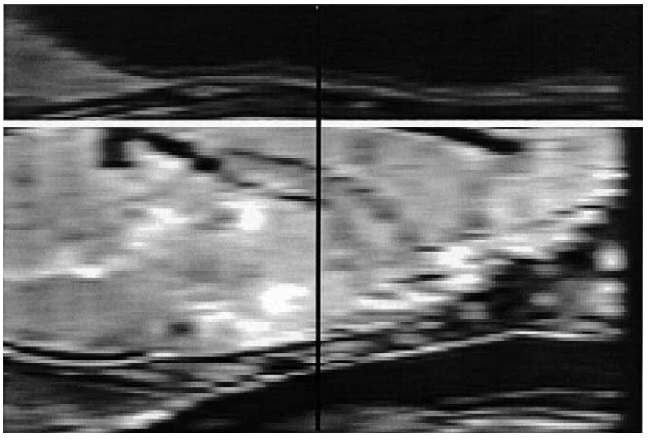

Fig. 1. Resliced sagittal image indicating the line connecting the superior end of the olfactory bulb with the superior end of the cerebellum (white line). A line perpendicular to this one (black line) was used to orient the coronal image acquisition creating the images used for the volumetric analysis (Fig. 2).

3.4. MRI sequence

A moderate spin-echo sequence (TR=4000 ms and TE 25 ms, four averages, 0.6 mm slice thickness with a 0.3 mm gap, slice interleave 2; bandwidth −25 kHz, RF pulse with shape of 5 lobe sinc and BW 3 kHz) optimized for reasonable signal-to-noise ratio and contrast in the considered brain structures was employed for acquisition of the images. The field of view (FOV) was 32×32 mm, the matrix was 256×256 resulting in an in-plane resolution of 125 μm. First a sagittal scout image was taken to control for proper image alignment. The 28 coronal sections used for the volumetric analyses were taken perpendicular to a line connecting the superior end of the olfactory bulb with the superior end of the cerebellum (Fig. 1).

3.5. Data transfer and description of the software

The MRI scans were transferred to a Sun computer station, where manual volumetric analysis was carried out using an in-house developed image analysis program, which uses Solaris as the operating system (MIDAS, Wai Tsui, NYU unpublished data). This program allows the user to manually outline regions of interest (ROIs) and afterwards calculates the volumes of a specific ROI. However the procedure described below is not restricted to this program, but can also be executed with other image analysis software (e.g. Analyze or NIH Image).

4. Detailed procedure

4.1. Protocol development, general comments

Pilot experiments revealed that a coronal orientation provided more slices of clearly visualized hippocampus and cingulate cortex than did either the axial or the sagittal orientation and thus images were collected in coronal orientation for the volumetric analysis.

As a first step, visible anatomical landmarks were identified using the Paxinos and Watson rat brain atlas [10]. Next, preliminary outlines of the ROIs of interest were marked and a stepwise procedure was established (see below). All measurements were performed from rostral to caudal. For the hippocampus, a conservative measurement approach was taken i.e. slices where the structure was not readily and reliably identifiable were not used for the analysis. In case of the hippocampus, the slice prior to that used for the start of the measurements consisted of a small piece of fornix and CA3 (Fig. 2, slice 3). Similarly the one slice after the last slice used for the measurements still contained a small part of the subiculum (Fig. 2, slice 6).

Drawings were made using a twofold magnification of the images. To check the correctness of each drawing, the ROIs were thereafter also viewed without magnification, in order to allow a better global overview.

4.2. Description of the outlined regions

The *cingulate cortex* (areas 1 and 2; [10]) was outlined by starting at the intersection of the corpus callosum with the midline and following the corpus callosum and cingulum to its most dorsolateral point. This point was then connected with a line to the most dorsal and medial intra-hemispheric point of the cortex (Fig. 2, slices 2 and 3). Using this landmark-based method, the cingulate cortex was measured on four slices starting rostrally at the closure of the genu of the corpus callosum (approx. 1.6 mm from Bregma) and terminating caudally at the rostral limit of the hippocampus (approx. −1.4 mm from Bregma). This measure rostrally excludes a certain amount of cingulate (area 1; see Ref. [10] and Fig. 2 slice 1), however, in order to assure high reliability, it was decided to only measure the slab mentioned above (see Fig. 2, slices 2 and 3 for an example of the cingulate cortex ROI).

The *retrosplenial granular cortex* (rsg; area b, sometimes also called posterior cingulate cortex) is the caudal continuation of the cingulate and was measured in a similar fashion as the cingulate gyrus. The retrosplenial granular cortex was measured on four consecutive slices starting rostrally at the rostral limit of the hippocampus (approx −2.12 mm from Bregma, see above). The caudal termination was defined as the slice prior to the opening of the corpus callosum, which is approx −5.3 mm from Bregma [10] (see Fig. 2, slices 4 and 5 for an example of the retrosplenial granular cortex ROI).

The *hippocampus* (cornu Ammonis, CA) and dentate gyrus (DG) were manually outlined on coronal slices from rostral to caudal. The starting rostral slice was defined by the CA and DG and coincided with the dorsal hippocampal commissure approximately −2.12 mm from Bregma [10]. Even though fimbria and possibly a small piece of CA3 were visible on an earlier slice (Fig. 2, slice 3), the reliable measurement of this portion of hippocampus was extremely difficult. Therefore, it was decided to only include those slices where the hippocampus was clearly visible (Fig. 2, slice 4).

Multiple features defined the caudal boundary: the loss of contrast between the external capsule and the subiculum, the absent DG and the clear separation of the two cerebral hemispheres. Moreover, the aqueduct opened up and became a clearly visible, large, round circle. The last hippocampal slice corresponds to ca. −6.8 mm from Bregma, whereas, the first non-hippocampal slice corresponds to ca. −7.8 mm from Bregma (Fig. 2, slice 6). Some subiculum is still apparent at this level, but was intentionally not included in the analysis.

In all animals the hippocampus was measured on six consecutive slices. In addition, the hippocampus was divided into its dorsal and ventral portion. The dorsal part ranged from ca. −2.12 mm from Bregma to −3.8 mm from Bregma, which corresponds to three slices on the MRI, while the ventral part ranged from ca. −4.5 mm from Bregma to ca. −6.8 mm from Bregma, which corresponds to three slices on the MRI (see Fig. 2 for examples of ROIs for the dorsal (slice 4) and ventral (slice 5) hippocampus).

4.2.1. Ventricular CSF volume

Using a threshold function with additional manual editing, the ventricular CSF volume (lateral ventricle, third ventricle, and aqueduct) was determined for each slice, which was used for volumetric analysis (10 slices).

4.2.2. Intracranial vault

In order to control for possible inter-individual differences in global brain volume, the entire brain was outlined on all the 10 slices, which were used for cingulate or hippocampal measurements [3].

4.3. Statistical analysis

4.3.1. MRI volumetric reliability

In order to assess intra-rater reliability, the same rater (OTW) outlined the ROIs of the seven animals again after a period of 1 week. Reliability was measured at the level of the total volume ($n=7$) and at the level of the slice ($n=21–70$ depending on the number of slices measured for the specific region) using the intraclass correlation coefficient (r_{icc}) [11].

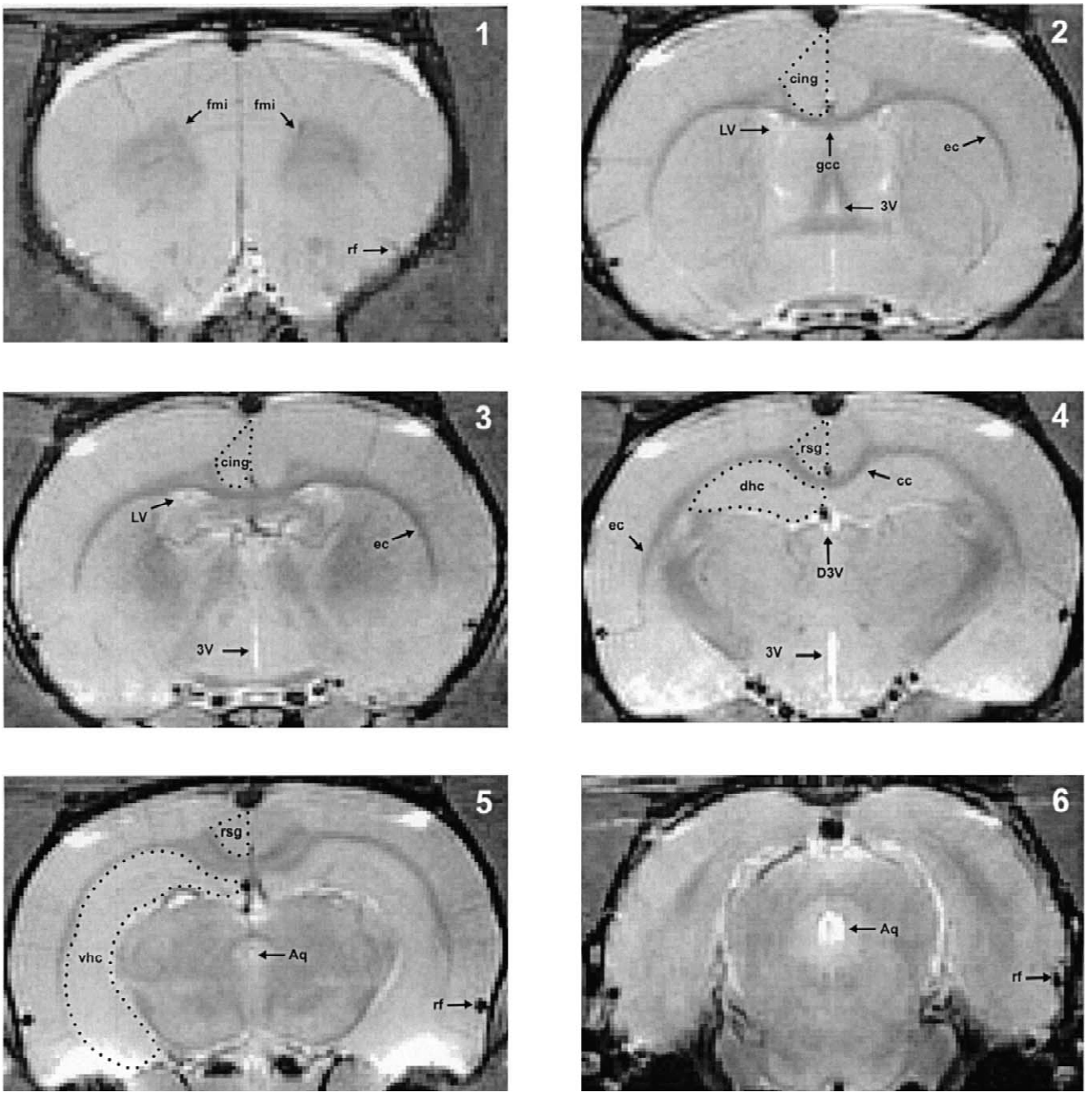

Fig. 2. Series of coronal MRI sections (from rostral to caudal) showing examples of the ROI drawings for the anterior cingulate cortex (cing), the retrosplenial granular cortex (rsg), the dorsal hippocampus (dhc), and the ventral hippocampus (vhc). For ease of presentation, the drawings are shown as dots in order to allow a view of the underlying anatomy. For the actual analysis, these dots are joined in order to create an outline of the region. (1) Approx 1.7 mm from Bregma, last slice (coming from caudally) before the anterior cingulate cortex is outlined; (2) Approx. −0.3 mm from Bregma, closure of the genu of the corpus callosum, anterior cingulate cortex measurements have started; (3) Approx. −1.8 mm from Bregma, the fimbria and first small parts of the dentate gyrus and CA3 are already visible, however they are not included in the hippocampus measurement, in order to improve the reliability. At this level, the LV is still superior to the fimbria; (4) Approx. −3.3 mm from Bregma mm, level of the dorsal hippocampus. Anterior cingulate cortex is now replaced by retrosplenial granular cortex; (5) Approx. −5.3 mm from Bregma, level of the ventral hippocampus, still containing retrosplenial granular cortex; (6) Approx. −7.8 mm from Bregma, opening of the two hemispheres. Some remains of the subiculum are visible, but are not included in the hippocampus measurements. Note the large aqueduct. Additional anatomical landmarks are indicated (cc, corpus callosum; gcc, genu of the corpus callosum; ec, external capsule; fmi, forceps minor corpus callosum; LV, lateral ventricle; D3V, dorsal third ventricle; Aq, aqueduct; rf, rhinal fissure).

Table 1
Intra-rater reliability as assessed with the intraclass correlation coefficients for the MRI-derived volumes

	r_{icc} whole volume	r_{icc} individual slice
Hippocampus	0.99 (n=7)	0.99 (n=42)
Dorsal hippocampus	0.99 (n=7)	0.99 (n=21)
Ventral hippocampus	0.98 (n=7)	0.99 (n=21)
Cingulate cortex	0.99 (n=7)	0.99 (n=28)
Retrosplenial granular cortex	0.98 (n=7)	0.97 (n=28)
Ventricular volume	0.96 (n=7)	0.94 (n=70)
Total brain volume	0.99 (n=7)	0.99 (n=70)

Table 2
MRI-derived brain structure volumes (right and left side combined) obtained from three 9-week-old male Sprague–Dawley rats

	Volume (mm^3)
Hippocampus	96.33 (91–99)
Dorsal hippocampus	28.67 (24–32)
Ventral hippocampus	67.67 (66–70)
Cingulate cortex	16.00 (15–17)
Retrosplenial granular cortex	8.67 (7–10)
Ventricular volume	4.00 (3–5)
Total brain volume (for the measured slab)	1064.67 (1038–1097)

Values are means with range in parentheses.

5. Results

High intra-rater reliability (r_{icc}>0.93) was obtained for the MRI-derived volumetric assessment of the regions (Table 1). The volumes (mean and range) for the brain regions assessed with the developed protocol are presented in Table 2. Data are presented for the three placebo-treated animals only.

6. Discussion

The present report described a volumetric method for the measurement of the rat hippocampus, two cortical brain structures (anterior cingulate cortex and retrosplenial granular cortex) and total ventricular volume. We imaged the rat brain with a high field strength (7 T) magnet and a sequence which resulted in a scan with sufficient anatomical contrast to determine regional brain volumes. However, this volumetric protocol can also be used for images acquired with lower magnetic field strength, given that they provide sufficient anatomical detail, to reveal the anatomical landmarks needed for the analysis. Groups working with other field strength magnets should first develop a sequence suitable for their scanner. There are multiple publications describing sequences leading to high quality images suitable for the volumetric work described in the current protocol (e.g. Refs. [5,6,9]).

This protocol can easily be adapted to images with thicker or thinner slices, since the protocol describes multiple anatomical landmarks which can readily be identified on MRI images of small rodents. Indeed thinner slices as used in the current experiment would be preferable, since thereby the anterior cingulate cortex and the retrosplenial granular cortex could be assessed on more than four slices. In addition, a 3D data set (without gaps) instead of the 2D multislice data set used in this paper would allow the use of 3D image analysis software and the parallel use of multiple image views (e.g. sagittal and coronal). The use of multiple image orientation for the determination of anatomical boundaries has proven to be extremely useful in our human volumetric work [3,4]. Finally, we used only one sequence and one resulting image for the analysis of regional brain structures as well as the CSF determination. Obviously the brain–CSF differentiation would be easier on images with a longer TE.

The current report focuses on rat brain images, but the same volumetric method can also be used for the assessment of the mouse brain since the same anatomical landmarks are readily detectable in the mouse [6].

Potential problems during the image acquisition are movement artefacts during the relatively long image acquisition. Therefore it is important to maintain deep anesthesia during the entire scanning time. The ideal dose of the anesthetics used has to be fine-tuned depending on age, sex and strain of the rats used. Alternatively, if no follow-up images or behavioral assessments are planned, the animals could be paralyzed and mechanically ventilated. If the coronal image despite the scout acquisition is not well aligned (e.g. right side more dorsal than the left side), than the anatomical decisions should be made separately for each hemisphere. In case of a 3D data set, the image could also be aligned and resliced correctly after the original acquisition [3,4].

The measurement of limbic and cortical regions allows the determination of the anatomic specificity of observed effects. For example, using this protocol, we could demonstrate in vivo a selective volume reduction of the hippocampus following kainic acid administration [12]. The measurement of the intracranial vault allows to ascertain that the observed regional volume differences are not caused by baseline differences in brain size and/or global effects on brain volume. If differences in total intracranial vault are observed between experimental groups, the intracranial vault should be used as a covariate [7]. The current protocol should stimulate the use of structural MRI for the in vivo investigation of rodent models of neurodegenerative diseases. Moreover, since similar protocols are available for the human brain [2,4], this protocol might facilitate the parallel MRI investigation of animal models and clinical patients.

7. Quick procedure

(A) Animal preparation for the scanner.
(B) Shimming, sagittal scout, coronal acquisition.
(C) Data transfer to the image analysis computer.
(D) First overview of the scan, identification of the landmarks.
(E) Determination of the first and last slice used for each region of interest.
(F) Manual outline of the ROIs.
(G) Volume computation and statistical analysis (possibly correcting for differences in total brain volume).

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