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A new approach to location of the dentate gyrus and perforant path in rats/mice by landmarks on the skull

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The hippocampus has important roles in learning and memory. Many *in vivo* experiments require accurate location of a certain region of the hippocampus, long-term potentiation (LTP) recording being one of them. *In vitro* and *in vivo* studies can be used to measure LTP in the hippocampus. It is more difficult *in vivo* to locate the specific brain region than *in vitro*. Location of the dentate gyrus (DG) and perforant path (PP) is usually achieved using brain stereotaxic atlasses. Because the data in the atlasses were obtained from a particular rat/mouse strain (Rat: adult Wistar, 290 g; Mouse: adult C57BL/J6, 26–30 g), the data in atlases could not be easily applied in all the different other strains of these species. We describe a method that uses landmarks on the skull to locate these structures in both species, which has been successfully applied in BALB/c mice, KM mice, SAMP8, SAMR1 and Wistar rats; making it a reliable and useful means of locating the DG and PP.

Key words: landmarks on the skull, hippocampus, relative location, dentate gyrus, perforant path

The hippocampus has important roles in the consolidation of information from short-term to long-term memory and spatial navigation. Long-term potentiation (LTP) has been promoted as a putative neural mechanism of associative memory formation or storage in the mammalian brain. LTP was first described by Bliss and Lomo (1973) in the rabbit, in which a stimulation electrode was positioned within the perforant path (PP) and a recording field electrode in the dentate area of the hippocampus. Although LTP has been used in many brain areas, most have been focused on the hippocampus. *In vitro* and *in vivo* methods can be used to measure synaptic plasticity in the hippocampus. The overwhelming majority of synaptic plasticity investigations in the hippocampus used slices, an *in vitro* procedure with several advantages: (1) greater success than *in vivo* recordings; (2) multiple slices can be used from a single animal; and (3) easier location of certain pathways. But the protocols have differed widely regarding the physiological and mechanical treatment of slices, e.g. varying buffer composition, different stimulation paradigms and recording criteria. Such differences lead to variability and even conflicting results (Moore et al. 2003, Kukley et al. 2005). Slices also lose much connectivity and have a significantly altered milieu compared with *in vivo* preparations. Since loss of connectivity and altered milieu may influence synaptic plasticity (Freudenthal et al. 2004), it is important whenever possible to test and confirm important conclusions found in slice preparations using the whole mouse model.

The use of *in vivo* models for LTP is limited, especially in mice. Over 11 000 papers in PubMed were found using "long-term potentiation" as the discriminator, 891 papers with "long-term potentiation"/"*in vivo*"/"rats", and 356 papers with "long-term potentiation"/"*in vivo*"/"mice" (to 2012-08-21). The difficulty in locating a certain pathway in the hippocampus has meant that most investigators have chosen to do *in vitro* studies. Stereotaxic coordinates were obtained from certain strains of animals [rat: adult Wistar, 290 g (Paxinos and Watson 2007); mouse: adult C57BL/J6, 26–30 g (Franklin and Paxinos 2008)], which could not simply be used for other strains. Compared to rat strains, mouse strains vary considerably more in weight and brain anatomy (Wahlsten et al. 1975); in rats the positioning error might even be 0.5 mm (Paxinos and Watson 2007). When a new strain of mouse/rat is to be

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used for *in vivo* studies, confirmation of the location parameters must first be obtained. If several strains of animals of different ages are used in a study, the confirmation of the location parameters presents considerable difficulty. Therefore we have devised a simplified method for locating the DG and PP, which can be used on different mouse stains and rats.

Experiments were conducted on seven adult male BALB/c mice (20 ± 2 g), six adult male KM mice (25 ± 2 g), five adult male Wistar rats (300 ± 10 g), five juvenile Wistar rats (200 ± 20 g), six 6-month-old Senescence-accelerated mouse/prone 8 (SAMP8) (25 ~ 30 g) and five 6-month-old senescence-accelerated mouse/resistance 1 (SAMR1) (35 ~ 40 g). BALB/c mice, KM mice, and Wistar rats were obtained from the animal center of our institute. SAMP8 and SAMR1 were provided by Dr. T. Takeda (Kyoto University, Japan), and cultivated in our institute. All animals were maintained in a 12 h/12 h light/dark cycle at 22 ± 1°C and with 50 ± 5% humidity; they were given free access to food and water. Animal treatment, husbandry and the experimental protocols were approved by the Institute Animal Care and Use Committee (IACUC) of National Beijing Center for Drug Safety Evaluation and Research (NBCDSER).

Animals were anesthetized intraperitoneally with urethane (1.2 g/kg for mice and 1 g/kg for rats) (Beijing Chemical Reagents Corp.), and placed in a stereotaxic frame fitted with ear cuffs (SR-6N, Narishige Ins.) to restrain head movement. An incision was made along the midline of the head, the skin freed and the skull tissue removed to make the sutures visible.

Several landmarks were first identified on the skull: "B" stands for Bregma; "L" stands for Lambda; "I" stands for the point where the Sagittal suture meets the Lambdoid suture; and "M" stands for the midpoint of the line BL. We ensured that the lateral length of line MD was equal to half the line BM, and that line MD was perpendicular to the sagittal suture through "M", "D" was the crosspoint of line BD and MD. Another perpendicular line to the sagittal suture was drawn through "I", crossing line BD at the point marked "P". "D" was used to locate the DG of the hippocampus, and "P" to locate the PP angular bundle

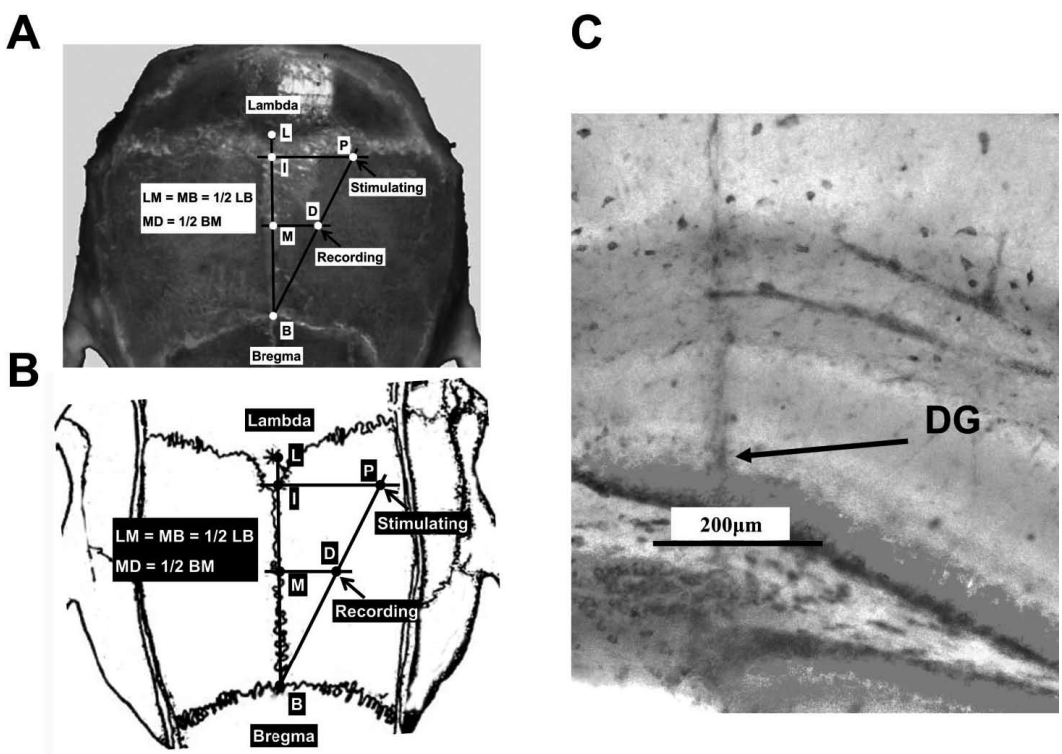

Fig. 1. A schematic diagram of electrode positioning and histology. (A, B) position on the skull for locating DG and PP. (A) a photograph of a skull of a mouse, (B) a scheme of a skull of rat. I is where the Sagittal suture meets the Lambdoid suture. M is the midpoint of line B–L. Lines M–D and I–P are perpendicular to the Sagittal suture (l_{MD}=1/2l_{BM}). Point D is where the recording electrode is located, and P the stimulating electrode location. (C) Histological confirmation of recording electrode location in the DG. This transverse slice (BALB/c mouse; 300 μm) was stained with methylene blue.

leading from the entorhinal cortex (EC) to the hippocampus (Fig. 1 A, B). The bregma position was determined at the point of crossing of the coronal and sagittal sutures on the dorsal surface of the skull.

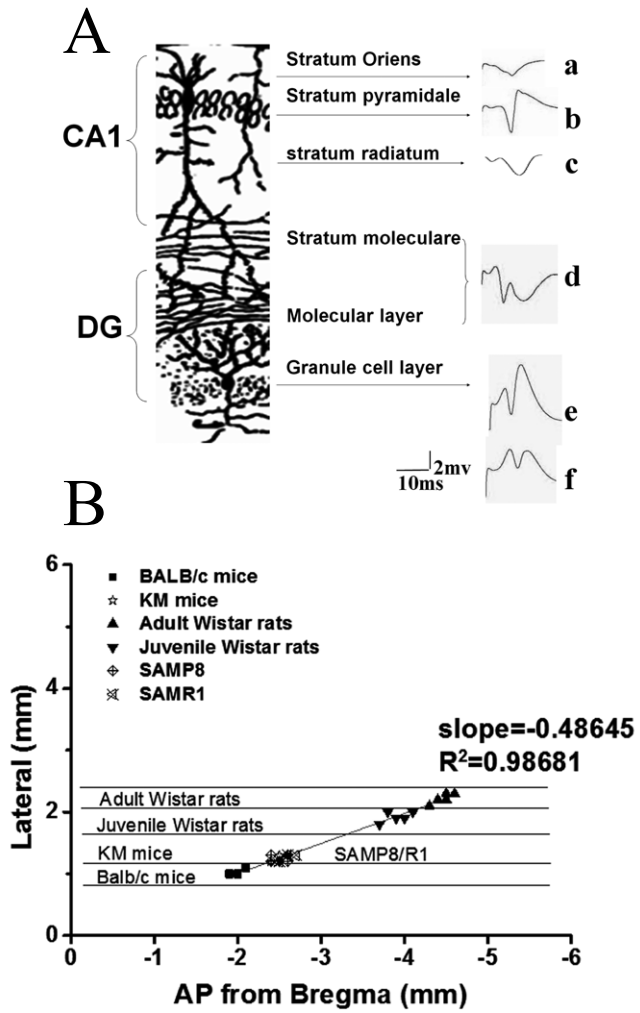

Fig. 2. Relationship between elicited responses and electrode depth. Traces are single responses from BALB/c mouse, with the responses from other groups being similar to these (not shown). (A) Different layers of the hippocampus where the recording electrode passes through and records the elicited responses. Trace *a* represents the elicited response in the Stratum oriens, trace *b* the elicited response in the Stratum pyramidale, trace *c* in the Stratum moleculare of CA1/molecular layer of DG, trace *d* in the area between molecular layer and granule cell layer, traces *e/f* in the granule cell layer of DG (trace *e* is the elicited response in the medial PP-DG pathway, and trace *f* the elicited response in the lateral PP-DG pathway; both of e and f were recorded in granule cell layer); (B) Linear fit of coordinates for the recording electrode (slope: 0.48645; R^2:0.98681).

All the electrodes were made from stainless steel needles (0.25-mm diameter), which were coated with insulated lacquer except at the tips. The recording electrode was a single needle, whereas the stimulating electrode was made of 2 needles with 2 tips separated by ~0.5 mm. The two electrodes were perpendicular to line BDP, with the longer one closer to point "I". A ground reference electrode was placed on the scalp with an alligator clip. Electrical stimuli were initiated by WinLTP (http://www.Winltp.com) and generated by a SEN-7203 stimulator (Nihon Kohden). The pulses (1/60 Hz, 0.1 ms, for baseline recording and after TS recording) passed through SS-102J isolator (Nihon Kohden) to provide a constant current. The responses were amplified (gain = 10) and low-pass filtered (1 000 Hz) (Axoclamp 2B, Axon Ins), and *via* a data acquisition system (sampling frequency is 500 KHz) (DIGIDATA 1322A, Axon Ins), data were recorded and analyzed by WinLTP (the gain was set as 10 in WinLTP).

The animals were killed at the end of experimentation by excess anesthesia. The brains were removed and weighed. Transverse slices (300 μm) were cut on a vibratome (Campden Instruments, Loughborough, UK) and placed in PBS, before being stained with methylene blue (0.1%). Electrode placement was confirmed by microscopy (Fig. 1C).

In the work of several investigators (McNaughton and Barnes 1977, Ito et al. 2001, Huang et al. 2008, Frey and Frey 2009), the shape of an elicited potential (EP) was used to determine the depth of the electrode tip within a particular layer of the DG, and on the PP fibers. We have used this approach to test the correctness of the electrodes' placement (see below). A monopolar recording electrode was lowered at "D" into the granule cell layer (~1.5 mm DV in mice and 2.5 mm DV in rats, the electrode depth needed for future adjustments according to EP) of the DG to record extracellular field potentials elicited by stimulating the PP. A biopolar stimulating electrode was lowered at "P" into the PP (~1.5 mm DV in mice and 2.5 mm DV in rats, the electrode depth needed for future adjustments according to EP). Details of the relation between elicited responses and electrode depth are shown in Figure 2A.

Four strains of mouse and one strain of rat were used for the verification of the method. There was no significant difference among the brain weight of KM mice (0.35 ± 0.01 g), SAMP8 (0.35 ± 0.01 g) and SAMR1 (0.36 ± 0.01 g). The brain weight of KM mice, SAMP8 and

SAMR1 was significantly heavier than for BALB/c mice (0.32 ± 0.00 g) (one-way analysis of variance with S-N-K *post hoc* tests; groups: $F_{3,19}$=6.696, P=0.003; KM mice vs. BALB/c mice: P=0.006; SAMP8 vs. BALB/c mice: P=0.006; SAMR1 vs. BALB/c mice: P=0.005; n=5). Adult Wistar rat brain weight (1.44 ± 0.03 g) was significantly heavier than those of juvenile Wistar rat (t-tests: P<0.001; n=5). The precise coordinates for the electrodes' implantation in the five strains of animals were different, but linear fit showed that the relative positions, according to Bregma, were similar (Fig. 2B). Except for four mice (2 BALB/c, 1 KM and 1SAMP8) with unstable responses, in five animals of each strain and size stable evoked responses were obtained in DG after stimulation of medial PP. Inserting the recording electrode from the brain surface elicited responses [such as those Figure 2A(a-e)] in all the animals. The lateral PP to DG granule cell layer responses (Fig. 2A-f) was elicited in 24 animals (3 BALB/c mice, 4 KM mice, 4 SAMP8, 3 SAMR1, five adult Wistar rats and five juvenile Wistar rats).

We have described an improved and effective method for locating DG and PP in mouse/rat brains. Responses like those seen in Figure 2A-b were recorded at the stratum pyramidale. In our previous experiments on slices (Qiao et al. 2005), we had recorded similar responses in Schaffer collateral-stratum pyramidale pathway. Responses like those seen in Figure 2A-c were recorded at the stratum radiatum of CA1, responses like those in Figure 2A-d were recorded at the stratum moleculare of CA1 and the Molecular layer of DG. With the recording electrode in the granule cell layer of DG, responses as seen in Figure 2A-e were recorded, which was the standard PS in medial PP-Granule cell layer of DG in many reports (Namgung et al. 1995, Bordi et al. 1997, Chapman et al. 1999, Freudenthal et al. 2004, Gureviciene et al. 2004, Criado et al. 2005, Koranda et al. 2008). Thus we used the responses as an index of the position of the electrode, and this made locating more convenient.

Lateral and medial perforant paths are the 2 parallel pathways leading from the entorhinal cortex to the DG (Lomo 1971, McNaughton and Barnes 1977, McNaughton 1980, van Groen et al. 2003) which terminate in different sectors of the DG. Activating them independently can initiate responses of different shapes; our method can activate them separately. In activating the lateral PP, the shape of responses was similar to those in Figure 2A-f (the simulating electrode was about 1.5 mm bellow the skull surface for mice, and about 3 mm for rats), and when the medial PP was activated, the shape was similar to those in Figure 2A-e (the simulating electrode was about 2.0 mm bellow the skull surface for mice and, and about 3.5 mm for rats). These shapes in different pathways agree well with previous studies (Ito et al. 2001, Huang et al. 2008). In our current work, the lateral PP-DG pathway could not be activated in every animal. Although it is unclear why the lateral PP-DG could not be activated in these animals, defining the reference point on the skull might be one of the reasons. Blasiak and coauthors (2010), in reporting a new method for defining bregma, showed data that more precisely located brain regions than the old method (a commonly used and convenient approach). The position of the bregma point, when located by old and new methods, could vary by as much as several hundreds of microns. The lateral PP seems to be more difficult to locate and as such is more susceptible to errors resulting from unprecise Bregma identification. The medial PP-DG pathway could be activated in every animal, indicating a higher success rate than our previous study (Huang et al. 2008). Ten SAMR1 (6-month-old) and 11 SAMP8 (6-month-old) were used in the previous study for long-term potentiation (LTP) evaluation. The locating parameters (recording: AP −2.0 mm from bregma, ML +1.4 mm, DV −1.5 mm; stimulating: AP −3.8 mm from bregma, ML +3.0 mm, DV −1.5 mm) referred to by Gureviciene and colleagues (2004), who evaluated the LTP in APP/PS1 transgenic mice, and only five SAMR1 and five SAMP8 were initiated good EP and LTP. These results indicated that Gureviciene's locating parameters might not be the optimal for SAMR1/P8.

In summary, our data indicate that our new method is effective and accurate in locating the PP-CA1/DG pathway. Location parameters in different animal strains vary, making for a time-consuming job that must be done first in confirming the location coordinates. Using relative coordinates avoids these strain differences and the time-consuming work. The improved method has successfully been used in different strains of anesthetized mice and rats.

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