

ORIGINAL RESEARCH ARTICLE

A new area in the human brain associated with learning and memory: immunohistochemical and functional MRI analysis

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Previous studies identified a new brain area, the marginal division (MrD), at the caudomedial border of the neostriatum in the brain of the rat, cat and monkey. The MrD was distinguishable from the rest of the striatum by the presence of spindle-shaped neurons, specific connections, and dense immunoreactivity for neuropeptides and monoamines in fibers, terminals and neuronal somata. Behavioral testing demonstrated that the MrD contributes to learning and memory in the rat. In the present study, the structure and the function of the MrD were investigated in the human brain. The presence of spindle-shaped neurons and the distribution of neurotransmitters in the MrD were evaluated by immunocytochemical methods. The function of the MrD was identified with functional magnetic resonance imaging (fMRI) of healthy volunteers tested with an auditory digital working memory task. Highly active areas were observed in the prefrontal cortex and MrD with left sided predominance during performance of the task, but other parts of the neostriatum were not excited and the MrD was not activated in a control test of non-working memory. The results of the present investigation therefore indicate the existence of a new area associated with learning and memory function in the human brain. The MrD probably plays an important role in the execution of digital working memory and appears to link the limbic system and the basal nucleus of Meynert. The MrD may also be involved in the mechanism of Alzheimer's disease.

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In previous studies we discovered a structure we termed the marginal division (MrD) on the basis of its location at the caudomedial margin of the neostriatum surrounding the rostral border of the globus pallidus in rat,¹ cat² and monkey³ brains. A three dimensional reconstruction from Nissl-stained sections of the rat brain showed that the MrD is a flat, pan-shaped structure. The MrD was easily distinguishable from the rest of the striatum by the presence of spindle-shaped neurons and a variety of intensely expressed neuropeptides and monoamines in nerve fibers, terminals and neuronal somata.⁴ Most of the neuropeptides in the

MrD have been considered to be functionally involved in learning and memory.^{5,6} Functional neuronal connections between the MrD and the hippocampus, amygdala and basal nucleus of Meynert (NBM) were identified by chemically induced c-fos protein (c-Fos) expression.⁷ The MrD was shown to contribute to learning and memory in the rat with the double-blind Y-maze test.⁸ The presence of the MrD in the neostriatum was confirmed by other investigators in immunohistochemical and physiological studies. Talley *et al* reported a higher level of expression of $\alpha 2A$ adrenergic receptors in the marginal division than in the rest of the striatum.⁹ Lavoie and Parent demonstrated that pedunculopontine nuclear projections to the basal ganglia terminated exclusively in the MrD,¹⁰ distinguishing it from other parts of the striatum. Nociceptive neurons were reported localized solely in the MrD of the rat striatum, using neurophysiological techniques,¹¹ and the MrD was described as part of the ventral striatal area by Heimer and coworkers.^{12, 13}

In the present study we wished to determine whether the MrD is also present in the human brain and to assess its possible function *in vivo* with a non-invasive imaging technique. Neuroanatomical and immunocytochemical studies were carried out on brains autopsied from 12 children and five adults to assess any differences between the young and the mature brain; no staining differences between them were observed. Brains were dissected and sectioned to study the structure of the neostriatum. The cytoarchitecture of brain sections was investigated with Nissl staining. Neurotransmitter distribution was identified with primary and secondary antibodies using the Avidin-Biotin Complex (ABC) technique and a glucose oxidase and nickel ammonium sulfate-intensified diaminobenzidine (GDN) staining method.¹⁴ A cellular zone consisting of fusiform neurons situated between the neostriatum (putamen) and the globus pallidus was detected in these sections of the human brain. The long axes of the neurons in this region ran dorsoventrally, parallel to the border between the putamen and the globus pallidus, in a fashion similar to that seen in other mammalian species. Numerous Leu-enkephalin- (L-Enk), neurotensin- (NT) and substance P- (SP) positive fibers and cells were found in this area (Figure 1). The

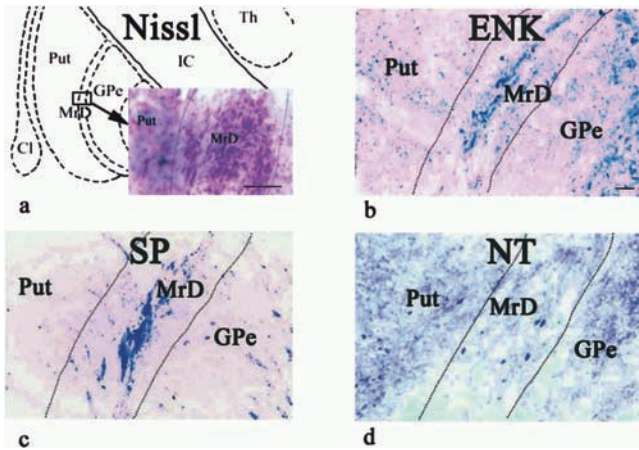


Figure 1 Neurons and neurotransmitters in the human marginal division (MrD). (a) The schematic drawing shows the MrD of the human brain as a narrow band located between the putamen (Put) and the external segment of the globus pallidus (GPe). The rectangle at lower right displays a high magnification view of Nissl-stained fusiform neurons in the MrD. Scale bar is 50 μm. (b–d) Enkephalin- (Enk), Substance P- (SP), and Neurotensin- (NT) immunoreactive fibers, terminals and fusiform neurons in the MrD visualized with ABC-GDN staining. Scale bar for (b–d) is 50 μm.

results of these studies confirmed that a new area, recognized by the presence of spindle-shaped neurons with dense expression of a variety of neuropeptides, is also present in the medial margin of the neostriatum (putamen) in the human brain. The MrD appears thicker and more developed in primate than in rodent brains, and is most highly developed in the human brain. The elaboration of the MrD in the human brain suggested that it could have a significant function.

Is the MrD of the human brain also involved in learning and memory as it is in the rat? Non-invasive methods are required to explore the function of the human brain *in vivo*. Functional imaging techniques, and particularly fMRI, provide a powerful and non-invasive means of linking brain function to structure *in vivo*. Because of its high spatial and rapid temporal resolution, fMRI is especially useful in detecting and localizing neural activity in the human brain, and was therefore used in the present study to investigate whether the MrD contributes to the function of learning and memory in human subjects.

Thirteen volunteers undertook both an auditory digital working memory task and a non-working memory control task separately monitored by fMRI. The working memory task required subjects to actively maintain and identify several stimuli at a time, while the non-working memory task only required subjects to perform an instant judgment. Eleven of 13 subjects achieved criterion performance on the working memory task with an average score of 22.7 (of a possible score of 30; SD = 1.74). Data from two male subjects with scores below 20 were excluded because of their failure to achieve a criterion performance in the task. In these 11 subjects highly activated areas were observed bilaterally in the prefrontal cortex (PFC, McNemar Test

$P < 0.001$) and left MrD ($P < 0.001$) during execution of the auditory digital working memory task (Figure 2b,d). Most of the activity in the PFC exhibited left predominance. However, the right MrD in one male subject was highly activated as well (Figure 2f), although other parts of his right striatum were not. Other areas, such as the right internal capsule (IC, $P > 0.05$), right thalamus (Th, $P > 0.05$), left temporal lobe (TL, $P > 0.05$) and genu of corpus callosum (GCC, $P > 0.05$) were occasionally activated in some subjects (Table 1). The PFC and MrD were not activated when these subjects were executing the non-working memory control task (Figure 2c, e, g).

It is widely accepted that the PFC is an important center for working memory, involved in the integration and temporary storage of information. Research on the PFC of monkeys and humans indicates that this region contributes to many mental processes and complex behaviors.¹⁵ fMRI studies have identified the role of the PFC in working memory and indicated that the mnemonic content of the task affects the relative weighting of hemispheric activation. When results from a group of previously reported human functional brain imaging studies were plotted onto a standardized brain, the hemispheric organization of the PFC was found to depend on the type of material held in working memory.¹⁶ Activity of the left PFC was greater during tasks involving nonspatial working memory (such as digital working memory) than during tasks using spatial working memory. This analysis was supported by other studies.^{17,18} In the present investigation, 11 of 13 subjects showed activation of the PFC with left predominance during the execution of auditory digital working memory. These results therefore support a prominent role of the PFC in working memory, while other cortical regions, such as the supplementary motor area, lateral premotor and the parietal cortex, appear to play subservient roles.¹⁶

There are few previous studies examining activation of subcortical regions of the brain using fMRI during tasks involving working memory. The results of the present investigation show that both the MrD and the PFC are activated during the execution of auditory digital working memory. The MrD was not activated in the same individuals during execution of the control task, suggesting that the MrD was not activated by auditory stimuli or finger movements during the auditory digital working memory task.

The MrD thus appears to be activated by working memory itself. It is interesting to note that the MrD showed obvious left predominance in 10 of the 11 subjects, which was the same hemispheric predominance of the PFC in these individuals. This suggests that the MrD should not be considered as merely a transfer station on the afferent or efferent pathways involved in working memory but as a subcortical center for learning and memory.

C-Fos immunohistochemistry labels functionally connected neurons activated by a variety of stimuli¹⁹ and was used to trace the functional connections of the MrD in our previous study.⁷ C-Fos-immunoreactive

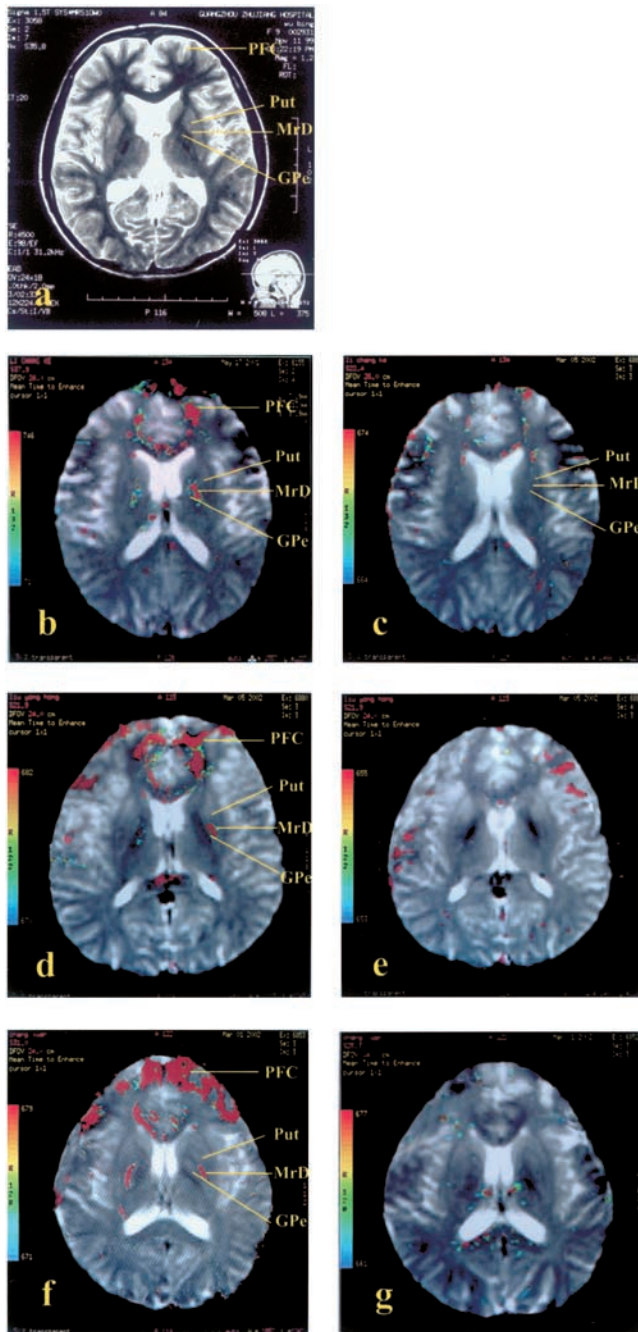


Figure 2 Selective activation of the human MrD during auditory digital working memory task, visualized with functional MRI (fMRI). (a) A T2 MRI image demonstrates the positions of the prefrontal cortex (PFC), the putamen (Put), the external segment of the globus pallidus (GPe) and the marginal division (MrD) in the normal human brain. (b) A T2* fMRI image shows a highly activated area in the bilateral PFC with left predominance and in the left MrD in the first subject's brain while he was performing the auditory digital working memory task. (c) The T2* fMRI image reveals no activation in the MrD in the same subject's brain (b) while he was performing the non-working memory control task. (d,e) Similar results to (c,d) in the second subject's brain. (f,g) Results similar to those in (b,c) and (d,e) demonstrating selective activation of areas in the left PFC and bilateral MrD of the third subject's brain.

Table 1 Summary of brain areas activated during the working memory task

Areas	MrD	PFC	IC	GCC	TL	Th
Left	11	11	2	5	5	1
Right	1	11	5		3	5

Numbers of subjects (total = 11).

MrD: marginal division; PFC: prefrontal cortex; IC: internal capsule; GCC: Genu of corpus callosum; TL: temporal lobe; Th: thalamus.

neurons were observed in the hippocampus, dentate gyrus, amygdala, NBM and the PFC following stereotaxic injection of 0.01% kainic acid into the MrD of the rat brain. C-Fos immunoreactivity was also expressed in neurons of the MrD, hippocampus, dentate gyrus, amygdala and PFC after injecting 0.01% kainic acid into the NBM. The positive expression of c-Fos was stained in a purple-black color with the ABC-GDN immunohistochemical staining method. The purple-black stained nuclei of activated neurons were observed only in the caudal margin of the striatum without expression in other parts of this structure. The c-Fos positive stained nuclei of activated neurons accumulated in a rainbow-shaped zone clearly distinguished the MrD from the rest of the striatum, following chemical stimulation of the NBM (Figure 3). The hippocampus, dentate gyrus and amygdala are considered to be part of the limbic system and important for learning and memory.²⁰ Pathological changes in the NBM induced an obvious decrease in cholinergic fiber density in the cortex, which could be of relevance to the changes in cognitive function seen

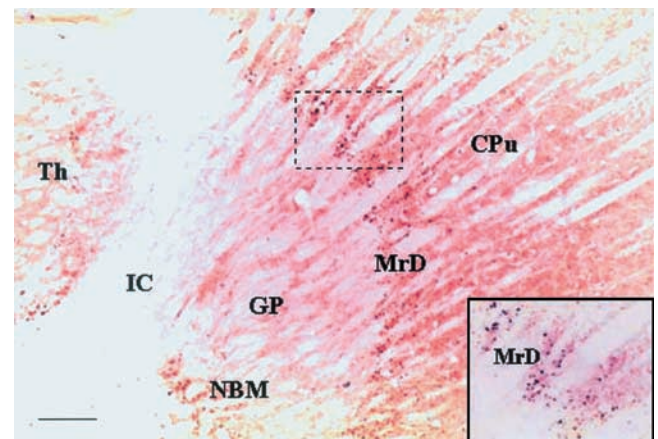


Figure 3 Sagittal section of the rat brain illustrating c-Fos expression in the marginal division following stimulation of the basal nucleus of Meynert with kainic acid. The MrD was distinguished from the rest of the striatum by a rainbow-shaped zone of c-Fos-positive nuclei at the caudal border of the striatum. Scale bar is 100 μ m. The bottom right corner shows a high magnification view of c-Fos-reactive nuclei of the MrD in the rectangular area above. NBM: basal nucleus of Meynert; GP: globus pallidus; IC: internal capsule; MrD: marginal division; St: striatum; Th: thalamus.

in Alzheimer's disease.²¹ The efferent projections from the MrD were further identified by electron microscopy to synapse on the cholinergic cell bodies of the NBM. A reduction in learning and memory was recorded following terminal degeneration of the MrD-NBM projection after a lesion of the MrD.²² These studies thus identify functional connections among the NBM, the MrD and the limbic system. The MrD was formerly considered as part of the limbic motor circuit²³ and thought to represent neurons of passage from the central division of the extended amygdala in the rat brain.²⁴ In the present study, functional connections between the MrD and the limbic system are demonstrated. Thus the MrD functions as a link between the NBM and the limbic system, and appears likely to modulate learning and memory in both parts of the brain (Figure 4).

In conclusion, the present study demonstrates the presence of the MrD in the human brain and provides direct evidence that the MrD contributes to learning and memory *in vivo*. The MrD as well as the PFC may play an important role in the execution of digital working memory. In addition, the MrD constitutes a link between NBM and the limbic system, suggesting involvement in the mechanism of Alzheimer's disease.

Materials and methods

Immunohistochemical techniques

Autopsy brains were obtained from 12 children, ages 9 to 12, and from five adults, ages 21–38, within 12 h of death from acute, non-nervous system diseases such as pneumonia. Standard protocols were followed for approval by relatives of the deceased and by the human subjects review committee of the hospital. Brains were removed from the skull and fixed with 4% paraformaldehyde for 14–20 days at 4°C. The neostriatum and glo-

bus pallidus were dissected and sectioned frontally at 50- μ m thickness with a Cryostat microtome (Cryocut 1800, Leica Instruments, Germany). Polyclonal anti-enkephalin (Enk) (1:3000; INCSTAR, Stillwater, MN, USA), anti-substance P (SP) (1:6000; INCSTAR) and anti-neurotensin (NT) (1:2000; INCSTAR) were used to examine peptide expression. Sections were processed with the ABC method with ABC kits (Vector Laboratories, Burlingame, CA, USA) and stained with a glucose oxidase and nickel ammonium sulfate-intensified diaminobenzidine (GDN) method.¹⁴ Positively stained structures exhibited purple-black reaction product in the sections. Control sections were processed without the primary antibody; these sections were not stained.

C-Fos immunocytochemistry experiments were performed on rats. A low concentration of kainic acid solution was used as the chemical stimulus to induce C-Fos expression in the brains of 61 animals. A total of 0.1–0.2 μ l of 0.01% kainic acid was stereotaxically microinjected into the NBM or MrD. Animals were killed 6 or 8 h after injection by anaesthesia with Nembutal and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) through the aorta. Brains were removed and sectioned frontally at 40- μ m thickness with a Cryostat microtome. Sections were reacted with an antibody to c-Fos (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). ABC and GDN methods were used to demonstrate the expression of c-Fos in the brain. Thirty brains with correct injection sites were analyzed. c-Fos-positive neurons exhibited purple black nuclear stain. No nuclear staining of neurons was observed when the primary antibody to c-Fos was omitted in reaction of control sections.

Behavioral tasks

Thirteen right-handed healthy volunteers (nine males and four females, aged 21–35 years) were asked to participate in both the auditory digital working memory task and the non-working memory control task. All subjects were undergraduates and graduates from the First Military Medical University of PLA, Guangzhou, China. They had no history of medical, neurological or psychiatric illness, nor were taking any type of prescription medication immediately prior to or during the period of the test. They gave their written, informed consent in accordance with the Declaration of Helsinki.

Stimuli consisted of a series of two-digit numbers, which were read by a man at a speed of one number per second followed by a 1-s interval. Each series was divided into two groups: a memory group and a test group. Each group had five numbers. In each series, two or three numbers in each group were the same while the others were different. The experiment contained six cycles, each of which consisted of a 20-s block of the memory task, alternating with a 20-s block of the interval phase. In each auditory working memory task cycle, subjects heard a randomly chosen series of stimuli. The subjects were instructed to memorize the numbers in the memory group and then determine whether the numbers in the corresponding test group

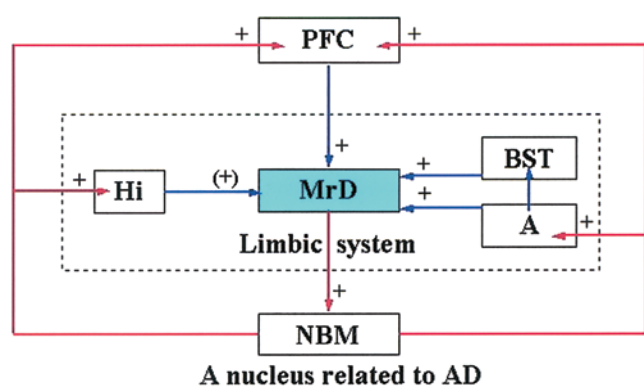


Figure 4 Schematic drawing illustrating the functional connections between the marginal division (MrD), basal nucleus of Meynert (NBM) and the limbic system. The MrD is shown as a linking area between the limbic system and the NBM. Afferent impulses from the prefrontal cortex (PFC), bed nucleus of stria terminalis (BST), amygdala (A) and hippocampus (Hi) provide input to the MrD. Efferent impulses from the MrD travel to the NBM, which sends efferent impulses to the limbic system and the cortex. AD: Alzheimer's disease; +: excitatory influence; (+): assumed excitatory influence.

had been presented. 'Yes' was indicated by raising the right thumb and 'no' was indicated by raising the left thumb, respectively. The score was recorded as the number of correct answers out of a total possible score of 30. Data from two male subjects, whose score was lower than 20, were excluded from analysis. In the non-working memory control task group the stimuli and process were the same as those in the auditory digital working memory task group; however, subjects were instructed to judge whether each number was greater than 50, instead of memorizing them and comparing them with numbers in the test group. Subjects were asked to perform the digital task first followed by the non-working memory control task.

fMRI techniques

Imaging was carried out on a 1.5 T SIGNA scanner (GE Medical Systems, Milwaukee, USA) equipped with a fast gradient system for echo-planar imaging. A standard radio frequency head coil was used and the head motion was restricted. High-resolution sagittal T₁-weighted images were obtained from every subject prior to localization experiments. A gradient echo, echoplanar sequence (repetition time = 2000 ms, echo time = 60 ms, flip angle = 90°) was used to acquire data sensitive to the blood-oxygen-level-dependent (BOLD) signal. Resolution was 128 × 128 lattice in each plane and 0 mm between planes (four plane slices). Activated areas were visualized after processing by the Functool program (supplied by GE Medical Systems) using the correlation coefficient algorithm method. Regions of interest were analyzed by the McNemar test (a non-parametric test for two related samples).

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