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Effect of inactivation of the intermediate cerebellum on overground locomotion in the rat: A comparative study of the anterior and posterior lobes

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HIGHLIGHTS

- Effect of the impaired intermediate cerebellum on locomotor movements was examined.
- The effect was compared between anterior and posterior lobes.
- Either impairment led to hyperflexion of joints and excessive toe elevation.
- No postural deficit was observed in either case.
- Impairment of anterior lobe more severely affected than that of posterior one.

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ABSTRACT

The importance of the cerebellum in control of locomotion is demonstrated by the ataxic gait of cerebellar patients. The intermediate cerebellum contains somatotopical representations for forelimbs and hindlimbs in both anterior and posterior lobes. However, it is not known whether these separate regions have discrete roles in control of limb movements during locomotion. Here we compared the effect of muscimol-induced inactivation of the anterior or posterior intermediate cerebellum on limb movements in walking rats. Inactivation of the anterior intermediate cerebellum had clear effects on limb movements during overground locomotion, resulting in excessive toe elevation and hyperflexion of joints in the swing phase. Inactivation of the posterior region resulted in similar but less pronounced deficits. Postural defects were not present in either group of rats. These findings suggest that the intermediate cerebellum of the anterior lobe has a greater influence on the ability to control limb movements during overground locomotion than the posterior lobe.

1. Introduction

The intermediate cerebellum, which is located between the medial and lateral cerebellum, plays an important role in control of limb movements [13]. It is anatomically defined by the olivo-cortico-nuclear organization with inferior olive (IO) and cerebellar nuclei (CN): more specifically, dorsal and medial accessary olive (DAO, MAO) and anterior and posterior interpositus nuclei (AIN, PIN). Electrophysiological [2,9,12,14] and neuroanatomical studies [17] have demonstrated that somatotopical representations for forelimbs and hindlimbs are present in the intermediate cerebellum of both anterior (lobule I–V) and posterior lobes (lobule VI–X). Forelimb representations are mainly present in lobule IV–V and the paramedian lobule (PMD) of lobule VII; hindlimb representations are located in lobule III–IV and the copula pyramidis (COP) of lobule VIII. However, it is unknown whether the anterior and posterior regions of the intermediate cerebellum have discrete roles in locomotor movements.

Clinical studies that have sought to address comparative roles of the anterior and posterior intermediate cerebellum encounter two difficulties. First, cerebellar lesions usually involve both the CN and cerebellar cortex [11]; second, volume of the lesion or atrophy,

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which influences the severity of motor deficits, is unequal among subjects [8,11]. These limitations essentially preclude identification of functional differences between the anterior and posterior regions of the cerebellar cortex in patient studies. It has been demonstrated in cats that cooling of the intermediate cerebellum of lobule V induces hyperflexion of the ipsilateral forelimb joints during locomotion [23]. Unfortunately, similar manipulation of the posterior region was not performed in the previous study, therefore the effect of impairment was not compared between the anterior and posterior intermediate cerebellum.

Previously, we established a system for the kinematic analysis of locomotion in rodents [1,19]. Here, we used this system to perform comparative analysis of the effect of impairment of the anterior or posterior intermediate cerebellum on limb movements in walking rats.

2. Material and methods

2.1. Ethical approval

The present study was approved by the Ethical Committee for Animal Experiments at the University of Tokyo, and was carried out in accordance with the Guidelines for Research with Experimental Animals of the University of Tokyo and the Guide for the Care and Use of Laboratory Animals (NIH Guide) revised 1996. All efforts were made to minimize the number of animals used and their suffering throughout experiments.

2.2. Animals

Male Wistar rats were used ($n = 20$; weight, 280–320 g). All animals were provided with food and water ad libitum, and housed under standard conditions (12 h/12 h light/dark cycle, at 22 °C). We prepared two groups of nine animals; the first group was used for treatment of the anterior region (lobule IV–V) of the intermediate cerebellum, and the second group was for the posterior region (lobule VII–VIII). In each group, two conditions were prepared: vehicle injection (saline) and muscimol-induced inactivation. Two remaining rats were used for histological identification of the injection sites and were not included in behavioral analyses.

2.3. Surgical procedures

The initial surgery, which was conducted 3 days before behavioral analyses, was performed on a stereotaxic frame under isoflurane anesthesia (initial, 3.5%; maintenance, 2.5–3%). A small plastic socket was installed on the skull covering the right cerebellum into which we later injected saline or muscimol unilaterally. For these injections, animals were lightly anesthetized with isoflurane (duration ~25 min). Saline or 2% muscimol (Tocris Bioscience, USA) was pressure-injected using a Hamilton syringe; the concentration was selected on the basis of the results of a previous study [5]. In most cases, we added 1% cholera toxin b subunit (CTb, Lot #104, List Biological Laboratories, USA) to saline or muscimol solutions for subsequent identification of the injection site. In a few animals, we injected 2% fluorescent muscimol (Muscimol, BODIPY® TMR-X conjugate, Life Technologies, USA) mixed with CTb, enabling to directly monitor the extent of diffusion of muscimol. Coordinates of the injection sites were determined by consultation of the rat brain atlas [15]. Because of the stacked structure of lobules in the target areas, two separate injections were made at different depths: from interaural or dural surface, AP: −1.2 mm, ML: 2.2 mm, and depth: 2.9 and 1.5 mm (anterior lobe); AP: −4.6 mm, ML: 2.3 mm, and depth: 3.0 and 1.6 mm (posterior lobe). The volume of each injection was 160 nl (total of 320 nl) for the anterior region and 180 nl (total of 360 nl) for the posterior region.

2.4. Behavioral analysis

Analysis of locomotor behavior was commenced in 30 min after saline or muscimol was injected. We recorded overground locomotion on the runway (140 cm) for a minimum of five successful trials for each animal. Two criteria were used to define successful trials: (1) the animal walked continuously without stopping; and (2) the double support phase was identified in each step cycle. Colored markers were placed on the shaved skin over various anatomical landmarks, namely, shoulder, wrist and toe of the forelimb, and iliac crest, great trochanter (hip), ankle, fifth metatarsophalangeal joint and toe of the hindlimb. We calculated elbow and knee positions by triangulation as skin slippage over these joints precluded their direct monitoring. Locomotor movements and reflected images on the mirror beneath the runway were captured at 200 frames/s using a high-speed digital camera (HAS-220, Ditect, Inc., Japan). The reflected images were used to measure inter-foot distances between left and right feet during walking. Movement analyses were restricted to the ipsilateral side of treatment (right side), and to the sagittal plane parallel to the walking direction. Limb trajectories were reconstructed by digitizing two-dimensional coordinates of the measured landmarks (Fig. 2). Maximal toe heights, stride lengths, inter-foot distances (width of the stance), and girdle heights (height of iliac crest and shoulder) were measured. Minimum joint angles of the hip, knee, ankle and elbow were also calculated. To analyze displacement of hindlimb joints (Fig. 3G), duration of the swing phase was normalized and averaged data of these joints were plotted in three dimensions as we previously performed in two dimensions [19]. These analyses were conducted with the motion analysis software DIPP-Motion Pro 2D (Ditect, Inc., Japan) and MATLAB (The Mathworks, Inc., USA).

2.5. Histology

Three days after completion of behavioral experiments, animals were perfused with 4% paraformaldehyde. Brains were extracted and post-fixed in the same fixative and later in 10% sucrose for a week. Coronal sections (40 μm) were prepared using a freezing microtome (REM-700, Yamato Kohki, Japan). We performed CTb immunohistochemistry to verify injection sites as described previously [16]. Briefly, sections were incubated overnight in a polyclonal anti-choleragenoid antibody (goat anti-CTb, Lot. #703, List Biological Laboratories, USA) and later incubated in biotinylated rabbit anti-goat IgG (Sigma, Japan), followed by an avidin-biotin complex (ABC Elite, Vector Laboratories, USA). Labeled structures were visualized using 3,3′-diaminobenzidine-tetrahydrochloride (DAB) and the sections were counterstained by cresyl violet. Using CTb labeling, injection sites, retrograde labeling of the IO and anterograde labeling of axon terminals in the CN were analyzed with reference to published information [15,17,24,25], in order to determine whether the injection was localized to the intermediate cerebellum.

In a few animals, we injected fluorescent muscimol with CTb to directly examine the area of diffusion. For fluorescent CTb labeling, sections were incubated overnight in the same primary antibody and later in a secondary antibody conjugated to Alexa fluor 488 (Alexa fluor® 488 donkey anti-goat IgG, Life Technologies, USA). The sections were mounted using anti-fade reagent containing DAPI (Prolong® Gold Antifade Reagent with DAPI, Life Technologies, USA). Different filters were used to visualize each fluorescent stain: fluorescent muscimol (544 nm excitation, 574 nm emission; XF-108-2, Opto Science, Inc., Japan), Alexa fluor 488 (470 nm, 535 nm; OP-66836, Keyence, Japan), and DAPI (360 nm, 460 nm; OP-66834, Keyence, Japan). Images of representative sections were captured using a digital microscope (BZ-9000, Keyence, Japan).

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