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Projections of glutamate decarboxylase positive and negative cerebellar neurons to the pretectum in the cat

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Abstract

The pretectum is one of the primary visual centers, and plays an important role in the visuomotor reflexes. It also receives projections from the cerebellar nuclei that are considered to regulate these reflexes. Gamma aminobutylic acid (GABA) and glutamate are supposed to be two major neurotransmitters of the projection neurons of the cerebellar nuclei. We double labeled the projecting neurons with a tracer, biotinylated dextran amine (BDA), and with an antiserum to glutamate decarboxylase (GAD), the enzyme that synthesizes GABA. The results indicated that about 40% of the pretectal-projecting neurons of the cerebellar nuclei were GAD immunoreactive. The GAD positive pretectal-projecting neurons were significantly smaller than the GAD negative projecting neurons. Our findings thus suggest the existence of two distinct cerebello-pretectal projection systems: one is mediated by GABAergic inhibitory projections, while the other is mediated by non-GABAergic, probably glutamatergic excitatory ones.

Keywords: Extrageniculate visual system; Visuomotor reflex; Eye movement; Gamma amino butylic acid; Cerebellum

The pretectum receives retinal inputs, and is involved in the pupillary light reflex [15] and the optokinetic nistagmus [11]. It also receives projections from the cerebellar nuclei that are supposed to regulate these reflexes [21]. The projections from the cerebellar nuclei mainly originate in the ventral part of the lateral nucleus [13] that receives projections from the flocculus and paraflocculus of the cerebellar cortex where the visuomotor responses are recorded [21]. The ventral part of the lateral nucleus is composed of small cells [9,16], and previous immunocytochemical studies using antibody against gamma aminobutylic acid (GABA) or glutamate decarboxylase (GAD, the enzyme that synthesizes GABA) showed that these small cells are GABAergic [4–6,14,17–19]. As a result, GABAergic neurons may send axons to the pretectum. It is still not known, however, whether GABAergic neurons project to the pretectum or not.

About one-third of the neurons in the cerebellar nuclei are GABA immunoreactive and are thus considered to be GABAergic [5]. On the other hand, 80% of the neurons in the cerebellar nuclei are glutamate immunoreactive, and are larger than the GABAergic ones [5]. Since glutamate is the precursor of several amino acids such as GABA and aspartate, not all the glutamate immunoreactive neurons are glutamatergic but some are supposed to be GABAergic and aspartatergic [5,14]. The immunoreactivity for glutaminase, which is considered to be glutamatergic neuronal marker, is also observed in neurons scattered throughout the cerebellar nuclei [12]. The GABAergic and the glutamatergic neurons, the principal inhibitory and excitatory sources, respectively, are both reported to project from the cerebellar nuclei [3,6,8,18]. Additionally, medium-sized and large neurons are immunoreactive to aspartate, and thus aspartate is also a candidate for neurotransmitter of output neurons of the cerebellar nuclei [18].

To elucidate whether GABAergic cerebellar nuclear neurons project to the pretectum or not, we injected a tracer, biotinylated dextran amine (BDA), into the pretectum and visualized the retrogradely labeled cells in the cerebellar nuclei and, at the same time, visualized the GAD-like immunoreactive neurons in the same sections. We herein report that both GABAergic and non-GABAergic cerebellar neurons project to the pretectum.

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Four adult cats weighing 3.0–4.2 kg were used in this study. All animals were cared for in accordance with the "National Institutes of Health Guide for the Care and Use of Laboratory Animals" and the guidelines for care and the use of animals established by the Animal Care and Use Committee of Gifu University.

The animals were administered ketamine hydrochloride (50 mg/kg) and then were anesthetized with sodium pentobarbital (20 mg/kg). The head of animal was mounted on a stereotaxic frame, a midline incision was made, and then a small opening in the skull was made by drilling over the stereotaxic coordinate of the pretectum. A glass micropipette filled with 20% BDA (BDA-10,000, Molecular Probes, Eugene, OR, USA) was placed at the stereotaxic coordinate of the pretectum, and 0.1–0.3 μl of the tracer was injected into the pretectum under intermittent pressure using Picospritzer 2 (General valve, Fairfield, NJ, USA). After 2 weeks, the animals were perfused through the ascending aorta with 500 ml of saline followed by 2 l of 4% paraformaldehyde solution in 0.1 M sodium phosphate buffer (PB, pH 7.4). The brain was blocked and immersed in 20% sucrose solution at 4 °C. After 2 days, the brain was cut into 50 μm thick coronal sections on a freezing microtome, and the sections were collected into PB.

One-in-four serial sections were treated with 0.5% Triton X-100, 0.5% bovine serum albumin, and 0.5% normal goat serum in PB overnight. The sections were then incubated in rabbit antiserum to GAD (RB X GAD65/67, Chemicon, Temecula, CA, USA) at 1:5000 dilution, 1% normal goat serum, 0.5% bovine serum albumin, and 0.3% Triton X-100 in PB at 4 °C. A few sections were incubated in the same solution without antiserum to GAD for control. After 3–5 days, all the sections were rinsed in PB, and then were incubated in fluorescein labeled anti-rabbit goat serum (Vector, Burlingame, CA, USA) at 1:100 dilution overnight at 4 °C. The sections were rinsed and then incubated in TexasRed labeled streptavidin (Vector) at 1:200 dilution overnight. Finally, the sections were rinsed, mounted on glass slides, and were dried without coverslipping for fluorescent observations.

For an analysis of the injection site, one-in-four serial sections, adjacent to those for fluorescent observations, were treated in 1% solution of hydrogen peroxide in PB for 3 h, and subsequently in 0.25% solution of Triton X-100 in PB overnight at 4 °C. The sections were then incubated in an avidin-biotin-peroxidase solution (*Elite* ABC kit, Vector) overnight at 4 °C. The sections were rinsed, and were then reacted in a 0.5% diaminobenzidine tetrahydroxide (DAB, Wako, Osaka, Japan) solution with 0.6% ammonium nickel sulfate and 0.7 ml 0.3% hydrogen peroxide for 10–20 min at room temperature. The sections were mounted on gelatinized slides, dried, counterstained with cresyl violet, and then were coverslipped.

The cells were observed under a fluorescent microscope with either 10× or 20× objectives (Axioplan 2, Zeiss, Jena, Germany) and were photographed with a 20× objective using a double exposure CCD camera system (AxioVision, Zeiss). We measured the size of the cells with the soma and dendrites clearly labeled with TexasRed. The outline of the cells was drawn using a data analyzing system (Neurolucida, MicroBrightField, Colchester, VT, USA), and the area size of the cells was measured using NIH image. The mean and standard deviation of these measurements were calculated, and the means were compared using Student's *t*-test for unpaired comparisons.

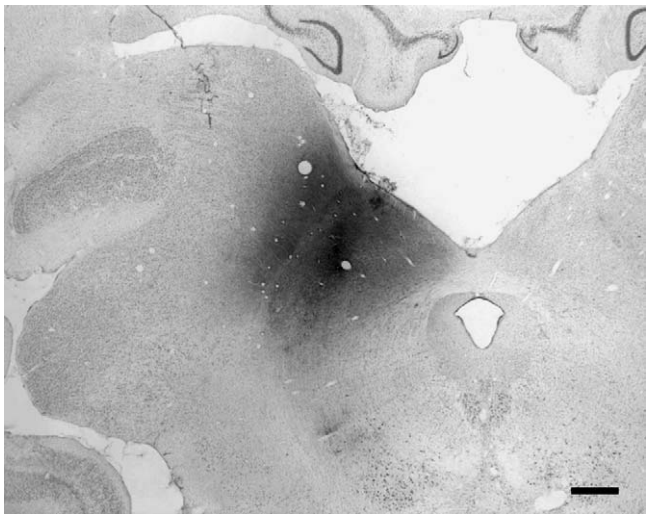

Fig. 1. Injection site of biotinylated dextran amine (BDA) in the pretectum. Scale bar: 1 mm.

Fig. 1 shows the typical injection site of BDA in one of the animals. In this case, the injection was centered in the reticular part of the anterior pretectal nucleus. The tracer covered most of the pretectum and diffused into the nucleus limitans and the lateral posterior nucleus of the thalamus, but did not diffuse into the superior colliculus, red nucleus, periaqueductal gray, or the contralateral pretectum. The labeled neurons in the cerebellar nuclei in this case thus showed projections to the entire pretectum. The second case had a large injection covering the pretectum with slight diffusion into the nucleus limitans, the lateral posterior nucleus, the periaqueductal gray matter, and the rostral end of the superior colliculus. The resultant labeling in the cerebellar nuclei thus included not only the pretectal projections but also some projections to the superior colliculus and the periaqueductal gray matter. In the third case, the injection covered the compact part of the anterior pretectal nucleus, the dorsal portion of the reticular part of the anterior pretectal nucleus, and the lateral part of the nucleus of the optic tract. The tracer diffused into the nucleus limitans and the lateral posterior nucleus along the tract of the injection micropipette. The labeling in the cerebellar nuclei in this case showed the projections mostly to limited parts of the pretectum, namely, to the reticular part of the anterior pretectal nucleus and the lateral part of the nucleus of the optic tract. In the fourth case, the injection was small and was centered in the compact part of the anterior pretectal nucleus. Tracer diffusion was restricted to the anterior pretectal nucleus, the nucleus of the optic tract, and the lateral posterior nucleus of the thalamus. The labeling in this case thus showed the cerebellar neurons that project mostly to the compact part of the anterior pretectal nucleus and partly to the reticular part of the anterior pretectal nucleus and the nucleus of the optic tract.

We found both GAD-positive (Fig. 2A–I) and negative (Fig. 2J–L) neurons in the cerebellar nuclei project to the pre-