Neuroscience 133 (2005) 1047–1059

ZONAL ORGANIZATION OF THE VESTIBULO-CEREBELLAR PATHWAYS CONTROLLING THE HORIZONTAL EYE MUSCLES USING TWO RECOMBINANT STRAINS OF PSEUDORABIES VIRUS

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Abstract—Many studies have documented the influence of the flocculus upon vestibulo-ocular reflex eye movements. Electrical stimulation of Purkinje cells in a central longitudinal zone evoked slow ipsilateral eye movements in the horizontal plane. Recently, the organization of neurons in the vestibulo-cerebellar pathways controlling single lateral rectus and medial rectus muscles was identified in rats using the transynaptic transport of pseudorabies virus. Overlapping distributions of neurons innervating single muscles were located predominantly in a central longitudinal zone of ventral paraflocculi/dorsal flocculi, and the rostral half of ventral flocculi. This study used two isogenic pseudorabies virus recombinants to determine whether individual cells in those brain regions have collateralized projections to motoneuron pools innervating the right lateral rectus and the left medial rectus muscles using different survival times and dual injection paradigms. The infected neurons were detected using dual-labeling immunofluorescence. Three populations of labeled neurons were observed: two populations replicated only one reporter while a third contained both viruses (i.e. dual-labeled). Most dual-labeled cells were located in a central longitudinal zone of the ventral paraflocculus, ipsilateral to the injection into the medial rectus, whereas very few were in the flocculus. This finding suggests that the flocculus and ventral paraflocculus may exert influence upon distinct vestibulo-cerebellar pathways. Most Purkinje cells in the ventral paraflocculus may influence the vestibulo-ocular reflex pathways through collateralization, whereas those in the flocculus may instead provide a monocular control of eye movements.

Key words: dual labeling, flocculus, ventral paraflocculus, microzones, binocular control, rats.

Prior physiological investigations determined the topographical organization of specific populations of cells of the flocculus that participate in control of vestibulo-ocular reflex (VOR) eye movements in different semicircular canal planes. For example, Purkinje cells in a central longitudinal zone, perpendicular to the long axis of the folia, were shown to inhibit monosynaptically the horizontal canal vestibulo-ocular pathways, and evoke slow ipsilateral eye movements in the horizontal plane (monkey: Balaban and Watanabe, 1984; Belknap and Noda, 1987; rabbit: Dufossé et al., 1977; Fukuda et al., 1972; Ito et al., 1977, 1982; Nagao et al., 1985; Van der Steen et al., 1994; Yamamoto, 1979a; cat: Sato and Kawasaki, 1984, 1990a,b; Sato et al., 1988). This flocculus zone was also determined to receive horizontal optic flow information from olivo-cerebellar climbing fibers originating in the caudal dorsal cap of Kooy (rabbit: Graf et al., 1988; Kano et al., 1990; Kusunoki et al., 1990; Leonard et al., 1988; Maekawa and Simpson, 1973; Simpson and Alley, 1974; Simpson et al., 1981). Anatomical studies using conventional tracers largely substantiated the concept of *longitudinal* organization of Purkinje cells in the flocculus/ventral paraflocculus based upon distinct vestibular Purkinje cell efferents (rabbit: Balaban, 1984; De Zeeuw et al., 1994; Tan et al., 1995; Yamamoto, 1978; Yamamoto and Shimoyama, 1977; monkey: Balaban et al., 1981; rat: Balaban et al., 2000; Sugihara et al., 2004; Umetani, 1992; cat: Sato et al., 1982) and olivocerebellar climbing fiber projections from distinct areas of the inferior olive (rat: Ruigrok et al., 1992; Sugihara et al., 2004; cat: Sato et al., 1983; rabbit: Yamamoto, 1979b). More recently, the use of retrograde transynaptic transport of viruses supported the microzone concept and identified the neurons in the flocculo–vestibulo–oculomotor circuitry innervating single medial rectus (MR, Graf et al., 2002; Billig and Balaban, 2004) and lateral rectus (LR, Billig and Balaban, 2004) muscles. Interestingly, Billig and Balaban (2004) found overlapping distributions of neurons controlling the LR of one eye and the MR of the other eye in a central zone of the ventral paraflocculi/dorsal flocculi and circumscribed areas of the rostral half of the flocculi. In the vestibular nuclei, the distribution of neurons influencing each muscle mostly overlapped in the parvicellular part of the medial vestibular nucleus (MVePC) and, to a lesser extent at the periphery of the superior vestibular nucleus (SuVe), dorsal Y groups and the nucleus prepositus hypoglossi. This study used two antigenically distinct recombinant strains of pseudorabies virus (PRV) to determine whether neurons in vestibulo-cerebellar circuits have collateralized projections to

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Abbreviations: EGFP, enhanced green fluorescence protein; EOMs, extraocular muscles; β-gal, beta-galactosidase; LR, lateral rectus; MR, medial rectus; MVe, medial vestibular nucleus; MVeMC, magnocellular part of the medial vestibular nucleus; MVePC, parvicellular part of the medial vestibular nucleus; OVAR, off-vertical axis rotation; pfu, plaque-forming unit; PRV, pseudorabies virus; SuVe, superior vestibular nucleus; VOR, vestibulo-ocular reflex.

0306-4522/05$30.00+0.00
doi:10.1016/j.neuroscience.2005.04.005

pathways innervating the LR motoneuron pool for one eye and the MR motoneuron pool for the other eye.

EXPERIMENTAL PROCEDURES

All animal procedures carried out in this study were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (publication No 80-23), and approved by the Institutional Animal Care and Utilization Committee and rDNA office of the University of Pittsburgh. The approved protocol included assurances that all efforts were made to reduce animal suffering and minimize the numbers of animals used, no alternatives to *in vivo* techniques were available, and that the present study did not duplicate previous research.

Recombinant strains of pseudorabies virus (PRV)

Isogenic recombinant strains of PRV from the parental Bartha strain were used in this study. This attenuated strain was developed originally as a vaccine (Bartha, 1961) and is commonly used to highlight multiple-synapse systems (see Card, 2001; see Enquist et al., 1999 for recent reviews). The organization of recombinant genomes has been published elsewhere (Billig et al., 2000; see Card, 2001). Briefly, PRV-Bablu contains the lacZ gene at the gG locus and produces beta-galactosidase (β-gal) under the control of the viral gG promoter. PRV-152 expresses the enhanced green fluorescence protein (EGFP) and carries an insertion at the gG locus such that EGFP is expressed using the cytomegalovirus immediate-early promoter. Prior studies in other motor pathways (e.g. Billig et al., 2000) have shown that the distribution of labeled neurons induced by injection of recombinant strains was similar to the distribution of labeled neurons following injection of the parental PRV-Bartha. Furthermore, the distribution of neurons expressing transgenes was coextensive with the distribution of infected neurons by the rabbit polyclonal antiserum. Viruses were grown in pig kidney (PK15) cells, and their final titer in plaque-forming units (pfu) (determined in PK15 cells) was 1×10^8pfu/ml for PRV-152 and PRV-Bablu. Those two PRV recombinants were shown to have a similar transport rate and equivalent neuroinvasiveness.

Design and selection of survival times

Dual injections into horizontal extraocular muscles (EOMs) were conducted in 10 Long-Evans male rats (280–320 g, Charles River Laboratories, Wilmington, MA, USA). Animals were allowed a minimum of 5 days acclimation to the animal facility before being injected with PRV.

Single PRV injection experiments were conducted in our prior study (Billig and Balaban, 2004) using 35 rats, for which either PRV-152 or PRV-Bablu was injected into single LR and MR muscles. A minimum survival time of 80-h was necessary to observe a consistent number of labeled Purkinje cells in flocculi/ventral paraflocculi following PRV injections into the MR, whereas an 88-h survival time was necessary to obtain an equivalent infection in flocculi/ventral paraflocculi after injecting PRV into the LR. This was true for either recombinant strain of PRV. The delayed progression of infection from the PRV injections into the LR was attributed to the greater length of the abducens nerve compared with the oculomotor nerve, totaling about 5 mm in rats. Furthermore, Billig and Balaban (2004) indicated that the first signs of lysis of Purkinje cells in flocculi/ventral paraflocculi appeared after 84-h and 96-h survival times following injections of PRV into single MR and LR, respectively. This factor was taken into account to determine the temporal window for the infection of neurons in the vestibulo-cerebellar pathways.

Three animals were injected initially with PRV-152 into the right LR muscle followed 8 h later by the injection of PRV-Bablu into the left MR muscle, after which the animals survived 80 h. Despite a robust infection of motoneurons from the transport of each PRV recombinant, a much smaller number of labeled neurons were observed in the vestibular nuclei compared with single injection paradigm at same survival times (Billig and Balaban, 2004). In those cases, few dual-labeled cells were located bilaterally in the MVePC and in one case out of three one dual-labeled cell was present in the magnocellular part of the medial vestibular nucleus (MVeMC), ipsilateral to the injection into the MR. In addition, few labeled Purkinje cells were present in the flocculi/ventral paraflocculi from the transport of each PRV recombinant, and no dual-labeled cells were ever detected. Hence, the other seven animals were subdivided into two groups. In a group of four animals, the two recombinant strains of PRV were injected simultaneously into the left MR and the right LR followed by an 84-h-survival time. In the second set of three animals, recombinant strains were injected with a delay of 8 h between the injections. In those cases, PRV-152 was first injected into the LR muscle of the right eye, and 8 h later PRV-Bablu was injected into the MR muscle of the left eye, after which the animals survived 84 h. Those two dual injection paradigms were intended to induce optimal conditions for infection of VOR pathways from each PRV recombinant transport.

Injection procedure

Injection procedures conformed to those detailed in our prior viral transneuronal analysis of the distribution of labeled neurons in the vestibular nuclei and the flocculi/ventral paraflocculi controlling single LR and MR muscles (Billig and Balaban, 2004). Animals were anesthetized with a dose of 0.85 ml/kg animal body wt. of a mixture of ketamine (100 mg/ml), xylazine (20 mg/ml) and acepromazine maleate (10 mg/ml), injected i.m. Cornea and conjunctiva were superfused with local anesthetic (2% Xylocaine) just before incision of soft-tissue. Under aseptic conditions, the conjunctiva of each eye was detached from the sclera to expose the distal insertion of the LR and MR into the eyeballs, which were left intact. A 7-0 Maxon monofilament polyglyconate ligature was looped around the insertion of each muscle to allow a gentle traction, and ease the insertion of a 5-μl Hamilton syringe equipped with a 33-gauge needle longitudinally into the muscles. An approximate volume of 4 μl of PRV-Bablu and PRV-152 were injected into the left MR and the right LR, respectively. Injections were performed under visual control with an operating microscope to optimize the uptake of the virus by the motor nerve endings preferably in the global layer. To reduce the possibility of spread to nearby orbital tissue, the injection needle was retained in place in the muscle for 1 min post-injection, and the area was subsequently swabbed using saline. In our prior study, we showed that injections of PRV into the orbital connective tissue of eyes induced labeling in areas to be known to innervate the sympathetic and parasympathetic nervous system (see Billig and Balaban, 2004). Upon completion of injections, animals were maintained under biosafety level II conditions for the balance of the experiments.

Tissue preparation

Upon completion of survival times, animals were killed with an overdose of sodium pentobarbital i.p. (100 mg/kg) and perfused transcardially with 250 ml of saline followed by 500 ml of paraformaldehyde–lysine–periodate fixative (McLean and Nakane, 1974). Brainstems were removed, post-fixed overnight at 4 °C, and cryoprotected in a 30% sucrose solution in phosphate buffered saline at 4 °C for 2 days. Transverse 30 μm sections of the brainstem were cut with a freezing microtome, collected sequentially in six wells of cryoprotectant (de Olmos et al., 1978), until processing for immunolocalization of viral antigens.