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TRANSSYNAPTIC TRACING OF CONDITIONED EYEBLINK CIRCUITS IN THE MOUSE CEREBELLUM

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Abstract—The eyeblink has long served as a model for motor learning and modulation. However, cerebellar pathways underlying conditioned blinks remain poorly studied in the mouse, and the location of blink-related neurons has never been transsynaptically mapped in the cerebellar cortex. This study aims to rectify this gap in our knowledge. By injecting GFP-expressing Pseudorabies virus (PRV-152) into the mouse orbicularis oculi muscle, neurons in the mouse eyeblink motor control circuit are transsynaptically labeled. In the facial nucleus, labeling was strictly ipsilateral to the injection site and restricted to the dorsolateral rim, consistent with previous studies. The red nucleus is bilaterally labeled at the lateral rim with clear contralateral preference. Previously unreported labeling was found in the ventrolateral red nucleus. Single-step tracing confirmed this area receives projections from eyeblink-related portions of the anterior interpositus and sends projections to eyelid-controlling portions of the facial nucleus. In the deep cerebellar nuclei, blink-related neurons were labeled both in areas associated with blink conditioning and in areas associated with other blink modulation. Finally, novel maps of the cerebellar cortex revealed a characteristic spatiotemporal pattern of labeling. Posterior vermal Purkinje cells were labeled first, followed by anterior vermal cells, then by hemispheric cells. © 2011 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: classical conditioning, circuit tracing, cerebellar cortex, deep cerebellar nuclei, eye blink, pseudorabies virus.

The latticed cell structure of the cerebellum houses highly conserved motor coordination, modulation, and learning mechanisms. As the cellular anatomy of the cerebellum is remarkably uniform (Nieuwenhuys et al., 1998; Kandel et al., 2000; Lowrie, 2004), much of its function likely arises from the intricacies of its connectivity. In this project, that functional anatomy was explored in one of the simplest forms of motor learning, the classically conditioned blink.

The overall layout of the blink conditioning pathway is well established. Purkinje cells of the cerebellar cortex (CbCtx) project to neurons of the deep cerebellar nuclei (DCN), which then project to the neurons of the red nucleus (RN). These in turn project to the primary motoneurons of the facial nucleus (7N) (Voogd and Glickstein, 1998; Mauk et al., 2000; Thompson and Steinmetz, 2009). However, significant uncertainty remains regarding the precise intranuclear distribution of eyeblink-related neurons, particularly in the cerebellum. Although most groups focus on the lobule simplex (HVI) of the CbCtx (Lavond and Steinmetz, 1989; Perrett et al., 1993; Woodruff-Pak et al., 1993) and the anterior interpositus nucleus (AIP) of the DCN (Lavond et al., 1984; McCormick and Thompson, 1984; Yeo et al., 1985; Racine et al., 1986; Steinmetz et al., 1992a; Krupa et al., 1993), others implicate the anterior lobules of the cerebellar cortex (Mauk and Donegan, 1997; Garcia et al., 1999; Medina et al., 2000; Kalmbach et al., 2010) and the posterior interpositus (PIP) and dentate nuclei (DN) in conditioning (Gruart et al., 2000; Delgado-Garcia and Gruart, 2005; Sanchez-Campusano et al., 2007, 2009).

Early explorations of the cerebellar eyeblink circuit employed lesions, inactivations, and electrophysiology. More recently, Morcuende et al. (Morcuende et al., 2002) used rabies virus to transsynaptically label the eyeblink pathway in rats. Because transsynaptic tracing identifies successive orders of neurons projecting to a downstream infusion site, inoculating the orbicularis oculi muscles (OOM) of the eyelid identifies eyeblink-related neurons across several nuclei. This technique is more specific than lesions/inactivations and more comprehensive than electrophysiological recordings. Morcuende's study provides a valuable mapping of rat eyeblink-related conditioning circuitry up to third-order DCN neurons.

Despite this, the field still lacks a rigorous mapping of eyeblink-related neurons in the CbCtx. Additionally, little work has targeted the fine anatomy of the mouse eyeblink circuitry at any level. Because the CbCtx is both a critical site of learning and the subject of considerable controversy, mapping the eyeblink circuit yields important information regarding both the location of the memory trace in the CbCtx and the overall functional anatomy of the cerebellum in motor learning. Furthermore, because the genetically versatile mouse is an increasingly important model of cerebellar memory, charting the locations of eyeblink-related neurons in the mouse builds a valuable foundation for future site-targeted investigations of the cellular and circuitry changes underlying memory acquisition. Using the GFP-expressing transsynaptic virus Pseudorabies (Bartha 152 strain), this project identified blink-related neurons from the CbCtx to the 7N. These results were then analyzed for novel labeling trends and consolidated into the

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Abbreviations: BTX, bungarotoxin; CbCtx, cerebellar cortex, including subdivisions; DCN, deep cerebellar nuclei, including subdivisions; DN, dentate nucleus; FN, fastigial nucleus; HVI, lobule simplex of the cerebellar cortex; IPN, interpositus nucleus (PIP, posterior interpositus and AIP, anterior interpositus); NMJs, neuromuscular junctions; OOM, orbicularis oculi muscle (eyelid muscle); PRV, pseudorabies virus, including PRV-152, the eGFP-expressing strain of PRV; RN, red nucleus; 7N, facial nucleus.

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first comprehensive stereotaxic maps of the mouse cerebellar eyeblink circuitry.

EXPERIMENTAL PROCEDURES

Viral culture

These experiments used the EGFP-expressing Pseudorabies virus, PRV-152, generously provided by Dr. Lynn Enquist via Dr. Botond Roska. A detailed overview of the PRV-152 virus can be found in Smith et al. (2000). Virus was grown in PK-15 cells using an adapted version of Card and Enquist's protocol (Card and Enquist, 2001). Immediately prior to usage, viral suspension was sonicated in a Misonix S3000 sonicator with cup attachment (Misonix Inc., Farmingdale, New York, NY, USA) to rupture any intact cells and disperse aggregated virus. If virus was to be injected intracranially, the aliquot was further centrifuged at $2000\times g$ for 5 min at room temperature to remove cellular debris. Viral batches were regrown every 6 months to maintain potency, and titers ranged between 8.9×10^8 to 1.2×10^9 IFU/ml.

Surgery

All experiments presented in this article conformed to local and international guidelines on the ethical use of animals. Experiments were designed to minimize the number of animals used and their suffering. Nine-week-old male C57Bl/6 mice were used throughout these studies. Prior to all surgeries, animals were anesthetized using a ketamine/xylazine cocktail administered intraperitoneally. For intramuscular injections, a model 7002KH Hamilton syringe with a 25 gauge beveled Style 2-point needle (Hamilton Company, Reno, NV, USA) was used to inject viral suspension along the edge of the eyelid between the tarsus and the skin, where fibers of the orbicularis oculi were observed to pass in histological slices. Each animal received three to five injections amounting to a total of 1.0 μl of virus distributed across the lower edge of the eyelid. After injection, animals were monitored daily for a viral incubation period of 3–10 days, depending on experimental purpose, during which virus propagates to higher-order brain structures. Of all experimental animals injected with PRV, approximately 50% showed staining and, if incubated for greater than 3–4 days, developed symptoms of encephalopathic infection. Only results from infected animals were included in mapping studies, neuron counts, and statistical analyses. Only labeled animals were included in N-counts. The number of animals infected and included in the study at each time point was as follows: 3 day, $n=18$; 5 day, $n=16$; 6 day, $n=18$; 7 day, $n=21$; 8 day, $n=24$; 9 day, $n=22$; 10 day, $n=15$.

For intracranial pressure injections and electroporations, each animal was mounted on a Kopf Model 900 stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). For pressure injections of Red Retrobeads (LumaFluor, Inc., Naples, FL, USA), a World Precision Instruments UltraMicroPump (UMP3) with a SYS-Micro4 Controller unit (World Precision Instruments, Sarasota, FL, USA) was mounted onto the stereotaxic frame, and a model 7001KH Hamilton syringe with a 25 gauge style 3 needle was inserted into the UMP3 unit. For electroporation of dextran-conjugated dyes, a glass pipette with inner diameter 2–4 μm was used to load dye. A Multichannel Systems STG 1004 (Multi Channel Systems MCS GmbH, Reutlingen, Germany) was used to generate all electroporation currents, and a standard Kopf electrode holder was used to mount the electrode and glass capillary onto the stereotaxic frame in preparation for surgery.

For all intracranial inoculations, a 1-cm incision was made along the midline of the scalp. The skull was leveled and a Foredom Micromotor 45000 rpm dental drill (Blackstone Industries, Inc., Bethel, CT, USA) was used to bore a small hole in the skull over the targeted area. The needle/electrode was then lowered down to the inoculation site. The tissue is allowed to relax for 5 min, after which injection or electroporation was initiated. Pressure injections were made at a rate of 50 nl per min. In all, 100–250 nl of RetroBeads were injected into target nuclei. In electroporation experiments, anionic, lysine-fixable fluorescein-conjugated 3000 MW dextran (Molecular Probes Invitrogen, Life Technologies Inc.) was prepared at a 10% w/v solution. The ground electrode was clipped to the base of the tail, and a -50 μA square-wave 50 ms current was pulsed at 2 Hz for 20 min. After injection/electroporation was complete, the needle/electrode was left in place for another 5 min and then slowly withdrawn. The wound was closed with VetBond (3M, St. Paul, MN, USA) and/or skull staples (Fine Science Tools (USA), Inc., Foster City, CA, USA). Animals were returned to holding cages for an incubation period of 2 days. Final experimental animal counts are as follows: $n=12$ for Red RetroBead red nucleus pressure injection experiments, and $n=9$ for electroporation into the anterior interpositus.

Additional controls

Microanatomy of the mouse eyelid was first established by excising, microtome slicing, and H&E staining two upper right eyelids of C57Bl/6 mice. The location of the orbicularis oculi muscles was closely examined; these slides were thereafter used as a reference for all eyelid injection site checks. To confirm initial injections were properly localized to the orbicularis oculi, PRV-152 and AlexaFluor 594-conjugated alpha-bungarotoxin (BTX, Molecular Probes Invitrogen, Life Technologies Inc., Grand Island, New York, NY, USA) were coinjected in approximately 20% of all eyelid injection experiments. At time of sacrifice, the right eyelid of the animal and adjacent areas of the scalp was excised along with the brain. The eyelid was immediately examined for AlexaFluor 594 labeling with a Leica M165 FC variable-magnification fluorescent stereo microscope (Leica Microsystems Inc., Buffalo Grove, IL, USA) to confirm location of labeled neuromuscular junctions as compared with prior H&E-stained eyelids. Additionally, the oculomotor nuclei, which innervate all extraocular muscles except the lateral rectus and superior oblique, the trochlear nuclei, which innervate the superior oblique, and the abducens nuclei, which innervate the lateral rectus of the eyes, were examined for viral eGFP labeling in all facial nucleus experiments.

Histology

After sacrifice, excised brains were fixed overnight in ice-cold 4% PFA, then washed for a minimum of 8 h in PBS before histology. Agarose gel (4%) was mixed fresh or reheated and used to embed fixed brains. A Leica VT1000S vibratome (Leica Microsystems Inc., Buffalo Grove, IL, USA) was used to take sequential 100 μm coronal slices. Each brain was sliced from the posterior margin of the cerebellum to the approximate level of bregma. For experiments using PRV, additional antibody staining was required. Slices were blocked in 1% w/v bovine serum albumin (BSA, Sigma-Aldrich Corp., St. Louis, MO, USA) and 3% goat serum (Sigma-Aldrich Corp.) in PBS for 1 h at room temperature. They were then incubated in 1% w/v BSA/1% goat serum/1:500 rabbit anti-GFP antibody (Molecular Probes Invitrogen, Life Technologies Inc.) in PBS for 48 h at 4 °C. Secondary antibody solution was prepared similarly using a 1:1000 dilution of AlexaFluor 488-conjugated goat anti-rabbit antibody (Molecular Probes Invitrogen, Life Technologies Inc.) and incubated for 60 min at room temperature.

Imaging and mapping

Slices were mounted using Fluoromount G (Southern Biotech, Birmingham, AL, USA) under #0 or #1 coverslips sealed and examined using a Leica M216 FA variable-magnification fluorescent stereo microscope. Epifluorescent images were taken using a Q-Imaging Retiga 4000RV camera. Confocal images were taken

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