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Localization of the cerebellar cortical zone mediating acquisition of eyeblink conditioning in rats

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ABSTRACT

Delay eyeblink conditioning is established by paired presentations of a conditioned stimulus (CS) such as a tone or light and an unconditioned stimulus (US) that elicits eyelid closure before training. The CS and US inputs converge on Purkinje cells in the cerebellar cortex. The cerebellar cortex plays a substantial role in acquisition of delay eyeblink conditioning in rabbits and rodents, but the specific area of the cortex that is necessary for acquisition in rodents has not been identified. A recent study identified an eyeblink microzone in the mouse cerebellar cortex at the base of the primary fissure (Heiney, Kim, Augustine, & Medina, 2014). There is no evidence that the cortex in this eyeblink microzone plays a role in rodent eyeblink conditioning but it is a good candidate region. Experiment 1 examined the effects of unilateral (ipsilateral to the US) lesions of lobule HVI, the lateral anterior lobe, or the base of the primary fissure on eyeblink conditioning in rats. Lesions of either the anterior lobe or lobule HVI impaired acquisition, but lesions of the base of the primary fissure produced the largest deficit. Experiment 2 used reversible inactivation with muscimol to demonstrate that inactivation of the putative eyeblink microzone severely impaired acquisition and had only a modest effect on retention of eyeblink conditioning. The findings indicate that the base of the primary fissure is the critical zone of the cerebellar cortex for acquisition of eyeblink conditioning in rats.

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1. Introduction

The cerebellum is necessary for acquisition and retention of associative eyeblink conditioning (McCormick, Clark, Lavond, & Thompson, 1982). Delay eyeblink conditioning consists of a conditioned stimulus (CS; e.g., a tone) paired with an unconditioned stimulus (US) that elicits an eyelid closure unconditioned response (UR) before training. Repeated CS–US pairings result in the development of an eyelid closure conditioned response (CR) which precedes the onset of the US. Numerous studies demonstrated the essential role of the cerebellum in acquisition and retention of the CR using lesions, reversible inactivation, electrical stimulation, unit recording, genetic manipulations, neuropharmacology, and quantitative neuroanatomy (for review see Freeman & Steinmetz, 2011).

Acquisition and retention of the eyeblink CR are completely abolished by lesions or inactivation of the anterior interpositus nucleus (Clark, Zhang, & Lavond, 1992; Freeman, Halverson, & Poremba, 2005; Krupa, Thompson, & Thompson, 1993; Lavond, Hembree, & Thompson, 1985; Lincoln, McCormick, & Thompson, 1982; McCormick et al., 1982; Steinmetz, Lavond, Ivkovich, Logan, & Thompson, 1992; Steinmetz, Logue, & Steinmetz, 1992; Yeo, Hardiman, & Glickstein, 1985a). Lesions or inactivation of the cerebellar cortex also produce deficits in acquisition; however, the severity of the deficit has differed between studies (Attwell, Rahman, & Yeo, 2001; Garcia, Steele, & Mauk, 1999; Hardiman, Ramnani, & Yeo, 1996; Lavond & Steinmetz, 1989). Mice with the Purkinje cell degeneration mutation (*pcd*) which lose most of their Purkinje cells during development are impaired during acquisition, but show an increase in CRs across training that is significantly greater than unpaired controls (Chen, Bao, Lockard, Kim, & Thompson, 1996; Chen, Bao, & Thompson, 1999). A similar set of results was found in rats that had Purkinje cells destroyed by OX7-saporin, an immunotoxin, as adults (Nolan & Freeman, 2006). The findings from the *pcd* studies in mice and the OX7-saporin study in rats suggest that the cerebellar cortex plays a substantial role in acquisition of eyeblink conditioning but the cerebellar interpositus nucleus can support modest learning without the cortex (Chen et al., 1999; Nolan & Freeman, 2006). Indeed,

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lesions of the interpositus nucleus abolish CRs in *pcd* mice (Chen et al., 1999). The rodent studies were informative regarding the role of the cerebellar cortex in eyeblink conditioning but they did not identify the area within the cortex that plays a critical role in acquisition.

A recent study that used optogenetic inhibition of Purkinje cells in mice that were not given eyeblink conditioning identified an "eyeblink microzone" at the base of the primary fissure (Heiney et al., 2014). Electrophysiology, electrical stimulation, and optogenetics were used to demonstrate that inhibition of Purkinje cells in this area produced eyelid closure. This mouse eyeblink microzone is therefore a very good candidate for the cortical area that is critical for acquisition of eyeblink conditioning in rodents. The current study used lesions (Experiment 1) and reversible inactivation (Experiment 2) to localize the area of the cerebellar cortex that is critical for acquisition of eyeblink conditioning in rats. Electrolytic lesions were made in the base of the primary fissure (PFb) or in the lobules on either side of the primary fissure: hemispheric lobule VI (HVI) or lateral anterior lobules IV/V (AIV/V). Reversible inactivation was used to determine whether the base of the primary fissure plays a role in acquisition and retention of eyeblink conditioning in a within subjects design.

2. Materials and methods

2.1. Subjects

Subjects were 78 male Long-Evans rats, 250–350 g at the beginning of the experiment. The rats were housed in Spence Laboratories of Psychology at the University of Iowa with a 12 h light–dark cycle and were given ad libitum access to food and water.

2.2. Surgery

One week prior to the onset of training, rats were removed from their home cage and anesthetized with isoflurane. For Experiment 1, unilateral lesions of the cerebellar cortex were produced by passing 1.0 mA of DC current for 10 s through an insect pin insulated with Epoxylite into the left cerebellar cortex. Rats in the control group had electrodes lowered into the cerebellar cortex but no current was passed. In Experiment 2, a 23-gauge guide cannula was implanted 1.0 mm dorsal to the targeted area in the cerebellar cortex. A 30-gauge stylet was inserted into the guide cannula and extended 1.0 mm from the end of the guide. The stereotaxic coordinates taken from bregma were 11.2 mm posterior, 2.6 mm (AIV/V), 3.0 mm (PFb), or 3.4 mm (HVI) lateral, and 3.2 mm ventral to the skull surface. For both experiments rats were implanted with differential electromyographic (EMG) electrodes in the left upper eyelid muscle (orbicularis oculi) and a ground electrode attached to a stainless steel skull screw. The EMG electrode leads terminated in gold pins held in a plastic connector, which was secured to the skull with dental acrylic. A bipolar stimulating electrode (Plastics One, Inc.) for delivering the periorbital stimulation US was implanted immediately caudal to the left eye. The bipolar electrode terminated in a plastic connector that was secured to the skull with dental acrylic.

2.3. Infusions

Prior to infusions, the 30-gauge stylet was removed and replaced with a 30-gauge infusion needle that extended 1.0 mm past the tip of the guide cannula. A 10 μL syringe (Hamilton, Reno, NV) was connected to the infusion needle by a polyethylene tube (PE 10, 110–120 cm) and the syringe was secured to an infusion pump (Harvard Apparatus, Hilliston, MA). Rats were infused with .5 μL of either muscimol (2.0 mM) or PBS at a rate of 6.0 μL/h 30 min in each site prior to the beginning of training.

2.4. Apparatus

The conditioning apparatus consisted of four small-animal sound attenuation chambers (BRS/LVE, Laurel, MD). Within each sound attenuation chamber was a small animal operant chamber (BRS/LVE, Laurel, MD) where the rats were located during conditioning. One wall of the operant chamber was fitted with two speakers through which the CS was presented. The electrode leads from the rat's headstage were connected to peripheral equipment. Computer software controlled the delivery of stimuli and the recording of eyelid EMG activity (JSA Designs, Raleigh, NC). The US (2–3 mA, DC constant current) was delivered through a stimulus isolator (Model number 365A, World Precision Instruments, Sarasota FL). EMG activity was recorded differentially, filtered (500–5000 Hz) and integrated by equipment (JSA Designs, Raleigh, NC) as described in other reports (Freeman et al., 2005).

2.5. Conditioning procedures

Rats recovered from surgery for 1 week prior to the initiation of training. Rats completed daily sessions of paired training. Each session consisted of 10 blocks of 9 paired CS–US presentations and 1 CS alone probe trial. The CS was a 400 ms tone (2 kHz; 85 dB). The CS terminated with a 25 ms periorbital stimulation US. The shock intensity was adjusted in each rat to elicit a blink and slight head movement prior to the first session and then it was unchanged for the remaining sessions (range = 2–3 mA). For Experiment 1, rats completed daily sessions until they reached two consecutive sessions at or above 80% CRs. Training was terminated after 15 sessions if the rat never reached criterion. In Experiment 2, rats underwent 12 paired sessions. Thirty minutes before each of the first 5 sessions, rats received infusions of muscimol or vehicle (0.5 μL). During the next five sessions rats received training without infusions in order to examine possible savings following infusions or to make sure that all rats reached asymptotic conditioning. On sessions 11 and 12 rats received muscimol or vehicle to assess inactivation effects on retention. CRs were defined as EMG activity that exceeded a threshold of 0.4 units (amplified and integrated units) above the baseline mean during the CS period after 80 ms. EMG responses that exceeded the threshold during the first 80 ms of the CS period were defined as startle responses to the CS. On CS-alone probe trials, the duration for scoring CRs was extended beyond the CS to the end of the trial period (1.0 s). URs were defined as responses that crossed the threshold after the onset of the US.

2.6. Histology

Following the last training session, rats were euthanized with a lethal injection of sodium pentobarbital (150 mg/kg) and perfused transcardially with 0.9% saline followed by 10% buffered formalin. Brains were extracted and then post-fixed and cryo-protected in a 30% sucrose formalin solution and subsequently sectioned at 50 μm with a sliding microtome. Sections were mounted on gel-coated slides and stained with thionin. The extent of lesions and cannula placements were then verified using light microscopy.

3. Results

3.1. Experiment 1: cerebellar cortical lesions before acquisition

Unilateral electrolytic lesions were made in the cerebellar cortex ipsilateral to the conditioned eye prior to acquisition of delay

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