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Developmental Changes in Eyeblink Conditioning and Simple Spike Activity in the Cerebellar Cortex

ABSTRACT: *The activity of neurons in the cerebellum exhibits learning-related changes during eyeblink conditioning in adult mammals. The induction and preservation of learning-related changes in cerebellar neuronal activity in developing rats may be affected by the level of maturity in cerebellar feedback to its brainstem afferents, including the inferior olive. Developmental changes in cerebellar plasticity were examined by recording the activity of Purkinje cells in eye regions of cerebellar cortical lobule HVI (lobulus simplex) in infant rats during eyeblink conditioning. The percentage and amplitude of eyeblink conditioned responses increased as a function of age. Analyses of Purkinje cell simple spike activity revealed developmental increases in the number of units that exhibited stimulus-evoked and learning-related changes in activity. Moreover, the magnitude of these changes exhibited a substantial age-related increase. The results support the view that the emergence of learning-specific cerebellar plasticity and the ontogeny of eyeblink conditioning are influenced by developmental changes in the synaptic interactions within brainstem–cerebellum circuits. © 2003 Wiley Periodicals, Inc. Dev Psychobiol 44: 45–57, 2004.*

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Characterizing the relationship between neurophysiological and behavioral development may provide important insights into the neural mechanisms and organization of behavior (Campbell & Spear, 1972; Carew, 1989; Carew, Menzel, & Shatz, 1998; Freeman & Nicholson, 2001; Stanton, 2000). The classically conditioned eyeblink response (CR) provides a robust and easily measured learned behavior (Gormezano, Kehoe, & Marshall, 1983) that emerges ontogenetically between postnatal Days (PND) 17 and 24 in rats (Stanton, Freeman, & Skelton, 1992).

Studying the ontogeny of the eyeblink CR in infant rats provides a unique opportunity to assess the influence that maturation of a well-characterized neural circuit has on the ontogeny of associative learning. The neural substrates of the eyeblink CR include the cerebellum and its interconnections with the pontine nuclei and inferior olive, which supply the necessary and sufficient information about the conditioned stimulus (CS) via the mossy/parallel fiber system and unconditioned stimulus (US) via climbing fibers, respectively (Thompson & Krupa, 1994). Recent neurophysiological experiments indicate that the ontogeny of eyeblink conditioning is likely related to the maturation of synaptic interactions within and between the cerebellum and brainstem (Freeman & Nicholson, 2000; Nicholson & Freeman, 2000, 2003).

Previous developmental studies have shown that the proportion of single units that exhibit learning-related plasticity in the interpositus nucleus increase with age in parallel with age-related increases in stimulus-evoked activity and eyeblink conditioning (Freeman & Nicholson, 2000). In subsequent studies, it has been suggested

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that immature inhibitory feedback from the cerebellum to the inferior olive might limit learning-related changes in cerebellar neuronal activity (Nicholson & Freeman, 2000, 2003). Activity in the deep cerebellar nuclei, which drives the production of the eyeblink CR (Freeman & Nicholson, 2000; McCormick & Thompson, 1984), regulates learning-related changes in climbing fiber activity through inhibitory feedback to the inferior olive (Andersson, Garwicz, & Hesslow, 1988; Kim, Krupa, & Thompson, 1998; Lang, Sugihara, & Llinas, 1996; Medina, Nores, & Mauk, 2002; Sears & Steinmetz, 1991). The deep cerebellar nuclei also regulate learning-related changes in mossy/parallel fiber activity through excitatory feedback to the pontine nuclei (Bao, Chen, & Thompson, 2000). Plasticity in the activity of Purkinje cells, the principal neuron of the cerebellar cortex, is influenced by activity in both the climbing fibers and the mossy/parallel fibers (Lev-Ram, Wong, Storm, & Tsien, 2002; Linden & Connor, 1995; Sakurai, 1987). It is possible then that the maturation of interactions between the cerebellum and brainstem is an important developmental event influencing the ontogeny of cerebellar plasticity and eyeblink conditioning. For example, developmental differences in Purkinje cell neuronal activity may play a role in the ontogeny of eyeblink conditioning by changing cortical inhibition of the deep nuclei, thereby influencing the induction and preservation of learning-specific cerebellar plasticity during conditioning (Freeman & Nicholson, 2001; Nicholson & Freeman, 2003).

Purkinje cells exhibit two different types of spikes depending on the source of the afferent input (Llinas, 1981). Complex spikes are elicited by input from the climbing fibers of the inferior olive. Simple spikes are elicited by the axons of granule cells (parallel fibers), which receive CS-related information from neurons in the pontine nuclei (Gould, Sears, & Steinmetz, 1993) and US-related information from neurons in the trigeminal nucleus (van Ham & Yeo, 1992). Accordingly, the CS and the US elicit simple spike activity. Moreover, Purkinje cell simple spikes exhibit learning-related changes following paired stimulation of the CS and US pathways in adult mammals (Berthier & Moore, 1986; Gould & Steinmetz, 1994; Hesslow & Ivarsson, 1994; McCormick & Thompson, 1984). Recording Purkinje cell simple spike activity in developing rats therefore offers the opportunity to assess the development of CS and US afferent pathways while monitoring the ontogeny of learning-related cerebellar plasticity.

This report describes the results of an examination of simple spike activity recorded from Purkinje cells in eye regions of cerebellar cortical lobule HVI of infant rats during eyeblink conditioning. Rat pups were given training sessions of paired or unpaired presentations of a tone CS and a periorbital shock US on PND17 and 18 or

PND24 and 25. The findings suggest that the parallel ontogenetic emergence of cerebellar plasticity and eyeblink conditioning may be attributable to interactions within the brainstem–cerebellum circuitry.

METHODS

Subjects

The subjects were 25 Long-Evans rat pups trained on either PND 17 and 18 (PND17: $n = 12$) or PND 24 and 25 (PND24: $n = 13$). Subjects were 15 males and 10 females taken from nine litters. The rats were housed in the animal colony in Spence Laboratories at the University of Iowa. The rats were maintained on a 12:12 hr light:dark photoperiod, with light onset at 7 a.m.

Surgery

The rat pups were anesthetized with an ip injection of ketamine hydrochloride (60 mg/kg) and xylazine (7.5 mg/kg), and given atropine (1.25 mg/kg) to reduce respiratory tract secretions and excess salivation during anesthesia. Supplements of ketamine hydrochloride (10 mg/ml) were given in 0.1-ml increments, if needed. The rat's head was positioned in an infant stereotaxic head holder and aligned in three planes to bring it into the orientation required for placement of the electrodes. The rats were fitted with differential EMG electrodes that were implanted in the left eyelid muscle (orbicularis oculi) and a ground electrode that was attached to a stainless-steel skull hook. The EMG electrode leads terminated in gold pins in a plastic connector, which was secured to the skull with dental acrylic. A bipolar stimulating electrode (for delivering the shock US) was implanted subdermally, immediately caudal to the left eye. The bipolar electrode terminated in a plastic connector that was secured to the skull by dental acrylic.

In each rat, a bundle of eight insulated stainless-steel microwire electrodes (25 μm) was implanted in the left cerebellar cortical lobule HVI (lobulus simplex). Previous recording (e.g., Berthier & Moore, 1986; McCormick & Thompson, 1984; Schreurs, Gusev, Tomsic, Alkon, & Shi, 1998), stimulation (e.g., Katz, Tracy, & Steinmetz, 2001; Swain, Shinkman, Nordholm, & Thompson, 1992), lesion (e.g., Lavond & Steinmetz, 1989; Yeo, Hardiman, & Glickstein, 1985), and inactivation studies (Attwell, Rahman, Ivarsson, & Yeo, 1999; Attwell, Rahman, & Yeo, 2001) indicated that lobule HVI plays a critical role in eyeblink conditioning in rabbits. The stereotaxic coordinates for HVI were taken from lambda (AP = -2.3 mm; ML = $+2.3$ mm; and DV = -1.5 – -3.0 mm). Coordinates did not vary with age.

The bundle of microwire electrodes was implanted deep in the molecular layer, around the region of the Purkinje cell bodies. Deep placements optimize the probability of recording reliable, long-lasting Purkinje cell neuronal activity with chronically implanted electrodes (Bell & Kawasaki, 1972; Welsh & Schwartz, 1999). The electrode bundle was slowly lowered in increments and affixed to the skull when the neuronal activity exhibited spontaneous simple spikes and complex spikes in response to periorbital stimulation (light corneal taps with a cotton-tipped probe soaked in warm saline; Sears & Steinmetz, 1991).

The electrodes were held in place by a microelectrode connector and dental acrylic applied to the skull. The surgical site was closed with sutures on both sides of the electrode plug. Lightweight cables that allowed the rats to move freely during conditioning connected the EMG electrodes, bipolar stimulating electrode, and microwire electrodes to peripheral equipment and a Pentium computer. Damage to the headstage incurred by the dam or littermates chewing/gnawing at components of the headstage was prevented by applying Thum (Oakhurst Company, Levittown, NY), a mixture of citric acid and cayenne pepper, to the infants' headstages after surgery.

Conditioning Apparatus

The conditioning apparatus consisted of an electrically shielded small-animal sound attenuation chamber (BRS/LVE, Laurel, MD). Within the sound attenuation chamber was a small-animal operant chamber (BRS/LVE, Laurel, MD) where the rats were kept during conditioning. One wall of the operant chamber was fitted with two speakers that independently produced tones of up to 130-dB SPL, with a frequency range of approximately 1000 to 9000 Hz. The back wall of the sound-attenuating chamber was equipped with a small light. Computer software controlled the delivery of stimuli and the recording of eyelid EMG activity. EMG activity was recorded differentially, filtered, amplified, and integrated by equipment that was described in previous studies (Skelton, 1988; Stanton et al., 1992).

Paired Conditioning Procedure

The rat pups in the paired group received training sessions that consisted of 100 trials each. Three sessions were run on each day of training at approximately 4-hr intervals. During the first session, the rats were given explicitly unpaired presentations of a tone CS (50 presentations; 2.0 kHz, 300 ms, 85-dB SPL) and a periorbital shock US (50 presentations; 60 Hz, 6 ms, 4 mA) to assess developmental differences in stimulus-evoked activity without the confound of possible learning-related changes attributa-

ble to paired training. The rats were then given five training sessions, which consisted of 80 paired presentations of the tone CS and the shock US, 10 CS-alone trials, and 10 US-alone trials. The five training sessions consisted of 10 blocks of 10 trials. Of the 10 trials in each block, there were eight trials consisting of paired presentations of the CS and US, one CS-alone trial, and one US-alone trial. During paired trials, the CS coterminated with the US, yielding an interstimulus interval of 294 ms.

The CS-alone trials were included to assess behavioral responses (integrated EMG activity) and neuronal activity related to the CS or the CR, uncontaminated by an unconditioned response (UR; Gormezano et al., 1983). The US-alone trials provided the ability to evaluate developmental differences in US-evoked complex spike activity throughout training, without the possible confound of increased inhibition during paired (CS-US) trials on which there was a CR (see Nicholson & Freeman, 2003). The duration of the US during US-alone trials was 1 ms.

All trials were separated by a variable intertrial interval that averaged 30 s (range = 18–42 s). Behavioral data were examined from computer records of EMG responses. Conditioned responses (CRs) were defined as responses that crossed a threshold of 0.4 units (amplified and integrated voltage units) above baseline during the CS period, but at least 80 ms after CS onset, to avoid contamination of the CR by the startle or alpha response (Skelton, 1988). Previous studies have shown that the paired training protocol used in this study established associative eyeblink CRs (e.g., Stanton et al., 1992).

Unpaired Conditioning Procedure

Purkinje cell neuronal activity has exhibited training-related changes during forward paired (i.e., CS precedes the US), backward paired (i.e., US precedes the CS), and unpaired presentations of the CS and US (Gould & Steinmetz, 1996; Katz & Steinmetz, 1997). Plasticity during the latter two protocols is not specific to stimulus conditions that promote the acquisition of eyeblink CRs (Note that interpositus neuronal activity exhibits plasticity only under conditions that promote the acquisition of eyeblink CRs; Gould & Steinmetz, 1996.) Five PND17 rats and 6 PND24 rats received six sessions of unpaired presentations of the CS and US to determine the extent of plasticity in Purkinje cell neuronal activity under conditions that do not promote excitatory eyeblink conditioning in infant rat pups. Each session consisted of 180 explicitly unpaired presentations of the CS and US (90 presentations each). The durations of the CS and US were 300 ms and 6 ms, respectively. The intertrial interval (average = 15 s; range = 9–21 s) was half that used in the paired group to control for the total time spent in the conditioning chamber and the approximate temporal distribution of

the stimuli. Data analysis and definition of eyeblink responses were the same as that used in the paired groups.

Stimulation Procedure

If the activity of Purkinje cells in the present study was specifically related to the production of the eyeblink CR (i.e., involved in the control of the orbicularis oculi muscle), rather than to gross-movement proprioception or other non-learning-related processes, then activation of their axons may generate eyelid EMG activity. Activation of Purkinje cell axons strongly hyperpolarizes neurons in the deep cerebellar nuclei, which then exhibit rebound depolarizations and subsequent bursts of action potentials (Aizenman & Linden, 1999). The bursts of action potentials activate neurons in the red nucleus, which are ultimately connected to the eyeblink reflex circuit (Thompson & Krupa, 1994). Stimulation of regions of the cerebellar cortex that are involved in the control of the orbicularis oculi muscle might therefore elicit eyelid EMG activity (Hesslow, 1994). A subset of rats at both ages received stimulation through the individual microwire electrodes after training to explore the possibility that some of the electrodes were placed in so-called eyeblink control regions (Hesslow, 1994). Each electrode in each rat was stimulated at two durations (54 ms and 108 ms) and at two current levels (perithreshold and 1.5X threshold). In all conditions, stimulation pulses of 100 μ s duration were presented at a rate of 333 Hz. Raw EMG responses were recorded and analyzed offline using pClamp 6 (Axon Instruments, Foster City, CA). Only data from electrode placements that exhibited complex spikes in response to the US were included in the present study. In addition, for rats that received cerebellar cortical stimulation, only data from electrodes that exhibited complex spike activity in response to the US *and* elicited EMG activity when stimulated were included in the present study.

Neuronal Recording Procedure

The activity of each microwire electrode (eight for each rat) was initially passed through a unity gain JFET pre-amplifier (NB Labs, Denison, TX). The outputs of the JFET preamplifier were fed into an eight-channel programmable amplifier (Lynx-8, Neuralynx, Tucson, AZ), band-pass filtered between 300 and 6000 Hz, and amplified at a gain of 10,000. The outputs of the amplifiers were fed into a computer-controlled acquisition system at 10.4 kHz per channel (Datawave Technologies, Workbench-32, Longmont, CO), where thresholding was used to detect and extract waveforms of units with signal-to-noise ratios of at least 3:1. Two voltage thresholds were used. Waveforms that crossed the lower threshold, but did not cross the upper threshold within any of the sample

points were recorded to computer disk. The lower threshold was set to sample only the largest two to four spikes on each electrode. The upper threshold was set to a level approximately twice the voltage value of the largest spike on the electrode. The waveforms were saved as separate 32-point data chunks, yielding a waveform length of 3.08 ms (32 data points/10.4-kHz sampling rate). The acquisition and analysis software displayed all of the waveforms that were recorded during a particular data collection period (usually one training session). A template-matching program was used to identify all spikes with similar waveform characteristics. This technique effectively isolated single units from multi-unit records (Freeman & Nicholson, 2000; Nicholson & Freeman, 2000, 2003).

Data Analysis

The behavioral data were examined for each training session. Repeated measures analysis of variance (ANOVA) was performed for the CR percentage, CR amplitude, CR latency, and UR amplitude. Significant differences were evaluated by Tukey–Kramer’s honestly significant difference (HSD) test ($ps < 0.05$).

An eye region of cerebellar cortical lobule HVI was defined as a region that exhibited short-latency (~ 20 ms) Purkinje cell complex spikes in response to periorbital stimulation (e.g., unconditioned stimulus). Purkinje cells satisfying this criterion were considered to have receptive fields near the left eye. All data in the present study derive from electrode placements that satisfied this criterion. Peristimulus time histograms of the simple spike firing rates of each unit were created for the entire period of the conditioning trial (1 s) for each trial type (i.e., paired, CS-alone, US-alone) for the pretraining, first, third, and fifth paired training sessions for the paired group; and for the first, third, and sixth unpaired training sessions for the unpaired control group. Onset and peak latencies during the different trial types for all sessions were determined for all simple spikes at each age.

During analysis of paired CS–US trials, simple spike activity from the pre-CS baseline period was compared with six 49-ms intervals during the CS (not including the 6-ms US period) using the Wilcoxon signed rank matched pairs test (Bruning & Kintz, 1997). Analyses of paired Session 5 simple spike data also compared CS-period activity from CR or NO CR trials with pre-CS baseline activity. Age- and training-related differences in onset and peak latencies were examined using ANOVA, and significant differences were evaluated by Tukey–Kramer’s HSD test ($ps < 0.05$).

The functional relationship between cerebellar cortical output and eyeblink EMG is still relatively unknown, but many models of eyeblink conditioning in rabbits suggest

that the correspondence between simple spike activity and eyeblinks is a form of associative plasticity representing the timing and topography of the eyeblink CR (e.g., Medina, Garcia, Nores, Taylor, & Mauk, 2000; Moore & Choi, 1997). To assess the neural-behavioral specificity between simple spikes and eyelid EMG during the ontogeny of eyeblink conditioning in rats, cross-correlations were calculated between each rat's averaged integrated EMG response during CR trials and each individual simple spike's cumulative mean response during CR trials in the fifth session (130 ms before US-onset). Cross-correlations were calculated for only the fifth session because younger rats failed to exhibit a sufficient number of CRs in earlier conditioning sessions. If simple spike activity reflects processing of proprioceptive information, then changes in simple spike activity should lag behind changes in integrated EMG activity (phase lag). If simple spike activity plays a role in shaping or timing the eyeblink CR, then changes in simple spike activity should precede or accompany changes in integrated EMG activity (phase lead). Accordingly, a phase lag relationship between simple spike activity and integrated EMG activity is indicated by significant cross-correlations at time lags greater than zero. A phase lead relationship between simple spike activity and integrated EMG activity is indicated by significant cross-correlations at time lags less than zero. The absence of significant cross-correlations would indicate that there is no reliable relationship between simple spike activity and integrated EMG activity.

Histology

On the day after training, the rats were sacrificed with a lethal injection of sodium pentobarbital (90 mg/kg) and transcardially perfused with 100 ml of physiological saline followed by 300 ml of 3% formalin. After perfusion, the brains were postfixed in the same fixative for a minimum of 96 hr, and subsequently sectioned at 50 μ m with a sliding microtome. Sections were then stained with cresyl violet. The locations of the recording electrodes were confirmed by examining serial sections.

RESULTS

Behavior

Rat pups in both age groups exhibited significantly more eyeblink CRs than their unpaired control groups on Sessions 3 to 5 (Figure 1; $F(4, 84) = 5.851, p < 0.01$). PND24 rats in the paired group exhibited significantly more CRs than PND17 rats in the paired group on Sessions 3 to 5. The CR onset latencies were analyzed from only Sessions 3 to 5 because most rats exhibited few CRs

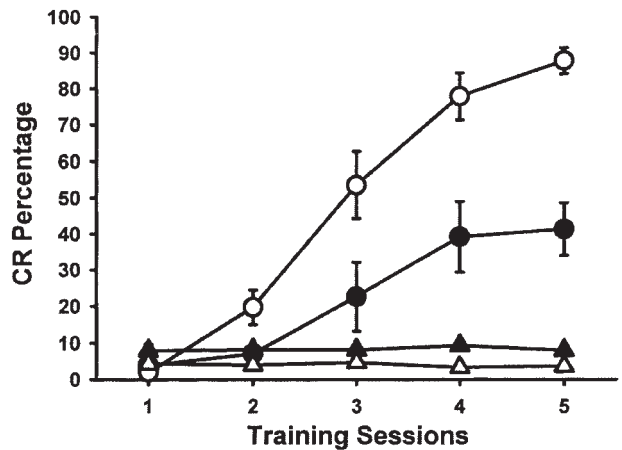


FIGURE 1 Eyeblink conditioning emerges between PND17 and PND24. Mean (\pm SEM) percentage of conditioned responses from infant rats trained on PND17 (black) and PND24 (white) across five sessions of paired training (circles) or unpaired training (triangles).

before Session 3. Rats receiving paired training on PND24 had earlier CR onset latencies than PND17 rats on Sessions 3 to 5, $F(1, 21) = 6.212, p < 0.05$. Peak latencies and amplitudes were determined for all CS-alone trials with CRs during paired Session 5. Eyeblink CRs at PND17 had earlier peaks and lower amplitudes than eyeblink CRs at PND24 (PND17 peak latency = 52 ms before US onset; PND24 peak latency = 1 ms before US onset; peak latency: $t(6, 7) = 2.318, p < 0.05$; amplitude: $t(6, 7) = 3.025, p < 0.05$). These data replicate the behavioral results of previous studies showing that the eyeblink CR develops between PND17 and PND24. There were no age-related differences in the amplitude, percentage, or latency of the UR at any point in training, which indicates that performance of the eyeblink response was not a factor in the developmental differences in eyeblink conditioning.

Purkinje Cell Neuronal Activity

The histological confirmation of electrode placements and the large potentials in the neuronal records confirmed that all electrodes were in the deep portions of the molecular layer near the Purkinje cell layer. Histological confirmation of electrode placements showed that the electrodes were never in the granule cell layer, which suggests that granule or Golgi cell activity was not recorded. Moreover, the window thresholding criterion likely eliminated the small size of granule cell and basket cell potentials and strongly argues against Golgi cell potentials (Bower & Kassel, 1990). These points and the presence of spontaneous and evoked complex and simple spikes indicated that the potentials recorded and analyzed in the present

experiment were from Purkinje cells in cerebellar cortical lobule HVI with periorbital receptive fields (see also Lobule HVI stimulation below). The complex spike data have been published as part of another study (Nicholson & Freeman, 2003).

The total number of isolated single units throughout all four analyzed sessions (i.e., pretraining, paired Sessions 1, 3, and 5) in the paired group was 311 from PND17 rats and 331 from PND24 rats. The total number of isolated single units from the unpaired control group throughout the three analyzed unpaired sessions was 210 from PND17 rats and 224 from PND24 rats. The distribution of single units was similar across sessions and groups for each age. While it is possible that some electrodes recorded the activity of the same Purkinje cell over two or more sessions, there is no reliable method to determine that the same unit is being examined from session to session. Therefore, units from each electrode during the different training sessions were treated as distinct units.

Simple Spike Activity During Unpaired Training

To directly assess nonassociative changes in Purkinje cell neuronal activity, rats at each age (5 PND17 rats; 6 PND24 rats) received six sessions of explicitly unpaired presentations of the CS and the US. Any changes in simple spike activity seen in both the unpaired control group and the paired group would likely be due to nonassociative influences such as habituation or sensitization to the CS and US. Conversely, any changes seen in the paired group, but not the unpaired controls, are likely attributable to paired training, and are therefore related to excitatory conditioning. As can be seen in Figures 2a–d, there was a developmental increase in the magnitude of CS- and US-period simple spike activity throughout unpaired training, main effect of age: $F(1, 856) = 5.63, p < 0.05$, predominantly confined to the first 150 ms after CS and US onset, main effect of bin: $F(99, 84744) = 140.308, p < 0.05$. Moreover, the proportion of simple spikes exhibiting significant changes in neuronal activity relative to baseline was higher in PND24 rats, $\chi^2 = 170.7, p < 0.05$. There were no decreases in the responsiveness to the CS or US either in the mean neuronal activity or at the single-unit level as a function of unpaired training sessions nor were there any significant effects of unpaired training on the peak or onset latency of simple spikes. There was no developmental difference in onset latencies during CS- and US-alone unpaired sessions throughout training (data not shown), but there was a developmental decrease in simple spike peak latencies throughout training, CS-peak: main effect of age, $F(1, 856) = 5.81, p < 0.05$; US-peak: main effect of age, $F(1, 856) = 6.044, p < 0.05$. Neuronal activity from the single session of CS- and US-alone trials in rats receiving paired training exhibited identical

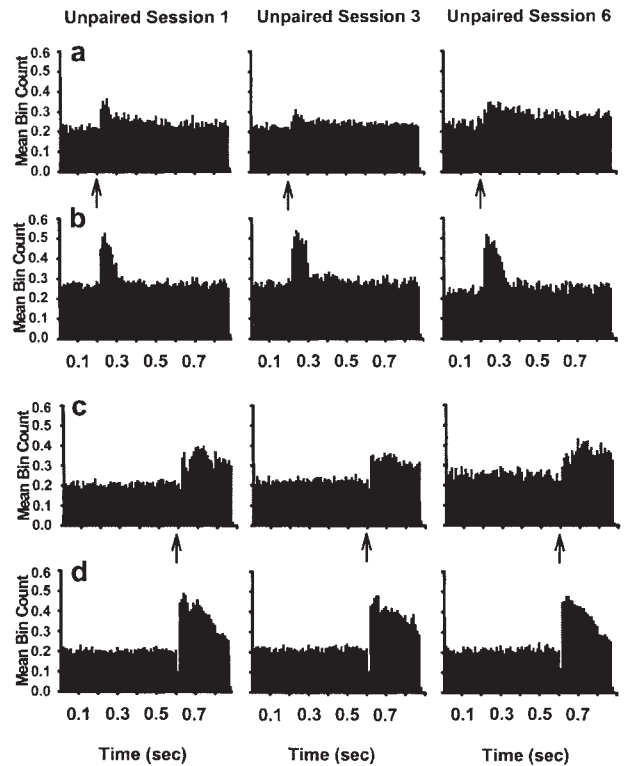


FIGURE 2 Simple spike activity in the unpaired control group is confined to the first 150 ms after CS and US onset. Mean neuronal activity for all simple spikes recorded from Purkinje cells in lobule HVI on PND17 (a, c) and PND24 (b, d) during Sessions 1, 3, and 6 of unpaired presentations of the CS alone (a, b) and US alone (c, d) in the unpaired control group. Arrows in (a) indicate CS onset; arrows in (c) indicate US onset.

developmental differences. There was no discernible evidence of habituation in the neuronal response to either the CS or US during unpaired training. Age-related increases in the proportion of units exhibiting changes and the magnitudes of those changes complement similar developmental differences in cerebellar interpositus neuronal activity (Freeman & Nicholson, 2000).

Simple Spike Activity During Paired Training

Figure 3 depicts the mean neuronal activity of simple spikes for paired Sessions 1 and 3. The histograms for Session 1 include the mean neuronal data from all simple spikes. The histograms for Session 3 include the mean neuronal data grouped according to whether the CS-period activity exhibited any significant decreases (histograms with the *minus* signs) or significant increases (histograms with the *plus* signs) compared to baseline in any of the last three 49-ms bins for PND17 rats (Figure 3a) and PND24 rats (Figure 3b). The neuronal activity from

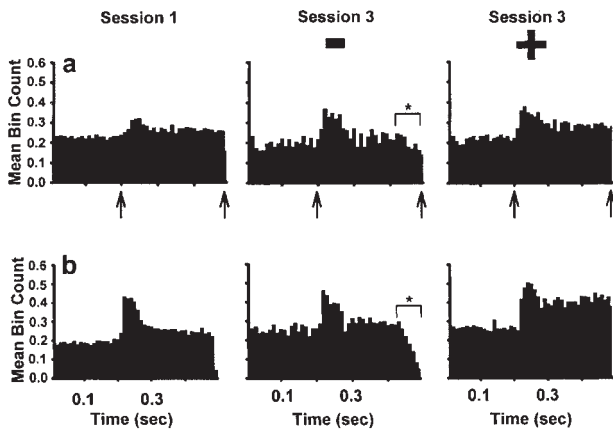


FIGURE 3 Training-related changes in simple spike activity during early stages of conditioning. Mean simple spike activity recorded from PND17 (a) and PND24 (b) rats during paired Sessions 1 and 3. Session-1 histograms represent all units recorded during paired Session 1. Session-3 histograms represent only those units that exhibited pairing-specific decreases (minus sign) or increases (plus sign) in activity in the second half of the CS period. Arrows indicate CS and US onset. The asterisks and brackets in a and b mark the point during the CS at which simple spike activity showed the greatest decrease relative to baseline.

simple spikes that did not exhibit any significant changes in activity in any of the last three 49-ms bins are not represented in the histograms, as they are very similar to the histograms from the unpaired control groups (see Figures 2a–d).

As can be seen in Figure 3, the developmental differences in stimulus-evoked simple spike response magnitude were maintained throughout training (also see Figure 5). The proportion of simple spikes that demonstrated significant CS-period activity increased as a function of training at both ages. However, a higher proportion of simple spikes in PND24 rats (Session 1: 21%, Session 3: 43%, Session 5: 68%) showed activity significantly different (i.e., increases and decreases) from baseline during the CS-period throughout training compared to PND17 rats (Session 1: 9%, Session 3: 17%, Session 5: 29%), $\chi^2 = 152.45$, $p < 0.05$. This suggests that the clustering of training-related modifications in simple spike activity in the second half of the CS period in Sessions 3 and 5 is associative in rats at both ages by the third paired training session, PND17: $\chi^2 = 23.6$, $p < 0.05$; PND24: $\chi^2 = 779.2$, $p < 0.05$. Even though some units from PND17 rats exhibited learning-specific changes in simple spike activity, the magnitudes of the changes in Sessions 3 and 5 were still significantly smaller compared to units from PND24 rats (Age \times Session \times Modification Type interaction, $F(2, 333) = 7.530$, $p < 0.05$). That is, simple spike activity in PND24 rats exhibited more robust

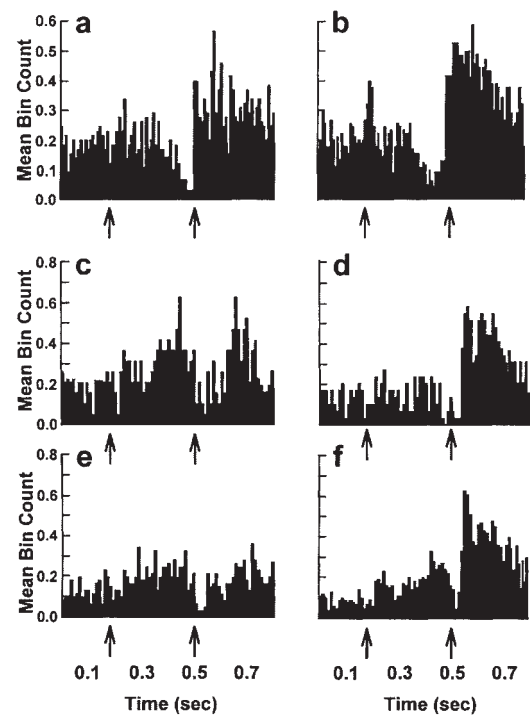


FIGURE 4 Simple spike activity exhibited conditioning- and response-specific changes. Histograms from four different single-unit simple spikes recorded from Purkinje cells in lobule HVI from four different rats that illustrate representative learning-related plasticity in PND24 rats from the paired group during paired Session 5. (a, b) illustrate two different units that exhibited significant decreases during the last 49-ms CS-period bin relative to baseline. (c–f) depict activity of two different single units during trials on which a CR was not (c, d) or was (e, f) displayed. The unit in (c, e) exhibited increased neuronal activity during trials on which no CR was displayed. The unit in (d, f) exhibited increased neuronal activity during trials on which a CR was displayed. Arrows indicate CS and US onset.

decreases and increases than PND17 rats during Sessions 3 and 5.

Figures 4a–f depict simple spike activity from 4 different PND24 rats during paired CS–US training on Session 5, which illustrate some of the general patterns found in the present study. The histograms in Figures 4a,b illustrate the activity of two different units that exhibited significantly less activity than baseline during the last 49-ms CS-period bin. Note that the inhibition appears to be maximal around the time of US onset, at which time the amplitude of eyeblink CRs tends to be at a maximum (Gormezano et al., 1983; Hesslow & Ivarsson, 1994). Figures 4c–f illustrate the activity of two units that exhibited behavior-specific increases in activity. The histograms in Figures 4c,e are from a unit that exhibited greater activity during trials on which no CR occurred (Figure 4c). The histograms in Figures 4d,f are from a

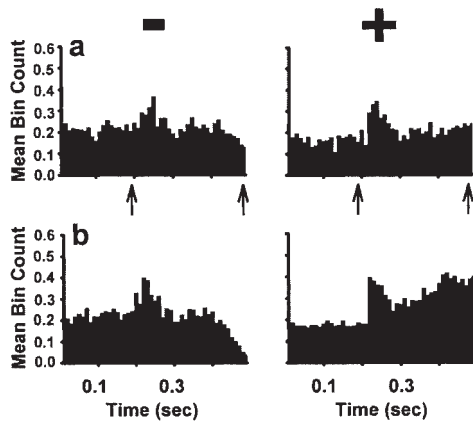


FIGURE 5 Age-related differences in the magnitude of learning-specific increases and decreases in simple spike activity. Mean neuronal activity for simple spikes in the PND17 (a) and PND24 (b) paired group during paired Session-5 trials. Histograms represent the mean activity from units that exhibited pairing-specific decreases (minus sign) or increases (plus sign) in activity relative to baseline. Arrows in a indicate CS and US onset.

unit that exhibited greater activity during trials on which a CR occurred (Figure 4f).

The patterns of simple spike activity during paired Session 5 were grouped into one of two patterns: units that showed a decrease compared to baseline in at least one of the last three 49-ms bins (Figures 5a,b, minus sign) or units that showed only an increase compared to baseline in at least one of the last three 49-ms bins (Figures 5a,b, plus sign). Simple spikes that exhibited only CS-elicited sensory responses (i.e., in the first three 49-ms bins, but not in any of the last three 49-ms bins) are not represented in the histograms. As shown in Figures 5a,b, there were age-related changes in the magnitudes of the increases and decreases in simple spike activity relative to baseline in addition to age-related increases in the proportion of units showing these changes (PND17, increase: 27%, decrease: 9%; PND24, increase: 58%, decrease: 31%). Very few simple spike single units exhibited any change in activity during the last three 49- ms CS-period bins at either age in the unpaired control group, and no simple spike single units from the unpaired control groups exhibited response-specific changes in activity.

Temporal Relationships Between Simple Spike Activity and Eyeblink CRs

Neuronal activity from Purkinje cells and neurons within the cerebellar deep nuclei exhibit increases in firing, time locked to the eyeblink CR (Berthier, Barto, & Moore, 1991; Berthier & Moore, 1990; Freeman & Nicholson, 2000; McCormick & Thompson, 1984), forming a so-called amplitude–time course model of the eyeblink.

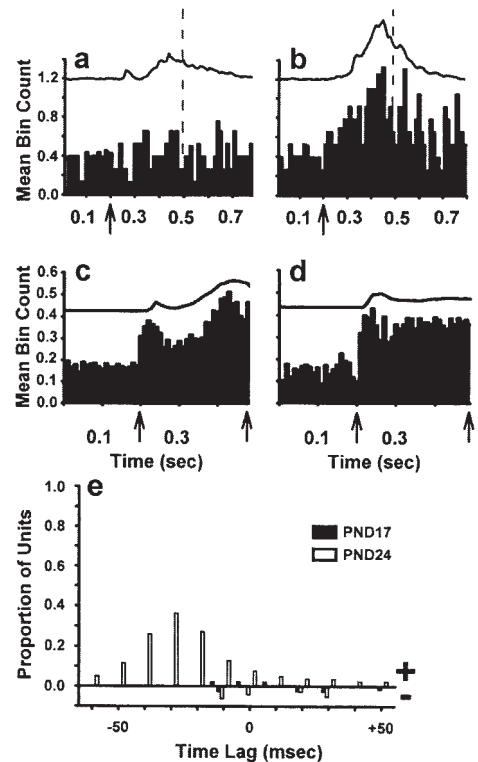


FIGURE 6 Eyeblink conditioned response topography is encoded in simple spike activity. Single-unit histograms of simple spike activity and averaged integrated EMG activity from CS-alone trials on which a CR occurred in a PND17 (a) and PND24 rat (b). Arrows indicate CS onset. The dashed vertical lines indicate the point in time of US onset during paired trials. Mean neuronal activity from all units in a PND24 rat that exhibited different CR topographies (c, d). Superimposed onto the histograms is the averaged integrated EMG activity from the two different CR trials. Note that simple spike activity exhibits changes that model the different CR topographies. Time scale in (c, d) is different than time scale in (a, b). Distribution of the proportions of simple spikes at PND17 (black bars) and PND24 (white bars) that exhibited significant positive (+) or negative (–) cross-correlations with the integrated EMG activity during CR trials (e). Significant correlations at time lags less than zero indicate that changes in simple spike activity preceded changes in integrated EMG activity.

Figures 6a–d depict simple spike single-unit histograms during CR trials from electrode placements that elicited eyelid EMG activity when stimulated. Figure 6a is a histogram of the activity of a single unit in a PND17 rat. Figure 6b is a histogram of the activity of a single unit in a PND24 rat. Superimposed on the unit histograms are the averaged integrated EMG records of the same trials from which the neuronal data were examined. As can be seen, the simple spike activity from the PND24 rat exhibited a more precise amplitude–time course relationship to the eyeblink CR.

In the beginning stages of eyeblink conditioning, rats at both ages (i.e., PND17 and PND24) exhibited two types of CR topographies. The first, and most prevalent topography in later stages of training, was a CR that showed a delayed onset and peaked in amplitude near the time of US onset (e.g., the CRs shown in Figures 6a,b). This type of topography demonstrates the adaptive nature of the eyeblink CR (Gormezano et al., 1983). The second type of topography, which was only transiently present in a subset of rats from any given experiment, showed an early onset, with a low sustained amplitude, showing no clear peak around the time of US onset. This second type of topography was nonadaptively timed, occurred only on ~20 to 30% of CR trials, and disappeared by the last training session. Very few of the responses (<10% of the total) in the unpaired control groups resembled these nonadaptively timed CRs. Figures 6c,d show the mean neuronal activity of nine simple spike single units from a PND24 rat during trials with adaptively timed CRs (Figure 6c) and trials with nonadaptively timed CRs (Figure 6d). Superimposed onto the histograms are the averaged integrated EMG records of the two eyeblink CRs, illustrating their different topographies. Only the simple spikes in the PND24 paired group exhibited a tight amplitude–time course model of both CR topographies. Analysis of archival cerebellar interpositus neuronal activity (Freeman & Nicholson, 2000) during the two types of CR trials indicated similar developmental differences in neural–behavioral specificity in that interpositus unit activity from the PND24 paired group exhibited a much tighter amplitude–time course model of both CR topographies than interpositus unit activity from the PND17 paired group (data not shown).

The temporal relationship between eyeblink CRs and simple spike single-unit activity was further investigated with cross-correlations. The temporal patterns of the spike trains in the present study were analyzed by calculating the cross-correlations of CS-period simple spike activity with the integrated EMG activity of the eyeblink CR. Cross-correlations were calculated only between unit data and averaged integrated EMG records from trials on which a CR was exhibited. Figure 6e shows the proportions of simple spike units that exhibited significant positive (plus sign) or negative (minus sign) cross-correlations with integrated EMG activity at each time lag indicated for PND17 (black bars) and PND24 (white bars) rats during trials on which a CR occurred. A positive cross-correlation indicates that simple spike activity and eyelid EMG activity exhibited parallel changes (e.g., both increased). A negative cross-correlation indicates that simple spike activity and eyelid EMG activity exhibited opposite changes (e.g., simple spike decreased and eyelid EMG activity increased). Very few simple spikes from PND17 rat pups exhibited significant cross-

correlations with the EMG response. In contrast, many simple spikes from PND24 rats exhibited significant cross-correlations with the EMG response. Moreover, the distribution of the proportions of simple spikes showing significant cross-correlations with the EMG response at each time lag indicated that most simple spikes lead the EMG response, phase lead; time lags less than 0: $\chi^2 = 202.075$, $p < 0.01$. No units from the unpaired control groups at either age exhibited significant cross-correlations with the integrated EMG response.

Lobule HVI Stimulation

Stimulation through the recording electrodes of rats at each age (8 PND17 rats; 9 PND24 rats) elicited EMG activity. Figures 7a,b show eight sweeps of overlaid raw EMG activity in response to stimulation of lobule HVI through the recording electrodes in a PND17 (Figure 7a) and a PND24 rat pup (Figure 7b). The stimulation frequency (333 Hz) and amplitudes (100–600 μA) used in the present study were comparable to previous studies (Hesslow, 1994). Figures 7a,b show raw EMG elicited in response to a 54-ms (top) and a 108-ms (bottom) stimulus train. Note that stimulation-evoked EMG activity is time locked to the offset of the stimulation.

A previous study in decerebrate cats demonstrated that regions of the cerebellar cortex that elicit eyelid EMG activity when stimulated exhibit short-latency complex spikes in response to periorbital stimulation (~15 ms; Hesslow, 1994). All simple spike units in the present study were recorded from electrode placements that exhibited evoked complex spike latencies of less than 20 ms for PND24 rats and less than 24 ms for PND17 rats (The longer latency criterion for younger rats was adjusted in light of the ongoing myelination of climbing fibers at this age; Lang & Rosenbluth, 2003.) It is possible, therefore, that the electrodes that elicited eyelid EMG activity when stimulated were placed in homologous regions of the rat cerebellum. However, the additional criterion of unilateral

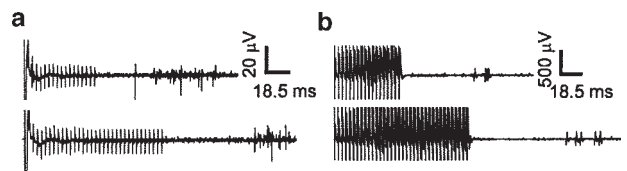


FIGURE 7 Stimulating through the recording electrodes produced eyelid EMG. Representative raw EMG activity evoked by cerebellar cortical stimulation through the recording electrodes in a PND17 (a) and a PND24 (b) rat pup. The time-locked relationship between current offset and EMG activity are depicted for perithreshold current levels for 54-ms (top)- and 108-ms (bottom)-duration stimuli.

receptive fields was not tested, and there was no systematic examination of thresholds in adjacent cortical areas.

Eyelid EMG activity was elicited when current was passed through 35 electrodes. Eyelid and eyeball movements were observed in some rats at higher current levels. There was a total of 56 simple spike single units isolated from these electrodes during the last session of paired training (PND17: $n = 22$, PND24: $n = 34$). The isolated complex spike units from these electrodes had latencies of less than 20 ms. In PND17 rats, 9.1% of the units exhibited decreased activity relative to baseline in at least one 50-ms time epoch during the last half of the CS period, and 22.7% exhibited increased activity relative to baseline in the same epoch. In PND24 rats, 45.2% of the units exhibited significantly less activity than baseline in at least one 50-ms time epoch during the last half of the CS period ms, and 48.6% exhibited significantly more activity than baseline during the same period. In PND24 rats, proportionally more units exhibited decreased activity during the CS period when recorded from eyeblink control regions compared to all placements (31% overall vs. 45.2% from eyeblink control regions; also see Hesslow & Ivarsson, 1994). The proportions did not differ among placements in PND17 rats.

DISCUSSION

Stimulus-evoked and learning-related modifications of Purkinje cell simple spike activity from eye regions within lobule HVI emerged in parallel with eyeblink conditioning between PND17 and PND24. Simple spike activity in the unpaired control groups exhibited a developmental increase in the percentage of responsive units and in the magnitude of the evoked activity in those units throughout unpaired training. During paired CS-US training, the proportion of simple spikes that showed CS-period learning-related increases or decreases in activity increased with age. Moreover, the learning-related changes in simple spike activity from PND24 rats were greater in magnitude than the learning-related changes that occurred in simple spikes from PND17 rats. The time patterns of single-unit activity, in which the eyeblink CR was modeled by simple spike activity, also demonstrated age-related increases in specificity. The developmental differences in stimulus-evoked and learning-related simple spike activity support previous conclusions that neural and behavioral plasticity during eyeblink conditioning depend on functional maturation at more than one site within the eyeblink neural circuit (Freeman & Nicholson, 2000, 2001; Nicholson & Freeman, 2000, 2003).

Previous studies in adult animals demonstrated that neural plasticity within the cerebellar cortex and deep nuclei accompanies or precedes behavioral plasticity

during eyeblink conditioning. The present study extends these findings to rats as young as PND17 (although the changes were substantially more robust at PND24), and demonstrates that stimulus-evoked and learning-related changes in neuronal activity within the cerebellar cortex mature at a similar point in development as stimulus-evoked and learning-related changes in neuronal activity within the cerebellar interpositus nucleus (Freeman & Nicholson, 2000) and inferior olive (Nicholson & Freeman, 2000). Developmental differences in stimulus-evoked and learning-related neuronal activity are correlated ontogenetically with anatomical (e.g., Altman, 1982; Bourrat & Sotelo, 1983; Nicholson & Freeman, 2003) and physiological (e.g., Crepel, 1974; Gardette, Debono, Dupont, & Crepel, 1985; Pettigrew, Crepel, & Krupa, 1988; Puro & Woodward, 1977a, 1977b) maturation within all three structures, which suggests that the ontogeny of the eyeblink CR likely involves maturation of interactions within and between the cerebellum and brainstem.

Despite the developmental changes in synaptic connectivity, cerebellar cortical plasticity was present in rats at both ages. However, the magnitude of the learning-related changes in simple spike activity and the proportion of units exhibiting these learning-related changes were much greater in PND24 rats. This pattern of results is similar to that seen in cerebellar interpositus neurons (Freeman & Nicholson, 2000) and indicates that the basic connections between the cerebellum and brainstem are established by PND17, but that these connections must be elaborated before robust synaptic plasticity can be established and maintained *in vivo*. In support of the presence of basic connections within the eyeblink neural circuit is the finding that cerebellar cortical stimulation elicited eyelid EMG activity in rats at each age, which may indicate the presence of cortical microregions specifically involved in the control of eyelid muscles. It is important to mention, however, that EMG activity was elicited only with relatively intense stimulation parameters (see also Hesslow, 1994), which may mean that the stimulation was not confined to the region near the recording electrodes. Moreover, the present study did not systematically test all of the necessary criteria to establish a region as an eyeblink control region as defined in decerebrate cats (Hesslow, 1994), in part because onset latencies for evoked complex spikes are more variable in unanesthetized animals (Nicholson & Freeman, 2003). Nonetheless, the latencies of US-evoked complex spikes (Nicholson & Freeman, 2003) indicate that the present study recorded simple spikes from Purkinje cells that were monosynaptically connected to neurons in the inferior olive with periorbital receptive fields. Future studies are needed to examine the relationship (if any) between such regions and eyeblink conditioning (e.g., localized permanent or

temporary cortical lesions), especially in regard to eyeblink control regions since the original study characterized these regions only on the surface of the cerebellar cortex (Hesslow, 1994). Given the close relationship between the various forms of plasticity and the topography of the eyeblink CR reported here, however, it is probable that at least some of the unit activity represented information processing specifically related to controlling the eyeblink CR. Moreover, the increases and decreases in simple spike activity may be related to the presence or absence of climbing fiber activity, which influences increases and decreases in parallel fiber–Purkinje cell synaptic strength and is under the control of the cerebellum itself (Andersson et al., 1988; Kim et al., 1998; Nicholson & Freeman, 2003).

The training-related increases in simple spike activity may be related to long-term potentiation (LTP) of parallel fiber synapses onto Purkinje cells, which occurs when parallel fibers are active in the absence of climbing fiber activity (Lev-Ram et al., 2002; Sakurai, 1987). Alternatively, increases in simple spike activity may be influenced by conditioning-specific increases in Purkinje cell membrane excitability (Schreurs et al., 1998). Training-related decreases in simple spike activity may be related to cerebellar long-term depression (LTD), which occurs when parallel fibers are active in the presence of climbing fiber activity (Ito, 1984; Linden & Connor, 1995) and may be related to eyeblink conditioning (Aiba et al., 1994; Albus, 1971; Hesslow & Ivarsson, 1994; Ito, 1984; Medina & Mauk, 1999; Thompson & Krupa, 1994). The emergence of learning-related changes in simple spike activity paralleled age-related increases in stimulus-evoked activity, suggesting that developmental changes in postsynaptic depolarization following presentations of the CS and US could influence the induction of cerebellar plasticity. It is possible, therefore, that developmental differences in the magnitude of learning-related increases and decreases in simple spike activity are influenced by the maturation of parallel and climbing fiber inputs to the Purkinje cells after PND17. Younger rats that exhibited weaker responses to the CS and US may have had postsynaptic responses that were not sufficient for inducing LTP/LTD or increased membrane excitability. Alternatively, the biochemical plasticity mechanisms within cerebellar neurons could be developing in parallel, or as a consequence of, developmental changes in cerebellar afferents.

Recent work indicates that cerebellar-feedback connections to the inferior olive play an important role in the induction and preservation of learning-specific cerebellar plasticity (Kim et al., 1998; Mauk & Donegan, 1997; Medina & Mauk, 1999; Medina et al., 2002; Sears & Steinmetz, 1991). Specifically, cerebellar feedback to the inferior olive regulates climbing fiber activity, which

establishes and maintains learning-related changes in cerebellar neuronal activity. Recently, Mauk and colleagues (Mauk & Donegan, 1997; Medina & Mauk, 1999; Medina et al., 2000; Medina et al., 2002) provided a possible mechanism whereby the deep cerebellar nuclei regulate bidirectional plasticity within Purkinje cells (i.e., LTP and LTD). Activity within the cerebellum (both deep cerebellar nuclei and Purkinje cells) maintains an essential equilibrium between spontaneous/random LTP and LTD such that learning-specific synaptic changes due to the convergence of the CS and US (LTP and LTD) are distinct from random changes and are preserved by zero net change in synaptic strength. It is possible, then, that cerebellar plasticity in PND17 rats is limited not only by weak afferent inputs but also by their inability to regulate climbing fiber activity (Nicholson & Freeman, 2003). The learning-related changes that did occur in PND17 rats were significantly weaker than the learning-related changes at PND24, which may reflect functional immaturity in the ability to establish (Medina et al., 2002) or preserve (Attwell, Cooke, & Yeo, 2002; Medina & Mauk, 1999) learning-specific changes in cerebellar plasticity.

The present results, combined with previous work, suggest that the ontogeny of the eyeblink CR depends on the interactions between and within the cerebellum and interconnected brainstem structures. The developmental emergence of cerebellar plasticity may originate in age-related changes in the cerebellum's ability to regulate its own afferent inputs. Further examination of age-related changes in neuronal activity within the framework of synaptic interactions known to exist in adult animals will not only provide insight into the neural mechanisms of the ontogeny of learning and memory but also may help to elucidate how memories are formed, stored, and forgotten.

NOTES

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REFERENCES

- Aiba, A., Kano, M., Chen, C., Stanton, M. E., Fox, G. D., Herrup, K., Zwingman, T. A., & Tonegawa, S. (1994). Deficient cerebellar long-term depression and impaired motor learning in mGluR1 mutant mice. *Cell*, 79, 377–388.

- Aizenman, C. D., & Linden, D. J. (1999). Regulation of the rebound depolarization and spontaneous firing patterns of deep nuclear neurons in slices of rat cerebellum. *Journal of Neurophysiology*, 82, 1697–1709.
- Albus, J. S. (1971). A theory of cerebellar function. *Mathematical Biosciences*, 10, 25–61.
- Altman, J. (1982). Morphological development of the rat cerebellum and some of its mechanisms. In S. L. Palay & V. Chan-Palay (Eds.), *The cerebellum: New vistas* (pp. 8–49). Berlin: Springer-Verlag.
- Andersson, G., Garwicz, M., & Hesslow, G. (1988). Evidence for a GABA-mediated cerebellar inhibition of the inferior olive in the cat. *Experimental Brain Research*, 72, 450–456.
- Attwell, P. J., Cooke, S. F., & Yeo, C. H. (2002). Cerebellar function in consolidation of a motor memory. *Neuron*, 34, 1011–1020.
- Attwell, P. J., Rahman, S., Ivarsson, M., & Yeo, C. H. (1999). Cerebellar cortical AMPA-kainate receptor blockade prevents performance of classically conditioned nictitating membrane responses. *Journal of Neuroscience*, 19, RC45.
- Attwell, P. J., Rahman, S., & Yeo, C. H. (2001). Acquisition of eye-blink conditioning is critically dependent on normal function in cerebellar cortical lobule HVI. *Journal of Neuroscience*, 21, 5715–5722.
- Bao, S., Chen, L., & Thompson, R. F. (2000). Learning- and cerebellum-dependent neuronal activity in the lateral pontine nucleus. *Behavioral Neuroscience*, 114, 254–261.
- Bell, C. C., & Kawasaki, T. (1972). Relations among climbing fiber responses of nearby Purkinje cells. *Journal of Neurophysiology*, 35, 155–169.
- Berthier, N. E., Barto, A. G., & Moore, J. W. (1991). Linear systems analysis of the relationship between firing of deep cerebellar neurons and the classically conditioned nictitating membrane response in rabbits. *Biological Cybernetics*, 65, 99–105.
- Berthier, N. E., & Moore, J. W. (1986). Cerebellar Purkinje cell activity related to the classically conditioned nictitating membrane response. *Experimental Brain Research*, 63, 341–350.
- Berthier, N. E., & Moore, J. W. (1990). Activity of deep cerebellar nuclear cells during classical conditioning of nictitating membrane extension in rabbits. *Experimental Brain Research*, 83, 44–54.
- Bourrat, F., & Sotelo, C. (1983). Postnatal development of the inferior olivary complex in the rat: I. An electron microscopic study of the medial accessory olive. *Brain Research*, 284, 291–310.
- Bower, J. M., & Kassel, J. (1990). Variability in tactile projection patterns to cerebellar folia crura IIA of the Norway rat. *Journal of Comparative Neurology*, 302, 768–778.
- Bruning, J. L., & Kintz, B. L. (1997). *Computational Handbook of Statistics* (4th ed.). New York: Addison-Wesley.
- Campbell, B. A., & Spear, N. E. (1972). Ontogeny of memory. *Psychological Review*, 79, 215–236.
- Carew, T. J. (1989). Developmental assembly of learning in *Aplysia*. *Trends in Neurosciences*, 12, 389–394.
- Carew, T. J., Menzel, R., & Shatz, C. J. (Eds.). (1998). *Mechanistic relationships between development and learning*. New York: Wiley.
- Crepel, F. (1974). Excitatory and inhibitory processes acting upon cerebellar Purkinje cells during maturation in the rat: Influence of hypothyroidism. *Experimental Brain Research*, 20, 403–420.
- Freeman, J. H. Jr., & Nicholson, D. A. (2000). Developmental changes in eye-blink conditioning and neuronal activity in the cerebellar interpositus nucleus. *Journal of Neuroscience*, 20, 813–819.
- Freeman, J. H. Jr., & Nicholson, D. A. (2001). Ontogenetic changes in the neural mechanisms of eye-blink conditioning. *Integrative Physiological and Behavioral Science*, 36, 15–35.
- Gardette, R., Debono, M., Dupont, J. L., & Crepel, F. (1985). Electrophysiological studies on the postnatal development of intracerebellar nuclei neurons in rat cerebellar slices maintained *in vitro*: I. Postsynaptic potentials. *Developmental Brain Research*, 19, 47–55.
- Gormezano, I., Kehoe, E. J., & Marshall, B. S. (1983). Twenty years of classical conditioning research with the rabbit. *Progress in Psychobiology and Physiological Psychology*, 10, 197–275.
- Gould, T. J., Sears, L. L., & Steinmetz, J. E. (1993). Possible CS and US pathways for rabbit classical eyelid conditioning: Electrophysiological evidence for projections from the pontine nuclei and inferior olive to cerebellar cortex and nuclei. *Behavioral and Neural Biology*, 60, 172–185.
- Gould, T. J., & Steinmetz, J. E. (1994). Multiple-unit activity from rabbit cerebellar cortex and interpositus nucleus during classical discrimination/reversal eyelid conditioning. *Brain Research*, 652, 98–106.
- Gould, T. J., & Steinmetz, J. E. (1996). Changes in rabbit cerebellar cortical and interpositus nucleus activity during acquisition, extinction, and backward classical eyelid conditioning. *Neurobiology of Learning and Memory*, 65, 17–34.
- Hesslow, G. (1994). Correspondence between climbing fibre input and motor output in eye-blink-related areas in cat cerebellar cortex. *Journal of Physiology*, 476, 229–244.
- Hesslow, G., & Ivarsson, M. (1994). Suppression of cerebellar Purkinje cells during conditioned responses in ferrets. *Neuroreport*, 5, 649–652.
- Ito, M. (1984). *The cerebellum and neural control*. New York: Raven.
- Katz, D. B., & Steinmetz, J. E. (1997). Single-unit evidence for eye-blink conditioning in cerebellar cortex is altered, but not eliminated, by interpositus nucleus lesions. *Learning and Memory*, 4, 88–104.
- Katz, D. B., Tracy, J. A., & Steinmetz, J. E. (2001). Rabbit classical eye-blink conditioning is altered by brief cerebellar cortical stimulation. *Physiology & Behavior*, 72, 499–510.
- Kim, J. J., Krupa, D. J., & Thompson, R. F. (1998, January). Inhibitory cerebello-olivary projections and blocking effect in classical conditioning. *Science*, 279, 570–573.
- Lang, E. J., & Rosenbluth, J. (2003). Role of myelination in the development of a uniform olivocerebellar conduction time. *Journal of Neurophysiology*, 89, 2259–2270.
- Lang, E. J., Sugihara, I., & Llinas, R. (1996). GABAergic modulation of complex spike activity by the cerebellar nucleoolivary pathway in rat. *Journal of Neurophysiology*, 76, 255–275.

- Lavond, D. G., & Steinmetz, J. E. (1989). Acquisition of classical conditioning without cerebellar cortex. *Behavioural Brain Research*, 33, 113–164.
- Lev-Ram, V., Wong, S. T., Storm, D. R., & Tsien, R. Y. (2002). A new form of cerebellar long-term potentiation is postsynaptic and depends on nitric oxide but not cAMP. *Proceedings of the National Academy of Sciences, USA*, 99, 8389–8393.
- Linden, D. J., & Connor, J. A. (1995). Long-term synaptic depression. *Annual Review of Neuroscience*, 18, 319–357.
- Llinas, R. (1981). Electrophysiology of cerebellar networks. In V. B. Brooks (Ed.), *Handbook of physiology*, Vol. 2 (pp. 831–876). Bethesda, MD: American Physiological Society.
- Mauk, M. D., & Donegan, N. H. (1997). A model of Pavlovian eyelid conditioning based on the synaptic organization of the cerebellum. *Learning and Memory*, 3, 130–158.
- McCormick, D. A., & Thompson, R. F. (1984). Neuronal responses of the rabbit cerebellum during acquisition and performance of a classically conditioned nictitating membrane-eyelid response. *Journal of Neuroscience*, 4, 2811–2822.
- Medina, J. F., Garcia, K. S., Nores, W. L., Taylor, N. M., & Mauk, M. D. (2000). Timing mechanisms in the cerebellum: Testing predictions of a large-scale computer simulation. *Journal of Neuroscience*, 20, 5516–5525.
- Medina, J. F., & Mauk, M. D. (1999). Simulations of cerebellar motor learning: Computational analysis of plasticity at the mossy fiber to deep nucleus synapse. *Journal of Neuroscience*, 19, 7140–7151.
- Medina, J. F., Nores, W. L., & Mauk, M. D. (2002). Inhibition of climbing fibres is a signal for the extinction of conditioned eyelid responses. *Nature*, 416, 330–333.
- Moore, J. W., & Choi, J. S. (1997). Conditioned response timing and integration in the cerebellum. *Learning and Memory*, 4, 116–129.
- Nicholson, D. A., & Freeman, J. H. Jr. (2000). Developmental changes in eye-blink conditioning and neuronal activity in the inferior olive. *Journal of Neuroscience*, 20, 8218–8226.
- Nicholson, D. A., & Freeman, J. H. Jr. (2003). Addition of inhibition in the olivocerebellar system and the ontogeny of a motor memory. *Nature Neuroscience*, 6, 532–537.
- Pettigrew, A. G., Crepel, F., & Krupa, M. (1988). Development of ionic conductances in neurons of the inferior olive in the rat: An in vitro study. *Proceedings of the Royal Society of London Biological Sciences*, 234, 199–218.
- Puro, D. G., & Woodward, D. J. (1977a). Maturation of evoked climbing fiber input to rat cerebellar Purkinje cells: I. *Experimental Brain Research*, 28, 85–100.
- Puro, D. G., & Woodward, D. J. (1977b). Maturation of evoked mossy fiber input to rat cerebellar Purkinje cells: II. *Experimental Brain Research*, 28, 427–441.
- Sakurai, M. (1987). Synaptic modification of parallel fibre-Purkinje cell transmission in in vitro guinea-pig cerebellar slices. *Journal of Physiology*, 394, 463–480.
- Schreurs, B. G., Gusev, P. A., Tomsic, D., Alkon, D. L., & Shi, T. (1998). Intracellular correlates of acquisition and long-term memory of classical conditioning in Purkinje cell dendrites in slices of rabbit cerebellar lobule HVI. *Journal of Neuroscience*, 18, 5498–5507.
- Sears, L. L., & Steinmetz, J. E. (1991). Dorsal accessory inferior olive activity diminishes during acquisition of the rabbit classically conditioned eyelid response. *Brain Research*, 545, 114–122.
- Skelton, R. W. (1988). Bilateral cerebellar lesions disrupt conditioned eyelid responses in unrestrained rats. *Behavioral Neuroscience*, 102, 586–590.
- Stanton, M. E. (2000). Multiple memory systems, development, and conditioning. *Behavioural Brain Research*, 110, 25–37.
- Stanton, M. E., Freeman, J. H. Jr., & Skelton, R. W. (1992). Eye-blink conditioning in the developing rat. *Behavioral Neuroscience*, 106, 657–665.
- Swain, R. A., Shinkman, P. G., Nordholm, A. F., & Thompson, R. F. (1992). Cerebellar stimulation as an unconditioned stimulus in classical conditioning. *Behavioral Neuroscience*, 106, 739–750.
- Thompson, R. F., & Krupa, D. J. (1994). Organization of memory traces in the mammalian brain. *Annual Review of Neuroscience*, 17, 519–549.
- van Ham, J. J., & Yeo, C. H. (1992). Somatosensory trigeminal projections to the inferior olive, cerebellum and other precerebellar nuclei in rabbits. *European Journal of Neuroscience*, 4, 302–317.
- Welsh, J. P., & Schwartz, C. (1999). Multielectrode recording from the cerebellum. In M. A. L. Nicolelis (Ed.), *Methods for neural ensemble recordings* (pp. 79–100). Boca Raton: CRC Press.
- Yeo, C. H., Hardiman, M. J., & Glickstein, M. (1985). Classical conditioning of the nictitating membrane response of the rabbit: II. Lesions of the cerebellar cortex. *Experimental Brain Research*, 60, 99–113.