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Rasmussen, Anders; Jirenhed, Dan-Anders; Zucca, Riccardo; Johansson, Fredrik; Svensson, Pär; Hesslow, Germund

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Number of Spikes in Climbing Fibers Determines the Direction of Cerebellar Learning

Anders Rasmussen,1 Dan-Anders Jirenhed,1 Riccardo Zucca,1,2 Fredrik Johansson,1 Pär Svensson,1 and Germund Hesslow1

1Associative Learning Group, Department of Experimental Medical Science, Lund University, 22184 Lund, Sweden, 2The Laboratory for Synthetic, Perceptive, Emotive, and Cognitive Systems, Universitat Pompeu Fabra, 138 08018 Barcelona, Spain

Cerebellar learning requires context information from mossy fibers and a teaching signal through the climbing fibers from the inferior olive. Although the inferior olive fires in bursts, virtually all studies have used a teaching signal consisting of a single pulse. Following a number of failed attempts to induce cerebellar learning in decerebrate ferrets with a nonburst signal, we tested the effect of varying the number of pulses in the climbing fiber teaching signal. The results show that training with a single pulse in a conditioning paradigm in vivo does not result in learning, but rather causes extinction of a previously learned response.

Introduction
The prevailing view of cerebellar learning is that climbing fiber (CF) impulses induce synaptic modification in the cerebellar cortex, such as long-term depression (LTD) of parallel fiber to Purkinje cell synapses. A much studied model of cerebellar learning is eyeblink conditioning, where repeated presentations of a neutral conditional stimulus (CS), often a tone, followed by a reflex eliciting unconditional stimulus (US), such as an air puff to the cornea, causes acquisition of a conditioned blink response (CR) (Gormezano et al., 1983). Eyeblink conditioning relies on the cerebellum (McCormick and Thompson, 1984), and acquisition of adaptively timed CRs requires the cerebellar cortex (Yeo et al., 1984; Hesslow and Yeo, 2002). During conditioning, Purkinje cells develop adaptively timed pause responses, “Purkinje cell CRs,” thought to be the physiological basis of learned eyeblinks (Jirenhed et al., 2007; Jirenhed and Hesslow, 2011a). Results from microstimulation experiments suggest that Purkinje cells receive information about the CS via the mossy/parallel fiber pathway (Steinmetz et al., 1985; Hesslow et al., 1999), and information about the US via the CFs (Mauk et al., 1986; Jirenhed et al., 2007).

Purkinje cell CRs can disinhibit the cerebellar nuclei and, in extension, generate motor output (Hesslow and Yeo, 2002). In addition, disinhibition of the cerebellar nuclei causes increased inhibition of the inferior olive (IO), via the nucleo-olivary pathway (Bengtsson and Hesslow, 2006). Thus, the Purkinje cell CR can potentially inhibit the IO and the US signal (Rasmussen et al., 2008).

Cells in the IO fire in high-frequency bursts (>250 Hz), typically consisting of one to six spikes (Armstrong and Rawson, 1979; Maruta et al., 2007; Mathy et al., 2009). Each spike elicits a corresponding EPSP in the Purkinje cell dendrite, but only a single complex spike will appear in extracellular recordings. Thus, additional spikes do not result in extra complex spikes, although they alter the appearance and duration of the complex spike (Mathy et al., 2009).

The burst nature of the IO response was discovered decades ago (Eccles et al., 1966), but virtually all research on cerebellar learning, in which climbing fibers were stimulated, has used single pulses as teaching signals, assuming they are of an “all-or-none” nature (Ito, 2001). Recently, the nature of the CF signal has received renewed interest and the bursting nature of the IO response has been reaffirmed several times (Maruta et al., 2007; Mathy et al., 2009).

Here we tested the effect of varying the number of climbing fiber stimulus pulses on the acquisition and extinction of Purkinje cell CRs. A burst-like stimulus reliably results in acquisition of Purkinje cell CRs. Here we show that single climbing fiber impulses do not result in learning, but actually cause extinction of previously acquired Purkinje cell CRs.

Materials and Methods
Surgery: Thirteen male ferrets were anesthetized in a mixture of O2 and air with 1.5–2% isoflurane (Baxter Medical), which was subsequently replaced intravenously by propofol. During anesthesia, a tracheotomy was performed, and the gas was led directly into a tracheal tube. The end expiratory CO2 concentration, arterial blood pressure, and rectal temperature were monitored and kept within physiological limits throughout the experiment. During the whole experiment, infusion was given intravenously [50 mg/ml glucose and isotonic acetate Kinger’s solution (proportion, 1:1) with 0.004 mg/ml albumin fraction V (from bovine serum; Sigma-Aldrich), 2 mg/kg/h]. After fixation of the head in a stereotaxic frame, the skull was opened on the left side, and the caudal half of the left cerebral hemisphere, together with a substantial part of the...
 impulses at 500 Hz, applied to the ipsilateral climbing fibers (15–90 μA). Before switching to a single pulse was made, the US consisted of two 10 ms impulses at 500 Hz and then to three impulses so that we could establish the threshold at which the climbing fiber signal induces learning. If at any point a cell was lost, we searched for another one. If another cell had to satisfy the following criteria: (1) confirmed periorbital climbing fiber input with a latency of <15 ms, (2) confirmed climbing fiber input from the inferior cerebellar peduncle with a latency of ~2 ms, and (3) sufficient recording stability to demonstrate a change in the response to CS during training. Intracellular Purkinje cell recordings (n = 3) were performed using pulled micropipettes filled with KNO3 (impedance, 10–20 MΩ). Recorded electrical signals were passed unfiltered through an amplifier and a Power1401 data acquisition analog-to-digital converter unit (Cambridge Electronics Design). The sampling rate was 40 kHz.

Data analysis. All spike sorting was performed using Spike2, version 7 (Cambridge Electronics Design), and subsequent data analysis was performed in custom Matlab software (MathWorks). To avoid misclassifying naturally occurring decreases in simple spike activity as Purkinje cell CRs, all analysis was based on normalized simple spike firing during the CS period. To calculate the normalized simple spike firing, we divided the simple spike frequency within the CS period with the frequency in the 600 ms before CS onset. Since reextinction in general required fewer trials than extinction, we also normalized the number of paired trials that had been given; that is, instead of using the absolute number of paired presentations, we looked at the percentage of completed training of the task before that was completed. To test whether the change in simple spike firing following training with a single climbing fiber impulse was statistically significant, we used a nonparametric Wilcoxon matched-pairs signed rank test.

Results

The decerebrate ferret preparation (Fig. 1A) has several advantages when studying the mechanisms of eyelink conditioning. Though the cell recordings presented here are from paralyzed animals, decerebrate ferrets can acquire conditioned eyelinks (Hesslow et al., 1999; Hesslow and Yeo, 2002), meaning that the

Figure 1. Experimental setup, field recordings, and intracellular recordings. A, Illustration of the experimental setup including relevant afferent and efferent cerebellar pathways. As the CS, we stimulated the forelimb, mossy fiber (MF), or superior colliculus (SC). As the US, we stimulated SFs, DN, Deep nuclei; GC, granule cell; NO, nucleo-olivary; PC, Purkinje cell. B, Typical examples of field potentials elicited on the cerebellar cortex following stimulation of cerebellar afferents. C, Representative sweeps from intracellular recordings showing that the number of elicited EPSPs in Purkinje cell dendrites corresponds to the number of stimulus pulses applied to the climbing fibers, and that peripheral, periorbital stimulation elicits multiple EPSPs.
neural network responsible for this learning remains intact. The relative stability of the setup allows for long single-unit recordings, which are necessary to follow the acquisition or extinction of Purkinje cell CRs. In addition, anesthesia can be excluded.

When we first tested IO/CF stimulation as a teaching signal, we used a protocol analogous to that which induces learning with a periorbital US (three pulses; 100–300 μA; 20 ms pulse interval, 50 Hz; 0.1 ms pulse duration). Although we always verified that CF stimulation elicited complex spikes in the Purkinje cell, this training protocol, with a nonburst CF stimulus, failed to induce any Purkinje cell CRs in nine consecutive experiments. To ensure that single CF pulses were indeed transmitted to the Purkinje cells and that these results not merely reflected insufficient signal strength, we used micropipettes to record EPSPs in Purkinje cell dendrites following one, two, or three CF stimulus pulses. The results confirmed that the number of EPSPs elicited corresponded to the number of CF stimulus pulses applied (Fig. 1C), which is also consistent with previous research (Mathy et al., 2009).

After these nine failed attempts to obtain acquisition of Purkinje cell CRs, we switched to a teaching signal that more resembled normal IO firing (Eccles et al., 1966; Maruta et al., 2007; Mathy et al., 2009), namely, two 500 Hz bursts with five stimuli in each. This reliably resulted in acquisition of Purkinje cell CRs (Jirenheds et al., 2007). Subsequently, nearly all Purkinje cells (more than 100 cells), provided they had input from the relevant climbing fibers and mossy/parallel fibers, acquired Purkinje cell CRs when using climbing fiber burst stimulation as the US.

Motivated by the observation that two sets of five CF pulses at 500 Hz resulted in learning, whereas three pulses at 50 Hz did not, we designed an experiment to test how the number of CF pulses in the US affects the acquisition and extinction of Purkinje cell CRs. Animals were conditioned with a US consisting of two sets of five pulses at 500 Hz (200–700 μA), delivered to the CF, paired with a CS consisting of a 300 ms pulse train at 50 Hz, delivered to the ipsilateral forelimb (n = 8; 0.6–1.5 mA; 1 ms), mossy fibers (n = 3; 100 μA; 0.1 ms), or superior colliculus (n = 1; 150 μA; 0.1 ms). When a Purkinje cell CR had been acquired, we switched from a burst to a single-pulse climbing fiber stimulus. Other variables were kept constant.

Fifteen sessions from 13 cells were recorded using a single-pulse CF stimulus after Purkinje cell CRs had been acquired. On three occasions, the recording was sufficiently long lasting to allow us to later switch back to a burst US, and then to a single pulse again. In 12 of 15 sessions, training with a single pulse CF stimulus resulted in extinction of the Purkinje cell CR (an increase in simple spike firing during the CS; Fig. 2). In two sessions there was no change in simple spike activity, and in one session there was a decrease in simple spike activity. The median change in simple spike activity was 41%, ranging from −17 to 116% (Fig. 2F). A Wilcoxon matched-pairs signed rank test confirmed that the simple spike frequency increased significantly during training with a single pulse (W = 104; p = 0.0015). Consistent with previous findings (Jirenheds et al., 2007; Jirenheds and Hesslow, 2011a,b), and with behavior (Gormezano et al., 1983), the de-
Occasionally, climbing fiber stimulation elicited simple spikes, possibly because of antidromic activation of mossy fiber collaterals. However, the rate of acquisition appears to be independent of this increase in simple spikes. Burst activation of climbing fibers or the inferior olive consistently results in Purkinje cell CRs, even if no simple spikes are elicited (Jirenheds et al., 2007). It is therefore unlikely that this increase in simple spike firing affected the present results.

Discussion

We have demonstrated that a US, or teaching signal, consisting of a single climbing fiber stimulus causes extinction, not acquisition, of a Purkinje cell CR. This was not because the stimulus failed to activate the climbing fibers since we always confirmed that the CF stimulus elicited a complex spike in the Purkinje cell. These results challenge the interpretation of research on cerebellar learning where an all-or-none teaching signal has been assumed (Ito, 2001).

For example, several studies have suggested that LTD, which is induced by pairing parallel fiber stimulation with CF stimulation, is the synaptic mechanism responsible for classical conditioning (Ito, 2001; Koekkoek et al., 2003). However, a single CF impulse, which has been shown to be sufficient for LTD induction (Ito et al., 1982; Ekerot and Kano, 1985; Ito, 2001), actually causes extinction of Purkinje cell CRs. These results, together with additional physiological evidence (Hesslow et al., 2013) as well as evidence from transgenic mice (Schonewille et al., 2011), suggest that LTD is not the critical mechanism for conditioning, although it is still possible that LTP plays an important role (Schonewille et al., 2010). In contrast to the LTD studies cited above, Mathy et al. (2009) observed potentiation of the parallel fiber–Purkinje cell synapse when pairing parallel fiber stimulation with a single CF impulse. Only when a CF burst was used did they get LTD. These recent discoveries, and the results presented in the present paper, raise some intriguing questions concerning the plastic changes underlying cerebellar learning. To what extent are depression and potentiation of the parallel fiber–Purkinje cell synapses responsible for the acquisition of Purkinje cell CRs, and for cerebellar learning in general? If depression and potentiation, which are induced within minutes of applying the stimulation protocol, are the underlying mechanisms of cerebellar learning, then why does acquisition of conditioned eyeblinks and Purkinje cell CRs take several hours? How exactly does the number of spikes in the climbing fiber signal influence the type of plasticity induced in the cerebellar cortex? These important questions should be addressed in future studies.

The reason that single CF impulses are insufficient to induce cerebellar learning may have to do with the fact that the IO serves multiple purposes. One function of the IO, besides relaying the teaching signal, is to regulate background activity in the cerebellar circuit (Cerminara and Rawson, 2004; Bengtsson and Hesslow, 2013). Interfering with the IO’s normal firing pattern has striking effects on cortical activity and, consequently, on cerebellar output (Bengtsson et al., 2004; Zbarska et al., 2007). Thus, to maintain normal functioning of the cerebellar network, it may be advantageous if modulation of learning only requires slight alterations of IO activity.

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al., 2008) may be able to push the strength of the US signal below the learning threshold. This would allow the cerebellar circuit to reach an equilibrium where the negative feedback from the Purkinje cell CR matches the strength of the teaching signal, thus preventing runaway synaptic plasticity in the cortex (Lepora et al., 2010). It would also explain why lowering the US intensity can lead to extinction of overt CRs (Kehoe and White, 2002). As long as the negative feedback generated by the Purkinje cell CR matches the strength of the US signal, neither acquisition nor extinction ought to occur. However, if the US intensity is reduced, the Purkinje cell CR could suppress the US signal below the learning threshold, causing extinction.

Placing our results in a broader context invites comparisons with learning in the hippocampus, where it has been known for a long time that opposite forms of plasticity can be induced by slight alterations of stimulus parameters (Malenka and Bear, 2004). In a similar manner, and consistent with the nonlinear relationship between cerebellar plasticity and calcium release in Purkinje cells (Vogt and Canepari, 2010), our results demonstrate that the exact stimulus parameters used determines whether you get acquisition or extinction of the Purkinje cell CR.

References