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A Mechanism for Extinction of Classically Conditioned Forelimb Movement in Mice

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A Circuit Mechanism

For Extinction of Classically Conditioned

Forelimb Movements in Mice

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Neuroscience

by

Alexander Michael Brian Reeves

2016

ABSTRACT OF THE DISSERTATION

A Circuit Mechanism

For Classically Conditioned Forelimb Movements

In Mice

by

Alexander Michael Brian Reeves

Doctor of Philosophy in Neuroscience

University of California, Los Angeles, 2016

Professor Thomas Stephen Otis, Chair

The cerebellum has long been hypothesized to be involved in the storage and expression of motor memories. Work presented in this thesis demonstrates that optogenetically conditioned motor memories are stored in the cortical and nuclear regions of the cerebellum and expressed via disinhibition-mediated bursting of the cerebellar nuclei. Building on this observation we sought to determine the impact of preventing disinhibition of the cerebellar nuclei when cueing an optogenetically conditioned forelimb movement. By transiently increasing inhibition of cerebellar nuclei during high-speed video recordings of mice responding to a previously conditioned auditory cue, we demonstrated that preventing disinhibition of cerebellar nuclei

prevents the expression of the learned forelimb movements. We conclude that disinhibition-mediated bursting of the cerebellar nuclei is necessary for retrieving the motor memory and the subsequent forelimb movement. Furthermore, this mechanism could be relevant for the expression of other kinds of motor memories.

The dissertation of Alexander Michael Brian Reeves is approved.

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2016

To Mom.

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Lang, E.J., Apps, R., Bengtsson, F. et al. (2016). The Roles of the Olivocerebellar Pathway in Motor Learning and Motor Control. A Consensus Paper. *Cerebellum* **doi:10.1007/s12311-016-0787-8**

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AMB Reeves, KH Lee, P Mathews, TS Otis. (2014) Facilitation and Suppression of an Optogenetically-induced Motor Memory *UCLA Neural Microcircuits Symposium 2014.*

KH Lee, P Mathews, AMB Reeves, RE Serrano, TS Otis. (2013) Classical Delayed conditioning of forelimb movements in Archaerhodopsin-1 mice using optogenetic approaches. *Gordon Research Conference on Cerebellum, August 2013; SfN, 2013.*

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AMB Reeves, E Shigetomi, BS Khakh. (2011) Bulk loading of calcium indicator dyes to study astrocyte physiology: key limitations and improvements using morphological maps. *Society for Neuroscience, November 2011; UCLA BRI Neuroscience Poster Day, November 2011.*

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CHAPTER I: INTRODUCTION

BRIEF HISTORY

Purkinje cells were discovered just a few years before Schwann proposed his Cell Doctrine. Although it is tempting to call Jan Purkinje's description of the cells which bear his name the first piece of evidence in support of the Neuron Doctrine, this would be stretching the truth. In reality, anatomists at the time were still debating whether neurons in the brain stood as exceptions to the Cell Doctrine. With their long, elaborate axons and dendrites, some anatomists proposed that neurons formed a continuous, interconnected reticulum. Other anatomists thought that the neurofibrils were not continuous with each other but merely contacted one another contiguously. Because of microscopy resolution limits at the time, the neurofibrils could not be observed at a sufficient level of detail to conclude whether the nervous system was a continuum or contiguum of neural tissue.

It wasn't until Ramon y Cajal employed the Golgi stain to survey the brain's cytoarchitecture that it became clear that cells—in particular, neurons—were the fundamental building block of the brain. In the years that followed, neuroanatomists agreed that the nervous system was indeed a contiguum of cellular tissue, and that neurons contacted each other but did not share cytoplasm.

One of the most important contributions to cerebellar research was Sir John Eccles' *The Cerebellum as a Neuronal Machine (Eccles et al., 1967)*. His book compiled three years of neurophysiological research across three different labs and gave the interested scientist a convenient resource for understanding cerebellar anatomy, physiology, and function. It was published in 1967, just in time for a young David Marr to help him answer a question his Ph.D. advisor, Giles Brindley, had posed him.

In 1964, Giles Brindley gave a lecture where he proposed an explanation for why the cerebellum contains so many granule cells (Brindley, 1964). In essence, he proposed that the

cerebellum is a principal agent of learning motor skills. He thought that the cerebrum recruited the cerebellum in order to automate its motor commands, so that a relatively simple or incomplete command from the cerebrum would be elaborated and completed by the cerebellum after motor learning. Further, he proposed that the cerebellum is a site of learning because it contains many neurons and synapses, most of which are probably modifiable, and that absent plasticity, one wouldn't need so many neurons to merely elaborate forebrain motor commands in some routine, unchanging way. This notion of treating the immense number of neurons in the cerebellum—in particular, the granule cells—as a reservoir of modifiable elements continues to permeate theories of cerebellar cortex to this day.

Following this lecture, Giles began working out the computational features of modifiable synapses in order to explain the basic properties of classical and operant conditioning (Brindley, 1969). Between the work of behaviorists like Pavlov (Pavlov, 1951) and Gormezano (Schneiderman and Gormezano, 1964) to establish the features of associative learning, Giles' theories of neural learning rules, and Eccles' recently-published compendium of cerebellar anatomy and neurophysiology, the stage was set for young David Marr to tackle the problem of how exactly the cerebellum leverages its huge reservoir of granule cells to learn motor skills.

In hindsight, we can see how this particular problem may have influenced his later philosophical approach to neuroscientific hypotheses (Marr, 1976) where he advocated understanding an information processing system at different but intersecting levels of analysis. At a basic level, behaviorists beginning with Pavlov had identified that the computational problem of motor skill learning is to associate an old behavior with a novel stimulus because those stimuli help the organism predict how to behave in changing environments. At an algorithmic level, Marr's advisor Giles had identified the rules by which associative learning could conceivably occur within a neural network. It was up to Marr to specify how these learning rules were implemented in an actual neural microcircuit to explain how associative motor

learning might happen in the cerebellum, using the connectivity and physiology established by Eccles' program of research.

Marr began with the observation that the Purkinje cell receives two very different types of excitatory input (Marr, 1969). One type of excitatory input comes from the axons of the granule cells called the parallel fibers, with each Purkinje cell having about 200,000 parallel fiber inputs, The other type of excitatory input comes from the axons of inferior olivary cells called the climbing fibers, each Purkinje cell receiving only a single, powerful climbing fiber input. Thus, Marr noted a conspicuous dichotomy between the huge number of parallel fibers and the single, large climbing fiber impinging on Purkinje Cells.

Marr's next insight was that Purkinje cells could learn to modify parallel fiber input in order to substitute for and recreate the activity induced by the climbing fiber. He proposed that the cue for the modification of parallel fiber inputs is that the relevant climbing fiber is active at the same time. He assumed that climbing fiber inputs represented an instruction from cerebral cortex whereas mossy fibers represented the current state of the organism and the environmental context the organism is in. (Actually, his use of the term "context" is somewhat vague in the original paper.) Consequently, Purkinje cells gradually learn to instruct movement independently of cerebral commands originally relayed by the climbing fibers.

Furthermore, Marr proposed that a sequence of movements can be learned if the Purkinje cells can recognize a sequence of contexts, with each movement eliciting the next context in the sequence. He showed that because the mossy fiber-granule cell-Purkinje cell arrangement essentially operates as a pattern separator, and because there are a large number of granule cell inputs impinging on a given Purkinje cell, each Purkinje cell could conceivably recognize about 200 different contexts.

Marr published his theory of cerebellar cortex in 1969. Just two years later, James Albus published a similar theory of cerebellar cortex (Albus, 1971), independently of David Marr's proposal but which agreed with Marr in several important ways. To take a few examples, they agreed that the mossy fiber-granule cell-Purkinje cell arrangement effectively acts as a pattern-recognition device, they also agreed that the climbing fiber modifies co-active parallel fiber inputs, and they even agreed that parallel fibers can substitute for and re-create the climbing fiber-evoked Purkinje cell activity.

However, Marr and Albus disagreed in some significant ways, particularly with respect to the modifications parallel fiber synapses underwent when paired with climbing fiber activity. Marr's main prediction was that parallel fiber-to-Purkinje cell synapses were strengthened during learning; in contrast, Albus postulated that the parallel fiber-to-Purkinje cell synapses would weaken (he also proposed other sites of plasticity). Albus defended his synaptic weakening argument by pointing out that the parallel fibers should be trained to emulate the post-complex spike pause and not the excitatory phase of the climbing fiber-induced complex spike, as Marr suggested. In order to emulate the post-complex spike pause, the parallel fiber synapses should weaken, and so the crucial mechanism for Albus' theory was a weakening of the parallel fiber synapse. Thus, although they agreed that the parallel fiber-to-Purkinje cell synapse was modifiable, they disagreed over the direction of change most relevant for storing motor memories in cerebellar cortex.

It wasn't until Masao Ito, a co-author of Eccles' compendium of cerebellar physiology, attempted the necessary experiments (Ito and Kano, 1982) that the issue of whether co-activation of parallel and climbing fibers led to facilitation or depression of synaptic strength could be tested directly. He conjunctively stimulated the parallel fibers and climbing fibers with microelectrodes in decerebrate rabbit and found that the parallel fibers had depressed their ability to excite the Purkinje cell. On the one hand, this validated both Marr's and Albus' theories

in that the parallel fibers modified their strength when co-active with the climbing fiber. On the other hand, Albus had correctly guessed that the plasticity would be depressive rather than facilitative, as Marr had guessed. It would have been remiss not to give Marr due credit for being the first to publish a full-fledged theory of cerebellar cortex. In consideration of the important contributions from each of Marr, Albus, and Ito, the notion that the cerebellar cortex is capable of storing motor memories is now known as the Marr-Albus-Ito theory of cerebellar cortex.

Further evidence in support of the Marr-Albus-Ito theory came from the laboratory of Richard Thompson in the early 1980s (McCormick et al., 1982; McCormick and Thompson, 1984). He and his colleagues had conducted a series of experiments investigating the neural basis of classically conditioned eyeblinks in rabbits by systematically recording from and then lesioning various brain structures before and after conditioning. Their goal was to discover brain locations whose activity correlated with the conditioned eyeblinks. Then, they would examine the effect of lesioning this location on the rabbit's performance of conditioned eyeblinks versus reflexive eyeblinks. Using this particularly tractable experimental preparation, they found an associative motor memory "engram" located in the cerebellum. This was a milestone in the biology of psychology because previous engram localization studies had failed.

After further investigation, Thompson refined the location of his hypothesized engram for conditioned blinking. He claimed that the essential brain structure was the interpositus nucleus and not it's associated, presynaptic cerebellar cortex. His results suggested that the while the cortex was not required for the response, it might be required for the amplitude time-course of the blink.

This led a disciple of Thompson, Michael Mauk, to begin an independent scientific project whose chief aim was to explain how the cerebellar cortex could be responsible for the

timing of the blink response (Mauk and Donegan, 1997). He, like Marr and Albus before him, saw the granule cell layer as a reservoir of neuronal elements that could be used to achieve a spectrum of different responses. His interpretation of the granule cell layer was that it not only encoded the context in which a response occurred, but also that it varied its activity across the duration of a stimulus. Furthermore, Mauk predicted that the time-evolution of the granule cell activity would be consistent for a given stimulus and duration even if it were presented at different occasions. He suggested that this consistent, time-varying pattern of activity in the granule cell layer is exploited by Purkinje cells to predict the interval between the onset of a salient cue that reliably evoked granule cell activity and the onset of a stimulus that reliably evoked a climbing fiber discharge. Thus, his answer to how the cerebellar cortex could be responsible for the timing of the blink response was that it leveraged the huge number of granule cells to encode and store the duration of biologically relevant stimuli.

During the same period, just east of Lomma Bay at Lund University, Germund Hesslow was performing experiments that suggested the Purkinje cells act as a gate on the expression of associative motor memories formed in the cerebellum. By electrically stimulating the cerebellar cortex in a putative eyeblink region just before a conditioned blink would normally happen, he could reliably prevent conditioned eyeblink responses (Hesslow, 1994). Since Purkinje cells are the sole output of the cerebellar cortex, Hesslow reckoned that Purkinje cell activation mediated the absence of the conditioned response, and suggested a suppression mechanism. A decade later, Hesslow's group demonstrated that direct co-activation of climbing and parallel fibers *in vivo* led to both to transient pauses in Purkinje cell activity as well as conditioned eyeblinks (Jirenhed et al., 2007). This was compelling evidence that Purkinje cells exhibit neural correlates of classical conditioning that resembles the climbing fiber-elicited pause, as Albus had predicted 35 years earlier. It also further corroborated the notion that Purkinje cells act as a gate on memories and that pauses in activity "opened" the gate.

CEREBELLUM ORGANIZATION

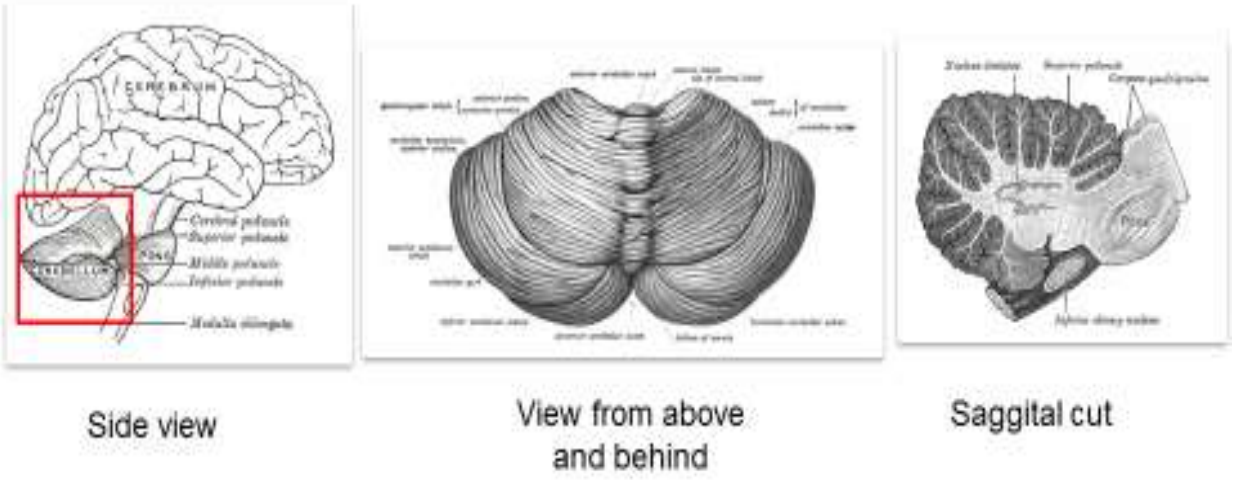

Figure I-1. Cartoons of the cerebellar anatomy adapted from Gray's Anatomy images.

If you were to reach behind yourself and grab the back of your head where the base of your skull meets your neck, your hand would be right above your cerebellum, assuming you haven't lost it to disease or that you never developed one due to congenital defect. Because you cannot see your hand reaching behind your head, you would have to rely on proprioceptive feedback from the reaching movement to guide your hand to the target. This action would make heavy use of your cerebellum, because it is chiefly involved in coordinating and sensing movement.

The cerebellum is a highly organized brain structure from gross anatomy all the way down to its individual cells. At a macroscopic level, along the medial-lateral axis, the cerebellum divides into two hemispheres of tissue with a thin worm-like region called the vermis situated in the middle between the two hemispheres. The hemispheres have ipsilateral motor control such that the left hemisphere has control over the left side of the body and the right hemisphere has

control over the right side of the body. Along the anterior-posterior axis, the cerebellum divides into a tri-lobed structure with the anterior, posterior, and flocullo-nodular lobes, in that order.

A cut along the sagittal plane reveals the cerebellum's "arbor vitae", or "tree of life." With this perspective, one can see a highly convoluted cortex at the outer-most layers and large tracts of white matter at the innermost layers. The white matter is a mixture of afferent axons originating from extra-cerebellar regions such as the pons and spinal cord and efferent axons originating from the cortex and the deep cerebellar nuclei. The deep cerebellar nuclei are clusters of cell bodies and are the main output of the cerebellum. They project rostrally with excitatory axons towards the red nucleus and the thalamus. They also project ventrally with inhibitory axons towards the inferior olive.

MICROCIRCUITRY OF THE CEREBELLUM

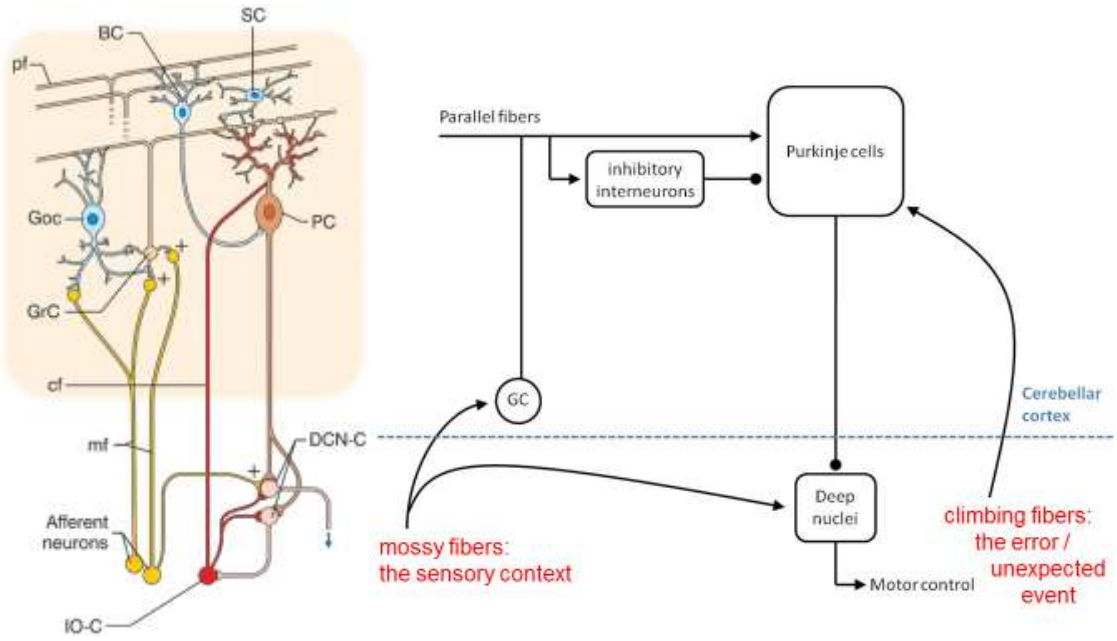

Figure I-2. (Left panel) Cartoon of the cerebellar microcircuit. Cerebellar cortex is delineated with the light pink shading. Mossy fibers (mf) make excitatory contact with Golgi cells (GoC) and Granule cells (GrC). Granule cells make excitatory contact via parallel fibers (pf) with Golgi cells, Basket cells (BC), Stellate cells (SC), and Purkinje cells (PC). Purkinje cells also receive powerful excitatory input from the climbing fiber (cf) whose cell bodies of origin lie in the inferior olive (IO). The deep cerebellar nuclei (DCN) comprise the output of the cerebellum and they receive powerful inhibitory input from the Purkinje cells as well as excitatory collaterals from the mossy and climbing fibers. From (D'Angelo and Casali, 2012). (Right panel) Simplified schematic of the same cerebellar microcircuit. Excitatory inputs are arrows and inhibitory inputs are filled circles.

At a microscopic level, the roughly *50 billion* neurons in the cerebellum consist of a small number of cell types connected in a highly stereotyped feed-forward circuit. >99% of the cells in the cerebellum are granule cells. In fact, cerebellar granule cells comprise two-thirds of the neurons in the entire brain! The granule cells are bunched together in a distinct layer of cerebellar cortex known as the granule cell layer. Also scattered throughout the granule cell layer are the inhibitory interneurons, the Golgi cells. Both granule cells and Golgi cells receive their inputs from the excitatory mossy fibers. Mossy fibers number roughly 200 million and convey diverse exteroceptive and proprioceptive information from spinal cord, brainstem, and

cerebral cortex. Therefore, granule cells and Golgi cells relay and convey diverse sensory information to their target neurons.

The Golgi cells make local inhibitory synapses with granule cells, and thus are capable of both feed-forward inhibition, via the mossy fiber—Golgi cell—granule cell connection, and feed-back inhibition, via the granule cell—Golgi cell—granule cell connection.

Each granule cell projects a single, ascending excitatory axon into the molecular layer. This projection bifurcates at a right angle, with each bifurcation continuing in opposite directions for several millimeters longitudinally and parallel to the granule cell layer. These bifurcations are the parallel fibers.

Parallel fibers synapse *en passant* with the dense forest of dendritic arbors within the molecular layer of the cerebellar cortex. Most of the molecular layer consists of the orthogonal intersection of parallel fibers with the flat dendritic arbors of the Purkinje cells. Each Purkinje cell receives about 200,000 parallel fiber inputs and each parallel fiber contacts about 100 Purkinje cells. However, in addition to parallel fiber-to-Purkinje cell synapses, the molecular layer also contains climbing fibers, Golgi cell dendrites, and two types of interneurons: the basket cells and the stellate cells. Unlike the Golgi cell, the basket and stellate cells are not capable of feedback inhibition because they do not project to their pre-synaptic partner, the granule cell. Instead, these cells exhibit lateral inhibition via stellate cell—stellate cell connections and feed-forward inhibition via the granule cell—molecular layer interneuron—Purkinje cell connection.

In contrast to the immense number of parallel fiber inputs, each Purkinje cell receives only a single climbing fiber, albeit a very powerful one—the climbing fiber is one of the most powerful excitatory synapses in the CNS. This conspicuous dichotomy between the single-yet-powerful climbing fiber input and the numerous-yet-weak parallel fiber inputs impinging on each Purkinje cell has inspired much speculation as to the function of such an odd pair of inputs. It is

widely hypothesized that climbing fibers convey a prediction error to Purkinje cells, and the Purkinje cells use the diverse sensory information conveyed to them by the numerous parallel fiber inputs in order to correct this prediction error.

Because the Purkinje cells are the sole output of the cerebellar cortex, an explanation for the function of the Purkinje cell layer would constitute an explanation for the function of the entire cerebellar cortex. Since this would in turn provide an explanation for the parallel fibers and their cell bodies of origin, the granule cells, it follows that an explanation for what Purkinje cells compute for the rest of the nervous system would constitute an explanation for nearly 2/3 of the neurons in the brain! In terms of the sheer number of cells such an explanation would account for, a hypothesis for Purkinje cell function is a worthy goal for neuroscientists.

The highly stereotyped cerebellar microcircuit repeats millions of times throughout the cerebellum. This has inspired a modular or parallel theory of cerebellar function, whereby the different regions of the cerebellum perform the same general neural computation on their respective inputs before generating their output. Decades of research have led to sophisticated and testable hypotheses regarding the neural mechanisms by which this microcircuit coordinates motor behavior.

CEREBELLAR PLASTICITY

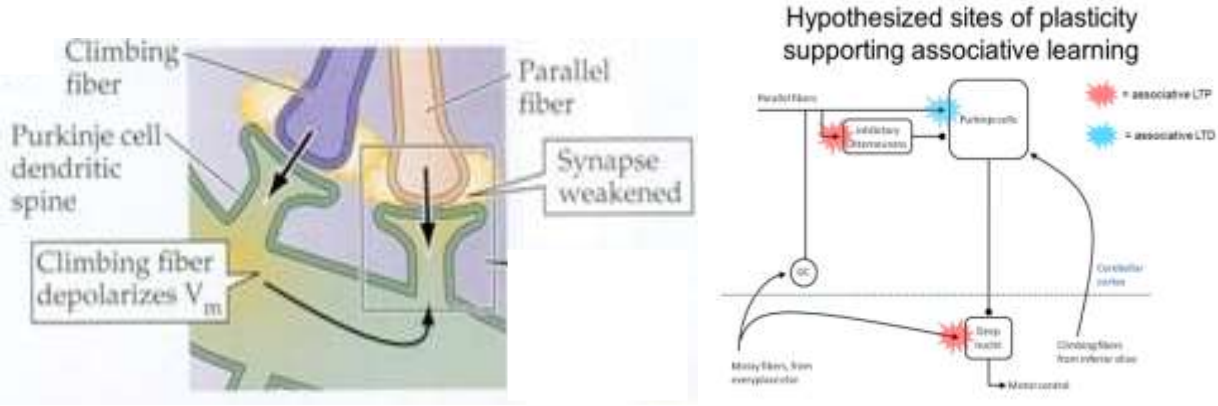

Figure I-3. (Left Panel) Parallel fiber LTD is the result of co-activation of the climbing fiber and parallel fiber synapses onto Purkinje cell dendrites. If activity at a parallel fiber synapse coincides with hetero-synaptic depolarization from a nearby climbing fiber synapse, then that parallel fiber synapse is weakened. Because the parallel fiber and climbing fiber inputs are driven by different sources, this is a form of associative plasticity. From Purves et al "Neuroscience" 2nd edition. 1999, p.552. (Right Panel) A simplified schematic of the cerebellar microcircuit with hypothesized sites of plasticity highlighted by fireworks. The blue firework indicates parallel fiber LTD.

If the cerebellar microcircuit merely performed the exact same routine computation repeatedly, there is no obvious reason why it would require so many granule cells and parallel fiber-to-Purkinje cell synapses. Brindley (Brindley, 1964) proposed an anatomical argument: the reason there are so many parallel fiber-to-Purkinje cell synapses is to support a plasticity function. With a large reservoir of elements to modify, the cerebellar cortex makes an excellent candidate site of learning and memory storage.

The canonical mechanism (Linden, 2003) for plasticity at the parallel fiber-to-Purkinje cell synapse is parallel fiber long-term depression, or "Parallel fiber LTD." Parallel fiber-to-Purkinje cell synapses modify their strength when they are active just before the climbing fiber-to-Purkinje cell synapse activates. In other words, parallel fibers depress most readily when their activity directly precedes climbing fiber activity. This makes sense from an adaptive standpoint because if the climbing fiber input represents an error or unexpected event, this

plasticity rule would modify the parallel fibers that are most predictive of the imminent arrival of a climbing fiber-mediated error signal.

The canonical modification of the parallel fibers is a decrease—or *depression*—in synaptic strength over a long period. This is counter-intuitive because it means that the parallel fibers most strongly associated with the onset of a climbing fiber input will have the weakest input. Donald Hebb popularized the concept of long-term synaptic plasticity with the aphorism "neurons that fire together, wire together." Yet with parallel fibers, the opposite is true. For this reason, depression of the parallel fibers is an example of anti-Hebbian plasticity.

Because the trigger for weakening of the parallel fibers comes from the activity of the climbing fiber input, it is a form of hetero-synaptic plasticity. Because the rate of the change in the strength of the parallel fiber depends on its correlation with the climbing fiber, and the parallel fiber and climbing fiber encode different events, this is a form of associative plasticity. Put together, the canonical form of parallel fiber plasticity is associative and hetero-synaptic.

Parallel fiber LTD is not the only type of plasticity found in the cerebellar cortex. If it were, parallel fiber synapses would eventually depress to the point where further plasticity is no longer possible. In order to balance parallel fiber LTD, long-term potentiation (LTP) of parallel fibers occurs when they are active in the absence of climbing fiber input (Coesmans et al., 2004). Because parallel fiber LTP only relies on a single input to trigger plasticity, this is a form of non-associative, homosynaptic plasticity. Thus a parallel fiber's strength is reflective of how correlated it is to the climbing fiber input: a parallel fiber synapse which is consistently active just before the climbing fiber input will be very weak whereas a parallel fiber synapse which is consistently active only when the climbing fiber input is absent will be very strong.

Many theories of cerebellar function propose that the granule cell layer acts a biological stopwatch for behavior at the scale of 10 – 1000 milliseconds (Ivry and Keele, 1989; Mauk and

Donegan, 1997). In other words, parallel fibers may be capable of conveying millisecond-level timing information about the organism's sensory context. Moreover, Purkinje cells may exploit this timing information in order to predict changes in the organism's environment. Thus, in order to exploit the computations of this biological stopwatch, parallel fiber-to-Purkinje cell synapse modifications should be sensitive to the time elapsed between their own activation and the climbing fiber activation.

In fact, there is evidence that, both *in vitro* (Safo and Regehr, 2008) and *in vivo* (Raymond and Lisberger, 1998; Wetmore et al., 2014), parallel fiber plasticity is most effective over a certain range of inter-stimulus intervals between the parallel fiber and climbing fiber. The largest changes in parallel fiber strength occur when the parallel fiber input precedes the climbing fiber input by roughly 200 milliseconds.

Parallel fiber LTD is optimal when it precedes the climbing fiber by 200 milliseconds because the organism needs some non-zero amount of time to prepare and execute a compensatory movement. Consider what would happen if the synapses changed most readily when active after the climbing fiber instead of before it. The Purkinje cell would not be able to predict the onset of the climbing fiber input, it would only be able to recognize from the parallel fiber inputs that a climbing fiber input had just happened, which would be redundant information. Furthermore, if the biggest modifications occurred when there was zero time lag between the parallel fibers and climbing fibers, then the parallel fibers still could not signal the arrival of the climbing fiber. Thus, it makes sense that the parallel fibers make their largest modifications when their activity precedes the climbing fiber at a behaviorally relevant timescale.

Because the anterior interpositus nucleus—one of the deep cerebellar nuclei—is supposed to be an essential site of associative memory storage, it must have a learning rule as well. Indeed, there is a hypothesized site of plasticity at the mossy fiber-to-cerebellar nuclei

synapse (Mauk and Donegan, 1997). This plasticity is similar to the cortical plasticity in that it is associative and hetero-synaptic. The two kinds of synapses being associated are the mossy fiber and the Purkinje cell input. Interestingly, the plasticity rule is that the mossy fibers are potentiated when active during the relief of otherwise persist Purkinje cell input (Pugh and Raman, 2006). Because this synapse is not privy to the stopwatch calculations done in the cerebellar cortex, these synapses are not as sensitive to the interval between mossy fiber and the dis-inhibitory burst.

DISINHIBITION HYPOTHESIS

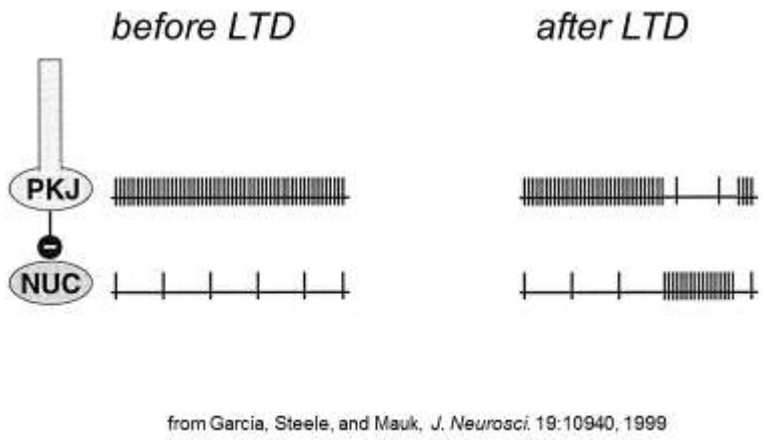

Figure I-4. The Disinhibition Hypothesis. Purkinje cells (PKJ) are tonically active with high firing rates and are thus constantly inhibiting their target nuclear cells (NUC) in the deep cerebellar nuclei. After a sufficient number of a Purkinje cell's parallel fiber inputs weaken, its firing rate will temporarily decrease. This leads to a transient pause in Purkinje cell firing which relieves the nuclear cell from otherwise tonic inhibition. This relief of inhibition—or "disinhibition"—produces a transient burst in firing rate in the nuclear cell. The burst of activity in the nuclear ceases once the Purkinje cell firing rate returns to normal.

A prominent explanation for how the Purkinje cell computes the output of the cerebellar cortex was popularized by Eccles' physiology studies (Eccles et al., 1967) and later elaborated by Albus in his theoretical work (Albus, 1971). This explanation begins with the observation that Purkinje cells tonically inhibit their target cells, the deep cerebellar nuclei, with highly active GABA-ergic axons. In spite of this descending inhibition, nuclear cells still exhibit spontaneous spiking activity (Thach, 1968) with firing rates of about 10-50 Hz (Jahnsen, 1986), largely because of a tonic cation conductance (Raman et al., 2000). In the event that a sufficient fraction of a Purkinje cells' active parallel fibers have been weakend due to parallel fiber LTD, there is a transient decrease in Purkinje cell activity and, consequently, a relief of inhibition onto the nuclear cells. This relief of inhibiton—or *dis-inhibition*—of the nuclear cell activity can produce a burst in cerebellar output if a sufficient number of Purkinje cells simultaneously pause their firing (Heiney et al., 2014). If all these conditions are met, then the burst in nuclear cell firing relays a messages to the cerebellum's associated downstream brain structures. In

summary, Purkinje cells may communicate their predictions to the rest of the nervous system by pausing their activity and dis-inhibiting their target nuclear cells. This is known as the "disinhibtion hypothesis" of cerebellar function.

Purkinje Cells are spontaneously active, so that even without excitatory input from the parallel or climbing fibers they will fire consistently at rates as fast as 100 spikes per second (Raman and Bean, 1997). This inconvenient truth makes it difficult to explain how parallel fiber LTD could produce a transient pause in Purkinje cell activity. A decrease in excitatory input alone would presumably bring the firing rate to spontanous firing rates. Yet, firing rates would need to decrease below this spontaneous rate to provide salient relief of inhibition to the target cells in the deep cerebellar nuclei. So, how exactly does parallel fiber LTD produce Purkinje cell pauses?

The most parsimonous explanation that I've encountered (Mauk and Donegan, 1997) begins with the observation that Purkinje cells and their afferent inhibitory molecular layer interneurons both are driven by the same parallel fiber input. This is an example of feedforward inhibition, a common circuit motif in the nervous system. The upshot of this synaptic arrangement is that for every parallel fiber impulse, the Purkinje cell receives a mixture of inhibitory and excitatory neurotransmitter. If the excitatory and inhibitory inputs are balanced, then their effects on Purkinje cell excitability cancel each other out. However, if there is more excitation than inhibition, the net effect is excitation; and if there is more inhibition than excitation, the net effect is inhibition.

Bearing in mind this feedforward circuit, if the excitatory parallel fiber inputs to a Purkinje cell weaken because of LTD, the balance of excitation and inhibtion would shift towards inhibition. With enough parallel fiber LTD, inhibition would end up dominating excitation such

that there would be a transient pause in Purkinje cell activity. Consequently, this Purkinje cell pause would produce a dis-inhibitory burst of activity in the nuclear cell.

Some cerebellar physiologists are uncomfortable with the notion that the balance of excitation and inhibition can only shift by changing the levels of excitation (Hesslow et al., 2013; Mittmann and Hausser, 2007), and this has led to proposals which invoke additional plasticity mechanisms beyond parallel fiber LTD (Hansel et al., 2001). One proposal with considerable—albiet indirect—evidence to support it is the notion that parallel fiber-to-molecular layer interneuron synapses exhibit long-term *potentiation* (Albus, 1971; Jorntell and Ekerot, 2002; Kenyon, 1997). This would allow the balance of excitation and inhibition impinging on a Purkinje Cell to change more flexibly since the balance could be alterned by not only changing the level of excitation, but also the level of inhibition.

Since parallel fiber-to-molecular layer interneuron plasticity is a type of cortical plasticity, it is postulated to have many of the same features as parallel fiber LTD: it would last a long time, it would be sensitive to the time interval between the parallel fiber activation and the climbing fiber activation, and its strength would reflect the degree to which it correlates with a climbing fiber-mediated error. This form of plasticity is also predicted to have additional computational benefits such as faster error-correction and increased capacity for pattern separation. Alas, one of the difficulties with this hypothesized site of plasiticity, besides the lack of direct evidence for it, is its biological plausibility. It is not yet clear how molecular layer interneurons can sense the interval between parallel fiber and climbing fiber activity since they do not receive direct climbing fiber contacts. However, recent evidence demonstrating climbing fiber spillover (Mathews et al., 2012) hints at a possible mechanism.

CEREBELLUM'S ROLE IN CLASSICAL EYELID CONDITIONING

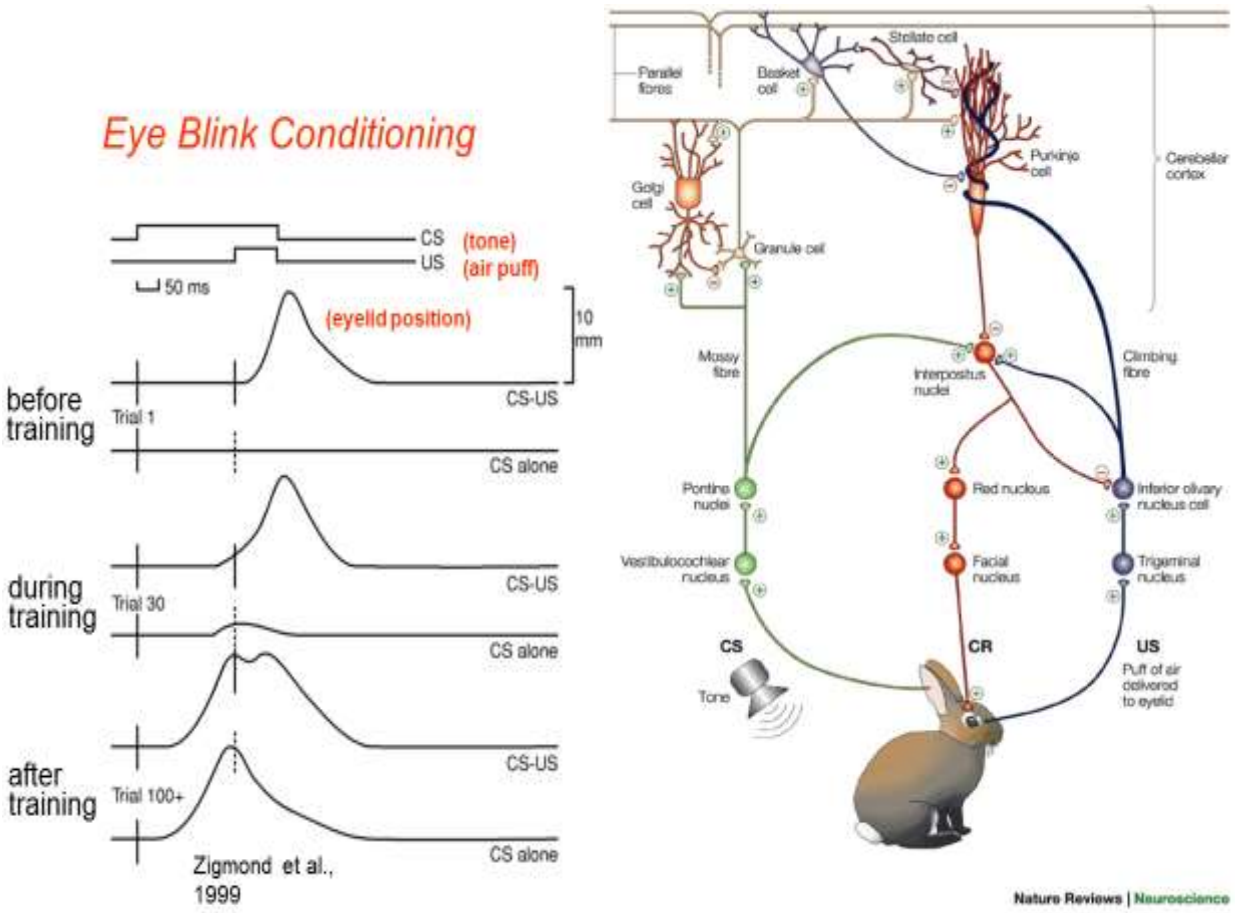

Figure I-5. The cerebellum's role in classical eyelid conditioning. (Left panel) In classical eyeblink conditioning, a behaviorally neutral conditioning stimulus (CS), usually an auditory tone, repeatedly cues the onset of a unconditioned stimulus (US), typically an airpuff that causes a unconditioned blink response (UR). Before training, the CS does not cause the eyelid position to change but the US does. During training, a small amplitude response develops to the CS. After several hundred pairings of the CS and US, the CS elicits a large-amplitude, anticipatory conditioned blink response (CR) from the subject, even without a subsequent airpuff. (Right panel) A cartoon of the relevant neural circuitry in classical eyeblink conditioning. The hypothesized CS pathway (green) and US pathway (blue) converge in the cerebellar cortex at the Purkinje cell. The spontaneously active Purkinje cell disinhibits the interpositus nucleus after training, which activates the CR pathway (red) and produces a blink. From (Medina et al., 2002b).

James Albus' original proposal for parallel fiber LTD in the early 1970s (Albus, 1971) served as an explanation for how the cerebellum might be able to support Pavlovian classical conditioning, a form of associative motor learning. In the early 1980s, two pieces of independent, yet inter-related, evidence emerged to corroborate the notion that the cerebellum

is a site of associative memory storage. The first piece of evidence was that the cerebellar cortex was capable of maintaining long-term changes in its synaptic efficacy after conjunctive stimulation of the climbing and parallel fibers (Ito and Kano, 1982). The second piece of evidence was that the cerebellum was found to be essential for classical eyeblink conditioning (McCormick and Thompson, 1984). By showing that the cerebellum exhibited a neural correlate of learning and demonstrating the necessity of the cerebellum for retaining an associative motor memory, these two pieces of evidence supported Albus' notion of the cerebellum as the site of associative motor memory formation and storage.

If the cerebellum is the site of associative memory formation and storage in classical eyeblink conditioning, then the cerebellar microcircuit should in principle exhibit neural features correlated with all the important aspects of the classical conditioning task. In order to list the important aspects of classical eyeblink conditioning that the cerebellar microcircuit should emulate, it is worthwhile to review the basics of classical eyelid conditioning.

In classical conditioning, two stimuli are repeatedly paired: a neutral stimulus (the conditioning stimulus or "CS", see Figure I-5) followed by a salient stimulus (the unconditioned stimulus or "US", see Figure I-5) which elicits a natural reflex (the unconditioned reflex or "UR"). After several hundred such pairings, the conditioning stimulus begins to elicit responses (conditioned responses or "CRs", see Figure I-5) which often resemble the natural reflex elicited by the unconditioned stimulus. If, after conditioned responses appear, the conditioning stimulus is then repeatedly presented without the unconditioned stimulus, the conditioned responses will gradually extinguish (see Figure I-6).

In classical eyelid conditioning, the conditioning stimulus is an auditory tone and the unconditioned stimulus is an airpuff (or shock) directed towards the eye. Initially, there is no response to the auditory tone and the airpuff elicits a blink response. After pairing the tone and

the airpuff several hundred times, the subject will blink in response to the tone even if the airpuff is not delivered (see Figure I-5). The conditioned blink can be extinguished by repeatedly presenting the tone without the airpuff.

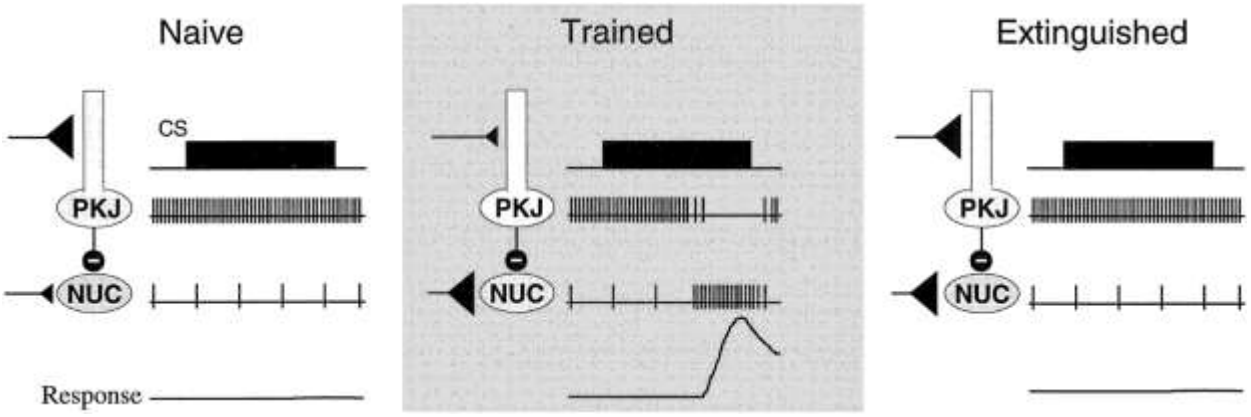

Figure I-6. Neural correlates of classical conditioning in the cerebellar microcircuit. In the naïve subject, tonic Purkinje cell (PKJ) activity mitigates CS-evoked mossy fiber input to the nuclear cells (NUC) and there is no change in nuclear cell activity during the CS. In the trained subject, after many pairings of the CS and the US, plasticity at the parallel and mossy fibers alters the response to the CS. In contrast to the naïve response, Purkinje cells pause their firing. This leads to a burst in nuclear cell activity, which in turn produces a blink response. If, after training, the CS is repeatedly presented on its own, then plasticity at the parallel fibers will again alter the response to the CS such that the CS-driven Purkinje cell pause will disappear. Without the Purkinje cell pause, the eyeblink response will extinguish. From (Garcia et al., 1999).

The explanation for how the cerebellum supports formation of associative motor memories goes as follows. The tone activates the auditory system, which activates a subset of mossy fibers in the cerebellum. The granule cell layer then begins a biological stopwatch that encodes time in an evolving population of active parallel fibers. Meanwhile, the airpuff directed at the subject's cornea activates the somatosensory system, which activates a subset of climbing fibers in the cerebellum. The tone encoded in the parallel fiber activity and airpuff encoded in the climbing fiber activity converge at a small cluster of Purkinje cells in the cerebellum. The cluster of Purkinje cells record the inter-stimulus interval by modifying their parallel fiber synapses that were active just prior to the climbing fiber input. This modification is in accordance with an associative, hetero-synaptic learning rule. Several pairings of the stimuli

produce a long-lasting change in tone-evoked Purkinje cell activity that is predictive of the airpuff onset. By a transient pause in their otherwise rapid and persistent firing rates, the small cluster of Purkinje cells transmit this airpuff onset prediction downstream to the nuclear cells in the anterior interpositus. These nuclear cells then command the eyelid to blink in anticipation of the airpuff by transmitting a dis-inhibitory burst of activity to the red nucleus. Several pairings of the tone-evoked mossy fiber input to the nuclear cells and the dis-inhibitory bursts of the nuclear cells themselves produce long-lasting changes in the strength of the mossy fiber inputs.

Another feature of classical conditioning is that if the conditioning stimulus is repeatedly presented on its own after the subject has learned to perform conditioned responses, then this will corrupt the predictive value of the conditioning stimulus and the conditioned response will eventually vanish. This phenomenon is known as extinction. In classical eyelid conditioning, the extinction procedure is performed by removing the airpuff from stimulus presentations once the tone reliably elicits conditioned blinks from the subject. After many presentations of the tone in the absence of an expected airpuff, the conditioned blinks subside and disappear.

Like acquisition, extinction of conditioned eyelid responses can be explained using the cerebellar microcircuit. Once conditioned blinks are reliably evoked by the tone, the parallel fibers are depressed and the mossy fiber collaterals are potentiated, which leads to a well-timed burst in the cerebellar nuclei in response to the tone. When the airpuff is omitted and the tone is played on its own, the well-timed burst in the cerebellar nuclei still happens, but parallel fibers also potentiate according to a non-associative, homo-synaptic learning rule. After many presentations of the tone on its own, the parallel fibers potentiate to the point that the balance of excitation and inhibition on the Purkinje cell shifts back towards excitation, and the Purkinje cell no longer pauses in response to the tone-evoked parallel fiber activity. Without the pause in Purkinje cell activity, the cerebellar nuclei fail to burst in response to the tone-evoked mossy fiber activity. And without a burst in the cerebellar nuclei, the tone no longer elicits a conditioned

blink, which is consistent with the result of an extinction procedure. In summary, extinction of classically conditioned eyelid responses could be the result of parallel fiber LTP eliminating a transient pause in Purkinje cell activity.

ELECTRICAL STIMULATION AS THE UNCONDITIONED AND CONDITIONED STIMULI

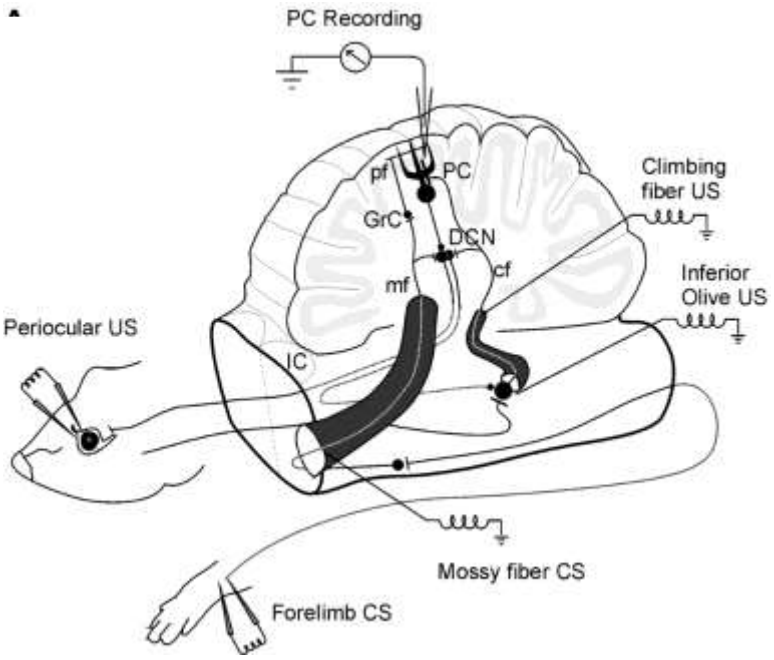

Figure I-7. A cartoon of Hesslow's experimental set-up for eliciting and recording Purkinje cell (PC) pauses in decerebrate ferrets. The brain is transected at the inferior colliculus (IC) to exclude the participation of forebrain structures in conditioning. The hypothetical CS and US pathways are stimulated either at their respective sensory organ or downstream closer to the cerebellum. With this arrangement, Purkinje cell activity can be recorded extracellularly long enough to watch the effects of classical conditioning on Purkinje cell spiking happen in real-time. From (Jirenhed et al., 2007).

The cerebellum is thought to form and store associative motor memories because: 1) It is required for expressing conditioned eyeblinks; 2) CS-evoked neural activity converges with US-evoked neural activity at Purkinje cells and nuclear cells; 3) The cerebellum's anatomy and physiology can be used to explain how the process of forming and storing associative memories could work in a biologically plausible way. However, if the cerebellum is the site of the associative memory, the cerebellar circuit, and its associated downstream brainstem motor structures, should not only be necessary but also sufficient for expression of the motor memory. In other words, forebrain structures should not be necessary for the retention and expression of the motor memory. However, because the neural activity representing the conditioning stimulus and the unconditioned stimulus could converge outside the cerebellum, there is the possibility that parts of the associative memory form outside the cerebellum, and that these extra-

cerebellar sites of memory storage are also necessary for expressing the conditioned responses.

In order to address this potential confound, the Hesslow group developed a preparation for testing the degree to which the cerebellum and its associated descending motor structures are sufficient for generating the conditioned responses (Hesslow et al., 1999). They used ferrets as the experimental subject because of the relatively easy electrophysiological access. In their preparation, they have electrical control over the mossy fibers and the climbing fibers as well as single-unit records of electrical activity in Purkinje cells. They transect the brain rostral to the inferior colliculus in order to isolate the hindbrain activity from forebrain structures such as thalamus, cerebral cortex, and basal ganglia. Thus, this is a reduced system in which the contributions of the cerebellum to the conditioned responses can be studied more directly than before.

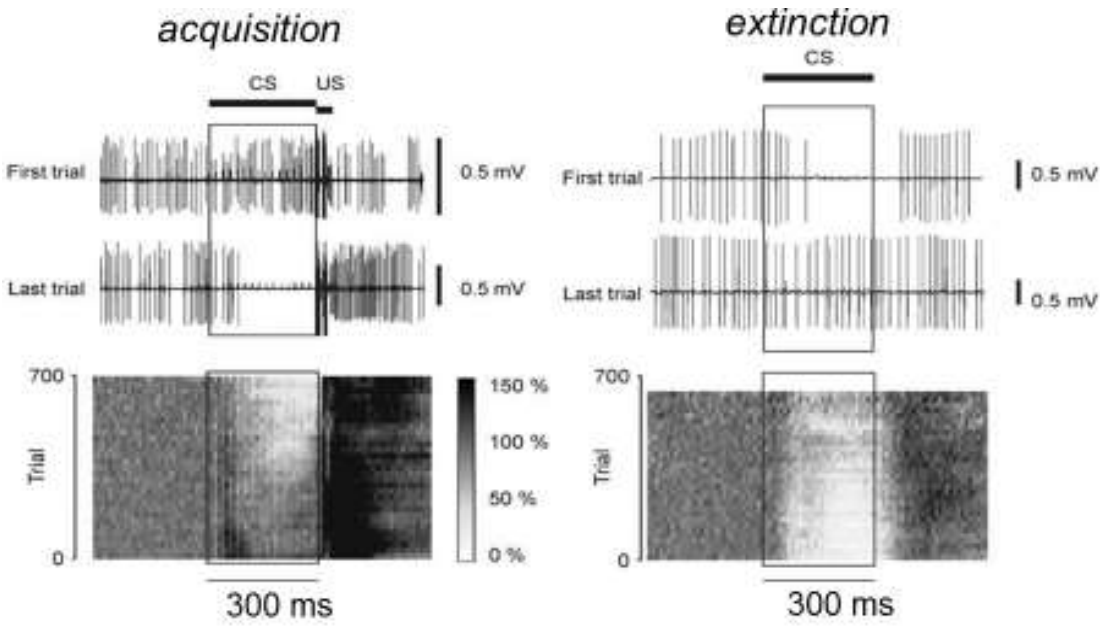

Figure I-8. Single unit Purkinje cell recording in decerebrate ferret as reported in (Jirenhed et al., 2007). Internal electrical activation of the mossy and climbing fibers substituted for the CS and US, respectively. (Left panel, top) After several hundred pairings of mossy fiber and climbing fiber activity, Purkinje cells begin to pause in response to the mossy fiber stimulus. (Left panel, bottom) This raster plot of Purkinje cell spiking activity shows a gradual development of the pause over 700 trials. (Right panel, top) If the mossy fiber stimulus is repeatedly presented in the absence of the climbing fiber stimulus, the mossy fiber-evoked pause disappears. (Right panel, bottom) A raster plot of Purkinje cell spiking activity shows the gradual disappearance the pause.

The Hesslow Lab's single unit recordings with electrical stimulation substituting for the CS and US demonstrate that Purkinje cells do in fact pause after acquiring the CS-US relationship. The pause happens gradually over several hundred pairings of mossy fiber and climbing fiber input with an inter-stimulus interval that matches those used in classical eyeblink conditioning. The Purkinje cell pauses also extinguish if the mossy fiber stimulus is repeatedly presented on its own, and will reappear if the climbing fiber stimulus is reintroduced with presentations of the mossy fiber CS (Jirenhed et al., 2007). For this reason, the Purkinje cell pauses have been called Purkinje cell CRs in view of the fact that so many of the features ascribed to classical eyelid conditioning are present in the spiking activity of a single Purkinje cell.

OPTOGENETICS ALLOWS CELL-TYPE SPECIFIC STIMULATION

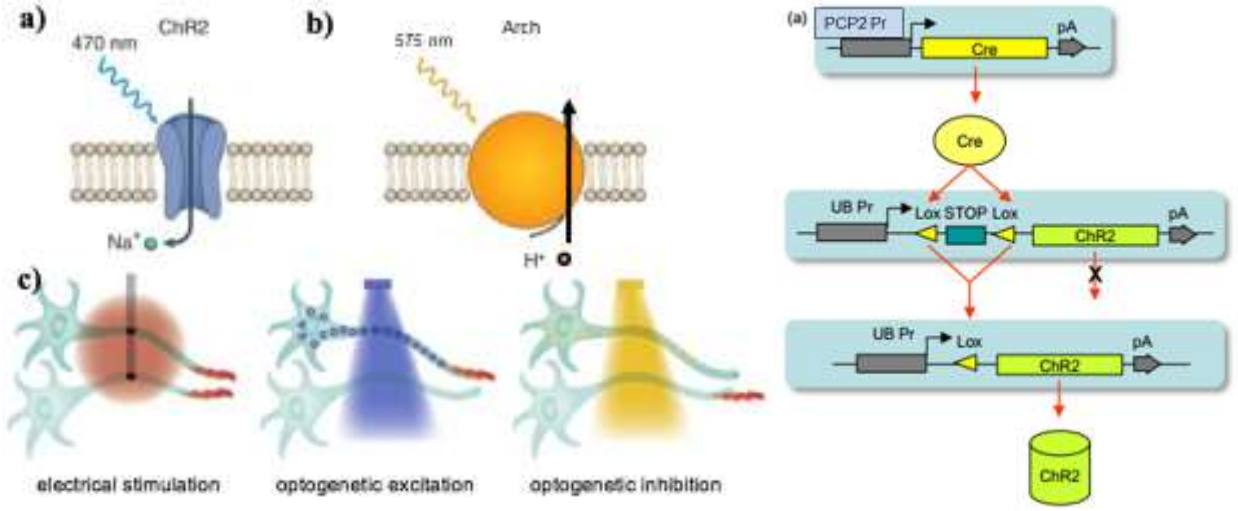

Figure I-9. Optogenetics allows cell type-specific activation of excitable cells by using light-sensitive ion channels and pumps. (Right panel, a) Channelrhodopsin-2 (ChR2) is a light-activated nonselective cation channel that allows neurons expressing it in their membrane to be excited by blue light. (b) Archaerhodopsin (Arch) is a light-driven proton pump that allows neurons expressing it in their membrane to be inhibited by yellow light. (c) Compared to electrical stimulation, which indiscriminately activates all nearby axons, opsins can selectively excite or inhibit some neurons without affecting others, even if tightly packed together. Adapted from (Deisseroth, 2011). (Left panel) Using the Cre/lox system for producing ChR2-expression selectively in Purkinje cells. One mouse line drives expression of the Cre protein under control of a Purkinje cell-specific promoter (PCP2 Pr). Another mouse line reports the presence of Cre protein by Cre-mediated deletion of the STOP cassette and subsequent expression of ChR2 protein. When the two mouse lines are mated with each other, the offspring's Purkinje cells express Cre and hence ChR2. Adapted from (Zeng and Madisen, 2012).

In recent years, the advent of optogenetics has been a huge boon to the field of behavioral neuroscience. The ability to selectively stimulate individual cells in a neural microcircuit has been a major asset in testing important theories of memory and descending motor control. With respect to the cerebellum, optogenetics has enabled direct tests of crucial aspects of the disinhibition hypothesis (Heiney et al., 2014; Nguyen-Vu et al., 2013).

In this thesis we use cutting-edge techniques to demonstrate that optogenetically conditioned motor memories are stored in the cortical and nuclear regions of the cerebellum and expressed via disinhibition-mediated bursting of the cerebellar nuclei. Building on this observation we aim to determine the impact of preventing disinhibition of the cerebellar nuclei when cueing an optogenetically conditioned forelimb movement. By transiently increasing

inhibition of cerebellar nuclei during high-speed video recordings of mice responding to a previously conditioned auditory cue, we demonstrated that preventing disinhibition of cerebellar nuclei prevents the expression of the learned forelimb movements. We conclude that disinhibition-mediated bursting of the cerebellar nuclei is necessary for retrieving the motor memory and the subsequent conditioned response.

Chapter II is a review presenting theoretical analysis of how the climbing fiber instructs movement and learning. Chapter III shows how optogenetic activation of Purkinje cells can act as an effective climbing fiber substitute by instructing changes in the cerebellar circuitry. Chapter IV shows how optogenetic activation of Purkinje cells can also act as an effective parallel fiber substitute by driving Purkinje cells to suppress the expression of associative motor memories. Chapter V is a reflection on the work and suggestions for future directions.

CHAPTER II: CONTRIBUTIONS OF CLIMBING FIBERS TO CEREBELLAR FUNCTION

Contributions of climbing fibers to cerebellar function

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Extensive evidence suggests that climbing fibers (CFs) play a pivotal role in cerebellar-dependent forms of associative learning due to changes in circuit function driven by CF activity (Jirenhed et al., 2007; Jorntell and Ekerot, 2002; Mauk et al., 1986; Medina and Lisberger, 2008; Yang and Lisberger, 2014). These findings raise a number of critical questions. What patterns of CF activity lead to learning? Which circuit elements do CFs alter? Are these alterations dependent on the stage or circumstance of learning?

Based on our own recent published (Maiz et al., 2012; Mathews et al., 2012) and unpublished work, as well as published work from many other labs (Cooke et al., 2004; Kassardjian et al., 2005; Okamoto et al., 2011; Shutoh et al., 2006; Titley et al., 2007), we favor the “trigger and storage” hypothesis of cerebellar learning (Medina, 2011). It posits that CFs trigger plasticity at distinct sites within the cerebellar cortex and deep cerebellar nucleus in separate stages: a rapid plasticity in the cortex followed by a slower plasticity in the nuclear cells driven by the changes that have occurred in cortex. The following section summarizes evidence and ideas pertaining to the role of error-associated CF activity in the “trigger and storage” hypothesis of cerebellar learning.

Originally, the “trigger and storage” hypothesis explained the mechanics of consolidation for eyeblink learning (Medina et al., 2002a). Since then, the hypothesis has expanded to include other forms of cerebellar-dependent learning (Kassardjian et al., 2005; Shutoh et al., 2006; Wulff et al., 2009). In all of these forms of learning, the Purkinje neuron (PN) and CF play

central roles, although the mechanisms involved in the initial plasticity and consolidation remain incompletely understood.

The “trigger-and-storage” hypothesis treats the cerebellum as an error-correcting machine, where the CF is a source for error information. Errors can be viewed as arising from a difference between expected and actual outcome of a sensory prediction or motor command, as unexpected events that pertain to poorly calibrated sensorimotor function, or simply as negative sensory events to be avoided (Gilbert and Thach, 1977; Kim et al., 1998; Simpson and Alley, 1974). For example, retinal slip, corneal airpuffs, and periorbital stimulation are maladaptive or aversive sensory stimuli that in associative learning paradigms the animal learns to anticipate and avoid.

Such errors evoke CF activity which is conveyed to the Purkinje neurons (PNs) as a complex spike—a salient, cell-wide signal—increasing calcium throughout the PN dendritic tree and cell soma (Kitamura and Hausser, 2011; Tank et al., 1988). Since we can record a CF’s activity in the post-synaptic PN, we can study its effects on PN excitability. Evoked CF activity differs from spontaneous CF activity in its firing rate, population activity (Bell and Kawasaki, 1972; Ghosh et al., 2011; Lang et al., 1999; Marshall and Lang, 2009; Mukamel et al., 2009; Ozden et al., 2009; Schultz et al., 2009; Welsh et al., 1995), and capacity for altering circuit function (Jirenhed et al., 2007; Jorntell and Ekerot, 2002; Mauk et al., 1986; Medina and Lisberger, 2008; Yang and Lisberger, 2014).

CFs can drive associative decreases in PN firing (Jirenhed et al., 2007; Lisberger, 1994; Medina and Lisberger, 2008). Several studies have established correlational relationships of CF activity to long-term changes in PN firing. Some of the best evidence comes from studies of decerebrate ferret in which co-activation of CF and mossy fiber input gradually leads to CS-evoked PN pauses in firing (Jirenhed et al., 2007). Lisberger and colleagues have developed a

smooth pursuit learning task in which the occurrence of a complex spike on one trial led to significant decreases in PN firing on the subsequent trial (Medina and Lisberger, 2008) and recent work using this paradigm demonstrates that the strength of complex spikes shows slight gradation and this is correlated with the magnitude of trial by trial learning (Yang and Lisberger, 2014). Strikingly, this “analog teaching signal” must be correlated at a population level because the strength of behavioral learning can be predicted based on recordings from a single PN. These and other findings are consistent with a unique effect of evoked, population CF activity to drive circuit changes in cerebellar cortex. Such associative decreases in PN firing are hypothesized to drive increases in nuclear cell activity, allowing cerebellum to exert control over descending motor pathways.

Learned pauses in Purkinje cell activity require mossy fiber activity but not CF activity. Typically, mossy fibers convey external stimuli like auditory or visual cues to evoke learned pauses in PNs. However, it is conceivable that internal activity, replay patterns of MF activity that occurred during conditioning, could later drive learned pauses and promote consolidation to DCN. Support for this idea is provided by a study done on human participants in which BOLD signals were measured during rest periods in between bouts of motor training (Albert et al., 2009). Resting state activity in fronto-parietal and cerebellar networks were significantly elevated after motor learning but not after sham learning (i.e. motor performance without training). This suggests that motor learning, but not motor performance, specifically alters a cerebellar resting state network which then remains active offline.

Such activity is a candidate mechanism for replay-mediated consolidation in cerebellum. Models of cerebellum energy use suggests that BOLD signals are chiefly the result of activity in the granule cells (Howarth et al., 2012). Thus, elevated BOLD signals during resting state may indicate self-generated replay of task-relevant granule cell activity. Replay would elicit learned

pauses in PNs in the absence of external cues and promote transfer of motor memories from PN to DCN. Consistent with this idea, lesioning or inactivating cerebellar cortex shortly after motor training disrupts consolidation of motor memories (Shutoh et al., 2006). More work remains to be done in order to better understand the role of learned PN pauses in motor memory consolidation.

What are the candidate circuit mechanisms underlying such CF-driven, learned reductions in PN firing? Parallel fiber long-term depression (PF LTD) is one proposed mechanism for associative decreases in PN firing (Ito, 2002; Safo and Regehr, 2008; Wang et al., 2000), however the necessity of this form of plasticity in associate learning is under debate (Schonewille et al., 2011; Welsh et al., 2005). In addition to PF LTD, some of the original theories of cerebellar function posited other sites of plasticity in cerebellar cortex (Albus, 1971), suggesting that CFs could drive LTP of PF inputs to molecular layer interneurons. Evidence in support of this mechanism is indirect. *In vivo recordings* show that CF stimulation leads to strong increase in inhibitory receptive fields in PNs (Jorntell and Ekerot, 2002) and genetic deletion of $GABA_A$ receptors from PNs leads to deficits in memory consolidation in associative learning tasks (Wulff et al., 2009).

The “trigger and storage” hypothesis predicts circuit changes downstream of the PN in the DCN (Medina, 2011; Medina and Mauk, 2000; Miles and Lisberger, 1981) and there is considerable evidence supporting the proposal that learning related plasticity occurs in DCN (Cooke et al., 2004; Ohyama and Mauk, 2001; Perrett et al., 1993; Welsh and Harvey, 1991). Does the CF play a pivotal role in instructing learning-related plasticity in DCN?

Perhaps. In addition to the learned reductions in PN firing discussed above, the CF can elicit acute, non-associative decreases in PN firing, termed post-complex spike pauses, that could in principle modulate DCN activity and drive plasticity (Otis et al., 2012). Even if there is no overt pause (i.e. an increase in inter-spike interval beyond the baseline interval), complex spikes reset the period of simple spike firing in PNs. Thus, given error-associated synchronous CF input to functional microzones, there will be a synchronous pause that could drive DCN excitability.

It is in this context that we interpreted experiments indicating that pharmacological prolongation of the post-complex spike pause enhances rate of eyeblink acquisition but not extinction (Maiz et al., 2012). These findings support the idea that post-complex spike pauses train circuit changes in DCN by selectively enhancing plasticity at MF to deep nuclear synapses. In this mechanism, the PN is less of a trigger cell and more a mouthpiece for CF instructions, providing a pathway for the error information to reach the DCN. Thus, both PN and DCN plasticity could occur simultaneously but at different rates (Medina et al., 2002a).

CFs convey errors to cerebellar cortex, but it is unknown whether the inferior olive or some upstream structure actually computes the error. Pharmacologically blocking synaptic inhibition of the inferior olive prevents extinction; conversely, pharmacologically blocking synaptic excitation of the inferior olive initiates extinction (Medina et al., 2002a). Importantly, these conditions maintain spontaneous CF activity suggesting that only evoked CF output serves as an acquisition signal, and that perhaps spontaneous CF activity can serve as an extinction signal. Recent findings indicate that projections from DCN inhibit gap junction coupling between IO neurons as well as their individual intrinsic oscillations (Lefler et al., 2014). This would prevent spatiotemporal synchrony among CFs within a single microzone as well as affect spontaneous PN firing rates.

One corollary of the “trigger and storage” hypothesis of cerebellar learning is that CF error signals do not alter nuclear synapses. Instead, errors adjust cortical synapses until the animal learns to avoid the error via disinhibition of its cerebellar nuclei. Successful patterns of nuclear disinhibition then consolidate by altering the strength of mossy fiber collaterals to DCN. Either acute or learned pauses in PN activity could drive MF-DCN plasticity, however, we favor the notion that CF instructed learned pauses in PN firing drive DCN plasticity.

To summarize, in the initial stage of associative learning, error-associated population activity in CFs leads to learned pauses in PN firing in response to the conditioned sensory stimulus. Climbing fiber error signals may also be relayed to DCN via acute actions on PNs, which could instruct changes in PN and DCN excitability to occur simultaneously, albeit at different rates. During consolidation, motor memories induced as pauses in PNs can then be transferred to the DCN in a CF-independent, serial manner via externally or internally-evoked PN pauses instructing LTP of collateral MF inputs to DCN neurons.

CHAPTER III: CIRCUIT MECHANISMS UNDERLYING MOTOR MEMORY FORMATION IN THE CEREBELLUM

Circuit Mechanisms Underlying Motor Memory Formation in the Cerebellum

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Running title: Learned Forelimb Movements Triggered by Purkinje Neuron Inhibition

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SUMMARY

The cerebellum stores associative motor memories essential for properly timed movement; however, the mechanisms by which these memories form and are acted upon remain unclear. To determine how cerebellar activity relates to movement and motor learning, we used optogenetics to manipulate spontaneously firing Purkinje neurons (PNs) in simplex lobe. Using high-speed videography and motion tracking, we found that altering PN activity produced rapid movement. PN inhibition drove rapid movements time-locked to stimulus onset, whereas PN excitation drove delayed movements that were time-locked to stimulus offset. Pairing PN inhibition or PN excitation with sensory stimuli triggered the formation of robust, associative motor memories. Moreover, training with PN excitation produced sharper timing in the learned movements. These findings implicate inhibition of PNs as a teaching signal in the cerebellar circuit, consistent with a model whereby learning leads to reductions in PN firing that subsequently instruct memory consolidation in the deep cerebellar nucleus.

HIGHLIGHTS

- Pauses in spontaneous PN firing drive DNN bursts and rapid forelimb movements
- Exciting or inhibiting PNs can drive robust, associative motor learning
- Movements trained by PN excitation are more accurately timed
- PN excitation and inhibition drive circuit plasticity at distinct sites in cerebellum

INTRODUCTION

To ensure coordination, the brain must make accurate predictions about how to direct movement (Medina, 2011). These predictions are constructed through a process of error-driven learning, and then stored as associative memories in the cerebellum (Albus, 1971; Marr, 1969; Medina, 2011; Raymond et al., 1996). Correlates of such memories have been observed in the firing patterns of cerebellar PNs as sensory-evoked reductions in the firing rate of PNs in advance of a learned movement (Jirenhed et al., 2007; Lisberger, 1994; Medina and Lisberger, 2008). PNs show high rates of spontaneous activity causing them to powerfully inhibit deep cerebellar nuclear and vestibular neurons (DNNs) which function as premotor neurons (Person and Raman, 2012). Thus, reductions in spontaneous PN firing in response to the predictive sensory stimulus could, in principle, drive learned movements. Although this disinhibition hypothesis was first suggested four decades ago (Albus, 1971), it is still actively debated (De Zeeuw et al., 2011; Heck et al., 2013; Ito, 1984; Medina, 2011), in part because PN firing patterns do not straightforwardly encode aspects of movement (Cao et al., 2012; Catz et al., 2008; Greger et al., 2004; Kojima et al., 2010; Popa et al., 2013). Moreover, evidence causally linking specific patterns of PN activity to discrete movements has been lacking until very recently (Heiney et al., 2014).

A related question concerns whether training- and or learning-related reductions in PN firing play a role not only in the learned movements but in subsequent memory consolidation (Maiz et al., 2012; Medina, 2011; Otis et al., 2012). Substantial evidence indicates that associative memories involve plasticity in the DNNs (Lisberger et al., 1994; Miles and Lisberger, 1981; Ohyama and Mauk, 2001; Ohyama et al., 2006; Perrett et al., 1993) and several studies suggest that memory formation occurs in two stages, first in the cerebellar cortex, followed by consolidation within the deep cerebellar nuclei (Cooke et al., 2004; Kassardjian et al., 2005; Okamoto et al., 2011; Shutoh et al., 2006; Titley et al., 2007). At a cellular level, disinhibition of

DNN is known to induce activity-dependent forms of long term potentiation that could support this type of learning (Pugh and Raman, 2006). Finally, genetic deletion of $GABA_A$ receptors in PNs results in a memory consolidation defect (Wulff et al., 2009), consistent with the notion that pauses in PN firing are required for the formation of cerebellar memories.

In order to determine how PN firing relates to movement and to explore whether certain patterns of PN activity could drive the formation of associative motor memories we developed a behavioral paradigm allowing direct manipulation of PN firing and precise measurement of forelimb movements. We find that inhibiting PNs drives short latency forelimb movements while exciting PNs results in movement delayed to the offset of excitation. Optrode experiments indicate that in both circumstances movement is linked to pauses in PN firing and bursts in downstream DNNs. By pairing PN activity with auditory tones, we demonstrate robust associative learning leading to tone-evoked, predictive forelimb movements. As suggested by a two stage model, paired excitation or paired inhibition of PNs drives learning, but the timing of learned movements trained by PN inhibition is less precise, consistent with observations that plasticity in the cerebellar cortex is required for well-timed, learned movements. These findings demonstrate that reductions in PN firing are sufficient to elicit discrete movements and that repeatedly pairing such reductions with sensory stimuli leads to the formation of associative motor memories, implicating inhibition of PNs as a consolidation mechanism within the cerebellar circuit.

RESULTS

To examine the underlying mechanisms by which PNs contribute to movement and to associative motor memory formation, we exploited Cre-conditional transgenic mice to direct expression in PNs of the excitatory opsin ChannelRhodopsin-2 fused to eYFP (ChR2) or the inhibitory opsin Archaerhodopsin-3 fused to eGFP (Arch). Expression of ChR2 or Arch was selective for PNs throughout the cerebellum and was apparent in axons projecting to DNNs (Figure 1A and S1). Electrophysiological experiments in brain slices showed that ChR2 activation generates large inward currents and rapid increases in PN firing followed by brief pauses in spontaneous firing activity, while Arch activation generates large outward currents and pauses in the spontaneous firing of PNs (Figures S2 & S3). Light evoked responses and fluorescence were absent from other types of neurons in the cerebellar cortex such as molecular layer interneurons, granule cells, and Golgi cells.

Inhibition of spontaneously firing PNs drives movement

To examine behavioral consequences of transient modulation of PN activity, we developed an awake, head-fixed mouse preparation amenable to simultaneous opsin excitation and extracellular recording. Activation of ChR2 for 75 ms led to robust increases in the simple spike (SSp) firing frequency of PNs (Figure 1B, left, peak freq. = 363.4 ± 40.7 Hz, n=11), and in most PNs the increase was followed by pauses in SSps occurring upon cessation of laser illumination (pause duration 35.0 ± 4 ms in n=9/11 PNs). Recordings of downstream DNN firing showed that ChR2-mediated activation of PNs strongly inhibits DNNs during laser illumination, and that large increases in firing rate occur upon cessation of laser illumination (Figure 1C, left, peak freq. = 212.2 ± 43.2 Hz, n = 11). In contrast to the ChR2 results, inhibiting PNs via Arch rapidly silenced PN SSp activity (Figure 1B, right, pause duration 99.1 ± 7.4 ms, n=16) and strongly excited DNNs (Figure 1C, right, peak freq. = 194.0 ± 38.6 Hz, n = 12) during laser illumination. Systematically varying pulse duration demonstrated that Arch stimulation led to DNN firing that

was time locked to the onset of illumination, while ChR2 stimulation led to DNN inhibition followed by excitation upon offset of laser illumination (Figures S4 & S5). Taken together, these findings indicate that the firing frequencies of PNs and DNNs are robustly and differentially modulated by optogenetic stimuli and that briefly inhibiting PNs is sufficient to produce high frequency firing of DNNs.

To test whether manipulation of PN/DNN excitability generates motor output we stereotaxically implanted optical fibers in the forelimb motor region of the anterior cerebellar lobe (Figure S1). Movement of the wrist joint was monitored in head fixed mice and measured using high-speed videography (Chettih et al., 2011; Heiney et al., 2014) and motion tracking analysis (Figure 2 & Movie S1). Inhibiting PNs via activation of Arch led to rapid and stereotyped upward forelimb movements during laser illumination that reached peak velocities of 0.3 to 1.5 m/s (Figures 2 & S6). By comparison, excitation of PNs via ChR2 led to delayed movement of similar magnitude but with onsets time-locked to the laser pulse termination (Figured 2 & S6). This relationship between stimulus onset/offset and movement onset can be visually compared by viewing Movie S2. Across animals, the average timing relative to laser illumination of peak DNN firing and peak movement speed in the two mouse lines indicates that by driving DNN firing, PN pauses lead to the observed forelimb movements (Figure 2D).

Robust motor memories are induced by pairing PN activation with auditory tones

Cerebellum-mediated, associative motor learning has been hypothesized to involve forms of synaptic plasticity triggered by teaching signals from olivo-cerebellar climbing fibers at multiple sites within the cerebellar circuit (Maiz et al., 2012; Medina and Lisberger, 2008; Raymond et al., 1996), yet the mechanisms underlying this learning are actively debated (Ke et al., 2009; Medina, 2011; Schonewille et al., 2011). Because both climbing fiber-dependent and

independent PN plasticity rely on PN depolarization, we tested whether synchronous PN depolarization can serve as a teaching signal. Mice were trained by pairing 2 kHz tones with pulses of laser illumination delivered 250 ms after tone onset. Each day, training consisted of 90 tone/laser pairings and 10 interleaved tone alone trials extended over five days of training. Following this acquisition training, mice were subject to extinction training in which they were presented 100 tone alone trials on each day. Within 2-3 days, acquisition training led to robust learned responses (LRs) evident as tone-evoked forelimb movements (Figure 3 & Movie S3), which occurred in 83 ± 4.4% of trials and were rapidly extinguished over 3-4 days of extinction training (Fig. 3F). Kinematic analysis showed that these learned movements were very similar to those evoked by the laser pulses, but occurred earlier in time, as expected for predictive cerebellar learning (Medina, 2011; Ohyama et al., 2003). These findings indicate that synchronous PN depolarization can trigger the formation of associative motor memories allowing sensory stimuli to drive predictive forelimb movements.

Pairing PN inhibition with auditory tones also leads to associative learning

Evidence suggests that cerebellum-dependent associative learning involves circuit changes not only in the cerebellar cortex, but also in deep cerebellar nuclei (Broussard and Kassardjian, 2004; Gao et al., 2012; Lisberger, 1994; Miles and Lisberger, 1981; Ohyama et al., 2006; Raymond et al., 1996). In principle, pauses in PN activity driven either by complex spikes (Maiz et al., 2012) or those developed in response to learning may instruct such changes by conveying transient periods of disinhibition to DNNs leading to potentiation of tone-driven inputs to DNNs. Mechanistically, NMDA receptor-dependent forms of long-term potentiation, which have been observed *in vitro*, could serve as the basis for this learning (Pugh and Raman, 2006). To test whether PN pauses are also sufficient to induce LRs we relied on an associative training paradigm similar to that described above for ChR2, but using Arch to synchronously inhibit PNs.

Pairing tones with Arch-driven PN pauses instructed tone-evoked forelimb movements (Figure 4). Although the percent of LRs was smaller than observed for ChR2 and the individual LRs were of smaller amplitude (Table S2), LRs in Arch mice developed over several days of acquisition training and extinguished in response to tone-alone training (Figure 4F). These results indicate that disinhibition of DNNs alone is sufficient to drive associative motor learning, and suggest that synchronous pauses in the firing of groups of PNs can trigger circuit changes in the DNN that underlie associative learning.

Learned movements trained with PN excitation versus inhibition show differences in timing

A critical aspect of associative motor memories is that they are adaptively timed, enabling predictive movements to be executed at precise times during sensory stimuli. Lesion studies suggest that these memories are stored in both the cerebellar cortex and deep cerebellar nucleus but that information related to the timing of learned movements resides in the cerebellar cortex (Ohyama et al., 2006; Ohyama et al., 2003; Perrett et al., 1993). To test whether ChR2 and/or Arch-training produces well-timed movements, we compared the timing of LRs for two different training intervals. For ChR2 trained animals, despite being similar in amplitude, the time courses of peak, average LRs were different, precisely anticipating the ends of the 250 or 500 ms training intervals (Figure 5 & Tables S1 & 2). In contrast, LRs resulting from 250 and 500 ms training in Arch animals were similarly timed such that the LRs in mice trained with 500 ms intervals showed peak movement velocity early in the stimulus (Figure 5). Comparison of the average movement speeds in time periods at the ends of the 250 ms and 500 ms training intervals (Table S1) further supported the conclusion that learned movements are significantly better timed in ChR2 trained animals.

The differences in timing of LRs described above suggest that learning resulting from PN inhibition occurs downstream of PNs and a likely mechanism for this learning involves

potentiation of tone-related mossy fiber inputs to DNNs (Boele et al., 2013; Miles and Lisberger, 1981; Ohyama et al., 2006; Raymond et al., 1996). Such a mechanism leads to the prediction that movements evoked by PN inhibition would be facilitated if accompanied by a sensory stimulus to which learning has occurred. We tested this prediction in Arch mice by comparing movements in response to the tone alone to those evoked by the same tone plus a laser pulse timed to elicit PN pauses at the peak of the LR (Figure 6A). As shown in Figure 4, prior to learning, tone alone stimuli evoked no movements. Following four days of paired training, PN inhibition (laser) facilitated LRs elicited by the tone by 361 ± 63% (n=3 mice, see Figure 6), leading to a significantly larger movement compared to the sum of movements evoked by laser stimulation and tone stimulation alone (sum=202 ± 72%, $p<0.005$, two-tailed, paired t-test). These results indicate that the contributions of PN pauses to movement are influenced by whether or not learning has occurred, and support the hypothesis that inhibition of PN firing can instruct memory-related changes in the deep cerebellar nucleus, a potential mechanistic substrate for “offline” consolidation from the cerebellar cortex to the deep cerebellar nuclei.

DISCUSSION

Here we combined optogenetics, high speed videography, and motion tracking to explore how modulation of the firing of groups of PNs in the simplex lobe acutely affect movement and how coupling changes in PN firing with sensory experience can drive motor memory formation. This novel paradigm allows for the creation of artificial memories, powerfully demonstrating the generality of basic principles of cerebellar associative learning uncovered in classical eyeblink conditioning and vestibuloocular reflex (VOR) plasticity. Our results also implicate critical functional roles for PN inhibition in the cerebellar circuit. On a fast time scale, reductions in PN activity lead to movement while on a longer time scale, reductions in PN activity participate in consolidation of learning to downstream circuit elements. Finally, by engaging distinct circuit

elements with ChR2 and Arch training we show that learned forelimb movements exhibit different timing profiles, providing support for the hypothesis that cerebellar learning results from circuit modifications in both cerebellar cortex and deep cerebellar nucleus with plasticity in the cerebellar cortex required for precisely timed learned movements.

Synchronous reductions in PN firing drive forelimb movements

Despite extensive study, direct links between PN activity and specific aspects of movement have been unclear. While electrical microstimulation of the deep cerebellar nuclei reliably triggers limb movements (Ekerot et al., 1995; Rispal-Padel et al., 1981, 1982; Schultz et al., 1976, 1979) and microstimulation of floccular and vermal regions of cerebellar cortex leads to eye movements (Cohen et al., 1965; Lisberger, 1994; Noda and Fujikado, 1987; Ron and Robinson, 1973), there are no reports in the literature of limb movements evoked by stimulation of cerebellar cortex. By contrast, microstimulation in cerebellar cortex has been reported to suppress learned eyeblink movements (Hesslow and Ivarsson, 1994).

Given the well-established correlation between reductions in PN firing and learned movements, the issue of how PN firing contributes to movement is critical (Jirenhed et al., 2007; Lisberger, 1994; Medina and Lisberger, 2008). Our findings comparing the effects of stimulating ChR2 and Arch in PNs indicate that synchronous pauses in spontaneous firing of PNs in the simplex lobe are sufficient to elicit rapid forelimb movements. These results are in line with recent reports showing facial movements in response to ChR2-elicited synaptic inhibition of PNs (Heiney et al., 2014) and delayed postural movements elicited by ChR2 stimulation of PNs (Witter et al., 2013). Taken together the findings imply that populations of functionally related PNs may promote movement by synchronizing the inter-spike intervals on a fast time scale thereby disinhibiting DNNs to drive movements (Person and Raman, 2012).

Modulation of PN activity in conjunction with sensory stimuli induces motor memories

We also show that specific PN activity patterns, when paired with sensory stimuli, can drive robust, associative motor learning. Learned movements closely resemble the acutely evoked movements, consistent with the somatotopic map known to exist in cerebellum (Apps and Hawkes, 2009). The ChR2-induced artificial memories described here have a number of properties similar to natural forms of associative, cerebellar learning such as eyeblink conditioning and VOR plasticity (Raymond et al., 1996). These include the rate at which learning and extinction occur, the predictive timing of learned responses, and the registration between training interval and learned movement timing. The findings suggest a conserved mechanism by which cerebellar circuits can make use of sensorimotor information to prompt adaptive movements.

The learning we describe in response to Arch stimulation demonstrates that PN inhibition can trigger the formation of associative motor memories. The most likely circuit mechanism for this learning is that disinhibition of DNNs leads to an enhancement in the strength of mossy fiber collateral inputs to DNNs. Brain slice studies have identified an NMDA receptor-dependent form of long term potentiation of excitatory inputs to DNNs that is triggered upon release of hyperpolarization (Pugh and Raman, 2006). Consistent with these *in vitro* observations, our findings indicate that release from hyperpolarization, rather than a collateral climbing fiber input, can serve as the teaching signal driving such plasticity.

Ensuring precise timing of movements is a hallmark of cerebellar function, as demonstrated for classically conditioned eyeblinks (Ohyama and Mauk, 2001; Ohyama et al., 2006; Perrett et al., 1993). Pharmacological inactivation of the cerebellar cortex after eyeblink training does not abolish learned responses but leads to rapid eyeblinks with time courses that are no longer in

register with the training interval. The Arch-induced learning described here shows a similar profile, consistent with learning being confined to the deep cerebellar nucleus.

A unifying hypothesis accounting for optogenetic and normal forms of learning is presented in Figure 7. We posit that pairing ChR2 excitation of PNs with sensory stimuli leads to the development of sensory-evoked changes in both PN and DNN excitability, but that pairing Arch stimulation with sensory stimuli leads only to changes in DNN excitability. Thus, Arch training instructs bursts in DNN firing but not pauses in PN firing in response to conditioned sensory stimuli (compare Figures 7B & 7C). This would explain why learned forelimb movements are smaller after Arch training. The model also accounts for the differences in timing of learned movements (Figure 5), as well as the facilitation of effects of laser evoked movement observed by previously conditioned sensory stimuli (Figure 6).

Functional implications

By generating artificial motor memories through optogenetic training, we show that the effects of PN pauses on movement are augmented by learning, providing support for models of cerebellar learning that hypothesize learning-related circuit changes in the deep cerebellar nucleus (Miles and Lisberger, 1981; Perrett et al., 1993). Our results further show that disinhibition of DNNs is sufficient to drive associative memory formation. The fact that PN pauses can instruct memory formation provides proof of concept support for theories of cerebellar memory formation which propose that learning-related changes in the cerebellar cortex precede and may be necessary for memory consolidation in the deep cerebellar nucleus (Cooke et al., 2004; Kassardjian et al., 2005; Ohyama et al., 2006).

EXPERIMENTAL PROCEDURES

Animals

All animal procedures were performed in accordance with National Institutes of Health standards and were approved by the University of California, Los Angeles Institutional Animal Care and Use Committee. Male and female mice homozygous for L7-Cre (B6.129-Tg(Pcp2-cre)2Mpin/J, Jackson Labs) were crossed with either an animal homozygous for ChR2-eYFP (Ai32, B6;129S-*Gt(ROSA)26Sor*$^{tm32(CAG-COP4*H134R/EYFP)Hze}$/J, Jackson Labs) or Arch-eGFP (Ai35, B6;129S-*Gt(ROSA)26Sor*$^{tm35.1(CAG-aop3/GFP)Hze}$*/J,* Jackson Labs*)*. Subsets of animals at the end of behavioral experiments were perfused (4% paraformaldehyde) and their brains removed. Fluorescent images of the whole cerebellum were obtained at 14x using a Zeiss (Stereo Discovery V12) dissecting microscope and camera (Axiocam MRm) in order to examine and document the sites of chronic fiber placement in each mouse.

In vitro electrophysiology

Adult mice 23-34g (L7-Cre/Arch or ChR2) in weight were anaesthetized and decapitated. Parasagittal cerebellar slices (300 µm) were cut in an ice cold (4°C), low-sodium cutting solution using a vibratome (Leica VT-1000). Slices were incubated for ~30 min. at 35°C and allowed to sit at room temperature before electrophysiological recordings at ~34°C. Cutting and recording media were bubbled with 95% O_2 and 5% CO_2. The low-sodium cutting solution consisted of (in mM): 82.7 NaCl, 2.4 KCl, 1.4 NaH_2PO_4, 0.5 $CaCl_2$, 6.8 $MgCl_2$, 23.8 $NaHCO_3$, 65 sucrose, 23.7 dextrose and the recording solution consisted of (in mM): 119 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1 $MgCl_2$, 26 $NaHCO_3$, 25 dextrose.

Cells were visualized using an upright microscope (Zeiss Axioskop II) with a 40X water immersion lens using infrared differential interference contrast microscopy. Optical activation of

ChR2 or Arch was achieved using an LED light source (532 nM, THOR labs) projected through the epifluorescence pathway of the microscope. Pulses of light were either triggered directly by TTL signals from pClamp to the LED controller or through a signal generator (Master 8, AMPI) triggered by pClamp. For all graphs, optical stimulation was elicited at t=0 ms. Data was acquired using pClamp9 (Molecular Devices) at 50 kHz for intracellular recordings and 5-10 kHz for extracellular recordings. A Multiclamp 700B was used to record electrophysiological signals. In voltage clamp, the pipette and cellular capacitance (≥ 80%) were compensated for using onboard circuitry. The pipette solution for PN voltage and current clamp recordings contained (in mM): 126 $KMSO_3$, 10 KCl, 4 NaCl, 10 HEPES, 0.5 EGTA, 14 Tris-phosphocreatine, 2 MgATP, 0.4 NaGTP. Experiments were analyzed using custom macros written for Igor Pro (Taro Tools, Dr. Taro Ishikawa, https://sites.google.com/site/tarotoolsregister/).

Surgical procedures

All surgical procedures were performed under isofluorane (1%) anesthesia, and performed at least 2 days prior to either *in vivo* electrophysiological recording or behavioral manipulation. Animals were placed into a stereotaxic device and custom made head-bars (FabtoOrder) were glued to the skull using Vetbond (3M) and dental cement (Bosworth Co.). For *in vivo* electrophysiological recordings large craniotomies were made over the medial or left cerebellum. The exposed site was then filled over with a silicon based elastomer (Kwik-cast, WPI) that was easily removed just prior to recording. For behavioral experiments, chronically implanted optic fiber cannulas (Doric lenses), dipped in DiI (Sigma), were stereotaxically positioned (RC:6.25mm, ML:1.9mm, DV:2mm) into the brain through small craniotomies, and fixed into place using Meta-Bond (Parkell). Whole mount fluorescence visualization (see Figure S1) allowed posthoc localization of the fiber.

In vivo electrophysiology

Head-fixed animals were allowed to move in place freely on a spinning disk (Ware Flying Saucer). Optrodes constructed of fiber optic cannulas (Doric lenses) glued ~400 µm behind 1-5 MΩ Parylene-C insulated tungsten electrodes (A-M Systems) were inserted into craniotomies and vertically driven into the cerebellum using a micromanipulator (Sutter). Electrical signals were recorded with an Axopatch 200B amplifier (V-clamp mode) and data acquired in pClamp9 (50 kHz). A TTL controlled, 100 W diode pumped solid state laser (532 nm, Opto-Engine) was coupled through a patch cable (Doric Lenses) to the optrode to deliver brief pulses of light. Spike detection and data analysis were performed in Igor Pro 6 using custom macros (Tarotools).

Behavior

All animals were habituated to the behavioral setup for at least 4 days prior to training. Animals were head fixed but allowed to move freely on a spinning disk (Ware, Inc.). A TTL controlled 100 W, 532 nm diode laser (CNI Laser, Optoengine), launched into a patch cable and connected to the fiber optic cannula were used to activate either ChR2 or Arch within the forelimb region of the cerebellum for the indicated durations determined by TTL control. Power output per unit area, measured regularly from a pristine fiber segment identical to that implanted, ranged from 320 to 1910 mW/mm^2. These values are calculated by dividing total power (10-60 mW) by the cross sectional area of the 200 µm diameter optical fiber used in our study. The values used here compare well to values of 142 to 16,000 mW/mm^2 reported by Heiney et al (2014) for the 20-30 µm diameter fibers used in their study.

Optical stimulation was paired with a tone coordinated using custom routines written in LabView and controlled via TTL pulses from a NIDAQ board (National Instruments). Epochs of high speed video (200 f/s) were also synchronized via TTL and obtained with a Giga-E camera (Allied). Kinematic measurements were made offline using Custom LabView routines that allowed for the tracking of an IR reflective button (Mocap solutions) adhered to the mouse's wrist. Data were further analyzed using custom macros in Igor Pro 6 allowing infrequent tracking artifacts to be excised. A subset of the kinematic data were also analysed by MTrackJ in ImageJ and the results were identical to the automated LabView routine. All speed-vs-time traces were smoothed by a binomial function with a factor of 3. All error bars or shaded errors in figures represent S.E.M.

All individual trials free of movement within a period 500 ms prior the onset of tone onset were analyzed. Rejected trials included those in which movement speed exceeded 0.04 m/s during this 500 ms epoch prior to tone onset (baseline period) or trials in which movement speed exceeded 0.04 m/s within 50 ms after tone onset (startle period). On average this resulted in variable rejection rates of 35-70% of trials. In trials with tone and laser stimuli, learned responses (LRs) were judged to occur when movement speed exceeded 0.04 m/s in the time epoch from 50 ms after tone onset until laser onset; in tone alone trials LRs could occur from 50 ms following tone onset until tone offset which co-terminated with the laser pulse offset. To determine percent LR, the number of LR trials was divided by total number of analyzed trials (excluding rejected trials). We chose the term "learned response" rather than the more conventionally used term "conditioned response" to reflect the fact that LRs in this study are generated by optogenetic stimuli rather than by behavioral error signals and because LRs in ChR2- and Arch-trained animals are likely to result from distinct circuit mechanisms.

AUTHOR CONTRIBUTIONS

P.J.M., K.H.L., and T.S.O. designed experiments. P.J.M. performed *in vivo* and *in vitro* recordings, made behavioral kinematic measurements with S.A.J., and generated the histological reconstructions with A.M.B.R. and K.Y.C. K.H.L. performed behavioral learning experiments in Figures 3 - 5. A.M.B.R. performed the augmentation experiment in figure 6. K.Y.C. conducted behavioral measurements on control mice. R.E.S. designed the custom software for acquisition and analysis of the behavioral data. P.J.M. and T.S.O. wrote the manuscript with help from K.H.L. and comments from A.M.B.R. and K.Y.C.

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FIGURES AND LEGENDS

Figure 1

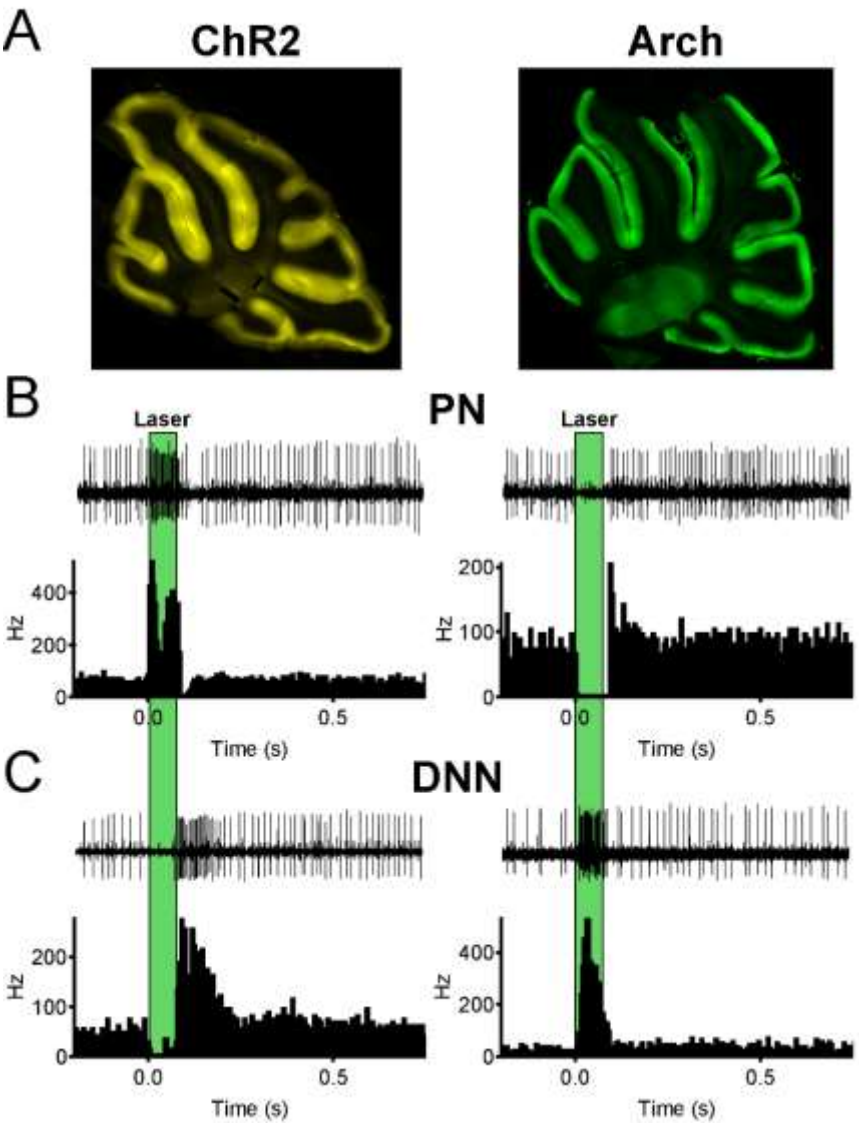

Figure III-1. Pauses in PN firing drive motor output. (A) Photomicrographs of parasagittal cerebellar sections indicating expression of ChR2-eYFP (left) and Arch-GFP (right) selectively in PNs. (B) *In vivo*, single unit optrode recordings from PNs in response to 75 ms activation of ChR2 (left) or Arch (right) in awake mice. Laser pulse duration is indicated by green boxes. Single extracellular traces are shown above the peri-stimulus time histograms (PSTH) for each cell. (C) *In vivo* recordings from DNNs in ChR2 (left) and Arch (right) mice in response to 75 ms light pulses.

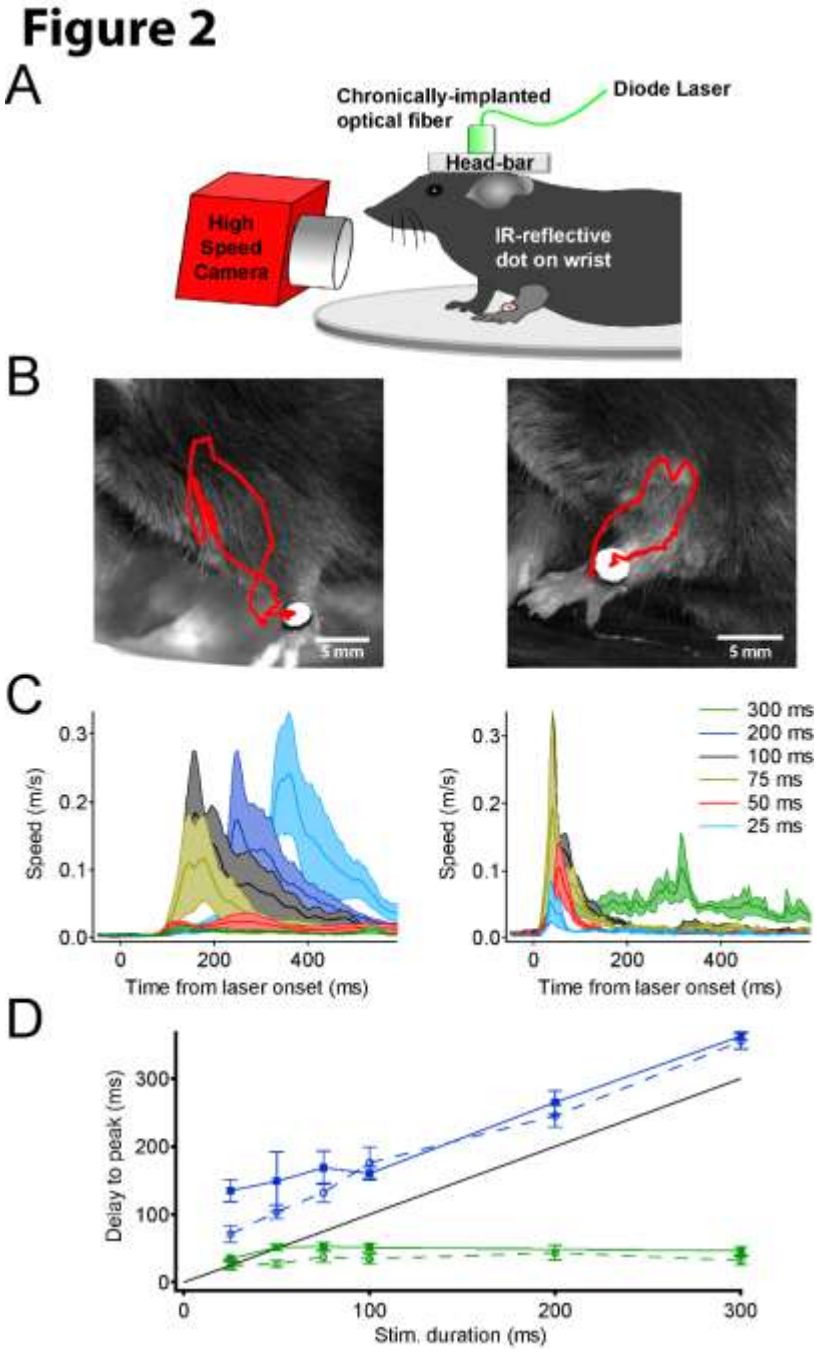

Figure III-2. Optogenetically elicited, rapid forelimb movements. (A) Illustration of head-fixed animal preparation used for behavioral studies. A high-speed video camera coupled with infrared illumination (IR) allowed movement of the wrist, tagged with an IR-reflective dot, to be tracked offline using motion tracking software. (B) The movement paths (red line) in response to 100 ms laser pulses delivered through the fiber optic chronically implanted in the forelimb region of the ipsilateral cerebellar cortex. ChR2 on left, Arch on right. (C) Mean forelimb speed versus time relative to the onset of laser pulses of the indicated durations. Note that in ChR2 mice (left) movement onset is time-locked to the end of laser illumination while in Arch mice (right) movement occurs independently of pulse duration with a fixed delay following illumination onset. (D) Delay to peak movement speed (solid lines) and peak DNN firing frequency (dotted lines) are plotted together as a function of laser pulse duration. Data are plotted for ChR2 (blue, n=7) and Arch (green, n=9) mice. Note that in ChR2 mice movement and peak firing occur with

a fixed delay from the end of the laser pulses (indicated by black line), whereas in Arch mice movement and peak DNN firing occur at a fixed delay from pulse onset.

Figure 3

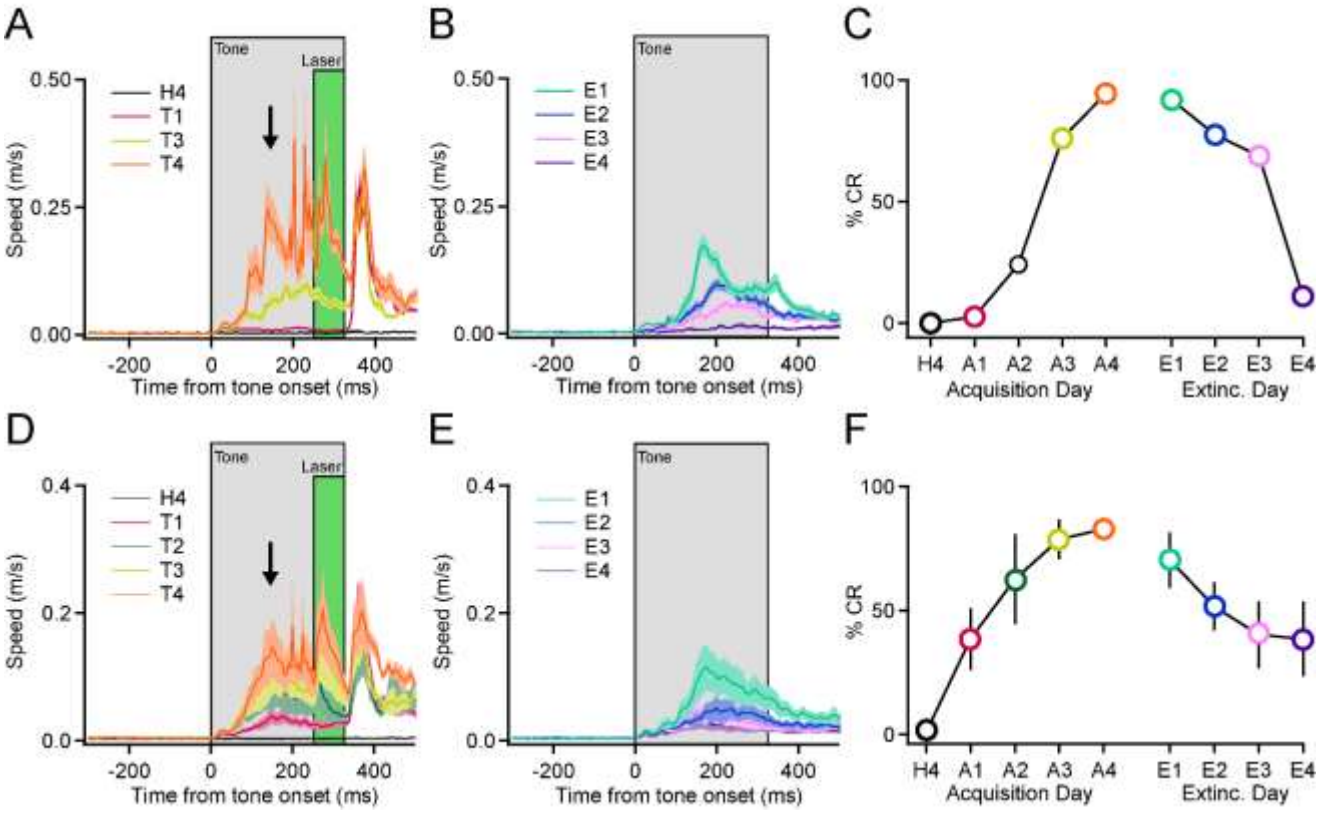

Figure III-3. Pairing ChR2 activation in PNs with auditory tones leads to predictive, tone-evoked forelimb movements. (A) Mean speed of wrist movement for a single animal on the last day of habituation and the indicated days of acquisition training (H4, habituation day 4; A1, 3, & 4, acquisition training days 1, 3 & 4). All analyzed trials are included and the lighter shading represents s.e.m. The timing of the tone and laser pulses are indicated with grey and green boxes, respectively, and the arrow indicates the unconditioned movement evoked by the laser pulse. Note the gradual appearance of tone-evoked movement with acquisition training and abolition of this movement with extinction training. (B) Mean movement speed during extinction training (E1 to 4 = extinction day 1 to 4) for the same animal. (C) Summary plot for this animal indicating percent LR across acquisition and extinction training. Colored symbols correspond to mean speed versus time traces in A and B. (D, E) Mean wrist speed profiles for four ChR2 mice

during acquisition (D), and extinction (E) Lighter shading indicates s.e.m. across animals . (F) Summary of percent LR+/- s.e.m. (n=4 mice) across days.

Figure 4

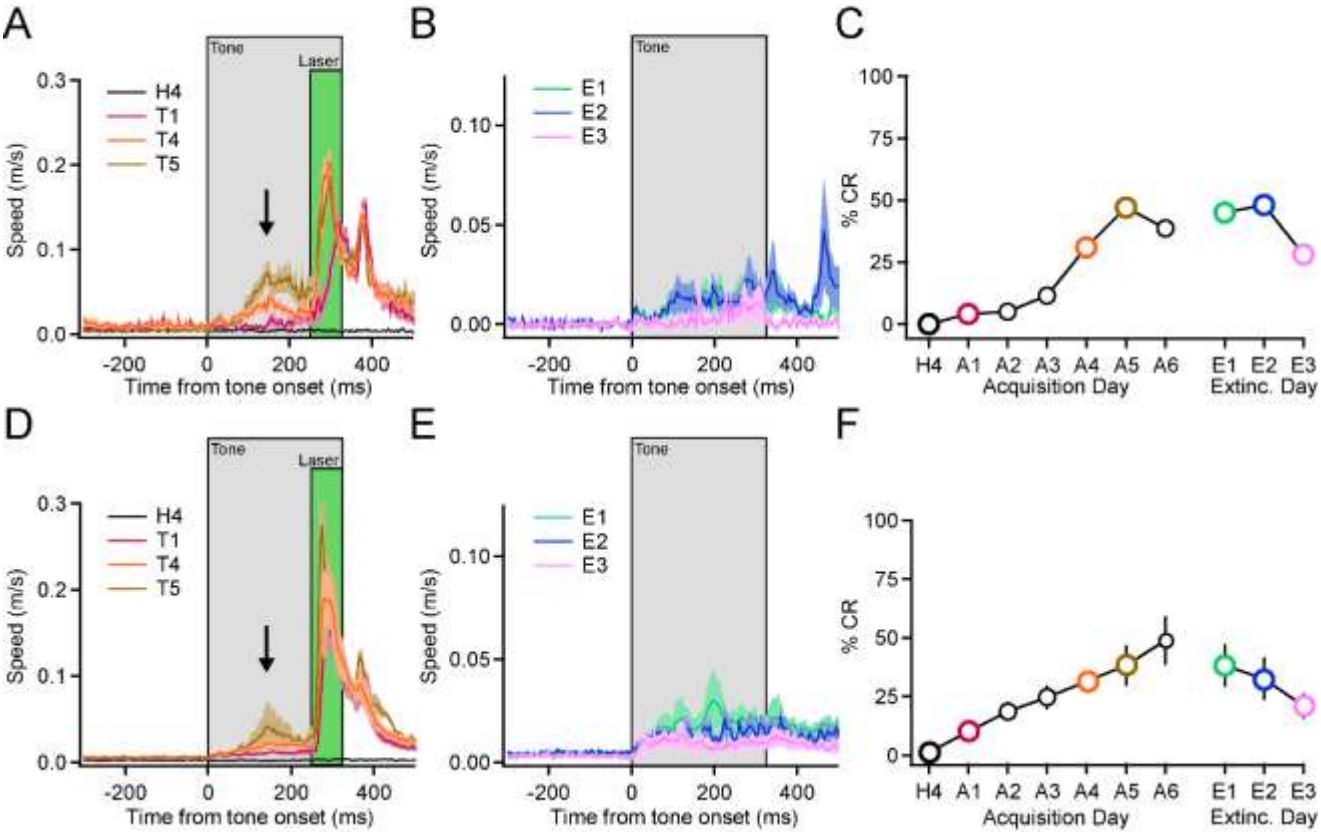

Figure III-4. Pairing Arch activation in PNs with auditory tones leads to predictive, tone-evoked forelimb movements. (A) Mean speed of wrist movement for a single animal on the last day of habituation and the indicated days of acquisition training (H4, habituation day 4; A1, 4, & 5, acquisition training days 1, 4 & 5). All analyzed trials are included and the lighter shading represents s.e.m. The timing of the tone and laser pulses are indicated with grey and green boxes, respectively, and the arrow indicates the unconditioned movement evoked by the laser pulse. Note the gradual appearance of tone-evoked movement with acquisition training and abolition of this movement with extinction training. (B) Mean speed during extinction training (E1 to 3 = extinction day 1 to 3) for the same animal. (C) Summary plot for this animal indicating percent LR across acquisition and extinction training. Colored symbols correspond to mean speed versus time traces in A and B. (D, E) Average wrist speed profiles for Arch mice during

acquisition (n=6 mice in D), and extinction (n=3 mice in E) Lighter shading indicates s.e.m. across animals. (F) Summary of percent LR+/- s.e.m. (n=2-6 mice) across days.

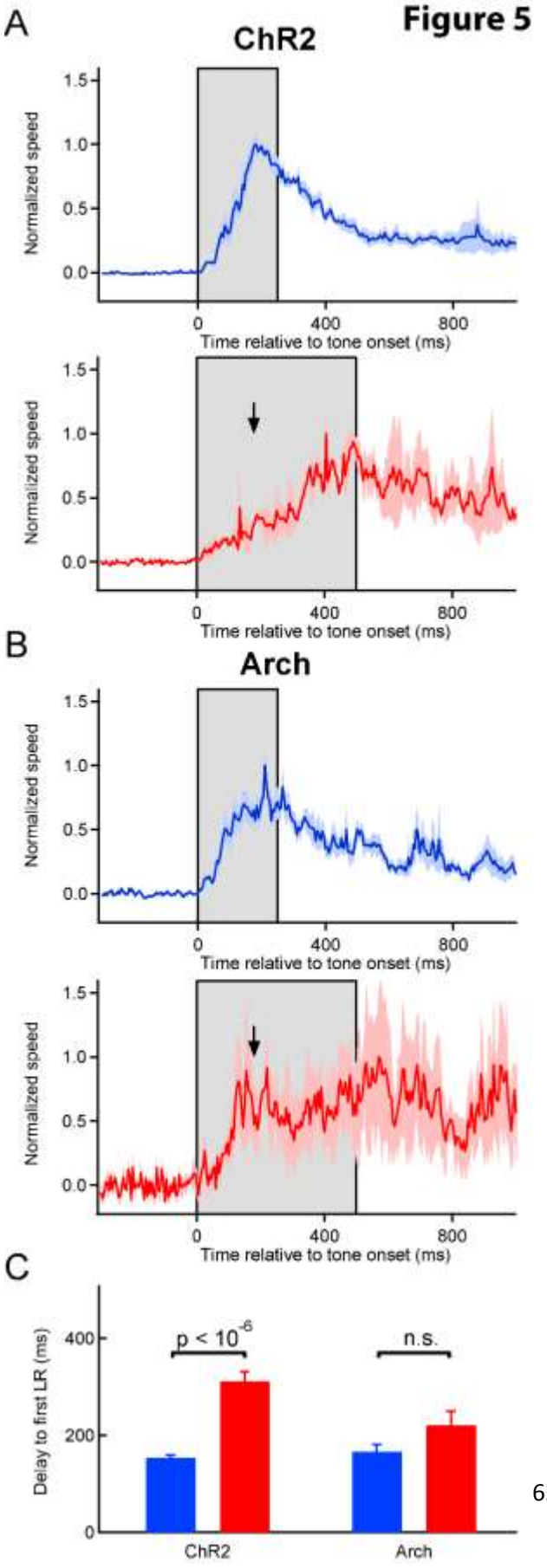

Figure III-5. ChR2 training, but not Arch training leads to well-timed learned movements.

(A) Average, normalized movement speed for ChR2 mice in tone-alone LRs occurring between acquisition day 3 and extinction day 1 trained with 250 ms (blue, 4 mice) or 500 ms intervals (red, 2 mice). Gray boxes indicate intervals between tone and laser pulse onset. Lighter shading represents ± s.e.m. across mice. Black arrows in 500 ms panels indicate the time of peak movement in corresponding 250 ms panels. (B) Movement speed in tone-alone LRs for Arch mice trained with 250 (blue, 7 mice) or 500 ms (red, 3 mice) intervals. (C) Mean ± s.e.m. of delays from tone onset to the first LR for all trials included in the averages in A and B. LR timing in ChR2-trained mice is significantly different between 250 and 500 ms intervals ($p<10^{-6}$, Mann Whitney U test), but not for Arch-trained mice (p=0.3, Mann Whitney U test).

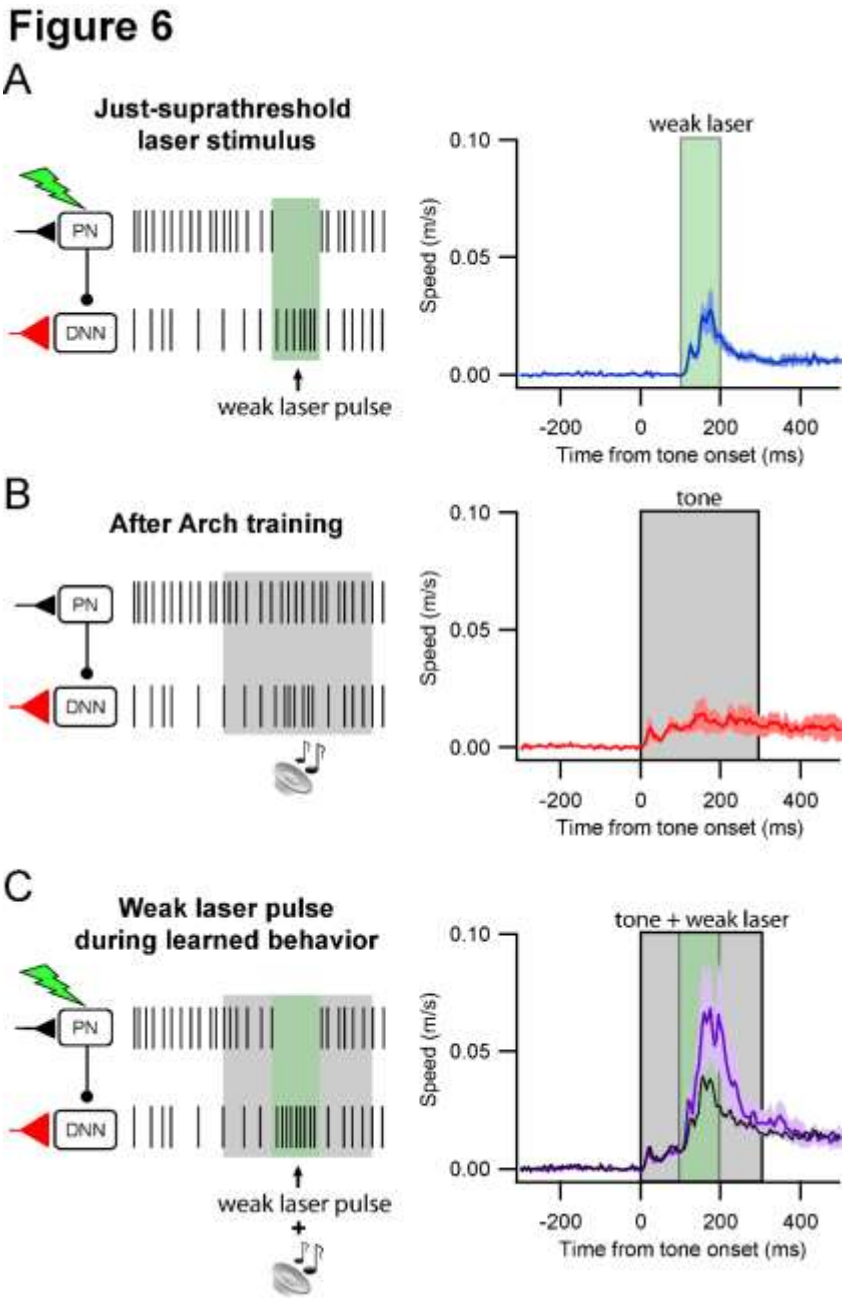

Figure III-6. Arch training alters the excitability of DNNs in a tone-dependent manner. Mice were associatively trained by pairing tones and Arch activation. Each panel indicates on the left the hypothesized effects on PN and DNN firing in response to the stimulus and on the right the mean movement speed ± s.e.m. for 3 mice. (A) Mean movements evoked by weak laser pulses. (B) Mean tone-evoked LRs after 4 days of training (C) Mean movements in response to the tone and weak laser pulse delivered simultaneously. Note that the resulting augmented LRs (purple) are larger than the arithmetic sum of the laser-evoked plus the learned movements (black).

Figure 7

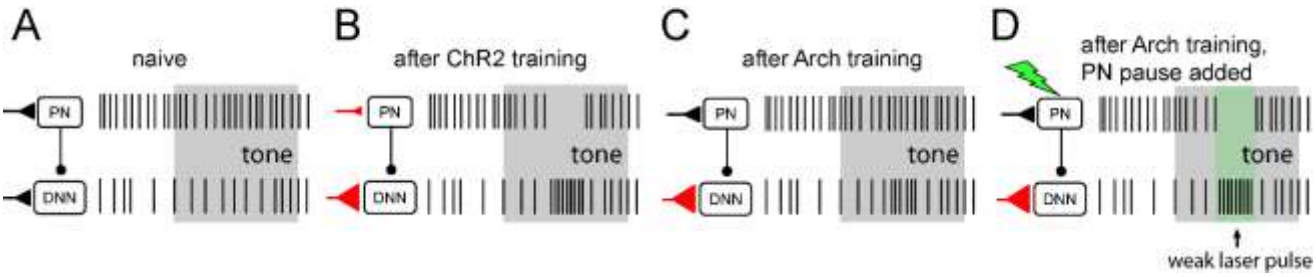

Figure III-7. Proposed circuit basis of ChR2- and Arch-driven learning. (A-D) Schematic of proposed model for associative learning in ChR2 and Arch animals. (A) Before learning, the tone (grey box) does not drive activity changes in PNs or DNNs. (B) After learning, PNs in ChR2 animals respond with tone-evoked pauses that disinhibit DNNs. Changes in synaptic strength of PN and DNN inputs (red triangles) are hypothesized to underlie learned changes in excitability. (C) After Arch-training, learning results only in increased excitability of DNNs to the conditioned response. (D) Weak Arch activation (green box), timed to the peak of the forelimb movement mimics disinhibition of DNNs, thereby producing stronger DNN-driven motor output.

CHAPTER IV: A CIRCUIT MECHANISM FOR EXTINCTION OF CLASSICALLY CONDITIONED FORELIMB MOVEMENTS IN MICE

SUMMARY

Our previous study established a form of classical conditioning where the unconditioned stimulus was optogenetically-triggered Purkinje neuron excitation. Like other forms of classical conditioning, such as eyelid conditioning, our opto-conditioning of forelimb movements displayed robust acquisition and extinction of conditioned responses. However, the mechanisms by which these opto-conditioned forelimb responses appeared and disappeared remained unclear. In order to determine the necessity of cerebellar activity for the conditioned response and, incidentally, test a widely hypothesized mechanism for cerebellum-dependent extinction, we used optogenetics to manipulate spontaneously firing Purkinje neurons in simplex lobe during opto-conditioned limb movements. By using high-speed videography to track limb kinematics with millisecond precision, we found that Purkinje neuron excitation during presentation of the conditioning stimulus rapidly suppressed conditioned responses. Moreover, this Purkinje neuron-mediated suppression of conditioned responses resembled the subsequent extinction of conditioned forelimb responses. These findings 1) imply that Purkinje neurons act as a gate on associative motor memories stored in the cerebellum and 2) corroborate a proposed mechanism of cerebellum-dependent extinction of classically conditioned skeletal muscle responses.

INTRODUCTION

Most classically conditioned behaviors exhibit a gradual decrease in their rate or amplitude of responding when the conditioning stimulus is repeatedly presented without the unconditioned stimulus (Medina et al., 2002a). This phenomenon is known as extinction and its mechanism remains unknown, despite having both plausible mechanistic suggestions ((Mauk and Donegan, 1997; Mauk and Ohyama, 2004; Robleto et al., 2004) and compelling electrophysiological evidence of proposed neural correlates (Jirenhed et al., 2007). In acquisition of a classically conditioned eyeblink, Purkinje neurons learn to pause their high rates of firing during the presentation of the conditioning stimulus which disinhibits their target cells in the interpositus nucleus (Heiney et al., 2014; Lee et al., 2015; Mauk and Donegan, 1997; Witter et al., 2013), which act as premotor neurons. A widely hypothesized mechanism of extinction proposes that the learned Purkinje neuron pauses that appear during acquisition disappear during extinction training (Jirenhed et al., 2007; Mauk and Donegan, 1997; Medina et al., 2002a). The reason this leads to extinction of the response is that without the relief of inhibition from the cerebellar cortex, the cells in the interpositus nucleus fail to respond to the conditioning stimulus with a disinhibitory burst of activity, which prevents a conditioned response. While pioneering studies for eyeblink have provided evidence for this mechanism (Hesslow, 1994), there is still a lack of detailed, purposeful exploration of the contributions of cerebellar cortex to the extinction of conditioned responses.

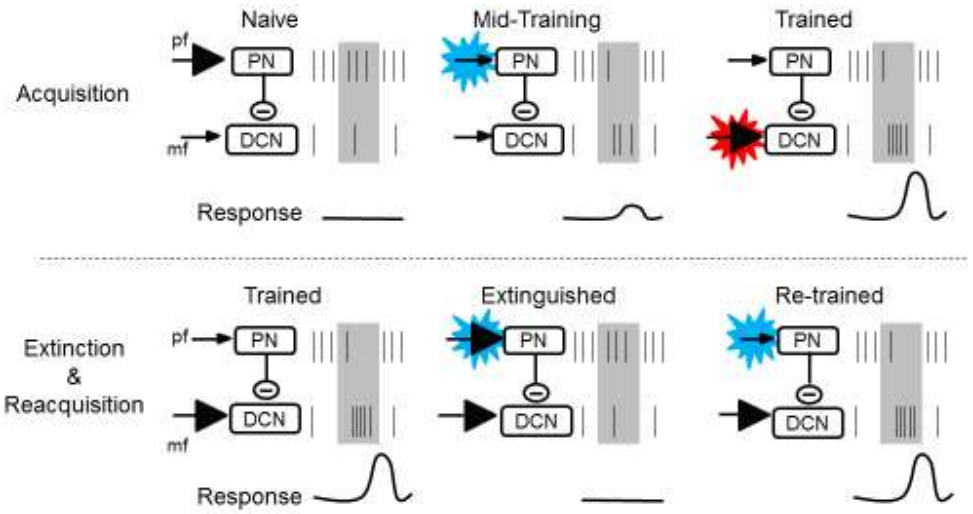

Figure IV-1. A cartoon hypothesis explaining how two sites of cerebellar plasticity can account for conditioned responses seen during acquisition, extinction, and re-acquisition. PF – parallel fiber, MF – mossy fiber, PN – Purkinje neuron, DCN – Deep cerebellar nuclei. The parallel and mossy fiber inputs are excitatory, whereas the Purkinje neuron input to the nuclear cell is inhibitory. Colored bursts denote plasticity, blue—parallel fiber plasticity, red—slower, extinction-resistant nuclear cell plasticity. Note that extinguished responses are hypothesized to be the result of increased ability of the parallel fiber to drive Purkinje neuron activity and mitigate the extinction-resistant mossy fiber plasticity.

A related question concerns whether extinction is the result of reversing the synaptic modifications made during extinction or is the result of new, inhibitory connections suppressing the modifications underlying acquisition (Mauk and Ohyama, 2004). There is little experimental evidence to distinguish between these two possibilities and, indeed, multiple authors have suggested both kinds of mechanisms apply (Kehoe and White, 2002; Mauk and Ohyama, 2004). However, given that acquisition produces new, excitatory learning in the cerebellar cortex and the cerebellar nuclei (Ohyama and Mauk, 2001; Ohyama et al., 2006) it is likely that Purkinje neurons must increase their conditioned stimulus-driven spiking response above their naïve firing rate in order to mitigate the new excitatory learning in the cerebellar nuclei (Medina et al., 2001).

To address these questions, we employed a behavioral paradigm that allowed us to directly manipulate Purkinje neuron firing while measuring forelimb kinematics with millisecond precision. We find that Purkinje neuron excitation during presentation of the conditioning

stimulus rapidly suppressed conditioned responses. Moreover, this Purkinje neuron-mediated suppression of conditioned responses resembled the subsequent extinction of conditioned forelimb responses. These findings 1) imply that Purkinje neurons act as a gate on associative motor memories stored in the cerebellum and 2) corroborate a proposed mechanism of cerebellum-dependent extinction of classically conditioned skeletal muscle responses.

METHODS

Animals

All animal procedures were performed in accordance with National Institutes of Health standards and were approved by the University of California, Los Angeles Institutional Animal Care and Use Committee. Male and female mice homozygous for L7-Cre (B6.129-Tg(Pcp2-cre)2Mpin/J, Jackson Labs) were crossed with an animal homozygous for ChR2-eYFP (Ai32, B6;129S-*Gt(ROSA)26Sor*$^{tm32(CAG-COP4*H134R/EYFP)Hze}$/J, Jackson Labs). Subsets of animals at the end of behavioral experiments were perfused (4% paraformaldehyde) and their brains removed.

Surgical procedures

All surgical procedures were performed under isofluorane (1%) anesthesia, and performed at least 2 days prior to behavioral manipulation. Animals were placed into a stereotaxic device and custom made head-bars (FabtoOrder) were glued to the skull using Vetbond (3M) and dental cement (Bosworth Co.). For behavioral experiments, chronically implanted optic fiber cannulas (Doric lenses), dipped in DiI (Sigma), were stereotaxically positioned (RC:6.25mm, ML:1.9mm, DV:2mm) into the brain through small craniotomies, and fixed into place using Meta-Bond (Parkell).

Behavior

All animals were habituated to the behavioral setup for at least 4 days prior to training. Animals were head-fixed but allowed to move freely on a spinning disk (Ware, Inc.). A TTL controlled 100 mW, 532 or 473nm diode laser (CNI Laser, Optoengine), launched into a patch cable and connected to the fiber optic cannula was used to activate ChR2 within the forelimb region of the cerebellum for the indicated durations determined by TTL control. Power output per unit area, measured regularly from a pristine fiber segment identical to that implanted, ranged from 320 to 1910 mW/mm^2. These values are calculated by dividing total power (10-60 mW) by the cross

sectional area of the 200 µm diameter optical fiber used in our study. The values used here compare well to values of 142 to 16,000 mW/mm^2 reported by Heiney et al (2014) for the 20-30 µm diameter fibers used in their study.

Optical stimulation was paired with a tone or a blue LED coordinated using custom routines written in LabView and controlled via TTL pulses from a NIDAQ board (National Instruments). Epochs of high speed video (200 f/s) were also synchronized via TTL and obtained with a Giga-E camera (Allied). Kinematic measurements were made offline using Custom LabView routines that allowed for the tracking of an IR reflective button (Mocap solutions) adhered to the mouse's wrist. Data were further analyzed using custom macros in Igor Pro 6 allowing infrequent tracking artifacts to be excised. All speed-vs-time traces were smoothed by a binomial function with a factor of 3. All error bars or shaded errors in figures represent S.E.M.

Identification of conditioned responses was automated through custom Igor procedures. We rejected those trials where we could not determine the presence or absence of a conditioned response. Rejected trials included those in which movement speed exceeded 0.04 m/s during the 500 ms prior to tone onset (baseline period) or trials in which movement speed exceeded 0.04 m/s within 50 ms after tone onset (startle period). On average this resulted in variable rejection rates of 35-70% of trials. In trials with tone and laser stimuli, conditioned responses (CRs) were judged to occur when movement speed exceeded 0.04 m/s in the time epoch from 50 ms after tone onset until laser onset; in tone alone trials CRs could occur from 50 ms following tone onset until tone offset which co-terminated with the laser pulse offset. To determine percent CR, the number of CR trials was divided by total number of analyzed trials (excluding rejected trials).

RESULTS

In order to test the hypothesis that extinction is the result of Purkinje neurons learning to increase their firing rate, we examined the effect of directly increasing Purkinje neuron firing rate during opto-conditioned forelimb responses by exploiting Cre-conditional transgenic mice to direct expression of the excitatory opsin ChannelRhodopsin-2 fused to eYFP (ChR2). In a previous study, we characterized the resulting mouse line by verifying both that the expression of ChR2 was selective for Purkinje neurons throughout the cerebellum and that light-evoked responses and fluorescence were absent from other types of neurons in the cerebellar cortex such as molecular layer interneurons, granule cells, and Golgi cells.

Conditioning Timeline

In order to test the prediction that learned forelimb movements will be suppressed with ChR2-mediated Purkinje neuron excitation, we trained mice with the following conditioning timeline:

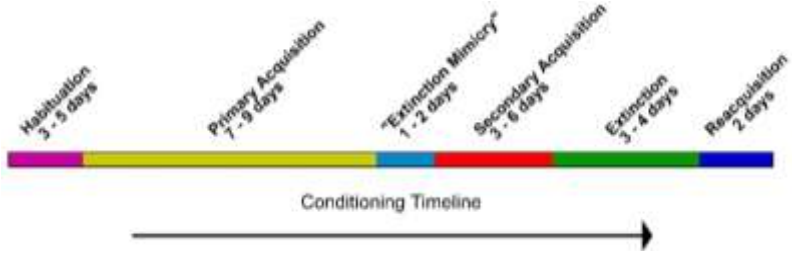

Figure IV-2. Conditioning timeline. An extended acquisition period increased the likelihood of a forelimb motor command during the subsequent extinction mimicry experiments. Following a second period of acquisition, extinction experiments were carried out to allow a direct comparison between mimicked and natural extinction responses. Subsequent re-acquisition experiments tests for the presence of residual, latent plasticity.

This timeline included acquisition, extinction, and re-acquisition conditioning procedures interspersed with a long laser procedure and a secondary acquisition procedure. Four out of twelve mice displayed conditioned forelimb responses elicited by the conditioning stimulus (either a 2kHz or a blue LED, see Methods section) at the end of the primary acquisition phase. These are the mice whose results we report.

Opto-conditioned Mice Exhibit Robust, CS-driven Forelimb Responses

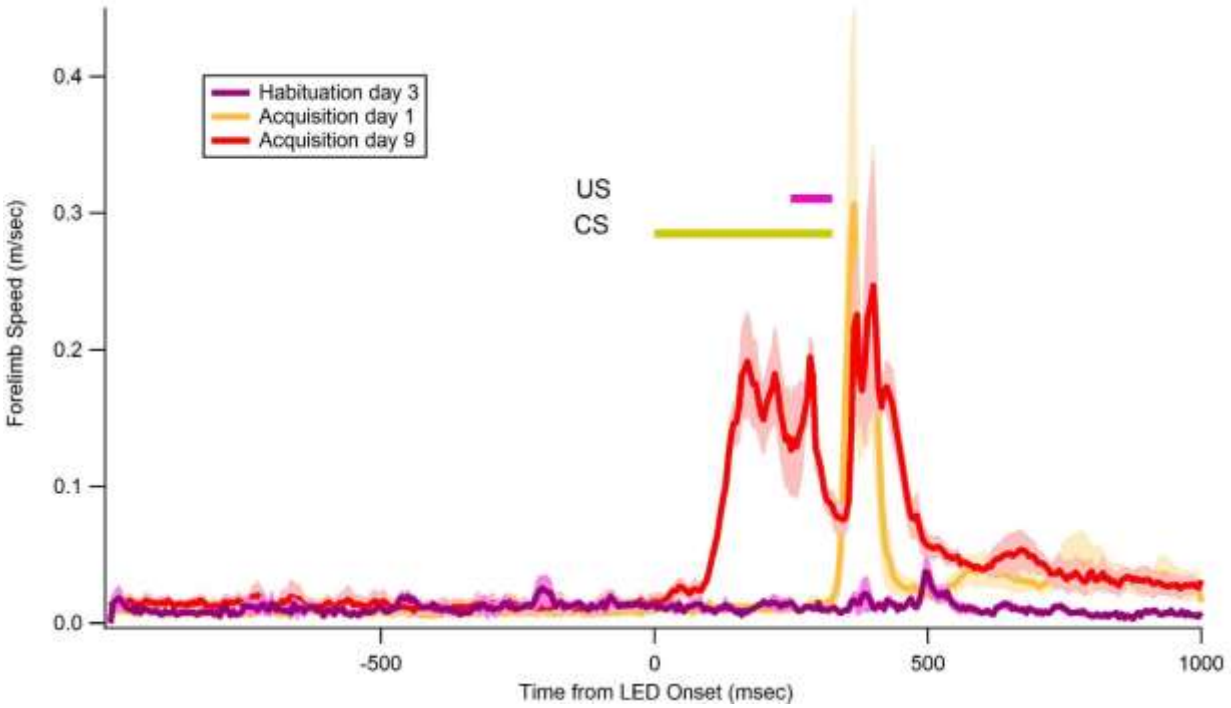

Figure IV-3. Mean speed (± s.e.m. indicated by lighter shading) of forelimb movement across all animals and across all trials on indicated days of training. All analyzed trials are included. The timing of the CS (325 ms) and laser ("US", 75 ms) pulses are indicated with gold and pink lines, respectively. Note the large difference in CS-elicited movement between the first and last day of acquisition training.

Studies of cerebellum-dependent associative learning suggest that Purkinje neurons modify their synapses in response to error-driven teaching signals and consolidate these changes by instructing synaptic modifications in their target cerebellar nuclear cells (Maiz et al., 2012; Medina and Lisberger, 2008; Nguyen-Vu et al., 2013; Raymond et al., 1996). Typically, the error-driven teaching signal is provided by climbing fiber activity (Gilbert and Thach, 1977; Kim et al., 1998; Simpson and Alley, 1974) although, in principal, direct depolarization of the Purkinje neuron dendrites could act as a substitute (Crepel and Jaillard, 1991), particularly for LTD of parallel fiber inputs.

Whether by depolarization-induced parallel fiber LTD or Purkinje neuron-instructed changes in the deep cerebellar nuclei, direct Purkinje neuron excitation is an important aspect of cerebellum-dependent motor learning. In order to define the extent to which Purkinje cell depolarization is sufficient to induce learning, we used a delayed conditioning protocol with ChR2-mediated Purkinje neuron depolarization as the unconditioned stimulus (Lee et al., 2015). We had the stimulus mimic the spatiotemporal dynamics of climbing fiber-mediated Purkinje neuron microzone depolarizations seen with traditional unconditioned stimuli and/or error-elicited signaling (Bell and Kawasaki, 1972; Ghosh et al., 2011; Lang et al., 1999; Marshall and Lang, 2009; Mukamel et al., 2009; Ozden et al., 2009).

ChR2 mice were trained by pairing a conditioning stimulus ("CS", either a 2 kHz auditory tone or a blue LED) with 75 millisecond pulses of laser illumination delivered 250 milliseconds after the CS onset. Each day, training consisted of 90 CS/laser pairings and 10 interleaved CS-alone trials, this continued for between 7 and 9 days. Conditioning with Purkinje neuron depolarization as the US produced robust, CS-driven movements when compared to the movement during the habituation phase and the naïve responses to the CS on the first day of acquisition (Fig. 2). Peak paw speeds during late-stage acquisition ("A9", 0.36 ± 0.01 m/sec, n = 189 trials) had larger averages than those from the first day of acquisition ("A1", 0.018 ± 0.002 m/sec, n = 296 trials). Kinematic analysis showed that these conditioned forelimb responses were similar to those elicited by the laser pulse, but occurred earlier in time, as expected for predictive cerebellar learning (Medina, 2011; Ohyama et al., 2003). Thus, Purkinje neuron depolarization is sufficient for conditioning

Animals exhibit slower mean speed with a long-laser pulse superimposed over the CS

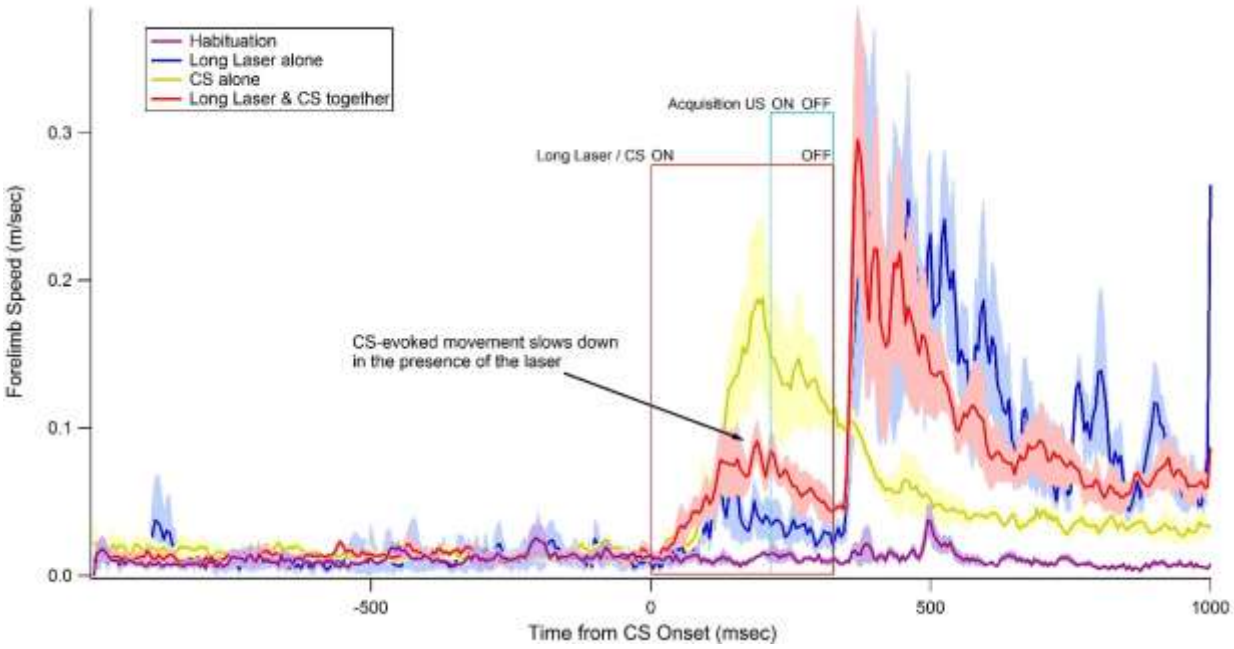

Figure IV-4. Mean speed (± s.e.m. indicated by lighter shading) of forelimb movement for all animals and across all trials for the indicated condition ("Habituation", from the 3rd day of habituation; "Long Laser alone", long-duration laser pulse presented without the CS; "CS alone", conditioning stimulus presented without the long-duration laser pulse; "Long Laser & CS together", the conditioning stimulus and long-duration laser pulse are presented simultaneously with identical onset and offsets). All analyzed trials are included. The timing of the CS and the laser pulse (both 325 ms) is indicated with a red box. The timing of the laser pulse during acquisition (75 msec) is indicated with a light blue box. Note the arrow indicates a diminished response in the "Long Laser & CS together" condition relative to the "CS alone" condition.

A widely hypothesized mechanism for extinction of cerebellum-dependent classical conditioning suggests that a gradual increase in Purkinje neuron activity suppresses the conditioned response (Jirenhed et al., 2007; Mauk and Donegan, 1997; Medina et al., 2002a) and masks extinction-resistant plasticity that developed in the cerebellar nuclei during acquisition (Medina et al., 2001). The increase in Purkinje neuron activity reflects a shift towards greater excitatory than inhibitory input due to enhanced parallel fiber strength (Medina et al., 2002a) which consequently enhances depolarization of the Purkinje neuron. In effect, this widely hypothesized mechanism suggests extinction is the result of CS-driven parallel fibers depolarizing their Purkinje neurons enough to suppress the conditioned response.

In order to test the hypothesis that Purkinje neuron depolarization mediates extinction, we defined the extent to which ChR2-driven Purkinje neuron depolarization can prevent the performance of opto-conditioned forelimb movement. Following acquisition, we supplemented the presentation of the CS with a ChR2-mediated Purkinje neuron depolarization in half of the trials of a CS-alone extinction protocol (Fig. 3). Because the laser pulse onset and offset matched that used for the conditioning stimulus, the ChR2 depolarization mimicked the spatiotemporal dynamics of the hypothesized parallel fiber-mediated Purkinje neuron depolarization underlying extinction.

In trials where the CS was presented on its own, the peak paw speed during the first 250 milliseconds of the CS was (0.35 ± 0.02 m/sec, n = 93 trials). Yet in trials where the CS was supplemented with a long-duration laser pulse, the peak paw speed during the first 250 milliseconds of the CS was much slower (0.20 ± 0.01 m/sec, n = 90 trials) and this difference was statistically significant (one-tailed, Mann-Whitney U test, $z = 4.47$, $p < 0.0001$). This result indicates that depolarization-mediated increases in Purkinje neuron activity hinders opto-conditioned forelimb responses.

In order to account for long-duration laser pulses affecting paw speed on their own, we recorded several trials of the animals responding to these long laser pulses just prior to the extinction mimicry experiments. The peak paw speed in these long laser pulse trials was (0.11 ± 0.02 m/sec, n = 12 trials), which indicates the presence of responses during the long-duration laser pulse being played on its own. Relative to peak paw speeds from trials in which the CS and long laser pulse were played together (0.20 ± 0.01 m/sec, n = 90 trials), these during-laser responses had different peak paw speeds (two-tailed, Mann-Whitney U test, $z=2.45$, $p = 0.0143$). This suggests that although there are residual movements during the “extinction mimicry” trials, the movements generated by the laser alone do not fully account for them.

Animals do not exhibit adaptation to the long-laser pulse trials

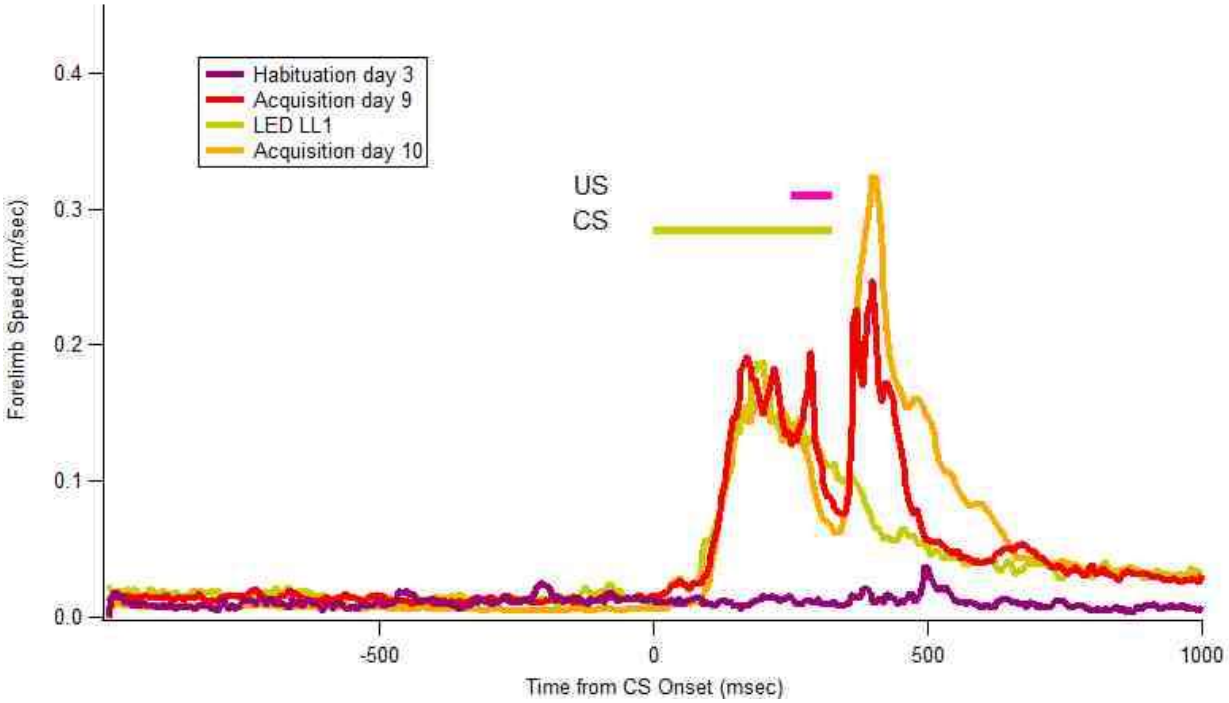

Figure IV-5. Mean speed of forelimb movement for all animals and across all trials for the indicated condition ("Acquisition Day 9", the last day of the primary phase of acquisition just prior to the extinction mimicry experiment; "LED LL1", the conditioned responses to the CS alone on the day of the extinction mimicry experiment; "Acquisition day 10", the first day of the secondary phase of acquisition following the extinction mimicry experiment. The time course of the conditioning stimulus and laser pulse are indicated with a gold and pink line, respectively. Note that the conditioned forelimb responses have similar time courses during the first 250 msec of the conditioning stimulus.

Because Purkinje cell depolarization can influence movement execution and motor memory formation, the long laser pulses may have promoted behavioral adaptations. If such adaptations took place, they should be present in the first few trials following a transition back to the delayed conditioning protocol.

In order to determine the extent of adaptation to the long laser pulse, we examined the learned forelimb movements before and after the long laser pulse experiments. There were no obvious differences between the paw speed time-courses on the last day of acquisition before the long laser pulse experiments and the first day of acquisition following the long laser pulse experiment. However, comparison of the peak speeds late in the primary phase of acquisition

and early in the secondary phase of acquisition revealed a moderate difference that was statistically significant (two-tailed, Mann-Whitney U test, $z = 4.32$, $p < 0.0001$) and indicated that peak speeds late in primary acquisition were somewhat faster (0.36 ± 0.01 m/sec vs. 0.24 ± 0.02 m/sec). Indicating that any effect of the altered CS-US contingency during long laser pulse experiments on the conditioned forelimb response was moderate, albeit extant.

Opto-conditioned Animals Exhibit Extinction of Forelimb Responses

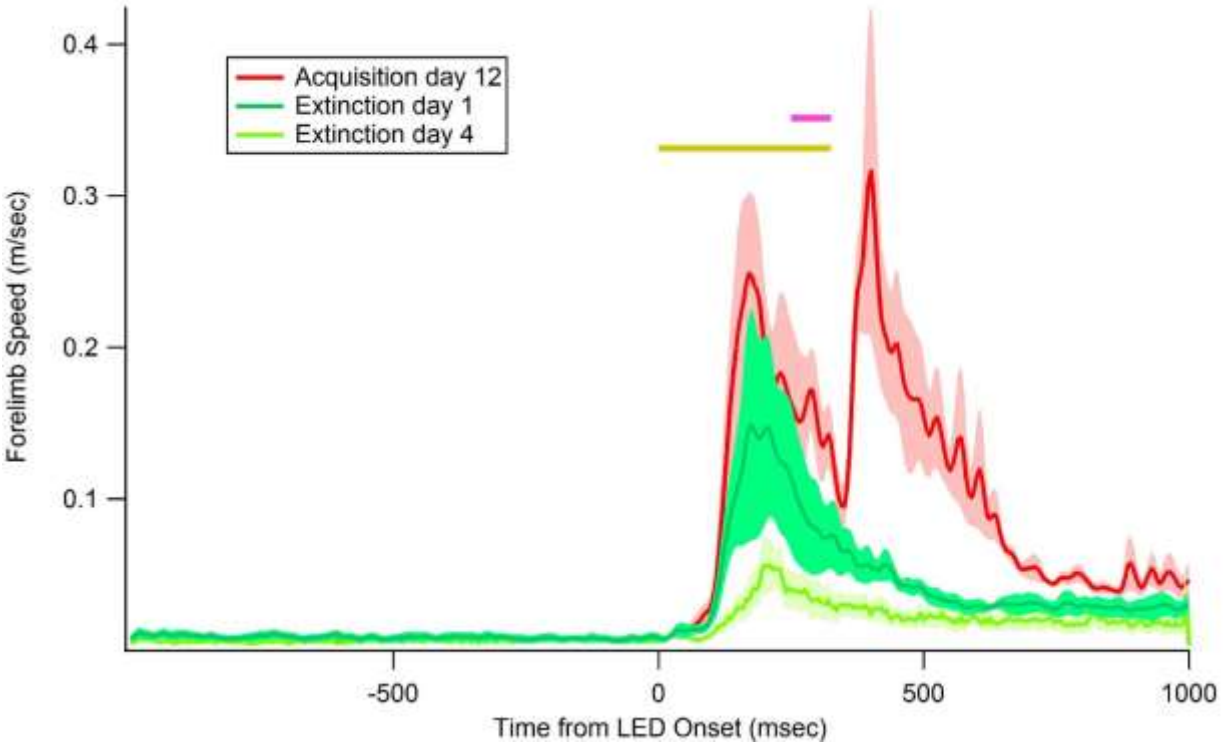

Figure IV-6. Mean speed (± s.e.m. indicated by lighter shading) of forelimb movement for all animals and across all trials for the indicated condition ("Acquisition Day 12", the last day of the secondary phase of acquisition just prior to extinction; "Extinction day 1" and "Extinction day 4" are the first and last days of extinction, respectively. In the extinction procedure, the conditioning stimulus (325 ms duration) is presented without the laser pulse for 100 trials daily. The time course of the conditioning stimulus is indicated with a gold line. A reminder of the timing of the laser pulse during acquisition (75 msec) is indicated with a pink line. Note that the forelimb speed is greatly diminished by the last day of extinction relative to the response seen during acquisition.

After the long laser pulse experiments, a second phase of acquisition ensued in order to re-establish the original CS-US contingency and to dilute any potential kinematic adaptation to the long laser pulse. The net effect of the second period of acquisition was that the patterns of

neural activity underlying the responses in the long laser pulse experiments and the CS-alone extinction experiments, in principle, act on similar initial behavioral conditions and similar states of cerebellar plasticity.

If ChR2-mediated Purkinje neuron excitation maintains conditioned forelimb responses by essentially acting as a substitute for US-elicited climbing fiber-mediated Purkinje neuron depolarizations, then ChR2-mediated Purkinje cell excitation should be necessary for maintaining the conditioned forelimb responses. Thus, a CS-alone extinction procedure is suitable for determining whether the learned forelimb movement depends on the continued presence of the ChR2-mediated Purkinje cell excitation.

In order to determine the necessity of the ChR2-mediated Purkinje cell depolarization in maintaining the conditioned forelimb responses, we withheld the ChR2 stimulus and presented the CS alone, as is done in traditional extinction procedures (Jirenhed et al., 2007; Medina et al., 2001; Medina et al., 2002a). Over a period of four days, the forelimb responses diminished in their speed time course (Fig. 5). The peak speed on the last day of acquisition was (0.43 ± 0.01 m/sec, n = 169 trials). By the final day of extinction, the peak speed during the first 250 msec of the CS was (0.095 ± 0.009 m/sec, n = 231 trials), indicating much slower movements at the end of extinction compared to the end of acquisition, and this difference was statistically significant (one-tailed, Mann-Whitney U test, $z=13.65$, $p < 0.0001$). In general, the gradual disappearance of the forelimb responses resembled that of other extinction results.

Reinstating the Laser Pulse Reinforces CS-driven Responses Faster than Original Learning

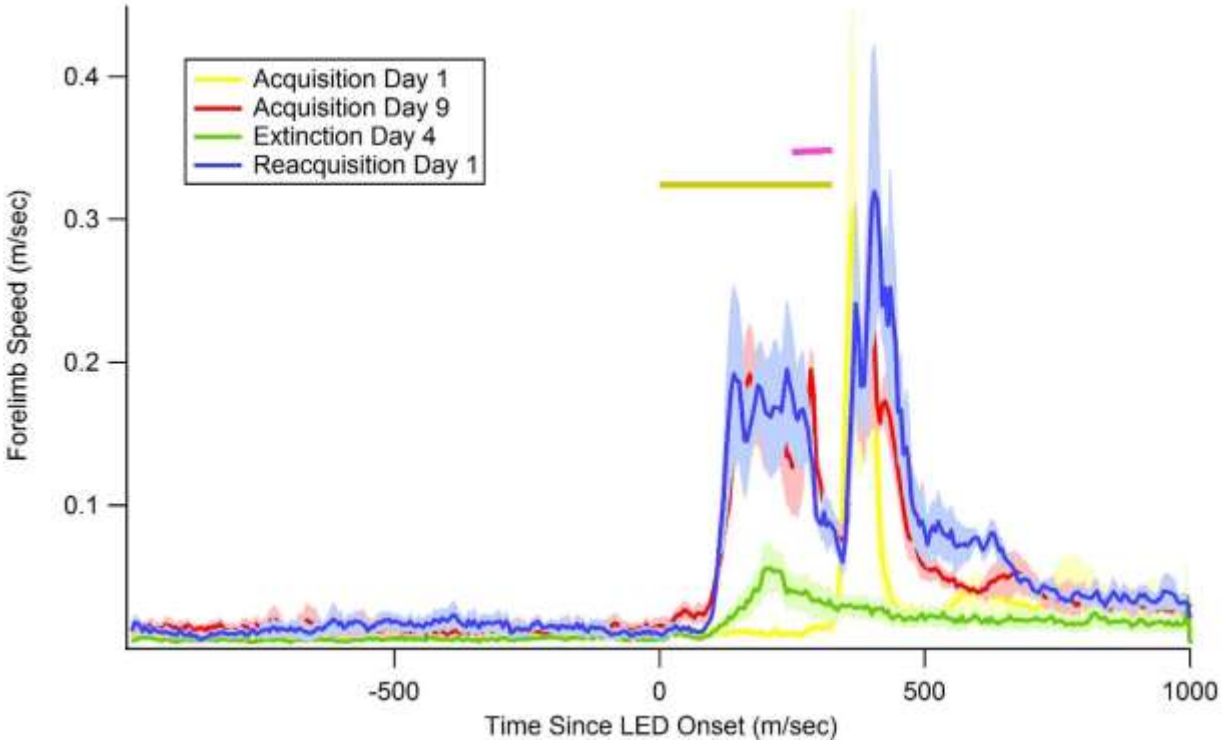

Figure IV-7. Mean speed (± s.e.m. indicated by lighter shading) of forelimb movement for all animals and across all trials for the indicated condition. All analyzed trials are included. The timing of the CS (325 msec) and the laser pulse (75 msec) are indicated with gold and pink lines, respectively. The laser pulse was not presented on extinction trials. Note the similar time course for late-stage acquisition and early-stage re-acquisition.

It is possible that erasure—and not suppression—of the motor memory mediates the extinction of the learned response. If this were true, a period of re-acquisition should reinforce a learned forelimb movement at the same rate as the original acquisition. Alternatively, the period of re-acquisition could reinforce learned responses at a faster rate than the original period of acquisition. This phenomenon is termed “savings” and is typically interpreted to mean that extinction is not the result of erasing the association but is instead suppressing the association with new learning. This is consistent with a mechanism where extinction-resistant synaptic modifications in the deep cerebellar nucleus are masked by inhibition from Purkinje neuron

activity. In this case, the period of re-acquisition could reinforce learned responses at a faster rate than the original period of acquisition.

If savings is evident, it makes comparing the extinction trials and long laser pulse trials more appropriate since both are conceivably mediated by a CR suppression mechanism.

In order to increase our confidence that a suppression mechanism mediates the extinction of the learned response, we initiated a re-acquisition period following extinction. On the first day of re-acquisition, the peak paw speeds were (0.29 ± 0.02, n = 163 trials) which, when compared to peak speeds late in the primary phase of acquisition (0.36 ± 0.01, n = 189 trials), were not significantly different from each other (two-tailed, Mann-Whitney U test, $z=1.06$, $p = 0.2891$). This indicates that the forelimb responses returned to late-stage acquisition speeds on the first day of re-acquisition. These results are consistent with the idea that extinction was the result of new learning rather than an unlearning of the adaptations during acquisition and that there is extinction-resistant residual plasticity that enhances the relative rate of re-acquisition.

DISCUSSION

Here we further explored a new type of classical conditioning in which the unconditioned stimulus is an optogenetic stimulus, in this case a rapid ChR2-mediated Purkinje neuron depolarization. This novel paradigm allows us to implant and modulate artificial motor memories that have compelling similarities with traditional classical conditioning. We combined our opto-conditioning approach with high-speed videography and motion tracking to measure the expression of these artificial motor memories at a millisecond timescale in the form of limb kinematics.

Because of the temporal precision and cell-specificity of stimulation afforded by our optogenetic approach to classical conditioning, we were able to mimic both climbing fiber-mediated Purkinje neuron depolarizations as well as parallel fiber-mediated Purkinje neuron depolarizations to reveal the contributions of the cerebellum and related downstream structures to conditioned forelimb responses. In particular, this allowed us to test a widely hypothesized circuit mechanism explaining the phenomenon of extinction in classical conditioning of skeletal muscle. While a previous study has presented similar results using electrical stimulation and eyeblinking (Hesslow, 1994) ours is the first to employ optogenetics to study the specific contributions of Purkinje neurons to extinction of conditioned forelimb responses.

In general, our results are consistent with the idea that extinction is the result of increases in CS-evoked Purkinje neuron firing which masks an extinction-resistant component of the motor memory. This extinction-resistant component could be plasticity in the deep cerebellar nuclei, which would be consistent with other reports (Lee et al., 2015; Medina et al., 2001; Ohyama and Mauk, 2001; Raymond et al., 1996).

Our study has caveats, and they are listed below:

Regarding ChR2 acquisition: Although the immediate effect of the laser is to depolarize Purkinje cells, we cannot rule out a possible laser-offset, burst-mediated teaching signal in the cerebellar nuclei. We also cannot rule out UR-induced proprioceptive feedback acting as an error signal, which could influence learning, perhaps even through extra-cerebellar pathways. Finally, we cannot rule out disinhibition of inferior olive during the laser pulse, which produces climbing fiber activity (Chaumont et al., 2013).

Regarding incomplete suppression: The residual movement during extinction mimicry trials could be due to an incomplete suppression of the nuclear cells. However, this is unlikely considering the laser power (320 to 1910 mW/mm^2).

The residual movement could be entirely due to the laser alone, since the laser does induce small movements during the pulse. For this not to be the case, the CR would have to exceed the small amount of movement seen during the laser alone. Statistical tests showed that CRs did in fact exceed the small amount of movement induced by the laser alone.

The laser alone may induce a co-contraction of the biceps and triceps through an unknown mechanism.

Alternatively, training might engage extra-cerebellar plasticity mechanisms. In this case, conditioned responses could conceivably originate from extra-cerebellar pathways such as M1 or SC. PN depolarization could still suppress an extra-cerebellar CR by “dis-facilitating” brainstem motor nuclei with convergent cerebellar and extra-cerebellar input. This would increase the threshold for activating the brainstem motor nuclei yet not necessarily prevent movement. If this were true, the results presented would still be interesting evidence of the role of the cerebellar contributions to descending motor control.

Regarding adaptations to the long laser + CS trials: Adaptations could result from several consequences of a long laser pulse super-imposed over the CS. For example, it alters the

timing of the CS→PN depolarization relationship established during acquisition; it alters the training-to-catch trial ratio from 9:1 to 1:1; it may produce error-related proprioceptive feedback in response to perturbed CR execution; it may disinhibit the inferior olive. Any one of these aspects or a combination thereof could have led to the observed difference in peak speeds for the end of the primary acquisition and beginning of the secondary acquisition.

*Regarding the possibility that savings reflects incomplete erasure of the motor memory rather than suppression of the motor memory: T*he animal may have learned faster because its response to the CS and the ChR2-stimulus have changed since the original acquisition period. An enhanced response to the CS indicates that the animal learned at the same rate but had a "head start" on its original acquisition period. An enhanced response to the ChR2-stimulus indicates a possibly more effective teaching signal, which means that the animal learned at a faster rate because of a better teaching signal and not because of latent plasticity.

Despite these caveats, we have made an important contribution to the field by increasing our understanding of the role of the cerebellum in forelimb motor control. The increased cell-type specificity and temporal precision of our approach is an advantage over earlier approaches (Hesslow, 1994; Kolb et al., 1997). Our findings 1) imply that Purkinje neurons act as a gate on associative motor memories stored in the cerebellum and 2) corroborate a proposed mechanism of cerebellum-dependent extinction of classically conditioned skeletal muscle responses.

CHAPTER V: CONCLUSION

That David Marr and James Albus independently and nearly simultaneously proposed very similar explanations for the cerebellum's role in motor learning was no coincidence. The stage had already been set by the behaviorists, who had established the principal features of associative learning, the theoreticians, who had suggested simple learning rules by which neural nets could build associations, and the physiologists and anatomists, who had compiled detailed evidence of structure and activity of the cerebellum. The cerebellar microcircuitry has continued to inspire neuroscientific explanations of motor learning, timing, and performance, and likely will continue to do so for quite some time.

In Chapter III, we concluded that forelimb movements can be initiated by bursts of activity in the cerebellar nuclei and that these burst-mediated forelimb movements are augmented by learning. This is consistent with the idea that learning-related in the deep cerebellar nuclei underlie associative motor memory formation. Furthermore, the fact that Arch-induced pauses in Purkinje neurons could instruct plasticity supports the notion that learning-related changes in the cerebellar cortex precede and may be necessary for memory consolidation in the deep cerebellar nucleus.

In Chapter IV, we extended the idea that bursts of activity in the cerebellar nuclei underlie opto-conditioned forelimb movements. In order to determine the necessity of cerebellar activity for the opto-conditioned response and, incidentally, test a widely hypothesized mechanism for cerebellum-dependent extinction, we used optogenetics to manipulate spontaneously firing Purkinje neurons in simplex lobe during opto-conditioned limb movements. We found that increases in Purkinje neuron activity suppress movement, but not to the same extent as subsequent extinction.

The recent technological advances in electrophysiology, behavioral measurement, and optogenetics have made the study of the cerebellum all the more exciting in recent years. This

thesis tested a widely hypothesized mechanism for extinction of classically conditioned skeletal muscle responses. It is the first result of its kind in that it gave causal evidence of the ability of Purkinje neurons to suppress conditioned forelimb responses. Future experiments might explore other mechanisms of extinction with the aim of more fully emulating the effects of extinction optogenetically. Alternatively, future experiments might examine the ability of Purkinje neurons to suppress other kinds of forelimb responses, such as reflexes and operant conditioned responses.

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