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The impact of the post-complex spike pause in cerebellum-dependent learning

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The impact of the post-complex spike pause in cerebellum-dependent learning

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Neurobiology

by

Miren Jaione Maiz Urtizberea

ABSTRACT OF THE DISSERTATION

The impact of post-complex spike pause in cerebellum-dependent learning

by

Miren Jaione Maiz Urtizberea

Doctor of Philosophy in Neurobiology

University of California, Los Angeles 2012

Professor Thomas Stephen Otis, Chair

The cerebellum plays a fundamental role in motor movement coordination and motor error correction. By using (and associating) sensory cues present during motor errors, the cerebellum learns to anticipate motor error mistakes and execute correct movements.

Damage to the cerebellum, either by trauma or disease, can impair or eliminate this ability, resulting in poor coordination and an inability to adapt or correct motor movements. In order to assist these patients, a detailed understanding of the mechanisms involved during error correction and adaptation learning is needed.

Many groups have worked to understand how this learning occurs. One identified area that requires more understanding is at the output of the cerebellum, the synapses made by Purkinje neurons on Deep Cerebellar Nucleus neurons.

The climbing fiber input to the cerebellum is believed to serve as a teaching signal during associative, cerebellum-dependent forms of motor learning. In Purkinje neurons the climbing fiber generates a complex spike, and a post-complex spike pause, that interrupts Purkinje neuron pacemaker firing. Because Purkinje neurons form inhibitory (GABAergic) synapses onto DCN neurons, coordinated pauses in Purkinje neuron firing lead to transient periods of dis-inhibition at the DCN, allowing them to be active. We hypothesize that windows of dis-inhibition relay information about the presence of a motor error to the DCN, since the DCN neurons do not actually see the climbing fiber-evoked complex spike that the Purkinje neurons see.

In addition to facilitating DCN neurons to activate their downstream targets (including the Red Nucleus and other motor targets), we propose that this relief from inhibition allows for learning-related plasticity mechanisms, like mossy fiber long term potentiation (LTP), to occur at the DCN. It is not entirely understood, however, how the climbing fiber signal is coordinating changes in cerebellar circuitry during learning.

The post-complex spike **pause** is the final component of the climbing fiber signal transmitted by Purkinje neurons to the DCN following a motor error. The pause itself may therefore play an important role in the motor learning process

In *in vitro* experiments described in Chapter 3, the post-complex spike pause is reliably prolonged by two different drugs acting by two distinct, and opposing,

mechanisms. The same drugs delivered *in vivo*, during classical eye-blink conditioning experiments described in Chapter 4, facilitated the onset of learning in this paradigm: rats receiving the drugs learned the behavioral association faster than their control counterparts.

These results elucidate an unappreciated aspect of the climbing fiber teaching signal, the post-complex spike pause, and support a model in which synchrony of post-complex spike pauses drives learning-related plasticity in the deep cerebellar nucleus.

The disseratation of Miren Jaione Maiz Urtizberea is approved.

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Alcino J. Silva

Thomas S. Otis, Committee Chair

University of California, Los Angeles

2012

Dedication - Eskaintza

Aita, ama, zuek erakutsi zenidaten,
benetan nahi nuena lortuko banuen,
lana gogotik egin beharko nuela ,
eta horra hemen ametsa egia bihurturik:
erronka berrietarako ateak irekiko
dizkidan doktoregoa.
Eskerrik asko aita, ama, eta muxu bana.

Zuentzat, bihotzez

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Atlantikoaz beste aldean
herri zaharra utzita,
hona etorri zineten biok
aurrera egin nahi ta.
amets ta nahiak lortze aldera,
zuen indarra ikusita
Norbere lana ta ahalegina
bide on bakarra baita
saiatu ezkero, ezinik ez da
hala nago ikasita,
maisu eta eredu izan zarete,
eskerrak, ama ta aita.

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CHAPTER 1

INTRODUCTION

CEREBELLUM

The cerebellum is a brain structure located at the base of the brain and is important in motor movement control and coordination. It receives varied inputs (somatosensory, visual, auditory, vestibular, and proprioceptive) from many brain regions, including from the cerebral cortex, visual and auditory cortices, and even the spinal cord.

The cerebellum shapes the timing and coordination of movements. It is especially important for, and good at, correcting motor errors. The cerebellum avoids motor errors by using sensory cues to anticipate and preemptively correct these errors.
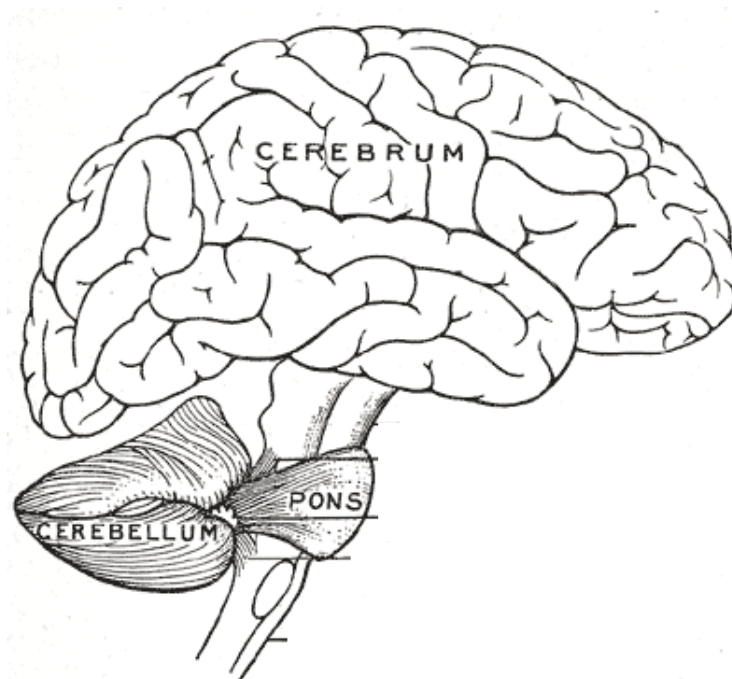
Figure 1-1. **Location of the cerebellum in a whole brain drawing.** From Gray's Anatomy, 20th Edition.

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PATHOLOGY

Patients with trauma or degeneration of the cerebellum can have gait, balance, and coordination problems. The most common result of cerebellar dysfunction is *ataxia*, which is essentially uncoordinated movement. Ataxic patients have difficulty controlling their movements, including the direction, force, range, velocity, etc (Fredericks, 1996). New evidence also describes cognitive problems, including executive, visual-spatial, language-related, and affective disturbances (Schmahmann, 2004) in cerebellar patients.

ANATOMY

The cerebellar cortex is composed of two hemispheres (**Figure 1-2**). Each hemisphere corresponds to same-side (called ipsilateral) body sides. The vermis lies along the midline of the cerebellum , with adjacent intermediate (paravermal), and lateral zones on either side. These regions are subdivided into "modules" or groups of cells with similar connections.

In the perpendicular (dorsal-ventral in humans, rostral-caudal in rodents) plane, the cerebellum is divided into three lobules: the anterior, posterior, and flocculonodular lobes in that order.
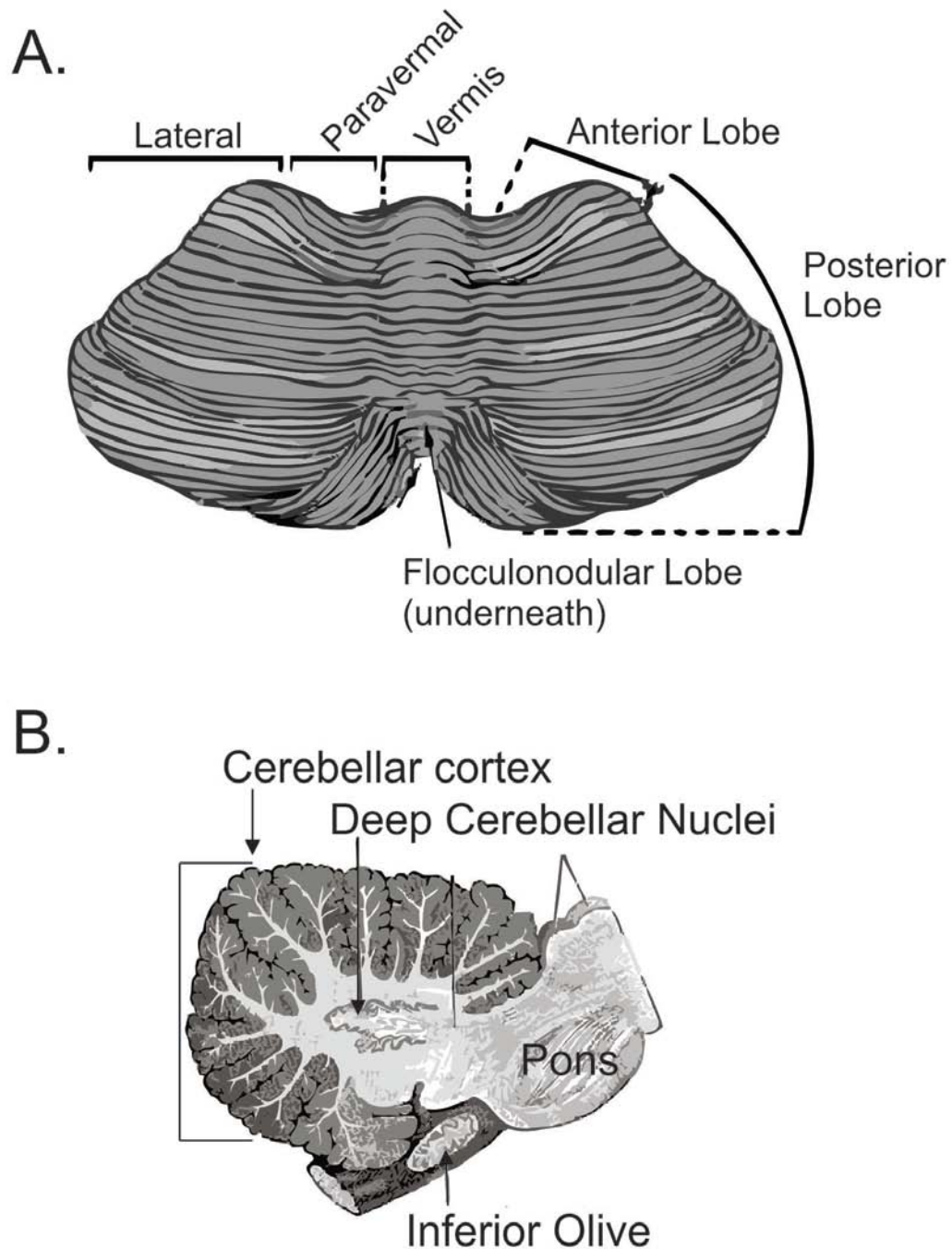
Figure 1-2. **Macroscopic organization of the cerebellum. A.** View from above. **B.** View through a parasagittal slice. Location of Deep Cerebellar Nuclei to the cerebellar cortex is seen, as well as the Inferior Olive and Pons, from which the majority of the two main inputs into the cerebellar cortex (climbing fibers and mossy fibers, respectively) arise. Cartoons are adapted from Gray's Anatomy images.

The output of the cerebellar cortex is composed of Purkinje neuron axons, which go to the three Deep Cerebellar Nuclei (DCN): dentate, fastigial, and interposed nuclei. DCN neurons are pre-motor neurons that send projections to the Red and Vestibular Nuclei, the Reticular Formation, and the Basal Ganglia, in addition to regulatory feedback projections onto Pontine Nuclei and the Inferior Olive.

While the cerebellum does appear to have somatotopic maps (brain areas directly related to specific body parts or regions), the somatotopy is fractured, meaning adjacent brain areas could relate to non-contiguous body regions.

Identifying cellular mechanisms responsible for memory formation is one of the central challenges in neuroscience. In the cerebellum, changes in circuit function are thought to occur in at least two locations during formation of associative motor memories: the cerebellar cortex, and the deep cerebellar nuclei (DCN) (Miles and Lisberger, 1981; Thompson and Krupa, 1994; Raymond et al., 1996; Mauk, 1997) (Raymond et al., 1996; Krupa and Thompson, 1997; Mauk, 1997)(Raymond et al., 1996; Krupa and Thompson, 1997; Mauk, 1997)(Raymond et al., 1996; Krupa and Thompson, 1997; Mauk, 1997)(Raymond et al., 1996; Krupa and Thompson, 1997; Mauk, 1997)

MICRO-CIRCUITRY OF THE CEREBELLUM
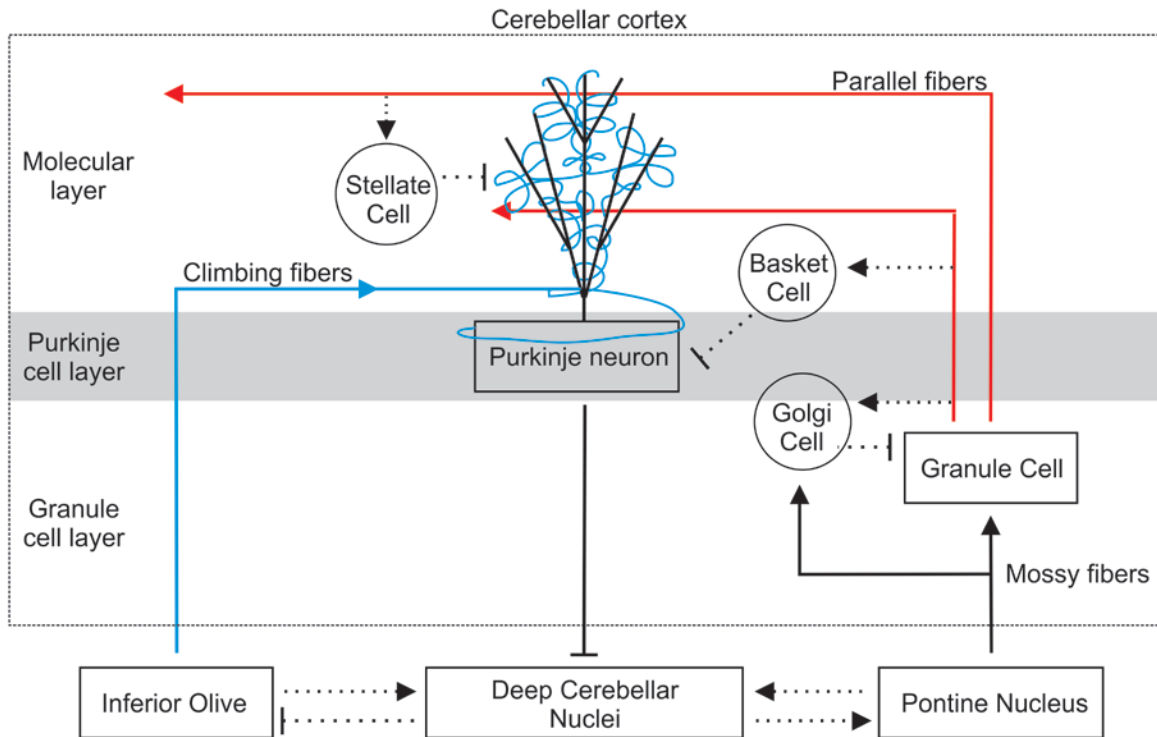
Figure 1-3. **Simplified cerebellar cortex circuitry including input and output axons.**

The cerebellar circuitry is one of the best described in the brain. **Figure 1-3**

shows the simplified circuit. The two main inputs into the cerebellar cortex are climbing

fibers and mossy fibers, originating from the Inferior Olive (IO) and Pontine Nuclei

respectively. Mossy fibers, via granule cell axons called parallel fibers, and climbing

fibers, both synapse onto Purkinje cell dendrites.

The only projections leaving the cerebellar cortex are Purkinje neuron axons.

Most Purkinje axons contact cells in the deep cerebellar nuclei (DCN). The exceptions

are Purkinje neurons in the flocculonodular lobe, which contact neurons in the vestibular nuclei. This report will focus only on Purkinje neuron axons terminating in the DCN.

The cerebellar cortex is divided into three layers: from inside outward, the granule cell layer, the Purkinje cell layer, and the molecular layer. The granule cell layer has about 50 billion granule cells (the smallest neurons in the brain), in addition to inhibitory interneurons (mostly Golgi neurons), but some unipolar brush and Lugaro cells. The Purkinje cell layer is a single layer of aligned Purkinje neurons, oriented with their axons into the granule cell layer, and their dendrites in the molecular layer. Purkinje neuron dendrites are extensive and all aligned in the parasagittal plane. The molecular layer has two types of inhibitory interneurons, basket and stellate cells, which synapse onto Purkinje cell dendrites. Climbing fibers and parallel fibers also synapse onto Purkinje neuron dendrites. One climbing fiber contacts about eight Purkinje neurons, and each Purkinje neuron receives input from a single climbing fiber which extensively wraps itself around the Purkinje neuron dendrites and cell body making approximately 1,000 synapses. Parallel fibers run orthogonal to the Purkinje neuron dendrites, through the cerebellar cortex, contacting hundreds of Purkinje neurons, but making only a few contacts with each.

CEREBELLAR LEARNING THEORIES

An influential theory regarding cerebellar learning came from David Marr in 1969. He proposed that Purkinje neuron firing produced a response that could be conditioned, with climbing fibers producing the unconditioned response. In his theory, external

stimuli-driven parallel fiber activity would act as the conditioning stimulus, and repeated pairing of parallel and climbing fibers would strengthen, in classical Hebbian fashion, parallel fiber synapses on climbing fiber-activated Purkinje neurons. The problem with this proposed mechanism is that stronger parallel fiber synapses would cause Purkinje neurons (already firing at high frequencies) to fire more, increasing the inhibition on downstream DCN neurons. DCN neurons must be active for downstream signaling, and movement, to occur.

Realizing this, James Albus modified the theory in 1971 (Albus, 1971). This new theory, called the Marr-Albus Theory, has been a pillar of cerebellar learning theory. The theory conjectured that the conditioning stimulus (via the parallel fibers) should cause a decrease in Purkinje neuron firing if a conditioning stimulus is to produce a movement. The parallel fiber-to-Purkinje neuron synapses should therefore become weaker in climbing fiber-activated Purkinje neurons during conditioning acquisition.

The theory was supported in 1982 when Ito and colleagues demonstrated that parallel fiber-to-Purkinje neuron synapses show long-term depression (LTD) following paired parallel and climbing fiber stimulation (Ito et al., 1982).

The Marr-Albus theory of cerebellar learning hypothesizes that all cerebellar learning is due to synaptic changes in the cerebellar cortex. If this were the case, lesions of the cerebellar cortex following training should abolish the learned condition response. It has been shown, however, that while the quality of the conditioned response, especially the timing, is compromised, the conditioned response is still

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present (Lavond et al., 1987; Yeo and Hardiman, 1992; Perrett et al., 1993; Thompson and Krupa, 1994; Garcia et al., 1999; Medina et al., 2000).

The Marr-Albus hypothesis also does not take into account plasticity occurring at the deep cerebellar nuclei. Inactivation of the interpositus nucleus of the deep cerebellar nuclei, specifically, eliminates the learned conditioned response, and if inactivated before training, prevents acquisition of the conditioned response (Clark et al., 1992; Krupa et al., 1993; Nordholm et al., 1993; Mauk and Donegan, 1997).

We now know that cerebellar learning occurs in stages, with plasticity occurring first in the cortex followed by plasticity in the DCN (Ohyama and Mauk, 2001; Kassardjian et al. 2005; Shutoh et al., 2006). These sites appear to encode distinct aspects of motor memories, with the DCN necessary for the expression of the memory (McCormick and Thompson, 1984b; Yeo et al., 1985; Clark et al., 1992) and for savings (Medina et al., 2001), while circuits in the cortex contribute more refined features such as the timing of learned movements (Perrett et al., 1993; Garcia and Mauk, 1998; Garcia et al., 1999).

CEREBELLUM-RELEVANT MOTOR LEARNING: CLASSICAL EYE-BLINK CONDITIONING

Classical eye-blink conditioning involves using Pavlovian reinforcement principles. Ivan Pavlov, a Russian physiologist, observed that dogs would salivate at the mere anticipation of food. Bringing the dogs food caused them to salivate, while the presentation of a sound (e.g. a metronome) would not (Pavlov, 1927). If the sound was

played each time the dogs were given their food, and the sound and the food presented together many times, however, the sound itself would eventually be sufficient to cause salivation, since it would signal that food was on its way.

Classical eye-blink conditioning uses similar principles. Any subject that receives a small air-puff or small electrical shock to the upper eye (*orbicularis oculi*) muscle will, unconsciously, react by closing their eye. Conversely, a random sound or tone will not cause an eye-blink. If the tone is played just preceding the air-puff to the eye, however, and these two stimuli are presented together many times, that particular sound, by itself, will cause an eye-blink. The tone becomes a warning signal for the upcoming air-puff. This association demonstrates the brain's ability to use contextual cues to anticipate noxious stimuli in our environment and create protective mechanisms against them. Because the air-puff elicits an overt reaction, it is called the 'unconditioned stimulus' (US), whereas the sound, which prior to pairing causes no reaction, is called the 'conditioning stimulus' (CS). The corresponding movements are called 'unconditioned response' (UR) for the air-puff-induced eye-blink, and 'conditioned response' (CR) for the sound-evoked eye-blink.

McCormick and colleagues determined that classical eye-blink conditioning is a cerebellum-dependent learning task (McCormick et al., 1982; McCormick and Thompson, 1984a, 1984b). In their experiments they lesioned the ipsilateral cerebellar cortex or deep nucleus (specifically dentate and interpositus nuclei) of well-trained rabbits (McCormick and Thompson, 1984b) and observed changes in the learned conditioned response (eye-blink). Deep nucleus lesions abolished the learned

conditioned response, while cerebellar cortex lesions resulted in altered time course of the learned conditioned responses (CRs) (McCormick and Thompson, 1984a). In all cases the unconditioned response (UR), the eye-blink that occurs as a reaction to the noxious air-puff, was unaffected, and they could successfully train the opposite eye (contralateral to the lesioned cerebellar hemisphere). They concluded, therefore, that at least part of the learned response is localized to the cerebellum. Neuronal recordings from these regions verified these findings: a subset of neurons in the ansiform and anterior lobules respond during the CS (tone) period, with some neurons firing specifically at the onset of the CS. Neurons in the dentate and interpositus nuclei of the DCN fire at the onset of, and during, the CS, in addition to the US, and some even demonstrate activity timed to the learned conditioned response (CR) (McCormick and Thompson, 1984a).

	Before learning	After learning	After learning: Lesion cortex	After learning: Lesion DCN
Unconditioned Stimulus (US)	Eye-blink	Eye-blink	Eye-blink	Eye-blink
Conditioned Response (CR)	None	Eye-blink	Poorly timed eye-blink	None

Table 1-1: **Summary of the first results looking at the effects of cerebellar cortex and DCN lesions on the conditioned response.**

LESION EXPERIMENTS

Using classical eye-blink conditioning as a model behavior paradigm,

experimenters addressed the Marr-Albus hypothesis by inducing lesions in different

areas of the cerebellum (see **Figure 1-4**).
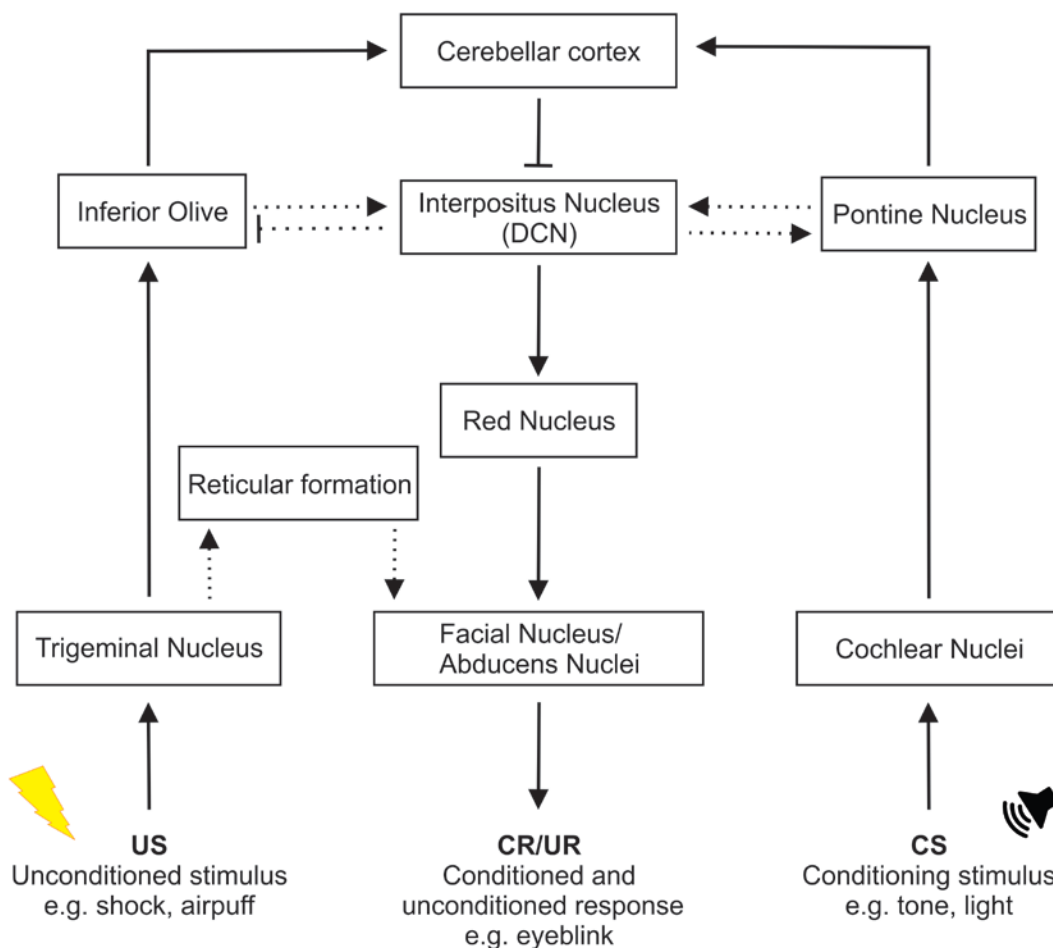
Figure 1-4. **Extended cerebellar circuit involved in cerebellum-dependent associative learning, including classical eye-blink conditioning.** Lesions in the various regions have identified nuclei and pathways important in classical eye-blink conditioning.

Cerebellar Cortex

Original cerebellar cortex lesions were crude and caused inconsistent results. The first tests with lesions of the cerebellar cortex done in well trained rabbits resulted in an abolished conditioned response (McCormick et al., 1982), although subsequent data indicate that this was likely due to a lesioned deep cerebellar nuclei (Thompson and Krupa, 1994). However, Yeo and colleagues specifically lesioned lobule HVI, without touching the underlying deep nuclei, and observed a similar disappearance of, and inability to reacquire, the conditioned response (Yeo et al., 1984).

Perrett and colleagues investigated lesions to parts or all of the anterior lobe, ansiform and/or paramedian lobules, and observed incorrectly timed (onset and peak) conditioned responses which they attributed to the anterior lobe. Furthermore, when conditioned responses are retained, they were seen as short-latency responses (SLR) the shortest being 60-70msec after the CS onset (therefore not auditory startle responses) (Perrett et al., 1993).

Thompson and Krupa resolved these discrepancies by observing that it is very difficult to remove large chunks of cerebellar cortex without also lesioning the deep cerebellar nuclei. The location, and importantly size, of the lesion could explain the inconsistent changes in conditioned responses observed by different groups (Thompson and Krupa, 1994). Garcia and colleagues addressed this controversy by injecting picrotoxin, a GABA antagonist, into the interpositus nucleus (effectively blocking all output from the cerebellar cortex) in well trained rabbits. Picrotoxin, as well as

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muscimol, a GABA agonist, infused into the interpositus nucleus abolished the

conditioned response entirely (Garcia and Mauk, 1998).

Timing-relevant information about the conditioned response was thus thought to

be localized to the cerebellar cortex. More discrete lesions, including aspiration or

electrolytically induced lesions, in the anterior lobe resulted in CR timing deficits (Garcia

et al., 1999). Furthermore, rabbits that displayed timing deficits could not be re-trained,

even using a new conditioning stimulus. They concluded, therefore, that the anterior

lobe of the cerebellar cortex is necessary for proper conditioned eye-blink response

acquisition, whereas the anterior interpositus nucleus is necessary for the expression of

the conditioned response (Garcia and Mauk, 1998; Garcia et al., 1999). Intact inputs

from the cerebellar cortex to the interpositus deep cerebellar nuclei are needed for

plasticity underlying proper conditioned response learning (Garcia et al., 1999) and

extinction (Perrett and Mauk, 1995).

Deep Cerebellar Nuclei

McCormick and Thompson investigated regions of the DCN. They observed that

electrical stimulation of the dentate and interpositus nuclei of the DCN, specifically,

cause eye closures in untrained animals (McCormick and Thompson, 1984a). They, as

well as Clark and Thompson, lesioned these regions in well-trained rabbits. This

eliminated the conditioned response (CR), in addition to preventing new learning, thus

pointing to direct DCN involvement in conditioning learning (Clark et al., 1984;

McCormick and Thompson, 1984a). As expected, if the lesion was made before any

training, the animal was unable to learn the conditioned response (Lincoln et al., 1982).

It should be noted that in all cases, new CR learning in the unaffected eye (contralateral to the lesioned hemisphere) was normal. Furthermore, they recorded from neurons in the medial dentate and lateral interpositus nuclei specifically and observed that some neurons respond in ways that predict the conditioned response (McCormick and Thompson, 1984a, 1984b).

Because electrical lesions are inherently non-specific, Lavond and colleagues used kainic acid to pharmacologically lesion these regions. They determined that lesioning the dorsal interpositus nucleus, specifically, is sufficient to abolish the conditioned response in well-trained animals (Lavond et al., 1985).

Other researchers, however, argued that these effects were general performance deficits not specific to the conditioned response. Welsh and Harvey lesioned the dorsal interpositus nuclei and observed deficits in unconditioned responses in addition to the expected conditioned response, although only when using lower conditioning stimuli intensities (Welsh and Harvey, 1989).

Steinmetz and colleagues later reported that they observed similar decreases in the amplitude and frequency of occurrence of the conditioned responses, in addition to increases in CR onset latency, but only when dorsal interpositus nuclei lesions were incomplete (Steinmetz et al., 1992).

Other reversible lesion methods, including muscimol (Krupa et al., 1993) or lidocaine (Nordholm et al., 1993) injections, and cooling (Clark et al., 1992), of the interpositus nucleus, inhibited conditioned response expression. In all cases new

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learning was possible following reversal of the lesion, and animals learned as if naïve to

the training stimuli (Thompson and Krupa, 1994).

Inferior Olive

The Inferior Olive (IO) sends axons, called climbing fibers, into the cerebellar

cortex, forming one of the two (the others being mossy fibers) input types. In trained

animals, lesions to the rostro-medial Inferior Olive (rmIO) caused the conditioned

response to slowly disappear, decreasing in amplitude and frequency similar to what

happens during extinction (McCormick et al., 1985). Yeo and colleagues saw immediate

abolishment of the learned conditioned response when they lesioned the rmIO and

Principal Olive[1] (Yeo et al., 1986). However, Thompson et al. claim that those lesions

were larger than theirs, and that smaller lesions more subtly affect conditioned

responses (Thompson et al., 1998). In all cases there were no effects to the

unconditioned response. The inferior olive, and more specifically the climbing fibers

originating in the rostro-medial regions of the IO are therefore needed for acquisition

and maintenance of the learned conditioned response. Reversible lesions, with NMDA

infusions into the dorsal accessory of the rmIO, prevented retention of, and new

learning in contralateral eye-blink conditioning during the temporary lesion only (Mintz et

al., 1994), supporting these conclusions.

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[1] Climbing fibers from these regions of the Inferior Olive innervate lobule HVI of the cerebellar
cortex

Pontine Nuclei (Middle Cerebellar Peduncle)

The middle cerebellar peduncles (MCP) are bilateral structures connecting the pontine nuclei to the contralateral cerebellar hemisphere. These fibers form the mossy fibers that compose the other main input into the cerebellar cortex (the climbing fibers are the other main input). Rabbits that had their MCP lesioned following classical eye-blink training were unable to retain the learned conditioned response, and were unable to learn a new conditioned response using a different conditioning stimulus (Lewis et al., 1987). This occurred regardless of the conditioning stimulus being auditory, visual, or tactile stimuli.

Red Nucleus

The Red Nucleus (RN) receives inputs from the Interpositus Nuclei of the Deep Cerebellar Nuclei, and sends axons to blink-producing motor neurons in the accessory abducens nuclei. As expected, lesions of the red nucleus reduce or prevent, depending on the lesion size and type, expression of the conditioned response (Rosenfield and Moore, 1983; Rosenfield et al., 1985). In experiments where the RN was reversibly inactivated following learning, using either lidocaine (Chapman et al., 1990), muscimol[2] (Krupa et al., 1993), or a cooling probe (Clark and Lavond, 1993), the learned conditioned eye-blink disappeared but resumed normally when the inactivation was

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[2] Krupa et al. also temporarily inactivated the cerebellar cortex using the GABA-receptor agonist muscimol, and prevented conditioned eye-blink learning only during muscimol inactivation (Krupa et al., 1993).

removed. The Red Nucleus is necessary for expression of the conditioned eye-blink but not proper acquisition.

Location	Lesion before learning	Lesion after learning	Reversal of lesion
Cerebellar cortex	No CR	Poorly timed CR	No (old or new) CR
Deep Cerebellar Nuclei	Can't learn CR	Lose CR	New learning possible
Inferior Olive	Can't learn CR	Lose CR	New learning possible
Pontine Nuclei (Middle Cerebellar Peduncle)	Can't learn CR	Lose CR	No (old or new) CR
Red Nucleus	No effect	Less robust CR/ lose CR	Normal CR / new CR possible

Table 1-2. **Summary table of cerebellar and extra-cerebellar lesions and their effects on acquisition of a conditioned eye-blink response (CR).**

ELECTRICAL STIMULATION AS UNCONDITIONED AND CONDITIONING STIMULI

Experiments where climbing or mossy fibers were electrically stimulated during conditioning, replacing external unconditioned (i.e. air-puff) and conditioned (i.e. tone) stimuli, demonstrate the direct involvement of these fibers in associative learning. A summary diagram showing the relay of learning-related information in the cerebellar circuit is in **Figure 1-4**.

Mauk and colleagues observed that high frequency electrical stimulation (100ms, 60μA/60Hz - 400μA/400Hz) in the IO elicited discrete movements, including eyelid closures in addition to head and forepaw movements depending on the location of stimulation (Mauk et al., 1986). Rabbits that exhibited eye-blinks (stimulation localized to the dorsal accessory of the IO; daIO) were trained with standard eye-blink conditioning, except that the unconditioned stimulus (i.e. air-puff) was replaced by high frequency electrical stimulation in the daIO. They observed conditioned responses comparable, in timing, acquisition rate and amplitude, to responses seen during eye-blink conditioning with an air-puff as the US (Mauk et al., 1986). The dorsal accessory of the IO, and the climbing fibers (IO axons) that extend to the cerebellar cortex, must therefore convey important information about the unconditioned stimulus during associative learning.

The Marr-Albus learning hypothesis predicts that cerebellar learning occurs via synaptic plasticity at the Purkinje neuron, the site of convergence of cerebellar inputs, and the sole output cells of the cerebellum. Direct electrical stimulation of the Inferior Olive can replace the external unconditioned stimulus during eye-blink conditioning, therefore Steinmetz and colleagues tested whether the conditioned stimulus could also be replaced by electrical stimulation (Steinmetz et al., 1985). They electrically stimulated in the Pontine Nucleus[3], the origin site of mossy fibers that will become the parallel fibers that reach the Purkinje neuron. They paired pontine stimulation with a

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[3] Specifically the Dorsolateral Pontine Nucleus (DLPN) and the Lateral Reticular Nucleus (LRN) (Steinmetz et al., 1985). The DLPN projects mostly to the cerebellar cortex with fewer projections to the cerebellar nuclei while the LRN projects heavily to the dentate and interpositus nuclei, as well as the cerebellar cortex.

standard air-puff (as the unconditioned stimulus) and observed normal acquisition[4] of a conditioned response, that is, the learned eye-blink timed with, normally, the tone that in this case was the pontine nuclei stimulation (Steinmetz et al., 1986).

Steinmetz, Lavond, and Thompson then classically conditioned rabbits without any external stimuli, using electrical stimulation of both the inferior olive (IO) and pontine nuclei (PN) as the unconditioned and conditioning stimuli respectively (Steinmetz et al., 1989). Conditioned responses were like those observed with standard external conditioning stimuli[5].

In some animals, blink-eliciting electrical stimulation in the IO was reduced to "sub-threshold," or non-blink eliciting, levels before undergoing IO-PN stimulation-type conditioning. Even though no unconditioned responses were observed, learned conditioned responses appeared during training, implying that information about the presence of an unconditioned stimulus is enough for associative learning. In all cases, however, ablation of the Interpositus Nucleus (of the Deep Cerebellar Nuclei) abolishes the learned conditioned response without affecting the "reflex" unconditioned response[6].

These electrical replacement results supplement the lesion experiments to strengthen our understanding about the neuronal circuit underlying classical eye-blink conditioning in particular, but cerebellum-dependent associative motor learning in general.

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[4] Normal extinction was also observed.
[5] Except peak latency which occurred 150 ms after US onset and did not change with further conditioning. Using standard conditioning stimuli, well-trained rabbits show peak latencies around the time of onset of the US.
[6] The reflex eye-blink to the US goes through the Trigeminal Nucleus, Reticular formation, and accessory abduncens nuclei, bypassing the upstream DCN and even Red Nucleus.
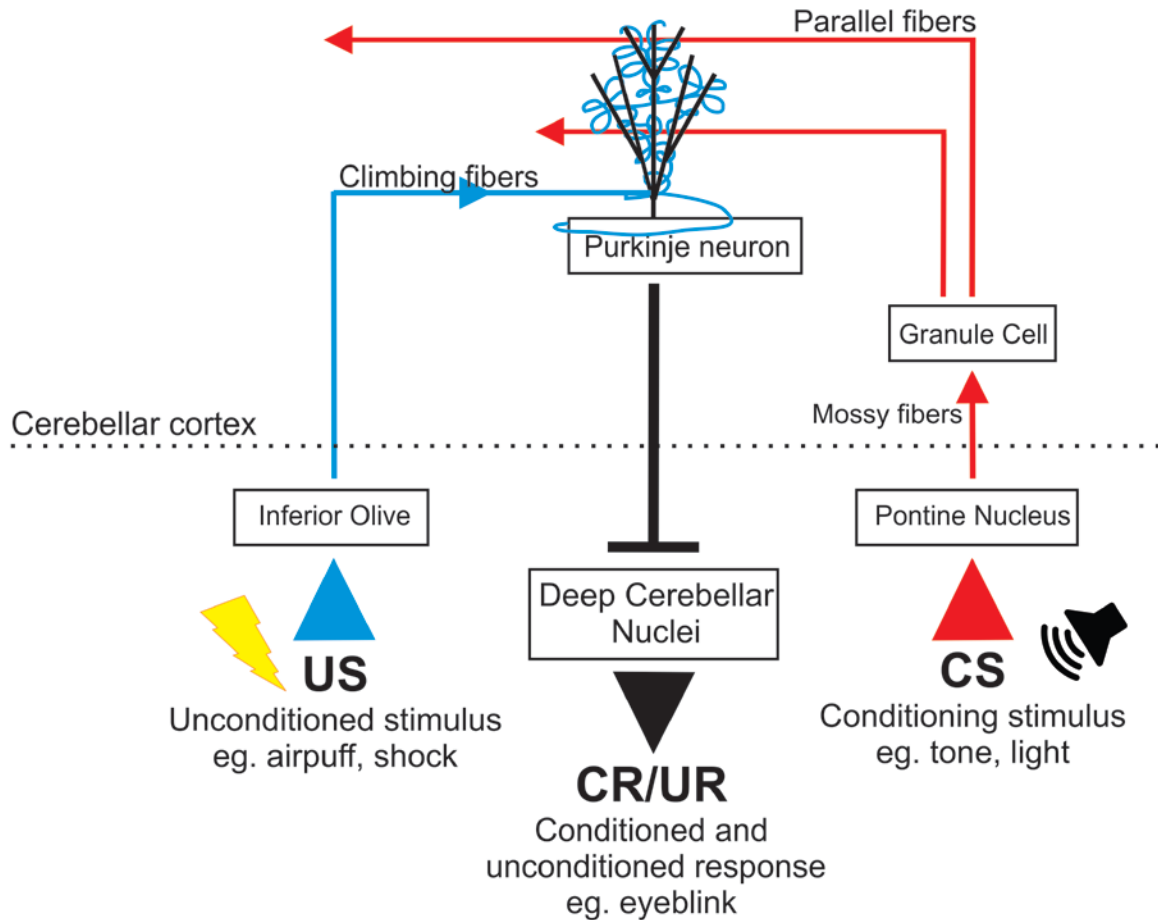
Figure 1-5. **Simplified cerebellar circuitry underlying classical eye-blink conditioning.** Information about the US reaches the cerebellar cortex via climbing fibers, whereas sensory information used as the CS during associative learning, reaches the cerebellar cortex via mossy fibers that then become parallel fibers.

MOLECULAR MECHANISMS OF LEARNING IN CEREBELLAR CORTEX AND DCN

Purkinje neurons lie at the center of information input into the cerebellar cortex, and form the only output. They integrate information entering the cerebellum, as well as information from the local micro-circuitry, and modulate their firing frequency in

response. Since Purkinje neurons continuously fire at high frequencies (30-100Hz) (Thach, 1968; Häusser and Clark, 1997; Raman and Bean, 1999), their post-synaptic counterparts in the DCN are inhibited (these are GABAergic synapses) during normal Purkinje neuron firing. Pauses in Purkinje neuron firing will be noticed by the DCN, especially synchronized pauses from multiple Purkinje neurons, like following a motor error.

Climbing fibers also fire spontaneously at around 1Hz. When a motor error signal, like a US during classical eye-blink conditioning, is received, the climbing fibers fire faster (Gilbert and Thach, 1977; Ojakangas and Ebner, 1992). Because the climbing fiber to Purkinje neuron synapse is so strong (~1000 synapses as the climbing fiber wraps around the Purkinje neuron cell body and dendrites), any climbing fiber activity is correlated directly with a complex spike in the Purkinje neuron. The complex spike is an all-or-nothing burst, lasting up to 10ms, that activates voltage-gated calcium channels in the Purkinje neuron. The complex spike is followed by an extended hyperpolarization, called the **post-complex spike pause**, until Purkinje simple spiking (high-frequency pace-making) resumes. The calcium influx drives molecular cascades including the ones that induce long-term depression (LTD) at specific parallel fiber-to-Purkinje neuron synapses (Coesmans et al., 2004; Safo and Regehr, 2005) that have been active up to 100ms preceding the complex spike (Ekerot and Oscarsson, 1981). While recent work has debated the importance of parallel fiber at Purkinje neuron LTD for motor learning (Schonewille et al., 2011), the field has long considered this plasticity an important component of cerebellum-related learning (Mauk et al., 1998). The climbing fiber signal is therefore considered the "teaching" signal during learning since the climbing fiber

evoked complex spike drives this and other plasticity mechanisms involved in learning (Marr, 1969; Albus, 1971; Raymond et al., 1996).

At the DCN, Purkinje neuron pauses relieve inhibition, causing post-hyperpolarization rebound excitability in DCN neurons (Pugh and Raman, 2006). This rebound firing can trigger increases in the strength of mossy fiber inputs to these same DCN neurons. Synchronous Purkinje neuron pauses could drive substantial long-term potentiation (LTP) at mossy fiber-to-DCN synapses that could underlie cerebellum-dependent associative learning, specifically the learned conditioned response (e.g. eye-blink in response to the previously irrelevant tone). Recent work suggest that the "teaching signal" is in fact encoded by synchronous climbing fiber activity (Ghosh et al., 2011). This supports earlier work (using *in vivo* imaging and multi-electrode arrays) that demonstrated that climbing fibers fire simultaneously in "modules" of Purkinje neurons (Welsh et al., 1995; Lang et al., 1999; Mukamel et al., 2009; Ozden et al., 2009).

THE ERROR SIGNAL

In the simplest sense, an error signal emerges from a difference between desired and actual outcome. A motor error signal could therefore be an incorrect or incomplete movement. The cerebellum coordinates time-adjusted changes in movements, based on relevant contextual sensory cues, to produce correctly-timed, learned movements that compensate for the movement error. The new movements, the conditioned responses to innocuous contextual conditioning stimuli, reduce the

differential readout between desired and actual actions, thereby eliminating the error signal as the learned "desired" movement becomes the actual movement.

In the context of eye-blink conditioning, the error signal is calculated in the Inferior Olive, based on information relayed from the body, in addition to information relayed from the Deep Cerebellar Nuclei, the cells receiving the output of the cerebellar cortex. When the differential between desired and actual is large, axons from the Inferior Olive (i.e. climbing fibers), synchronously provoke a complex spike in corresponding Purkinje neurons, followed by a post-complex spike pause. The complex spike induces plasticity mechanisms in the cerebellar cortex that learn correctly timed associations to conditioning stimuli preceding the onset of the error. The post-complex spike pause (and learned Purkinje neuron pauses as learning progresses), relieves robust Purkinje neuron inhibition from downstream Deep Cerebellar Nuclei neurons, allowing them to be active. Relief from Purkinje neuron inhibition allows plasticity mechanisms to occur in the Deep Cerebellar Nuclei, and consequent expression and storage of the learned conditioned response.

Deep Cerebellar Nuclei neurons send inhibitory projections back to Inferior Olive neurons, however, meaning that their activity will suppress consequent climbing fiber activation. The error signal is the difference between the information coming into the Inferior Olive and the feedback signal from the Deep Cerebellar Nuclei, as new corrected movements become more like desired movements. In *instrumental learning*, or operant conditioning, the new movements could evade the error-causing stimulus, but in Pavlovian conditioning, where the stimulus is unavoidable, such as a peri-orbital electrical shock, the new learned movement is unable to avoid the stimulus. In the latter

scenario, the association between tone and shock still provides the animal with predicting power for the onset of the stimulus, and so the circuit learns to expect, for example, the eye-shock. Once the noxious signal can be predicted, it no longer produces an error signal, since it is no longer an unexpected signal. Continued presentation of conditioned and unconditioned stimuli, even after associative learning saturates, would therefore result in a null error signal in either scenario: one because the subject learns to avoid the error with a new learned movement, and two, because the subject learns to expect the error, thereby abolishing any alerting error signal to the system. Based on such considerations, it has been suggested that climbing fibers signal unexpected events rather than errors. Throughout the thesis I will refer to error signals, and this phrase can be interpreted as synonymous with unexpected event signals.

BLOCKING

The phenomenon of **blocking** is consistent with the notion that the complex spike encodes unexpected events that the circuit must learn to predict. Kim, Krupa, and Thompson, demonstrate blocking, that is: the cerebellar circuit associates an unconditioned stimulus (US) to a singular conditioning stimulus (CS), and that once that association is made, the training of the same US with a new CS does not produce a conditioned response to the second CS (Kim et al., 1998). Essentially:

If A = CS1, and B = CS2

And the subject is trained with A + US, then presentation of A leads to CR,

If the subject is then trained with A + B + US, the presentation of A leads to CR, but just B ≠ CR

Following a proper association between a tone (CS), and an airpuff (US), for example, the introduction of a new CS, such as a light, offers no new information to predict or avoid the US, and will therefore not associate with the airpuff.

As a tone and airpuff are associated during classical conditioning, activity in the Deep Cerebellar Nuclei neurons facilitates feedback mechanisms via inhibitory, GABAergic connections back to the Inferior Olive, the nucleus for cells whose axons are the climbing fibers that innervate the cerebellar cortex. The climbing fibers transmit information regarding the occurrence of an external motor error by causing concerted complex spikes in Purkinje neurons. As the cerebellum learns the association between conditioning and unconditioned stimuli, however, these inhibitory feedback mechanisms through the Inferior Olive suppress consequent complex spikes. Essentially the differential "error" signal is reduced as the once-noxious airpuff becomes less noxious, since the animal has learned to use the context of the tone to close its eye prior to, and thereby preventing, the uncomfortable airpuff.

Kim et al. eliminated this blocking effect by inhibiting GABAergic feedback from the Deep Cerebellar Nuclei with picrotoxin injections in the Inferior Olive. Picrotoxin prevented learning-induced inhibition of Inferior Olive neurons (by DCN neurons), and therefore prevented the suppression of climbing fibers (and complex spikes) that occurs

during proper associative conditioning. This resulted in an ability to associate the same

airpuff US with two different conditioning stimuli: tone and light.

CHAPTER 2

DISSERTATION PURPOSE AND OBJECTIVES

DISSERTATION PURPOSE AND OBJECTIVES

This dissertation uses 1) two different pharmacological manipulations during *in vitro* electrophysiological experiments and 2) an *in vivo* behavioral assay to explore a mechanism by which climbing fibers transiently inhibit Purkinje neurons and regulate associative learning in the cerebellum. Our strategy is to lengthen the climbing-fiber evoked post-complex spike pause in Purkinje neurons, increasing the window of dis-inhibition on Deep Cerebellar Nuclei cells. We find that this effectively facilitates plasticity mechanisms underlying cerebellar associative learning at the Purkinje neuron to Deep Cerebellar Nuclei synapse.

I have used two different drugs to prolong the pause: 1-EBIO and ZD7288, which both lengthen the post-complex spike pause but by opposing mechanisms on two different receptors. This strategy ensured that the observed effect is related to the length of the pause and not to some other unforeseen drug-related effect.

The *in-vivo* experiments at UCLA required the development of an eye-blink conditioning study set-up for rats, modeled after one existing at USC. Many improvements in the hardware, software, and data analysis were necessary to improve system robustness, efficiency, and improved signal to noise ratio. With this improved equipment I showed that modulating the post-complex spike pause by pharmacological means affects the learning rate during associative eye-blink conditioning, implying that information important to learning is encoded in the **length** of the post-complex spike pause.

These results are the first to describe enhanced learning rate during a cerebellum-dependent associative learning paradigm. These findings introduce a potentially novel mechanism by which instructive error signals could shape associative learning-dependent plasticity. Detailed understanding of these circuit mechanisms may provide guidance in the development of strategies that combat cerebellar diseases like cerebellar ataxias.

CHAPTER 3

TWO INDEPENDENT PHARMACOLOGICAL STRATEGIES
THAT PROLONG THE POST-COMPLEX SPIKE PAUSE *IN-VITRO*

SUMMARY

The first step in addressing the role of the post-complex spike pause during cerebellum-dependent associative learning is to determine a way to specifically modulate the duration of the post-complex spike pause *in vitro*. The ideal set of drugs would affect pause length only, without affecting either the complex spike or simple spiking (frequency or spike properties). Several drugs that modulate the length of the pause by distinct mechanisms are desirable to ensure that the observed results can be attributed to the modulation of the pause length, and not to other drug related effects. Preliminary experiments in the lab, as well as evidence in the literature, led to the identification of two potential drug candidates: 1-EBIO and ZD7288. These two drugs work via entirely different mechanisms, and in fact are opposite modulators: 1-EBIO is a positive modulator of SK (small conductance potassium) channels whereas ZD7288 is an inhibitor of HCN (hyperpolarization activated cation; I_h) channels. At the concentrations used, both drugs resulted in longer post-complex spike pauses without significantly affecting the other features mentioned (complex spike waveform, simple spiking).

METHODS

All experimental procedures were approved by the UCLA Institutional Animal Committee on Use and Care (IACUC).

<u>Electrophysiology</u>

Cerebella from one to two month old C57Bl/6 mice were used. Acute parasagittal slices, 200-300µm thick, were made and kept in artificial cerebrospinal fluid (aCSF). The precise composition is in **Table 3-1.**

Product	Concentration (mM)
NaCl	119.0
$NaHCO_3$	26.0
Glucose	11.0
KCl	2.5
$CaCl_2$	2.5
$MgCl_2$	1.3
NaH_2PO_4	1.0
Saturated with 95% O_2 and 5% CO_2	

Table 3-1. **Artificial cerebrospinal fluid (aCSF) composition.** Cerebellar slices were cut and kept in this solution throughout experiments. All drugs used were added to this solution.

All experiments were conducted at near physiological temperatures (32-34°C), and in the presence of the GABA$_A$ receptor antagonist picrotoxin (100 μM) to block inhibitory interneuron-evoked pauses. Extracellular recordings in either voltage clamp ($V_{pipette}$ = 0 mV) or current clamp ($I_{pipette}$=0) were made with 2-5 MΩ resistance pipettes. A bipolar theta stimulation electrode was used to directly stimulate single climbing fiber axons, taking special care to place the stimulation as far away as possible from the Purkinje neuron, up to ~200um away. 1-EBIO (20 μM, a positive modulator of SK channels), ZD7288 (1 μM, an HCN (I_h) channel antagonist), and DNQX (20 μM, an AMPA-receptor antagonist) were obtained from Tocris Biosciences (Ellisville, MO) and bath applied as indicated.

A photo of the microscope set-up used for the electrophysiology experiments is below.
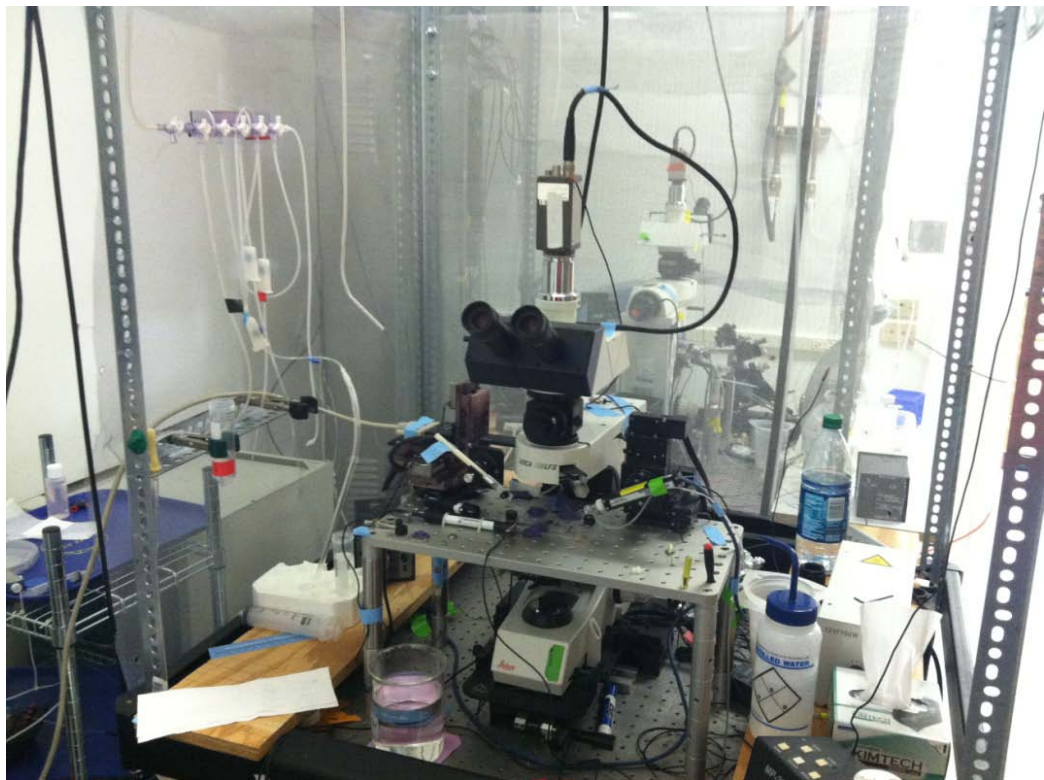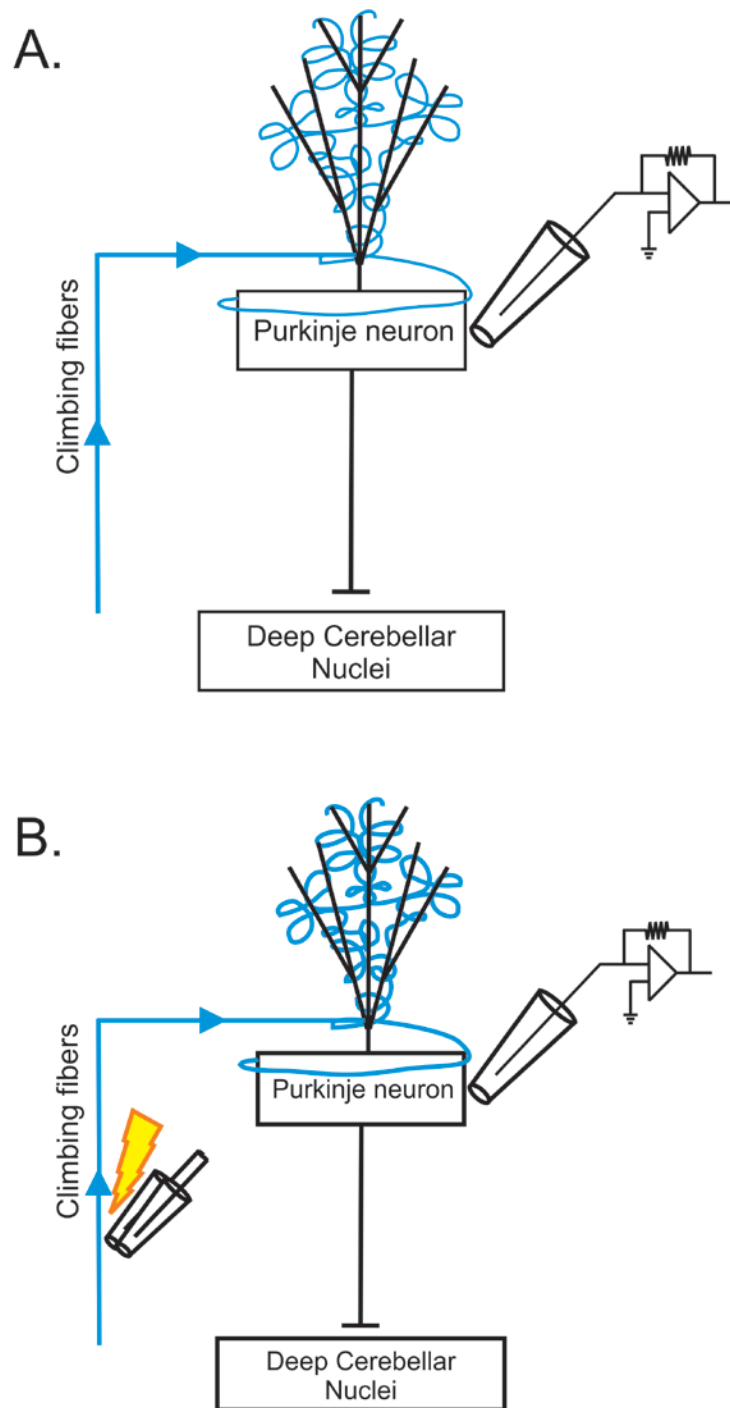
Figure 3-1. Placement of electrical recording and stimulation electrodes in acute brain slices to record Purkinje neuron activity.
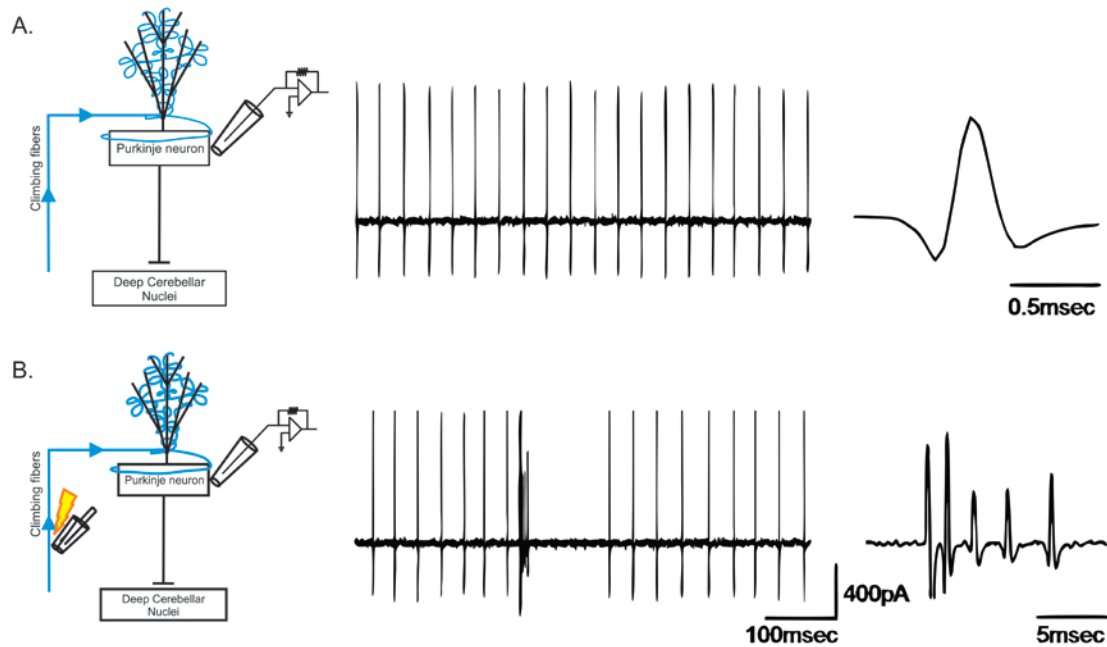
Figure 3-2. **Extracellular recordings from Purkinje neurons. A.** the Purkinje neuron fires action potentials spontaneously at high frequencies (30-100Hz).Right most panel is a single action potential zoomed in. **B.** Stimulation of the climbing fiber produces a complex spike (zoomed in: bottom right panel) and consequent post-complex spike pause before simple spiking resumes.

Data and statistical analyses

Electrophysiology data was collected using pClamp (Axon Instruments, Union City, CA), and analyzed using Igor Pro (Wavemetrics, Lake Oswego, OR) and Microsoft Excel. To compare pause lengths between cells with different baseline firing frequencies, an analysis method described in Mittman and Häusser, 2007 was used.

The Mittman et al. method determines the number of spikes "lost" during the post-complex spike pause. A post-stimulus time histogram is calculated
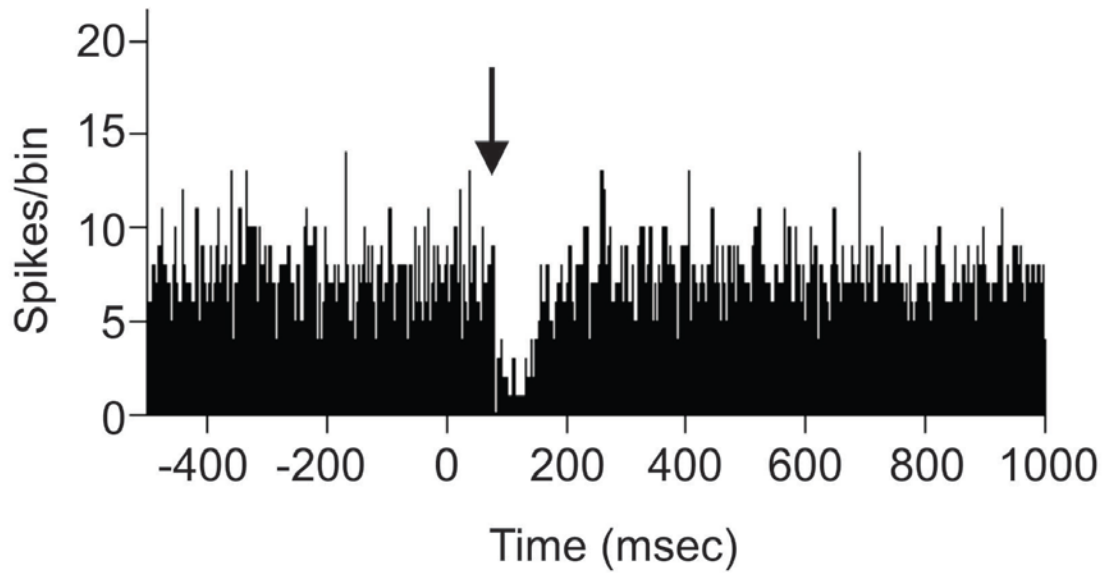
and integrated. Arrow marks time point when the climbing fiber was stimulated.
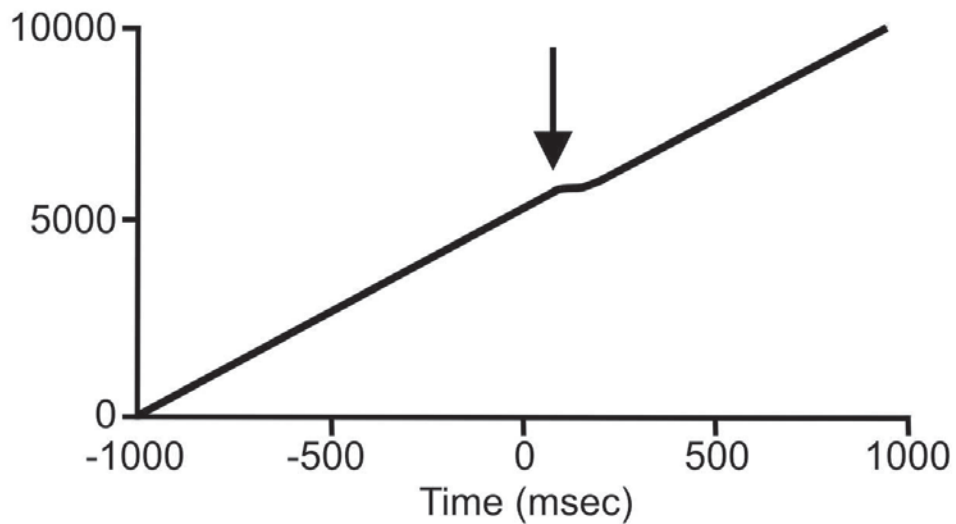
The pre-stimulus baseline is calculated from the slope of the pre-stimulus (-100 to -5 ms window), and extrapolated.
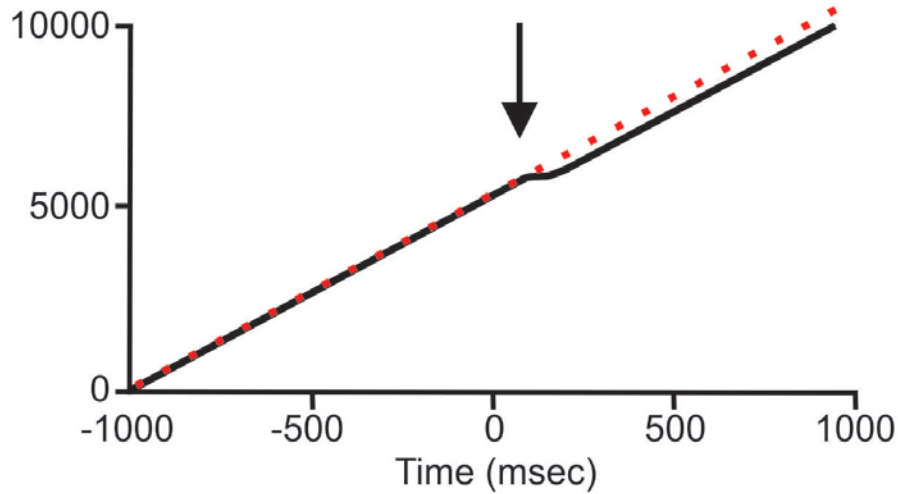
The integrated line is subtracted from the (baseline) extrapolated line, resulting in a net "spikes lost" (during the pause) count.

Averaged data was compared by using student's t-test in Microsoft Excel.

Pharmacology

1-EBIO is a nonselective positive modulator of small conductance calcium-dependent potassium (SK) channels. SK channels in Purkinje neurons are activated by calcium entering through voltage-gated calcium (P/Q-type) channels during the complex spike. 1-EBIO increases SK-channel affinity for calcium (Pedarzani et al., 2001), making SK channels stay open longer. 1-EBIO therefore results in an extended period of hyperpolarization following the complex spike in the Purkinje neuron, called the post-complex spike pause.

1-EBIO was found to be therapeutic in mouse models of episodic ataxia type-2 (EA2), a cerebellar disease arising from mutations in P/Q-type calcium channel genes, by recovering the SK conductance to levels observed in normal animals (Otis and Jen, 2006; Walter et al., 2006).

ZD7288 blocks the hyperpolarization-activated cyclic nucleotide-gated (HCN) channel, preventing the I_h current and thereby extending the time in which the cell remains in a hyperpolarized state. . While high concentrations of ZD7288 can result in Purkinje neuron bi-stability, low concentrations of ZD7288 [1-5 μM used in these work] did not affect Purkinje neuron membrane stability even though it did result in a longer post-complex spike pause.
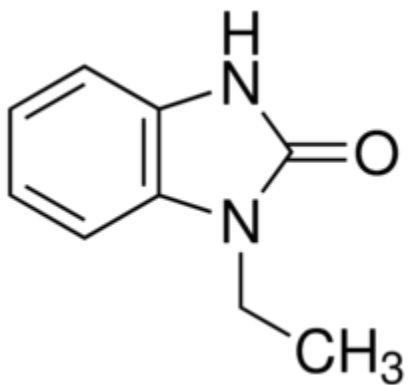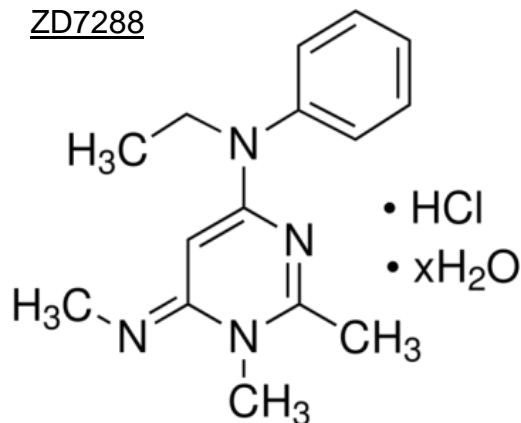
Figure 3-3. **Molecular diagrams of 1-EBIO and ZD7288.**

Channel	SK	HCN
Activated by	Ca^{2+}	• Hyperpolarized potentials • Cyclic nucleotides (i.e. cAMP)
Permeable to	K^+	$Na^+/K^+/(Ca^{2+})$
Net effect	Hyperpolarization	Depolarization
Drug used	1-EBIO	ZD7288
Effect of drug	• Positive modulator • Keeps channel open longer	• Negative modulator • Blocks channel
NET EFFECT	Longer post-complex spike-pause	Longer post-complex spike pause

Table 3-2. SK and HCN channels, and the impact of 1-EBIO and ZD7288 on post-complex spike pause duration. 1-EBIO and ZD7288 both result in a longer post-complex spike pause, even though 1-EBIO activates SK channels, whereas ZD7288 block HCN channel.

In order to provide a control, DNQX, an AMPA and kainite receptor antagonist, was added at the end of every experiment. The climbing fiber evoked complex spike in the Purkinje neuron is mediated by AMPA receptors on the Purkinje cell dendrites and cell body. In the presence of DNQX these receptors are blocked, and no complex spike will occur. Since the post-complex spike pause is inherently dependent on the presence of the complex spike, the pause, along with the complex spike, is eliminated. The addition of DNQX, and the observation of its effect, was used to ensure that the pause measured was indeed a post-complex spike pause and not the result of some unwanted artifact.

RESULTS

Two independent and opposing pharmacological strategies for prolongation of the post-complex spike pause

The hypothesis for this dissertation's work is that the length of the post-complex spike pause has a direct impact on cerebellum-dependent learning. In an attempt to isolate the effects of the pause length, I needed pharmacological tools that would lengthen the pause in fundamentally different ways. 1-EBIO and ZD7288 proved to both lengthen the post-complex spike pause, even though they act in opposite ways (1-EBIO enhances, while ZD7288 block) on entirely separate channels (SK and HCN channels, respectively).

Small-conductance, calcium-activated potassium channels (SK channels) are activated by the large calcium influx that occurs during a complex spike (Swensen and Bean, 2003). SK channels are also likely mediating the effects of complex spike-dependent dendritic calcium spiking and its influence on the post-complex spike pause (Davie et al., 2008). The enhancement of SK channel function by 1-EBIO, a positive modulator of SK channel function (Pedarzani et al., 2001; Walter et al., 2006), was therefore a likely useful candidate to prolong the post-complex spike pause in Purkinje neurons.

In order to non-invasively monitor complex spikes and firing, I recorded extracellularly from single Purkinje neurons in acute cerebellar slices. In the presence of the $GABA_A$ receptor antagonist picrotoxin (100 μM), I electrically stimulated single climbing fibers (0.125 Hz) to elicit complex spikes and transient post-complex spike

pauses in simple spike firing. Bath application of 20 µM 1-EBIO prolonged these pauses

(**Figure 3-4**). The mean pause duration prior to 1-EBIO application was 32 ± 9 ms, and

1-EBIO increased the pause duration by 120 ± 44% (n= 6, p< 0.02). Concluding each

experiment, application of the AMPA-receptor antagonist DNQX (20 µM) confirmed that

the pauses were in fact post-complex spike pauses, since DNQX blocks the climbing

fiber evoked complex spike, and, therefore, its associated pause.
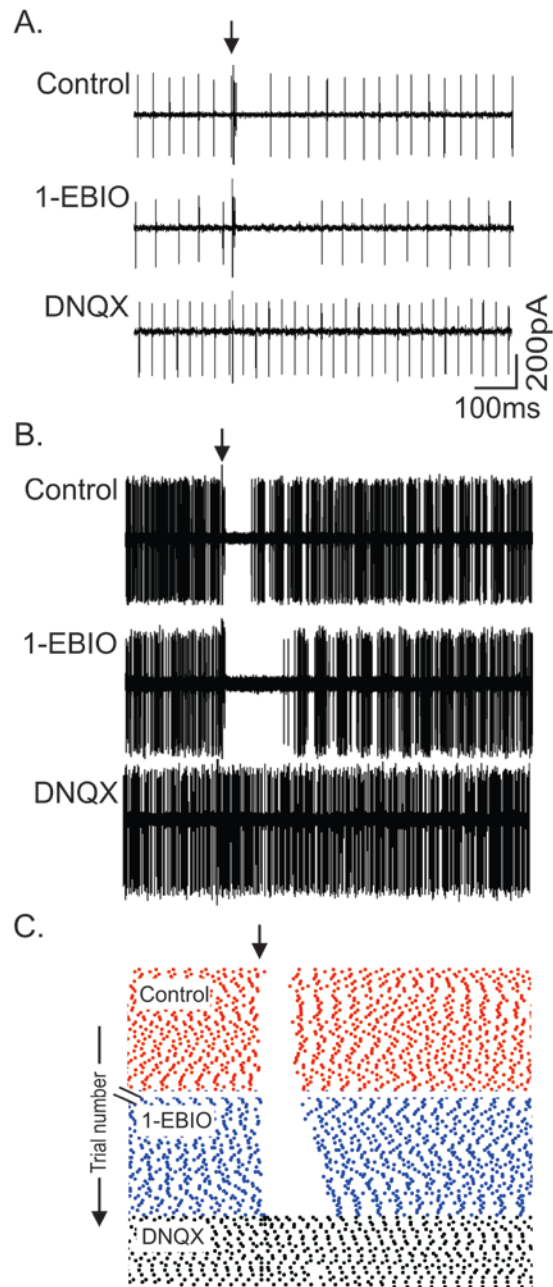
Figure 3-4. 1-EBIO prolongs the post-complex spike pause. A. Example traces showing single trial responses to climbing fiber stimulation (arrow) under the indicated conditions. **B.** Ten superimposed consecutive single traces showing robust pause duration in each condition. **C**. Spike rasters aligned to the climbing fiber stimulus (arrow) demonstrating control responses in red, and in 1-EBIO (blue). The hash marks indicate a 13.3 minute gap between control and 1-EBIO conditions. DNQX (black) was applied at the end of the experiments to confirm that all pauses were the result of climbing fiber evoked complex spikes. Arrow marks time point when the climbing fiber was stimulated.

To capture the complicated dynamics with which Purkinje neurons can resume regular spiking after a post-CS pause, I quantified the prolongation of post-complex spike pause by calculating the net number of "spikes lost" during each pause. This analysis demonstrated that 1-EBIO significantly lengthened the post-CS pause (3.85 ± 1.35 spikes versus 1.70 ± 0.58 spikes in control, $p<0.0001$, n=6). Additional analysis confirmed that the pauses were due to complex spikes as no net change in spike count was observed in the presence of DNQX (**Figure 3-5**).
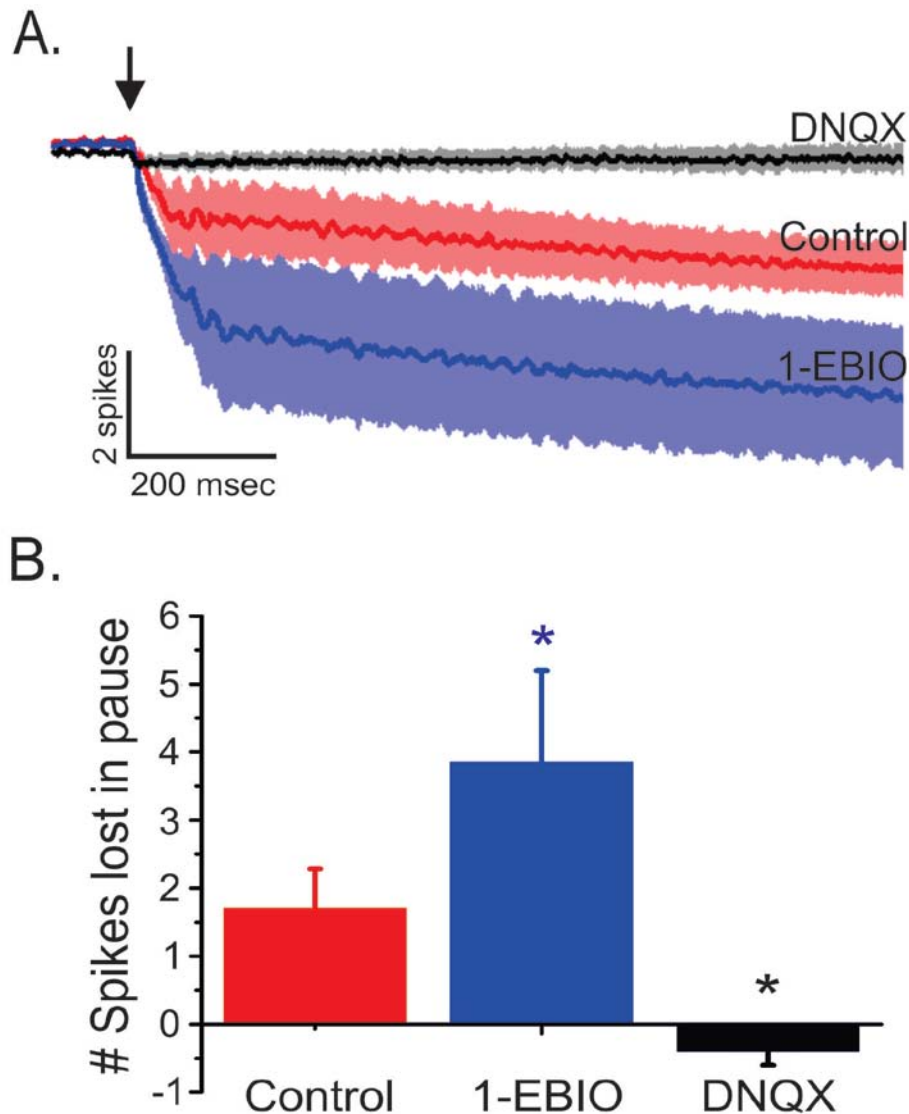
Figure 3-5. **More spikes are "lost" (i.e. longer pause) in 1-EBIO. A.** Average cumulative net spike counts (see Methods) for 6 Purkinje neurons indicate that significantly more spikes are "lost" in response to the climbing fiber input in 1-EBIO (blue) versus control (red). Responses are blocked entirely by DNQX (black). Light shading represents standard error of the mean (SEM), and arrow marks time point when the climbing fiber was stimulated. **B.** Steady state changes (t= 400-600 ms after the climbing fiber stimulus) in net spike count. Asterisks denote p-values of p < 0.0001 compared to control, using the student's t-test.

We conducted additional measurements to show that 1-EBIO has specific effects on the post-complex spike pause. 1-EBIO has no observable effect on the complex spike waveform, as neither an analysis of the number of spikelets per complex spike (control, 3.5 ± 0.29; 1-EBIO, 3.75 ± 0.48; p=0.64) nor of the complex spike duration (control, 4.5 ± 0.6 ms; 1-EBIO, 5.2 ± 0.9 ms; p=0.21) showed significant differences. We also determined that 1-EBIO has only moderate effects on baseline Purkinje neuron spiking rate, reducing it from 47 ± 0.7 Hz in control to 40 ± 5.5 Hz (**Figure 3-6**). We attribute this moderate slowing to the fact that regular complex spike input is likely to elevate basal calcium levels slightly explaining our results; prior work has found that 1-EBIO under some conditions has no effect on basal spike rate (Walter et al., 2006) but in other experiments a small reduction in firing rate is observed in the absence of CF input (Womack and Khodakhah, 2003).
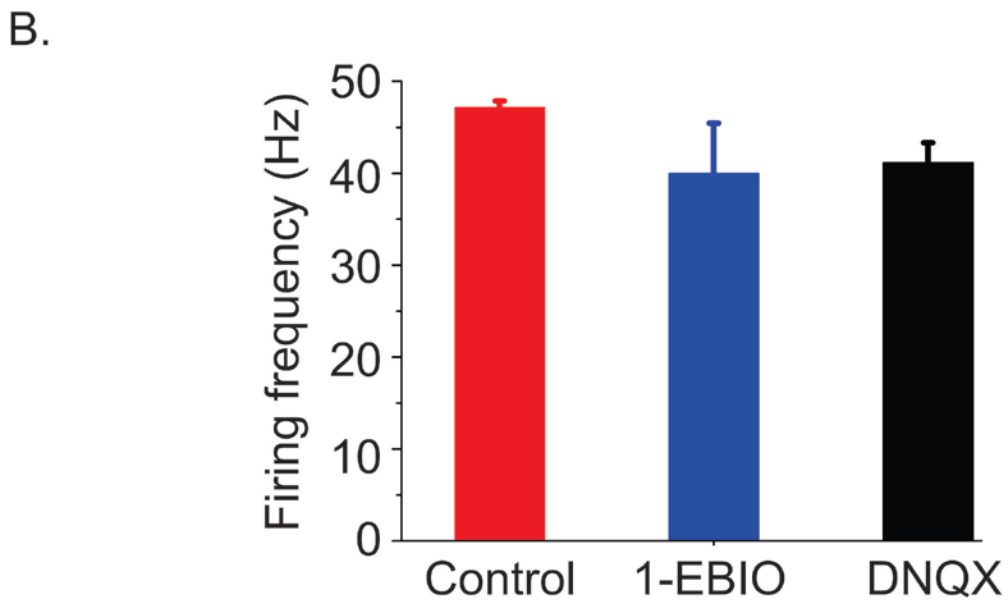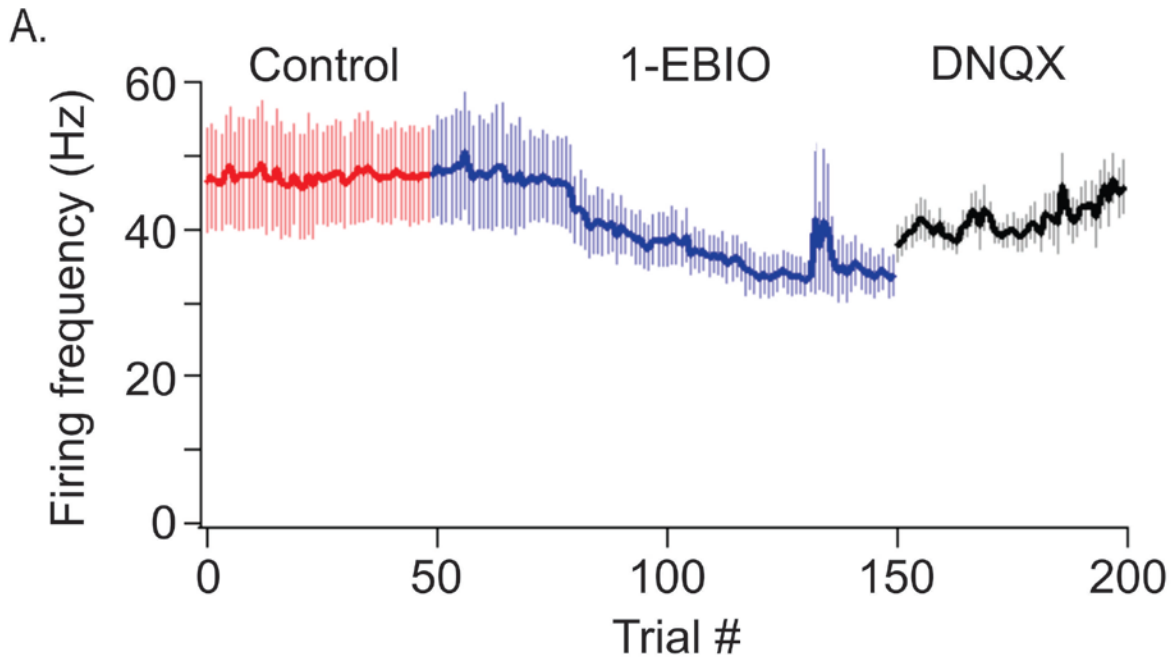
Figure 3-6. The effect of 1-EBIO on basal firing frequency of Purkinje neuron. A. 1-EBIO was bath applied for 100 trials (about 13 minutes), during which the frequency of Purkinje neuron simple spiking decreased. **B.** A histogram summarizing the average firing frequencies in each drug condition.

The second drug used to prolong the post-complex spike pause was ZD7288, a HCN channel blocker. Hyperpolarization-activated cation (HCN) channels are highly expressed in cerebellar Purkinje neurons, and both HCN1 gene deletion and blockade of HCN1 channels leads to bi-stable firing in Purkinje neurons (Williams et al., 2002; Nolan et al., 2003). This bi-stability is characterized by alternating periods of normal pace-making and periods of silence. These observations suggest that upon strong hyperpolarization, Purkinje neurons rely on HCN1 channels to help resume the more depolarized membrane potential at which they pacemake. With this in mind, low concentrations of ZD7288 (1 µM) were used. This concentration was determined, in separate experiments, to cause approximately 50% inhibition of the HCN-mediated current (I_h). As summarized in **Figure 3-7**, the presence of ZD7288 resulted in an increase in post-complex spike pause duration. On average ZD7288 lengthened the post-complex spike pause by $216 \pm 59\%$, $p < 0.0$
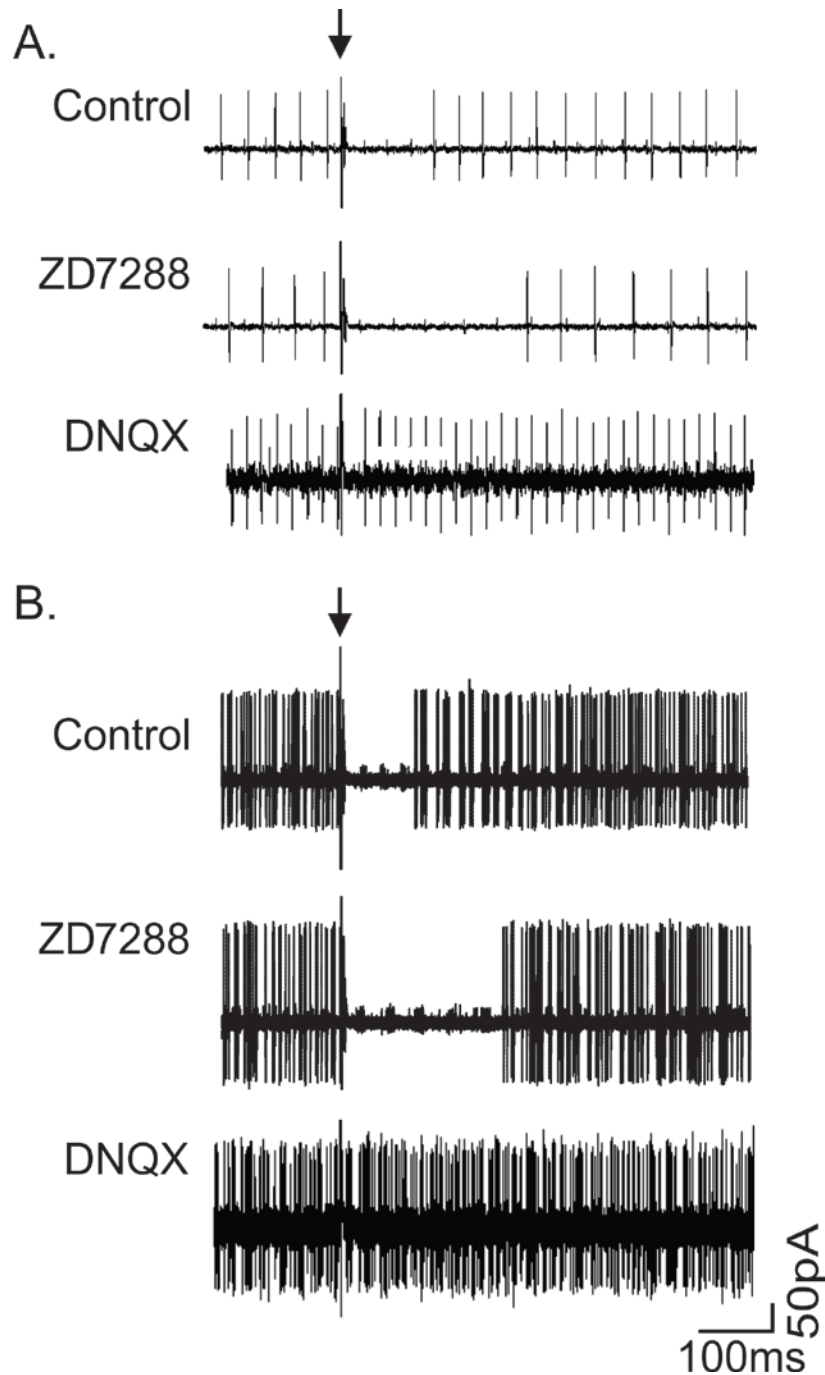
Figure 3-7. A longer post-complex spike pause is seen in ZD7288 compared to in control. A. Example traces showing single trial responses to climbing fiber stimulation (arrow) in control and ZD7288 conditions. **B.** 10 superimposed trials in control, ZD7288, and DNQX (no complex spike or pause) conditions to demonstrate robust and reliable changes in pause length.

The longer pause duration in ZD7288 was reflected in the more spikes "lost" analysis as well, losing about 11.4 ± 3.2 spikes in ZD7288 versus 2.06 ± 0.79 spikes in control, p<0.001, n=6; (**Figure 3-8**). This concentration of ZD7288 did not affect baseline firing frequency (control: 27.1 ± 4.2 Hz; ZD7288 23.1 ± 4.6 Hz) nor did it affect the complex spike waveform (number of CS spikelets: control, 2.75±0.5; 1-EBIO, 3.25±0.5; p=0.5; CS duration: control, 4.7±0.67 ms; 1-EBIO, 4.5.2±0.72 ms; p=0.9).
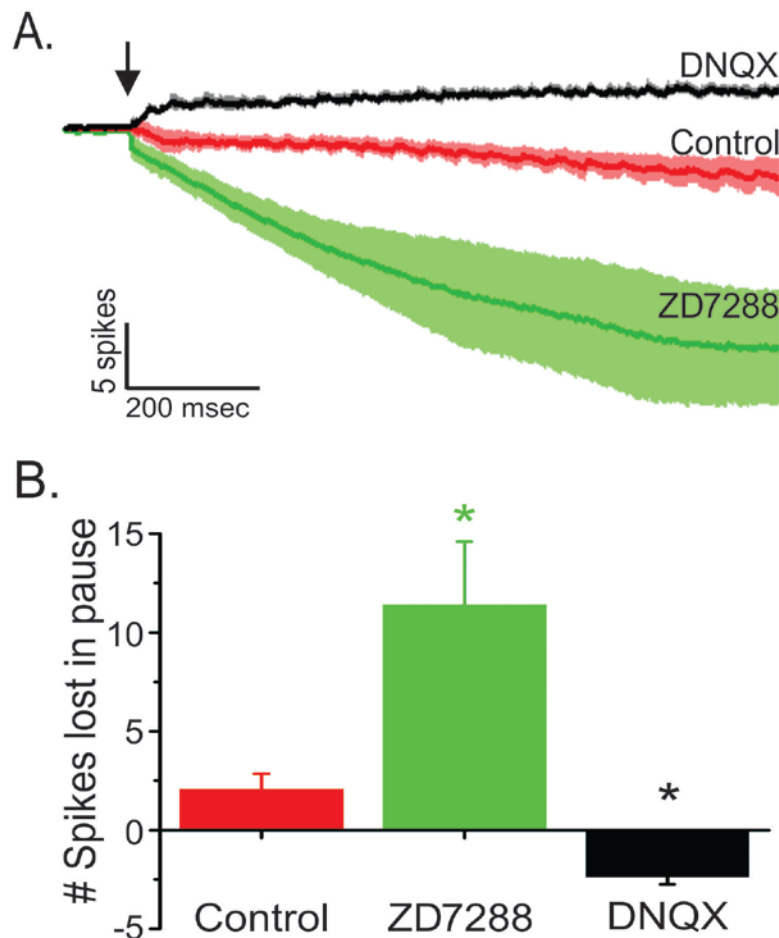
Figure 3-8. **More spikes are "lost" (i.e. longer pause) in ZD7288. A.** Average cumulative net spike counts for 6 PNs exposed to ZD7288 (green) compared to control (red) and DNQX (black) conditions. Light shading represents standard error of the mean (SEM). **B.** Steady state changes (t= 400-600 ms after the climbing fiber stimulus) in net spike count. Asterisks denote p-values of p < 0.0001 compared to control, using the student's t-test.

The overall results are summarized in **Figure 3-9.**
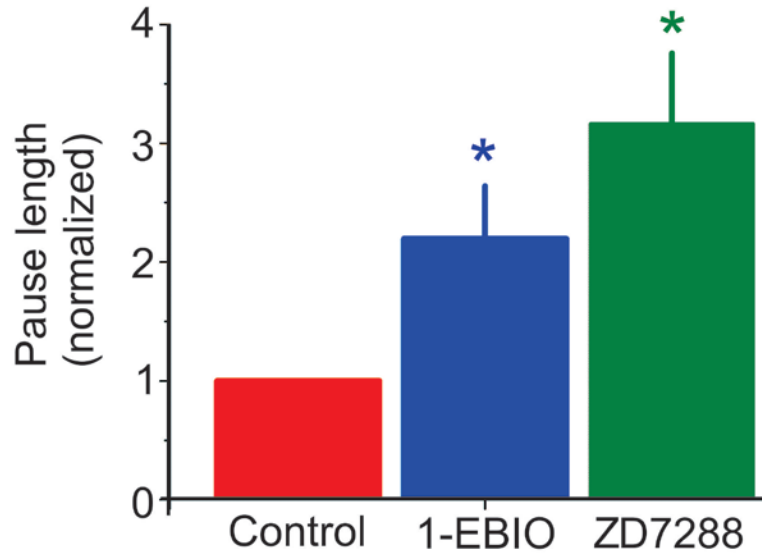
Figure 3-9. **Summary graph showing the effects of 1-EBIO and ZD7288 on prolonging the post-complex spike pause.** Averaged pause lengths in drug conditions were divided by averaged pause lengths in control aCSF conditions to normalize. $p < 0.02$ for 1-EBIO, $p < 0.0075$ for ZD7288.

Taken together these *in vitro* experiments validate two mechanistically distinct

pharmacological strategies for prolonging the post-CS pause that can be used to test

how manipulations of the post complex spike pause affect the learning rate in-vivo .

CHAPTER 4

DEVELOPMENT OF EYE-BLINK CONDITIONING

HARDWARE, SOFTWARE, AND DATA ANALYSIS TOOLS

PURPOSE AND REQUIREMENTS

The purpose of the eye-blink conditioning experiments were to validate, *in vivo*, whether the use of the drugs shown to prolong the post-complex spike pause *in vitro* would affect cerebellum-dependent learning. We suspected that a longer post-complex spike pause would facilitate, or speed up, learning, and tested this by delivering the same drugs, 1-EBIO and ZD7288, during eye-blink conditioning, the cerebellar-dependent learning task.

To complete the classical eye-blink conditioning experiments I built a set-up that would be able to collect and analyze electromyogram (EMG) data from two rats simultaneously. The system was modeled after an existing set-up in Dr. Richard F. Thompson's lab at the University of California, although I made many upgrades in the hardware, software, and data analysis procedures.

The conceptual requirements of the system were as follows:

1. Keep it as simple as practical.
2. Make it as comfortable as possible for the animal subjects.

The technical requirements of the system were as follows:

1. Allow for simultaneous data collection from two animals at a time.
2. Minimize the electrical noise for improved signal-to-noise and reliable data collection.
3. Design a rat-proof animal to system interface.
4. Maintain flexibility in the variation of key experimental parameters.

5. Enable substantial automation of the data analysis.

6. Abide by all IUCAC and ARC animal welfare protocols

INSTRUMENTS AND DESIGN

The flow diagram for the experimental set-up is shown in **Figure 4-1**.
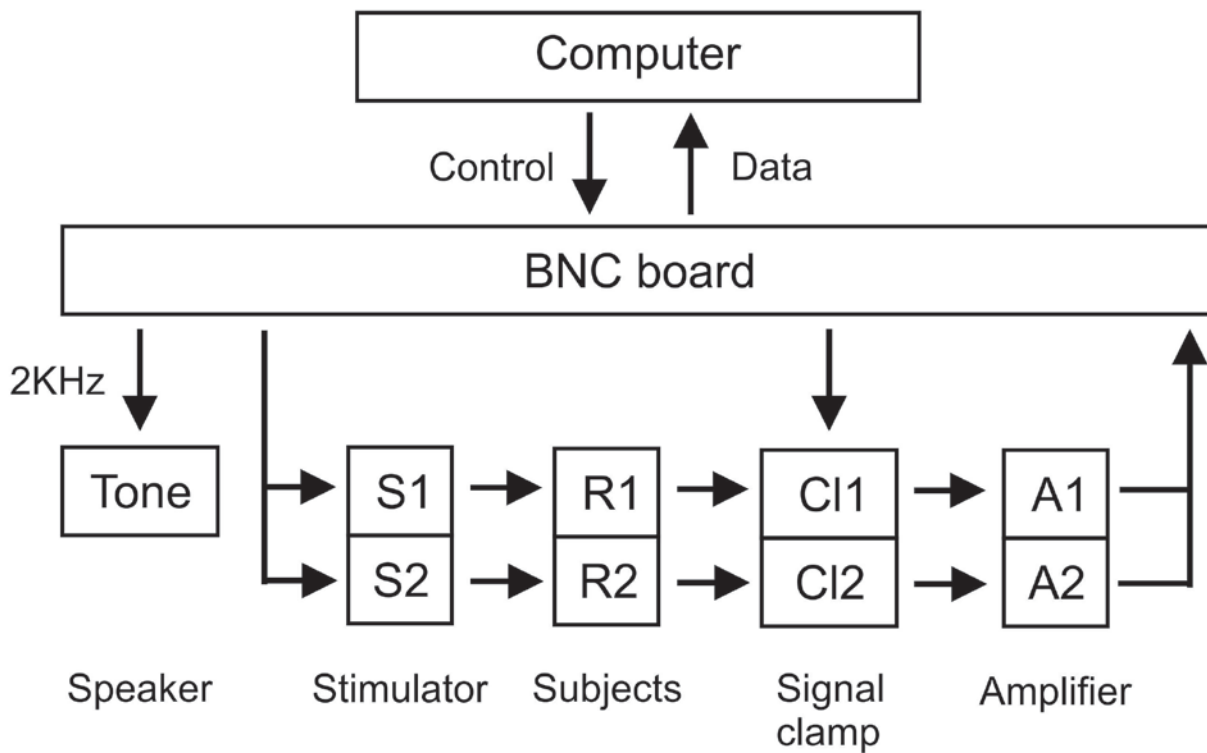
Figure 4-1. **Flow diagram for the eye-blink instrumentation set-up**

The key components of the set-up are as follows:

- A computer program purposefully developed for these experiments and loaded

  into a notebook computer controls the experiment.

- A BNC data acquisition board that controls the routing of the control signals as well as the acquisition of data.

- A 2KHz tone (generated digitally in the software) to be used as the conditioning stimulus

- An electrical pulse generator (stimulator) to deliver a 10msec electrical shock to the upper eyelid muscle to be used as the unconditioned stimulus.

- A signal clamp box that blocks the stimulation artifact in the EMG response, preventing the stimulation signal from reaching, and potentially overloading, the amplifier. The EMG recorded activity signals of the animal's eye muscle movements are thus routed through this clamp box before reaching the amplifier.

- An amplifier to filter the noise and amplify the EMG signals before sending the data to the computer via the BNC board.

The equipment description is as follows.

- BNC acquisition board: National Instruments BNC-2090 USB DAQ

- Stimulator: Grass S88 dual stimulator

- Clamp box: Cambridge Electronic Design (CED) 2804

- Amplifier: AM Systems 1700 differential AC amplifier

- Two Plexiglas boxes contained within a sound isolating cabinet. The Plexiglas cages have an SLC6 6-channel commutator (Plastics1, Roanoke, VA) fit in to the ceiling panel. The sound damping cabinet was padded with Styrofoam and

surrounded by a Faraday cage made of a grounded wire mesh to minimize electromagnetic noise.

- A 363-363 cable (with a spring cover, Plastics One Inc., Roanoke, VA) with one end attached to the outer fitting of the SLC6 commutator and the other end cut and modified to have banana plug endings. An un-modified 363-363 cable attaches to the inner fitting of the SLC6 commutator (inside the Plexiglass cage). The unattached end of this inside cable will attach to the plastic pedestal (housing the implanted electrodes) mounted on the rat's head during surgery.

Figure 4-2 shows a functional connectivity diagram implementing the instruments described above and in **Figure 4-1.**
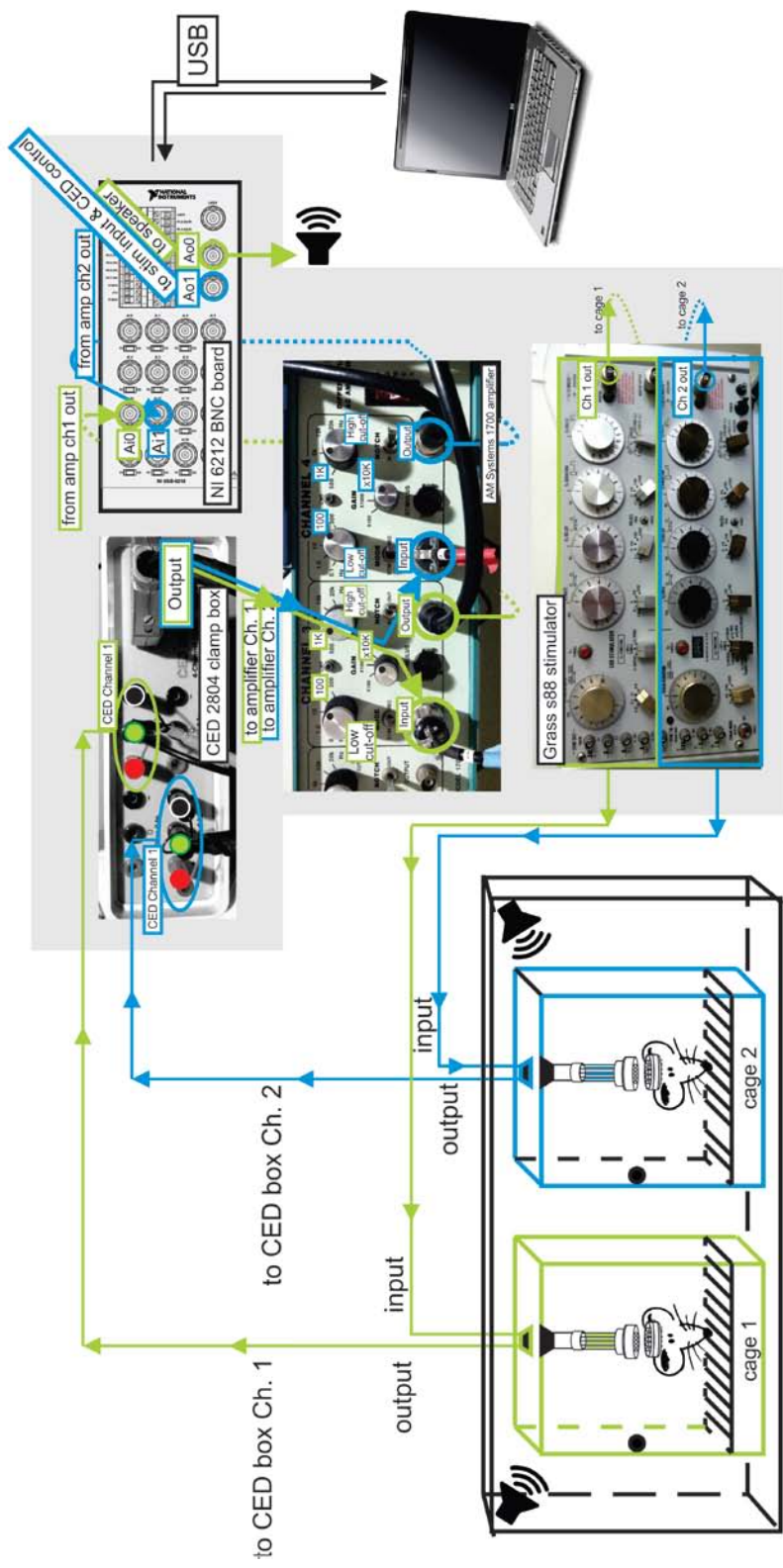
Figure 4-2. Functional connectivity diagram for the eye-blink instrumentation set-up. The connections between the eye-blink set-up instruments are shown. On the BNC board, Ai0-1 are the analog inputs (from the amplifier) and Ao0-1) are the outputs (to the speaker, and stimulator and CED box control (using a T-connector) respectively).
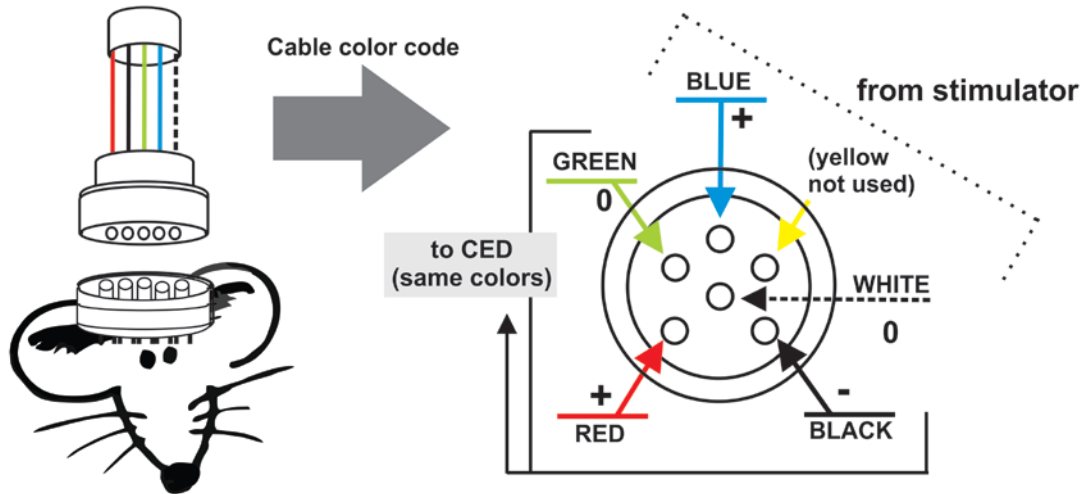
Figure 4-3. Connecting the animal subject to the eye-blink equipment. Each cable wire was a different color, and corresponded to a specific implanted electrode. Specific colors were maintained throughout the set-up for continuity. Of the five wires used, two (blue and white) corresponded to the incoming stimulation signal (+/0), and the other three wires(red, green, and black) corresponded to the EMG signal (+/-/0) that is sent to the CED box.
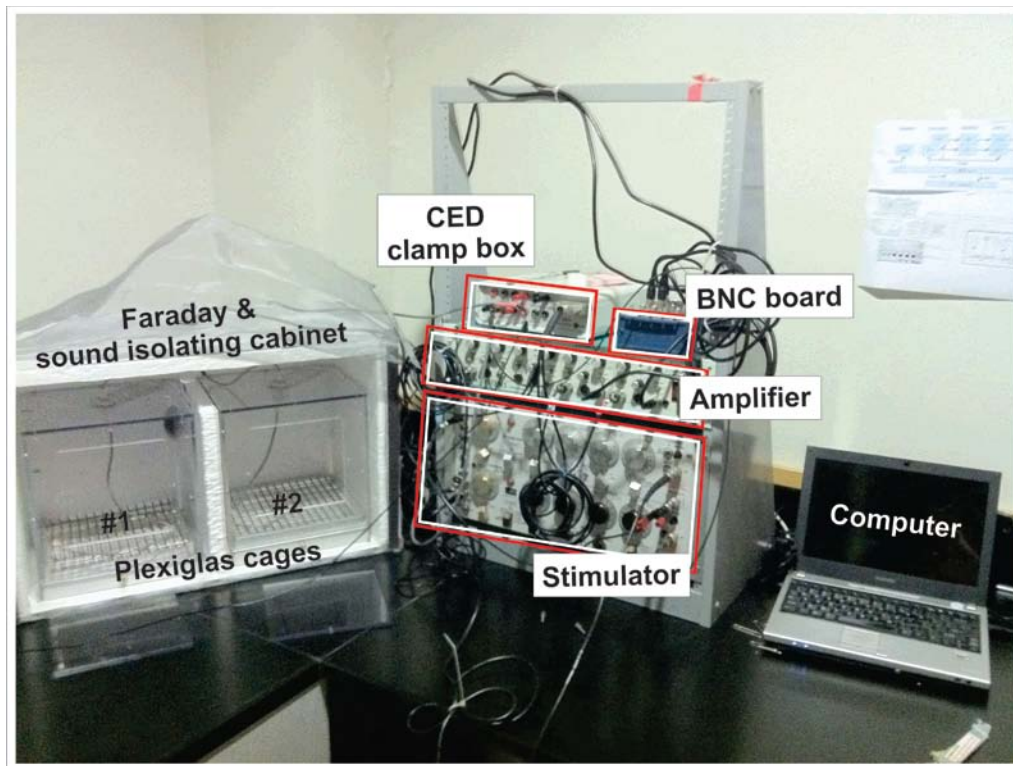
Figure 4-4. A photograph of the completed eye-blink set-up.

EMG Electrodes

The implanted electrodes used to deliver the electrical shock to, and record electromyogram (EMG) data from the upper eyelid muscle are Teflon-coated stainless steel wires (A-M Systems #791000, 0.003" bare, 0.0055" coated) with the Teflon stripped at contact points. In the first step a wire 1.5-2" long is cut from the spool and 1 centimeter of Teflon stripped from the end using fine forceps. Simultaneously, a drop of solder is added at the small end of a female Amphenol pin (FHC #30-40-1). The Teflon-stripped centimeter is folded to make a small hook at the end of the stainless steel wire. Using the soldering iron to heat up the Amphenol pin (and solder already in the hole at the end of the pin), the hook at the end of the stainless steel wire is inserted into the solder drop in the pin. Because stainless steel does not solder, the small hook serves to mechanically prevent the electrode from being pulled out of the pin. Once the solder had cooled, another centimeter of the Teflon coating was stripped at the other end of the electrode and a power meter was used to confirm that the electrode is electrically connected to the gold pin. The stripped electrode end will be the part inserted into the upper eyelid muscle. A detailed drawing is provided in **Figure 4-5**.
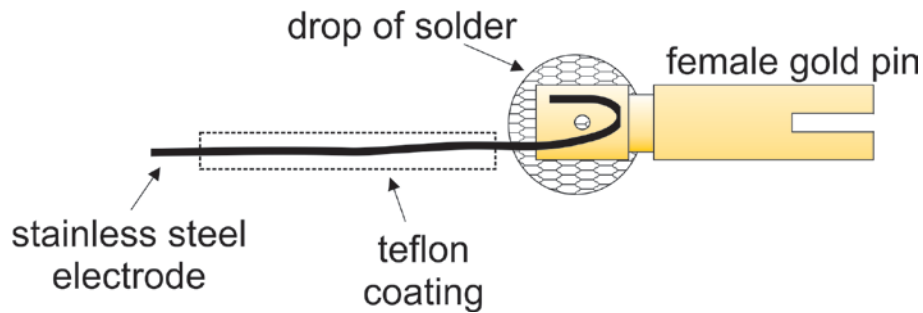
Figure 4-5. **Diagram detailing the stainless steel electrode connected to the gold pin.** Even with the solder, the hook is a necessary mechanical trick to prevent the electrode from being pulled out of the gold pin since stainless steel does not solder with conventional electronic solder products.

Head-stage

5 of the gold pins, each with an electrode attached, are fitted individually into five of six holes in a circular 6-hole plastic pedestals (Plastics One Inc., #MS363 Pedestal 2298 6-pin white; specify "with holes drilled through" when ordering). The gold pins are inserted into the plastic pedestal non-soldered side up, such that the electrodes hang out from below. The result should be that the bottom of the pedestal shows the soldered ends of the gold pins and their corresponding electrodes. The non-soldered ends of the gold pins reach the "top" surface of the pedestal. Dental cement is added to the bottom of the pedestal to cover the gold pins and ensure the wires do not cross. Four of the wires (excluding the ground wire) should face in the same general direction since these four will be inserted into the same eye muscle. It is important to keep track of which wires connect to which pins if it is desired to insert them in a specific order. This finished head-stage will be mounted onto the bare rat skull (with the dental cemented side down) using dental cement. See surgery methods for more details. **Figure 4-6** shows the details on the pedestal fabrication.
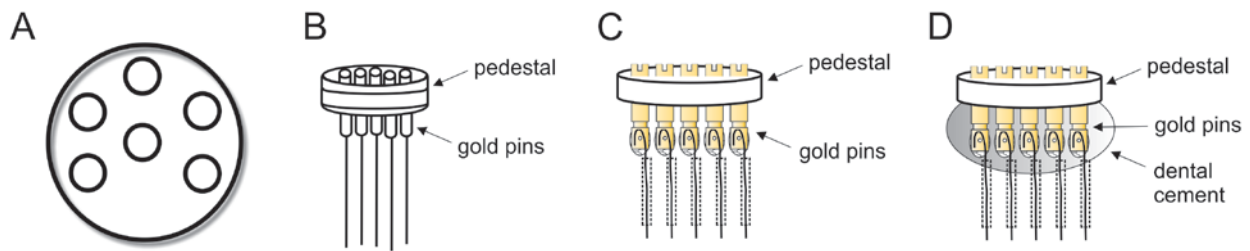
Figure 4-6. **A diagram of the finished head-stage: a plastic pedestal with electrode-containing gold pins. A**. View of pedestal from above. **B**. Side view of the pedestal showing how the electrodes protrude from underneath. **C.** Diagram depicting electrode and gold pin orientation, and **D.** extent of dental cement.

Connecting the head-stage to the eye-blink hardware

A 363-363 cable (with a spring cover, Plastics One Inc., Roanoke, VA) stays connected to the inside connector of the commutator (secured into the top panel of the Plexiglass behavior box), hanging. Because of the asymmetrical nature of the pedestal, the orientation of the hanging cable must correspond to the orientation of the rat's head-stage. The sixth, and unused pin, can be cut if it gets in the way (the unused hole will sometimes fill with dental cement). The gold pins in the rat's head-stage are female, whereas the pins in the connecting cable are male, and so the cable simply fits into the pedestal and screws on to secure. This prevents the rat from removing the cable during the duration of the experiment. The spring covering prevents the rat from chewing through the cable (see below).

Another 363-363 cable stays connected to the outer connection of the commutator. The end of this cable was cut and the endings re-soldered to necessary terminals. Three of the wires (red, green, and black) terminate in banana plugs that connect to the CED clamp box, and two of the wires (blue and white) terminate in a female BNC terminal (the white wire corresponds to the BNC shield) that will connect to the stimulator box. The wire from the sixth, and unused channel, is cut.

CED Clamp Box

The CED clamp box (model 2804 Cambridge Electronics Design, Cambridge, UK) precedes the amplifier and exists to "blank" the stimulation artifact recorded, to prevent that signal from reaching the amplifier. While the stimulator sends a shock signal only to the eye muscle, the implanted electrodes delivering the shock are in the same muscle as the implanted electrodes recording the EMG signals. As a result, the EMG wires pick up the stimulation artifact as they record muscle activity, encoding it in the EMG signal that leaves the rat.

The clamp box was triggered 5ms before the stimulation, and grounds any stimulation signals until 5ms after the shock ends. This served to prevent the EMG wires from picking up the stimulation artifact and consequently overloading the amplifier with the large stimulation signals.

The problem with this is that the triggering and release of the clamp itself saturate the amplifier. Under these conditions the amplifier saturated at both onset and offset of the stimulus, with the saturation occurring faster than the amplifier response speed (2V/sec). This saturation caused the amplifier filter to oscillate, resulting in a fluctuation, or "ringing" in the signal, whose duration was dependent on the voltage of the stimulus.
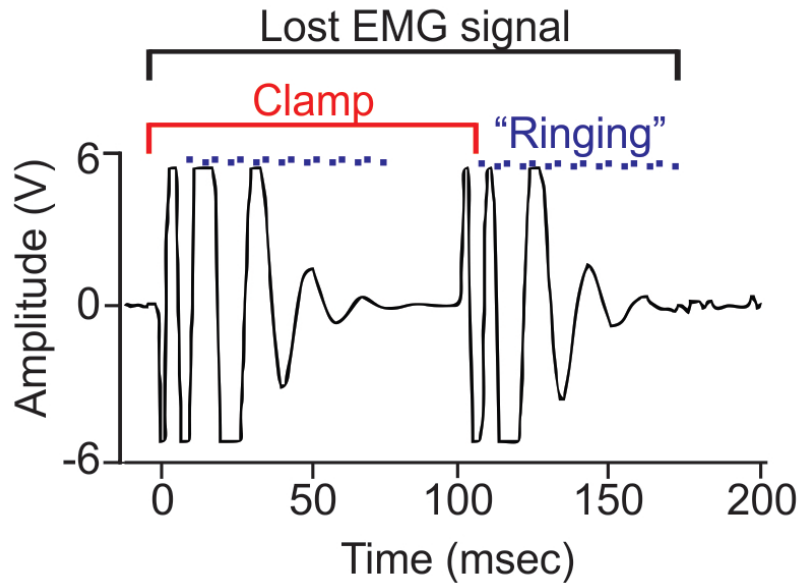
Figure 4-7. Example trace of amplifier ringing. The clamp box signal saturates the amplifier both at onset and offset, causing the amplifier filter to oscillate. The oscillation, or "ringing" at the offset extends the window of "lost" data, to almost double the actual stimulation signal (100msec).

The EMG signal is occluded during the time the clamp is active. The additional filter oscillations occlude any EMG signal that should be recorded during and just following the delivered eye-shock. This problem was resolved by decreasing the shock time to 10 ms (rather than 100 ms), which still evoked a full eye-blink but reduced the duration of the ringing and consequently the amount of data that had to be blanked during analysis.
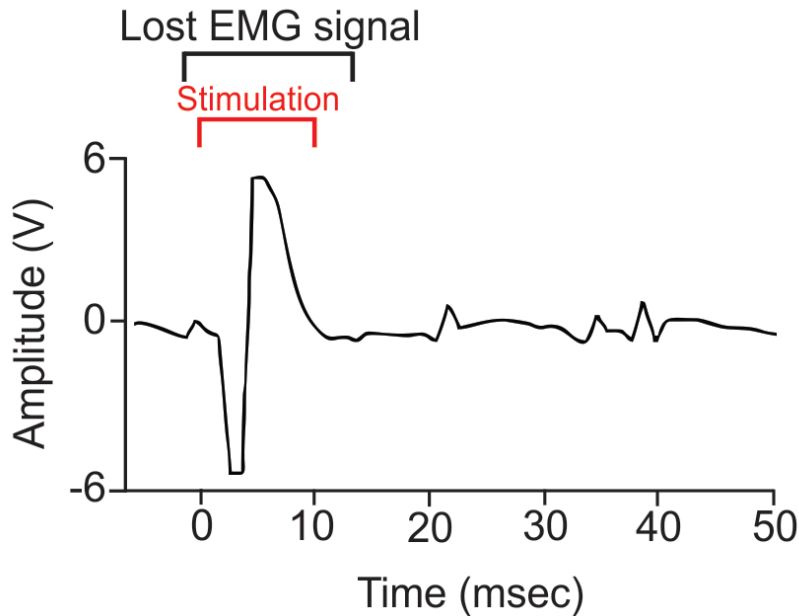
Figure 4-8. **Reduction in the amount of stimulus-induced artifact.** A shorter stimulation pulse (10ms instead of 100ms) prevents excessive amplifier filter oscillation, even in the absence of the clamp box. The time window that must be "blanked" due to stimulus artifact is greatly reduced.

The original clamp box had 2mm safety socket inputs, but because these terminals were difficult to match, I replaced the panel with female banana plugs inputs (red, green, and black to match the wires). The clamp box is connected via its "trigger" input (back panel) to the analog output 1 (AO1) of the BNC board via BNC cable. The output of the CED clamp box is a multi-pin cable that terminates in 4, 5-contact male Amphenol connectors. The cable was ordered from CED at the same time as the clamp box. I modified the 5-pin connectors to ground the ground input from the CED box (to maintain continuity) (**Figure 4-9)**.

5-pin connector terminal from CED clamp box
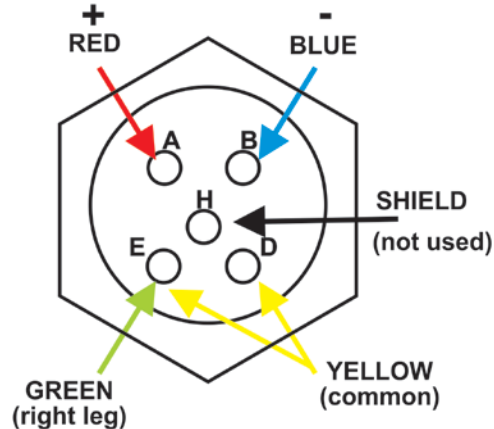
Figure 4-9. **5-pin connector terminal from CED clamp box.** The wiring was modified to ground the incoming EMG signal. These terminals connect at the amplifier inputs, bringing the EMG signals from the CED clamp box.

Signal Amplifier

The amplifier used was a differential AC pre-amplifier model 1700 from A-M Systems. Although it has four channels available, only channels number 3 and 4 were used (for reasons that are unknown, channels 1 and 2 looked noisy during initial troubleshooting). The band pass filter settings are set at 100Hz for low cutoff, 1 KHz for high cutoff, and the notch filter on "off" mode. The notch passes all frequencies except those in a determined "stop band" range of frequencies. In this amplifier the notch filter centers on 60Hz, so while it sounds like a useful approach to block electrical noise associated with the AC power grid and fluorescent lights, the inclusion of the notch filter causes an increase in stimulus artifact-related oscillation. By building a faraday cage around the sound-attenuating box I could keep the notch filter out and still prevent 60Hz electrical noise infiltration.

The EMG signals were recorded at gain x10K. Gain x1K can be used in cases where baseline noise is higher than desired but the signal-to-noise is still adequate.

A summary diagram of the timing for all the key signals is provided in **Figure 4-10.** The timing parameters for the stimuli were as follows:

- Total trial length: 2,000ms (2 seconds)
- Baseline: -1,000 to 0ms
- Tone (CS) onset at: 0ms
- Tone duration: 300ms
- Electrical shock (US) onset at: 290ms
- Shock duration: 10ms
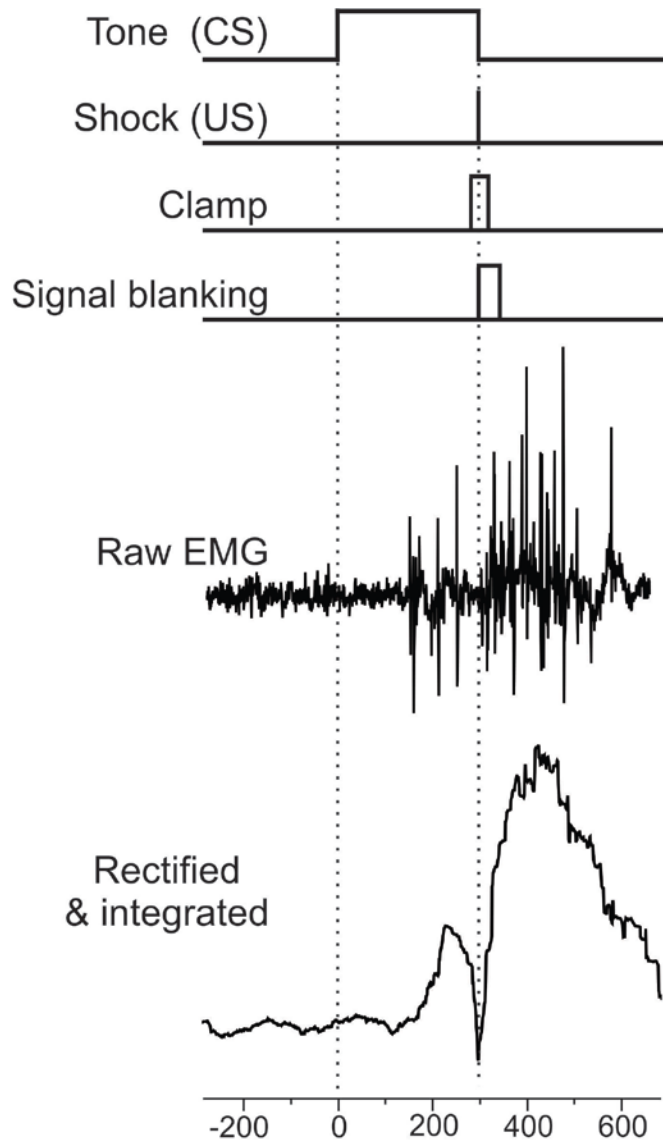
Figure 4-10. **Diagram showing the timing of the key signals during EMG acquisition.** An EMG signal trace is shown for reference.

Drug Infusions

Instruments needed:

- 5 or 10 µL Nanofil syringe (World Precision Instruments Inc., Sarasota, FL)

- 11Plus micro-injector (Harvard Apparatus, Holliston, MA)

- Polyethylene tubing (catalog #427406, Intramedic Clay Adams Brand, Becton Dickinson and Company, Sparks, MD)

  - Inner diameter = 0.38mm (0.015"), outer diameter = 1.09mm (0.043"), 30.5m (100 feet)

- 27 ½ gauge syringe needles

  - (PrecisionGuide, Becton, Dickinson and Company, Franklin Lakes, NJ)

- 1 mL syringes (Becton, Dickinson and Company, Franklin Lakes, NJ)

- Cannula internal (catalog #C312I, Plastics One, Roanoke, VA)

  - For clarity, the cannula piece implanted in the rat is called the "cannula guide." The "cannula internal" is a smaller cannula (also hollow, like the guide) that fits into the cannula guide.

To prepare:

- Prepare drugs: 1-EBIO and ZD7288 were made up in sterile 0.9% saline (APP Pharmaceuticals, Schaumberg, IL)

- Cut a piece of tubing ~2 feet long

- The cannula internal has a short side and a long side: fit the short end into one end of the tubing (see **Figure 4-11**)
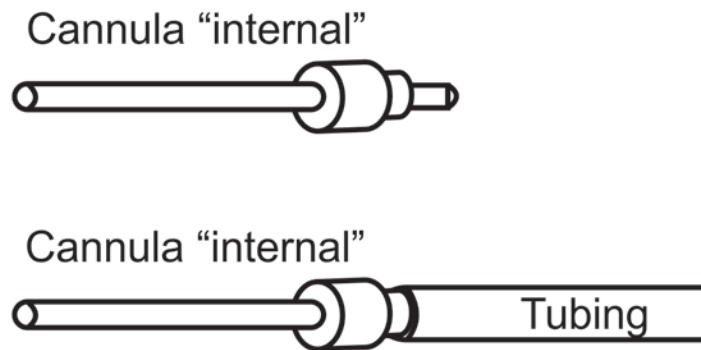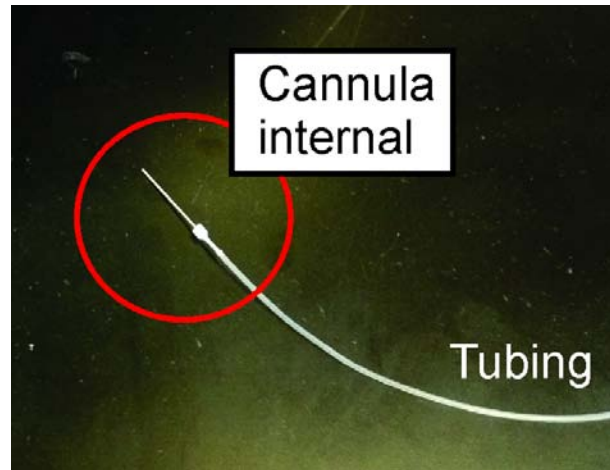
Figure 4-11. Photo and cartoon of cannula and tubing connection for drug infusions.

- Fill a 1 mL syringe with sterile water

- Fit the syringe needle into the free end of the tubing (with the cannula internal at the other end), and fill the tubing completely with water

- Pull up slightly on the syringe plunger to create a small air pocket at the cannula end of the tubing (this will be used to visually confirm that that the infusion is working, since such small volumes are being used)

- Replace the attached 1 mL syringe with the 10uL Nanofil syringe (plunger should be completely down)
    - o Once the Nanofil syringe is attached, pull plunger up a bit if the air pocket at the cannula guide end is too small (want at least a half-inch or so)
- Insert the cannula internal into the drug solution and pull the Nanofil syringe plunger all the way up slowly
- Using a sharpie marker, mark, on the tubing, the position of the air bubble with a line (this will allow the experimenter to confirm that the liquid inside the tubing is fact moving)
- Position and secure the Nanofil syringe in the Harvard Apparatus micro-injector (see **Figure 4-12**)
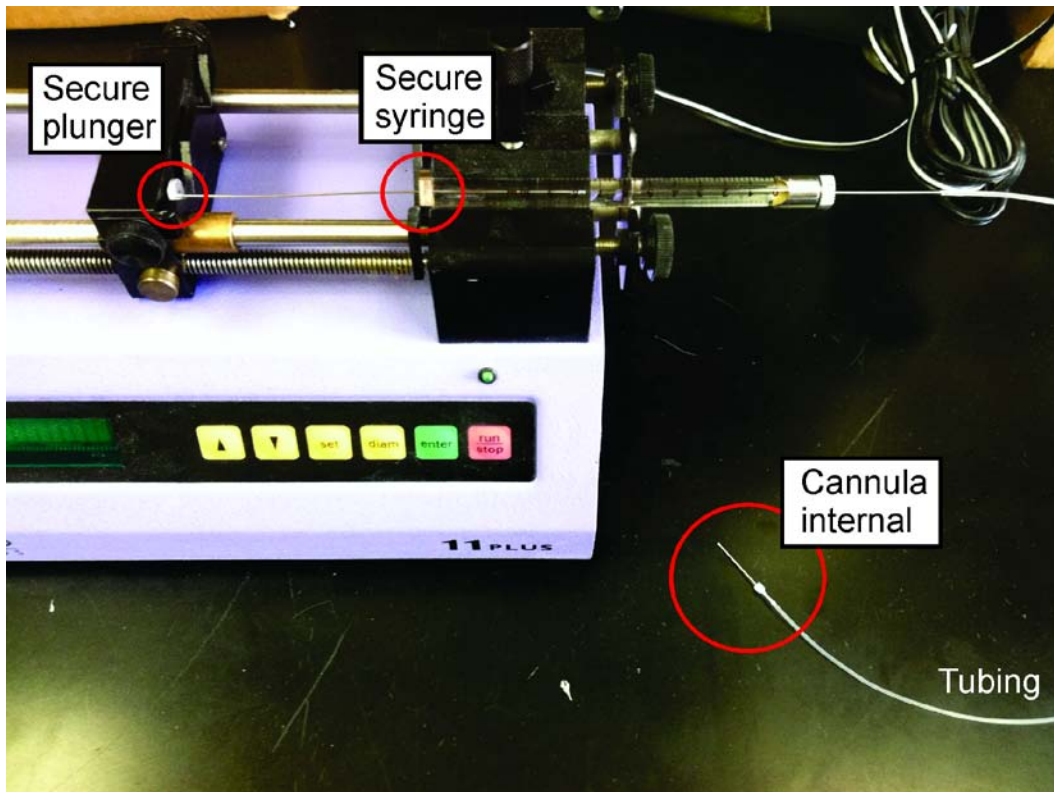
Figure 4-12. **A photo showing the prepared Nanofil syringe secured to the micro pump for drug infusion.**

- Set micro-injector to injection rate of 1 μL/min

To inject:

- Hold the rat securely

- Remove the dummy cannula (screws on and off)

- Insert the cannula internal (the piece at the end of the tubing), into the cannula guide (the piece that is implanted in the rat's head)
    - Tubing should be long enough so that rat can move and won't pull on tubing

- Start the infusion, and following infusion let the cannula stay in for another minute or two before removing.
    - It is easiest if you hold the rat during the injection since sharp head movements, or curiosity, can partially or completely remove the cannula and tubing during infusion.

- To clean the tubing flush sterile water and then air through the tubing using a 1 mL syringe. Each drug should have different tubing, and each animal its own internal cannula (these can be cleaned in ethanol but dry overnight).

Notes:

- Test micro-injector once before connecting to rat

- "Handling" (i.e. holding) the animals for up to a week before surgery and in between surgery and training make holding the rat to remove the dummy cannula

and during the drug infusion possible , since they have become comfortable with your holding them.

Acquisition and analysis software

The acquisition and analysis programs was written from scratch in LabView (National Instruments, Austin, TX) with the help of Raul Serrano, an electrical engineer in Julio Vergara's lab (UCLA, Department of Physiology). The aim for both programs was to make them as flexible as possible to have complete parameter freedom for both acquisition and analysis of any and all aspects of the EMG signal.

The acquisition software interface allows for digital control of timing parameters needed for eye-blink conditioning. These include the shock and tone onset and duration, total trial duration and inter-trial period (20-40sec random), data sampling rate, the number of trials, and the frequency of tone-only trials. In these trials the following parameters were used:

- Total trial length: 2,000ms (2 seconds)

- Baseline: -1,000 to 0ms

- Tone (CS): 0 to 300ms

- Electrical shock (US): 290 to 300ms

The analog output numbers for the tone (AO0) and shock (AO1) refer to the output numbers on the BNC acquisition board. The US stimulus can be delivered in single pulses or trains, making the software adaptable to different experiment needs. Upon running the program the user will be asked to choose the directory where the file will be saved, as well as the name of the file. The program is defaulted to running both

channels simultaneously, so two "save file as" prompts will appear, for channel 1 and 2

respectively. Files are saved as LabView TDMS files although through the analysis

program the user is able to extract either raw or processed data traces for separate

analysis in Microsoft Excel, Igor, etc.
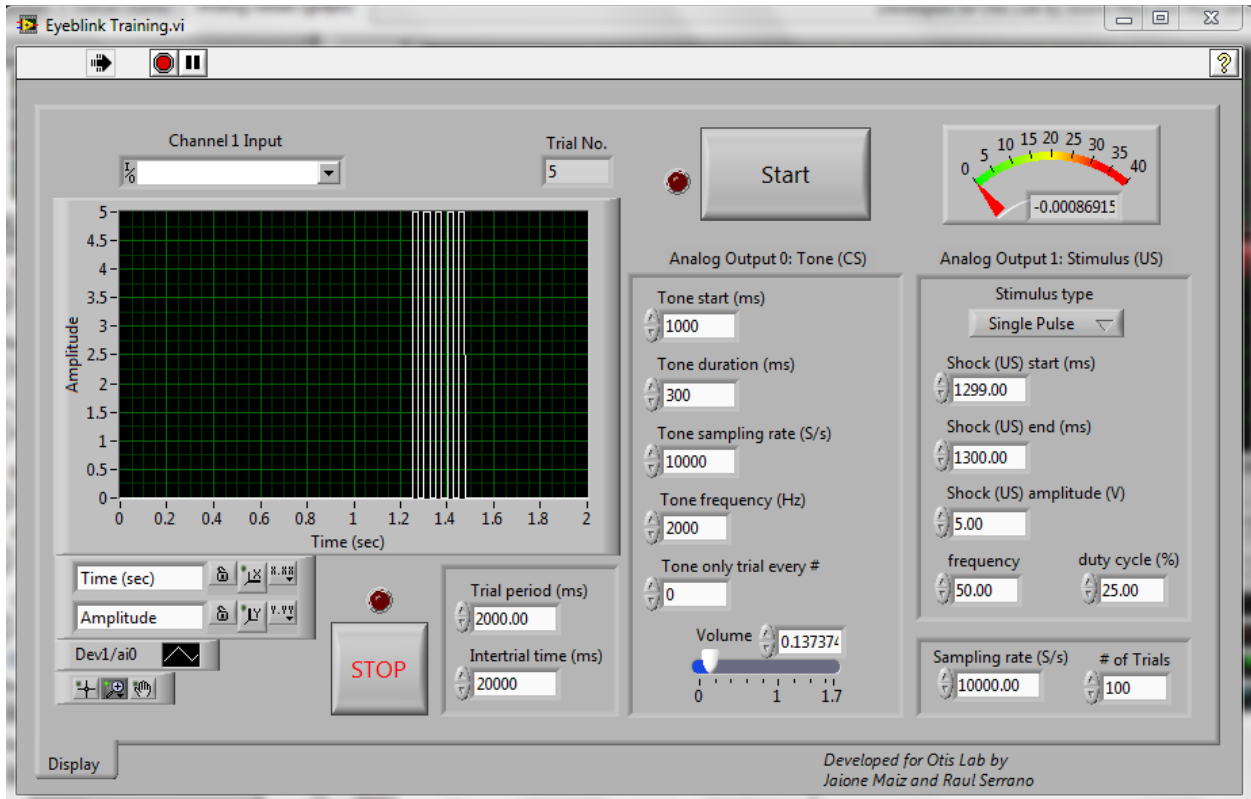
Figure 4-13. Acquisition software front panel. Digital control of stimuli parameters, including number of trials, the duration of each trial, the frequency of tone-only trials, the tone and shock onset and duration, and the data sampling rate. The program is set up to control three different outputs (two analog and one digital) and flexible for delivering single pulses or trains of stimuli. When running two animals simultaneously two graphs will appear to monitor EMG signals from both animals simultaneously.

The analysis program:
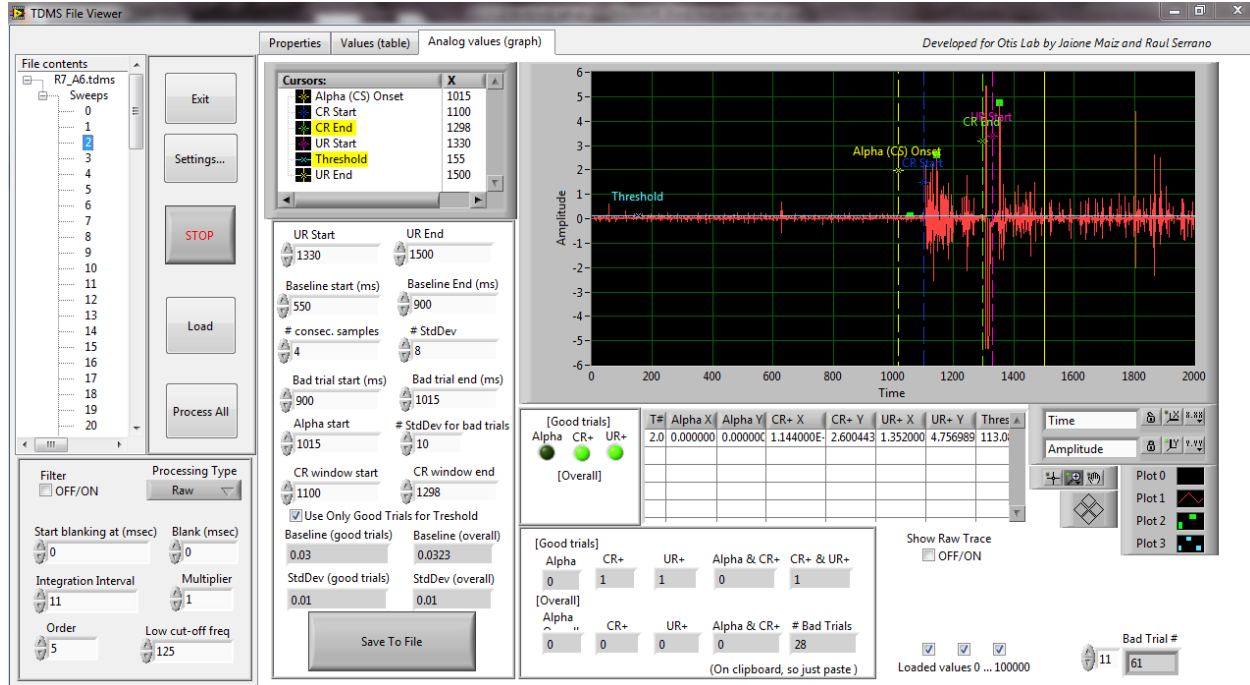
Figure 4-14. Analysis software front panel. Digital control of analysis criteria, including processing, detection of peaks, baseline parameters, and bad trial criteria. All analysis results (number of trials in each condition, peak timing and values, raw and integrated data values) can be extracted from the program.

In the analysis program data is loaded by the "load" button, although some of the analysis parameters, like "bad trial" criteria, should be set before loading a file since they are calculated upon file loading. The other analysis parameters can be changed after a file is loaded by using the "process all" button after changing the criteria.

The analysis program loads one file at a time, allowing the user to display any of the 100 trials (or "sweeps") in each file. The parameters for the different windows of interest, including baseline, alpha, conditioned response (CR), and unconditioned (UR) (**Figure 4-14)**, are entered manually.

In the lower part of the first column, the analysis type (raw, rectify and integrate, root mean squared (RMS), or integration algorithm from USC) can be selected, and those parameters (integration value, smoothing, filtering) determined. The "blanking" onset and duration can be set to remove the stimuli artifact data points.
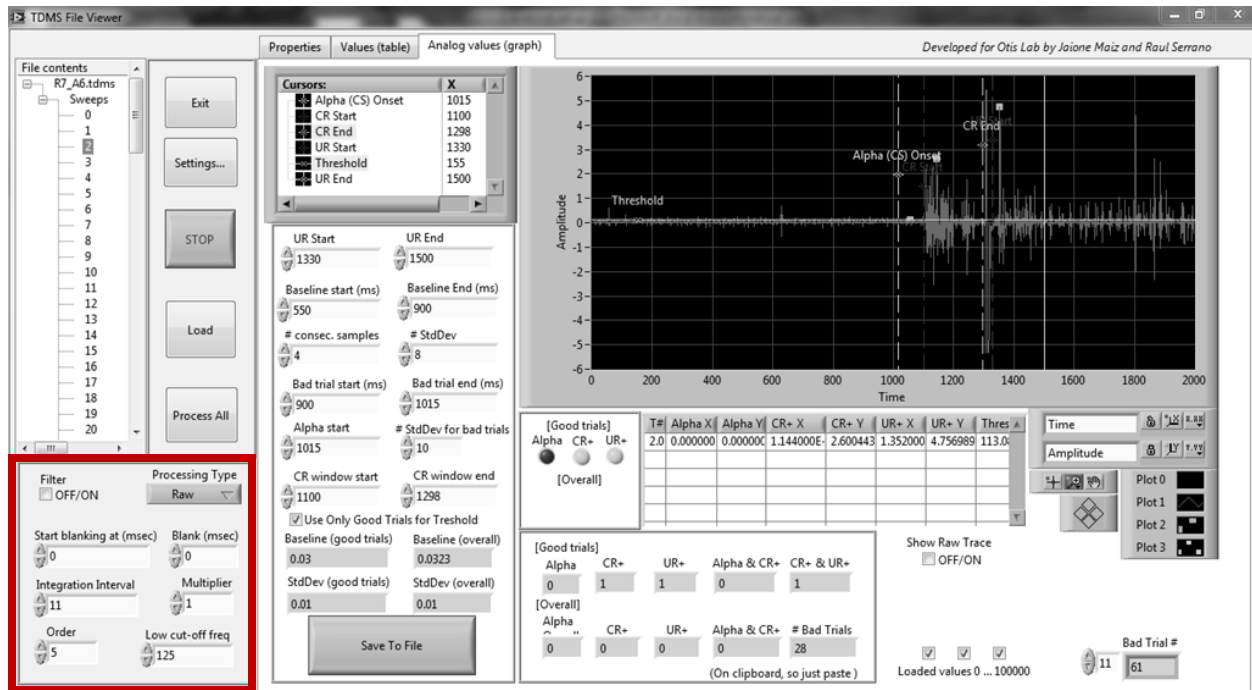
The ability to select between various types of processing was used for initial troubleshooting, and to be able to compare new data with preliminary data acquired at USC. Ultimately the RMS method was chosen, however, based on classical literature, including work by Joseph Steinmetz and colleagues (Lavond and Steinmetz, 2003).

The integration method used was a "boxcar" averaging method, essentially the average of a moving interval. The integration interval needs to be an odd number: the program uses an equal number of values on either side of any selected time point. For example, if an integration interval of 11 is used at time point 20ms, the integral value

assigned at 20ms will be the average integral of time points 15-25 (11 points total). If a data point is taken every millisecond, the next point in the analysis would be at 21msec, at which point the value assigned would be the average integral of time points 16-26, and so on.

The timing criteria for the rest of the conditions can be entered manually, and the cursors (for visualization) changed simply by changing the numbers. As a reminder, the stimuli parameters were:

- Total trial length: 2,000ms (2 seconds)

- Baseline: -1,000 to 0ms

- Tone (CS): 0 to 300ms

- Electrical shock (US): 290 to 300ms

So for these experiments the following analyses windows were used (refer to **Figure 4-15**):

- Baseline (to determine threshold): -450 to -100ms

- Bad trial consideration window: -100 to 15ms

- Alpha ("startle") response window: 15 to 100ms

- Conditioned response window: 100 to 290ms

- Unconditioned response onset: ~330ms (depends on stimulus blanking)
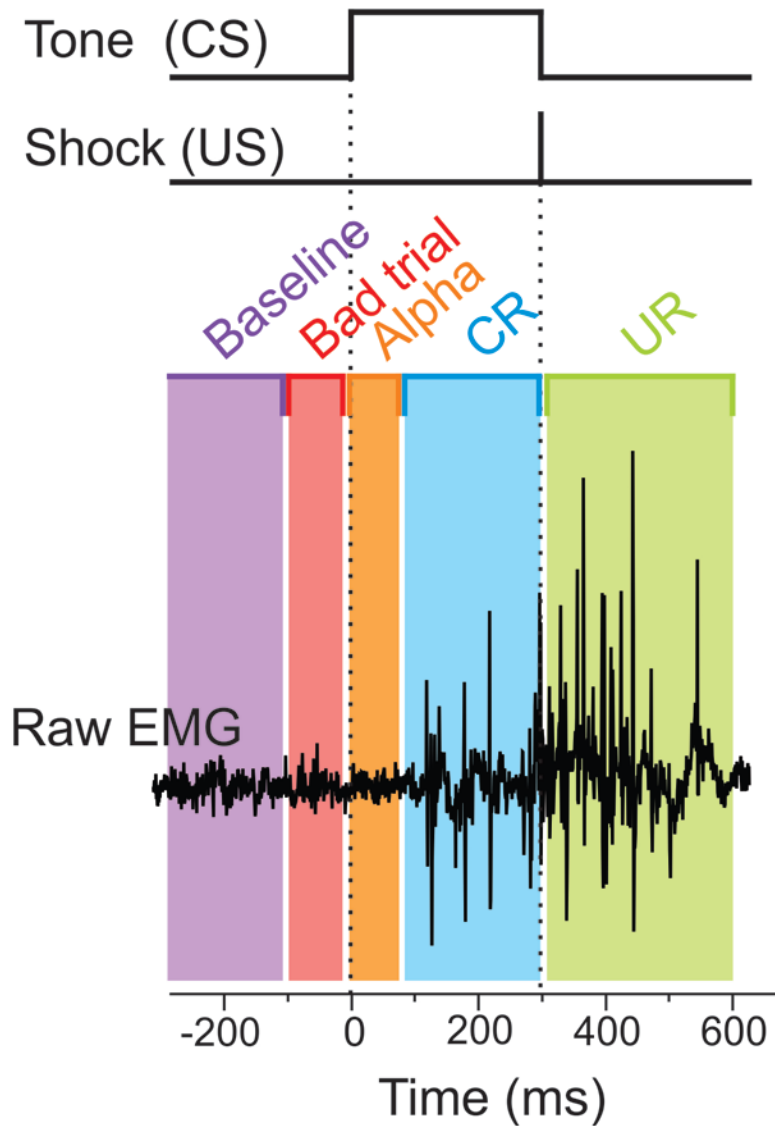
Figure 4-15. Windows of analysis for the different peak detections.

Bad trials are determined by spontaneous activity in the time period preceding tone onset (-100 to 15ms), and are excluded. They correspond to random blinking by the rat in a way that is unrelated to the experiment. Once the bad trials have been

determined, a window at the bottom right corner will show which trial numbers were determined "bad."

Even though the tone (CS) begins at 0msec, the conditioned response (CR) window begins at 100ms to eliminate "startle" or "alpha" responses that are quick reflex eye blinks that can occur with tone onset (Boele et al., 2010). Alpha responses (15 to 100ms) are recorded but alpha-positive trials are eliminated from CR analysis. We had very few alpha responses, but it should be noted that more alpha responses are observed if the volume of the tone is very loud.

The threshold above which CRs, URs, and bad trials were counted was determined by a number of consecutive points (we used 4) that surpassed the average baseline plus a certain number of standard deviations. The number of standard deviations used to determine the CR and bad trial thresholds were determined separately for each animal, each day, depending on the specific signal-to-noise ratio, and were always above five.

Once the analysis values are set, the "process all" button in the first column will process all traces. After processing all, information about the number of alpha-positive, CR- or UR-positive, and combination CR and UR traces from all trials (overall) and from "good" trials only (bad trials excluded) are shown in a box directly below the graph. The values are also automatically placed on the virtual clipboard and can be pasted into another document (such as Microsoft Excel) just by using the "paste" function in that other program.
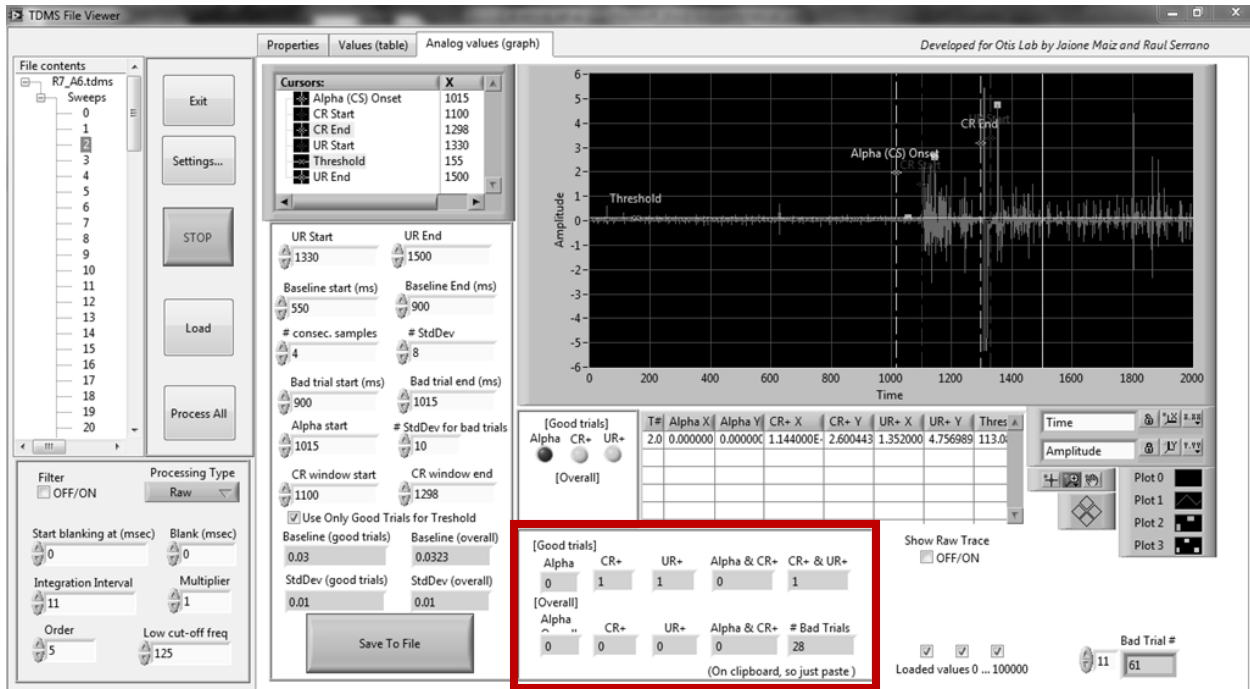
The "save to file" button at the bottom of the second column will save, in .DAT

format, the x and y values of alpha, CR, and UR peaks of an individual trial or all trials.

Once saved this table can be opened in Microsoft Excel as well.
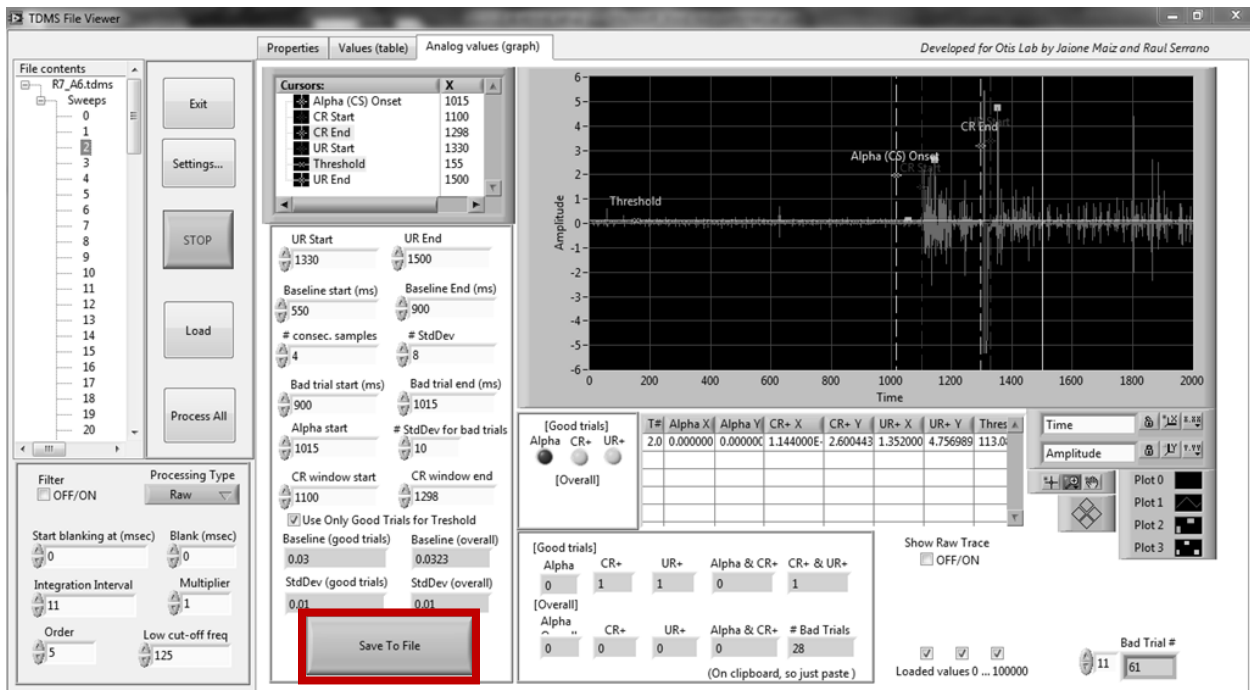
Once the data has been processed, raw, integrated, or CS-only trace values can be copied here to be pasted into the program of your choice, including Microsoft Excel. To recreate the graphs in any other program, chose the "values (table)" tab (the middle of the three) at the top of the interface.
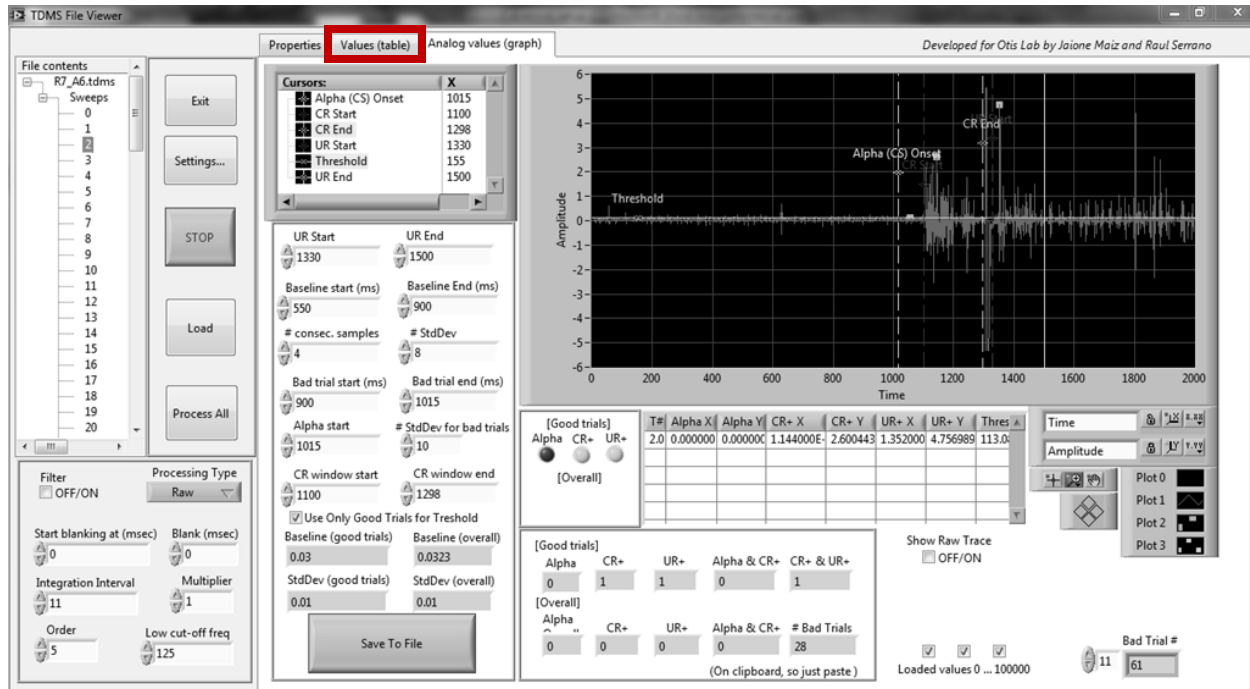
Once in the values tab, the user can copy data from all raw traces or all integrated (processed) traces. If the user selects a specific sweep from the left column then values from that sweep only will be copied. Once the data has been copied it can be pasted just by opening a spreadsheet and using the "paste" function.

TDMS File Viewer

Properties | Values (table) | Analog values (graph)

File contents
- Sweeps
  - 0
  - 1
  - 2
  - 3
  - 4
  - 5
  - 6
  - 7
  - 8
  - 9
  - 10
  - 11
  - 12
  - 13
  - 14
  - 15
  - 16
  - 17
  - 18
  - 19
  - 20
  - 21

Exit

Settings...

STOP

Load

Process All

Filter
OFF/ON

Processing Type
Raw

Start blanking at (msec)
1299

Blank (msec)
35

Integration Interval
11

Multiplier
1

Order
5

Low cut-off freq
125

Sweeps
9
-0.065515
-0.066009
-0.054659
-0.041171
-0.027519
-0.015676
-0.014690
-0.015347
-0.009755
0.010148
0.017385
0.020346
0.018701
0.009654
0.011792
0.048637
0.084494
0.097324
0.081369
0.059328
0.033011
0.010312
-0.007288
-0.013045
0.004226

Copy all raw Traces Copy all Integrated Traces 2
OK   OK

SUMMARY

The development of this set-up and software programs allowed for data collection and analysis of the classical eye-blink conditioning experiments. The set-up delivers timed unconditioned and conditioning (US: shock, and CS: tone) stimuli while simultaneously recording clean EMG signals relevant to the animal's upper eyelid muscle movements. The acquisition and analyses software programs allow for user-friendly, customizable parameter exploration, quantification, and exportation without having to go into the program code, resulting in flexible data handling.

CHAPTER 5

***IN-VIVO* EFFECT OF 1-EBIO OR ZD7288 ON**

CEREBELLUM-DEPENDENT CLASSICAL EYE-BLINK CONDITIONING

SUMMARY

Classical eye-blink conditioning is a cerebellum-dependent associative motor learning task. The task creates an association between an innocuous auditory tone, and a mild electrical shock to the eyelid muscle (*orbicularis oculi*) that results in an eye-blink. Following repetitions of this association, the audio tone by itself will result in an eye-blink.

The underlying cerebellar circuitry driving this learning is fairly well understood. The eye-shock, and tone information, reach the Purkinje neurons via the climbing fibers (the "error" signal), and mossy fibers respectively. Purkinje neurons then modify their firing patterns, namely via timed pauses in firing that occur following the "error"-driven complex spikes. These post-complex spike pauses are windows of dis-inhibition on downstream Deep Cerebellar Nuclei cells, allowing them to activate their downstream motor targets.

The two drugs, 1-EBIO and ZD7288, which both lengthen the post-complex spike pause, were used during eye-blink conditioning to explore the effect of pause length on cerebellar learning. Both drugs, even though they work via two completely distinct, and opposing, mechanisms, enhance the learning rate during the early part of the conditioning protocol, thus reducing the onset time for learning. That is, the rats learn to associate the tone with the shock faster than their control counterparts, although all subjects reach the same maximum level. Therefore a longer post-complex spike pause does not result in more learning, but instead faster onset of learning. The fact that the two different drugs produce the same effect in enhanced learning onset increases

confidence in the hypothesis that post-complex spike pause length is directly relevant to the enhanced onset of cerebellum-dependent associative motor learning.

METHODS

Cannula and eyelid electrode implant surgery

All stereotactic surgeries were carried out using aseptic techniques approved by the UCLA IACUC.

Two to three month old male and female Sprague-Dawley rats were used based on classical experiments done in Richard F. Thompson's lab at USC and in the literature. All animals were handled for up to a week before surgery, and again following surgery and until training began. Essentially the rats were removed from their cages and held for 15-20 minutes so that they become comfortable with the experimenter. This prevents the handler from being bitten during drug infusions and while connecting the rat to inside the behavior cage, and also results in a more relaxed rat during experiments, resulting in better (less noisy) EMG recordings.

For surgery, all animals were anesthetized using a ketamine (75-85mg/kg) and xylazine (5-10mg/kg) cocktail delivered via an intra-peritoneal injection. Once anesthetized, the head was shaved and disinfected, and the animal was secured in a stereotaxic apparatus (David Kopf). As seen in **Figure 5-1**, two stainless steel anchoring screws were secured to the skull, and a stainless steel cannula guide (Plastics One, Roanoke, VA) was inserted through a small craniotomy into the left

cerebellum lobule HVI (stereotaxic coordinates from Bregma: AP=-10.85, ML=3.0, DV=-4.0) (Paxinos and Watson, 1998) and secured with dental cement. A homemade head-stage composed of a plastic pedestal (Plastics One, Roanoke, VA) with 5 stainless steel electrodes emerging, was secured with a drop of dental cement, and four of the electrodes passed through the upper left eyelid muscle (*orbicularis oculi*), with a small hook at the end to secure the electrode wire in the muscle. The fifth electrode was wrapped around one of the anchoring screws to serve as a ground. The skull was then covered with a layer of dental cement, and a sterile suture was used to close the skin around the implants. Finally, triple antibiotic gel was applied the wound. A dummy cannula (Plastics One, Roanoke, VA) was inserted and secured to the cannula guide to prevent clogging. Rats were given Buprenex (anti-pain, 0.05mg/kg) and carpofen (anti-inflammatory, 5mg/kg), and a week to recover. As seen in the photo in **Figure 5-1**, the operated eye should appear as if un-operated (fully open). The rat should appear completely normal (including pain and lesion-free) to be included in training trials.
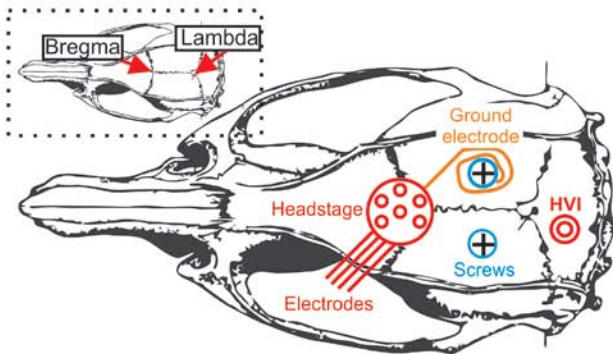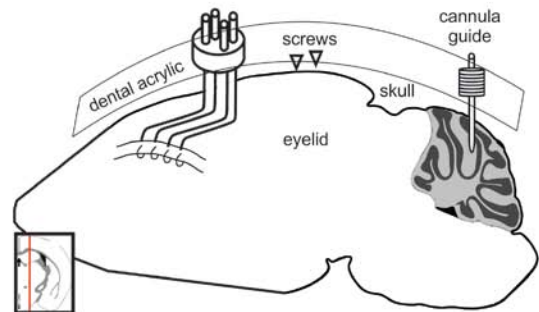
Figure 5-1. **Photos and cartoons showing implanted cannula and eyelid electrodes. A.** Top view; location of headgear relative to bregma and lambda. Biggest circle with six little circles corresponds to the pedestal with the five gold pins (attached to electrodes) inserted. The two red circles with black + correspond to the surgical anchor screws, and the double red circle corresponds to the cannula placed into the cerebellum. **B.** Side view; cartoon shows cannula placement in cerebellum lobule HVI. Eyelid wires run under the scalp but over the skull, and are inserted into the *orbicularis oculi* upper eyelid muscle, forming a small hook which secures them into the muscle.

<u>Delay eye-blink conditioning</u>

The following stimulus parameters were used: conditioning stimulus (CS) was a

300 ms, 2 KHz tone, and the unconditioned stimulus (US) was a 10 ms peri-orbital

unipolar electrical stimulation (via implanted electrodes) that co-terminated with the tone

(**Figure 5-2**). The inter-trial interval was randomized between 20-40 seconds.
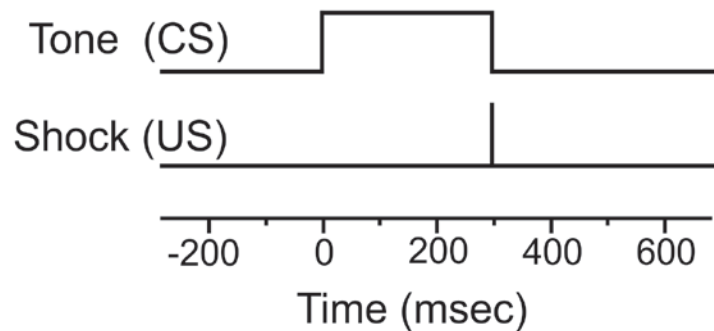
Figure 5-2. **Co-terminating stimuli during paired CS-US training trials.** Each trial
consisted of a 300msec 2 KHz tone whose onset precedes, and offset co-terminates
with a 10 ms peri-orbital shock. Consecutive trials were separated by randomly
generated 20-40 second intervals.

Training occurred over 11 days: two days of habituation (no stimuli), six days of

acquisition training (CS-US paired trials), and three days of extinction (CS only). During

training, nine CS-US paired trials preceded a CS-only trial, and this set of 10 trials was

repeated 10 times for a total of 100 trials per day (**Figure 5-3**).
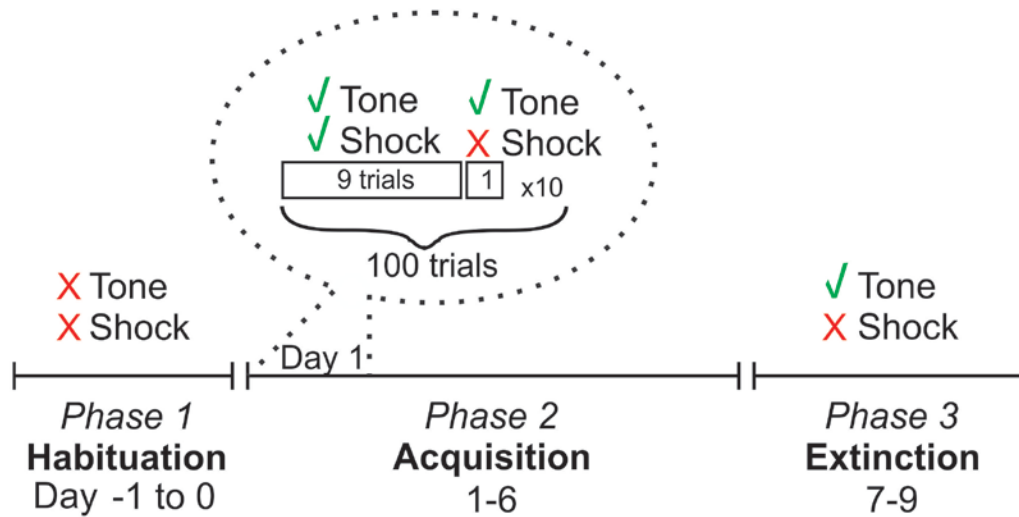
Figure 5-3. **Delay eye-blink conditioning training schedule.** Subjects undergo 100 trials each day. During habituation EMGs were recorded but no stimuli were delivered. During acquisition the 100 trials are divided into ten blocks of ten trials, with nine paired CS-US trials preceding a CS-only trial in each block. During extinction only the tone is delivered.

Days	Purpose	Protocol
1-2	Habituation	• No CS (tone) • No US (electrical shock)
3-8	Acquisition	• 100 trials per day (10 sets of 10) ○ 9 paired CS-US trials ○ 1 CS-alone trial • 20-40 sec random inter-trial interval
9-11	Extinction	• CS (tone) alone • No US (shock)

Table 5-1. **Table summarizing eye-blink training schedule with corresponding stimuli.**

Eye movements were detected by electromyogram (EMG) signals recorded by the electrodes implanted into the upper eyelid muscle during surgery (**Figure 5-1**). EMG signals were amplified at x10K gain, digitized at 10 KHz, and filtered between 100 and 1,000 Hz.
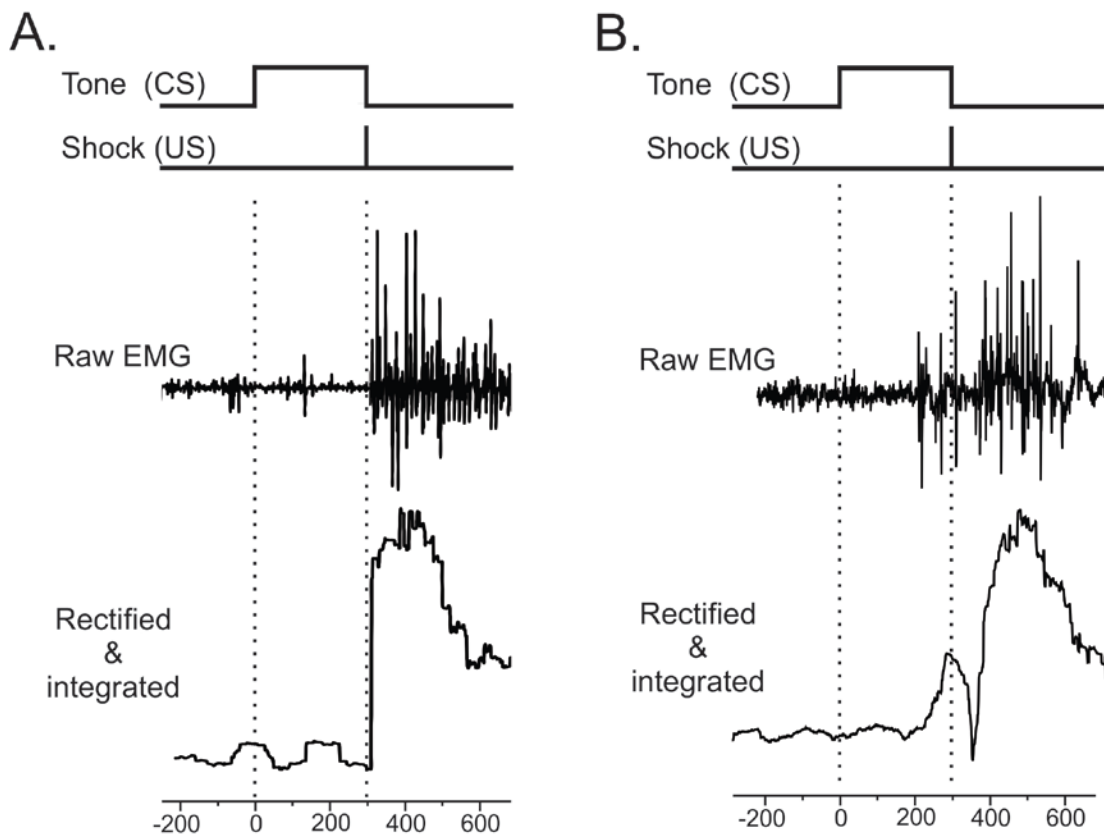
Figure 5-4. Example EMG traces showing an unconditioned response (UR) and the emerging of a conditioned response (CR) during paired CS-US trials. Raw EMG data is processed (described in Chapter 4) for analysis. The eye-blink setup, with its robust cabling system, electrode fabrication, precise surgery placement, and faraday cage covering results in traces with good signal to noise ratio.

<u>Drug infusions</u>

On acquisition days only, 1 μL of either [20 mM] 1-EBIO (a positive modulator of SK channels), [5 mM] ZD7288 (an HCN (I_h) channel antagonist), or 0.9% saline was microinjected via the implanted cannula, over one minute, ten minutes before beginning the training session. The details of this infusion are described in Chapter 4.

<u>Data and statistical analyses.</u>

Eye-blink conditioning experiments were done in a custom built system at UCLA, with some preliminary experiments completed in the lab of Dr. Richard F. Thompson at the University of Southern California. Custom routines, written in LabView (National Instruments, Austin, TX) were used for EMG acquisition and analysis. A small subset of trials (2% per animal on average) were excluded on the basis of

1. spontaneous activity in a 100 ms pre-tone window or

2. startle (alpha) responses determined by above threshold activity within the first 100 ms following tone onset.

Conditioned responses were counted when integrated EMG traces exceeded a threshold criterion in the last 200 ms of the conditioned stimulus. Repeated measures ANOVA was done in Stata (College Station, TX).

Figure 5-5. Diagram of analyses time-frames.

RESULTS

Infusion of each drug during eye-blink training results in faster acquisition of conditioned eye-blink
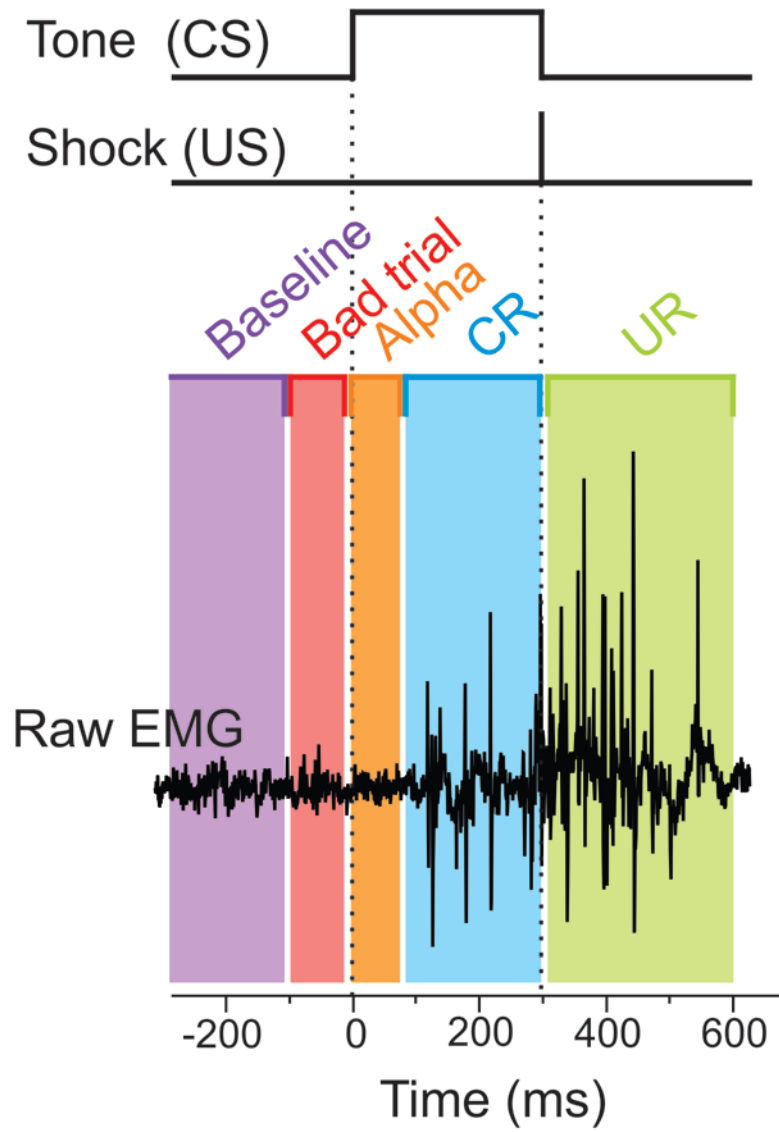
In order to assess the effects of prolonging post-CS pause on associative cerebellar learning we tested the influence of 1-EBIO and ZD7288 in an eye-blink conditioning paradigm. Rats were trained in a classic 300 ms-delay conditioning paradigm (**Figure 5-2**), using a co-terminating tone as the conditioning stimulus and a peri-orbital shock as the unconditioned stimulus.

Ten minutes prior to training each day the rats were injected with 1 µL of either 1-EBIO (n=12), ZD7288 (n=8), or a saline control (n=8) into lobule HVI of the cerebellum via the surgically implanted cannula. Electrodes implanted into the *obicularis oculi* muscle relayed electromyogram signals that corresponded to eyelid muscle movements allowing us to monitor eye closure.

Training sessions consisted of 6 days of associative conditioning with 90 tone plus shock pairs and 10 tone alone trials delivered each day as described in **Figure 5-3** and **Table 5-1**. By day 6, all rats, regardless of the drug they received, reached the same performance plateau in terms of the percentage of conditioned response (59% 1-EBIO, 65% ZD7288, and 65% saline control, **Figure 5-6**). During the first four days of training, however, the rats receiving 1-EBIO and those receiving ZD7288 had significantly higher % CR, as determined by repeated measures ANOVA analyses (1-EBIO: days 1-3 $p<0.01$, day 4 $p<0.05$, and ZD7288: days 1-3, $p<0.05$), when compared to saline control animals. Early in the training paradigm, animals receiving either 1-EBIO or ZD7288

showed a higher rate of acquisition of conditioned responses as compared to the saline-injected control counterparts, an effect that was most significant for 1-EBIO in the first two days of conditioning.
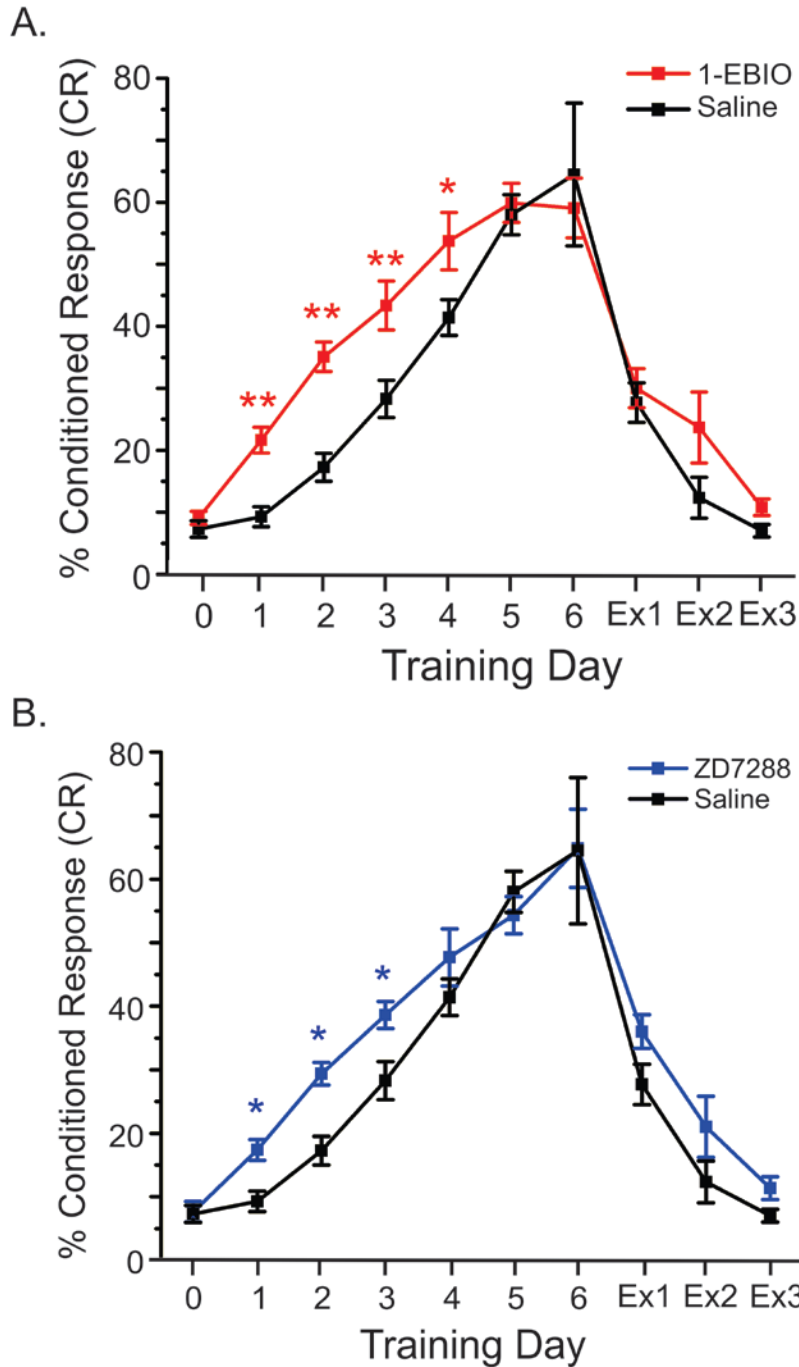
Figure 5-6. **1-EBIO or ZD7288 delivered before training results in faster acquisition of conditioned responses in delay eye-blink conditioning.** Percent conditioned responses (CRs) for each day. Day 0 is a habituation day, days 1-6 are training (acquisition) days, and Ex1-3 are extinction days. Animals received either **A.** 1-EBIO (n=12), **B.** ZD7288 (n=8), or saline (n=8) via an implanted cannula 10 minutes before training on acquisition days. Asterisks denote significant differences from control at $p < 0.05$, and double asterisks at $p < 0.01$, by repeated measures ANOVA.

The rate at which Conditional Responses were acquired is shown in **Figure 5-7**. This was calculated as the change in CR per day (derivative).
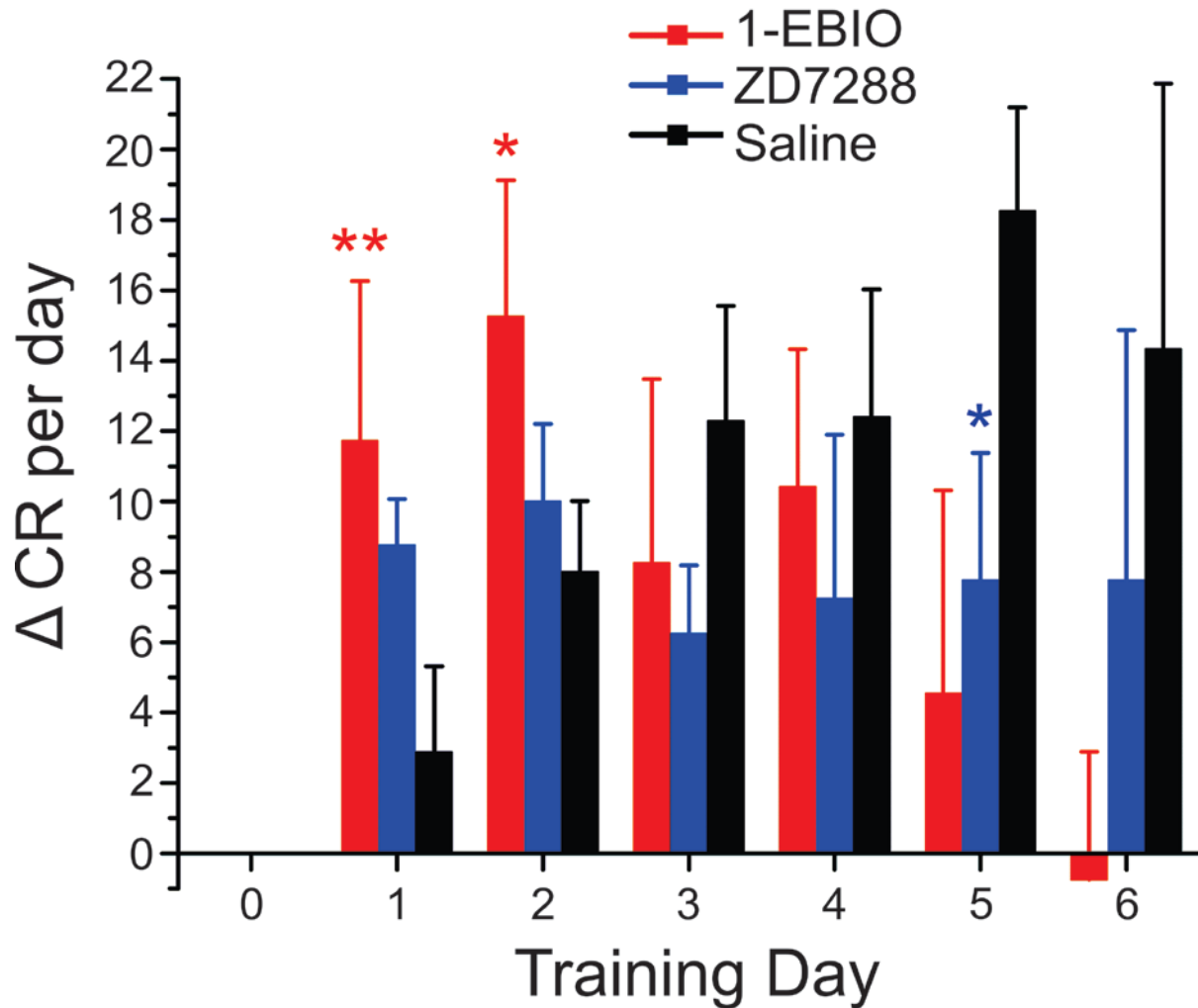
Figure 5-7. **Conditioned response (CR) acquisition rates with 1-EBIO or ZD7288.** Mean rate of CR acquisition on each training day as a function of experimental group. The double asterisk indicates a significant difference from saline at $p < 0.01$ and the single asterisk at $p < 0.05$.

EMGs were examined daily in an attempt to understand the details underlying changes in CR timing and amplitude between days as well as between drugs. Example EMGs are shown in **Figure 5-8**. Unfortunately this traditional method for obtaining eye-blink responses, via implanted EMG electrodes, is not a sensitive enough measure to examine subtle changes across days. Regardless we are able to see learning occur throughout training.
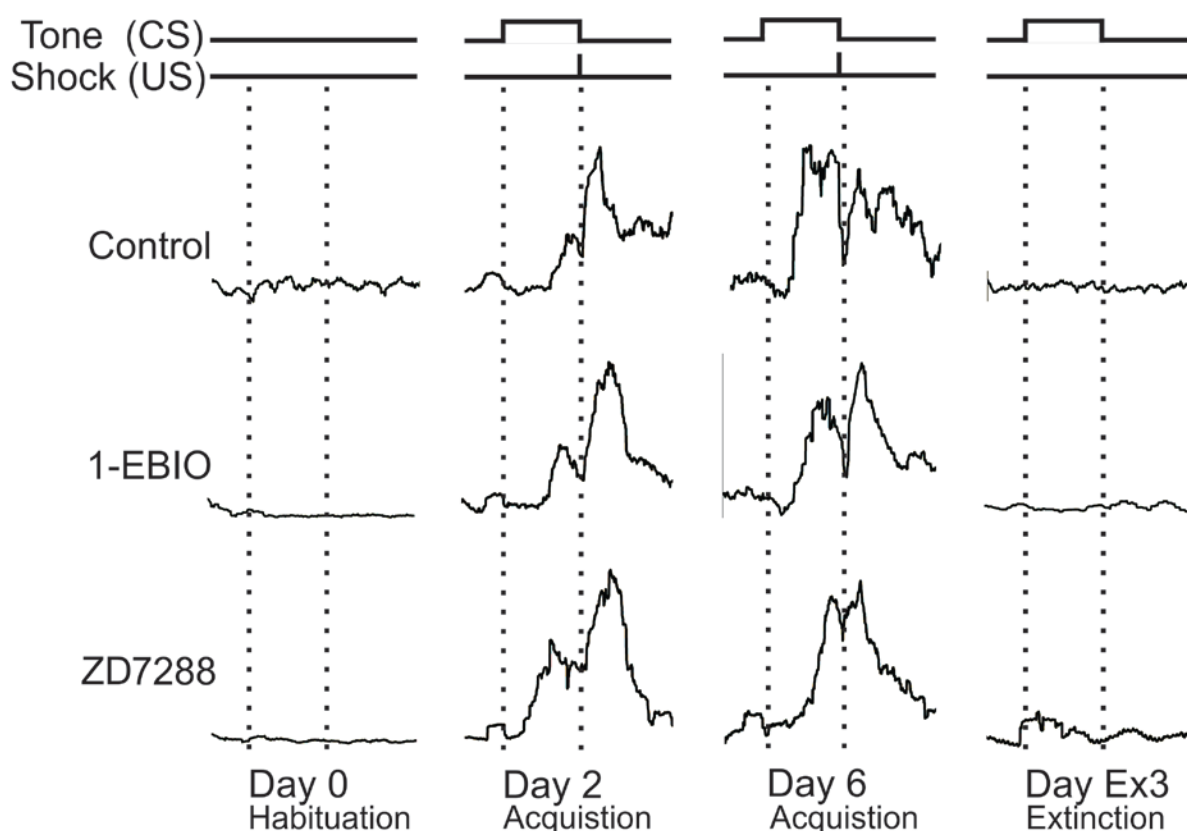
Figure 5-8. Example (processed) EMG recordings from the last habituation day, acquisition days two and six, and the last day of extinction. Conditioned responses occur between the two dotted lines. On the last day of habituation, as on the last day of extinction, no CRs or URs are observed. On all acquisition days, and in each trial, a UR (an eye-blink in response to the shock) is seen. On day 2 of acquisition, as the subject begins to learn the association, a small CR begins to emerge on some trials. By acquisition day 6 the CR is as big as the UR.

Enhancements in learning are rare, and yet the use of two mechanistically distinct drugs result in a similar facilitation of learning onset. Subjects receiving either drug, prior to acquisition days, show similar faster association of the tone (CS) with the shock (US), compared to their saline receiving counterparts. The drug effects to prolong the post-complex spike presumably play a role in facilitating associative learning onset in cerebellum-dependent learning.

Chapter 6

DISCUSSION OF RESULTS

Our results identify an important contribution by the post-complex spike pause to the rate of cerebellum-dependent learning. We find that enhancement of this specific component of the climbing fiber response enhances learning. Classical cerebellar learning theory proposes that the climbing fiber input to the cerebellar cortex carries unconditioned stimuli (US), or "teaching" information, which trigger forms of circuit plasticity that underlie learning (Marr, 1969; Albus, 1971; Raymond et al., 1996). However, at a cellular level, most attention has focused on only one aspect of climbing fiber-mediated signals, the complex spike burst in Purkinje neurons. Complex spikes in Purkinje neurons are believed to be essential for the induction of several forms of synaptic plasticity within Purkinje neurons, including parallel fiber-to-Purkinje neuron LTD (Raymond et al., 1996). Our findings suggest that a slower, inhibitory phase of the climbing fiber signal, the post-complex spike pause, plays a significant role in role in circuit plasticity.

Our data support the idea that climbing fiber-induced synchronous pauses in Purkinje neuron spontaneous firing may be important inductive signals for associative forms of plasticity in the deep cerebellar nuclei. It is known from *in vivo* imaging and multi-electrode array measurements that complex spikes synchronously discharge in modules of Purkinje neurons within the cerebellar cortex (Welsh et al., 1995; Lang et al., 1999; Mukamel et al., 2009; Ozden et al., 2009), and recent evidence is consistent with the idea that the climbing fiber-associated teaching signal is encoded by synchronous complex spike activity (Ghosh et al., 2011). The axons of Purkinje neurons are inhibitory and converge on single DCN neurons, thus prolonged post-complex spike pauses occurring synchronously in a module of Purkinje neurons could provide an extended

window of dis-inhibition to the DCN cells receiving such input. This pattern of activity would have appropriate properties for driving LTP in mossy fiber-to-DCN synapses (Pugh and Raman, 2006). Moreover, convergent post-complex spike pauses to DCN neurons would render climbing fiber teaching signals distinctive from spontaneous climbing fiber input or strong mossy fiber inputs, an important property for climbing fibers to confer an unambiguous teaching signal.

By their nature, pharmacological manipulations raise concerns due to the possibility of non-specific effects; in addition, HCN and SK channels are present on neurons other than Purkinje neurons. The fact that both drugs had a similar effect on learning makes the possibility of non-specific actions unlikely. In considering whether the drugs might act on other neurons within the circuit it is important to realize that both compounds reduce rather than increase excitability and all forms of synaptic plasticity in the cerebellar cortex that have been described to date are triggered by increases in excitability (Hansel et al., 2001). For these reasons it is difficult to conceive of alternative mechanisms that would lead to increased learning.

Although we have targeted the drugs to the cerebellar cortex, it is possible that the Deep Cerebellar Nuclei could be affected. Within the DCN, rebound excitability following hyperpolarization can trigger a robust increase in the strength of mossy fiber inputs (Pugh and Raman, 2006) and HCN channels could in principle promote such rebound excitability. However, if HCN channels are involved, their inhibition would be predicted to impair plasticity. Thus as for the cerebellar cortex, it is difficult to identify alternative mechanisms of action common to both 1-EBIO and ZD7288 that would explain our findings.

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In summary, we have validated two pharmacological tools and have used them in an eye-blink conditioning paradigm to show that the duration of post-complex spike pauses in Purkinje neuron firing impact the rate of learning. These results provide additional insight into the cellular signals that drive learning in a canonical associative learning circuit and suggest a network basis for delivery of a teaching signal to the DCN, a long hypothesized site of circuit plasticity in cerebellar learning (Miles and Lisberger, 1981; Raymond et al., 1996).

Chapter 7

SUMMARY AND CONCLUSION

This work has investigated the hypothesis of whether cerebellum-dependent associative learning can be altered by modulating the duration of the post-complex spike pause. We modulated the post-complex spike pause through the use of two distinct pharmacological agents that work via opposing mechanisms on two different membrane receptors. *In vitro* electrophysiological experiments, in acute cerebellar brain slices, validated the effectiveness of both drugs in significantly prolonging the post-complex spike pause. *In vivo* associative eye-blink behavior experiments, with rat subjects receiving an infusion of either drug prior to acquisition training days, demonstrated an earlier onset of conditioned responses compared to their control counterparts. Essentially, rat subjects learned the association between the conditioned and unconditioned stimuli earlier than control animals, even though all subjects reached the same plateau of learning. The use of two distinct drugs, that both accomplish the same goal of prolonging the post-complex spike pause, result in increased confidence that a longer post-complex spike pause indeed facilitates the onset of associative learning.

This work also designed and successfully implemented the first associative eye-blink learning set-up capability at UCLA, modeled after a similar set-up at USC. The set-up required EMG measurement and analysis tools, and incorporated significant updates in hardware and software from the modeled set-up to improve experimental and data robustness, in addition to more objective and flexible data analysis.

We can conclude that the same drugs that prolong the post-complex spike pauses in acute cerebellar slice experiments result in faster onset of associative learning during the cerebellum-dependent associative eye-blink conditioning task.

These results are the first to describe enhanced learning rate during a cerebellum-dependent associative learning paradigm. These findings introduce a potentially novel mechanism by which instructive error signals could shape associative learning-dependent plasticity. Detailed understanding of these circuit mechanisms may provide guidance in the development of strategies that combat cerebellar diseases like cerebellar ataxias.

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