A Purkinje cell Timing Mechanism. On the Physical Basis of a Temporal Duration Memory.

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FREDRIK JOHANSSON

The neural doctrine universally ascribes the encoding of memories to modification of synaptic strength. By making some neuron-to-neuron connections stronger and others weaker, neural networks rewire themselves to produce learned output.

For most behaviors the brain must learn to produce precisely timed patterns of activity. Learned response timing is indispensable but it has been problematic to explain memorization of the temporal relationships between different input signals in terms of changes in synaptic strength. Theorists have resorted to complex network models in which thousands to millions of neurons construct codes that represent the passage of time but none conform to the observed spiking of cerebellar Purkinje cells in timing-dependent tasks.

Here, I offer evidence for an alternative in the form of a novel single neuron learning mechanism. Purkinje cells can learn the temporal relationship between inputs delivered immediately to its pre-synaptic fibers under experimental control. With no role for a complicated network code to instruct the cell's response timing the precisely timed Purkinje cell activity must depend upon an intrinsic cellular timing mechanism that measures and stores temporal duration. Beyond demonstrating the phenomenon, pharmacological manipulations and theory presented herein provide a window into the molecular machinery that is capable of this function.

This marks a departure from the doctrine of learning and memory and shows that the capacity for information storage in the brain is many times larger and vastly more energy efficient than previously realized.

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Abstract

The standard view of neural signaling is that a neuron can influence its target cell by exciting or inhibiting it. Learning is thought to involve strengthening or weakening synaptic connections. For most behaviors, the brain must learn to produce precisely timed activity patterns. Learned response timing is indispensable for a wide range of tasks and requires learning of interstimulus intervals (ISIs). The learning mechanism thought to accomplish this combines time-varying patterns of activity in the pre-synaptic neural network with changes in synaptic strength between the pre-synaptic neurons active at the end of the ISI and the post-synaptic neuron.

Timing-dependent learning can be studied in eyeblink conditioning. If a neutral conditional stimulus is paired with an unconditional blink-eliciting stimulus, at an ISI of fixed duration, it acquires the ability to elicit a blink that peaks near the end of the ISI. Cerebellar Purkinje cells that control the blink acquire adaptively timed pauses in spontaneous firing, conditioned Purkinje cell responses, that interrupt their tonic inhibition of cerebellar nuclear cells and cause excitatory output that generates the overt blink.

Most models assume the generation of a time code instantiated in varying patterns of activity in the presynaptic granule cells that represent the passage of time. However, we show here (paper I) that a cerebellar Purkinje cell can learn to respond to a specific input with adaptively timed pauses without such a temporally patterned input. Training Purkinje cells with direct stimulation of their presynaptic fibers, and pharmacological blocking of interneurons shows that the timing mechanism is intrinsic to the cell itself and not an emergent property of the network.

That an individual neuron can learn temporal relationships suggests the existence of intracellular temporal duration memory. We demonstrate that this Purkinje cell memory is triggered by the metabotropic glutamate receptor 7 (paper II) and that the timed voltage response in large part is produced by the G-protein activated K^+ channel family Kir3/GIRK (paper III). The implication is that a learned and adjustable timing of a metabotropic signaling cascade constitutes a physical memory of temporal duration. A theoretical model (paper IV) describes how this could be accomplished by a learning mechanism that selects among a finite number of regulatory proteins, those which bestow the intracellular signaling cascade with latencies to activation and deactivation that matches the ISI.

The results presented in this thesis show that the traditional view of learning as a change in synaptic strength is insufficient. Finally, because Purkinje cells directly control the conditioned eyeblink we believe that, to our knowledge, this is the first time that a causal link can be shown between a learned and timing-dependent behavior and not only a single neuron's memory, but also the specific activating receptor of said memory and the specific ion channel that puts it into effect.

Key words: Cerebellum, Purkinje cell, Timing, Temporal, Memory, Eyeblink conditioning, mGluR7, Kir3, GIRK

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"If at first the idea is not absurd, then there is no hope for it".
- Albert Einstein

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1) **Johansson, F.,** Jirenhed, D.-A., Rasmussen, A., Zucca, R., and Hesslow, G. (2014). Memory Trace and Timing Mechanism Localized to Cerebellar Purkinje cells. *Proceedings of the National Academy of Sciences of the United States of America* 111(41): 14930-4

2) **Johansson, F.,** Carlsson, H.A.E., Rasmussen, A., Yeo, C.H., and Hesslow, G. (2015). Activation of a temporal memory in Purkinje cells by the mGluR7 receptor, *Manuscript*

3) **Johansson, F.,** Carlsson, H.A.E., and Hesslow, G. Programmable Activation of Metabotropic K+ Channels Constitutes a Physical Basis of Temporal Duration Memory in Purkinje cells. *Manuscript*

4) **Johansson, F.**, and Hesslow, G. (2014). Theoretical Considerations for Understanding a Purkinje cell Timing Mechanism, *Communicative and Integrative Biology* **7**(6): e994376

Introduction

Neural signaling and memory

Neuroscientists are confident that we now know a great deal about the mechanisms that enable nerve fibers to carry messages around our brains. However, despite a century of intense research, several fundamental questions remain to be answered. One question that has remained particularly elusive concerns the structural changes in biological tissue that mediate memory formation. Nervous impulses are of constant amplitude and shape regardless of whether the strength or the quality of a stimulus is changed or not. Hence, it is believed that informational detail is determined by varying the frequency of impulses in a fiber on the one hand and the number and kind of fibers in action (excitatory or inhibitory), on the other hand. As information is acquired and behaviors are learned, some physical modification within this system must take place. Regardless of how much information is fed to it, the brain of a newborn does not know what the category of feline creatures represent, nor can it play the piano, until it is mechanically modified by the information feed.

Inspired by 18th century philosophers such as David Hume (Hume, 1739), and later by behaviorist learning theory and experimentation, came the neurobiological theory of learning that still persists today. The central tenet for all three paradigms is that events occurring regularly and closely conjoined in time form associations in mind and brain. The first formal proposals that the connections between neurons – the synapse – could be the physical place of experience-driven changes was introduced to the scientific community when the neuron doctrine itself was still being debated (Cajal, 1894; Tanzi, 1893).

An elaborate suggestion for how a large network of modifiable connections could be the foundation of complex neural memory came from an unexpected source (forgotten and/or overlooked as perhaps the true prior of neural connectionism). Before becoming an economist, Freidrich von Hayek in 1920 realized that, essentially, the brain had to be a self-ordering classification system in the form of a network with modifiable connections. Memory consisted in a distributed pattern of synapses of varying strengths. This would be left in lecture and manuscript form ('Contributions to the theory of evolution of consciousness') until published three decades later as 'The Sensory Order' (Hayek, 1952).

As for the first explicit proposal on different ways in which synapses could be made stronger or weaker Donald Hebb is often credited (Hebb, 1949). Hence, synapses that behave as or near-as he described (virtually all synapses studied) are called Hebbian synapses. His original proposition has been expanded upon by theory and plentiful observation in the half-century that ensued. The Hebbian synapse and the Tanzi-Cajal-Hayek-Hebb learning paradigm can be simplified as follows.

Electrical impulses travelling down the axon of neuron A in figure 1 below cause release of excitatory transmitter molecules at the synapse, which excites the neuron D. This increases the probability that neuron D will respond and/or increases the number of impulses it responds with (i). Inhibitory transmitter from neuron C conversely inhibits neuron D (ii). If neurons A and B repeatedly excite neuron D near-simultaneously, or if neuron A repeatedly and strongly excites neuron D, the synapses are made stronger such that activity in the excitatory presynaptic cells becomes more likely to excite neuron D. It will respond with more impulses as seen in (iii). This is referred to as long-term potentiation (LTP).

If the activity in neurons A and B is not conjoined or if neuron A only weakly and not very often excites neuron D, the opposite result (long-term depression, LTD) is obtained (iv). In this way, learning and information storage consists in changing the efficacy of synapses, in making excitation or inhibition more or less efficient. The essential concept that distinguishes LTP and LTD is correlated versus uncorrelated pre-synaptic activity.

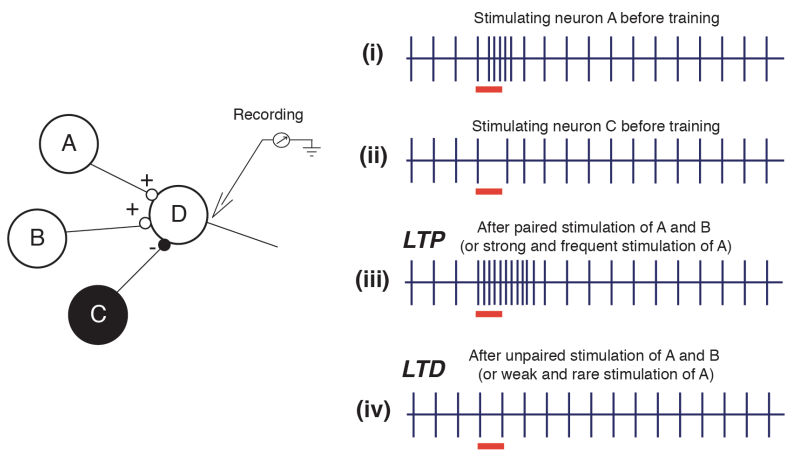

Figure 1. The standard view of neural signaling and memory.

In trying to define the effective time windows between pre- and post-synaptic activity in which spikes (nerve impulses) induce either strengthening or weakening of the synapse, the last decade has brought an expansion to this scheme called spike-timing dependent

plasticity, abbreviated as STDP (Caporale & Dan, 2008; Dan & Poo, 2004; Karmarkar, Najarian, & Buonomano, 2002). In different neural circuits, varying the order and timing of weak and strong synaptic inputs in different ways induces plasticity of different signs. For various combinations of parameter values, either LTP or LTD is obtained. An important clarification here is that what is meant by timing in STDP is only that the strong and weak inputs from the pre-synaptic neurons vary over a range of a few tens of milliseconds, and dependent upon this a connection is 'potentiated' or 'depressed'. It does not mean that neuron D in figure 1 exhibits any meaningful timing in the spikes is responds with. Irrespective of what the parameter values are, neuron D will, after training, still respond immediately, just more strongly or weakly than it used to do.

When STDP learning rules are incorporated, some brain functions can reliably be simulated in computer programs. While there is plentiful evidence that these phenomena exist and that computer simulations can accomplish functions ostensibly similar to brain functions, it is for reasons elaborated upon in subsequent chapters less clear whether, and to what extent, these are the relevant learning rules to study in the brain. As will become apparent, learning the temporal precision in neural signaling that is essential to most behaviors constitutes a particularly challenging problem to solve in terms of the Hebbian synapse.

Timing

On temporal precision

Temporal precision in neural signaling is not only important but also essential to control any aspect of behavior. From driving a car or pressing piano keys to anticipating the next step of a dance partner or tilting a coffee cup just so that you get coffee in your mouth and not in your lap, timing is everything. Accurate movements require a specific temporal pattern of activity in the neurons that control a large number of muscle fibers that act across multiple joints. A concrete example is that temporal precision of exactly when you open your fingers in a ball throwing movement is critical for, and perhaps even the most important component of, spatial accuracy in terms of where the ball will land (Ivry & Keele, 1989).

The same is true on the perception side. For example, the physical sound waves produced by uttering the letters that constitute the words BIDDEN and BITTEN are practically identical. We perceive them differently mainly because they are distinguished on the basis of a temporal cue, the time between the identical components of the sound wave (Ackermann, Graber, Hertrich, & Daum, 1997). The estimation of temporal duration and the judgement whether one event or the other extended further in the temporal dimension are also crucial to the normal function of any mammal (Timmann, Watts, & Hore, 1999). Neural actions similar to perception and motor control underlie cognition, so the same is of necessity true for our inner mental lives. From the perspective of neurons, *when* to do something just as important as *what* to do.

Given the title and topic of this thesis it should come as no surprise that all of the examples given above have clear cerebellar dependencies. Specific examples in a clinical setting include cerebellar temporal processing deficits with several links to dyslexia (Farmer & Klein, 1995) and some schizophrenic patients seem to misinterpret the very phenomenon of causality due to faulty temporal estimations of events (Waters & Jablensky, 2009). The importance of accurate timing might be best illustrated by the fact that finger tapping variability, a test that measures how well a subject can follow the beat of a metronome by tapping his or her index finger, is a better predictor of general intelligence (Raven G score) than most standard IQ tests (Ullen, Forsman, Blom, Karabanov, & Madison, 2008). Indeed, cerebellar patients are significantly impaired in this simple task (Ivry & Keele, 1989).

Because the use of the term ‘timing’ is most often used rather loosely and indistinctively, some clarification is necessary. The term is used widely for any of (or combinations of) the following: (a) estimation of temporal duration, (b) prediction of event onset and/or offset, (c) judgment of whether an event occurred before or after some other, (d) the

planning of sequential actions in temporal order, and (e) performing an action at a favorable time point in a relatively large time window (suprasecond ranges to days and weeks). The important distinction is that these phenomena place very different constraints on the neural functions that support it. Specifically, only (a)-(b), and some cases of (c) require some form of actual 'measuring' (in the metric sense) of the passage of time. These are the kind of timing functions that this thesis concerns. Notice that the behaviors that depend upon it place three important constraints:

1) The passage of time must be 'measured'.
2) Such 'measurements' must be stored by some physical change in brain tissue.
3) The brain must be able to use these stored 'measurements' in order to produce the necessary temporal dynamics in its output. The stored information must be accessible.

The consequential point to make is that the underlying temporal relationships between different inputs have to be learned. We are not born with the sense of duration that is needed for any of the exemplified behaviors that are dependent on precise timing. The natural question becomes, how is this learning accomplished?

On the physical basis of storing a memory of duration

Because memory formation is near to universally ascribed to changes in synaptic strength, it is no surprise that that is the case for theories of learning temporal durations as well. However, this has been a particularly vexing task. There are no sensory receptors that detect the flow of time in the same sense that we see, hear and feel, so which are the connections that should be made stronger or weaker when an interval of 400 milliseconds is to be stored?

The vast majority of timing models have the following assumptions for pre- and post-synaptic neurons in common. The passage of time is represented by different sub-populations of pre-synaptic neurons that vary in the time course with which they respond to a common input. Activity will peak early in some sub-populations, with a slight delay in some others and even later in yet others. As an alternative to delayed activity peaks, all the sub-populations could go through series of slightly different oscillations or random fluctuations in activity during a temporal interval. Hebbian learning mechanisms are assumed to act on the different subpopulations so that the pre-synaptic neurons whose activity happens to peak at the end of a target interval in learning a timed behavior achieves greater control over the post-synaptic neurons. When learning is complete, post-synaptic output becomes appropriately timed because it is dominated by the pre-synaptic neurons whose activity peaks at the "right" time. Thus, the neural network rewires itself so as to produce the target interval when activated by the same input that was acting during training. How such a time code is instantiated has been the subject of extensive

debate. In a broad perspective, two schools of thought have developed. In one, there are specialized mechanisms for representing temporal relationships (*dedicated timing*). In the other, representation of duration is not a specific mechanism but emerges from neural network dynamics (*emergent timing*) (Ivry & Schlerf, 2008).

The earliest dedicated model was the pacemaker-accumulator framework (Gibbon, 1977; Treisman, 1963). Here, a sensory stimulus triggers a centralized internal clock, which starts to emit pulses that are then counted by an accumulator. The tally can then be stored, compared and so on. Another alternative is tapped delay line theory (Valentino Braitenberg, 1984; Desmond & Moore, 1988; Ivry, 1996). Here, due to a suggested architecture of sequentially connected and activated neurons, or neurons with axons of different length, duration is represented by the time it takes for a sensory signal to reach a particular destination. A third class of dedicated models is spectral timing models. In this scenario, certain neurons are pre-programmed with variably slow membrane time constants such that they respond to an input signal with a firing rate that always reaches a peak with a certain delay (100, 250, 400 ms etc.) and then decays. Pre-synaptic neurons that happen to have properties that match the training interval would become preferentially associated with the output neurons. Finally, a bank of oscillator neurons could also encode for intervals in a dedicated way. If the cells have slightly different beat frequencies, combinations thereof can serve as a representation of duration. An output neuron excited by a group of pacemaker neurons could via Hebbian learning rules select a subgroup of oscillators (whose oscillation period represents e.g. 200 ms) with which synaptic connections would be altered (Miall, 1989).

In contrast, emergent timing models rely on time being an inherent property of network dynamics. Networks composed of excitatory and inhibitory neurons with slightly different synaptic time constants can, with distributed plasticity here and there, represent durations as unique spatial patterns of activity (Karmarkar & Buonomano, 2007). Time is not actually monitored, in the metric sense, anywhere. There are many such models wherein information about temporal duration is somehow stored in the global pattern of changes in synaptic strength within a large and complex neural network. One cannot easily extract what specific structural changes underlie the learning, e.g. point to a specific pattern of Hebbian changes that represents a learned duration of 200 ms. The network just rewires itself to produce the desirable outcome given a specific input.

Both dedicated and emergent models for neural timing can be boiled down to the same end result. Due to time-varying signals in the input pathway, output neurons learn response timing by altering synaptic conductances for selected pre-synaptic neurons (Laje & Buonomano, 2013; Mauk & Buonomano, 2004; Yamazaki & Tanaka, 2009). The contemporary debate concerns which model of passage of time representation combined with Hebbian learning rules is correct.

Classical conditioning of cerebellar motor responses

Introduction to classical conditioning

As will become apparent, classical conditioning of motor responses is an excellent experimental model of associative learning (Gormezano, Schneiderman, Deaux, & Fuentes, 1962) that can be used to study the acquisition of timed behavior. *Conditioning* derives its name from Pavlov's original experiments with dogs (Pavlov, 1927). He discovered that paired presentations of a metronome and delivery of food caused the normal reflexive response to food, to start salivating, to become *conditional* upon presentation of the tone. The dogs started to salivate when only the tone was presented.

In eyeblink conditioning, a subject is repeatedly presented with a behaviorally neutral conditional stimulus (CS) followed by a blink-eliciting unconditional stimulus (US). This neutral conditional stimulus is typically auditory or electrical stimulation of the skin. The unconditional stimulus is often an airpuff directed to the periocular area or electrical stimulation of the periocular skin, both of which evokes a blink. This reflex response is called the unconditioned response (UR). After a number of paired presentations, or "trials", and time for learned changes in the brain to take place, a conditioned blink response (CR) to the previously neutral conditional stimulus develops (Gormezano & Moore, 1969; Kehoe & Macrae, 2002). Figure 2 shows a typical conditioned eyeblink response as recorded from the *musculus orbicularis oculi* that controls the external eyelids in the ferret.

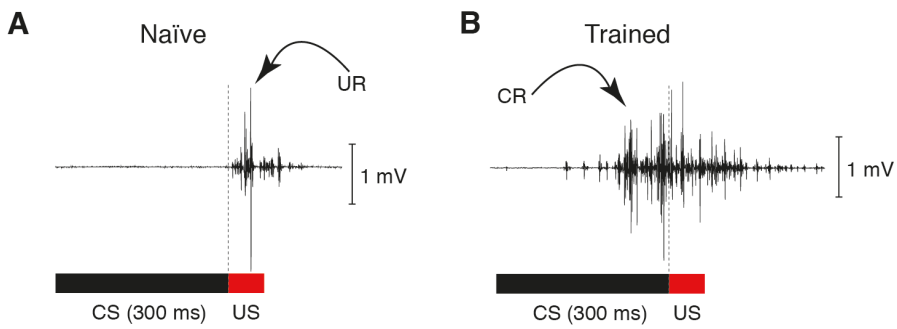

Figure 2. Electromyogram of unconditioned and conditioned eyeblink responses. ***A.*** Before training the US elicits a reflex blink, an unconditional response (UR). ***B***. After training, the CS elicits a conditioned blink response (CR).

Notice in figure 2 that the animal not only blinks in response to the conditional stimulus, but that it does so just in advance of the unconditional stimulus. In the paradigm called

delay eyeblink conditioning, the onset of the conditional stimulus and the onset of the unconditional stimulus are separated by a fixed temporal interval (the interstimulus interval). The timing of the conditioned response varies systematically with the duration of the interstimulus interval in such a way that the latencies to onset, peak and offset of the blink varies in proportion with the duration of the interstimulus interval used in training. Maximal closure of the eyelid always occurs near the point in time at which the onset of the unconditional stimulus is expected (Gallistel, 1990; Kehoe & Macrae, 2002; Mauk & Buonomano, 2004).

What is most interesting in classical conditioning of motor responses is not that animals learn to respond to neutral stimuli, but just this, that they learn to respond at the right time. This is what importantly sets it apart from, for instance, classical conditioning of fear responses that are not, and do not need be, precisely timed. Understanding the interstimulus interval dependent timing of the conditioned response is crucial for understanding the mechanisms of learning and memory, both in general and for the specific case of temporal memory.

Learning occurs in the cerebellum

Eyeblink conditioning has been extensively used as a model system to study the physical changes in biological tissue that underlie both associative learning and adaptive timing. Early theories on cerebellar learning by Marr (Marr, 1969) and Albus (Albus, 1971) suggested that classical conditioning relies on the cerebellum. The conditional stimulus could be signaled to the cerebellum via mossy fibers originating in pontine nuclei and the unconditional stimulus via the climbing fibers originating in the inferior olive. If there were a convergence of the two pathways upon Purkinje cells in the cerebellum, learning could take place in cerebellar cortex.

The theory later gained substantive support from early lesion studies. First, the entire cerebrum could be removed in cats without disrupting learning (Norman, Buchwald, & Villablanca, 1977). The cerebellum therefore became the prime candidate structure for memory storage. Indeed, it was shown that both major ipsilateral lesions of the rabbit cerebellum (Lincoln, McCormick, & Thompson, 1982; McCormick, Clark, Lavond, & Thompson, 1982) and of its relevant output pathway, the *pedunculus cerebellaris superior*, abolished previously acquired nictitating membrane conditioned responses in the rabbit (McCormick, Guyer, & Thompson, 1982). The neural circuit of interest was then further delimited by lesion studies in which damage to one and only one of the four deep cerebellar nuclei, the *nucleus interpositus anterior* (AIP), abolished previously acquired conditioned responses and prevented reacquisition (Yeo, Hardiman, & Glickstein, 1985a). Reversible inactivation of the AIP (Hardiman, Ramnani, & Yeo, 1996; Krupa, Thompson, & Thompson, 1993) later confirmed the conclusion that the AIP is part of

the relevant circuit. The four nuclei are the only output neurons from the cerebellum to the rest of the nervous system. This means that the cortical area of interest is determined by the anatomical connections of the AIP specifically.

The next step of restricting the area within which the learning underlying conditioned responses could take place was the observation that lesions within only one part of the inferior olive, the medial rostral part of the dorsal accessory olive (DAO), led to exactly the same outcome (Yeo, Hardiman, & Glickstein, 1986).

Projection neurons in the inferior olive send their axons, the climbing fibers, to the cerebellum so that they form an organization of longitudinal bands, or "zones", in the cerebellar cortex (Apps & Garwicz, 2005; Armstrong, 1974; Oscarsson, 1980; Oscarsson & Iggo, 1973; Voogd & Glickstein, 1998). For associative learning to be possible there needs to be an anatomical substrate for convergence of the conditional and unconditional stimulus pathways. Such a convergence of mossy fibers from pontine nuclei and climbing fibers from this identified critical part of the inferior olive onto Purkinje cells was indeed demonstrated (Yeo, Hardiman, & Glickstein, 1985b).

Specifically, the AIP receives afferents from the C1 and C3 zones in lobule HVI of the cerebellar cortex, wherein Purkinje cells receive climbing fiber input from the identified relevant part of the inferior olive. Indeed, local lesions and reversible inactivation restricted to this cortical area abolished previously acquired conditioned responses in the rabbit (P.J. Attwell, Cooke, & Yeo, 2002; P. J. Attwell, Ivarsson, Millar, & Yeo, 2002; P. J. Attwell, Rahman, Ivarsson, & Yeo, 1999; Hardiman et al., 1996; Hardiman & Yeo, 1992; Yeo & Hardiman, 1992; Yeo et al., 1985b).

The CS and US pathways

There is now a large body of physiological evidence that the conditional and unconditional stimuli are indeed signaled by the mossy and climbing fibers, respectively. Replacing the behavioral conditional stimulus with direct electrical stimulation of mossy fibers and the behavioral unconditional stimulus with direct electrical stimulation of the inferior olive (Mauk, Steinmetz, & Thompson, 1986; Steinmetz, Lavond, & Thompson, 1989; Steinmetz, Rosen, Chapman, Lavond, & Thompson, 1986) both produced reliable conditioned responses. In perfect agreement with the hypothesis, inactivating the inferior olive during training (Welsh & Harvey, 1998) and blocking excitatory input to the olive (Medina, Nores, & Mauk, 2002), which, importantly, spares normal spontaneous firing of its neurons and selectively prevents transmission of the unconditional stimulus, both prevented acquisition of conditioned responses. These findings have also been corroborated in the ferret (Hesslow, 1995; Hesslow, Svensson, & Ivarsson, 1999; Hesslow & Yeo, 2002; Ivarsson, Svensson, & Hesslow, 1997).

Based on somatic receptive fields cerebellar zones can be further subdivided into microzones, small groups of Purkinje cells that receive climbing fibers from a shared subset of olivary neurons and project to a shared subset of deep cerebellar nuclear cells (Andersson & Oscarsson, 1978; Apps & Garwicz, 2005; Ekerot, Garwicz, & Schouenborg, 1991; Garwicz & Ekerot, 1994). In the cat and ferret a few highly delimited such microzones (figure 3) exhibit all the hallmarks of an area controlling a muscle. Microzones in the C1 and C3 zones of lobule HVI (as well as two more microzones in other lobules) exhibit short-latency climbing fiber responses with a periocular receptive field. Stimulation of these and only these cortical areas elicits a blink and, importantly, an electrical stimulus applied to the microzones just when a conditioned response in a trained animal would be emitted completely aborts the response (Hesslow, 1994a, 1994b; Hesslow & Ivarsson, 1994). These observations clearly define these microzones as eyeblink microzones. This causal link between Purkinje cell activity and eyeblinks has recently also been replicated in mice with optogenetic stimulation (Heiney, Kim, Augustine, & Medina, 2014). Further, not only does local inactivation of lobule HVI prevent acquisition and expression of conditioned responses but, vitally, post-training consolidation is disrupted by only cortical, not nuclear, inactivation (Kellett, Fukunaga, Chen-Kubota, Dean, & Yeo, 2010). The evidence that microzones in the C1 and C3 zones control the conditioned blink response and that the cerebellar cortex is the site of memory storage is overwhelming.

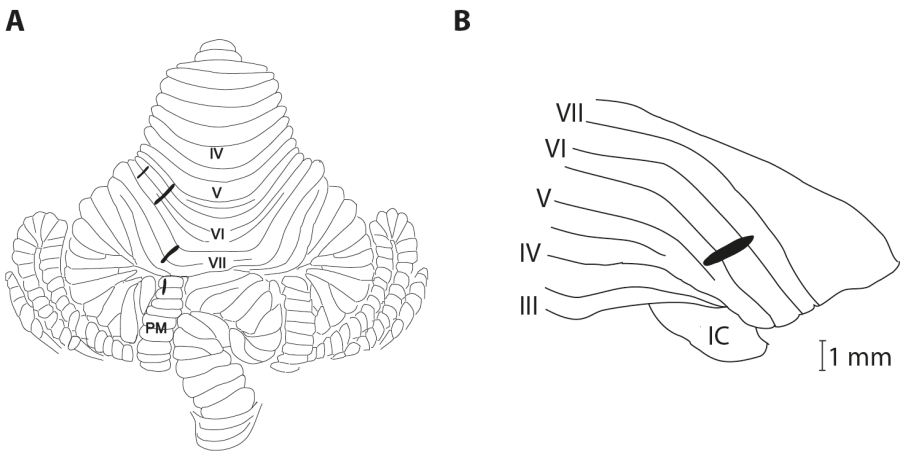

Figure 3. Localization of eyeblink microzones.

A. Schematic drawing of cerebellar microzones that control the eyeblink ***B.*** The eyeblink microzone in the C3 zone.

Purkinje cell behavior during classical conditioning

Mossy fibers primarily make synapses on granule cells, which in turn send ascending axons up into the molecular layer of cerebellar cortex where they bifurcate to form the archetypical parallel fibers that run parallel to the cortical folia. A single Purkinje cell is contacted by up to several hundred thousand parallel fibers (Harvey & Napper, 1991; Napper, 1988), dependent upon the species examined. In stark contrast, each Purkinje cell is contacted by one and only one climbing fiber that forms hundreds of synapses with the Purkinje cell (Eccles, Llinas, & Sasaki, 1966). Both types of input are excitatory but the climbing fiber input is orders of magnitude stronger and causes a large and long-lasting kind of action potential called a complex spike, unique to Purkinje cells. Apart from these glutamatergic inputs, Purkinje cells also receive input from GABA-ergic interneurons in the molecular layer, which are also excited by the parallel fibers. However, both Purkinje cells and molecular layer interneurons are spontaneously active, their firing is not driven by the parallel fiber excitation. Because the Purkinje cell is the sole output neuron in the cerebellar cortex, these three pre-synaptic inputs represent the penultimate modulators of cortical output. Figure 4 illustrates the cerebellar circuit.

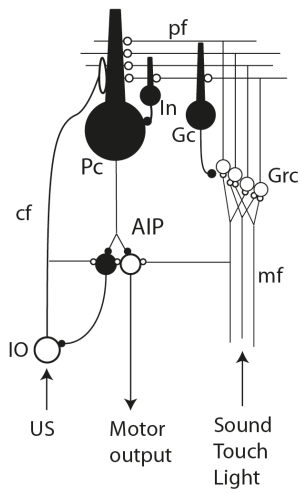

Figure 4. The cerebellar circuit involved in eyeblink conditioning.
The CS (here in the form of touch, sound or light) is signaled via mossy fibers (mf) that synapse with a large number of granule cells (Grc). Their ascending axons form parallel fibers in the molecular layer and contact Purkinje cells (Pc), Golgi cells (Gc) and molecular layer interneurons (In). The US is signaled to the inferior olive (IO) and via their climbing fibers (cf) to the Purkinje cells. Purkinje cells project to the nucleus interpositus anterior (AIP), which project to nucleus ruber and in turn to the facial nucleus and nerve. Shown is also an inhibitory pathway from the Purkinje cells to the inferior olive, a feedback pathway that regulates learning. White cells represent excitatory neurons and black cells represent inhibitory neurons.

The early theories (Albus, 1971; Marr, 1969), the anatomical connections and the physiology described above all lead to the prediction that the spontaneously active inhibitory Purkinje cells will decrease or even cease their firing during performance of conditioned responses. This would, in turn, disinhibit the excitatory pathway that causes an eyeblink. Indeed, Purkinje cells in the C3 zone that, as previously shown, control the blink consistently develop such pause responses, conditioned Purkinje cell responses, after paired conditioned stimulus – unconditional stimulus presentations, with either behavioral stimuli or direct electrical stimulation of mossy and climbing fibers (Hesslow, 1995; Hesslow & Ivarsson, 1994; Jirenhed, Bengtsson, & Hesslow, 2007). There are reports of Purkinje cells behaving differently, either decreasing their firing, increasing their firing or not modulating their firing at all (Berthier & Moore, 1986; Gould & Steinmetz, 1996; Kotani, Kawahara, & Kirino, 2003, 2006; Tracy & Steinmetz, 1998). However, recall the precise organization of Purkinje cells into small microzones that control a specific action. A likely explanation for these conflicting observations is that they are due to the fact that microzonal identification was not performed in most studies. A Purkinje cell that does not control eyeblinks does not need to learn conditioned eyeblink responses.

An example of a typical conditioned Purkinje cell response recorded in the blink controlling microzone of the ferret is illustrated in Figure 5. While it is often said that the conditioned Purkinje cell response is a reduction or suppression in firing, most times, at least as seen in the data presented later herein, it is a complete cessation in firing, a pause, such as that seen in Figure 5. A perceived gradual reduction in firing may simply be an artifact of averaging across trials, either visually or statistically, arising from the fact that the response does not start and end at the exact same millisecond trial after trial.

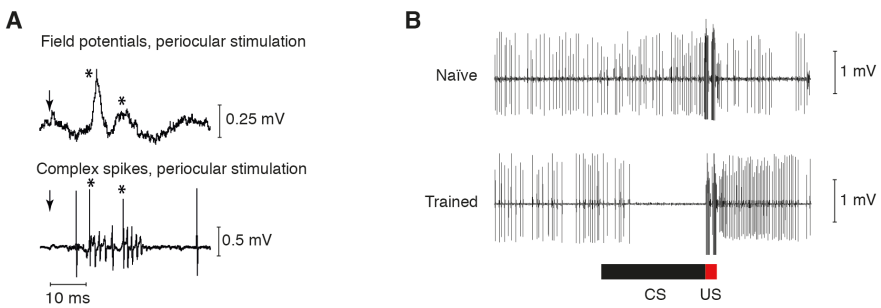

Figure 5. Typical Purkinje cell responses in the eyeblink microzone.
A. Periocular stimulation (1 pulse, 300 μA) elicits short-latency field potential responses on the cerebellar surface (*top*). Single-cell Purkinje cell recording (*bottom*) of two complex spikes elicited by the periocular stimulation. Arrows indicate stimulation and asterisks indicate responses. ***B.*** Typical naïve and conditioned Purkinje cell responses to the conditional stimulus (300 ms forelimb stimulation).

The conditioned Purkinje cell response shares a remarkable number of features with the overt conditioned blink response. Before conditioning there are never any pause responses to the conditional stimulus. With mossy fiber stimulation or behavioral conditional stimuli Purkinje cells either respond with no modulation in firing or a slight increase in firing. The conditioned Purkinje cell responses develop with paired conditional stimulus-unconditional stimulus presentations over one to four hours and are extinguished by unpaired presentations. Upon re-retraining after extinction, reacquisition is much faster, often accomplished in less than five minutes (Jirenhed et al., 2007), a phenomenon referred to as "savings" in the behavioral literature. Another important similarity that we will return to later in this thesis is that both at the behavioral level (Gormezano & Moore, 1969; Schneiderman & Gormezano, 1964) and at the Purkinje cell level (Wetmore et al., 2014), no learning takes place if the interstimulus interval is <100 ms.

The parallels between what can be read out and explained by Purkinje cell behavior almost seem unending. For instance, take the canonical notion from learning theory that as the probability of a learned response increases, the reinforcing value of subsequent pairings of conditional and unconditional stimuli should fall (Rescorla, Wagner, Black, & Prokasy, 1972). It turns out that Purkinje cells have a feedback pathway to the olivary neurons that provide its own climbing fiber input. As learning progresses, Purkinje cells pause more and more, disinhibiting the feedback pathway and turning off the instructive signal (Andersson, Garwicz, & Hesslow, 1988; Bengtsson, Jirenhed, & Hesslow, 2007; Hesslow, 1986; Rasmussen, Jirenhed, Wetmore, & Hesslow, 2014; Svensson, Bengtsson, & Hesslow, 2006). Evolution has even designed this feedback loop with signaling latencies so cleverly that even if the conditioned response is produced, but too early or too late, feedback inhibition of the inferior olive does not arrive at the precise time it would need to, in order to block the unconditional stimulus, and so learning continues. The properties of Purkinje cells and its feedback loop to its very own instructive signal even seem able to explain complex behavioral learning phenomena such as Kamin blocking and overexpectation (Bengtsson & Hesslow, 2013; Rasmussen & Hesslow, 2014).

One could go on, but most important for the present purposes is the adaptive timing of the conditioned Purkinje cell response. When using electrical stimulation of the mossy fibers as the conditional stimulus in a decerebrate ferret, the timing of the response (onset, maximum and offset) is closely related to the interstimulus interval used in training (Jirenhed & Hesslow, 2011a). At different interstimulus intervals in the range of hundreds of milliseconds the conditioned Purkinje cell response always occurs near the anticipated unconditional stimulus onset. The cerebellum clearly learns the temporal interval.

This study further demonstrated two important phenomena. First, once conditioned to a given interstimulus interval the same Purkinje cell can be re-trained to a new interstimulus interval ("ISI-shift"). Second, even though a conditional stimulus that

outlasts the interstimulus interval with several hundred milliseconds was used, the offset of the conditioned Purkinje cell response was still always close to the anticipated onset of the unconditional stimulus. The Purkinje cell pause ends with the learned interstimulus interval despite the fact that parallel fiber input continues to reach the cell for many hundreds of milliseconds.

Further, once a conditioned eyeblink response has been learned, its time course depends mainly on the initial part of the conditional stimulus. Once an animal has been trained with a 300 ms interstimulus interval, a conditional stimulus that only last 20 ms, or sometimes even a single pulse, can elicit a well-timed overt blink (Svensson & Ivarsson, 1999) and Purkinje cell pause (Jirenhed & Hesslow, 2011b). It seems as if only a trigger signal is necessary to elicit the full time course of the acquired response.

This rather extensive introduction has served the purpose to explain why, at the outset of this thesis, it is already clear that Purkinje cell behavior controls the blink and that most aspects of classical conditioning of motor responses can be read out from Purkinje cell firing. Despite these findings over the last half-century, while we do know that the output neuron produces the appropriate output, we do not know where and how learning takes place in the cerebellar circuit. Most daunting, compared to the acquisition of an association of two stimuli as such, is that it is especially difficult to account for the monitoring and storage of temporal duration information.

Models on cerebellar learning

Before conditioning, Purkinje cells respond to the conditional stimulus with an insignificant change or an increase in firing. For there to be a conditioned Purkinje cell response two things need to change. First, the learning mechanism has to adjust the net effect on Purkinje cell firing to a decrease (often a complete cessation or "pause" in firing). Second, the decrease must occur at the right time. This section will deal with these two aspects, in turn.

Mechanisms for generating a cessation in Purkinje cell firing

What causes the suppression in Purkinje cell firing during the conditioned response? The most commonly invoked mechanism is long-term depression induced by spike-timing dependent plasticity (STDP) learning rules. Parallel fiber and climbing fiber input that occur close together in a conditioning protocol is assumed to cause LTD at the parallel fiber-to-Purkinje cell (pf-PC) synapse and this is believed to cause the decrease in Purkinje cell firing towards the end of the interstimulus interval (Aiba et al., 1994; Albus, 1971; Buonomano & Mauk, 1994; Hansel, Linden, & D'Angelo, 2001; Ito, 1982; Ito & Kano, 1982; Lepora, Porrill, Yeo, & Dean, 2010; Li, Hausknecht, Stone, & Mauk, 2013; Marr, 1969; Medina & Mauk, 2000).

From a theoretical perspective this view is troublesome. It was long thought that the high levels of spontaneous firing in Purkinje cells was caused by continuously ongoing parallel fiber input to the Purkinje cell. Had this been the case, removing excitation through LTD at the pf-PC synapse could very well silence the Purkinje cell. However, it has been convincingly shown that the spontaneous firing is generated by an intrinsic pacemaker mechanism, not by pf input. First, granule cells have no or very low spontaneous activity (Chadderton, Margrie, & Hausser, 2004; Jörntell & Ekerot, 2006). Further, up to 98% of pf-PC synapses are electrically silent, i.e. even if the granule cell fires it does not cause excitatory current in the Purkinje cell (Ekerot & Jorntell, 2001; Isope & Barbour, 2002; Wang, Khiroug, & Augustine, 2000). Any background level of granule cell activity in the remaining 2% would have to be extremely powerful to set Purkinje cells going at 40-80 Hz. Moreover, Purkinje cells continue to fire at high rates even after blockade of AMPA-kainate receptors, i.e. in the absence of excitatory synaptic input (Cerminara & Rawson, 2004). Lastly, the Purkinje cells are spontaneously active at high rates *in vitro* even with the entire dendritic tree removed, leaving only the cell body (Raman & Bean, 1997). The spontaneous firing is clearly not generated by parallel fiber input, but by an intrinsic spike generator. This is important because it has the following implications.

Simply decreasing excitatory input through long-term depression cannot by itself suppress cell firing below the spontaneous rate. To silence the cell as occurs during conditioned Purkinje cell responses requires an *active* suppression mechanism. Transiently removing excitatory parallel fiber input is not sufficient. Indeed, genetically modified mice precisely targeted to lack long-term depression at the pf-PC synapse do not show impaired learning of the conditioned response (Schonewille et al., 2011). Alternatively, the pause in firing could result from net inhibition from interneurons after removal of balancing excitation. This rests on a strong and highly improbable assumption. In this scenario there would have to be, before conditioning, a perfect balance between excitation and inhibition that cancel each other out exactly. However, there is often, in particular with a behavioral conditional stimulus, no net effect upon Purkinje cell firing by the conditional stimulus before conditioning. If this is because parallel fiber input only has a negligible excitatory effect, LTD at pf-PC synapses cannot be the mechanism. Removing a negligible excitation cannot cause a drop from 40-80 to 0 Hz. The lack of excitation in the naïve state is also not likely due to such a balance of excitation and inhibition because of the fact that monosynaptic excitatory input produces an EPSP and simple spike before the di-synaptic inhibitory input even reaches the Purkinje cell (Eccles, Ito, & Szentagothai, 1967). Thus, such a balance does not seem possible. Yet another option, suggested in one model, is that the relevant learning that occurs is long-term potentiation at parallel fiber-to-interneuron synapses (Jörntell, Bengtsson, Schonewille, & De Zeeuw, 2010), resulting in an acquired active inhibition of the Purkinje cell. However, this would suggest a remarkable departure from what the architecture of the circuit suggests. It is the large Purkinje cell, not the small interneurons, which appears ideally suited for the learning of associations. It is here that a couple of hundred thousand parallel fibers can signal almost any imaginable contextual input, and it is here that a single powerful climbing fiber purposefully wraps around the cell and forms hundreds of synapses that control learning. In contrast, the interneurons receive far fewer parallel fibers and are excited by multiple climbing fibers, seemingly only through diffusion of spillover glutamate from climbing fibers that contact Purkinje cells (Szapiro & Barbour, 2007).

Despite the above considerations, LTD at the pf-PC synapse is the predominantly suggested mechanism in models of classical conditioning. LTD and LTP certainly are real and observable phenomena at all cortical synapses (Crepel & Jaillard, 1991; Hirano, 1990, 1991; Ito & Kano, 1982; Jörntell & Ekerot, 2002; Linden, 1999; Sakurai, 1987) but using them to explain the particular form of learning that occurs in classical conditioning has additional inherent problems than those already mentioned. These problems all concern the fact that LTD and LTP are observed under conditions that are very different from those of conditioning, and the training protocols that induce the changes are also markedly incongruent.

(i) The most striking difference between Hebbian plasticity and conditioning is the time it takes to induce learning. As described earlier in this thesis, conditioning takes hours to establish. On the other hand, LTD and LTP is always induced in minutes, sometimes less than two minutes, always within ten minutes (Chen & Thompson, 1995; Ekerot & Kano, 1989; Hirano, 1990; Ito & Kano, 1982; Jörntell & Ekerot, 2002; Safo & Regehr, 2008; Wang, Denk, & Häusser, 2000).

(ii) LTD at the pf-PC synapse was in all of the above mentioned studies induced with a single stimulus pulse to the climbing fibers. This is the standard protocol. However, the olivary neurons fire in bursts and no conditioning is possible with a 1 cf pulse protocol. In fact, using 1 cf pulse as the unconditional stimulus even causes extinction of conditioned responses previously acquired with a burst stimulus (Rasmussen et al., 2013).

(iii) The computer models based on STDP rely on spike-timing rules that render LTD at certain parallel fiber (pf)-climbing fiber (cf) input intervals (the LTD window), and LTP for parallel fiber inputs that fall outside of this window. What happens in the simulations is crucially dependent on these exact parameters. The LTD window in published simulations is set to perplexingly different pf-cf intervals. For instance, two example values are pf spikes occurring within 50 ms *before* the cf spike (Yamazaki & Tanaka, 2007) and 50 ms *after* (referenced as -50 ms) the cf spike (Medina & Mauk, 2000). In some, but not all, iterations of the latter model by the same group the LTD window is set to pf input 100 ms *before* the cf spike (Kalmbach, Voicu, Ohyama, & Mauk, 2011). The models are critically dependent on millisecond precision that enables differential operation of LTD and LTP for these specific windows. Without a precise differential operation there can be no timed conditioned pause responses. This is problematic for the following reasons.

What does the empirical literature indicate that the LTD window is at this synapse? First, LTD is most effective at short pf-cf intervals. Most work on LTD has even been done with an interstimulus interval of 0 ms, or so-called "conjunctive stimulation", and early comparisons showed that this was the most optimal paradigm (Ekerot & Kano, 1989; Ito, 2001; Karachot, Kado, & Ito, 1995). There have been later claims that a pf input preceding a cf input at intervals more similar to those used in conditioning is optimal. LTD optima of 50 ms (Wang, Denk, et al., 2000), around 80 ms (Safo & Regehr, 2008) and 250 ms (Chen & Thompson, 1995) have been reported. However, in all three cases it was still appreciable at both 0 ms and at negative values. This is not the only thing that is problematic for the STDP models of conditioning. They require an LTD window that is fixed. Take the Chen & Thompson study for instance: after two minutes of training, LTD was optimal at 250 ms and there was no effect with backwards pairing. However, given enough training, in this case 10 minutes which is still orders of magnitude less than

in conditioning, the LTD window grew to reach all the way from 250 ms to negative 250 ms.

What do the empirical data on conditioning show? Not only is there no conditioning at 0 ms where LTD is very effective, but conditioning does not occur with interstimulus intervals shorter than 80-100 ms. At the Purkinje cell level, not only does no conditioned pause response develop but, surprisingly, a response consisting of *increased* firing does (Wetmore et al., 2014). This response is not timed but consists of an increase in firing that lasts as long as the conditional stimulus does. Recall that a conditioned pause response ends at the end of the learned interstimulus interval even if the conditional stimulus continues for several hundred milliseconds. With hours of training, this seems incompatible with a growing LTD window that is much wider than the conditioned response. Notice that in conditioning the interstimulus interval is calculated between the onset of a uniform repetitive stimulus and the onset of the unconditional stimulus. It is this onset that must precede the cf input with 80-100 ms, otherwise the opposite result is obtained. In STDP rules, the intervals in question are those between each pf input and the cf input. With a 50 Hz conditional stimulus the majority of the individual pulses that are delivered every 20 ms have much shorter pf-cf intervals than the stated interstimulus intervals. As an example, take the case with an interstimulus interval of 300 ms with a conditional stimulus that lasts 800 ms (Jirenhed & Hesslow, 2011a). Relative to climbing fibre input, the Purkinje cell receives parallel fiber input every 20^{th} ms at t = 300, 280, 260, 240 and so on, including 0 and all the negative values up to -500. By all accounts of LTD optima, the LTD window essentially lies at the only STDP intervals where conditioning is *not* possible and the opposite, an increase in firing, is induced.

If one wants to invoke LTD as the mechanism one cannot say that pf excitation is negligible because then depressing it would have no effect. If it is not negligible, differential operation of LTD and LTP seems impossible. In the referenced computer models all specific pf-cf intervals induce either LTD or LTP, but how could this work? When the cf input arrives, the Purkinje cell will have received pf-cf input at any and each interval. Hence, only one of LTD and LTP can be operative, resulting in decreased firing or increased firing as long as the conditional stimulus continues, not a pause timed to the interstimulus interval. The only way to get around this is if at each time step a unique subpopulation of granule cells and hence a specific set of pf-PC synapses is active. This necessitates a function of the pre-synaptic granule/Golgi cell network as generators of temporal patterns of activity and this is where we turn to in the next section of this thesis. Disregarding this last point, notice the multitude of crucial incongruences here. There is mismatch between computer models and the STDP phenomena that they rely on, particularly as the LTD window seems impossible to define and is neither precise nor constant as it grows with training. There is a mismatch between the models and the

empirical conditioning data, and there is a mismatch between the empirical STDP phenomena and the conditioning data.

An entirely different proposal, yet only theoretically modeled and rarely paid much attention to is the concept that both adaptive timing and cessation of firing is controlled within the Purkinje cell itself (Fiala, Grossberg, & Bullock, 1996; Steuber & Willshaw, 2004) through delayed activation of hyperpolarizing ion channels. These models are also discussed below.

Mechanisms for adaptive timing

What causes the conditioned Purkinje cell pause in firing to be accurately timed? The cerebellar instantiation of the general timing idea presented earlier in the introduction is that their pre-synaptic neurons, the granule cells, have variable activity states during the interstimulus interval and thus provide time-varying input to the Purkinje cell. In response to a common mossy fiber drive, the activity of different granule cell subpopulations is suggested to vary such that their activity peaks at different times during the interstimulus interval. Computer models of classical conditioning based on STDP rules only output properly timed conditioned responses when they assume such variable long-lasting granule cell activity states during the interstimulus interval. The terminology for what is needed by the majority of contemporary models of conditioning is that granule cells are assumed to function as temporal pattern generators. The plausibility of this assumption is evaluated below.

Some of the conditioning models were devised before some critical anatomical and physiological facts were known, and some more contemporary models have disregarded them, especially when it comes to the electrophysiological properties of granule cells and Golgi cells. Because strong assumptions with a wide scope for freedom of thought on how these two cell types may behave are crucial to virtually all contemporary models of conditioning, a few notes on their actual properties warrant elaboration. Granule cells are notoriously difficult to record from because of their small size (it is the smallest cell type in the nervous system) and *in vivo* observations have only become available in the last ten years or so. However, the data that are now available are very informative when it comes to understanding how classical conditioning may work.

Granule cells *in vivo* have a short response latency to peripheral stimuli (5-6 ms) and a very fast membrane time constant (Chadderton et al., 2004), in non-anaesthetized animals as fast as 2 ms (Jörntell & Ekerot, 2006). Neither parameter exhibits any significant variability between cells. These studies also showed that granule cells fire at very low spontaneous rates, if they fire at all, due to tonic inhibition from Golgi cells. Activated by repeated and uniform stimulation pulses, exactly the kind of stimulation

used in conditioning, granule cells consistently respond to mossy fiber input with a short-latency EPSP, exactly timed across different cells. Further, no temporal summation occurs up to mossy fiber input as high as 1000 Hz (Jörntell & Ekerot, 2006). This strongly suggests that during eyeblink conditioning there is no variable response latency and no temporal summation of EPSPs, and so each mossy fiber impulse causes exactly the same granule cell response.

Both studies demonstrate that sensory stimulation evokes EPSPs but virtually no IPSPs from Golgi cells on any relevant time-scale for granule cells to interact with Golgi cells in such a way as to produce any meaningful temporal patterns of activity that could be read out by Purkinje cells. IPSPs from Golgi cell input are always small and do not contribute substantially to synaptic integration on a short time scale. This palpable lack of fast IPSPs agree with earlier *in vitro* observations that the role of Golgi cell inhibition of granule cells is to set the excitability level on long time scales by means of tonic inhibition (Brickley, Cull-Candy, & Farrant, 1996; Hamann, Rossi, & Attwell, 2002; Rossi & Hamann, 1998; Wall & Usowicz, 1997). This facilitates the role of granule cells as excellent signal-to-noise enhancing elements. A recent *in vivo* study with simultaneous recordings (dual loose-patch) of interconnected granule cell/Golgi cell pairs convincingly showed that the main time constant over which Golgi cells influence granule cells does not match that which is needed in the contemporary models of classical conditioning. It is counted in seconds rather than tens or hundreds of milliseconds (Bengtsson, Geborek, & Jörntell, 2013). This means that the temporal patterns of granule cell activity assumed by models simply, not only have never been observed, but are explicitly contradicted by experiments. *In vivo* studies conclude that the function of granule cells, essentially, is to serve as signal-to-noise enhancing elements, not as temporal pattern generators.

In addition to the questions raised for pf-PC LTD there is now a whole set of further constraints for any model on adaptive timing. Combining what is known of conditioned responses with the limitations of the STDP models and the physiological properties of the cerebellar circuit, there are 10 essential challenges that limit the feasibility of explanations for the learning that takes place in classical conditioning:

1) The anatomical organization must be feasible (*Anatomical organization*).
2) Direct stimulation of mossy fibers must be an effective conditional stimulus (*Mossy fiber CS*).
3) Presenting only a few pulses to the mossy fibers must be able to elicit the full response (*Mossy fiber brief CS*).
4) Granule cells do not exhibit variable long-lasting response patterns (*No variably slow granule cells).*
5) Granule cells exhibit no fast EPSP/IPSP temporal summation (*No granule cell summation)*.
6) Golgi cell inhibition is not fast enough to allow the generation of temporal patterns by granule cells (*Slow Golgi cell inhibition*).
7) Conditioning does not occur with interstimulus intervals of <100 ms (*Short ISI*).
8) The conditioned Purkinje cell response is appropriately expressed and terminated near the unconditional stimulus even if the conditional stimulus outlasts the interstimulus interval by hundreds of milliseconds (*Extended CS*).
9) Any Purkinje cell (in the C3 zone) can learn any interstimulus interval (*Any ISI*).
10) Once trained with one interstimulus interval, Purkinje cells can be re-trained to a new interstimulus interval and exhibit double-peaked responses (*ISI shift*).

Dedicated timing: Tapped delay lines

Tapped delay line models depend on a sequential activation of neurons by the conditional stimulus. In the models by Desmond and Moore (Desmond & Moore, 1988; Moore, Desmond, & Berthier, 1989) pre-cerebellar neurons A, B, C, and so on, are activated by the conditional stimulus in sequence because they excite each other. Upon activation each of A, B, C … sends a signal to a specific granule cell or granule cell population so that they get differently delayed conditional stimulus signals. Associations were then thought to be formed with and only with the "delay line" whose activity coincides with the unconditional stimulus (hence the name "tapped" delay line). The anatomical organization of the brainstem does not however support this, as there are no such connections. No version of these models can account for the fact that direct electrical stimulation of the mossy fibers works just fine as a conditional stimulus. Other early ideas were based on explicit propagation delays in which the parallel fibers themselves conduct signals at different speeds, would be of different length or contact multiple Purkinje cells such that unique Purkinje cells sequentially receive the CS signal at each time point (Valentino Braitenberg, 1984; V. Braitenberg, Heck, & Sultan, 1997; Chapeau-Blondeau

& Chauvet, 1991) but these face the same problem of not finding support in neither anatomy nor physiology (Pichitpornchai, Rawson, & Rees, 1994; Vranesic, Iijima, Ichikawa, Matsumoto, & Knopfel, 1994). Furthermore, learning at short interstimulus intervals would occur because granule cells respond to peripheral stimuli as early as after 5-6 ms. There is no 100 ms delay.

Critical model challenges:

- (1) Anatomical organization
- (2) Mossy fiber CS
- (3) Mossy fiber brief CS
- (7) Short ISI

Dedicated timing: A spectrum of variably slow pre-synaptic neurons

Slow pre-synaptic neurons are envisioned in the spectral timing models of Grossberg and colleagues (Bullock, Fiala, & Grossberg, 1994; Grossberg & Schmajuk, 1989). While the specifics vary between versions of these and kindred models they are all based on a granule cell population within which there would be a spectrum of response kinetics. Once the conditional stimulus starts, different granule cells are active at different time steps because of their widely different membrane time constants. An individual granule cell becomes active only once during the interstimulus interval because it is immediately inhibited by a Golgi cell in a sustained fashion for the remainder of the interval. In this way, the Purkinje cell can undergo selective synaptic alterations with the granule cell that fires at the right time. The models can be tweaked so that no granule cells are active within the first 100 ms. However, it is proven beyond doubt that there are no such variable granule cell properties and certainly no granule cell with a response latency of 100 ms. Further, there is no such fast granule cell-Golgi cell feedback loop. There are also spectral timing models in which the spectrum of response kinetics is found among the Purkinje cells instead of the granule cells. These are discussed further on.

Critical model challenges:

- (4) No variably slow granule cells
- (6) Slow Golgi cell inhibition
- (7) Short ISI

Emergent timing: Temporal evolution of activity in the network

Most contemporary models are based on a suggested mechanism by which the Purkinje cell selectively forms an association with a random pattern of pre-synaptic activity that represents a particular interval. Timing passively "emerges" from the inherent dynamics of the network. Due to varying activity in granule cells during the conditional stimulus, each time point is uniquely represented by a specific pattern of granule cell activity.

In oscillation models, by e.g. Gluck et al. (Gluck, Reifsnider, & Thompson, 1990), once activated by the conditional stimulus, granule cells start to randomly oscillate with different firing frequencies and time lags. The unconditional stimulus then identifies the particular oscillation pattern at the time and these pf-PC synapses undergo LTD. When the same pattern appears after training the Purkinje cell ceases to fire around the anticipated time of the unconditional stimulus. However, no such oscillations have ever been observed. Granule cell recordings directly contradict the suggestion. As pointed out by Buonomano and Mauk (Buonomano & Mauk, 1994), it is not difficult to develop hypothetical systems that generate time-varying CS representations when any and all assumptions can be made freely. The trick is to create a model system that is biologically feasible. The oscillation model has no empirical grounding. Improving on this, they constructed a model that is more grounded in physiology. Theirs was the first model that does not depend on conduction delays, a spectrum of variably slow pre-synaptic neurons or speculative oscillations. Common to the original model and its later versions based on the same principle is that it is the instantaneous granule cell population activity vector that represents different time steps (Buonomano & Mauk, 1994; Li et al., 2013; Medina, Garcia, Nores, Taylor, & Mauk, 2000; Medina & Mauk, 2000). The time-variance in granule cell activity derives from a fast negative feedback connection with Golgi cells. A particular mossy fiber input pattern would activate a subset of granule cells, which in turn activates a subset of Golgi cells. These Golgi cells inhibit a second and partially overlapping subset of Granule cells. The result is that granule cells undergo random transitions between active and inactive states during the conditional stimulus. On a population level, a unique pattern of granule cell activity would represent each time step. Learning a particular duration equals learning to recognize the random pattern of activity that, essentially "accidentally", represents it.

In terms of generating a representation of time, the most severe issue with this model is that its entire foundation, fast Golgi cell inhibition of granule cells, is directly contradicted by physiological data. There is no relevant granule cell summation of neither multiple EPSPs nor EPSPs and IPSPs that could generate these random patterns. Even if one were to disregard this there are other challenges. One is that the model system is very sensitive to noise. The authors noted that spurious activity in just a few granule cells alters the population dynamics to such an extent that the timing signal is lost. Thus, the exact same random pattern would have to appear every time that the conditional stimulus is

presented. In contrast, the conditioned Purkinje cell response is remarkably robust and not very sensitive to changes in stimulus parameters. This is exemplified by the ability of a very brief conditional stimulus to elicit the full response. It is impossible for such a conditional stimulus (1 instantaneous pulse or 2 stimulation pulses in 20 ms) to elicit the same granule cell activity pattern as the full stimulus (e.g. 16 pulses at 50 Hz in a 300 ms CS). The only way to rescue the model would be to re-introduce the unfounded slowly activating granule cells. Further, it is inherently difficult to explain why these models would not predict learning at short interstimulus intervals. The concept is founded upon each time point being represented by a unique input pattern, including the 0-100 ms where conditioning does not occur, that reaches the Purkinje cells. These patterns reach the Purkinje cell in the time window where the STDP rules predict induction of LTD.

Noting that the actual mechanism by which the sequence of active populations of granule cells in the Buonomano and Mauk models arises remained unclear, Yamazaki and Tanaka analyzed the theoretical dynamics of the principle (Yamazaki & Tanaka, 2005). While they subsequently were able to generate a more stable computer model based on the same principle (Yamazaki & Tanaka, 2007) what they found to be a necessary condition for this entire category of models is that temporal integration of input signals over a long time scale occurs in the granule cells. As noted above, we know that it does not.

Critical model challenges:

- (3) Brief mossy fiber CS
- (4) No variably slow granule cells
- (5) No granule cell summation
- (6) Slow Golgi cell inhibition
- (7) Short ISI

Dedicated timing: Timing models based on Purkinje cell Ca^{2+} transients

Noting the problem of a critical sensitivity to noise in the above kind of models Fiala and colleagues (Fiala et al., 1996) developed the first model of cellular adaptive timing in this circuit. A slow neuron response would be the most robust mechanism (and of most computational use) and because it was already then indirectly demonstrated that it is not likely that granule cells are capable of delayed responses, the simplest explanation would be that delayed Purkinje cell responses is the operative mechanism for adaptive timing. Their model is based on slow intracellular calcium signaling mediated by the metabotropic glutamate receptor 1 (mGluR1). This is also a spectral timing model but instead of a spectrum of kinetics in the granule cell layer the spectrum is instantiated at

the Purkinje cell layer. A variation in the number of mGluR1s expressed by different Purkinje cells (preset and constant) bestows them with different latencies of calcium concentration increase to a threshold level where hyperpolarizing current through calcium-activated potassium channels is turned on. Specific Purkinje cells are prepared to learn a particular interstimulus interval. The model is not feasible however, because any individual Purkinje cell can learn and re-learn any and multiple interstimulus intervals. Steuber & Willshaw improved on this model by suggesting that the number of mGluR1s adaptively changes with learning (Steuber & Willshaw, 2004). With paired CS-US presentations the learning mechanism thus adjusts the response latency of the Ca^{2+} increase in the individual Purkinje cell to match the interstimulus interval. However, the model cannot explain double peaked responses as observed after an ISI-shift.

There are two further problems with both alternatives. First, they both predict learning with short interstimulus intervals. Second, mechanisms that depend on adjusting the latency of a concentration rise of a given ion will be sensitive to the duration of the conditional stimulus. It is therefore unlikely that a brief conditional stimulus could evoke the same, if any, response. Further, when the conditional stimulus extends beyond the interstimulus interval with several hundred milliseconds, both in the training setting, and during performance, the Ca^{2+} current cannot easily be adjusted to match one and only one time point. As with the improbable scenario where 2 pulses in 20 ms and 16 pulses in 300 ms would need to produce exactly the same unique pattern of granule cell activity, here the same Ca^{2+} current would have to be produced by the widely different inputs.

Critical model challenges:

- (3) Brief mossy fiber CS
- (7) Short ISI
- (8) Extended CS
- (9) Any ISI [Fiala et al.]
- (10) ISI-shift

Summary of cerebellar timing models

Figure 6 summarizes the range of models for cerebellar timing. Fig. 6A illustrates how all models depend on different pre-synaptic neurons (a, b, c, d) becoming active at different time steps during the interstimulus interval. Here, granule cell c is active when the US arrives and LTD is induced. This neuron will always become active at the same time step and reduce excitation. Fig. 6B illustrates the delay line that could underlie the sequential activation of granule cells a-d. At the other end of the range (fig. 6C), the mossy fiber drive sets all granule cells going in random patterns. Unique subpopulation vectors represent each time step. An LTD-association is somehow formed between the appropriate vector and the Purkinje cell.

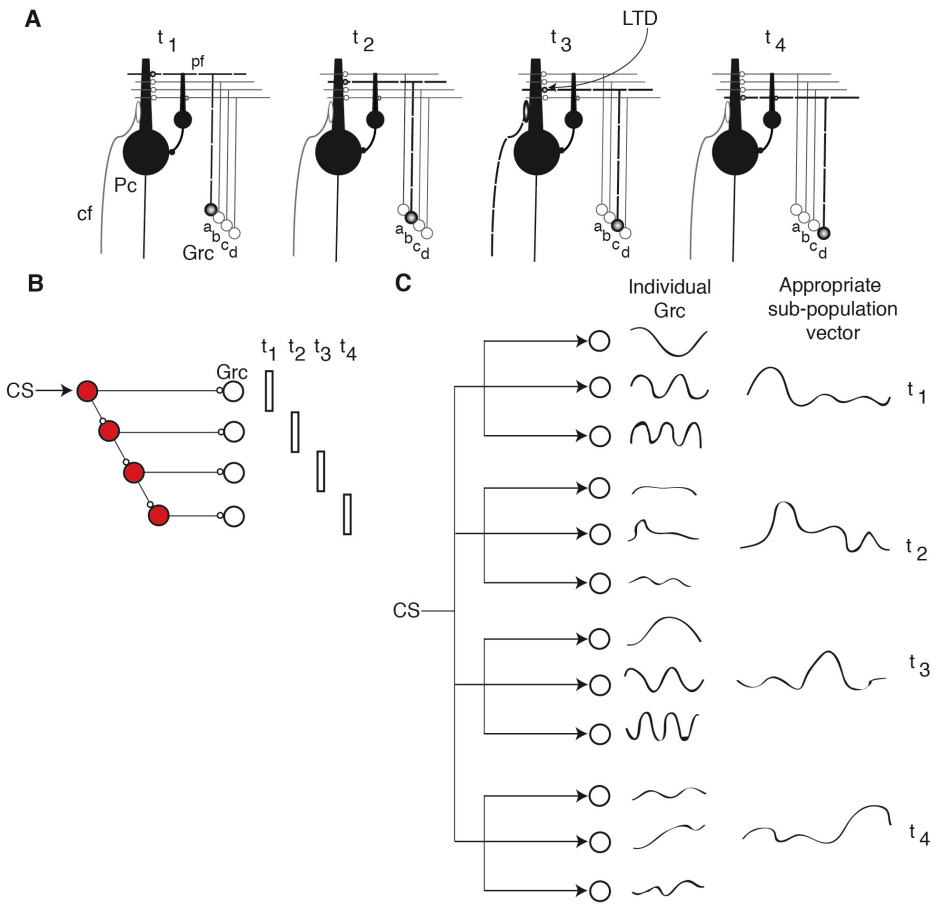

Figure 6. Summary of models for cerebellar timing.

While cellular models of adaptive timing have their challenges and while they may appear difficult to envision because we are unaware of what the mechanism might be, they nevertheless appear to be more promising than the network models discussed above. Such a mechanism would be the most robust, of most computational use and able to explain more of the empirical conditioning data. Because both STDP rules and the existing models on pre-synaptic generation of time codes have so many critical shortcomings, this thesis directly tests a hypothesis that the learning of temporal duration occurs within the Purkinje cell itself. If this is the case, it should be possible to classically condition a Purkinje cell using direct electrical stimulation of the presynaptic parallel fibers themselves as the conditional stimulus. If it is possible, clues about how intracellular molecular machinery could accomplish this should be obtainable from pharmacological manipulation of receptors and ion channels expressed by the Purkinje cell.

Strategy and methods

Strategy

The purpose of this thesis is to investigate what kind of learning mechanism allows the precise and peculiar clockwork-like timing of conditioned Purkinje cell responses. Is the learning of temporal relationships and memory storage of temporal duration spread out over a large number of synaptic strength changes or is it intrinsic to the Purkinje cell itself?

We first test the hypothesis that the learning resides within the Purkinje cell by using direct electrical stimulation of the parallel fibers as the conditional stimulus. With experimental control of the pre-synaptic input to the Purkinje cell one can by-pass the network thought to generate a time code and thus obtain a direct test for whether timing in the granular layer is necessary. If it is not, timing and memory must be located within the Purkinje cell.

Using selective antagonists of the neurotransmitter receptors expressed by Purkinje cells we then try to determine what kind of signal (ionotropic, metabotropic, glutamatergic, GABA-ergic) triggers the conditioned Purkinje cell response. Next, by investigating the role of ion channels we can also determine which conductance generates the delayed voltage response and thus get a better picture of the signaling cascade from receptor activation to voltage response.

Lastly, a theoretical study discusses how a cellular mechanism for timed responses could work in principle.

Experimental procedure

What follows is a description of the general methods common to all studies in this thesis. Hence, some details are left out and the reader is referred to the appendices where a full method description accompanies each individual study.

Surgery

All surgery was performed on 10-14 month old male ferrets weighing 0.75-1.5 kg. Following a brief period of habituation to the laboratory environment, the animals freely walk into an anesthesia chamber that is filled with isoflurane (Baxter Medical, Kista, Sweden) in a mixture of O_2 and air. At this point they fall asleep and never regain consciousness, why our procedure is close to unique in that the subjects literally never experience any discomfort. A tracheotomy was then performed and the subjects were artificially ventilated through a tracheal tube. A catheter for blood pressure measurement was placed in the *arteria femoralis* and a central venous line was placed in the *vena femoralis*. At this point, isoflurane anesthesia was discontinued and replaced by propofol (10 mg/ml, AstraZeneca, Södertälje, Sweden), intravenously. The arterial blood pressure, rectal temperature and end-expiratory CO_2 concentration were all monitored continuously and kept within physiological limits throughout the experiment.

Physiological homeostasis was maintained by intravenous infusion of either 50 mg/ml glucose and isotonic acetate Ringer's solution in proportion 1:1 with 0.004 mg/ml albumin fraction V from bovine serum (Sigma-Aldrich, St. Louis, MO) or 50 mg/ml glucose, isotonic acetate Ringer's solution and Macrodex solution in proportions 1:1:1. The infusion rate was in both cases 6 ml $\times$ kg^{-1} $\times$ h^{-1}.

The subject's head (*meatus acusticus externus*, bilaterally) was fixated in a custom made stereotaxic frame after which the ipsilateral epicranial masticatory muscle and the medial segments of the suboccipital muscle group on both sides were reflected to expose the cranium.

A first craniectomy, sized ~2 $\times$ 1.5 cm, centered on an approximate midpoint between the superior temporal line of the parietal bone and the vertex allowed access to remove the caudal 2/3 of the left cerebral hemisphere together with a substantial part of the thalamus by aspiration. This procedure exposed part of the superior *lobus cerebellaris posterior* and the left *colliculus inferior et superior*. Sectioning the brainstem with a blunt spatula 1-2 mm rostral to the *colliculus superior* (sparing *nucleus ruber*) then decerebrated the subjects. Anesthesia was at this point discontinued. Decerebration is advantageous compared to continued anesthesia because the latter interferes with cerebellar function (Bengtsson &

Jorntell, 2007). Anesthesia can be discontinued because at this point the subject will not regain consciousness because the cerebrum has been removed.

The resection surface of the rostral ipsilateral cerebral hemisphere was walled off with absorbable hemostatic sponges (Spongostan™, Johnson & Johnson, New Brunswick, N.J., U.S.A.). A second craniectomy, sized 1 × 0.5 cm, between the lambda and the caudal end of the neurocranium, was performed to allow electrode access to the *pedunculus cerebellaris inferior*. With the left cerebellum and left colliculi exposed, walls of cotton reinforced agar solution around exposed brain tissue and neurocranium constructed a pool that was filled with warm perfluoro carbon liquid of high-density (FC-40 Fluorinert; 3M, Zwijndrecht, Belgium). This ensured a pseudo-physiological environment in terms of humidity and intracranial pressure for the exposed brain.

In order to increase mechanical stability three further actions were taken. First, the animals were kept curarized with vecuronium bromide (Norcuron®, Organon Pharmaceuticals, N.J., U.S.A.) or rocuronium bromide (Rocuronium Kabi®, Fresenius Kabi, Bad Homburg, Germany) at 0.3-2.5 mg × kg^{-1} × h^{-1}. Second, the musculature external to the spinous processes of the vertebral column at the superior thoracic and inferior lumbar levels were uncovered, clamped and elevated. Third, a bilateral pneumothorax diminished chest movements and consequent nervous tissue movements that occur from pressure propagation during the respiratory cycle.

The falx cerebelli and dural, arachnoid and pial membranes covering the rostrolateral parts of cerebellar lobules IV-VII were removed. Exposed cerebellar surface was covered with agarose gel (10-20 mg/ml) in order to provide recording stability and mitigate potential oedema development in the area of recording and stimulation.

All procedures were reviewed and approved by the local Swedish Ethical Committee.

Electrical stimulation sites and recording

In order to localize the microzone within the C3 zone that controls eyelid musculature, insulated stainless steel electrodes were placed bilaterally in the eyelids (fig. 7). While applying single electrical pulses (1 mA), field potential responses on the surface of hemispheral lobule VI were recorded (fig. 5, page 22). The microzone was located by using the established criteria explained earlier. Two needle electrodes were placed subcutaneously in the ipsilateral forelimb for experiments with a peripheral conditional stimulus.

To stimulate climbing fibres in the ipsilateral *pedunculus cerebellaris inferior* an electrode was lowered into the brain 4.0-6.0 mm deep of the caudal cerebellar surface, approximately 4 mm lateral to the midline and 4 mm rostral to the caudal end of the cerebellar vermis, at an angle of ~45°. Climbing fibres were located by applying single pulses (20-100 μA) while moving the stimulation electrode and recording elicited field potential responses in the above-identified zone. The response latency considered as an effective electrode placement ranged between 2.0-2.3 ms.

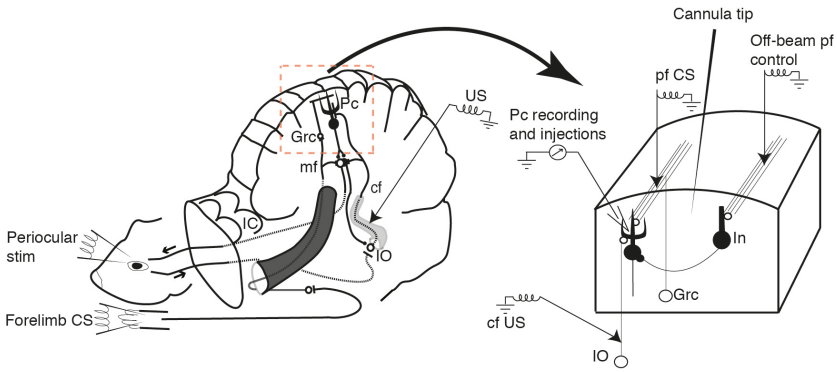

Figure 7. Experimental setup. Periocular stimulation was used to locate the correct Purkinje cells. Direct electrical stimulation of parallel fibers and forelimb stimulation were used as conditional stimuli. Direct electrical stimulation of the climbing fibers was used as the unconditional stimulus. Interneurons in the cortical molecular layer were stimulated by off-beam parallel fiber stimulation. Drug injections were made both locally from barrels with openings at the tip of the recording electrode (0.5-2 nano liters) and as infusions (2 micro liters) 1-2 mm away from the recorded cell through a separate cannula tip.

Parallel fibers were stimulated by placing a single electrode or a fork of two to four electrodes (home-made) in the superficial cortical sheet of the above-identified microzone. On-beam location (Purkinje cell afferent stimulation) was confirmed by eliciting Purkinje cell simple spikes. Off-beam location (interneuron afferent stimulation) was confirmed by suppressing Purkinje cell simple spikes.

Direct stimulation of cerebellar afferents was performed with etched and lacquered tungsten electrodes (wire diameter, 30 μm; de-insulated tip, ~50 μm) or platinum-tungsten electrodes with pulled and ground tips (25 μm metal core diameter). The effectiveness of stimulation at various sites was confirmed for each Purkinje cell recording.

Extracellular single unit recordings were made using quartz glass-coated platinum-tungsten microelectrodes with pulled and ground tips with a 25 μm metal core diameter (Thomas Recording GmbH, Giessen, Germany) or carbon fibre (10 μm core diameter) microelectrodes (Kation Scientific, Minneapolis, M.N., U.S.A.). All Purkinje cells were identified by the presence of complex spikes and were located in the eye blink controlling area of the C3 zone of the ipsilateral lobule VI as confirmed by eliciting short-latency complex spikes with periocular stimulation.

The electrode signal was fed through a NL100 headstage to a pre-amplifier (NL104) with a filter module (NL125) from Digitimer Ltd. (Cambridge, U.K.) A Power 1401 analog/digital converter interface sampled the signal at 43 kHz and passed it on to a computer running Spike2 v7 software (Cambridge Electronics Design, Cambridge, U.K.). On- and offline spike sorting was performed using Spike2 v7 and all data analysis and statistical calculations were done using custom-made Matlab scripts (MathWorks, Natick, M.A., U.S.A.).

Training protocol

The conditioning protocol was similar to those used previously in our lab (Hesslow & Ivarsson, 1994; Hesslow et al., 1999; Jirenhed et al., 2007; Jirenhed & Hesslow, 2011a). The conditional stimulus either co-terminated with the unconditional stimulus or outlasted it for several hundred ms, the latter of which was more commonly used here. Peripheral conditional stimuli consisted of electrical stimulation of the ipsilateral forelimb skin (50 Hz, 400 ms, 1 ms pulse duration) and parallel fiber conditional stimuli most often consisted of 100 Hz stimulus trains (230-800 ms, 2-20 μA, 0.1 ms pulse duration). The increase to 100 Hz as compared to 50 Hz with mossy fiber stimulation (Jirenhed et al., 2007) was motivated by the fact that granule cells respond to mossy fiber stimulation with more than one spike (Chadderton et al., 2004; Jörntell & Ekerot, 2006). The unconditional stimulus always consisted of two electrical stimulus trains, each consisting of five 0.1ms pulses at 500Hz, separated by 10 ms, applied to ipsilateral climbing fibers. The olivary neurons naturally respond to peripheral stimuli with bursts (Simpson, Wylie, & De Zeeuw, 1996), peripheral stimuli often elicits two complex spikes separated by 10-20 ms (Ekerot, Gustavsson, Oscarsson, & Schouenborg, 1987) and burst-stimuli of climbing fibers is necessary for conditioning to occur (Rasmussen et al., 2013). The intertrial interval was in all cases 15 ± 1 s.

Pharmacology

Several different pharmacological compounds and two different methods for their application where used in the studies included in this thesis. Pipette infusions in the microlitre range allows agonizing and antagonizing a given receptor on multiple cells and cell types in cubic millimeters of nervous tissue, thus permitting investigation into its relevance for the entire local neuronal circuit for a particular function. Micro pressure ejections from fine barrels in the subnano- to nano liter range into the microenvironment of a specific target neuron offer several advantages. Direct infusion of the extracellular space surrounding a Purkinje cell's dendritic tree with a pharmacological substance circumvents diffusional barriers, limits enzymatic breakdown and, most importantly, confines the effects of a substance to a single or a couple of neurons. This method of application was the most commonly used method in the investigations reported here. In experiments using micro pressure ejections, 4-, 6- and 7-barreled carbon fibre electrodes were used (Kation Scientific, Minneapolis, MN, U.S.A.). This allows testing the effect of multiple compounds, alone or combined, on the same neuron.

The details of the pharmacological manipulations made are found in other chapters of this thesis and in the appendices.

Summary of results

Conditioning with a parallel fiber conditional stimulus

The purpose of the first study was to determine if the timing of the conditioned Purkinje cell responses depends upon a time code generated in the pre-synaptic network. The results show that the timing does not depend upon such a code. Parallel fibers make synaptic contacts with Purkinje cells and cortical interneurons without any intermediate synapses. Direct stimulation of parallel fibers as the conditional stimulus thus bypasses the pre-synaptic network and makes a time code other than the repetitive pulses under experimental control impossible. Despite this, we observed the acquisition of conditioned Purkinje cell responses (n = 23) timed to interstimulus intervals of 150, 200 and 300 ms. Longer intervals were not studied because learning is slower and therefore difficult to obtain in this novel experimental setup that is more fragile due to the introduction of more foreign material in a very small volume of nervous tissue.

In eight cells, the conditional stimulus co-terminated with the unconditional stimulus and in fifteen cells the conditional stimulus duration outlasted the interstimulus interval by 150-600 ms. This design allowed investigation into the response properties that are intrinsic to the conditioned response as such, in contrast to direct effects of conditional stimulus duration.

Naïve cells (n = 19) responded to the conditional stimulus with no change, moderate increases in firing or powerful increases in firing (figs. 8A, 8F, 9D). As seen in figure 9, all cells acquired conditioned responses during training. Considering the robust differences between naïve and trained responses we also included four additional neighboring Purkinje cells, along the same parallel fiber beam, encountered after training.

In order to determine whether Purkinje cells trained in this paradigm behave as those trained with a forelimb or mossy fiber conditional stimulus, as described in the introduction, we performed a series of post-acquisition manipulations. When it was possible, extinction with unpaired conditional stimulus was attempted. As expected, the conditioned Purkinje cell responses disappeared with time (example in Fig. 8E). In one case, it was also possible to attempt to re-train the Purkinje cell by shifting the interstimulus interval. After emitting responses to an interstimulus interval of 200 ms,

subsequent training with a new 350 ms interstimulus interval caused the cell to acquire a bimodal conditioned response, with a second pause response close to the end of the new interstimulus interval (fig. 8H).

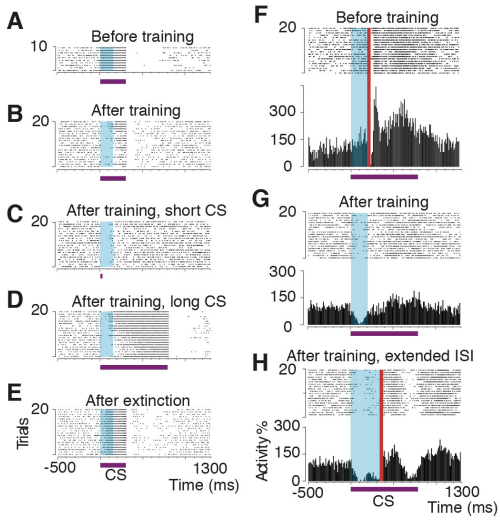

Figure 8. Conditioned Purkinje cell responses timed to interstimulus intervals.
A-B. Raster plots showing a typical Purkinje cell response to a 300 ms conditional stimulus before (*A*) and after (*B*) training with a 150 ms interstimulus interval (blue shading). ***C-D***. Responses of the same cell as in *A-B* to 17.5 ms and 800 ms conditional stimuli after training. ***E***. Response to a 300 ms conditional stimulus after extinction. ***F-H***. Raster plots and histograms illustrating responses of a Purkinje cell that was first trained with a 200 ms interstimulus interval and subsequently with a 350 ms interstimulus interval. Red bars in *F* and *H* denote unconditional stimulus artifacts (data from paired conditional stimulus-unconditional stimulus trials). Purple bars indicate the conditional stimuli.

As when conditioning has been accomplished with peripheral or mossy fiber conditional stimuli, the conditioned Purkinje cell responses varied systematically with the interstimulus interval duration. There was a significant effect of interstimulus interval on latencies to onset, maximum and offset ($p < 0.0006$, Kruskal-Wallis one-way analysis of variance). For all interstimulus intervals, response maxima appeared <75 ms before the anticipated unconditional stimulus onset.

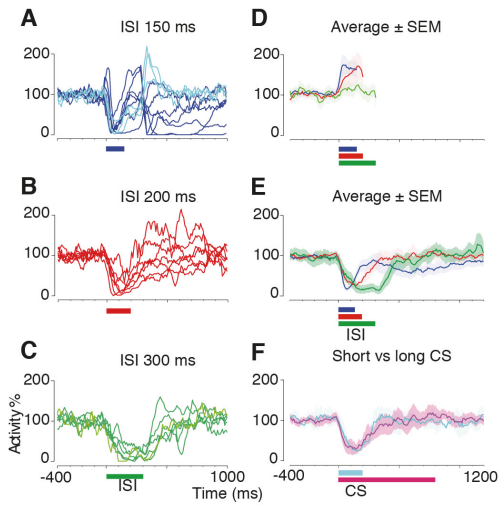

Figure 9. Time courses of conditioned responses after training with different interstimulus intervals and using conditional stimuli of different durations.
A-C. Smoothed and averaged simple spike activity after training with 150 ms (blue, n = 10), 200 ms (red, n = 7) and 300 ms (green, n = 6) interstimulus intervals. Traces with lighter shading represents cells for which we lack naïve data. Colored bars indicate the interstimulus interval. ***D***. Activity ± SEM for each interstimulus interval before training. Traces end at the onset of the unconditional stimulus artifact, which prohibits identification of spikes. Abrupt downward inflections at the end of some traces reflect an effect of smoothing (0 identified spikes during the unconditional stimulus artifact). ***E***. Activity ± SEM for each interstimulus interval after training. ***F***. Activity ± SEM for cells trained with a 200 ms interstimulus interval and a co-terminating conditional stimulus (cyan, n=2) or an 800 ms conditional stimulus (magenta, n=5).

When we manipulated the duration of the conditional stimulus (17.5-800 ms, 100-400 Hz) on a series of probe trials after training, it still elicited a response timed to the learned duration of the interstimulus interval. Examples of this phenomenon are given in figure 8C-D. Further, the duration of the conditional stimulus used during training does not appear to have any effect on the temporal profile of the conditioned response. Cells conditioned to an interstimulus interval of 200 ms using a co-terminating conditional stimulus or a conditional stimulus that outlasts the interstimulus interval by 600 ms show similar temporal response profiles (fig. 9F). Thus, the timing of the conditioned Purkinje cell response does not depend on a time coded input to the cell signaled by a time-varying pattern in the conditional stimulus.

Ionotropic GABA receptors

The above findings show that the memory trace must reside either in the Purkinje cells or in molecular layer inhibitory interneurons. In order to determine the site of learning and memory storage, we tested the effect of an ionotropic GABA receptor antagonist upon conditioned Purkinje cell responses acquired with a peripheral conditional stimulus. Before local injection of Gabazine, off-beam parallel fiber stimulation, that is stimulation of parallel fibers that do not terminate on the recorded Purkinje cell but which do excite interneurons that innervate that Purkinje cell, effectively silenced the simple spike activity (figs. 10A and E). Injection of the antagonist blocked interneuron inhibition from off-beam stimulation (figs. 10B and E) but the most important features of the conditioned responses remained essentially the same (figs. 10C-D, F-G).

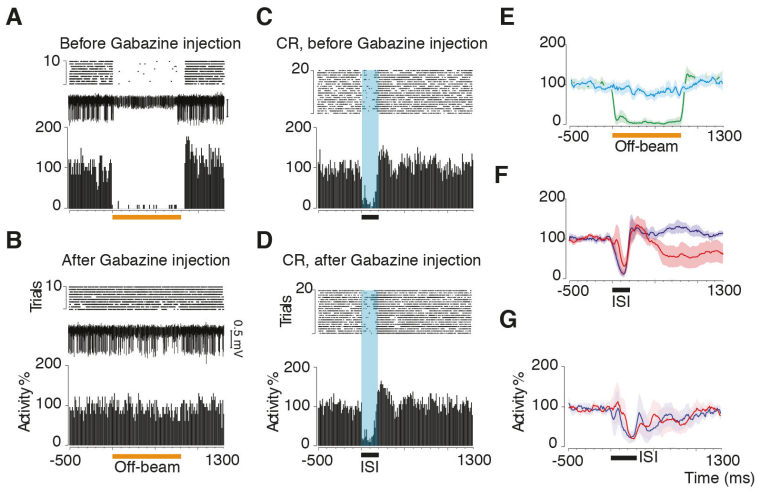

Figure 10. Gabazine blocks interneuron inhibition of Purkinje cells but leaves conditioned responses intact.
Orange bars indicate off-beam stimulation (800 ms, 81 pulses, 100 Hz). Black bars and blue shading indicate the interstimulus interval (200 ms). ***A-B***. A representative Purkinje cell's responses to interneuron activation before and after Gabazine injection. ***C-D***. Conditioned responses before and after Gabazine injection in the same Purkinje cell as in *A-B.* **E**. Average (n = 4) responses ± SEM before (green) and after (cyan) Gabazine injection. ***F.*** The average response profile of cells trained with a 200 ms interstimulus interval before (blue) and after (red) injection in the same cells as in *E*. ***G.*** The average response profile of cells trained with a 300 ms interstimulus interval before (blue) and after (red) injection (n = 3)

To the same end, a limited number of experiments (due to the technical limitations imposed by the placement of three electrodes and injection barrels in 0.3 mm^3 of nervous tissue *in vivo*) were also performed using a direct parallel fiber conditional stimulus (Fig. 11). As is perhaps best seen in Figs. 11B, E and H, the suppression in firing during the interstimulus interval is not caused by GABA-ergic inhibition from interneurons. Importantly, the concentration used in most of the experiments with both peripheral and parallel fiber stimulation as the conditional stimulus was sufficient to block all ionotropic GABA receptor ($GABA_A$, $GABA_C$ and the glycine receptor).

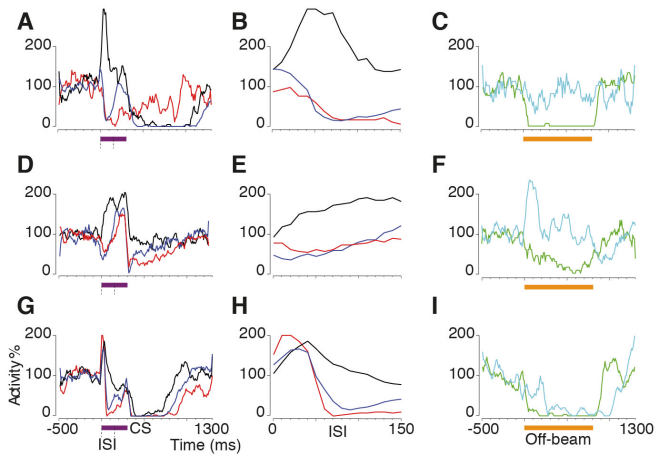

Figure 11. Effects of different concentrations of Gabazine on conditioned Purkinje cell responses to a parallel fiber conditional stimulus.

Each row indicates one cell. ***A, D, G.*** Naïve (black) and conditioned responses before (blue) and after (red) Gabazine injection Purple bars indicate a 300 ms conditional stimulus. Dashed lines indicate a 150 ms interstimulus interval. ***B, E, H.*** Magnification of the response during the interstimulus interval. ***C, F, I.*** Purkinje cell responses to interneuron activation by off-beam parallel fiber stimulation before (green) and after (cyan) Gabazine injection. 100 µM Gabazine distinctly blocks inhibition (*C* and *F*) whereas 10 µM Gabazine blocks inhibition for the first 200 ms (*I*). Orange bars indicate off-beam stimulation (800 ms, 81 pulses, 100 Hz).

Ionotropic glutamate receptors

The learned Purkinje cell response is not caused by interneuron inhibition so it seems that there is an intrinsic temporal memory within the Purkinje cell. The trigger for the response, or the activator of the memory if you will, must depend upon glutamate release from parallel fibers. Given the theory and results presented herein so far, one should not expect that the responses be mediated by the excitatory ionotropic AMPA-kainate receptors.

Cortical infusions of the AMPA-kainate receptor antagonist CNQX has been shown to prevent behavioral conditioned nictitating membrane responses in rabbits (P. J. Attwell et al., 1999; Mostofi, Holtzman, Grout, Yeo, & Edgley, 2010). However, those orders-of-magnitude larger infusions may have blocked transmission of the conditional stimulus at the mossy fiber-granule cell synapses. We suggest that extremely local blockades of AMPA-kainate receptors at the recorded Purkinje cell, by applying sub-nanoliter volumes of 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) (Wilding & Huettner, 1996) 15 μm from the tip of the recording electrode, should leave the cellular delayed responses intact.

To establish an effective concentration of NBQX at the recorded Purkinje cell, we first stimulated parallel fibers to generate an excitatory response. When a sufficient dose of NBQX (~ 0.5-1nl, 25 μM) had been injected, the excitatory response to parallel fiber stimulation disappeared (n = 8) (fig. 12A) and later returned. In three cases an initial excitatory response to parallel fiber stimulation was replaced by a suppression of Purkinje cell firing (fig 12B, *top*). This was probably because the beam of activated parallel fibers could still drive inhibitory interneurons (lateral to the Purkinje cell) further away. Consistent with this interpretation was the finding that this suppression faded after a few minutes during which time the drug would diffuse laterally (fig. 12B, *bottom*). The average response to parallel fiber stimulation fell from >200% of simple spike firing (relative to background) to no increase (Fig. 12C, n = 8).

Conditional stimulus trials were then interspersed with the control parallel fiber stimulation trials, so that conditional stimulus data was only sampled in the time during which we could confirm that the AMPA-kainate receptors were blocked. Conditioned pause responses were unaffected and normal after applying NBQX (Fig 12D-E).

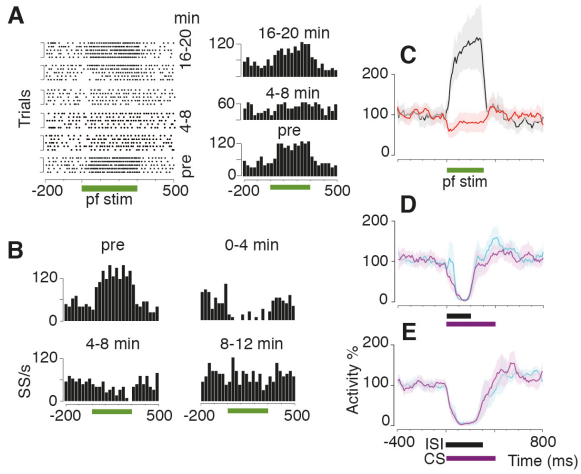

Figure 12. Purkinje cell responses to parallel fiber stimulation and to the conditional stimulus after injection of ionotropic glutamate receptor antagonist.

A. Typical Purkinje cell response to 100 Hz parallel fiber stimulation before and during 0-4, 4-8, 8-12, 12-16 and 16-20 minutes (bottom-up) after local injection of 25 µM NBQX. ***B.*** Example case of NBQX briefly un-masking inhibition elicited by the parallel fiber stimulation. ***C.*** Averaged and smoothed response profiles +/- SEM to parallel fiber stimulation before (black) and after (red) local injection of NBQX (n = 8). ***D-E.*** Averaged and smoothed response profiles +/- SEM to the conditional stimulus before (cyan) and after (purple) local injection of NBQX (*d*: 200 ms interstimulus interval, n = 4; *e*: 300 ms interstimulus interval, n = 4).

Metabotropic glutamate receptor 1 and Ca^{2+}-activated K^+ channels

The mGluR1 is thought to be necessary for parallel fiber-Purkinje cell LTD (Knöpfel & Grandes, 2002). In the context of LTD-induction, intracellular Ca^{2+} cascades has raised significant interest because timing-dependent and associative supralinear Ca^{2+} signals can be measured following subsequent parallel and climbing fiber input to the Purkinje cell (Safo & Regehr, 2008; Wang, Denk, et al., 2000). Alternatively, earlier models of intracellular Purkinje cell timing in eyeblink conditioning (Fiala et al., 1996; Steuber & Willshaw, 2004) are based on mGluR1-elicited Ca^{2+} influx that activates calcium-activated K^+ channels (the models do not specify which K_{Ca} channels). As discussed in the introduction there are critical incongruences between those models and empirical observations.

However, it is possible that alternative versions of those models, in theory, could work. It is also possible that K_{Ca} channels through a different mode of action than that in the models could cause the Purkinje cell pause response. This is unlikely because the rapid kinetics and primary roles of the two major K_{Ca} channels, $K_{Ca}1.1$ and $K_{Ca}2.2$, in afterhyperpolarization following single action potentials and acceleration of EPSP repolarization make them ill-suited for delayed and long-lasting cessations in spontaneous firing. K_{Ca} channels could nevertheless be ruled out as effectors of a cellular timing mechanism on the basis of these considerations alone. For these reasons we antagonized both mGluR1 and all K_{Ca} channels expressed by Purkinje cells.

To investigate potential mGluR1 contributions to the conditioned pause responses, 13 Purkinje cells were recorded in 4 subjects after a 10 μM cortical infusion of the mGluR1 antagonist JNJ16259685 and 4 more cells were recorded from 2 additional subjects after local injections of 1 or 10 μM. This allowed 8 direct comparisons before and after application of the antagonist (within-subject, fig. 13A). Because of the similarity between the conditions all 17 cells are reported together in Fig. 13B. The conditioned pause responses remained unchanged at all applied concentrations of the antagonist.

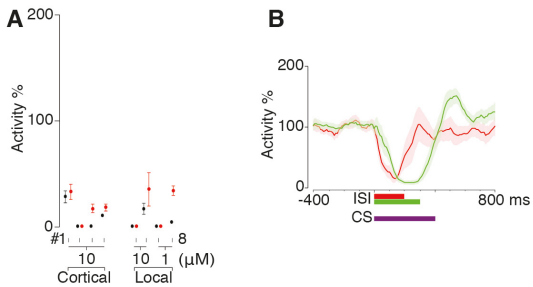

Figure 13. Purkinje cell responses to the conditional stimulus after injection of mGluR1 antagonist. ***A***. All Purkinje cell responses during the last 100 ms of the interstimulus interval before (black) and after (red) injection of JNJ16259685. X-axis indicates individual cells. ***B.*** Averaged response profiles after infusion/injection of JNJ16259685 (red, 200 ms interstimulus interval, n = 6; green, 300 ms interstimulus interval, n = 9).

Purkinje cells express three types of K_{Ca} channels, $K_{Ca}1.1$ (Edgerton & Reinhart, 2003; Gahwiler & Llano, 1989; Womack & Khodakhah, 2002), $K_{Ca}2.2$ (Cingolani, Gymnopoulos, Boccaccio, Stocker, & Pedarzani, 2002; Edgerton & Reinhart, 2003; Stocker & Pedarzani, 2000; Womack & Khodakhah, 2003) and $K_{Ca}3.1$ (Engbers et al., 2012). Because these large, small and intermediate conductance K^+ channels hyperpolarize Purkinje cells, they could potentially contribute to conditioned Purkinje cell responses. In order to investigate any contributions from K_{Ca} channels we antagonized these three with 60 nM Penitrem A, 1 μm Apamin and 1 μm TRAM34, respectively.

Consistent with *in vitro* findings (Edgerton & Reinhart, 2003; Womack & Khodakhah, 2002), blocking $K_{Ca}1.1$ had a dramatic effect on Purkinje cell firing. As expected, the cells exhibited highly aberrant behavior with bursts up to 600 Hz and long periods of silence (fig. 14A). This volatile firing is also apparent in the population average (fig. 14B). Despite this, the suppression elicited by the conditional stimulus is clearly not removed (n = 5, fig. 14A-C). Blocking $K_{Ca}2.2$ also increased firing rate and irregularity but to a lesser extent. No important change in the conditioned responses was detected (fig. 14D-F). To establish an effective concentration of the selective $K_{Ca}3.1$ antagonist TRAM34 we stimulated parallel fibers with five pulses at 100 Hz at low intensities, below 100% spiking probability to 1 pulse, to moderately excite the Purkinje cells. Consistent with the suggested function of $K_{Ca}3.1$ channels to suppress temporal summation of excitatory inputs and *in vitro* findings (Engbers et al., 2012), injection of the antagonist (1 μM) led to an increased firing probability in response to the second to fifth parallel fiber stimulation pulses (Δ+56% to 102%, see fig. 14G). On average, injection of TRAM34 had a moderate effect on the conditioned response with slightly increased firing towards the end of the interstimulus interval (n = 5, Fig. 14H-I). Because we used a reliable criterion for ion channel block and because the effect upon the conditioned response was only moderate it is prudent to conclude that it is not the chief effector of the timed

response. The partial effect of blocking $K_{Ca}3.1$ could result from the resulting excessive temporal summation of parallel fiber input in the Purkinje cell dendrites after each conditional stimulus pulse, which could corrupt the expression mechanism to some extent. It is also possible that this minor effect is due to actions of the vehicle. In contrast to all other drugs used in these studies, TRAM34 was dissolved in 100% DMSO, and a control experiment with pure vehicle needs to be done.

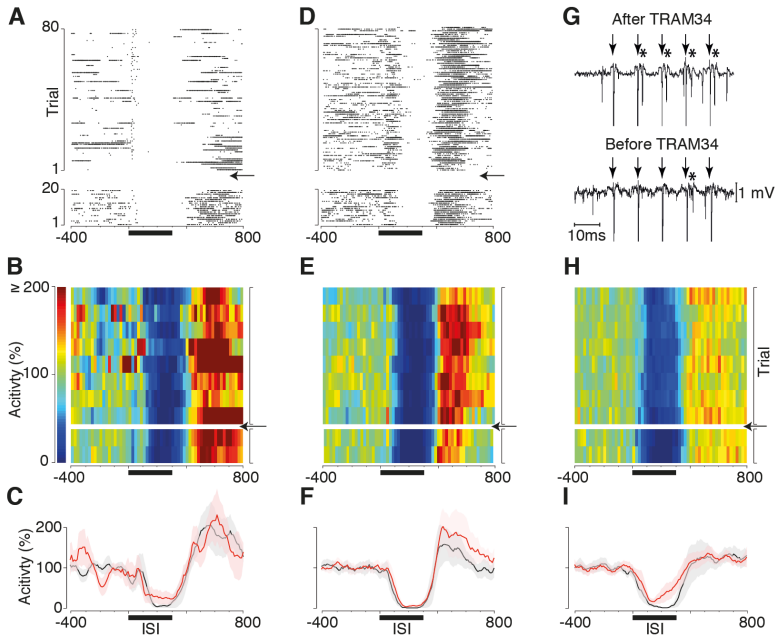

Figure 14. Effects of K_{Ca} channel block on conditioned Purkinje cell responses.

A. Raster plot of a Purkinje cell's responses to the conditional stimulus before (20 trials) and after (80 trials) injection of 60 nM Penitrem A. **B.** Population average (n = 5) of Purkinje cell activity before and after injection of 60 nM Penitrem A. The color of each square represents the average simple spike activity in a 10 trial, 10 ms bin divided by the average pre-trial frequency in the same bin. **C.** Averaged temporal response profiles before (black) and after (red) injection of Penitrem A. **D**. Raster plot of a Purkinje cell's responses to the conditional stimulus before (20 trials) and after (80 trials) injection of 1 µM Apamin. **E.** Population average (n = 5) of Purkinje cell activity before and after injection of 1 µM Apamin. **F.** Averaged temporal response profiles before (black) and after (red) injection of Apamin. **G.** A Purkinje cell's responses to sub-threshold parallel fiber stimulation before (bottom) and after (top) injection of 1 µM TRAM34. Arrows indicate stimulation artifacts and asterisks indicate elicited simple spikes. **H**. Population average (n = 5) of Purkinje cell activity before and after injection of 1 µM TRAM34. **I**. Averaged temporal response profiles before (black) and after (red) injection of Tram 34.

Metabotropic glutamate receptor 7 and protein-activated K^+ channels

An inhibitory response to glutamate contrasts with the normally excitatory effects of glutamate. Indeed, as expected, both the excitatory mGluR1 (a group I metabotropic glutamate receptor) and the excitatory AMPA-kainate receptors are not the generators of the response. However, Group II/III metabotropic receptors have been shown to have inhibitory effects in some neurons (Cox & Sherman, 1999; Dutar, Vu, & Perkel, 1999; Lee & Sherman, 2009) and an unusual hyperpolarizing effect of glutamate on Purkinje cells has been reported (Inoue, Miyakawa, Ito, Mikoshiba, & Kato, 1992) but never studied further. Because Purkinje cells express a group III receptor, the mGluR7b splice variant of mGluR7 (Kinoshita, Shigemoto, Ohishi, van der Putten, & Mizuno, 1998; Phillips et al., 1998) we suggested that this receptor could be involved in generating the conditioned Purkinje cell response (Johansson, Jirenhed, Rasmussen, Zucca, & Hesslow, 2014).

To evaluate the mGluR7 hypothesis, we used the allosteric selective mGluR7 antagonist 6-(4-Methoxyphenyl)-5-methyl-3-(4-pyridinyl)-isoxazolo[4,5-*c*]pyridin-4(5*H*)-one hydrochloride (MMPIP). Cortical infusions (2 µl, 300-600 µM applied 1-2 mm from the recorded cell) distinctively removed the pause response (n = 4, Fig. 15A) and even replaced it with excitation. Local sub-nanoliter injections, 15 µm from the tip of the recording electrode, (n=7) also removed most of the pause response but some (n=3) revealed a tendency of the drug to preferentially disturb the pause response at its normal maximum (the last 100 ms of the interstimulus interval). In one of these cases, additional local injections (i.e. an increasing dose) were possible, and these progressively flattened the temporal response profile as shown in Fig. 15B-D. For all 10 cells and all concentrations of MMPIP used (6-600 µM) the pause response at the anticipated maximum towards the end of the interstimulus interval was diminished (Fig. 15E). An example of a residual early pause following a single sub-nanoliter injection, is seen in Fig. 15F.

As an additional test, we applied the orthosteric mGluR7 antagonist LY341495 (5 µM) in another group of cells (n = 5) and obtained similar results (Fig. 15G-H) although this less selective antagonist (with higher affinity for other mGluR subtypes, including mGluR2 expressed by Golgi cells (Knöpfel & Grandes, 2002)), appeared less efficient with 3/5 cells maintaining a partial pause response early in the interstimulus interval.

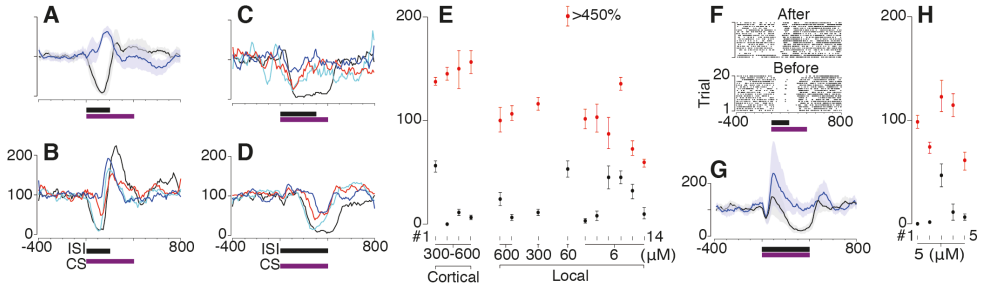

Figure 15. Purkinje cell responses to the conditional stimulus after injection of mGluR7 antagonist. Key: y-axes indicate simple spike firing (% of baseline activity) ***A-E, G-H*; trials *F***. x-axes indicate time in ms (-400 to 800 ms) ***A-D***, ***G***, ***H;*** individual Purkinje cells ***E*** *and* ***H***. Purple and black indicate conditional stimuli and interstimulus intervals, respectively. All traces represent smoothed response profiles. Shading and error bars indicate SEM. **A**. Averaged response profile before (black) and after (blue) cortical infusions of MMPIP (300-600 μM; n = 4). **B-D.** Response profiles of individual Purkinje cells, one in each panel, before (black) and after one (cyan), two (red) and three (blue) sub-nanoliter local injections of 6 μM MMPIP. **E.** All Purkinje cell responses during the last 100 ms of the interstimulus interval before (black) and after (red) injection of MMPIP. For cortical infusions: cell #1: 300 μM, cells #2-4: 600 μM. **F**. Raster plot of a Purkinje cell where a short latency inhibition remained after injection of MMPIP. **F.** Averaged response profile before (black) and after (blue) local injection of LY341495 (5 μM; n = 3). **H**. All Purkinje cell responses during the last 100 ms of the interstimulus interval before (black) and after (red) local injection of LY341495.

The finding that the conditioned Purkinje cell response is elicited by glutamate release from parallel fibers acting on mGluR7 suggests a plausible mechanism for eliciting timed responses. Gβγ protein dimers produced upon activation of mGluR7 (Saugstad, Segerson, & Westbrook, 1996) can directly activate the G protein-gated inwardly-rectifying K^+ channel family Kir3 (or *GIRK*) which, despite its historical name, mediates postsynaptic inhibition (Dascal, 1997; Whorton & MacKinnon, 2013), suggesting that these channels could constitute the effector component of the intrinsic temporal memory. To test this hypothesis, we have pharmacologically blocked Kir3 in trained Purkinje cells and ruled out alternative sources of Gβγ, besides mGluR7, for their activation.

In order to evaluate the hypothesis that Kir3 channels are the effectors of conditioned Purkinje cell responses, we used the antagonist $Tertiapin_{LQ}$. This choice was made because the more traditionally used $Tertiapin_{Q}$ has an undesirable high affinity for large conductance $K_{Ca}1.1$ channels (Kanjhan, Coulson, Adams, & Bellingham, 2005). The effect of this drug was in perfect agreement with the hypothesis of this thesis. On average, local injections of 5 (n = 7), 25 (n = 9) and 200 μM (n = 5) $Tertiapin_{LQ}$ considerably diminished the conditioned response (figs. 16A-F).

In addition to mGluR7, Purkinje cell Kir3 channels can also be activated by Gβγ produced upon activation of the $GABA_B$ receptor ($GABA_BR$) (Tabata, Haruki, Nakayama, & Kano, 2005). Kir3 channels are also expressed pre-synaptically in parallel fiber terminals and interneurons where they are activated by the endocannabinoid CB1 receptor (CB1R) and the $GABA_B$ receptor (Aguado et al., 2008; Daniel, Rancillac, & Crepel, 2004; Fernandez-Alacid et al., 2009). In order to tie our results specifically to mGluR7-activated Kir3 channels on Purkinje cells, we continued with blocking the $GABA_BR$ with 50 µM CPG5548 (n = 2) and the CB1R with 80 µm AM251 (n = 3). As seen in Fig. 16G-H neither drug distinctly affected the conditioned response.

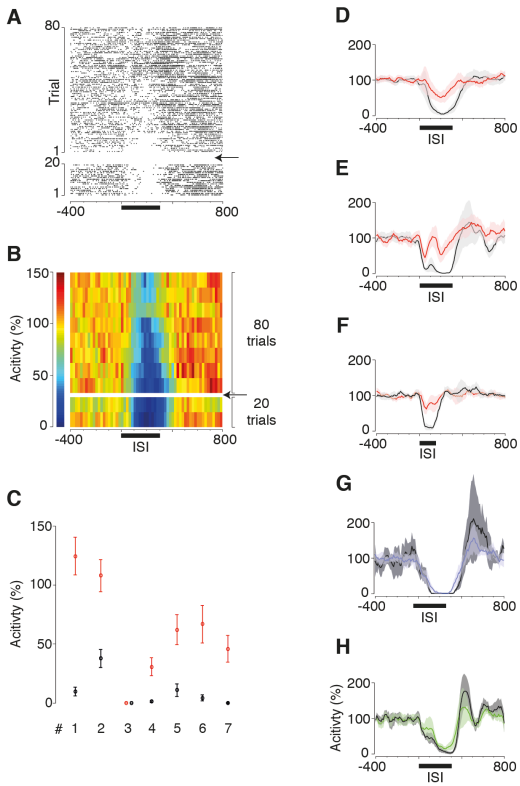

Figure 16. Kir3 supports conditioned Purkinje cell responses.
Arrows in ***A-B*** indicate injection. **A**. Raster plot of a Purkinje cell's responses to the conditional stimulus before (20 trials) and after (80 trials) injection of 5µM $Tertiapin_{LQ}$. **B**. Population average (n = 7) of Purkinje cell activity before and after injection of 5 µM $Tertiapin_{LQ}$. **C**. All Purkinje cell responses during the last 100 ms of the interstimulus interval in the same population as in ***B***, before (black) and after (red) injection of $Tertiapin_{LQ}$. ***D-F***. Averaged response profiles before (black) and after (red) injection of $Tertiapin_{LQ}$ (***D***, ISI 300 ms, 5 µM, n = 7; ***E***, ISI 300 ms, 200 µM, n = 5; ***F***, ISI 150 ms, 25 µM, n = 3). **G**. Averaged temporal response profile before (black) and after (blue) injection of 50 µm CGP5548 (n = 2). **H**. Averaged temporal response profile before (black) and after (green) injection of 80 µm AM251 (n = 3).

Discussion

The timing mechanism is intrinsic to the Purkinje cell

According to the view of learning that has dominated psychology and neuroscience for more than half a century (cf. Introduction) is that synapses undergo potentiation and depression such that neural networks rewire themselves so as to produce appropriate outputs. Memory takes the form of LTP and LTD distributed across synapses in the network. The learning and memory storage of temporal duration combines such Hebbian plasticity with hypothesized generation of time-varying patterns in the input pathway, which passively represent temporal duration. We show here that cerebellar Purkinje cells can learn to respond to a specific input with a temporal pattern of activity, consisting of temporally specific decreases and increases in firing. The Purkinje cell is capable of learning and storing temporal relationships between different stimuli. This marks a clear departure from the Hebbian learning paradigm thought to explain memory.

Most models assume temporal variation in the activity of granule cells arising from integration of mossy fiber excitation and Golgi cell inhibition. This integration produces vectors of activity peaks that generate a time code for the transmission of duration information to the Purkinje cell. In the present investigation no such mechanism is possible because we obtain direct experimental control of the pre-synaptic spike train by stimulating the parallel fibers directly, thereby bypassing the network. There can be no time code except for the regular repetition provided by the train of parallel fiber stimuli.

Here, the naïve Purkinje cell shows an immediate and sustained increase in firing as the conditional stimulus is presented. It lasts for as long the conditional stimulus is on. With training the Purkinje cell develops a distinct pause in firing that lasts as long as the interstimulus interval and resumes firing even if the conditional stimulus continues for hundreds of milliseconds beyond the interstimulus interval. In perfect agreement with our hypothesis, that the Purkinje cell itself learns the duration, it stores the temporal relationship between the onset of the conditional stimulus and the onset of the unconditional stimulus. At different interstimulus intervals, the cells learn pause responses timed to the specific interval. Importantly, the pause response is determined only by the interstimulus interval and is independent of the duration and frequency of the conditional stimulus. The cells respond with a pause timed to a trained, e.g., 200 ms

interstimulus interval, irrespective of whether the conditional stimulus used in training is 200 ms or 800 ms. After training, the Purkinje cell produces a response timed to the interval irrespective of whether we present 8 pulses at 400 Hz (17.5 ms), 31 pulses at 100 Hz (300 ms) or 81 pulses at 100 Hz (800 ms). The conditioned Purkinje cell response is clearly determined by the onset of the conditional stimulus and is insensitive to any temporally patterned input during the main part of the interstimulus interval and conditioned response (a temporal pattern we anyway experimentally annulled in this study).

We can pinpoint the learning to the Purkinje cell, as the response is resistant to pharmacological blockade of interneurons. This suggests that a glutamate trigger from parallel fibers activates a cellular mechanism that turns on a hyperpolarizing response with a specific learned time course. What molecular machinery is capable of such a sophisticated function?

The glutamate receptor experiments show that neither AMPA-kainate receptors nor mGluR1 are involved in the expression of conditioned responses. These receptors are excitatory and so were unlikely to cause the Purkinje cell suppression in firing. Their assumed role in learning is that AMPA-kainate receptors are internalized from the membrane as a structural substrate for long-term depression and the primary role of mGluR1 is thought to be the induction and maintenance of said long-term depression (Knöpfel & Grandes, 2002). The mGluR1 receptor had also been hypothesized to be able to suppress firing in some theoretical models (Fiala et al., 1996; Steuber & Willshaw, 2004) through delayed activation of calcium-activated K^+ channels. This has been challenged on theoretical grounds (Hesslow, Jirenhed, Rasmussen, & Johansson, 2013; Johansson & Hesslow, 2014; Yamazaki & Tanaka, 2009) and to our knowledge no such mGluR1 action has been observed. Here, we nevertheless controlled for this possibility by blocking all three K_{Ca} channels expressed by the Purkinje cell. Neither for AMPA-kainate receptor nor for mGluR1 nor for K_{Ca}, did blockade importantly affect the conditioned Purkinje cell response.

This leaves the mGluR7 as the only possible known glutamate receptor. Because it has been shown that it can mediate post-synaptic inhibition in other cell types through unknown mechanisms we suggested that it might activate the intrinsic temporal memory and trigger the conditioned Purkinje cell response. In striking contrast to all the other receptors tested, blocking mGluR7 consistently impaired and most of the time even completely abolished the pause response. Because mGluR7 can activate the protein-gated K^+ channel family Kir3, an expression mechanism is suggested. Activation of mGlur7 catalyzes the production of G-protein subunits $G\beta\gamma$ that activate Kir3 and leads to a voltage response.

That this could be the case is indicated by the fact that out of all four K^+ channels tested, the Kir3 family stands out as the most important contributor to the voltage response. These channels are constituted by tetramers of different combinations of subunits with different electrophysiological properties (Krapivinsky et al., 1995; Wischmeyer et al., 1997). In addition to the inherent different properties of these tetramers, Kir3 kinetics are regulated *in vivo* (Xie et al., 2010) over a wide span in the hundreds of milliseconds by the regulator of G protein signaling (RGS) family of proteins, Gα and some members of the Gβ family. The timing, amplitude and duration of Kir3-mediated inhibitory synaptic transmission could vary greatly in this context depending on which RGS proteins (more than 20 have been identified) associate with the channel complex. A plausible RGS-protein-dependent range of latencies to onset and offset of their active state has been established (Doupnik, Davidson, Lester, & Kofuji, 1997) and experience-dependent changes in RGS expression has been demonstrated after ~1h following electrical stimulation (Ingi et al., 1998). For example, association of Gβ5-RGS7 to Kir3 channels shape vision and some forms of motor control by ensuring timely inactivation of G-protein responses (Anderson, Posokhova, & Martemyanov, 2009). It is not impossible that what the learning mechanism in classical conditioning does is the following. Determined by the interstimulus interval, it selects for translation or activation the components among these regulatory protein families and/or mGluR7s and/or Kir3 subunits, those that bestow the suggested mGluR7-Kir3 signaling cascade with the appropriate kinetics that matches the interstimulus interval. Further discussion on a potential learning mechanism is found later in this thesis.

The standard models of conditioning rely on ionotropic glutamatergic and GABA-ergic receptors, depressed or potentiated with the assistance of mGluR1, CB1R or $GABA_BR$ signaling. The theoretical models by Fiala (Fiala et al., 1996) and Steuber (Steuber & Willshaw, 2004) rely on mGluR1-K_{Ca}1.1/2.2/3.1 signaling. The present results show that all of these components can be blocked without removing the conditioned response. In contrast, in our suggested mGluR7-Kir3 cascade, blocking either component individually diminishes or completely abolishes the response.

In addition to demonstrating the existence of memory of temporal duration in some intracellular Purkinje cell structure, these data considerably restrict the possible molecular mechanisms that underlie the timed Purkinje cell response. The results point to mGluR7-catalyzed production of Gβγ that activates Kir3. It is suggested that the regulation of timed responses takes place at the protein level within Purkinje cells, not in making synaptic connections stronger or weaker. The implication is that a learned and adjustable kinetic of a metabotropic signaling cascade is involved in the structural changes that mediate the physical memory of temporal duration.

Plausibility of alternative explanations

What are the limitations of these studies and the plausibility of alternative explanations? Virtually all neural timing models postulate that neurons learn to time their responses by altering the strength of synaptic connections for selected sub-populations of pre-synaptic neurons. In conditioning, the idea has been that the common mossy fiber input sets the granule cells going. They then exhibit activity peaks at different time steps, which come to represent the passage of time. The synapses active when the unconditional stimulus arrives undergo LTD. In this way, those granule cells that peak at the appropriate time gain control over the Purkinje cell output.

Here, this mechanism is bypassed. There is no common mossy fiber drive that sets the granule cells going. It could be argued that stimulation of parallel fibers caused antidromic activation of granule cells and that a temporal input pattern to the Purkinje cell could be generated via this route. However, this is extremely implausible. First, antidromic activation should have been seen in the recording in the form of a second simple spike a couple of milliseconds after the first one. Second, identical electrical stimuli were delivered to the parallel fibers at 100 Hz, up to 81 times. The immediate effect of such stimulation is certain to corrupt any temporal pattern generated by the granule cells. Recall that spurious activity in a few granule cells causes loss of the timing signal in those models. Third, because granule cells do not exhibit any delayed activity, no meaningful temporal summation, and no fast Golgi cell feedback connection, they could not generate a temporal pattern even if there was an undetectable antidromic activation of granule cells. Fourth, even if there was antidromic activation and even if granule cells despite all the evidence could generate temporal patterns, the unlikely implication is that three such different stimuli as 8 pulses at 400 Hz (17.5 ms), 31 pulses at 100 Hz (300 ms) and 81 pulses at 100 Hz (800 ms) all generated the same granule cell population activity vector. Particularly in the case of <20 ms stimuli, there would have to be a whole set of unknown anatomical connections and electrophysiological properties that enormously contradict what is known of granule cells and Golgi cells, in order for these pulses to antidromically propagate backwards and generate a time code that represents an interstimulus interval of, say, 150 ms presented to the Purkinje cell. This is extremely improbable.

Moreover, the contemporary STDP models assume that the learning mechanisms are LTD and LTP. A change in the balance of glutamatergic and GABA-ergic input to the Purkinje cells is what is stipulated to cause the voltage response. This can obviously not be the case since both AMPA-kainate and GABA receptors can be blocked without affecting the conditioned Purkinje cell response. Even if, for argument's sake, this was wrong, the latitude of the LTD and LTP windows assumed by theorists clearly make differential operation of LTD and LTP impossible in this situation. Here, the same presynaptic fibers are repeatedly stimulated. Our stimulation clamps the input from all stimulated fibers.

There are no sub-populations of fibers with activity peaks at different intervals. In this case the Purkinje cell does receive pf-cf input at any and all STDP intervals, *at the same synapses*. There can be no differential operation of LTD and LTP as needed by the models. Only one of the two could possibly operate. What would the necessary consequence of this be? If LTD is operative there would be an 800 ms long pause when the conditional stimulus is 81 pulses at 100 Hz. If LTP is operative, there would be tremendous excitation throughout the 800 ms. It is impossible to explain a dip in spiking locked to the previously experienced interstimulus interval followed by a return of firing above baseline. Either way, it seems to be a null point since neither AMPA-kainate nor GABA receptors contribute to the response. In summary, the likelihood that the conclusion that the memory of temporal duration is formed within Purkinje cells is wrong, and that the prevailing models are correct, is the product of so many improbable assumptions that they are difficult to count.

I turn now to the limitations that face the specific suggestion that the conditioned Purkinje cell response is regulated by an mGluR7-Kir3 signaling cascade. First, it is seen in the data that conditioned response is not always completely removed by antagonists of mGlur7 and Kir3. There are several probable explanations for this. The results with mGluR7 blockade are remarkably consistent but there are cases where the initial part of the conditioned response is not entirely removed. This resistance of the pause response to suppression in the early part of the interstimulus interval may relate to its dynamics. The lowest instantaneous firing rate occurs in the later part of the interstimulus interval. However, the rate-of-change of firing is greatest in the earlier part of the interstimulus interval. The mechanism that drives the suppression in Purkinje cell firing is therefore likely to be more potent in this early part, and so more resistant to the antagonists. In most cases, this appeared to be a dose issue.

Perhaps an even more likely explanation is that the small residual responses are due to the fact that neither MMPIP nor LY341495 at concentrations orders of magnitude larger than those used here completely block all mGluR7 effectors, in particular and not surprisingly, Kir3 channels (Niswender et al., 2008; Niswender et al., 2010). That the response is not completely removed (although in some cases it is) by the Kir3 antagonist used could be due to the fact that it is not known whether the drug can effectively block Kir3.2/3.3 heteromultimers. The binding domain of Kir3 antagonists is subunit specific and because the antagonist used blocks Kir3.1/3.3 and 3.1/3.2 its binding domain might reside in the Kir3.1 subunit. Another possibility is that Kir3 channels are not the sole effectors of the response.

Next, both mGluR7 (potentially) and Kir3 are expressed by other cell types than the Purkinje cell and so, the observed effect of blocking these could potentially be due to altered functions of these cells. In both cases, this is unlikely because the injections were

made only microns away from the dendritic tree of the Purkinje cell recorded from. The drugs do diffuse somewhat but this consideration places strong restrictions on alternative interpretations. The very local drug effects with this technique of drug delivery, micro pressure ejection near the tip of the recording electrode, is illustrated by the fact that neighboring Purkinje cells were often unaffected. For instance, in the AMPA-kainate experiments, injection of the drug could cause excitation to turn into inhibition because the parallel fiber stimulation could still excite interneurons, a short distance away, connected to the Purkinje cell. Nevertheless, the matter of whether the observed results are due to post-synaptic action of these drugs warrants discussion.

In many cell types, group III metabotropic glutamate receptors are believed to function as presynaptic autoreceptors that mediate feedback inhibition of glutamate release, probably via reduced Ca^{2+} entry into the nerve terminals (Millan, Lujan, Shigemoto, & Sanchez-Prieto, 2002). So, in principle, blocking mGluR7 might lead to increased glutamate release from parallel fibers and so counteract simple spike suppression during the conditioned pause. Five considerations argue strongly against this interpretation.

First, we are not aware of any studies that convincingly show that mGluR7 is expressed in parallel fiber terminals. Second, in vitro studies in rodents reveal that parallel fiber glutamate release is regulated by mGluR4 and not mGluR7 (Abitbol, Acher, & Daniel, 2008). Third, excitatory ionotropic glutamate receptors and mGluR1 are the other postsynaptic candidates for mediating a conditioned pause response consequent upon decreased glutamate release and both are now excluded by our findings here that blocking them had no significant effects on the learned pause. Fourth, there were no significant excitatory effects of mGluR7 block outside the learned pause period. Because we in these experiments used a standard duration for the conditional stimulus of 400 ms, it outlasted the interstimulus interval and the main part of the pause response by 200 ms in most cases. If the mGluR7 antagonists had acted to increase simple spike firing by increasing glutamate release, rather than by interfering with a specific mechanism for eliciting the pause responses, this should be reflected in an increased firing rate during the conditional stimulus presentation beyond the pause duration. This was clearly not the case. The simple spike firing level during the conditional stimulus presentation after the pause is not increased, rather there is some rate decrease. Fifth, with a peripheral conditional stimulus there is no marked excitation of the Purkinje cell in the naïve state. The instantaneous excitatory effect seems negligible. If the learning mechanism were LTD, this excitatory effect would be even weaker after training. Hence, it seems improbable that slightly increased glutamate release from the parallel fibers would cause the conditioned pause response to be removed upon pre-synaptic mGluR7 block. In summary, the findings do not support the suggestion that the mGluR7 antagonist effects on the conditioned pause depend upon enhanced glutamate release by presynaptic action. A post-synaptic action is strongly indicated.

Could the effects observed after Kir3 block be due to disturbed Kir3 function in other cell types in the circuit? Most likely it cannot. Kir3 channels are expressed in parallel fiber terminals and in interneurons where they are activated by CB1 and $GABA_B$ receptors (Aguado et al., 2008; Daniel et al., 2004; Fernandez-Alacid et al., 2009). If the disturbance of the conditioned Purkinje cell response after blockade of Kir3 channels was due to interference of their function at these sites, blocking their activator should have a similar effect. However, the preliminary data presented here shows that neither CB1R nor $GABA_BR$ antagonists have significant effects upon the conditioned response. This indicates that also the effects of Kir3 block are due to the effect it has on Purkinje cells, not on parallel fiber terminals or interneurons.

The lack of effect upon the conditioned response after blocking CB1Rs and $GABA_BRs$ is not surprising for the following reasons. The presumed effect of blocking these receptors is a slightly stronger conditional stimulus signal due to an increased probability of glutamate release from parallel fibers (Daniel et al., 2004; Fernandez-Alacid et al., 2009) in the first case. However, the conditioned response is clearly not very sensitive to changes in the conditional stimulus properties. In the second case, a slightly increased spontaneous firing rate of molecular layer interneurons is expected (Kreitzer, Carter, & Regehr, 2002). This should not diminish the conditioned Purkinje cell response either. Further, the majority of $GABA_B$ receptors are expressed post-synaptically at excitatory pf-PC synapses (Tabata & Kano, 2010) where they are activated by spillover from inhibitory synapses. Activation of $GABA_BR$ keeps the spontaneous firing rate down but does not shape Purkinje cell response patterns (Dizon & Khodakhah, 2011). The receptor also facilitates mGluR1 signaling (Hirono, Yoshioka, & Konishi, 2001) but mGluR1 is not needed for expression of the conditioned Purkinje cell response.

In our view, the data that indicates the existence of intrinsic memory of temporal duration within the Purkinje cell is very strong. Alternative explanations of the data are highly improbable. As for the hypothesis of the specific nature of the molecular machinery that underlies the remarkable temporal precision of Purkinje cell response, we believe that the mGlur7-Kir3 cascade hypothesis is promising and warrants further investigation.

What is the molecular learning mechanism?

The data presented and the theoretical considerations that have accompanied it invite speculation into how an intracellular learning mechanism could implement this peculiar form of neural signaling and memory. After training, the neuron receives a trigger signal that has acquired the properties to induce cessation of firing with a slightly delayed onset, pronounced and accurately timed maximum and critically timed offset. It has learned the temporal relationship between two input sources and acquired the ability to cease firing with a duration proportional to the learned interval. It has stored a memory of temporal duration. How can a neuron learn to respond with a particular time course in the range of hundreds of milliseconds between cell surface receptor activation and voltage response?

Cellular timing mechanisms

It seems two cellular functions are needed. First we need a mechanism for measuring or recording the time interval between the two inputs (the first in a series of parallel fiber inputs and the climbing fiber input). This is the recorder mechanism. Second, we need a mechanism to generate the timed voltage response itself. This is the "effector mechanism". A single mechanism that learns that contextual input X implies that a voltage response of a certain kind is needed cannot explain the full story. The response would be immediate and repetitive for each presentation of the input, which in our conditioning protocol is every 10^{th} or 20^{th} millisecond. The results would be continued firing or lack thereof as long as the conditional stimulus is present. The "recorder mechanism" is necessitated from a logical standpoint. If the temporal duration between the two inputs is not recorded or stored in some way, each presentation of the temporal interval counts as the first presentation of the interval. This would not be a problem if the Hebbian explanation for the phenomenon were true. Then each CS-US presentation would induce a little bit more of LTD or LTP for each presentation. However, since that is not the case, this recorder mechanism is obligatory.

Interpreting and adapting the cellular timing theories that exist (Fiala et al., 1996; Steuber & Willshaw, 2004) for this context, the first mechanism, the recorder, involves some cumulative biochemical process that is terminated by the unconditional stimulus. This is similar to the pulse-accumulator theory of timing explained in the introduction. There could be a scenario in which neurotransmitter released by the pre-synaptic terminals leads to a gradual build-up in the concentration of a given ion or in the concentration of a second messenger molecule. This build-up would eventually reach some threshold value. The second mechanism, the effector mechanism, could be some phenomenon that causes this accumulation to acquire the ability to render a voltage response that silences the cell.

If the relevant trigger receptor is coupled to a rise in the concentration of a substance *x* that can acquire the ability to elicit a voltage response, the time delay between receptor activation and voltage response will depend upon the number of receptors that are activated. If there is an x-dependent feedback connection that adjusts the number of available receptors, the neuron can learn to adjust the delay.

The most plausible version of such models (Steuber & Willshaw, 2004) suggests that the key mechanism is calcium-dependent phosphorylation of receptors and ion channels (in biology phosphorylation is most often synonymous with activation). Such regulation could in theory be a plausible way to implement adjustable response kinetics. Activation of many different receptors leads to an intracellular increase in $[Ca^{2+}]$ (the concentration). The time course of this increase can in theory range over hundreds of milliseconds depending upon the number of neurotransmitter receptors and second messengers that are available for activation, the number of steps between receptor activation and a rise in $[Ca^{2+}]$, as well as the rate constants of these different steps. Fewer available receptors would cause the build-up in $[Ca^{2+}]$ to be slower and more available receptors would cause it to be faster. Any delay to a threshold level at which this $[Ca^{2+}]$ would cause a response could then be learned if two antagonistic biochemical processes control the number of receptors that are available for activation and hence the rate of rise in Ca^{2+} concentration.

The suggested mechanism in this model is that in the naïve state Ca^{2+} influx caused by the conditional stimulus leads to PKC synthesis and depolarization because most Ca^{2+}-activated hyperpolarizing channels are inactivated. During training, the unconditional stimulus evokes PKG synthesis, which decreases the number of available receptors by de-activating them. This causes a slower rise in $[Ca^{2+}]$. Because the conditional stimulus also evokes PKC synthesis, there is a Ca^{2+}/PKC peak that moves towards the unconditional stimulus evoked PKG peak. When they overlap, equilibrium in the number of available receptors is reached and the latency of the $[Ca^{2+}]$ rise matches the interstimulus interval. Further, coincident PKC and PKG activity is hypothesized to phosphorylate and activate Ca^{2+}-activated K^+ channels. The conditional stimulus response is transformed from an excitatory response into a hyperpolarization response around the anticipated onset of the unconditional stimulus.

This and any model that depends on an adjustable concentration rise encounters several challenges. First, because each conditional stimulus pulse produces an increase in intracellular [X], the mechanism will be highly sensitive to the parameters of the conditional stimulus. Eight pulses at 400 Hz and eighty-one pulses at 100 Hz could not produce the same time course of an increase in [X]. Second, very much for the same reason it is not possible to account for double-peaked responses. A single synapse with a single direct delay adaptation mechanism can never learn more than one time delay at a time, as the authors of the model used as an example here themselves conclude (Steuber

& Willshaw, 2004). Third, learning at intervals shorter than 100 ms is predicted, but as we know is not possible.

A mechanism based on recorder proteins and timer units

We suggest an alternative conceptual framework for how an intracellular mechanism capable of adaptive timing could work. The central tenet is that the learning mechanism is one of interstimulus interval-dependent selection of different components for translation or activation. Let us imagine the existence of what we can name *timer units* that provide the components of the response expression machinery with distinct temporal activation profiles. The learning process would select, among a finite number of such timer units, a combination that matches the temporal interval.

As an alternative to the standard idea in both the above model and in STDP of Ca^{2+} currents (or some other current) being responsible for tracking the time passed since input onset, we suggest the following. The conditional stimulus onset triggers a cascade of second messenger molecules, a series of some unknown molecular switches, or proteins to start changing their conformation over time. The logic of the concept does not require specification of either one so let it be a family of proteins changing their conformation over time. We can call these the recorder proteins.

At input onset, the recorder proteins start to change in a predictable way. We assume four possible conformational states: '-' (null or inactive), *A*, *B* and *C*. Suppose that for the first 100 ms, all are in the '-' state. Between, say, 100-250 ms most of them are in the *A* state, between 250-350 ms most are in the *B* state and between 300-400 ms in the *C* state. Assume further that the recorder proteins interact with the unconditional stimulus signal dependent on in which conformational state they are in, i.e. dependent on how much time has passed since conditional stimulus onset. When they are in the '-' state there is no effect. However, when they are in the *A*, *B*, or *C* states, different activation sites are available for the unconditional stimulus to interact with. This activation may then cause the translation or activation of particular timer units (the components which bestow the signaling cascade from receptor activation to voltage response with the appropriate latencies). In this way, the recorder proteins function as molecular switches. Note that, due to the combinatorial nature of the components, one would need neither many different states of the recorder proteins nor a large number of timer units to select among in order to learn many different temporal intervals.

Suppose that training with an interstimulus interval of 150 ms cause the recorder proteins to only become activated in the *A* state, which translates/activates the pool of timer units *A*A*A*A**. Training with an interstimulus interval of 215 ms might lead to a pool of

A*A*B*B* and training with an interstimulus interval of 400 ms might lead to *C*C*C*C**. This process could produce response maxima around, say, 150, 200 and 350 milliseconds. Such a mechanism could explain more of the experimental data. (i) There is no learning at less than 100 ms or so because all the recorder proteins are in the '-' state and no timer units are chosen. (ii) There is no reason why a Purkinje cell could not produce multiple responses. If it is alternately trained with interstimulus intervals of 150 ms and 400 ms, every other trial will result in the recorder proteins selecting timer units *A*A*A*A** and *C*C*C*C** respectively. Eventually two responses will appear. Recorder proteins are never activated in the *B* state. (iii) The ability of a very short conditional stimulus to elicit the full response becomes less of a problem. After learning, once the conditional stimulus sets the machinery going it runs its course with a particular delay. Onset, duration and offset of the response will be the same regardless of variations in the conditional stimulus parameters. The signaling cascade from activation of neurotransmitter receptor to the voltage response generator need not be critically affected by variations in the incoming spike-train if the first pulse(s) trigger the machinery in analogy to lighting a bomb fuse.

Induction of a learned timed response based on mGluR7-Kir3 signaling

From the data presented in this thesis it appears that mGluR7 is the trigger receptor and that Kir3 could be the ion channel that causes the voltage response. As far as we are aware, Kir3 is the only ion channel family where it is known that the latency to activation and de-activation can be varied over large time spans by regulatory proteins. The timing and amplitude of Kir3 activity depends on the inherent different properties of their subunit composition and their association with regulatory G proteins and members of the regulator of G protein signaling (RGS) family. Albeit speculative, these components seem to fit the criteria of timer units that could be selected by recorder proteins.

Take vision as an example for how RGS proteins can regulate the duration of G protein signaling. High temporal resolution in vision is critically dependent on photoreceptors returning to the resting state with an average time constant of 200 ms (not 100 or 300 ms). The association of RGS9-1 to the photoreceptors accomplishes this temporal precision. With a two-fold increase over wild-type photoreceptors it is ~115 ms (Krispel et al., 2006). An example specific for Kir3 channels is that in hippocampal neurons association of Gβ5-RGS7 to Kir3 channels cuts the latency to activation by 120 ms or so (Xie et al., 2010). The function of both Kir3 channels and in particular the function of all of these regulatory proteins (>20 different identified) is far too unexplored at present time to devise a specific model, but as an illustration of the principle we can imagine the following scenario.

In the naïve Purkinje cell, there is either no response to the conditional stimulus or there is an increase in firing. As opposed to rapid LTD and LTP processes, the time it takes to induce conditioned responses implies that protein synthesis is needed. This could mean that any or all of the components in the signaling cascade that generates the response need to be produced: components of the triggering receptor, timer units selected by the recorder proteins (regulatory molecules that adapts the time course of Kir3 activity), or the Kir3 channel components that generate the voltage response themselves. In the simplest scenario, the lack of a response before training could for instance be due to something similar to the binary function of RGS4 in other cell types, which at low levels inhibits activation of Kir3 and at high levels stimulates it (Keren-Raifman et al., 2001). In these cell types it also bestows the Kir3 activation-deactivation cycle with a particular time course. In this case the same protein accomplishes both tasks of setting the channels to a mode where they can be activated by metabotropic receptors *and* bestows the signaling cycle with a particular time course. Kir3 channel activity could also be insignificant in the naïve state because other G-proteins than those catalyzed by mGluR7 inhibit the Kir3 channels.

The Purkinje cell learning mechanism would hence need to accomplish two things: (i) cause the expression of Kir3 channels, or change the gating of existing Kir3 channels to a mode where they can be more readily activated by mGluR7, and (ii) bestow the signaling cascade with the appropriate time course. In the simplest scenario, translation/activation of particular RGS proteins (selected by the unknown recorder proteins dependent on their conformation at US-onset) both changes the Kir3 gating and adapts their time course of activation-deactivation. Recall that what is here called a recorder protein is a proxy for any thermodynamically stable molecule with switch-like properties. After learning, the trigger signal (glutamate via mGluR7) lights the "fuse" that is the intracellular signaling cascade that generates the voltage response. The expression mechanism is insensitive to whether the conditional stimulus is brief or long, because once activated the early substrate molecules in the machinery need to be replenished before a new trigger can initiate a response.

It should be pointed out that even though RGS-induction has been observed *in vivo*, no attempt has been made to measure experience-driven adaptive changes in the time course of Kir3 activity. However, the need to has never presented itself. The true story is near certain to be more complicated, in particular because RGS proteins mainly regulate duration and time to offset, and only to a lesser extent time to onset. Although the time to onset of Kir3 activity following metabotropic receptor activation is adjustable, the regulating function of this aspect is to date unknown. Either way, this consideration implies that something also needs to change with the mGluR7 receptors and/or the interactions of Gβγ (the mGluR7 signal which activates Kir3) with its antagonistic G

proteins. Note however in study I of this thesis that time to onset is the least adaptable parameter of the time course of the response.

Persistence: extinction and savings

The rapid re-acquisition of conditioned responses suggests that the same likely process of *de novo* protein synthesis undertaken at the time of original learning does not need to take place with re-learning. This implies that what is usually referred to as memory extinction in classical conditioning, the disappearance of conditioned responses after persistent presentations of the conditional stimulus without the unconditional stimulus, more likely involves a secondary mechanism that masks or overshadows the expression machinery of the voltage response, not extinction of the memory itself. This means that extinction in classical conditioning involves more of retrieval failure than forgetting. It is much more efficient to temporarily mask a memory currently not needed than to extinguish the full memory. Consistent with this notion is the behavioral observation that while re-acquisition is fast and becomes progressively faster with successive cycles of acquisition and extinction, successive extinctions are of essentially the same rate (Kehoe, 2006). Also, the learned duration of the conditioned response does not change with extinction and re-acquisition (in the current models the duration is expected to contract and expand), it is the amplitude of the response that changes (Kehoe, Ludvig, & Sutton, 2014), consistent with a secondary and separate masking mechanism involved in so called extinction. The memory of the duration is not forgotten. This is relevant in the context of this speculative model because it implies that the process of learning to express timer units is not reversed with an extinction protocol. In contrast to LTD models, where LTP could reverse the learning, our model is explicitly not even capable of actively reversing it. There is no fifth conformational state in our recorder proteins which signals to reverse the learning. This leads to the predictions that a separate masking mechanism should be identifiable and that even in the "extinguished" state, the timer units should be observable. As opposed to the LTP and LTD models where memory is saved in synaptic strength changes, which do not persist over time and cannot explain more rapid re-acquisition, the phenomenon of memory persistence could in this model take the form of timer unit proteins, and/or for instance their mRNA strands, at rest within the cytoplasm. Of course, neither of these components persist indefinitely either, so there has to be some additional molecular change that lasts longer.

Non-trivial predictions

This model, albeit necessarily vague due to the novelty of the phenomenon, conforms to most aspects of what is observed of the Purkinje cell behavior. In addition, the following non-trivial predictions are derived from it.

(i) In contrast to the LTD/LTP models where each paired presentation renders a little bit more of one or the other until a sufficient number of pairings produce the desired response, this model is not based on a large number of repetitions. It is predicted that the reason why conditioning takes time is due to translation or activation of the timer units, not that a large number of pairings is needed. This means that it should be possible to condition Purkinje cells with either or both of long intertrial intervals or a short block of normally spaced pairings followed by time at rest for the molecular changes to take place, without increasing the total time needed for learning.

(ii) After training with different interstimulus intervals, including after an extinction protocol has masked expression of the response, different timer unit proteins and/or mRNA should be measurable in the Purkinje cell. Note that while RGS and G proteins are the most likely candidates we are aware of, the general model is not obliged to this being the case.

(iii) A separate mechanism for masking of conditioned responses should be identifiable. This could involve separate machinery, which reversibly inactivates or overpowers the triggering mGluR7 receptor or the Kir3 channels while leaving the time-adapted mGluR7-Kir3 signaling cascade as such mostly intact.

(iv) Due to the nature of the expression mechanism, exemplified by the fuse analogy, there should be a minimum intertrial interval with which conditioned responses can be expressed, which should increase for progressively longer interstimulus intervals. This is derived from the fact that Kir3 current deactivation is determined by the reaction rate for $G\beta\gamma$ sequestration. The longer that $G\beta\gamma$ keeps the Kir3 channel open, the longer before it can re-associate into the inactive $G\alpha\beta\gamma$ heterotrimer and again be able to trigger a response. A similar phenomenon would be true even if some other channel than Kir3 turns out to be the effector of the conditioned response. In the standard LTD/LTP models there is no reason for a refractory period to be dependent on the interstimulus interval in this way.

Conclusion

In our experiments, the stored information of temporal duration is expressed in the complex temporal response of the Purkinje cell to the onset of a conditional stimulus delivered to its immediate afferents. The circumstances are such that the temporal information cannot be in the activating pre-synaptic spike train or in the synaptic conductances between parallel fiber and Purkinje cell.

The conditioning paradigm with parallel and climbing fiber stimulation causes an enormous change in the input-output characteristics of the Purkinje cell. In the extreme case the naïve cell responds to parallel fiber stimulation with spiking at 200 % of pre-trial baseline. After training it often goes completely silent at the time it has learned to pause. The onset, maximum, offset (and duration) of the learned response increases in proportion to the duration of the interstimulus interval used in training. These characteristics are dependent on the interstimulus interval alone. Despite vast changes in the duration and frequency of the input signal train, the timing of the pause does not change. After training, the input delivered directly to its immediate afferents can be varied over more than an order of magnitude but the response remains essentially the same. The timing of the response clearly does not depend on a temporal input pattern. The memory is not in the synapses, i.e. in the mechanisms or transmitter release or post-synaptic binding thereof. It is within the post-synaptic neuron itself. An individual neuron can memorize the time interval between two incoming stimuli.

In stark contrast to prevailing theory, these results pinpoint the locus of the learned timing to the Purkinje cell. This changes our views on the nature of neural signaling and memory. The storage of information, the physical change in nervous tissue, in this case resides in some intracellular structure. Purkinje cells are capable of intrinsic memory of temporal duration, which is far more complex than learning to regulate excitability. After training, the onset of a simple input signal appears to trigger adequately configured molecular machinery capable of converting a trigger signal into a temporally complex output signal. In other words, the trigger causes some form of read-out of an intracellular memory of what the temporal duration during training was, and starts intracellular machinery capable of converting the stored information into a complex output signal.

As illustrated in figure 17, the notion that individual neurons can memorize time intervals is a clear departure from the traditional view of learning and memory as changes in the efficacy of excitation and inhibition. Not only this, we believe that it demonstrates a new principle of neural signaling. Neurons can signal to other neurons not just by increasing or decreasing the target neuron's firing rate, but also by sending a complex timing signal. Importantly, this implies that the capacity for information storage in the brain is many times larger than previously realized.

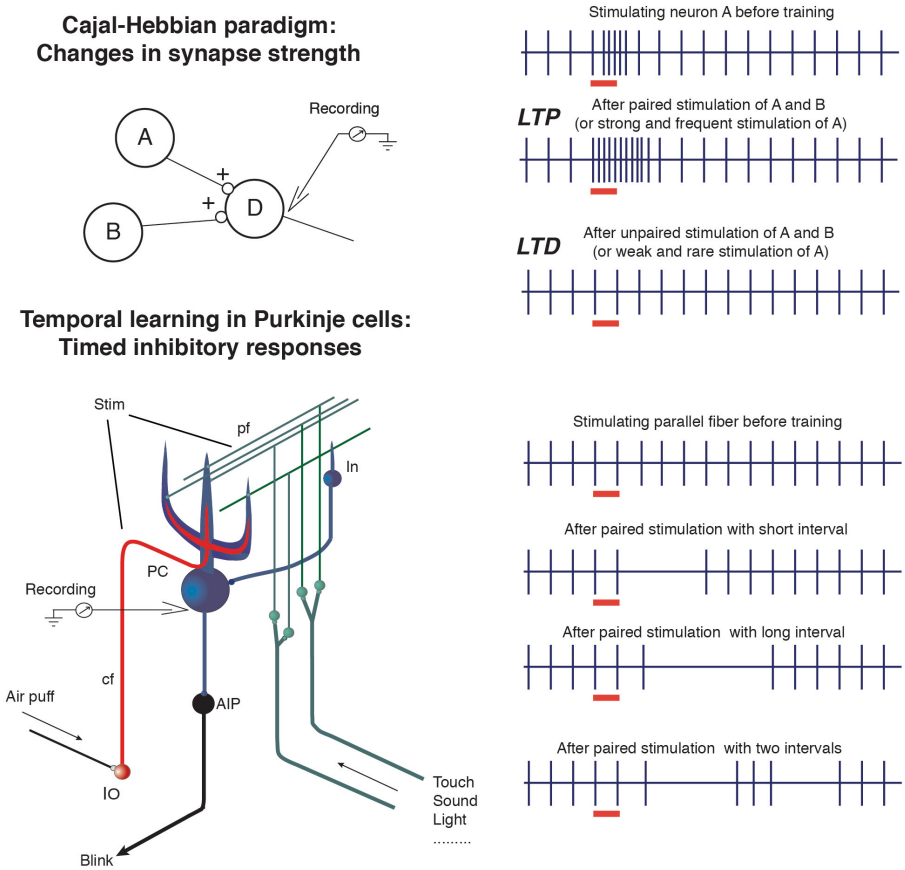

Figure 17. A new kind of learning in the nervous system.

The present studies further demonstrate that the mGlu7 receptor likely serves as the trigger signal in this particular case of classically conditioned temporal memories. Further, the timed voltage response appears to be produced by the protein-activated K^+ channel family Kir3. This considerably restricts the possibilities for how the intracellular molecular machinery might work. It seems likely that the learning mechanism selects for translation or activation those regulatory proteins that bestow the signaling cascade with latencies to activation and de-activation that match the temporal duration of the interstimulus interval during training. This set of investigations has also produced non-trivial and testable predictions for the future.

Finally, because Purkinje cells directly control the conditioned eyeblink we believe that, to our knowledge, this is the first time that a causal link can be shown between a learned and timing-dependent behavior and not only a single neuron's memory, but also the specific activating receptor of said memory and the specific ion channel that puts it into effect.

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Populärvetenskaplig sammanfattning på svenska

Standardsynen på hur signalering mellan nervceller fungerar är att en nervcell påverkar sin mottagarcell genom att öka eller minska mottagarcellens aktivitet. Inlärning och minne tros bestå i att kontakterna mellan nervcellerna förstärks eller försvagas så att effektiveten i hur mycket den signalerande cellen påverkar mottagarcellen ökar eller minskar. Genom att vikta tusentals olika kontakters styrka i stora nätverk av nervceller tros nätverket förändras på ett sådant vis att samma indata efter inlärning och bildandet av minnen leder till modifierad utdata. I den här avhandlingen visas dock att dessa minnesmekanismer inte är tillräckliga och därmed att gängse teorier är felaktiga. Kortfattat innebär resultaten att en ny inlärningsmekanism för enskilda nervceller har upptäckts.

Det förelagda problemet för detta arbete var att undersöka hur precis tidsinställning av hjärnans reaktioner är möjlig. För många beteenden måste hjärnan lära sig tidsrelationer mellan händelser i yttervärlden och lära sig att utföra kommandon perfekt koordinerade i tid. Ett begripligt tal är bara möjligt att förstå och producera genom tidsinställda pauser mellan olika språkljud. Förståelsen av orsakssamband härrör från förmågan att bedöma tidsrelationen mellan A och B. Att vicka en kaffekopp precis så att kaffet hamnar i munnen och inte i famnen beror på precis sekvensering av motoriska kommandon längs en tidsaxel.

Inlärning av tidsrelationer kan studeras med hjälp av så kallad klassisk betingning. Principen kommer från Ivan Pavlov som i början av 1900-talet visade att hundar kan lära sig att koppla ihop ett visst ljud med att de skulle få mat. Så småningom började de drägla när de hörde ljudet, precis innan maten kom. På samma vis kan man även ”betinga” reflexer. En vanlig forskningsmodell idag är att lära djur och människor att koppla samman en ton med en luftpuff riktad mot ögat som utlöser en blinkningsreflex. Så småningom lär sig djuret eller människan att blinka som en reaktion på tonen. Det intressanta fenomenet är att tidsintervallet mellan ton och luftpuff bestämmer precis när blinkningen kommer. Oavsett hur långt intervallet är kommer blinkningen alltid precis innan man lärt sig att luftpuffen kommer.

Tidigare resultat från bland annat vårt laboratorium visar att blinkningen styrs av så kallade Purkinjeceller i lillhjärnan. Dessa celler är spontant aktiva och skickar omkring 50 nervimpulser i sekunden som i utgångsläget blockerar en signalväg till muskler som styr ögonlocken. Som ett resultat av upprepade parningar av ton och luftpuff förändras Purkinjecellens aktivitet så att en paus uppstår i det annars kontinuerliga skickandet av blockerande nervimpulser. Om tiden mellan ljud och luftpuff är, säg, 200 ms, 400 ms eller 600 ms uppstår pausen i Purkinjecellens fyrning på ett tidsinställt vis så att djuret alltid blinkar vid precis rätt tillfälle. Det är dock inte känt hur denna inlärning går till och hur minnet lagras.

Gängse teorier försöker att förklara detta på följande vis. Tolkningen har varit att det finns två problem som måste lösas av hjärnan. Dels måste styrkan av kontakterna mellan Purkinjeceller och de celler som skickar information om tonen förändras på ett sådant vis att Purkinjecellerna tystnar (en försvagning av kontakter som i utgångsläget gör att Purkinjecellens aktivitet ökar). Dels måste tidsintervallet på något vis mätas och mätningen måste lagras i hjärnan. De vanligaste idéerna bygger på spekulationer i att en tidskod genereras av det nätverk utav nervceller som skickar information om tonen. På grund av tänkta variationer i egenskaperna bland dessa celler och att informationen om tonen studsar runt genom en serie återkopplingar tänker man sig att varje unik tidpunkt representeras av att en unik grupp celler i nätverket råkar vara aktiv. Tidsflödet mäts inte explicit utan representeras passivt per automatik tack vare hur man spekulerar att nätverket fungerar. När luftpuffen kommer förstärks eller försvagas kontakter mellan de nervceller som råkar vara aktiva vid tillfället. På så vis genereras en tidskod. Det bör påpekas att detta har varit just spekulationer. Det finns inte några observationer som stödjer en sådan tidsmekanism.

Här visar vi dock att Purkinjeceller kan lära sig att tidsinställa sina reaktioner utan att den får en tidskod från nätverket. De mekanismer man har trott ligger bakom är i vår experimentella uppställning inte möjliga då vi kortsluter nätverkskretsen genom att byta ut en riktig ton mot att elektriskt stimulera de nervfibrer som leder direkt till Purkinjecellen. Ingen tidskod är möjlig. Innan träning svarar Purkinjecellen på stimuleringen omedelbart med en ökning i aktivitet som varar lika länge som "tonen" presenteras. Med träning utvecklar cellen en tydlig paus som varar precis så långt som intervallet mellan ton och luftpuff har varit och fortsätter sedan att fyra så lång tid som återstår av tonen. Latenstid till start och avslut av pausen (durationen) ökar proportionerligt med hur långt tidsintervallet som används vid träningen är. Med andra ord lär sig cellen att avbryta sin spontana aktivitet precis vid rätt tillfälle. Efter träning kan "tonens" längd varieras i mycket stor utsträckning men ändå svarar cellen bara precis vid den tidpunkt som den har lärt sig att luftpuffen brukar komma. Tidsinställningen av Purkinjecellens reaktion kan därför inte bero på att det finns en tidskod som levereras till cellen. Istället lär sig Purkinjecellen själv tidsrelationen mellan ton och luftpuff. Den

lagrar ett minne av durationen för att kunna tidsinställa sin signalering när den detekterar tonens början.

Vi visar att inlärningen sker inuti just Purkinjecellen genom att elektriskt aktivera just de nervfibrer som leder direkt dit och samtidigt blockera andra nervceller med särskilt utvecklade läkemedel. Upptäckten att enskilda nervceller kan memorera tidsintervall utgör ett tydligt avsteg från dagens teorier om inlärning och minne som säger att mekanismerna utgörs av förstärkning eller försvagning av kontakter. Detta förändrar radikalt vad vi tror om hjärnans funktioner.

Resultaten demonstrerar en ny princip för signalering. Nervceller kan inte bara öka eller minska mottagarcellers aktivitet utan även skicka komplicerad tidsinformation. Detta innebär en drastisk ökning av hjärnans inlärningskapacitet och en drastisk effektivisering av dess energiförbrukning. Information kan lagras i molekylära strukturer inuti nervceller, vilket tillåter mycket mer komplex informationshantering jämfört med att bara kunna reglera hur enkelt det är att öka eller minska en cells aktivitet. Det som upptäckts här är en slags klockfunktion där nervcellen mäter och lagrar tidsduration. Efter inlärning orsakar en enkel startsignal aktivering av minnet (utläsning av den lagrade informationen som säger hur långt tidsintervallet var) och startar ett intracellulärt maskineri som omvandlar minnet till en komplex utsignal.

De resultat som presenteras i den här avhandlingen visar att den gängse synen på hur inlärning och minne fungerar inte är tillräcklig. Utöver att påvisa existensen av en helt ny inlärningsmekanism och cellulärt minne, innehåller denna avhandling även resultat som avsevärt begränsar vad de möjliga molekylära mekanismerna inuti cellen kan vara. Vi har identifierat både de nödvändiga receptorerna på cellens yta som registrerar signalen (tonen) och de jonkanaler som behövs för att utföra cellens beteende. Tidsinställning av reaktioner tycks bestämmas på protein-nivå inuti Purkinjeceller, inte av förändrad styrka i nervcellers kontakter. I princip alla modeller som beskriver hjärnan beskrivs celler som enkla enheter som enbart summerar inkommande signaler i realtid. Detta arbete visar att denna doktrin är felaktig. En nervcell är en mycket mer komplicerad entitet än någon tidigare har anat.

De celler vi studerar styr blinkreflexer men det finns många celler av samma typ som styr helt andra saker. Dessutom är det troligt att andra celltyper på flera olika platser i hjärnan har liknande mekanismer då tidsinställning är precis lika viktigt där. Dessa upptäckter kan i framtiden få tillämpningar för de tillstånd hos människor som tros bero på felaktig funktion av lillhjärnan eller förmågan att tidsinställa nervcellers signalering. Språkstörningar, autism, ADHD och rehabilitering efter en hjärnblödning (stroke) kan komma i fråga.

Avslutningsvis, på grund av att de Purkinjeceller vi studerar kontrollerar ett specifikt beteende är detta första gången som ett orsakssamband kan påvisas mellan ett inlärt och tidsinställt overt beteende och inte bara (i) en enskild nervcells minne men även (ii) den specifika receptor som aktiverar nervcellens minne och den specifika jonkanal som utför det som minnet programmerar.

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Paper I

Memory trace and timing mechanism localized to cerebellar Purkinje cells

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The standard view of the mechanisms underlying learning is that they involve strengthening or weakening synaptic connections. Learned response timing is thought to combine such plasticity with temporally patterned inputs to the neuron. We show here that a cerebellar Purkinje cell in a ferret can learn to respond to a specific input with a temporal pattern of activity consisting of temporally specific increases and decreases in firing over hundreds of milliseconds without a temporally patterned input. Training Purkinje cells with direct stimulation of immediate afferents, the parallel fibers, and pharmacological blocking of interneurons shows that the timing mechanism is intrinsic to the cell itself. Purkinje cells can learn to respond not only with increased or decreased firing but also with an adaptively timed activity pattern.

cerebellum | eyeblink conditioning | temporal control | glutamate transmission

Timing is an integral aspect of all movements, from tilting a coffee cup to pressing a piano key. Fine motor timing involves the cerebellum (1), as illustrated by eyeblink conditioning. If a neutral conditional stimulus is followed repeatedly at a fixed temporal interval (an interstimulus interval) by an unconditional blink-eliciting stimulus, the conditional stimulus acquires the ability to elicit a blink that will be timed to occur just before the unconditional stimulus. If the interstimulus interval is increased or decreased, the timing of the conditioned response will change accordingly after additional training (2). The cerebellar cortex is necessary for the generation of such timed conditioned responses (3, 4). The conditional stimulus is transmitted to the cerebellar cortex by the mossy and parallel fiber system, and the unconditional blink-eliciting stimulus is transmitted by the climbing fibers (5). During conditioning, tonically active Purkinje cells in a blink-controlling area of the cerebellar cortex acquire learned pauses in firing. These pauses, conditioned Purkinje cell responses, cause disinhibition of the cerebellar nuclei and thereby generate the overt conditioned response (6, 7). The conditioned Purkinje cell responses share a number of features with the overt conditioned blink responses. For instance, they are extinguished by unpaired presentations of conditional and unconditional stimuli and show savings on retraining after extinction (7), they are adaptively timed (8), their latencies respond to changes in stimulus parameters in the same way (9, 10), and they are not acquired with interstimulus intervals below about 100 ms (11).

In accordance with current views on learning, long-term depression of synapses between parallel fibers and Purkinje cells usually is considered to be the mechanism underlying conditioning. Strengthening or weakening of synapses alone cannot explain the timing of neural responses, however (12). Therefore the timing of conditioned Purkinje cell responses generally is believed to depend on a temporal code carried by the parallel fibers. If different parallel fiber afferents are active at different times during the interstimulus interval, and Purkinje cells could learn to respond differentially to particular parallel fibers, timing would follow automatically (1, 13).

The purpose of the present work was to determine if the timing of the conditioned Purkinje cell response depends on such a temporally patterned input. We show that it does not do so. Parallel fibers make synaptic contacts with Purkinje cells and cerebellar cortical interneurons without any intermediate synapses. By using direct stimulation of parallel fibers as the conditional stimulus, we can bypass any delays in the conditional stimulus signal to the Purkinje cells and ensure that no time code in the parallel fiber signal is possible. Nonetheless, we observed the acquisition of conditioned Purkinje cell responses, adaptively timed to a range of different interstimulus intervals from 150–300 ms.

Results

We first made extracellular recordings from 23 Purkinje cells in 19 decerebrate male ferrets, while using direct electrical stimulation (50 or 100 Hz) of parallel fibers as the conditional stimulus and stimulus of climbing fibers (500 Hz) as a proxy for the unconditional blink-eliciting stimulus (Fig. 1).

We monitored activity of Purkinje cells in an area in the C3 zone that controls the conditioned blink response (14, 15) for several hours during training to three different interstimulus intervals (150, 200, and 300 ms). Longer intervals were not studied because learning would be very much slower and difficult to obtain in the time span available in the decerebrate preparation.

In eight cells, the conditional stimulus coterminated with the unconditional stimulus, and in 15 cells the duration of the conditional stimulus outlasted the interstimulus interval by 150–600 ms. In the standard conditioning protocols, the conditional stimulus is terminated at the time of the unconditional stimulus. The fact that the conditioned Purkinje cell response ends at that time simply might reflect the termination of the conditional stimulus. By using long conditional stimuli, we can distinguish response features that are intrinsic to the conditioned response,

Significance

The standard view of neural signaling is that a neuron can influence its target cell by exciting or inhibiting it. An important aspect of the standard view is that learning consists of changing the efficacy of synapses, either strengthening (long-term potentiation) or weakening (long-term depression) them. In studying how cerebellar Purkinje cells change their responsiveness to a stimulus during learning of conditioned responses, we have found that these cells can learn the temporal relationship between two paired stimuli. The cells learn to respond at a particular time that reflects the time between the stimuli. This finding radically changes current views of both neural signaling and learning.

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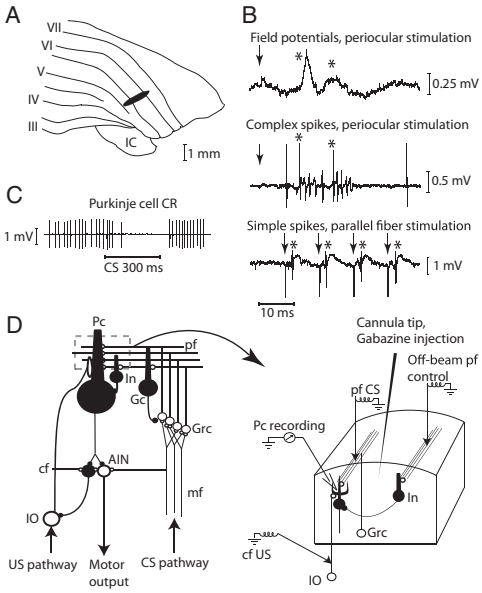

Fig. 1. Experimental setup. (*A*) Blink-controlling area in cerebellar cortex. IC, inferior colliculus; Roman numerals indicate cerebellar lobules. (*B*) Periocular stimulation (1 pulse, 300 μA) elicits short-latency field potential responses on the cerebellar surface. Below are single-cell recordings of two complex spikes elicited by the periocular stimulation (1 mA) and simple spikes elicited by parallel fiber stimulation (4 μA). Arrows indicate stimulation; asterisks indicate responses. (*C*) Typical conditioned Purkinje cell response (CR). (*D*) Neuronal wiring diagram with stimulation, recording, and injection sites. AIN, anterior interpositus nucleus; CS, conditional stimulus; cf, climbing fiber; Gc, Golgi cell; Grc, granule cells; In, interneuron; IO, inferior olive; mf, mossy fibers; Pc, Purkinje cell; pf, parallel fibers; US, unconditional stimulus.

in particular response offset, from direct effects of the conditional stimulus duration (8).

Naive cells ($n = 19$) responded to the conditional stimulus with increases or no change in simple spike firing (Figs. 2 *A* and *F* and 3*D*). All cells acquired conditioned responses during training, i.e., a significant reduction in simple spikes in response to the conditional stimulus during the interstimulus interval ($P < 0.00001$), illustrated in Fig. 3. Considering the robust difference between naive and trained responses both here and in hundreds of Purkinje cells in our previous publications (6–8, 10, 11, 16, 17), we felt justified in including four additional neighboring cells encountered after training, for which there were no naive data.

To determine whether Purkinje cells trained with a parallel fiber conditional stimulus behaved as those trained with a forelimb or mossy fiber conditional stimulus (7, 8, 16), we performed a series of postacquisition manipulations. For three cells, recording conditions permitted additional hours of training with conditional stimulus only. As expected, the conditioned Purkinje cell responses gradually disappeared (see the example in Fig. 2*E*). For one cell, recorded for almost 10 h, we also were able to shift the interstimulus interval. After emitting conditioned responses to an interstimulus interval of 200 ms, subsequent training with a new 350-ms interstimulus interval caused the cell to acquire a bimodal conditioned response, with a second pause response close to the end of the new interstimulus interval (Fig. 2*H*). A similar observation has been made previously using a mossy fiber conditional stimulus (8).

Training to increasingly longer interstimulus intervals resulted in responses with increasingly delayed onsets, maxima, and offsets (Fig. 3). Conditioned response maxima ±1 SD for the different interstimulus intervals (79 ± 34, 146 ± 32, and 260 ± 36 ms, respectively) appear <75 ms before the anticipated onset of the unconditional stimulus. The estimated latencies to the conditioned response onset were 13 ± 11, 48 ± 34, and 73 ± 18 ms, respectively. The latencies to offset were 193 ± 62, 298 ± 82, and 477 ± 64 ms, respectively. There was a significant effect of interstimulus interval on latencies to response onset, maximum, and offset ($P = 0.0006$, 0.0002, and 0.0005, respectively; Kruskal–Wallis one-way ANOVA).

If, after training, the conditional stimulus was lengthened ($n = 4$) or shortened ($n = 4$) on a series of probe trials, it still elicited a response timed to the previously trained interstimulus interval (see the examples in Fig. 2 *C* and *D*). Also, the duration of the conditional stimulus used during training does not appear to have any effect on the temporal profile of the conditioned response. Cells conditioned to an interstimulus interval of 200 ms using a coterminating conditional stimulus or a conditional stimulus that outlasts the interstimulus interval by 600 ms show similar temporal response profiles (Fig. 3*F*). Thus, the timing of the conditioned Purkinje cell response does not depend on a time-coded input to the cell signaled by a temporal pattern in the conditional stimulus.

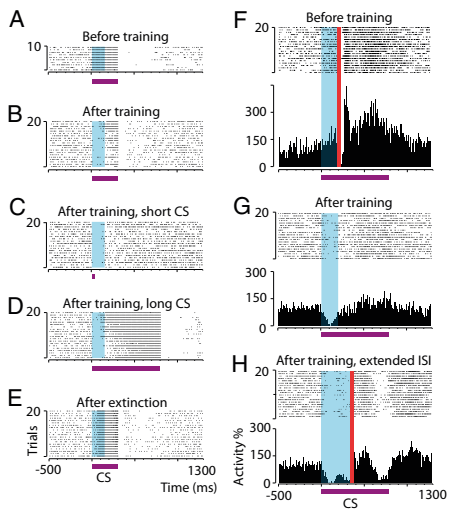

Fig. 2. Conditioned Purkinje cell responses timed to interstimulus intervals. (*A* and *B*) Raster plots showing a typical Purkinje cell response to a 300-ms conditional stimulus before (*A*) and after (*B*) training with a 150-ms interstimulus interval (blue shading). (*C* and *D*) Responses of the cell in *A* and *B* to 17.5-ms (*C*) and 800-ms (*D*) conditional stimuli (CS) after training. (*E*) Response to a 300-ms conditional stimulus after extinction. (*F–H*) Raster plots and histograms illustrating responses of a Purkinje cell that was trained first with a 200-ms interstimulus interval and subsequently with a 350-ms interstimulus interval. Red vertical bars in *F* and *H* denote unconditional stimulus artifacts (data from paired conditional stimulus–unconditional stimulus trials). Purple horizontal bars indicate the conditional stimuli. ISI, interstimulus interval.

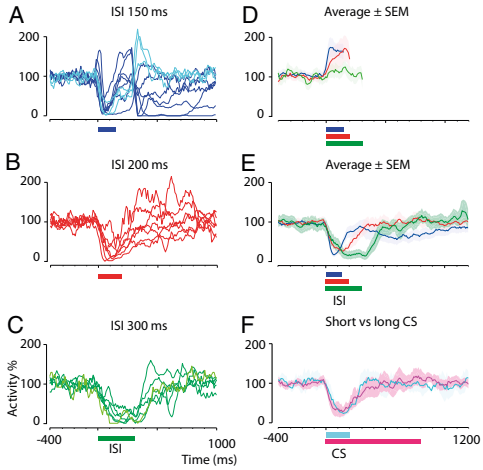

Fig. 3. Time courses of conditioned responses after training with different interstimulus intervals and using conditional stimuli of different durations. (*A*–*C*) Smoothed and averaged simple spike activity after training with 150-ms (blue, $n = 10$) (*A*), 200-ms (red, $n = 7$) (*B*), and 300-ms (green, $n = 6$) (*C*) interstimulus intervals. Traces with lighter shading represent cells for which naive data are lacking. Colored horizontal bars indicate the interstimulus intervals. (*D*) Activity ± SEM for each interstimulus interval before training. Traces are truncated at the onset of the unconditional stimulus artifact, which prohibits identification of spikes. Abrupt downward inflections at the end of some traces reflect an effect of smoothing (0 identified spikes during the unconditional stimulus artifact). (*E*) Activity ± SEM for each interstimulus interval after training. (*F*) Activity ± SEM for cells trained with a 200-ms interstimulus interval and a coterminating conditional stimulus (cyan, $n = 2$) or an 800-ms conditional stimulus (magenta, $n = 5$).

Therefore, the memory trace must reside either in the Purkinje cells or in molecular layer inhibitory interneurons. To examine the role of the latter, we tested the effect of a GABA-antagonist on conditioned Purkinje cell responses using two different interstimulus intervals (Figs. 4 and 5). Seven cells were trained until they reliably emitted conditioned responses (in this case with a forelimb conditional stimulus). Before local injection of the $GABA_A$ receptor antagonist gabazine, off-beam parallel fiber stimulation [i.e, stimulation of parallel fibers that do not terminate on the recorded Purkinje cell but which do excite interneurons that innervate that Purkinje cell (Fig. 1*D*)] effectively silenced the simple spike activity (Fig. 4*A*). Injection of the antagonist blocked interneuron inhibition from off-beam stimulation (Fig. 4 *B* and *C*), but the most important features of the conditioned responses remained essentially the same (Fig. 4 *D* and *F*). In two cases, the stimulation activated both excitatory and inhibitory input to the Purkinje cell, and the effect of gabazine was to remove inhibition and unmask an excitatory response (visible in Figs. 5*A* and 6*F*). In this case, too, there was no effect on the conditioned pause response. Similar experiments were performed using a direct parallel fiber conditional stimulus instead of the forelimb stimulation (Fig. 6).

The gabazine experiments demonstrate that the main part of the conditioned Purkinje cell response is not mediated by interneuron inhibition but must be an effect of parallel fiber input to the Purkinje cells. Parallel fibers are glutamatergic, and a pause in firing might seem an unexpected response to this normally excitatory transmitter, but glutamate-evoked hyperpolarization through group II and III metabotropic glutamate receptors has been described previously (18).

Discussion

Although many different mechanisms, such as long-term depression or potentiation or changes in intrinsic excitability in Purkinje cells, perhaps working in synergy, probably participate in many forms of cerebellar motor learning in the behaving animal (19), the synaptic mechanism usually invoked to account for the learning of a Purkinje cell conditioned response is long-term depression of the parallel fiber to Purkinje cell synapses (13). Long-term potentiation of parallel fibers to interneurons that inhibit the Purkinje cell also has been suggested (20). Modulation of these synapses has been demonstrated with parallel and climbing fiber inputs that occur in close temporal proximity to each other (21–23). However, a challenge for both theories has been to explain how learning of conditioned responses could be adaptively timed and dependent on the conditional stimulus–unconditional stimulus interval. Mere strengthening or weakening of these synapses cannot account for the time course of the conditioned pause response (onset, maximum, offset) (12).

Most models (1, 13), with some notable exceptions (24), assume that delays in the granule cells, perhaps through interactions with Golgi cells, generate a temporal spike pattern in the granule cell responses to the mossy fiber input carrying the conditional stimulus

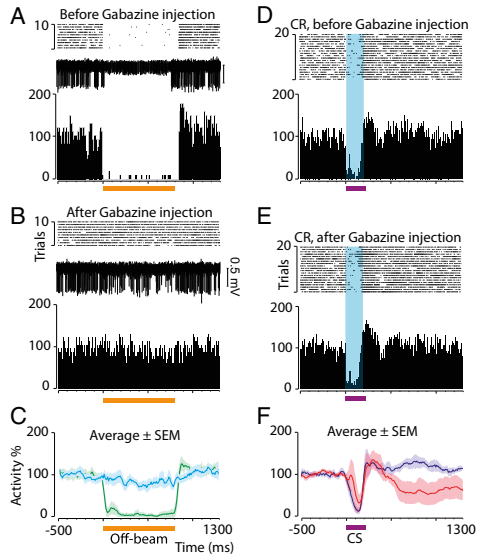

Fig. 4. Gabazine blocks interneuron inhibition of Purkinje cells but leaves conditioned responses to a 200-ms interstimulus interval intact. Orange horizontal bars indicate off-beam stimulation (800 ms, 81 pulses, 100 Hz). Purple horizontal bars indicate the conditional stimulus, and blue shading indicates the interstimulus interval (200 ms). (*A* and *B*) Purkinje cell responses to interneuron activation by off-beam parallel fiber stimulation (compare with Fig. 1*D*) before (*A*) and after (*B*) gabazine injection. Stimulation artifacts are masked. (*C*) Average ($n = 4$) responses ± SEM before (green) and after (cyan) gabazine injection. (*D* and *E*) Conditioned responses before (*D*) and after (*E*) gabazine injection in the same Purkinje cell shown in *A* and *B*. (*F*) The average response profile before (blue) and after (red) injection in the cells shown in *C*.

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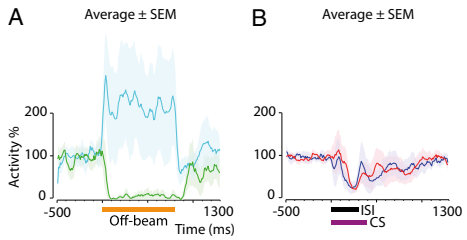

Fig. 5. Gabazine blocks interneuron inhibition of Purkinje cells but leaves conditioned responses to a 300-ms interstimulus interval intact. (*A*) Average Purkinje cell responses ($n = 3$) ± SEM to interneuron activation by parallel fiber stimulation before (green) and after (cyan) injection of gabazine. The horizontal orange bar indicates off-beam stimulation (800 ms, 81 pulses, 100 Hz). (*B*) The average response profile to a 400-ms conditional stimulus before (blue) and after (red) in the cells shown in *A*. Black and purple horizontal bars indicate the interstimulus interval (300 ms) and conditional stimulus (CS, 400 ms), respectively.

signal. If granule cells have long-lasting variable activity states during the interstimulus interval, some cells in the population will have an activity peak with a temporal relation to the climbing fiber input that is maximally conducive to depression or potentiation of molecular layer synapses. When the same temporal pattern appears after learning, these synapses will automatically generate an appropriately timed conditioned response.

In the present investigation the conditional stimulus was delivered directly to the parallel fibers, thus bypassing any possible delays or temporal patterns in the granule cells. Therefore there can be no time code in the temporal pattern of the input to the Purkinje cells except the regular repetition provided by the train of parallel fiber stimuli. It could be argued that stimulation of parallel fibers caused antidromic activation of parallel fibers and granule cells and that a temporal input pattern might be generated via this route. This notion is extremely implausible, however. Identical electrical stimuli were delivered to the parallel fibers up to 81 times every 10 ms (800 ms, 100 Hz). The immediate effect of such stimulation is almost certain to drown or corrupt any specific temporal activity pattern in granule cell responses elicited by antidromic activation. Furthermore, in vivo recordings of granule cells and Golgi cells show that these cells do not exhibit the delayed signals that are necessary for the models (25, 26).

Furthermore, in agreement with previous findings on both overt and Purkinje cell conditioned responses (27, 16) using mossy fiber conditional stimuli, we observed the same response on posttraining probe trials whether we delivered eight pulses over 17.5 ms, 31 pulses over 300 ms, or 81 pulses over 800 ms, to the parallel fibers, suggesting that the temporal profile of the conditioned Purkinje cell response is determined by the initial part (less than 20 ms) of the conditional stimulus and therefore is insensitive to any temporally patterned input during the main part of the interstimulus interval and conditioned response (Fig. 2 *B–D*). If the granule cell network were necessary for the adaptive timing, the unlikely implication is that three such different stimuli would elicit the same temporal activity pattern in the parallel fibers.

Instead, the data strongly suggest that the main timing mechanism is within the Purkinje cell and that its nature is cellular rather than a network property. Parallel fiber input lacking any temporal pattern can elicit Purkinje cell responses timed to intervals at least as long as 300 ms. Other mechanisms likely contribute to cerebellar motor learning and response timing (19). However, our data demonstrate that one important associative memory trace, exemplified by eyeblink conditioning, resides in the Purkinje cell. In addition, the data show that a main part of the timing of the conditioned response relies on intrinsic cellular mechanisms rather than on a temporal pattern in the input signal.

Materials and Methods

Surgery and Stimulation Sites. Animal experiments were approved by the Malmö–Lund animal experimentation ethics committee. Twenty-six male

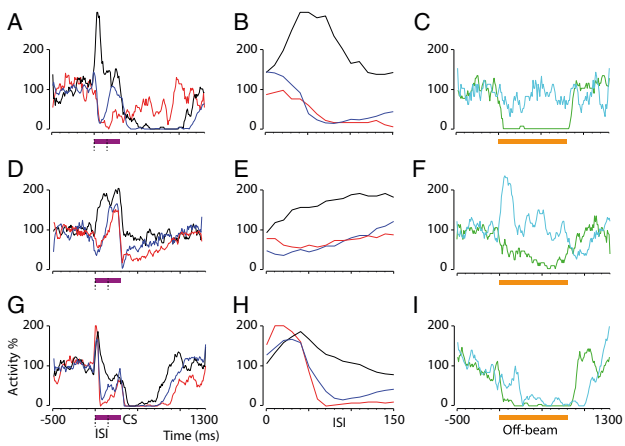

Fig. 6. Effects of different concentrations of gabazine on conditioned Purkinje cell responses to a parallel fiber conditional stimulus. Each row indicates one cell. (*A*, *D*, and *G*) Naive (black) and conditioned responses before (blue) and after (red) gabazine injection. Purple horizontal bars indicate a 300-ms conditional stimulus. Dashed lines indicate a 150-ms interstimulus interval. (*B*, *E*, and *H*) Magnification of the response during the interstimulus interval. (*C*, *F*, and *I*) Purkinje cell responses to interneuron activation by parallel fiber stimulation before (green) and after (cyan) gabazine injection. At 100 μM, gabazine distinctly blocks inhibition (*C* and *F*), whereas 10 μM gabazine blocks inhibition for the first 200 ms (*I*). Orange horizontal bars indicate off-beam stimulation (800 ms, 81 pulses, 100 Hz).

1-y-old ferrets were surgically prepared with electrical stimulation sites as previously described (7). Parallel fibers were stimulated with platinum-tungsten electrodes (pulled and ground tips, 25-μm core diameter). Eliciting or suppressing Purkinje cell simple spikes confirmed on-beam and off-beam location, respectively.

Training Protocol. For the conditional stimulus, 100- or 50-Hz stimulus trains (230–800 ms, 2–20 μA, 0.1-ms pulse duration) were applied to parallel fibers, or 50-Hz stimulus trains (230–400 ms, 0.6–1.2 mA, 1-ms pulse duration) were applied to the ipsilateral forelimb. For the unconditional stimulus, two five-pulse 500-Hz stimulus trains (30–400 μA, 0.1-ms pulse duration) separated by 10 ms were applied to ipsilateral climbing fibers 150–350 ms after the onset of the conditional stimulus onset. The intertrial interval was 15 ±1 s (randomized). Acquisition sessions with paired conditional stimulus–unconditional stimulus or conditional stimulus-alone stimulation lasted 1–5 h.

Recordings and Data Analysis. Recording technique and analysis software were as previously described (7). Training effect was defined by a significant reduction in spike frequency in the last third of the interstimulus interval after training (paired sample *t* test, spikes averaged over 20 or 10 trials and normalized to activity 600 ms pretrial). Data were quantified in 10-ms bins. The first and last bins in a series of consecutive bins with spike activity below the spontaneous activity defined response onset and offset. The last bin in the block of bins with the lowest activity during the interstimulus interval defined response maximum (7). This procedure was motivated by the expected post-synaptic effect on nuclear cells (maximal response at the end of maximal dis-inhibition). Traces of cell activity in all figures are smoothed using a five-point moving average.

Pharmacology. Gabazine (Tocris Bioscience) (10 μm–8.97 mM) was injected ~0.1–1.0 mm away from the recording electrode in steps until stimulation of interneurons no longer caused inhibition of Purkinje cells.

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Paper II

Title: Activation of a temporal memory in Purkinje cells by the mGluR7 receptor

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Abstract

Cerebellar Purkinje cells can learn to respond to a conditional stimulus with an adaptively timed pause in firing. When the stimulus is a train of electrical pulses delivered directly to the pre-synaptic fibers, there is no temporal variation in the input signal to instruct timing of the learned response. This must therefore depend upon an intrinsic timing mechanism rather than on changes in synaptic strength, such as long-term depression. The learned Purkinje cell response is resistant to blockade of GABAergic inhibition. Here we test whether it depends upon glutamate release. We show that the response can be abolished by antagonists of the mGlu7 receptor, but is not significantly affected by antagonists of other glutamate receptors. These results support the existence of a novel learning mechanism, different from changes in synaptic strength. They also demonstrate *in vivo* post-synaptic inhibition mediated by glutamate and show that the mGlu7 receptor is involved in activating intrinsic temporal memory.

Introduction

To control any aspect of behavior, the brain must produce complex temporal activity patterns. Temporal precision is necessary for a wide range of tasks, from driving a car to anticipating the next step of a dance partner. A simple timing-dependent learning can be studied in eyeblink conditioning. If a neutral conditional stimulus (CS) is repeatedly paired with an unconditional blink-eliciting stimulus (US), with a fixed temporal delay (the interstimulus interval, ISI), it acquires the ability to elicit a timed blink response that peaks near the US (Kehoe and Macrae, 2002). Such timed conditioned responses depend upon the cerebellar cortex (Yeo et al., 1984). Purkinje cells receive information about the CS and US via mossy/parallel fibers and climbing fibers, respectively (Hesslow and Yeo, 2002; Mauk et al., 1986; Steinmetz et al., 1986). During

conditioning, Purkinje cells in a blink-controlling area of the cerebellar cortex acquire a learned suppression of firing in response to the conditional stimulus (Heiney et al., 2014; Hesslow, 1994b; Hesslow and Ivarsson, 1994; Jirenhed et al., 2007). This firing rate reduction releases tonic inhibition of cerebellar nuclear cells, which increase their firing rate to generate an overt, conditioned blink (Heiney et al., 2014; Hesslow, 1994b; Hesslow and Ivarsson, 1994; Jirenhed et al., 2007).

A crucial property of the conditioned Purkinje cell responses is that they mirror the behavioral responses in that they are adaptively timed. They reach their maximum amplitude just before the anticipated onset of the US and end shortly after even if the conditional stimulus lasts only a few milliseconds or if it outlasts the interstimulus interval by several hundred milliseconds (Jirenhed and Hesslow, 2011a, b; Johansson et al., 2014). The timing of the conditioned Purkinje cell responses has usually been ascribed to a temporal code in the input signal in the parallel fibers arising from network dynamics that create time-varying activation of different granule cell subpopulations (Medina and Mauk, 2000; Yamazaki and Tanaka, 2009). Pairing the parallel fiber inputs with a US-elicited climbing fiber input would cause long-term depression of those parallel fiber-to-Purkinje cell synapses that were activated at a particular time, relative to the CS and US. Only these parallel fibers would then contribute to the conditioned response, which would therefore automatically be correctly timed. However, we have recently shown that even when the conditional stimulus is a direct train of repetitive stimuli to the Purkinje cell's immediate afferents, the parallel fibers, and therefore no such temporal code is possible, the cell still learns an adaptively timed response (Johansson et al., 2014). The timing of the response must therefore be due to a mechanism intrinsic to the Purkinje cell. Furthermore, according to

the traditional view, the Purkinje cell firing is determined by the balance between excitatory input caused by glutamate from parallel fibers acting on AMPA-kainate receptors and inhibitory input via GABAergic interneurons, which are themselves driven by the parallel fibers. This view is also challenged by the demonstration that the conditioned Purkinje cell responses are resistant to blocking GABAergic interneuron inhibition of the Purkinje cell, raising the intriguing possibility that they depend upon glutamate release from parallel fibers (Johansson et al., 2014).

In addition to the excitatory ionotropic (AMPA-kainate) glutamate receptors, there are two types of metabotropic glutamate receptors on Purkinje cells, mGluR1 and mGluR7 (Knöpfel and Grandes, 2002). The mGluR1 is thought to be necessary for pf-PC LTD (Knöpfel and Grandes, 2002). It has been suggested (Fiala et al., 1996; Steuber and Willshaw, 2004), though later challenged (Hesslow et al., 2013; Johansson and Hesslow, 2014; Yamazaki and Tanaka, 2009), that it triggers conditioned responses through calcium-activated potassium channels.

Although the timed inhibitory pause in Purkinje cell activity appears to contrast with the normally excitatory effects of glutamate, group II/III metabotropic receptors have been shown to have inhibitory effects in some neurons (Cox and Sherman, 1999; Dutar et al., 1999; Lee and Sherman, 2009) and an unusual hyperpolarizing effect of glutamate on Purkinje cells has been reported (Inoue et al., 1992). Purkinje cells express the mGluR7b splice variant of mGluR7 (Kinoshita et al., 1998; Phillips et al., 1998) and we suggested that this group III metabotropic glutamate receptor could be involved in generating the conditioned Purkinje cell response (Johansson et al., 2014). To test this hypothesis, here we have pharmacologically blocked

mGluR7 after conditioning and also tested possible contributions from the other Purkinje cell glutamate receptors by antagonizing AMPA-kainate receptors and mGluR1.

Results and discussion

The activity of 48 Purkinje cells from a blink-controlling area in the C3 zone (Hesslow, 1994a, b) was recorded in 33 decerebrated male ferrets. The cells had been trained with interstimulus intervals of 200 ms (n = 22), 300 ms (n = 21) or 400 ms (n = 5), with trains of electrical stimuli (50 Hz, 400 ms) to the ipsilateral forelimb as the conditional stimulus and direct electrical stimulation of climbing fibers (two stimulus trains of five pulses at 500 Hz separated by 10 ms (Jirenhed et al., 2007)) as the unconditional stimulus (Fig. 1).

After sufficient training, the Purkinje cell exhibited conditioned responses. The selective mGluR7 antagonist 6-(4-Methoxyphenyl)-5-methyl-3-(4-pyridinyl)-isoxazolo[4,5-*c*]pyridin-4(5*H*)-one hydrochloride (MMPIP) was then applied. Cortical infusions (2 µl, 300-600 µM applied 1-2 mm from the recorded cell) distinctively removed the pause response (n = 4, Fig. 2A) and even replaced it with excitation. Local sub-nanoliter injections (n=7) also removed most of the pause response but some (n=3) revealed a tendency of the drug to preferentially disturb the pause response at its normal maximum (the last 100 ms of the interstimulus interval, fig. 1C). In one of these cases, additional local injections (i.e. an increasing dose) were possible, and these progressively flattened the temporal response profile as shown in Fig. 2B-D. For all 10 cells and all concentrations of MMPIP used (6-600 µM) the pause response at the anticipated maximum towards the end of the interstimulus interval was diminished (Fig. 2E). An example of a residual early pause following a single sub-nanoliter injection, is seen in Fig. 2F. As an additional test, we

applied the orthosteric mGluR7 antagonist LY341495 (5 μM) in another group of cells (n = 5) and obtained similar results (Fig. 2G-H) although this less selective antagonist (with higher affinity for other mGluR subtypes, including mGluR2 expressed by Golgi cells (Knöpfel and Grandes, 2002)), appeared less efficient with 3/5 cells maintaining a partial pause response early in the interstimulus interval.

The resistance of the pause response to suppression in the early part of the interstimulus interval may relate to its dynamics. Although the lowest instantaneous firing rate occurs in the later part of the interstimulus interval, the rate-of-change of firing in the pause response is greatest in the earlier part of the interstimulus interval. Hence, the mechanism driving the Purkinje cell inhibition is likely to be more potent in this early part, and so more resistant to the antagonists. The small residual responses may be due to dose effects or it may be that neither MMPIP nor LY341495 optimally block all mGluR7 effectors, such as Kir3 (GIRK) channels (Niswender et al., 2008; Niswender et al., 2010).

To investigate potential mGluR1 contributions to the conditioned pause responses, 13 Purkinje cells were recorded in 4 subjects after a 10 μM cortical infusion of the mGluR1 antagonist JNJ16259685 and 4 more cells were recorded from 2 additional subjects after local injections of 1 or 10 μM. This allowed 8 direct comparisons before and after application of the antagonist (within-subject, fig. 3A). Because of the similarity between the conditions all 17 cells are reported together in Fig. 3B. The conditioned pause responses remained unchanged at all applied concentrations of the antagonist. This antagonist has no reported effects upon fast parallel fiber EPSPs and climbing fiber responses up to high concentration (10 μM) (Fukunaga et al., 2007), so

no changes in spontaneous firing rate were anticipated. Thus it is not straightforward to verify physiologically, *in vivo*, that the mGluR1s were fully blocked. But the possibility that these receptors contribute importantly to the conditioned response here is unlikely since the highest concentration of mGluR1 antagonist was greater than 500 times the *in vitro* IC_{50}). In contrast, similar applications of low mGluR7 antagonist concentrations (~10 times *in vitro* IC_{50}) significantly diminished the conditioned response.

The importance of AMPA-receptor mediated activity for conditioned responses is supported by the finding that cortical infusions of the AMPA-kainate receptor antagonist CNQX prevents behavioral conditioned nictitating membrane responses in rabbits (Attwell et al., 1999; Mostofi et al., 2010). However, CNQX may have blocked AMPA-mediated transmission in the granule cell layer in the first of these studies (Attwell et al., 1999), though the smaller infusions (600-800 nl) adjacent to recorded Purkinje cells and simultaneous suppression of complex spike activity in the later (Mostofi et al., 2010), are consistent with infusions focused on, but perhaps not restricted to, the molecular layer. However, if those results were due to the conditional stimulus signal not adequately reaching the Purkinje cell (mossy fiber-granule cell transmission block) then we suggest that extremely local blockades of AMPA-kainate receptors at the recorded Purkinje cell, by applying sub-nanoliter volumes of 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) (Wilding and Huettner, 1996) 15 μm from the tip of the recording electrode, should leave the cellular delayed responses intact. To establish an effective concentration of NBQX at the recorded Purkinje cell, we first stimulated parallel fibers to generate an excitatory response. When a sufficient dose of NBQX (~ 0.5-1nl, 25 μM) had been injected, the excitatory response to parallel fiber stimulation disappeared (n = 8) (Fig 4A) and

later returned. In three cases an initial excitatory response to parallel fiber stimulation was replaced by a suppression of Purkinje cell firing (Fig 4B, *top*). This was probably because the beam of activated parallel fibers could still drive inhibitory interneurons (lateral to the Purkinje cell) further away. Consistent with this interpretation was the finding that this suppression faded after a few minutes during which time the drug would diffuse laterally (Fig. 4B, *bottom*). The average response to parallel fiber stimulation fell from >200% of simple spike firing (relative to background) to no increase (Fig. 4C, n = 8). Conditional stimulus trials were then interspersed with the parallel fiber stimulation trials, so that conditional stimulus data was only from trials where the AMPA receptors were shown to be blocked. Conditioned pause responses were unaffected and normal after applying NBQX (Fig 4D-E). Given that only excitatory responses to AMPA receptor activation have been described, this need not be a surprising result and it is also consistent with our other data showing that the learning mechanism is unlikely to involve altered strength of AMPA receptor mediated synapses (Hesslow et al., 2013; Johansson et al., 2014).

In many cell types, group III metabotropic glutamate receptors are believed to function as presynaptic autoreceptors that mediate feedback inhibition of glutamate release, probably via reduced Ca^{2+} entry into the nerve terminals (Millan et al., 2002). So, in principle, blocking mGluR7 might lead to increased glutamate release from parallel fibers and so counteract simple spike suppression during the conditioned pause. Three considerations argue strongly against this interpretation. First, in vitro studies in rodents reveal that parallel fiber glutamate release is regulated by mGluR4 and not mGluR7 (Abitbol et al., 2008). Second, excitatory ionotropic glutamate receptors and mGluR1 are the postsynaptic candidates for mediating a conditioned pause response consequent upon decreased glutamate release and both are now excluded by our

findings here that that blocking them had no significant effects on the learned pause. Third, there were no significant excitatory effects of mGluR7 block outside the learned pause period. Because we used a standard duration for the conditional stimulus of 400 ms, it outlasted the interstimulus interval and the main part of the pause response by 200 ms in most cases. If the mGluR7 antagonists had acted to increase simple spike firing by increasing glutamate release, rather than by interfering with a specific mechanism for eliciting the pause responses, this should be reflected in an increased firing rate during the conditional stimulus presentation beyond the pause duration. This was clearly not the case. Inspection of Fig.2 A shows that the simple spike firing level during the conditional stimulus presentation after the pause is not increased, rather there is some rate decrease. The findings do not support the suggestion that the mGluR7 antagonist effects on the conditioned pause depend upon enhanced glutamate release by presynaptic action.

Conditioned Purkinje cell responses have been attributed to timed input signals in parallel fibers acting on Purkinje cells through excitatory ionotropic glutamate receptors and inhibitory GABAergic interneurons. The present results suggest instead that the conditioned responses are mediated by a metabotropic glutamate receptor, the mGluR7. Although, perhaps surprising, this is actually much more plausible than the traditional view. Here, the conditioned pause responses are elicited by a uniform repetitive parallel fiber input to the Purkinje cell. The responses have a learned temporal profile with specific onset, peak and termination times that are mostly independent of the duration of the impulse train in the parallel fibers (Jirenhed and Hesslow, 2011b; Johansson et al., 2014; Svensson and Ivarsson, 1999). To explain these properties in terms of ionotropic receptors is intrinsically difficult (Hesslow et al., 2013) but the recent

demonstration that the pause responses are independent of GABA makes it virtually impossible. The mGluR7 receptor would seem to be a much more plausible initiator of a biochemical signal cascade that could account for essential properties of the conditioned pause response.

In other learning situations, peripheral mechanisms provide a temporally varying input to the granule cell layer and thus permit temporal coding in the parallel fiber input to Purkinje cells. Under these conditions, synaptic weight changes at parallel fiber synapses may play their part in establishing a temporal profile to the learned responses. It will be important to determine whether they operate in parallel with the mGluR7-mediated postsynaptic temporal learning mechanism identified here.

Experimental procedures

Surgery and training protocol

33 male one-year old ferrets were surgically prepared with electrical stimulation sites as previously described (Jirenhed et al., 2007) and approved by the local ethics committee. The conditional stimulus was a 400 ms stimulus train (50 Hz, 1 ms pulse duration, 0.8-1.4 mA) applied to the ipsilateral forelimb. The unconditional stimulus consisted of two 5-pulse 500 Hz stimulus trains (0.1 ms pulse duration, 100-400 μA) separated by 10 ms, applied to ipsilateral climbing fibres 200, 300 or 400 ms after conditional stimulus onset. The intertrial interval was 15 +/-1 s (randomized). Acquisition sessions with paired conditional stimulus - unconditional stimulus trials lasted 100-180 minutes.

Recordings and data analysis

Recording technique and analysis software were as previously described (Jirenhed et al., 2007; Johansson et al., 2014) with the addition of using Carbostar-4 and Carbostar-6 multibarrel electrodes (Kation Scientific, Minneapolis, U.S.A.) for recording Purkinje cell activity. All data is quantified in 10-ms bins and cell activity reported as a percentage is normalized to activity 600 ms pre-trial and averaged over 20 trials for responses to the conditional stimulus or 5 trials for responses to parallel fiber stimulation. Raster plots and histograms show raw data and traces of cell activity in all figures are smoothed using a five point moving average.

Pharmacology

All drugs were from Tocris Bioscience (Bristol, UK). Stock solutions of MMPIP hydrochloride, LY341495 and JNJ16259685 were prepared by dissolving in fresh DMSO, then diluted in

physiological saline to final DMSO concentrations of 0.1-0.5%. NBQX (disodium salt) was dissolved in H_2O and diluted in physiological saline. All drugs were kept frozen until use and injected either with a pipette 1-2 mm away from the recording electrode or with pressure micro-ejections through the multibarrel Carbostar electrodes. The micro-ejections were calibrated by ejecting saline into paraffin oil and measuring the droplet size under a microscope against a calibration scale. Pressures were set to achieve a droplet size of (0.25-0.5 nl).

Author contributions: F.J. conceived the initial experiment. F.J., G.H., C.H.Y. and H.A.E.C. developed the protocols. F.J. performed all surgery and experiments. H.A.E.C. assisted with experiments. F.J., H.A.E.C. and A.R. carried out data analysis. F.J., G.H., C.H.Y. and H.A.E.C. interpreted experiments. F.J. wrote the manuscript. G.H., C.H.Y., H.A.E.C. and A.R. edited the manuscript.

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Figures and figure legends

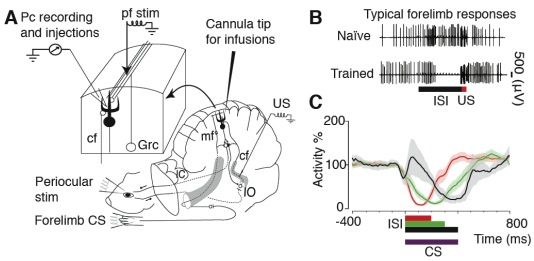

Fig. 1. Experimental setup and conditioned responses.

A. Simplified neural circuitry with stimulation, recording and injections sites. **B.** A typical example of naïve and conditioned Purkinje cell responses to a forelimb conditional stimulus. **C.** Averaged and smoothed response profile +/- SEM (% of background activity) to the conditional stimulus for all of the Purkinje cells included in this study (ISI 200, n = 22; ISI 300, n = 21; ISI 400, n = 5). **Key**: CS: conditional stimulus. US: unconditional stimulus. ISI: interstimulus interval. IC: Inferior colliculus. IO: inferior olive. cf: climbing fiber. mf: mossy fiber. Pc: Purkinje cell. Gc: Golgi cell. pf: parallel fibers. Grc: Granule cell.

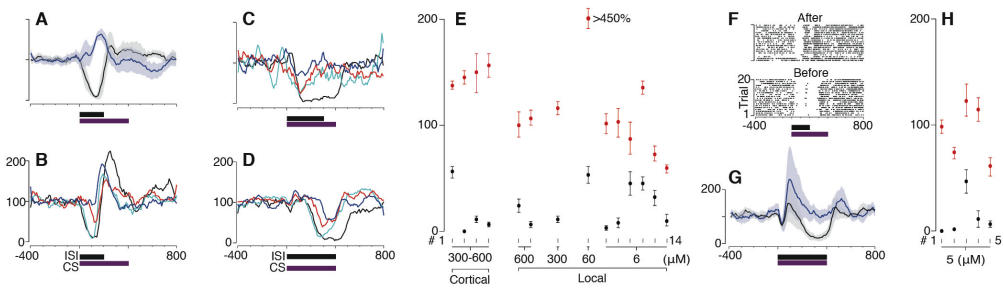

Fig. 2. Purkinje cell responses to the conditional stimulus after injection of mGluR7 antagonist.

Key: y-axes indicate simple spike firing (% of baseline activity) ***A-E, G-H*;** trials ***F***. x-axes indicate time in ms (-400 to 800 ms) ***A-D, G, H;*** individual Purkinje cells ***E*** *and* ***H***. Purple and black indicate conditional stimuli and interstimulus intervals, respectively. All traces represent smoothed response profiles. Shading and error bars indicate SEM. **A**. Averaged response profile before (black) and after (blue) cortical infusions of MMPIP (300-600 μM; n = 4). **B-D.** Response profiles of individual Purkinje cells, one in each panel, before (black) and after one (cyan), two (red) and three (blue) sub-nanoliter local injections of 6 μM MMPIP. **E.** All Purkinje cell responses during the last 100 ms of the interstimulus interval before (black) and after (red) injection of MMPIP. For cortical infusions: cell #1: 300 μM, cells #2-4: 600 μM. **F**. Raster plot of a Purkinje cell where a short latency inhibition remained after injection of MMPIP. **F.** Averaged response profile before (black) and after (blue) local injection of LY341495 (5 μM; n = 3). **H**. All Purkinje cell responses during the last 100 ms of the interstimulus interval before (black) and after (red) local injection of LY341495.

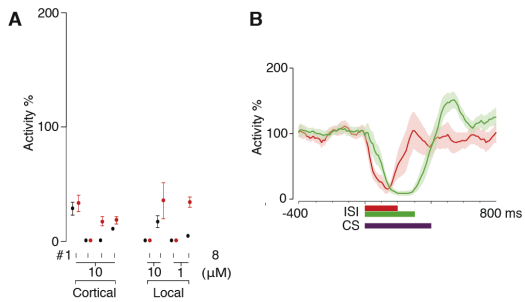

Fig. 3. Purkinje cell responses to the conditional stimulus after injection of mGluR1 antagonist.

A. All Purkinje cell responses during the last 100 ms of the interstimulus interval before (black) and after (red) injection of JNJ16259685. X-axis indicates individual cells. **B.** Averaged response profiles after infusion/injection of JNJ16259685 (red, 200 ms interstimulus interval, n = 6; green, 300 ms interstimulus interval, n = 9).

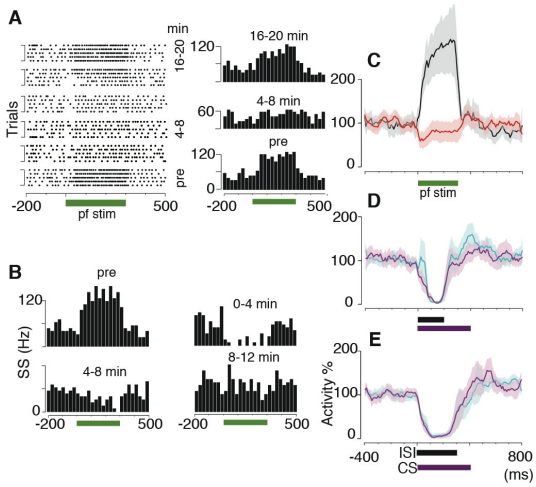

Fig. 4. Purkinje cell responses to parallel fiber stimulation and to the conditional stimulus after injection of ionotropic glutamate receptor antagonist.

A. Typical Purkinje cell response to 100 Hz parallel fiber stimulation before and during 0-4, 4-8, 8-12, 12-16 and 16-20 minutes (bottom-up) after local injection of 25 μM NBQX. **B.** Example case of NBQX briefly un-masking inhibition elicited by the parallel fiber stimulation. **C**. Averaged and smoothed response profiles +/- SEM to parallel fiber stimulation before (black) and after (red) local injection of NBQX (n = 8). **D-E**. Averaged and smoothed response profiles +/- SEM to the conditional stimulus before (cyan) and after (purple) local injection of NBQX (*d*: 200 ms interstimulus interval, n = 4; *e*: 300 ms interstimulus interval, n = 4).

Paper III

Programmable Activation of Metabotropic K+ Channels Constitutes a Physical Basis of Temporal Duration Memory in Purkinje cells

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Summary

Cerebellar Purkinje cells can learn to respond to activity in its parallel fiber input with an adaptively timed pause in spontaneous firing. The response requires activation of the metabotropic glutamate receptor 7 (mGluR7), which catalyzes the production of G-protein subunits Gβγ that are capable of directly activating the metabotropic K^+-channel family Kir3. Here we show that Kir3 is necessary for Purkinje cell responses timed to different temporal intervals. The results suggest that learned and adjustable latencies to activation and deactivation of Kir3 constitute part of a physical basis of temporal duration memory in single neurons.

Introduction

Precise timing in neural signaling is essential to control any aspect of behavior. Timing-dependent learning can be studied in eyeblink conditioning. If a neutral conditional stimulus is repeatedly paired with a blink-eliciting unconditional stimulus, with a fixed temporal delay (the interstimulus interval), it acquires the ability to elicit a conditioned blink response. The interstimulus interval determines the timing of the blink in such a way that the response peaks near the unconditional stimulus [1]. This learning depends upon cerebellar cortex [2] where Purkinje cells receive information about the conditional stimulus signal via the mossy/parallel fiber system and about the unconditional stimulus signal via the climbing fibers [3-6]. As a result of conditioning, Purkinje cells that control the blink learn to suppress their spontaneous firing in response to the conditional stimulus. This conditioned Purkinje cell response disinhibits the cerebellar nuclei and generates an appropriately timed overt blink [7-10]. The conditioned Purkinje cell response is adaptively timed in the same way as the behavioral response; it reaches its maximum just before the onset of the unconditional stimulus and ends shortly after even if the conditional stimulus continues [9, 11, 12].

It was previously thought that the time course of the conditioned Purkinje cell response was determined by a temporal code in parallel fiber signals acting on Purkinje cells directly and indirectly via inhibitory GABAergic interneurons. We have recently shown that no temporal code in the parallel fiber signal is necessary, that GABAergic interneurons can be blocked, and that the cell can learn to respond with different time courses to the same short-lasting parallel fiber input [13]. Furthermore, the conditioned Purkinje cell response is elicited by glutamate release from parallel fibers acting on metabotropic glutamate receptor 7 (mGluR7) [14]. This finding suggests a plausible mechanism for eliciting timed responses. Gβγ dimers produced upon activation of mGluR7 [15] can directly activate the G protein-gated inwardly-

rectifying K^+ channel family Kir3 (or *GIRK*) which mediates postsynaptic inhibition [16, 17], suggesting that these channels constitute the effector component of this intrinsic temporal memory. To test this hypothesis, we have pharmacologically blocked Kir3 in trained Purkinje cells and ruled out alternative sources of Gβγ, beside mGluR7, for their activation. As a control we also blocked alternative hyperpolarizing K^+ channels in the calcium-activated family K_{Ca}. We show that only Kir3 contributes importantly to the conditioned Purkinje cell response and hence that a time-programmable mGluR7-Kir3 cascade constitutes part of a physical basis of temporal duration memories in Purkinje cells.

Results

Extracellular recordings of 47 Purkinje cells in the blink-controlling C3 zone of cerebellar lobule VI [7, 18] were made in 16 decerebrate ferrets. The cells had been trained with interstimulus intervals of 150 ms (n = 3) and 300 ms (n = 38), with electrical stimulation of the ipsilateral forelimb as the conditional stimulus (50 Hz, 400 ms) and direct electrical stimulation of climbing fibers (two stimulus trains of five pulses each at 500 Hz separated by 10 ms) as the unconditional stimulus (Fig. 1). When the Purkinje cells exhibited clear conditioned responses (see Fig. 1) we applied drugs in a nanoliter range (~0.5-1.5 nl) injected 15 μm from the tip of the recording electrode.

Kir3 channel block disrupts conditioned responses

In order to evaluate the hypothesis that Kir3 channels are the effectors of conditioned Purkinje cell responses, we used the antagonist $Tertiapin_{LQ}$. This choice was made because the more traditionally used $Tertiapin_Q$ has an undesirable high affinity for large conductance $K_{Ca}1.1$ channels [19]. The effect of this drug was in perfect agreement with the hypothesis of this paper. On average, local injections of 5 (n = 7), 25 (n = 9) and 200 μM (n = 5) $Tertiapin_{LQ}$

considerably diminished the conditioned response (Fig. 2A-F). In 3 out of the 21 cells there was however no effect of the injection.

The observed effect of Kir3 block is post-synaptic and mGluR7 is the source of Gβγ

In addition to mGluR7, Purkinje cell Kir3 channels can also be activated by Gβγ produced upon activation of the $GABA_B$ receptor ($GABA_BR$) [20]. Kir3 channels are also expressed pre-synaptically in parallel fiber terminals, in molecular layer interneurons and in Golgi cells where they are activated by the endocannabinoid CB1 receptor (CB1R) and the $GABA_B$ receptor [21-23]. In order to tie our results specifically to mGluR7-activated Kir3 channels on Purkinje cells, we continued with blocking the $GABA_BR$ with 50 μM CPG5548 (n = 2) and the CB1R with 80 μm AM251 (n = 3). As seen in Fig. 2G-H neither drug distinctly affected the conditioned response.

K_{Ca} channels are not the primary contributors

Purkinje cells express three types of K_{Ca} channels, $K_{Ca}1.1$ [24-26], $K_{Ca}2.2$ [25, 27-29] and $K_{Ca}3.1$ [30]. Because these large, small and intermediate conductance K^+ channels down-regulate the excitability of Purkinje cells through hyperpolarization, they could potentially contribute to conditioned Purkinje cell responses. In order to investigate any contributions from K_{Ca} channels we antagonized these three with 60 nM Penitrem A, 1 μm Apamin and 1 μm TRAM34, respectively.

Consistent with *in vitro* findings [24, 25], blocking $K_{Ca}1.1$ had a rapid and dramatic effect on Purkinje cell firing. As expected, the cells exhibited highly aberrant behavior with bursts up to 600 Hz and long periods of silence (Fig. 3A). This volatile firing is also apparent in the population average (Fig. 3B). Despite this, and in good agreement with our hypothesis, the

suppression elicited by the conditional stimulus is clearly not removed (n = 5, Fig. 3A-C). Only rarely did a high-frequency burst overpower the hyperpolarizing effect of the conditional stimulus. Blocking $K_{Ca}2.2$ also increased firing rate and irregularity but to a lesser extent. No important change in the conditioned responses was detected (Fig. 3D-F).

To establish an effective concentration of the selective $K_{Ca}3.1$ antagonist TRAM34 we stimulated parallel fibers with five pulses at 100 Hz at low intensities, below 100% spiking probability to 1 pulse, to moderately excite the Purkinje cells. Consistent with the suggested function of $K_{Ca}3.1$ channels to suppress temporal summation of excitatory inputs and *in vitro* findings [30], injection of the antagonist (1 μM) led to an increased firing probability in response to the second to fifth parallel fiber stimulation pulses (Δ+56% to 102%, see Fig. 3G). On average, injection of TRAM34 had a moderate effect on the conditioned response with slightly increased firing towards the end of the interstimulus interval (n = 5, Fig. 3H-I). Fig. 4 summarizes the resulting change in simple spike firing (normalized to pre-trial) during the interstimulus interval after Kir3, $K_{Ca}1.1$, $K_{Ca}2.2$ and $K_{Ca}3.1$ block, respectively.

Discussion

Kir3 importantly contributes to conditioned Purkinje cell responses

Of all four K^+ channels tested, the Kir3 family stands out as the most important hyperpolarizing K^+ channel that is activated by the conditional stimulus. Neuronal Kir3 channels are tetramers of different combinations of Kir3 subunits 1-3 with different electrophysiological properties [31, 32]. That the response is not completely eliminated by $Tertiapin_{LQ}$ could be due to the fact that is not known whether the drug can effectively block Kir3.2/3.3 heteromultimers or that Kir3 channels are not the sole effector of the response. Nevertheless, these results indicate that Kir3 channels mediate the major part of the

conditioned response. Because mGluR7 can activate Kir3 and because mGluR7 block [14] and Kir3 block both alone diminishes the response while other sources of Kir3 activation (CB1R and $GABA_BR$) can be antagonized without effect, we suggest that an mGluR7-Kir3 signaling cascade is the most likely mechanism for the expression of a timed conditioned Purkinje cell response.

The lack of effect upon the conditioned response after blocking CB1Rs and $GABA_BRs$ is not surprising because the presumed effect of blocking these receptors is a slightly stronger conditional stimulus signal due to an increased probability of glutamate release from parallel fibers [21, 23] in the first case and a slightly increased spontaneous firing rate of molecular layer interneurons [33] in the second, neither of which should diminish the Purkinje cell response. Further, the majority of $GABA_B$ receptors are expressed post-synaptically at excitatory pf-PC synapses [34] where they are activated by spillover from inhibitory synapses. Activation of $GABA_BR$ keeps the spontaneous firing rate down but does not shape Purkinje cell response patterns [35]. The receptor also facilitates mGluR1 signaling [36] but mGluR1 is not needed for expression of the conditioned Purkinje cell response [14].

K_{Ca} channels are not the primary effectors of conditioned Purkinje cell responses

As an alternative mechanism for cellular timing, intracellular Ca^{2+} cascades has raised significant interest because timing-dependent and associative supralinear Ca^{2+} signals can be measured following subsequent parallel and climbing fiber input to the Purkinje cell [37, 38]. Earlier models of intracellular Purkinje cell timing in eyeblink conditioning [39, 40] are based on mGluR1-elicited Ca^{2+} influx that activates non-specified K_{Ca} channels. These models have however already been challenged both theoretically and empirically [14, 41-43]. The rapid kinetics and primary roles of $K_{Ca}1.1$ and $K_{Ca}2.2$ in afterhyperpolarization following single

action potentials and acceleration of EPSP repolarization make them ill-suited for delayed and long-lasting cessations in spontaneous firing. K_{Ca} channels could nevertheless be ruled out as effectors of a cellular timing mechanism on the basis of those findings alone. Here we provide empirical support that $K_{Ca}1.1$ and $K_{Ca}2.2$ do not contribute importantly to the expression of conditioned Purkinje cell responses. This study does however not address whether these two channels are important during learning. Given the loss of much of normal function it would not be surprising if learning would not take place during $K_{Ca}1.1/2.2$ block even if they are not part of the learning or expression mechanism as such. Less is known of the $K_{Ca}3.1$ channel. Because we used a reliable criterion for ion channel block and because the effect upon the conditioned response was only moderate it is prudent to conclude that it is not the chief effector of the timed response. The partial effect of blocking $K_{Ca}3.1$ could result from the resulting excessive temporal summation of parallel fiber input in the Purkinje cell dendrites after each conditional stimulus pulse, which could corrupt the expression mechanism to some extent.

Conclusion

The standard models rely on ionotropic glutamatergic and GABAergic receptors, depressed or potentiated with the assistance of mGluR1, CB1R or $GABA_BR$ signaling. The theoretical models by Fiala [39] and Steuber [40] rely on mGluR1-$K_{Ca}1.1/2.2/3.1$ signaling. All of these components can be antagonized without removing the response. In contrast, in our hypothesized mGluR7-Kir3 signaling cascade, blocking either of the components individually diminishes the timed Purkinje cell response. These findings considerably restrict the possible molecular mechanisms underlying the timed Purkinje cell response and points to mGluR7-catalyzed production of Gβγ that activates Kir3. Kir3 channels are in comparison to the K_{Ca} channels better suited for a timed cellular response. In addition to the inherent different

properties of Kir3 multimers, Kir3 activity is regulated *in vivo* [44] over a wide span in the hundreds of milliseconds by the regulator of G protein signaling (RGS) family of proteins, Gα and some members of the Gβ family. For example Gβ5-RGS7 complexes shape vision and motor control by ensuring timely inactivation of G protein responses [45]. The timing, amplitude and duration of Kir3-mediated inhibitory synaptic transmission could vary greatly dependent on which RGS proteins associate with the channel complex [46] and experience-dependent changes in RGS expression has been demonstrated within <1h [47]. Potentially, the learning mechanism could, determined by the interstimulus interval, select for translation or activation the components among these that would bestow the suggested signaling cascade with the appropriate kinetics [43] that matches the interstimulus interval.

Here, our data suggests that at least part of the regulation of timed responses takes place at the protein level within Purkinje cells. If Kir3 activity, perhaps in synergy with other plasticity mechanisms, supports conditioned Purkinje cell responses at multiple interstimulus intervals the implication is that a learned and adjustable kinetic of a metabotropic signaling cascade constitutes a physical memory of temporal duration.

Author contributions

F.J.: Performed surgery, performed research, designed experiments, analyzed data and wrote the manuscript. H.A.E.C.: Contributed to research, experiment design, data analysis and manuscript revisions. G.H.: Contributed to experiment design and manuscript revisions.

Acknowledgements

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Figures

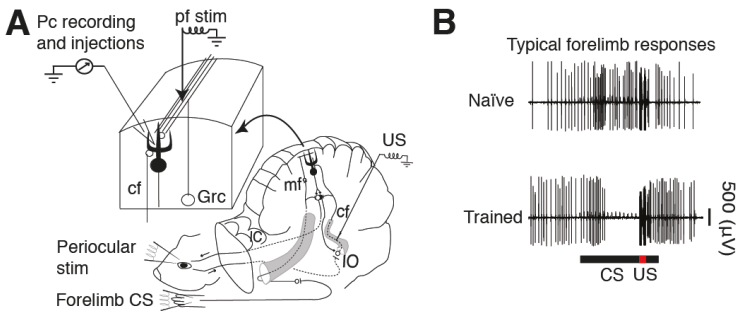

Figure 1. Experimental setup

A. Simplified neural circuitry with stimulation, recording and injections sites. ***B.*** A typical example of naïve and conditioned Purkinje cell responses to a forelimb conditional stimulus. **Key**: CS: conditional stimulus. US: unconditional stimulus. IC: Inferior colliculus. IO: inferior olive. cf: climbing fiber. mf: mossy fiber. Pc: Purkinje cell. Gc: Golgi cell. pf: parallel fibers. Grc: Granule cell.

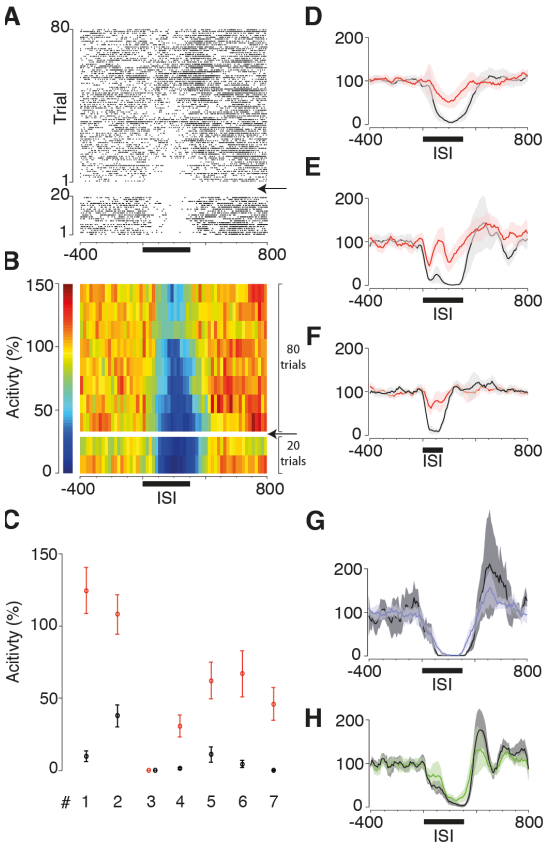

Figure 2. Kir3 supports conditioned Purkinje cell responses

Key: y-axes indicate simple spike firing (% of baseline activity) ***A-B, D-H*;** trials ***A***. x-axes indicate time in ms (-400 to 800 ms) ***A-B, D-H;*** individual Purkinje cells ***C.*** Bars indicate interstimulus intervals (ISI). All traces represent smoothed response profiles. Shading and error bars indicate SEM. Arrows in ***A-B*** indicate injection. **A**. Raster plot of a Purkinje cell's responses to the conditional stimulus before (20 trials) and after (80 trials) injection of 5μM Tertiapin$_{LQ}$. **B**. Population average (n = 7) of Purkinje cell activity before and after injection of 5 μM Tertiapin$_{LQ}$. The color of each square represents the average simple spike activity in a 10 trial, 10 ms bin divided by the average pre-trial frequency in the same bin. **C**. All Purkinje cell responses during the last 100 ms of the interstimulus interval in the same

population as in ***B***, before (black) and after (red) injection of Tertiapin$_{LQ}$. ***D-F***. Averaged temporal response profiles before (black) and after (red) injection of Tertiapin$_{LQ}$ (***D***, ISI 300 ms, 5 μM, n = 7; ***E***, ISI 300 ms, 200 μM, n = 5; ***F***, ISI 150 ms, 25 μM, n = 3). **G-H**. Averaged temporal response profile before (black) and after injection of 50 μM CPG5548 (***G***, blue, n = 2) or 80 μm AM251 (***H***, green, = 3).

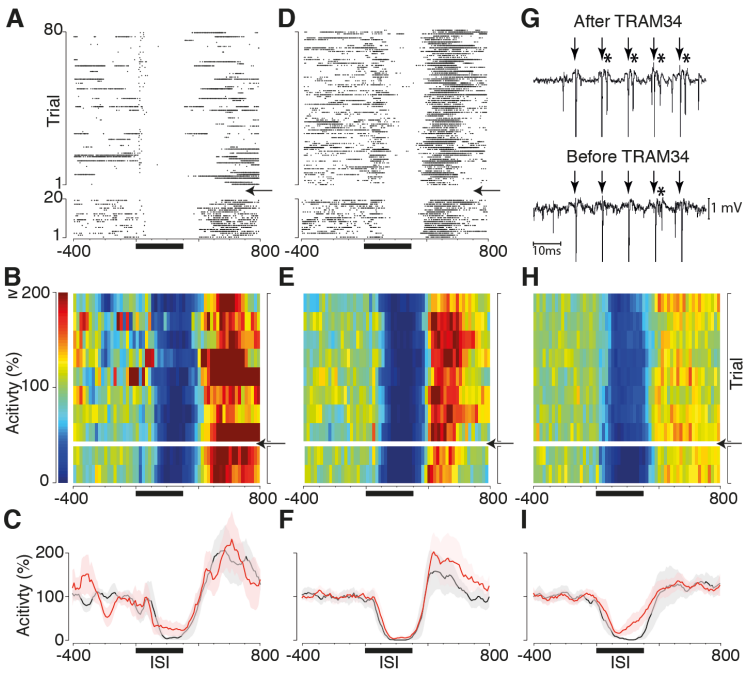

Figure 3. Effects of K_{Ca} channel block on conditioned Purkinje cell responses.

A. Raster plot of a Purkinje cell's responses to the conditional stimulus before (20 trials) and after (80 trials) injection of 60 nM Penitrem A. **B.** Population average (n = 5) of Purkinje cell activity before and after injection of 60 nM Penitrem A. **C.** Averaged temporal response profiles before (black) and after (red) injection of Penitrem A. **D**. Raster plot of a Purkinje cell's responses to the conditional stimulus before (20 trials) and after (80 trials) injection of 1 μM Apamin. **E.** Population average (n = 5) of Purkinje cell activity before and after injection

of 1 μM Apamin. **F.** Averaged temporal response profiles before (black) and after (red) injection of Apamin. **G.** A Purkinje cell's responses to sub-threshold parallel fiber stimulation before (bottom) and after (top) injection of 1 μM TRAM34. Arrows indicate stimulation artifacts and asterisks indicate elicited simple spikes. **H**. Population average (n = 5) of Purkinje cell activity before and after injection of 1 μM TRAM34. **I**. Averaged temporal response profiles before (black) and after (red) injection of Tram 34.

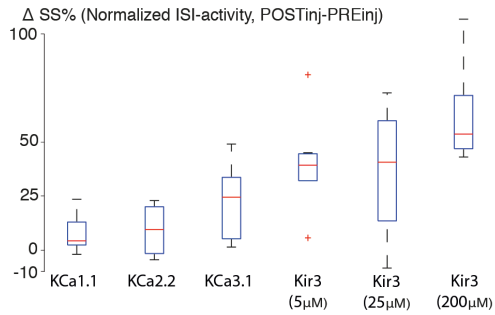

Figure 4. Summary of changes in interstimulus interval activity upon K_{Ca} and Kir3 channel block.

Box plot showing how much cells increased their relative firing during the interstimulus interval after the respective ion channel blocks. The change in firing was calculated by subtracting the average normalized simple spike firing at maximal effect after drug injection with the average normalized simple spike firing before injection.

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Experimental procedures

Surgery and training protocol

15 male one-year old ferrets were surgically prepared with electrical stimulation sites as previously described [9, 13] and approved by the local ethics committee. The conditional stimulus was a 400 ms stimulus train (50 Hz, 1 ms pulse duration, 0.8-1.4 mA) applied to the ipsilateral forelimb. The unconditional stimulus consisted of two 5-pulse 500 Hz stimulus trains (0.1 ms pulse duration, 100-400 μA) separated by 10 ms, applied to ipsilateral climbing fibres 150 or 300 ms after conditional stimulus onset. The intertrial interval was 15 +/-1 s

(randomized). Acquisition sessions with paired conditional stimulus - unconditional stimulus lasted 100-180 minutes.

Recordings and data analysis

Recording technique and analysis software were as previously described [9] with the addition of using Carbostar-4 and Carbostar-6 multibarrel electrodes (Kation Scientific, Minneapolis, U.S.A.) for recording Purkinje cell activity. All data was quantified in 10-ms bins and cell activity reported as a percentage is normalized to activity 600 ms pre-trial and averaged over 20 trials. Raster plots show raw data and traces of cell activity in all figures are smoothed using a five point moving average.

Pharmacology

All drugs were from Tocris Bioscience (Bristol, UK). Stock solutions of Tertiapin$_{LQ}$, Apamin and Penitrem A were prepared by dissolving in H_2O and then diluted in physiological saline. TRAM34 was dissolved in DMSO. All drugs were kept frozen until use and injected with pressure micro-ejections through the multibarrel Carbostar electrodes The micro-ejections were calibrated by ejecting saline into paraffin oil and measuring the droplet size under a microscope against a calibration scale. Pressures were set to achieve a droplet size of (0.5-1.5 nl).

Paper IV

ARTICLE ADDENDUM

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Theoretical considerations for understanding a Purkinje cell timing mechanism

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In classical conditioning, cerebellar Purkinje cells learn an adaptively timed pause in spontaneous firing. This pause reaches its maximum near the end of the interstimulus interval. While it was thought that this timing was due to temporal patterns in the input signal and selective engagement of changes in synapse strength, we have shown Purkinje cells learn timed responses even when the conditional stimulus is delivered to its immediate afferents.[1] This shows that Purkinje cells have a cellular timing mechanism. The cellular models of intrinsic timing we are aware of are based on adapting the rise time of the concentration of a given ion. As an alternative, we here propose a selection mechanism in abstract terms for how a Purkinje cell could learn to respond at a particular time after an external trigger.

Keywords: cerebellum, eyeblink conditioning, glutamate transmission, purkinje cell, temporal timing, control

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In classical conditioning, preceding an unconditional blink-eliciting stimulus with a neutral conditional stimulus at a fixed temporal delay, an interstimulus interval, gives the conditional stimulus the ability to elicit a blink that is timed to that interval. The blink occurs just before the unconditional stimulus.[2] In this learning paradigm, cerebellar Purkinje cells that control the blink learn to respond with a timed pause[3-5] in their tonic inhibition of cerebellar nuclear cells, leading to an excitatory signal that generates the overt blink.[6-8] The conditional and unconditional blink-eliciting signals reach the Purkinje cell via the mossy-parallel fiber system and climbing fibers respectively.[9]

The Timing Mechanism is Intrinsic to the Purkinje Cell

Virtually all neural timing models postulate that neurons learn to time their responses by altering the strength of synaptic connections for selected subpopulations of pre-synaptic neurons.[10,11] Following the onset of a stimulus, different pre-synaptic neurons are assumed to have activity peaks at different times during the interval. The signals in the parallel fibers with a peak towards the end of the interstimulus interval would coincide with the unconditional stimulus and climbing fiber activity. The synapses active at that time would be selectively recruited for long-term depression or long-term potentiation. When learning is complete, those granule cells that peak at the appropriate time control the timing of the Purkinje cell output. Thus, the timing of conditioned Purkinje cell responses would depend on a time code in the parallel fiber afferents transmitting the conditional stimulus.

However, as we have recently shown, adaptively timed responses also occur when the conditional stimulus is direct stimulation of parallel fibers, demonstrating that the response timing does not reflect a temporal code in the input signal, but must be due to a cellular timing mechanism that cannot be explained by changes in synapse strength.[1,12] The Purkinje cell pause response was also shown to be

resistant to pharmacological blockade of inhibitory interneurons. Our finding that the adaptive time course of the Purkinje cell conditioned response depends on a mechanism in the cell itself suggests that a glutamate trigger from parallel fibers activates a cellular mechanism with a particular delay after which a hyperpolarizing response with a specific duration is turned on.

What is the Learning Mechanism?

How can a neuron learn to respond with a particular delay in the range of hundreds of milliseconds between receptor activation and voltage response? Notice that we need both a mechanism for "recording" the time interval between the conditional and unconditional stimuli and a mechanism for generating the delayed response itself.

The first mechanism could involve some cumulative biochemical process that is terminated by the unconditional stimulus. One may envision that neurotransmitter receptor activation leads to a gradual build-up in the concentration of a given ion or second messenger molecule until some threshold level is reached. The second mechanism could be that this accumulation somehow also acquires the ability to hyperpolarize the cell.

If a receptor is coupled directly or indirectly to a rise in the concentration of a substance *x* that can acquire the ability to trigger a voltage response, the time delay between receptor activation and voltage response will depend on the number of receptors that are activated. If there is an *x*-dependent feedback connection that adjusts the number of available receptors, the neuron can learn to adjust the delay.

In an implementation of this theory, Steuber and Willshaw[13] proposed that Ca^{2+} dependent phosphorylation of receptors could implement adjustable delays in this way. Activation of many different receptors produces a temporary increase in post-synaptic $[Ca^{2+}]$ and the latency of this response can range widely depending on the number of available receptors and second messengers, the number of steps between receptor activation and Ca^{2+} rise and the rate constants at the different steps. Decreasing the number of available receptors leads to an increase in the latency of the Ca^{2+} rise. Any delay to a threshold level of $[Ca^{2+}]$ can then be learnt if two antagonistic biochemical processes control the number of available receptors.

Simplified, the specific mechanism here is that before training the number of available receptors is large and Ca^{2+} influx causes regular depolarization because most Ca^{2+} activated hyperpolarizing channels are inactivated. The conditional stimulus also evokes PKC synthesis, which increases the number of receptors available.

During training, presentation of the unconditional stimulus evokes PKG production that decreases the number of available receptors, rendering a slower $[Ca^{2+}]$ rise. The conditional stimulus evoked Ca^{2+}/PKC peak moves towards the unconditional stimulus evoked PKG peak until they both overlap and equilibrium between PKG induced receptor decrease and PKC induced receptor increase is reached. The $[Ca^{2+}]$ rise latency now also matches the interstimulus interval. Coincident PKC and PKG activation further leads to phosphorylation and activation of Ca^{2+} activated K^+ channels. The conditional stimulus response is thus gradually transformed into a hyperpolarization response around the time of the unconditional stimulus presentation.

This model, indeed any model that depends on an adjustable concentration rise, raises several difficulties.

First, a learning mechanism that depends on adjusting the latency of a [*x*] rise will be sensitive to the duration and frequency of the conditional stimulus. However we showed, consistent with data on both overt and Purkinje cell conditioned responses,[14,15] that for instance a conditioned response, that was timed to a 150 ms interstimulus interval, was the same on post-training probe trials whether we delivered eight pulses at 400 Hz (17.5 ms) or 81 pulses at 100 Hz (800 ms). It is difficult to see how such disparate receptor activations could render the same [*x*] rise in the Purkinje cell.

Second, if the interstimulus interval is changed after learning, these models predict that the response will move in time to the new location of the unconditional stimulus. This is not what occurs, however. Both at the behavioral[2] and at the Purkinje cell[1,5] level, the old response is extinguished and the new response at the new time is acquired separately.

Third, both in behaving animals trained with alternating interstimulus intervals[16] and in Purkinje cells re-trained to a new interstimulus interval[1,5] double peaked responses can be observed. As noted by Steuber and Willshaw, it is difficult to account for this with a model based on adjustable concentration rise latencies.

Fourth, whereas models such as these predict that conditioning should occur in Purkinje cells with short interstimulus intervals (<100 ms), we have shown that it does not.[17]

As an alternative to earlier timing models, we would like to propose a selection mechanism. Let us imagine the existence of what we might name "timer units" (receptor subunits, proteins, channels. . .) that would provide receptors (or molecular structures that are activated by them) with distinct temporal activation profiles. The learning process would then select, among a finite number of such units, a combination that matches the temporal interval. These timer units are the effector components that generate a response at the right time.

Instead of the time tracking that starts with the onset of the conditional stimulus being a rise in the concentration of a given ion, we can envision either a cascade of second messengers, a protein changing its conformation over time or a series of molecular switches. The logic of the hypothesis does not require specification of either one of these so let us call it the 'recorder' and let it, for the sake of argument, be a protein changing its conformation over time.

At the onset of the conditional stimulus the 'recorder' proteins start changing in a predictable way. We assume four possible conformational states: '-', *A, B* and *C*. Suppose that for the first 100 ms they are all in the '-' state, between, say, 100-250 ms most are in the *A* state, between 200-350 ms most are in the *B* state and between 300-400 ms in the *C* state. Whether '-', *A*, *B* and *C* in fact are different conformational states of a protein,

different molecules in a second messenger cascade transiently being present or some form of hitherto unknown molecular switch does not matter.

Assume further that the recorder proteins interact with the unconditional stimulus in different ways depending on when it arrives. Suppose that when they are still in the ' -' state there is no effect, but when they are in either the *A*, *B* or *C* states, different activation sites are available for the unconditional stimulus. Activating the recorder proteins in one of the states may then cause translation or activation of particular timer units. In this way the learning mechanism selects appropriate timer units (the effector components that generate a response at the right time).

Note that one would not need many different states of the recorder proteins nor a large number of timer units they select from when activated in particular states, in order to learn many different temporal intervals. Recall that in the retina, a combination of only three types of cones is enough to represent the entire visible color spectrum.

Suppose that the timer units A*, B* and C* generate responses with maximum amplitudes at 150 ms, 250 ms and 350 ms respectively. Training with an interval of 150 ms might only lead to activating the recorder in the *A* state, which translates/activates the pool of timer units *A*A*A*A** that in turn produces a response with a maximum amplitude at 150 ms. Training with an interval of 300 ms would lead to a pool of units *B*B*B*B** with a maximum at 250 ms. Training with 215 ms might lead to a pool of *A*A*B*B** with a maximum somewhere between 150 ms and 250 ms, say 200 ms and training with 400 ms would lead to *C*C*C*C** with a maximum at 350 ms.

Such a mechanism could explain more of the experimental data such as the ability of very short conditional stimuli to elicit full responses. After learning, once a glutamate trigger has started a timer unit, it runs its course with a particular delay. On and offset of the response is the same regardless of variations in the conditional stimulus parameters. Concentration rise models would by necessity be affected by further input after the initial trigger. That is however not automatically the case here. If the timer units work like a kitchen timer they would not necessarily be re-started by further input.

There is also no need for the conditioned response to gradually move in time when a cell is re-trained to a new temporal interval. During initial training selection of timer units *A*A*A*A** leads to a response latency of 150 ms. When the unconditional stimulus is moved to 400 ms the learning mechanism starts selecting *C*C*C*C** instead. A sufficient number of timer units with delays of 200-300 ms are never selected so the response does not gradually move in time from 150 ms to 400 ms. Furthermore, there is no reason why a Purkinje cell could not harbor multiple responses at once. If it is alternately trained with interstimulus intervals of 150 ms and 400 ms, every other trial will result in the recorder selecting timer units *A*A*A*A** and *C*C*C*C** respectively. Eventually two responses will appear. If the unconditional stimulus arrives in <100 ms, the 'recorder' is in the ' -' state and no timer units are selected.

At this point, we cannot speculate further on the exact nature of the hypothetical timer units but we suggest that it could be worthwhile to try to identify them. However, given the surprising existence of a temporal memory, we expect the explanation to have more surprises in store for us.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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