Learned response sequences in cerebellar Purkinje cells

Dan-Anders Jirenhed[a,b,1], Anders Rasmussen[a,b], Fredrik Johansson[a,b], and Germund Hesslow[a,b]

[a]Associative Learning Group, Department of Experimental Medical Science, Lund University, 221 84 Lund, Sweden; and [b]The Linnaeus Centre Thinking in Time: Cognition, Communication and Learning, Lund University, 221 00 Lund, Sweden

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Associative learning in the cerebellum has previously focused on single movements. In eyeblink conditioning, for instance, a subject learns to blink at the right time in response to a conditional stimulus (CS), such as a tone that is repeatedly followed by an unconditional corneal stimulus (US). During conditioning, the CS and US are transmitted by mossy/parallel fibers and climbing fibers to cerebellar Purkinje cells that acquire a precisely timed pause response that drives the overt blink response. The timing of this conditional Purkinje cell response is determined by the CS–US interval and is independent of temporal patterns in the input signal. In addition to single movements, the cerebellum is also believed to be important for learning complex motor programs that require multiple precisely timed muscle contractions, such as, for example, playing the piano. In the present work, we studied Purkinje cells in decerebrate ferrets that were conditioned using electrical stimulation of mossy fiber and climbing fiber afferents as CS and US, while alternating between short and long interstimulus intervals. We found that Purkinje cells can learn double pause responses, separated by an intermediate excitation, where each pause corresponds to one interstimulus interval. The results show that individual cells can not only learn to time a single response but that they also learn an accurately timed sequential response pattern.

cerebellum | Purkinje cells | learning | timing | classical conditioning

Playing the piano, typing on your keyboard, and uttering a sentence are all typical examples of complex behaviors. Although they involve simple movements as building blocks, they need to be executed with great temporal precision and in a specific sequential order. Utter phonemes in the wrong order or with incorrect timing, and the result is incomprehensible.

Learning of accurately timed movements is exemplified by classical conditioning of motor responses such as the eyeblink response (1–3). In the simplest case, a neutral conditional stimulus (CS), usually a tone, is followed by a blink-eliciting unconditional stimulus (US), for example, a puff of air to the cornea, after a fixed interval (the interstimulus interval, ISI). The subject learns to emit a conditional blink response (CR) that is timed so that the maximum amplitude is reached close to the time of US onset. Eyeblink conditioning depends on the cerebellar cortex (4–6) and the overt CRs are driven by learned pause responses in the spontaneously active cerebellar Purkinje cells (7–9). These cells receive the CS signal via the mossy fiber/parallel fiber system, and the US signal via the climbing fibers from the inferior olive (10, 11). The conditional Purkinje cell response (Purkinje cell CR, or PcCR) is elicited by input from the parallel fibers (12), triggering a delayed and adaptively timed pause in the Purkinje cell's simple spike firing (13), illustrated in Fig. 1*A*. Importantly, the PcCR is accurately timed even when the CS consists of a uniform and repetitive train of electrical pulses applied directly to the mossy fiber or parallel fiber afferents; that is, when the CS input signal to the cell contains no temporal code (14).

Eyeblink conditioning with mixed ISIs, that is, using different intervals between CS and US on alternating trials, produces more complex temporal patterns in the blink CRs. The responses are less stereotypical than those obtained with a single ISI, often consisting of long-duration blinks or multipeaked blinks that form response sequences (see Fig. 1*B* for illustrations), with temporal profiles adapted to the ISIs (15, 16). Excitatory response patterns that match double-peaked blink responses have also been observed in the anterior interpositus nucleus, the downstream target of the blink controlling areas in the cerebellar cortex (17).

Several important questions are raised by these findings. First, it may be asked whether a Purkinje cell can learn more than one interval; that is, can the cell learn to respond to a uniform repetitive CS containing no temporal code, with sequences corresponding to the long-duration or double-peaked eyeblink CRs, after training with alternating ISIs? There are data that suggest this is the case, but they consist only of unsystematic observations in a very small number of subjects: three decerebrate ferrets (12, 13) and two intact rabbits (18). Second, if a cell can learn a response sequence, the question may be asked of how the components of such a sequence are related to each other. For instance, is a double pause response a composite of separate response components, or should the whole response sequence be regarded as a single unit? If they are composites of separate components, then it should be possible to learn each component independently. Conversely, if it is a single response unit, then the whole sequence conceivably also could be elicited with shorter versions of the CS, as has previously been shown for single PcCRs (19) and eyeblink CRs (20).

To answer these questions, we studied Purkinje cells in decerebrate ferrets during training with a classical conditioning paradigm, using a mossy fiber CS and climbing fiber US, with alternating ISIs.

Results

We made extracellular recordings in vivo, lasting for 3–12 h, of activity in 27 Purkinje cells in 22 decerebrated and immobilized male ferrets. Behavioral data were thus not collected, as the long extracellular recording sessions required immobilization for sufficient tissue stability. All recordings were made in a microzone within the cerebellar C3 zone that controls the conditional blink response (7). The CS was a uniform and repetitive stimulus consisting of direct electrical stimulation of mossy fibers at 50 Hz for 600 ms (or 800 ms in two cases). Climbing fibers were

Significance

Learning is thought to rely on the strengthening or weakening of synapses. However, we have previously shown that a neuron can also learn when to time its response so that the timing reflects the interval between two stimuli, without any temporal information in the input signal. Here, we report that a neuron can even learn a sequence of at least two, and probably more, accurately timed responses. A single cell is in a sense "programmable," and can encode a temporal response pattern. This means that the nature of what a cell can learn is very different from the traditional view, and that the information storage capacity may be far greater.

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[1]To whom correspondence should be addressed. Email: dan-anders.jirenhed@med.lu.se.

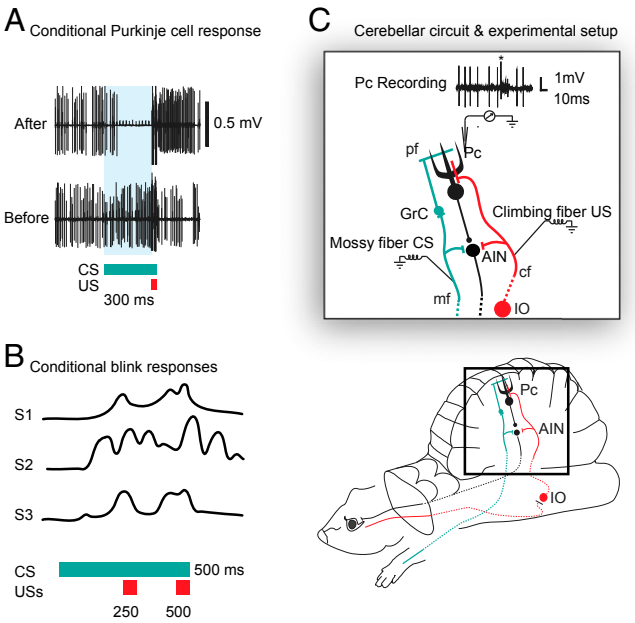

Fig. 1. Eyeblink conditioning and Purkinje cell recording. (*A*) Extracellular records from sample Purkinje cell in the C3 zone of the cerebellum that responded with a weak excitation to the CS before training, but acquired a Purkinje cell CR (a pause) after 2 h of training (ISI, 300 ms). (*B*) Learned blink responses in rabbits. Sample traces adapted from Hoehler et al. (15) illustrate learned sequential blink responses in three subjects, S1–S3. After training with alternating trials with 250- and 500-ms ISIs, blink responses displayed complex temporal profiles that were shaped by the two ISIs used. (*C*) Cerebellar circuit and experimental setup. Illustration of a ferret face and forelimb and decerebration transection just rostral to the superior colliculi. Above the illustrated electrode is a typical Purkinje cell (Pc) record with simple spikes and a complex spike (*). Mossy fibers (mf) and climbing fibers (cf) were stimulated electrically as proxies for a forelimb CS and periocular US, respectively. US signals (red) from the periocular area, via the inferior olive (IO) and cf, converge on blink controlling Purkinje cells with CS signals (green) transmitted via mossy fibers (mf), granule cells (GrC), and parallel fibers (pf). Inhibitory interneurons are omitted for clarity. Since Purkinje cells are inhibitory, a conditional pause response produces a blink by disinhibiting the anterior interpositus nucleus (AIN), which in turn activates the red nucleus and the facial nucleus.

electrically stimulated for 10 ms at 500 Hz as a proxy for the blink-eliciting periocular US (21, 22) (Fig. 1*C*).

During training, we alternated between two ISIs so that the US was presented after either 150 or 450 ms after CS onset. On every tenth trial, the CS was presented alone to probe for learning effects.

The choice of ISIs was determined by two considerations. If the difference between the two ISIs is too small, there is a risk, suggested by the behavioral literature (3, 15, 16, 23) and our own pilot experiments, that two PcCRs may merge together, rather than form two distinct pauses. Using very different ISIs, in contrast, means the second has to be quite long. Because learning to long ISIs is considerably slower than learning to shorter ones (3, 13, 24), it is difficult to obtain learning in the limited time available in the acute decerebrate preparation.

To study the acquisition of PcCRs, we recorded the activity of seven cells during training from the naive state in which the CS elicited an excitatory simple spike response. As the CS was repeatedly paired with the US, at alternating ISIs, we observed acquisition of reliable PcCRs over the course of 2.5–5.5 h of training.

Of the seven cells, four acquired a response sequence, with two distinct pauses separated by an excitatory response (Fig. 2 *A* and *B*). In one case, where a cell was lost, recording from a second cell started after 1.5 h of training, but before any PcCRs had been acquired (Fig. 2*C*).

In two of the seven experiments, we wanted to test whether cells could learn a third interval, so an additional US after 300 ms was introduced, thus alternating between three ISIs. One example of training with three ISIs is illustrated in Fig. 2*D*. After

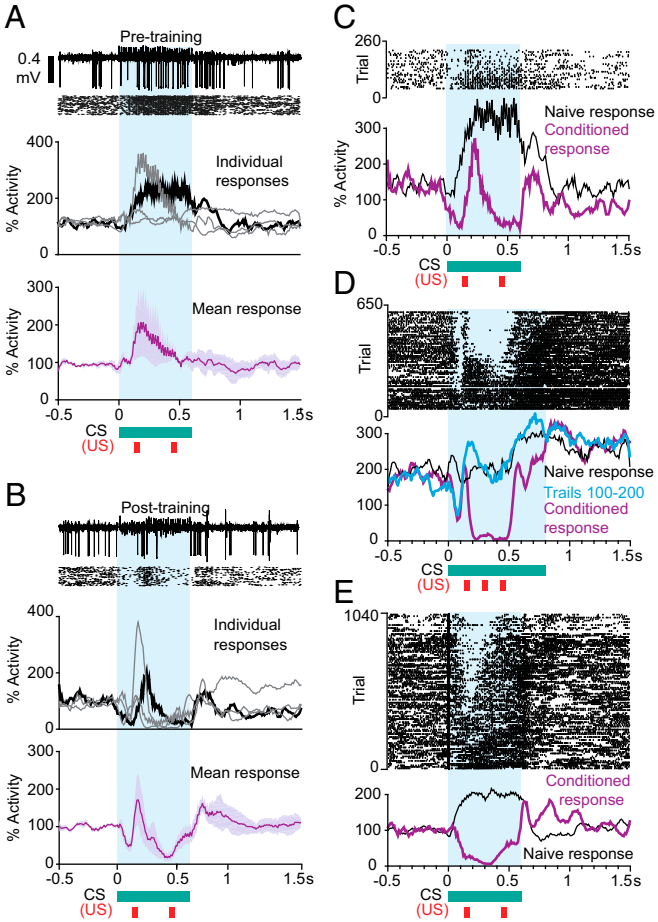

Fig. 2. Acquisition of Purkinje cell CRs. All illustrations represent simple spike activity during trials in which the CS was presented alone. Presentation of the CS (green) and time of US presentations on paired CS–US trials (red). Time window during CS presentation (blue shading). Response profiles (line graphs) illustrate simple spike activity relative to background activity. (*A* and *B*) Pretraining responses (*A*) and posttraining responses (*B*) from four cells that acquired sequential PcCRs. (*Upper*) Record showing single-cell response to CS presentation on a single trail. Stimulus artifacts have been edited from the record. Below that is a raster plot of simple spikes in the same cell during 20 consecutive CS presentations. The highlighted response profile (black) shows the same cell's mean activity relative to background. Gray traces show response patterns from the other three Purkinje cells that acquired sequential PcCRs. Lower panels in *A* and *B* illustrate the mean response profiles (dark purple line) ± SEM (purple shading) for the same four cells, pretraining (*A*) and posttraining (*B*), respectively. (*C–E*) Raster plots illustrate simple spikes during CS-alone probes presented on every tenth trial during training. Response profiles below raster plots illustrate simple spike activity relative to background activity. Black graphs illustrate response profiles before training (Naive) and purple graphs after training (Conditioned). (*C* and *E*) Alternating ISIs were 150 and 450 ms. (*D*) An additional ISI of 300 ms was used. (*C*) Before the cell was found, 2 h of training with paired CS and US presentations had already taken place. It had not yet acquired a conditional response, but did so within the 260 trials that were recorded. (*D*) Three ISIs of 150, 300, and 450 ms were used. The additional blue response profile illustrates that the first response component, timed to the shortest interval, was acquired first and was present already on trials 100–200. The cell in *E* acquired a long-duration pause PcCR (instead of a sequential PcCR) during 4.3 h of training.

almost 3 h of training and recording from the naive state, two distinct pauses and an intermediate excitation were observed. The second pause had a long duration and started before the end of the second ISI (300 ms) and lasted beyond the third ISI (450 ms). However, the difference between the ISIs may have been too small to produce three clearly separable pause components, and therefore this line of experimentation was not pursued further.

In the four cases in which sequential PcCRs were acquired during training while recording, the early pause was always acquired before the late pause (compare Fig. 2 *C* and *D*). Furthermore, the response property that was learned first was the latency to pause onset.

Two of the seven cells acquired a single long-duration pause response starting before the first anticipated US (ISI 150 ms) and ending after the last anticipated US (ISI 450 ms). One is illustrated in Fig. 2*E*. Finally, one cell acquired an early pause response timed only to the first ISI.

In addition to the seven cells that were studied during learning from the naive state, we recorded activity in 20 cells that had learned reliable PcCRs after 5.5–12 h of training.

All cells displayed PcCR pauses, but with varying characteristics in their temporal profiles. Based on the timing of pause onsets, maxima, offsets, and occurrence of excitatory response components, we divided the observed PcCRs into four categories.

The most frequent response type (observed in 13 of the 27 cells) was a sequence of two pauses separated by an excitatory response, here called a sequential PcCR, as it was not simply a dual pause response (compare Fig. 3 *A* and *B*).

The latencies of the response components varied between cells (Fig. 3*A*, *Bottom*). This significantly affected the mean response profile and smoothed out different components, although the general pattern was still clear (Fig. 3*B*). The mean response profile was characterized by a short-latency pause (P1) followed by spiking activity (here called the middle peak), followed by a second pause (P2). The two pauses were timed to the two ISIs;

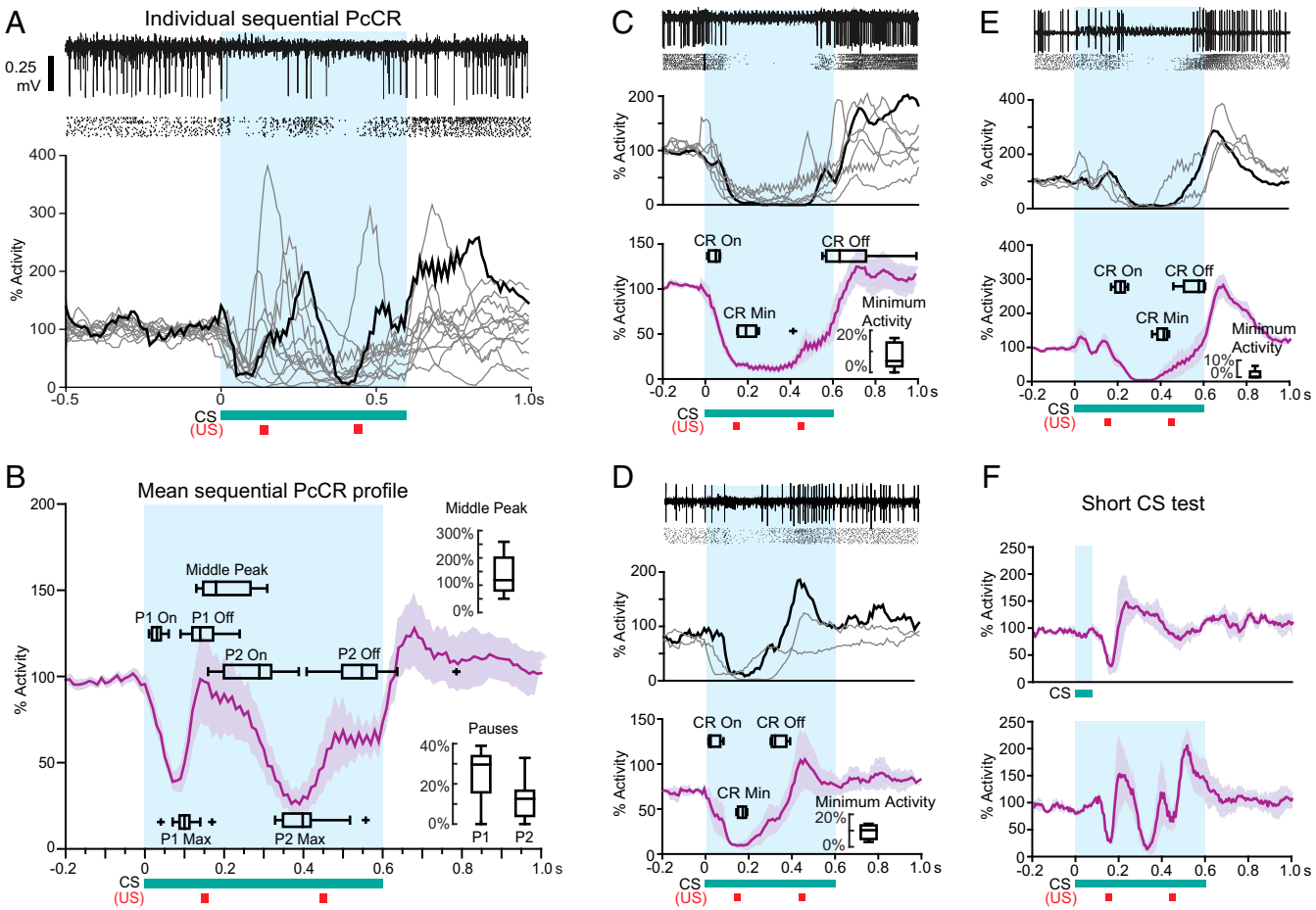

Fig. 3. Purkinje cell CR types. (*A*) Sample Purkinje cell that acquired a sequential PcCR. (*Upper*) Record showing sequential PcCR in response to CS presentation on a single trail. Stimulus artifacts have been edited from the record. (*Middle*) Raster plot of simple spikes in the same cell during 20 consecutive CS presentations. (*Bottom*) The highlighted response profile (black) shows the cell's mean activity relative to background. Gray traces show response patterns from the other 12 Purkinje cells that acquired sequential CRs. (*B*) Population data for all 13 Purkinje cells that acquired sequential PcCRs. Mean response profile (magenta) ± SEM (purple shading) and boxplots (black). The onset, maximum, and offset of the first pause (P1) and second pause (P2) and their respective activity levels relative to background. The pauses are separated by a middle peak of spiking activity ranging from 50% to 250% of background. Boxplots show median (line) and 25th and 75th percentiles (box edges); whiskers extend to the most extreme data points not considered outliers, and outliers are plotted individually. (*C–E*) Other Purkinje cell CRs. (*C*) Long-duration PcCR ($n = 7$ cells). (*D*) Early PcCR ($n = 3$ cells). (*E*) Late PcCR ($n = 4$ cells). Each panel includes example records of individual cells producing a PcCR in response to CS presentation on a single trail (*Upper*) and raster plot of simple spikes in the same cell during 20 consecutive CS presentations. The highlighted response profile (black) shows the cell's mean activity relative to background. Gray traces show response patterns from the other Purkinje cells of the same response category. (*C–E*, *Bottom*) Population mean response profiles (magenta) ± SEM (purple shading) and boxplots (black) for the response components. (*F*, *Top*) Response to presentation of a short-duration (80 ms) CS consisting of the first five stimulus pulses. In comparison with the full duration CS (*Bottom*), the short CS elicited the first pause and the following excitation, but not the second pause.

that is, P1 appeared near ISI A and P2 near ISI B, and they were separated by a 100–400-ms period of spiking activity that often exceeded baseline level.

This interpretation of the response profile was confirmed in a second analysis in which the latencies to different response components were identified for each individual cell, and their distributions were visualized as boxplots, illustrated in Fig. 3*B*. The response component latency medians were in sequence: P1 onset, maximum, and offset, followed by the middle peak and P2 onset, maximum, and offset. Mean activity levels for P1 and P2 maximum ranged from 0% to 40% of baseline. A majority of the middle peaks exceeded the pretrial baseline activity, sometimes reaching up to 380%.

The second most common response type (observed in seven of the 27 cells) was a single long-duration pause that covered both the short and the long ISIs; that is, the pause started earlier than 150 ms after CS onset and ended later than 450 ms after CS onset, as illustrated in Fig. 3*C*. Compared with the sequential PcCRs, the temporal profiles of long-duration responses were less variable with regard to their latencies to onset and offset. In addition, minimum activity during the long-duration PcCR was on average lower than for most sequential responses, with mean activity levels at less than 10% of baseline.

The third and fourth types of conditional responses were timed to either the shorter or the longer ISI. The early PcCR (Fig. 3*D*) was observed in three cells across three animals. This pause response was timed only to the short ISI; that is, it started with a short latency after CS onset, reached its activity minimum within 150 ms, and had a duration shorter than 300 ms, thus ending before the time of US presentation on long ISI trials.

The late PcCR (Fig. 3*E*) was observed in four cells across four animals. It was a pause response that was timed to only the long ISI. The response had an onset latency that was longer than the short ISI (150 ms), but reached its activity minimum before the long ISI (450 ms) and sometimes had a duration that extended beyond the long interval.

With regard to the properties of the sequential PcCRs, we wanted to test the possible independence of the different response components within a sequence. To probe this, we performed a test in which we presented of a short version of the CS.

In previous work (19), in which we studied the temporal characteristics of single-pause PcCRs, we were often able to elicit the full PcCRs with a short version of the CS. We wanted to investigate whether this was also possible for sequential PcCRs. We presented a short CS, consisting of only the first five electrical pulses (i.e., a stimulus with an 80-ms duration), while we recorded the activity in four cells that displayed clear sequential PcCRs. In all four cases, the short CS elicited the first two response components (i.e., the early pause and the following excitation, but not the late pause component; Fig. 3*F*).

Discussion

The purpose of this study was to investigate what responses Purkinje cells learn when they are trained with a uniform CS and a US that is presented after mixed temporal intervals. In eyeblink conditioning, such training protocols are known to produce complex response sequences and long-duration responses (15, 16).

The first question we wanted to answer was whether a Purkinje cell can learn more than one ISI and respond with temporal sequences that could generate double-peaked or long-duration overt blink CRs. The results show that Purkinje cells were indeed capable of learning more than one interval. Most cells learned a sequential response pattern with pauses timed to each of the two ISIs (13 of 27 cells), or a long-duration pause that extended over both ISIs (seven of 27 cells). In the remaining seven cases, cells acquired pause responses that were timed to either one of the two ISIs used during training.

To promote learning of clearly separable response components, we wanted to use ISIs that were sufficiently different that separate pause responses would not merge into one. This meant the second ISI had to be relatively long compared with the first. However, conditioning with long ISIs requires more time (3, 13, 24), and the acute setup in these experiments provides only a limited window for studying learning effects. In this trade-off, we settled on a paradigm with a 300-ms difference between the two ISIs (150 and 450 ms). This factor is a plausible cause of the observed long-duration pauses.

Importantly, the particular ISIs used here are not the only ones that can produce adaptively timed sequential PcCRs. This is illustrated by a case in a previous study (13), in which a cell had first been conditioned with a mossy fiber CS to an interval of 200 ms that was later shifted to 600 ms, after which it learned a response sequence that was adaptively timed to both intervals (compare Fig. 4c in ref. 13). Similarly, Fig. 2H in ref. 12 illustrates a case with well-timed response sequences to a parallel fiber CS after an ISI shift from 200 and 350 ms. In addition, double Purkinje cell pauses in two conditioned rabbits after training with ISIs of 200 and 700 ms have been reported by Halverson et al. (18).

These observations of sequential PcCRs, together with the observation of dual responses in the anterior interpositus nucleus reported by Choi and Moore (17), strongly suggest it is sequential PcCRs that produce the complex overt response patterns seen in eyeblink conditioning with mixed ISIs and after ISI shifts.

The second question we wanted to answer was how response components are related to each other. Let us first consider the possibility that two pauses occur because the CS elicits the same pause response twice. This interpretation can be rejected because the different pauses were not acquired simultaneously during training: The early pause was always learned before the late pause (see raster plots in Fig. 2 *C* and *D* for examples). The latter was thus not merely a repetition of the first, as it should have been elicited twice by the continuous CS once it had been learned. Instead, we believe the response pattern should be interpreted as a sequence with two different pause responses, separated by a brief period of simple spike firing. Further evidence in support of this interpretation comes from previous studies in which, when training with a single ISI, we have applied long-duration CSs that outlast the ISI by several hundred milliseconds (12, 13, 19, 25), but almost never observed more than one pause except when two ISIs have been used during training.

If the second pause in the sequential PcCR was not a repetition of the first pause, could the first pause be elicited independent from the second? We have previously found, after training with single ISIs, that a mossy fiber CS lasting only a few milliseconds or tens of milliseconds can elicit a full-blown, accurately timed PcCR (19), as well as a complete and accurately timed blink CR (20). If the sequential PcCR is a single response unit, then a CS that elicits the early components could potentially also elicit the later ones. However, short CSs that elicited the first pause, and the peak in spiking activity afterward, did not elicit the second pause. Because the second pause was not elicited by the early part of the CS, or as a consequence of the early pause or the middle peak, the second pause component was most likely elicited by signals arriving at the Purkinje cell later than 80 ms after CS onset.

What could be the source of these late signals? In an awake animal that has been conditioned to blink twice, the first blink will elicit sensory feedback that could potentially act as a CS for a second blink. However, with regard to the PcCRs in our experiments, such sensory feedback was excluded as a second CS, as the animals were immobilized and could not blink. Nevertheless, one could imagine that the first Purkinje cell pause produced neural activity downstream in the anterior interposed nucleus, the red nucleus or the facial nucleus, that eventually was fed back

up to the Purkinje cell via mossy fibers or nucleocortical projections (26), as an efference copy that could act as a CS for the second pause. The trials with short CSs are a test of this idea as well. The short test CS elicited the first pause and the following excitation, but did not elicit a second pause. An efference copy or feedback signal was thus unlikely to play any role in eliciting the second pause, as it should have been present also in the short test CS case. Rather, it suggests additional (i.e., later) stimulus pulses in the mossy fiber CS were necessary to elicit the second pause component. The second pause was thus a separate response, and it was in response to a particular part (i.e., a later part) of the CS. A behavioral phenomenon that may be related to this PcCR property is the observation that a short (50 ms) CS could elicit blink responses after training with a 250-ms ISI, but could not elicit long latency responses after training with a 500-ms ISI (27).

The long-duration pause responses that were sometimes observed could plausibly be interpreted as double-pause response sequences lacking the intermediate simple spike firing. They were thus probably two differently timed pause responses that had merged into one, meaning the long-duration response profile illustrated in Fig. 3*C* could be a summation of short-latency and long-latency responses with profiles resembling those illustrated in Fig. 3 *C* and *D*, respectively. As was the case for the Purkinje cells that learned sequential responses, the cells we observed as they learned long-duration pauses also acquired the short latency pause onset before the longer one. Again, the first response property that was learned was the latency to onset.

The excitatory simple spike components that followed pauses were observed in all cells except two, regardless of whether the response profile was sequential, a long-duration pause, or timed to only one of the intervals. The offset of the CS and the offset of pause often overlapped, making it hard to tell which was the cause. However, the short CS tests also elicited an excitatory component after the pause in all cases, suggesting rebound excitation after pauses was the main cause of increased activity, although a delayed and adaptively timed excitatory response to the initial parts of the CS may also have been involved. In previous investigations of single PcCRs (12, 13, 19, 28), we sometimes observed excitation after the pause when the CS was delivered to the mossy fibers or parallel fibers and extended beyond the CS duration. Similar excitation was observed here. This suggests a direct mossy/parallel fiber CS may recruit more parallel fibers than a peripheral CS. In addition, in cases in which PcCR offset and CS offset did not coincide, the increased activity coupled to CS offset and not PcCR offset suggests tonic inhibition from molecular layer interneurons may also have been elicited by the CS.

One characteristic property of PcCRs is that they can be acquired to CSs consisting of repetitive trains of electrical stimuli delivered for several hundreds of milliseconds. In all cases, the PcCRs are timed to the ISI (7, 13), even though the CS is uniform. Importantly, Purkinje cells acquire the same PcCR regardless of where in the signal pathway the CS is applied. The CS can be a peripheral sensory stimulus consisting of subcutaneous electrical stimulation of the forelimb, or it can be direct electrical stimulation of mossy fibers projecting to the cerebellum, or even direct stimulation of the parallel fibers (12). In all cases, the PcCR displays a characteristic adaptively timed temporal profile that is shaped by the ISIs used during training (7). The most parsimonious explanation for this, in our opinion, is that the different CSs elicit similar parallel fiber activation in response to each electrical pulse in the CS, regardless of where it is delivered.

Earlier theories of cerebellar response timing have generally assumed that temporal information in the CS is transmitted to the Purkinje cell by numerous parallel fibers with different time-varying activity patterns that span several hundreds of milliseconds (29–31). The mechanism by which a Purkinje cell learns when to respond was supposed to be realized by selectively modifying the strength of those parallel fiber synapses that are active around the time of the US.

We have previously shown that a Purkinje cell could learn an adaptively timed PcCR to a uniform parallel fiber CS where no temporal information in the CS signal was present (12). Furthermore, expression of the PcCR was independent of GABAergic transmission from inhibitory molecular layer interneurons. Instead, the PcCR seems to be elicited by glutamate from parallel fibers acting on type 7 metabotropic glutamate receptors (32). The temporal memory is therefore unlikely to be encoded exclusively in synaptic connection strength, but probably also involves a timing mechanism within the Purkinje cell (14).

The question now arises whether a Purkinje cell that has acquired a sequential PcCR has memorized more than one time interval. We think the observations presented here suggest this is the case, and that they constitute additional evidence challenging the adequacy of the standard theory of temporal learning as relying exclusively on strengthening and weakening of synaptic connections (see e.g., ref. 33 for a discussion). The results are in better agreement with theories suggesting the existence of additional intracellular mechanisms for storage of temporal relations in associative learning and for response timing (34–36). However, the inadequacy of synaptic weight changes to account for temporal memory traces does not mean the learning is not synapse specific. Most likely, the memory trace is coupled to a specific subset of synapses, or dendritic compartments (37), but it is not exclusively reliant on a change in the strength of those synapses. The memory trace also probably includes a postsynaptic intrinsic mechanism for response timing that can produce timed pauses in simple spike activity by delayed hyperpolarization.

In conclusion, individual Purkinje cells are able to learn sequential response patterns that include both pauses and increased simple spike activity. Such sequential Purkinje cell responses may underlie learned motor sequences that have been shaped by sensory feedback signals arriving with different delays, such as, for example, the musical tones that are produced by finger movements while playing the piano. The ability of single Purkinje cells to learn response sequences suggests cerebellar control and coordination of motor behaviors may rely more on intracellular mechanisms and less on neuronal network properties than previously thought. It also suggests the capacity for information storage in the individual neuron is vastly greater and of a very different nature than suggested by the dominant paradigm.

Materials and Methods

Surgery and Stimulation. Twenty-two adult male ferrets (1–2 kg) were surgically prepared, decerebrated, and implanted with stimulation electrodes for direct activation of cerebellar afferents (mossy fibers in the middle cerebellar peduncle and climbing fibers in the inferior cerebellar peduncle), as described previously (28). The experiments were approved by the Malmö–Lund animal experimentation ethics committee.

Training Protocol. A stimulation protocol analogous to classical conditioning was used during training. The CS consisted of electrical pulse stimulation trains with 50–100 μA intensity, applied to the mossy fibers. Each stimulation train consisted of 31 pulses delivered at 50 Hz (duration, 600 ms), except in two cases, in which 41 pulses were used instead (duration, 800 ms). The US consisted of an electrical stimulation train of six pulses delivered at 500 Hz (duration, 10 ms) applied to the climbing fibers with an intensity of 20–700 μA. This was intended to mimic a strong olivary response that occurs in response to a periocular US (21, 28).

The ISI, that is, the time between presentation of the CS and the US, was alternated between trials. In all but two experiments, the ISIs were alternated between 150 and 450 ms on every other trial. In the other two experiments (using the 800 ms CS), the ISIs were alternated among 150, 300, and 450 ms on every third trial. In addition to the paired stimulus trials, every 10th trial was a probe trial in which the CS was presented alone. The intertrial interval was 15 ± 1 s in all experiments.

Purkinje Cell Recordings and Data Analysis. Extracellular recordings of Purkinje cells, identified by the presence of complex spikes, were performed using 25-μm metal core diameter, quartz glass-coated platinum–tungsten fiber microelectrodes with an impedance ranging from 5 to 10 MΩ (Thomas Recording GmbH). The signal from the microelectrode was fed through a preamplifier and filter module from Digitimer before entering a Power 1401 data acquisition and AD converter (Cambridge Electronic Design Ltd), which passed the signal on to a PC running Spike2 v.7 software. Online and offline spike sorting was performed in Spike2, and subsequent data analysis was performed in MATLAB (MathWorks).

Purkinje cell simple spike activity was normalized to the background activity during 500 ms preceding stimulus onset. The onset and offset of the conditional Purkinje cell response was defined as the first and last bins in a series of consecutive bins with spike activity below 50% of the background activity. The response maximum was defined as the last bin in the series of bins with the lowest activity during the ISI defined (28). This procedure was motivated by the expected postsynaptic effect on nuclear cells (maximal response at the end of maximal disinhibition). Plots of response profiles in all figures are smoothed using a 5-point moving average.

Responses to the CS were classified as conditional PcCRs if simple spike activity decreased below 50% of the cell's background activity level for a period of 40 ms or longer. This is a stricter criterion compared with previous studies (28), and was chosen to more clearly identify and separate different response components in the PcCRs.

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