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2007

Document Version:
Publisher's PDF, also known as Version of record

Lund University, Faculty of Medicine Doctoral Dissertation Series 2007:61

ISSN 1652-8220
ISBN 978-91-85559-39-8

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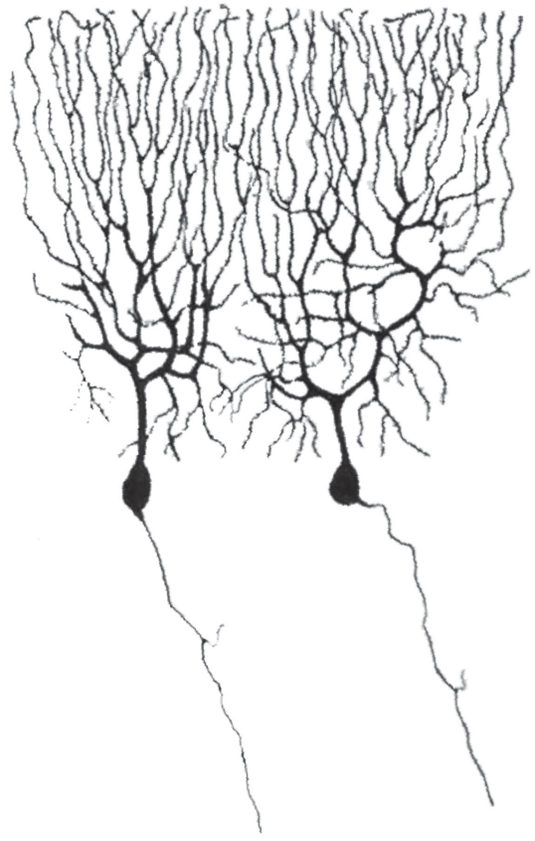

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An Associative Memory Trace in the Cerebellar Cortex

Akademisk avhandling

som med vederbörligt tillstånd av Medicinska fakulteten vid Lunds Universitet för avläggande av doktorsexamen i medicinsk vetenskap kommer att offentligen försvaras i Segerfalksalen, Wallenberg Neurocentrum lördagen den 28 april 2007, kl. 10.00.

av

Dan-Anders Jirenhed

Sektionen för Neurovetenskap
Institutionen för Experimentell Medicinsk Vetenskap

Fakultetsopponent: Dr Paul Dean,
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Lund 2007

Organization	Document name
LUND UNIVERSITY Dept. of Experimental Medical Science Division for Neuroscience BMC F10 221 84 Lund, Sweden	DOCTORAL DISSERTATION
	Date of issue April 28, 2007
	Sponsoring organization
Author(s) Dan-Anders Jirenhed	

Title and subtitle

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Abstract

Classical conditioning of motor responses, e.g., the eyeblink response, depends on the cerebellum. In the theoretical works of David Marr (1969) and James Albus (1971), it was proposed that Purkinje cells in the cerebellar cortex learn to associate the neutral conditioned stimulus with the response. Since their work, several studies have provided data that are consistent with this suggestion, but definitive evidence has been lacking. Information on how Purkinje cells change their activity during learning has been ambiguous and contradictory and there has been no information at all about how they behave during extinction and reacquisition.

The electrical activity of single Purkinje cells was recorded with microelectrodes in decerebrate ferrets during learning, extinction, and relearning. We demonstrate that paired peripheral forelimb and periocular stimulation, as well as paired direct stimulation of cerebellar afferent pathways (mossy and climbing fibres) consistently causes a gradual acquisition of an inhibitory response in Purkinje cell simple spike firing. The response also displays gradual extinction to unpaired presentations of the stimuli, and reacquisition with substantial savings when paired stimulus presentation is reinstated. This conditioned Purkinje cell response thus has several properties that match known features of the conditioned eyeblink response across training trials.

The temporal properties of the conditioned Purkinje cell response were also investigated. The response maximum was adaptively timed to precede the unconditioned stimulus. The latencies to response onset, maximum, and offset varied with the interstimulus interval used during training. Further training with changes in the interstimulus interval caused new learning of response latencies. Finally, short-term manipulations of the conditioned stimulus after training had effects on the Purkinje cell response that match effects on the conditioned eyeblink response.

These data suggest that many of the behavioural phenomena in eyeblink conditioning can be explained at the level of the single Purkinje cell.

Key words: Cerebellum, Classical Conditioning, Purkinje cell, Mossy fibre, Climbing fibre

Classification system and/or index termes (if any):

Supplementary bibliographical information:		Language
ISSN and key title: 1652-8220		ISBN 978-91-85559-39-8
Recipient's notes	Number of pages	Price
	Security classification	

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Cover illustration: Drawing of Purkinje cells from pigeon cerebellum by Santiago Ramón y Cajal, 1899. Instituto Santiago Ramón y Cajal, Madrid, Spain. (Adapted)

To my Mother and the memory of my Father

Contents

List of original papers

I. Jirenhed DA, Bengtsson F, Hesslow G. (2007) Acquisition, extinction, and reacquisition of a cerebellar cortical memory trace. *The Journal of Neuroscience*. 27(10):2493-2502.

II. Jirenhed DA and Hesslow G. Temporal Characteristics of the Classically Conditioned Purkinje Cell Response. *Manuscript*.

Aims

The aim of this work was to test a cerebellar cortical model of classical conditioning of the eyeblink response. In paper **I** we investigated how paired and unpaired presentations of different conditioned and unconditioned stimuli cause acquisition, extinction, and reacquisition of a conditioned Purkinje cell response. The temporal characteristics of this conditioned Purkinje cell response were further investigated in paper **II**.

Introduction

It will be argued that [...] the Purkinje cell can learn all the 'situations' in which the olive cell movement is required, and later, when such a situation occurs again, can implement that movement itself.
- David Marr (1969, p. 439)

...the effect of learning should be that eventually the particular mossy fiber pattern (CS) should elicit a pause (CR) in Purkinje activity similar to the inactivation response (UR) that previously had been elicited only by the climbing fiber burst (US).
- James S. Albus (1971, p. 44)

Classical conditioning, or Pavlovian conditioning, is an experimental paradigm for the study of associative learning. It is called conditioning because the procedure, described by Ivan Pavlov (1927), causes a response to become conditional upon the presentation of an associated stimulus. For example, an eyeblink response can be made to be conditional upon the presentation of a tone, even though a tone would not elicit such a response under normal circumstances. However, if the tone is presented just prior to a blink-eliciting stimulus, say a puff of air to the cornea, and this paired presentation of the two stimuli is repeated over several trials, the tone will gradually acquire the power to elicit the eyeblink on its own (Fig. 1). In Pavlov's terminology, the tone is called a conditional stimulus (or CS) and the acquired eyeblink response is called a conditioned response (or CR). The puff of air to the cornea is called an unconditional stimulus (or US), since it requires no training to elicit an eyeblink. Consequently, an eyeblink in response to the US is called an unconditional response (or UR).

Though Pavlov suggested that the cerebrum was responsible for classical conditioning, later work showed that eyeblink responses could be conditioned also after decerebration (Norman et al., 1977). Since the report of McCormick and Thompson (1984), a large body of work has produced converging evidence that classical conditioning of motor responses is dependent upon the cerebellum.

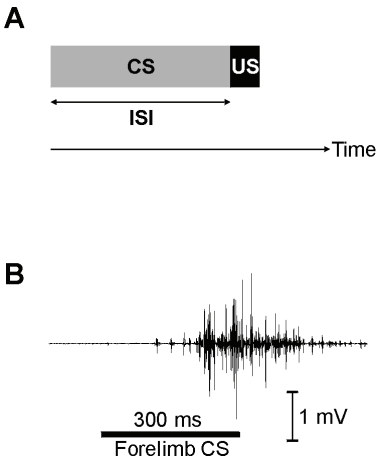

Figure 1. Classical conditioning. A During acquisition training a conditioned stimulus (CS) is presented before the unconditioned stimulus (US). The time period between the onset of the two stimuli is called the interstimulus interval (ISI). **B** Sample eyelid EMG record of a typical CR on a CS alone trial. The onset latency is about 100 ms and the duration is about 350 ms.

The cerebellum

The cerebellum (or small brain), located dorsal to the brainstem, is an evolutionarily old structure, preserved in all vertebrate species, and it is involved in many aspects of motor control.

Like the cerebrum, the cerebellum in birds and mammals is a foliated and layered structure that can be divided into cortical and sub-cortical parts (Eccles et al., 1967; Voogd and Glickstein, 1998). However, the only sub-cortical structures in the cerebellum are the so called deep nuclei that contain the only outward projecting neurons from the cerebellum. Afferent and efferent connections are organised separately and form three cerebellar peduncles or brachia. The superior cerebellar peduncle (or *brachium conjunctivum*) contains all efferent fibres that project from the cerebellum to other parts of the central nervous system. Afferent mossy fibres project from all major areas of the central nervous system and enter the cerebellum via the middle cerebellar peduncle (or *brachium pontis*) or via the inferior peduncle (or *corpus restiforme*). The inferior peduncle also contains the so-called climbing fibres that project exclusively from the inferior olive, located in the pons. Mossy fibres terminate upon granule cells in the deepest of the three cortical layers, the granule cell layer (that also contains Golgi cells), while climbing fibres terminate upon Purkinje cells in the middle layer, the Purkinje cell layer. Collaterals of mossy and climbing fibres also contact cells in the nuclei. The most superficial of the cortical layers is the

molecular layer that contains interneurons (stellate/basket cells) and the granule cell axons, called parallel fibres after they bifurcate near the surface.

The olivo-cortical projection of climbing fibres is organised such that its forms longitudinal bands or zones (Armstrong, 1974; Oscarsson, 1973; Oscarsson, 1980; Voogd and Glickstein, 1998) in the cerebellar cortex, where the Purkinje cells in a zone receive climbing fibre input from a specific part of the inferior olive. Within each of these zones, the climbing fibre projections are further sub-divisible into microzones (Andersson and Oscarsson, 1978; Apps and Garwicz, 2005; Ekerot et al., 1991; Garwicz and Ekerot, 1994; Hesslow, 1994), based on their receptive fields on the body. For example, there are at least four microzones that receive climbing fibre input from the face via the inferior olive, and one of these microzones is located in the C3 zone (Fig. 2A). The Purkinje cells in a microzone target a specific set of nuclear cells, where many of them project out to motor pathways, while some project back to the olivary cells that send climbing fibres to the Purkinje cells in that microzone. These olivo-cortico-nuclear cells with connections that are microzonally arranged, form so called, microcomplexes (Ito, 1984; Ito, 2006; Apps and Garwicz, 2005), or micromodules including the nucleo-olivary projection (Hesslow and Yeo, 2002), as illustrated in Figure 2B.

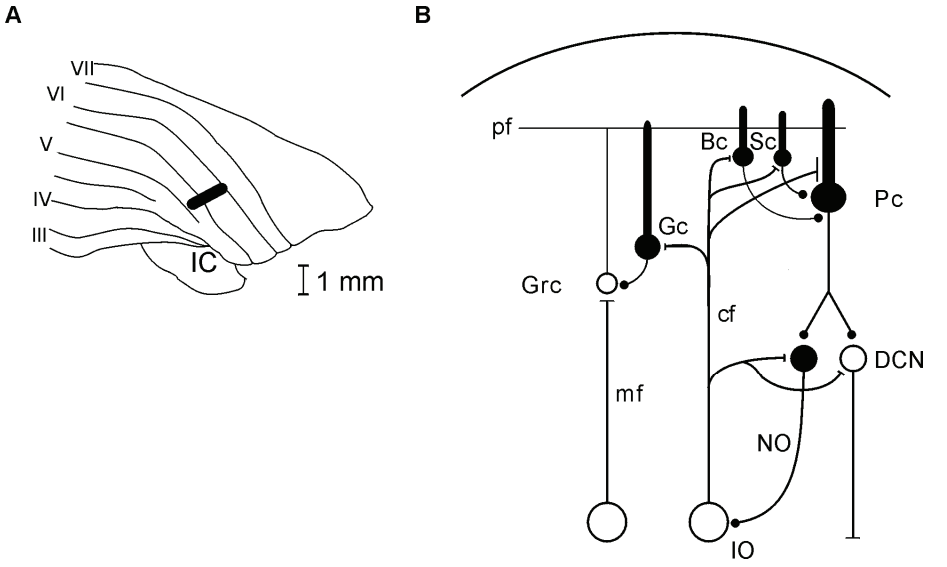

Figure 2. The cerebellum. A Hemispheral lobules III to VII of the cerebellar cortex. The blink-controlling area in the C3 zone is indicated in black. **B** Wiring diagram of the cerebellar micromodule Excitatory neurons in white and inhibitory neurons in black. Parallel fibre (pf), granule cell (Grc), mossy fibre (mf), Golgi cell (Gc), Basket cell (Bc), Stellate cell (Sc), Purkinje cell (Pc), climbing fibre (cf), Deep cerebellar nucleus cells (DCN), Nucleo-olivary pathway (NO), Inferior olive (IO).

The entire cerebellar cortex and the underlying structures are organised as a uniform and repeated arrangement of micromodules where the cortico-nucleo-olivary connections respect the microzonal organisation. Within each micromodule, the Purkinje cell is the point of greatest sensory convergence. A single Purkinje cell receives input from only one climbing fibre, but also from hundreds of thousands of parallel fibres (Napper, 1988). The strong convergence of sensory input onto the Purkinje cell, from the many parallel fibres and the single climbing fibre, makes it a strong candidate locus of associative learning.

Models of cerebellar conditioning

Two theoretical articles, published independently by David Marr (1969) and John Albus (1971), suggested that the cerebellar physiology described by Eccles et al. (1967) implied that the function of the Purkinje cell was to learn associations between parallel fibre and climbing fibre activity. Olivary (and thus climbing fibre) activity transmitted to the Purkinje cell, was known to be related to movements. It was argued that the parallel fibre input, with its diversity of sensory information, signals information about the situations in which these movements were performed. Through associative learning, the Purkinje cell would be able to elicit those movements on its own. Marr speculated that the Purkinje cell could accomplish this by learning to increase its activity in response to the parallel fibre input, while Albus suggested that the Purkinje cell would instead learn a pause response.

The Cerebellar Cortical Conditioning (CCC) model (Hesslow and Yeo, 2002), is a modified version of the Marr-Albus theory of cerebellar function, adapted to the context of cerebellar mechanisms underlying eyeblink conditioning (for an illustration, see Figure 3). The CCC assumes that (i) learning occurs in eyeblink controlling Purkinje cells; (ii) mossy fibres and climbing fibres transmit the conditioned stimulus and learning involves changes at parallel fibre to Purkinje cell synapses; (iii) the synaptic change is dependent on climbing fibre transmission of the unconditioned stimulus; and (iv) the CS is processed at mossy fibre – granule cell synapses. To briefly put the current investigation into context, it addresses the validity of assumptions (i-iii).

The CCC model makes no assumptions about the sufficiency or necessity of plasticity at mossy fibre – nuclear cell synapses to account for data on eyeblink conditioning. Some phenomena, like the rapid reacquisition with savings that is observed when paired CS-US presentations are introduced after extinction, have led to suggestions that plasticity at nuclear synapses is a necessary mechanism (Mauk, 1997; Medina et al., 2001; Robleto et al., 2004). The proposed cerebellar models implement learning in two stages; first the synapses on the Purkinje cell

change, and later learning takes place at the nuclear cell synapses under influence of the Purkinje cell pause (Pugh and Raman, 2006). Rapid reacquisition with savings, according to this view, is the effect of uncovering an intact memory trace in the nuclei. This intact nuclear memory originated in the cortex, was transferred to the nucleus, and later during extinction training, it was suppressed by learned inhibition of the eyeblink CR, possibly by recruitment of excitatory Purkinje cell responses to the CS. After the associative memory has been transferred to the nucleus, the role of the Purkinje cell in CR generation is merely to supply a timing signal. This is one possible interpretation of incorrectly timed CRs observed after cortical lesions in conditioned animals (Medina et al., 2001).

Regardless of the specific assumptions made in the different models for cerebellar involvement in eyeblink conditioning, a few physiological properties of the micromodule must be considered. First, most of the cells that are part of the micromodule seem to have internal mechanisms for generating spontaneous firing of action potentials. Second, connections between these spontaneously active cells make them interdependent upon each other for normal functioning. In the case of the microzonally organised interconnections between Purkinje cells, nuclear cells, and olivary cells, there is strong evidence for a feedback regulation mechanism (Bengtsson et al., 2004; Bengtsson and Hesslow, 2006). This means that spontaneous nuclear cell activity is constantly regulated by the spontaneously active Purkinje cell, that in turn is regulated by the spontaneous climbing fibre activity produced by olivary cells, that in turn are under influence of nucleo-olivary transmission. This means that any manipulation of one part in the network will have downstream effects on all other parts of the network, and additionally feedback effects at the place of manipulation via the network. So, for instance, will lesions, inactivations or blocking of transmission disrupt the stable background states of activity that are under feedback regulation, rendering the whole micromodule dysfunctional. The first point here is that any and all manipulations made must be within reasonable physiological limits if the effects are to be clear and possible to interpret. The second point regards the Purkinje cell response. The CCC model predicts that Purkinje cells should learn to decrease their firing during the later part of the CS-US interval. If the Purkinje cell is to decrease its firing, and potentially pause completely for any longer time periods, hyperpolarisation (perhaps via inhibitory input) is necessary since the Purkinje cell is spontaneously active. It is possible that this assigns an important functional role to the cortical interneurons.

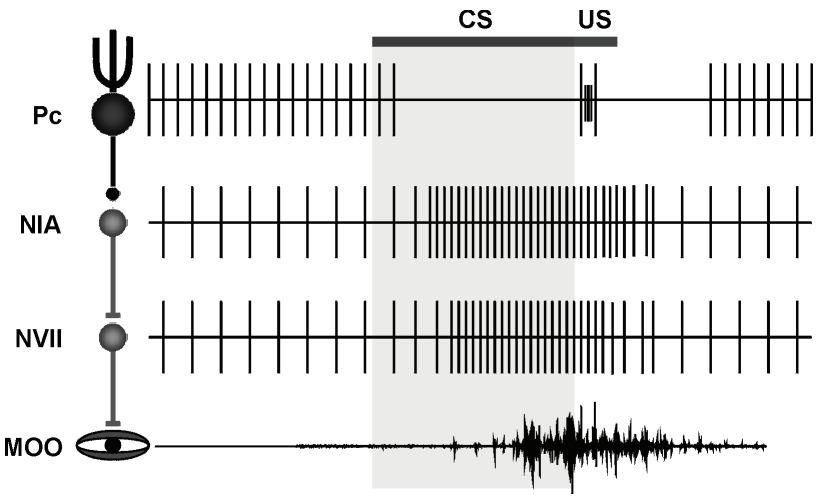

Figure 3. Predicted cell responses. According to the CCC model, the Purkinje cell (Pc) pauses in response to the CS. This disinhibits the cells in the *nucleus interpositus anterior* (NIA) and causes increased activity that drives the activity of cells in the *nucleus facialis* (NVII). Via the facial nerve the *orbicularis oculi* muscle (MOO) is activated and causes an eyeblink.

Main problems and aims of this thesis

The aim of the work presented in this thesis was to test of the validity of the CCC model assumptions about Purkinje cell behaviour during conditioning. To test a number of specific hypotheses, we made extracellular *in vivo* recordings of Purkinje cell activity in a decerebrate ferret preparation during different phases of training. Points 1-4 were addressed in paper **I**, and 5-7 were addressed in paper **II**.

1. The CCC model predicts that Purkinje cells should decrease their firing during the later part of the CS-US interval. To test this prediction we observed Purkinje cell behaviour during training with paired CS-US presentations from a naïve state until a stable cellular response to the CS had been acquired.

2. The CCC model states that the CS is transmitted to the Purkinje cell via the mossy fibres. To test this prediction we used an electrical pulse CS that was applied either peripherally, to the forelimb skin, or directly to the mossy fibres in the middle cerebellar peduncle. The effects of using the different sites of stimulation was then compared with regard to the effects on Purkinje cell behaviour.

3. The CCC model asserts that the change at parallel fibre to Purkinje cell synapses is dependent on climbing fibre transmission of the unconditioned stimulus. To test this prediction we used an electrical pulse US that was applied either peripherally, to the periorbital skin, or to the cells in the inferior olive or directly to the climbing fibres in the inferior cerebellar peduncle. The effects of using the different sites of stimulation was then compared with regard to the effects on Purkinje cell behaviour.

4. If learned Purkinje cell responses display extinction behaviour and also rapid reacquisition with savings, then there is no need to assume nuclear plasticity to account for extinction and reacquisition of eyeblink CRs. These phenomena may then be explained by purely cortical mechanisms. The CCC model makes no assumptions about, and does not exclude, changes at mossy fibre – nuclear cell synapses as an essential mechanism to account for extinction of eyeblink CRs, or to account for the rapid reacquisition of CRs that is observed when paired CS-US presentations are reinstated after extinction. We therefore investigated if any cellular response changes similar to these eyeblink CR phenomena could be observed in the Purkinje cell.

The results reported in paper **I** showed that Purkinje cells acquire a conditioned inhibitory response. This finding raised a number of questions regarding the temporal characteristics of the response. Results from previous investigations of eyeblink conditioning in the decerebrate ferret preparation allow for comparisons between data on the Purkinje cell behaviour and existing data on conditioned eyeblink responses.

5. Eyeblink CRs are timed in accordance with the interstimulus interval (ISI). What is the relation between ISI and Purkinje cell CR profile, i.e., the latencies to CR onset, maximum, and termination?

6. Eyeblink CRs acquired to one interval continually adapt to new temporal contingencies, e.g., when training with a new interval follows. Does the Purkinje cell CR adapt to new ISIs?

7. Eyeblink CRs are affected by temporal manipulations of the CS. How are Purkinje cell CRs affected by temporal manipulations of the CS?

Strategy and Methods

One of the experimental strategies that can be used to investigate the neural mechanisms underlying a certain behaviour is to record the electrical activity in neurons and analyse correlations between the neuronal activity and the behaviour. But what do neural correlates tell us? Correlations may appear for many different reasons and neural correlates of stimuli and responses may be found in many places, since the CNS consists of many interacting structures that communicate with each other. Recordings alone are not sufficient to clarify the links in the causal chain from stimulus to response. Lesions and inactivations supply critical clues to where one may look for the relevant correlates. The hippocampus, for example, is not an essential structure for delay eyeblink conditioning while the cerebellum is, as decerebration (Norman et al., 1977) does not prevent acquisition or expression while lesion (Yeo et al., 1985b; Yeo et al., 1986; Yeo et al., 1985a) and inactivation (Hardiman et al., 1996) of relevant cerebellar structures do. Still, hippocampal CA1 pyramidal cell activity is observable during delay eyeblink conditioning (Green and Arenos, 2007).

Evidence from lesion and inactivation studies have converged on two possible cerebellar loci of associative learning, the anterior interpositus nucleus and/or the cerebellar cortex in hemispheral lobule VI (or HVI). One way to address the problem of dissecting the relative roles of these two loci is to record from the structure closest to the response end of the stimulus-response causal chain, i.e. the anterior interpositus nucleus, and then move back to the stimulus end. Initially, one should find very strong correlations with the overt responses and also the gradual learning of these responses. Such observations have been made in recording from interpositus cells (Berthier and Moore, 1990; Choi and Moore, 2003) where cell activity was found to strongly match blink responses. However, if such matches can also be found in cells closer to the stimulus end of the causal chain (e.g. in Purkinje cells), then it is reasonable to assume that the activity in these cells is the cause of the responses that were observed in the interpositus cells.

In order to investigate this, single unit recordings of Purkinje cell activity were made. Single unit recordings allow for definition of input in a way that (single-channel) multi-unit recordings do not. Since the aim was to study associative learning in single cells, it was essential to verify that the CS and US inputs converge on the same cell. A multi-unit recording makes this verification much more difficult, since it is possible that one of the cells responds to one stimulus and another cell responds to the other stimulus. The convergence of mossy fibre CS-input and climbing fibre US-input on the same Purkinje cell was defined as

critical for data inclusion, since radically different results were expected if Purkinje cells receive non-converging CS or US input.

Decerebration rather than anaesthesia was chosen for this investigation, as well as in previous investigations in our lab, since anaesthesia is known to influence transmission in the cerebellum and since it prevents behavioural observations. Decerebration also provides the opportunity to study cerebellar learning mechanisms in isolation from learning mechanisms in other structures, e.g. the hippocampus. The disadvantage with respect to cerebellar physiology is that the decerebrate lacks the cerebral input via mossy fibres that is present under normal circumstances.

The investigation presented in paper **I** was intended to test the hypothesis that Purkinje cells learn to respond to CS input transmitted via mossy fibre afferents when it is repeatedly paired with US input transmitted via the climbing fibres. It was therefore important to control for possible inadvertent effects of stimulation. For example, a periorbital US will elicit both mossy and climbing fibre activity. Direct stimulation of the inferior olive as the US may also activate some mossy fibres in addition to climbing fibres, and the same problem can arise when the stimulation is applied to the inferior cerebellar peduncle. However, using three different sites of stimulation as the US for different cells, circumvents problems of activating other structures, since those other structures can hardly be identical in all cases. The only common factor was climbing fibre activation. The same strategy was used for CS presentation by using two different sites of stimulation. Additionally, applying low stimulus intensities close to (but always above) threshold reduces the risk of inadvertently activating other structures than intended.

The investigation presented in paper **II** was intended to test the hypothesis that temporal characteristics of the eyeblink CR is matched by the behaviour of the Purkinje cell. Among the manipulations made, the ISI was shifted after Purkinje cell CRs had been acquired. Often the CS duration is also changed along with the ISI, and that poses a possible problem for interpreting the effects of training to different ISIs and subsequent ISI-shifts. Also, it makes it hard to investigate the mechanisms underlying the termination of the CR. For this reason, a long duration CS that overlaps US presentation was used to keep all properties of the CS and US constant, and only varying the interval between CS and US.

Results

The observations presented in papers **I** and **II** revealed a strong match between several well known eyeblink CR phenomena and the behaviour of the single Purkinje cell response.

A cerebellar cortical memory trace (I)

We performed extracellular Purkinje cell recordings *in vivo* in the decerebrate ferret during training with paired and unpaired CS-US presentations. The CS was an electrical pulse train applied either peripherally, to the forelimb skin, or directly to the mossy fibres in the middle cerebellar peduncle. The US was an electrical pulse train applied either peripherally, to the periorbital skin, or to the cells in the inferior olive, or directly to the climbing fibres in the inferior cerebellar peduncle (see Fig. 4).

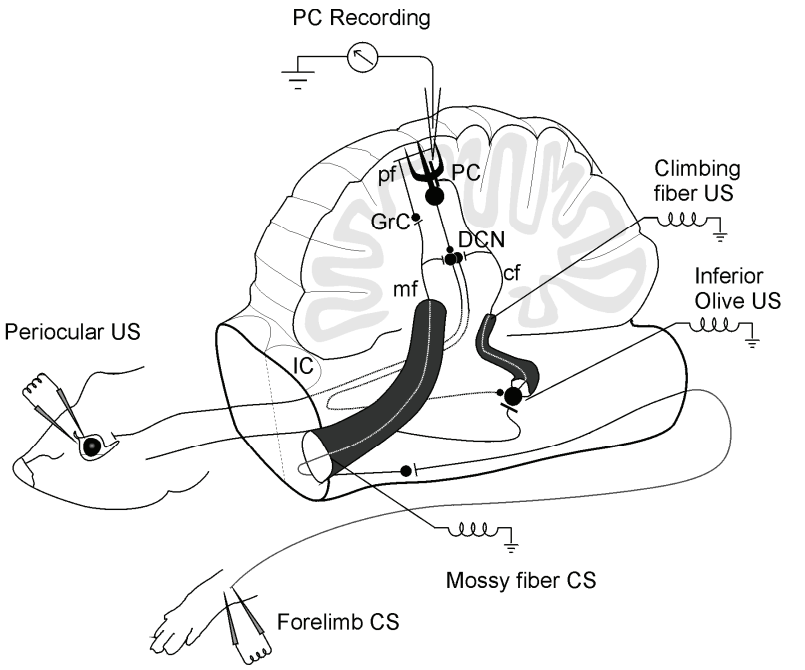

Figure 4. Experimental setup. Purkinje cell activity was recorded during training. The CS was applied either to the forelimb or to mossy fibres in the middle cerebellar peduncle. The US was applied either to the periocular area, to the inferior olive, or to the climbing fibres in the inferior cerebellar peduncle.

Observation of Purkinje cell responses to the CS (illustrated in Figure 5) were made from a naïve state during paired CS-US presentations for up to 900 trials. Initially, the CS elicited a weak excitatory response, but after 600 trials, the Purkinje cells had acquired a reliable inhibitory response to the CS. This response was acquired regardless of where the CS and US were applied. When the CS was repeatedly presented on its own or explicitly unpaired with the US, the Purkinje cell response displayed extinction behaviour. After about 600 trials, the inhibitory Purkinje cell response was almost completely gone. When paired CS-US presentations were again introduced after extinction, inhibitory Purkinje cell responses to the CS reappeared rapidly, often within less than ten trails. Finally, Purkinje cells trained with a 200 ms ISI displayed shorter latencies to response onset and maximum when compared to cells trained with a 300 ms ISI, thus showing that the response is timed relative to the ISI during training. Together, these data strongly suggest that the inhibitory response has all the characteristics of conditioned responses and can therefore be called a Purkinje cell CR.

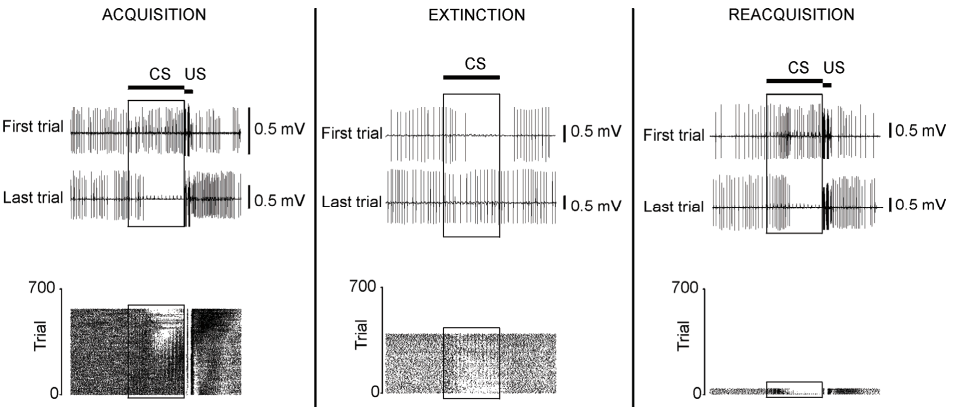

Figure 5. Results from paper I. Single unit records from three Purkinje cells during different phases of learning. Top row illustrates Purkinje cell responses on individual trials. Bottom row illustrates simple spike activity during learning. Each dot is one simple spike. Sweep length is 0.9 s.

Temporal characteristics of the memory trace (II)

In this investigation (see Fig. 6 for illustration of setup), we did a detailed follow-up on the finding in paper **I,** that the Purkinje cell CR seems to be well timed in accordance with the ISI. We also wanted to test what factors control the temporal profile of the response, i.e. the latencies to onset, maximum and offset. Finally, we were interested in studying how the Purkinje cell CR is affected by acute manipulations of temporal properties in the CS.

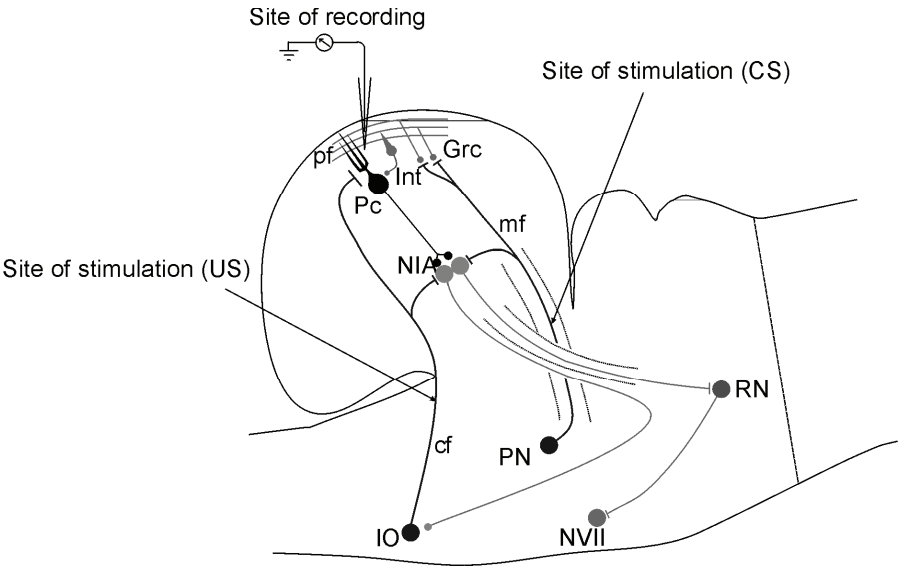

Figure 6. Experimental setup. Experimental set-up and cerebellar wiring diagram, showing the recording site, the two CS and US stimulation sites, the deep cerebellar nuclei and the inferior olive. Purkinje cell (Pc), Interneuron (Int), Climbing fibre (cf), granule cell (Grc), mossy fibre (mf), parallel fibre (pf), anterior interpositus nucleus (NIA), inferior olive (IO), red nucleus (RN), facial nucleus (NVII), pontine nuclei (PN).

Purkinje cells acquired CRs that terminated independent of CS-duration. After the CR had been elicited, mossy fibre pulses did not elicit further inhibition of the Purkinje cell activity. Thus, there was a differential effect on the Purkinje cell by the individual mossy fibre pulses. The CR was timed in accordance with the ISI. Shorter ISIs during conditioning caused Purkinje cells to acquire CRs that had shorter latencies to onset, maximum and offset, compared to CRs conditioned to longer ISIs (Fig. 7A-B). Also, CRs conditioned to one ISI adapted to new temporal contingencies when training with a longer ISI followed (Fig. 7D). When the new ISI was sufficiently greater, bimodal responses were

acquired by the Purkinje cell (Fig. 7C). Manipulations of the CS duration after training revealed that presenting a shorter or a longer CS did not disrupt CR timing. In fact, the first two CS pulses (i.e. first 20 ms of the CS) sufficed to elicit normal CRs (compare Figs. 7F and 7G). However, when the mossy fibre CS frequency was increased, the response latency decreased in some cells, though this was not always the case. Together, these data show temporal characteristics in the Purkinje cell CR that match available data on the eyeblink CR.

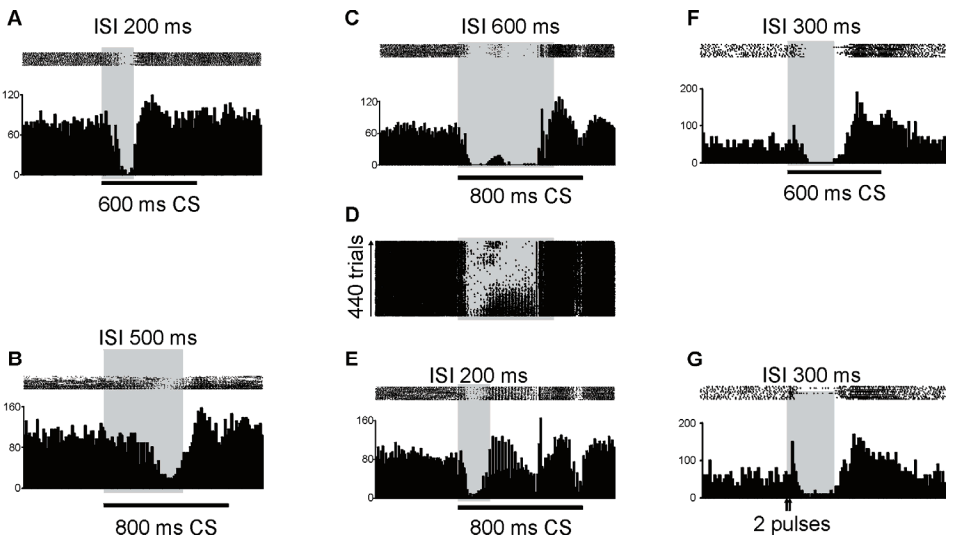

Figure 7. Results from paper II. Temporal profiles of Purkinje cell responses. (**A-B**) Two Purkinje cell CRs that were acquired to different interstimulus intervals; in **A** 200 ms and in **B** 500 ms. Notice the differences in latencies to CR onset, maximum, and offset. (**C-E**) ISI-shift manipulation causes learning of new CR timing. (**E**) Before the shift, the CR was timed in accordance with the 200 ms ISI. (**D**) During training with the 600 ms ISI the Purkinje cell CR gradually develops a biphasic temporal profile. (**C**) The biphasic temporal profile after ISI-shift. (**F**) Purkinje cell CR elicited by CS alone presentations. (**G**) Similar CRs elicited by presenting only two electrical pulses to the mossy fibre (same cell as in **F**).

Discussion

A cortical memory trace

The investigation reported in paper **I** showed that an associative memory trace can be formed in the cerebellar cortex. The observation that Purkinje cells can acquire an inhibitory CR in response to mossy fibre activity is in perfect agreement with the Cerebellar Cortical Conditioning model (Hesslow and Yeo, 2002). According to the CCC model, this cellular CR, in Purkinje cells that control blink related muscles, is the critical memory trace that underlies the eyeblink CR.

Though the memory trace is observable in the behaviour of Purkinje cells, this observation does not allow for a more exact localisation of the memory trace within the cerebellar cortex, in terms of where the synaptic changes have occurred. The Purkinje cell is clearly hyperpolarised in response to the mossy fibre CS by some mechanism, but the location of this mechanism is unknown. It must however be confined to the cellular network between granule cells and Purkinje cells, i.e., within the cerebellar cortex.

CS and US pathways

According to the CCC model, Purkinje cells should acquire an inhibitory CR when CS activity in mossy fibres is paired with US activity in climbing fibres. In paper **I** we therefore wanted to make sure that it was indeed paired mossy fibre CS activity and climbing fibre US activity that led to the acquisition of the Purkinje cell CR paired. Test stimuli presented while recording the activity of a Purkinje cell provided important information about what input the cell received. Short-latency complex spikes were elicited when the climbing fibres were activated, and simple spike activity was increased in response to mossy fibre activation. But since it is very difficult to exclusively activate only one of the two afferent systems at any stimulation site, we also chose to systematically present the CS and US at different sites, with the only common factor for the CS application sites being mossy fibre activation and the only common factor for the US application sites being climbing fibre activation. The Purkinje cell CR could be acquired to, and then elicited by, either forelimb stimulation or stimulation in the middle cerebellar peduncle. The Purkinje cell CR was acquired when the US consisted in stimulation of the periorbital area, the inferior olive or the inferior cerebellar peduncle. This is in line with previous investigations (Hesslow et al., 1999; Mauk et al., 1986) and provides further evidence that, in eyeblink conditioning, the CS is transmitted to the cerebellar cortex via the mossy fibres and the US via the climbing fibres.

Extinction

After an eyeblink response has been conditioned to a CS, repeated presentations of the CS on its own, or presentations of the CS explicitly unpaired with the US, causes extinction of the CR. During extinction, the CS will elicit the CR with gradually decreasing reliability (Kehoe and Macrae, 2002).

It has been suggested that extinction could be a result of a different population of Purkinje cells learning to suppress the CR (Robleto et al., 2004). Our results instead suggest a mechanism for extinction that involves the same Purkinje cells that are involved in acquisition. The match between eyeblink CR extinction and Purkinje cell CR extinction is very strong, in terms of the time-course and the transient recoveries of CR expression.

The results presented in paper **I** is the first demonstration that a conditioned Purkinje cell response undergoes extinction during CS alone and unpaired CS–US presentations (see Fig. 5, middle panels). This observation provides very suggestive evidence for the hypothesis that the associative memory is localised in the cerebellar cortex.

Reacquisition

After an eyeblink CR has been extinguished, reinstated pairing of the CS and US will cause the CR to reappear (Kehoe and Macrae, 2002). Compared to the time and number of trials that are required for CRs to appear during acquisition, reacquisition of CRs is much more rapid, a phenomenon known as savings. The savings in reacquisition has been ascribed to learning mechanisms with different time characteristics in the cerebellar cortex and nuclei (Kehoe, 1988; Medina et al., 2001). Our results instead suggest purely cortical mechanisms behind reacquisition.

The results presented in paper **I** is the first demonstration that a conditioned Purkinje cell response is reacquired with substantial savings when paired presentations of the CS and US are reinstated after extinction (see Fig. 5, right panels). This observation provides further evidence that suggests an associative memory in the cerebellar cortex.

Climbing fibre control of Purkinje cell CR timing

The conditioned eyeblink response is adaptively timed to reach its maximum at the expected time of US presentation. This means that the interstimulus interval (ISI) shapes the temporal profile of the conditioned response. Short ISIs cause

learning of eyeblink CRs that rapidly increase in magnitude from the time of onset to maximum. Training with long ISIs cause acquisition of CRs that rise more slowly. Any change in ISI after a CR has been acquired will induce learning of new response timing, such that the CR will adapt to the new interval (Kehoe and Macrae, 2002).

Purkinje cell CRs were acquired with different ISIs, as described in both papers **I** and **II**. The resulting CRs displayed latencies to onset, maximum and offset that were related to the ISI during acquisition, i.e., longer ISIs caused Purkinje cells to acquire longer onset, maximum and offset latencies.

Conditioning to new ISIs after acquisition was studied in the ISI-shift experiments reported in **II**. In all cases the ISIs were increased relative to the interval used during acquisition, and in all cases CR latency variables increased and the magnitude of increase was in direct proportion to the magnitude of the ISI-shift.

Together, these observations provide strong evidence for climbing fibre control of Purkinje cell CR timing. The interval between CS onset and US onset determines the temporal profile of the Purkinje cell CR in a way that matches the effects seen on eyeblink CRs.

Mossy fibre control of the Purkinje cell CR

In paper **II** we investigated what factors in the mossy fibre CS are relevant for the temporal profile of the Purkinje cell CR. This was motivated by observations made in previous investigations of eyeblink CR timing in relation to CS input manipulations.

Eyeblink CR timing is affected by acute manipulations of the CS. For example, when either intensity or frequency of a 300 ms electrical pulse train CS to the forelimb was increased after training, eyeblink CRs were elicited earlier, with regard to latency to both onset and maximum (Svensson et al., 1997). This effect could also be replicated when the forelimb CS was replaced by direct mossy fibre stimulation and the stimulus frequency, rather than intensity, was increased (from 50 Hz to 100 Hz).

The Purkinje cell CR was also affected when the mossy fibre CS frequency was increased to 100 Hz. However, the effects were not systematic in the 10 cells that were observed. Four cells responded faster, i.e., the latencies to CR onset and maximum were reduced. In two cells there was no change in latency to onset or maximum, but instead a reduced latency to offset that was coupled to

the CS offset. In two cells there was no effect at all, and finally, in two cells the stimulation elicited excitatory responses instead of inhibitory CRs.

After conditioning to a forelimb CS, eyeblink CRs can be elicited by applying a single electrical pulse to the mossy fibres (Svensson and Ivarsson, 1999). In paper **II** we investigated if this would also be the case with Purkinje cell CRs.

With the normal mossy fibre CS used during training, 10 stimulus pulses were presented during the 200 ms interval before the US was presented. In four cells that had acquired CRs with the 200 ms ISI (in 3 cells using a 400 ms CS; in 1 cell using a 600 ms CS), we investigated the effects of presenting a very brief mossy fibre stimulus. Stimulus intensities and frequencies were the same as during conditioning. In all four cases, a mossy fibre stimulus of two pulses elicited reliable and accurately timed CRs. In one of these cells, CRs could also be elicited after an ISI-shift to 300 ms. In two of the cells, even a single pulse proved sufficient to elicit CRs.

Purkinje cell control of the eyeblink CR

Paired CS and US presentations caused acquisition of Purkinje cell CRs. The kinds of conditioned and unconditioned stimuli that were used (excluding the inferior cerebellar peduncle US) have previously been shown to cause acquisition of eyeblink CRs (Mauk et al., 1986; Hesslow et al., 1999; Svensson and Ivarsson, 1999).

There are three facts suggesting that the direction of causality must be from Purkinje cell to behaviour. First, the animals were curarized, so behavioural (i.e., sensory) feedback from the periorbital area could not have caused the Purkinje cell response. Second, the suppression of simple spike firing started earlier than the eyeblink CRs observed in similarly trained animals in previous investigations (Svensson and Ivarsson, 1999; Hesslow et al., 1999). Third, the response was elicited by direct mossy fibre stimulation, which excludes all extracerebellar sources of input, including premotor structures.

The data on Purkinje cell CR extinction and reacquisition with savings suggests that there is no need to assume nuclear plasticity to account for extinction and reacquisition of eyeblink CRs. These phenomena may be explained by purely cortical mechanisms. This interpretation is further supported by the data presented in paper **II** that show strong matches between the temporal properties of the Purkinje cell CR and eyeblink CRs.

It is, however, possible that nuclear plasticity is needed for the Purkinje cell CR to be expressed behaviourally as an eyeblink CR. Excitatory responses in

interpositus cells are known to be correlated with eyeblink CRs (Berthier and Moore, 1990; Choi and Moore, 2003). But it is quite reasonable to expect that the Purkinje cell CR alone can cause such excitation of interpositus cells, since they are spontaneously active (Thach, 1968; Raman et al., 2000). Furthermore, blocking of excitatory mossy fibre input to the interpositus does not affect CR expression (Aksenov et al., 2005). The Purkinje cell CR thus seems to have all the properties necessary to explain the eyeblink CR.

Mechanisms

There are several features of the Purkinje cell CR that pose challenges to any account of the learning in terms of known mechanisms. Long term changes in efficacy of synaptic transmission is the standard mechanism whereby learning is thought to be realised in the central nervous system. Mechanisms of synaptic plasticity, like long term depression (LTD) and potentiation (LTP), have been shown at parallel fibre – Purkinje cell synapses and parallel fibre – interneuron synapses (Ito et al., 1982; Ekerot and Kano, 1985; Jorntell and Ekerot, 2002; Jorntell and Ekerot, 2003).

The standard account for the inhibitory Purkinje cell response that is elicited by mossy fibre activity assumes that a balance exists between the direct excitatory input to the Purkinje cell, via parallel fibres, and the indirect inhibitory input via the interneurons (Mauk, 1997). This may solve the problem of silencing the spontaneously active Purkinje cell, but requires that the excitatory and inhibitory inputs are properly balanced at all times and that the balance is shifted adaptively during training.

A common assumption used to account for Purkinje cell CR timing, is that of delay-lines (Mauk and Buonomano, 2004; Zipser, 1986) in granule cells and parallel fibres. The hypothesis is that different parallel fibres signal to the Purkinje cell during different parts of the CS, because granule cells respond with different patterns; some with short latencies and other with long latencies and some continue firing for several hundreds of milliseconds while others only fire briefly. From the perspective of the Purkinje cell then, the CR timing problem becomes a matter of responding to certain parallel fibres, that are co-incident with the climbing fibre signal, and not to the other parallel fibres. This can be accounted for by synapse specific plasticity mechanisms, that allow some synapses to undergo LTP while others undergo LTD. However, a recent investigation (Jorntell and Ekerot, 2006) of granule cell responses to mossy fibre input has shown that granule cells respond to mossy fibre stimuli with just short bursts of spikes that continue for no more than 10 ms. There seems to be no delayed or prolonged granule cell or parallel fibre activity and thus no delay-lines. Considering that the mossy fibre CS that was used here consisted of

identical electrical pulses repeated every 20 ms, it is reasonable to think that the elicited repetitive granule cell bursts are fairly similar during the whole CS period. In light of this, there are some Purkinje cell behaviour phenomena that need explaining and may pose challenges to the LTD and LTP models of cerebellar learning.

Appearance of early CRs during acquisition. In paper **I** it was shown that mossy fibre activity and thus parallel fibre activity acquires the ability to silence simple spike output in the Purkinje cell. In many models of cerebellar learning, e.g. (Medina and Mauk, 2000), LTD at parallel fibre – Purkinje cell synapses, induced by co-active parallel and climbing fibre input, is suggested as the prime mechanism behind the Purkinje cell CR. According to this view, the Purkinje cell CR should emerge near the time of US presentation (i.e., the time of co-active input) and gradually increase in duration by starting earlier and earlier. However, the weak early Purkinje cell CR does not begin to appear at the end of CS period, but rather in the middle, about 150 ms into the 300 ms stimulus train (see Fig. 5, lower left). As learning continues, the duration of pause response becomes gradually longer and approaches the time of US presentation. In other words, the direction of gradual change is opposite to the way LTD-based models of learning predict.

Long latency CRs. In paper **II** it was shown that the Purkinje cell CR, just as the eyeblink CR, is timed in accordance with the interstimulus interval or ISI. Even when the CS consisted of 25 repetitive (identical) mossy fibre stimuli over a period of 500 ms, the CR onset had a reliable latency of at least 350 ms after CS onset (see Fig. 7B). This means that the Purkinje cell received input from 17 identical mossy fibre stimuli, but did not respond in any observable way until the 18th stimulus pulse was presented. It is far from trivial to explain how the Purkinje cell discriminates between the different parts of the 500 ms CS if the granule cells respond to each of the 25 pulses in a fairly similar way.

CR termination and bimodal CRs. A third phenomenon that may be non-trivial to account for is the observed termination of the inhibitory Purkinje cell response. Purkinje cell CRs were systematically timed in accordance to the ISI used during training, such that the response onset and maximum preceded the expected time of US presentation. But even though the CS continued for several hundred milliseconds, CRs were terminated independent of the continuous CS input. Some inhibitory Purkinje cell CRs were also preceded and followed by strong excitatory responses (see Fig. 7F). Again, all stimulus pulses applied to the mossy fibres were identical, but some appear to have elicited hyperpolarisation while the others elicited depolarisation. Perhaps the most striking case of regulated CR termination was the bimodal Purkinje cell CRs that were observed after ISI-shifts of 300 and 400 ms magnitude (see Fig 7C). Both

pause responses were reliably elicited and they were timed in accordance with the two ISIs used during the different phases of training. Between the pauses, the Purkinje cell fired simple spikes. During the whole time, 30 identical stimuli were applied to the mossy fibres. It is hard to intuitively grasp how identical stimuli can elicit such a pattern of variable responses when one looks at a single trial, while at the same time this response pattern is reliably repeated over a large number of trials.

Brief CS-presentations. Finally, paper **II** also showed that well-timed Purkinje cell CRs could be elicited by very brief mossy fibre stimuli after conditioning. For example, two pulses elicited well-timed CRs after acquisition to 300 ms ISI (see Fig 7G). A single pulse was sufficient to elicit CRs after conditioning to a 200 ms ISI. This suggests that the critical part of the CS is confined to the first 20 ms after CS onset. How the temporal gap (of ~70 ms) is bridged, between the last mossy fibre pulse and the CR onset, is an intriguing question.

It is a reasonable speculation that each of the mossy fibre pulses that were applied as part of the CS, elicited very similar input to the Purkinje cell via the parallel fibres. If this is indeed the case, then the mechanism(s) responsible for correct timing of the Purkinje cell CR may be located either in the stellate and/or basket cells or within the Purkinje cell itself, as has been suggested by Steuber and Willshaw (2004), rather than in the granule cells.

Future work

The observations reported in papers **I** and **II** answer a number of questions, but also raise several other questions that need to be addressed with future experiments. Some of the more important problems that remain to solve concern the mechanisms behind Purkinje cell CR timing and the localisation of synaptic changes. Below are some suggestions on future work to address these issues.

Conditioning to a parallel fibre CS. Well-timed inhibitory Purkinje cell CRs could be acquired either to a forelimb CS or a mossy fibre CS consisting of an electrical stimulus pulse train. If there are no delay-line mechanisms in the granule cells, then Purkinje cell CRs should also be acquired to a CS applied directly to parallel fibres. And if so, then will these CRs retain all the temporal characteristics described in paper **II**? Will, for example presentations of a brief CS elicit well-timed Purkinje cell CRs? Such an investigation may shed light on the functional role of the granule cell/parallel fibre system.

Pharmacological manipulation of synaptic transmission. What synapses undergo changes during conditioning? Is the hyperpolarisation of the Purkinje cell caused by other cells? If so, is it LTP in parallel fibre – interneuron synapses

that underlies the acquisition of an inhibitory Purkinje cell response, or is it LTD of parallel fibre – Purkinje cell synapses? Parallel fibre CS presentation would be a suitable technique for investigating effects on the Purkinje cell CR combined with pharmacological manipulations of synaptic transmission, in order to investigate what transmitter systems are sufficient and necessary to elicit the response.

Recording from interneurons. Extracellular recordings from the stellate and basket cells may contribute with observations that would be helpful in dissociating between response mechanisms inside the Purkinje cell, and potential mechanisms that involve inhibition via synaptic input from the interneurons.

Finally, *is the Purkinje cell CR a long-term memory?* It is of some importance to investigate if the cortical memory trace described here changes over longer time periods. The Purkinje cell CR was only investigated for a few hours (12-15 in some cases) after acquisition. It is not known what the Purkinje cell CR looks like at later time-points, say one or two days after acquisition. Since much of the literature on eyeblink conditioning describes learning effects over the course of several days, this question will have to be addressed at some point in order to fully appreciate the role of the Purkinje cell CR as a mechanism underlying the eyeblink CR.

Populärvetenskaplig sammanfattning på svenska

Inlärningen av nya associationer är ett välstuderat fenomen. Redan kring det förra sekelskiftet utvecklades en metod som används än idag, kallad *klassisk betingning*. Den används för att studera en typ av associativ inlärning som är väldigt grundläggande. Det var den ryske fysiologen Ivan Pavlov som upptäckte att de försökshundar som han studerade i sitt laboratorium uppvisade tecken på associativ inlärning i samband med de serverades mat. Mat är en typ av *stimulus* som utlöser, bland annat, en saliverings*respons* då den kommer in i munnen. Denna saliveringsrespons är inte inlärd, utan är en medfödd reflex som fyller en viktig funktion i matsmältningsprocessen. Det som Pavlov noterade, och framförallt var först med att systematiskt undersöka, var att saliveringsresponsen utlöstes redan vid åsynen av mat. Genom att upprepade gånger presentera mat tillsammans med ett nytt stimulus, som t.ex. ljudet av en metronom eller bubblande vatten, så kunde Pavlov få hundarna att salivera till dessa nya stimuli. Det var som om hundarna lärde sig att ljudet signalerade att mat skulle serveras. Maten benämndes *obetingat stimulus* medan det associerade ljudet benämndes *betingat stimulus*. Den nya, inlärda, saliveringsresponsen som efter upprepad träning kunde utlösas av tonen benämndes *betingad respons*.

Ett numera ofta använt sätt att studera klassisk betingning är att betinga blinkreflexen. Vanligen presenteras en ton som följs av en luftpuff mot ögat. Från början utlöser enbart luftpuffen en blinkning men efter träning, då tonen och luftpuffen presenterats tillsammans upprepade gånger, så kommer även tonen att utlösa en blinkning. Numera vet vi att denna association lärs in i *lillhjärnan*, en struktur belägen mellan storhjärnans nacklob och hjärnstammen.

Nervcellerna i lillhjärnan kan indelas i två huvudsakliga grupper; de celler som utgör de s.k. *djupa kärnorna* som (liksom namnet antyder) ligger belägna djupt inne i lillhjärnan, samt de celler som utgör den s.k. *lillhjärnbarken*, som ligger nära ytan. Cellerna i lillhjärnbarken får signaler från andra delar av nervsystemet och de sänder sina signaler till de djupa kärnorna, som i sin tur skickar ut signaler till andra delarna av nervsystemet. Särskilt en typ av celler i lillhjärnbarken har väckt mycket intresse, den s.k. *Purkinjecellen* som är den enda av de ytliga cellerna som kommunicerar med de djupa kärnorna. Det är en i flera avseenden imponerande cell. Den är störst av alla celltyper i lillhjärnan och har framförallt ett oerhört stort antal utskott (ett s.k. *dendritträd*) där den tar emot signaler från uppemot 200.000 andra celler via *mosstrådar* och *parallelltrådar* som kontaktar dendriterna. Förutom alla dessa parallelltrådar, tar den även emot en enda *klättertråd* som slingrar sig kring Purkinjecellens kropp och dendritträd. Den synaptiska kopplingen mellan klättertråden och Purkinjecellen anses vara den kraftigaste i hela nervsystemet. Eftersom Purkinjecellen uppvisar en så enorm *konvergens* av inkommande signaler, så

kanske det inte är förvånande att det spekulerats länge kring om det inte skulle kunna vara just där som klassiskt betingade associationer lärs in. Mosstrådar/parallelltrådar skulle kunna signalera t.ex. ljudet av en ton, medan klättertrådarna skulle kunna signalera att en luftpuff träffat ögat. Purkinjeceller som styr ansiktsmuskler skulle då kunna lära sig att utlösa en blinkning till tonen. David Marr (1969) och senare James Albus (1971), vilka citeras i inledningen av denna avhandling, förslog just detta i lite olika ordalag och med lite olika syn på detaljerna. Sedan dess har det blivit en alltmer accepterad uppfattning att det nog är så. Dessutom har en lång rad experiment utförts som gjort detta troligt. Dock har ingen hittills visat att Purkinjecellen faktiskt kan betingas att svara på signaler i mosstrådar/parallelltrådar då de åtföljs av en signal i klättertrådarna.

I mitt första delarbete gjorde jag mätningar av den elektriska aktiviteten hos Purkinjeceller under tiden som betingning pågick. Vi betingade blinkreflexen med både perifera (dvs. vanliga) stimuli och genom stimulering direkt i de nervbanor som konvergerar på Purkinjecellen, dvs. mosstrådar och klättertrådar. Oavsett om vi stimulerade perifert eller direkt i nervbanorna så fick vi samma resultat – Purkinjecellerna lärde sig en ny respons. Den respons som betingades (dvs. som Purkinjecellerna lärde sig) var dessutom sådan att den med mycket stor sannolikhet skulle orsaka en blinkning. Vår tolkning av detta resultat är enkel: i lillhjärnans bark skapades ett minnesspår för associationen mellan mosstrådsaktivitet och klättertrådsaktivitet. Efter att Purkinjecellresponsen betingats, så undersökte vi om den även kunde släckas ut (dvs. avläras) och sedan återinläras. Det kunde den, och till yttermera visso skedde detta på ett sätt som ser ut som det gör när man mäter blinkresponsen. Utsläckningen var gradvis och långsam, medan återinlärningen gick mycket fort.

I mitt andra delarbete undersökte jag den betingade Purkinjecellresponsen i mer detalj med avseende på tajming. Betingade blinkningar är synnerligen vältajmade i förhållande till det tidsintervall som passerar mellan det att man börjar presentera tonen och till dess att luftpuffen träffar ögat. Den inlärda blinkningen utlöses precis innan luftpuffen och skyddar på så vis optimalt. Vi kunde se precis samma sak i Purkinjecellerna. De hade lärt sig att svara i förhållande till tidsintervallet. Om man t.ex. ändrade tidsintervallet så lärde sig cellerna detta och ändrade sin responstid. Just detta att Purkinjecellresponsen är tajmad utgör ett fynd som är svårförklarat utifrån nuvarande kunskaper om hur inlärning i nervceller fungerar. Framtida forskning kommer förhoppningsvis att ge oss mer kunskaper om hur en cell kan lära sig att tajma sina responser.

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