The Cerebellar Cortex and Motor Learning

Laurie Millar

Department of Anatomy and Developmental Biology
University College London

2004
Abstract

Classical conditioning of the rabbit eyeblink/ nictitating membrane response (EB/NMR) is dependent on the cerebellum and associated structures. Acquisition of conditioned responses (CRs) is prevented when normal function of either the anterior interpositus nucleus (AIP), lobule HVI of the cerebellar cortex or the inferior olive is functionally disrupted during conditioning. These three regions are connected to form the olivo-cortico-nuclear loop (OCN). So, disrupting normal processing at any one site within the OCN loop has functional consequences throughout the circuit, so the relative roles of HVI and the AIP cannot be dissociated. Some models suggest that HVI regulates the learned timing of conditioned responses, whereas others suggest HVI is where the critical association occurs and so is the principal site for learning-related plasticity.

Here, three different approaches are used to understand the function of cortical HVI in conditioning. The first experimental study used electrophysiology to identify and characterise an EB/ NM microzone within the inferior region of HVI and this microzone was mapped in relation to patterns of Zebrin II expression revealed by immunocytochemistry. The studies presented in chapters 3 and 4 use temporary functional inactivation of HVI to investigate its role in extinction learning and consolidation. It was shown that normal function of HVI is critical for extinction learning and that the adaptive timing of CRs remains constant throughout both acquisition and extinction training. Cortical infusions of the GABA_A receptor antagonist (SR 95531) intended to disrupt post-training consolidation processes impaired the acquisition of CRs 24 hours following the infusion but acquisition and post-training consolidation effects could not be dissociated.

These studies provide evidence for the critical involvement of HVI. The findings are consistent with the suggestion that there is memory storage in the cerebellar cortex but essential plasticity at other levels is not ruled out.
Acknowledgements

Firstly, I would like to express my gratitude to my supervisor, Professor Christopher H. Yeo. Throughout three years of research Chris taught me a great deal by way of lab techniques and writing skills. I have learnt that in both disciplines the need to be critical (of myself and others), thorough (the devil is in the detail) and to try and have a full understanding of the subject matter, is not just important but crucial.

Secondly, I would like to thank all of my colleagues from Chris Yeo’s research group. Dr. Attwell for teaching me many techniques, for showing me invaluable computational skills and for his help with my experimental work. Dr. Ivarsson who I enjoyed working with on the electrophysiology study and Dr. Cooke and Dr. Rahman for their companionship throughout my first year. Dr. Rahman also contributed to the extinction study. Finally, I would like to thank Dr. Krals-Hans who provided help with anatomical experiments as well as encouragement and friendship.

I would also like to thank my friends and my family who have been, and continue to be, so supportive.
Chapter 1: The Cerebellar Cortex and Motor Learning

The Cerebellar Cortex and Motor Learning
Classical conditioning
Features of classical conditioning
Classical conditioning of the EB/NMR of the rabbit
Classical conditioning of the EB/NMR is a good learning model

The Cerebellum
The function of the cerebellum
The anatomy of the cerebellum
Gross anatomy
The cerebellar cortex
Inhibitory neurones with the cerebellum
The olivo-cortico-nuclear loop
Parasagittal zones and microzones

Learning Theories
Hebb (1949)

Cerebellar Plasticity
Plasticity within the cerebellar cortex
LTD of parallel fibre–Purkinje cell synapses
LTD of climbing fibre–Purkinje cell synapses
<table>
<thead>
<tr>
<th>Page</th>
<th>Section</th>
</tr>
</thead>
<tbody>
<tr>
<td>39</td>
<td>Non-conjunctive parallel fibre–Purkinje cell LTD</td>
</tr>
<tr>
<td>39</td>
<td>LTP of parallel fibre–Purkinje cell synapses</td>
</tr>
<tr>
<td>40</td>
<td>Mossy fibre–granule cell synaptic plasticity</td>
</tr>
<tr>
<td>41</td>
<td>Interneurone–Purkinje cell and parallel fibre synaptic plasticity</td>
</tr>
<tr>
<td>42</td>
<td>Plasticity within the deep cerebellar nucleus</td>
</tr>
<tr>
<td>42</td>
<td>Non-synaptic plasticity</td>
</tr>
<tr>
<td>43</td>
<td><strong>Circuitry involved in classical conditioning of the rabbit NMR</strong></td>
</tr>
<tr>
<td>45</td>
<td>Lesion studies</td>
</tr>
<tr>
<td>45</td>
<td>The anterior interpositus nucleus (AIP)</td>
</tr>
<tr>
<td>47</td>
<td>Lobule HVI of the cerebellar cortex</td>
</tr>
<tr>
<td>51</td>
<td>The inferior olive (IO)</td>
</tr>
<tr>
<td>53</td>
<td>Reversible inactivation studies</td>
</tr>
<tr>
<td>55</td>
<td>Summary of results from reversible inactivation studies</td>
</tr>
<tr>
<td>56</td>
<td>Potential problems with reversible inactivation studies</td>
</tr>
<tr>
<td>58</td>
<td>Transmission of CS and US information: pathways involved</td>
</tr>
<tr>
<td>58</td>
<td>CS information: mossy fibres</td>
</tr>
<tr>
<td>59</td>
<td>US information: climbing fibres</td>
</tr>
<tr>
<td>61</td>
<td>Summary of results from studies into the circuitry involved in rabbit eyeblink/NMR conditioning</td>
</tr>
<tr>
<td>61</td>
<td><strong>Physiological evidence for learning in the cerebellum</strong></td>
</tr>
<tr>
<td>62</td>
<td>Purkinje cell firing rates during eyeblink conditioning</td>
</tr>
<tr>
<td>64</td>
<td><strong>Neuronal and Synaptic mechanisms suggested for rabbit EB/NMR conditioning</strong></td>
</tr>
<tr>
<td>69</td>
<td>Studies presented in this thesis</td>
</tr>
</tbody>
</table>

---

**Chapter 2: Mapping Rabbit EB Microzones**

Why is it important to locate EB/NM microzones in the rabbit?
Evidence that lobule HVI contains regions critical for EB/NMR conditioning

Anatomical studies
Climbing fibre responses

Parasagittal zones of the rabbit cerebellar cortex
Parasagittal zones and lobule HVI
Connections of the C zones
Microzones

Why is it important to use Zebrin II compartmentation to identify EB microzones?

Zebrin II/ aldolase C immunocytochemistry

Summary

Materials and Methods

Surgical Procedures
Criteria defining C2 and C3 zone responses
Stimulation of the periorbital region
Recording of CF responses

Experimental design criteria
C2 zone responses
C3 zone responses
Analysis of C2 and C3 zone responses

Histology and reconstruction of tracks
Zebrin II immunocytochemistry

Results

Recordings from C2 and C3 EB control regions
Laterality
Onset latency

C2 zone responses provide a boundary for the C3 zone
Summary of C2 and C3 zone EB/ NM responses
Chapter 3: Cerebellar Cortical Function in Extinction Learning

What is extinction?

Theories of extinction

Internal inhibition

The CS-US association is unlearned

Is extinction a new learning?

Extinction and eyeblink conditioning

Current theories for the mechanism of extinction in eyeblink/ NM conditioning

1) Inhibition of the climbing fibres is the signal for extinction learning

2) Inhibition of the classically conditioned response during extinction training completely prevents extinction learning

The cerebellar cortex and learned response timing

This study

Materials and Methods
Surgical procedures

Conditioning apparatus

Experimental design

Habituation phase

Acquisition

Extinction phase 1

Extinction phase 2

Reacquisition

Performance test

Behavioural Analysis

CR frequency

CR onset-to-peak

Perfusion and histology

Autoradiography

Analysis

Statistical analysis for CR frequency

Analysis: latency-to-peak of the CR

Statistical analysis

Coefficient of variation

Results

Principal findings

Criteria for inclusion of subjects

Infusions of CNQX in the cortex impaired extinction

Acquisition

Extinction phase 1

Extinction phase 2

Is different circuitry involved in extinction and acquisition/performance of the NM CR?

 Learned timing of CRs remained constant during acquisition and extinction training
The coefficient of variation remained constant during acquisition and extinction training

Discussion

Cerebellar cortical function is critical for extinction learning

Regions of the cortex involved in extinction learning

Mechanisms of extinction learning

1) Inhibition of the climbing fibres is the signal for extinction learning

2) Inhibition of the classically conditioned response during extinction training completely prevents extinction learning

Is extinction learning-related plasticity located in the cortex

Learned timing of CRs

Summary

Chapter 4: Cerebellar Cortical Function in Consolidation of a Motor memory

What is consolidation?

Consolidation and motor learning

Consolidation and the cerebellum

Consolidation and EB/NMR conditioning

Possible site and action of SR 95531

Experimental design

Materials and Methods
Surgical procedures
Conditioning apparatus
Experimental design
Habituation phase
Infusion procedure for SR-control group
Acquisition phase
Infusion procedure for PBS-control group and SR-experimental group
Rest period
Performance test
Behavioural analysis
CR frequency
Perfusion and histology
Autoradiography
Statistical analysis for CR frequency

Results
Principal findings
Criteria for inclusion of subjects
Location and spread of SR 95531
Pre-training and post-training infusions of SR 95531 in the cerebellar cortex impaired acquisition of CRs
Habituation phase
Acquisition session 1
Acquisition session 2
Acquisition session 3 and 4
SR 95531 prevented performance of CRs
Summary

Discussion
SR 95531 in the cortex impaired acquisition
Did SR 95531 target consolidation processes?
Index of Figures

Chapter 1: Introduction

<table>
<thead>
<tr>
<th>Page</th>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>1.1</td>
<td>Trace and delay conditioning</td>
</tr>
<tr>
<td>25</td>
<td>1.2</td>
<td>Gross anatomy of the cerebellum</td>
</tr>
<tr>
<td>27</td>
<td>1.3</td>
<td>Anatomy of the cerebellar cortex</td>
</tr>
<tr>
<td>30</td>
<td>1.4</td>
<td>Inhibitory connections within the cerebellum</td>
</tr>
<tr>
<td>38</td>
<td>1.5</td>
<td>Summary of cerebellar synaptic and non-synaptic plasticity</td>
</tr>
<tr>
<td>44</td>
<td>1.6</td>
<td>Cerebellar circuitry involved in EB/NMR</td>
</tr>
</tbody>
</table>

Chapter 2: Mapping rabbit EB microzones

<table>
<thead>
<tr>
<th>Page</th>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>78</td>
<td>2.1</td>
<td>Parasagittal zones and connections to cerebellar nuclei</td>
</tr>
<tr>
<td>80</td>
<td>2.2</td>
<td>Parasagittal zones within lobule HVI</td>
</tr>
<tr>
<td>83</td>
<td>2.3</td>
<td>Zebrin II compartmentation of lobule HVI and parasagittal zone orientation</td>
</tr>
<tr>
<td>90</td>
<td>2.4</td>
<td>Recording data (subjects A-I)</td>
</tr>
<tr>
<td>98</td>
<td>2.5</td>
<td>Reconstruction of tracks and recording sites</td>
</tr>
<tr>
<td>97</td>
<td>2.6</td>
<td>Summary of recording sites on schematic standard images of lobule HVI</td>
</tr>
<tr>
<td>100</td>
<td>2.7</td>
<td>Zebrin II immunohistochemistry and recording sites</td>
</tr>
<tr>
<td>99</td>
<td>2.8</td>
<td>Schematic of zebrin II compartmentation and EB/NMR control zone within lobule HVI</td>
</tr>
</tbody>
</table>

Chapter 3: Cerebellar cortical function in extinction learning

<table>
<thead>
<tr>
<th>Page</th>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>119</td>
<td>3.1</td>
<td>Conditioning apparatus for rabbit NM</td>
</tr>
<tr>
<td>120</td>
<td>3.2</td>
<td>Experimental design</td>
</tr>
</tbody>
</table>
Chapter 4: Cerebellar cortical function in consolidation of a motor memory

149 4.1a Muscimol action within cerebellar circuitry
149 4.1b SR 95531 action within cerebellar circuitry
152 4.2 Experimental design
159 4.3 SR 95531 Autoradiographs
159 4.4 Acquisition of CRs
165 4.5 Drug activity and performance of CRs

183 A1.1 Appendix 1: Complete data set (chapter 2)
190 A.2.1 Appendix 2: Examples of unconditioned and conditioned responses
## Index of Tables

<table>
<thead>
<tr>
<th>Page</th>
<th>Table</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>89</td>
<td>2.1a</td>
<td>Latency of C2 zone responses</td>
</tr>
<tr>
<td>89</td>
<td>2.1b</td>
<td>Latency of C3 zone responses</td>
</tr>
<tr>
<td>135</td>
<td>3.1</td>
<td>Coefficient of variation taken from White et al (2000)</td>
</tr>
<tr>
<td>135</td>
<td>3.2</td>
<td>CR coefficient of variation taken from experimental results</td>
</tr>
<tr>
<td>166</td>
<td>4.1</td>
<td>Frequency of CRs following infusion of SR</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction
The Cerebellar Cortex and Motor Learning

This thesis is about learning and memory. Learning can be defined as 'an experience-dependent generation of enduring internal representations' and/or 'experience-dependent lasting modification in such representations'. Memory can be defined as 'the retention of experience-dependent representations over time' (Dudai 1989). Learning can be divided into two types, one that is related to events and factual knowledge and one that is related to procedures and is reflected in skilful behaviours. Similarly, memory can also be divided into two main types. Conscious memory, that is memory of facts or events and non-conscious memory, that is memory for a skill or habit. In order to study memory and learning in the laboratory it is useful that learning is the result of conditions set and controlled by the experimenter and that it can be identified by an overt, well-defined, measurable behavioural response.

Classical conditioning is a form of learning that is particularly suited to experimental analysis. During a classical conditioning task, a learned association is formed between two stimuli and this learning can be measured as the performance of a conditioned response. Classical conditioning of the rabbit eyeblink (EB)/ nictitating membrane response (NMR) is a simple model of learning and because it involves the production of a simple conditioned movement, is considered to be a simple form of motor learning. Motor learning can be defined as the acquisition of a novel motor response or adaptation of an existing motor reflex (Doyan and Ungerleider 2002). During EB/NMR conditioning, the experimenter has full control of all stimulus parameters, the unconditioned and conditioned behavioural response is robust and easily identifiable and the technique allows for good controls (this chapter, page 21). Using this simple learning model investigations into separate components of the learning process such as acquisition, storage, performance and extinction, are possible.

Lesion and inactivation studies have revealed that classical conditioning of the rabbit EB/NMR is dependent on the cerebellum and associated structures (see Yeo and Hesslow 1998, for review). The anatomy of the cerebellum is well characterised and the
neuronal structure of the cerebellar cortex has prompted models of cerebellar function that involve a motor learning rule (e.g. Marr 1969, Albus 1971, Gilbert 1974, Ito 1982a). Simple implementations of these early theories for EB NMR conditioning have been suggested (Kim and Thompson 1997, Yeo and Hesslow 1998).

There is strong empirical evidence defining the essential cerebellar circuitry for conditioning of the rabbit EB/NMR, but many issues still need to be resolved. The neuronal and synaptic mechanisms underlying this learning remain largely unknown. Current, system level theories of this conditioning suggest possible mechanisms, but to date there is no direct evidence to support them (this chapter, page 64). To characterise the neural plasticity underlying cerebellar-dependent learning, it is first necessary to identify where within the cerebellum the association between the two stimuli is made during conditioning. Current research focuses on two candidate sites for this essential plasticity: the cerebellar cortex and the cerebellar nuclei. Normal function in both these sites is critical for the acquisition, extinction and performance of conditioned EB/NM responses (see, Yeo and Hesslow 1998), but the functional interactions between these sites during conditioning is unknown. Recent research provides evidence that there is at least some learning-related plasticity within the cerebellar cortex (Attwell et al 2002b) but it is possible that memory transfer may occur and extra-cerebellar regions may also store motor memories. Locating and identifying plasticity responsible for learning will contribute to understanding the relative roles of the cerebellar cortex and nuclei in this and, it is hoped, other types of cerebellar-dependent learning.

**Classical Conditioning**

I.P Pavlov, the Russian physiologist, was studying the digestive system of the dog, and in particular the fluids secreted from the stomach wall to aid digestion, when he observed a phenomenon that he later called classical conditioning (Pavlov 1927). During the original experiment food was placed in the dog’s stomach to stimulate digestive fluid secretion. However, Pavlov went on to discover that the act of placing the food dish in front of the subject or even the appearance of the experimenter who usually provides the food could also act as the stimulus for reflex fluid secretion in the stomach. This led Pavlov to conclude that the stimulus for digestive fluid secretion,
such as the presentation of the food dish, could be an anticipatory signal. Pavlov termed the reflex response elicited by one of these signals the *conditioned response* and the process responsible for it he called *classical conditioning*.

During classical conditioning an unconditional stimulus (US) that reliably elicits an unconditioned response (UR) is paired with a conditional stimulus (CS) that at first does not elicit any response. After repeated presentations of paired unconditioned and conditioned stimuli the conditioned stimulus gradually comes to elicit a conditioned response (CR). It is the process of forming an association between the CS and US that Pavlov termed conditioning (Pavlov 1927). Pavlov went on further to investigate conditioned responses and many important phenomena associated with them.

**Features of classical conditioning**

Many features of classical conditioning originally noted by Pavlov (1927) must be taken into account when analysing neural mechanisms underlying acquisition, consolidation and extinction processes during classical conditioning.

Pavlov described all behaviour in terms of reflexes. For example, behaviours such as growling or biting he defined as ‘self-defence reflexes’ that are used to defend the animal. Another reflex described by Pavlov is the ‘investigatory reflex’, which refers to the attention a subject will pay to novel stimuli. The investigatory reflex fades on repeated presentation of the once novel stimulus and Pavlov called this decrease in response, habituation. Habituation forms an important part of many conditioning experiments. Subjects are typically given pre-training habituation to the environment where the conditioning sessions will take place, so as to prevent interference by the investigatory reflex in the acquisition of CRs (Bolles 1978a).

Pavlov also discovered generalisation. If conditioning occurs to a CS so that it reliably elicits a CR, then a second CS that is similar to the first CS can also elicit a similar CR. Generalisation is an important phenomenon as there may be slight variations in the stimuli within the conditioning apparatus during training, for example, slight fluctuations in the perceived intensity of a tone CS. However, provided the
stimuli are perceived as more similar than they are different during training sessions, CRs can occur. Discrimination can be considered as the opposite of generalisation, and occurs when the subject responds differently to two similar stimuli. If CS₁ is paired with stimulus X and CS₂ (that is similar to CS₁), is never paired with stimulus X then initially, CRs will occur to both CS₁ and CS₂. However, with repeated pairings eventually CRs will only occur to CS₁.

Pavlov also described and studied extinction, which is the gradual decrease of the CR to the CS. Extinction occurs after conditioning when the CS is presented alone, that is, without the US. Extinction and associated phenomena will be discussed in more detail in chapter 3, ‘Cerebellar Cortical Function in Extinction Learning’.

The optimal parameters for successful conditioning vary widely according to the response that is being conditioned. Pavlov did not study conditioning of the rabbit EB/NMR but studies by Smith and colleagues provide information on the optimal temporal relationship between the CS and US, which is fundamental to successful EB/NMR conditioning. Varying the interval between the CS and US onsets, a parameter known as the inter-stimulus interval (ISI), results in differing rates of acquisition of the CR. If the ISI is too long or too short then conditioning is weak. With a corneal airpuff US, the upper and lower limits of the ISI in NMR conditioning are about 500 ms and 100 ms, respectively (Smith et al 1969). An ISI of 250 ms results in the highest percentage of CRs during acquisition (Smith 1968).

It is not just contiguity that is important - contingency is also necessary for conditioning. If the CS and US are presented completely randomly, inevitably some pairings will result in a number of trials with contiguous stimuli but no conditioning occurs. Conditioning only occurs if, in addition to stimulus contiguity, there is also stimulus contingency in the trials. A reliably predictive relationship between the CS and US, where the CS consistently predicts the US, is necessary for maximal conditioning.

Classical conditioning of the rabbit EB/NMR is clearly demonstrated using two paradigms described by Pavlov (1927) - delay and trace conditioning. The standard
procedure for EB/NMR conditioning is delay conditioning, where the US onset occurs after the onset, but before (or simultaneously with) the offset, of the CS (figure 1.1). In trace conditioning the CS onset and offset occurs before the onset of the US and a typical interval during EB/NMR trace conditioning is 500 ms (Thompson and Kim 1996, for review). Studies have shown that subcortical structures are capable of supporting this type of conditioning as acquisition of NMR conditioning is relatively normal in decorticate subjects (Oakley and Russell 1972, Oakley and Russell 1975). Other studies demonstrated that the hippocampus and amygdala, recognised as important in other forms of learning, are not essential for delay conditioning of the rabbit NMR (Schmaltz and Thios 1972, Kemble, Albin and Leonard 1972). In contrast hippocampal lesions impair acquisition during trace conditioning (Soloman et al 1986). For the studies presented and discussed here, unless otherwise stated, the delay paradigm is used for rabbit EB/NMR conditioning.

Figure 1.1

Trace conditioning

Delay conditioning

Figure 1.1 Conditional Stimulus (CS) and Unconditional Stimulus (US) onset (▲) and offset (▼), for trace conditioning, the onset of the US occurs following the CS offset, a typical period of time between the CS and US for eyelink conditioning is 500ms. The bottom trace shows the CS and US onset and offset pattern for delay eyelink/ NMR conditioning, the US and CS offset at the same time although the CS onset (in the conditioning studies presented in this thesis the CS onset occurs 350ms before the US onset).
Classical conditioning of the rabbit EB/NMR

Conditioning studies described in this thesis investigate learning using delay classical conditioning of the rabbit NMR. The NM, or third eyelid, is a cartilaginous membrane that sweeps horizontally across the eyeball from the nasal canthus towards the lateral canthus. This movement occurs during eyeblink.

Conditioning of the EB of the rabbit is a widely used learning model and studies have shown that the CS and US parameters, commonly used, result in conditioning of both the NM and EB response. EMG recordings from the muscles controlling EB correspond with the extension of the NM during URs and during acquisition and extinction of CRs. Additionally, the frequency and amplitude of NM and EB responses condition at an almost parallel rate (McCormick et al 1982a, Lavond et al 1990). Importantly, the close correspondence between the two learned responses suggests a common locus for the pre-motor commands.

During conditioning subjects are restrained in a Perspex holding box and placed in a soundproof conditioning chamber for the duration of a training session. Currently, standard CSs used in this type of conditioning are the presentation of a tone, light or weak electrical skin stimulation. The US used is typically an airpuff directed to the cornea or an electrical stimulus to the peri-orbital area; both are stimuli that reliably elicit the EB/NM responses. In the studies described in this thesis the CS used is an auditory tone and the US used is an electrical stimulus to the skin behind and below the eye. Movements of the NM in response to the US and CS are referred to as the UR and CR respectively.

Classical Conditioning of the rabbit NMR is a good learning model.

Classical conditioning of the rabbit NMR has several features that make it a good model to investigate learning and memory:

- Classical conditioning of the rabbit EB/NMR is associative and depends upon two distinct and identifiable stimuli, the CS and the US. The parameters of each stimulus
can be defined by the experimenter to allow a large degree of control during conditioning. The behavioural responses, the UR and CR, are robust and easily measurable.

- Rabbits are docile so they are easy to restrain for the duration of the conditioning session (typically 30-60 minutes). Using appropriately designed restraining boxes the subjects remain still and importantly, their heads move very little for the duration of the session. This is an important for reliable conditioning as head and ear position affect the perceived intensity of the tone CS. Rabbits also have widely opened eyes with an easily accessible NM and periorbital region.

- The NM has a very low rate of spontaneous movement. During conditioning sessions the majority of movement is elicited by the US or CS and can be counted as a true UR or CR, allowing accurate measurement of learning. The NM reflex shows very few non-associative responses.

- The rate of acquisition of CRs can be manipulated depending on the requirement of the study. For example, for recording studies that require stable maintenance of the physiological and behavioural variables of the individual subjects during conditioning, CRs can be acquired within one training session (Thompson 1976). However, studies that manipulate the circuitry involved in EB/NMR conditioning commonly use pharmacological agents that have a known ‘active’ period. These studies require multiple, short sessions that reflect the activity time of the drug and result in a slower rate of acquisition. Typically, CRs are acquired over two or three training sessions.

- Once learned the CS reliably elicits CRs with a consistent temporal profile allowing true CRs to be easily distinguishable from alpha responses. Alpha responses (see Appendix Two, page 190) are responses that occur to the CS before training and that can become intensified during conditioning, these responses are defined by their onset latency and are rejected as true CRs (see Chapter 3, Behavioural Analysis, CR Frequency, page 119).
The CS (tone) and the US (peri-orbital shock or air puff to the eyeball) when applied at intensities used to achieve good conditioning.

Classical conditioning of the EB/NMR is critically dependent on the cerebellum (McCormick et al 1982b). The anatomy of the cerebellum is well understood and the neuronal structure of the cerebellar cortex is similar throughout all regions. The neuronal architecture of the cerebellar cortex prompted early learning theories that are still useful (Marr 1969, Albus 1971).

It will be valuable to describe the functional anatomy of the cerebellum in some detail before discussing the Marr (1969) and Albus (1971) learning theories.

The Cerebellum

The function of the cerebellum

The cerebellum has been shown to be involved in a variety of different functions. Early studies, such as those by Flourens (1824) and Luciani (1915), demonstrated that lesions of the cerebellum severely affected many aspects of, but did not prevent, voluntary and non-voluntary movement, indicating that the cerebellum is involved in integrating and controlling movements. The cerebellum receives inputs from the periphery and from all levels of the nervous system, including motor areas of the cerebral cortex. The cerebellum also receives cutaneous and proprioceptive information via the spinocerebellar tracts. Cerebellar outputs project to the brainstem and motor regions of the cerebral cortex via the midbrain and thalamus. These feedback systems allow the cerebellum to modify ongoing movements via projections to the descending motor pathways.

A role for the cerebellum in motor learning has been revealed by studies of classical conditioning of the rabbit EB/NMR and investigation of the adaptation of the vestibulo-ocular reflex (VOR). The VOR is responsible for keeping images stable on the retina during head movements by generating eye movements that are equal in
magnitude and opposite in direction to the head movement. When the head changes position, the VOR stabilises the eye against the head changes. These two types of motor learning are critically dependent on the cerebellum and associated structures (Ito 2002, for review).

A more recent suggestion is that the cerebellum is involved in non-motor, cognitive and language processes. The circuitry proposed to be involved in cerebellar language processes contains a feedback system from the cerebral cortex to the cerebellum. Because of this it has been proposed that the cerebellum could be part of a language-learning loop (Leiner et al 1993). Furthermore, there is evidence of a cognitive role for the cerebellum from patients with cerebellar lesions, who display cognitive and perceptual difficulties as well as motor control problems (Ivry and Baldo 1992, Schmahmann 1998).

The anatomy of the cerebellum

Gross anatomy

The cerebellum is composed of: (i) the cerebellar cortex - an outer, highly foliated layer of grey matter, (ii) internal white matter and (iii) three pairs of cerebellar deep nuclei, the fastigial, interpositus and dentate nucleus, located medially to laterally respectively.

The cerebellum can be divided into three lobes. The primary fissure divides the anterior and posterior lobe and the flocculo-nodular lobe is separated from the posterior lobe by the posterolateral fissure. Shallower fissures divide the anterior and posterior lobe into nine smaller lobules and the flocculo-nodular lobe accounts for one lobule. The most commonly used nomenclature for these ten lobules are the roman numerals I to X with hemispheral regions prefixed with an H, identified and named by Larsell (1970). The anterior lobe consists of lobule I to V, the posterior lobe consists of lobules VI to IX and the flocculo-nodular lobe is lobe X, (figure 1.2).
The cerebellum is also divided into medial and lateral regions. There is a longitudinal strip at the midline of the cerebellum called the vermis that is separated from the hemispheres at either side by two longitudinal furrows. The hemispheres are divided into the intermediate and lateral hemispheres. These three regions the vermis, the intermediate hemisphere and the lateral hemisphere project to the three cerebellar nuclei. The vermis projects to the fastigial nucleus that projects to descending pathways controlling the proximal muscles. The intermediate hemisphere projects to the interpositus nucleus and projections from this region influence the lateral descending pathways controlling distal muscles. Finally, the lateral hemispheres project to the
dentate nucleus that in turn, projects to the motor and pre-motor regions of the cerebral hemispheres (Ito 1984) (figure 1.2).

The cerebellar cortex

The cerebellar cortex consists of three layers, an outer molecular layer, a Purkinje cell layer and an inner granule cell layer, (figure 1.3). The large Purkinje cells (Pcs) have a cell body diameter reported in the rabbit as approximately 50µm (Sanchez et al 2002) lie side by side in a monolayer and are the sole outputs of the cerebellar cortex, projecting to the cerebellar or vestibular nuclei. These cerebellar nuclei also receive collaterals from cerebellar excitatory inputs, mossy fibres (mfs) and climbing fibres (cfs) (Somogyi et al 1986, van der Want 1989). Pcs project to a discrete region of the cerebellar nuclei and are innervated by climbing fibres that have collateral projections to the same region of the cerebellar nuclei (Ruigrok and Voogd 2000). The Pc dendrites aborise extensively in a single plane in the molecular layer, perpendicular to the main axis of the folium. Pcs are inhibitory and use γ-aminobutyric acid (GABA) as their principal neurotransmitter (Ito 1964).

The cerebellar cortex receives two types of excitatory afferent input -mossy and climbing fibres. All inputs travel to the cerebellum via one of three peduncles. The middle cerebellar peduncle carries mfs from the pontine nuclei exclusively. The inferior cerebellar peduncle carries mfs from the spinal cord and brainstem reticular nuclei as well as cfs originating from the inferior olive (IO). The majority of outputs from the cerebellum travel via the superior cerebellar peduncle and some via the inferior cerebellar peduncle.

Mfs travel into the granule layer of the cerebellar cortex where they branch to contact many granule cells and form pre-synaptic enlargements (or rosettes) and terminals from granule cells form claw-like structures around these mf rosettes (Sultan 2001). The ascending axon of the granule cell contacts the Pc and the granule cell axons travel into the molecular layer where they bifurcate and run along the axis of the cerebellar folia, perpendicular to the Pc dendritic tree, these are called parallel fibres (pf). From the point of bifurcation, the length of pf extend is species dependent, but in
the cat are reported to extend to approximately 3.5mm (Brand et al. 1976) and in the monkey, can contact as many as 300 Purkinje cells (Larsell and Jenson 1972). Pfs only synapse once onto any given Pc and many different pfs synapse upon one Pc. In the rat, each Pc can receive inputs from 175,000 pfs (Napper and Harvey 1988).

Figure 1.3

Longitudinal Axis of folium

Figure 1.3 The cerebellar cortex consists of three layers, the molecular, Purkinje cell and granule cell layer. The two afferent inputs, climbing fibres and granule cells synapse on to Purkinje cell dendritic tree in the molecular layer. Purkinje cells receive inputs from many parallel fibres but only one climbing fibre. The Purkinje cell is the only output of the cerebellar cortex and projects to the deep cerebellar nuclei. Lugaro cells have been excluded as their connections are not fully understood.

(ML = molecular layer, PcL = Purkinje cell layer, GL = granule cell layer, Bc = basket cell, Cf = climbing fibre, DCN = deep cerebellar nuclei, Grc = granule cell, Goc = Golgi cell, Mf = mossy fibre, Pf = parallel fibre, Sc = stellate cell)
Cfs, the other excitatory input to the cerebellar cortex, originate from the IO in the brainstem. Cfs travel through the granule cell layer and Pc layer into the molecular layer. Pcs receive input from only a single cf but cfs contact the soma and dendritic spines of the Pc making numerous synaptic connections (Ito 1984), the majority of which are on the proximal Pc dendrites. Cfs can branch up to 10 times, each contacting a single Pc (Voogd and Bigare 1980).

The two afferent inputs to the Pcs are excitatory and the principal neurotransmitter in both fibres is thought to be glutamate (Cuenod et al 1989, for review see Ito 2000). The cf input to the Pc fires at low frequencies, approximately 1 Hz but can reach up to 10 Hz (de Montigny and Lamarre 1973, Armstrong 1974, Lang et al 1999). When a cf fires it causes a sustained depolarisation of the Pc, called a complex spike, which consists of a primary large depolarisation followed by a train of up to five much smaller secondary depolarisations. The long-lasting plateau potential of the complex spike is due to Ca\(^{++}\) influx to the Pc, following the initial depolarisation (Schmolesky et al 2002, for review). The complex spike dominates the Pc and silences the simple spikes, evoked by pf inputs, for up to 15 to 30ms (Granit and Phillips 1956).

**Inhibitory neurones within the cerebellar cortex**

Inhibitory receptor agonists and antagonist are commonly used to manipulate the cerebellar circuitry during and after EB/NMR conditioning. Recently and in this thesis, this has been done to locate the site of post-training consolidation processes (Attwell et al 2002b and chapter 4). So these inhibitory neurones of the cerebellar cortex are considered in detail here.

There are four types of GABAergic inhibitory interneurone within the cerebellar cortex. Stellate and basket cells are found in the molecular layer, Golgi cells are found in the granule cell layer and finally, a fourth less well characterised type of inhibitory interneurone, Lugaro cells, are found in or just below the Pc layer. One Lugaro cell contacts many Pcs as well as Golgi cells and possibly stellate and basket cells (Dean et al 2003).
The anatomy of the network is well characterised. The Pc itself is inhibitory and projects to the neurones of the cerebellar nuclei, it has also been revealed that Pcs also project back into the cortex (Chan-Palay 1971). Pcs receive inhibitory inputs from stellate cells, basket cells and Lugaro cells. Golgi cells too receive inhibitory inputs from Lugaro cells and Lugaro cells may also have inhibitory projections to stellate and basket cells (Dean et al 2003). Golgi cells are the sole source of inhibitory input to granule cells and are thought to be important in the pre-processing of information before it reaches the Purkinje cell via the parallel fibres (Ito 1984). In a study that selectively ablated Golgi cells in mice, they were shown to be essential for normal cerebellar function (Watanabe et al 1998) (see figure 1.4).

The physiology of the cortical inhibitory network is understood to some extent. Pcs and interneurones of the molecular layer have spontaneous activity that results in tonic inhibition of their target cell. It has been suggested that transmission of information is achieved by modulating, either increasing or decreasing, this tonic activity (Häusser and Clark 1997). A recent study shows that Lugaro cells are normally silent but, when activated via serotonin, they inhibit Pcs and Golgi cells and also possibly stellate and basket cells. Lugaro cell-Pc inhibition is mediated exclusively by GABA_A receptors and only occurs when other dominant inhibitory inputs are silenced (Dean et al 2003). One Lugaro cell contacts many Pcs, and in certain conditions the inhibitory output from these cells will be dominant and may synchronise the firing of many Pcs.

The contribution of these four types of inhibitory neurone to cerebellar motor learning is not understood. The Pc is influenced by all four inhibitory interneurone types and from the two excitatory afferent inputs to the cortex. Therefore a detailed description of the physiology of the cerebellar cortical microcircuitry will be very important for our understanding of cerebellar mechanisms in learning.

The Pc inhibitory synaptic projections to the neurones of the cerebellar nuclei make up approximately 75% of the total synaptic input to nuclear neurones (De Zeeuw and Berrebi 1995), all other inputs to the nuclear neurones are excitatory. One cerebellar
nuclear neurone can receive inputs from up to 30 Pcs. However, it is largely unknown how the Pcs transfer information to the nuclear cells. In agreement with the hypothesis that it is the change in tonic inhibition of the Pc that is critical for information transmission to the nuclei neurones, a recent study has revealed that Pc-cerebellar nuclei synapses display a larger sensitivity to dynamic and not steady Pc activation (Pedroarena et al. 2003). Additionally, it has been shown that nuclear neurones fire intrinsically at 10-20 Hz \textit{in vivo}, in the resting state, i.e. under tonic inhibition (Telgkamp and Raman 2002) and that nuclear neurone excitatory post-synaptic currents (EPSCs) have a significant NMDA component. Therefore, NMDA receptors may contribute to the resting activity of these neurones and may be involved in normal synaptic signalling and information transfer (Anchisi et al. 2001).

\textbf{Figure 1.4}

![Diagram of inhibitory connections within cerebellar circuitry involved in EB/NMR conditioning. Black lines with black circles indicate inhibitory projections, dashed line with black arrows indicate probable inhibitory projections. Black lines with white squares indicate excitatory projections. (AIP- anterior interpositus nucleus, Gr c- granule cell, Pc- Purkinje cell, Pf- parallel fibre, Sc- stellate cell, Bc- Basket cell, Lc- Lugaro cell, Go c- Golgi cell)](image)
The olivo-cortico-nuclear loop (OCN)

The connections within the cerebellum form many modules each containing olivo-cortico-nuclear loops (OCN). Each specific region of the IO gives rise to cfs that innervate Pcs in a specific parasagittal zone and these cfs have collaterals to that division of the cerebellar nuclei that itself receives Pc projections from that parasagittal zone. The specificity of the loop is completed as the cerebellar nuclei project to the region of the IO from which they receive collaterals (Ruigrok and Voogd 2000). These connections form OCN loops, (figure 1.6).

Parasagittal zones and microzones

Early studies analysing the corticonuclear and olivocortical connections within the cerebellum of the cat have shown that the three longitudinal regions of cerebellar cortex, the vermis, intermediate and lateral hemisphere, can be divided into eight parasagittal zones (Voogd and Bigaré 1980, for review). These zones receive cfs from a specific region of the IO and project to a specific target region of the cerebellar nuclei.

Based on anatomical evidence, originally only seven zones were identified in the cat and ferret, A, B, C1, C2, C3, D1 and D2 (or Y). Zone A is the most medial zone followed laterally in order by zones B, C and D (Voogd 1969). Further work in cats described nine zones in the anterior lobe with the addition of the X zones, A, X, B, C1, Cx C2, C3, D1 and Y, (Ekerot and Larson 1977, 1979, Trott and Apps 1991) but zone X has not been located in all species. The division of the zones is not always distinct and the C zone can be separated into four sub divisions. The C1 zone has overlapping connections with the Y zone, as does the medial C1 zone with the lateral C3 zone, indicating a functional coupling of zones (Oscarsson 1980).

Electrophysiological studies of the cerebellar cortex have shown that electrical stimulation of particular limb nerves elicits cf responses (cfrs) with particular response latencies and convergence patterns in the parasagittal zones (Oscarsson 1969). Importantly, cfrs have characteristic latencies that relate to the number of synapses in
the spino-olivary pathways and have distinctive receptive field properties. Functional specificity of the zones is also proposed as the IO receives specific information via spino-olivary paths that terminate in specific regions of the IO. Further evidence suggesting a functional difference between zones is that specific regions of the cerebellar nuclei modulate different descending motor control pathways (Oscarsson 1980).

The parasagittal zones can be subdivided into microzones that are orientated parallel to the Pc dendritic tree. Each microzone controls a single muscle or muscle group. The microzones controlling eyeblink have been mapped in the cat and have been located in four regions within hemispheral lobules V, VI and VII (Hesslow 1994a). The parasagittal organisation of the cerebellar cortex is discussed in detail in Chapter 2, Mapping Rabbit Eyeblink Microzones.

**Learning Theories**

**Hebb (1949)**

Donald Hebb first proposed synaptic modification (or synaptic plasticity) in 1949, in his book ‘The organisation of Behaviour: A Neuropsychological Theory’. Hebb proposed the idea of cortical ‘cell assemblies’, that reverberated with self-sustaining activity and that could activate neighbouring cortical areas. In order to maintain the activity in the cell assemblies Hebb suggested that the strength of the connections between neurones within a cell assembly could change by an activity-dependent mechanism for synaptic plasticity.

> 'When an axon of cell A is near enough to excite cell B and repeatedly and persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased'  
*Hebb 1949*

These ideas contributed to subsequent cerebellar learning theories (this chapter, page 64). Marr (1969) considered this Hebbian plasticity as a possible mechanism for synaptic modification during learning and Albus (1971) suggested that the process
underlying synaptic modification during learning is based on coincidence detection as described by Hebb.

Marr (1969), Albus (1971)

In 1969 Marr proposed a cerebellar cortical learning model. Marr assumed that olivary cells are driven by instructions for a movement to occur and that each olivary cell corresponds to an ‘elemental movement’. Marr defined ‘elemental movements’ as actions or instructions that are necessary to have under control during movement. Therefore every movement can be represented as an ordered pattern of ‘elemental movements’ and every movement has a defining representation as a sequence of firing patterns in the olive. Marr proposes that every olivary cell is represented by a Pc, and therefore Pcs can learn all the ‘situations’, (that require ‘elemental movement/s’) corresponding to all of the olivary cells.

The cerebellum could therefore learn to carry out rehearsed movements in the following way. As the movement occurs, the context for the next stage of the movement would be created by the specific parallel fibre input (see paragraph below) and recognised by the appropriate Pcs. This pattern would continue until the action was completed and could eventually be performed automatically. The context is learned and when it occurs again, mossy fibres (mfs) activate the relevant Pcs that in turn evoke the relevant movements.

Marr assumed that information about the context of the movement is transmitted via the mf- granule cell- Pc pathway and that this pathway acts as a pattern discriminator. This means that the differences between contexts are transmitted via different populations of parallel fibres (pfs), and one could learn many different contexts by modifying or strengthening the relevant pf-Pc synapses. Synapses are strengthened when the relevant climbing fibres (cfs) are also active.

Marr suggested that the change in efficacy of the pf-Pc synapse could occur for two possible reasons. Firstly, the cfs could release a ‘change’ factor that modifies active
synapses and secondly, the modification is due to simultaneous pre- and post- synaptic activity, as first suggested by Hebb (1949).

Two years later Albus (1971) published his paper on "A Theory of Cerebellar Function". This theory assumes that Pcs responses can be conditioned by cf inputs. Intriguingly, he likened this process to classical conditioning where the cf s act like a US and the mfs act like a CS, although he did not propose this as a mechanism for classical conditioning of overt behaviours.

Albus suggests that conditioning occurs via a three-way coincidence mechanism between, the 'inactivation response', (this refers to the depolarisation and pause in firing of the Pcs following cf input), activation of the Pcs due to pf excitation and pf synaptic activity. Importantly instead of facilitating or strengthening the synapses as Marr suggests, Albus proposed that the pf-Pc synapses are weakened by incorrectly firing during cf activity.

Cerebellar Plasticity

The learning models proposed by Marr (1969) and Albus (1971) suggest the cerebellar cortex as the location for plasticity responsible for motor learning. Lobule HVI of the cerebellar cortex is considered to be a critical component of the cerebellar circuitry (see page 47) necessary for acquisition and performance of rabbit EB/NMR conditioning (Attwell et al 1999, Attwell et al 2001). CS and US information converge onto Pcs in lobule HVI, so it is a potential site of essential plasticity for EB/NMR conditioning.

Early studies (Ito et al 1982b) found an important synaptic plasticity in the Pcs of the cerebellar cortex. Long-term depression (LTD) of pf-Pc synapses is produced by co-activation of cf and mf inputs and is still the principal candidate plasticity for the learning process. However, recent in vitro studies have characterised up to seven other types of plasticity that can occur within the cerebellar circuitry and which could, therefore, underlie EB/NMR classical conditioning. A non-conjunctive pf-Pc LTD, (pf induced LTD), has been characterised (Hartell 1996). Cf-Pc LTD has been observed and
identified as post-synaptic and may affect pf-Pc LTD (Hansel and Linden 2000). Pre-synaptic long-term potentiation (LTP) of pf-Pc synapses has been produced by stimulation of the pfs alone and, more recently, a post-synaptic LTP of pf-Pc synapses has been characterised (Lev-Ram et al 2002). LTP of the mf-granule cell synapses is also a possible mechanism for learning-related plasticity and interneurone-Pc synapses (Hansel et al 2001 for review) and interneurone-pf synapses are also sites with identified plasticities, (Kenyon 1997, Rancillac and Crepel 2003).

Normal function of the AIP is also critical for acquisition, performance and extinction of CRs (Hardiman et al 1996, Attwell et al 2002a and see page 45) and so the AIP is another candidate site for cerebellar learning-related plasticity. Different types of cerebellar nuclear plasticity have also been demonstrated including, LTP and LTD of the Pc-nuclear neurone synapses, excitatory synapse formation and increases and decreases in intrinsic excitability (Aizenman et al 1998, Aizenman and Linden 2000, Hansel et al 2001 for review, Kleim et al 2002). Figure 1.5 shows a summary of cerebellar cortical and nuclear plasticities discussed here.

In order to understand the synaptic and neuronal mechanisms responsible for learning and possibly unlearning during conditioning of the EB/NMR, it is important to identify all plasticities within the EB/NMR circuit and demonstrate involvement or non-involvement of these in the conditioning process.

**Plasticity within the cerebellar cortex**

**LTD of parallel fibre-Purkinje cell synapses**

Masao Ito and colleagues (Ito et al, 1982b) demonstrated LTD in vivo in the rabbit cerebellum and discovered that Pcs developed a persistent reduction in sensitivity to glutamate. The parameters used to induce this form of LTD specifically at the pf-Pc synapses, were iontophoresis of glutamate, replacing pf activation, and activation of cfs or conjunctive vestibular mf and olivary stimulation (Ito et al 1982b). However, because glutamate iontophoresis could also activate cf synaptic inputs, direct pf activation in conjunction with cf stimulation, was also used (Ito and Kano, 1982). Ito and colleagues
also provided evidence that this plasticity was post-synaptic. In vitro LTD in rat PCs can be induced following stimulation of cfs and pfs at low frequency (Karachot et al 1994).

Four initial requirements are necessary for the induction of LTD (for a review see Ito 2001). Firstly, cf activation that causes a Ca\(^{++}\) influx via voltage gated calcium channels (VGCCs) consistent with the finding that Ca\(^{++}\) ion chelators prevent the induction of LTD (Sakurai 1982). Secondly, release of glutamate from pfs activating AMPA and mGluR1 receptors (Ito 2002, for review). Thirdly, activation of protein kinase C (PKC) is also critical for pf LTD in vitro (De Zeeuw et al 1998) and transgenic mice that express a PKC inhibitor in all of their PCs have impaired LTD and show impaired EB conditioning (Koekkoek et al 2003). Fourthly, the nitric oxide (NO)-cyclic guanosine monophosphate (cGMP)- protein kinase G (PKG) signalling cascade is necessary for in vitro LTD (Daniel et al 1993). There is recent in vivo evidence that this signalling pathway is engaged and essential in rabbit EB/NMR conditioning (Rogelj et al. 2003). LTD of pf-Pc synapses is expressed as a reduction in the number of post-synaptic AMPA receptors produced by clathrin-mediated endocytosis (Wang and Linden 2000).

There is no direct evidence that pf–Pc LTD is the plasticity responsible for cerebellar motor learning and specifically rabbit EB/NMR conditioning. Further work is required to conclusively demonstrate whether LTD is the essential plasticity responsible for this type of conditioning. However, there is indirect evidence suggesting that pf-Pc LTD may be involved in EB/NMR conditioning.

The induction of LTD, in vitro in rat Purkinje cells, has been shown to depend on the temporal stimulus parameters. It has been shown that PF stimulation following CF stimulation, with intervals up to 1.75 seconds, are effective in inducing LTD. This is possible if CF stimulation induces a long-lasting process that interacts with another PF-evoked process in inducing LTD. Alternatively, the authors suggest CFs may release a factor such as a peptide, in addition to the neurotransmitter responsible for CF responses, that may account for these long-lasting processes. PF stimuli preceding CF stimuli was effective in inducing LTD but decreased as the PF-CF delay increased. This
study did not induce LTD when the temporal parameters previously used for eyeblink conditioning were replicated, although the authors note that this does not mean LTD is not involved in EB conditioning as complex processing in neural circuitry may alter the timing of PF and CF impulses to induce LTD (Karachot et al 1994). A later study demonstrated LTD induction in rabbit cerebellar slices where the temporal relationship between the cf and pf firing are consistent with those used for the stimuli in EB conditioning (Schreurs et al 1996).

Another study in the rat slice also demonstrated that LTD induction has a distinct temporal specificity but in contrast to Karachot et al (1994) the temporal specificity resembles that for eyeblink conditioning. In this study LTD was induced with PF stimulation preceding CF stimulation by 250ms (see page 19 for optimal temporal parameters during EB conditioning) and LTD could not be induced with an inter stimulus delay of 125ms, 0ms or ~250ms (Chen and Thompson 1995). In agreement with these findings a more recent study also found that induction of LTD was greatest when PF stimulation preceded CF stimulation by 50ms -200ms (Wang et al 2000). Together these studies demonstrate a possible relationship between cerebellar synaptic plasticity and behaviour.

Furthermore, evidence of increased PKC levels in lobule HVI following EB conditioning (Freeman et al 1998), and a deficit in EB conditioning following systemic infusions of nitric oxide synthase (NOS) inhibitor have been shown (Chapman et al 1992), suggesting the involvement of LTD in EB/NMR conditioning. However, direct evidence is still needed to implicate LTD as the mechanism underlying motor learning.

But LTD at the pf-Pc synapses is not the only synaptic plasticity that occurs in the circuit involved in conditioning the EB/NMR. The essential plasticity underlying motor learning need not be in the cerebellar cortex. There may be plasticity at two or more different locations and several types of LTD and LTP may be involved in learning.
LTD of Climbing fibre-Purkinje cell synapses

LTD of the cf-Pc synapses by brief stimulation of cfcs in rat cerebellar slices requires the same post-synaptic conditions as pf-Pc LTD (see above) briefly these are elevation in post-synaptic Ca$$^{++}$$, activation of mGluR1 receptors and activation of PKC. Cf-Pc LTD is expressed as a sustained depression of cf EPSCs and a decrease in the second depolarising current of the complex spike (Hansel and Linden 2000). A functional role for cf LTD has not been suggested in any theory of motor learning but it
should be noted that cf LTD may have consequences for pf LTD. The cf induced Ca\(^{++}\) transient necessary for pf LTD may be reduced during cf LTD and so effect pf LTD (Hansel and Linden 2000). Therefore, cf LTD must be taken into account when considering synaptic mechanisms in cerebellar processing and pf LTD regulation.

**Non-conjunctive parallel fibre-Purkinje cell LTD**

Repetitive stimulation of pfs at a frequency of 1 Hz and at an intensity to produce excitatory post synaptic potentials (EPSPs) of between 8-10 mV can induce LTD at the pf-Pc synapse *in vitro* in cerebellar rat slice. Using these parameters, stimulating pfs alone produced post-synaptic Ca\(^{++}\) levels that almost matched those induced by cf stimulation and were therefore capable of producing long-term synaptic depression. This type of LTD is dependent on post-synaptic Ca\(^{++}\) but does not require NO (Hartell 1996).

There is no *in vivo* evidence of non-conjunctive pf-Pc LTD and the physiological significance of this type of LTD is not fully understood. It may be that this plasticity contributes to the regulation of Pc output so the Pc is maximally responsive to changes in pf inputs (Hartell 1996).

**LTP of parallel fibre-Purkinje cell synapses**

If, as some evidence suggests, pf-Pc LTD is involved in learning (or acquisition) of the EB/NM CR, then LTP of the same synapses could be responsible for unlearning (or extinction) of the CR. Early *in vitro* research demonstrated pre-synaptic pf LTP in the rat cerebellum, induced by low frequency stimulation (4-8 Hz, 120 times) of pfs (Salin et al 1996). This pre-synaptic form of LTP cannot reverse post-synaptic LTD, the two processes are expressed at different locations and so it is unlikely that this pre-synaptic plasticity is responsible for ‘unlearning’.

However, a recent study characterised post-synaptic, NO dependent, pf-Pc LTP *in vitro* in the rat cerebellum (Lev-Ram et al 2002) providing a potential mechanism that would allow pf LTD to be reversed and for unlearning occur. In comparison to the pre-synaptic LTP described above, induction of post-synaptic LTP requires pf
stimulation at 1 Hz for 300s and an absence of Ca^{++} elevation. Induction of post-synaptic LTD is also elicited by stimulation at 1 Hz (and post-synaptic depolarisation Ca^{++} elevation). Therefore it likely that this post-synaptic LTP, and not pre-synaptic LTP described above, is capable of reversing LTD. pf-Pc LTD and post-synaptic pf-Pc LTP could be the plasticities underlying acquisition and extinction of CRs respectively.

Additionally, post-synaptic LTP would also provide a mechanism to prevent saturation of LTD at synapses. It should be noted that synapses that undergo LTD and LTP do not subsequently function as naïve synapses, that is, synapses that have not undergone any learning-related plasticity changes. However, this is in keeping with the implication that the circuitry involved in conditioning processes is permanently altered following acquisition and extinction training, as suggested by phenomena observed during extinction, such as savings (Hansel et al 2001).

**Mossy fibre-granule cell synaptic plasticity**

LTP of the mf-granule cell synapses was demonstrated _in vitro_ in rat cerebellum and is expressed as an increase in AMPA and NMDA receptor mediated synaptic currents (D'Angelo et al 1999). In addition to LTP at mf-granule cell synapses, potentiation of intrinsic excitability can also occur and when combined these forms of synaptic and non-synaptic plasticity can enhance EPSPs and spike generation (Armano et al 2000).

LTD at the mf-granule cell synapse has also been reported (Hansel et al 2001). Plasticity at these synapses may be important in motor learning, together, with modulation from Golgi cell input to granule cells, LTP, LTD and intrinsic excitability changes may control mf population coding and therefore, the mf pattern that is relayed to the cerebellar cortex (Hansel et al 2001). Additionally, in EB conditioning it is possible that an association between the CS and US is formed here as mfs carry information about both the CS and US stimuli.
Interneurone-Purkinje cell and Parallel fibre synaptic plasticity

The contribution that inhibitory cerebellar cortical stellate and basket cells make during EB/NMR conditioning is not understood and current models of this type of conditioning do not allocate a role for them. However, the inhibitory input from these interneurones to the Pc must play an important role in regulation of Pc firing.

Plasticity at the pf-stellate and basket cell interneurones has been proposed as a site for long-term reversible memory storage. This is based on a mathematical model of the cerebellar cortex with reciprocal connections to the IO and the assumption that plasticity at the pf-stellate and basket interneuone synapses follows pf-Pc plasticity. The model predicts that early acquisition conditioning is mediated by pf-Pc plasticity but with further training this plasticity is transferred to pf-stellate and basket interneurone synapses. Consequently, memories are stored at stellate and basket cell-pf synapses and they are therefore, protected from degradation due to ongoing learning at overlapping pf-Pc synapses and random fluctuations in cf input. Therefore learned motor adaptations can be safely stored while novel ones explored. (Kenyon 1997). However, there is no empirical evidence to support this prediction.

A very recent study in vitro in the rat cerebellum provides evidence for plasticity at pf-stellate cell excitatory synapses and suggests a synergistic relationship between the plasticity at these synapses and at pf-Pc synapses (Rancillac and Crepel 2003). Low frequency stimulation of pfs can induce LTD at the pf-stellate cell synapses and low frequency stimulation coupled with post-synaptic depolarisation can induce LTP at the same synapses.

The authors suggest that during conditioning co-activation of pfs and cfs may induce LTP at the pf-stellate cell synapse simultaneously with LTD at the pf-Pc synapse. The pf–stellate cell LTP would increase the inhibition to the Pc from the stellate cell and reinforce the decrease in responsiveness of Pcs due to LTD. This relationship would be reversed when for example, during extinction training; pf stimulation alone would cause LTP of the pf-Pc synapses and LTD of the pf-sc. The pf–
stellate cell LTD would decrease the inhibition to the Pc, reinforcing the increase in responsiveness of the Pc due to LTP (Rancillac and Crepel 2003).

Finally, inhibitory LTP (or rebound potentiation) at the stellate cell-Pc inhibitory synapses has been reported when cfs are repetitively stimulated (Kano et al 1992). Again, the function of this plasticity is not understood but alterations in synaptic strength will have a considerable effect on Pc output.

Plasticity within the Deep Cerebellar Nuclei

Current theories of EB/NMR conditioning in the rabbit suggest that the AIP is the location for the formation of the ‘basic’ association between the US and CS (this chapter, page 64). The AIP is a candidate cerebellar region for the location of learning-related plasticity as it receives collaterals from cfs and mfs transmitting information about the US and CS, respectively.

Until recently only one study had demonstrated plasticity within the cerebellar nuclei and that was a claim for LTP at mf-nuclear neurone synapses (Racine et al 1986). Stimulation of the white matter, where the inferior cerebellar peduncle enters the cerebellum, resulted in enhancement of a component of field potentials recorded from the interpositus nucleus.

However, more recent studies in rat cerebellum have demonstrated LTP of inhibitory Pc-nuclear neurone synapses. Stimulation of these synapses results in summating trains of inhibitory post-synaptic potentials (IPSPs) and eventually a rebound depolarisation and associated spike burst. If, in addition to stimulation, the amount of post-synaptic excitation is reduced, LTD at these synapses can also be seen (Aizenman et al 1998) (figure 1.5).

Non-synaptic plasticity

A type of non-synaptic plasticity has been observed in the neurones of the deep cerebellar nuclei. Activation of mfs using an electrode placed in the white matter adjacent to the cerebellar nuclei caused a synaptic tetanus to the nuclei neurones,
resulting in a Ca$^{++}$ dependent increase in excitability that stabilised over 15 to 20 minutes (Aizenman and Linden 2000). Whether this non-synaptic plasticity has any role in EB/NMR conditioning is not known. It may contribute to the effects of LTP (as suggested for increased intrinsic excitability in granule cells) and it will influence nuclear activity driven by mf and cf collateral inputs. Although some theories of cerebellar function in EB conditioning suggest a changes in mf-nuclear synapse efficacy (Mauk and Donegan 1997, Mauk 1997), the requirement for a learning-related change at the level of the cerebellar nuclei might be served by these intrinsic excitability changes rather than synapse specific plasticity.

Recent studies have demonstrated excitatory synaptogenesis in the cerebellar nuclei following rat EB classical conditioning (Kleim et al 2002). In this study, Kleim et al (2002) suggests that development of CRs during EB conditioning is associated with an increase of the number of excitatory synapses per neurone within the interpositus nucleus. No change was observed in the number of inhibitory synapses. It was suggested that the increase in synapse number involves mfs and not cfs, but there is no direct evidence to support this and there is, as yet, no evidence for a causal relationship between learning and interpositus synaptogenesis (discussed in chapter 4).

As discussed, in vitro studies in the AIP characterise plasticities such as, changes in neuronal excitability, or post-synaptic LTP and LTD at the Pc–nuclear neurone synapses, although as yet there is no evidence of these plasticities in EB/NMR conditioning. A non-synaptic plasticity, synaptogenisis within the cerebellar nuclei, has been demonstrated in vivo, and although this type of neuronal plasticity can not be replicated in vitro, appears to be correlated with rabbit EB/NMR conditioning (Kleim et al 2002).

**Circuitry Involved in Classical Conditioning of the NMR**

Lesion, tracing, stimulation and reversible inactivation studies have contributed to identification of the cerebellar regions and pathways involved in conditioning of the rabbit EB/NMR. Although mostly there is agreement about this circuitry there remains some dispute over precisely which regions of the cerebellar cortex are involved.
Additionally, the relative roles of the cerebellar cortex and cerebellar nuclei in learning are not understood. Figure 1.6 shows a proposed circuitry for this conditioning.

Figure 1.6

Figure 1.6 Cerebellar circuitry involved in EB/NMR conditioning. Inhibitory interneurones have been excluded for simplicity. Red dashed line indicates the olivo-cortico-nuclear loop (OCN). Inhibitory synapses are indicated with a black circle, excitatory synapses are indicated with a white box.

(AIP- anterior interpositus nucleus, Cf- climbing fibre, Grc- granule cell, DAO- dorsal accessory olive, Mf- mossy fibre, OCN loop- olivo-cortico-nuclear loop, Pc- Purkinje cell, Pf = parallel fibre, PN- pontine nuclei, RN- red nucleus).
Early evidence implicating the cerebellum as a critical structure in classical conditioning of the EB/NMR came from lesion studies. Following removal of the lateral cerebellar cortex and both the dentate and interpositus nuclei ipsilateral to the conditioned NMR, the previously well learned EB/NM CR of the rabbit was completely abolished and reacquisition of the response was prevented. The conditioned NMR develops ipsilateral to the applied US. The UR was not affected, suggesting that the loss of CRs was due to a memory, and not a motor, impairment (McCormick et al. 1982b). Lesions of the superior cerebellar peduncle, the output pathway of the cerebellar nuclei, also abolish the rabbit EB/NM CR ipsilateral to conditioning (McCormick et al. 1982c) as do lesions of the contralateral red nuclei, the target of efferent fibres from the cerebellar nuclei (Rosenfield and Moore 1983).

These studies provided the first evidence that classical conditioning of the EB/NMR of the rabbit is critically dependent on the cerebellum and its associated circuitry. Subsequent studies confirmed this finding and located the precise regions involved.

Lesion studies

The anterior interpositus nucleus (AIP)

Lesions of the deep cerebellar nuclei ipsilateral to EB/NMR conditioning and in particular the dentate and interpositus nuclei were shown to abolish the performance of the previously learned NM/EB CR, but they did not appear to affect the UR (McCormick and Thompson, 1984a). Histological examination provided evidence that the cortical regions remained intact in subjects with a lesion of the dentate and interpositus nuclei. In other subjects, where various parts of the cerebellar cortex were lesioned while the nuclei remained intact, previously learned CRs were not abolished, although a decrease in their amplitude and a disruption to their learned timing were observed.

Further evidence supporting the involvement of the dentate and interpositus nuclei in EB/NMR conditioning includes findings that neuronal activity within regions of these nuclei correlates with performance of the CR and in some cases, when these
regions are stimulated closure of the eyelid and extension of the NM would occur in both trained and untrained subjects. Because the response could be produced in untrained animals suggests the neuronal circuitry from the cerebellar nuclei to the motor neurones producing the response is not dependent on the learning of the response (McCormick and Thompson 1984b). The conclusions drawn from these studies were that learning and performance of the EB/NM CRs are critically dependent on a region of the medial dentate and/or the lateral interpositus nucleus and not on any cortical region.

In order to dissociate the involvement of the dentate and interpositus nuclei, kainic acid was used to produce neurotoxic lesions of the interpositus nuclei cells alone, additionally kainic acid has the advantage that damage to efferent and afferent fibres would be prevented. The results from this study show that lesions of the dorsal interpositus nucleus are sufficient to abolish EB/NM CRs in the rabbit and prevent further learning on the ipsilateral side (Lavond et al 1985). However this study does not dissociate between the anterior or posterior regions of the interpositus nucleus, it must be assumed that the kainic acid infusions destroyed cells throughout both these functionally significant divisions of the interpositus nucleus. Additionally the specificity of these lesions is questionable. The lesion reconstructions show that there was damage extending into the white matter below lobule HVI and above the cerebellar nuclei, it must be assumed that fibres of passage were affected, possibly due to insertion of the infusion cannula.

The specificity of connections within the cortical circuitry (as discussed on page 31), mean that it is vital to locate the precise region of the cerebellar nuclei involved in this type of conditioning. Discrete lesions of the individual cerebellar nuclei indicated that it is only the anterior interpositus nucleus (AIP) that is necessary for NMR conditioning. Lesions of the posterior interpositus nuclei, fastigial and dentate nuclei had no little or no effect on CRs, (Yeo et al 1985a). It is now widely accepted that classical conditioning of the EB/NMR of the rabbit is critically dependent on the anterior interpositus nucleus (AIP).
Lobule HVI of the cerebellar cortex

The controversy over which, if any, regions of the cerebellar cortex are critical for conditioning of the EB/NMR is a result of many cortical lesion studies. A brief summary of these studies and a current opinion of the precise region necessary for this type of conditioning is given below.

Thompson and colleagues (1984b) originally reported that some areas of the cortex may participate in the production of learned responses but that, overall, the cortex is not a critical structure for classical conditioning of the EB/NMR of the rabbit. Neuronal activity related to the performance of the CR has been recorded within regions of the ansiform lobe and the anterior lobe and the dentate and interpositus nuclei. Additionally, the responses recorded from the dentate and interpositus nuclei develop in relation to the learning of the CR. The authors concluded that the cortex is involved in the production of the CR but the dentate and/or the interpositus nucleus are independently capable of learning and producing the learned response (McCormick and Thompson 1984b).

This view that the cerebellar cortex is not critical for this conditioning is supported in work by Lavond and colleagues who demonstrated that following acquisition of CRs and subsequent removal of the cerebellar cortex, re-acquisition of the CR can still occur. Large lesions of the cerebellar cortex, including large parts of lobule HVI (which, as we shall see, could be very important in this conditioning), only transiently abolished previously learned CRs (Lavond et al 1987). It must be noted that although lesion reconstructions of each subject were provided, these were schematic images of the cerebellar cortex and were not anatomically detailed. From these schematic reconstructions it is difficult to assess to what extent, and whether the potentially critical regions of lobule HVI, were lesioned or spared. In a second lesion study that also included lesions of lobule HVI, the ability to acquire CRs was retained but CRs were severely retarded. CRs were produced with small amplitudes and disrupted timing. Additionally, subjects took on average, seven times longer to learn than to relearn following cortical lesions (Lavond et al 1989).
Further evidence to support the proposal that the cerebellar cortex is involved in, but not crucial for, the retention of eyelid CRs comes from a study by Perrett and colleagues (1993). The principal findings of this study are that ipsilateral cortical lesions including large areas of the ipsilateral cortex as shown in the lesion reconstructions, did not abolish CRs but did disrupt the learned timing of CRs, resulting in responses with shorter onset latencies, called short latency responses (SLRs). Analysis of the location of the lesions resulted in the conclusion that most effective cortical lesions always included the anterior lobe but not paramedian lobe or the ansiform lobe (Perrett et al. 1993). It must be noted that the lesions are schematically represented only from the surface view. It is impossible to assess the inferior-superior extent of the lesions.

Mauk argues that the presence of SLRs following cortical lesions is evidence that the cerebellar nuclei are intact, as SLRs are abolished with lesions of the interpositus nucleus (Perrett and Mauk 1995). A later study demonstrated that lesions of lobule HIV/V of the anterior lobe were sufficient to disrupt the learned timing of the CRs and prevent acquisition of CRs to a novel CS. However, lesions of the cortex did not prevent the performance of the short latency CR. Furthermore, lesions of the cortex that included lobule HVI but not HIV/V had no effect on the timing of the CRs and subjects were able to acquire CRs to a novel CS (Garcia et al. 1999).

Lesion studies by Yeo and colleagues that specifically targeted lobule HVI, powerfully contradicted these other cerebellar cortical lesion studies. The rationale for targeting lobule HVI was based on the known anatomy and physiology. HVI contains parasagittal zone C and it is known that Pcs from zones C1 and C3 project to the AIP, which is crucial for conditioning. Additionally, the face has a large cf representation in hemispheral region of lobule VI (Miles and Wiesendanger 1975a). Discrete lesions of lobule HVI abolished the NM CR and prevented re-acquisition and no disruption of the learned timing of the CRs was reported (Yeo et al. 1984). The results from this study were confirmed and extended in further lesion studies of lobule HVI and crus I and II of the ansiform lobe. (Yeo et al. 1985b, Yeo and Hardiman 1992). Extensive lesions of the posterior lobe that spared lobule HVI did not impair conditioning of the NMR (Yeo et al. 1985b). Critically, in all of these studies, lesion sites were examined from serial
transverse sections through the cerebellum and the full extents of the cortical damage were plotted. This histological examination showed that the lesions did not extend into the cerebellar nuclei.

Subjects were also histologically examined for evidence of olivary degeneration following cortical lesions. In subjects where only lobule HVI was lesioned, degeneration was seen in dorsal accessory regions (DAO) of the contralateral olive, and the medial part of the principal olive (PO) (Yeo et al 1985b). In order to study the involvement of lobule HVI of the cortex without possible degeneration of the IO, kainic acid was used to lesion lobule HVI, crus I and crus II and these produced no detectable degeneration of the IO. In cases where cortical cell loss had occurred deficits in the frequency and amplitude of CRs were observed. Not all Purkinje and granule cells were destroyed within lobule HVI but the extent of the CR deficits depended on the amount of damage within the cortex. In cases where lobule HVI was spared, the kainic acid lesions had either no effects or only transient and small effect upon CR frequency and amplitude (Hardiman and Yeo 1992).

The use of kainic acid to lesion lobule HVI provided evidence that the cerebellar nuclei alone are not capable of retaining the NM CR. At appropriate doses kainic acid spares afferent cfs and mfs, so the collaterals from these fibres to the cerebellar nuclei would also have been spared. Thus, even when the nuclei and their collateral inputs are not damaged, lesions of lobule HVI are sufficient to abolish the CR. The CR should still have been present if the nuclei alone were responsible for retention, indicating that the cortical input to the nuclei is essential for the expression of existing CRs.

Three factors are significant when considering the inconsistencies between the studies that identify lobule HVI as a critical region for classical conditioning (Yeo et al 1984: 1985b) and those that do not (e.g. Lavond et al.1987, Garcia et al 1999). Firstly, sparing of the critical EB/NM control regions of the cortex, including sparing of critical regions within lobule HVI may have occurred allowing CRs to remain undisturbed. Secondly, the nomenclatures used in cortical lesion studies discussed here describe relatively large regions of the cortex and do not provide information on the exact extent
of lesions in all directions. Therefore, precise anatomical comparisons between cerebellar cortical lesion studies are difficult to assess fully. Thirdly, Yeo and colleagues found that complete unilateral lesions of lobule HVI and crus I and crus II of the ansiform lobules is sufficient to abolish CRs but some slight recovery is possible with extended retraining, although CRs always maintain frequency and amplitude deficits. It is suggested that reacquisition following cortical lesions is dependent on the amount of pre and post-operative training, particularly the amount of pre-operative training that subjects receive (Yeo and Hardiman 1992, Harvey et al 1993). Additionally, bilateral involvement in EB/NMR conditioning could account for reacquisition following unilateral cortical lesions. And there is experimental evidence that contralateral learning contributes to learning a unilaterally trained CR (Gruart and Yeo 1995).

In support of this finding, a recent rabbit fMRI study demonstrated bilateral involvement of lobule HVI during unilateral EB conditioning and CS alone performance trials (Miller et al 2003). The fMRI technique overcomes some of the inherent problems with lesion and reversible inactivation studies, firstly only one area can be analysed at any one time and secondly these are invasive techniques that may effect the normal function of the cerebellum. The fMRI technique is non-invasive, allows multiple brain regions to be studied simultaneously in conscious, behaving subjects and is suitable for repeat applications. The authors note that activity is observed in the vermis and suggest this is due to learning-specific changes in the autonomic system, this finding highlights the advantage of analysing the complete cerebellum simultaneously throughout training (Miller et al 2003). It should be noted that activation due to sensory input may be responsible for part of the activation observed and therefore, cannot attribute all changes as learning specific.

Although there is still controversy about the region of the cerebellar cortex involved in the conditioning of EB/NMR, the empirical evidence from Yeo and colleague’s discrete lesion studies consistently demonstrate that lobule HVI is critical for acquisition and retention of the CR. This evidence is further supported by studies that use drug infusions temporarily to inactivate lobule HVI (this chapter, page 53). In
this thesis cerebellar cortical input to the nuclei, specifically input from lobule HVI to the anterior interpositus lobe of the cortex is considered to be critical for classical conditioning of the NMR of the rabbit.

**The inferior olive (IO)**

The mammalian IO is composed of the principal olive (PO) the dorsal accessory olive (DAO) and the medial accessory olive (MAO) (Brodal 1940). The IO receives somatosensory information from the face directly from the trigeminal system. In rabbits, the trigemino-olivo-cerebellar connections have been described in detail using wheatgerm agglutinin-horseradish peroxidase (WGA-HRP) to trace projections (van Ham and Yeo 1992). Cortical lobule HVI receives face somatosensory inputs mainly from cfs originating in the DAO and ventral leaf of the PO (Yeo et al 1985c, van Ham and Yeo 1992). These rostromedial regions of the IO receive somatosensory information mainly from the principal trigeminal nucleus and spinal trigeminal nucleus pars interpolaris (van Ham and Yeo 1992).

The precise region of the IO involved in conditioning of the EB/NMR has been mapped using discrete lesions. Lesions restricted to the most rostral region of the DAO abolished CRs and prevented re-acquisition; the UR remained intact (Yeo et al 1986).

Following DAO lesions, Yeo and colleagues (1986) reported an immediate and complete loss of previously well learned CRs, when tested 1 day, 2 weeks or 3 weeks post-operatively. However, contrasting results were seen in two other studies. In the first study, McCormick and colleagues (1985) reported that when tested 12 to 24 hours following lesions of the 'rostro-medial part of the IO' the CRs gradually decreased in size and amplitude in a profile that replicated extinction learning. In the second and more recent study, NMDA was used to lesion the rostral medial DAO, CRs were immediately and permanently abolished except in one animal that displayed evidence of extinction (Mintz et al 1994). In the latter study (Mintz et al 1994), the authors reveal that the subjects had undergone different acquisition and extinction procedures. Because some subjects had undergone acquisition training *ipsilateral* to the lesion they has also undergone extinction training *contralateral* to the lesion and therefore following
contralateral olivary lesions the contralateral side showed immediate abolition of CRs. In comparison the subject that exhibited extinction of CRs following the lesion had undergone acquisition training only, on the side contralateral to the lesion.

Additionally, Mintz and colleagues highlight two different procedures that they claim account for the differences seen in CR abolition following olivary lesions in these three studies. Firstly, the lesions were small in McCormick et al (1985) study compared to large lesions in the studies by Yeo et al (1986), and Mintz et al (1994). Secondly, subjects were only allowed a short post-operative recovery period of 12 to 24 hours in McCormick et al. (1985) study compared to a much longer recovery period of 7 days in Yeo et al. (1986) study and 2 to 5 days in Mintz et al. (1994) study.

In fact, in Yeo et al’s (1986) study, the lesion sizes were smaller than those in the McCormick (1985) study. The lesion reconstructions in Yeo et al’s (1986) study show that the different regions of the IO were histologically identified and the full extent of the discrete lesions could be mapped. This allowed identification of the precise regions of the olive necessary for CR performance. Furthermore, incorrectly reported by Mintz and colleagues (1994), in Yeo et al’s (1986) study the recovery period for one group of subjects was 1 day, replicating McCormick et al’s (1985) study that had recovery times of 12 to 24 hours following lesions.

Alternative reasons must be the cause of the different profiles of CR abolition. Yeo (1989) suggests that in some subjects the olivary lesions in McCormick et al’s (1985) study caused performance deficits, as demonstrated by a decrement in the UR amplitudes that appears to match the decrease in CR amplitudes. Furthermore, the location of the lesions in McCormick et al’s (1985) study is in contrast to those in Yeo et al’s (1986) study. In Yeo et al (1986) the contralateral olive- that projects to the conditioned side- is lesioned, whereas in McCormick et al's (1985) study the olivary lesions are medial and in one case ipsilateral to the conditioned side, because of this, the effects of these lesions on CRs could have been the result of retrograde loss following climbing fibre transection (Yeo 1989).
The dorsal accessory olive (DAO) and adjacent medial part of the rostral principal olive (PO) are critical for conditioning the eyeblink/NMR.

**Reversible inactivation studies**

Lesion studies have allowed the identification of regions of the cerebellar circuitry critically involved in conditioning of the rabbit EB/NMR. But different techniques are required to determine which structures are involved in different components of the learning process such as, acquisition, performance and extinction. Infusions of compounds that block excitatory or inhibitory transmission in discrete, identifiable regions for a temporary period of time can reveal which component of memory or performance depends upon normal function in the region inactivated.

For example, if a region is inactivated during acquisition training and CRs are seen to have developed after the inactivation is lifted, then it can be argued that the region inactivated was not necessary for the acquisition process. But, if during an inactivation the learning was blocked, no CRs will be present during subsequent training after the inactivation is lifted. Then it can be argued that normal function of the inactivated region is necessary for acquisition. Discrete, temporary, functional inactivation studies have been used to begin to dissociate the roles of the cortex and the cerebellar nuclei during acquisition, extinction and performance of CRs.

Reversible inactivation of the red nucleus with muscimol (a GABA$_A$ receptor agonist) (Krupa et al 1993) or with cooling (Clark and Lavond 1993) or of the superior cerebellar peduncle with TTX (Krupa and Thompson 1995) during acquisition training did not prevent learning but they did prevent the expression of CRs. CRs were not seen during the inactivation but they were present after the drug effects dissipated. These cerebellar efferent structures are, therefore, not critical for the acquisition of CRs but they are critical for the performance of CRs. In contrast however, inactivating the lateral cerebellar cortex and AIP did prevent the acquisition and the expression of conditioned responses (Krupa et al 1993). These results are consistent with the idea that memory storage is upstream from the red nucleus and superior cerebellar peduncle, either in the cerebellum or precerebellar structures.
Studies have consistently shown that inactivation of the AIP during acquisition (and extinction) training prevents learning (Hardiman et al 1996 (acquisition and extinction), Clark et al 1992 (acquisition only)) indicating that normal function of the AIP is necessary both for acquisition and extinction. However, although inactivation of the AIP prevents acquisition and extinction it is not possible to conclude that all or even some of the necessary plasticity is within it because of the function of OCN loop. Inactivation of the AIP has functional consequences throughout the entire loop, so it is possible that inactivation of the AIP disrupts or prevents processing, or plasticity, throughout the loop. Consistent with this idea, acquisition is also prevented when the IO or cortical lobule HVI is inactivated (Welsh and Harvey 1998, Attwell et al 2001). So the OCN loop must be taken into account in any inactivation study that targets the olive, cerebellar cortex or AIP.

Conflicting results concerning the involvement of the cerebellar cortex and the expression of CRs have been reported. Mauk and colleagues have reported that CRs are still present when the cortical input to the nuclei is blocked, using infusions of the GABA_A antagonist, picrotoxin, in the interpositus nucleus (Garcia and Mauk 1998). These responses appear to be unmasked by blocking the Pc inputs to the interpositus nucleus. Compared with normal CRs, they have diminished amplitudes and disrupted timing that results in short latency responses (SLRs). Similar SLRs were also seen in cortical lesion studies (Garcia et al 1999). It is claimed that the results from the reversible inactivation study (Garcia and Mauk 1998) suggest plasticity in both the cerebellar cortex and interpositus nuclei are necessary to produce adaptively timed CRs. Plasticity in the nuclei is indicated by the presence of SLRs when the cortico-nuclear input is blocked and cerebellar cortical plasticity is indicated by the loss of learned response timing with cortical lesions. It is suggested the cerebellar cortex is not critical for the basic CS-US association in EB/NMR conditioning but that it is necessary for performance of adaptively timed CRs.

In contrast, Yeo and colleagues have shown that infusions of drugs into lobule HVI, such as, CNQX to block AMPA/kainate receptors and prevent excitatory
transmission to Pcs or the GABA_A antagonist, SR 95531, to disrupt inhibitory transmission within the cortex, blocks the expression of CRs (Attwell et al 1999, Attwell et al 2001, Attwell et al 2002b). Performance of CRs is also prevented when inhibitory inputs to the AIP are blocked using the GABA_A antagonists (picrotoxin and SR 95531), but CRs are not prevented when the AMPA-mediated excitatory inputs to the cerebellar nuclei are blocked. Therefore, AMPA mediated excitatory inputs to the cerebellar nuclei are not necessary for the performance of CRs (Attwell et al 2002a).

The disparity in findings may be due to a number of reasons. The precise location and spread of the drugs used could vary considerably between studies, as could the concentration of drug within the critical eyeblink control regions. In Garcia and Mauk’s (1998) study, the volume of picrotoxin infusions to the interpositus nuclei was deliberately adjusted between subjects until the timing of the CR was disrupted. Importantly, the spread of the drug was not assessed in the subjects, so the drug may have reached the critical eyeblink control regions in the cerebellar cortex. This would mean the results seen in this study were not a result of blocking cortical input to the interpositus nuclei alone but also due to inhibition of inhibitory inputs within the cerebellar cortex. It must also be noted that picrotoxin infusions in the cerebellar nuclei will also block the local inhibitory interneurones within the nuclei, and therefore have additional effects upon information processing at this level. So the full effects of the infusion may be greater than as simple disconnection of cortical input, as has been proposed.

**Summary of results from reversible inactivation studies**

Results from these reversible inactivation studies have complemented the findings from lesion studies, and confirm the localisation of cerebellar regions involved in EB/NMR conditioning. However, these studies have also demonstrated that normal function in lobule HVI of the cortex, the AIP and the DAO is necessary for normal acquisition and performance of CRs.
Potential problems with reversible inactivation studies

There are some problems with the reversible inactivation technique that must be taken into consideration. It is important to check for any neuronal damage that could have resulted from the insertion of the cannula into a critical region or permanent damage from the drug itself. In studies where the red nucleus was inactivated by muscimol (Krupa et al 1993) or cooling (Clark and Lavond 1993) the cannula position was aimed lateral to the target, to prevent damage. However, histological evaluation of the position of cannula tips demonstrates that in both studies the red nucleus was invaded numerous times, so it is possible that permanent damage could have resulted from this. In other studies, the region penetrated by the cannula tips was critically evaluated for any damage and subjects rejected if damage was located in target areas (Clark et al 1992, Attwell et al 1999, 2001, 2002a and 2002b).

As the reversible inactivation technique has been used particularly to dissociate roles of the cortex and of the cerebellar nuclei in conditioning, it is critical to establish the extent of the spread of the drug. For example, if the cortex is inactivated and the drug has spread into the underlying nuclei then the effect of the drug on the cortex and nuclei cannot be dissociated. In previous studies, such as, the red nucleus inactivation study, (Clark and Lavond 1993) the spread of the effects of their inactivations was not assessed, although it is very unlikely that regions of the cerebellar cortex were inactivated by cooling because the red nucleus is well anterior to the cerebellum.

The most effective technique used for estimating the spread of a drug infusion is autoradiography of a radiolabelled version of the drug. In Krupa et al (1993), the extent of inactivation of the red nucleus, lateral cerebellum and AIP with muscimol was checked using autoradiographical techniques. In subjects that received muscimol infusions in the cerebellum, no drug was found to have spread into other brain regions. Welsh and Harvey (1998) also used autoradiography to establish the location of the infusions to the IO and reported that effective inactivations were located close to the DAO. In other studies quantitative autoradiographical techniques have provided good evidence that inactivations restricted to the cortex can prevent performance of

As an alternative, which is not as accurate in showing the anatomical regions invaded by drug infusions, some studies have located optimal cannula placements and estimated the spread of drug based on previous reports (Garcia and Mauk 1998, Attwell et al 2002a).

It is also important to establish the duration of effect of the drug used. It is vital that the effects from a drug infusion have completely dissipated if a subsequent non-drug conditioning session is required. If the drug is still effective during a non-drug training session then the behaviour may be a result of long-term drug effects. Analysis of extinction training overcomes some of these problems. Acquisition of CRs is a prerequisite for extinction learning therefore, any damage due to cannulation would be apparent, as acquisition learning would be retarded. Additionally, the test for any disruption or prevention of extinction learning following inactivation during extinction training, is the presence of CRs, another indication that there is no damage due to the drug infusion (Clark et al 1992, Hardiman et al 1996). Finally, if, following drug inactivation CRs are present during extinction training, then extinction learning has been prevented and then the drug infusion can not have caused any motor or general deficits (Hardiman et al 1996).

Another possible problem with the interpretation of inactivation studies is that state dependent learning (SDL) could occur, (Ramnani and Yeo 1996, Attwell et al 1999), where the drug could alter the sensory properties of the CS and US due to diffusion of the drug into other brain areas. Learning would occur to two CSs, the CS perceived in the drug-free state and the CS perceived in the ‘inactivation’ state.

The reversible inactivation technique is used in two of the experimental chapters presented here, chapter 3 and 4 and therefore, it is important to highlight the problems that can arise during reversible inactivation studies. In the studies presented in this thesis, control procedures have been used to address these potential problems. Finally, it
is crucial that the limitations of these studies, because of the OCN loop, are understood and considered when results are analysed. In the studies discussed here and in chapter 3, the reversible inactivation technique can only demonstrate if a region is needed for conditioning and it can not indicate a site of plasticity. However, in a recent study (Attwell et al 2002b) and in chapter 4, the reversible technique is used with a critical difference. Drug infusions are used to disrupt processes immediately following acquisition training. This design means that the question of where memories are stored can be directly addressed, without the problem caused by the OCN loop.

Transmission of CS and US information: pathways involved

The cerebellar learning models proposed by Marr (1969) and Albus (1971) can be applied to classical conditioning of the rabbit EB/NMR. Both theories suggest that the input from the pfs to the Pc is modified by the cf input. The mfs convey information concerning the context that a movement is made in and the cf input to the Pc changes the efficacy of the currently active pf-Pc synapses. So, in EB/NMR conditioning it has been suggested that US information is transmitted via cf's to Pcs and CS information is relayed via mfs to granule cells and to Pcs via pfs (e.g. Yeo and Hesslow 1998).

CS information: mossy fibres

There is good evidence suggesting that mfs relay information about the CS typically, a light or an auditory tone. Lobule HVI receives major auditory and visual inputs indirectly via the caudal parts of the dorsolateral and lateral pontine nuclei and from the nucleus reticularis tegmenti pontis (NRTP) (Yeo et al 1985c).

Electrical stimulation of the pontine nuclei or the middle cerebellar peduncle (MCP, that carries mfs exclusively), can be used as the CS and paired with an airpuff US directed at the eye, or inferior olivary stimulation as the US. Paired stimulation at these sites produces conditioning of the EB/NMR, and subsequent extinction training with stimulation CS alone trials resulted in extinction learning. Further acquisition conditioning using a stimulation CS and air-puff US, demonstrated major savings, (savings refers to an increased CR acquisition rate compared to the initial acquisition rate, due to some remaining residual learning that contributes to the faster acquisition
rate (Medina et al 2001)). Finally, conditioning with a new tone CS demonstrated strong transfer effects because CRs were acquired to the tone CS at a much faster rate than in naïve subjects. Pontine stimulation alone caused extinction of the CRs, suggesting that the CRs were real (Steinmetz et al 1986, Steinmetz et al 1989). Following extinction with pontine stimulation alone, CRs were not tested for extinction savings or transfer effects by presenting the tone CS alone. These tests are important because following extinction caused by pontine stimulation, if CRs were immediately absent when the CS tone alone was presented, this would have confirmed the CS pathway is via the pontine nuclei. If CRs were present during CS tone alone trials, then this would have demonstrated that separate pathways were responsible for the CRs.

Transfer effects, such as those seen in the studies reviewed above were also observed in a study that used forelimb stimulation as the CS and periorbital stimulation as the US during EB conditioning in decerebrate ferrets. Acquisition of the CR was normal and when stimulation of the forelimb as the CS was exchanged for stimulation of the MCP, CRs were produced immediately. If extinction training using stimulation of the MCP as the CS was given following acquisition of the CR to the same CS, CRs extinguished normally. CRs also extinguished if subjects acquired to MCP stimulation as the CS but during extinction training MCP stimulation was substituted for forelimb stimulation. The fact that CRs could be extinguished and reacquired, and have adaptive timing is strong evidence that the CRs were real (Hesslow et al 1999).

Importantly the latter study controlled for antidromic activation by blocking mfs ventral to the stimulation site using lignocaine. This ensures that other afferent inputs to the cerebellum, such as those from the spinal cord and brainstem were not activated and acting as the CS. This study confirmed that the information about the CS is transmitted via mf inputs from the MCP (Hesslow et al 1999) during normal conditioning with peripherally applied stimuli.

**US information; climbing fibres**

It has been suggested that information about the US is transmitted via cfs from the IO. The DAO receives projections from the spinal trigeminal nucleus (Berkley and
Hand 1978, van Ham and Yeo 1992) that receives afferents from the face and cornea. Neurones within the DAO respond to somatosensory stimuli and the rostral-medial portion of the DAO is activated by stimulation of the face (Gellman et al 1983). The medial DAO provides a major projection to lobule HVI and there are also projections to lobule HVI from the medial parts of the rostral DAO and the medial part of the ventral leaf of the PO. CF responses have been recorded from lobule HVI in the cat in response to direct stimulation of trigeminal afferents and from tactile stimulation to the face area (Miles and Wiesendanger 1975a). Providing further evidence that CFs carry information about stimuli applied to the face to the HVI region known to be critical for EB/NM conditioning.

Lesions and acute inactivations of the IO abolish CRs, although as discussed previously different profiles of CR abolition have been reported (McCormick et al 1985, Yeo et al 1986, Mintz et al 1994, Welsh and Harvey 1998). One study reported that CRs gradually decreased in amplitude and frequency as in extinction training (McCormick et al 1985). If this result had been replicated by other studies then it would have been strong evidence that information about the US is transmitted via the CFs. However, three other IO lesion studies reported a complete and immediate loss of CRs (Yeo et al 1986, Mintz et al 1994, Welsh and Harvey 1998). These differences are discussed in more detail (see page 51). So these studies do not provide direct evidence that CFs transmit US information, but only that there is a critical involvement of the IO in conditioning.

If stimulation of the DAO is substituted for the US and paired with a tone CS, acquisition of CRs developed at a rate comparable to acquisition in naïve subjects. Unpaired presentation of the tone and DAO stimulation resulted in extinction of the CR (Mauk et al 1986, Steinmetz et al 1989). A problem with this technique is that the IO cannot be isolated as the pathway transmitting the US information, as stimulating the IO may also cause antidromic activation of other precerebellar nuclei.

A recent reversible inactivation study provided a more direct test that the olive functions as the US pathway. NBQX, an AMPA receptor antagonist, was infused into the DAO of the rabbit to block excitatory inputs during paired tone CS and electric
shock US conditioning. The CRs gradually declined even though CS and US presentations were paired. This was extinction learning and its rate was comparable to that seen during tone CS alone presentations (Medina et al 2002).

There is good evidence that information about the US is transmitted via the cfs. It is important to note that the cerebellar cortex receives somatosensory information directly and indirectly via the pontine nuclei and it is possible that the mfs may also provide the cerebellar cortex with information about the US as well as the CS. However, empirical evidence strongly supports the theory that information about the US is transmitted via the cfs and this is assumed to be true for the purposes of this thesis.

Summary of results from studies into the cerebellar circuitry involved in EB/NMR conditioning

Lesion studies can dissociate between critical and non-critical structures involved in motor learning and reversible inactivation studies can dissociate their roles in acquisition, consolidation, performance and extinction of CRs. These studies, together with recording and stimulation studies have identified the critical regions of the cerebellum involved in classical conditioning of the EB/NMR. There is also evidence to suggest the pathways involved in the transmission of CS and US information. Based on these findings and the known anatomy of the cerebellum, a model of the circuitry involved in this specific motor learning has been proposed (see Yeo and Hesslow 1998, for review) (figure 1.6).

Physiological evidence for learning in the cerebellum

A major piece of physiological evidence for the involvement of the cfs and mfs in motor learning in the cerebellum comes from the classic study by Gilbert and Thach (1977). This study required monkeys to learn to compensate in a simple perturbation task. A handle was to be held at a steady position and was subject to occasional perturbations in the horizontal plane. The perturbation force was either with or against wrist flexion and had fixed values in either direction. The subjects learned to return the handle to the reference position as quickly as possible against the perturbations. Then the force applied to the handle was changed in order to increase or decrease the load.
placed on the flexor or extensor muscles and this change in load required the subject to
adapt its response. Pcs with simple spike activity related to the task were recorded
during the movement and the period of time while the new position was held.

Following an increase or decrease in force, subjects took up to one hundred
trials to relearn the task to the previous performance level. Initially, during this
adaptation period, the complex spike rate increased and the simple spike rate decreased.
As performance improved, the complex spike rate returned to normal but the long-term,
task-related simple spike activity decreased.

The results suggest that the cf input changes when a new movement is learned
and that this may cause a change in strength of the pf to Pc synapses, consistent with the
decreased rate of simple spikes. This study provides evidence supporting the basic
learning rule proposed by Marr (1969) but more consistent with the learning model
proposed by Albus (1971) who suggested a cf mediated decrease in strength at the pf to
Pc synapse.

**Purkinje cell firing rates during eyeblink conditioning**

A crucial feature of electrophysiological analyses of cerebellar function in
learning, is to establish whether the recording is from Pcs that have a direct relationship
with the behaviour. In order to establish such a relationship it is first necessary to map
the EB control regions within lobule HVI of the rabbit, the cortical area critical for
conditioning the EB/NMR. A mapping study of the EB control areas in the rabbit is
presented in chapter 2 of this thesis. EB control regions have also been mapped in the
C3 zone of the ferret (Hesslow and Ivarsson 1994) and have been found in four
locations in the cat, including two regions of lobule HVI, These control regions have
been shown to have a direct influence on EB CRs (Hesslow 1994a).

Activity from Pcs within lobule HVI has been recorded during classical
conditioning of the NMR (Bethier and Moore 1986) but it unknown whether these Pcs
were in a subregion directly involved in the control of the EB/NMR. However, it is
reported that Purkinje cells demonstrated activity that appeared to be related to CRs.
Some Purkinje cells showed an increase in simple spikes that appeared to be correlated with CRs but more importantly there was a small population that displayed a decrease in simple spike activity that occurred before the onset of the CR by 20-200ms, these findings are in agreement with Gilbert and Thach (1977) and later studies e.g Gould and Steinmetz (1996). Furthermore the authors report that the majority of cells were;

'located in HVI, mostly deep within this structure.' (Berthier and Moore 1986)

This is in good agreement with previous results from reversible inactivation and lesion studies that demonstrate that the medial-rostral inferior region of lobule HVI was found to be critical for EB/NMR conditioning (Yeo et al 1985b, Attwell et al 1999, Attwell et al 2000).

A recording study in decerebrate ferrets analysed Pc behaviour located in EB control regions. These cells showed an increase in activity during the initial production of a CR and were suppressed during the late stage of a CR, suggesting that the Pc may be involved in generating the CR. If the inhibitory Pc output to the AIP is suppressed, activity in the AIP would increase and a CR could be produced via excitatory projections to the red nucleus. Again, the latency of Pc suppression varied between 50-200ms and occurred before the CR. In addition, the initial increase in activity of the Pc fits in with the characteristic timing profile of CRs that is, the CR begins towards the latter part of the CS-US interval (Hesslow and Ivarsson 1994). This theory is in agreement with findings from a recording study in the rabbit, that also suggests that depression of cortical Purkinje cell activity occurs together with increased activity in the AIP to produce CRs (Gould and Steinmetz 1996).

There is an inhibitory, GABAergic pathway from the cerebellar nuclei to the IO (Nelson and Mugnaini 1989). In well-trained subjects, presentation of the CS evokes a CR and IO activity is inhibited, probably via this nucleo-olivary pathway. But at the start of acquisition training, presentation of the CS does not evoke a CR and it does not inhibit the IO. Subsequent extinction of the CR results in dis-inhibition of the IO once again, indicating that inhibition of the IO is conditioning-dependent (Hesslow and
Ivarsson 1996), and that the nucleo-olivary pathway may regulate the size of the CR (Andersson et al 1988). These studies provide further physiological evidence that the olive and cf system is a critical component of the neural circuitry for conditioning of the EB/NMR.

**Neuronal and synaptic mechanisms suggested for rabbit EB/NMR conditioning**

Mechanisms responsible for conditioning of the rabbit EB/NMR have not yet been defined. Based on knowledge of the discrete anatomical regions and pathways critical for EB/NMR conditioning and what is understood of the physiology and candidate plasticity of the neurones within this circuitry, several mechanisms have been proposed.

The evidence so far suggests that the memory is formed and stored within the cerebellum. There are three critical questions to answer in order to understand the mechanisms responsible for memory acquisition during conditioning of the EB/NMR. Firstly, what are the relative roles of the cerebellar cortex and cerebellar nuclei? Secondly, where are the mechanisms responsible for acquisition, consolidation and storage of the memory? Thirdly, what are the synaptic or non-synaptic plasticities that underlie these processes?

Although numerous plasticities that can occur within the proposed circuitry for EB/NMR conditioning have been characterised (and are described previously, page 34), few have been incorporated into models of EB/NMR conditioning. Several forms of plasticity have been identified and characterised recently and so were not considered in these models and others such as cortical interneurone-Pc or interneurone-pf synapses are in advance of our understanding of inhibitory neuronal control in the target behaviours. The models of cerebellar function in EB/NMR conditioning, described below, focus on pf LTD and cerebellar nuclear LTD and LTP. These plasticities are well characterised and occur at sites where CS and US information converge, the cerebellar cortex and interpositus nucleus. All mechanisms for learning during
EB/NMR conditioning discussed in this thesis agree with the circuitry proposed for this conditioning (described on page 43, Chapter 1).

A mechanism based on computational simulations and empirical findings suggests that a distributed synaptic plasticity between the cerebellar cortex and cerebellar nuclei is responsible for learning. Synaptic plasticity occurs at the pf-Pc synapses in the cortex and at mf synapses in the interpositus nucleus (Mauk and Donegan 1997). They suggest that plasticity is regulated so that a relatively constant net strength of pf-Pc synapses is maintained in the cortex except during acquisition and extinction training. Without this regulation the strength of these synapses would saturate. The firing rate of the cf is kept at an equilibrium level and this stable equilibrium is achieved by coupling the average pf-Pc synaptic weight, indexed by the activity of the Pcs, to the modulation of cf activity by negative feedback. The negative feedback would occur via the nucleo-olivary pathway (Mauk and Donegan 1997).

It is suggested that bi-directional plasticity is responsible for learning at both sites. In the cortex LTD would occur when pf-Pc synapses are co-active with cfs and LTP would occur in the absence of cf input. In the interpositus nuclei, bi-directional plasticity of the mf-nuclei synapses is dependent on the inhibitory input from the Pc. When mf-nuclei synapses are active during transient decreases in Pc inhibition, LTP occurs, and when these synapses are active during Pc strong inhibition, LTD occurs (Mauk and Donegan 1997, Mauk 1997).

This model proposes that initial acquisition of CRs is due to LTD at the pf-Pc synapses in the cortex. Following a number of trials, a decrease in Pc firing would result in a phasic disinhibition of the interpositus cells during the CS, that could allow induction of LTP at CS activated mf-nuclei synapses. LTP in the nuclei coupled with the pause in inhibition from the Pcs would cause the inhibition of cfs and result in the production of a CR. The excitatory input to the cfs from the US is counteracted by the CR-related inhibition of the cfs and the probability of pairing during a CS–US trial would be the same as when the cfs are at equilibrium.
Following acquisition of CRs, responses with consistently short onset latencies can be revealed by blocking the cortical input to the cerebellar nuclei (Perrett and Mauk 1993, Garcia and Mauk 1998). This is evidence for the presence of cortical plasticity, related to the learned timing of a CR and learning-related plasticity in the interpositus nucleus capable of driving responses, albeit lacking the learned timing typical of CRs. It is proposed that the cerebellar cortex is responsible for temporally-specific learning by producing appropriately timed increases and decreases in Pc activity that modulate the output from the cerebellar nucleus (Medina et al 2000).

Extinction of CRs can also be explained by this model. During extinction training the CS is presented alone, in the absence of cfs and LTP occurs at the active pf-Pc synapses. The phasic disinhibition of the nuclei would no longer occur, as the pause in Pc firing during the CS is eliminated, so this would promote the induction of LTD in the nuclei. The changes in plasticity at the cortex and nuclei would act to diminish the ability of the CS to produce a CR. During extinction, in the absence of the US input, the inhibition of the cfs would cause cf firing to fall below the cf equilibrium. As CRs diminish so would the CR-related inhibition of the cfs allowing the cfs to return to equilibrium (Mauk and Donegan 1997).

There are similarities between the empirical findings and mechanisms proposed by Mauk, and those of Thompson and colleagues. Thompson also suggests a parallel or distributed learning system, where memory traces could occur in both the cerebellar cortex and interpositus nuclei and assigns different CR development functions of the CR to the cortex and nuclei. The cortex is responsible for the learned timing of a CR and the interpositus nucleus stores the ‘basic’ memory trace of a CR (Bao et al 2001, Thompson and Raymond 2002). Thompson reports that Pcs and interpositus neurones develop amplitude and time course models of the CR that predict and precede the occurrence of a CR within trials and over training trials (Thompson and Kim 1996).

Typically the Pc simple spike firing rate decreases during a trial and the decrease in Pc firing rate will lead to an increase in interpositus activity and the production of a response. The neuronal and synaptic mechanism suggested for learning is as follows,
during conditioning the pf-Pc synapses are weakened when co-active with cf-Pc synapses. This may lead to Pcs increasing their firing during the early CS stage and decreasing firing during late CS stage. This pattern of firing of the Pcs controls the timing of the CR and shapes the firing of the cerebellar nuclei neurones. During the period when inhibition from the Pcs to the cerebellar nucleus is absent the mf and cf inputs to the cerebellar nuclei may lead to activated NMDA receptors and VGCCs resulting in LTP at these synapses (Bao et al 2001).

These two layer learning ideas (Mauk and Donegan 1997, Medina 2000, Bao et al 2001) are consistent with suggestions in other studies. A recent report suggests that the basic association between the CS and US is made in the interpositus nucleus and that ancillary learning occurs in the cerebellar cortex and possibly extra-cerebellar regions, such as the brainstem and forebrain (Lavond 2002). It is also suggested that different aspects of the CR, for example, learned timing and amplitude of the CR is learned in the cerebellar cortex and the interpositus nucleus acts as a co-ordinator for all the different aspects of learning that occur in the cerebellar cortex and extra cerebellar regions (Lavond 2002).

These proposals suggest the cerebellar cortex is important, but not critical for conditioning. Furthermore, it has been suggested that multiple cortical sites may be involved in conditioning, reflecting the fractured somatotopy of the mf inputs (Thompson 1986). In comparison with the evidence for cortical function discussed so far, empirical evidence provided by Yeo and colleagues consistently indicates that lobule HVI of the cortex is a critical structure for conditioning (Yeo et al 1985b, Attwell et al 1999, Attwell et al 2001). In an attempt to elucidate the roles of the cortex and nuclei in conditioning, a recent reversible inactivation study used AMPA/ kainate receptor antagonists or GABA_A receptor antagonists to disrupt normal function of the cortex or the nuclei. This study provides no evidence that the cortex is independently responsible for the adaptive timing of a CR or that the nuclei are independently capable of driving an adaptively timed CR or even a short latency response. The results indicate that normal function at both sites is necessary for production of CRs (Attwell et al 2002a).
A cerebellar cortical conditioning (CCC) model was proposed and suggests that the main site of plasticity occurs within a set of EB control Pcs within cortical lobule HVI. This is a crucial difference from previously described models that suggest the plasticity underlying the association between the CS and US occurs in the cerebellar nuclei. The CCC model is in agreement with theories and empirical evidence suggesting that CS information is transmitted by mfs via the pontine nucleus and the pf-Pc synapses are modulated by cf input from the DAO, that transmit information about the US (Yeo and Hesslow 1998). Again, the CCC model predicts that during conditioning the CS related simple spike firing in the Pcs decreases.

The CCC model differs crucially from the mechanisms suggested previously in that, plasticity of the mf-interpositus nuclei cells is unlikely to be an essential mechanism in conditioning. Empirical evidence demonstrates that blocking the mf AMPA receptor mediated collateral input to cerebellar nuclei has no effect on CR expression (Attwell et al 2002a). If mf to nuclear neurone synapses were the site for the learning related plasticity capable of driving CRs, it would be expected CR expression would be prevented when these mf AMPA mediated synapses were blocked. Importantly, however, the CCC model does not rule out the possibility of plasticity in the cerebellar nuclei contributing towards learning during conditioning (Attwell et al 2002b), but it assigns no specific function to this possible plasticity.

The crucial feature of the CCC model, that affects the interpretation of inactivation studies involving the proposed circuitry of EB/NMR conditioning, is the suggestion that a fully functional olivo-cortico-nuclear loop (OCN) is necessary in order for all of the characteristics of the conditioned response to be learned and expressed (Attwell 2002a). The essential plasticity responsible for learning could be distributed at any one level or between all three levels (cortical lobule HVI, the AIP or the IO) within the OCN loop. Reversible inactivation studies result in a destabilisation of the OCN loop and could effect processing at the cortical, nuclear and olivary levels. Therefore the relative roles of the cortex and nuclei have not yet been fully dissociated.
Studies presented in this thesis

The mechanisms involved in EB/NMR conditioning are not fully understood and critical issues need to be resolved. One of the most crucial of these is to discover where, within the cerebellum, are memories formed and stored? As discussed in detail above, lobule HVI of the cerebellar cortex and the AIP are two likely sites for the formation and storage of motor memories. Experimental evidence demonstrates the necessity for normal function of these two sites in EB/NMR conditioning and characterises synaptic and non-synaptic plasticity that could be responsible for learning at these sites. The studies described in the following three chapters contribute towards understanding the functional roles of lobule HVI of the cortex in EB/NMR conditioning.

It is important to understand cortical and nuclear neuronal behaviour during rabbit EB/NMR conditioning however, to achieve this understanding, it is crucial that the recordings are from neurones with a direct relationship to the behaviour. Therefore, for an understanding of cortical activity in learning, the EB/NMR microzones in the rabbit cerebellar cortex must be mapped, (study presented in chapter 2).

Although cerebellar cortical EB control regions have previously been mapped in the ferret and cat (Hesslow 1994a, Hesslow and Ivarsson 1994) the precise location of these important control regions is not known in the rabbit. In addition to mapping the EB control region/s with respect to the mediolateral, rostrocaudal and inferior–superior extent, EB/NMR control regions can be put in register with zebrin II/ aldolase C immunocytochemistry. The pattern of zebrin II staining is consistent throughout all species previously described (Sanchez et al 2002). Importantly by locating the EB control regions within lobule HVI with respect to zebrin II staining, the EB control microzones of other species can be located and across species comparisons made.

Understanding extinction learning and the neuronal and synaptic mechanisms responsible for extinction learning is as important as understanding acquisition during conditioning. It is vital to discover if the same cerebellar regions and cerebellar circuitry are responsible for both extinction and acquisition learning. Previous studies have
shown that normal function of the AIP is necessary for extinction to occur normally (Ramnani and Yeo 1996, Hardiman et al 1996), however, the role of the cortex in extinction is not known. Identifying whether normal function of the cortex, and in particular lobule HVI, is necessary for normal extinction learning is a critical step in identifying the circuitry necessary for extinction learning and locating a possible site for extinction–related learning plasticity. Chapter 3 presents a study investigating the role of lobule HVI in extinction.

Finally, the reversible inactivation procedure can be used to target post-conditioning consolidation processes, providing a technique that allows full functional dissociation between the cortex and AIP, and may provide an answer to the essential question of where within the cerebellum memories are stored.

A recent NMR conditioning study has provided evidence that the post-acquisition training consolidation processes are mediated by lobule HVI of the cerebellar cortex (Attwell et al 2002b). That study used the GABA\(_A\) agonist muscimol and demonstrated that consolidation was prevented by cortical, but not nuclear, muscimol treatments. However, because the balance of excitabilities in the cerebellar nuclei would be different following cortical and nuclear inactivations, there was a possibility that consolidation processes are sensitive to this difference and so may, to some extent, depend upon the cerebellar nuclei. This possibility was tested in the final study presented in chapter 4 by using local infusions of GABA\(_A\) antagonists to complement the Attwell et al (2002b) study.

These three studies target different questions concerning the precise anatomy, circuitry and mechanisms involved in rabbit EB / NMR conditioning. Independently, none provides a solution to the key issues such as, what the relative roles of the cortex and nuclei are in conditioning. But they provide important evidence towards understanding these issues and contributing to a resolution.
Chapter 2

Mapping Rabbit EB Microzones
2

Mapping Rabbit EB Microzones

The study presented in this chapter aims to locate and map the full extent of the microzones that are involved in the control of the EB within lobule HVI of the rabbit cerebellar cortex. Once these microzones have been anatomically located and shown to be directly involved in classical conditioning of the rabbit EB/NMR, detailed functional studies during EB/NMR classical conditioning may occur.

Why is it important to locate EB/NM microzones in the rabbit?

EB control regions have been mapped in the cat and ferret (Hesslow 1994a, Hesslow and Ivarsson 1994) but not in the rabbit. The rabbit is a widely used and good subject for EB/NMR classical conditioning and can be used in the naïve state to investigate motor learning during this type of conditioning. In previous ferret and cat mapping studies, all subjects were decerebrated and curarised during recordings of cf responses (Hesslow 1994a, Hesslow and Ivarsson 1994). Decerebration and curarisation may have critical effects on neuronal behaviour or on recording of neuronal behaviour and consequently, mapped EB control areas may be misrepresented for a non-curarised and non-decerebrated subject. It is critical to locate the EB/NM control regions in subjects that have not undergone decerebration and curarisation, as EB/NM conditioning studies in the rabbit do not require this preparation for successful EB/NMR conditioning. Furthermore, there may be species differences in the location of these EB control microzones.

Lesion and reversible inactivation techniques have provided evidence for the discrete regions and circuitry involved in EB/NMR conditioning in the rabbit (chapter 1, page 43). However, in order to understand the neural mechanisms involved in EB/NMR conditioning processes different techniques must be employed. Recording from Purkinje cells that are related to the learned behaviour during conditioning may help understand the neural behaviour underlying learning processes such as acquisition and extinction learning. Furthermore, a dual technique could be used to analyse the relative
roles of the cerebellar cortex and cerebellar nuclei in EB/NMR conditioning. For example, reversible inactivation of either the cortex or nuclei when recording from nuclei neurones or cortical EB control Pcs respectively, would allow analysis of neural behaviour within one region during pharmacological manipulation of the other.

Molecular studies can identify cellular changes that occur during this type of conditioning. Pf-Pc LTD is a good candidate plasticity for cerebellum-dependent motor learning and if molecular changes involved in its induction could be identified during behavioural learning, this would be strong evidence in support of LTD as a mechanism of EB/NMR conditioning. However, in order to test such ideas we need to know the precise location of the Pcs involved in the control of these reflex and conditioned eyeblinks.

**Evidence that lobule HVI contains regions critical for EB/NMR conditioning**

**Anatomical studies**

Periorbital somatosensory information reaches the cerebellum through the trigeminal system. Anatomical tracing studies in the rabbit have found that trigeminal information is transmitted to lobule HVI via climbing fibres (cfs) from the inferior olive (IO), via mossy fibres (mfs) from the pontine nuclei and directly from the trigeminal nuclei. Although trigeminal mfs terminate widely throughout the cortex, including lobule HVI, trigemino-olivary fibres terminate most heavily within lobule HVI (van Ham and Yeo 1992).

Lobule HVI receives inputs from the medial part of the rostral DAO and adjacent medial part of the PO via the olivo-cortical pathway and has a major projection to the AIP via the cortico-nuclear pathway (Yeo 1985c). Both of these regions- the DAO and AIP- have been found to be critical for acquisition and retention of CRs (Yeo et al 1985a, Yeo et al 1986, Ramnani and Yeo 1996). Parasagittal zones C1 and C3 have connections with the DAO and AIP, so it is these zones, within HVI, that probably contain the EB/NM microzones crucial for conditioning.
Lobule HVI has many other inputs and outputs. Projections to the PIP indicate the presence of a C2 zone. Additionally, lobule HVI has been reported to project sparsely to the lateral VN indicating that a narrow B zone may be present at certain rostro-caudal levels, furthermore at rostral levels of lobule HVI there is also evidence of a D zone. However, there is no evidence of the A zone being present (Yeo et al 1985c).

Lesion and reversible inactivation studies of the rabbit cerebellar cortex have provided differing results concerning the role of lobule HVI in conditioning of the EB/NMR (chapter 1, page 45). However, Yeo and colleagues have consistently found that normal function of lobule HVI is necessary for acquisition, and performance of CRs (Yeo et al 1985b, Attwell et al 1999, Attwell et al 2001, and Attwell et al 2002a). Further evidence that lobule HVI is involved in learning during conditioning is that lobule HVI plays a critical role in mediating consolidation processes (Attwell et al 2002b). These studies suggest that the EB/NM control regions are within lobule HVI.

**Climbing Fibre Response**

Activation of a cf causes a strong depolarisation of the Pc; this cf response is called a complex spike and consists of a primary large depolarisation followed by a train of up to five much smaller secondary depolarisations (Eccles et al 1964), this long lasting plateau potential is due to Ca\(^{++}\) influx. When recording single unit responses individual climbing fibre responses are seen, with characteristic latencies corresponding to the zone the Pc is located in (see section below). When recording climbing fibre field potentials many responses are recorded from multiple PCs, the characteristic complex spike shape is still seen although the onset latency can vary slightly.

**Characteristics of zones**

The different zones can be distinguished by the characteristics of the cf responses that are evoked by nerve stimulation. There are two important defining characteristics of a zone:

1. **Onset latency of cf responses**

   Within each zone, the onset latency of the cf response is consistent, but these latencies are distinguishably different in different zones. So the cf response onset latency provides a reliable characteristic for defining the zone.
The cf responses within each zone are mediated by a particular spino-olivary or trigemino-olivary pathway. The different onset latencies of cf responses in different zones relates to the number of synapses in the afferent pathway. Onset latencies ranging from 11 to 25 ms were reported for cf responses in lobule HVI evoked with trigeminal stimulation but the onset latency of cf responses within the C2, C1 and C3 zones is not differentiated (Miles and Weisendanger 1975a). Hesslow’s (1994a) study reports cf onset latencies for C2 and C1/3 zones in the cat, in response to ipsilateral and contralateral periorbital stimulation. Cf responses in the C2 zone have a mean onset latency of 15 ms when evoked by ipsilateral stimulation and 18 ms when evoked by contralateral stimulation. Ipsilateral periorbital stimulation evokes cf responses in the C1/3 zone, with an onset latency of approximately 9 to 12 ms (Hesslow 1994a). In the study described below these cf response onset latencies were used as a guide for the onset latencies that may be expected in the rabbit C1, C2 and C3 zones in response to ipsilateral and contralateral periorbital stimulation.

2. Inputs to the zones

Within lobule HVI only the B zone and the C2 zone have bilateral peripheral and cortical inputs and cf responses can be evoked by ipsilateral and contralateral stimulation. All other zones, including the C1 and C3 zones, are activated only by ipsilateral peripheral input and contralateral cortical input (Oscarsson 1980). Cf responses in the C1 and C3 zones can be evoked with ipsilateral stimulation only.

In an early study in cats it was shown that stimulation of the trigeminal nerve, gentle mechanical stimulation of the facial skin, stroking hairs or tapping the skin results in cf evoked potentials in specific regions of the cerebellar cortex. Trigeminal nerve stimulation resulted in cf potentials mostly seen in ipsilateral lobule HVI but also in lobule HV and crus I. Cf potentials to ipsilateral and contralateral trigeminal stimulation were seen in a small area of lobule HVI. These potentials have the same amplitude to ipsilateral and contralateral stimulation but, compared to ipsilateral stimulation, contralateral stimulation increased the onset latency by 2 to 6 ms (Miles and Wiesendanger 1975a Miles and Weisendanger 1975b). This area of lobule HVI (with cf responses to bilateral input) is probably within the C2 zone.
In cats, EB control regions have been found by recording cf responses in the cerebellar cortex in response to periorbital electrical stimulation (Hesslow 1994a). A total of four areas were mapped, two in lobule HVI, one in medial lobule HVI within the C3 zone and one in lateral lobule HVI that could be in the C3 zone or possibly the Y zone. The two other areas were found outside lobule HVI, one was located in lobule HV and one in the C3 zone of the paramedian lobe (Hesslow 1994a). It is important to note that although it is reported that these EB control regions are within the C3 zone, there is no conclusive evidence to indicate that this is the case, it is possible they are within the C1 zone. In order to be sure which zone these responses are recorded from it necessary to locate the C2 zone border, allowing anatomical definition of the C3 zone.

Stimulation of these four areas elicited delayed EMG activity in the orbicularis oculi muscle, time locked to the termination of the stimulus train. This delayed EMG activity is reported as being specific to C3 zone stimulation. The author suggests that the delayed-EMG may be due to hyperpolarisation of the AIP neurones followed by rebound excitation (Hesslow 1994b). Using the same technique as that used to identify the C1 or C3 EB areas in the cat cerebellum, an EB area was also found in the C1 or C3 zone of lobule HVI of the ferret (Hesslow and Ivarsson 1994).

Recently, in the cat, microelectrode mapping techniques have been used to identify zones C2, C3 and D1 within the medial folium of hemispheral crus 1. By combining the results from the mapping experiments with a bi-directional tracing technique, parasagittal zones and their projections were identified. Three distinct areas were identified electrophysiologicaly and each area has specific olivo-cerebellar and cortico-nuclear connections. The C2 zone was found to receive inputs from the medial accessory olive and project to the posterior interpositus nucleus and the C3 zone received inputs from the rostral DAO and projects to the AIP (Edge et al 2003). This study also provides the anatomical location of an EB control region, however, there is little evidence to suggest that crus 1 is critical for EB/NMR conditioning and so is not targeted in the study presented in this chapter. The results from this study are further discussed in the discussion (Chapter 2, page 102).
Parasagittal zones of the rabbit cerebellar cortex

The parasagittal zones of the cerebellar cortex have been mostly studied in the anterior lobe of the cat. Anatomically, seven zones were originally delineated: A, B, C1, C2, C3, D1 and D2 (Voogd 1969). Van Rossum (1969), using anterograde degeneration of the corticonuclear projections found the same longitudinal pattern in the rabbit cerebellum and, to date, this is the only study of zonal organisation in the rabbit. Parasagittal zones have also been defined according to the physiological properties of the olivocortical pathways. An electrophysiological investigation of the zonal organisation of the anterior zone of the cat (Oscarsson 1969, 1976) identified parasagittal zones similar to those reported by Voogd.

An eighth zone, zone X, was found between zones A and B in the anterior lobe in the cat (Ekerot and Larson 1979) and in the ferret (Garwicz 1997). Lateral C1 is also referred to as the Cx zone because collaterals of cfs that innervate the X zone also innervate the Cx zone (Trott and Apps 1991). However, there is no evidence from Van Rossum's 1969 study for the X or Cx zone in the rabbit cerebellum, (figure 2.1). The longitudinal divisions of the cerebellum have also been described for the rat (Buisseret-Delmas and Angaut 1992) and it is important to note that there are some differences in the zonal organisation between species. For example, based on electrophysiological properties, the A zone of the rat cerebellar cortex can be divided into the al and lateral ax zone (identified and denoted as A1 and A2 in Buisseret-Delmas and Angaut 1992), in addition the C3 and Y zone are absent (Jörntell et al 2000). The authors suggest that skilled movement in the rat is controlled via the A1, Ax and C1 zones whereas in the cat and ferret the control of skilled movement is distributed over the C1, C3 and Y zones. It can not be assumed that parasagittal zonation is identical across species.
Figure 2.1. A schematic image showing the parasagittal zones, A, B, C1, C2, C3, D1 and Y of the cerebellum and the connections to the cerebellar nuclei, (modified from (Andersson et al 1987)).

(DN = dentate nucleus, FN = fastigial nucleus, AIP = anterior interpositus nucleus, PIP = posterior interpositus nucleus, VN = vestibular nucleus).

In later studies of lobule V of the anterior lobe of the cat, the D2 zone was renamed the Y zone and it was discovered that cfs that innervate the lateral C3 zone branch and also innervate the Y zone. Furthermore, cfs that innervate the medial C3 zone also innervate the C1 zone (for review see, Oscarsson 1980, Voogd and Bigaré 1980).

Parasagittal zones and lobule HV1

Olivocortical and corticonuclear connections determine the same parasagittal zonal pattern (Groenewegen et al 1979, Voogd and Bigaré 1980). The vermis of the
hemisphere contains zones, C1, C2, C3 and D1 and Y zones. Therefore in accordance with Larsell’s (1970) nomenclature, lobule HVI contains zones C1, C2, C3 and possibly D1 or Y zones, (figure 2.2 and 2.3).

**Connections of the C zones**

Each zone has highly specific connections with the inferior olive and the cerebellar nuclei. A zone receives cfs from a specific region of the olive and projects to a specific region of the nuclei (Apps and Garwicz 2000). The nuclei also receive collaterals from cfs, and have a reciprocal inhibitory projection to the inferior olive (De Zeeuw et al 1997). Each cerebellar parasagittal zone receives inputs from a specific subdivision of the IO. Brodal reported that the intermediate zone that represents Voogd’s C1, C2 and C3 zone is supplied by fibres from the rostral medial accessory olive and the rostral part of the DAO (Brodal 1980).

Detailed studies have shown that the C1 and C3 zones efferents project to the AIP and receive cf inputs from the rostral DAO, the collaterals from the cfs also project to the AIP (Groenwegen et al 1979, Yeo et al 1985c) (figure 2.2). The C2 zone projects to the posterior interpositus nucleus (PIP) and receives inputs from the medial accessory olive (Voogd and Bigaré 1980).

**Microzones**

The parasagittal zones can be further divided into narrow longitudinal microzones of approximately 50-150μm, each microzone controls a single muscle or muscle groups and has private afferent and efferent inputs. The projections from microzones converge onto the cerebellar nuclei and each microzone exerts a regulation of the red nucleus (Ekerot 1995), part of the circuitry responsible for the production of CRs and URs. An individual microzone contains cf inputs that have very similar receptive fields as the local cf (Garwicz et al 1998).
Figure 2.2 Lobule VI contains zones C1, C2, C3 and possibly D1 and Y. C1 and C3 zones are innervated by climbing fibres from the dorsal accessory olive and project to the anterior interpositus nucleus. The DAO and AIP have been shown to be critical for eyeblink/NMR conditioning.
Within lobule HVI the C2 microzones are notable in having a wide receptive fields, including all four limbs and the head, whereas the C1 and C3 microzones have smaller receptive fields.

The microzones of zone B and C3 of the anterior lobe of the cat have been delineated using electrophysiological techniques. In zone B of the anterior lobe of the cat, five microzones have been identified by their inputs. Each microzone receives cfs from a specific set of olivary cells and projects to a specific set of neurones in the vestibular nucleus (Oscarsson 1980).

Why is it important to use Zebrin II compartmentation to identify EB microzones?

A useful way to compartmentalise the cerebellar cortex is to use zebrin II/aldolase C immunocytochemistry (Brochu et al 1990). Approximately 50% of Pcs in the cerebellar cortex express zebrin II, resulting in a symmetrical sagittal banding pattern from the midline of the cerebellum to the lateral hemispheres (Sanchez et al 2002). Zebrin II staining has been shown to be consistent throughout many species, for example, rat, mouse, opossum and rabbit (Brochu et al 1990, Eisenman and Hawkes 1993, Dore et al 1990, Sanchez et al 2002). If the EB/NM microzones of lobule HVI of the rabbit are mapped and put into register with the zebrin II banding pattern then the EB/NM microzones of all species can be determined using zebrin II/aldolase C immunocytochemistry alone, allowing across species analysis.

Zebrin II/aldolase C immunocytochemistry

Zebrin staining results in symmetrically arranged, longitudinal, positively and negatively stained bands from the midline of the vermis to the hemispheres of the cerebellum. However, due to the structure of the rabbit cerebellum the arrangement of zebrin II patterning in the lateral hemispheres is orientated at a different angle to that of the zebrin II patterning in the vermis. The nomenclature used to label zebrin II bands in the rabbit is the same as that used for the rodent; positive zebrin II bands in the vermis are labelled P1+ to P4+, and in the hemispheres, P4a+, P4b+, P5+, P6+ and P7+ medially to laterally with P- (negative) bands lateral to their positive neighbours.
In rabbits, the banding pattern resulting from zebrin II staining has been described in detail for lobule HVI of the cerebellar cortex, (figure 2.3). Zebrin banding within lobule HVI is at an approximate 45° angle to the rostralcaudal axis and has three positive bands, P4a+, P4b+ and P5+ orientated from the superior to inferior extent of lobule HVI (Sanchez et al 2002). Assuming that lobule HVI is an extension of vermal VI it is thought that the longitudinal parasagittal zones defined by the olivocortical and corticonuclear pathways are in alignment with the zebrin II bands, (figure 2.3). It is important to note that this does not mean that the division of zebrin II bands and parasagittal zones are the same, there may be overlap between the parasagittal zones and the zebrin II banding.

Summary

In the rabbit, lobule HVI of the cerebellar cortex may contain C1, C2 and C3 zones and possibly, D1 or Y zones. Due to the connections of the C1 and C3 zone to the AIP and from the DAO it is assumed the critical EB control regions are contained within the C1/ C3 zone of lobule HVI. This rationale is also supported by lesion and reversible inactivation studies that identify normal function of lobule HVI as critical for EB/NMR conditioning processes, as well as anatomical studies that show that there is a heavy input to lobule HVI from the trigeminal system.

Once a C3 EB microzone is located within lobule HVI anatomical immunocytochemical analysis is used to reveal the location of the microzone with respect to the zebrin II banding pattern. Zebrin II immunocytochemistry alone will be the only technique required to locate the C3 EB/NM microzone within lobule HVI in future studies and within a range of species.
Figure 2.3 A schematic diagram of zebrin II immunocytochemistry in lobule HVI of the rabbit. Positive zebrin bands are illustrated in orange and labelled accordingly P4a+, P4b+ and P5+. Superimposed is the probable orientation and order of hemispherical parasagittal zones (indicated by dashed black line). The zones are labelled B?, C1, C2, C3 and D1/ (Y), at the most inferior region of HVI. There is some evidence for a narrow B zone although only at certain rostro-caudal levels, there is also some evidence for the presence of the D1/Y zone at rostral levels (Yeo et al 1985c). Although in this study, no recordings were found with C1 and C3 zone characteristics at a superior level to recordings with C2 zone characteristics, the C1 zone is included in this diagram as there is no real evidence to prove the C1 zone is absent in lobule HVI. The boundary between C2 and C3 zones is based on the boundary between C2 and C3 eyeblink control regions identified in the study presented in this chapter. (modified from Sanchez et al 2002).
Materials and Methods

All subjects (A to I, n=9) received identical treatment during surgery, electrophysiological stimulation, recording, analysis of the C2 zone and C3 zone responses and reconstruction of tracks. Brain sections from subjects C, D and E were additionally processed for zebrin II immunocytochemistry and the reconstructed recording sites were put in register with zebrin II banding patterns. The electrophysiological data for subject I was collected with assistance from Dr. G. Hesslow and colleagues under conditions similar to those for the other subjects.

Surgical procedures

9 male Dutch belted rabbits (1.8-2.5 kg) were anaesthetised using a fentanyl/fluanisone mixture (Hypnorm, Janssen; 0.1/5.0mg kg\(^{-1}\), i.m.) with benzodiazepam (Valium, Roche; 0.5mg kg\(^{-1}\), i.v.) as a relaxant. Each subject was intubated with an endotracheal tube. Mannitol (20% w/v 25ml/30 min, i.v.) was given to facilitate exposure of the cerebellum. The head was then placed in an atraumatic head holder and anaesthesia was maintained with fluothane (1-2%) in oxygen/nitrous oxide (2:1). The scalp was then cut and bone and dura removed to expose a small region of the right cerebellar cortex. The most superior part of lobule HVI was identified visually.

Anaesthesia was maintained until the end of the recording session; although fluothane has been reported to depress spontaneous action potential firing and amplitude in \textit{in vitro} cerebellar Pc cells (Antkowiak et al 1997) the critical criteria for defining C3 and C2 cfrs i.e. the onset latency and characteristic tri-phasic shape are not effected. All subjects were sacrificed with an overdose of pentobarbitone sodium (90mg/ kg, i.v.). Subjects A, C and I were perfused transcardially with 0.9% saline (1 litre) followed by 4% formaldehyde solution (2 litres), the brain was then removed and put in 4% paraformaldehyde solution. For subjects (not A and I) the brain was immediately removed and post-fixed in 4% paraformaldehyde. For all subjects the brain was embedded in 10% gelatin and placed in a solution of 20% sucrose formalin for 3 days. Frozen sections were cut at 50\(\mu\)m in the transverse plane.
Electrophysiological techniques

Stimulation of the periorbital region

Pairs of stainless steel microwires, spaced approximately 3mm apart, were implanted subcutaneously in the ipsilateral and contralateral periorbital area, situated directly above the upper eyelid. Pairs of stainless steel microwires were also placed in the ipsilateral forelimb, situated immediately behind the paw. A 4mA, 300μs, monopolar electric stimulus was used to evoke cf responses.

C2 and C3 zone cf responses were tested as true cf responses by delivering paired pulse stimulation (interpulse interval 40 or 50 ms). It is a characteristic of evoked field potentials, within olivo-cerebellar pathways, that the second evoked cf response is absent or greatly diminished when stimulated with interpulse intervals of up to 150 ms (Armstrong and Harvey 1968, Edge et al 2003) (figure 2.4). If a complex spike occurs following paired pulse stimulation it can be assumed that this is not a true response to stimulation.

Recording Cf responses

Cf field and unit potentials were recorded from lobule HVI and surrounding regions (band-pass filtered 300-10000 Hz, using a DAGAN filter) using epoxylike insulated tungston microelectrodes, 0.008 inch diameter (Frederick Hare). The electrodes were gradually lowered into the cerebellar cortex until cf responses conformed to the experimental design criteria stated below. Data were sampled with a 12-bit A-D converter (CED 1401) and saved.

Criteria defining C2 and C3 zone responses

C2 zone responses

- Responses with large, bilateral receptive fields were defined as from the C2 zone i.e. cf responses were evoked in response to ipsilateral and contralateral periorbital stimulation and ipsilateral forelimb stimulation.

- C2 responses have long (approximately 15- 22 ms) onset latencies.
C3 zone responses

- Zones adjacent to C2 with ipsilateral and discrete receptive fields were defined as C1 (medial-dorsal to C2) or C3 (lateral-ventral) to C2 zone. Responses were only evoked in response to ipsilateral periorbital stimulation.

- C1/C3 responses have shorter onset latencies (approximately 9-15 ms).

After recordings were completed a current was passed through the electrode (10μA for 10 s) to mark recording sites.

Analysis of C2 and C3 zone responses

Peristimulus sweeps were averaged (5-10 sweeps) using SIGNAL (CED). Every recording site for each subject was analysed and onset latencies reported were taken from the averaged sweep.

Averaged climbing fibre field potential onset latencies were determined by eye and defined as: a point of departure from baseline (0mv) with a latency greater than 5-8 milliseconds.

Recordings were taken for responses to all stimulation sites. The averages of recordings from these sites were used as evidence for the laterality of zones. The previously defined criteria were used for identifying a C2 or a C3 zone response (see section above). The expected absence of C3 zone cf responses to contralateral periorbital stimulation and ipsilateral forelimb stimulation was also used as critical data for defining the zone from which the responses are recorded.

Histology and reconstruction of tracks

For each subject every second section was stained with cresyl violet. These sections were used to reconstruct the electrode tracks. For subjects E, F, G and I an image was produced of the stained section showing brain edges and granule cell layer boundaries. The path of the electrode track was reconstructed on these images with the C2 and C3 zone recording sites marked. This allowed precise anatomical relocation of
the recording sites for tracks that both C2 and C3 zone responses were recorded, (figure 2.5).

For all subjects the electrode tracks with C2 and/or C1/ C3 zone responses were reconstructed and marked on schematic images of lobule HVI and surrounding areas. A summary of all C2 and C3 zone recording sites illustrates the anatomical location of recording sites with respect to the rostro-caudal position of recording sites, (figure 2.6).

**Zebrin II immunocytochemistry**

In subjects C, D and E the sections were processed for zebrin II immunocytochemistry to reveal a characteristic set of landmark bands (Sanchez et al 2002). The location of the recording sites from these three subjects was put into register with the zebrin II staining and composite images created to reveal the C2 and C3 zone recording sites with respect to the zebrin II banding pattern.

**Immunohistochemistry**

Sections from each subject, possibly containing the electrode track marks, were left un-mounted following cressyl violet immunocytochemistry.

Throughout, all dilutions and washes used: 0.01M PBS containing 0.5% Triton X-100 and 0.05% thimerosal (Sigma, St.Louis, MO), unless otherwise stated.

Sections were incubated in 0.1% hydrogen peroxide for 15 minutes and then placed in 5% normal horse serum (NHS) for 30 minutes. They were incubated for 14 hours at 4°C in 0.1% Zebrin II antibody (kindly provided by Dr. Hawkes). The sections were then washed and placed in 5% NHS for 30 minutes and incubated in in biotinylated horse anti-mouse IgG (1:200) for 2 hours at room temperature. Sections were washed again and immersed in streptavidin-HRP (1:200) for 2 hours. Then they were washed, buffered with Tris (Sigma, pH 7.4, 1:20) and incubated with diaminobenzidine (0.03% w/v, in 0.01% hydrogen peroxide-Tris buffer) until zebrin positive stripes were revealed. The sections were washed, mounted and coverslipped.
Zebrin II staining is reported as being weak in lobule HVI of the rabbit cerebellum and therefore each section was visually examined through a light microscope for confirmation of the pattern of zebrin staining. An image of each section was recorded and image analysis, using Adobe Photoshop v.6.0, was used to increase the zebrin II staining for presentation. Outline of the zebrin II stained areas were reconstructed and verified with microscopy and overlaid on the image of the section. It should be noted that sections underwent two immunohistological processes and therefore sections do not maintain their original shape. This results in less clear presentation of zebrin II staining.

2

Results

A C3 zone area, within lobule HVI of the cerebellar cortex, that demonstrated cf responses to periorbital stimulation was identified and the extent of this microzone was mapped. Based on the results from Hesslow (1994a, 1994b) it could be assumed that this area may be involved in the control of the rabbit EB/NM. In all cases, this C3 microzone was found inferior to a C2 zone region that also demonstrated cf responses to periorbital and forelimb stimulation. The C3 zone region extends from the medial to the lateral lobe of lobule HVI and, in one case, on the medial boundary of crus I. This C3 zone region extends throughout the rostral to caudal areas of lobule HVI. No responses with C1/C3 characteristics were recorded at locations superior to C2, so this is evidence against the presence of C1 regions demonstrating cf responses to periorbital stimulation in lobule HVI. All responses with C1/C3 characteristics were located inferior to C2 and therefore were considered to be C3 responses.

Recordings from C2 and C3 EB control regions

Laterality

Recordings from 11 sites within lobule HVI satisfied criteria for C3 microzones. Cf responses were observed in the C3 zone to ipsilateral periorbital stimulation only. No cf responses were seen in response to contralateral periorbital stimulation or ipsilateral forelimb stimulation, (figure 2.4). These results are consistent with C3 zone characteristics, with small receptive fields for ipsilateral periorbital stimulation only.
Recordings from 10 sites within lobule HVI satisfied criteria for C2 microzones. These Cf responses were activated by ipsilateral and contralateral periorbital stimulation and by ipsilateral forelimb stimulation (figure 2.4) for an example, (see appendix 1 for complete data set). These results are consistent with the wide receptive field and bilateral input characteristics previously reported for the C2 zone.

Onset latencies

The onset latency for the C2 zone responses ranged from 12–25 ms and 14-27 ms for ipsilateral and contralateral periorbital stimulation respectively and from 18-33 ms for ipsilateral forelimb stimulation. The onset latencies for the C3 microzone ranged from 11-20 ms, with average response latency of 15 ms, see table 2.1. No responses were recorded for contralateral periorbital or forelimb stimulation.

Table 2.1a

<table>
<thead>
<tr>
<th>n = 10 (ms)</th>
<th>Ipsilateral eye</th>
<th>Contralateral eye</th>
<th>Ipsilateral forelimb</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Range</strong></td>
<td>12-25</td>
<td>14-27</td>
<td>18-33</td>
</tr>
<tr>
<td><strong>Mean±SEM</strong></td>
<td>19±1.4</td>
<td>21±1.4</td>
<td>26±1.4</td>
</tr>
</tbody>
</table>

Table 2.1b

<table>
<thead>
<tr>
<th>n = 11 (ms)</th>
<th>Ipsilateral eye</th>
<th>Contralateral eye</th>
<th>Ipsilateral forelimb</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Range</strong></td>
<td>11-20</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Mean±SEM</strong></td>
<td>15 ± 0.8</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Averaged climbing fibre field potential onset latencies were determined by eye and defined as: a point of departure from baseline (0mV) with a latency greater than 8 milliseconds.

Figure 2.4a Averaged (5-10 sweeps) climbing fibre field potentials recorded from a C3 region that showed responses to unilateral periorbital stimulation. These possible C3 EB/ NMR control regions were found in subjects, A, B, C, D, E (1 and 2), F (1 and 2), G (1 and 2) and I.

The left-hand panel shows an image of lobule HVI and surrounding areas, the red circle indicates the location that the recordings were taken from, the approximate depth of the recording site is given below the image. The right hand panel shows the averaged sweeps for ipsilateral and contralateral periorbital stimulations, ipsilateral (and in subject I, contralateral) forelimb stimulation and finally ipsilateral periorbital stimulation with a 40 ms or 50 ms interstimulus interval. The onset-latency is indicated using a vertical dashed line and the latency is given below the averaged sweep.
Subject B

- CF responses recorded at 4.3 mm -0.5 mm caudal to lambda

Subject C

- CF responses recorded at 4.5 mm -1.0 mm caudal to lambda

Stimulation

- Ipsilateral periorbital region
- Contralateral periorbital region
- Ipsilateral Forelimb
- Double Pulse Ipsilateral periorbital region
Subject D

Stimulation

Ipsilateral periorbital region

Contralateral periorbital region

Ipsilateral Forelimb

Double Pulse

Ipsilateral periorbital region

Subject E (1)

Stimulation

Ipsilateral periorbital region

Contralateral periorbital region

Ipsilateral Forelimb

Double Pulse

Ipsilateral periorbital region
Subject E (2)

- CF responses recorded at 4.5 mm -1.5 mm caudal to lambda

Subject F (1)

- CF responses recorded at 3.6 mm -1.5 mm caudal to lambda

Stimulation

- Ipsilateral periorbital region
- Contralateral periorbital region
- Ipsilateral Forelimb
- Double Pulse Ipsilateral periorbital region
- Ipsilateral Forelimb
- Double Pulse Ipsilateral periorbital region
Subject F (2)

Stimulation

Ipsilateral periorbital region
Contralateral periorbital region
Ipsilateral Forelimb
Double Pulse Ipsilateral periorbital

Subject G (1)

Ipsilateral periorbital region
Contralateral periorbital
Ipsilateral Forelimb
Double Pulse Ipsilateral periorbital region

Cf responses recorded at 4.0 mm
-2.0 mm caudal to lambda

Cf responses recorded at 5.6 mm
0 mm caudal to lambda
Stimulation

Subject G (2)

Cf responses recorded at 5.6 mm
-0.5 mm caudal to lambda

Subject I

Cf responses recorded at 4.1 mm
-1.5 mm caudal to lambda

Ipsilateral periorbital region

Contralateral periorbital region

Ipsilateral Forelimb

Double Pulse

Ipsilateral periorbital region

Ipsilateral periorbital region

Contralateral periorbital region

Contralateral Forelimb

Double Pulse

Ipsilateral periorbital region

0 mV

8 ms

14 ms

lmv

< 50 ms >
Figure 2.4b. Subject H and one C2 eyeblink control zone recording site. The left hand panel shows the climbing fibre field potentials evoked with ipsilateral (onset latency 20ms) and contralateral (onset latency 22ms) periorbital stimulation and ipsilateral forelimb stimulation (28 ms).
C2 zone responses provide a boundary for the C3 zone

In 4 subjects, electrode recording tracks (2 in subject E) recorded responses from both the C2 zone and the C3 regions. Figure 2.5 (on the following page), shows these tracks for all 4 subjects. In all 5 cases where C2 and C3 zone responses were recorded in the same track, responses recorded from the C2 zone are located superior to the C3 zone responses. This pattern is consistent and when all C2 and C3 zone response locations were plotted on a standard image of lobule HVI, in no case are the C2 zone responses found at a level of lobule HVI inferior to the C3 zone responses, (figure 2.6). Therefore the C2 zone responses provide a reliable boundary for the inferior- superior extent of the C3 microzone.

Summary of C2 and C3 zone EB/NM responses

All tracks that had C2 zone and/ or C3 zone control response sites were reconstructed and the anatomical location of recordings sites were marked on standard schematic images of lobule HVI and surrounding areas. Individual structural difference and locations of the recording site with respect to the rostro-caudal level within the cerebellum were taken in to account and are shown in (figure 2.6). The C3 zone microzone was found within the inferior extent of lobule HVI immediately inferior to all C2 recording sites and extended from the medial to the lateral lobe of lobule HVI and caudal to rostral HVI.

Figure 2.6

Figure 2.6. Standard schematic images of lobule HVI showing the positions where C2 zone (blue circles) and C3 zone (red circles) responses were recorded from. Taken from all subjects A to I. The standard transverse sections begin at the rostral level 0 mm anterior to 2.0mm posterior skull lambda.
Figure 2.5 Lobule HV1 and surrounding areas of subjects E, F, G and I (two tracks recorded both C2 and C3 climbing fibre responses to periorbital stimulation, in subject E) are shown with the track traversing the lobule. The track is represented by the thick black straight line, with small perpendicular lines indicating recording sites. The blue circle indicates the site where C2 zone responses were recorded and the red circle indicates the site where the C3 zone responses were recorded. C2 zone responses are anatomically superior in all three cases.
The C3 microzone and zebrin II immunocytochemistry

Three subjects (C, D, and E) were processed for zebrin II/aldolase C immunocytochemistry and the position of C3 microzone recordings were put in register with the zebrin II staining, (figure 2.7, due to the size of this image these are presented on the following 2 pages). The pattern of zebrin staining found in all three subjects conforms to the pattern of zebrin staining described for lobule HVI previously (Sanchez et al 2002), (see chapter 2, page 65). It has previously been noted that staining within lobule HVI is weak and can be difficult to see (Sanchez et al 2002). Although this is the case for zebrin band P4a+ in three of the sections presented (figure 2.7, subjects C, D and E2, please see the following pages), in all subjects zebrin bands P4b+ and P5+ are easily identifiable and are located at superior to inferior regions of lobule HVI respectively. The C3 microzone, that showed cf responses to periorbital stimulus, was found within the P4b- and P5+ zebrin zones, (figure 2.8, shown below).

Figure 2.8

Figure 2.8 A standard transverse section (-1.0mm caudal to lambda) of the zebrin II zones found within lobule HVI (positive zebrin staining is shown in orange) and the C3 EB/NM control microzone (shown in red). In all case C2 climbing fibre responses are anatomically superior to C3 climbing fibre responses. It must be noted that the C3 EB/NM microzone may extend further in the medio-lateral direction. C3 EB/NM control sites were located at the most inferior region of lobule HVI. The C2 EB/NM microzone is not fully mapped and so is excluded from this image.
Figure 2.7 Lobule HV1 stained for zebrin II aldolase C. Identified zebrin bands are outlined in orange (dashed line). Red circles indicate the position of the recording from the identified C3 microzone and blue circles indicate the position of recording from identified C2 microzones. In subject C, D and E2 P4a+ can not be identified. Although in: subject C, P4b+ of the lateral and medial lobule, subject D, P4b+ of the medial lobule, subject E1 P4a+ of the lateral lobules and subject E2 P4b+ of the medial lobule cannot be seen these regions of zebrin II immunostaining were verified using microscopy.
Figure 2.9 continued. Two C3 eyeblink/ NM control regions were located in subject E. In section E1, P4a+, P4b+ and P5+ can be identified. In E2, P4a+ can not be identified, but P4b+ and P5+ zebrin bands are identified and highlighted using the orange dashed line.
Discussion

Lobule HVI C3 EB/NM microzone

A C3 microzone was found within lobule HVI that demonstrated cfr responses to periorbital stimulation and extends through rostrocaudal HVI and from the medial leaf to the lateral leaf of lobule HVI, inferior to all C2 zone recording sites. This putative EB/NM microzone may extend somewhat beyond these inferior and medio-lateral extents because areas surrounding lobule HVI were not fully and systematically investigated. However, some tracks penetrated these regions and no C3 control regions were found beyond lobule HVI except in one subject where cf responses were recorded in medial crus I.

Another important possibility is that there is more than one microzone. Involved in the control of the EB/NMR. Intentionally, microzones involved in the control of the rabbit EB/ NMR were only mapped in lobule HVI but in previous studies of the cat up to four EB control regions have been reported, two in lobule HVI, one in lobule HV and one in the paramedian lobe (Hesslow 1994a). Cfs can branch up to 10 times and it has been found that cfs originating from the hindlimb region of the olive project to the lateral C3 zone and branch into lobules II and III of the anterior lobe (Voogd and Bigaré1980). It may be that cfs transmitting somatosensory information from a discrete periorbital receptive field, transmit the information to Pcs of many microzones spread throughout lobules. However, there is discrepancy concerning which cortical lobules are necessary for EB/ NMR conditioning, in agreement with Yeo and colleague's findings, as stated in the Introduction throughout this thesis it is assumed that lobule HVI is the only cortical lobule critical for EB/ NMR conditioning, so the EB/NMR microzone mapped within it is possibly the major contributor to EB/ NMR conditioning. Further studies will be needed to determine this and to investigate if there are additional, subsidiary areas outside HVI.
The C3 EB/NM microzone and learning

Lesion and reversible inactivation studies

The C3 microzone appears to be the only C3 region in lobule HVI that shows cf responses to periorbital stimulation and therefore, this region may be critically involved in EB/NMR conditioning. The extent of this microzone corresponds well with regions of lobule HVI that have been found to be critical for conditioning processes. Studies that have lesioned lobule HVI (including the most inferior part), following acquisition training, report a complete loss of CRs and an inability to reacquire CRs. However, if a small, inferior part of lobule HVI remains, CRs are notably less impaired; (Yeo et al 1984, Yeo et al 1985b).

'In the 3 cases in which the conditioning was not impaired, the lesion did not include the whole of HVI, there was a sparing of cortical tissue at the depths of the lobule’ (Yeo et al 1984).

Consistent with these lesion studies, the C3 EB/NM microzone located here is in the most inferior region of lobule HVI.

Reversible inactivation studies have infused various drugs into lobule HVI during conditioning procedures and on completion of training, the spread of the drug has been mapped using autoradiographical techniques. Normal function of the medial aspect of rostral lobule HVI is necessary for the acquisition and performance of the CRs (Attwell et al 2001, Attwell et al 1999). Although the map of the drug spread may not include the entire region necessary (and it may include regions unnecessary) for the performance and acquisition of CRs it can be concluded that an area of the critical EB control region is within the spread of the drug.

It is reported that the medial aspect of rostral HVI is involved in conditioning processes, (acquisition) and performance of CRs (Attwell et al 2001, Attwell et al 1999) in all cases the majority of drug spread is towards the medial-inferior extent of lobule HVI and has not spread to the superior regions of the lobule. This region, found in the
inferior medial aspect of rostral HVI, corresponds to the C3 EB/NM microzone mapped in this study. However, the microzone mapped here also extends to the inferior lateral extent and medial and lateral regions of inferior caudal HVI, an area of lobule HVI that the drug did not invade.

The area of lobule HVI found to be critical for performance of HVI was put into register with zebrin II banding patterns (Attwell et al 1999). The nomenclature of zebrin II staining has changed from that cited in this study (Attwell et al 1999) to that reported in this chapter, (page 65). According to the new nomenclature the region critical for the performance of the CR is found in zebrin II zones P4b+ and P5+, the same zebrin zones in which C3 EB/NM microzone is found. The region of lobule HVI critical for performance has been found to correlate closely with the site critical for acquisition (Attwell et al 2001). Therefore because the site necessary for performance is located in the same zebrin II banding pattern as the C3 microzone mapped here, it can be concluded that this C3 region may be involved in the acquisition of CRs.

**Characteristics of C3 EB/NM microzone**

The results found from the mapping study presented here display the bilaterality and wide receptive field characteristics previously seen for the C2 zone and also the ipsilateral and small receptive field characteristics of C3 zone responses.

The onset latencies found in this study vary slightly from those previously reported. This may be explained as onset latencies of cf responses to cutaneous stimulation vary due to the number of synapses in the spino-olivary or spino-trigeminal pathways. Short onset latency times result from monosynaptic pathways and longer onset latency times result from polysynaptic pathways. An explanation for the variance in different cf response onset latency times may result from studies using different species with varying lengths and size (diameter) of neurones within these pathways, for example cat (Hesslow 1994a), ferret (Hesslow and Ivarsson 1994) rat (Jörntell et al 2000) and rabbit (this study).
There is some overlap between the C3 cf onset latencies reported here (11–20 ms) and those reported for C3 EB control regions in the cat (approximately 9-12 ms), (Hesslow 1994a). However the differences found in cf onset latencies may be due to species differences or differences in the preparation and experimental techniques of the recording studies, Hesslow used decerebrate cats in comparison to the non-decerebrated rabbits used in this study. In order to prevent any walking movements from the cats a muscle relaxant was used to eliminate gross movements whilst still able to obtain EMG recordings from the obicularis oculi muscle. Cf responses were recorded from the surface of the cerebellar cortex using a silver ball electrode, the recording electrode could still pick up other muscle activity, as the subjects were not fully curarized. Stimulation parameters for the two studies were the same.

Recently in the cat, onset latencies for evoked cf field potentials were recorded from the most medial folium of crus I, and specifically from areas identified as C2 and C3 zones. Cf responses could only be evoked with ipsilateral periorbital stimulation within the C3 zone but with ipsilateral and contralateral forelimb stimulation and ipsilateral periorbital stimulation in the C2 zone (Edge et al 2003). In response to ipsilateral periorbital stimulation C3 climbing fibre field potential onset latencies ranging from 11 to 15 ms were recorded in crus 1, in close agreement with the findings in the study presented here. Moreover, C3 zone cf field potential onset latencies were recorded to periorbital stimulation, from the lateral lobe of lobule HVI and reported as being 13-14 ms, the range is notably smaller, probably because these results are from only 2 recording sites. However, the mean onset latency from the study presented here is 15 ms in good accordance with findings reported by Edge and colleagues (Edge et al 2003).

In the rabbit, Pc activity during EB/NMR conditioning has been reported (Berthier and Moore 1986). Extracellular recordings were made from Pcs within lobule HVI and specific attention was paid to the change in firing rate during the CS-US interval. Although the authors identified Pcs within lobule HVI that appeared to respond in relation to NM CRs, it is not possible to know if these Pcs have a direct relationship with the behaviour. Therefore, the results from this study may not contribute to the understanding of the physiology of the Pcs involved in this behaviour alone. However,
the authors do present findings that could be from cells directly involved in this behaviour, and these findings are discussed in detail in the Introduction (page 62, Pc firing rates during EB conditioning). Additionally, the anatomical reports of the location of Pcs recorded from are in agreement with the C3 EB zone identified here.

Summary

A C3 control region that shows cf responses to ipsilateral periorbital stimulation only, has been located in lobule HVI (and into crus I) within zebrin II zones P4b- and P5+, in agreement with mapping studies in the cat (Hesslow 1994a, Edge et al 2003) and ferret (Hesslow and Ivarsson 1994) and human (Dimitrova et al 2002). Lesion and reversible inactivation studies provide evidence that this region is critical for conditioning processes such as, acquisition and performance and also for the extinction of CRs (Attwell et al 1999, Attwell et al 2001 and this thesis, chapter 3). This evidence supports the suggestion that this C3 control microzone may be critically involved in the learning process during classical conditioning of the rabbit EB/NMR.
Chapter 3

Cerebellar Cortical Function in Extinction Learning
3

Cerebellar Cortical Function in Extinction Learning

What is extinction?

Extinction was first observed and described by Pavlov (1927) as a gradual weakening of the CR. A procedure to obtain extinction is to present the CS alone, after previously establishing CRs with conditioning. Presentations of the CS alone result in a gradual decrement of the CR until the CS no longer elicits a response. Pavlov recognised that the ability to extinguish a response is as important as the ability to acquire a response, in order for an animal to adapt to an ever-changing environment.

As part of describing the extinction process Pavlov also observed and named an associated phenomenon called *spontaneous recovery*. If extinction proceeds to a point where the CR has disappeared and the subject is subsequently given a rest period then, when tested again, presentation of the CS will, once again, elicit a CR. A second extinction training session will result in faster extinction and the amount of spontaneous recovery will decrease over successive training sessions (Pavlov 1927). It has been suggested that during extinction training a residual excitatory strength remains that can account for spontaneous recovery (Kehoe 1988).

Extinction learning appears to be sensitive to the context in which the extinction training occurs. Renewal is a characteristic re-occurrence of the extinguished CR when the subject is placed in a different context to that within which previous extinction training has occurred (Bouton and Bolles 1979, Bouton 1993). The finding that extinction is context-specific has been used to explain another feature of extinction - *rapid relearning* (or savings). Rapid relearning is an increased rate of acquisition of the CR following extinction of the CR (Hoehler et al 1973, Napier et al 1992). During acquisition, contextual cues related to the US are present, and during extinction the contextual cues are still present despite the absence of the US. It has been suggested that these contextual cues coupled with the CRs produced during spontaneous recovery may contribute to rapid relearning (Napier et al 1992). Rapid relearning and spontaneous
recovery are important features of extinction and they influence the theories proposed for the mechanism of extinction.

Extinction and acquisition learning have features in common. During extinction and acquisition, CRs are being driven to a minimum and maximum level respectively and as they reach this level the change in responses becomes progressively smaller. Generalisation is also applicable to extinction as well as acquisition. Extinction of the CR to a specific CS will generalise to produce reduced CR frequency and amplitude to similar CSs. Lesion and reversible inactivation studies provide evidence that it may also be the case that the same cerebellar circuitry is involved in acquisition and extinction learning (Ramnani and Yeo 1996).

Theories of extinction

Internal inhibition

Pavlov (1927) proposed his theory of internal inhibition as the mechanism underlying extinction learning. Pavlov suggested that when spontaneous recovery is seen, the association between the CS and US must have been still present and is not entirely weakened or lost during the extinction procedure but instead it is actively blocked by an internal inhibition process. If internal inhibition is temporary during the extinction procedure then it could account for spontaneous recovery. However extinction training will eventually prevent spontaneous recovery, demonstrating a real loss of the association between the CS and US during extinction training.

The mechanism for internal inhibition relies on independent excitatory and inhibitory processes. During acquisition learning the CS has positive or excitatory properties that elicit a CR. During extinction learning the CS comes to have inhibitory or negative properties that evoke the inhibition of the response instead of the usual excitation.

The CS-US association is unlearned

In 1972 Rescorla and Wagner proposed a mathematical model of conditioning (Rescorla and Wagner 1972). The R-W model assumes that, as each trial occurs there
are changes in the associative strength between the CS and the US. That is, for each trial the amount of conditioning that can occur depends on the amount of conditioning that has already occurred. At the beginning of a training session when most conditioning is possible, the associative strength between the CS and US will be large, when conditioning is nearing asymptote the associative strength between the CS and US becomes progressively smaller (Bolles 1978b).

During acquisition, the change in associative strength of the CS-US decreases with successive trials. This is because the amount of conditioning that can occur is greatest on the first trial, on the subsequent trials the amount of conditioning that can occur is less than previously and this trend continues for all successive trials. The strength of the CS-US association increases, limited by the magnitude (or intensity) of the US.

During extinction the US is not present and the change in associative strength decreases with successive trials as it does for acquisition but the associative strength between the CS-US decreases with successive trials. Therefore the association between the CS and US is unlearned (Rescorla and Wagner 1972). Rescorla and Wagner (1972) suggested that rapid relearning is evidence that no inhibition is involved in extinction. If inhibition produces extinction then, relearning after extinction would take longer than learning from the naïve state.

Recent empirical and computational support for the unlearning theory of extinction, implemented for EB/NMR conditioning, is presented by Mauk and colleagues, who propose a dual-site hypothesis for the mechanism of extinction and for the occurrence of savings. This hypothesis is based on partial unlearning and partial retention of the association between the CS and US in the cortex and interpositus nucleus respectively (Medina et al 2001, Medina et al 2002). This is discussed in the discussion (Chapter 3, page 136).
Is extinction a new learning?

One possibility is that extinction could be new learning, completely separate from the previous acquisition learning. So, in extinction, the CS predicts no US, and a new association is formed alongside the original association (see Bouton 2002, Myres and Davis 2002, for reviews) and the CS acquires a second meaning that is, it predicts no US. This new association co-exists with the association formed during acquisition and it is not known if the same circuitry is involved for the extinction and acquisition mechanisms.

The phenomena of spontaneous recovery and rapid relearning support this separate learning idea. They indicate that extinction is not a complete unlearning of the original association, on the contrary, they suggest that some of the original association still exists after extinction training. Spontaneous recovery, rapid relearning and renewal provide evidence that the CS can still drive a CR following extinction.

Extinction and eyeblink/ NMR conditioning

Discrete, permanent and reversible lesions of lobule HVI and its associated circuitry have indicated that this region of the cerebellum is crucial for the acquisition and performance of CRs in NM conditioning (see Yeo and Hesslow 1998 for review). These techniques have also been used to investigate whether the same circuitry is involved in extinction and in acquisition.

Acquisition and extinction are prevented during infusions of muscimol (a GABA\textsubscript{A} receptor agonist) directed at the AIP during conditioning (Hardiman et al 1996, Ramnani and Yeo 1996). Following recovery from inactivation, subjects acquire and extinguish responses at rates comparable to those for control subjects. So the same basic cerebellar circuitry appears to be involved in extinction and acquisition. Two recent studies, Medina et al (2002) and Krupa and Thompson (2003) provide evidence for mechanisms suggested to underlie extinction and are discussed in detail below.
Current theories for the mechanism of extinction in EB/NMR Conditioning

1) Inhibition of the climbing fibres is the signal for extinction learning

The ideas that the same circuitry is used in acquisition and extinction and that extinction involves the process of unlearning in the cerebellar cortex, is supported empirically and theoretically in the study discussed below (Medina et al 2002).

The fundamental theory that cfs provide a teaching signal to the Pc that modifies the efficacy of the currently active pf-Pc synapses (Marr 1969, Albus 1971) forms the basis for this theory of the mechanism underlying extinction (Medina et al 2002). During acquisition the US activates cfs from the DAO (Yeo et al 1985c). When CRs occur, olivary activity is inhibited via a pathway from the AIP to the DAO (Andersson et al 1988). It is suggested that, during extinction training, this inhibition of the IO and consequent decrease of the firing of cfs, in the absence of the US excitatory drive is the signal for the induction of extinction learning (Medina et al 2002). It is important to emphasise the role the climbing fibres play in this mechanism i.e it is the inhibition from the IO that occurs due to previous acquisition training and consequent CR production, in addition to the absence of the US that decreases the firing rate of the climbing fibres and this is the signal for the induction of extinction learning.

Medina and colleagues used a localised infusion of the GABA_A receptor antagonist, picrotoxin, to block the inhibitory pathway to the IO during extinction training and prevented extinction of CRs. The responses reported to occur throughout the extinction training session maintain their learned timing and therefore are considered to be CRs.

It is suggested that deviation from cf baseline firing activity signals the induction of plasticity for acquisition or extinction. An increase in cf firing is the signal that promotes acquisition of the CR. A decrease in cf firing to below baseline, due to the CR-related inhibition of the IO baseline activity, is the signal that promotes extinction of the CR (Medina et al 2002). Following this logic, picrotoxin infusions to the DAO
would increase the firing rate of cfs well above their intrinsic firing rate of approximately 1Hz. Not only would extinction be prevented, as demonstrated in this study, but acquisition learning could also occur and this would result in super-conditioning.

It is proposed that bi-directional modulation of cf activity is responsible for acquisition and extinction via LTD and LTP respectively and it is suggested that LTD and LTP occur at the same site of plasticity in the cortex. The most extensively researched plasticity in the cortex is LTD of the pf-Pc synapse, expressed postsynaptically and induced due to co-activation of pfs and cfs at the granule cell-Pc synapses (see Chapter 1, page 40). More recently a post-synaptic LTP induced with pf stimulation alone (Lev-Ram et al 2002) has been demonstrated. The characterisation of a post-synaptic LTP provides a possible mechanism for the reversal of LTD and, therefore, a reversal or ‘unlearning’ of motor learning.

In order to account for the phenomenon of savings in extinction, Mauk and colleagues suggest there is plasticity outside the cerebellar cortex that is resistant to extinction and a region of the cerebellar nuclei is proposed for this site. This proposal relies on evidence that there are two sites of plasticity, the cerebellar cortex and interpositus nucleus. Plasticity changes that occurred during acquisition training undergo a reversal during extinction training. During acquisition, plasticity in the cortex is followed by plasticity in the interpositus nucleus, and so, during extinction, there is a ‘reversal’ of plasticity firstly in the cortex and subsequently in the interpositus nucleus. Therefore, plasticity in the interpositus nucleus is more resistant during extinction training and it is the residual plasticity retained in the interpositus nucleus that contributes to savings (Medina et al 2001, Medina et al 2002).

The suggested mechanism underlying savings is as follows. During both acquisition and extinction learning cortical plasticity is under the control of the cfs, consequently, LTD occurs when the pf-Pc synapse is active and there is cf input. LTP occurs if the pf-Pc synapse is active in the absence of cf input. Cerebellar nuclear plasticity is under the control of the Pc, so, LTD of the mossy fibre to nuclear cell
synapse occurs during inhibition from the Pc and LTP of these synapses occurs during transient pauses in this inhibition. Therefore, during extinction, the reduction of cf activity during the CS, due to the increased CR related nucleo-olivary inhibition, induces LTP at pf-Pc synapses, this restores the CS induced activity of Pcs. Once this Pc activity is restored, LTD occurs at the mossy fibre-nuclear synapses. Therefore, there is a time period when nuclear plasticity lags behind cortical plasticity. So, CRs appear completely extinguished but there is residual plasticity at the mossy fibre to nuclear cell synapses remaining. This mechanism suggests that the amount of savings is reduced slowly as the residual plasticity is reversed.

2) Inhibition of the classically conditioned response during extinction training completely prevents extinction learning

The response-extinction theory is based on the assumptions that, during extinction learning, the original association between the CS and US is retained (Pavlov 1927), and that extinction training produces a new learning that interferes with performance of the CR. The response-extinction theory suggests that there is a strong correlation between the amount of CRs during extinction training and the amount of real extinction learning that occurs. For example, if CRs are prevented during extinction training the amount of extinction learning is reduced (Rescorla 1997).

Thompson and colleagues recently used the rationale of the response-extinction theory in a rabbit NMR conditioning study (Krupa and Thompson 2003). In order to block performance of previously acquired CRs during extinction training, muscimol (a GABA_A receptor agonist) was infused in the brainstem in the region of the facial nerve (FN), accessory abducens nuclei (ACC) and adjoining reticular formation. Subsequent extinction training after the muscimol effects had dissipated revealed a high frequency of CRs, indicating extinction learning had been prevented. The results may provide evidence in support of the response-extinction theory and they are considered together with the results from the study presented in this chapter (see, this chapter, discussion, page 140).
In an earlier study it was shown that inactivation of the same brainstem regions during acquisition training do not disrupt acquisition learning (Krupa et al 1996), suggesting that acquisition and extinction learning have different behavioural properties and neuronal substrates. It is suggested that inactivation of the motor nuclei to prevent the performance of CRs may prevent extinction because feedback from tactile stimulation of the cornea, muscle and tendon receptors or reafference from motor nuclei directly or via the inferior olive to the cerebellum is prevented. The authors suggest further experiments to test that this feedback would cause an increase in cerebellar cortical inhibition of the interpositus nuclei during extinction training to provide evidence for an inhibitory process during extinction learning.

In contrast to Mauk and colleagues (Medina et al 2001), Krupa and colleagues (2003) suggest that the site for savings seen during reacquisition following extinction training, is the cerebellar cortex and not the AIP. This proposal is based on recording studies that analyse single and multiunit responses in the cerebellar cortex and interpositus nucleus during acquisition and extinction training (Gould and Steinmetz 1996). During acquisition and extinction training, activity in the interpositus nucleus correlated with the behavioural CR (the onset of neural activity correlated with the onset of the conditioned eyeblink), importantly, there is an absence of increased activity when the CR was fully extinguished. In the cerebellar cortex however, activity increased during acquisition of the CR but did not decrease significantly during extinction, indicating the cortex as the site for residual plasticity (Gould and Steinmetz 1996) and also supporting the suggestion that inhibition of the AIP occurs during extinction. However, it is important to note that these findings are based on recordings from 13 Pcs which, although they had activity correlated with the behaviour, they were not in a region of the cerebellar cortex identified as an EB/NMR control region. Additionally, Pcs from the same region of lobule HVI demonstrated various patterns of increased and decreased activity during acquisition and extinction. Therefore, this cortical theory for savings is based on a subset of Pcs with behaviours that vary in different ways during conditioning.
The cerebellar cortex and learned response timing

Important implications for the learned timing of CRs comes from experimental studies that show that lesions of the cerebellar cortex or pharmacological blockade of cortical output, disrupted the learned timing of CRs but did not abolish CRs (Perrett et al 1993, Garcia and Mauk 1998). In fact, it is the presence of responses with disrupted timing- short latency responses (SLRs)- that indicate a subsequent failure to extinguish following lesions (Perrett and Mauk 1995). It is proposed that the cortex contains the site of plasticity responsible for the regulating the acquisition, extinction and timing of the CR, and the AIP is the site that contains the plasticity responsible for driving the expression of CRs (Garcia et al 1999).

In agreement with these findings is a study in which lesions of large areas of the cerebellar cortex caused a change in timing of the CRs but not their abolition (McCormick and Thompson 1984a). However, studies report different effects on CR adaptive timing following cortical lesions. Lesions of lobule HVI, crus I, crus II and rostral paramedian lobe, either uni- or bilaterally to the conditioned eye, affected CR timings. Following unilateral lesions the CR onset latency and CR latency-to-peak showed variable timings and following bilateral lesions there was an increase in this variance and not just a reduction of CR onset latency (Gruart and Yeo 1995).

The function of the cerebellar cortex in extinction learning: this study

By using drug infusions reversibly to inactivate lobule HVI of the cortex we can attempt to understand more clearly the role of the cerebellar cortex in extinction. Attwell and colleagues have shown that infusions of CNQX, (an AMPA/ Kainate receptor antagonist), prevents acquisition and performance of the CR indicating the critical role the cortex plays in acquisition of the CR (Attwell et al 1999, 2001).

In order to investigate a putative role for the cortex in extinction the present study has used CNQX infusions to disrupt the normal function of the cortex during extinction training. By undertaking this study a number of issues are addressed. Disrupting normal function of the cortex during extinction training may reveal the
extent of the involvement of the cerebellar cortex in extinction learning and may provide evidence for a site of extinction learning-related plasticity.

This study will also provide an indication of the regions of lobule HVI of the cerebellar cortex that may be involved in extinction learning. The regions identified can be compared to those found, in previous inactivation studies, to control performance and acquisition and with the results from the lobule HVI eyeblink control region mapping studies.

To explore the learned timing of the CR, the latency-to-peak of CRs was analysed during acquisition and extinction training. This analysis indicated whether the absence of the US input via the cfs, as in extinction training, causes a disruption in the learned timing of the response.

3

Materials and Methods

This study builds upon a previous incomplete set of data. That data set included the incomplete experimental group (n=3) and the complete control group (n=5). More subjects were necessary for the experimental group in order to be able to perform statistical analysis. The previous subjects, from both the experimental and control groups, are included in all analysis of the results and discussion.

Surgical procedures

6 male Dutch belted rabbits (1.8-2.5 kg) were anaesthetised using a fentanyl/fluanisone mixture (Hypnorm, Janssen; 0.1/5.0mg kg⁻¹, i.m.) with benzodiazepam (Valium, Roche; 0.5mg kg⁻¹, i.v.) as a relaxant, although benzodiazepams are GABA<sub>A</sub> receptor modulators and enhance the action of GABA these effects are not long-lasting and should not have an effect during the following experimental procedures. Each subject was intubated with an endotracheal tube. Mannitol (20% w/v 25ml/30 min, i.v.) was given to facilitate exposure of the cerebellum. The head was then placed in an atraumatic head holder and anaesthesia was
maintained with fluothane (1-2%) in oxygen/nitrous oxide (2:1). The scalp was then cut and bone and dura removed to expose a small region of the right cerebellar cortex. A 26 gauge, 11mm stainless steel guide cannula was then lowered into lobule HVI under visual guidance. The exposed brain was covered with sterile absorbable gelatin foam and the guide cannula fixed to the skull with acrylic cement. A 33 gauge dummy cannula with a screw cap was fitted into the guide cannula as a seal. The scalp was sutured around the cannula tip and the subject removed from the head holder.

Each subject received three days of post-operative treatment. The subjects were given buprenorphine (100 ug/kg/day, i.m.) as analgesic cover and chloramphenicol, 30mg/ kg/day, i.m.) as antibiotic cover. Subjects were kept on a 12 hour day/night cycle with ad libitum access to food and water. Subjects were allowed one week to recover.

**Conditioning apparatus**

During the habituation session a suture was placed in the right nictitating membrane of each subject under local anaesthesia (proxymetacaine hydrochloride, 0.5% w/v), applied directly on to the NM. The subject was placed in a Perspex restraining box, head and ear movements were prevented using an ear clamp and by securing the head within the Perspex box with an adaptable secure neck clamp. A potentiometer, mounted on a platform that fitted securely around the muzzle of the subject, was hooked onto the suture of the NM to allow isotonic transduction of the NM movement. Two 12mm stainless steel Michel clips were attached centrally, below the lower eyelid and behind the temporal canthus of the right eye. The subject was placed in a ventilated, sound attenuated conditioning chamber facing a centrally mounted loudspeaker, see figure 3.1. Previous studies have demonstrated that the duration of restriction used here does produce discomfort for the subjects (Thompson 1976).

The CS was a 1kHz sine wave tone with an intensity of 81dBA and duration of 410 ms. The US was periorbital electrical stimulation applied through the stainless steel clips. Each US was a 60 ms train of three biphasic current pulses (2.5mA). Ventilation

---

1 Previous work, experimental subjects (n=3) and control subjects (n=5) done by Dr. S. Rahman.
fans provided background noise at an intensity of 57 dBA. On paired trials the inter-stimulus interval (ISI) was 350 ms. There was no ISI on unpaired trials as the CS was presented alone. The inter-trial interval (ITI) was randomly selected between 17 and 23 seconds in order to prevent conditioning between trials that would allow the subject to predict the onset of the stimulus of the subsequent trial. Therefore conditioning only occurs between the CS and US of a trial.

**Experimental design**

Figure 3.1

![Conditioning Apparatus](image)

Figure 3.1 Conditioning Apparatus. Within the conditioning chamber the subject is restrained in a Perspex box specifically designed to prevent head and ear movement. The potentiometer (used to measure NM movement) is held in a constant position on a head stage and attached to the nictitating membrane (NM). The CS is an auditory tone delivered via the centrally mounted loud speaker and the US is an electric shock to the periorbital region.

Inset. A detailed image of the rabbit eye and NM. The black arrow indicates direction of movement of the NM. The degree of movement is measured by a potentiometer hooked onto a nylon suture inserted into the NM. The electric shock (2.0 mA) is delivered via Michel clips positioned underneath the lower eyelid and behind the nasal canthus.
**Experimental design**

All protocols used here for experimental subjects are identical to those previously used for experimental and control subjects (incomplete data set, see footnote¹), and are described below.

All conditioning phases were performed at approximately the same time of day (± 30 minutes).

![Diagram](image)

Figure 3.2 Experimental design. H = habituation session, A = acquisition session, E = extinction session, R = reacquisition session. Solid black line indicates training sessions on consecutive days. Black arrow indicates time of PBS infusion (2µl, 2 minutes, sterile PBS, pH 7.4), purple arrow indicates time of CNQX infusion (2µl, 2 minutes 3mM CNQX, pH 7.4). Arrows representing infusion times are post-fixed with X3, indicating the number of infusions/subject. Please see Material and methods for more details.

Previous control subjects (n=5), underwent the same procedures except in,

- Extinction, Phase 1, control subjects received infusions of sterile phosphate buffered saline (2µl infused over 2 min, pH 7.4)
- Extinction, Phase 2, control subjects only underwent extinction training sessions 7, 8 and 9 and not 10, 11 and 12.
Habituation phase

All subjects received 3 daily habituation sessions. Each subject was placed in the Perspex restraining box for 35 minutes, a duration equivalent to one conditioning session. The NM transducer was attached to the suture and the US shock leads were clipped on to the periorbital clips. The background noise was present during the habituation sessions but there was no presentation of the CS or US.

Acquisition (sessions 1, 2 and 3)

All subjects received three daily acquisition sessions. These consisted of 100 trials lasting 35 minutes. Every 10th trial the CS was presented alone, in order to monitor the development of CRs without the UR. These data were not analysed. All other trials were paired CS-US presentations.

Extinction phase 1 (sessions 4, 5 and 6)

Following session 3 of acquisition training, the two experimental subjects received infusions of CNQX (disodium salt, Tocris, IC50 = 0.3 ± 0.15μM). The dummy cannula was removed and a 33 gauge infusion cannula was inserted through the guide to protrude approximately 1.5 mm below the guide cannula. CNQX (3mM, 2μl in phosphate buffered saline (PBS), pH 7.4, infused over 2 min) was infused 5-10 minutes before each session. 2μl of 3mM CNQX was chosen based on previous studies (Attwell et al 1999). As the placement of the infusion cannula is performed visually there is variability in the region the drug spreads to so, to ensure a large region of lobule HVI received the drug at an effective concentration, a large volume (2μl) and high concentration (3mM) of CNQX is used. This concentration and volume has been used successfully previously (i.e. there was no spread to nuclear regions) Attwell et al (1999, 2000). Subjects then received extinction training of 100 CS alone trials lasting for 35 minutes.
Extinction phase 2 (sessions 7, 8, 9, 10, 11 and 12)

All remaining experimental subjects received a further six daily extinction sessions of 100 CS alone trials lasting for 35 minutes. No drug was infused during these sessions.

Reacquisition

All remaining experimental subjects received two daily sessions of acquisition training, 100 trials (90 paired CS-US, 10 CS alone) lasting 35 minutes.

Performance test

Following reacquisition, experimental subjects underwent a performance test in which CNQX was infused during acquisition training in order to determine if the location and dose of drug infusion was sufficient to abolish performance of responses. Subjects received 20 trials, (18 paired CS-US, 2 CS alone) to establish a baseline. Each subject received infusions as in Extinction, Phase 1, (CNQX 3mM, 2µl in PBS, pH 7.4 infused over 2 minutes). Acquisition training immediately followed infusions, 100 trials (90 paired CS-US, 10 CS alone) 35 min, and at 1 hour and 2 hour time points, each of these two sessions consisting of 20 trials (18 CS-US, 2 CS alone). This allowed the occurrence and frequency of CRs to be recorded during the drug inactivation and as the drug effects dissipated.

Behavioural analysis

CR frequency

During acquisition and extinction training a CR was defined as a NMR within the CS-US interval with amplitude ≥0.5mm and with an onset latency > 35ms from CS onset. These parameters for defining CRs are standard and have been described previously (Ramnani and Yeo 1996).

Analysis for acquisition and extinction phases were carried out in the same way. CR frequency was calculated for each block of 10 trials, including CS alone trials during acquisition training.
CR onset-to-peak

The onset-to-peak of every CR was measured: CS onset (amplitude ≥0.5mm and with an onset latency > 35ms from CS onset) to the maximum amplitude of the CR. These latencies were measured for all CRs during extinction sessions and on all CS alone trials during acquisition and re-acquisition sessions.

Perfusion and histology

After the final phase of the experiment all subjects were infused with $[^3]$H]CNQX (Tocris, U.K) in PBS (3mM, containing 1μCi/μl, 2μl infused over 2 min) at the same depth as for infusions during training sessions. All subjects were sacrificed with an overdose of pentobarbitone sodium (90mg/ kg, i.v.), 21.5min after the end of the infusion time. This time point corresponds to the middle of a training session. Each subject was perfused transcardially with 0.9% saline (1 litre) followed by 4% formaldehyde solution (2 litres).

The brain was removed from the skull, embedded in 10% gelatin and placed in a solution of 20% sucrose formalin for 3 days. Frozen sections were cut at 50μm in the transverse plane.

 Autoradiography

For each subject, (subjects A-E PBS-group, n=5 and A-E CNQX-group, n=5), every 6th brain section, including lobule HVI and the cerebellar nuclei, together with tritium standards (Microscales, Amersham) were opposed to tritium-sensitive film (Hyperfilm, Amersham). These were stored at 4°C for 6 weeks with no exposure to light, after this time period the film was developed.

An image of the autoradiograph of every brain section was taken using a monochrome CCD camera and these were analysed using densitometry techniques (AIS; Imaging research, St.Catherines, Ontario, Canada). The images were density calibrated as picomoles of CNQX per milligram of tissue equivalent, and colour coded against the tritium standards.
The brain sections were stained with cresyl violet and these were aligned with the autoradiographs of each section allowing the precise location and spread of the drug to be revealed. An image of the stained section was produced to show brain edges and granule cell layer boundaries, this was then used to create a composite image of the colour-coded densitometry autoradiograph and the outline of each brain section.

Analysis

Statistical analysis for CR frequency

CR frequencies were not normally distributed and could not be transformed to a normal population distribution so a non-parametric test (Mann-Whitney U test) was used to compare the control and experimental data across sessions.

Analysis for onset-to-peak of the CR

Statistical analysis

For each phase of the experiment, the onset-to-peak times for each CR were plotted in bin widths of 50ms. Any responses for the bin width 0-25ms were ignored, as these would have included all trials in which no CR occurred. Control and experimental groups were analysed using an unpaired t-test to test for any statistical significant differences during acquisition and extinction. Only onset-to-peak data could be used from CS alone trials during acquisition and re-acquisition phases and for all trials during extinction phases.

Coefficient of variation in timing of the classically conditioned eyeblink in Rabbits(CV)

The CV of CR timing, (a ratio of the mean and standard deviation of timing of the CR onset to peak) has been found to depend on the inter-stimulus interval (ISI) (White et al 2000). In the study, White and colleagues report CVs for a range of ISIs from 200–300 ms. These ratios provide a stable measure of CR timing for certain ISIs.

As an alternative analysis of CR timing, CV of CR timing, calculated from acquisition and extinction phases, were compared to those reported in (White et al 2000). A direct comparison of these CVs was not possible as White and colleagues do
not have any data for the ISI used in our study, 350 ms, however they do report CVs of CR timing, for ISI’s of 300 and 400 ms. The CVs of CR timing calculated from our data were compared to the CVs for both ISIs calculated by White et al (2000).

To obtain enough data points for valid statistical analysis for the extinction phase, data could only be taken from the first 3 extinction sessions for the control group and the first 3 no drug extinction sessions for the experimental group. Although only the CS and no US, is present during extinction training, there is a nominal ISI, due to previous acquisition training sessions.

For statistical analysis, z-tests were performed to test for any significant differences between CVs reported by White et al (2000) and those found here and t-tests were performed to compare CVs from our data across phases.

3
Results

Principal findings

• CNQX infusions in the cerebellar cortex during extinction training impaired extinction of NM CRs.

• The learned timing of the CR remained constant during acquisition and extinction training.

Criteria for inclusion of subjects
Subjects were included in the study and analysis unless they failed to satisfy one or more of the following criteria:

1. Subjects were required to reach an asymptotic level of ≥85% CRs in the last 20 trials of acquisition training in order to continue with the extinction phases of the study.
2. During performance testing CRs must be abolished for at least one block of training, post drug infusion, (one block is the equivalent of 10 trials, 9 paired CS-US and 1 CS alone).

3. Histological examination must reveal no damage to the cerebellar cortex other than immediately surrounding the guide cannula.

4. Autoradiographs must reveal that the drug is restricted to cortical areas and has not spread to the cerebellar nuclei (figure 3.3). If the drug spreads to the nuclei then cortical effects can not be dissociated from nuclear effects. In all subjects from the previous data set, (see, page 118, footnote\(^1\)) the drug spread was restricted to the cerebellar cortex with no drug found in the deep cerebellar nuclei.

Four of the six current subjects were rejected on criterion 1. Two subjects were accepted after histological examination showed no extensive damage of the cortex and autoradiographical examination revealed only cortical spread of the drug. The data from these 2 current subjects was added to the previous experimental group, (n=3, see, page 118, footnote\(^1\)), to create an n of 5.

Analysis of data from the complete experimental group (CNQX-group, (n=5)) and previous control group (PBS-group, n=5, see page 118, footnote\(^1\)) follows.

**Infusions of CNQX in the cerebellar cortex impaired extinction**

See appendix 2 for raw data examples of a UR and CR, (page 190).

**Acquisition (sessions 1, 2 and 3)**

Following 3 daily habituation sessions all subjects received 3 daily sessions of acquisition. The learning curves for acquisition for the PBS-group and the CNQX-group were not significantly statistically different (Mann-Whitney U-test, T=23835, n=150, n=150, p=0.093), (figure 3.4).
Figure 3.3. Autoradiographs for individual subjects. The left hand panel shows schematic images of the cerebellum, 0.5 mm anterior -3.0 mm posterior to skull lambda. The cerebellar nuclei are highlighted in pink note that no drug is found in the nuclei. This page (127) shows autoradiographs for the CNQX group and the opposite page (128) shows autoradiographs for the PBS group.

Densitometry calibration: pmol CNQX/mg tissue equivalent.

( PM - paramedian lobe, DPLF - dorsal paraflocculus, VPLF - ventral paraflocculus, ND - dentate nucleus NI - interpositus nucleus)
Figure 3.1 continued. Autoradiographs for control-group subjects.
Extinction phase 1 (sessions 4, 5 and 6)

PBS-group subjects received infusions of saline before each extinction session during Phase 1. The PBS-group displayed characteristic extinction learning behaviour, an average of 35.6% CRs in session 4 decreased to 13.1% in session 6, (figure 3.5). CNQX-group subjects received infusions of CNQX before each training session. During CNQX inactivation performance of the CRs was blocked, so it is not possible to determine whether CNQX prevents extinction in this group during this phase of the experiment. Once the inactivation is lifted in the subsequent phase, the presence of CRs would show whether CNQX had prevented extinction.

Extinction phase 2 (sessions 7, 8 and 9)

No infusions were given during Extinction Phase 2. PBS-group subjects continued to display extinction learning with low levels of % CRs. The behaviour displayed by CNQX-group subjects during this phase revealed that extinction had been impaired during the inactivation. CNQX-subjects had high CR frequencies at the beginning of session 7, (figure 3.5) and then they displayed a characteristic extinction learning profile.

Phase 2 extinction learning curves for the CNQX group subjects were compared with Phase 1 extinction learning curves for the PBS-group subjects. If these were seen to be significantly different, then it could indicate that the suspension of extinction produced by cortical CNQX was incomplete. There was not a statistically significant difference (Mann-Whitney U-test, T=24009, n=150, n=150, p=0.056) therefore, the extinction learning curves for the CNQX group following inactivation are not statistically different from the extinction learning curves for the PBS-group. However these learning curves are tested to see if they differ, there is no finding that they are identical, therefore some extinction learning could have occurred in the CNQX group. Figures 3.3a and b show that there is a trend towards a faster extinction learning in the CNQX-group than that in the PBS-group following inactivation. There are a number of possible reasons for this effect. Contralateral extinction learning could have occurred to some extent and this may have contributed towards the production of CRs (Gruart and
Yeo 1995) or CNQX might not have sufficiently inactivated NM control regions and some extinction learning may have occurred in some subjects.

The main result is that infusions of CNQX in lobule HVI of the cerebellar cortex, during extinction training, significantly impaired extinction learning but it is difficult to estimate whether the impairment of extinction was complete.

**Figure 3.4**

![Graph showing frequency of CRs during sessions 1, 2 & 3 of acquisition training.](image)

Figure 3.4 Frequency of CRs during sessions 1, 2 & 3 of acquisition training. • CNQX-group (n=5) ○ PBS-group (n=5). Each point represents 10 trials (9 CS-US paired and 1 CS alone). SEMs excluded for clarity.
Figure 3.5a Frequency of CRs during Extinction Phase 1, sessions 4, 5 & 6. • CNQX-group (n=5) ○ PBS-group (n=5) Control subjects showed characteristic extinction learning. CR performance is blocked in experimental subjects. SEMs are excluded for clarity for both figure 3.5a and b.

The dashed red arrows on figures 3.5 a and b indicate the extinction data used for statistical comparison i.e PBS group sessions 4, 5 and 6 and CNQX-group, sessions 7, 8 and 9.

Figure 3.5b Frequency of CRs during Extinction. ● CNQX-group (n=5), session 7, 8 & 9 ○ PBS-group (n=5), session 4, 5 & 6. Control subjects continued to show characteristic extinction learning with low levels of CRs. After inactivation of the cortex in experimental subjects, high levels of CRs occur at the start of session 7 and normal extinction behaviour is seen in subsequent sessions.
Is different circuitry involved in extinction and acquisition/performance of the NM CR?

All subjects were given two daily sessions of reacquisition training to allow CRs to reach asymptotic performance levels following extinction training. A baseline was established and CNQX was re-infused in each subject and subsequent CR frequencies were assessed. In all subjects, performance of the CR following infusions was abolished for a minimum of one block of trials.

It has been proposed that the severity of CR performance impairments is a measure of the completeness of the inactivation of cortical eyeblink control areas (Attwell et al 1999). A strong correlation between performance block and inactivation effects on acquisition has been seen and is taken as evidence that the same cortical eyeblink microzones mediate acquisition as well as performance (Attwell et al 2001). The measure used for the performance block was the number of 10-trial blocks with 0% CR over a 50 trial session, where drug had been infused immediately before the session. The measure used for drug effects on acquisition was expressed as the number of trials needed, after inactivation has been lifted, to reach a criterion of >90% CR within a 10-trial block.

Figure 3.4 shows a correlation between inactivation effects on extinction and inactivation effects on performance of CRs. The inactivation effects are expressed as, (1) for extinction: % CRs during extinction, phase 2, session 7, 8 and 9 and (2) for performance: (100) -number of CRs during the first 100 trials immediately following drug infusion. These measures were chosen to demonstrate a correlation between subjects with a large impairment on extinction and a good block of performance.

Thus, there is no evidence to suggest that different microzones are involved in extinction and performance. More restricted inactivations might reveal such differences, but the present findings are consistent with the idea that the same cerebellar circuitry is engaged in acquisition and extinction of CRs.
Each point represents a subject. No correlation ($R^2=0.23$) was found between the performance impairment following CNQX inactivation and the severity of impairment on extinction following CNQX inactivation, (Pearson product moment correlation, no significant relationship between variables, $p>0.05$).

**Learned timing of CRs remained constant during acquisition and extinction training**

The onset-to-peak of the CR was used to analyse timing during acquisition and extinction learning. This measure was chosen because it gives the time taken for the NM to reach maximum closure, this is achieved simultaneously with the onset of the US.

Frequency histograms (bin width 50ms) were used to display the population distributions of CR onset-to-peak latencies during acquisition and extinction (figures 3.7a and b). There are no statistically significant differences between population distributions between the CNQX-group and PBS-group. For acquisition phase ($t$-test, $t=1.392$, $df=8$, $p=0.201$), for extinction phase ($t$-test, $t=0.398$, $df=8$, $p=0.701$).
Figure 3.7a and 3.6b Distribution of CRs latency-peak during a) acquisition and b) extinction training for CNQX-group and PBS-group. The CNQX-group is represented by the black bars and the PBS-group is represented by the white bars. Only CRs from unpaired trials during acquisition training were used. CRs from all trials during extinction training were used.
The coefficient of variation (CV) of CR timing remained constant during acquisition and extinction training

CV of CR timing, reported for EB conditioning with ISIs of 300 and 400 ms, taken from White et al (2000), (table 3.1) are statistically compared to CVs of CR timing calculated for CNQX-group and PBS-group during acquisition and extinction training (table 3.2).

There are no statistically significant differences between PBS-group acquisition and extinction or CNQX-group acquisition and extinction and the CVs reported at 300 or 400 ISI from White et al 2000. (Z-tests: Acquisition Phase; CNQX-group and CV-300 ISI, CV-400 ISI, z=-0.669, p=0.503: z=-0.385, p=0.7 respectively. PBS-group and CV-300 ISI, CV-400 ISI, z=-0.778, p=0.437: z=-0.77, p=0.442 respectively. Extinction Phase; CNQX-group and CV-300 ISI, CV-400 ISI, z=-0.012, p=0.9: z=-0.5, p=0.617 respectively. PBS-group and CV-300 ISI, CV-400 ISI, z=-0.917, p=0.319: z=-0.547, p=0.585 respectively.)

Table 3.1
CVs of CR timing (calculated from mean peak latencies/sd), during acquisition training at 300 and 400 ISIs, data taken from White et al 2000. See Materials and methods for more details on ISIs (inter stimulus intervals) (page 117).

<table>
<thead>
<tr>
<th>ISI ms</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>300</td>
<td>0.09</td>
</tr>
<tr>
<td>400</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Table 3.2
CVs (calculated from the mean of: onset-to-peak latencies/ sd), for CNQX-group and PBS-group during acquisition and extinction at 350 ms ISI.

<table>
<thead>
<tr>
<th>350 ISI ms</th>
<th>Acquisition</th>
<th>Extinction</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNQX-group</td>
<td>0.12</td>
<td>0.1</td>
</tr>
<tr>
<td>PBS-group</td>
<td>0.18</td>
<td>0.25</td>
</tr>
</tbody>
</table>
Tests confirm that there is no statistically significant difference between CVs of CR timing for CNQX-group and PBS-group during acquisition or extinction training. For CNQX-group and PBS-group acquisition phase (z-test, z=-0.620,p=0.53), for CNQX-group and PBS-group extinction phase (z-test, z=-0.208,p=0.835). More importantly there is no statically significant difference between CVs during acquisition and extinction phases (Mann-Whitney U test, T=5, n= 2, n= 2, p=1.0).

In summary, CVs of CR timing from the study here are comparable with those reported by White and colleagues (2000). In addition, the CV of CR timing in the acquisition and extinction phases are comparable with each other indicating that the CR timing remained constant throughout all phases.

3 Discussion

Cerebellar cortical function is critical for extinction learning

Infusions of CNQX into the cortex of the cerebellum during extinction training impaired extinction learning. The high CR frequencies observed after inactivation, and with further training, indicate that there has been a real extinction learning deficit during inactivation and no simple performance impairment as a result of repeated CNQX infusions.

CNQX is an AMPA/kainate receptor antagonist and infusions in the cortex block ionotropic/non-NMDA receptor-mediated transmission, presumably resulting in the reduction of pf-evoked simple spikes and cf-evoked complex spikes in Pcs. However, because CNQX does not effect inhibitory inputs to the Pc, spontaneous activity of the Pc may not be affected i.e Pc activity that is not a result of pf or cf input, so the cerebellar nuclei may still receive some inhibitory modulation from the cortex, although at reduced levels.

Impairment of extinction learning may be due directly to the local, intracortical disruption of normal processing in the cerebellar cortex. The action of CNQX on Pcs
may prevent CS information being transmitted to the cell and therefore no learning can occur. CNQX infusions into lobule HVI of the cerebellar cortex prevented acquisition learning (Attwell et al 2001), in this case CNQX may have blocked CS and US information being transmitted to the relevant Pcs during acquisition training. It may be that no learning, acquisition or extinction, occurs as information about the stimuli cannot reach the necessary Purkinje cells. However, in the present study, CNQX infusions in the cortex would also result in a decrease in Pc activity and consequent decrease in inhibition of the AIP. Therefore, the impairment in extinction learning may be due to a disruption of normal function in the AIP.

Previous studies have demonstrated that inactivation of the AIP with muscimol, during extinction training, prevented extinction learning (Hardiman et al 1996, Ramnani and Yeo 1996). It is not possible from these studies, and the study presented here, to dissociate whether just lobule HVI of the cerebellar cortex or just the AIP, is critical for extinction learning. The anatomy of the cerebellum explains these results; disruption at any level of the OCN loop has functional consequences throughout the loop. Inactivations of the AIP will affect normal function within cortical lobule HVI, and CNQX infusions in lobule HVI will affect normal function of the AIP, (although because CNQX does not ‘inactivate’ the cortex the function of the AIP may be less affected as when muscimol is used to ‘inactivate’ it, as in Ramnani et al (1996) and Hardiman and Yeo (1996)). However, normal processing is disrupted at all three levels of the OCN loop, lobule HVI, the AIP and the IO. Therefore, it may be that normal function of both lobule HVI and the AIP is necessary and both areas contribute to extinction learning.

**Regions of the cortex involved in extinction learning**

As discussed in the chapter 1, (page 15), there is controversy over the precise region of the cerebellar cortex involved in classical conditioning of the rabbit EB/NMR. Numerous studies provide empirical evidence that lobule HVI of the cerebellar cortex is critical for conditioning processes and because of these, and anatomical studies (see Yeo and Hesslow 1998, for review), this region of the cortex was targeted in the reversible inactivation study presented here.
Here, post-training, autoradiographical imaging of the spread of the drug in individual subjects shows that the drug spread is mainly within the medio-rostro-inferior extent of lobule HVI. It is important to note that the drug may have spread into regions that are not involved in conditioning processes. Furthermore, the amount of drug that reached critical regions of lobule HVI involved in extinction learning may have varied between subjects. However, in all cases in the present study, the results indicate that the critical regions for extinction learning were successfully inactivated.

The area critical for extinction learning is similar to the area of lobule HVI critical for performance and acquisition of CRs (Attwell et al 1999, Attwell et al 2001). In these studies, the medial/rostral extent of lobule HVI was shown to be critical for conditioning. In addition, experiments reported in chapter 2 of this thesis show that a C3 EB/NM microzone extends within the inferior part of lobule HVI throughout the caudal/rostral extent and from the medial to the lateral lobule of lobule HVI. Taken together, these studies indicate that the same, eyeblink control region of lobule HVI, is critical for acquisition, performance and extinction of conditioned responses.

In contrast to the results reported here and in other studies (Chapter 1, page 46, for review) previous lesion studies indicated that lobule V of the anterior lobe is critical for extinction learning and for controlling the timing of CRs (Perrett et al 1993, Perrett and Mauk 1995). In these studies (Perrett et al 1993, Perrett and Mauk 1995), all subjects had lesions in lobule HVI and in lobule V, so conclusions must be drawn cautiously. Other lesion studies report a complete loss of CRs with lesions of the lobule HVI and ansiform lobe but never in cases where the ansiform lobe was lesioned alone with lobule HVI spared (Yeo et al 1985b, Hardiman and Yeo 1992). Although lobule V was not specifically targeted in these studies (Yeo et al 1985b, Hardiman and Yeo 1992), they provide strong evidence for the involvement of lobule HVI in conditioning.

In order to gain further evidence that the same region is involved in extinction learning and performance and acquisition of the CRs, a correlation between the impairment of performance following reacquisition of CRs and the severity of the effect
of inactivation during extinction learning was determined. No significant correlation was found, so any inference that the same region of lobule HVI is involved in acquisition and extinction learning would be premature. However, the nature of extinction learning means that the number of CRs is small and available only for the first three post-inactivation extinction sessions. Additionally, due to the large performance impairment found in all cases, the data range is compressed - 4 of the 5 subjects have ≤ 20% CRs following an infusion of CNQX. Thus, the power of the correlation is weak and further evidence will be needed to verify whether the same cortical region regulates acquisition and extinction processes.

According to some models of learning (Mauk and Donegan 1997, Thompson and Raymond 2002), the mossy fibre collateral to the AIP drives CRs. CNQX infusions into the cortex would not prevent CS information being transmitted to the AIP and, if these models are correct, they may be capable of driving the production of a CR, even if the intrinsic activity of the Pc still has an inhibitory effect on the nuclei. However, no CRs were observed during inactivation of the cortex. These results presented here provide evidence against plasticity in the nuclei being responsible for the drive of CRs.

Mechanism of extinction learning

1) Inhibition of the climbing fibres is the signal for extinction learning

The mechanism of extinction learning proposed by Mauk (chapter 3, page 111), suggests that, in the absence of the US and in the presence of inhibition via the nucelolo-olivary pathway, the climbing fibre firing rate is driven below the baseline. It is suggested that the negative correlation between pf and cf activity in the cortex produces extinction learning (Medina et al 2002).

The study presented here also suggests a critical role for the cerebellar cortex in extinction learning. Infusions of CNQX into the cortex will result in an increase of nuclear activity and a consequent increase in inhibition upon the olivary cells resulting in a decrease of cf activity. Under these conditions extinction is impaired. Additionally, previous studies completely inactivated the cerebellar nuclei resulting in a decrease of inhibition on the olivary cells and a possible increase of cf activity and under these
conditions extinction learning is still prevented (Hardiman et al 1996, Ramnani and Yeo 1996).

It appears that regardless of the activity-state of the nuclei, and therefore the inhibitory input to olivary cells, and the consequent activity state of the cf, extinction is prevented or impaired in studies that disrupt the normal function either in the cortex or cerebellar nuclei. These results suggest again that normal function of the nucleus and the cortex is necessary for normal extinction learning. A mechanism for the extinction learning process cannot be elucidated due to the nature of the connections in the cerebellum forming the OCN loop, as disruption of one site will lead to disruption of all other sites.

2) Inhibition of the classically conditioned response during extinction training completely prevents extinction learning

Based on the results from the recent Krupa and Thompson (2003) study, it is claimed that extinction was prevented in the AIP inactivation studies (Hardiman et al (1996) and Ramnani and Yeo (1996)), because inactivation blocked the performance of the CR and therefore prevented extinction learning (Krupa and Thompson 2003).

However, the results from the Krupa and Thompson (2003) study must be interpreted cautiously. It is probable that, because of the placement of the cannulae in the brainstem at the level indicated, muscimol has spread to regions of the superior olive (SO), the major brainstem auditory relay nucleus. In fact, histological reconstructions confirm one cannula placement within the SO and the close proximity to the SO of other cannulae. The CS used during extinction training, is an auditory tone and so prevention of extinction learning may be due to disruption of normal function in the So, preventing the subject from hearing the tone-CS.

Thompson and colleagues also report an earlier study that used muscimol to inactivate the brainstem at the same levels reported in Krupa and Thompson (2003), and acquisition of the CR was not prevented (Krupa et al 1996), suggesting that the SO was not inactivated. However, in this earlier study, (Krupa et al 1996), it is possible that
muscimol did not spread to the SO, allowing normal acquisition of the CR, whilst regions of the SO may have been inactivated in the later Krupa and Thompson (2003) study.

Furthermore, if the results from the Krupa and Thompson (2003) study are correct, then extinction learning, in the study here, should have been completely prevented following a block of CR performance. In the study presented here, CNQX infusions in the cortex did completely block the performance of the CR during extinction, phase 1, sessions 4, 5 and 6 (see figure 3.5a). However, CR frequency during extinction, phase 2, session 7, 8 and 9 demonstrate that a small amount of extinction learning had occurred, therefore, extinction learning was not completely blocked but impaired (see figure 3.5b).

**Is extinction learning-related plasticity located in the cortex?**

The results from the study presented in this chapter indicate that plasticity necessary for extinction learning may be located in lobule HVI of the cerebellar cortex because CNQX infused in the cortex may result in a disruption of normal function of cortical synapses. Normal function of the AIP has also been shown to be necessary for extinction learning (Hardiman et al 1996, Ramnani and Yeo 1996), and so it is possible that extinction-related plasticity is located here.

*In vitro* studies have demonstrated a cortical post-synaptic LTP (Lev-Ram et al 2002), that could be responsible for extinction learning assuming acquisition occurs due to cortical post-synaptic LTD (see chapter 1, page 40). However, cerebellar nuclei plasticity has also been demonstrated (Aizenman and Linden 2000, Aizenman et al 1998) and could underlie acquisition and extinction learning within the AIP. Therefore candidate synaptic mechanisms have been characterised for both the cerebellar cortex and nuclei and it is possible that lobule HVI or the AIP are the sites for essential extinction-related plasticity or alternatively, plasticity could be distributed between them.
In summary, the cerebellar cortex and AIP are candidate sites for extinction learning-related plasticity. However because of the OCN loop, it is not possible to dissociate the function of these two regions during extinction conditioning, with reversible inactivation techniques alone and therefore it is not possible to identify where extinction-related plasticity is located.

Learned timing of CRs

The latency-to-peak of CRs was analysed using two distinct methods, a direct statistical comparison and comparison of the CV’s from the data presented here with those previously reported by White et al (2000). The latency-to-peak of CRs remained constant between groups and throughout acquisition and extinction training and they were not statistically significantly different from that reported by White et al (2000).

It has previously been suggested that pharmacological block of the cerebellar cortical output to the interpositus nucleus results in short-latency responses (SLR) (Garcia et al 1998). A study that replicated the parameters used by Garcia et al (1998) found that all CRs were completely abolished, in agreement with the findings presented here. A possible reason for the discrepancy found in these studies is that Garcia and colleagues may be reporting responses that are not strictly CRs. Characteristics of true CRs include the adaptive, learned timing and the ability to extinguish. SLRs have lost the learned timing component of the CR and are reported as being present following extinction due to latent plasticity in the nuclei (Medina et al 2000). It is possible that SLRs may be alpha responses or the result of pseudo-conditioning.

Summary

Normal function of the cerebellar cortex and, in particular, regions within lobule HVI, plays a critical role in extinction learning. But because disruption of normal function of the cortex has functional consequences for all levels within the OCN loop therefore, normal function of the AIP and DAO may also be critical for extinction learning (Hardiman and Yeo 1996, Ramnani and Yeo 1996, Medina et al 2001). It is not certain, at this stage, how plasticity for extinction learning is partitioned between the different levels. In order to resolve this question it will now be necessary to analyse
neural activity in the AIP, DAO and lobule HVI during extinction training in normal subjects and, additionally, in subjects with discrete inactivated sites.

There is no evidence, from this study, that the cerebellar cortex plays a distinct and separable role in the control of the learned timing of CRs. The latency-to-peak of CRs remained constant throughout all training and cortical inactivation.
Chapter 4

Cerebellar Cortical Function in Consolidation of a Motor Memory
What is consolidation?

In 1900 Müller and Pilzecker proposed that permanent memories are not produced instantaneously but that they are consolidated slowly over time. Consolidation processes are progressive and responsible for transforming newly acquired information from a fragile, temporary state into a stable and long-lasting state (Lechner et al 1999, for review).

Investigations of time-dependent consolidation processes support the idea that there are at least two stages of memory, short-term and long-term memory, although it is not known if they occur sequentially or in parallel. Müller and Pilzecker (1900) suggested that there is a time-window within which consolidation processes are vulnerable to disruption and when acquired information can be altered or lost.

Consolidation and motor learning

A human study has shown that consolidation processes may last several hours. Subjects were required to guide a cursor, displayed on a monitor, to a series of targets using the stick of a manipulandum hinged at two places. Throughout the task the subjects had to adapt to a series of velocity-dependent forces applied to the manipulandum.

The subjects were randomly assigned to five groups. Four groups learned a second task after the following time delays: immediately, 5 minutes, 1 hour or 4 hours and repeated the original task 24 hours later. The best retention of the original task was seen in the group that did not learn the second task. Subjects that learned the second task displayed better retention of the first task as the time period between learning the first and second task increased. Importantly, subjects that learned the second task following a 4 hour delay demonstrated very little disruption in retention of the original task. It was suggested that learning the second task disrupted the retention of the motor skill learned during the first task. This indicates that consolidation processes are
necessary for retention of the original task, and therefore, there are time-dependent processes vulnerable to disruption (Brashers-Krug et al 1996).

**Consolidation and the cerebellum**

Consolidation processes have been demonstrated in a fear-conditioning task in rats. A single conditioning trial is sufficient for rats to learn the association between the CS (acoustic or contextual stimuli) and US (painful electrical foot-shock), resulting in a CR. In this study the CR was freezing, defined as, 'the complete absence of somatic motility except respiratory movements' (Sacchetti et al 2002). Specific regions of the cerebellum were reversibly inactivated at several delays after acquisition training and there was subsequent retrieval testing. Reversible inactivation of the AIP and vermis disrupted post-training consolidation processes for up to 192 hours post-training. This study suggests a cerebellar role in consolidation processes (Sacchetti et al 2002).

As has been reviewed previously, inactivation of discrete regions at any level of the olivo-cortico-nuclear (OCN) loop prevents the acquisition of EB/NM CRs (Yeo and Hesslow 1998, for review) but inactivation of the superior peduncle -the output pathway of the cerebellum- (Krupa and Thompson 1995) does not. Taken together, these studies indicate the cerebellum or pre-cerebellar structures are the sites for formation and storage of these motor memories.

Possible sites of memory storage can be more directly analysed by targeting consolidation processes. Reversible inactivation of discrete cerebellar regions immediately after EB/NMR acquisition training can reveal if consolidation processes have been disrupted. If CRs are absent when the subjects are tested following post-training inactivations, then it can be assumed that the inactivated site was involved in consolidation and is a potential site for memory storage. Critically, this technique appears to overcome the problems caused by the OCN loop (Attwell et al 2002b), seen in previous performance and acquisition studies (Attwell et al 1999, Attwell et al 2001), and therefore allows functional dissociation between cortical lobule HVI and the AIP.
Therefore, pharmacological manipulations of discrete cerebellar regions after training has been used to dissociate the relative roles of the cerebellar cortex and AIP in consolidation (Attwell et al 2002b) and the same technique is employed in the study presented here to continue this investigation.

**Consolidation and EB/NMR conditioning**

Processes sensitive to disruption subsequent to acquisition training of the EB/NMR in the rabbit have been demonstrated. Amphetamine, scopolamine and chlorpromazine, systemically administered immediately after daily conditioning, retarded the rate of subsequent conditioning, whereas ketamine accelerated conditioning. If administration of the drugs was delayed by 2 hours there was no effect on subsequent conditioning (Scavio et al 1992). These results provide evidence for a 120 minute time window when consolidation processes are vulnerable to disruption. Furthermore, this 120 minute time window is in agreement with another study of the time course for consolidation processes involved in conditioning of the rabbit EB/NMR, that provides evidence for a time window of 90 to 135 min (Cooke et al 2004).

Currently, there is evidence for a cortical role in consolidation involved in conditioning of the rabbit EB/NMR (Attwell et al 2002b). Muscimol (a GABA$_A$ agonist) was infused immediately after four consecutive daily acquisition sessions to either the AIP or lobule HVI of the cerebellar cortex (Attwell et al 2002b), (figure 4.1a). In subjects that received muscimol infusions in the AIP, subsequent acquisition of CRs was not prevented, indicating that consolidation processes were not disrupted. In subjects that received infusions of muscimol to lobule HVI of the cortex subsequent acquisition of CRs was prevented, indicating that post-training consolidation processes had been disrupted or prevented.

A simple explanation for this result, Attwell et al (2002b), is that muscimol infusions in the cortex disrupt consolidation processes that occur within cortical lobule HVI. Although cortical muscimol would have changed Pc modulation of target neurons in the AIP, this changed modulation appears to be unimportant, as direct inactivation of the AIP with muscimol had no effect upon consolidation. It would seem that disruption of cortical and not nuclear consolidation processes prevent acquisition of CRs.
However, another possibility must be considered. Perhaps consolidation processes are nuclear and they are sensitive to the direction of excitability change. Cortical muscimol would produce an up-regulation of nuclear excitability whereas muscimol directly infused in the AIP would produce a down-regulation of excitability. Perhaps consolidation is impaired only by the up-regulation so that, in fact, the cortical inactivation has produced an effect upon nuclear consolidation processes.

To resolve this question, cortical and nuclear interventions that reverse these excitability increases would be helpful. This reversal can be achieved by infusing a GABA<sub>A</sub> receptor antagonist in the AIP and in lobule HVI of the cortex (figure 4.1b). A GABA<sub>A</sub> receptor antagonist infused into the cortex will result in an increase in Pc activity and a down-regulation of cerebellar nuclei activity. Further evidence to support a cortical role in consolidation processes will be provided if a GABA<sub>A</sub> antagonist in the cortex disrupts consolidation processes and consequently prevents acquisition.

A GABA<sub>A</sub> receptor antagonist infused into the AIP would complete the study. If GABAergic modulation from the cortex is decreased (as with muscimol in the cortex) and consolidation were not be disrupted and acquisition occurs normally then there would be strong evidence for cortical consolidation processes.

**Possible site and action of SR 95531**

All cerebellar cortical neurones, (Pcs, Golgi cells, granule cells, Lugaro cells and possibly stellate and basket cells), have GABA<sub>A</sub> receptors and therefore, the infusion of a GABA<sub>A</sub> receptor agonist or antagonist will have a significant effect on normal processing in the cortical network. The sites and effects of action of SR 95531, [2-(3'carbethoxy-2'proply)-3-amino-6-paramethoxy-phenyl-piridazinium bromide], a competitive post-synaptic GABA<sub>A</sub> receptor antagonist (Johnston 1996), are considered here.

GABA<sub>A</sub> receptors are defined as sensitive to antagonism by bicuculline and insensitive to baclofen (Hill and Bowery 1981). SR 95531, (gabazine) is a more
Figure 4.1a Muscimol in lobule HVI of the cerebellar cortex or in the AIP may disrupt post-training consolidation processes and therefore conditioning, as a result of disrupting GABAergic transmission at the site of infusion or the general excitability levels within the OCN loop. Blue and red arrows indicate the direction of excitability in lobule HVI, the AIP and IO depending if muscimol is in the cortex or AIP.

Figure 4.1b. SR 95531 (a GABA_A antagonist) in lobule HVI of the cerebellar cortex will result in excitability changes within the OCN loop, indicated by the purple arrows.

(AIP = anterior interpositus nucleus, Bc = basket cell, Cf = climbing fibre, Goc = Golgi cell, Grc = granule cell, DAO = dorsal accessory olive, IO = inferior olive, Mf = mossy fibre, Pc = Purkinje cell, Pf = parallel fibre, PN = pontine nuclei, RN = red nucleus, Sc = stellate cell).
potent and specific GABA<sub>A</sub> receptor antagonist than bicuculline, although SR 95531 and bicuculline are thought to bind to identical or at least overlapping sites on the GABA<sub>A</sub> receptor (Uchida et al 1996). SR 95531 binds with high affinity to both high and low affinity sites via allosteric modulation of the GABA<sub>A</sub> receptor (Ueno et al 1997) and binds with equal densities in the molecular and granule cell layer of the cerebellar cortex (Laurie et al 1992). At the concentrations used in the study presented here, SR 95531 would almost certainly antagonise GABA<sub>A</sub> receptors at both high and low affinity sites throughout the cerebellar cortex.

The effect of SR 95531 in the cortex will be to disinhibit the Pc by inhibiting the inhibitory input to the Pc from stellate, basket and Lugaro cells and disinhibiting the granule cells. The Pc will have an increased excitability and in turn have a greater inhibitory effect on the neurones of the deep cerebellar nucleus, (figure 4.1b).

The main effect of SR 95531 in the AIP would be to block inhibitory transmission from the Pcs to the nuclear cells. Inhibitory post-synaptic potentials in the cerebellar nuclei neurones can be blocked by bicuculline, providing evidence for GABAergic transmission via GABA<sub>A</sub> receptors between Pcs and nuclear neurones (Mouginot and Gähwiler 1995). GABA<sub>A</sub> receptors are exclusively responsible for transmission between the cortex and nuclei and therefore, infusion of GABA<sub>A</sub> receptor agonists or antagonists will have a major effect on the transmission of information from Pcs to cerebellar nuclei neurones.

**Experimental design**

Here, SR 95531, was used reversibly to temporarily block GABA transmission within lobule HVI of the cerebellar cortex, immediately following acquisition training. Subsequent acquisition rates were analysed.

Ideally the experimental design for this study would replicate Attwell et al (2002b), described previously, and use multiple infusions of SR 95331 in lobule HVI following each of four training sessions. However, pilot studies had shown that multiple infusions of low doses of the GABA<sub>A</sub> receptor antagonist SR 95531 have adverse
behavioural effects that may, in themselves disrupt consolidation processes. These adverse behavioural effects included, head and paw twitching, whole body circling movements and fast, erratic movements. The animals remained in an excitable state for up to 3 hours with repeated doses of 0.3mM and 1.5mM SR 95531 infused in lobule HVI of the cortex. In addition, these behavioural effects increased in severity (onset of behavioural effects was faster and duration was increased) with multiple doses. Consequently it was not possible to dissociate direct drug effects from general behavioural effects on consolidation processes. Therefore, the experimental design is similar to that of the Attwell et al (2002b) but it uses a single infusion of GABA\textsubscript{A} antagonist after the first of four acquisition training sessions in order to avoid the behavioural problems associated with multiple infusions.

4

Materials and Methods

Surgical procedures

15 male Dutch belted rabbits (1.8-2.5 kg) were used in this study. All surgical procedures are as before (see chapter 3: Materials and Methods, Surgical procedures).

Conditioning apparatus

All procedures are as before (see chapter 3: Materials and Methods, Conditioning Apparatus) except here, the CS was a 1kHz sine wave tone with an intensity of 85\textit{dB} and duration of 410ms. The US was periorbital electrical stimulation applied through the stainless steel clips, each US was a 60 ms train of three biphasic current pulses (2.0 mA).

Experimental design (see figure 4.2)

Conditioning training was performed at approximately the same time of day (± 30 minutes) for all subjects. Subjects were randomly assigned to one of three groups:
- A control group that received an infusion of sterile PBS after the first acquisition session (PBS-control group, n=4, A-D).
- A second control group that received an infusion of SR 95531 24 hours before the first acquisition training session (SR-control group, n=4, E-H).
- An experimental group that received an infusion of SR 95531 after the first acquisition session (SR-experimental group, n=4, I-L).

Figure 4.2

PBS-control group

SR-experimental group

SR-control group

Figure 4.2 Experimental design. H = habituation session, A = acquisition session. Solid black line indicates training sessions on consecutive days. Black arrow indicates time of PBS infusion (2μl, 2 minutes, sterile PBS, pH 7.4), purple arrow indicates time of SR 95531 infusion (2μl, 2 minutes 0.15mM SR 95531, pH 7.4).

Habituation phase

All subjects received 2 consecutive daily habituation sessions (figure 4.2). Each subject was placed in the Perspex restraining box for 35 minutes - a duration equivalent to one conditioning session. The NM transducer was attached to the suture and the US shock leads were clipped on to the periorbital clips. The background noise was present during the habituation sessions but there was no presentation of the CS or US.
Infusion procedure for SR-control group

Immediately following the second habituation session the SR-control group received an infusion of SR 95531 (Ki = 150nm, displacement of [H]GABA from rat membranes Tocris, Bristol, U.K.) (figure 4.2). The dummy cannula was removed and a 33 gauge infusion cannula was inserted through the guide to protrude approximately 1.5 mm below the guide cannula. SR 95531 (1.5mM, 2μl in phosphate buffered saline (PBS), pH 7.4, administered over 2 min) was infused. The infusion cannula was left for 15 minutes before removal and then replaced with the dummy cannula. The subject was left in the Perspex training box during the infusion procedure and for 15 mins following the end of the infusion.

 Acquisition phase

All subjects received 4 consecutive daily acquisition sessions (figure 4.2). Each consisted of 100 trials lasting for a total of 35 minutes. Every 10th trial was the CS alone and all other trials were paired CS-US presentations.

Infusion procedure for PBS-control group and SR-experimental group

Immediately following the first acquisition session, SR-experimental group subjects received an infusion of SR 95531. The dummy cannula was removed and a 33 gauge infusion cannula was inserted through the guide to protrude approximately 1.5 mm below the guide cannula. SR 95531 (1.5mM, 2μl in phosphate buffered saline (PBS), pH 7.4, administered over 2 min) was infused. The infusion cannula was left for 15 minutes before removal and then replaced with the dummy cannula.

The PBS-control group received an infusion of PBS (2μl phosphate buffered saline pH 7.4, administered over 2 min) immediately following the first acquisition session. As with the previous two groups the infusion cannula was left in place for 15 minutes before removal and then replaced with the dummy cannula. For both the SR and experimental control groups, 3 further training sessions were given over the following days.
Rest period
Subjects did not receive any training for 3 consecutive days after the 4 days of training.

Performance test
All 15 subjects underwent a performance test in which SR 95531 was infused during final training sessions, when performance was at asymptote. This was done in order to determine if the location of the drug was correct and the dose of drug infusion was sufficient to abolish performance of CRs. Subjects received 20 trials, (18 paired CS-US, 2 CS alone) to establish a baseline. Each subject received infusions of SR 95531 (identical to those given during the experimental phase, SR 95531, 0.15mM, 2µl in PBS, pH 7.4 administered over 2 minutes). Immediately following infusions, each subject received 7 acquisition sessions at the following time points: 5 minutes, 35 minutes, 1 hour, 2 hour, 4 hour, 6 hour and 24 hours. Each of the sessions consisted of 20 trials (18 CS-US, 2 CS alone), except for the 5 min time point session that consists of 50 trials, (45 CS-US and 5 CS alone). This allowed the occurrence and frequency of CRs to be recorded immediately following drug infusion and as the drug effects dissipated.

Behavioural analysis
CR frequency
The parameters for defining CRs are standard and as described before (see chapter 3: Materials and Methods, Behavioural analysis, CR Frequency, page 119). CR frequency was calculated for each block of 10 trials, including CS alone trials.

Perfusion and histology
After the final phase of the experiment all subjects were infused with [3H] SR 95531 (Tocris, Bristol, UK) in PBS (1.5mM, containing 1µCi/µl, 2µl administered over 2 min) at the same depth as for infusions during training sessions. All subjects were sacrificed with an overdose of pentobarbitone sodium (90mg/ kg, i.v.) 15 minutes after the end of the infusion time. This time point corresponds to the time when...
the drug has maximum effect on performance of CRs. Each subject was perfused transcardially with 0.9% saline (1 litre) followed by 4% formaldehyde solution (2 litres).

The brain was removed from the skull, and stored at −20°C. Frozen sections were cut at 25μm in the transverse plane and mounted on glass slides.

**Autoradiography**

For each subject, every 14th mounted section, including lobule HVI and the cerebellar nuclei were placed on a storage-phosphor screen (Molecular Dynamics, U.K) and sealed in a cassette. These were stored at room temperature for 5 days with no exposure to light, after this time period the phosphor screen was scanned using a phospho-imager. The scan revealed the spread of the radioactive drug for each section.

Autoradiography of the brain sections was imaged to allow the precise location and spread of the drug to be revealed. An image of the cresyl violet-stained section was produced to show brain edges and granule cell layer boundaries, this was then used to create a composite image containing the autoradiograph, the granule cell boundaries and borders for each section.

It should be noted that this technique differs from that described in Chapter 3, Autoradiography. This is because material necessary to perform quantitative autoradiography is no longer available. For these autoradiographs, phosphor-imaging is a new technique and here, has been used to measure the spread ONLY of the drug, it is not a quantitative measure. This images are presented as in Chapter 3, including the abbreviations.

**Statistical analysis for CR frequency**

CR frequencies were not distributed normally and could not be transformed to a normal distribution, so a non-parametric test was used to analyse the data. For each session, a Kruskall–Wallis one way ANOVA was used to compare group data and Dunn’s all pairwise comparison, post hoc test, was used to compare all groups.
4

Results

Principal findings

• SR 95531 infusions in the cerebellar cortex immediately after acquisition training impaired acquisition of CRs.

• SR 95331 infusions in the cerebellar cortex 24 hours before acquisition training impaired acquisition of CRs.

• SR 95531 infusions in the cerebellar cortex abolished or greatly reduced the performance of CRs.

Criteria for inclusion of subjects

Subjects were included in the study and analysis unless they failed to satisfy one or more of the following criteria:

1. CR frequency must be reduced by ≥85% CRs for at least one block of training post drug infusion, during performance testing (one block is 20 trials, 18 paired CS-US and 2 CS alone).

2. Histological examination must reveal no damage to the cerebellar cortex other than that immediately surrounding the guide cannula.

3. Autoradiographs must reveal that the drug is restricted to cortical areas and has not spread to the cerebellar nuclei, (figure 4.3). Subjects were rejected from the study if there was evidence that nuclear areas had been invaded by the drug.

4. Subjects displayed no adverse behaviour following drug infusion Adverse behavioural effects included, fast head twitching, whole body circling, excitable erratic movements.
Three subjects were rejected on criterion 1. All other subjects were included after histological examination showed no damage to the cortex except that caused by insertion of the guide cannula. Following the single infusion of SR 95531 no adverse behavioural affects were seen in any subjects.

**Location and spread of SR 95531**

Autoradiography revealed that, in all subjects, the spread of SR 95531 was restricted to the cortex and did not extend into the underlying cerebellar nuclei. In all subjects the majority of the drug spread was within lobule HVI of the cortex.

In all groups the drug spread extended from caudal to rostral HVI, although not to the most rostral regions of lobule HVI. The drug was mainly within the inferior part of lobule HVI except in two cases, C and E, where the drug has spread in both the inferior and superior regions. Within each group, drug spread was seen in the medial and lateral lobes of lobule HVI and into surrounding areas including lobule V and crus I.

**Pre-training and post-training infusions of SR 95531 in the cerebellar cortex impaired acquisition of CRs**

**Habituation phase, session 1 and 2**

All subjects received 2 days of habituation training followed by 4 daily sessions of acquisition training. Immediately following the second habituation session the SR-control group received an infusion of SR 95531.

**Acquisition, session 1**

No CRs were seen during the first acquisition training for any group, (figure 4.4). Following the first acquisition session the PBS-control group immediately received an infusion of saline and the SR-experimental group immediately received an infusion of SR 95531.
Acquisition, session 2

During session 2 of acquisition training the PBS-control group acquired CRs, reaching a group mean of between 38-48% of CRs during the last 40 trials. In comparison the SR-control and SR-experimental groups achieved a group mean of 1% and 16% CRs in the last 20 trials, respectively. The rate of acquisition of CRs for the PBS-control group is significantly different from the other 2 groups (SR-control group and SR-experimental group), (figure 4.4), (Kruskall–Wallis one-way ANOVA, H = 13.7, df = 2, p<0.001; Dunn’s post-hoc test, p<0.05).

Acquisition, sessions 3 and 4

During the first 20 trials of session 3 the PBS-control group immediately performed at an asymptotic level (asymptote is defined as a frequency of ≥85% CRs) and maintained this frequency of CRs throughout sessions 3 and 4, (figure 4.4), PBS-control group).

During session 3, the SR-control group and SR-experimental group acquired CRs. During the last 20 trials of session 3, the SR-experimental group reached a mean frequency of 87% CRs whereas the SR-control group reached a mean frequency of 30% CRs.

There is no statistically significant difference between the SR-experimental group and PBS or SR-control groups, but there is statistically significant difference between the PBS- and SR-control groups (Kruskall-Wallis one way ANOVA H = 25.9, df = 2, p< 0.001; Dunn’s post-hoc test, p<0.5). The SR-experimental group appears to have a slower rate of acquisition than that of the PBS-control group by approximately one session (100 trials). During session 4 of acquisition training the SR-experimental group maintained asymptotic performance of CRs throughout the session.
Figure 4.3 SR 95531 Autoradiographs. The left hand panel shows schematic images of the cerebellum (level -1.0 - 3.0, rostral to caudal respectively). The coloured circles show the cannula tip position for each subject and for both the schematic images and autoradiographs, the nuclei are highlighted in pink. This page shows autoradiographs for the PBS-control group (A-D).
Figure 4.3 continued. Autoradiographs for the SR 95531-control group (E-H).
Figure 4.3 SR 95531 Autoradiographs for SR-experimental group (I-L)
The SR-control group continued to acquire CRs throughout session 4. The group mean reached a maximum of 71% CRs in the last 20 trials but did not perform at asymptote. Individual subject data shows that 3 of the 4 SR-control subjects did perform at asymptote within the last 20 trials and the group mean is decreased because one subject did not develop more than 6% CRs during the four acquisition sessions. With an n=4, this group appears to acquire CRs at a rate that is approximately more than one session slower than that of the SR-experimental group. During session 4, statistically there is only a significant difference between the PBS-control and SR-control groups (Kruskall-Wallis one way ANOVA H = 24, df = 2, p, 0.001; Dunn’s post-hoc test, p< 0.5), although the trend demonstrates a difference between the SR-experimental and SR-control groups, (figure 4.4, on next page).
Figure 4.4 Acquisition over four daily training sessions for each group. The arrows indicate the time point at which groups received an infusion. The PBS-control group is represented by the clear symbols, the SR-experimental group is represented by the black symbols and the SR-control group is represented by the red symbols.

The SR-control group received an infusion of SR 95531, 24 hours before the first acquisition session.

The PBS-control group reach asymptote by the beginning of session 3, the SR-experimental group reach asymptote towards the end of session 3 and the SR-control group reached 72% at the end of session 4.
SR 95531 prevented performance of CRs

Following acquisition training, subject E in the SR-control group required a further acquisition training session to perform at asymptote, once all subjects were performing at asymptote a baseline frequency of CRs was established over 20 trials. SR 95531 was re-infused and subsequent CR frequencies were analysed for each subject.

Performance of CRs was reduced to ≤ 15% CRs for at least one block of trials immediately following infusion of SR 95531 in all subjects. The length of time the performance of CRs was abolished ranged between subjects from 0.5 to 12 hours, with all subjects performing at asymptote between 0.5 and 12 hours post-reinfusion, (figure 4.5). The SR-control group appear to have the most immediate severe effects, the CR’s are abolished faster in this group than the SR- experimental or PBS-control groups. However, the trend shows that for some subjects in SR-control group the recovery time is longer the in the other two groups (Table 4.1).

Summary

Subjects that received an infusion of the GABA_A antagonist, SR 95531, have a subsequent impairment of acquisition of CRs. The results show that a single infusion of the GABA_A receptor antagonist 24 hours before acquisition training, or immediately following the first acquisition training session, impaired the subsequent acquisition of NM CRs.

In comparison to control subjects, subjects that received SR 95531 immediately following the first acquisition training session appear to have delayed acquisition of CRs by approximately one session. Subjects that received an infusion of SR 95531 before acquisition training have a delay in acquisition of CRs by more than one session. So, an infusion of SR 95531 appears to have a greater effect on acquisition when infused before training than when infused following a period of no training.

SR 95531 (2:1, 1.5 mM) abolished established CRs when infused into lobule HVI of the cortex, consistent with earlier findings that lower doses of SR 95531 in the cortex (0.1mM, 2:1) and AIP (1mM, 1:1) also abolish performance of CRs (Attwell et al 2002a)
Figure 4.5 Effect of SR 95531 on the performance of conditioned responses. Each point represents the group mean frequency of CRs over 10 trials (9 paired CS and US, 1 CS alone). The black arrow indicates the time of the infusion. Clear circles represent the PBS-control group, black circles represent the SR-experimental group and red circles represent the SR-control group. In all groups SR 95531 reduces the performance of CRs to <3% for at least one block of 10 trials.
Table 4.1

<table>
<thead>
<tr>
<th>Time when % CRs &gt; 85</th>
<th>PBS-control</th>
<th>SR-experimental</th>
<th>SR-control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hour</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>4 hour</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.5 hours</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>6 hours</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2 hours</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>12 hours</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

* % based on 20 trials/time point

Table 4.1. For each group (PBS-control, SR-experimental and SR-control) individual data for 2 subjects is shown. The data set is from a subject where CRs were abolished but returned with the shortest time period and the second data set is from a subject where CR’s were abolished and returned within the longest time period. The SR-control group demonstrates the greatest range with all subject’s CR’s returning within 2-12 hours compared with 1-4 and 0.5-6 hours from the PBS-control and SR-experimental groups, respectively.
Discussion

SR 95531 in the cortex impaired acquisition

Subjects that received cortical SR 95531 immediately following the first acquisition training session showed impaired acquisition of CRs in comparison to the saline control group. Considered alone, this result would indicate a cortical role in the consolidation of a cerebellar motor memory. Critically however, the second control group that received SR 95531 24 hours before acquisition training also displayed impaired acquisition of CRs. In this group, the drug could not have affected post-training consolidation processes because acquisition training was not given before the infusion of the drug. So the effects of the SR 95531 must have been anterograde to affect acquisition processes on the subsequent training sessions.

These anterograde effects on acquisition during conditioning, 24 hours following an infusion, confound any demonstration of potential effects of SR 95531 on post-training consolidation processes. The results demonstrate that a single infusion of SR 95531 in the cortex affected the ability of the subjects to acquire CRs even after a rest period of 24 hours. In the subjects that received SR 95531 to disrupt post-training consolidation processes, consolidation, acquisition or a mixture of both consolidation and acquisition processes may have been disrupted.

Because of the confounding of acquisition and consolidation effects, it was not possible to determine the effect of a GABA\textsubscript{A} receptor antagonist on consolidation processes in the cortex. Thus, the hypothesis that there are memory consolidation processes within the AIP, and that, these can be disturbed by increases but not decreases in GABA-mediated inhibition, was not tested.

Did SR 95531 target consolidation processes?

It may be that the SR 95531 infusions here were located within an area of the cerebellar cortex not responsible for consolidation but still critical for acquisition.
However, the SR 95531 spread seen here corresponds closely with the spreads of muscimol infusions reported in an earlier consolidation study (Attwell et al 2002b) where the muscimol was restricted to the cortex and found mostly within lobule HVI, as for the SR 95531 infusions here.

The region investigated here and in an earlier study (Attwell et al 2002b) also corresponds with the region of lobule HVI responsible for performance and acquisition of CRs (Attwell et al 1999, 2001). It is likely then, that the area affected by SR 95531 in the present study could have affected acquisition as well as putative consolidation processes.

Re-infusion of SR 95531 abolished CRs in all subjects but interestingly the SR-control group had the most immediate severe performance effects (not shown in Table 4.1 or figure 4.5) and long lasting, 12 hours compared with 4 and 6 hours for the PBS-control and SR-experimental groups, respectively. The infusions in this group may have been more accurate in targeting the critical region of lobule HVI responsible for performance and therefore, more effective in impairing acquisition and putative consolidation, than in the PBS-control and SR-experimental groups.

**Drug activity and inhibitory neurones**

Once subjects performed at asymptote, SR 95531 was re-infused to assess the effect of the drug on the performance of CRs. SR 95531 abolished or greatly reduced the frequency of CRs in all subjects but the frequency of CRs returned to asymptotic levels 30 minutes to 6 hours later. This is an indication of how long the drug is active in the cortex. Once CR levels recover it is assumed that the drug has completely dissipated and that the action of the drug in regions of the cortex responsible for performance lasts between 30 minutes and 6 hours. But it is important to note that the period of time that the drug affects the performance of CRs is not necessarily the same for the period of time that the drug affects acquisition or putative consolidation processes. Performance may be the most robust aspect of cerebellar function in this behaviour- acquisition and consolidation are potentially more sensitive.
The time window of activity of SR 95531, indicated by the performance losses is 30 minutes to 6 hours. This period encompasses a time window for consolidation processes mediated by the cortex of approximately 90 minutes (Cooke et al. 2004). A previous muscimol inactivation study (Attwell et al. 2002b) provides evidence that a post-training infusion of muscimol does not affect performance and, by implication, acquisition and consolidation, twenty-four hours later. Clearly, this was not true for the SR 95531 infusions here.

Why did SR 95531 infusions before training impair acquisition? One possibility is that long-lasting changes to the circuitry occur with an infusion of SR 95531, to produce impaired acquisition seen in the SR-control group. It has previously been shown that repeated subacute doses of bicuculline can cause an up-regulation in GABA\(_A\) receptors (Ito et al. 1988). So it is possible that the single infusion may have also caused a change in GABA\(_A\) receptors, and this disturbance in receptor population could account for the long-term effects of the antagonist. The SR-control group appeared to have greater acquisition impairments than the SR-experimental group and the SR-control group required the most acquisition training to perform at asymptote. This may be explained if cortical SR 95531 alters receptor populations within the cortex or, consequently, within the AIP. An up-regulation of cortical GABA\(_A\) receptors following an infusion of the GABA\(_A\) receptor antagonist could result in a longer time course for acquisition of CRs.

A final possibility is that subjects that received SR 95531 after the first session of acquisition training may have undergone a limited amount of acquisition and consolidation immediately after the first trial of the session, and continued consolidating throughout the conditioning session, resulting in a small amount of established learning. Infusions of SR 95531 subsequent to training might not affect memories already consolidated. If this is true then these subjects may have retained a small amount of residual learning that may have contributed to the faster rate of CR acquisition seen in these SR-experimental subjects compared to the subjects that received SR 95531 before any acquisition training. This would be consistent with the earlier study (Attwell et al. 2002b) in which post-training, reversible inactivations of the cortex produced major, but
not complete, loss of learning. These findings suggest that some consolidation can occur either during the training session itself, or in regions outside the inactivated area.

Further studies

It is necessary to establish if the impairment of acquisition seen in the SR-experimental subjects is due to a disruption of acquisition or consolidation processes. This may be possible by using a single infusion of SR 95331 then resting the subjects for 24 hours and progressively longer periods of time before resuming acquisition training. During subsequent acquisition training the frequency of CRs can be analysed and compared to subjects that receive an infusion of saline. At the time point that there is no significant difference between groups it can be assumed that the long-term drug effects are no longer present. Putative consolidation processes can then be disrupted using SR 95531 immediately following acquisition training and delaying retraining until the drug effects have dissipated. This design should allow dissociation of the effects of the drug on consolidation and acquisition processes in the cortex.

Alternatively, the effects seen here may be due to the dose of SR 95531 used. The potency of SR 95531 must be taken in to account when considering these studies. It is well documented that SR 95531 is threefold more potent than bicuculline (also a post-synaptic GABA\_A receptor antagonist), (Uchida et al 1996). Lower doses of the same or a smaller volume of SR 95531 are sufficient to prevent the performance of CRs when exactly applied to EB/NM control regions of lobule HVI and the AIP (1.0mM in the AIP and 0.1mM in lobule HVI) (Attwell et al 2002a). But dose response curves would be needed to evaluate the concentration required to disrupt consolidation processes and not acquisition processes. Using the same experimental design as used here but with a lower dose could result in disruption of consolidation immediately following training but not of acquisition 24 hours following infusion.

Finally, the study could be repeated using a different pharmacological agent. To achieve an increase in nuclear excitability, CNQX could be infused in the cortex, (an AMPA/kainate receptor antagonist) in order to prevent glutamate transmission to PCs, consequently the activity of PCs is decreased, reducing the inhibition to the nucleus and therefore increasing nuclear excitability.
Where is the site for cerebellar consolidation processes?

The results of the study presented here do not finally resolve where is the site of consolidation processes in the cerebellum during conditioning of the rabbit EB/NMR. It will still be necessary to complete this study by reversing the direction of cortico-nuclear modulation whilst reversibly inactivating lobule HVI of the cortex and the AIP.

It is assumed that consolidation occurs at the same site as memory formation and can be considered as a continuation of acquisition. Because of this, it can also be assumed that the same mechanisms are involved in both processes. So pharmacological manipulations, post-training (consolidation) or during training (acquisition), impinge on identical synaptic or neuronal mechanisms, although possibly at different temporal phases.

One theory of consolidation suggests that,

‘activity-dependent synaptic plasticity is induced at appropriate synapses during memory formation, and is both necessary and sufficient for the information storage underlying the type of memory mediated by the brain area in which that plasticity is observed’ (Martin and Morris 2002)

As discussed previously, many types of synaptic plasticity have been identified in vitro within the cerebellar circuitry proposed to be involved in conditioning (chapter 1, page 35) and according to the theory above, consolidation and acquisition may involve plasticity at any of these synapses. One probable candidate site is the pf-Pc synapse. These synapses are the focus of several cerebellar learning theories, (e.g. Marr 1969, Albus 1971), and long-term depression (LTD) of these synapses could be responsible for both consolidation and acquisition of CRs. However, cortical plasticity at other synapses such as LTP and LTD at excitatory pf-stellate cell synapses (Rancillac and Crepel 2003), LTD at excitatory cf-Pc synapses (Hansel and Linden 2000) or LTP at inhibitory stellate cell-Pc synapses (Kano et al 1992) may also be involved.
An alternative site for memory consolidation and acquisition may be the AIP, as this region has also been suggested as the location for the primary association formed between the CS and US (chapter 1, page 63). Plasticity at inhibitory Pc-neurone synapses has been seen (Aizenman et al 1998); both LTP and LTD occur at these synapses and are therefore, candidate plasticities responsible for acquisition and consolidation.

There is anatomical evidence for non-synaptic plasticity that may be responsible for memory storage in the AIP. A study investigating the long-term encoding of the CS and US association in the AIP during rat EB conditioning reported that, following conditioning, there was an increase in the number of excitatory synapses per neurone; changes in size of synapses was not analysed. The authors suggest it is the mf-nuclear neurone synapses that increase in number, because the CS alone can elicit a CR and information about the CS is transmitted via mfs. Additionally, the production of a CR is associated with a decrease in activity of the IO and therefore CRs are associated with a reduced input from the IO and cfs (Kleim et al 2002, see chapter 1, page X). Kleim and colleagues find no evidence of a change in the number of inhibitory synapses in the AIP and suggest that conditioning does not affect this receptor population (Kleim et al 2002).

Evidence from the study by Attwell et al (2002b), supports a role for the cortex in mediating consolidation processes. It may be the case that consolidation processes occur in the cerebellar cortex and encoded memories are transported to the AIP for storage, this may explain the reports of synaptogenesis in the AIP following learning (Kleim et al 2002). However, there is no causal evidence presented that learning causes synaptogenesis in the AIP and an increase in excitatory synapses may occur for a number of different reasons, for example, due to inputs via the cf or mf collaterals transmitting CS and US information. Further studies are necessary to establish that synaptic change in the AIP following EB/NMR conditioning is related to learning.

**Summary**
A single pre or post-acquisition training infusion of SR 95531 impaired learning but effects of the drug on acquisition or consolidation processes cannot be dissociated,
so whether the memory is consolidated within HVI remains an open question. Furthermore, it remains to be seen how different inhibitory interneuron types contribute to these effects, as SR 95531 does not discriminate between them. More discrete, molecular tools are needed.

Future studies, based on the experimental design described here, but using tools such as protein synthesis inhibitors, that can dissociate consolidation and acquisition processes should be able to identify whether motor memories are stored in cortical lobule HVI, the AIP or distributed between them.
Chapter 5

Discussion
5 Discussion

The functional roles of the cerebellar cortex and deep cerebellar nuclei in conditioning of the rabbit EB/NMR are still not fully understood. Previous studies show that both sites are necessary for acquisition, extinction and performance of CRs (Hardiman and Yeo 1996, Attwell et al 1999, Attwell et al 2001). Based on numerous experimental studies, EB/NMR conditioning theories have assigned different responsibilities to the cortex and nuclei (see Chapter 1, page 64), but there is no conclusive evidence that supports such differences and the particular function of these sites during processes such as acquisition, performance and extinction remain unknown.

Three different experimental approaches were used in an attempt to understand the function of lobule HVI and because of this, the implications of the findings from each study are discussed in the relevant context (Chapter 2, page 71, Chapter 3, page 107 and Chapter 4, page 144). Here, a short summary of the principal results is given and how these contribute to our previous knowledge of cortical lobule HVI function in rabbit EB/NMR conditioning.

The study presented in chapter 3 shows that normal function of lobule HVI is necessary for extinction learning and it has previously been shown that normal function of the AIP is also important for extinction learning (Hardiman et al 1996, Ramnani and Yeo 1996). However, because of the problems associated with reversibly inactivating a region within the OCN loop (potentially disrupting normal function through out the entire circuitry, Introduction) it is has not been possible to identify whether only one of these sites is necessary. It may be that both the AIP and lobule HVI are needed for extinction.

Importantly, the results from these reversible inactivation studies show that the same circuitry is involved in the extinction and acquisition of CRs (Ramnani and Yeo 1996, Hardiman at el 1996, chapter 3). This finding implicates a common locus for the plasticity underlying acquisition and extinction learning. But again, because of the
functional properties of the OCN loop, a final identification of the site of extinction learning-related plasticity is not possible. However, the finding that acquisition and extinction rely upon common circuitry supports that suggestion that extinction may involve the reversal of the original plasticity for acquisition, albeit incompletely where there is evidence of spontaneous recovery and later savings effects.

These extinction studies used a temporary inactivation technique to manipulate cerebellar sites. This technique can be used in conjunction with others in order to understand the neural mechanisms involved in EB/NMR conditioning. One possibility would be to record from cortical Pcs or from AIP neurones, directly related to the behaviour during acquisition and extinction conditioning. Then, functions of the cerebellar cortex and AIP could be dissociated by monitoring neural activity in these cells while inactivating the complementary site during conditioning. The mapping of an eyeblink microzones in parasagittal zone C3 of lobule HVI, as described in Chapter 2, now make such an analysis feasible.

Empirical evidence (discussed previously, Chapter 2, Introduction) shows that the C3 zone of lobule HVI receives cf and mf inputs from the dorsal accessory olive (DAO) and the pontine nuclei respectively, and Pcs of lobule HVI project to the AIP. Both the DAO and AIP have been shown to be critical in EB/NMR conditioning (Yeo et al 19985a, Yeo et al 1986). Coupled with these findings is evidence that information about the CS is transmitted via the mf -pf -Pc pathway and information about the US is transmitted via the cf-Pc pathway. Together with identifying an EB/NMR microzone, these results support the idea that CS and US information are provided by mf and cf, respectively, and give general support to the idea that a learning algorithm similar to that proposed by Marr (1969) Albus (1971) and others underlies this and other types of motor learning.

The study in chapter 4 was an attempt to dissociate nuclear and cortical functions in consolidation processes. This strategy could have provided a reasonably direct indicator of the localisation of memory storage. However, due to the adverse action and possible long-term effects of SR 95531, it was not possible to dissociate the
effects of the drug on acquisition and consolidation but, importantly, the study provided further evidence that normal function of lobule HVI is critical for normal acquisition and performance of the CR.

**Is the AIP the site of essential learning-related plasticity?**

There is no dispute that the AIP is essential for conditioning. Lesion and reversible inactivation studies of the AIP demonstrate that normal function of this region is critical for acquisition of CRs (Lavond et al 1985, Yeo et al 1985a), extinction learning (Hardiman et al 1996, Ramnani and Yeo 1996) and performance of CRs (Attwell et al 2002a). Anatomically the AIP is also a suitable candidate for essential plasticity as collaterals from both mfs and cfs project here. Although the anatomy of the cerebellar nuclei is simple compared to the cerebellar cortex, learning in the AIP can not be ruled out because of its’ anatomical simplicity; conditioning can occur in the spinal cord and that also has a simple anatomy (Lavond 2002, for review).

It is acknowledged that the AIP is a candidate site for learning-related plasticity. Supported by experimental evidence that the cortex is not necessary for conditioning, some authors suggest that the AIP *is* the site for essential plasticity (see Lavond 2002, for review). Theories of AIP learning mechanisms responsible for EB/NMR conditioning are supported by findings from a number of research groups (chapter 1, page 64). The suggestion common to these theories is that the AIP is the site for a ‘basic’ association between the CS and US and so it contains a memory that “drives” the CR. These theories further suggest that the cerebellar cortex contains a memory that controls other aspects of the CR (such as amplitude and learned timing). In this dual-level scheme there is essential plasticity in the AIP and ancillary plasticity in the cortex.

This idea is in contrast to a theory that suggests essential plasticity in cortical lobule HVI (Yeo and Hesslow 1998) and which is supported by substantial experimental evidence. In a study that used picrotoxin and SR 95531 to block the Pc input to the AIP, CRs were completely abolished (Attwell et al 2002a) suggesting that the mf collateral to the AIP is not capable of driving a CR and that the ‘basic’ association between the CS and US is not formed in the AIP.
In vitro studies have demonstrated various plasticities in the cerebellar nuclei that could be involved in conditioning (Introduction, page 42). A recent study showed an increase in number of excitatory synapses in the AIP following conditioning, so this result could be seen as evidence for plasticity during motor learning (Kleim et al 2002). However, there are other interpretations. Firstly, there is no evidence of a causal relationship between this increase in synapse number and learning. Secondly, the authors do not find a change in number of inhibitory synapses in the AIP. This is unexpected as the Pc–nuclear neurone inputs are the sole inputs to the nuclei from the cerebellar cortex. However, there is no evidence that synapse numbers have to change to implicate a significant role for the Pc inputs in learning.

Changes in intrinsic excitability of nuclear neurones and LTP/ LTD of the Pc-nuclear neurones synapses are candidate plasticities for conditioning (Aizenman and Linden 2000, Aizenman et al 1998). A recent stimulus generalisation study analysed the expression of SLRs following functional cortical disconnection (Ohyama et al 2003). It was suggested that, if cell-wide excitability changes alone are sufficient to mediate the expression of SLRs, they should generalise to other stimuli capable of supporting the learning. However, when tested, SLRs displayed robust stimulus specificity and therefore, at least one type of input-specific plasticity mechanism (possibly in conjunction with cell-wide excitability) is necessary. However, other groups have not been able to demonstrate SLRs following the same cortical disconnection parameters (Attwell et al 2002a) and so their mechanism remains obscure. Furthermore it is questionable if SLRs are true CRs because they do not have adaptive timing.

Different types of nuclear plasticity may account for different aspects of EB/NMR conditioning. Input specific plasticity may be responsible for the stimulus specificity necessary for the production of CRs, (as demonstrated by in the study described above (Ohyama et al 2003) and cell-wide excitability changes may account for the phenomenon of savings during rapid reacquisition following extinction (Medina et al 2001). The latter suggestion is only feasible if theories that suggest the association between the CS and US during acquisition training occurs within the AIP are correct. If
the AIP is the site of essential plasticity then, alternatively, increases in cell excitability may contribute to input specific changes in synaptic strength. It has been suggested that this additional level of change could result in a more flexible system for information storage (Aizenman and Linden 2000).

Is cortical lobule HVI the site of essential learning-related plasticity?

Reversible inactivation studies, lesion studies and the study presented in chapter 2, provide persuasive evidence for the critical role of lobule HVI in conditioning (Chapter 1, page 47 and chapter 2). These findings contradict other studies that have demonstrated the cortex is not necessary for conditioning but is involved in regulating the adaptive timing of CRs. Theories that dismiss lobule HVI as critical for conditioning must be reviewed.

A number of recording studies support the theory that learning-related plasticity may be located in the cerebellar cortex. The C3 EB microzones have been mapped in the cat (Hesslow 1994a) and ferret (Ivarsson and Hesslow 1993). In the cat two EB microzones were located in lobule HVI and found to have a direct influence on CRs, stimulation of these EB regions completely suppresses CRs (Hesslow 1994b). Recordings from Pcs located in the ferret C3 EB microzone following conditioning show that there is a suppression of simple spikes in response to the CS, this would cause a disinhibition of nuclei cells and consequently activate the red nucleus, leading to a blink (Hesslow and Ivarsson 1994). In addition to this, a conditioning-dependent increase in the inhibition of olivary cells via the nucleo-olivary pathway has been demonstrated (Hesslow and Ivarsson 1996). Furthermore, recording studies have demonstrated learning related changes in cf generated complex spikes and pf generated simple spikes in the Pc (Gilbert and Thach 1977), (Chapter 1, Introduction, page 61).

It has been shown that lobule HVI either mediates, or is wholly responsible for, consolidation processes (Attwell et al 2002b). Although the results from this study do not exclude the possibility that the AIP may be also be a site for memory storage, the findings do support the theory that learning-related plasticity occurs within the cortex. Further studies are necessary to resolve this issue.
Multiple types of plasticity have been demonstrated in the cerebellar cortex, (see, Chapter 1, Cerebellar plasticity, page 34, for review) and most work has concentrated on cortical in vitro LTD of the pf-Pc synapses as the foremost candidate plasticity responsible for EB/NMR conditioning. Transgenic mice expressing the PKC inhibitory peptide within Pcs showed impaired pf-Pc cell LTD (and not cf-Pc LTD) and demonstrated disrupted EB conditioning (Koekkoek et al 2003). Furthermore, recent evidence from Yeo and colleagues has shown the involvement of the nitric oxide cascade of LTD in EB/NMR conditioning of the rabbit (Rogelj et al 2003). This is the first direct in vivo evidence that a mechanism associated with pf-Pc LTD is engaged during motor learning and the most powerful evidence to date that essential plasticity is located within lobule HVI of the cerebellar cortex.

Sites of learning related plasticity in other types of cerebellar dependent motor learning

Coincident with the development of the theory that dual-site learning-related plasticity is responsible for EB/NMR conditioning, a multiple-site theory was proposed for another type of cerebellar dependent learning, adaptation of the VOR (Miles and Lisberger 1981).

Vestibular signals (or information about the context) are transmitted via parallel fibres and ocular signals (or information about the error) are transmitted via climbing fibres to cortical Purkinje cells. Adaptation of the VOR occurs, for example, when subjects wear minimising or maximising lenses, these changes in conditions produce errors so that the gain of the VOR is required to adapt in all directions. A change in gain of the VOR depends upon the flocculus, which projects to, and can inhibit, regions of the vestibular nuclei to provide a control on the reflex (Ito 2002, for review).

A widely accepted hypothesis for VOR learning suggests roles for the flocculus and for flocculus target neurones (FTN) of the vestibular nuclei in the brainstem. There is experimental evidence for response changes during VOR adaptation in the flocculus
Pcs and FTNs, (Hirata and Highstein 2001). As with EB/NMR conditioning, transgenic studies have suggested the involvement of cortical pf-Pc LTD in adaptation of the VOR. LTD and adaptation of the reflex is impaired in transgenic mice expressing a PKC inhibitory peptide in the Pcs (De Zeeuw et al 1998). Although it has not yet been finally established, it is assumed that changes in synaptic strength of the vestibular nucleus may also underlie plasticity responsible for adaptation of the VOR. Various types of plasticity have been demonstrated in the medial vestibular nucleus neurones (VNs), some of which are FTNs, and can be considered analogous to the deep cerebellar nuclei neurones. Changes in VN neurons have been seen in both adaptation of the VOR and in vestibular compensation following the loss of peripheral vestibular input. Briefly, studies in the rat have provided evidence that following unilateral labyrinthectomy there is an increase in the intrinsic firing properties of VNs and a decrease in the efficacy of GABA receptors and both may potentially mediate VOR plasticity (Him and Dutia 2001). Critically, flocculectomy subsequent to compensation resulted in a decrease in the efficacy of GABA receptors but not increased intrinsic excitability of VNs seen following loss of peripheral function (Johnston et al 2002). Recently, in vitro studies have shown long-lasting excitability increases (or firing rate potentiation) within VNs due to inhibition and the authors suggest this type of plasticity could contribute to adaptation of the VOR (Nelson et al 2003).

Although the specific location for the essential plasticity responsible for adaptation or compensation of the VOR has not been identified, two regions the flocculus and FTNs of the vestibular nuclei are considered candidate sites for plasticity responsible for these types of learning (Lisberger 1998, for review).

Summary

The answers to some of the problems that run through this thesis remain unknown. What are the specific mechanisms responsible for EB/NMR conditioning? And what are the relative roles that lobule HVI and AIP play in this type of motor learning? The studies in this thesis contribute to what is understood about the function of lobule HVI in conditioning. The precise anatomical location of EB control Pcs has been identified (chapter 2) and the critical role that cortical lobule HVI plays in
conditioning has been repeatedly demonstrated (chapter 3 and 4), as has the necessity of normal function of lobule HVI for extinction learning (chapter 3).

Future work requires analysis of neural processes during EB/NMR motor learning, identification of the site or sites of the learned association between the CS and US and characterisation of the synaptic or non-synaptic plasticities responsible. The functions of lobule HVI, the AIP and the IO in conditioning can then be fully understood.
Appendix One
Appendix 1

The data set presented here shows the averaged sweeps for C2 recordings from subjects, D, E, F, G, H and I and the anatomical location within lobule HVI from where the recordings were taken. Including the image from figure 2.4 (page 12, Chapter 2), a total of 10 C2 recordings sites were mapped providing a boundary for the C3 region that demonstrated responses to ipsilateral periorbital stimulation. The latencies are shown using a black, dashed vertical line.
Appendix 1: Figure A1.1

Subject H (2)

Cf responses recorded at 4.0 mm
● -2.0 mm caudal to lambda

Subject G

Cf responses recorded at 4.0 mm
● -0.5 mm caudal to lambda
Subject F (1)

Cf responses recorded at 3.2 mm
- 1.5 mm caudal to lambda

Subject F (2)

Cf responses recorded at 3.9 mm
- 1.0 mm caudal to lambda

Ipsilateral Eye

Contralateral Eye

Ipsilateral Forelimb

Double Pulse
Ipsilateral Eye

Ipsilateral Eye

Contralateral Eye

Ipsilateral Forelimb

Double Pulse
Ipsilateral Eye
Subject F (3)

Cf responses recorded at 4.8 mm
-2.0 mm caudal to lambda

Subject E (1)

Cf responses recorded at 4.5 mm
-1.0 mm caudal to lambda
Subject E (2)

Cf responses recorded at 5.0 mm
✓ 1.5 mm caudal to lambda

- Ipsilateral Eye
- Contralateral Eye
- Ipsilateral Forelimb
- Double Pulse

Subject D

Cf responses recorded at 3.8 mm
✓ 1.0 mm caudal to lambda

- Ipsilateral Eye
- Contralateral Eye
- Ipsilateral Forelimb
- Double Pulse

Ipsilateral Eye
Contralateral Eye
Ipsilateral Forelimb
Double Pulse
Ipsilateral Eye
Subject I

Cf responses recorded at 1.8 mm
-2.0 mm caudal to lambda

Subject H (1)

Cf responses recorded at 2.9 mm
-2.0 mm caudal to lambda

Ipsilateral Eye

71x712

V r

0 mV

15 ms

0 mV

18 ms

0 mV

25 ms

0 mV

23 ms

Contralateral Eye

Ipsilateral Forelimb

Contralateral Forelimb

Ipsilateral periorbital region

Contralateral periorbital region

Ipsilateral Forelimb

Double Pulse Ipsilateral periorbital region
Appendix Two
Figure A 2.1

Figure A 2.1 An example of an unconditional response during a paired trial at the beginning of training captured using the programme Blink. The conditional stimulus (CS) has a duration of 350 ms and the unconditional stimulus (US) has a duration of 60 ms. The movement of the nictitating membrane begins during the US. The Y-axis (mm) shows the distance moved by the nictitating membrane from baseline and X-axis shows the time (msec). The horizontal line is the threshold for a response above baseline.
Figure A 2.2

Figure A 2.2. An example of a conditioned response (CR) during a paired trial.

Figure A 2.3

Figure A 2.3. An example of a CR during an unpaired, CS alone, trial.

Figure A 2.3 and example of an alpha response and Figure A 2.4 shows an example of a rejected pre-CS response.
References
Reference List


52. Flourens P (1824) Reserches experimentales sur les proprietes et les fonctions de

conditioning increases membrane bound protein kinase C in rabbit cerebellum.
Neuroreport 9: 2669-2673.

contributions to the timing and expression of conditioned eyelid responses.
Neuropharmacology 37: 471-480.

acquisition of conditioned eyelid responses. The Journal of neuroscience 19:
10940-10947.

56. Garwicz M (1997) Sagittal zonal organisation of climbing fibre input to the

topography of mossy fibres and climbing fibres projecting to cat cerbellar C3


61. Gould TJ, Steinmetz JE (1996) Changes in rabbit cerebellar cortical and
interpositus nucleus activity during acquisition, extinction and backward classical
eyelid conditioning. Neurobiology of Learning and Memory 65: 17-34.

individual Purkinje cells of the cerebellum in cats. J Physiol (Lond) 133: 520-547.

the Olivocerebellar Projection II Climbing fibre distribution in the intermediate and
hemispheric parts of the cat cerebellum. Journal of Comparative Neurology 183:
551-602.


65. Hansel C, Linden DJ (2000) Long-Term Depression of the Cerebellar Climbing


69. Hartell NA (1994) cGMP acts within cerebellar Purkinje cells to produce long-term depression via mechanisms involving PKC and PKG. Neuron 5: 913-916.


**Common Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIP</td>
<td>Anterior interpositus nucleus</td>
</tr>
<tr>
<td>Bc</td>
<td>Basket cell</td>
</tr>
<tr>
<td>Ca++</td>
<td>Calcium ions</td>
</tr>
<tr>
<td>CCC</td>
<td>Cerebellar cortical conditioning model</td>
</tr>
<tr>
<td>CF</td>
<td>Climbing fibres</td>
</tr>
<tr>
<td>CFRS</td>
<td>Climbing fibre responses</td>
</tr>
<tr>
<td>CR</td>
<td>Conditioning response</td>
</tr>
<tr>
<td>CS</td>
<td>Conditioning/ al stimulus</td>
</tr>
<tr>
<td>DAO</td>
<td>Dorsal accessory olive</td>
</tr>
<tr>
<td>DCN</td>
<td>Deep cerebellar nuclei</td>
</tr>
<tr>
<td>D</td>
<td>Dentate nucleus</td>
</tr>
<tr>
<td>EB/ NMR</td>
<td>Eye blink/ nictitating membrane response</td>
</tr>
<tr>
<td>EPSPs</td>
<td>Excitatory post-synaptic potentials</td>
</tr>
<tr>
<td>fMRI</td>
<td>Functional magnetic resonance imaging</td>
</tr>
<tr>
<td>F</td>
<td>Fastigial nucleus</td>
</tr>
<tr>
<td>Gl</td>
<td>Granular layer</td>
</tr>
<tr>
<td>Goc</td>
<td>Golgi cell</td>
</tr>
<tr>
<td>Grc</td>
<td>Granule cell</td>
</tr>
<tr>
<td>IP</td>
<td>Interpositus nucleus</td>
</tr>
<tr>
<td>IPSP</td>
<td>Inhibitory post-synaptic potential</td>
</tr>
<tr>
<td>IO</td>
<td>Inferior olive</td>
</tr>
<tr>
<td>ISI</td>
<td>Inter-stimulus interval</td>
</tr>
<tr>
<td>Lc</td>
<td>Lugaro cell</td>
</tr>
<tr>
<td>LTD</td>
<td>Long-term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>MAO</td>
<td>Medial accessory olive</td>
</tr>
<tr>
<td>MF/ mf</td>
<td>Mossy fibres</td>
</tr>
<tr>
<td>ML</td>
<td>Molecular layer</td>
</tr>
<tr>
<td>PC</td>
<td>Purkinje cell</td>
</tr>
<tr>
<td>PcL</td>
<td>Purkinje cell layer</td>
</tr>
<tr>
<td>PIP</td>
<td>Posterior interpositus nucleus</td>
</tr>
<tr>
<td>PO</td>
<td>Principal olive</td>
</tr>
<tr>
<td>RN</td>
<td>Red nucleus</td>
</tr>
<tr>
<td>Sc</td>
<td>Stellate cell</td>
</tr>
<tr>
<td>SLR</td>
<td>Short latency response</td>
</tr>
<tr>
<td>UR</td>
<td>Unconditioned response</td>
</tr>
<tr>
<td>US</td>
<td>Unconditioning/ al stimulus</td>
</tr>
<tr>
<td>V</td>
<td>Vestibular nucleus</td>
</tr>
<tr>
<td>VOR</td>
<td>Vestibular ocular reflex</td>
</tr>
<tr>
<td>WGA-HRP</td>
<td>Wheatgerm agglutinate- horse radish peroxidase</td>
</tr>
</tbody>
</table>