The Cerebellum and Motor Learning: Anatomical and Behavioural Studies of the Conditioned Eyeblink.

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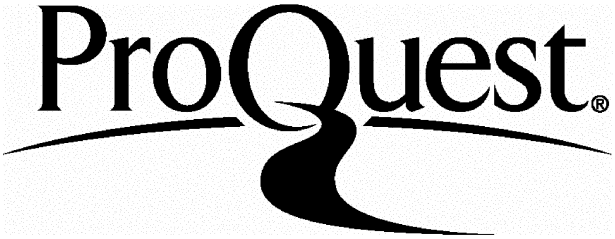

Abstract

The hypothesis that plasticity essential for motor learning is resident in the cerebellum is supported by studies showing that the integrity of parts of the olivo-cerebellar system (in particular, anterior interpositus nucleus, AIP) is critical for the acquisition and retention of conditioned nictitating membrane responses (CRs) - a simple form of motor learning. Some have argued that such lesions prevent performance, rather than learning. While permanent lesions cannot differentiate between these functions, reversible inactivations of AIP with muscimol (GABA-A agonist), reveal that AIP is essential for both. Experiments here evaluate the role of the AIP in extinction, the effects of AIP inactivation first on acquisition and then on extinction, and in post-conditioning consolidation processes.

Experiment 1: Rabbits that had previously acquired, were first given four sessions of extinction training during inactivation, and then a further four sessions of extinction training without inactivation. They showed no CRs during inactivation, and CRs were at unextinguished levels after inactivation. Responses then extinguished normally. Extinction was therefore prevented.

Experiment 2: Rabbits were given acquisition training first with inactivation of the AIP, and then without. They showed no CRs under muscimol, but acquired CRs gradually after inactivation. Muscimol therefore prevented acquisition. Subsequently, inactivations of the same site, AIP, in these animals also prevented extinction of CRs.

Experiment 3: Pharmacological interventions after conditioning often disrupt acquisition. If the AIP is associated with learning-related plasticity, will AIP inactivations prevent post-conditioning consolidation processes associated with such plasticity? AIP was inactivated immediately after conditioning and learning was unaffected. When muscimol was infused subsequently during training it effectively blocked performance of CRs. So the blockades were effective, but they did not prevent muscimol-dependent consolidation processes in AIP.

Inactivations of AIP with muscimol prevent extinction and acquisition, but not post-training consolidation processes. These findings are discussed in the context of the cerebellar learning hypothesis, and the anatomy and physiology of the cerebellum.

To my parents.

Acknowledgements

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Chapter 1
General Introduction

1. Neural Processes of Learning and Memory: Historical Analysis

The earliest records that document discussion on the nature of learning and memory were set in the context of the "nature vs. nurture" debate. It was Plato's belief that all knowledge is innately endowed, and that learning is a process of drawing this knowledge out (the "nativist" position). In other words, he viewed learning not as a process of acquisition, but as a process of *recollection.* In stark contrast to such ideas were those of Aristotle, whose model of memory was empiricist - he believed that learning depends on the flow of information into the mind. The "tabula rasa", an initially blank writing tablet, was considered as an analogy to the mind, on which experience was subsequently recorded. The origins of current empiricist ideas concerning the nature of learning and memory can be traced back at least to the time of Aristotle (*De Motu Animalium; De Memoria; 400 BC*) who also believed that the organ of human experience was the heart. Galen (200 AD) challenged this view, and proposed that it was the brain, and not the heart, that was the seat of mental function. It was not until much later that the neurophysiological basis of memory became an important consideration. The localisation of memory within structures of the brain has been the subject of vigorous debates, which are constituted from two, more general issues - the **localisation of brain function** and **brain processes of memory formation**.

1.1 Localisation of function

Among the earliest localisationists was Gall (1825), who asserted that the nervous system consists of localised components, each of which has an identifiable behavioural function. Experimentally, this idea was supported by the work of Broca, Wernicke and others, who showed that specific, localised lesions of the cerebral cortex resulted in highly specific effects on language, vision, and movement. The other extreme of the debate is perhaps best represented by Haller (1762) who supported the view that all parts of the brain were functionally equivalent. Here arose the concept of 'equipotentiality', or the equal contribution of brain areas to any particular function. Flourens (1822) was

credited with finding a middle way between these extreme views. From a series of experiments using ablation and stimulation techniques, Flourens (1822) came to the following conclusion:

> "In the final analysis, the cerebral hemispheres, the cerebellum, the quadrigeminal bodies, the medulla oblongata, the spinal cord, and the nerves, all of these essentially different parts of the nervous system have specific properties, appropriate functions, and clear effects; and in spite of this remarkable diversity of properties, functions and effects, they form nothing less than a single system".

> "There are...no diverse seats, neither for the various faculties, nor for the different perceptions. The faculty of perceiving, of judging, or of willing one thing, resides in the same locations as the faculty of perceiving, of judging, of willing another; and consequently this essentially unitary faculty resides in essentially a single organ."

In contrast to Haller, he asserted that each of the major subdivisions of the brain were functionally distinct. So, for example, the cerebral cortex was functionally distinct from the cerebellum. But in contrast to Gall's 'mosaic' concept of cerebral cortical function, Flourens admitted of no discrete locations of function within the cerebral cortex itself.

In relation to the cerebellum, a structure with which this thesis is concerned, Flourens concluded that one of its basic properties was to coordinate movements - a theme that persists even today. Such a conclusion was based on lesion studies which showed that "The slightest disturbance of the cerebellum alters the harmony of coordinated movements... the coordination of these movements, therefore, derives from the cerebellum".

He went on to partition the control of movement into constituent factors, and to suggest that each is localised to different brain structures:

> "I have shown that all movements persist after the ablation of the cerebellum; they lack only being regular and coordinated. From this I have been induced to conclude that the *production* and *coordination* of movements constitute two classes of essentially distinct phenomena, and that they reside in two classes of organs also essentially distinct; to wit: *coordination* in the cerebellum and *production* in the spinal chord and medulla oblongata"

As early as 1890, William James was concerned with interpreting subjective mental phenomena in terms of brain activity, and describing brain activity in terms of neurally mediated associations. In his seminal work "The Principles of Psychology" (1890), he discussed several issues concerning brain and behaviour that have formed the basis of modern thought in the fields of neuroscience and psychology. In his chapter entitled "The functions of the brain" he described the localisation of sensory and motor functions in great detail. He noted that "cortical irritations", or electrical stimulation applied to certain parts of the cerebral hemispheres, produced well defined movements of different parts of the body, and that these occurred contralaterally to the stimulated side. Furthermore, he noted that lesions of these areas affected the motion of the same body part, and to this extent, James' analysis of localisation and lateralisation of motor function was largely correct. James also commented on the localisation of visual function, and commented on Seguin's descriptions of visual neuroanatomy. Although rudimentary by today's standards, the description of pathways originating in the temporal and nasal hemispheres of each retina, that projected to various subcortical centres and on to the visual cortex, were also largely correct in relation to our current understanding of visual neuroanatomy. James noted that lesions to parts of this system induced quite different effects on visual function. Lesions to lower centres induced

devastating effects on the ability to see. However, subtler, higher order aspects of visual processing were affected by lesions to higher centres. For example, "mental blindness" is discussed as a "cortical disorder...[consisting] not so much in insensibility to optical impressions, as in an inability to understand them".

Thus, there was an awareness that sensory and motor systems were organised hierarchically, and that this was reflected anatomically. So, "lower centres" consisting of subcortical structures were involved in basic functions, and "higher centres" such as parts of the cerebral cortex, were involved in more complex functions. Localisation of sensory and motor functions in the brain were therefore early developments.

1.2 Brain processes and memory formation.

Another interesting and perplexing problem in behavioural neurobiology concerns the way in which neural systems acquire information about the environment (learning), and use it to the organism's advantage when the situation demands (memory). In "The making of memory", Rose (1995) states the problem quintessentially: "Something, somewhere, has to change...". A primary aim of learning and memory research in neurobiology has been to understand the nature of this change. Typically, the physical process of change is referred to as neural "plasticity", and the physical manifestation of memory is referred to as the "trace" or the "engram". References made to "the trace" or "the engram" should not be taken to imply a single physical system or location in the nervous system. The nature of neural change is likely to vary according to what is learned, and also according to what systems are engaged in learning. Therefore, in this thesis, the terms refer to the *collective* processes that may occur in any part of the nervous system, at any level, which are causal to learning and result in the formation of memory.

As with his ideas on the localisation of functions, James showed remarkable prescience in his views on how memories might be encoded in the brain. One school of thought to

which he made a significant contribution, asserted that memory is distributed in networks of neurons, such that experience is encoded in the strength of connections between neurons (deterministic or connectionistic school). In his chapter entitled "Association", James discussed "the law of neural habit" in deterministic terms - a thesis credited more to Hebb (1949) than to James (1890). Here follow three descriptions that are remarkably similar in their awareness of association:

> "When two elementary brain-processes have been active together in immediate succession, one of them, on reoccurring, tends to propagate its excitement into the other."
>
> (James, 1890/1983; pg534)

> "When an axon of cell A is near enough to excite a cell B and repeatedly or 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, The organisation of behavior, 1949).

> "When unit A and unit B are simultaneously excited, increase the strength of the unit between them"
>
> (McClelland and Rumelhart, 1986)

In a chapter entitled "Habit", James (1890) included a further discussion about the relationship between learned behaviour and the physiology of the brain.

> "Plasticity, then, in the wide sense of the word, means the possession of a structure weak enough to yield to an influence, but strong enough not to yield all at once. Each relatively stable phase of equilibrium in such a structure is marked by what we may call a new set of habits. Organic matter, especially

nervous tissue, seems endowed with a very extraordinary degree of plasticity of this sort".

(James, 1890/1983; pg110)

James (1890), Hebb (1949), and McClelland and Rummelhart (1986), to name but a few workers, proposed that experience-dependent modifications in the excitabilities of neurons may form the neural basis of memory formation. The idea, developed over a century ago, is still the dominant hypothesis for the neural basis of memory formation. The relationship between behavioural manifestations of the engram and 'Hebbian' neural plasticity may therefore be explored experimentally by testing for experience dependent changes in the excitabilities between neurones. Modern methods (e.g. those used for examining Long-Term Potentiation and Long-Term Depression) have been developed to explore synaptic function and to assess whether isolated networks of neurons can store information using the simple rules of association mentioned above.

Despite the dominance of this idea, no study has been able to demonstrate conclusively that experience-dependent changes in synaptic strength underlie behavioural manifestations of learning in intact preparations. Indeed, any analysis of learning at synaptic and sub-cellular levels in behaving animals is completely dependent on knowing the location of brain areas in which such changes occur. The localisation of memory traces in the brain is therefore a fundamental prerequisite to discovering the nature of such changes.

1.3 The localisation of traces and engrams.

Historically, a spectrum of views has developed in relation to the localisation of memory, which has two historical schools of thought at its extremes. One school argues that engrams are localised to particular brain structures. The other is the deterministic or connectionistic school (see above). An extreme form of this tradition would claim that the engram is not physically localised to individual parts of the brain, but is instead

widely distributed in networks of neurons. The most extreme form of the deterministic tradition was that of the Gestalt school, which even rejected the synapse as the unit of memory. Instead, it was proposed that the pattern of electrical activity over large cortical areas that resulted in perception and memory. Field theory, as this was known, suggested that perception was related to the patterns, or *fields* of current distributed across the cortex, and not to specific neural connections.

Lashley (1929), for example, postulated that Haller's views on equipotentiality might extend to memory, and suggested that the engram was probably distributed widely and equivalently throughout the cerebral cortex. Thus, the participation of a particular neuron or synapse in learning and memory was probablistic rather than specific. His position appeared to be vindicated at first, by an experiment that showed a correlation between the size of cortical lesions and deficits in the acquisition, retention and re-learning of a maze habit in rats. Neocortical lesions appeared to affect maze learning similarly independently of location. It emerged that an anti-localisationist interpretation of the results was simplistic and problematic because of design flaws in the study: the sizes of basic functional units in the cortex were smaller than Lashley had supposed. The size of the lesions may have correlated with the behavioural effects because the probability of encroachment upon areas of significance may have increased with lesions size. Indeed, subsequent work has demonstrated that maze learning is highly dependent upon the integrity of highly specific areas of the neocortex and hippocampus, some of them being very small in size (Eames and Oakley, 1985).

The tractability of the localisation problem, as far as memory is concerned, is partly determined by which aspects of the engram we choose to investigate. As has been discussed, the engram is not necessarily restricted to a single system, level or structure of the nervous system. Yet, it is probably not so widely distributed as to encompass the entire nervous system. There must be some regions of the nervous system which are, to varying degrees, important for learning and memory, and other parts which are not. In

addition, while parts of the nervous system may be important for learning and memory to take place, these may not be sites of plasticity. The experimental localisation of plasticity is easily confounded by these factors. Clearly, given this complexity, it would be unproductive to try to elucidate the nature of the engram in its entirety with the methods currently available to us.

Thus, it is important to focus on a specific aspect of the engram. If we assume that, distributed within the nervous system, there are locations which vary in degrees of importance for learning and memory, there must be a subset of locations which are essential, without which the organism cannot learn. Such locations are more easily found than ones with less importance, because preventing these areas from functioning, will, necessarily, prevent learning. Such results cannot be taken to mean that these regions are ones in which there is learning related plasticity, but one can assume that these are *candidate* structures in which there may be ***essential plasticity.*** It is therefore the goal of work presented in this thesis to experimentally explore and discuss the localisation of essential plasticity in classical conditioning.

1.3.1 Observational and interventionist approaches to the localisation of plasticity.

Two approaches have been adopted to localise sites of plasticity in the brain. One is observation of the effects of anatomical or physiological ***interventions*** on learning, and the other is the ***observation of learning-related changes*** in the physiology and biochemistry of neural tissue that correlates with learning.

1.3.1.1 Observational approaches:

As argued previously, memory traces must comprise a set of physical changes in the brain. Learning related plasticity must necessarily be manifested as a physical change in structures which are essential for learning. Several methods allow the observation of

learning related changes in the brain, each having is own merits. These include electrophysiological recordings of single or multiple neurones, autoradiographic and histochemical analysis of specific changes in gene expression or biochemistry, and functional imaging of brain activity. The potential for a structure to have learning related plasticity can be established by showing that changes that correlate with learning occur in a given structure. If such a correlation were found, this would lend considerable support to the idea that these changes form the basis of learning in this structure. While such evidence lends considerable weight to hypotheses predicting the localisation of functions such as plasticity to parts of the nervous system, it does not demonstrate a causal relationship between learning and physiological change. In the case of localisation of plasticity, it is necessary to establish that learning cannot occur without normal activity in such structures.

1.3.1.2 Interventionist approaches:

A traditional method of intervention is the use of permanent lesions of the brain. It is important to note that while lesions may profoundly impair or abolish the behavioural manifestation of the learned response, this finding does not necessarily indicate that there is plasticity in this structure in the intact state. In learning, sensory, plastic and motor processes may each be affected by lesions. If one or more of these processes are damaged by lesions, the behavioural manifestation may be the same in each case - the learned response may be prevented, or, learning may not occur. In instances where permanent lesions do not abolish learning, such an approach can reveal with certainty, areas in which are *not* essential for learning. Permanent lesions cannot reveal structures in which there *is* essential, learning related plasticity, but can reveal, with degrees of reservation, whether structures are candidates which *may* support essential plasticity.

It is clear that it is difficult, if not impossible, to establish a causal relationship between essential plasticity for learning, and specific neural circuitry in the intact animal. However, convergent evidence from studies using permanent lesions and reversible

inactivations during and after training, may strongly support or completely reject the argument for localisation of essential plasticity in a particular region of the brain. For this strategy to work effectively, it is important to use preparations in which indices of learning are easily measured and in which learning can easily be manipulated.

1.4. Motor Learning: Classical Conditioning can be used to study simple associative learning of motor skills.

Motor learning is an experience-dependent increase in the efficiency of motor performance, and the localisation of motor memory in the brain has been the subject of controversy and debate. The acquisition of simple, skilled reflexive movement is perhaps the simplest form of motor learning that can be studied in animals. Such behaviours are easily quantifiable, and can be measured objectively and unambiguously. A simple form of motor learning might, for example, involve temporally pairing two stimuli. One of these may always evoke a specific reflex, and the other would not. After a number of pairings, the latter would eventually come to evoke the reflex. In other words, a simple form of motor learning would occur. If James was instrumental in laying the foundations of modern psychology, Pavlov's (1927) work on conditioned reflexes was equally important to modern analyses of simple learned behaviour and its neural mechanisms. Pavlovs unique contribution to the science of observing behaviour was to provide a means of bringing stimuli and responses under stringent experimental control, so as to minimise the effects of variables which may confound the analysis of learned behaviour. In Pavlovian conditioning the learned response is specific and quantifiable. The animal is presented with a stimulus (unconditioned stimulus, US) that unconditionally elicits a defined response (the unconditioned response, UR). If the US is paired with another stimulus (conditioned stimulus, CS), then after a sufficient number of pairings, the CS will come to elicit the response (conditioned response, CR), even when presented alone. Such methods have been employed in the study of simple reflexes, their adaptive qualities and neural

mechanisms. The eyeblink reflex, for example, has certain specific advantages for the study of motor learning. The anatomy and physiology of afferent and efferent pathways of the trigeminally evoked reflex itself are extremely well characterised in a variety of species (see below). The behaviour is relatively easy to measure and interpret, and is extremely well characterised. This reflex has proved to be extremely consistent in studies of classical conditioning (see below). Properties of the conditioned eyeblink which make it suitable for the analysis of motor learning include the specificity of the response to the CS used in training, and the timing of the response in relation to CS onset (see below). Response timing and context specificity are important facets of motor control generally and motor learning specifically. These factors are easily controlled in classical conditioning.

1.5 Classically Conditioned Eyeblink and Nictitating Membrane Reflexes: A model used to investigate the neural mechanisms of motor learning.

In nictitating membrane reflex (NMR)/eyeblink conditioning, stimulation of periocular areas and cornea is the US and these evoke the UR - closure of the eyelids. Circuitry essential for production of the CR and UR overlap, because the motoneurones and muscle groups are the same. However, there are pathways which are essential for production of the CR but not for the UR (see below). It would be instructive to review the anatomy and physiology of both the CR (later section) and the UR (this section), to develop an understanding of how these systems operate.

In some species, in addition to the external eyelids, there is also a cartilagenous membrane to protect the eye from aversive stimuli - the third eyelid, or nictitating membrane (NM) is located beneath the external eyelids. Periocular and corneal stimuli which cause the external eyelids to close also result in a sweep of the NM horizontally across the eye thus protecting it from potentially harmful stimulation.

The closure of the external eyelids is mediated by the orbicularis oculi muscle group, and the sweep of the NM is mediated by the contraction of the retractor bulbi muscle, which retracts the eye into the orbit. This action forces fatty tissue located intraorbitally to move towards the temporal canthus. The mechanical action of this displaces the nictitating membrane, causing it to sweep across the eye. In addition, the levator palpebrae muscle group, which raises the upper external eyelid, is innervated by motoneurones from the oculomotor nucleus. This muscle is tonically active, but is inhibited when the orbicularis oculi muscle is activated. So, while the motor nuclei of these muscle reflexes are different, they operate co-operatively. It is likely that these motor nuclei receive common inputs from common premotor blink interneurons, which may serve to co-ordinate the blink reflex (see below).

1.5.1 Circuitry of the external eyelid blink and nictitating membrane reflex.

How do cutaneous and corneal receptors project to the motoneurones of muscle groups involved in the UR? The trigeminally evoked external eyelid blink is composed of two components. There is a short latency response, R1, followed by a longer latency response, R2. The R1, by virtue of its short latency (6-8ms), is thought to be disynaptic. It must therefore be the case that some trigeminal neurones project directly to motoneurones involved in the eyeblink. Therefore, the trigeminal areas to which receptors project must in turn project directly to orbicularis oculi motoneurones in the facial nucleus. While eyelid and NM conditioning have been investigated in many species, the issue of the neural basis of motor learning has been explored most extensively in the rabbit. The trigeminal system has been subdivided into two components. These are the nucleus principalis (Vp) and the nucleus spinalis, which itself may be divided into three subcomponents. The most rostral is pars oralis (Vo), pars interpolaris (Vi) is more caudal, and the most caudal is pars caudalis (Vc). Van Ham and Yeo (1996a) have studied projections of corneal, periocular and conjunctival afferents to these areas of the trigeminal system, and projections of the trigeminal system to the

eyeblink/NM motoneurones in rabbit (Van Ham and Yeo, 1996b). It is reported that periocular and conjunctival afferents project mainly to Vc and the dorsal horn of spinal segment C1 (dhC1). There were also weak projections from periorbital receptors to Vo and Vp, but no conjunctival afferents were seen in these areas. Corneal afferents distribute to the ventral parts of pars interpolaris (Vi), to caudal Vc and to dorsal horn of spinal segment C1. This finding is also consistent with studies of corneal afferents in rat and cat (Marfurt and Del Toro, 1987; Panneton and Burton, 1981; Shigenaga *et al.* 1986). Van Ham and Yeo (1996a) also found weak projections to Vp and Vo.

There must be a direct projection from areas of the trigeminal system which receive corneal and periocular afferents to motoneurones of the orbicularis oculi muscle in the facial nucleus. There must also be projections to retractor bulbi motoneurones in the accessory abducens nucleus. Van Ham and Yeo (1996b) found projections from principalis (Vp) and adjacent areas of pars oralis (Vo) of the spinal trigeminal nucleus to both the orbicularis oculi facial subnucleus and to the accessory abducens nucleus and contralateral levator palpebrae motoneurones in the oculomotor nucleus (see figure 1.1).

In agreement with findings in the rabbit, Holstege *et al.* (1986) have examined trigeminal afferents to accessory abducens in cat, and have found two main projections to the accessory abducens nucleus, which arise from the Vp and rostral spinal trigeminal. These authors also report that a subset of neurons from these areas also project to facial intermediate subnucleus, from which motoneurones innervate the orbicularis oculi muscles.

The R2 response is polysynaptic. Holstege *et al* (1986) have identified two pathways that mediate the R2 response in the cat. The first passes from the spinal trigeminal nucleus, to the intermediate and deep layers of the superior colliculus. From here, neurons project to the parapontine reticular formation, and on to a premotor blink area in the

medulla that projects to retractor bulbi motoneurones in the accessory abducens nuclei. The second pathway projects from spinal trigeminal nucleus to red nucleus, and from here to a pontine pre-motor blink area. These neurons project to retractor bulbi motoneurones. Both of these pathways also project to motoneurons involved in the external eyelid blink, in the facial nucleus. However, it is interesting to note that lesions of the superior colliculus (Wells 1987) and the red nucleus (Rosenfield and Moore, 1983) do not impair the amplitudes of the blink reflex in rabbit. It is unlikely that these pathways serve a major function in this reflex. Harvey *et al* (1984) claim to have identified a pathway that might serve as an R2 pathway in rabbit, that projects from spinal trigeminal nucleus to reticular areas. These project directly to accessory abducens nucleus. Such areas may correspond with the medullary and pontine pre-motor blink areas reported by Holsteige *et al* (1986) in cat. Van Ham and Yeo (1996b) have proposed a model that may account for the R2 response in terms of the interconnections within the trigeminal nuclei. Since Vp and Vo receive inputs from Vc, and these areas receive cutaneous input from periocular and corneal areas, the R2 response may be mediated, at least in part, by Vc projections to Vo and Vp, and projections from these areas to the motoneurones.

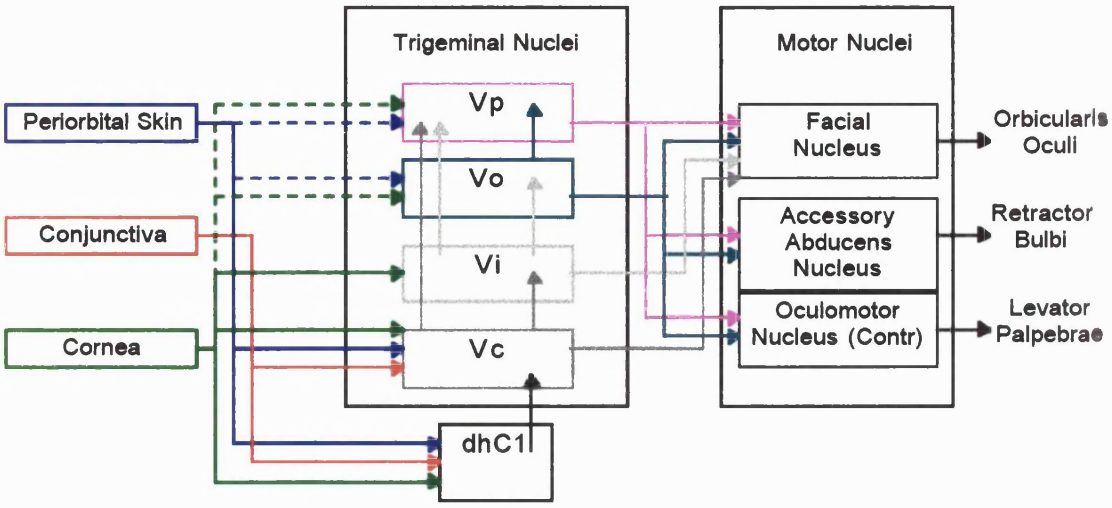


Figure 1.1: Simplified schematic diagram of projections involved in the trigeminally evoked blink reflex in the rabbit (results of Van Ham and Yeo, 1996a,b). Projections to the oculomotor nucleus from the trigeminal system are thought to be mainly contralateral. Projections from the trigeminal nuclei to the motor nuclei via reticular areas are not shown. Solid lines, strong projections. Broken lines, weak projections.

1.6 The Classically Conditioned Eyeblink and NM Response.

Gormezano and his colleagues (1962) have pioneered the eyeblink as a method with which to examine classical conditioning. Using classical conditioning, it is possible to explore the neural mechanisms which underlie the adaptation of the trigeminally evoked eyeblink and NM reflex. Periocular or corneal stimuli (US) unconditionally produce the eyeblink/NM reflex. If they are preceded by other, behaviourally neutral stimuli that do not produce the eyeblink reflex, such as auditory or visual stimuli, after a sufficient number pairings these conditional stimuli (CS) come to elicit a well timed response in anticipation of the US, that will peak at a time that is coincident to the US onset. This is a conditioned response (CR) - a simple, learned motor response, which can be regarded as a simple form of motor learning (see figure 1.2). For this response to come about, experience dependent changes must occur in the nervous system. While the nature of such changes is not known, a plausible hypothesis is that Hebbian mechanisms may be involved. Perhaps these take the form of altered neuronal excitabilities to the CS, within circuitry involved in NMR conditioning.

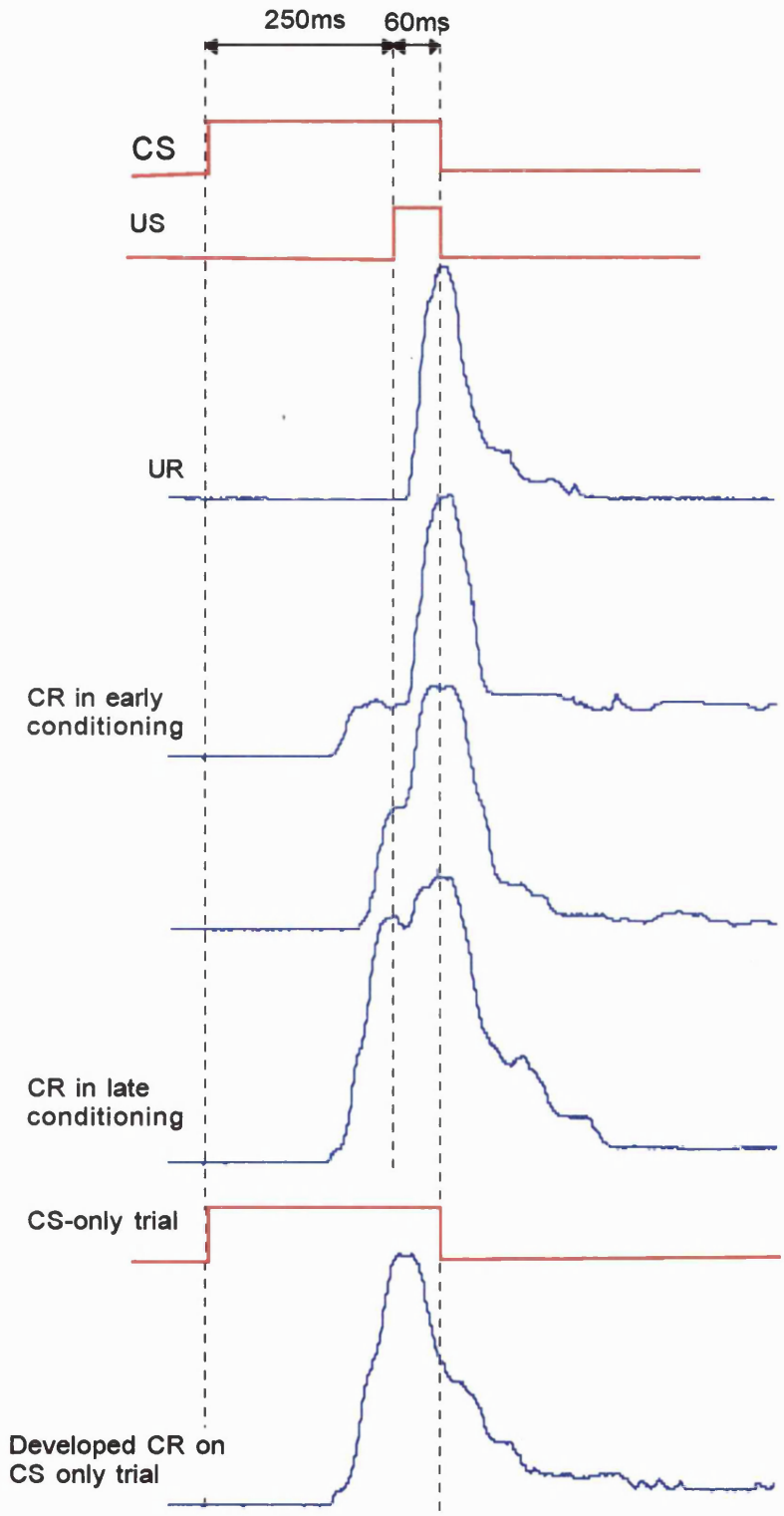


Figure 1.2: Stimuli (in red) and NMR responses (in blue) involved in NMR conditioning. CRs are well timed, and develop during paired trials. After a sufficient number of pairings, a CS presented alone elicits a CR. Closure of the NM is represented by upward displacements of the curves. Studies in this thesis have used parameters shown in this figure.

Although the external eyelid and NM reflexes are tightly coupled and occur simultaneously in response to trigeminally mediated stimulation, their neural pathways are not identical (McCormick and Thompson, 1982a). There are voluntary and reflexive movements of the external eyelid, but the NM response is purely reflexive and shows a very low rate of spontaneous activity. The low background blink rate produced by the NM response makes this the preferred reflex for classical conditioning.

There are principally two types of classical conditioning, which differ in the way that the CS and US are paired. In both types, the CS onset precedes the US onset. However, in 'trace' conditioning, the CS offset occurs before the US onset. In 'delay' conditioning, the CS overlaps the US (see figure 1.2). A review of literature concerning trace conditioning is beyond the scope of this thesis, which is concerned with delay conditioning of the nictitating membrane response, except to say that the essential neural circuitries in delay and trace conditioning are not identical. References made to NMR conditioning in this thesis will refer to delay conditioning.

1.7 Brain pathways essential for conditioned NMRs.

The problems associated with localising essential plasticity in the intact animal are due, at least in part, to the lack of methods which enable us to restrict the effects of interventions to the specific circuitry being examined. The spread of effects which are intended to remain restricted to one location forces us to extend assumptions about interventions more widely, to pathways related to the target structure. The localisation of function is made difficult by the dependency of associated pathways upon the target structure. In order to minimise the range of locations that effects of interventions can spread to, behavioural models have been 'reduced' - that is, parts of their nervous system have been systematically removed until only the basic circuitry for learning remains functional and intact.

Circuitry which is necessary and sufficient for conditioning can be determined by removing large parts of the brain, and observing the animal's ability to learn or express CRs. If reduced systems are able to learn, then it follows that the plasticity required for learning is within the remaining circuitry. Decortication, decerebration and cerebellectomy have been used to assess whether or not remaining structures retain the ability to support conditioning. Learning in the rabbit conditioned NMR preparation has proved to be remarkably resistant to removal of large brain areas. This section will describe these early attempts to identify which brain areas are essential for NMR conditioning

Oakley and Russell (1977) demonstrated that NMR conditioning could proceed normally in decorticate rabbits. Further work demonstrated that NMR conditioning could proceed in rabbits decerebrated above the level of the red nucleus and superior colliculus (Mauk and Thompson, 1987). Such findings confirmed that the necessary and sufficient circuitry for conditioning may be contained within the tectum, brainstem and cerebellum. Indeed, the use of decerebrate ferrets in eyeblink conditioning is a standard procedure in some laboratories (Hesslow, 1994a,b).

Would conditioning be supported if further brain areas were removed? Removal of the cerebellum would essentially leave only the brainstem and tectum. Given that the cerebellum may be a site of essential plasticity, could decerebrate rabbits in which conditioning can be established, retain CRs even when the cerebellum is removed? Kelly *et al.* (1990) addressed this issue directly, by attempting to classically condition decerebrate animals (n=3), and observing the effects on continued conditioning after cerebellectomy. In two other animals, decerebration and cerebellectomy were performed *before* training, to assess the ability of such preparations to acquire from the naive state. Eyeball retraction (which causes movement of the NM) and EMG responses of the orbicularis oculi muscles were measured during conditioning. Results showed that all animals which achieved successful conditioning after decerebration (n=5) showed

retention or acquisition of CRs in the cerebellectomised state. The authors report data from some animals in which repeated acquisition and extinction could be achieved, although CR frequencies were quite variable throughout conditioning. The authors argued that if such preparations can achieve acquisition and extinction, then the cerebellum is not necessary for conditioning to occur. They further suggested that lesions to some parts of the cerebellum in the intact animal abolished conditioning because the cerebellum provides tonic support for brainstem circuitry essential for NMR conditioning, and not because the cerebellum is a site of plasticity (Kelly *et al*, 1990). However, the training procedures used in Kelly *et al* (1990) study were very different to those used in conditioning of intact preparations, and Yeo (1991) and Thompson and Krupa (1994) argue that responses to the CS may not have the associative properties of true CRs. Using standard conditioning procedures, Yeo (1991) conducted a study in which decerebrate animals acquired CRs, but after cerebellectomy, they generally lost the ability to express or re-acquire CRs. However, some CRs *did* develop in some decerebrate-cerebellectomised animals, although these were small in amplitude. The findings in both studies were that CRs could be established in decerebrate animals, and that cerebellectomy severely disrupted CRs - more so in Yeo (1991) than in Kelly *et al* (1990). The difference between the results of Kelly *et al.* (1990) and Yeo (1991) only appears to be one of degree. These studies appear to provide evidence for an important role for the cerebellum in supporting conditioning, but suggest that the cerebellum may not be essential for conditioning in decerebrate animals. Such studies support the idea that extracerebellar circuitry in reduced preparations has the potential for plasticity in NMR conditioning.

The comparisons between greatly reduced preparations and intact preparations must be conducted with some caution. While reductionist methods have been relatively successful in localisation of essential circuitry, it must be remembered that processes in simple, reduced preparations may not generalise easily to processes in intact preparations of the same model. Studies reported above show that decerebrate-

cerebellectomised preparations are capable of some form of weak associative learning of conditioned NMRs. But, such results do not necessarily mean that the same processes occur in the anatomically intact preparation. Work subsequent to these studies in standard preparations, using standard conditioning parameters (reported later), has shown that conditioning is permanently abolished with small cerebellar lesions (Gruart and Yeo, 1995) and argue strongly in favour of essential cerebellar involvement in conditioning.

A growing body of literature has explored the notion that the there may be learning-related plasticity that is essential for NMR conditioning within this circuitry. Since the cerebellum has been a prominent structure in the analysis of learning related plasticity for NMR conditioning, its anatomy and physiology are important considerations.

1.8 Modularity in Olivo-Cerebellar Structure and Function: Implications for the conditioned eyeblink.

The mammalian cerebellum consists of a cortical surface, and beneath it, white matter in which the cerebellar nuclei are embedded. As in all mammalian non-primates, in the rabbit, the cerebellar nuclei consist of the fastigial nucleus most medially, the interpositus nucleus which is more lateral, and more lateral still is the dentate nucleus.

The anatomical organisation of the cerebellar cortex follows a highly ordered and stereotypical cytoarchitectonic pattern. The cerebellar cortex is laminated into three layers. The middle layer consists of the cell bodies of Purkinje cells (the Purkinje cell layer), which extend their dendrites into the upper, molecular layer above. Their axons extend through the granular layer below, and through the white matter until they synapse with the cells of the cerebellar nuclei. The primary neurotransmitter of Purkinje cells is gamma-amino-butyric acid (GABA), that acts on GABA receptors on the surfaces of cells in the cerebellar nuclei (Obata, 1976). The dendritic trees of Purkinje cells

spread out into a fan-shaped plane in the molecular layer, perpendicular to the transverse fissures and parallel to each other. These cells are considered to be the main computational units of the cerebellum, and their axons form the sole output of the cerebellar cortex. There are two important afferent systems to the cerebellum. Mossy fibres, which originate mostly from the pontine nuclei, and climbing fibres, that originate in the inferior olive (see below).

The granular layer contains the most numerous cell type in the brain - the granule cell (Eccles *et al.*, 1967). These receive synapses from mossy fibres. Granule cell axons ascend into the molecular layer where they bifurcate to form parallel fibres. Each fibre extends parallel to the plane of the folia and perpendicular to the plane of Purkinje cell dendritic trees with which it forms a synapse. Each parallel fibre may synapse with many Purkinje cells, and in man each Purkinje cell may receive up to 250,000 synapses from parallel fibres (Eccles *et al.* 1967), although this figure varies across species. The primary neurotransmitter at this synapse is glutamate. The granule cells also have 3-5 dendrites, and mossy fibres from outside the cerebellum form complex synapses called glomeruli with them.

Climbing fibres are very different to the parallel fibre system. They arise from a single source - the inferior olivary nucleus in the brainstem. A cell in the inferior olive may give rise to an axon that bifurcates up to several times to form climbing fibres, each of which synapses with a single Purkinje cell many times across the cell body and the dendritic tree. The identities of the excitatory neurotransmitters at these synapses are not known, but glutamate is thought to be a likely neurotransmitter at this synapse. This is one of the most powerful synapses in the brain, and one of the most significant in the computational operations of the cerebellar cortex (see figure 1.3).

The cerebellar cortex also has a series of intrinsic inhibitory cell types. Most of these appear to be positioned so as to provide lateral inhibition and sharpening of spatial

patterns of excitation. Basket cells for example, act on the output of the cerebellar cortex. They are excited by parallel fibres, and inhibit parasagittal rows of Purkinje cells with synapses that are situated at the cell bodies of Purkinje cells. Golgi cells are also inhibited by parallel fibres, but inhibit granule cells, thus acting on the input of the cerebellar cortex.

Both the cerebellar cortex and the cerebellar nuclei receive a convergence of sensory information. Generally, the cerebellar nuclei receive climbing fibre and mossy fibre collaterals, as well as Purkinje cell synapses. Purkinje cells, in particular, are well suited to integrate sensory information provided by climbing fires and parallel fibres. While parallel fibres induce Purkinje cells to respond with simple spikes, the massive depolarisation of Purkinje cells by climbing fibres result in complex spikes, followed by a short refractory period in which there are no simple spikes. The frequency of simple spikes then increases to previous levels. It is likely that each Purkinje cell receives diverse sensory information from parallel fibres all the time. In contrast, climbing fibres fire at a low rate - often below 4Hz. The information content in such signals is therefore low. It is beyond the scope of this thesis to speculate upon the functions of such a system, but it is important to mention that the anatomy and physiology of this system has inspired suggestions that the cerebellar cortex can support plasticity related to motor learning, (Marr, 1969; Albus, 1974). Such models have specifically suggested that plasticity for motor learning may be supported by the cerebellar cortex.

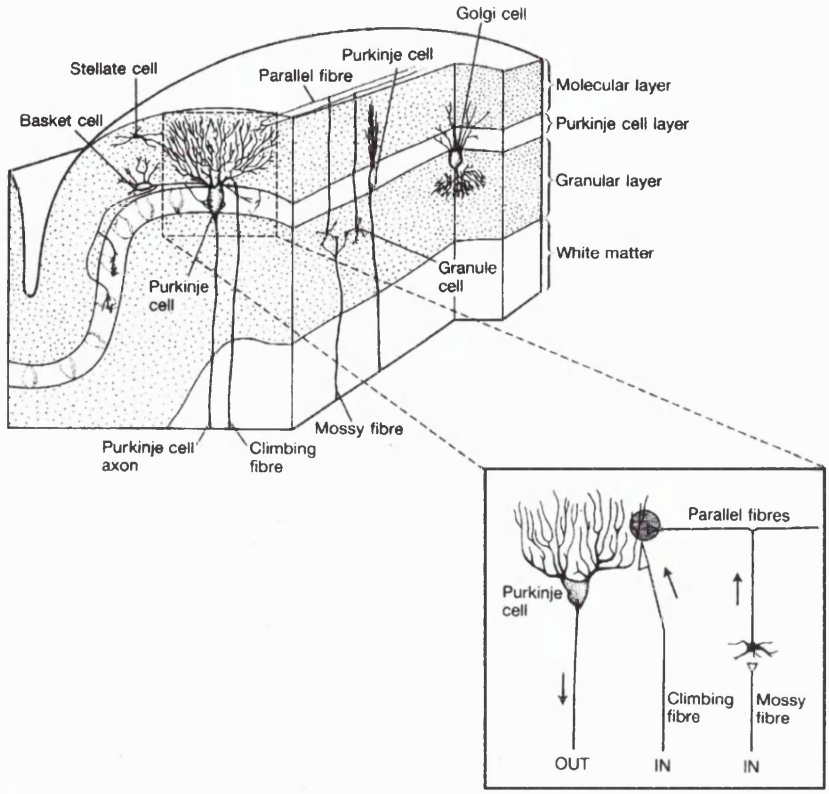


Figure 1.3: Schematic representation of cytoarchitecture of the cerebellar cortex. Adapted from Dudai (1991), with permission from Oxford University Press.

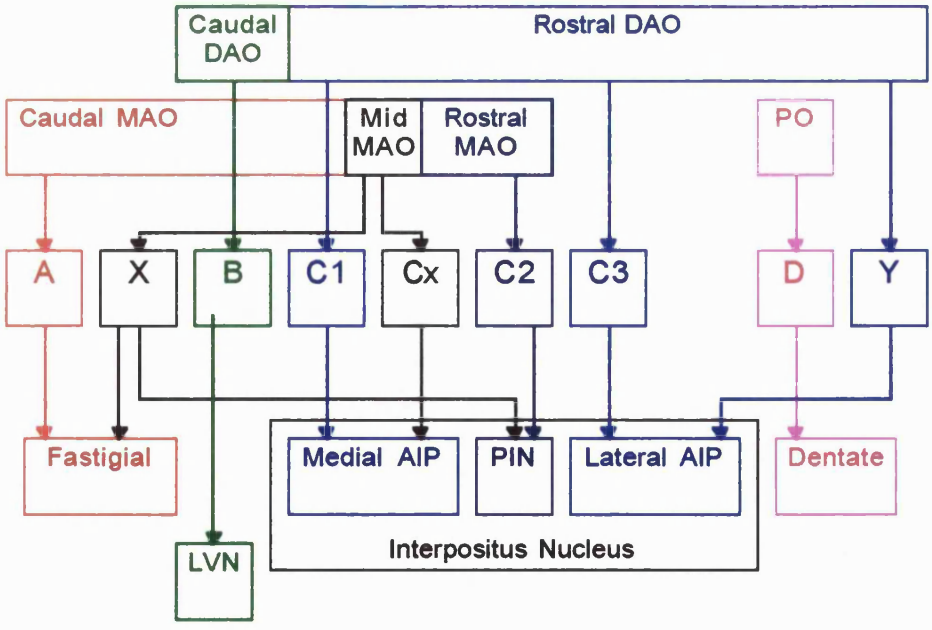


Figure 1.4: Schematic diagram of organisation of olivo-cortico-nuclear circuitry. Top two rows, divisions of the inferior olive (DAO, dorsal accessory olive, dark grey; MAO, medial accessory olive, white; PO, principal olive, light grey. Third row, zones of cerebellar cortex (A to Y; zones X and Y are less well characterised than the others, and have been found relatively recently); Bottom row, divisions of the cerebellar and vestibular nuclei (LVN, lateral vestibular nuclei; AIP, anterior interpositus nucleus; PIN, posterior interpositus nucleus). Colours represent anatomically distinct groups of modules.

The cerebellar cortex has numerous fissures that divide the cortex into several parts called 'folia'. These run transverse to the midline. Some fissures are much deeper, and divide the cortex into ten lobules (such that each lobule is composed of several folia). The midline of the cerebellum is called the vermis. The more lateral parts of the cortex are called the hemispheres. According to Larsell's classification (Larsell, 1970), each lobule is referred to in terms of its position from the first lobule, and whether it is hemispheral or not (so the hemispheral part of the sixth lobule from the anterior end of the cerebellum, is referred to as Lobule HVI). The deepest fissure (Primary fissure) separates the vermian and hemispheric parts of the anterior five lobules (Larsell's Lobules I to V) from the rest. The horizontal fissure separates Lobules VI to VIIa, and Lobules VIIb to Lobule X are situated posterior to this fissure (see figure 1.5).

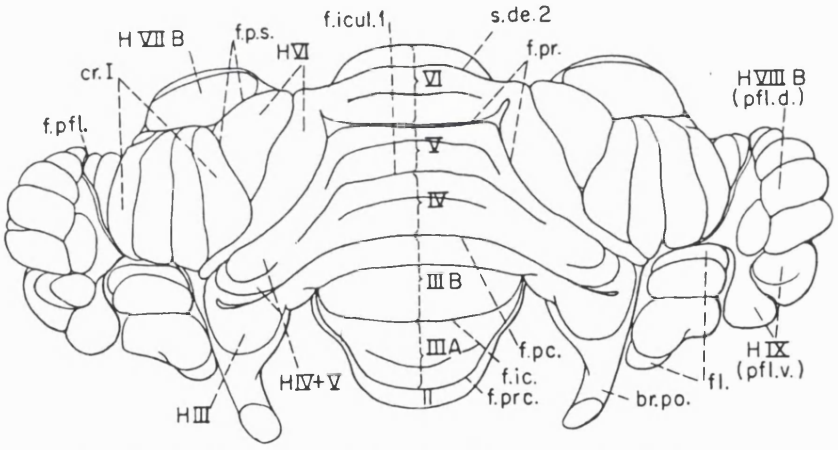


Figure 1.5: Anterior view of the rabbit cerebellum. Lobule HVI is critical for NMR conditioning. Reproduced with permission from O. Larsell (1970), University of Minnesota Press. Abbreviations: br. po., brachium pontis; cr.I, crus I; f.ic., intraculminate fissure; f.icul 1,2,3 intraculminate fissures 1,2,3; f.p fl, paraflocculer fissure; f.p.s, posterior superior fissure; f.pc., preculminate fissure; f.pr., fissura prima (primary fissure); f.prc., precentral fissure; fl., flocculus; HIII-HIX, hemispheral lobules III to IX; HVII B, paramedian lobule; III-VII, vermal lobules III-VII; pfl.d, dorsal paraflocculus (HVIIB); pfl.v, ventral paraflocculus (HIX); s.de 2, declival sulcus 2.

While the transverse organisation of the cerebellar cortex is obvious from the gross morphology, the cerebellum is *functionally* organised in a longitudinal, rostrocaudal direction. The longitudinal organisation of the cerebellar cortex is determined by climbing fibre afferents to Purkinje cells, and the efferent, inhibitory connections of Purkinje cells to the cerebellar nuclei. Individual 'zones' (longitudinal strips of Purkinje cells that extend rostrocaudally), project to specific areas of the cerebellar nuclei. Zones which are medial project to the fastigial nuclei (zones A and B). More lateral

zones project to the interpositus nucleus (zones C1, C2 and C3), and the most lateral zones (zones D1 and D2) project to the dentate nucleus (other lateral zones have also been discovered but are less well characterised). These zones in turn, receive climbing fibre afferents from specific regions of the inferior olive (see figure 1.4). Therefore, the cerebellum is composed of highly specific, compartmentalised olivo-cortico-nuclear modules which are relatively independent of each other, but whose modes of operation must be very similar. Evidence has also emerged to show that the cerebellar nuclei project back to the inferior olive (Graybiel, 1973), so as to preserve the distinctions between modules. Each zone contains somatosensory representations of the body surface, termed microzones (Oscarsson 1979). These modules have been proposed as functional units of the cerebellum.

There are less numerous but significant reciprocal connections that extend from the nuclei to the cerebellar cortex. Evidence suggests that these pathways may be inhibitory (Chan-Palay *et al.*, 1979; Tolbert and Bantli, 1980) but these findings remain controversial. Nucleo-cortical fibres appear to terminate in mossy fibre rosettes. Interestingly, these fibres appear to be collaterals of cerebellar efferents to the brainstem. The output of the cerebellar nuclei would serve to inhibit the activity of the nuclei via inhibitory feedback from Purkinje cells of the cerebellar cortex.

There are also nucleo-olivary fibres that serve to inhibit the activity of the inferior olive. It has been shown that such pathways are reciprocally organised and therefore preserve the zonal modularity of the olivo-cerebellar system and do not cross between modules (Brodal and Kawamura, 1980). It has been proposed that the nucleo-olivary pathway serves to regulate the excitability of olivary neurons (Andersson *et al.* 1988).

1.9 Permanent and reversible lesions of olivo-cerebellar circuits.

An important finding was made by R.F.Thompson and his colleagues (McCormick *et al.* 1981), when they reported that large, unilateral lesions of the cerebellum that included the cerebellar cortex and the underlying nuclei, abolished conditioned NMRs on the side ipsilateral to the lesion, leaving the URs intact. Such lesions also prevented re-acquisition. This result confirms the cerebellum as a site that is essential for NMR conditioning. Importantly, consistent with theoretical models of the cerebellar cortex (Marr, 1969; Albus, 1971; Gilbert, 1974), it supports the idea that the cerebellum may be a locus of plasticity that is required for motor learning. Since then, several studies have focused on the cerebellum as a potential locus of plasticity, particularly with reference to its modular architecture. Several studies have attempted to identify the circuitry which mediates the classically conditioned nictitating membrane response in the rabbit. Lesions of several structures have revealed the circuitry through which the CR is expressed.

1.9.1 Localised lesions of the cerebellar cortex

Given the highly specific and zonal organisation of the cerebellum, it was important to extend the findings of Mc Cormick et al. (1981), to define areas of significance for NMR conditioning at a modular level. In order to achieve a more refined analysis of the cerebellar anatomy essential for the NMR conditioning, Yeo and his colleagues carried out a systematic series of studies in which lesions were placed in small, restricted areas of the olivo-cerebellar system. In an early study, Yeo *et al.* (1984) demonstrated that part of the cerebellar cortex was specifically involved in NMR conditioning. Aspiration lesions of a localised area of the cerebellar cortex (Lobule HVI of Larsell, see figure 1.6) completely abolished the expression of conditioned NMRs. In addition, animals with such lesions were unable to re-acquire CRs with further training. When the US was transferred to the side contralateral to the lesion, there was rapid

acquisition of CRs. This was the first demonstration that the ipsilateral cerebellar cortex was critical for NMR conditioning. In order to further demonstrate the specificity of this lesion, it was shown that large lesions of Lobules VII - IX, but not HVI, do not disrupt NMR conditioning (Yeo *et al*, 1985b).

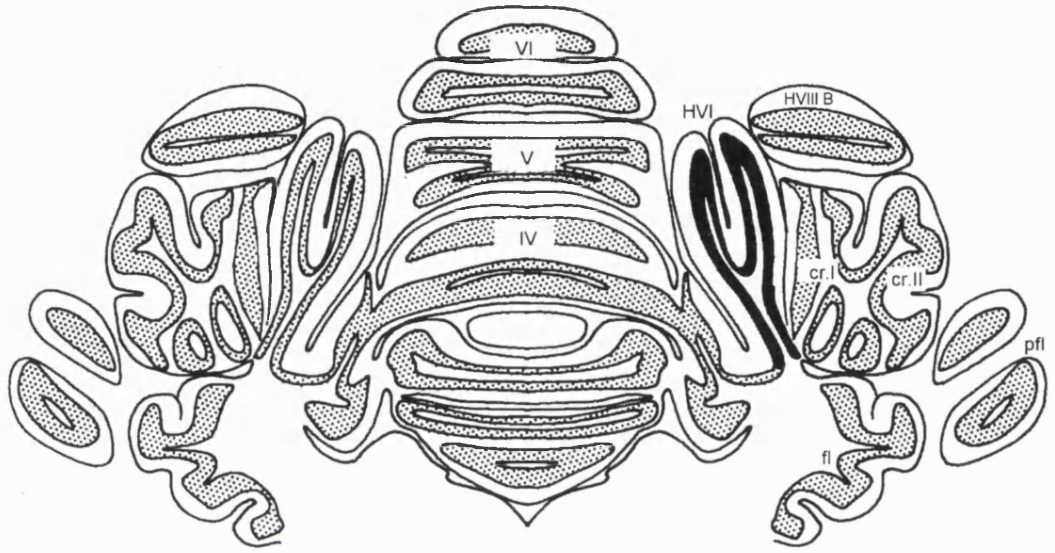


Figure 1.6: Transverse, standard serial section through the rabbit cerebellum (1mm caudal from λ). Hatching depicts the molecular layer of the cerebellar cortex. On the right side of the figure, the granular layer in Lobule HVI of Larsell is shaded black. In this thesis, the plane of section through the cerebellum is such that Lobule HVI can be identified as a the lobule adjacent to vermis medially, and to the paramedian lobe (HVIII B) and crura I (cr.I) and II (cr.II) laterally. At most rostro caudal levels, it appears shaped as a 'fork'.

Yeo *et al.* (1986) have argued that electrophysiological and anatomical studies demonstrate that the cerebellar cortex receives convergence of US and CS information, and that Lobule HVI may be a site of essential plasticity for NMR conditioning. In 1985, a comprehensive study of cerebellar cortical lesions was published (Yeo *et al*, 1985b), which also demonstrated the critical nature of Lobule HVI to NMR conditioning. Consistent with Yeo *et al* (1984), large unilateral lesions of the cerebellar cortex which spared Lobule HVI had no effect upon NMR conditioning, but small, restricted lesions of Lobule HVI abolished NMR conditioning. Also, the cerebellar nuclei were spared - there was no retrograde degeneration of the cerebellar nuclei (these lesions did however, cause degeneration of climbing fibres from parts of the inferior olive, revealing olivo-cerebellar projections that were essential for NMR conditioning).

In two further studies, Hardiman and Yeo (1992), and Yeo and Hardiman (1992) addressed some important issues. It was important to confirm that cerebellar cortical lesion effects were not due to degeneration of neurons in pre-cerebellar nuclei, such as the inferior olive. Therefore, Hardiman and Yeo (1992) made lesions to areas of the cerebellar cortex using fibre-sparing, excitotoxic concentrations of kainic acid. It was found that such lesions to Lobule HVI, Crus I and Crus II resulted in a loss of CRs. In subjects with less complete lesions, CRs gradually recovered with re-training. The extent of this deficit depended on the extent of the lesion, and subjects with the most complete lesions showed very few CRs (subjects with lesions to both Lobule HVI and Crus I and II, had a more pronounced deficit that subjects with only HVI lesions). This result confirms the critical nature of the cerebellar cortex in NMR conditioning, since there was no degeneration of pre-cerebellar nuclei.

Yeo and Hardiman (1992) further examined the effect of cortical aspiration lesions on NMR conditioning. In order for the lesion to be effective in abolishing CR frequency, the entire lobule HVI had to be lesioned. Lesions to this area result in a loss of CRs that recovered with extended retraining. However, if lesions were extended to ansiform and rostral paramedian lobe, effects on NMR conditioning were more complete and did not recover.

It has been suggested that cerebellar cortical lesions may not result in the loss of CRs, but in extended latency to onset into the post-US period. If this were the case, then these CRs would be masked by the onset of the US. In order to assess this possibility, paired training was punctuated at regular intervals by CS-only trials, so that latencies which were extended up to 1000 ms after the onset of the CS would be detected. It was found that CRs did not extend beyond the US period in the CS only trials.

Both Hardiman and Yeo (1992) and Yeo and Hardiman (1992) addressed the issue of performance deficits accounting for lesion effects. It has been suggested that lesions of the cerebellar cortex do not impair learning, but simply impair the animals' ability to 'perform' CRs (Welsh and Harvey, 1989). In order to address this view, Hardiman and Yeo (1992) and Yeo and Hardiman (1992) exhaustively tested the effects of the lesions on the unconditioned responses. In both studies, animals were given periocular stimulation at a range of intensities, and the size of the resulting UR was measured. After conditioning, Yeo and Hardiman (1992) found that animals with cerebellar cortical lesions produced significantly enhanced UR amplitudes, but only in animals where such lesions were completely effective in abolishing CRs. The effects of such lesions were also tested on naive animals, so that conditioning-related effects on UR amplitude could be ruled out. There were highly significant differences between UR amplitudes of lesioned and non-lesioned sides, particularly at lower US intensities - lesions significantly enhanced UR amplitudes. Hardiman and Yeo (1992) found that overall, with fibre-sparing lesions of the cerebellar cortex, there were no differences between subjects which were not lesioned and subjects which had differing degrees of CR deficits. Therefore, both studies found a dissociation between CR deficits and UR deficits. However, there were discrepancies between more subtle aspects of UR amplitude analyses. While Yeo and Hardiman (1992) have found that UR amplitudes are enhanced by lesions that completely abolish NMR conditioning, Hardiman *et al.* have found not found this to be the case in kainate lesioned animals (however, there were a small number of subjects in this study). In both cases, there is evidence to suggest that CR deficits are unlikely to be caused by performance deficits because URs are not impaired.

Both Yeo and Hardiman (1992) and Hardiman and Yeo (1992) have reported that lesions to HVI and Crus I and II have a more permanent and pronounced effect on NMR conditioning than lesions to HVI alone. What then, is the role of Crus I and II in NMR conditioning? Yeo and Hardiman (1992) reported that lesions of these areas alone,

have very little effect on NMR conditioning. It is important to consider that aspiration lesions of Lobule HVI used in earlier studies (Yeo *et al.* 1984, 1985b) may have additionally caused degeneration of climbing fibre collaterals projecting to related areas of the cerebellar cortex which also have eyeblink microzones. These include Crus I and II. Therefore, the functional effects of aspiration lesions would amount to lesions of HVI, and Crus I and II. Hence, kainic acid lesions that do not result in such degeneration, would have to be applied to all of these areas to have the same effect.

The studies reported above are all consistent with the idea that specific areas of the cerebellar cortex are essential for NMR conditioning. However, studies from other laboratories have produced alternative and controversial, but inconsistent results.

McCormick and Thompson (1984b) reported that lesions to the cerebellar cortex that included Lobule HVI, Crus I and II, did not impair CRs - instead, their timing was changed. In contrast to this study, Lavond *et al.* (1987) attempted to replicate Yeo *et al.* (1984) exactly. Large areas of the cerebellar cortex, including Lobule HVI, abolished CRs - but they recover with postoperative training. Hence they conclude that the cerebellar cortex is not essential for NMR conditioning.

Lavond and Steinmetz (1989) reported that aspiration lesions to larger areas of the ipsilateral cerebellar cortex did not abolish acquisition. Rather, it was proposed that such lesions affected "how well rabbits learn a classically conditioned nictitating membrane response". Also, they concluded that "The cerebellar cortex is not essential for classical conditioning", and "the rabbit is better off with the cerebellar cortex than without" because "the cerebellar cortex normally plays an important role in learning". It is not made clear whether they refer to NMR conditioning specifically, or to learning and memory models generally. Also, it significant that acquisition was severely impaired and CR amplitudes were small.

The findings of other laboratories are now in broad agreement with the findings of Yeo and colleagues. However, there is no agreement that lesions to adjacent areas (Crus I and II) abolish NMR conditioning without recovery. While this dispute remains unresolved, Gruart and Yeo (1995) have conclusively demonstrated bilaterality in the essential role of the Lobule HVI in NMR conditioning. While unilateral lesions of HVI and ansiform lobe impaired conditioning in highly overtrained subjects, they did not prevent some relearning with post operative retraining. However, bilateral lesions of HVI and ansiform lobe gave similar impairments, but substantially prevented re-learning. Consistent with previous studies, unilateral lesions gave enhanced UR amplitudes. However, bilateral lesions gave less enhanced UR amplitudes, indicating that UR amplitudes are more sensitive to lateral imbalances of excitabilities in the brainstem. There was no correlation between UR enhancement and CR loss in bilateral group, indicating that lesions probably impaired learning rather than performance. Interestingly, the CRs that were produced by animals with bilateral lesions, appeared to have more variable timing, indicated by the more variable latencies to onset and peak. These results implicate both ipsilateral and contralateral Lobule HVI in NMR conditioning - this is consistent with the suggestion that plasticity associated with NMR conditioning may be bilaterally distributed in the cerebellar cortex.

1.9.2 Localised lesions of the cerebellar nuclei

Anatomical studies have shown that the interpositus nucleus receives afferents from the C zones of the cerebellar cortex. Common to many species is the finding that zones C1 and C3 project to AIP, while zone C2 projects to posterior interpositus nucleus (PIN) (Voogd and Bigaré 1980). Yeo *et al* (1995c) have shown that AIP and Lobule HVI share the olivo-cortico-nuclear module essential for NMR conditioning. It is perhaps not surprising then, that early reports identified the interpositus nucleus (or the area known to Thompson and colleagues as 'dentate-interpositus border'), as being essential for NMR

conditioning (Clark *et al.* 1984; Glickstein *et al*, 1983; McCormick and Thompson, 1984).

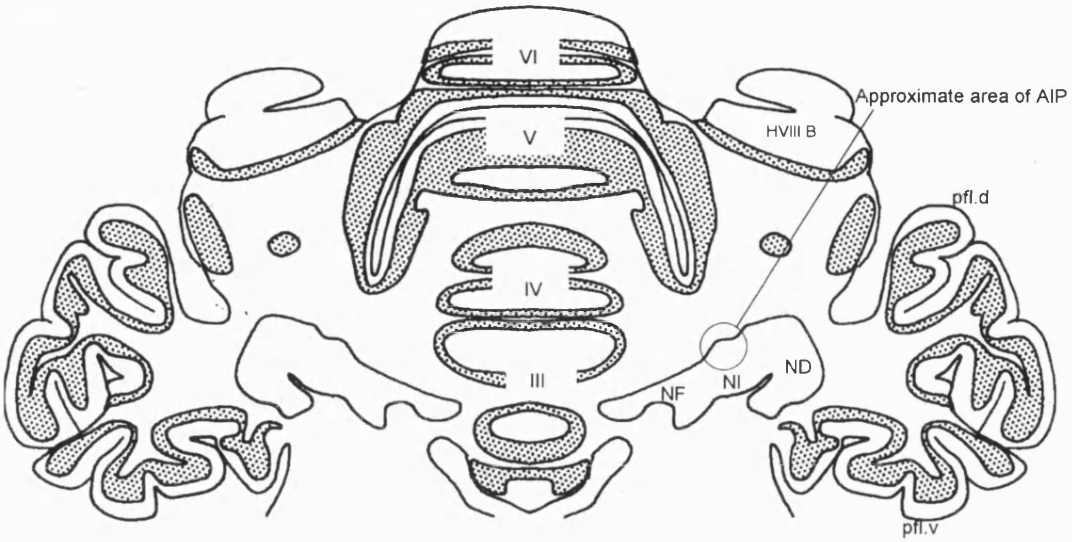


Figure 1.7: Transverse, standard serial section through the rabbit cerebellum (3mm caudal from λ). Hatching depicts the granular layer of the cerebellar cortex. The approximate location of AIP is represented by the encircled region of the interpositus nucleus. Lesions to this area abolish NMR conditioning. Abbreviations: III-V, lobules II-VI of vermis; HVIII B, paramedian lobe; ND, dentate nucleus; NI, interpositus nucleus; NF, fastigial nucleus; AIP, anterior interpositus nucleus; pfl.d, dorsal paraflocculus; pfl.v, ventral paraflocculus.

Yeo *et al.* (1985a) used small lesions in each of the cerebellar nuclei to determine the precise areas of the nuclei that are important for NMR conditioning. It was found that lesions to the fastigial, dentate nuclei and PIP did not affect NMR conditioning. However, CRs were lost when lesions were applied to AIP. These did not recover on retraining, unless the US was transferred onto the contralateral side of the lesion. Results were clearly that animals lost retention of CRs after the lesion. Also, there was no re-acquisition on retraining. This suggests that the AIP too, has an important role in NMR conditioning. This is entirely consistent with findings in other species that Lobule HVI contains eyeblink microzones that project to AIP (Ivarsson and Hesslow, 1993). This finding has been consistently replicated in other laboratories (Lavond *et al* 1985, 1994), and is particularly important in the context of the functional modular anatomy of the olivo-cerebellar system.

While there is no controversy attached to the finding that lesions of AIP abolish NMR conditioning, the interpretations of this result are diverse. One set of interpretations claim that AIP lesions impair learning, and the other set claims that these lesions impair the execution of CRs.

Of those that explain the result in terms of a learning impairment, one explanation is that the AIP is a site of plasticity, and that a loss of CRs results from a loss of an area of essential plasticity (Lavond *et al.* 1987). Another explanation asserts that such lesions may abolish learning by causing impairments to sensory mechanisms essential for NMR conditioning. The cerebellum is known to regulate aspects of trigeminal function though the red nucleus, and may also indirectly modulate auditory function. It is proposed that AIP lesions may impair learning by impairing sensory input to intact sites of plasticity. This is more fully discussed in a later chapter.

A third explanation relating to learning impairments, and one which is consistent with experiments reported in this thesis, is that plasticity for NMR conditioning is probably not localised to a single structure. The cerebellar nuclei may support plasticity, but in addition, it is suggested here that cerebellar cortical sites are also able to support learning related plasticity. The types of plasticity resident in AIP and cerebellar cortex may be qualitatively different. A hypothesis proposed in this thesis is that plasticity resident in AIP is active when conditioning is taking place, but longer term processes which outlast these may be engaged in the consolidation of motor learning in the cerebellar cortex. This hypothesis is discussed further in a later chapter.

Similarly, normal AIP activity may be essential to support plasticity in other sites, such as brainstem nuclei. There may be points of convergence in the brainstem where periocular (US), auditory and visual stimuli (CS) supply areas of plasticity. Lesions of AIP might disrupt possible plasticity in the cerebellar cortex. It has also been suggested that AIP lesions do not disrupt plasticity at all, but simply mask ongoing learning by

impairing the expression of CRs. Welsh and Harvey (1989) refer to this as a "performance" deficit, which is discussed later.

Permanent lesions of AIP cannot distinguish between these hypotheses. However, it is of importance to note that theories suggesting a cerebellar cortical locus of essential plasticity are substantiated by anatomical and electrophysiological evidence, and computational models of cerebellar cortical function that account for the complex physiological properties of cerebellar cortical circuits, their afferents and efferents. Theories suggesting brainstem loci of plasticity have to date, been of a purely hypothetical nature, with little specific anatomical evidence to support them.

1.9.3. Localised lesions of the Inferior Olive

Yeo *et al.* (1985b,c), have shown that aspiration lesions to areas of the cerebellar cortex which abolish NMR conditioning, result in retrograde degeneration to specific areas of the inferior olive. Such results have shown that highly restricted regions of the olive, medial parts or rostral DAO, project to lobule HVI of the cerebellar cortex. It has also been shown that this region of the inferior olive is essential for NMR conditioning. Yeo *et al* (1986) have shown that lesions which were restricted to medial parts of rostral DAO and PO abolished conditioning and prevented subsequent acquisition. URs were unaffected, and subsequent training could not re-establish conditioning on either side. It has been shown that medial parts of rDAO, AIP and Lobule HVI belong to the same module, and Yeo *et al* (1986) have proposed that these anatomically linked areas form a functional olivo-cerebellar module which is essential for NMR conditioning.

McCormick et. al (1985) have also lesioned the inferior olive in animals that have acquired CRs. In contrast to Yeo *et al.*, they have concluded that such lesions result not in an abolition of NMR conditioning, but that paired training after the lesion results in the extinction of CRs. These results have prompted Thompson and his colleagues to

suggest that an association is formed in the dentate-interpositus border, resulting from US information from climbing fibres, and CS information from parallel fibres. Lesions of the inferior olive would therefore result in a loss of US information, but not CS information, resulting in extinction of CRs.

This result is inconsistent with that of Yeo *et al.* (1986), and also with what is known about the physiological properties of the olivo-cerebellar system. There are indeed trigeminal inputs into medial parts of rostral DAO. Such information is likely to reach AIP. However, there are no studies which have demonstrated that CS information (auditory or visual) also converges at AIP. In addition to this, lesions of the olive are likely severely to disrupt the functions of the cerebellar cortex. It has been shown that a loss of climbing fibre activity in the cerebellar cortex results in greatly enhanced simple-spike activity induced by parallel fibres. This would lead to abnormally inhibited activity in the cerebellar nuclei, whether the inferior olive functioned as the US channel or not.Thus, one would expect that lesions of the inferior olive to abolish NMR conditioning.

These studies have been reconciled in Yeo (1989), where from anatomical tracing studies, it is suggested that McCormick *et al.* effectively deafferented the inferior olive.

1.9.4 What have permanent lesions of the olivo-cerebellar system revealed?

Lesion studies have revealed that there are areas which are essential for the expression of the CR, but not essential for UR expression. Neural pathways essential for the conditioned NMR are now relatively well characterised. While there is some overlap between the CS and US pathways, they are not identical. Of course, the motoneurones for the CR are the same as those of the UR.

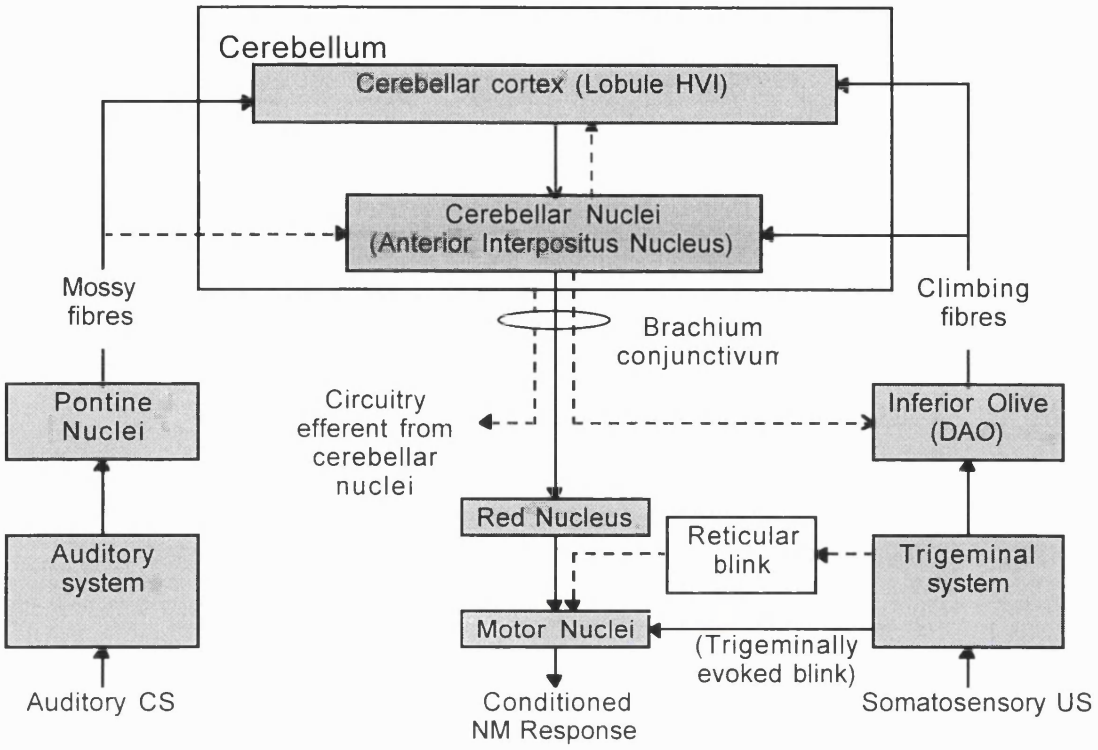


Figure 1.8: Simplified diagram of circuitry involved in NMR conditioning. Unbroken lines, pathways essential in NMR conditioning; broken lines, pathways which may have roles in NMR conditioning; open arrows, inhibitory pathways; closed arrows, excitatory pathways.

Studies reported above have shown that essential parts of the cerebellar cortex (Lobule HVI) project to essential parts of the cerebellar nuclei (AIP). In addition, AIP is known to project, via superior cerebellar peduncle (McCormick and Thompson, 1982c), to the magnocellular division of the contralateral red nucleus (Rosenfield and Moore, 1983). The magnocellular red nucleus projects principally to the retractor bulbi motoneurones in the ipsilateral accessory abducens (Desmond *et al.* 1983; Rosenfield *et al.* 1985). Lesions of structures in the pathway from the cerebellar cortex to the red nucleus abolish conditioned NMRs, but fail to abolish URs. Since these structures are essential for CR expression, this pathway is thought to be that through which the CR is expressed (Thompson and Krupa, 1994). A second, less direct pathway to these motoneurones, via reticular pre-motor blink areas has also been proposed (Rosenfeild and Moore, 1983).

Lesions of the olivo-cerebellar system have generated interesting and sometimes controversial results. In summary, lesions of the cerebellar cortex and the inferior olive have yielded inconsistent results. Generally, Yeo and colleagues have found that highly specific lesions to localised areas of the olivo-cerebellar system abolish NMR conditioning. These areas are related anatomically, and are components of a functional module. In contrast to this, Thompson *et al.* have found that lesions to parts of the olivo-cerebellar system affect NMR conditioning in different ways. Lesions to cerebellar cortex have been found not to abolish NMR conditioning permanently. Lesions of the dentate-interpositus border abolish NMR conditioning (and are to this extent, consistent with other studies), and lesions of the inferior olive have apparently resulted in extinction of CRs. All of these studies support the cerebellar learning hypothesis, because they implicate elements in the olivo-cerebellar system as a set of candidate structures that may support essential plasticity.

A great deal of evidence has now accumulated to suggest that normal function of the cerebellar nuclei is essential for normal function in both the inferior olive and for the cerebellar cortex. Physiological evidence shows that lesions of the olive result in a loss of climbing fibre-induced complex-spike activity that is normal in Purkinje cells of the cerebellar cortex. There is also a significant rise in parallel fibre induced simple-spike activity (Montarolo *et al.*, 1982). Also, inactivations of the inferior olive result in decreased excitabilities of cells in the cerebellar nuclei (Benedetti *et al.*, 1983). It is also significant that such effects are long-term (Colin *et al.* 1980). Rawson and Tilokskulchai (1981) reported that high frequency stimulation of the inferior olive resulted in the suppression of simple spikes in Purkinje cells. This would disinhibit the cerebellar nuclei, the activity of which would suppress activity in the same set of olivary neurons. Normal activity in the cerebellar nuclei is also important for maintenance of normal activity in the inferior olive. Andersson, Garwicz and Hesslow (1988) demonstrated that 'post-conditioning' inhibition is indeed mediated by the nucleo-

olivary fibres that run through the brachium conjunctivum. Small lesions of the brachium conjunctivum abolished the effects of high frequency climbing fibre stimulation on the excitability of olivary neurons. Furthermore, microinjections of bicuculline into the inferior olive did not have any effect on climbing fibre responses, suggesting that olivary inhibition is not mediated locally by olivary interneurones. In classical conditioning too, it is likely that lesions to AIP would disrupt normal function in the inferior olive through nucleo-olivary pathways.

The reciprocal projections to the cerebellar cortex may also be essential for supporting normal function and plasticity in cerebellar cortical circuitry. Anatomical and physiological data are limited, but support the view that lesions of AIP disrupt cortical function. So, lesions of AIP or inferior olive probably therefore result in functional lesions of the whole olivo-cortico-nuclear circuit. Such lesions must impair nucleo-olivo-cortical *function.*

None of these studies prove that olivo-cerebellar circuitry supports plasticity for NMR conditioning. In order to determine the role of this system in NMR conditioning, it is important to first distinguish between the effect of lesions on *learning* and their effects on the *execution* of CRs. Permanent lesions cannot achieve this, and so other methods have been employed.

1.10 Inactivations of the olivo-cerebellar system.

Local inactivation experiments provide a means of *reversibly* lesioning specific brain structures. This methodology allows interpretative problems, inherent in methods employing permanent lesions, to be overcome. In a typical experiment, a brain structure is inactivated during classical conditioning. This is achieved by infusing a pharmacological agent directly into the brain structure through an implanted cannula. When the effects of the drug have dissipated, the animal is tested to assess the extent to

which it has profited from the conditioning. If the animal has not profited from conditioning during the inactivation, revealed by substantial levels of conditioned responses after the inactivation, it may be concluded that the animal has not learned, and that the structure in question is essential for such conditioning. Loss of conditioned responses cannot be attributed to motor deficits.

Such results would considerably strengthen the case for the structure as a candidate for essential plasticity, but other explanations must be taken into consideration. An alternative hypothesis could be that the structure in question is indeed essential for learning but not because it is a site of plasticity. It may, for example, provide indispensable input to sites of essential plasticity. This may be input that tonically supports the general activity of such a site. Or, the inactivated site may directly or indirectly affect sensory processing that is required for sites to undergo learning related change. Reversible lesions also suffer from some interpretative problems, including state-dependency (see later).

The importance of temporary and permanent lesion studies lies in their ability to demonstrate that a particular structure is essential for the expression of learned responses. Temporary lesions during classical conditioning, but not permanent lesions, can demonstrate that a disappearance of CRs is due to *learning* deficits rather than a deficit in the execution of CRs by a motor deficit. An important strength of such studies, is that they can reveal with absolute certainty which structures are *not* involved in learning and memory. If Inactivations do not prevent learning, then the structure is definitely not a site of plasticity. In this way, it may be possible to further restrict the range of structures that are regarded as possible sites of plasticity.

Lesion studies have been instrumental in demonstrating the essential role of olivo-cerebellar and related circuitry in NMR conditioning. They have implicated specific areas of the cerebellar cortex and cerebellar nuclei in modules within the olivo-

cerebellar system as candidate sites of learning-related plasticity. In order to investigate whether a loss of CRs is due to learning or to execution, reversible lesions have been used. Since permanent lesions of AIP have reliably abolished NMR conditioning, several studies have attempted to use pharmacological agents to inactivate the AIP during NMR conditioning. Some important studies are discussed.

The importance of AIP and lobule HVI in NMR conditioning have been established by permanent lesion studies, but the results have often been inconsistent. Mamounas, Thompson and Madden (1987) have used reversible inactivations of AIP and lobule HVI to assess the involvement of cerebellar GABAergic, strychnine independent processes in NMR conditioning. They have shown that infusions of the GABA antagonists bicuculline methiodide and picrotoxin, into AIP, completely and reversibly abolish the expression of conditioned NMRs in trained animals in a dose dependent manner. Interestingly, microinfusions of these GABA antagonists did not affect the baseline neural activity of deep nuclear cells. There were also no effects of UR amplitudes. The authors report a single case in which microinfusions of picrotoxin were infused into lobule HVI of the cerebellar cortex. These infusions, made into the deeper layers of the mid to rostral region of lobule HVI, reversibly and completely abolished CRs, with no effect on URs. No histology for this case is presented.

This study confirms the findings of Yeo *et al* (1985a), which indicate that AIP is essential for NMR conditioning. The essential involvement of lobule HVI in NMR conditioning is also confirmed, consistent with Yeo *et al.* (1985b). This result conclusively ends the controversy surrounding the involvement of Lobule HVI in NMR conditioning. While this study has confirmed earlier findings which have implicated AIP and lobule HVI in the expression of conditioned NMRs, it has not been able to demonstrate the roles of these structures in learning. Such inactivations may, like permanent lesions, abolish CRs for several reasons, including those which suggest an impairment of motor expression of CRs.

Welsh and Harvey (1989) have argued that AIP lesions impair acquisition and retention of NMR conditioning by inducing 'performance' deficits. In order to evaluate this hypothesis, they performed a 3 phase experiment in which reversible pharmacological inactivations were used rather than permanent lesions (Welsh and Harvey, 1991). In the first phase, rabbits acquired CRs to a flashing light CS and an airpuff US. Rabbits acquired CRs in this phase. In the second phase, lidocaine, a sodium channel blocking anaesthetic, was infused into AIP while rabbits were given paired training using a tone CS, punctuated by presentations of the light CS to test for completeness of the inactivation. In this phase, animals were categorised by the effectiveness of the blockade of CRs to light CS. Welsh and Harvey argued that if learning was impaired by lidocaine infusion into the cerebellar nuclei, then animals in which the blockade was effective would not learn CRs to the tone CS. In Phase 3, subjects were tested with both light and tone CS. They gave CRs to both light and tone. Welsh and Harvey (1991) interpreted these findings as evidence that learning is not impaired by inactivations of the AIP. They conclude that

> "the AIP can neither be a site where memories for Pavlovian conditioning are stored nor a site that is essential for sensory processing which leads to associative learning. The AIP must be involved in maintaining the excitability of brainstem motor centres that are directly responsible for the expression of the nictitating membrane reflex".

In another study, Krupa *et al* (1993) have inactivated AIP, and in a different set of animals, red nucleus, during classical conditioning. They have used a very different, 2-stage experimental design. In stage 1, AIP or Red Nucleus was inactivated with 14nm of muscimol in 1 microlitre, and given delay conditioning to a tone CS and airpuff UR. In stage 2, subjects were given more acquisition training, after the effects of muscimol were presumed to have dissipated. In Phase 1, animals in both groups showed no conditioned responses. In phase 2, animals with AIP inactivations initially showed no

CRs, but then acquired CRs in a way identical to controls doing so for the first time. However, animals with red nucleus inactivations immediately showed the same level of CRs as animals which had learned to asymptotic levels. Since red nucleus inactivations were effective in preventing performance but not learning, and inactivations of AIP prevented both performance and learning, the plasticity associated with NMR conditioning could not have been efferent from the cerebellum. Krupa *et al.* (1993) have interpreted this result to mean that muscimol blockade of the AIP prevents acquisition of CRs, and that "the memory trace for eyeblink conditioning must be localised to the ipsilateral cerebellum".

Krupa and Thompson (1995) have reasoned that if inactivations of AIP prevent learning, then plasticity for NMR conditioning must lie in the cerebellum or structures efferent to it. The output of AIP to efferent structures is via the superior cerebellar peduncle. In order to assess the involvement of efferent structures in NMR conditioning, Krupa and Thompson (1995) inactivated the superior cerebellar peduncle using TTX, a sodium channel blocker, in phase 1 during conditioning. After the inactivation (phase 2), animals were tested to see whether they had acquired CRs in phase 1. Thompson *et al* reasoned that if they had acquired under these conditions, they would immediately show CRs in phase 2. However, if efferent pathways were essential for NMR conditioning, there would be evidence of impaired NMR conditioning. When TTX effects had dissipated, further conditioning revealed immediate production of CRs. TTX inactivation of the superior cerebellar peduncle had not prevented acquisition. Thompson *et al.* have interpreted this finding as demonstrating that the essential memory trace for the classically conditioned eyeblink is localised within the cerebellum, and that cerebellar influences on efferent pathways cannot be essential for NMR conditioning.

The objections to the inactivation studies reported above can be conceptualised as of two types. Firstly, there are logical objections concerning the validity of interpretations

of results. Secondly, there are methodological objections concerning the adequacy of designs and methods used in these studies. These objections will now be considered.

There is an incongruence between Welsh and Harvey's (1991) lidocaine inactivation study and Krupa *et al*'s (1993) muscimol inactivation studies. While both claimed to have fully inactivated AIP, Welsh and Harvey found that cerebellar inactivation did not prevent learning, and Krupa *et al.* claim that they did. How can this difference be explained? There is a methodological objection to the design of Welsh and Harvey (1991) - there may have been transfer of learning from phase 1 to phase 2. In NMR conditioning, cross-modal transfer of learning is known to occur. Kehoe and Holt (1984) have tested for "immediate transfer". On presenting a few initial tone CS test trials, they found that there was little or no immediate transfer of learning from an initial light CS to a later tone CS. Welsh and Harvey (1991) verified this result - there was no immediate generalisation of the light CS to the tone CS in their study either. Interestingly, Kehoe and Holt (1984) went on to test for "general transfer", revealed by subsequent training to the new, tone CS. Acquisition was extremely rapid, revealing substantial cross-modal general transfer. Could the CRs in phase 3 of Welsh and Harvey (1991) be due to general transfer, if not immediate transfer? If general transfer is dependent upon the presentation of stimuli rather than learning and performance, then it is feasible that by the time subjects were tested in Phase 3, associations learned in phase 1 to the light CS had transferred to the tone CS, particularly in view of the fact that light CS was concurrently presented with tone CS in close temporal association. This argument is made more feasible by a study in which substantial general transfer was demonstrated when CRs were previously absent or expression of overt performance of CRs was decreased. Kehoe, Morrow and Holt (1984) showed that animals which first acquired to a light CS, and then extinguished to it, subsequently demonstrated robust general transfer to a new CS. The behavioural strength of the original CR cannot be used as a predictor of the magnitude of the transfer.

The difference between the results of Welsh and Harvey (1991) and Krupa *et al.* (1993) have also been explained in terms of the differential pharmacological properties of lidocaine and muscimol. Lidocaine acts to block sodium channels in axons as well as cell bodies. Thompson and Krupa (1994) and Krupa and Thompson (1995) have suggested that Welsh and Harvey may have inactivated AIP efferents rather than AIP itself, thus achieving a result comparable to inactivation of the superior cerebellar peduncle. This would account for impairments of expression but not of learning. Thompson and Krupa (1994) have suggested, on the basis of findings of Nordholm *et al.* (1993), that ventral sites in AIP, where they claim Welsh and Harvey infused muscimol, are not regions in which there is plasticity. Rather, they propose that these areas contain efferent projections that leave AIP. Since efferent structures are thought by Krupa and Thompson (1995) not to be involved in plasticity for NMR conditioning, such inactivations would not prevent learning. Muscimol acts by allosteric modulation of the chloride channel to allow influx of chloride ions into the cell (Sieghart, 1995). Infusion of muscimol into AIP would result in a hyperpolarisation of cells in AIP, without having its effects on axons. Krupa *et al.* (1993) did indeed find that learning was prevented with such inactivations.

Such an explanation would be plausible if efferents from AIP did not project to structures other than the red nucleus that were essential for NMR conditioning. The fact that the AIP also sends inhibitory, GABAergic projections to parts of the inferior olive confounds this analysis, and forms the basis of an important logical objection to their conclusion. In several species, AIP is known to project to medial parts of dorsal accessory olive (DAO) (monkey: Kalil, 1979; cat: Tolbert *et al.* 1976; Angaut and Cicirata, 1982, Deitrichs and Walberg, 1986). Ruigrok and Voogd (1990) have, for example reported that in rat, injections of PHA-L into AIP result in heavy label in ipsilateral DAO, and that this projection is carried in the superior cerebellar peduncle. Interestingly, label in ipsilateral parts of the olive was complemented by label in the same sites in contralateral parts of the olive. This is not to say that such labelling arose

from descending nucleo-olivary projections. Fibres were seen to cross the midline from ipsilateral to contralateral parts of the olive.

Something of the physiology of the nucleo-olivary pathway has already been mentioned, and it must be the case that it is functionally significant in NMR conditioning, as are all other anatomically associated parts of the olivo-cerebellar modules involved in NMR conditioning. Indeed, Thompson and Krupa (1994) acknowledge the importance of this connection and Gluck, Reifsnider and Thompson (1990) and Gluck, Myers and Thompson (1994) have incorporated this pathway into a connectionist model of NMR conditioning, in which feedback from the AIP to DAO plays a critical role in NMR conditioning. There is a logical flaw in the conclusion of Krupa and Thompson (1995), because the anatomy and physiology of the nucleo-olivery pathway are inconsistent with the assertion that cerebellar efferents from AIP are not essential for NMR conditioning, and consequently inconsistent with Krupa and Thompson (1995) because inactivations of the superior cerebellar peduncle would also have inactivated inhibitory projections to the inferior olive.

Krupa *et al.* (1993) report results which are clearly suggestive of a learning impairment related to cerebellar function, but not red nucleus function. They have interpreted the results of their study as having localised plasticity for NMR conditioning to the cerebellum. There are both logical and methodological objections to this study. Logically, such a study cannot rule out alternative possibilities (mentioned above) that can account for learning deficits. Methodologically, the amount of muscimol used in their study was about an order of magnitude more than the amounts used in other studies (discussed in a later chapter). Krupa *et al.* (1993) have autoradiograpically demonstrated the extent of diffusion of muscimol throughout the cerebellum, and claim that there was no diffusion outside the cerebellum. While the autoradiograph indicates that muscimol did not diffuse into brainstem areas, it is clear that it diffused dorsally, beyond the cortical surface and into the CSF. From here, muscimol may have spread

into various brain regions in low levels. Given the large amounts of muscimol injected, levels reaching CSF may have been significant. Autoradiography is not capable of showing the amount of muscimol that became incorporated into CSF.

Pharmacological inactivations, particularly using large quantities of the drug, are likely to have long-term effects if made repeatedly. There is now substantial evidence that the pharmacological properties of receptors and ion channels are subject to change if they are repeatedly exposed to ligands (Sieghart, 1995). Prolonged exposure to muscimol causes sequestration of receptors from the cell surface to internal pools. This is often accompanied by decreased receptor gene expression. Internalised receptors are either recycled to the cell surface or degraded. The latter is more likely to occur if exposure continues for hours rather than minutes. Such down-regulation is also dose dependent - higher concentrations of muscimol are likely to result in faster down-regulation. Interestingly, a discontinuation of application of the agonist after prolonged exposure, typically induces receptor upregulation. It is also significant that other GABA-A ligands (e.g. benzodiazipines, barbituates, ethanol), have been shown to cause a development of tolerance to the drug in animals and in humans, presumably mediated by GABA-A receptor regulation. In sufficient quantities, muscimol is also likely to cause tolerance in behaving animals. In Krupa and Thompson's (1993) study, pathological receptor down-regulation may have occurred. Large, repeated doses of muscimol may have caused prolonged desensitisation of GABA-A receptors. Thus, the absence of CRs in phase 2 may have been due not to an absence of learning in phase 1, but to partial motor deficits in phase 2, that required continued training and time to recover. Did tolerance to muscimol develop? If tolerance to muscimol did develop, it did not exceed the threshold at which performance is manifested. Claims made by Krupa and Thompson (1995) need to be re-assessed in light of what has been discussed here (Chapters 2 and 3).

The studies reported here, indicate that it is learning rather than execution that is affected by inactivations of AIP. However, some aspects of these studies are methodologically problematic. Logically, their interpretations are sometimes inconsistent with the anatomy and physiology of the cerebellum, and avoid analyses of important alternative explanations. If indeed learning were prevented by cerebellar inactivations, impaired cerebellar plasticity need not be the only explanation for such a result. The effects of drugs on sensory processing may affect learning quite independently from their effects on plasticity.

If muscimol affected the sensory properties of the CS, then State Dependent Learning (SDL) may have occurred. Here, subjects learn in the drug condition but cannot retrieve this information unless they are returned to the drug condition. If a drug alters the properties of the CS, subjects will first associate the CS and US. But then, after the drug effects have dissipated, the CS will be perceived as being novel, and subjects will need to re-learn the association in the drug-free state. Two independent sets of associations may therefore be formed - one in the drug condition, and the other in the no-drug condition. This possibility will be discussed more fully in Chapter 2.

One way in which such confounds can be overcome, is to use experimental designs that can impair plasticity without affecting sensory processing - this is discussed below.

1.11 Consolidation of motor learning?

A key feature of memory formation in many behavioural models of learning and memory is the progression from a short-lived labile form of memory, to a long-lasting stable form. This progression is susceptible to interventions that impair or prevent the formation of stable memory. A variety of interventions after training, such as systemic or intracranial administration of drugs, electroconvulsive shock, hypothermia or hypoxia cause amnesia to varying degrees. It is also apparent that these interventions impair memory

at different times after learning, and in different neural pathways. Such results support the hypothesis that there are distinct processes in the post-training period, each being disruptable by a subset of interventions. These are collectively referred to as consolidation.

Interventions *after* classical conditioning (see chapter 4), such as intracranial or intravenous infusions of certain drugs, often disrupt learning, revealing processes which typically occur for some hours *after* training and which are essential for memory. Like inactivation studies that reversibly lesion structures during classical conditioning, such studies have the advantage of being able to show that a structure is essential for learning and memory, and that motor deficits cannot account for loss of CR expression. In addition to this, post-inactivation conditioning can demonstrate that memory formation is disrupted independently of sensory processing necessary for classical conditioning - CS and US are absent during the consolidation period, so pharmacological interventions cannot affect these, as they might in studies using inactivations *during* classical conditioning. Such studies can lend considerable weight to the hypothesis that a structure contains essential plasticity. It is possible however, that if learning is impaired or prevented, then structures efferent from the inactivated area might also be involved, because normal activity in these target areas may also be affected.

The methods *used* to disrupt consolidation may not be *capable* of disrupting consolidation. If specific post-conditioning interventions fail to prevent memory formation, one can only reject the localisation hypothesis if it can be demonstrated that the memory formation in the behavioural model being used is susceptible to post-conditioning intervention in the first place. It is important to establish the approximate temporal limits of consolidation processes. The behavioural model itself may have consolidation processes which are very short-lived, occurring after each trial in a classical conditioning session rather than after each session. Such processes will reach

completion after the intervention takes effect if applied after the session. Indeed, some very long-term consolidation processes may only start after the effects of an intervention have receded and so interventions will only work when applied at this stage.

In rabbit eyeblink conditioning, studies indicate that consolidation processes are disrupted by intravenous injections of several drugs (reviewed in Chapter 4). In addition, muscimol is not only known to affect consolidation processes when injected into various brain structures in other behavioural preparations, but it also affects NMR conditioning when injected into the cerebellum. If muscimol is effective in blocking normal AIP function, then it should impair consolidation of NMR conditioning if such plasticity is resident in AIP. This possibility has been directly tested in chapter 4, and is discussed in more detail therein.

If post-training inactivations do not impair learning, then the implication is that the AIP is not involved in consolidation processes. Essential plasticity for NMR conditioning must therefore be independent of AIP function. However, alternative possibilities should be considered. One is that AIP may not have not been inactivated for the period in which consolidation occurs. Secondly, muscimol may not target biochemical processes which underlie consolidation. Both of these are alternative explanations are unlikely, since it is known that consolidation processes for NMR conditioning are active for some hours after conditioning. Also, muscimol infusions into AIP are known to affect the physiology of conditioned NMR circuitry for periods of several hours. Muscimol is likely to have general and widespread effects on the physiology of AIP, rather than processes which are more specific. If post-training infusions of muscimol affect NMR conditioning, then it is likely to affect processes involved in consolidation and plasticity, should they be dependent on normal function in AIP.

1.12 Is AIP required for learning and consolidation?

This chapter has discussed localisation of function generally, and the potential for a cerebellar locus of learning-related plasticity in particular. Studies using permanent lesions have now established that Lobule HVI, AIP and associated parts of the inferior olive are critical for NMR conditioning. These findings are in broad agreement with theories of motor learning which predict that cerebellar plasticity is essential for such learning.

However, studies of permanent lesions have not been able to rule out other possibilities. Cerebellar lesions may affect conditioning not only by impairing plasticity, but also by impairing the expression of learned responses. Although plasticity may be intact, perhaps in extracerebellar circuitry, it is possible that animals may be unable to express CRs due to a motor deficit induced by the lesion. In addition, sensory processing essential for conditioning may also be impaired by cerebellar lesions. These factors confound the interpretation that the cerebellum is a locus of essential plasticity in NMR conditioning. Some of these problems can overcome by using reversible inactivations, rather than permanent lesions.

Previous inactivation studies which have targeted cerebellar structures in NMR conditioning have reported conflicting results, and have been problematic both logically and methodologically for reasons already discussed. The aims of experiments in this thesis is to resolve these conflicts and to overcome some of these problems.

Lesion and inactivation studies reported above are open to the following interpretations:

1. Lesions and inactivations of the cerebellum induce motor deficits, not learning or sensory deficits (Welsh and Harvey, 1991).

2. Cerebellar lesions and inactivations impair normal sensory processing by removing essential modulatory influences on pathways involved in CS and US processing (possibly resulting in SDL).

3. Cerebellar lesions and inactivation may impair essential plasticity in extracerebellar circuitry.

4. Lesions and inactivations of the cerebellum prevent conditioning because sites of plasticity have been destroyed/inactivated.

The series of experiments reported in this thesis examine the involvement of the cerebellum in the commonest aspects of classical conditioning. Chapter 2 examines whether or not extinction learning is dependent on normal activity in AIP. Very low doses of muscimol infused during conditioning have revealed that the cerebellum is essential for *learning*, and that performance deficits cannot account for loss of learning during inactivations. The results are discussed in the context of the possibilities of cerebellar plasticity and state-dependency. Chapter 3 examines the involvement of cerebellar circuitry in acquisition of CRs, and reports that acquisition is also dependent upon normal activity in AIP. In the same animals, further subsequent infusions of muscimol are reported to prevent extinction. The results are discussed in terms of common mechanisms of acquisition and extinction in NMR conditioning, and the problem of establishing acceptable control procedures. Chapters 2 and 3 clearly show that learning is prevented in both acquisition and extinction conditioning. Performance deficits are unable to account for these findings. Is learning prevented because plasticity is impaired, or because muscimol infusions into the cerebellum induce state-dependency effects? If AIP inactivations impair plasticity, then post-training consolidation processes, known to be important in NMR conditioning, will be impaired by post-training inactivations of AIP. Chapter 4 reports an experiment in which this

hypothesis was tested. Learning was unaffected by immediate post-training muscimol infusions, even though muscimol was highly effective in disrupting normal function in AIP for several hours. Reasons for such a result include the possibility that there is no plasticity for NMR conditioning in AIP, or cerebellar circuitry generally. However, other explanations are possible - these are also discussed.

Chapter 5 attempts a synthesis of these results, and discusses their implications for cerebellar theories of NMR conditioning specifically, and motor learning generally.

Chapter 2

Reversible inactivations of the cerebellum prevent the extinction of conditioned nictitating membrane responses in rabbits.

2.1 Introduction

In the previous chapter, the effects of localised lesions of the olivo-cerebellar system on NMR conditioning have been reviewed. It is clear that the neural circuitry essential for NMR conditioning includes parts of DAO, Lobule HVI of the cerebellar cortex, and anterior parts of the AIP - structures which are anatomically connected. One of the aims of this thesis is critically to evaluate the reasons for the dependency of NMR conditioning on the integrity of this circuitry. Many hypotheses may explain the absence or impairment of conditioning after lesions to these areas, and some of these have already been discussed in Chapter 1. Briefly,

1. The cerebellum provides tonic support of the neural excitabilities in brainstem pathways critical for expression of CRs, but is not itself a site of plasticity. Lesions induce motor deficits, not learning or sensory deficits (Welsh and Harvey, 1991).

2. The cerebellum modulates extracerebellar pathways involved in non-plastic, sensory processing essential for conditioning. Cerebellar lesions therefore impair normal sensory processing by removing essential modulatory influences on pathways involved in CS and US processing.

3. The cerebellum modulates extracerebellar pathways critical for plasticity, but is not itself a site of plasticity. Cerebellar interventions may therefore leave sites of plasticity anatomically intact, but affect physiological processes in these sites, which depend on normal cerebellar function. Cerebellar lesions may therefore impair essential plasticity in extracerebellar circuitry.

4. Essential circuitry of the olivo-cerebellar system contains sites of plasticity essential for NMR conditioning. Lesions of the cerebellum prevent conditioning because sites of plasticity have been destroyed.

Studies employing permanent lesions cannot differentiate between the above hypotheses, since each would predict the same outcome (i.e. abolished or impaired conditioning). Therefore, this method cannot localise plasticity to any part of circuitry that is essential for NMR conditioning.

The use of *reversible* lesions has been used extensively to assess the involvement of specific neural pathways in functions such as motor control, sensory processing, learning and memory. In NMR conditioning, reversible inactivations may be used to rule out at least some of the hypotheses set out above. When applied to cerebellar structures, this technique can reveal which *processes* (e.g. plasticity, sensory processing) are dependent upon normal cerebellar activity (although, these studies are not sufficient to *localise* processes such as plasticity to pathways which have been inactivated). Such an approach can therefore reveal whether normal activity in the cerebellum is essential for acquisition, expression, extinction and consolidation of conditioned NMRs. Essential circuitry in the cerebellum may be inactivated when these processes occur. The consequences of cerebellar inactivations upon these processes may be examined after the inactivation, when normal activity returns to the cerebellum. The involvement of the cerebellum in aspects of conditioning can therefore be verified. If normal cerebellar activity is essential for these, then it is *possible*, but not *proven*, that the cerebellum is a site of plasticity for learning. However, if acquisition, extinction or consolidation processes are found to be independent of normal activity in the inactivated cerebellar circuitry, then it follows that this circuitry cannot contain sites in which these processes occur. In this event, the hypothesis that essential plasticity is localised in cerebellar circuitry could be rejected with confidence.

The previous chapter has reviewed the studies in which localised and reversible inactivations have been used in NMR conditioning. In one study, CRs were found after training during cerebellar inactivation (Welsh and Harvey, 1991), but in other studies,

there has been no evidence for acquisition in previously naive subjects during cerebellar inactivations (Krupa *et al.* 1993; Nordholm *et al.* 1993). In a further study, reversible inactivation of the brachium conjunctivum does not prevent NMR conditioning (Krupa and Thompson, 1995). These authors have concluded that the essential memory trace for NMR conditioning is within the cerebellum.

The interpretation of these studies is problematic for reasons discussed in Chapter 1. Briefly, problems general to these studies include the cerebellar localisation of drug effects, and the high doses and volumes of drugs infused into the cerebellum. Studies reporting acquisition to be abolished by drugs infused into the cerebellum have used relatively high doses or large volumes of drugs. To some extent both dose and injection volume determine the degree of drug dissipation through tissue and so these studies cannot confine spread of muscimol effects to the cerebellum because it is likely that these drugs spread into extracerebellar circuitry.

It is clear that localised infusions of low doses at small volumes must be used in order to restrict the direct action of muscimol to cerebellar circuitry in NMR conditioning. This is one of the aims of the present study. In instances where large doses are used, measures should be taken to establish where the drug has spread (see chapter 4).

Acquisition of CRs is the process best characterised in NMR conditioning and has been tested in all studies where cerebellar circuitry is inactivated during NMR conditioning. In these studies, an essential cerebellar contribution to conditioning is judged by the absence of CRs after the inactivation. A problem with these experiments is that factors other than learning may produce this result. For example, partial cannula or surgical damage to critical areas of the cerebellum would confound the effect of muscimol inactivations on CR expression. After sufficient training and recovery time, animals may begin to acquire CRs. A less likely possibility concerns persistence of drug effects into the post-inactivation phase in target areas essential for response expression (AIP or

Lobule HVI). Initial failure to produce CRs would relate to such extended drug effects and learning may have occurred during the inactivation. In the study by Krupa and Thompson (1993), for example, muscimol effects may have extended from the initial muscimol phase and into the test phase in which muscimol was not infused. In such event, muscimol would have had lasting effects on CR expression from an hour before the last conditioning session under muscimol (injection time), and through the three-day rest period between conditions. It would then have gradually lost its efficacy from exactly the time when conditioning started in the control subject, proceeding at a rate exactly proportional to acquisition in the control subjects. Such an occurrence is rather unlikely but cannot be ruled out. These problems may be overcome by analysing extinction learning. In extinction learning, it is the *absence* of CRs which demonstrates learning, in contrast to acquisition in which the presence of CRs indicates learning. In NMR conditioning, extinction is observed as the gradual waning of CRs with repeated presentation of the CS alone in the absence of the reinforcing US after previous acquisition. The possible effects of incidental cannula damage and the declining efficacy of muscimol in inactivating critical cerebellar circuitry will be evident before the experimental phase (extinction), during the acquisition phase. Animals with damage to cerebellar structures will not acquire like controls - these animals may be rejected from the experimental group. The effectiveness of muscimol in animals with no cannula damage can be assessed, by observing for the *immediate absence of CRs* during subsequent repeated presentations of the CS.

Several features of extinction learning, including the phenomena of spontaneous recovery and rapid re-acquisition after extinction (see Mackintosh 1974) indicate that extinction is an active learning process. The AIP forms a part of the essential circuitry for the expression of the acquired CR. A low dose of muscimol which reversibly and completely inactivates AIP might also prevent extinction of CRs, as it would with acquisition. The presence of CRs in the post-inactivation phase, despite sessions of extinction training, would not only rule out cannula damage and extended drug effects

as explanations of the absence of CRs in the drug phase, but would also demonstrate that the cerebellum is essential for learning and execution of CRs, rather than just the execution of CRs.

Does such a result imply that plasticity has been affected by inactivations? Learning may also be affected by drug effects on stimulus processing. Experimental designs which require drug infusions during some, but not other, training sessions using the same stimulus parameters are prone to a dissociation of stimulus perception, resulting in state-dependent learning (SDL; Overton, 1991). Learned responses to stimuli may occur differently in the drug and no-drug conditions because the drug may cause conditioning stimuli to be perceived differently in each condition. Studies of NMR conditioning in which conditioning is apparently prevented by intracerebellar drug infusions, have not considered the possibility that impaired acquisition is explained equally by impaired plasticity and state-dependent learning. This possibility has been discussed briefly in the previous chapter, and is analysed further in this chapter. The evidence for cerebellar control of sensory processing, particularly of auditory stimuli, is central to the issue of SDL in studies in this thesis and is discussed below.

2.1.1 Do cerebellar inactivations induce state-dependent learning (SDL)?

There is evidence which suggests that altered CS processing in NMR conditioning is specifically susceptible to the influence of drugs, and is the basis of SDL. There is also evidence suggesting that the cerebellum has a modulatory effect upon normal function in the auditory system.

2.1.1.1 CS specific drug effects in NMR conditioning?

One way in which a drug may affect conditioning is by its effects of CS processing in NMR conditioning. It is remarkable that every systemically injected drug found to affect NMR conditioning has also been found to change the processing of auditory stimuli (Harvey, 1987). Such effects can be studied by training animals to criterion levels of

CRs, and then subject the animals to a range of CS intensities. The results are observed as frequency-intensity plots, which may be shifted by drugs, in relation to no-drug control conditions. Schindler *et al.* (1984) have specifically addressed this issue in NMR conditioning by using morphine and naloxone, while using an auditory CS at various intensities. Morphine significantly depressed the probability for CR expression with different intensities of a tone CS. Naloxone completely antagonised this effect. Haloperidol (Harvey and Gormezano, 1981) and scopolamine (Harvey *et al.* 1983) were also found to increase the CS threshold and decrease the rate of acquisition. Gormezano and Harvey (1980) reported that LSD considerably *lowered* the CS threshold for CR elicitation, and also increased the rate of acquisition. Schindler *et al* (1984) have examined the relationship between drug influences on acquisition and drug-induced shifts in auditory tone thresholds for eliciting CRs across a range of studies. There was a linear relationship between the number of trials to criterion and magnitude of the drug induced shifts in CS tone thresholds. While these effects were found with drugs injected systemically, it is also possible that such effects may occur on an auditory CS when drugs such as muscimol are injected intracranially. Muscimol infusions into the cerebellum may also have induced such shifts in the CS threshold through actions on the auditory system. This possibility is discussed in detail later.

2.1.1.2 How have drugs affected extinction in NMR conditioning?

Extinction learning is prone to drug effects on CS processing that may confound drug effects on plasticity. However, extinction learning presents an opportunity to examine such effects on the CS in the absence of effects on the US. Cholewiak *et al.* (1968) investigated the effects of strychnine on NMR conditioning, which significantly potentiated acquisition relative to saline controls (phase 1). Subjects were then given extinction training (phase 2). Those which received saline in phase 1 and received strychnine in phase 2, showed potentiated CR frequencies in which there was resistance to extinction. If subjects received strychnine in phases 1 and 2, there was no extinction of behaviour in phase 2 - CRs were maintained at a high level throughout extinction

training. Strychnine in phase 1 and saline in phase 2 resulted in normal extinction from a comparatively high CR frequency. These results strongly suggest that there were no state-dependent effects, but strychnine may have depressed the CS threshold since CR frequencies under strychnine were resistant to extinction whether they had been acquired under strychnine or saline.

The effects of LSD on auditory CS thresholds have already been reported. LSD is known to increase the rate of acquisition in NMR conditioning, but there is little or no effect of LSD on the ability of the US to elicit a UR. As discussed above, in contrast to this, the intensity threshold at which the CS elicits a CR is considerably reduced (Gormezano and Harvey, 1980; Gormezano, Harvey and Aycock, 1980). This demonstrates that increased acquisition rates are probably attributable to altered perception of the auditory CS intensity. Schindler, Gormezano and Harvey (1986) reported that rabbits given acquisition under LSD (phase 1) reached asymptotic levels of CR frequency much more quickly than controls. Further acquisition (phase 2) without LSD found CR frequency to be at greatly reduced, pre-asymptotic levels at the beginning of Phase 2, implying slight state-dependency effects due to drug effects on the CS. In a different group, there was no dissociation between phase 1 and phase 2 if LSD was also given in phase 2. Subjects given acquisition under LSD in phase 1 and then extinction under LSD in phase 2 (LSD-LSD), acquired quicker than controls in phase 1, but extinguished at the same rate as controls in phase 2. Those given LSD in phase 1 but no LSD in phase 2 (LSD-control), also acquired quickly in phase 1 but showed baseline CR frequencies throughout phase 2. These were maintained at a constant level throughout extinction training. In contrast, subjects given no LSD in phase 1, but LSD in phase 2 (control-LSD) acquired and extinguished normally. This demonstrates substantial state-dependency effects in which there is a dissociation between the drug and no-drug state, but only if the drug state was followed by no-drug state. This is in keeping with findings of many other studies using other models of learning, in which CRs learned normally generalise to drug conditions which follow, but CRs learned in the drug condition are

generally not retained in the following no-drug condition (Overton, 1991). The mechanisms of such effects are not clear, but may be strongly related to altered CS processing.

In order for state-dependency to occur by altered auditory processing, there should be evidence that the cerebellar activity has a bearing upon auditory processing, since drug-induced changes in CS perception are likely to be the basis of SDL. Traditionally, the cerebellum has been regarded as a structure involved in the control of movement. It is widely accepted that the cerebellum *uses* sensory information to guide movement (Stein and Glickstein, 1992; Snider and Stowell, 1944), consistent with the massive amount of sensory input and output which correlates with motor expression. But what of the role of the cerebellum in the *regulation* of sensory information? The view that the cerebellum is involved in the regulation of sensory information processing is supported by an old and substantial literature.

One of the earliest studies to investigate this possibility was that of Wolfe (1972), in which depression of activity recorded in the cat posterior vermis under low doses of pentobarbitone resulted in correspondingly enhanced evoked potentials to pure tone stimuli in the auditory cortex. Wolfe and Kos (1975) then examined the effects of cerebellar interventions on auditory function in rhesus monkey. This study used conditioned shock avoidance to demonstrate that lesions of the posterior cerebellar vermis resulted in permanently decreased thresholds of responsivity to auditory stimuli. Such results suggest that the general effects of cerebellar lesions are to remove inhibition on the auditory system, including the auditory cortex. The effects of cerebellar interventions also have effects at much lower levels of the auditory system. Velluti and Crispino (1979) have found that electrical stimulation of the guinea pig anterior vermis or the simplex lobule of cerebellar cortex decreased the amplitudes of cochlear microphonic responses and of action potentials in the auditory nerve in response to both click and pure tone pip acoustic stimuli. The influences that pre-

receptorial components would have on auditory nerve action potentials were eliminated by curarising the middle ear muscles and delivering the stimuli directly to the bulla, and so central control of middle ear muscles and auditory nerve potentials could be assessed independently. The extent of the depression varied in proportion to the amount of stimulation. Cooling the cerebellar cortex resulted in increased amplitudes of auditory nerve action potentials and cochlear microphonic responses. Inhibiting the cerebellar cortex enhances responsivity in the lowest levels of the auditory system, and activity in the cerebellar cortex reduces responsivity of the auditory system at these levels. A study by Misrahy *et al.* (1961) examined the effects of cerebellar interventions on acoustically driven myoclonic reflex responses of the cat forearm under chloralose anaesthesia. Electrical stimulation of the anterior vermis of cerebellar cortex greatly diminished or abolished this response. A higher pulse stimulation rate was more effective in inhibiting responses than lower rates of stimulation. This study provides evidence that cerebellar activity inhibits acoustically driven reflexes.

Teramoto and Snider (1966) have complemented Snider's identification of sensory (including auditory) receiving areas of the cerebellar cortex by demonstrating that the same areas affect sensory processing in remote, central sensory structures. They have found that stimulation of the *tuber vermis* in cat resulted in responses evoked in the ipsilateral lateral inferior colliculus, the ventral portion of medial geniculate nucleus, mesencephalic reticular formation and the auditory cortex. When animals were given auditory click stimuli, the there was a biphasic response in the inferior colliculus that was depressed by cerebellar electrical stimulation. Cerebellar stimulation depressed these responses for 100ms after the end of stimulation. In the medial geniculate nucleus, click stimuli resulted in a negative wave with an onset latency of 4ms, followed by a slow positive wave. Stimuli applied to the cerebellar cortex caused the medial geniculate nucleus response to click stimuli to be depressed. Click-evoked responses in the midbrain reticular formation consisted of a negative wave followed by a positive one. Cerebellar stimulation resulted in these being depressed. Stimulation of the

cerebellar cortex also resulted in click-evoked responses being depressed in the auditory cortex. Such results clearly show that cerebellar activity is capable of modulating acoustically evoked activity in circuitry known to include major sites of auditory processing.

In a more recent study, Crispino and Bullock (1984) have also studied the effects of cerebellar interventions on auditory processing in central pathways. This study examined the effects of cerebellar stimulation on stimulus processing in different sensory modalities. In order to examine effects on auditory processing, auditory responses were recorded as far-field auditory brainstem responses (ABR's). Auditory click stimuli are known to provoke a series of evoked ABRs of different latencies. The earliest to occur, Wave I, reflects the compound action potential of the cochlear nerve. Waves II and III reflect activity from the cochlear nucleus, superior olive and lateral leminiscus. Wave IV reflects activation in the inferior colliculus. The cortical component of this response occurs well after these and was not studied in this experiment. Cerebellar stimulation reduced the amplitudes of components of the ABR. However, each was differentially affected and Wave I was affected the most. This study provides further evidence that activity in the cerebellar cortex results in the changes in auditory processing at several loci of the auditory system. While such effects are physiologically unambiguous, the anatomical basis of cerebellar influences upon auditory processing are less clear and sometimes controversial.

Henneman, Cooke and Snider (1950) reported the existence of cerebellar projections to the auditory areas of the cerebral cortex. Steriade and Stoupel (1960) have confirmed these projections electrophysiologically. Some have suggested that the cerebellum exerts its influence on the auditory system though its action on the olivo-cochlear bundle (OCB) - a pathway which originates in the ipsilateral and contralateral superior olive and terminates in the ipsilateral hair cells of the organ of Corti. Rossi *et al.* (1967) reported the existence of fibres originating from the cerebellum and projecting to the

cochlear nuclei, the retro-olivary nucleus and the pre-olivary nuclei. This study assessed the degeneration in auditory pathways induced by cerebellectomy and large cerebellar lesions. However, the results of this study were not universally accepted. Gacek (1973) attempted much more detailed analysis, and reported projections from the posterior cerebellar vermis to the fastigial nuclei, and on to the ventral cochlear nucleus. Other pathways from the fastigial nuclei have also been proposed. Carpenter, Brittin and Pines (1958) found projections from the fastigial nuclei in the cerebellum to the inferior colliculus and medial geniculate nucleus.

These studies clearly demonstrate that

i) NMR conditioning is susceptible to state-dependency effects by way of drug action on CS processing.

ii) auditory processing may be altered by cerebellar interventions.

Taken together, such studies reveal the potential for muscimol to induce state-dependency effects by way of its action on cerebellar circuitry. This possibility is explored further in the Discussion of this chapter.

The purpose of the present study is, therefore, to examine the role of the AIP in extinction of NMR conditioning. After acquisition training, experimental subjects were given a period of extinction training during inactivation of AIP with a low dose and volume of muscimol, and then a period of extinction training without AIP inactivation. Control subjects received the same behavioural training but without the muscimol blockade. Results are considered in the context of impaired plasticity and muscimol-induced SDL, since either or both of these could be involved.

2.2 Methods

Methods common to all chapters are reported in detail in Appendix I.

2.2.1 Subjects

Subjects were 15 male Dutch belted rabbits weighing from 1.8kg to 2.5kg. They were housed individually, allowed *ad libitum* food and water and maintained on a 12hr light/dark cycle.

2.2.2. Surgery

Nine rabbits underwent surgery for implantation of a guide cannula directed towards the right AIP. Surgical procedures are reported in Appendix 1. The approximate position of the cerebellar nuclei was defined stereotaxically (λ-5mm AP, λ+4mm ML, λ-10mm DV), and their exact position was then determined by electrophysiological recording using tungsten microelectrodes. A 24G stainless steel cannula guide was then implanted 1mm caudal and 1mm dorsal to the position of the recorded cells. All subjects were allowed 2 weeks for recovery.

2.2.3 Preparations for Conditioning

All cannula-implanted and non-operated control rabbits were prepared for measurement of nictitating membrane movements (details in Appendix 1).

2.2.4 Conditioning and Stimuli Parameters

The conditioned stimulus (CS) was a 1 kHz sine wave tone of 310 ms duration and an intensity of 63 dBA (re $20\mu N.m^{-2}$). Background noise produced by ventilation fans was 54 dBA (re $20\mu N.m^{-2}$). The unconditioned stimulus (US) was periorbital electrical stimulation. Each US was a 60ms train of 3 biphasic pulses of intensity 2.5 mA applied to the periorbital region of the face through the stainless steel clips described in

Appendix 1. On paired trials the interstimulus interval between the CS and US onset was 250 ms. The intertrial interval was randomly selected between 25-35 seconds.

2.2.5 Behavioural Training

2.2.5.1 Session Types

There were 3 different types of sessions.

(i) Adaptation: Subjects were placed in the restraining stock, fitted with the nictitating membrane transducer cradle and the Michel clips (see Appendix 1) and placed in the conditioning chamber for one hour - equivalent to the duration of one conditioning session. There were no presentations of either the CS or the US during this period.

(ii) Acquisition training: Subjects were placed in the restraining stock, fitted with the nictitating membrane transducer cradle and placed in the conditioning chamber. Each session consisted of 100 trials. In 90 trials the CS and US were paired and in 10 trials the CS was presented alone. A CS alone trial was presented on every tenth trial.

(iii) Extinction training: Subjects were placed in the restraining stock, fitted with the nictitating membrane transducer cradle and placed in the conditioning chamber. Each session consisted of 100 trials. The CS was presented alone on every trial.

2.2.5.2 Experimental Design

Two weeks after surgery, a monofilament loop was sutured in the right nictitating membrane and three days later all subjects underwent a session of adaptation. On the following day, they entered the experiment in Phase 1. There were 3 Phases, and three days between each phase.

(i) Phase 1. All subjects received 4, daily sessions of acquisition.

(ii) Phase 2. All subjects received 4, daily sessions of extinction training. Experimental subjects received muscimol infusion prior to each session. 1.54 nanomoles of muscimol in 1μl of solution (muscimol hydrobromide, RBI, in 50 mM phosphate buffered saline, pH7.4) was injected over one minute into the right cerebellum of each cannulated subject. This dose was based on prior pilot studies, conducted to establish a dose which was as low as possible, but high enough to be effective at sites close to, but not in, AIP. The injection was via a 36G cannula inserted through the 24G implanted guide cannula and protruding 0.5 mm below the tip of the guide. The target of the injection was therefore 0.5 mm above cells with activity related to eyeblinks. The cannulated subjects were then left for one hour in their home cage before training.

(iii) Phase 3. All subjects received 4, daily sessions of extinction training. If experimental subjects had extinguished in phase 2, they would show the same frequency of CRs as control subjects in Phase 3. However, if muscimol infusions prevented extinction, then subjects would extinguish at the same rate as controls extinguishing for the first time (control subjects in phase 2).

2.2.6 Subject Groups

Nine subjects underwent surgery in which a guide cannula was implanted above the right cerebellar nuclei. 5 subjects in the cannulated group had very low frequencies of CRs during the first extinction session with muscimol blockade in Phase 2 (see Results) and so were assigned to the Effective Group. The remaining 4 animals had much higher CR frequencies during this session (see Results) and were assigned to the Ineffective Group (N=4). The control group consisted of 6 subjects.

2.2.7. Histology

At the end of the experiment rabbits with implanted cannulae were injected with heparin sodium (500 $Units.kg^{-1}$, i.v.) and an overdose of pentobarbitone sodium (90 $mg.kg^{-1}$, i.v.). Each rabbit was perfused through the aorta with 0.9% saline followed by

4% formaldehyde and the brain was removed from the skull. The brain was then embedded in 10% gelatin and placed in a solution of 20% sucrose formalin for 3 days. 60μm thick frozen sections were cut in the transverse plane. Alternate sections were mounted onto gelatinized slides and stained for Nissl substance with cresyl violet.

2.2.8. Data analysis

2.2.8.1 Conditioned Responses

A conditioned response (CR) was defined as a nictitating membrane response with amplitude greater than or equal to 0.5mm and with onset latency greater than 35ms from CS onset (Hardiman and Yeo, 1992). All responses occurring from CS onset to 1000ms after CS onset were recorded. Responses on paired and unpaired trials occurring between 35ms and 250ms from CS onset were defined as CRs. The frequency of responses whose latency to onset was between 250ms and 1000ms in the extinction phases was calculated for each group, in order to determine whether response onset latency changed during extinction training. CR frequencies were expressed as the percentage of CRs expressed a function of the number of trials presented. In order to show within-session effects, data were plotted graphically in 10 trial blocks (9 paired trials + 1 CS alone trial for Phase 1, and 10 unpaired trials for Phases 2 and 3). Where necessary, within-session frequency graphs were superimposed on block frequency graphs. In Phase 1, data from the paired and unpaired trials were treated similarly, (i.e. occurrence of CRs could only be determined within the CS-US interval). We applied the same analysis to extinction learning trials (Phases 2 and 3) which, although consisting entirely of CS alone presentations, were analysed for CR frequency during the same time window. Statistical analysis was performed on session and block CR frequency. CR topography was assessed by observing the distributions of latencies to peak amplitude of CRs occurring between 35ms and 1000ms after CS onset (see appendix 1, figure A1.1).

2.2.8.2. Data analysis

A-priori comparisons of CR frequencies of groups across blocks and sessions were made using 3-Way Analysis of Variance (ANOVA). In addition, a 2-Way Repeated Measures ANOVA and Newman-Keuls test were applied for *post-hoc* analysis where required. Latencies to peak amplitude were examined using frequency histograms. Some control data from Phase 1 is incorporated into Chapter 4, and so Phase 1 histogram analyses of CR topographies are reported in chapter 3, since these are concerned with acquisition. Phase 2 and 3 histograms are reported in the current chapter, since these are concerned with extinction.

2.3 Results

The principal finding of this study was that muscimol infusions into the interpositus nucleus completely prevented the extinction of conditioned responses.

2.3.1 Histological verification and group assignment

There were nine cannulated subjects. 5 cannulated subjects (E1-E5) were included in the Effective Group. They showed very few CRs under muscimol inactivation during the first extinction session of Phase 2 (range 0%-2.5%). Cannula placements of these subjects were located between approximately 0.5mm and 1.5 mm from anterior parts of the interpositus nucleus (see figures. 2.1 and 2.2). In 4 of these subjects (E1 - E4), the guide cannulae traversed white matter, slightly dorsal to or just below the surface of posterior parts of the interpositus nucleus. Damage in this area, including posterior parts of the interpositus nucleus, does not impair conditioned responses (Yeo, Hardiman and Glickstein, 1985a). In one other case (E5), the cannula tip was located in the caudal aspect of the interpositus nucleus.

The remaining 4 subjects showed high CR frequencies in session 1 of Phase 2 (range 42% - 100%). Cannula placements of these subjects were located approximately between 2 mm and 5 mm from AIP. Placements in two of these subjects were located close to the surface of the cerebellum, in the white matter between vermis and paramedian Lobe, dorsal to AIP (I6 and I7). In another subject, a cannula tip was located in the cerebellar cortex of the Paramedian Lobe (I8), and another at the base of Lobule VI of the cerebellar cortex (I9). These subjects constituted the Ineffective Group.

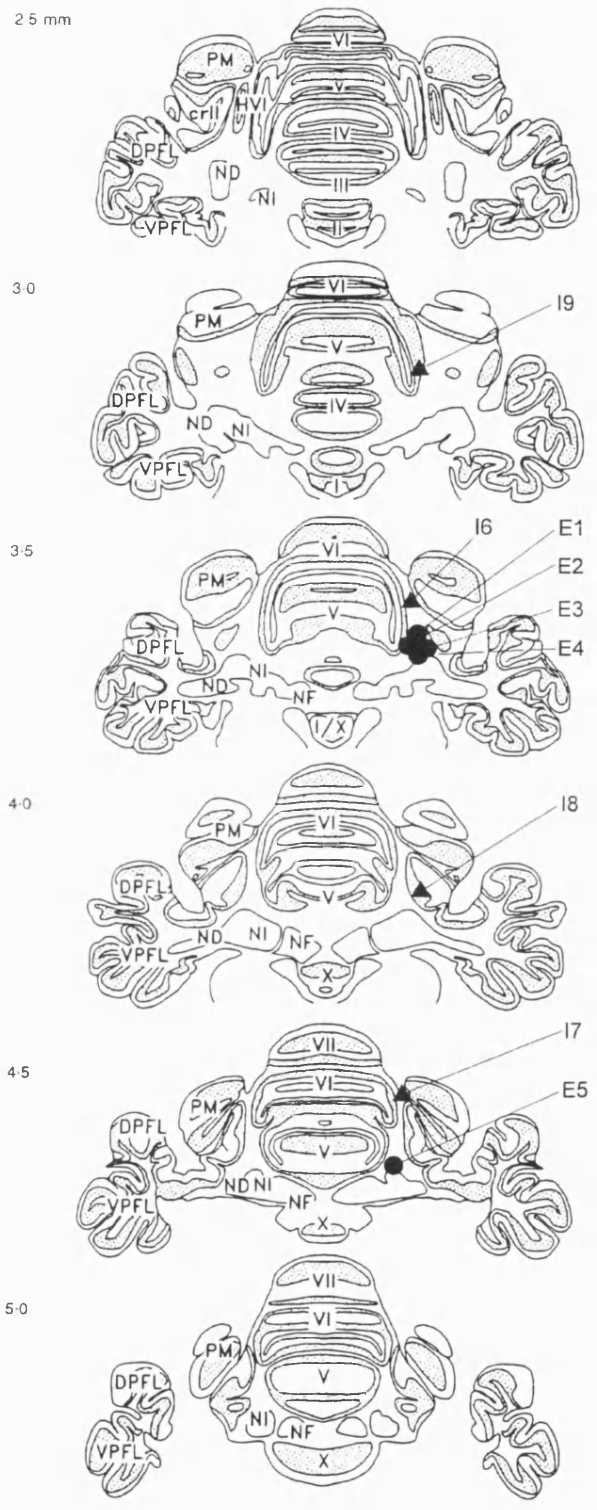


Figure 2.1: Reconstructions of cannulated subjects. Transverse sections through the cerebellum and brainstem at 0.5mm intervals. Section identifier is distance from λ. Each symbol represents the site of injection in a cannulated subject. Black circle, effective placements; black triangle, ineffective placements. Identities of individual subjects, see text. DPFL, dorsal paraflocculus; crII, crus II; HVI, lobule HVI; ND, dentate nucleus; NF, fastigial nucleus; NI, interpositus nuclues; PM, paramedian lobe; VPFL, ventral paraflocculus.

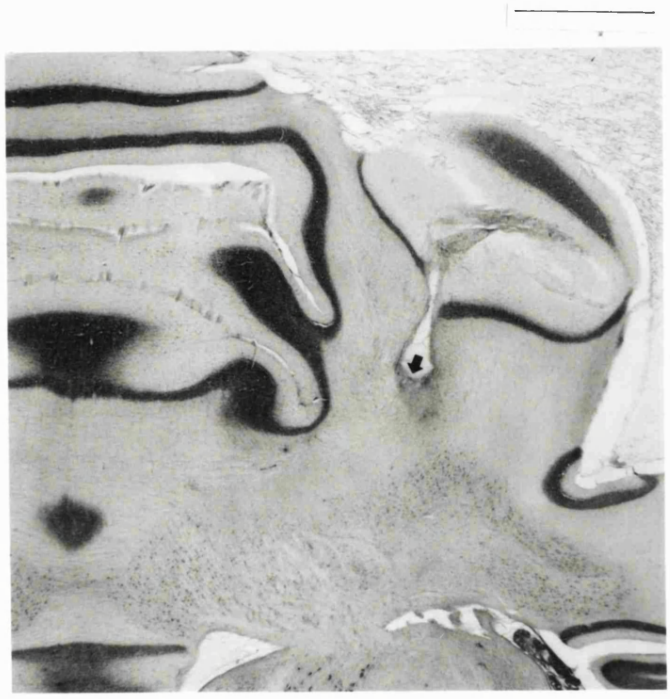


Figure 2.2:

Photomicrograph of transverse, Nissl stained section through the cerebellum and brainstem of subject E1 (approximately 3.5mm caudal to lambda). The cannula track penetrated through paramedian lobe, terminating just above lateral parts of posterior interpositus nucleus. Bar, 2mm.

Arrow marks the position of the cannula tip.

2.3.2 Behavioural Results

All probability values result from Analysis of Variance tests unless stated otherwise.

Phase 1

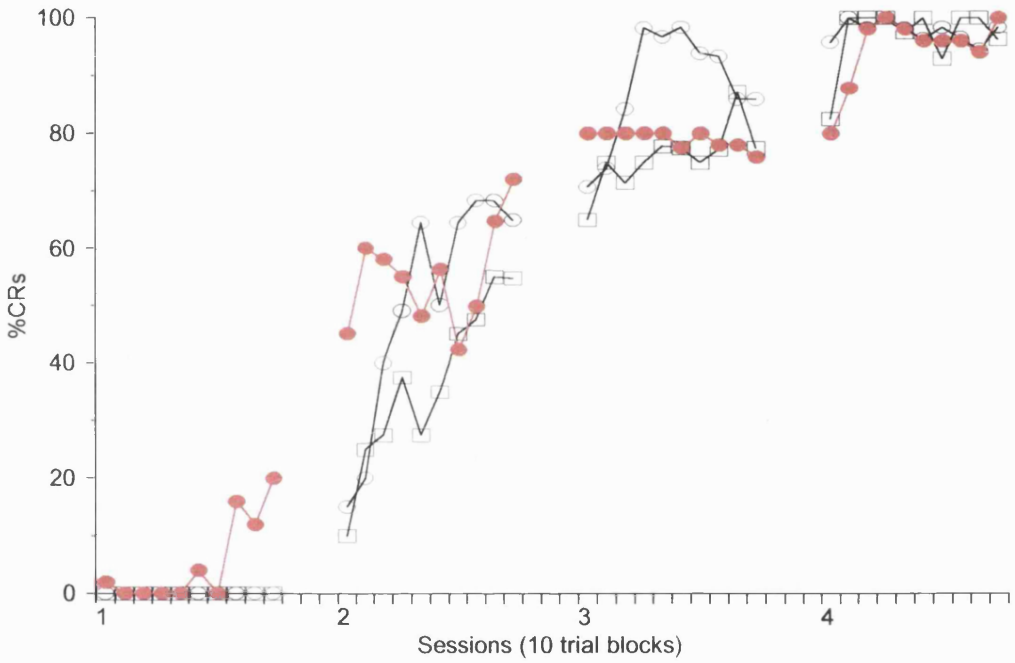


Figure 2.3: Each marker represents CR frequency across a block of 10 trials. Closed red circles joined by red lines, represent the Effective Group. Open circles represent the Control Group, and open squares represent the Ineffective Group.

There were significant effects of session ($F(3,33)=40.5$; $p<0.05$) and block ($F(9,18)=6.15$; $p<0.05$), consistent with increases in CR frequency during learning. Groups ($F(2,11)=0.26$, $p>0.05$) and all interactions were not significant factors, indicating that the three groups learned at similar rates (figure 2.3).

Phase 2

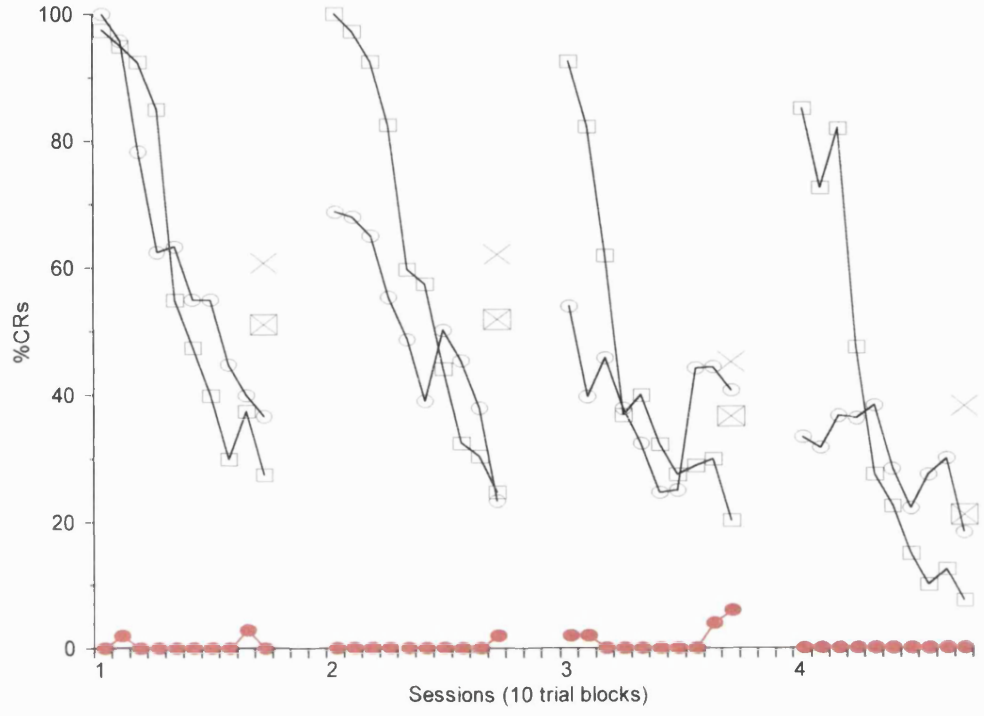


Figure 2.4: Extinction training during muscimol blockade. Closed red circles joined by red lines, Effective Group; open circles joined by black lines, Control Group; open squares joined by black lines, Ineffective Group. Large markers at end of each session represent mean session CR frequencies. Crossed squares, Control Group; crosses, Ineffective Group. CRs in the Effective Group are virtually absent in Phase 2, and so the data points are close to the x-axis.

Before each session of Phase 2 all cannulated subjects received injections of muscimol. Figure 2.4 shows that subjects in the Effective Group produced very few CRs during this phase (0.52%).

Subjects in the Control Group showed a pattern of extinction typical for this type of conditioning. There was no significant group x session interaction between the CR frequencies of the Ineffective Group and the Control Group ($F(3,24)=0.22$; $p>0.05$), but there was a significant group x block interaction ($F(9,72)=7.10$; $p<0.05$) for these groups. Therefore, subjects in the Ineffective Group extinguished normally over sessions (see

session CR frequencies in figure 2.4), but had different patterns of CR frequencies within the sessions. Inspection of figure 2.4 reveals that the Ineffective Group had elevated levels of CR expression at the beginning of each session (see *block* CR frequencies in figure 2.4).

Phase 3

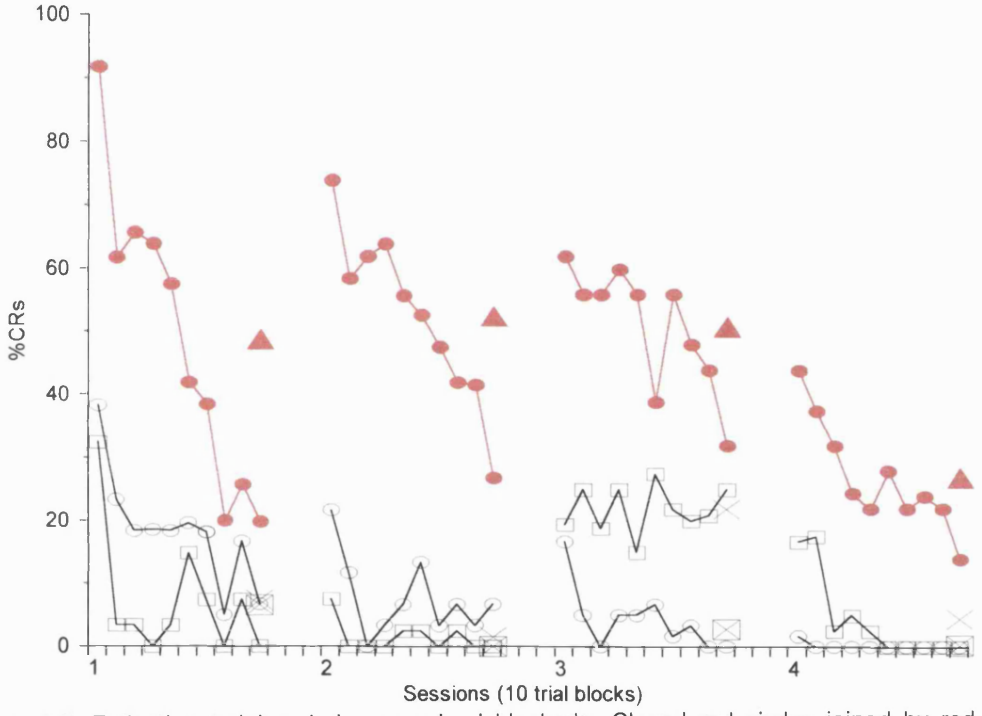


Figure 2.5: Extinction training during muscimol blockade. Closed red circles joined by red lines, Effective Group; open circles joined by black lines, Control Group; open squares joined by black lines, Ineffective Group. Large markers at end of each session represent mean session CR frequencies. Closed red triangles, Effective Group; Crossed squares, Control Group; crosses, Ineffective Group. CRs in the Effective Group are virtually absent in Phase 2, and so the data points are close to the x-axis.

All subjects were given continued extinction training for 4 more sessions (figure 2.5). The cannulated subjects did not receive muscimol in this phase.

At the beginning of session 1, the Ineffective Group and Control Group showed low CR frequencies which extinguished to baseline levels by session 4. However, subjects in the Effective Group showed very high CR frequencies, which then extinguished with continued training (see comparison with control group below). There were significant main effects for

groups ($F(2,11)=8.14$; $p<0.05$), confirming that, CR frequencies were different between the three groups.

Consistent with the very low session CR frequencies in both the Ineffective and Control Groups in this phase, the main effect for sessions was not significant ($F(3,33)=2.58$; $p>0.05$), and there was no significant interaction between groups and sessions ($F(6,33)=1.44$; $p>0.05$). The significant main effect for blocks ($F(9,99)=10.55$; $p<0.05$), and significant interaction between groups and blocks ($F(18,99)=3.22$; $p<0.05$) clearly relate to large, within-session differences across groups - the cannulated group extinguished from high CR frequency to a low CR frequency, in contrast to the CR frequencies of the Ineffective and Control groups, which showed low levels of extinction within each session.

By inspection of figure 2.5, it is clear that the main effect of groups was due to greater CR frequencies produced by the Effective Group. Over sessions, a Student-Newman-Keuls test revealed that there was no significant difference between the Ineffective Group and the Control Group (2-Way ANOVA, $F(2,58)=0.025$, $p<0.05$; Student-Newman-Keuls, $p=2$, $q=0.04$, $p>0.05$), but there was a significant difference between the Effective and Control Groups (Student-Newman-Keuls, $p=3$, $q=4.07$, $p<0.05$), and between the Effective and Ineffective Groups (Student-Newman-Keuls, $p=2$, $q=3.60$, $p<0.05$).

Comparison of Effective Group (Phase 3) and Control Group (Phase 2)

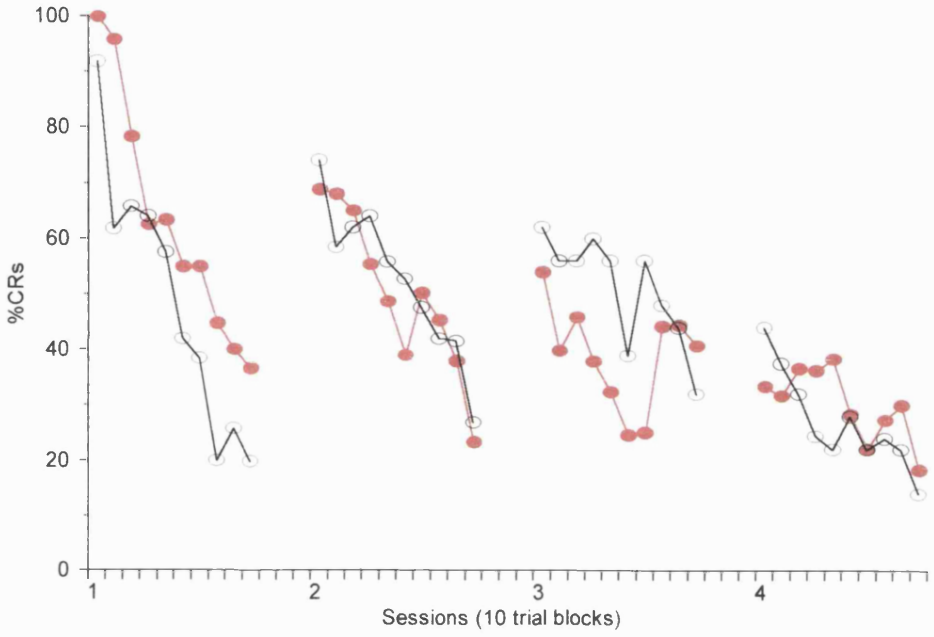


Figure 2.6: Extinction training during muscimol blockade. Closed red circles joined by red lines, Effective Group; open circles joined by black lines, Control Group; open squares joined by black lines, Ineffective Group.

A comparison between Effective Group in Phase 3, and Control Group in Phase 2 (figure 2.6), revealed no significant main group effect ($F(1,9)=0.2$, $p>0.05$). There were also no group x session ($F(3,27)=1.29$, $p>0.05$) or group x block interactions ($F(9,81)=0.7$, $p>0.05$). There were significant block ($F(9,81)=16.7$; $p<0.05$) and session ($F(3,27)=4.76$; $p<0.05$) main effects. Therefore, subjects in the Effective Group extinguished at a rate very similar to that of Control Group subjects extinguishing for the first time .

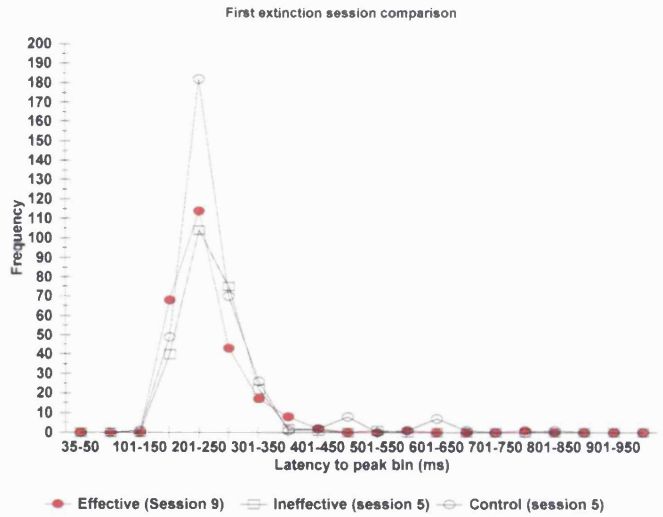
CR topography

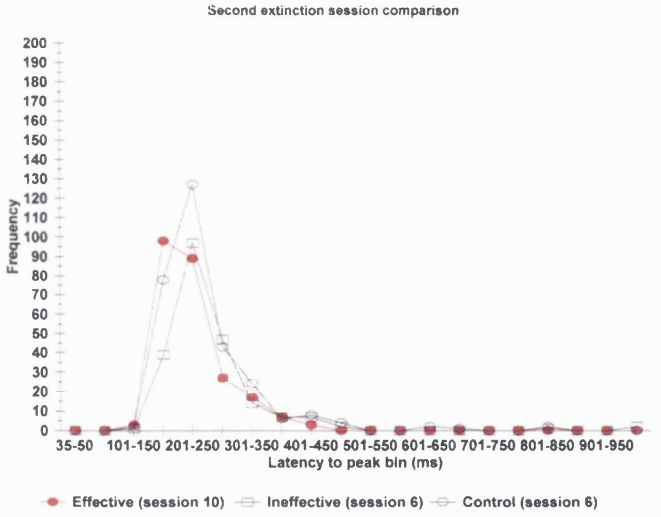
Effective Group subjects in phase 3 showed peak latency distributions similar to those of Ineffective and Control Group subjects in phase 2 (figure 2.7). These distributions were similar in all sessions, the peaks occurring between 151-250ms. It is evident that at the beginning of extinction, the peak started in the 201-250ms bin and in the last extinction session, had shifted 50ms earlier to the 151-200ms bin. The timing of CRs during extinction in control animals extinguishing for the first time, and animals extinguishing in the Effective Group in Phase 3 was comparable.

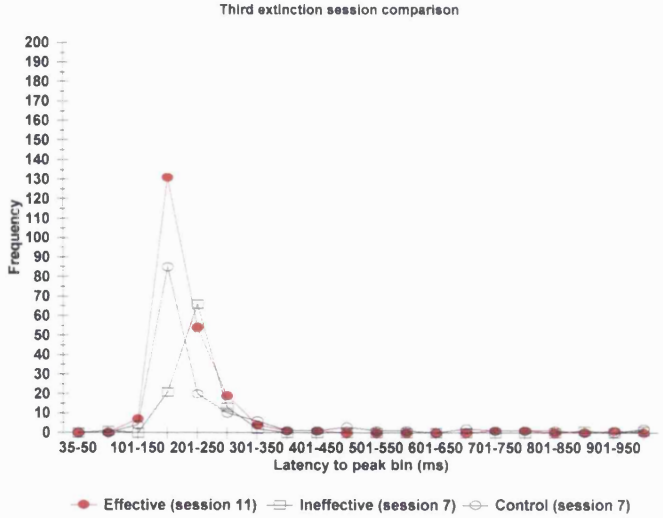
It is interesting to note that muscimol infusions in subjects in the Ineffective Group did not appear to affect timing of CR latencies to peak, since their distributions appeared to be very similar to those of the Control Group.

This analysis shows that while extinction progressed, the timing of CRs remained similar between groups, and consistent between sessions. Distributions of all groups in each extinction session are shown in Appendix 3 as supplementary data.

Distributions of latencies to peak
Comparisons between phases 2 and 3







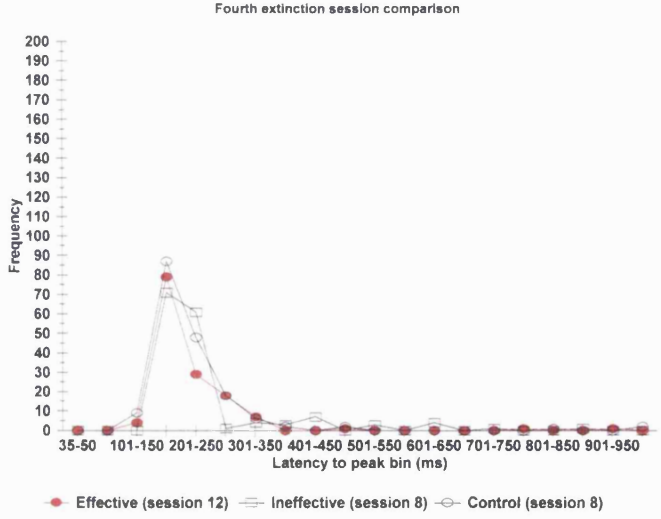


Figure 2.7: Session by session comparison of Phases 2 and 3.
In each group, CRs from all subjects are pooled to contribute to distributions of latencies to peak.

Analysis of responses occurring between 250ms and 1000ms in Phases 2 and 3.

The number of responses occurring after stimulus offset was very low. The tendency for CR onset latency to extend beyond 250ms in the extinction phases was negligible.

	Control	Ineffective	Effective
Phase 2	1.04 (2.11)	0.95 (1.65)	2.1 (3.08)
Phase 3	0.4 (0.64)	1.27 (1.25)	0.5 (1.52)

Table 2.1: Frequency of responses occurring between 250ms and 1000ms during extinction. Percentages (standard deviations in brackets) as a function of the number of trials.

In summary, these results show that the muscimol blockade of the cerebellum appears to prevent the extinction of conditioned NMRs. In terms of CR frequency and timing, CRs that emerge after the blockade are indistinguishable from CRs of control subjects extinguishing for the first time.

Discussion

It is now well established that destructive lesions of the cerebellum and its associated circuitry can abolish or impair conditioned responses of the nictitating membrane. Whether cerebellar lesions impair learning or whether they produce deficits in execution of conditioned responses has been controversial. The results of the present experiment demonstrate that deficits in the execution of conditioned responses occur when normal activity in the AIP is prevented. They further demonstrate that such deficits are not sufficient to explain why CRs are absent in the post-inactivation phase. Possible effects of cannula damage and extended muscimol effects on conditioning can be ruled out. This study clearly shows that extinction of the classically conditioned NMR is prevented by cerebellar inactivation and so is critically dependent upon the cerebellum.

Drugs may have three kinds of effect on classical conditioning. Firstly, they may impair motor expression of CRs through effects on motor systems. Secondly, they may alter the sensory properties of the CS and US, which would in turn affect learning and performance of CRs. Thirdly, they may impair learning-related plasticity (Schindler and Harvey, 1990). The results of this study are considered in the context of these possibilities.

Does muscimol infusion in the cerebellar nuclei affect the motor expression of CRs ?

All subjects in the effective group, with cannula locations close to the AIP, were trained to asymptotic levels of CR frequency in Phase 1 before administration of the muscimol. Since the cannulated groups acquired indistinguishably from controls, cerebellar circuitry essential for NMR conditioning in these subjects was intact. Cannula damage

to such circuitry can therefore be ruled out. In the Effective Group, muscimol infusions in Phase 2 completely abolished the motor expression of CRs. However, the high frequency of CRs present immediately upon testing in Phase 3, after the effects of muscimol had dissipated, indicates that there were no deficits of motor expression extending into Phase 3. This validates the reversible cerebellar lesion technique as free from performance deficits during the post inactivation phase, at the muscimol dose used in this study.

The Ineffective Group extinguished at the same rate as the Control Group across sessions, but showed enhanced expression of CRs at the beginning of each session in Phase 2, though not in Phase 3. The cannula placements in this group were not as close to AIP as those in the Effective Group and most were dorsal to it. Muscimol infusions would have invaded cortical areas dorsal to AIP more quickly in the Ineffective Group and agonised GABA-A mediated transmission by cortical interneurons. It is possible that muscimol spread was not optimally localised to block cortical function in all NM response-related areas but was sufficient to produce some disinhibition of the NM-related areas in the cerebellar nuclei resulting in small enhancements of reflex expression and thus the enhanced CR expression. The increased CR frequency relative to controls could, therefore, be attributed to increased excitability of motor pathways (however, see below), and may be similar to "performance" changes reported in other studies (Hardiman and Yeo, 1992; Gruart and Yeo, 1995).

Does muscimol infusion in the cerebellar nuclei affect sensory properties of the CS or US?

We have considered and rejected the possibility of cannulation damage and residual muscimol effects upon motor expression in the critical post-inactivation sessions. The remaining possibility, other than that the drug impaired learning-related plasticity, is that the drug affected the sensory properties of the CS and US. In the present extinction learning study, there is no presentation of the US during the muscimol phase, so any

possible effects that muscimol may have upon the sensory properties of the US would not be relevant to extinction learning. However, it is possible that such effects would also extend to US processing in acquisition, a possibility considered in the next chapter.

If muscimol affected the sensory properties of the CS, then SDL may have occurred (see Introduction). Here, subjects learn in the drug condition but cannot retrieve this information unless they are returned to the drug condition. If a drug alters the properties of the CS, subjects will first associate the CS and US. But then, after the drug effects have dissipated, the CS will be perceived as being novel, and subjects will need to re-learn the association in the drug-free state. Two independent sets of associations may therefore be formed - one in the drug condition, and the other in the no-drug condition.

To observe SDL, behaviour in the drug condition is often compared with behaviour afterwards in the no-drug condition. However, in our study, CR performance was abolished by the drug in the Effective Group so overt behaviour during muscimol inactivation could not be observed. If SDL had taken place, then subjects in the Effective Group may have extinguished covertly under muscimol in a state dependent manner in Phase 2, and may not have shown retention for the extinction of CRs because there was no muscimol in Phase 3. Thus, CRs may have been unextinguished at the beginning of Phase 3. Alternatively, subjects may not have extinguished in Phase 2 because muscimol inactivation of the cerebellum impaired learning-related plasticity. Evidence has been reported earlier which suggests that NMR conditioning is susceptible to drug effects on the CS, and that cerebellar interventions result in altered auditory processing.

It is possible that in the present study, intracerebellar injections of muscimol affected auditory processing. In the Effective Group, the action of muscimol would have been complete. If this occurred, then the perception of the CS in phase 2 would be different from that of phases 1 and 3. Effective group subjects in this study would not have

extinguished in phase 2 because muscimol may have altered the CS in some way. If state-dependency occurred because the properties of the CS were qualitatively changed in phase 2 for the Effective Group, then impaired plasticity and state-dependency effects would yield the same result in phase 3 (no evidence of extinction in phase 2). Is this account of state-dependency compatible with observations of specific drug/no-drug 'transfer effects' common some other studies? The LSD study of Schindler *et al.* (1986) clearly shows that CRs in the no-drug state generalise to the subsequent drug state, but CRs in the drug state do not generalise to a subsequent no-drug state, thus inducing a dissociation. In the present study, state-dependency would not have occurred between phases 1 and 2, but would have occurred between phases 2 and 3. So CRs acquired normally in phase 1 would have extinguished normally in phase 2 if plasticity was unaffected. What was the status of these CRs in phase 3? The CRs acquired in phase 1, having extinguished in phase 2 would have been at very low levels throughout phase 3, and so no state-dependency effects would have occurred. In this event, CR frequency of the Effective Group would be low at the beginning of phase 3. Since CR frequencies were at high, and not low levels, drug/no-drug transfer effects were absent. State-dependency effects of this type did not occur. Either plasticity was impaired, or muscimol induced state-dependency effects through altered auditory processing in phase 2.

There is no study which has specifically reported anatomical projections from AIP to the auditory system, and so if muscimol had effects on auditory processing by acting only at AIP, these are likely to have occurred through indirect projections. However, muscimol infusions in Ineffective subjects (more dorsal placements) may have depressed activity in cortical areas (auditory receiving areas of Snider and Stowell, 1944) such as anterior vermis and vermian simplex (Larsell's lobule VI), resulting in a decreased auditory threshold and possibly changing the qualitative properties of the stimulus. This would result in the observed increased rate of CS evoked conditioned NMRs relative to controls. If muscimol spread to such areas beyond AIP in the Effective Group subjects,

then auditory processing may also have been affected in this group. However, would these effects have resulted in SDL? The studies reported above clearly demonstrate that cerebellar cortical activity, affects auditory processing at many levels of the auditory system, generally depressing activity in these pathways. There is scope for both intensity and auditory frequency coding of the CS to be altered by cerebellar interventions. In NMR conditioning, there is no evidence that the specificity of the CS *intensity* is learned. Frequency-specific CRs are produced to an auditory CS at *all* detectable intensities (Moore, 1972). So, if muscimol simply affected the perceived *intensity* of the CS in the Effective Group, then muscimol is unlikely to have induced state-dependent effects. If, however, muscimol induced other, CR specific changes (e.g. perceived frequency changes), then state-dependent effects are more likely to occur because in effect, the same auditory stimulus would be processed as a 'new' CS. Data from the Ineffective Group shows that during Phase 2, subjects showed initially elevated CR frequencies. In this group, the perception of the CS intensity could have been affected, but CR-specific properties, were left unaffected. These CRs were well timed and extinguished normally despite initially elevated CR frequency. Also, there was no dissociation between Phases 2 and 3 in the Ineffective Group confirming that SDL did not occur in this group. If muscimol had produced SDL effects in the Ineffective Group, then subjects would have extinguished in Phase 2, and would show no signs of having extinguished in Phase 3 (i.e. high CR frequencies which would have declined with further training). However, there was a continual decline in CR frequency in Phases 2 and 3, so extinction in Phase 2 was continuous with, and not dissociated from, extinction in Phase 3. If muscimol had the same effects on auditory processing in the Effective Group, then state-dependency could *not* have occurred in phase 2.

What if the effects of auditory effects of muscimol in target areas (Effective Group) and in surrounding cortical areas (Ineffective Group) were different? Perhaps muscimol in AIP affected CR-specific properties such as frequency, but muscimol infusions into cortical areas (e.g. auditory receiving areas) only affected low-level processing of non-

associable properties such as intensity. In this event, SDL effects would be restricted to the target area of the infusion (AIP). In the present experiment, in the Effective group, extinction learning may have occurred during muscimol inactivation of the AIP, but CRs would not be expressed because AIP activity is essential for expression. If associable aspects of auditory function (e.g. frequency properties) were also dependent upon normal AIP activity, then it is possible that altered CS processing in this group would result in SDL. Thus, with inactivation studies, we cannot distinguish between the possibility that SDL is caused by effects of muscimol on AIP-dependent processing of auditory stimuli, or the possibility that learning within cerebellar circuitry itself is disrupted.

Anatomical and Temporal Specificity of Inactivation

The approximate spread of muscimol and the time taken for behavioural effects to dissipate have been assessed. Evidence from several sources has been considered. Muscimol doses varied, but in all cases, the injection volumes were 1 μl. Mink and Thach (1991) injected 8.8 nanomoles of muscimol into the dentate nucleus in monkeys (approximately 6 times the dose used in our study). Behavioural effects began about 3 minutes after the injection and disappeared between 7 and 12 hours later. Robinson, Straube and Fuchs (1993) also injected 8.8 nanomoles of muscimol into caudal fastigial nucleus of monkeys and found behavioural changes starting 10 minutes after injection and lasting for 2 to 3 hours, with no residual effects 24 hours later. Their estimated radius of spread was up to 2.5mm. In their analysis of NMR conditioning in rabbits, Krupa *et al.* (1993), injected tritiated muscimol into the interpositus nucleus (AIP) and on the basis of subsequent autoradiography they claim that their dose of 14 nanomoles did not spread outside the cerebellum within 2 hours (but see Introduction).

When muscimol was injected up to 2mm from AIP, CRs were abolished consistent with permanent lesion studies (Yeo *et al.* 1985a). However, if muscimol was injected further away from AIP, CRs were not abolished. Assuming that muscimol abolishes CRs by

acting at AIP, it is highly unlikely that the injection bolus spread beyond 2mm or, in significant concentrations, beyond the cerebellum. Our preliminary data indicates that an injection of 1.54 nanomoles takes effect within 5 minutes. Behavioural effects (depressed CR frequency or absent CRs) persist for approximately 7 hours in overtrained animals, after which CRs recover completely, and there are no effects on CRs after 24 hours (see chapter 4).

Therefore, the time course of behavioural muscimol effects in the present study was considerably less than 24 hours. The effects of muscimol in one session of training could not have intruded into the next. The dose of muscimol effective in the cerebellar nuclei spread approximately 2mm from the site of injection. However, lower but sufficient doses could have spread into areas of the cerebellar cortex involved in the regulation of plasticity or auditory processing.

If such procedures do not produce SDL, cannula damage to critical circuitry, or significant post-inactivation performance deficits, then the cerebellum is implicated in plasticity essential for acquisition of NMR conditioning.

Is cerebellar efferent circuitry required for NMR conditioning?

Important objections to the hypothesis of cerebellar plasticity for NMR conditioning are based on the possible effects that lesions and reversible inactivations may have upon efferent circuitry. The role of cerebellar efferent circuitry in NMR conditioning is therefore of critical importance in interpreting the inactivation studies discussed so far in this thesis. If it can be shown that normal activity in cerebellar efferent circuitry is not required for NMR conditioning, then it can be concluded that state-dependent effects due to impaired CS processing do not occur outside the cerebellum, and that sites efferent from the cerebellum do not contain cerebellar-dependent sites of essential plasticity for NMR conditioning. In an important study, Krupa and Thompson (1995) reported that inactivation of the brachium conjunctivum during conditioning does not

prevent acquisition (see Chapter 1). These findings are consistent with the suggestion that acquisition learning is critically dependent upon cerebellar or pre-cerebellar plasticity and that state-dependent effects do not occur. The effectiveness of the inactivation was judged by the absence of CRs after TTX infusion. Is it possible that TTX invaded areas essential for conditioning, other than the brachium conjunctivum? A pathway almost adjacent to the brachium conjunctivum in the rabbit, is the rubrobulbar tract - the pathway projecting from the red nucleus to the accessory abducens that is also essential for CR expression. Krupa and Thompson have argued that the spread of TTX was restricted to a radius of approximately 0.75mm from the brachium conjunctivum. It is conceivable that TTX spread from the injection site to the rubrobulbar tract. However, there are many factors which make this possibility difficult to assess. The only study to have characterised the anatomy of this pathway in the rabbit (Rosenfield, Dovydaitis and Moore, 1985) has shown that the RT is close to the BC at dorsal levels. However, since no scale is present in this paper, the distances are difficult to resolve. In addition, the plane of section of the cerebellum and brainstem reported in Krupa and Thompson (1995) is very different to that of Rosenfield *et al.* (1985). It is therefore difficult to establish whether or not the injection sites are more than 0.75mm away from the RT. This leaves open the possibility that incomplete BC inactivations and complete inactivations of RT account for the results of Krupa and Thompson (1995).

In conclusion, while inactivation methodology is useful for identifying which processes are impaired by inactivations (e.g. learning, execution of responses or plasticity) such studies by themselves, cannot localise these processes to specific circuitry. This experiment shows that learning in NMR conditioning is a process critically dependent on normal activity in AIP. It is unlikely that state-dependency can account for this result. In addition, the possibilities of cannulation damage and temporally extended effects of muscimol can be ruled out, since these were stringently controlled for in the study. While it is clear that the process of learning is dependent on AIP, the possibility that state-dependent effects occurred cannot be ruled out. The critical involvement of AIP

in extinction of NMR conditioning suggests that AIP and related cerebellar circuitry may be sites at which essential plasticity occur. This possibility needs investigation using more direct tests of plasticity (see chapter 4).

Chapter 3

Reversible Inactivations Of The Same Cerebellar Sites Prevent Acquisition And Subsequent Extinction Of Conditioned Nictitating Membrane Responses.

3.1 Introduction

Chapter 1 has reviewed the effects of permanent lesions and reversible inactivations on the acquisition and retention of conditioned NMRs. There is a consensus that specific parts of the cerebellum are essential for conditioning and there are several explanations which could account for this result.

3.1.1 Inactivations of AIP prevent execution and learning in extinction training.

The 'performance deficit' hypothesis (Welsh and Harvey, 1989) has been a prominent argument against the idea that cerebellar lesions result in learning deficits. In Chapter 2 it was shown that a low dose of muscimol used to reversibly inactivate the AIP, prevented the execution of CRs after the animals had acquired CRs. But such deficits in performance of CRs are insufficient to explain why extinction training during this blockade appeared not to result in extinction *learning.* Although processing of the conditioned stimulus may have been altered during muscimol blockade, it has been argued in Chapter 2, that this is unlikely to have produced state-dependent effects. It is more likely that the AIP regulates plasticity essential for extinction.

3.1.2 Is the AIP also required for acquisition learning in NMR conditioning?

Krupa *et al.* (1993) have reported that muscimol infusions used to inactivate the cerebellum during acquisition, were effective in preventing acquisition. However, the interpretation of this study is problematic. Firstly they used 1μl muscimol infusions (14nm per 1μl, given daily in six successive days). They report that the inactivations prevented acquisition, and that subsequent acquisition occurred at the same rate as in naive controls (i.e. subjects with no previous experience of the stimuli or conditioning environment). This large dose of muscimol may have had its direct action at sites outside the cerebellum. Krupa *et al.* (1993) have infused muscimol six times into the

cerebellum. Secondly, it is clear from in-vitro and in-vivo studies (Sieghart, 1995) that repeated exposure of GABA-A receptors to GABA-A agonists results in long term, often irreversible and pathological consequences. Additionally, the cannula may have partially damaged critical circuitry in the cerebellum which retarded acquisition, and which required extended training to overcome. These alternative interpretations of Krupa *et al* (1993) confound the interpretation that muscimol infusions impaired cerebellar plasticity. To address this issue, it is necessary to determine if fewer infusions and much lower doses of muscimol prevent acquisition. One aim of the present study is directly to address the first two issues. The potential problem of cannula damage has been ruled out in the extinction study (Chapter 2). Since animals first acquired CRs before muscimol infusions, it was possible to determine whether or not there was cannula damage to circuitry essential for NMR conditioning before the experimental phase. Since the decline of CR frequency to baseline levels is an index of learning in extinction, there can be no confounding of learning with extended muscimol effects, or the long-term detrimental effects of muscimol on the production of CRs.

The interpretation of data from experimental groups in such studies is determined in part by the nature of control data. Krupa *et al*, (1993) predicted that experimental animals would only begin to acquire when the muscimol blockade was lifted. The control subjects were therefore those which started conditioning when the blockade was lifted, and acquired indistinguishably from the experimental subjects. It would appear that learning was completely prevented. However, these controls did not match the experience of the experimental group in the inactivation phase. The experimental group experienced auditory and somatosensory stimulation in the muscimol phase, but the control animals did not. Experimental animals were also present in the conditioning chamber during the inactivation phase, but the control animals were not. These factors by themselves may cause changes in conditioning subsequent to the inactivation phase. A useful control group would therefore consist of subjects which experienced the same stimulation in the same conditioning environment, but which do not acquire. However, any kind of stimulation, paired, explicitly unpaired or randomly paired, would inevitably establish learning gradients which would contribute to rates of subsequent acquisition. Practically, it is more feasible to use a control group which received no

stimulation, but which did experience all other aspects of the conditioning environment. The present study used just such a control group. To determine the effects of this 'Sit Control' procedure, a second control group (similar to the one used by Krupa *et al.* 1993) was also used, in which subjects simply remained in their home cages during the inactivation phases.

3.1.3 Is cerebellar circuitry essential for acquisition also critical for extinction?

In the previous chapter, an extinction design has overcome some of the problems associated with other studies that have examined the involvement of AIP in acquisition. Acquisition and extinction are similar, but distinct. How similar are neural processes in acquisition and extinction? Inactivation studies cannot examine all aspects of these processes, but it is possible to examine whether or not acquisition and extinction depend on the same cerebellar circuitry. While the previous study has established that AIP is required for extinction, no inactivation studies of acquisition have attempted to restrict the direct effects of muscimol to AIP. The equivalence of acquisition and extinction as learning processes which are dependent upon normal activity in the same circuitry, is therefore another issue which warrants investigation. In order to directly test this possibility, the same sites, in the same animals, were inactivated first during acquisition and then during extinction. If both acquisition and extinction are prevented by inactivations of the same circuitry, then it is possible that plasticity for acquisition and extinction require normal activity in the same circuitry. The results of the study are discussed in the context of the cerebellar learning hypothesis.

3.2 Methods

Methods were the same as those used in the last chapter, and are described in Appendix 1.

3.2.1 Subjects

Subjects were 14 male Dutch belted rabbits weighing from 1.8kg to 2.5kg. They were housed individually, allowed *ad libitum* food and water and maintained on a 12hr light/dark cycle. All subjects in the Control Group of the previous study were used as control subjects in this study (Naive Group - see below) since their training was identical to that required of Naive Group subjects in the present study.

3.2.2 Surgery

A 24G stainless steel cannula guide was surgically implanted 1mm caudal and 1mm dorsal to eyeblink related cells of the cerebellar nuclei, located electrophysiologically. The placement of the guide tip avoided damage to the connections of these cells and the orientation of the guide avoided damage to parts of the overlying cortex that are important for NMR conditioning (Yeo and Hardiman 1992). This strategy was particularly important in this experiment, because unlike the previous chapter, damage to critical structures would not have been manifested behaviourally in this experiment (see design below). All cannulated rabbits were allowed 2 weeks for recovery.

3.2.3 Experimental Design

Session Types

There were 4 different types of sessions.

(i) Adaptation: The first session in which the subjects were placed in the conditioning chamber was always an adaptation session. The subject was fitted with the nictitating membrane transducer and periorbital clips and placed in the conditioning chamber for

one hour - equivalent to the duration of one conditioning session. There were no presentations of either the CS or the US during this period.

(ii) Sit: Procedurally, Sit sessions were identical to Adaptation sessions.

(iii) Acquisition training: Each session consisted of 100 trials. In 90 trials the CS and US were paired and in 10 trials the CS was presented alone - there was no US in these trials. A CS alone trial was presented on every tenth trial.

(iv) Extinction training: Each session consisted of 100 trials. The CS was presented alone on every trial. The US was not presented.

Subject Groups

Subjects were randomly assigned to one of three groups.

(i) Cannula Group: Six rabbits were assigned to this group. Each underwent surgery in which a guide cannula was implanted above the right cerebellar nuclei. Two weeks after surgery a suture was placed in the right nictitating membrane. Three days later all subjects underwent a session of adaptation. They then entered the experiment in Phase 1 (see figure 3.1). In the muscimol phases, 1µl of muscimol solution (RBI, 1.54mM [0.3µg.µl^{-1}] muscimol hydrobromide in 50 mM phosphate buffered saline pH7.4) was injected over one minute into the right cerebellum of each subject. The injection was via a 36G cannula inserted through the 24G implanted guide cannula and protruding 0.5 mm below the tip of the guide. The injection was, therefore 0.5 mm above and 1 mm caudal to cells with activity related to eyeblinks. The subjects were then left for one hour in their home cage before training.

(ii) Sit Group: Four rabbits were assigned to this group. Three days after suturing the nictitating membrane all subjects underwent a session of adaptation and then they entered the experiment in Phase 1.

(iii) Naive Group: Six rabbits were assigned to this group. This group is the same as the control group in Chapter 2. So, all subjects underwent a session of adaptation and then they entered the experiment in Phase 2.

Experimental Phases

Group		Phase 1	Phase 2	Phase 3	Phase 4	Phase 5
Cannula	Adaptation →	Acquisition* →	Acquisition →	Extinction* →	Extinction	
Sit	Adaptation →	Sit →	Acquisition →	Sit →	Extinction →	Extinction
Naive	Adaptation →	→	Acquisition →	→	Extinction →	Extinction

Figure 3.1: Experimental design. All groups received an adaptation session on day 1. On the next day, they entered the appropriate experimental phase. Asterisk indicates training under muscimol. Thin arrows indicate that treatments were on successive days. Thick arrows indicate a 3 day interval.

On the first experimental day, all subjects had a single session of adaptation to the conditioning chambers during which no training stimuli were presented. All subjects entered their appropriate experimental phase one day after their adaptation session.

There were 5 Experimental Phases. There were always three days between each experimental phase but each group did not enter all phases of the experiment.

(i) Phase 1. This was the first experimental phase of the experiment, and subjects in the Cannula Group subjects received 4, daily sessions of acquisition training under muscimol inactivation of the cerebellar nuclei. Since the Sit Group was required to control for experience of the conditioning environment, these subjects were placed in the conditioning chambers for 4, daily sessions but they received no training stimuli.

(ii) Phase 2. Cannula, Sit and Naive Group subjects received 4, daily sessions of acquisition training. Any learning by the Cannula group that had taken place during Phase 1 would be revealed as higher initial levels of CRs compared with the Sit or Naive Groups.

(iii) Phase 3. Cannula Group subjects received 4, daily sessions of extinction training under muscimol inactivation of the deep cerebellar nuclei. Sit Group subjects were placed in the conditioning chambers for 4, daily sessions but they received no training stimuli. These subjects form a control for any factors unrelated to extinction training over the equivalent sessions in the Cannula Group.

(iv) Phase 4. Cannula, Sit and Naive Group subjects received 4, daily sessions of extinction training. Any extinction by the Cannula group that had taken place during Phase 3 would be revealed as lower initial levels of CRs or more rapid extinction compared with the Sit or Naive Groups.

(v) Phase 5. Sit and Naive Group subjects received 4, daily sessions of extinction training. If there had been full extinction by the Cannula group during Phase 3 then the pattern of CR responses in Cannula Group would be similar to those in Sit and Naive Groups.

3.2.4 Histology

At the end of the experiment rabbits with implanted cannulae were injected with heparin sodium (500 U.kg-1, i.v.) and an overdose of pentobarbitone sodium (90 mg.kg-1, i.v.). Each rabbit was perfused through the aorta with 0.9% saline followed by 4% formaldehyde and the brain removed from the skull. The brain was embedded in 10% gelatin and placed in a solution of 20% sucrose formalin for 3 days. Frozen sections were cut at 60μm in the transverse plane. Alternate sections were mounted onto gelatinised slides and stained for Nissl substance with cresyl violet.

3.2.5 Data analysis

CR frequency

CR frequencies were expressed as the percentage of CRs expressed as a function of the number of trials presented. In order to show within-session effects, data were plotted graphically in 10 trial blocks (9 paired trials + 1 CS alone trial for Phases 1 and 2, and 10 unpaired trials for Phases 3, 4 and 5). All responses occurring from CS onset to 1000ms after CS onset were recorded. A conditioned response (CR) was defined as a nictitating membrane response with amplitude greater than or equal to 0.5mm, with onset latency greater than 35ms from CS onset and which occurred between 35ms and 250ms from CS onset on paired and unpaired trials (Hardiman and Yeo, 1992). On paired trials this period corresponded to the inter-stimulus interval.

CR topography in Phase 2 (see appendix 1, figure A1.2)

Secondary, topographical analyses of CR latencies in Phase 2 (acquisition) were conducted to compare CRs of the Cannula Group after muscimol blockade, with CRs of subjects acquiring *de novo*. This analysis was restricted to Phase 2 (acquisition) because a similar analysis for extinction after muscimol blockade has already been reported in chapter 2.

Latency measurements (see Appendix 1, figure A1.1) were taken only from responses made in probe (CS alone) trials, because on paired trials, the full form of the CR is obscured by the NM response to the UR in paired trials. For latency measurements, a CR was defined as a response that attained an amplitude greater than or equal to 0.5mm in the period 35-1000ms from CS onset. CR latency to peak was recorded as the time from CS onset to the peak amplitude of the CR. CR latency to onset was obtained by extrapolating backwards from 0.5mm to the point at which the a CR exceeded baseline noise by 0.15mm.

Latencies to peak and onset for each group were compared *session by session* (group mean of every probe trial), and *distributions* of latencies to peak and onset of each group were compared over the whole of Phase 2.

3.3 Results

The principal findings of this study were that muscimol infusions into the AIP prevented both the acquisition and extinction of conditioned responses.

3.3.1 Histological verification and group assignment

Cannulated subjects were included in the Cannulated Group if they did not show CRs under muscimol blockade, and if the cannula tip was located near the deep cerebellar nuclei. There were six cannulated subjects (C1-C6). Cannula placements in four subjects (C1, C2, C3, C4) were located at very similar sites, close to the anterior part of the interpositus nucleus (see figures 3.1 and 3.2). In two cases (C1 and C2), the placements were within posterior parts of the interpositus. It has previously been shown that damage in this area does not impair conditioned responses (Yeo *et al* 1985a). In two other cases (C3 and C4), the cannula tip was located in white matter just dorsal to caudal parts of the interpositus nucleus. These four subjects produced very few CRs during Phase 1, but did show URs in response to the US. These four subjects constituted the Cannulated Group.

In one of the two other cannulated subjects (C5), the cannula guide tip was incorrectly positioned in the paramedian lobe of the right cerebellar cortex due to a stereotaxic error (see figure 3.2). Injections of muscimol did not prevent the appearance of CRs. This subject was excluded from the Muscimol group. The other subject (C6) showed abnormal CR amplitudes and very retarded acquisition. The cannula tip in this subject was located in, and damaged anterior parts of the interpositus nucleus (see figure 3.1) where lesions impair or abolish CRs (Yeo *et al* 1985a). Because of these factors, behavioural data from these subjects is not considered further. In subject C4, muscimol no longer abolished CRs in Phase 3, session 4. Extinction data from this subject, was excluded from the analysis, so for the extinction Phases 3 and 4, the Cannulated Group consisted of subjects C1-C3.

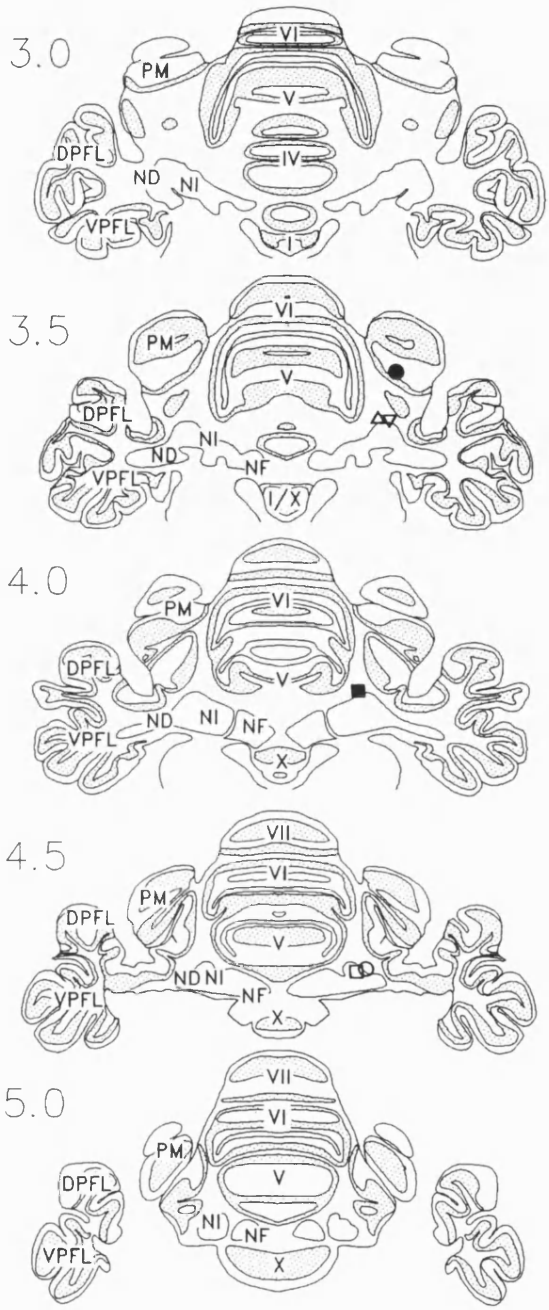


Figure 3.2: Positions of injection cannula tips. Standard transverse serial sections at intervals of 0.5mm through the cerebellum. Sections are numbered with caudal distance from λ. Locations of cannula tips for each subject is shown by a different symbol. C1, open triangle; C2, open circle; C3, filled square; C4 inverted open triangle; C5, filled circle; C6, open square; A key to the major subdivisions of the cerebellum is shown on the left side of each section. DPFL, dorsal paraflocculus; crII, crus II; HVI, lobule HVI; ND, dentate nucleus; NF, fastigial nucleus; NI, interpositus nucleus; PM, paramedian lobe; VPFL, ventral paraflocculus; V-X, lobules V-X of the vermis.

Figure 3.3: Transverse section through the right cerebellum of subject C1 at approximately 3.5mm caudal to λ. The cannula track passed through the paramedian lobe and the tip was situated immediately dorsal to the junction between the dentate and interpositus nuclei. Scale bar represents 2mm.

Arrow marks the position of the cannula tip.

3.3.2 CR Frequency

Acquisition

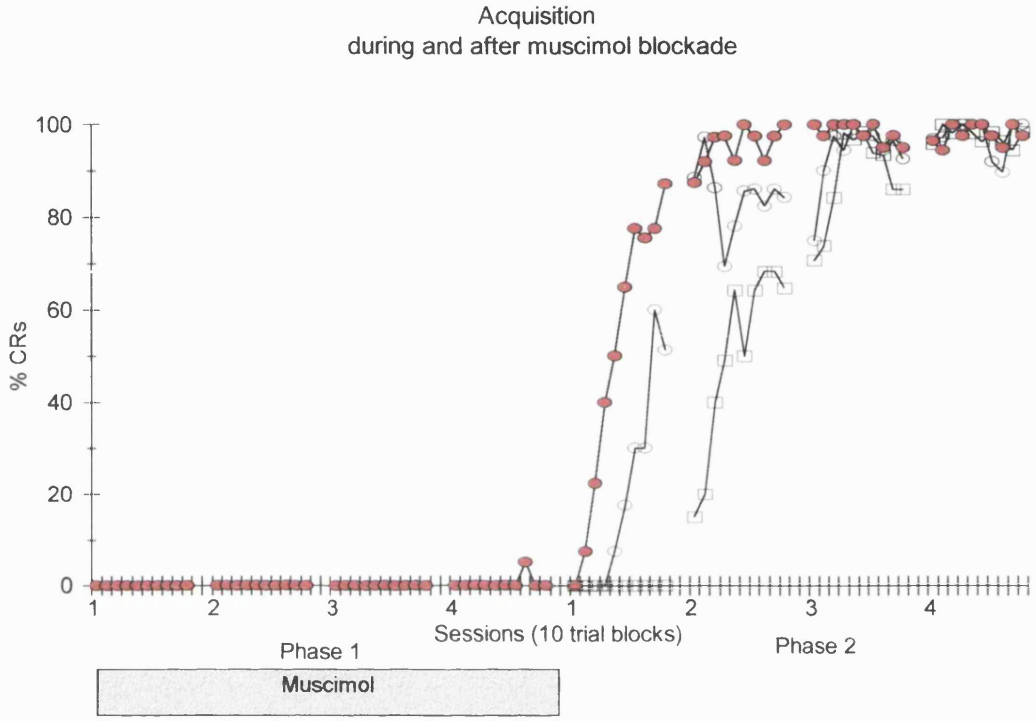


Figure 3.4: Acquisition (Phases 1 and 2). Each data point marks the percentage of CRs for each group, over a block of 10 trials. In Phase 1, muscimol was injected an hour before each session into the cerebellar nuclei of subjects in the Cannula Group. Almost no CRs were expressed in this group. In Phase 2 (three days after Phase 1), these subjects acquired CRs when trained without muscimol. Filled red circles, Cannula group; open circles, Sit group; open squares, Naive group.

Figure 3.3 shows that in Phase 1, cannula subjects produced very few CRs during the muscimol blockade (0% in sessions 1,2 and 3; 0.53% in session 4). In Phase 2, when the effects of muscimol had dissipated, subjects were given further acquisition training. Subjects in the Cannula Group acquired CRs. In the first block of 10 trials, there were no CRs. As training progressed, CR frequency increased. There was no evidence of learning in the previous phase. Had these subjects acquired during Phase 1, CR frequencies during session 1 of Phase 2 would have been similar to CR frequencies in the final block of acquisition

training for subjects in the Sit (100%) and Naive (100%) Groups. The Sit and Naive groups also started acquiring CRs in Phase 2.

A 2-Way Repeated Measures Analysis of Variance was conducted, with Groups as one factor, and Session CR frequencies (repeated) as the other factor. There is a statistically significant difference between groups ($F(2,11)=5.92$, $p<0.05$). To isolate which groups differed from each other, a Student-Newman-Keuls test was used (see below). Consistent with the fact that all groups acquired CRs, there was a significant rise in CR frequency across sessions in Phase 2 ($F(6,33)=65.2$, $p<0.05$). The significant interaction between group and session ($F(6,33)=3.48$, $p<0.05$ indicates that groups acquired differently across sessions.

The power (with $\alpha=0.05$) of each part of the analysis of variance was high.

Group: 0.735

Session: 1.000

Group x Session: 0.754

The Student-Newmann-Keuls test showed that the difference between groups was attributed to the difference between the Muscimol and Naive groups ($p=3$, $q=5.0$, $P<0.05$). There was no significant difference between the Muscimol and Sit groups ($p=2$, $q=2.02$, $P>0.05$) or between the Naive and Sit group ($p=2$, $q=2.80$, $P>0.05$).

By inspection, figure 3.4 indicates that the Cannula and Sit Groups produced more CRs than the Naive Group. Mean CR frequency in the Sit Group began to increase in session 1, as did the mean CR frequency of the Cannula group. In contrast, mean CR frequency in the Naive Group did not begin to increase until session 2. This would suggest that prior exposure to the conditioning environment significantly enhances rates of acquisition. Statistical tests fail to reveal any significant differences between the Cannula and Sit Groups but inspection of figure 3.4 reveals a small but consistent trend towards higher CR frequencies in the Cannula Group. It is likely that prior exposure to the conditioning environment accelerates subsequent learning, and that prior exposure to stimuli may also contribute to subsequent rapid learning.

Extinction

During Phase 3 muscimol was infused in the Cannula Group subjects and they showed few CRs (mean CR frequency, 0.66%; see figure 3.4).

When the effects of muscimol had dissipated by the beginning of Phase 4, Cannula Group subjects showed a very high CR frequency in the first 10 trials of the first session (86.7%), which gradually declined as extinction training progressed (figure 3.5). A 2-way repeated measures ANOVA was conducted to assess whether or not the three groups differed from each other. One factor was Group, and another was Session CR frequency (repeated).

There was no statistically significant difference between the session CR frequencies of the three groups ($F(2,10)=1.56$, $p>0.05$). Consistent with the session-by-session decrease of CR frequencies of all groups, there is a statistically significant difference between session CR frequencies across sessions ($F(3,33)=9.06$, $p<0.05$). The Group x Session interaction is not significant ($F(6,33)=2.15$, $p>0.05$). The statistical power of the ANOVA was much diminished by the loss of one subject from the experimental group ($\alpha=0.05$, power for the Group statistic was 0.116). Despite this, all experimental subjects in this phase of the experiment showed very high CR frequencies (figure 3.6), consistent with unextinguished CRs. These then declined in frequency as extinction training progressed.

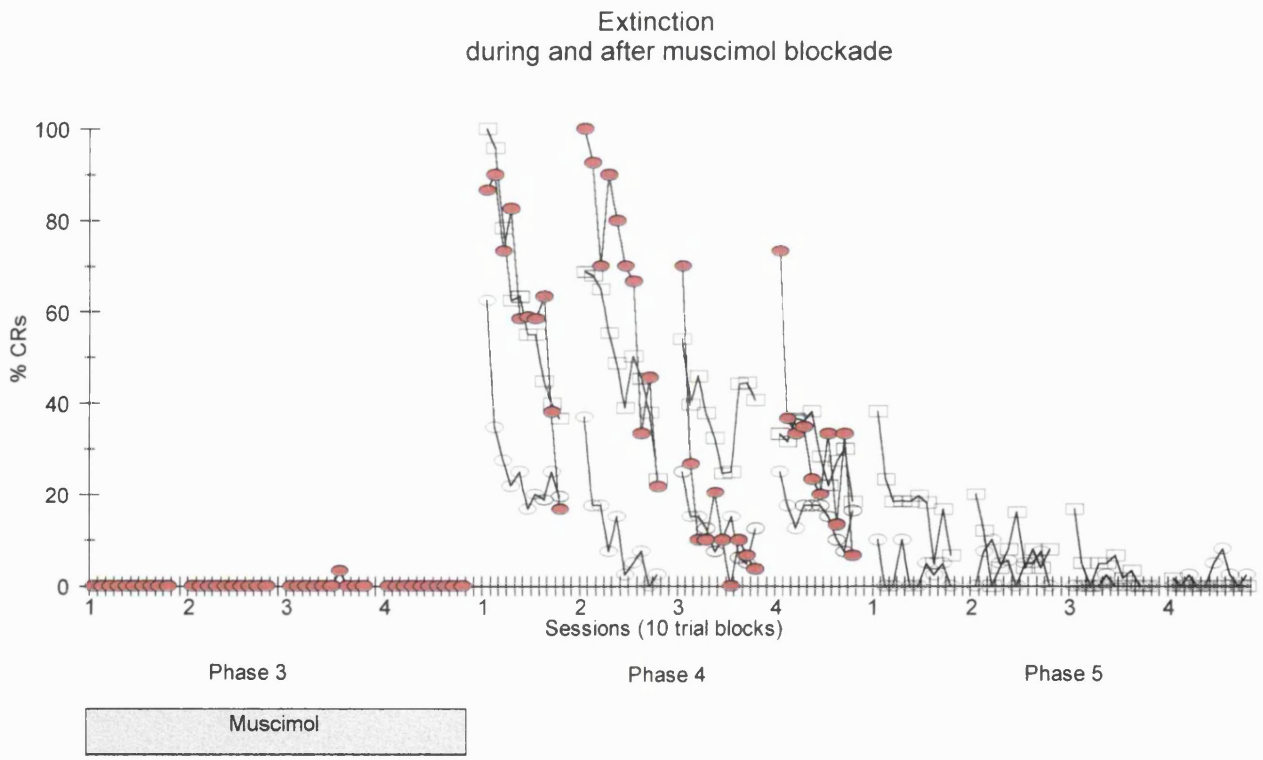


Figure 3.5: Extinction (Phases 3, 4 and 5). In Phase 3, subjects in the cannula group were given muscimol injections an hour before each training session, and then given extinction training. Very few CRs were produced. In Phase 4 (three days later) these subjects were again given extinction training, without muscimol. They initially showed very high CR frequencies, and CRs then extinguished with further training. Control subjects extinguished to very low CR frequency levels in Phase 5. Legend as for figure 3.4.

Figure 3.6 shows CR frequencies of individual subjects. All subjects in the experimental group showed high CR frequencies at the start of phase 4, and these extinguished to very low levels at the end of phase 2. Subject C4 was excluded from the experimental group because muscimol infusion failed to prevent CRs in the last session of Phase 3. CRs extinguished after this in this subject.

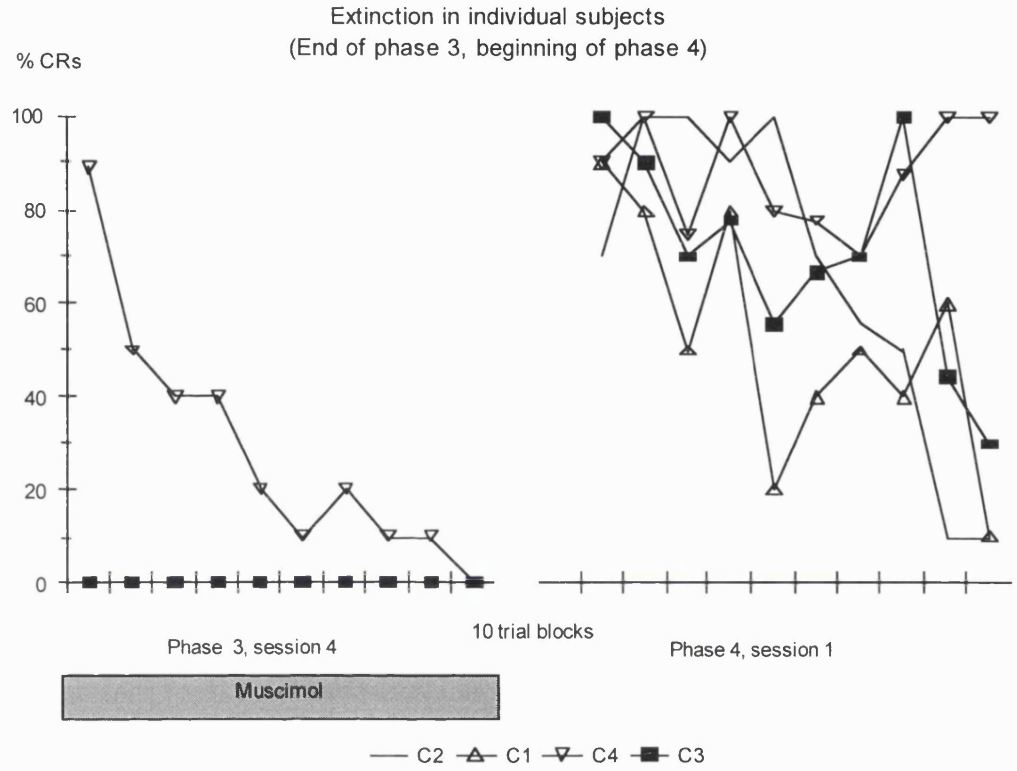


Figure 3.6: Extinction for individual subjects. Last session of Phase 3 and first session of Phase 4. In Phase 3, there were no CRs for three subjects, and so data points are superimposed and lie very close to the x-axis. Muscimol had no effect on subject C4 in the last session of Phase 3, and began extinction in this phase. Other subjects in the cannula group began extinction at the beginning of Phase 4.

These results suggest that the Cannula Group subjects had not extinguished during the muscimol blockade in Phase 3, but that they did extinguish when the blockade was lifted. At the end of this extinction phase, CR frequencies were similar in all groups. This is consistent with the results of the previous chapter. If subjects in the Cannula group had extinguished during muscimol blockade (Phase 3), then continued extinction training in the following phase would have resulted in CR frequencies similar to subjects given two phases of extinction conditioning, such as those achieved by Naive and Sit groups in Phase 5 - they achieved low levels of spontaneous recovery, and produced few CRs.

3.3.3 CR Topography

The topography of CRs in the cannula group which emerged during acquisition (Phase 2) after muscimol blockade, were compared with those of animals acquiring for the first time.

Latencies to Peak:

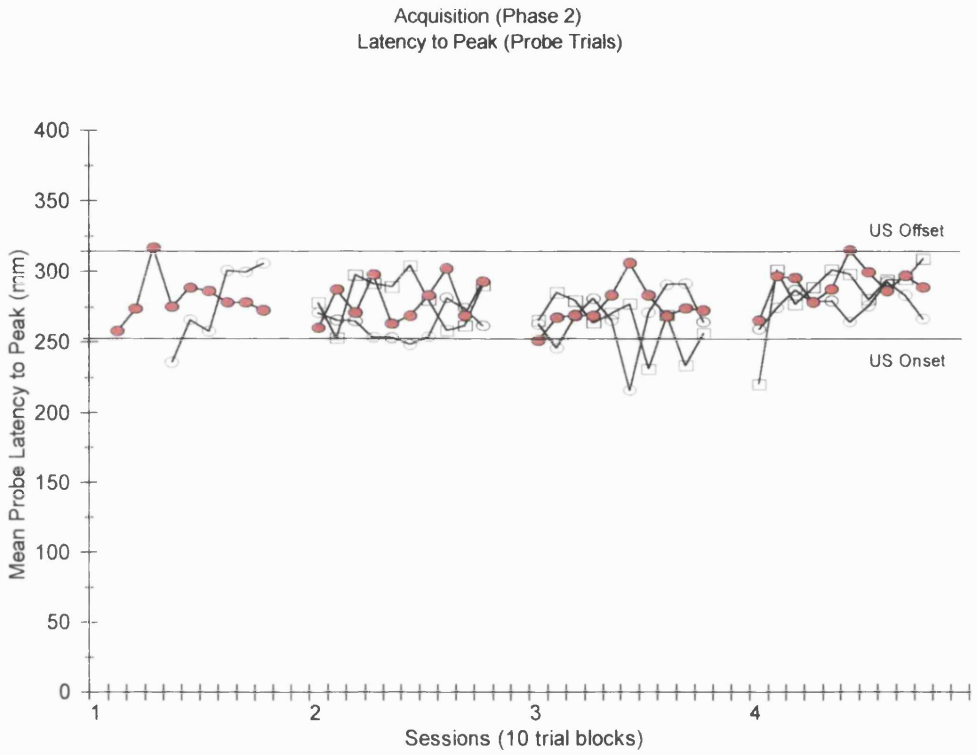


Figure 3.7: Latency to peak of probe (CS only) trials in Phase 2. Each data point represents the group mean value of CR latencies to peak during probe trials, in the period 35-1000ms from CS onset. Legend as for figure 3.4.

A characteristic of a well timed conditioned NMR is the occurrence of the peak at a time coincident with the delivery of the US (Gormezano *et al.* 1983). Figure 3.7 shows that during acquisition in phase 2, latencies to peak of CRs produced by the experimental group were comparable with those of both control groups. They were well timed and stayed within a range of 250ms to 310ms (US interval).

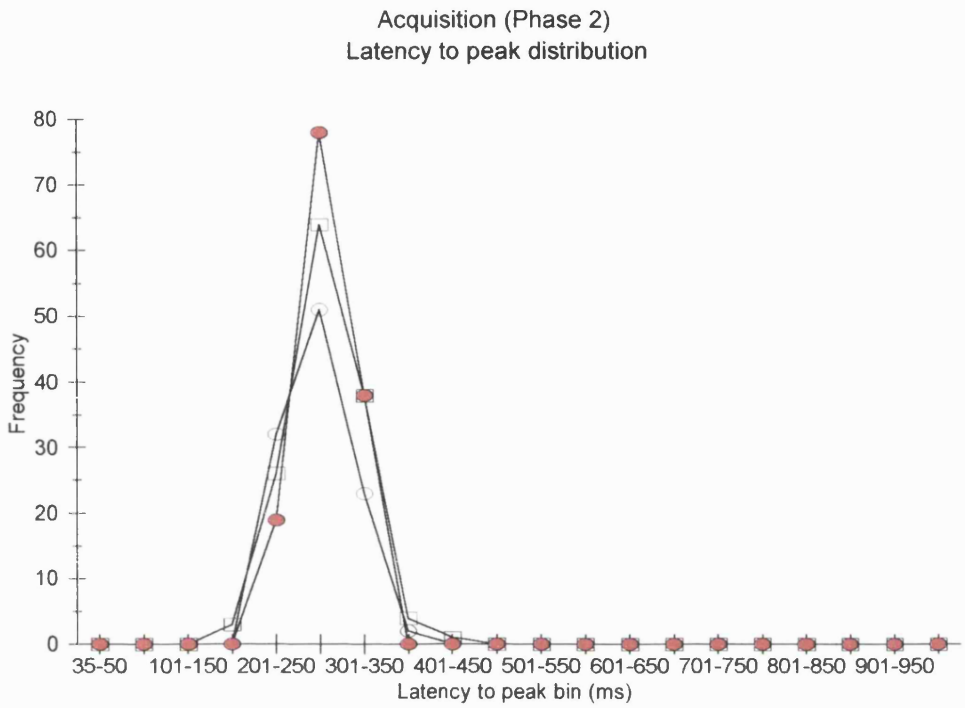


Figure 3.8: Distributions of latencies to peak of probe trials in Phase 2. Bin width, 50ms (except earliest bin width, 15ms). Legend as for figure 3.4.

Figure 3.8 shows the distributions of CR latencies to peak over a wide range of latencies. For the three groups, distributions are narrow and all peak in the 251ms-300ms bin. The distribution of the Cannula Group is therefore comparable to those of the control groups.

CR latencies to peak of subjects in the Cannula Group are therefore comparable with those of subjects in the control groups.

Latencies to onset:

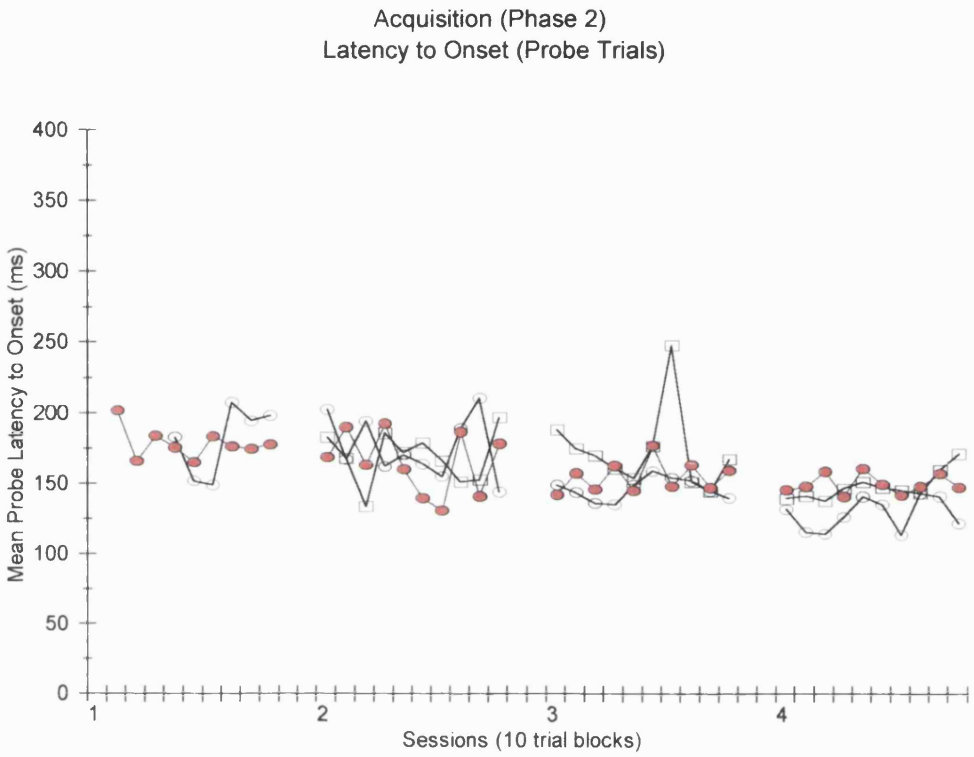


Figure 3.9: Latencies to onset of probe trial CRs in Phase 2. Each data point is the group mean latency to onset for all CRs made during the period 35-1000ms from CS onset. Legend as for figure 3.4.

Latencies to onset typically shorten as a function of acquisition in NMR conditioning (Gormezano, 1962). Figure 3.9 shows that latencies to onset for the control groups did indeed decline with training. The Cannula Group showed the same pattern of declining latencies to onset as the control groups, indicating that learning in the Cannula Group was likely to have progressed from a state similar to controls which had not acquired CRs.

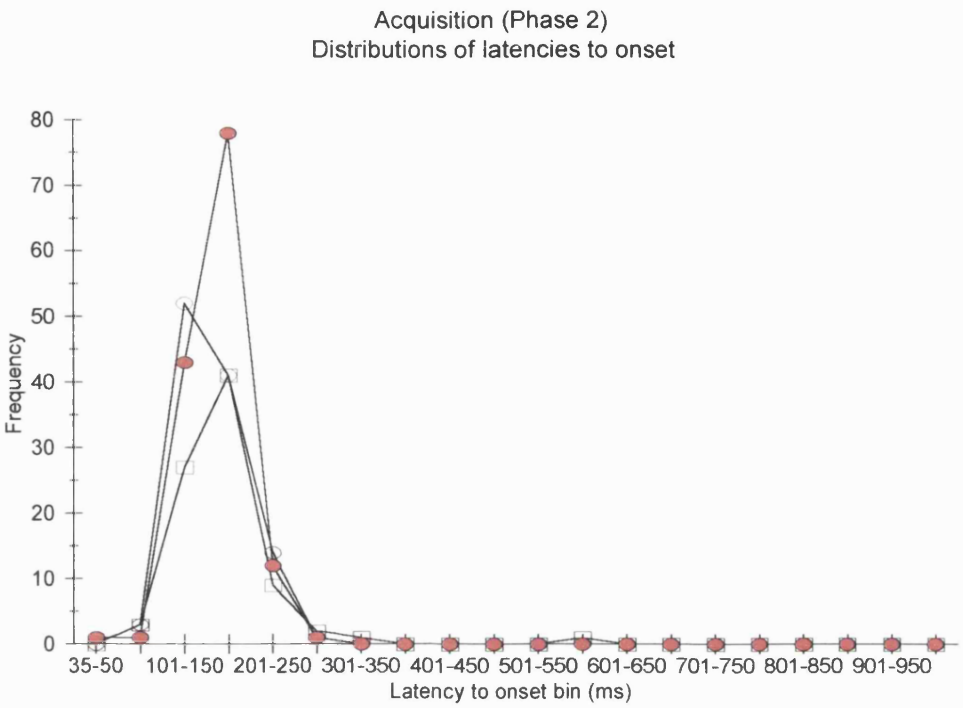


Figure 3.10: Distributions of latencies to onset for probe trials in Phase 2. Legend as for figure 3.4.

The distributions of these latencies (figure 3.10) show that they peaked between bins 101-150ms and 151-200ms. So, onset latencies of control groups and the Cannula groups are similar (the Sit Group distribution peaks only 50ms earlier than those of the other groups). It is evident from figure 3.10 that the number of responses exceeding 250ms was negligible.

CR latencies to onset and CR latencies to peak across Phase 2 were similar in all groups. There was a characteristic decrease in the latency to onset of CRs as training progressed. CRs in all subjects tended to peak in the US period between 250 and 300 ms after CS onset. Subjects in all groups produced appropriately and accurately timed conditioned responses confirming that they were likely to have been truly associative, and that they were not produced by sensitisation.

Overall, there is clear evidence that Cannula Group subjects did not acquire or extinguish during muscimol inactivation. That apparently normal acquisition and extinction began only

after these blockades, confirms the AIP as essential for both acquisition and extinction of conditioned NMRs.

3.4 Discussion

Successful inactivation of the cerebellar nuclei during NMR conditioning with the GABA-A agonist muscimol prevented normal acquisition - there were no conditioned responses at the beginning of training in phase 2. In phase 3, the same circuitry was again inactivated and subjects were given extinction training. Consistent with the hypothesis that acquisition and extinction require normal activity in the same circuitry, CRs remained unextinguished at the start of phase 4. These then extinguished normally in phase 4 with further extinction training. The results have implications for theories of cerebellar learning.

3.4.1 Acquisition is prevented by a low dose and few infusions of muscimol

The results of this study confirm that normal activity in AIP is essential for acquisition. In the present study, using low doses of muscimol, the pattern of response development by Cannula Group subjects in Phase 2 is consistent with real acquisition. The topography of CRs in the Cannula Group developed similarly to that of Naive and Sit Group subjects. Initially CR's were of long onset latency, just anticipatory of US onset, but these progressively shortened. The conditioning-related shortening of onset latencies from just before US onset towards the CS onset is the classic pattern for normal NMR conditioning (see Smith 1968). CR latencies to peak in all groups in Phase 2 were characteristically well timed, occurring between the US onset and offset in the probe trials. This is in contrast to some studies, in which timing of the conditioned external eyelid blink (Perret *et al.*, 1993) and conditioned NMR (Gruart and Yeo, 1995) was disrupted by permanent lesions of the cerebellar cortex. We suggest that the development of CRs by Cannula Group subjects in Phase 2 is characteristic of proper associative conditioning. In Phase 2, Sit controls acquired a little faster than controls which had not been previously exposed to the conditioning environment. It is likely that prior exposure habituated the sit group, so that any adverse effects on learning that a

new environment might have would have been minimised. This rapid acquisition is not likely to be due to associative learning in Phase 1, since associative learning would have resulted in the presence of CRs immediately in Phase 2.

The issue of using an appropriate control group is of some importance to this study specifically, but also to inactivation studies generally. The hypothesis under investigation in the present study is that learning is prevented by reversible inactivations of the AIP. Training subsequent to the intervention should result in learning from a naive state. So, the control for this situation is one in which animals do not acquire CRs in the first phase, and begin acquisition from a naive state in the second phase. The simplest control would be animals which start conditioning in second phase, having no experience of conditioning in the first. Phase 2 learning in experimental subjects would then match controls acquiring CRs, assuming that all other factors independent of associative processes remain normal, as was the case in the previous chapter. In the present study, the situation is made more complex by the fact that conditions of the Naive group in Phase 1 did not *exactly* match those of the experimental condition. Although the animals were naive to the pairing of stimuli, they were also naive to other important factors such as experience of the conditioning environment and auditory and somatosensory stimulation. This was partially overcome by using a second control group where experience of the conditioning environment was controlled for (Sit group). Acquisition of CRs was prevented in the experimental group. Acquisition subsequent to the inactivation was much faster than that of the Naive Group, but only a little faster than animals in the Sit Group. Why should this be so? Sit and Cannula groups had been experienced the conditioning environment in Phase 1. There was an opportunity here to habituate to the unfamiliarity of the situation. This habituation, not granted to the Naive group, is likely to have removed adverse effects on subsequent conditioning.

Ideally, both exposure to the conditioning environment and sensory stimulation should have been incorporated into the design. How can one best expose control animals to the same stimulation without any effects on subsequent learning? This ideal may not be possible. The process one trying to control for is the very one under investigation, and so

one can only speculate at how the CS and US can be presented to the animal in a way that might match CS and US processing when the AIP is inactivated. In NMR conditioning, any exposure to the CS and US results in learning about the relationship between these stimuli. Thus explicitly unpaired (Prokasy *et al.* 1978; Welsh and Harvey 1991; Napier *et al.* 1992 - exp 2) or backwardly paired (Plotkin and Oakley 1975) presentations of the CS and US result in the retardation of learning when the stimuli are subsequently paired. In other classical conditioning tasks, even randomly paired CS-US presentations can influence subsequent conditioning (see Mackintosh 1974). At present, it is not possible to resolve which is the appropriate control procedure for assessing any possible learning during reversible inactivations. The Sit Group control may be the least inappropriate of a wide range of possibilities. In contrast to the present study, Krupa *et al* (1993) found that experimental subjects which had received high doses of muscimol and frequent infusions, acquired at the same rate as their 'naive' control group. Notwithstanding slight differences in experimental design, this discrepancy can only be accounted for if Phase 2 acquisition in their study was impaired by the long term effects of large concentrations of frequently injected muscimol, or partial cannulation damage to the cerebellar nuclei. We have controlled for these by using fewer injections of a much lower muscimol dose. Chapter 2 has already established that deficits in extinction learning induced by muscimol infusions cannot be accounted for by cannula damage or long term muscimol effects. This validation may be extended to studies of acquisition, if it can be shown that acquisition and extinction of NMR conditioning both depend upon the same cerebellar circuitry.

3.4.3 Inactivations that prevent acquisition also prevent extinction.

No conditioned responses were evident during extinction presentations of the CS to Cannula Group subjects during Phase 3, and so muscimol inactivations were sufficient to block all CRs that had developed during training in Phase 2. After muscimol effects had dissipated, immediately upon presenting the first CS in Phase 4, CRs were present and they then began to extinguish. This finding is clear evidence that the same nuclear inactivation that had prevented acquisition learning also prevented extinction learning.

This result confirms and extends the findings of the previous study, and opens the possibility of acquisition and extinction in NMR conditioning being dependent on common mechanisms, or common sites of plasticity.

Pavlov (1927) argued that CRs are extinguished by an active inhibition of the original CS/US association. This idea can explain spontaneous recovery during extinction training and the rapid relearning of the original memory during re-acquisition training. Others have suggested that extinction is the loss of all, or part, of the original memory (Napier *et al.* 1992). The present findings do not distinguish between these possibilities, but they confirm that cerebellar circuitry is critical for both acquisition and extinction. Is post-muscimol extinction of CRs in the present experiment comparable to that of experimental subjects in the previous study? In terms of the common requirement of normal activity in AIP, they are directly comparable. Also, CRs in Phase 4 have the same topographical properties as experimental subjects in the previous study. However, the experimental designs differed, and the experience of experimental subjects in the present study *before* extinction was more complex that that of subjects in the previous study. This experience may have unavoidably resulted in uncontrolled interactions between acquisition and extinction in the present experimental design, so that the experience of experimental subjects in Phase 1 impinged on learning in phases 3 and 4 in ways that it did not in the control subjects. It would therefore be inappropriate to compare rates of extinction in the experimental group in the present study.

3.4.4 What effects could muscimol have outside AIP and beyond conditioning sessions?

The intended effects of muscimol were to inactivate AIP for the duration of conditioning. However, the effects of muscimol may have extended beyond AIP, and beyond the duration of conditioning. It is therefore important to consider the present results in light of these possibilities.

Muscimol infusions inactivated AIP during conditioning.

Muscimol has prolonged effects upon normal function of the cerebellum (Van Neerven *et al.* 1989) (see Chapter 2). Even at the low dose used here, its effects were of sufficient duration for the conditioning experiments. In Phase 4, previously acquired CRs were prevented from expression throughout each training session of one hour. The complete absence of conditioned responses to the CS alone indicates that the muscimol block was as effective as unilateral destructive lesions of the cerebellar nuclei (Yeo *et al.* 1985a). All effective injections were immediately above the cerebellar nuclei. Consistent with the electrophysiological recordings, these placements were just above the interpositus nucleus, or approximately on, or just caudal to, the border of the anterior and posterior divisions. From lesion studies (McCormick and Thompson 1984; Clark *et al.* 1984; Welsh and Harvey 1989; Yeo *et al.* 1985a) it is clear that cells in the anterior interpositus nucleus or at the border of the interpositus and dentate nuclei are required for the production of conditioned NMRs. Consistent with results of the previous chapter in which effective cannula placements were centred around AIP, and with electrophysiological and anatomical evidence reported in Chapter 1, muscimol infusions in the present study would have primarily agonised GABAergic receptors on cells of the AIP. It is likely that the most important effect of muscimol was to inhibit the cerebellar nuclei directly.

Could muscimol be effective outside AIP?

A recurring issue in this thesis concerns effects of muscimol on circuitry other than the AIP. As far as NMR conditioning is concerned, permanent lesions have shown that there are a restricted set of pathways which are necessary for NMR conditioning. In the cerebellum, the action of muscimol is likely to be on AIP and parts of the cerebellar cortex including Lobule HVI. Since Chapters 1 and 2 have discussed this issue in detail, the arguments will be re-stated here only briefly. We cannot discount the possibility that some of the effects may have been due to the direct action of muscimol on important

parts of the cerebellar cortex. Injections into the cerebellar nuclei of doses of muscimol considerably greater (14 nanomoles of muscimol in 1μl of saline) than those used here spread quite widely through the cerebellar cortex but it is claimed that muscimol did not spread outside the cerebellum within 90 minutes (Krupa *et al.* 1993). It is highly probable that the much smaller doses of muscimol used here did not spread outside the cerebellum and may not have invaded such wide areas of the cerebellar cortex and other parts of the deep nuclei. No subject showed vestibular symptoms that would have arisen if significant concentrations of muscimol had spread into the fastigial nucleus or anterior vermis. In one subject (C5) the cannula tip was located in the Paramedian lobe and muscimol was ineffective in disrupting CRs. The distance from that cannula tip to the centre of an area covering the anterior interpositus and dentate nucleus boundary was 3.2 mm at its greatest, and results from the previous chapter suggest that as an indication of distance, this figure is a liberal estimate of muscimol spread.

The indirect, but equally important consequences of AIP inactivations on the cerebellar cortex must also be considered. One cannot assume normal activity in anatomically related parts of the olivo-cerebellar system, since perturbations of normal activity in AIP would inevitably have consequences for cerebellar cortical and olivary function. Learning deficits arising from AIP inactivations could be attributed to impaired function in any or all parts of the olivo-cerebellar module of which AIP is a part.

Could muscimol affect learning after conditioning?

Cerebellar inactivations may have lasted beyond the duration of one conditioning session. Would this have implications for the interpretations of the current study? An important aspect of memory formation in classical conditioning is its dependence on processes which occur after training. These are more closely associated with plasticity than acquisition and extinction for reasons explained in chapters 1, 2 and 4. Is it possible that learning was prevented because muscimol disrupted such essential processes in cerebellar circuitry? It is conceivable that muscimol may have impaired cerebellar plasticity by acting on mechanisms of consolidation, which are susceptible

to disruption even after conditioning has ended. This outcome would be consistent with the hypothesis that there is essential plasticity for NMR conditioning in cerebellar circuitry, and has been directly tested in the following chapter.

Alternatively, it is possible that muscimol spread beyond the cerebellum after conditioning, and disrupted consolidation processes in extracerebellar circuitry. This is certainly a possibility in Krupa *et al.* (1993), given the very large dose of muscimol used. However, in the present study, this possibility is much less likely because a very small dose of muscimol was used. If muscimol spread to the surface of the cerebellum, its concentration would have been decimated when it left the surface and entered the cerebro-spinal fluid. Concentrations reaching other structures would therefore be very low. The next chapter examines this issue more directly, by infusing a large dose of muscimol into the cerebellum immediately after conditioning.

3.4.5 Cerebellar circuitry and NMR conditioning

What has this study revealed about the role of the cerebellum in NMR conditioning? This study has shown that the circuitry required for extinction is also required for acquisition, and that long term effects of muscimol or cannula damage to AIP cannot account for learning deficits seen in the present study. It is now certain that normal activity in the AIP is required for learning in NMR conditioning. The study strongly supports the hypothesis that plasticity essential for NMR conditioning is in cerebellar circuitry. The main objection to this hypothesis is that learning observed in the current study is state-dependent. The possibility that this might occur through the altered perception of the CS has been discussed, and it has been argued that this possibility is remote but cannot be ruled out. It is equally possible that in acquisition, muscimol infusions into the cerebellum might affect processing of the US. There is some evidence that the cerebellum might have a role in attenuating the excitabilities of neurones in the sensory trigeminal nuclei, through cerebello-rubro-trigeminal pathways (Donegan and Thompson, 1991). These issues cannot be resolved by inactivations during conditioning. So, work in the next chapter attempts to investigate the potential

for cerebellar plasticity by inactivation of AIP during the consolidation period - after conditioning - when no stimuli are present.

In conclusion, this study has confirmed a role for the AIP in both the acquisition and extinction of NMR conditioning, supporting the idea that there are common mechanisms of learning for both processes. This result also strengthens support for the idea that essential plasticity for NMR conditioning is based in the cerebellum.

Chapter 4

Post-trial inactivations of the cerebellar nuclei do not prevent the consolidation of conditioned responses.

4.1 Introduction

Preceding chapters report experiments that have demonstrated that activity in cerebellar circuitry is critical for NMR conditioning. Inactivations of the cerebellum that are effective in preventing the expression of conditioned NMR's are also effective in preventing acquisition (chapter 3) and extinction (chapter 2). So, parts of the cerebellum that are essential for the expression of conditioned NMRs, are also essential for acquisition and extinction.

These results support the postulate that cerebellar circuitry is essential for this kind of motor learning. The issue that is as yet unresolved concerns the *reasons* for critical cerebellar involvement in NMR conditioning. The long-standing cerebellar learning hypotheses (Marr, 1969; Albus, 1972; Ito, 1972) are one prominent set of ideas that explains these results, and are centred on the idea of cerebellar plasticity. However, there are alternative hypotheses. As discussed in previous chapters, studies involving drug infusions during presentation of stimuli may be problematic for localisation of learning related plasticity because such studies are not free from drug influences on sensory, motor, and other factors which may confound localised drug effects on such plasticity. In the cerebellum, learning may be prevented by AIP inactivations for reasons other than impaired plasticity. Impaired sensory processing of the CS and/or the US in the drug condition may result in a dissociation between the properties of the stimuli in the drug state and in the no-drug state. This would yield state-dependent learning (SDL). In chapter 2, in which SDL is discussed more fully, it is argued that either altered CS processing or impaired plasticity could account for learning deficits resulting from AIP inactivations. Whether there is learning-related plasticity in AIP must therefore be examined independently of confounding factors introduced by interventions during conditioning.

An important facet of memory formation is its dependence upon processes that occur after training. In conditioning experiments, the CS and US are absent at this time. Spaced training experimental designs, that incorporate rest intervals spaced between

regular training sessions are known to result in stronger learning than designs which have massed training, without rest intervals (Mackintosh, 1974). Processes that facilitate memory formation must occur in these intervals. In many investigations of learning, post-training interventions between sessions such as electroconvulsive shock, administration of drugs (either systemic or intracerebral) or electrical brain stimulation, have the effect of impairing or preventing learning (McGaugh and Herz, 1972). The most plausible account of this phenomenon involves the 'consolidation hypothesis', which asserts that physiological, time dependent processes occur after training and act to stabilise relatively labile memory acquired during training. Post-training interventions damage consolidation processes.

In support of the consolidation hypothesis, studies have shown that memory can be disrupted by post-training interventions in species across the phylogenetic scale, and in many different preparations. Some studies have shown that motor learning in humans requires consolidation. In one of these, Brashers-Krug, Shadmehr and Bizzi (1996) have shown that there are indeed post-training, time dependent memory processes essential for human motor learning. In their study, subjects were given a motor task to learn (task A; reaching movements to different targets using a force-producing manipulandum within a specified time). Different groups of experimental subjects were given another similar task, in which movement was required to be learned in the opposite direction (task B). Subjects who were given task B immediately after task A showed no improvement in task A on day 2, compared with controls (no task B) who did improve. Subjects who were given task B five minutes or an hour after task A showed some improvement, but only the group given task B after 4 hours showed improvements on task A comparable to controls. This demonstrated that a 4 hour consolidation period was required for this task, in which consolidation gradually progressed.

There is also evidence that memory formation in NMR conditioning can be affected by post-training interventions, although this literature is sparse. There are four studies in particular which have examined this issue, and show that NMR conditioning is susceptible to post-training interventions. There appear to be no studies in which post-training interventions leave NMR conditioning intact. The earliest study to examine

post-training effects on NMR conditioning (Cholewiak *et al.* 1968) examined the effects of strychnine, (glycine antagonist) a substance found to enhance learning when administered after training in a number of other behavioural preparations (McGaugh and Herz, 1972). Rabbits received systemic strychnine injections just before delay conditioning. This treatment had the effect of significantly enhancing conditioning compared to saline controls (effects on extinction are discussed in chapter 2). When strychnine was injected just after conditioning, the effect was to depress acquisition. Acquisition was significantly retarded in the post-training strychnine group compared with the post-training saline group.

Not all interventions after conditioning have impaired NMR conditioning. Hernandez and Powell (1983) reported the effects of naloxone on NMR conditioning. When intravenous naloxone injections were administered immediately after each conditioning session, conditioning was facilitated over a number of sessions. This apparently suggests that naloxone accelerates consolidation of NMR conditioning.

Scavio *et al.* (1992) examined the post-training effects on NMR conditioning of scopolamine, ketamine, amphetamine, chlorpromazine and saline. In this consolidation study, when injected intravenously immediately after each session, all depressed acquisition except ketamine, which enhanced acquisition. When injected 2 hours after conditioning, all these had very little effect. The authors argue that the drugs affected time-dependent, associative processes occurring within 2 hours of conditioning.

These studies have also examined the effects of these same drugs on acquisition, extinction and on sensory processing - issues particularly pertinent to studies in this thesis. These aspects of the experiments have been discussed in previous chapters. Also of interest is the fact that some drugs can enhance acquisition if injected after training. Possible reasons for this are beyond the scope of this chapter, but are discussed in the concluding chapter of this thesis.

These studies indicate that motor learning generally, and NMR conditioning specifically, require consolidation processes which last for some hours. So, there is an

opportunity to examine the effects of post-training inactivations of the cerebellum on processes assumed to be causal to the establishment of long-term plasticity, without the influence of drugs on sensory or motor processing. Testing for consolidation deficits using post-training interventions, provides a more stringent criterion for determining the involvement of plasticity in circuitry essential for NMR conditioning, since consolidation processes are important for the formation of motor memory.

NMR conditioning and post-training inactivations of AIP with muscimol

In this chapter, experiments are reported which directly test for possible plasticity in essential cerebellar circuitry in consolidation of NMR conditioning, by muscimol inactivations of this circuitry immediately after conditioning. Muscimol is the preferred drug for inactivations because it has been effectively used to inactivate the cerebellar nuclei in numerous studies (Mink and Thach, 1991; Robinson, Straube and Fuchs, 1993; Krupa and Thompson, 1993). Muscimol is also known to have dose-dependent behavioural consequences that last for many hours and which completely dissipate within 24 hours of intracranial microinjection (this will be confirmed in the present study and is discussed further in chapter 2). Its spread is also known to be restricted to small areas if small doses are used (Martin, 1991) and, because it acts on GABA-A receptors to hyperpolarise neurons, it only affects cell bodies and not fibres of passage. In addition, the availability of radiolabelled muscimol makes possible the autoradiographic evaluation of its spatial distribution when injected into the cerebellum. Muscimol is known to be effective in impairing post-training processes essential for memory formation in other behavioural preparations and models. Post-training, bilateral muscimol injections into the amygdala cause retrograde amnesia for inhibitory avoidance learning (Brioni *et al.* 1989; Izquierdo and Medina, 1991). Also, If injected bilaterally into the hippocampus after training, memory formation is disrupted for both inhibitory avoidance and habituation to novel environments (Izquierdo, 1991). In the entorhinal cortex, post-training muscimol infusions cause amnesia for both of these if given 90 to 120 minutes after training, but not before or after this. If similar

GABAergic processes exist in cerebellar circuitry essential for NMR conditioning, then muscimol would also be appropriate to use in the present set of studies.

In the present study, experimental subjects were required to meet two criteria for inclusion in the experimental group. The first was that muscimol had to be completely effective. The effectiveness of the muscimol injection after each acquisition session was inferred by subsequent testing of a further injection after conditioning had developed. The blockade of CR expression in this phase indicated that earlier blockades have been similarly successful. A second criterion related to cerebellar damage produced by the cannula. Damage to critical structures (AIP and HVI) would produce impairments in conditioning - a result indistinguishable from consolidation impairments (Yeo *et al*, 1985 b,c). So, subjects were only included in this group if histological examination revealed no significant cannula damage to either AIP or Lobule HVI. It is also important to determine the temporal limits of the inactivations, to be certain that measurable drug effects do not extend into later periods in which training occurs. Localised, intra-cerebellar muscimol injections which prevent learning also prevent the expression of CRs if the dose of muscimol is sufficient, and if the cannula position is close enough to critical cerebellar structures. So, perhaps uniquely to NMR conditioning, it is possible to assess the completeness of inactivations in individual animals, by observing CR frequency immediately after muscimol injections.

The target of inactivtions in these studies is AIP. Muscimol is know to be highly effective at preventing acquisition, extinction and expression at very low doses when microinjected into or near this area (see previous chapters; Krupa and Thompson, 1993). However, it is not possible to preclude the possibility that the drug also has its effects in critical parts of the cerebellar cortex. AIP is situated near and ventral to HVI, and it is possible that low but sufficient concentrations of muscimol migrated to HVI in previous studies. Also, given the connectivity between these structures, it is feasible that muscimol injections which inactivate HVI are likely to affect normal activity in AIP. Another, though less likely possibility, is that HVI is significantly affected by AIP inactivations through minor nucleo-cortical connections, and indirectly through nucleo-olivocortical connections. The functional consequences of such secondary effects is

unclear. If post-training inactivations of AIP succeed in impairing or preventing conditioning, it is clear that AIP is involved in the consolidation of plasticity, but this plasticity cannot be localised to AIP since there is some chance that effect of inactivations may extend to cerebellar cortex. However, in the event that acquisition proceeds normally despite post-training inactivations of AIP, it can be unequivocally concluded that AIP is not involved in consolidation processes for NMR conditioning and therefore cannot be a site of plasticity.

4.2 Methods and Design

Details of methods common to experiments in all chapters are detailed in appendix 1.

Subjects

18 rabbits were used in this study. They were divided into two experimental groups (High dose (HD) n=12; Low dose (LD) n=6). There were two control groups, each derived from previous chapters. The cannula control group and unoperated control group were derived from experimental subjects and control subjects respectively, from chapter 2, phase 1.

Experimental Design

Groups	*Conditions*	*Phase 1*	*Phases 2a & 2b*	*Phase 3*
		Acquisition	*Muscimol Testing*	*Histology and analysis of muscimol spread*
Experimental Groups	1) Low dose, LD. (n=12 initially; n=3 after rejections)	1.54nm	(a) 1.54nm (b) 7.7nm	Histological analysis
	2) High dose, HD. (n=6 initially; n=5 after rejections)	7.7nm	(a) 7.7nm (b) 1.54nm	Histological analysis, [^{3}H]muscimol injection and autoradiography.
Control Groups	1) Cannulated	no muscimol	no muscimol	Histological analysis
	2) Unoperated	no surgery, no muscimol	no surgery, no muscimol	-

Table 4.1: Experimental design

Phase 1: Cerebellar inactivations immediately after conditioning.

Prior to Phase 1, subjects underwent a session of adaptation. At the start of Phase 1, experimental and control subjects received 4, daily sessions of classical conditioning (100 trials per session, 90 paired CS and US, 10 CS alone; see appendix 1 for details). The experimental groups received a 1 microlitre microinjection of muscimol into the cerebellum within 5 minutes of the end of each session (for injection protocol, see appendix 1). There were two experimental groups - one group received a low dose (LD group, 1.54nm) and the other a high dose of muscimol (HD group, 7.7nm). At the start of Phase 1, the LD group consisted of 12 animals and the HD group consisted of 6 animals (n_s). In order to ensure specificity of AIP inactivations, in the LD group cannulae were targeted as close as possible to AIP so that low levels of muscimol could achieve maximal inactivations. However, in the event of a negative result, a low dose of muscimol would leave the experiment prone to a type II error, in which insufficient levels of muscimol may have resulted in a false negative result. This group was also prone to type I error, in which a false positive result could have resulted from cannula damage to cerebellar structures critical for NMR conditioning. To counteract such effects, the HD group was used in which a much higher dose of muscimol would avoid false negative result due to insufficient muscimol levels. To avoid false positive results in this group, cannulae were implanted more dorsal to the cerebellar nuclei. This would, of course, be achieved at the cost of because the larger volume of muscimol would migrate further, perhaps to cerebellar cortical structures known to be critical for NMR conditioning.

In summary, the LD group achieved anatomical specificity but was prone to both type I and type II errors. The HD inactivations were much less prone to these, but were less specific. In this group, it would be important to estimate the maximum spread of muscimol (see below) so that problems of specificity may be overcome to some extent.

Acquisition of CR's in the experimental groups was compared with two control groups. The cannula control group was derived from subjects in chapter 1 (Effective group in phase 1 of chapter 1). These animals had been implanted with cannulae and had then

received NMR conditioning with parameters identical to those of the experimental group. No muscimol was infused during Phase 1 conditioning. Data from chapter 1 indicates that cannula placements of these animals were known to be sufficiently close to AIP so that infusions of the low dose (1.54nm) prevented the expression of CRs during unpaired CS presentations. The other group was derived from the Unoperated control group in chapter 1, some of which were also used in the Naive group in chapter 2. Comparison of the cannula control group and the unoperated group established whether or not general cannula damage resulted in impaired conditioning. Comparison of the experimental groups with these groups established whether or not the experimental treatment (i.e. infusions of post-trial muscimol infusion) had any effects on conditioning.

Phase 2: Muscimol testing

Another measure used to avoid false negative results was to introduce phase 2, in which the behavioural efficacy of muscimol inactivation could be evaluated in each animal. Three days after phase 1, animals were a given a further 4 daily sessions of training to bring CR frequencies of all experimental animals to post-asymptotic levels. They were then given muscimol infusions at the experimental dose (phase 2a), in the same way and at the same dose as in Phase 1. Animals were then immediately given one session of training (100 conditioning trials). After an interval of one hour, in which they were returned to home cages they were given hourly training sessions consisting of 20 trials per session (18 paired ; 2 unpaired), for 6 hours. 24 hours after the injection, the animals were once again given 20 trials to assess whether or not the behavioural efficacy of cerebellar muscimol injections extended beyond a 24 hour period. In Phase 2b, This procedure was repeated with the LD group receiving 7.7nm of muscimol (equivalent to that in the HD group), and the HD group receiving 1.54nm of muscimol (equivalent to that of the LD group). Phase 2a revealed the time course and behavioural efficacy of the experimental dose, and Phase 2b revealed whether this was dose-dependent and therefore specific to muscimol, or whether any decrement in CR frequency was due to

effects unrelated to muscimol effects, such as fatigue or stress from repeated training sessions.

Any animal in which the experimental dose of muscimol did not reduce CR frequency to zero during Phase 2a, was rejected from the study. LD and HD subjects were grouped according to this criterion, and for the HD group, the time point at which no CRs were present in any subject was established (t_{max}). This phase therefore ensured that muscimol was effective in inactivating cerebellar circuitry essential for NMR conditioning in all experimental subjects.

Phase 3: Histological verification and autoradiograph

In order to assess the approximate spread of the experimental dose of muscimol from the time of injection to $t_{max.}$, subjects in HD group were given microinjections of [^{3}H]muscimol (7.7nm), and all subjects were killed and perfused at $t_{max.}$.

Serial sections cut from their brains (see below) were subjected to autoradiography and image analysis, from which the approximate spread of muscimol was established in each case.

After histological processing, serial sections from animals of LD and HD groups were assessed for damage to cerebellar cortical and nuclear areas known to be essential for NMR conditioning. Any animal in which there was damage to significant areas of the cerebellum were rejected from the study. This measure was to ensure that false positive results (impaired conditioning due to factors other than post-trial muscimol injection) did not bias towards confirming effects upon consolidation.

Surgery

18 rabbits underwent surgery for implantation of a guide cannula directed towards the right AIP. Surgical and stereotaxic procedures used are similar to those used in previous chapters, and are reported in appendix 1. The right cerebellar cortex was exposed and

the approximate position of the cerebellar nuclei was defined stereotaxically (-5mm AP, +4mm ML, -10mm DV). Their exact position was then determined by electrophysiological recording. In the LD group, a 24G stainless steel cannula guide was implanted 1mm above the estimated position of AIP, so as to avoid damage to AIP and parts of cerebellar cortex important for NMR conditioning. In the HD group, the cannulae were implanted 2mm above the estimated position of AIP. Surgical subjects were maintained in a recovery room for 3 days and allowed 4 more days to recover in their cages.

Conditioning

All rabbits were prepared for measurement of nictitating membrane movements. Preparation methods were identical to those reported in chapters 2 and 3. The rabbits were trained using techniques and parameters in the previous chapters.

Analysis of behavioural data

Criteria for CRs were the same as those in another study (7). Statistical analysis was performed on session CR frequency in Phase 1 using a 2-way Analysis of Variance.

Tritium Injections

Tritiated muscimol (7.7nm) was prepared (appendix 2). Injections were carried out in a registered radiation protection area. Rabbits were placed in a stock individually, and microinjected with tritiated muscimol with procedures similar to those in phases 1 and 2. They were then placed in a large, waterproof box until t_{max} at which time they were immediately killed and perfused (appendix 1). Their brains were removed and processed for histology.

Histology and Autoradiography

Histology

In order to assess cannula damage, injection sites and spread of muscimol, 50 micron sections cut from brains of HD and LD subjects and (see appendix 1). Every fourth

section from each case was mounted onto glass slides and stained for Nissl substance to reveal the cerebellar nuclei and the path of the guide cannula and injector. Incidental damage to the cerebellum that may have impaired conditioning, was also revealed.

Autoradiography

Sections from the HD group adjacent to those stained, were prepared for autoradiography. These were selected on the basis of Nissl stained histology. About 8 sections from each case were selected. Sections which contained the path of the cannula, the injector site, parts of lobule HVI and AIP were chosen. Tritium standards (Amersham) containing 8 polymer sections (increasing levels of tritium across this range) were each mounted onto a glass slide. Each standard contained 8 separate polymer sections each with a different level of tritium. The mounted sections and standards were glued onto cards that were cut to exactly fit into two Amersham autoradiography cassettes, such that each cassette contained one standard.

Autoradiography and image analysis methods are reported in Appendix 2.

4.3 Results

Groupings

Subjects were first grouped according to histological analysis for damage to critical cerebellar structures (lobule HVI and AIP) and CR execution after muscimol injections (Phase 2). The results are summarised below (behavioural data of individual subjects are presented in appendix 3). Phase 1, 2 and 3 results are presented below.

Table 4.2: Categorisation of subjects in LD Group

Subject	Histological Analysis	Effect of muscimol on CR frequency	Grouping
G3201	Significant damage to caudal parts of HVI.	Incomplete inactivation	Reject
G3202	No significant damage	Incomplete inactivation	Reject
G3203	No significant damage	Incomplete inactivation	Reject
G3204	No significant damage	Incomplete inactivation	Reject
G3205	No significant damage	Incomplete Inactivation	Reject
G3206	No significant damage	Reduced to zero	Include
G3207	Significant damage to caudal parts of HVI	Reduced to zero	Reject
G3208	Extensive damage to dorsal parts of caudal HVI and to most of paramedian lobe.	Incomplete inactivation	Reject
G3209	No significant damage	Reduced to zero	Include
G3210	No significant damage	Reduced to zero	Include
G3211	No significant damage.	incomplete inactivation	Reject
G3212	Extensive damage to caudal and rostral HVI.	Reduced to zero	Reject

Table 4.3: Categorisation of subjects in HD Group

Subjects	Histological Analysis	Effect of muscimol on CR frequency	Grouping
G3213	Significant damage to caudal HVI	Reduced to zero	Reject
G3214	No significant damage	Reduced to zero	Include
G3215	No significant damage	Reduced to zero	Include
G3216	No significant damage	Reduced to zero	Include
G3217	No significant damage	Reduced to zero	Include
G3218	No significant damage	Reduced to zero	Include

3 subjects in the LD group and 5 subjects in the HD group met the required criteria for inclusion as experimental subjects. The low number of subjects in the LD group favoured analysis of data as individual cases rather than grouped data, since the power of statistical tests in such a group is considerably reduced. Behavioural results of HD subjects are grouped.

It is useful to assess how many trials it took for subjects to start showing CRs (typically, when subjects first start showing CRs, they reach asymptote very quickly). The first of two consecutive non-zero 10 trial blocks, is taken as the point at which acquisition began. The number of blocks to this criterion for each subject was calculated, and is represented in figure 4.1. The mode, median and mean are located at the beginning of session 2 (blocks 10 - 20), so subjects in all groups typically started showing CRs at this time. The scores of all groups (except the LD group), appeared to be normally distributed because of the near coincidence of the mean, median and mode.

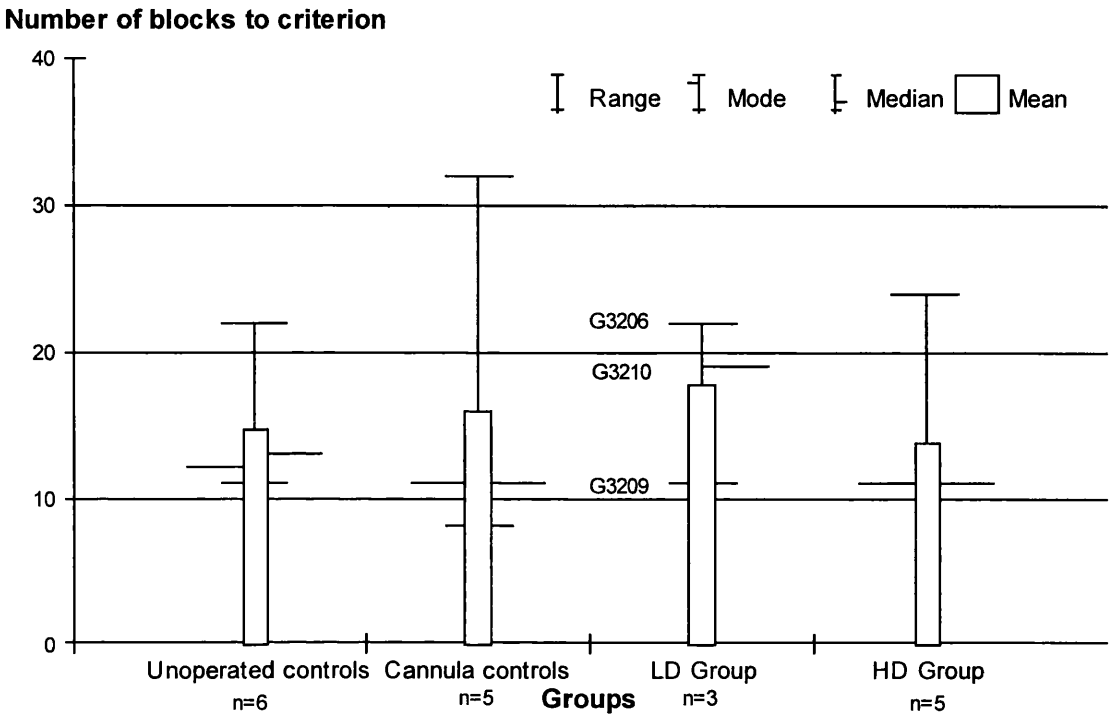


Figure 4.1: Number of blocks to criterion. The criterion to acquisition is defined as the first of two consecutive blocks with non-zero values.

LD Group

Phase 1:

Subjects G3206, G3209 and G3210 acquired CRs in the same range as controls. From figure 4.1, it is evident that the three subjects in the LD group (represented as the maximum, minimum and median) started to acquire CRs at about the same time as subjects in all other groups. Their actual acquisition profiles are shown in figure 4.2. These results show that post-training injections of muscimol did not affect acquisition.

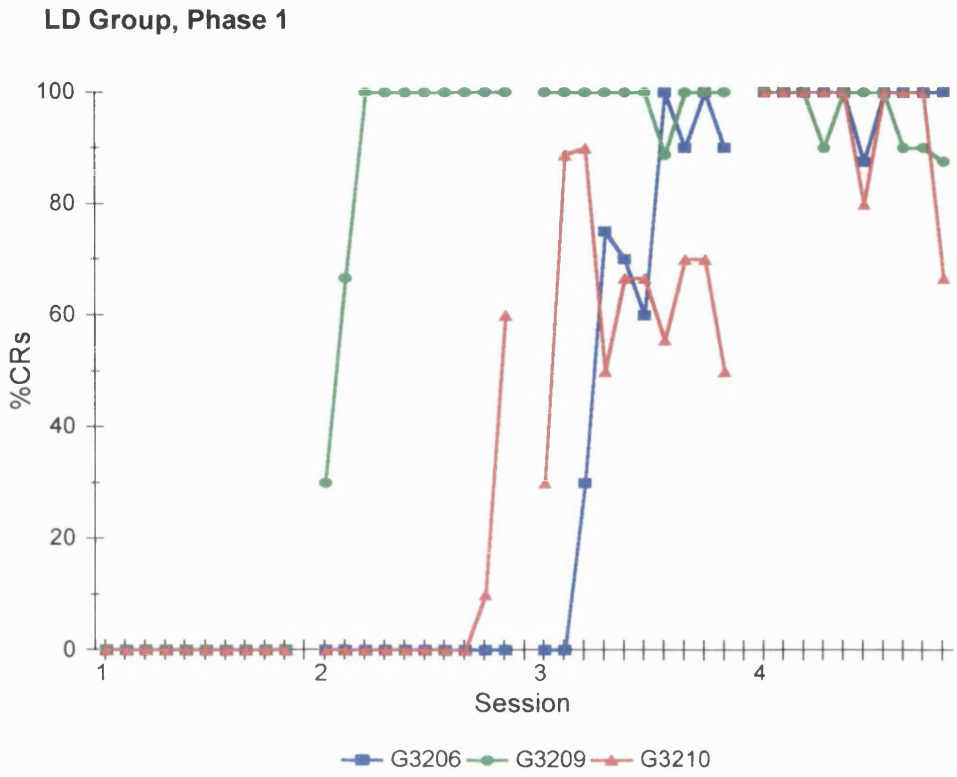


Figure 4.2: Phase1, LD Group. Data points represent 10 trial blocks. Data from individual subjects are plotted.

Phase 2:

CR frequency in all 3 subjects were quickly reduced to zero immediately after injections into or near the AIP. The inactivation was fully functional for at least two hours, after which CRs began to recover (Graph 4.3; thick lines, closed symbols). The higher dose reduced CR frequency to zero for longer (Graph 4.3; thin lines, open symbols). The effect was profound for two cases (G3209 and G3210). In G3206, CRs were prevented for an hour longer than with the low dose. 24 hours after injection, subjects in the LD group showed high CR frequencies.

These data indicate that in this phase, the absence of CRs was dose-dependent.

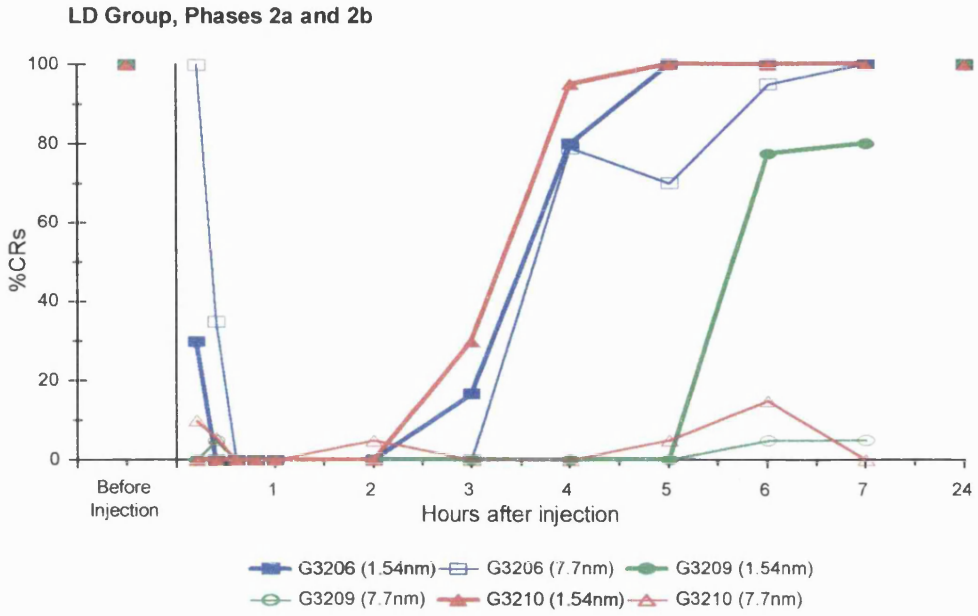


Figure 4.3: LD group, Phase 2. Animals were infused with muscimol, and then tested immediately for CRs for 1 hour. They were then tested at hourly intervals. Each data point represents the percentage of CRs in a 20 trial block.

Phase 3:

Histological verification shows that injections were made into or very close to the AIP.

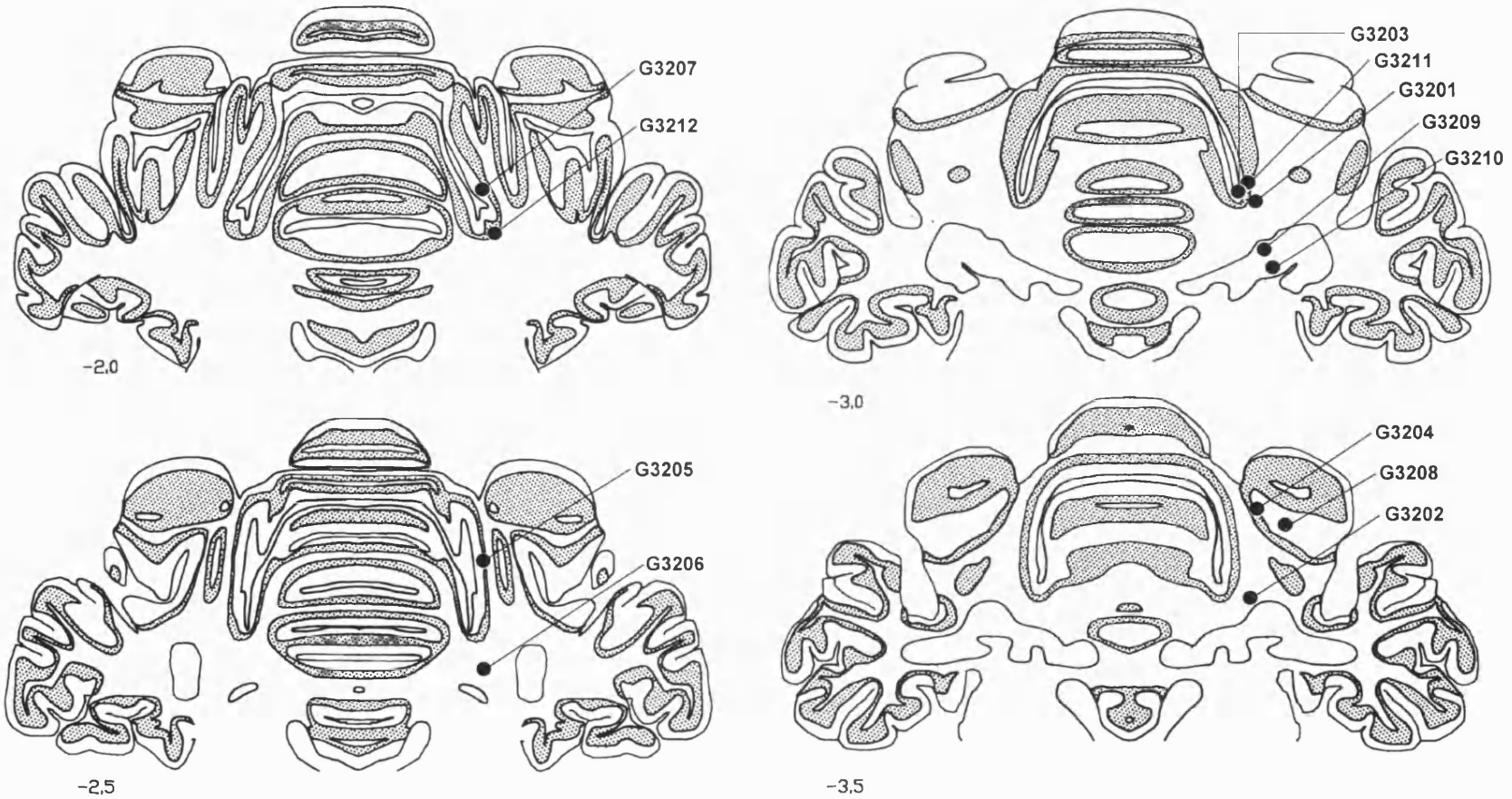


Figure 4.4: Cannula tip positions of cannulated subjects which received 1.54nm muscimol in Phase 1(G3201-G3212). Tip positions are represented on standard serial sections through the rabbit cerebellum at 0.5mm intervals. Section identifiers are the caudal distance of the sections from λ. Key to main anatomical features is the same as that used for figures 1.6 and 1.7.

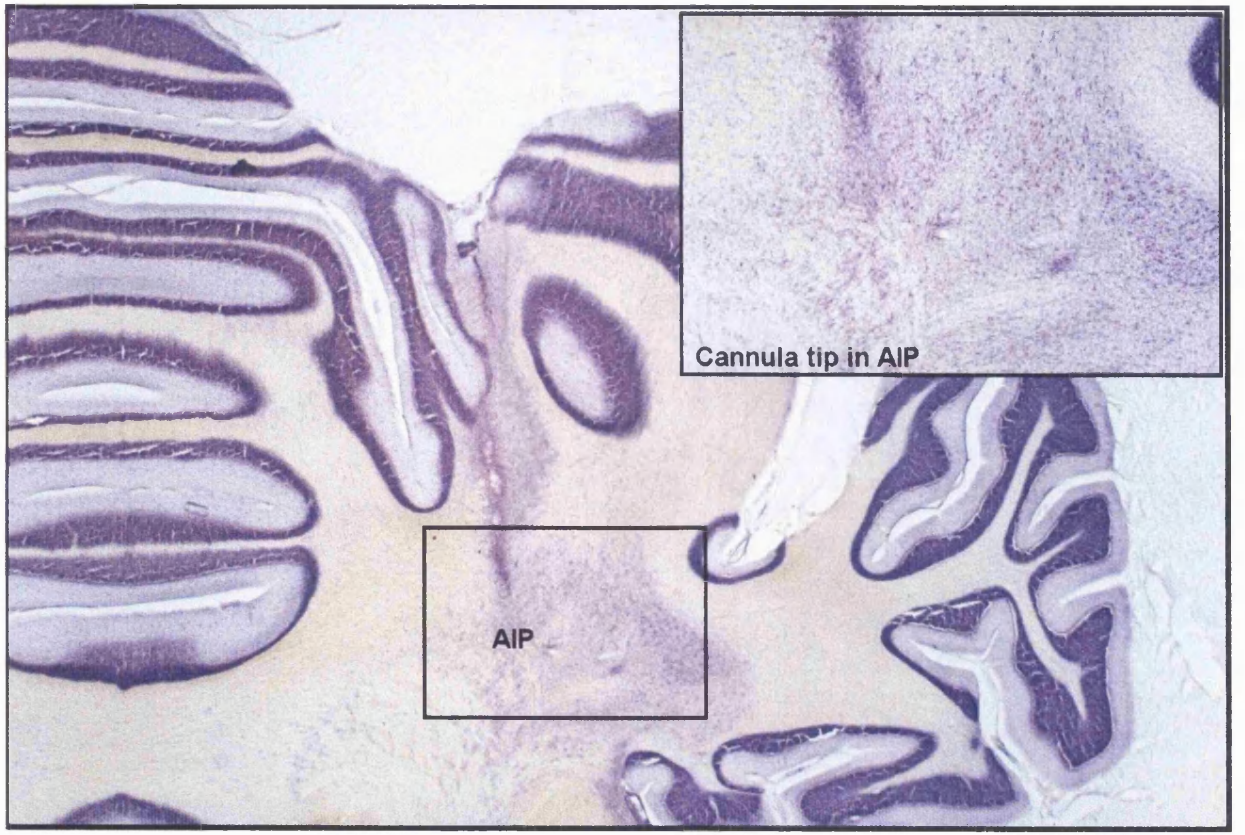


Figure 4.5: Transverse, Nissl stained section through the cerebellum of subject G3209. Cannula penetrated the cerebellar surface between vermis and paramedian lobe. Gliosis marks the cannula track. The cannula tip penetrated dorsal parts of AIP (see inset depicting high power photomicrograph).

It is clear that in each case, the cannula was located in or very near AIP. There can be no doubt that muscimol was delivered to AIP.

In this study, It is clear that AIP inactivations were complete but post-trial inactivations of AIP with muscimol has no effect upon the consolidation of NMR conditioning.

HD Group

Phase 1:

An analysis of variance (2 way repeated measures ANOVA) was used to compare acquisition of CRs in the unoperated controls, cannula controls, and experimental groups (Figure 4.6). Groups was one factor, and session CR frequency was the other (repeated).

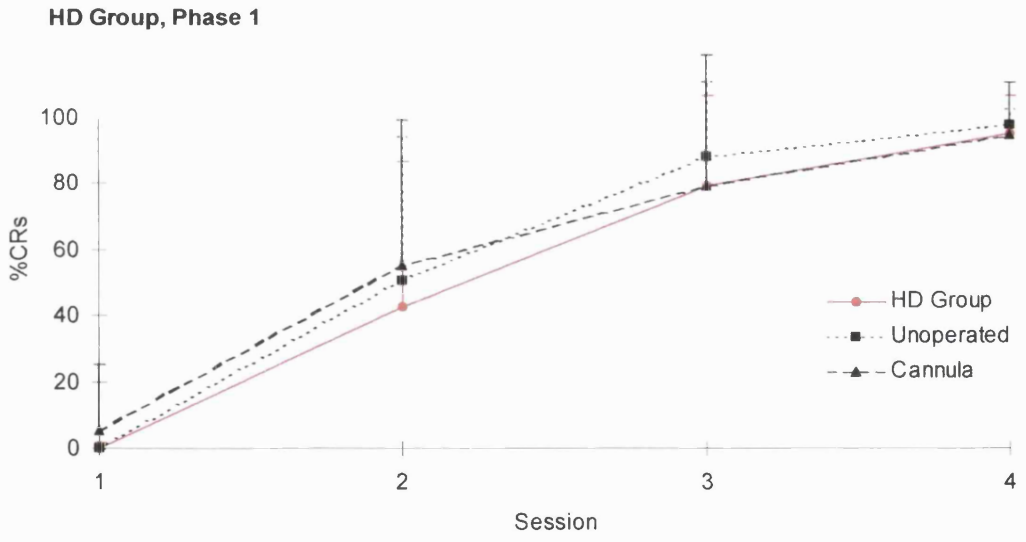


Figure 4.6: Phase 1, HD group. Data points represent session means. Error bars are standard deviations. Subjects in the HD Group received muscimol infusions (7.7nm) after each session of conditioning.

Groups acquired CRs similarly, there being no statistically significant difference between them ($F(2,13)=0.11$, $p>0.05$). There was a significant increase in CR frequency across the 4 conditioning sessions indicating learning in both groups ($F(3,39)=56.4$, $p<0.05$), and no group-by-session interaction ($F(6,39)=0.19$, $p>0.05$), indicating that groups acquired CRs similarly. Since neither infusion of muscimol nor cannulation affected acquisition, a carrier control group was not thought to be necessary.

Phase 2:

Behavioural data from the HD Group in Phase 2 are grouped (individual data are presented in Appendix 3).

a) Before muscimol injection, subjects showed no impairment in CR expression since CR frequency was high. When 7.7nm muscimol was injected into AIP, CR frequency declined rapidly to very low levels. There were very few CRs one hour after injection. After 4 hours (t_{max}), CRs were completely abolished in all subjects. CRs then began to recover, and 24 hours later, subjects showed no impairments in CR expression (figure 4.7).

b) In order to verify that the effect of drug infusion was dose-dependent and therefore muscimol-specific, and not confounded by other factors unrelated to pharmacological properties of muscimol (e.g. repeated training sessions, stress etc.), this was repeated using a much lower dose of muscimol (1.54nm in 1 microlitre). In the first hour, there was a slight decline in CR frequency, but 2 to 7 hours later CR frequencies were normal, as they were 24 hours later. The effect was therefore dose-dependent (figure 4.7).

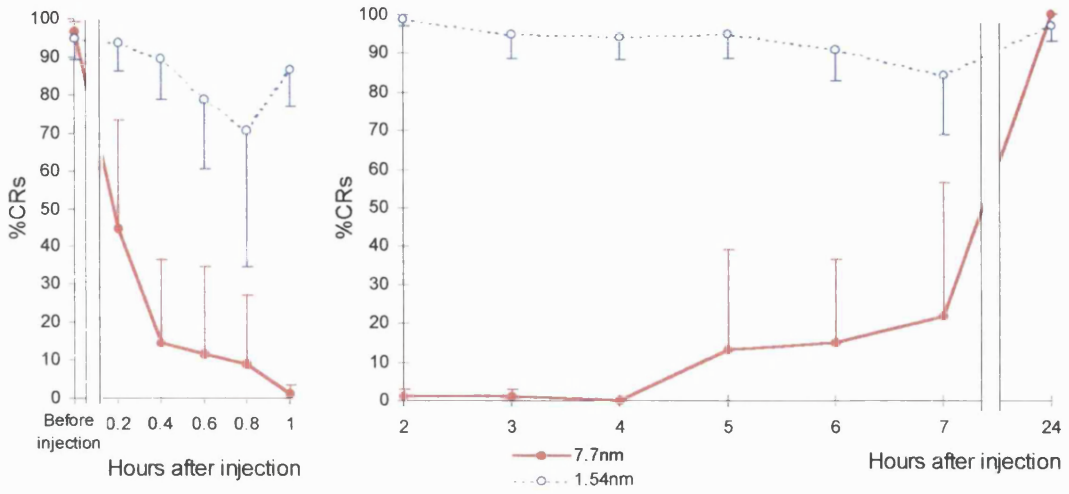


Figure 4.7 : Phase 2, HD Group. Animals were infused with muscimol, and then tested immediately for CRs for 1 hour. They were then tested at hourly intervals. Each data point represents the percentage of CRs in a 20 trial block.

Phase 3:

Histological data for individual cases formed the basis for histological grounds for rejections, and is reported above in Tables 4.1 and 4.2. Cannula tip positions are given in figure 4.8. Given that post-trial infusions of muscimol have no effect on the consolidation of CRs at either dose, it was of interest to know the approximate spread of the high dose of muscimol, to assess the extent to which muscimol was present in the cerebellar cortex. However, certain points should be borne in mind when interpreting the autoradiography. Tritium autoradiography has certain limitations, mostly concerned with the inefficient transfer of emissions to film. This problem is common to low energy emitters (^{3}H, ^{14}C and ^{35}S), and is caused by the re-absorption of emissions into the section (quenching). Unfortunately, this problem is greatest for tritium because it is the weakest emitter. It is particularly problematic for areas of tissue containing myelin, because beta particles are reabsorbed more much in myelinated tissues than non-myelinated tissues, so the distriubtion of silver grains on the film is determined in part by the distribution of myelin. This is problematic for brain areas which contain high myelin levels. In the present study, tritium levels in sections will inevitably be under represented in areas of high myelin content, such as cerebellar white matter. Herkenham and Sokoloff (1984) have reported that cerebellar white matter quenched 75% of tritium emissions relative to grey matter, and that this difference is eliminated by defatting in

xylene. Geary and Wooten (1985) have calculated regional quenching coefficients for different brain regions. Their estimate for cerebellar grey matter was 35.3%, which represents the ratio between optical density of ordinary, unprocessed tissue and the optical density of tissue from which myelin has been extracted using chloroform.

There are two ways of overcoming this problem. The first is to remove the lipids which are responsible for reabsorption by chloroform or xylene extraction. The second method is to calculate the degree of quenching from grey/white matter brain paste standards containing known amounts of tritium. These observations can then be used to mathematically readjust the optical density values obtained from film. A full discussion of these methods is beyond the scope of this thesis, except to say that they are necessary for quantitative analyses. If amounts of muscimol on different parts of the sections were required to be known, then one of these methods would be employed. However, this information would not extend our understanding of functional aspects of the inactivations, since nothing is known of the actual amounts of muscimol required to achieve functional inactivations, and so quantitative analyses were not conducted. The presence of any amount of muscimol in cortical or nuclear areas of sections revealed the potential to inactivate these areas in the present study.

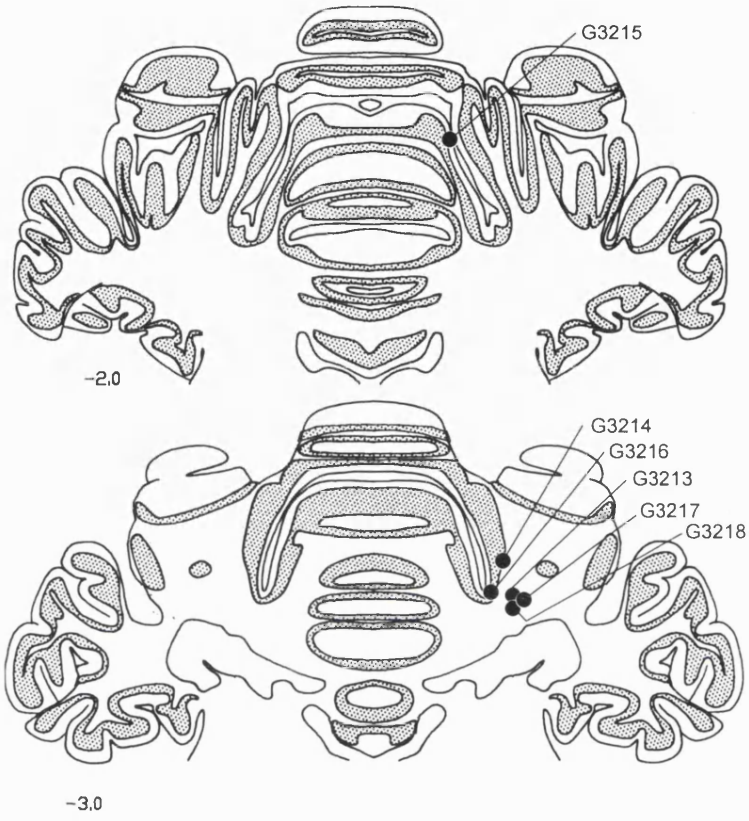


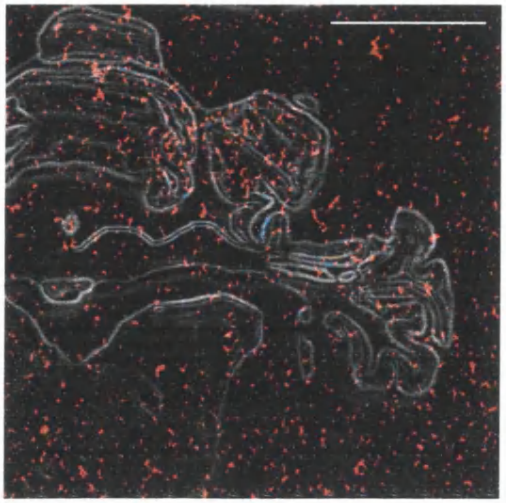
Figure 4.8: Cannula placements of subjects in the HD Group. Black filled circles represent the locations of cannula tips. Note that most are very near AIP (see figure 1.7 for location of AIP). See figures 1.6 and 1.7 for legend and locations of major subdivisions of the cerebellum.

The purpose of autoradiography and image analysis in this study was, therefore, to establish areas in each in section in which muscimol was present at all (i.e. where there is a high probability tritium being present above background levels). It should also be noted that the threshold level (grey level 220) selected to differentiate signal from background noise, was above the highest background value (grey level 221). Noise was therefore effectively subtracted from the images. Background noise statistics are presented in appendix 2. Inevitably, low levels of signal, representing lowest levels of muscimol at the periphery of the muscimol spread, may also have been subtracted from these images as a result of the conservative threshold setting.

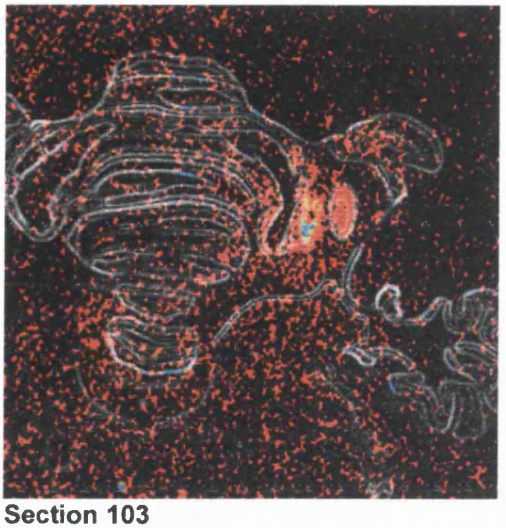
The data are presented in the following way. Merged images of all sections are presented for each case. In addition, sections of importance are presented in their uncombined form, so that colour coded autoradiography can be compared with colour coded standards.

After following the procedures in the Methods section and Appendix 2, the following images (figure 4.9) were produced (note that the scale bar at the top right of the first image in each case, represents a distance of 5.0mm).

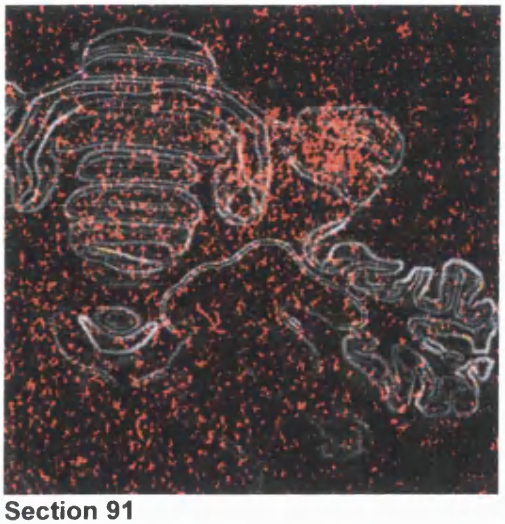
Case: G3213



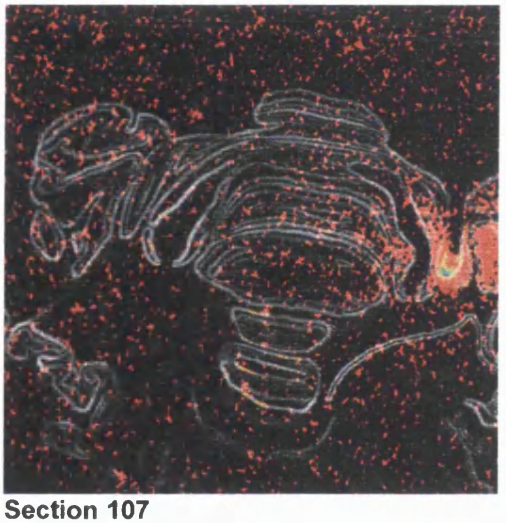
Section 75



Section 103

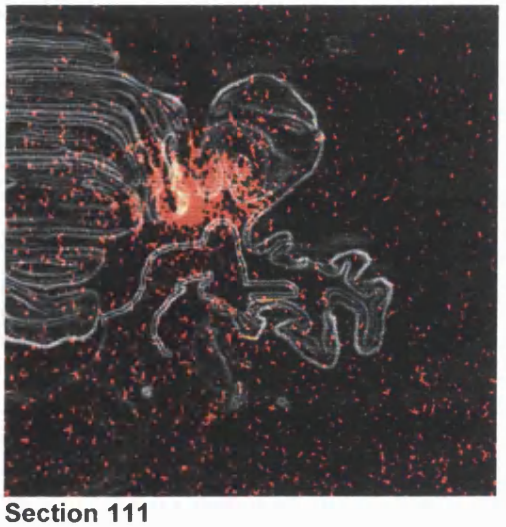


Section 91

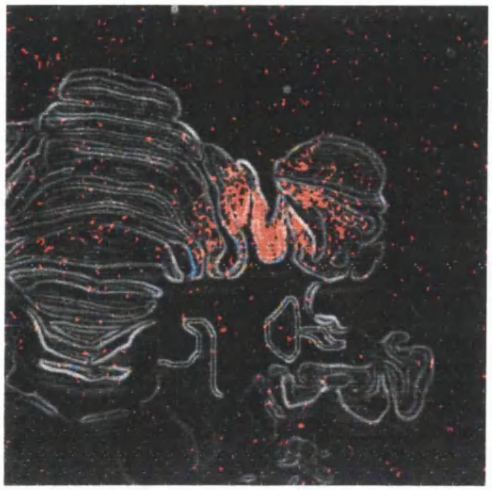


Section 107

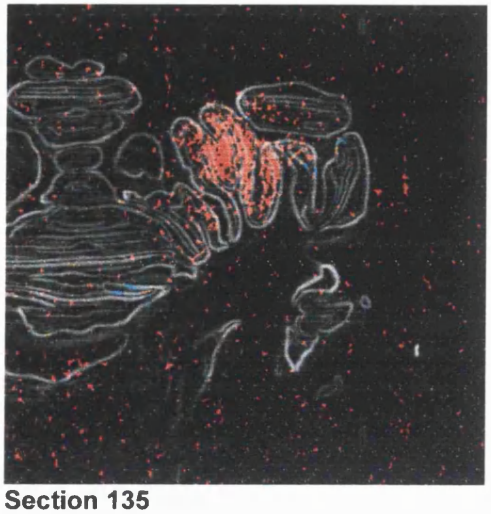
Section 99



Section 111

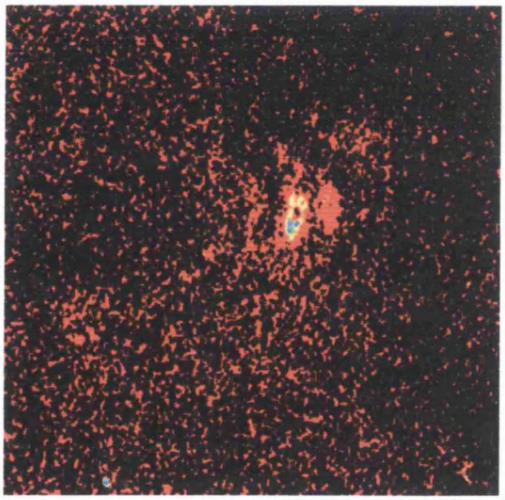


Section 119



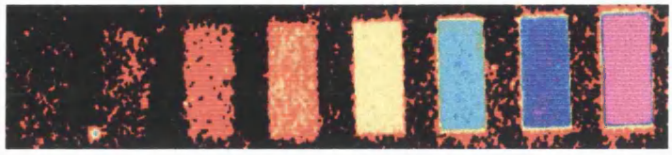
Section 135

Negative greyscale image of nissl stained section 103.

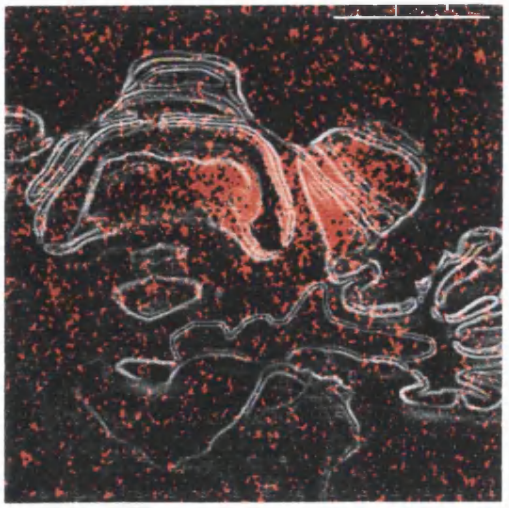


Unmerged image of processed autoradiograph of section 103.

Processed autoradiographic image of exposed [^{3}H] standards

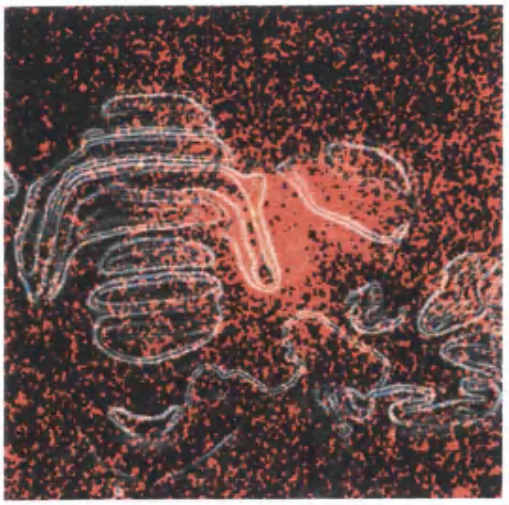


Case: G3214



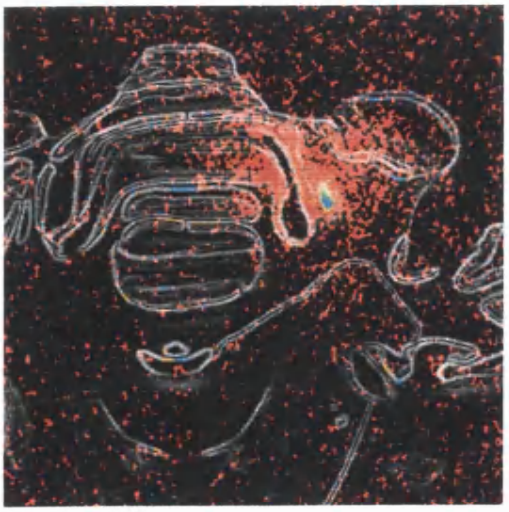
Section 107

Section 123

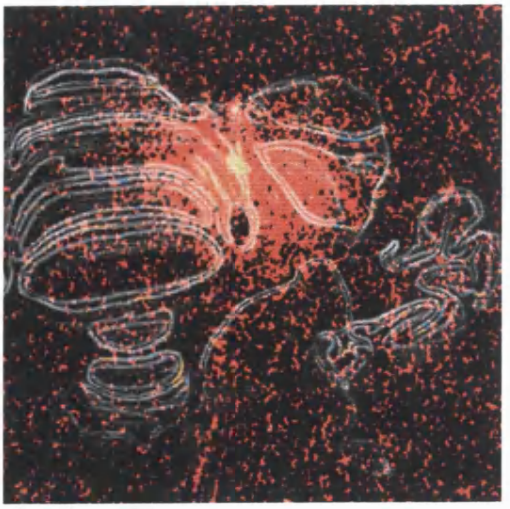


Section 115

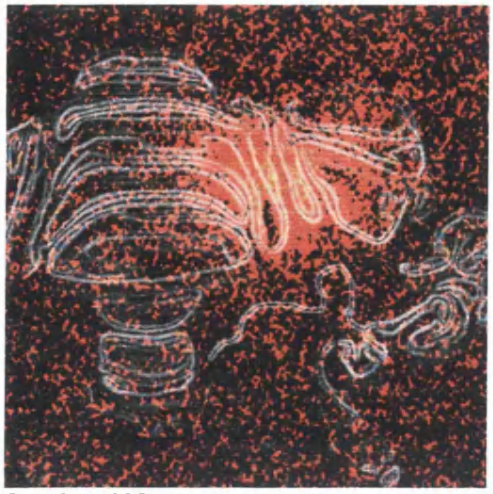
Section 127



Section 119

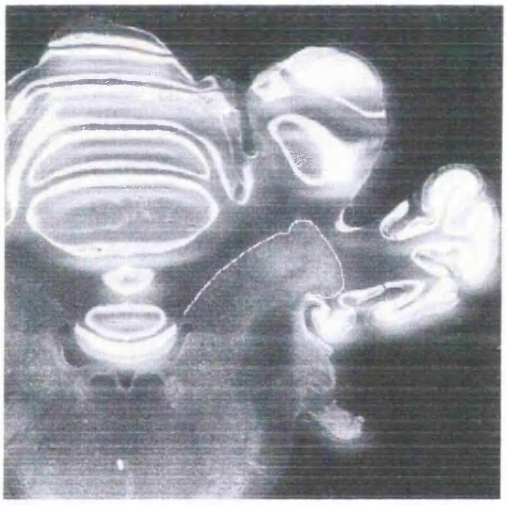


Section 131

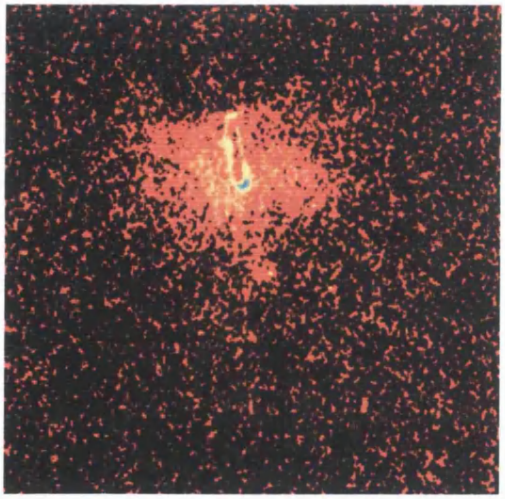


Section 135

Section 143

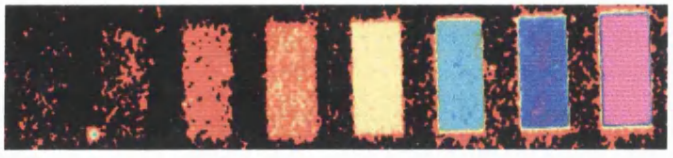


Negative greyscale image of nissl stained section 127.

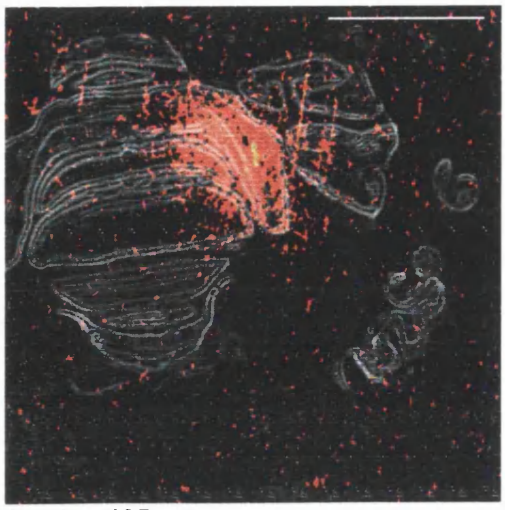


Unmerged image of processed autoradiograph from section 127.

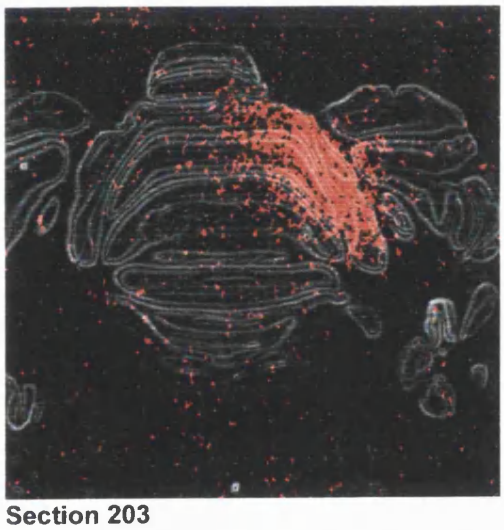
Processed autoradiographic image of exposed [^{3}H] standards



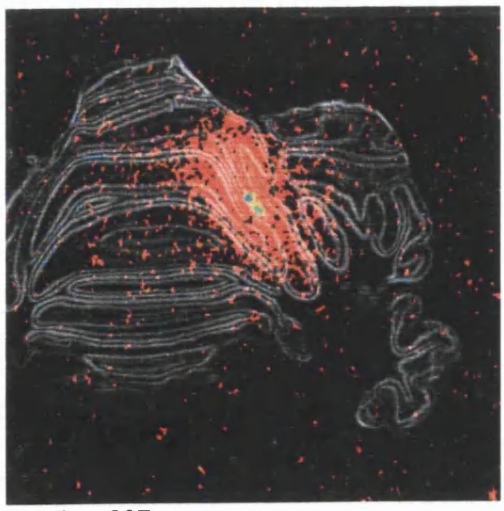
Case: G3215



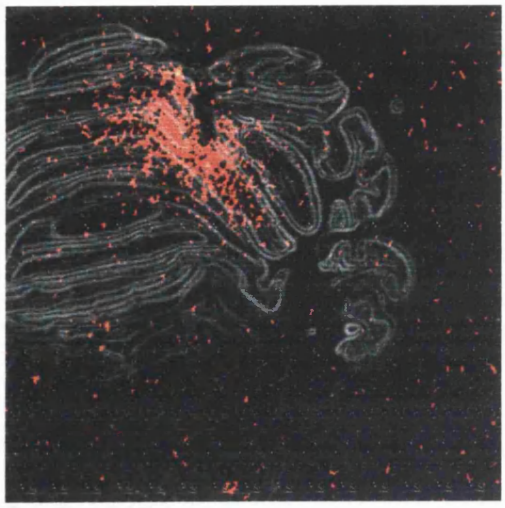
Section 195



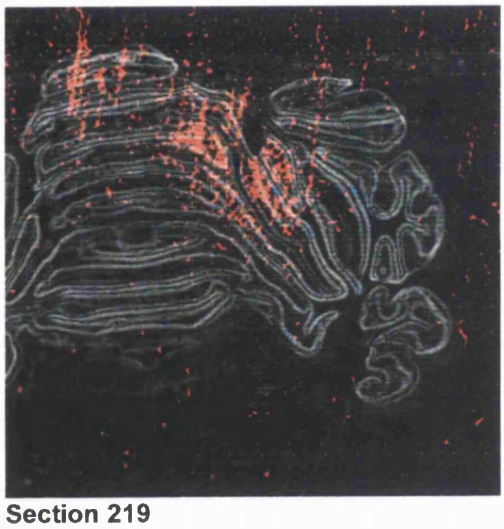
Section 203



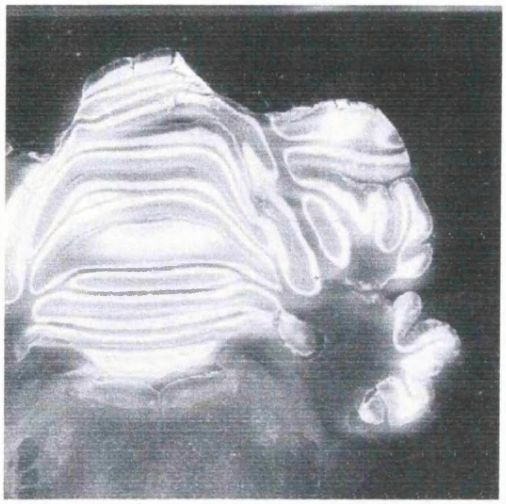
Section 207



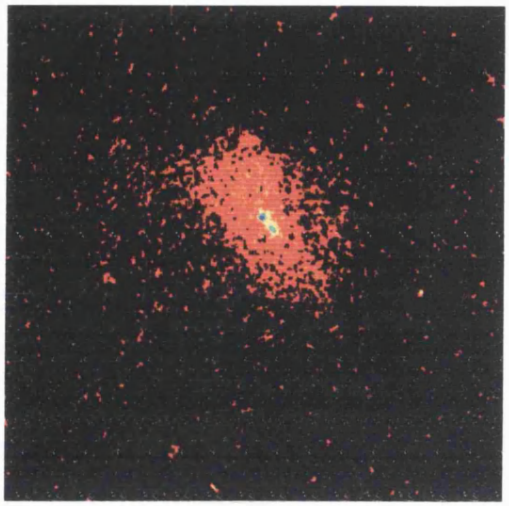
Section 215



Section 219

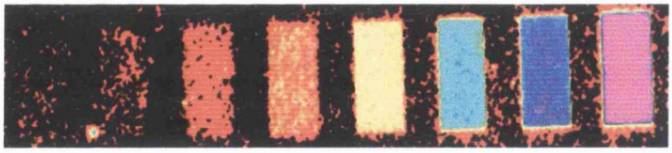


Negative greyscale image of nissl stained section 207



Unmerged image of processed autoradiograph of section 207

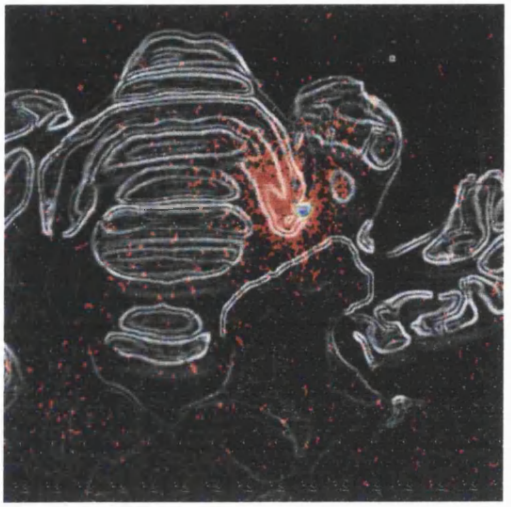
Processed autoradiographic image of exposed [^{3}H] standards



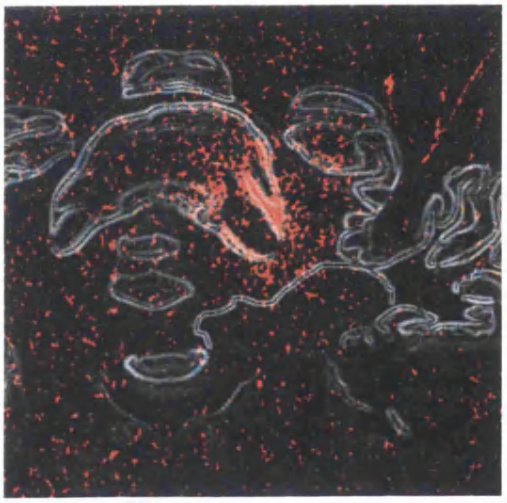
Level	1	2	3	4	5	6	7	8	
Known polymer activity	2.9	4.9	8.1	13.8	22.8	38.1	65.5	105.6	(nCi/mg)

Case: G3216

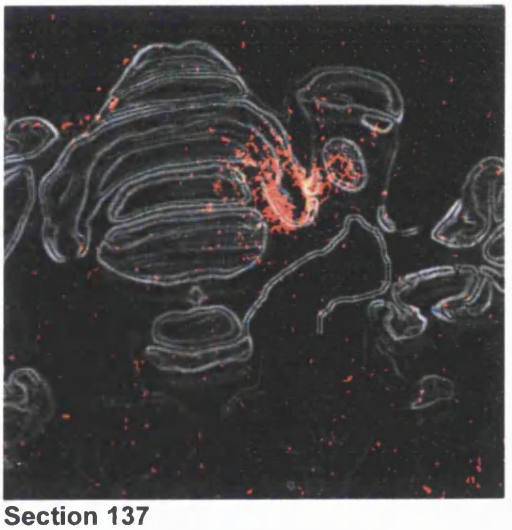
Section 110



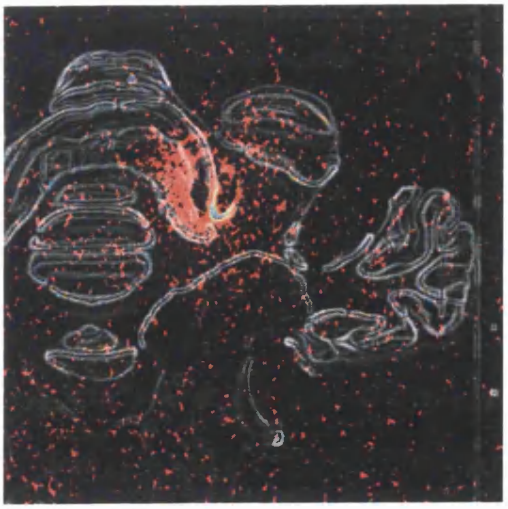
Section 133



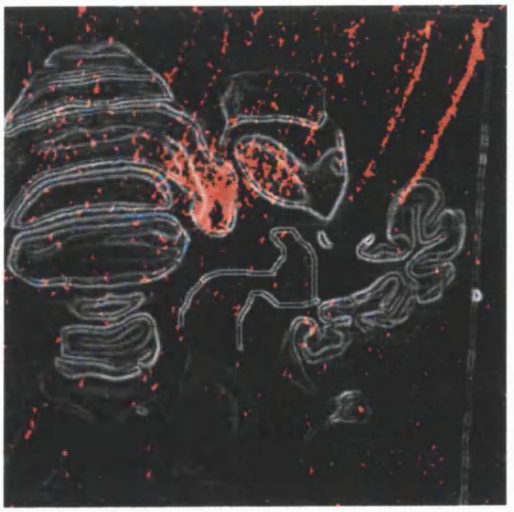
Section 122



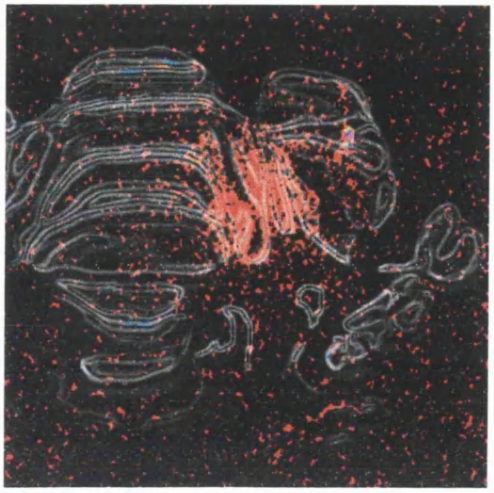
Section 137



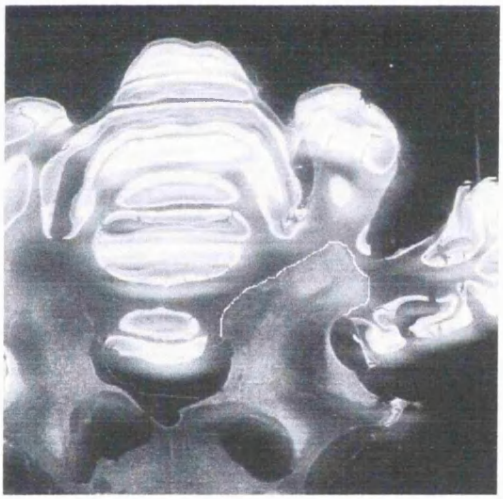
Section 129



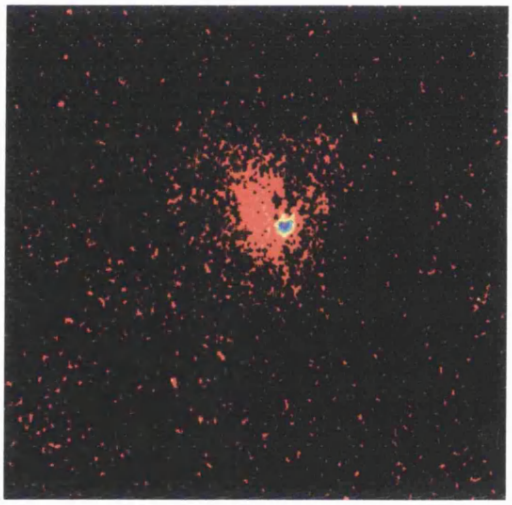
Section 141



Section 149

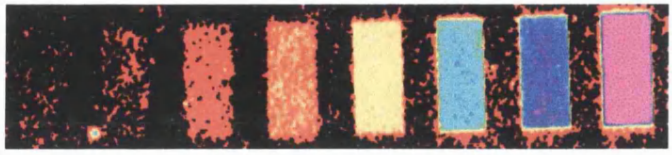


Negative greyscale image of nissl stained section 133



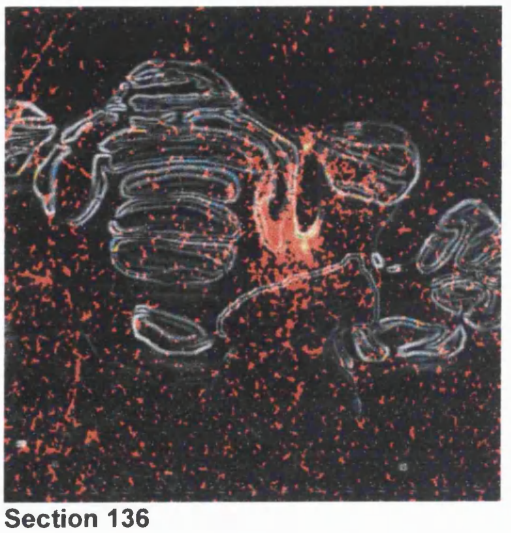
Unmerged image of processed autoradiograph of section 133

Processed autoradiographic image of exposed [^{3}H] standards



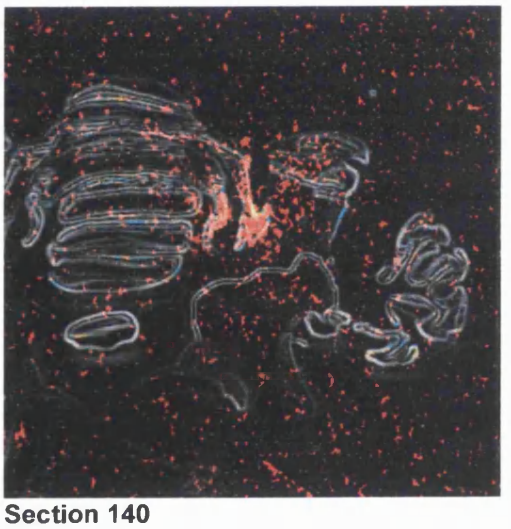
Case: G3217

Section 104



Section 136

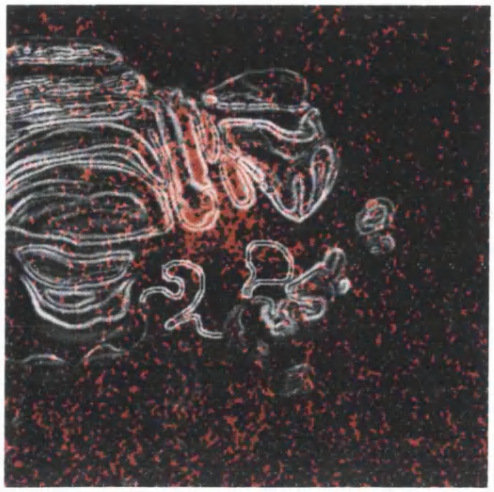
Section 120



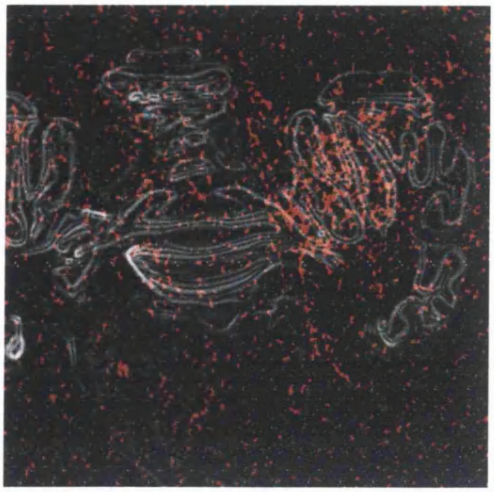
Section 140

Section 132

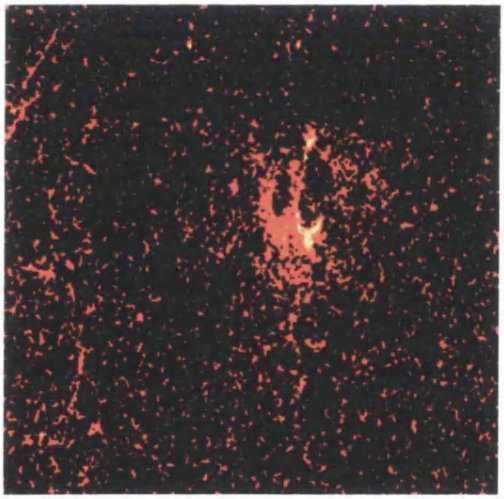
Section 144



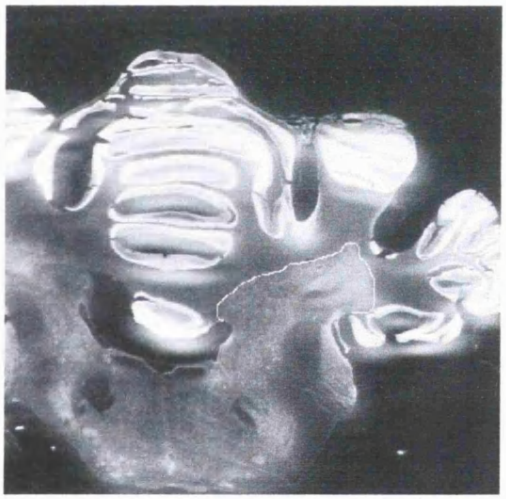
Section 156



Section 172

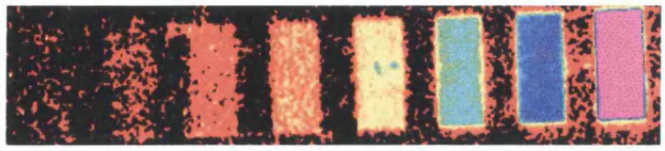


Unmerged image of processed autoradiograph of section 136

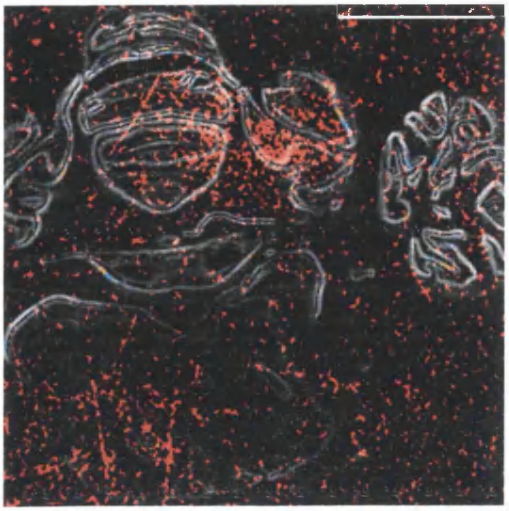


Negative greyscale image of nissl stained section 136

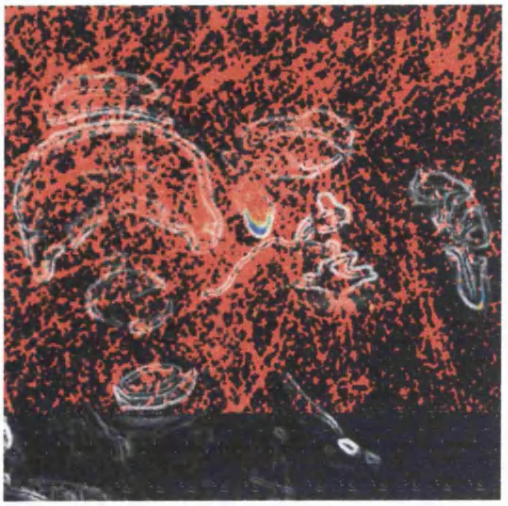
Processed autoradiographic image of exposed [^{3}H] standards



Case: G3218

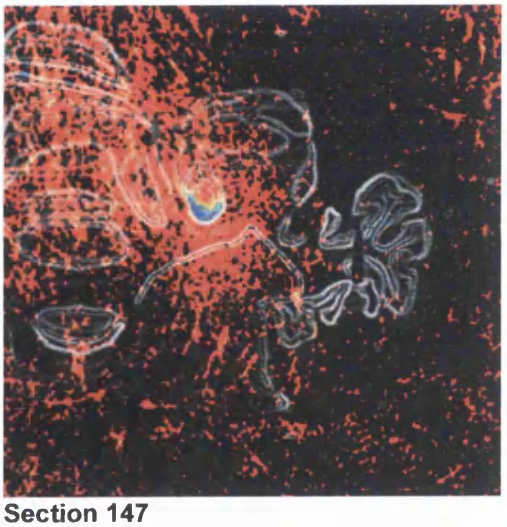


Section 108

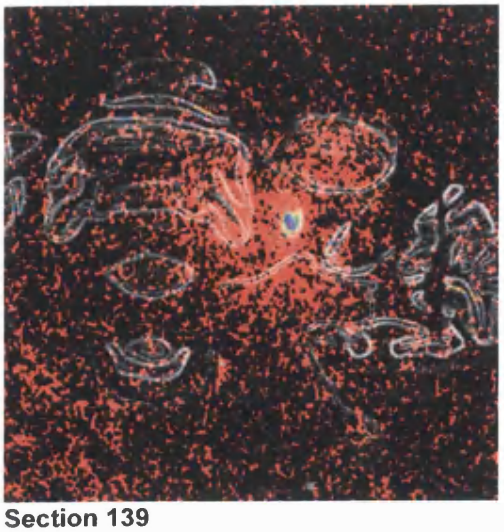


Section 143

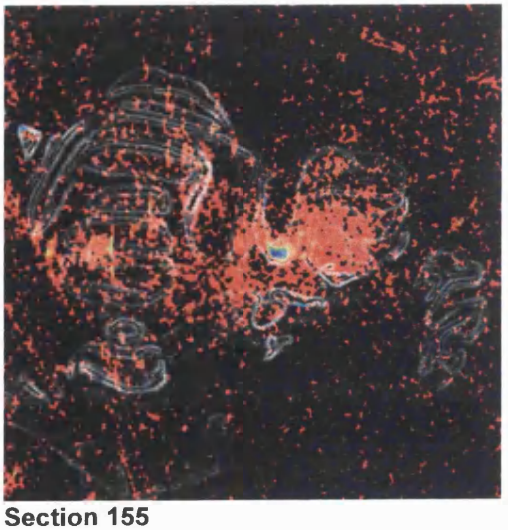
Section 124



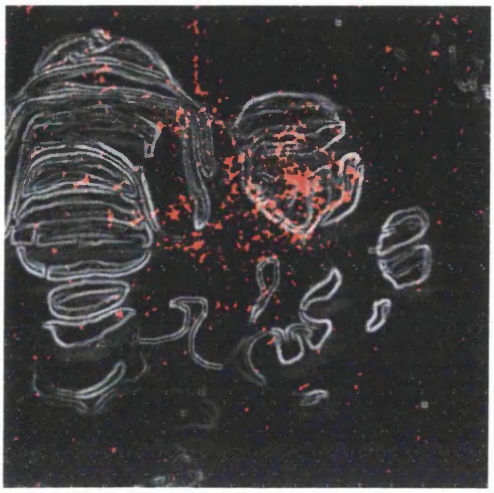
Section 147



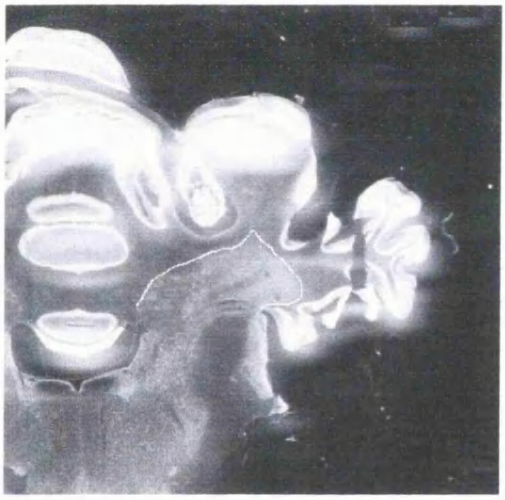
Section 139



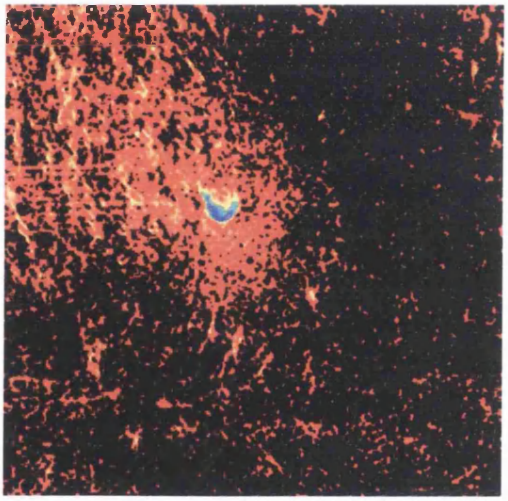
Section 155



Section 167

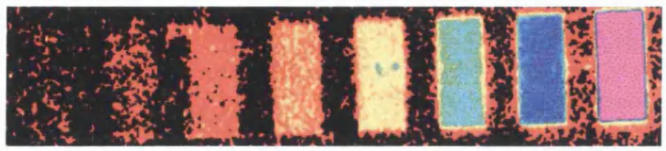


Negative greyscale image of nissl stained section 147



Unmerged image of processed autoradiograph of section 147

Processed autoradiographic image of exposed [^{3}H] standards



Summary of autoradiography:

G3213: This case was rejected from the study because of cannula damage to lobule HVI. However, muscimol spread in this subject was interesting because the muscimol inactivation was complete. Little muscimol was evident in the cerebellar nuclei. In contrast, there were levels of muscimol considerably above threshold in most parts of lobule HVI, particularly in caudal parts of the lobule. The behavioural consequences of inactivations were very significant, and very likely to be due to effects of muscimol in the cerebellar cortex rather than the cerebellar nuclei. It is also interesting that acquisition proceeds much more rapidly during further training after the muscimol phase is over. It may be the case that in this subject a combination of a cortical lesion and muscimol have impaired consolidation to some extent. This case is comparable with a rejected case in the LD group (G3201).

G3214: The injection was quite dorsal in this case (section 119), but muscimol was present in the AIP (section 127) and also in caudal parts of lobule HVI, paramedian lobe and crus II. There were also detectable levels in vermian lobules V and VI in cerebellar cortex. While AIP was certainly inactivated, loss of CRs in phase 2 may have to be due to a combined effect of muscimol on both cortex and nuclei. It is also evident from section 127 that high concentrations of muscimol ascended up the path of the cannula. The spread of muscimol in this case was comparatively large.

G3215: The injection site (section 207) was in Lobule VI/caudal part of HVI. Muscimol spread included Vermian Lobule V and VI and caudal parts of HVI. The extent of the inactivation was limited to the cerebellar cortex, and ventral spread stopped short just before the most ventral part of the primary fissure. No muscimol was detected in the cerebellar nuclei.

G3216: The spread of muscimol was comparatively small in this case. The injection site (section 129) was very close to the base of HVI and relatively close to the cerebellar

nuclei. There were small areas of muscimol detectable in AIP and the base of HVI. There were also some levels detectable in vermian lobules IV, V and VI.

G3217: The injection site (section 132) was very close to AIP, and muscimol was present in detectable levels in AIP (section 136) and most parts of lobule HVI, although it is clear that more rostral parts of Lobule HVI had very little muscimol present.

G3218: The autoradiographic image had high levels of noise in some sections because in these areas the film were scratched. However, it is clear that the injection site was very close to the cerebellar nuclei, and the spread was relatively large. Despite noise in some of these images, it is clear that muscimol was present in AIP and caudal parts, but not rostral parts of HVI.

Are these complete inactivations of AIP?

In all cases in the HD Group, muscimol infusions fully inactivated circuitry necessary for expression of CRs. Autoradiographic images have shown that there is not much radioactivity in AIP, but G3214, G3216, G3217 and G3218 all have some muscimol present in the cerebellar nuclei. A simplistic interpretation is that there is very little muscimol in the cerebellar nuclei. However, it should be borne in mind that firstly, AIP has a relatively low cell density. Secondly, although the amount of muscimol required to achieve a complete inactivation is not known, concentrations as low as 1.54nm are enough to reduce CR frequency to zero for several hours (chapters 2 and 3). If the cell density is low, then the GABA-A receptor density in this area would also be relatively low. Bound muscimol in this area would therefore inevitably result in a low grain density (of course, some proportion of signal would represent *unbound* muscimol which must be cross-linked to membranes by fixative, although much of this will have been washed out during perfusion). While it is certain that muscimol is present in areas where image analysis and autoradiography have revealed the presence of tritium, low concentrations of muscimol are very likely to be present in more peripheral regions. These levels are likely to be undetectable because of factors discussed above.

The experiments reported in this chapter have shown that low and high doses of muscimol used to inactivate AIP, are sufficient to prevent the expression of CRs, but do not prevent consolidation of conditioned NMR's.

4.4 Discussion

One of the aims of this thesis is to systematically explore issues related to the localisation of plasticity related to motor learning. It is clear that the AIP is involved in acquisition and extinction of conditioning but for reasons already discussed, methods used in these studies are necessary but not sufficient to localise plasticity to AIP. The purpose of the current study was to examine the role of AIP specifically and cerebellar circuitry generally in processes causal in long-term plasticity. Results demonstrate that in the LD group, muscimol was delivered to the AIP, and was completely effective in abolishing CRs. Since conditioning proceeded normally even with post-training injections, AIP inactivations cannot prevent consolidation. In the HD group, muscimol was effective in abolishing the expression of CRs, and so circuitry essential for CR expression was inactivated. 7.7nm of muscimol was effective for longer in the HD subjects than 1.54nm in the LD subjects. While it is clear that muscimol was effective in the HD group, the locus of the effects in the cerebellum is less clear.

Are these complete inactivations of HVI?

It is clear from the autoradiographic results of the HD group that there is substantial signal in the cerebellar cortex, partly reflecting the very high density of GABA-A receptors here. It is particularly of interest that parts of lobule HVI in the cerebellum are saturated with signal. Although the whole of HVI was not represented in all cases, the caudal portion of HVI up to the point where the lobule forms a fork is shown to be saturated with muscimol in all cases. In G3215, a case in which muscimol was only present in the cerebellar cortex, there was very little signal in HVI beyond this point (section 219). In G3217, the signal is present in appreciable levels in the most caudal part of the lobule, and a little rostral to the point where HVI forks, but more rostrally there is very little signal. What are the significant areas of HVI? Lesions of all parts of HVI affect conditioning, although lesions of the 'stem' of the lobule are known be most effective in affecting conditioning (Yeo *et al.* 1985b). Locations of significant areas in lobule HVI (zones C1 and C3 which project to AIP) are not known, and so to be sure that

these areas are inactivated, muscimol must reach the whole of the lobule for any cortical inactivations to be complete. As a group, these subjects homogeneously show muscimol spread in caudal parts of HVI, but beyond this location the extent of muscimol spread is not uniform, and in some cases is not known. In the absence of information about the location of important areas, and the specific significance of caudal parts of HVI, these must be regarded as incomplete inactivations of HVI.

The relative importance of HVI and AIP in these inactivations is not clear, however, what is clear is that in all subjects CRs were completely abolished in phase 2, and cerebellar circuitry essential for conditioning was inactivated. In cases where HVI was inactivated, normal activity in AIP would also have been disrupted. In cases where AIP was inactivated, the disruption to HVI is less clear because reciprocal projections from AIP to HVI are very minor, and the nature of secondary effects through indirect, nucleo-olivocortical projections on NMR conditioning or cerebellar cortical physiology is unknown. Whether inactivations were direct only or had an indirect component as well, the absence of CRs in phase 2 is indicative of disruption of normal activity in AIP.

Cerebellar plasticity and consolidation.

As a candidate site for motor learning-related plasticity, the cerebellum presents many advantages to those who model its operations. At their heart, most of these models incorporate the notion that the cerebellar cortex is capable of storing motor memory by altering the efficacies of parallel fibre synapses on Purkinje cells (Marr, 1969, Albus, 1971). All of these models work on the basis that synaptic strength changes during the course of the training event, but none of these have suggested mechanisms by which the cerebellum continues to operate processes which strengthen these synapses in a time-dependent manner. This is probably because the relationship between post-training, time dependent consolidation processes and plasticity is not fully understood. Interpreting results from interventions *during* conditioning is complex and less straightforward than interpreting results from that arise from interventions which specifically target the post-training, 'consolidation period'. The former involve process

which affect conditioning but are not directly associated with plasticity. The latter are much more strongly associated with plasticity.

With regard to localisation of plasticity, it has been argued in chapter 1 that if interventions such as those used here were effective in affecting conditioning, the structure being inactivated would be strongly implicated as one in which plasticity might occur. It would not be possible however, to localise plasticity to that structure. If such interventions proved to be ineffective (as are results in the current study), then the one could reject the hypothesis that plasticity is localised in this structure with greater certainty. Methods classically used to demonstrate the critical nature of cerebellar circuitry have failed to show that the AIP has a role in the consolidation of NMR conditioning. The AIP may therefore not have a role in plasticity for NMR conditioning.

Is the whole olivo-cerebellar circuit implicated, or only AIP?

Previous studies have clearly shown that AIP is implicated as a site of plasticity, and have confirmed that it is required for *learning* in NMR conditioning. These conclusions are re-inforced by the study by Krupa and Thompson *et al* (1995) in which the brachium conjunctivum was inactivated during conditioning. Since conditioning was normal despite possible cerebellar influences on efferent circuitry, it is argued that plasticity must be in cerebellar or pre-cerebellar circuitry. Studies in previous chapters have argued that AIP inactivations cannot be considered in isolation from the circuitry in which AIP exists. It is possible that inactivations of AIP disrupt information processing in the whole circuit, and not just AIP. The conclusions of such studies may extend not just to AIP, but to associated parts of the olivo-cerebellar system. So any or all parts of this circuitry (AIP, lobule HVI and rostral parts of medial rDAO) could be implicated as sites of plasticity. The same analysis must also be applied to the current study, in which AIP inactivations left consolidation processes intact. So, it is arguable that no part of the olivo-cerebellar circuit is required for consolidation. This may be particularly true for the current study in which there is strong evidence that muscimol invaded parts of lobule HVI in the HD group. Indeed, there was one subject (G3215) in which muscimol was effective, and was present only in the cerebellar cortex, and yet conditioning in Phase 1

was unaffected. However, there are two problems with this argument. Firstly, one can only speculate about the physiological cerebellar cortical consequences of AIP inactivations, because there is no available data on which to base conclusions about this question. Secondly, direct inactivations of the cerebellar cortex with muscimol cannot be regarded as complete in this study, because it is uncertain whether appropriate areas of HVI were inactivated in every case. Parenthetically, it is worth noting that the presence of muscimol only in caudal parts of HVI were effective in abolishing CRs (G3215). Zones involved in conditioning may therefore be present in this part of the lobule. If this were the case, then in all subjects in the HD group would have cortical inactivations in addition to nuclear inactivations. However, this must remain a speculation.

A straightforward conclusion in this instance is that AIP is not involved in consolidation of conditioned NMR's. So, where might such processes occur?

Consolidation in the cerebellar cortex?

It is probable that physiological processes which underlie memory formation are multi-phasic, complex and interdependent (McGaugh and Herz, 1972). In NMR conditioning, perhaps there are multiple traces of varying duration, localised in different pathways. The cerebellar cortex and nuclei are each likely to receive a convergence of CS and US information. Since it has been argued that the status of cerebellar cortical inactivations are uncertain in this study, the results are not inconsistent with the hypothesis that the consolidation of NMR conditioning occurs in the cerebellar cortex. The cerebellar nuclei may only be involved in learning *during* presentation of stimuli, consistent with inactivation studies. On-line information may be processed here so that a short-term, labile trace is formed and decays quickly. This information may be used to generate CRs during conditioning. The cerebellar cortex may also receive this information during conditioning and gradually undergo consolidation. After stimulation, when conditioning has ended, the trace in the cerebellar cortex may continue to consolidate over time, in keeping with the typical characteristics of consolidating traces (Tully *et al.* 1994). But inactivations of AIP during conditioning prevent conditioning, so if this were true, then the long term trace in the cerebellar cortex would have to depend

on the progress of the short term trace in the cerebellar nuclei, during conditioning. For consolidation to take place, the cerebellar cortex may rely on feedback about the cerebellar nuclear output (during NMR conditioning, this is the generation of the CR 'model'). Such a role could be fulfilled by AIP projections to pre-cerebellar nuclei which project back to the cerebellum. Pathways from the AIP to DAO are known to exist and function during conditioning (Ivarsson and Hesslow, 1994). Nucleo-pontine pathways are also know to exist, and project specifically from AIP to dorsolateral parts of the pontine nuclei (Brodal, 1981). This hypothesis is completely consistent with data from numerous behavioural studies. In particular several studies have shown that inactivations of the cerebellar nuclei are completely effective in preventing conditioning including studies in this thesis. However, while unilateral lesions of lobule HVI are able to abolish CRs initially, they less effective in preventing re-learning. Bilateral lesions of the cerebellar cortex are completely effective in preventing re-learning Gruart and Yeo, 1995). This is consistent with inactivations of the cerebellar nuclei preventing the short-term, labile trace from forming, and therefore also the formation of long-term motor memory. Re-learning may be possible with unilateral cortical lesions because the trace consolidates contralaterally. Lesions to both contralateral and ipsilateral HVI may therefore abolish conditioning completely by removing all capacity for long-term storage of this motor memory.

Long-term physiological processes in the cerebellar cortex.

While in-vitro studies of cerebellar and hippocampal function are to some extent 'pathological' situations in which the circuit properties of these structures are not represented, they are nevertheless able crudely to demonstrate the nature of more local operations. If learning related consolidation depends on the stabilisation of synaptic efficacies, it would be important to demonstrate that the stabilisation of LTD and LTP is time-dependent. It is known that hippocampal LTP in the CA1 and CA3 areas undergo two phases after stimulation. The early phase lasts one to three hours and is independent of protein synthesis. A later, more persistent phase (L-LTP) requires protein synthesis and is mediated by cAMP (Nguyen and Kandel, 1996). The time required for this process to be completed is about 30 minutes. If a similar mechanism operates in cerebellar cortical LTD, it is possible that LTD may provide a mechanism by which

consolidation for motor learning takes place. Linden and Connor (1995) have argued that the important mechanisms underlying the LTD are post-synaptic, and have developed a model of LTD that facilitates observation of intracellular properties of Purkinje cells during LTD. Linden (1996) reports that if protein synthesis inhibitors are applied immediately after induction of LTD, the depressed responses were only maintained for 50 minutes, compared with 130 minutes for controls (analogous to STD, short term depression). This intervention did not work if anysomycin was applied 30 minutes after induction, indicating a critical time window in which essential protein synthesis occurs to facilitate LTD. Although this is one of very few studies conducted on the late phase of cerebellar LTD, it clearly demonstrates that there are consolidation processes in Purkinje cell which are time-dependent, and result in the stabilisation of synaptic efficacy. Along with the weight of behavioural evidence, this lends support to the hypothesis that the cerebellar cortex is may be a site at which motor memory is stored.

Alternative explanations of consolidation study

Although the explanation given above best accounts for the results of this study, other possibilities should also be considered. These are discussed below.

1. Postponement hypothesis:

The 'postponement hypothesis' suggests that perhaps the inactivated circuitry is involved in consolidation, but that the injected drug *postponed* consolidation rather than preventing it. There is some evidence in the literature (Rose, 1995) which shows that biochemical processes which correlate with learning can be held off with inactivations, but then occur normally once the inactivation is lifted. Did this occur in cerebellar circuitry? It should be noted that in most models of learning used to investigate consolidation processes, including NMR conditioning, intravenous injections of drugs profoundly alter consolidation processes (see Introduction in this chapter) for a specific time period, after which they have no effect. This makes the postponement hypothesis an unlikely explanation.

2. Incomplete inactivations:

The drug may initially have failed to sufficiently inactivate AIP in time to prevent consolidation. In HD subjects (except for G3215), muscimol profoundly retarded CRs within 30 minutes of the injection in Phase 2, and then abolished them for many hours. It is possible that consolidation occurred in these 30 minutes. However, in the LD study, this cannot be the case, since inactivations were effective immediately. If the dose of muscimol is sufficient not only to depress CR frequency, but to abolish CRs altogether for several hours, then it is highly unlikely that this dose is insufficient to completely inactivate AIP. In two subjects (G3209, G3210) CRs were abolished immediately. In a third case (G3206) CRs were reduced to 30% in the first 20 minutes, and to zero after that. An insufficient time course for the inactivations cannot account for the results.

In the HD group, although CRs were not immediately abolished, they were immediately reduced to sufficiently indicate a profound impairment of cerebellar circuitry including AIP and possibly HVI. If consolidation processes did occur in AIP, then this would have had *some* effect on consolidation processes, if not the maximal effects. This may require further testing, using a design in which a high dose is immediately effective. This will require placements in or very near AIP, and using 7.7nm muscimol.

3. Too many trials per session?

A more serious concern relates to the number of trials in each session. Common to other NMR conditioning drug studies which have investigated consolidation, is the use of much fewer trials per session than those designed into this study. Hernandez *et al.* (1983) used 40 trials per session. Scavio *et al* (1992) used 30 trials per session. Cholewaik *et al* (1968) used 15 trials per session. These are in contrast to 100 trials per session used in the current study, chosen for consistency with the other studies in this thesis. It may be argued that consolidation begins after the first trial, and gains strength cumulatively as the number of trials increases, and after many trials, it becomes much more difficult to disrupt. Consistent with this is the fact that maximum drug effects on consolidation in Scavio *et al.* (1992) were observed early (session 2), and there were no differences

between groups in the last session. Was training in this experiment too strong to be significantly disrupted? It must be noted that like control subjects in studies in this thesis, those of Scavio *et al* (1992) also reached asymptote on session 4. In this sense the strength of conditioning in the current study was certainly similar in to that of Scavio *et al.* (1992). By this account, muscimol would have disrupted conditioning to some extent if consolidation occurred in AIP. Since no such effects were observed, it must be concluded that the strength of conditioning in the present study was not too great for consolidation deficits to be shown, and that consolidation processes do not occur in AIP.

4. Was muscimol incapable of affecting consolidation processes?

It may be argued that consolidation processes do occur in the cerebellum, but the pharmacological properties of muscimol are not capable of impairing these processes. Such an argument must be predicated by the assumption that the sub-cellular actions of muscimol on learning and expression of CRs are different to those on potential cerebellar consolidation processes, because muscimol affects the former, but not the latter. The following arguments make this possibility unlikely. The effects of muscimol on intracellular biochemistry are known to be wide-ranging, and affect biochemical pathways involved in the most fundamental cellular processes. Obviously, the first process to be affected inside the cell, is the homeostasis of chloride ions. Muscimol causes a massive enhancement of chloride influx into the cell which results in the hyperpolarisation of the cell membrane. This process affects the polarity of the cell membrane and its ability to propagate action potentials. There are examples of muscimol-induced hyperpolarisation which result in cascades of intracellular events that influence many other processes. Gene expression, a process known to be necessary for consolidation processes in many models of learning (Davis and Squire, 1984), is know to be severely affected by muscimol induced chloride influx. Much of this effect is probably mediated by the action of muscimol on immediate early genes (IEG) such as *c-fos*, which play critical roles in the transcription of several other genes. These are known to be specifically affected by muscimol (Berninger et al. 1995 Shim and Wirtshafter, 1996) and specifically involved in consolidation (Rose, 1991). Hence, by its

action on *c-fos*, muscimol affects the transcription of several other genes, and an important process involved in consolidation in many other models of learning.

Another processes known to be critical for cell function is that of calcium homeostasis. Intracellular calcium pools are affected quite dramatically by muscimol induced hyperpolarisation (Lorsignol et al. 1994). Calcium influx into neurons resulting from muscimol binding to GABA-A receptors, is specifically associated with the enhanced phosphorylation of GAP-43 and MARCKS by PKC (Fukura et al, 1996). GAP-43 and MARCKS are also specifically implicated in consolidation of memory processes in other preparations (Meberg, et al. 1996; Sheu et al. 1993). Given the heavy dependency of most intracellular cascades on calcium homeostasis, in particular those associated with learning and memory, it seems very unlikely that muscimol would leave these processes unaffected if they were active in the cerebellum during the consolidation of NMR conditioning.

Given the effects of muscimol on at least two very general processes known to be involved in consolidation in other models, the effects of muscimol are likely to be general rather than specific, and will have affected memory consolidation had it been present in the cerebellum. This argument is strengthened by the fact that there are specific processes affected by muscimol which are common to memory consolidation in many behavioural preparations. There are reports showing that muscimol injections in the medial septal area impair acquisition and retention of inhibitory avoidance and spatial learning in rats, but do not affect these forms of learning if injected after training (Brioni et al. 1990; Nagahara and McGaugh, 1992; Nagahara et al, 1992). This situation parallels the findings of experiments in this thesis. In contrast, inhibitory avoidance was severely affected by post-training muscimol infusions into the amygdala (Brioni et al. 1989). By this example, it is quite feasible that post-training infusions of muscimol into extracerebellar circuitry may impair consolidation of NMR conditioning. These facts strongly argue against the case that muscimol failed to affect cerebellar consolidation processes.

In conclusion, post-trial inactivations of the AIP and possibly associated cerebellar circuitry have not resulted in impaired conditioning. It can be concluded that AIP is unlikely to be a site of consolidation related plasticity, because inactivations were well targeted, occurred immediately after conditioning and lasted for several hours. The involvement of the cerebellar cortex is less certain. In the LD group, AIP inactivations may have resulted indirectly in the disruption of olivo-cerebellar circuitry, implicating the cerebellar cortex also. However, the effects of AIP inactivations on cerebellar cortical physiology has not been formally tested and so are not known. In the HD group, there was strong evidence that muscimol invaded parts of HVI involved in the regulation of CRs, because cases in which there were high concentrations of muscimol in HVI showed an abolition of CRs in phase 2. Most of these cases had low concentrations of muscimol in the cerebellar nuclei, and one case showed no evidence of muscimol in the cerebellar nuclei. However, the single case in which muscimol was shown not to be present in the nuclei showed a retention profile where CR frequency declined much more slowly than the others. There may have been time for consolidation to occur in the cerebellar cortex before the effects of muscimol had their maximum effects. In the other cases, the effects of muscimol in Phase 2 cannot be differentially attributed to HVI or AIP because critical locations within HVI are unknown.

The role of the cerebellar cortex needs to be addressed more specifically in a separate study, in which HVI inactivations are shown to be complete.

Chapter 5
General Discussion

The principal concern of this thesis has been to investigate the role of the cerebellum in classical conditioning of the rabbit NMR - a model widely regarded as a simple form of motor learning. Theories of motor learning have predicted that the formation of motor memory occurs within cerebellar circuitry (Marr, 1969; Albus, 1971; Gilbert, 1974; Ito, 1972). To this end, the studies reported in the previous chapters have investigated the possibility that normal activity in the cerebellum is required to support plasticity essential for NMR conditioning.

Consistent with theories of cerebellar plasticity, lesion studies (reviewed in Chapter 1) have shown that specific parts of the cerebellar cortex and nuclei are required for the expression of conditioned NMR's. Lesions to these areas abolish the expression of CRs and prevent re-learning. However, data from these studies has spawned other hypotheses that can offer alternative explanations of these results, without recourse to the concept of essential cerebellar plasticity. Briefly, alternative explanations include the following:

1. The cerebellum provides tonic support of neural excitabilities in brainstem pathways critical for ***expression*** *of CRs, but is not itself a site of plasticity. Lesions induce motor deficits, not learning or sensory deficits (Welsh and Harvey, 1991).*

2. The cerebellum modulates extracerebellar pathways involved in ***non-plastic, sensory processing*** *essential for conditioning. Cerebellar lesions therefore impair normal sensory processing by removing essential modulatory influences on pathways involved in CS and US processing.*

3. The cerebellum modulates ***extracerebellar pathways critical for plasticity****, but is not itself a site of plasticity. Cerebellar interventions may therefore leave sites of plasticity anatomically intact, but affect physiological processes in these sites, which depend on normal cerebellar function. Cerebellar lesions may therefore impair essential plasticity in extracerebellar circuitry.*

An objective of studies in this thesis is to evaluate these alternative hypotheses. In Chapters 1 and 2, results have demonstrated conclusively that if normal activity in AIP is disrupted, not only is execution of CRs prevented, but learning is also prevented. The data are consistent with the cerebellar learning hypothesis, and permit the exclusion of hypothesis 1 (above), because AIP is essential for learning, irrespective of muscimol effects on execution. However, these findings do not reveal *why* learning was prevented. Even if muscimol impaired *plasticity,* rather than other processes essential for conditioning (e.g. sensory processing), the results of inactivation studies still do not confirm a *cerebellar* locus of essential plasticity. Before the localisation problem can be resolved, it should first be determined whether cerebellar muscimol infusions prevented learning-related plasticity at all, or whether they simply prevented *sensory processing* essential for conditioning. Inactivations during conditioning cannot rule out the remaining hypotheses above. To assess whether the process of plasticity can be prevented by AIP inactivations independently of effects on sensory processing, the third study (Chapter 4) targeted the process of consolidation. Inactivations immediately after conditioning, when no stimuli were presented, but when consolidation processes are known to occur in NMR conditioning, did not affect conditioning.

Therefore, although normal activity in AIP that is required for acquisition and extinction, it may not be required for consolidation. These results suggest that AIP is unlikely to be a site of plasticity, and that normal activity in AIP is unlikely to be essential for consolidation to take place in sites of plasticity in circuitry efferent from the cerebellum.

The results from Chapters 2 and 3 argue in favour of the cerebellar learning hypothesis, but results from Chapter 4 argue against the hypothesis that the AIP is a site of essential plasticity for NMR conditioning. How can these apparently conflicting findings be

reconciled? The evidence for cerebellar and extracerebellar plasticity is discussed below.

Is the cerebellum a candidate site of essential plasticity for NMR conditioning?

It has been suggested that CS and US information may be integrated at the parallel fibre-Purkinje cell synapse in the cerebellar cortex (Yeo, 1986) and cerebellar nuclei (Thompson and Krupa, 1994), and that the instruction which guides the modification of this synapse occurs when error signals are received from the inferior olive. The view that such a process occurs in the cerebellar cortex is consistent with several models of cerebellar cortical plasticity (Marr, 1969; Albus, 1971), and with studies of LTD showing *in-vitro* evidence that plasticity at the PF-PC synapse is possible (Linden and Connor, 1995). The notion that plasticity occurs in the cerebellar nuclei is supported by evidence showing that learning is abolished if the cerebellar nuclei are inactivated (Chapters 2 and 3; Krupa and Thompson, 1993), although the physiological basis of such plasticity is not known. Consistent with these ideas, studies have shown that there is conditioning-related activity in the parts of the cerebellum known to be essential for conditioning. McCormick and Thompson (1984a) have shown that neuronal multiple-unit activity in AIP (referred to as 'medial dentate-lateral interpositus' by these authors) correlates with NM movements during the execution of conditioned NMRs. Berthier and Moore (1986) have also shown that cells in this region discharge in a manner correlated to the execution of the conditioned NMR. Furthermore, they have also reported the existence of Purkinje cells in lobule HVI that typically, also discharged in a manner correlated with the movement of the conditioned NMR, but do so 20-200ms prior to its onset (Berthier and Moore, 1990). This evidence clearly suggests that the cerebellum may be a site in which there is learning-related plasticity for NMR conditioning.

Does work in this thesis also support the cerebellar learning hypothesis? The finding that normal activity in AIP is *not* required for consolidation, is inconsistent with this view that

AIP might be a site of essential plasticity. How might these explanations be reconciled? One explanation which might reconcile findings in this thesis has already been discussed in Chapter 4. Briefly, consolidation processes might occur in the cerebellar cortex, and not in the cerebellar nuclei. AIP may process conditioning stimuli *during* conditioning, forming a trace that is temporary and which decays quickly. It is proposed that slower, less labile consolidation processes may occur in areas of the cerebellar cortex, which are initially dependent on the progress of shorter term processes in AIP during conditioning. Inactivations of AIP during conditioning would therefore prevent both long-term processes in the cerebellar cortex, and the short-term processes in the cerebellar nuclei. If these are independent of each other after training (during consolidation), then inactivations of the AIP after training will not impair consolidation (but see below). An important step would therefore be to test for cerebellar consolidation processes by post-training inactivations of areas of the cerebellar cortex known to be important for conditioning (i.e. Lobule HVI). Impairment of conditioning in such a study would confirm the essential contribution of the cerebellar cortex to plasticity for NMR conditioning.

Do essential memory traces for NMR conditioning exist in extracerebellar circuitry?

The evidence for cerebellar plasticity in NMR conditioning is strong, but indirect and inconclusive. Since plasticity has not been localised to the cerebellum by experiments to date, it is important not to exclude the possibility that essential plasticity for NMR conditioning may exist outside the cerebellum. It has been argued in this thesis that plasticity for NMR conditioning is likely to be distributed (see Chapters 1 and 2), and if such plasticity is resident at all in the cerebellum, it must only be in part, since there are operations essential for conditioning which the cerebellum seems incapable of performing (see below). There must be other structures which also form neural substrates of plasticity of NMR conditioning.

Inactivation studies have used anatomically intact rabbit conditioned NMR preparations, limiting the direct, reversible drug effects to physiological processes occurring in small areas. Unlike reduced preparation and lesion studies, inactivation studies can examine whether or not the output of the cerebellum is required to support efferent circuitry that may contain essential plasticity, under relatively normal conditions.

Krupa and Thompson (1995) found that TTX inactivations of the brachium conjunctivum during conditioning do not prevent acquisition of conditioned NMRs, and concluded that circuitry efferent from the cerebellum cannot be critical for learning in NMR conditioning. Notwithstanding some reservations about their study (see Chapter 2), their result strongly argues in favour of the conclusion that learning-related plasticity must be resident in cerebellar circuitry. However, the results of Krupa and Thompson (1995) cannot exclude the possibility that there may be essential plasticity in circuitry *afferent* to the cerebellum. The cerebellum is only one of many sites in the mammalian nervous system in which auditory and face somatosensory stimuli are processed. If such areas are unaffected by TTX inactivations of the brachium conjunctivum, plasticity in these sites may make essential contributions to learning in NMR conditioning, perhaps in addition to essential cerebellar contributions.

Some studies have attempted to identify the brain areas in which learning-related changes occur in completely intact preparations during NMR conditioning. All have used an auditory CS during delay conditioning, and to this extent, the studies are comparable with experiments in this thesis. The deoxyglucose method (Sokoloff,1978) has revealed learning-related changes bilaterally in the dorsal cochlear nuclei, but with stronger changes ipsilateral to the conditioned side compared with pseudoconditioned control animals (Harvey *et al.*, 1988). In another study, Irwin *et al.* (1992) reported that if Fos protein was used as an index of neuronal activation, conditioning induced learning-

related decreases in expression of Fos protein in many areas, including the spinal trigeminal nucleus. Carrive *et al.* (1997) have also used Fos protein as an index of neuronal activation, and have found learning related decreases of Fos expression in locus coeruleus, relative to unpaired controls.

These studies have revealed that primary sensory brainstem relays (spinal trigeminal nucleus and dorsal cochlear nucleus) which are mandatory for conditioning, exhibit learning related changes. The trigeminal nuclei receive inputs from the auditory system (Brodal, 1981), and the dorsal cochlear nucleus is know to receive inputs from the trigeminal nuclei (Brodal, 1981). Such structures therefore have the potential for plasticity in NMR conditioning. However, lesions and inactivations of these structures cannot reveal whether they are sites of plasticity, because impaired learning could unambiguously be attributed to impaired sensory processing of the CS and US. The potential for these structures to contain essential plasticity may be explored by attempting to impair consolidation processes in these structures immediately after conditioning. While sensory processing will be impaired after conditioning, this will not confound any impairments in conditioning that might arise from impaired time-dependent consolidation processes.

What could be the contribution of primary sensory structures to plasticity for NMR conditioning? An important characteristic of NMR conditioning to an auditory CS, is that CRs are relatively specific to the frequency of the CS (Moore, 1972), and so plasticity must exist which is capable of learning frequency specificity. However, studies which have specifically examined frequency selectivity of single units of the cerebellar cortex, including Purkinje cells, have concluded that auditory information conveyed to the cerebellar cortex has very poor frequency specificity (rat: Huang *et al.*, 1991; cat: Aitkin and Boyd 1975; Altman *et al*, 1976; Huang and Burkard, 1986). Huang *et al.* (1991) concluded, "the tuning characteristics of cerebellar auditory neurons ensures

that, whatever it may be, it will not be dependent on the frequency content of the sound". In agreement with these studies, Boyd and Aitkin (1976) reported that the single units in the dorsolateral pontine nuclei, to which several auditory centres project, and from which there are major auditory afferents to the cerebellum (see Yeo *et al*, 1985c), also have very little frequency selectivity in rat, suggesting that frequency information is probably absent from auditory signals even before they reach the cerebellar cortex or nuclei. Interestingly, these units fired only at the *onset* of tones at very short latencies, and so the cerebellum would still be able to use timing of CS onset to generate appropriately timed CRs. It is therefore rather unlikely that CS frequency specificity can be learned in the cerebellum and plasticity for frequency selectivity in NMR conditioning is likely to be extracerebellar. When auditory CS's have been used in other forms of conditioning in other species, plasticity related to auditory frequency specificity has been observed in many parts of the auditory system including the medial geniculate nucleus (Edeline, 1990) and the auditory cortex (Weinberger, 1993; Weinberger, Hopkins and Diamond, 1984). Other studies have shown anatomical dissociations where some lesions leave the ability for frequency learning intact, but others do not. In studies using conditioned avoidance in cats, lesions of auditory cortices, with accompanying degeneration to the medial geniculate nucleus, resulted in loss of auditory frequency discrimination, but these cats could re-learn frequency discrimination (Goldberg and Neff, 1961a). However, other cats, which had been sectioned at the level of the brachium of the inferior colliculus, could not re-learn the same frequency discrimination (Goldberg and Neff, 1961b). These studies argue in favour of learning for frequency specificity in sites of plasticity below the level of the inferior colliculus. Perhaps the specificity of the CS frequency in NMR conditioning, is also learned in lower auditory circuitry, such as the dorsal cochlear nucleus. Such a conclusion argues in favour of essential plasticity that is afferent to the cerebellum.

The finding that there are learning related decreases in activation of locus coeruleus one hour after conditioning (Carrive *et al.*, 1997), argues in favour of the hypothesis that

locus coeruleus is involved in the consolidation of learning related plasticity in the cerebellar cortex. This result would also support Gilbert (1975) who argued that locus coeruleus projections to the cerebellar cortex provide important noradrenergic input to Purkinje cells, which contributed to the stabilisation of an otherwise labile trace formed during motor learning. However, McCormick and Thompson (1982b) reported that lesions of locus coeruleus left acquisition of conditioned NMRs completely intact, but induced resistance to extinction. The locus coeruleus therefore cannot be mandatory for acquisition of CR's, and so cannot contribute in any way to plasticity.

Analysis of observational and reduced preparation studies suggests that there is potential for extracerebellar plasticity for NMR conditioning, particularly in sensory pathways which are afferent to the cerebellum. Learning related changes, by themselves, are insufficient to localise plasticity, so it is fundamental to determine the 'essential' contribution to learning of candidate structures by using reversible inactivations.

What problems have been overcome by inactivation studies in this thesis?

Work presented in this thesis has resolved two issues. Firstly, there is conclusive evidence that the AIP is critically involved in *learning* in NMR conditioning, irrespective of the effects of inactivations on the execution of CRs. There is also evidence which strongly argues against the notion of plasticity for NMR conditioning in AIP, since consolidation was not impaired by highly effective inactivations of AIP. This work raises a number of issues, and leaves others unresolved (see below).

Future directions for localisation of plasticity.

Lesion and inactivation studies have revealed the essential contribution of specific parts of the olivo-cerebellar system in NMR conditioning. Inactivations of cerebellar circuitry prevent expression of CRs (Mamounas *et al.* 1987; Krupa *et al.*, 1993), but more importantly, such inactivations prevent learning, confirming the cerebellum as a potential site of plasticity in NMR conditioning. However, no study of NMR conditioning has been able to prove the cerebellar learning hypothesis in the context of NMR conditioning. For inactivation studies to show that there is learning related essential plasticity in cerebellar circuitry, studies must show that

i) Cerebellar inactivations which prevent learning, do so by preventing *plasticity* rather than other processes essential for conditioning.

ii) Effects on plasticity of such inactivations remain restricted to *cerebellar* circuitry, and do not generalise to extracerebellar circuitry.

The first requirement may be met by inactivations which can impair or prevent consolidation. In Chapter 4, this approach has identified the AIP as a structure unlikely to be involved in consolidation, and therefore unlikely to be involved in long-term plasticity. The use of inactivations to target potential consolidation processes is an important first step, and should be now applied to important areas of the cerebellar cortex (i.e. lobule HVI) and to structures known to be mandatory for conditioning which also express learning related changes (e.g. dorsal cochlear nucleus).

If these post-training inactivations prove effective in impairing or abolishing conditioning, is this enough evidence to prove that they are essential sites of plasticity? This result would localise plasticity to the cerebellar cortex, only if the effects of inactivations were restricted to cerebellar cortical areas essential for conditioning.

In relation to the second requirement, the major interpretative problem which arises from cerebellar inactivations studies is that it is difficult to demonstrate whether indirect effects of AIP inactivations affect cerebellar-dependent processes in efferent circuitry. It is also difficult to show that effects on one part of the olivo-cerebellar system (e.g. cerebellar nuclei) remain restricted to it and do not affect the excitabilities of cells in anatomically related areas (i.e. cerebellar cortex or inferior olive). Negative results of two inactivation experiments become important in this context. Firstly, Krupa and Thompson (1995) demonstrated that inactivations of cerebellar output leaves conditioning unaffected. So, there can be no efferent, cerebellar-dependent processes essential for conditioning. If this study is correct, this provides further incentive for future work to test for consolidation using inactivations of cerebellar cortical circuitry. Secondly, it is argued above that the negative results from Chapter 4 clearly show that inactivations of AIP do not prevent consolidation, and so if inactivations of the cerebellar cortex do prevent consolidation, this cannot be attributed to indirect effects of cerebellar cortical inactivations on the cerebellar nuclei, which are immediately efferent from the cerebellar cortex. In this event, plasticity will be localised to the cerebellar cortex. However, if these inactivations fail to prevent consolidation, then the cerebellar cortex cannot be a site of plasticity. In such an event, it would be important to determine why cerebellar inactivations prevent learning, and to find the sites of essential plasticity.

It is important to bear in mind that the localisation of plasticity is a means to an end, and not simply an end in itself. The ultimate purpose of this excersise must be to evaluate the mechanisms of plasticity, once its locations have been identified. An important step in the analysis of plasticity is its examination at a sub-cellular level. Other preparations have been remarkably effective in achieving this goal in intact preparations. Prominent examples include avoidance learning and imprinting in the chick, in which lesions of specific brain areas - intermediate hyperstriatum ventrale (IMHV) and lobus parolfactorius (LPO) - are known to prevent learning and consolidation (Patterson, Gilbert and Rose, 1990). Learning related biochemical changes have also

been identified and characterised in these sites (Meburg, McCabe and Routtenberg, 1996; Anokhin et al, 1991). It would be instructive to use the same methods in rabbit NMR conditioning to characterise learning specific changes at the sub-cellular level, in structures known to be likely sites of plasticity.

What strategies might be used in future to analyse plasticity at a sub-cellular level in NMR conditioning? It is possible to track the progress of specific, learning related cellular events such as gene transcription and mRNA translation during conditioning, and during post-training consolidation. Gene transcription into mRNA, and mRNA translation into protein are fundamental cellular processes, and must be involved in learning and memory. Injections of radiolabelled nucleotides - mRNA precursors (e.g. [^{3}H]-Uracil) - into the blood stream results in rapid active transport across the blood-brain barrier into neurons (Alberts, 1994). The molecular and cellular mechanisms which form mRNA, can then requisition and incorporate these nucleotides into mRNA. If injections are made just before conditioning, then conditioning specific changes in gene transcription can be examined using autoradiographic techniques on serial brain sections. mRNA from tissue expressing significant changes can then be isolated by electrophoresis and subjected to analysis to determine its composition and sequence. In this way, one can detect which brain areas are involved in learning related gene-transcription and determine the composition of mRNA formed during learning or consolidation in NMR conditioning. Similar methods may be used to identify learning related changes in protein synthesis during conditioning. The precursors of proteins are amino acids. Radiolabelled amino acids (e.g. [^{3}H] lysine) injected into the bloodstream are, like nucleotides, actively and rapidly transported across the blood-brain barrier. They are then incorporated into proteins. If injected during learning or consolidation, amino acids will be incorporated into proteins which are formed during these processes. Autoradiography can then be used to detect areas in which there is specific protein synthesis. These proteins can then be extracted and analysed using methods similar to those mentioned above.

Such methods are capable of revealing the cellular and molecular correlates of memory formation and their timecourses. However, in order to establish these processes as necessary for memory formation, it must be shown that memory formation is prevented when these processes are blocked. It is possible, for example, to block the translation of mRNA sequences into proteins by injecting antisense mRNA (synthesised RNA sequences which are complimentary to the target mRNA sequence) into brain areas critical for conditioning, in which this mRNA is expressed as a function of learning. If antisense mRNA prevents or impairs conditioning, and is shown to be specific to the target mRNA, then it can be concluded that the synthesis of the target mRNA is critical for conditioning and takes an active role in memory formation. It is also possible to block the function of proteins independently of their synthesis from mRNA. Many proteins require a change in their phosphorylation state to become functionally active. Protein phosphorylation is tightly regulated by a class of enzymes called protein kinases. In the cerebellum, for example, there is a protein kinase quite unique to Purkinje cells that is absent from virtually all other brain cells - Protein kinase G (PKG). This is involved in the phosphorylation of proteins which have critical roles in cerebellar LTD. The functions PKG and other parts of the PKG cascade can be specifically blocked by using PKG inhibitors which also prevent LTD (e.g. KT5823; Lev-Ram et al, 1997).

If the cerebellar cortex was found to be a site of plasticity in NMR conditioning, it would be instructive to determine the extent to which learning specific mRNA and protein changes are constituted from components of the PKG cascade, using the methods above. If significant components were to be found, it would be feasible to block the PKG cascade by infusions of inhibitors into critical areas of the cerebellar cortex (e.g. Lobule HVI). These would only affect PKG and its biochemical components, exclusively in Purkinje cells. If this prevented learning and consolidation, this approach

would not only demonstrate that the PKG cascade is specifically altered during learning, but it would also show that PKG in Purkinje cells is critical for learning. Such a strategy would reveal the essential contribution of specific biochemical pathways and cells types in critical areas.

In conclusion, permanent lesion studies have provided a gross understanding of the nature of memory formation in NMR conditioning. Essentially, they have localised structures which are critical for NMR conditioning. However, greater functional specificity has been achieved using inactivations of critical structures, because it has been clearly shown that AIP is specifically required for learning in NMR conditioning. However, still greater specificity is required to show why this learning is affected by inactivations. If sites of plasticity are identified in the future, observational and interventionist strategies discussed above may be applied. These will enhance our understanding of plasticity still further by describing how sub-cellular processes are causally involved in the formation of memory.

Appendix 1:

General Methods

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1. Behavioural Methods

1.1 Subjects

Subjects were male Dutch belted rabbits weighing from 1.8kg to 2.5kg. They were housed individually, allowed *ad libitum* food and water and maintained on a 12hr light/dark cycle.

1.2 Preparations for Conditioning

Restraint:

Rabbits were placed in a Perspex restraining stock and a rubber foam padded end-stop was positioned to hold each subject firmly. The ears were gently held between rubber foam pads to restrain head movements.

Attachment of suture to nictitating membrane:

2-3 drops of ophthalmic anaesthetic, proxymetacaine hydrochloride 0.5% w/v (Opthaine, Squibb) were administered to the right eye so as to wet the surface of the nictitating membrane and the sclera. After 5 minutes, a sterile, fine monofilament suture (Ethicon, Prolene, 5/0) was passed through the superficial soft tissue on the lateral edge of the right nictitating membrane and tied to form a very small loop (diameter 1.5 mm). This loop afforded a simple, atraumatic coupling for the nictitating membrane movement transducer (see below) and so it was left in place throughout the experiment. Topical chloramphenicol eye ointment 1% w/w (Daniels Pharmaceuticals) was applied to the right eye of each subject on each day throughout the experiment and no subject developed infection or irritation of the eye or periocular areas. The rabbits were then returned to their home cages where they resumed normal feeding and grooming behaviours. Rabbits were observed for signs of discomfort resulting from attachment of the suture, but none were seen.

Attachment of Michel clips to facial skin for US delivery:

Two standard 12 mm stainless steel Michel clips served to deliver stimulation the facial skin surrounding the eyelids. One was placed approximately 5 mm behind the lateral

canthus of the eye and the other was placed approximately 5 mm below the centre of lower eyelid. The 12 mm clips were gently attached to the superficial skin layer using a standard applicator without the need to remove underlying fur. Again, when the subjects were returned to their home cages at the end of the adaptation session the rabbits made no attempts to remove the clips and there was no evidence of discomfort.

1.3 Conditioning Apparatus and Stimuli

The rabbits were trained using techniques similar to those first developed by Gormezano, Schneiderman, Deux and Fuentes (1962) and described in Yeo and Hardiman (1992). At the beginning of each, daily behavioural testing session, each subject was placed in the Perspex restraining stock. Subjects adapt well to this restraint and remain passive during the behavioural testing sessions of up to one hour duration. A low-torque potentiometer mounted upon a simple alloy cradle was positioned above the dorsal aspect of the head. Posteriorly, the cradle included a smooth, curved alloy ring which passed around the base of the ears and anteriorly, smooth alloy extensions stabilised the cradle on each side of the muzzle. This arrangement allowed the body of the potentiometer to be atraumatically fixed relative to the head. The shaft of the potentiometer was then coupled to the nictitating membrane, via a lever and miniature universal joint, with a small, stainless steel hook that was passed through the suture loop (Gormezano and Gibbs 1988). This coupling of the potentiometer and suture allowed isotonic transduction of NM movement and eliminated the need for a restoring force on the transducer. This isotonic transduction is directly comparable with that used in recent studies by Welsh and Harvey (1989, 1991) and in those from our own laboratory (Gruart and Yeo, 1995).

Each subject was then placed in a ventilated, sound-attenuating chamber facing a loudspeaker mounted centrally. Studies in this thesis used the following conditioning parameters. The conditioned stimulus (CS) was a 1 kHz sine wave tone of 310 ms duration and an intensity of 63 dBA (re 20$\mu N.m^{-2}$). Background noise produced by ventilation fans was 54 dBA (re 20$\mu N.m^{-2}$). The unconditioned stimulus (US) was periorbital electrical stimulation. Each US was a 60ms train of 3 biphasic pulses of intensity 2.5 mA applied to the periorbital region of the face through the stainless steel clips described above. On paired trials the interstimulus interval between the CS and US onset was 250 ms. The

intertrial interval was randomly selected between 25-35 seconds. This US level is sufficient to evoke a clear blink of the external eyelids and a closure of the nictitating membrane that does not habituate with repeated presentations. On initial stimulation, small movements of the neck and limb muscles can sometimes be seen but these habituate rapidly over a few trials

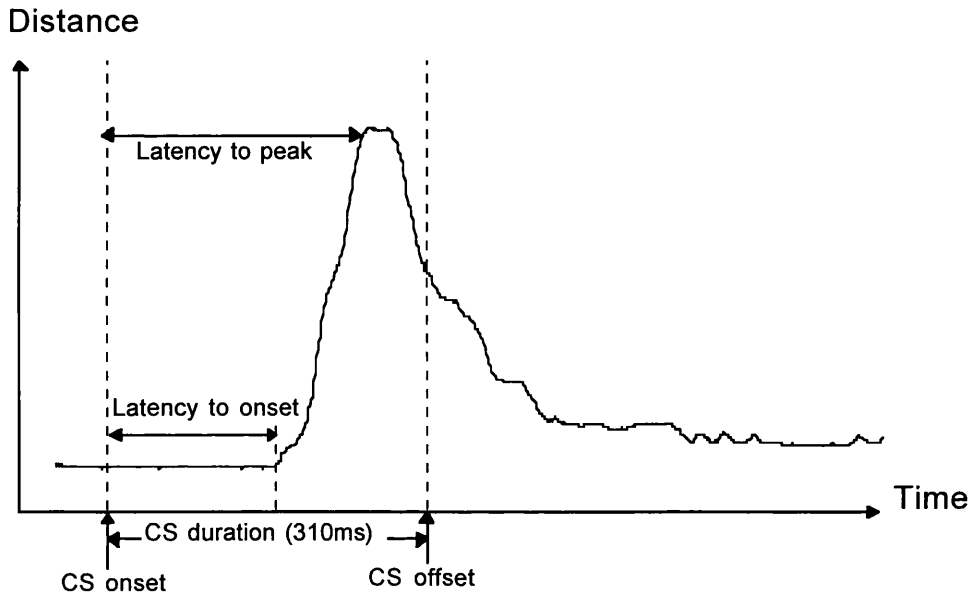


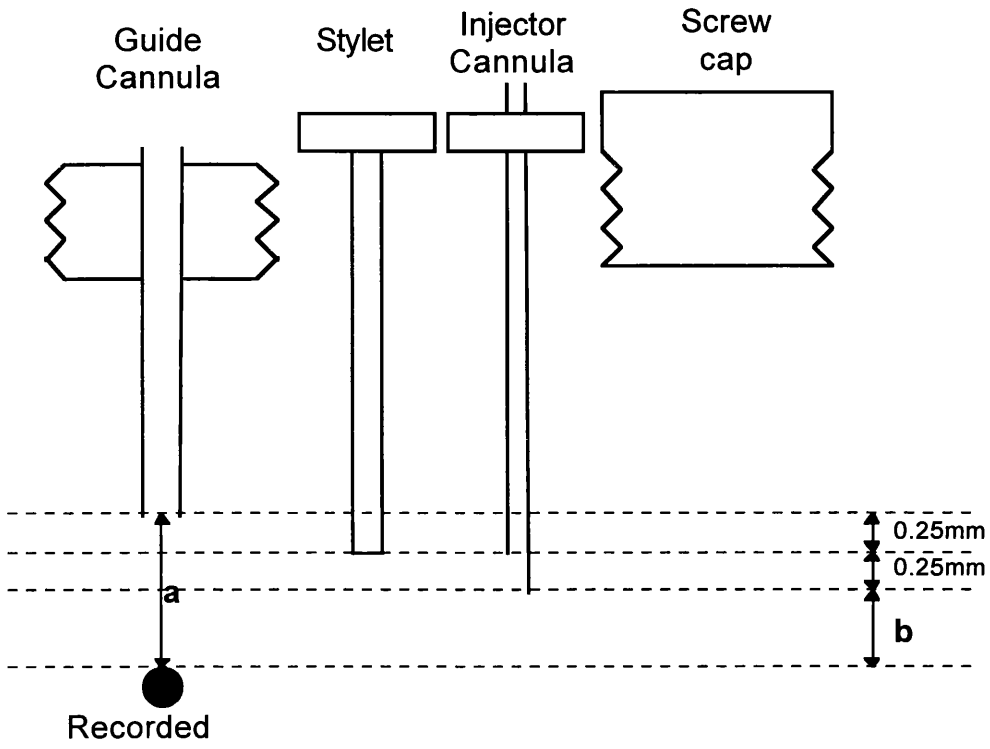
Figure A1.1: Latency measurements on probe trials. Latency to peak is the time taken for a CR to reach its maximum displacement from baseline. The latency to peak of a well timed CR typically coincides with the interstimulus interval. Latency to onset is the time taken for the movement of the nictitating membrane to exceed 0.15mm from baseline levels, from CS onset.

2. Surgical methods

Studies in this thesis required rabbits to undergo surgery for implantation of a guide cannula directed towards the right anterior interpositus nucleus (AIP). Each rabbit was anaesthetized using a fentanyl/fluanisone mixture (Hypnorm, Janssen; 0.1/5.0 $mg.kg^{-1}$, i.m.) with a supplement of benzodiazepam (Valium, Roche; 0.5 $mg.kg^{-1}$, i.v.) and was then intubated with an endotracheal tube and the head was placed in a stereotaxic instrument (Kopf), aligned with the stereotaxic coordinate system of Matricali (1961). Naloxone hydrochloride (Narcan, Du Pont; 5-7 $mg.kg^{-1}$, i.v.) was used to antagonize the fentanyl and anaesthesia was maintained throughout the operation using halothane (1.5-2.5%) in a nitrous oxide/oxygen mixture (1:3). The scalp was reflected and bone and dura removed to expose the right cerebellar cortex. The approximate position of the cerebellar nuclei was defined stereotaxically (λ-5mm AP, λ+4mm ML, λ-10mm DV), and their exact position was then determined by electrophysiological recording using tungsten microelectrodes. Rapid cellular activity below the deepest cortical layer ventral to the rostro-medial part of the paramedian lobe indicated that the electrode tip was in the cerebellar nuclei. The cerebellar nuclei were then explored for eyeblink related activity. Microelectrode tracks were made until cellular activity that correlated with eyeblinks evoked by gentle tactile stimulation of the cornea periocular area was recorded. In order to evoke blinks whilst recording, a level of anaesthesia was maintained that completely suppressed the toe-pinch response but which did not entirely suppress the corneal blink reflex. A 24G stainless steel cannula guide was then implanted near the activated cells (see figure A1.2 for details of cannulae).

Figure A1.2: Guide, stylet and injector cannulae. Measurements and relative positions (not to scale).

*Measurements **a** and **b** are discussed below.*



The distance of the injector cannula tip from the recorded cells (b) varied according to requirements of each experiment. In Chapters 2 and 3, the guide was positioned 1mm above (a) and 1mm caudal to the recorded cells. The injector cannula protruded 0.5mm from the guide cannula when inserted, and was therefore at a distance of 0.5mm above the level of the recorded cells (b), but 1mm caudal to them. In Chapter 4, the HD Group were implanted in the same way. In the LD Group, the aim was to target the injection cannula tip very close to the AIP (i.e. 0.5mm *directly over* the recorded cells, not 1mm caudal to them).

The stylet was placed in the guide cannula during surgery, and protruded 0.25mm from the end of the guide. It was left in place until injections were made, and replaced again after injections.

Care was taken to avoided damage to these cells and to parts of the overlying cortex that are important for NMR conditioning (Yeo *et al.*, 1985c). Exposed brain was covered with sterile absorbable gelatin foam and the cannula guide was fixed to the skull with dental cement. The scalp was sutured around the cannula guide and the animal removed from the stereotaxic instrument. Sodium chloride/glucose (0.18%/4%, 10 - 20 ml s.c.) was given to supplement fluid and electrolyte levels. Postoperative analgesia was provided by buprenorphine (Temgesic, 50µg/kg) each day for 3 days and antibiotic cover was by chloramphenicol (Chloromycetin succinate, 7.5 mg/kg) twice per day for 3 days. All surgical subjects were maintained in a quiet recovery room separate from the main colony for these 3 days and their behaviour was closely monitored. All cannulated rabbits resumed normal feeding, drinking and grooming within 12 hours and they exhibited no obvious motor deficits. All subjects were allowed 1 to 2 weeks for recovery.

3. Muscimol infusion procedure

A stock solution of muscimol hydrobromide (RBI) was prepared by dissolving the lyophilised form in 50mM phosphate buffered saline (PBS). This was further diluted to the required concentrations using more PBS. In Chapters 2, 3 and 4, 1 microlitre of muscimol was used in each infusion. The amount of muscimol in this injection volume was 1.54nm. In Chapter 4, animals additionally received 7.7nm in the same injection volume.

Before infusion, a 10µl Hamilton syringe was cleaned with sterile saline containing a few drops of 30% hydrogen peroxide solution (Sigma) had been added. The syringe was then rinsed thoroughly and with fresh sterile saline. A length of plastic tubing (internal diameter, 0.28mm) with a 36G stainless steel injector cannula attached to one end was similarly cleaned by flushing the same solutions through these. The syringe was filled with muscimol solution and the other end of the plastic tubing was attached to the tip of the syringe, taking care not to puncture the tubing with the sharp syringe tip. A small volume of muscimol solution was injected through the tubing and injector to check that the flow of muscimol was not impeded by a blockage, and that there was no leakage of muscimol from the syringe or plastic tubing. At the time of injection, the external surface of the injector cannula was carefully cleaned with sterile saline. The screw cap was unscrewed and removed. The stylet previously inserted inside the implanted guide cannula at the time of surgery was then removed, and the injector cannula was carefully inserted in its place. 1µl of muscimol was then infused over 1 minute. The injector remained inserted in position for a further minute after injection, to allow time for muscimol to disperse from the site of injection. The injector was then withdrawn and the stylet was inserted back into the guide cannula, and the screwcap was replaced.

4. Histological Methods

3.1 Termination and perfusion fixation:

At the end of each experiment rabbits with implanted cannulae were injected through the marginal ear vein with heparin sodium (500 Units.kg^{-1}, i.v.) and an overdose of pentobarbitone sodium (90 mg.kg^{-1}, i.v.). Each rabbit was perfused through the aorta with 1 litre of 0.9% saline followed by 1 litre of 4% formaldehyde. The cannula was gently dislodged and removed, and the brain was then removed from the skull.

3.2. Pre-embedding procedure:

Brains were then stored in post-fixative solution until the brains had sunk to the bottom of the container (usually, this occurred after about 72 hours). 1 litre of post-fixative solution was composed of 300g of sucrose dissolved in 840ml of 4% formaldehyde diluted in distilled water. Brains were then washed in running water overnight to remove as much formaldehyde as possible. The arachnoid tissue was gently removed from the surface of the brains.

3.3. Embedding Procedure:

Brains were placed in 5% gelatin solution. 100ml of 5% gelatin was composed of 5g gelatin powder (Sigma, 225 bloom) dissolved in 100ml warm distilled water (the water temperature was maintained below 70°C, the level at which gelatin polymerizes). The vessel containing the brain and gelatin was placed in a vacuum oven (maintained at 37°C), and a vacuum (800 mbar) was applied by air extraction. The brains were left in this condition for 1 hour, so that remaining air pockets in the fissures of the brains would be filled with gelatin. The gelatin solution was removed, and substituted with 10% gelatin solution (10g in 100ml, 225 bloom), and placed under vacuum for 30 mins. The brains and gelatin were then removed from the vacuum oven and placed in small, rectangular cardboard vessels and allowed to cool. The resulting gelatin block containing the brain was then trimmed and placed in post-fixative solution to harden for 72 hours.

3.4. Sectioning, mounting and coverslipping procedure:

Gelatin embedded brains were then prepared for sectioning. The block was sectioned with a blade parasaggitally at the widest part of the forebrain, and the rostral part was discarded. The resulting block, consisting of gelatin embedded cerebellum and brainstem, was rinsed with water to remove surface post-fixative solution. A dry ice-cooled platform was prepared on a microtome. The brain block was affixed to this platform by freezing, such that the flat, anterior part of the block rested on the platform, and the ventral part of the brain faced the blade (to cut ventrodorsally). 50 micron (Chapter 4) or 60 micron (Chapters 2 and 3) sections were cut, and placed into distilled water containing trace amounts of formaldehyde. Glass slides were prepared. These were first washed thoroughly in detergent and hot water. They were then rinsed thoroughly in tap and distilled water. Subbing solution was prepared by heating a litre of water to 60°C and adding 5g of gelatin and then 0.5g of Chromic Potassium Sulphate. Slides were then dipped into the solution while still warm and the left to dry in an aerated oven. A subset of sections taken at regular intervals (see Chapters 2, 3 and 4 for details) were then mounted onto glass slides pre-prepared glass slides. They were left to dry onto the slides overnight.

3.5 Nissl staining procedure:

Troughs containing the following solutions were prepared using distilled water.

1. Distilled water
2. 70% ethanol.
3. 95% ethanol.
4. 100% ethanol.
5. Xylene.
6. Cresyl Violet stain solution

    I) 2g Cresyl Violet stain in 950ml distilled water at 50°C.

    ii) 50ml acetate buffer (pH 3) added. This was composed of

Sodium acetate	27.2g
Hydrochloric acid (1M)	190ml
Distilled water	810ml

iii) Solution was left overnight at 37°C, and then filtered.

7. Distilled water
8. 70% ethanol, acidified with a few dropsof acetic acid (1M).
9. 95% ethanol.
10. 100% ethanol.
11. Xylene.
12. Xylene.

Sections were first rinsed briefly in water, and placed in increasing concentrations of ethanol to remove water, and then placed in xylene to remove fat (1-5). Sections were then rehydrated by placing them in decreasing concentrations of ethanol and then into water, for about 5 minutes in each solution (5-1). They were then left in Cresyl Violet stain until darkly stained, and then rinsed in water (6-7). Sections were dehydrated again by placing them in increasing concentrations of ethanol (7-10). Acidified 70% ethanol was used (8) to differentially extract more stain from white matter (at this stage, sections were continuously observed while in 70% acidified ethanol, until differentiation was optimal). Sections were then placed in two changes of Xylene to complete differentiation (11-12). After leaving briefly in xylene, slides were removed one at a time, and DPX mountant (BDH) was applied to the section. A coverslip was cleaned and placed on the section, taking care to remove air bubbled from the mountant fluid. These were left to dry overnight, and were then ready for inspection.

Appendix 2:

Autoradiographic and Image Analysis Methods

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1. Autoradiography

1.1 [methylamine-3H]Muscimol (Amersham Life Sciences; Batch 48)

Radioactive muscimol used in chapter 4 had the following specifications:

Pack size	250 μCi
Specific activity	511 GBq/mmol, 13.8 Ci/mmol 4.44 GBq/mg, 120 mCi/mg
Molecular Weight	114 (unlabelled) 115 (at this specific activity)
Radioactive concentration	37 MBq/ml, 1.0 mCi/ml
Conc. of Muscimol	0.072mM
Supplied quantity	250 μl = 250 μCi
Required concentration	7.7 mM (1.54 μg/μl, or 7.7nmoles/μl)

To obtain the required concentration of [^{3}H]muscimol (7.7mM), 250μl of tritiated muscimol was added to 250μl of an appropriate concentration of muscimol hydrobromide. The resulting solution had a specific activity of 1μCi/μl.

Slides with mounted sections from the HD Group (Chapter 4) were prepared using normal histological methods (Appendix 1), but were not stained or coverslipped. The lower surface of the slides were then glued onto stiff cardboard, which was cut to exactly fit the internal space of a light tight cassette (Hypercassette, Amersham). Sections were on the upper surface of the slides (onto which the autoradiographic film would be apposed). A strip of tritium standards (Amersham, RPA 506, batch 10b) were mounted onto a slide, and this slide was also glued onto the cardboard. The card was then inserted into the cassette, ensuring that there was no space for movement within the cassette.

In a clean, light-tight darkroom illuminated by light brown filters (Ilford S902) suspended a little over a meter above a work bench, a package containing Tritium Hyperfilm was carefully opened, taking care to avoid contact with the emulsion layer. Film was apposed to the sections and standards, and the cassettes were sealed shut. Each cassette was tightly wrapped with 2 layers of plastic to protect their contents from moisture. These were then placed in a refrigerator at 4°C.

After 12 weeks, the cassettes were removed and unwrapped in the darkroom, under the same darkroom conditions described above. The darkroom temperature was maintained below 20°C during film processing.

Films were removed from the cassettes and gently washed for 5 minutes sequentially, in developer (100ml Phenisol (Ilford) + 400ml tap water), tap water, and fixing solution (500ml Hypam (Ilford) + 100ml tap water). Finally, they washed again in tap water for several minutes.

The aim of the image analysis procedures was to find approximate boundaries of muscimol spread in each section. Tritium emits weak, low energy beta particles which cause several silver ions in silver halide crystals in tritium hyperfilm, to be deposited as black silver grains to be deposited in the path of the beta particle emissions. These silver atoms catalyze the production of several more when the film is developed after exposure. The greater the levels of tritium reaching the film, the greater the density of silver grains that appear on the corresponding area of the film. The density of silver grains in film positioned on and around parts of the section injection sites therefore reflect relative levels of muscimol present in these areas (but see Chapter 4). The advantages conferred on tritium by the short particle lengths of the beta emissions, include high resolution images relative to high energy emitters. Also, variations in thickness of the sections do not affect the density of the autoradiographic image (Herkenham and Sokoloff, 1984). The disadvantages of using tritium include the long exposure times required and the problem of differential autoabsorption (see Chapter 4).

Typically, injection sites, where levels of muscimol are highest, have high levels of radiation reflected by high grain density. These areas appear very dark. As the radial distance from these areas increase, grain density decreases, and the film appears lighter reflecting decreasing concentrations of muscimol. The points at which decreasing levels of signal meet background noise levels may be regarded as the detectable boundary of muscimol spread. The analysis in this study has therefore focused on determination of background levels, and optimal visualization of signal through colour coding while preserving the spatial properties of the signal as much as possible.

Alignment

When the sections (still glued onto card) were in the cassettes, the film fitted into the cassette tightly so that there was no possibility of movement. This allowed easy realignment of the film while sections were still in the cassettes. The standard was also used for realignment. It consisted of 8 rectangular areas of polymer with differing levels of tritium, which produced 8 corresponding rectangular images on film, each image having uniform grain density, and each rectangle image decreasing in grain density from the next. By comparing the edges in the standards with the edges of the resulting images on the film, the film could be accurately aligned against the sections. These methods were useful for making preliminary observations of muscimol spread.

For a complete analysis, it was necessary to process the films and sections in the following way. Images of the film were first to be acquired in digital form by using an appropriate scanner (see below). Images of *stained* sections were required so that the cerebellar nuclei, white matter and the cerebellar cortex could be distinguished. Images of the sections and the film were to be acquired separately. Processed, digital images of the film were to be superimposed on images of the stained sections. To achieve this, slides had to be removed from the card for staining, and so positions of sections relative to each other, to edges of the cassette and to the standard would be lost. Due to scanner limitations, it was not possible to scan the entire film into a single image. Each slide therefore needed its own points of reference that exactly corresponded with reference points on the film. So a method was developed to re-align individual slides with corresponding areas of film, using three alignment markers on individual slides and areas of film. Before removing slides from the card, they were marked with three small crosses using with a glass cutting diamond marker. The film was re-apposed to the slides, and a permanent black ink marker with a fine tip was used to mark the film with a small dot, over the intersection of the crossed lines. Each area on the film that corresponded to a particular section was surrounded by three black dots.

Image acquisition

Image acquisition was achieved by using a Sharp JX-330P scanner with a transparency adapter. All images were acquired in greyscale format (Bitmap, BMP) in exactly the same resolution (600dpi) and therefore the same scale. Each image incorporated three reference marks and therefore the area of the film to which the section was apposed, and were therefore very similar in size.

Scanner composition

The scanner is composed of a glass table on which transparencies are placed. Above this is the scan head, and below the glass table are the light sources (red, green and blue). In between the glass table and the transparency is a diffusion tablet which ensures that light passing through the transparency is completely even. A white plate is permanently fixed to the glass table, and is used to measure white balance (see below). The scanner was operated using a Viglen Genie personal computer (Intel Pentium 100 processor; 16MB RAM).

Scanner operation

When scanning proceeds, light passes from red, green and blue sources through a system of mirrors and filters, and then through the transparency to be scanned. Light transmitted from the film then passes to a lens in the scan head that focuses the light onto a high density CCD sensor (5150 elements). The three light sources blink cyclically, not together, so that RGB light frequencies reaching the transparency do not intrude onto another. This provides true, three colour information to the CCD. The CCD converts the image into pixels, which encode intensity information for each colour. This data is subjected to the certain corrections before digitisation by the analogue-to-digital converter in the scanner hardware. Firstly, before scanning starts, the CCD supplies an output signal when no light is present. This represents noise levels inherent in the scanner when it operates, and is subtracted from the CCD output. Secondly, prior to scanning, the scan head moves to the white plate. Red, green and blue light sources are cyclically pulsed and the intensity of each is adjusted so that white balance is achieved. Thirdly, the "shading correction" is performed. Since the CCD receives light

through a curved lens, the peripheral areas of the CCD receive light that has lost intensity due to the reduced thickness of the lens at its periphery. When the white plate is scanned, the voltage of each pixel is recorded, and the correction factor for each pixel is calculated and applied to analogue scans of subsequent images. The CCD image is then digitised. These corrections are mandatory for scanner operation and are necessary for the faithful reproduction of the film image. Other corrections , such as the gamma function, may be applied at the discretion of the user, but since these would result in transforming fundamental properties of the image, they were not applied. Once acquired, the images were saved onto Iomega ZIP Disks in Bitmap (BMP) format.

Image processing

After image acquisition, images were processed on a PowerPC using NIH Image 1.6. Before this, it was necessary to convert images from BMP to Tagged image File (TIF) format, using conversion software (Graphic Converter). These were saved on the hard disk of the Power PC. TIF is the only format recognised by NIH Image 1.6. Also, the TIF format files acquired on the Viglen PC were not recognised by any image viewing/converter program on the Power PC. The conversion was therefore necessary.

Threshold

Each image was opened in NIH Image 1.6, and a rectangular area outside that apposed to the section was selected. The mean grey value was measured and recorded for each section. The range was calculated, and a value a little above this range was chosen as background level (see figure 2A.1). This value was used to set a threshold. Values above this threshold were regarded as signal, and values below this were regarded as noise. This value was incorporated into a look up table (LUT), with which NIH Image converted grey values into colours. The LUT colours were constructed in such a way that greylevels below threshold were all black and those immediately above started with red (see figure A2.2), thus providing a very clear contrast, revealing a conservative estimatate of the boundary of muscimol spread.

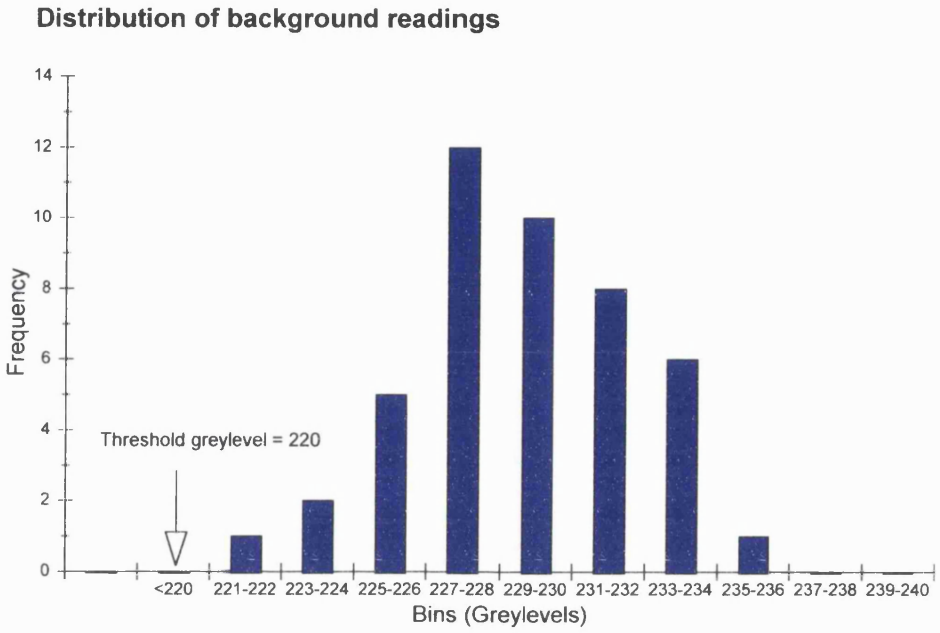


Figure A2.1: Graph of distributions of background readings. 45 samples taken (one from each slide). The distribution peaks at the bin for greylevels 227 and 228. No background value was observed below greylevel 221. The threshold was therefore set at greylevel 220.

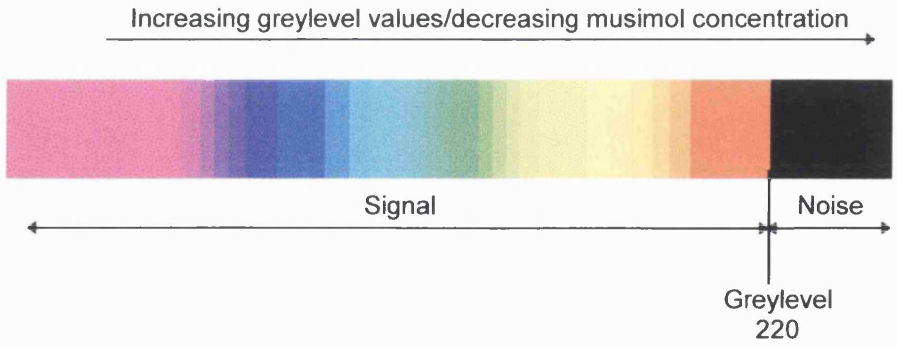


Figure 2A.2: Look-up-table (LUT) constructed for autoradiographic images. Each of the 256 greylevels is assigned an individual colour (although all are not visible in this schematic representation). The LUT shows the order of colour transition. Low greylevels represent the darkest areas of the film, and the highest levels of muscimol. The point of transition between red and black (greylevel 220) represents the threshold chosen to distinguish signal from noise.

Noise reduction

Unlike some other filters, median filters are commonly applied to images to reduce noise *without distortion of boundaries*. A median filter was therefore applied to the images after thresholding and applying the look up table in NIH Image. This worked by taking a group of 9 pixels (3 x 3 square arrays across the image). The pixel with the median grey value of these pixels was calculated and then applied to all the pixels. This strategy removes peaks of high and low greylevels in the greylevel histogram of the image without flattening greylevel steps that separate greylevel regions. The images were saved in TIF format, and were ready to be superimposed on images of the sections.

Image Enhancement of Stained Sections

After analysis of the films was complete, slides were carefully removed from the card and sections were Nissl stained and coverslipped (see Appendix 1). Each slide was individually scanned at 600 dpi. to produce a digital image of the section, taking care to ensure that the cerebellar nuclei, cerebellar cortex, edge of section and reference marks were clearly visible. This was converted to a negative image resulting in a black background, in Paint Shop Pro (v3.0).

In order to obtain an accurate outline diagram of cerebellar sections, in which there were no light areas which could obscure the coloured representation of the autoradiography, the negative image was again processed on a PC in Ad Oculos, an image processing software package.

Although the boundaries of the cerebellar nuclei were clearly visible on the untransformed negative images of the sections, Od Oculos transformations could not detect these. These were drawn in by hand in Paint Shop Pro (v3.0) before transformation in Ad Oculos, and so the resulting image was an outline of the cerebellar section with an outline of the cerebellar nuclei.

In order to superimpose the processed autoradiographic image over the negative image of the section accurately, software was used which could achieve interactive, pixel by pixel movement of one image relative to another (*i2i*, v1.0). The opacity of each image

was set to 50%. An image of the processed autoradiography was moved until reference points on both images were accurately superimposed. The final image was saved as a TIFF file.

Appendix 3:
Supplementary Data

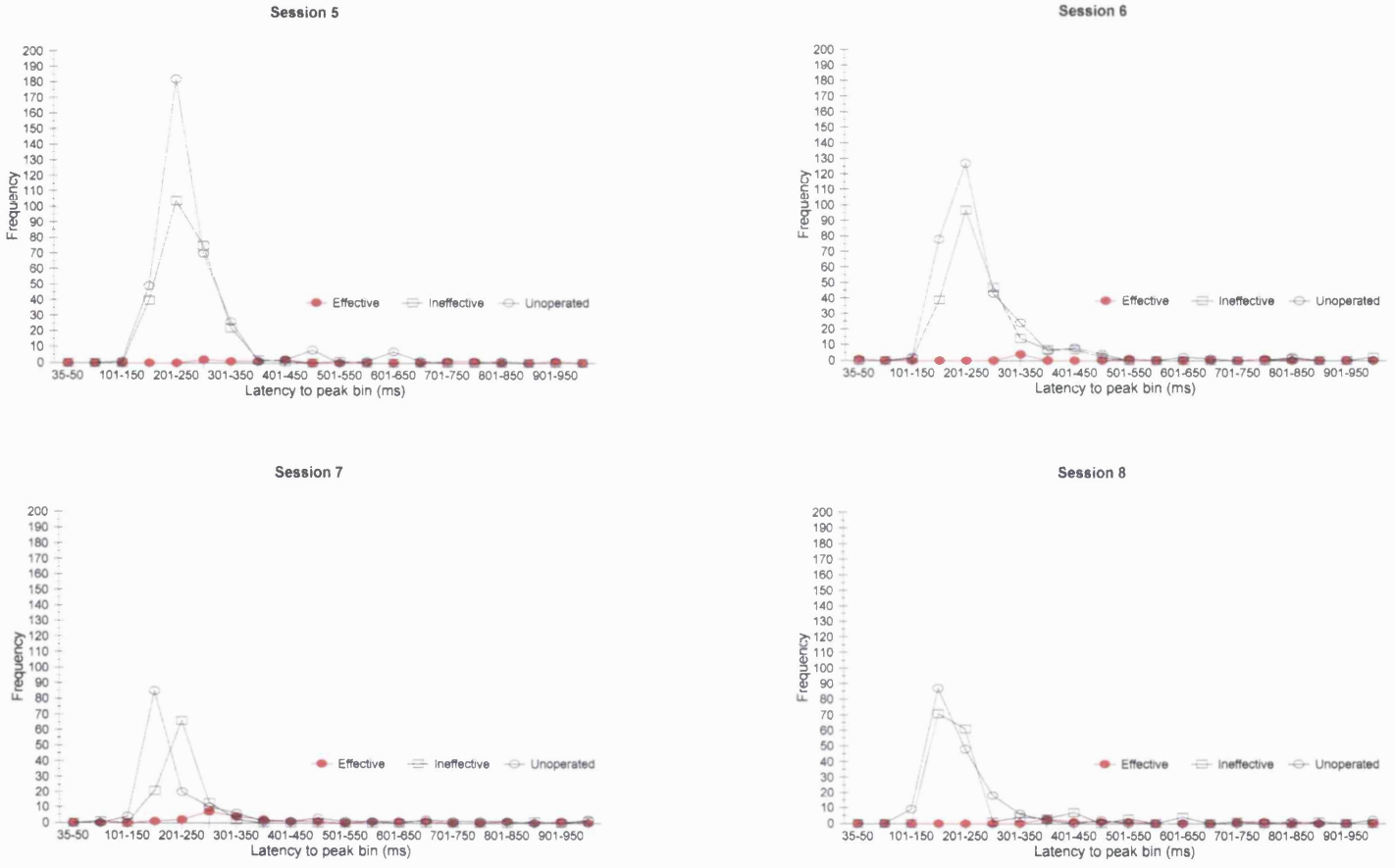


Figure A3.1: Phase 2 latencies to peak of all groups, session by session.
In each group, CRs from all subjects are pooled to contribute to distributions.

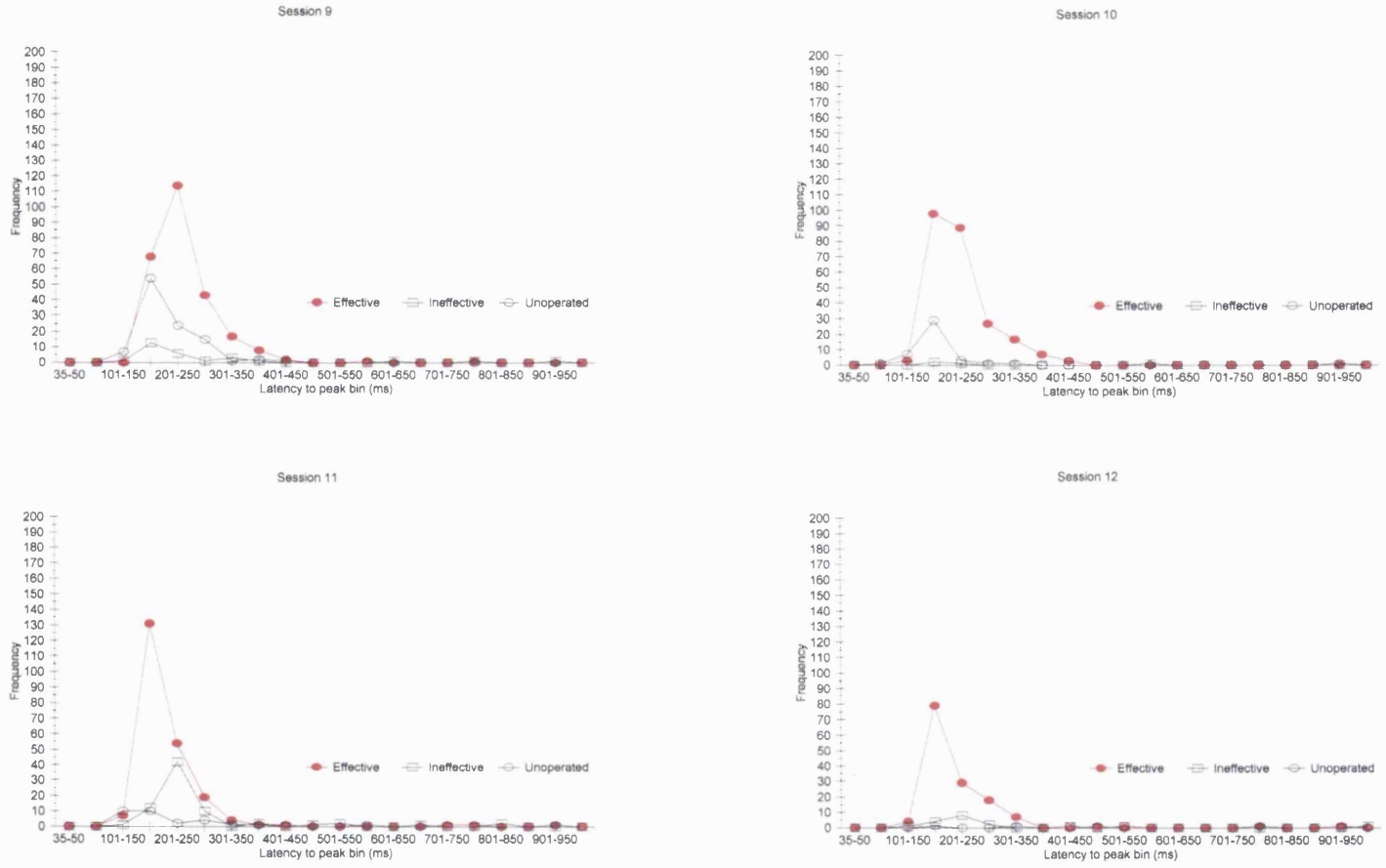


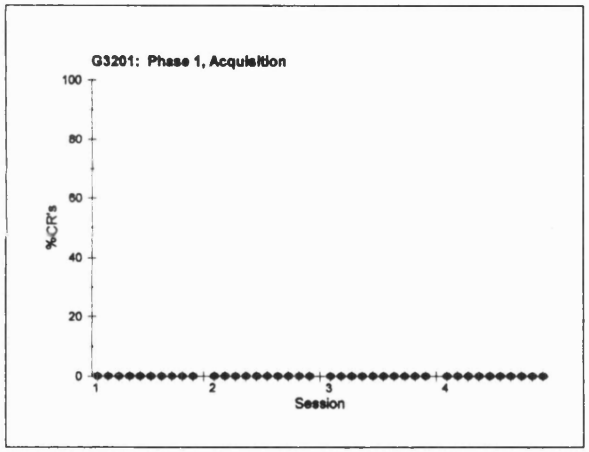
Figure A3.2: Phase 3 latencies to peak of all groups, session by session.
In each group, CRs from all subjects are pooled to contribute to distributions.

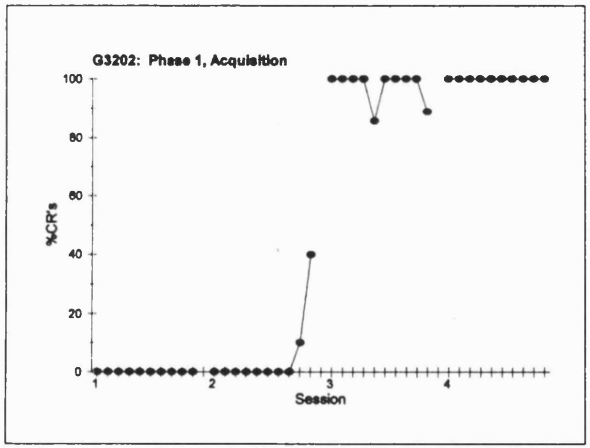
Figure A3.3:

Phase 1 and 2 data for individual subjects in LD Group.

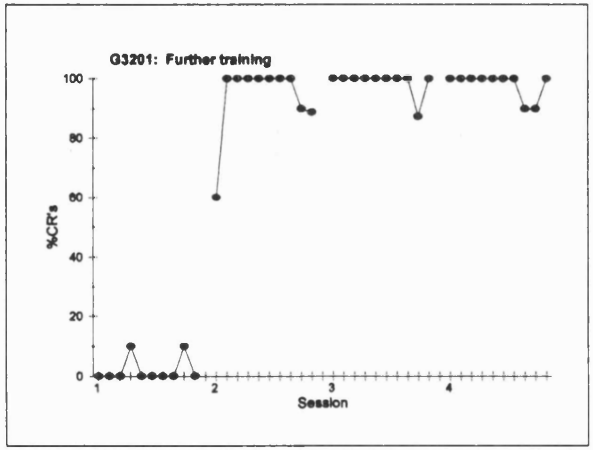
All Low Dose Subjects

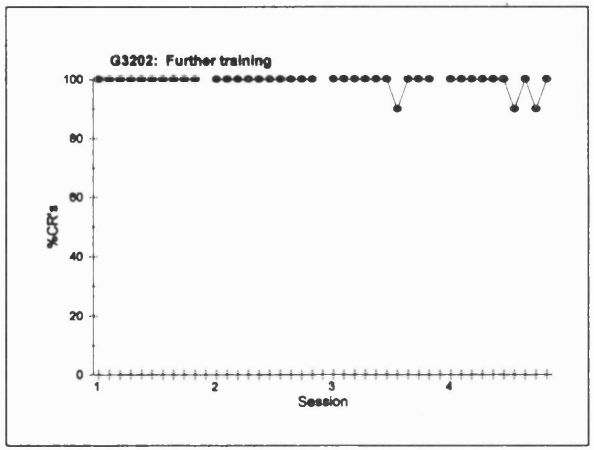
Phase 1
Acquisition



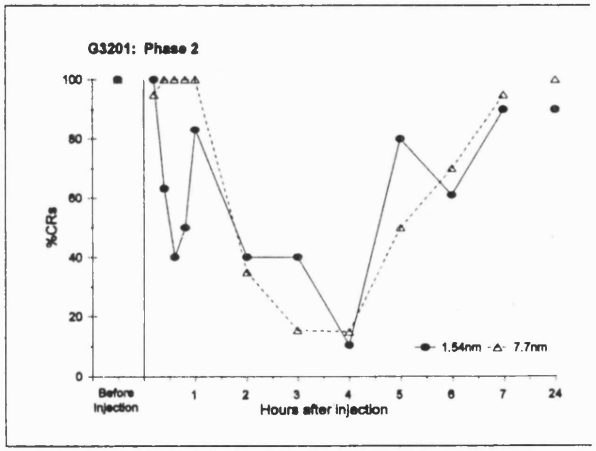


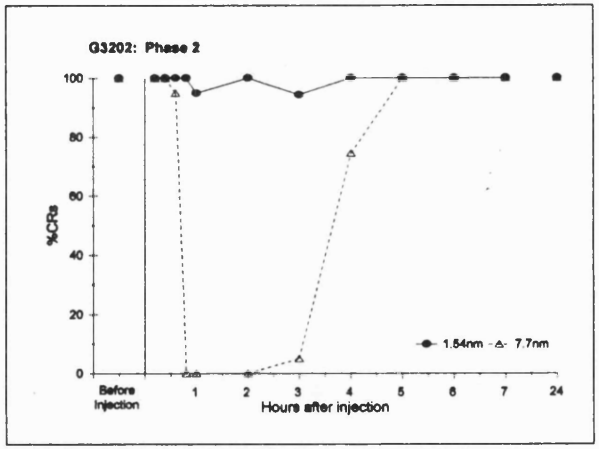
Further training





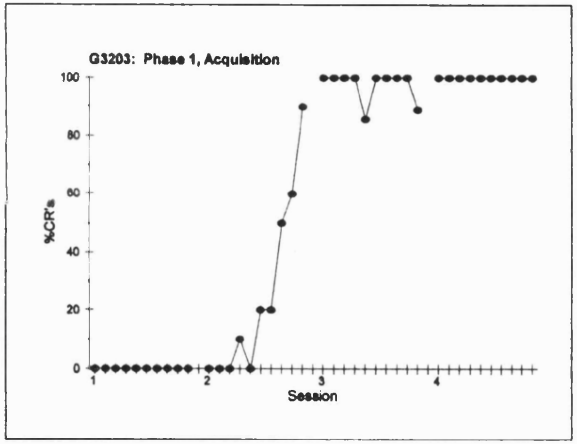
Phase 2

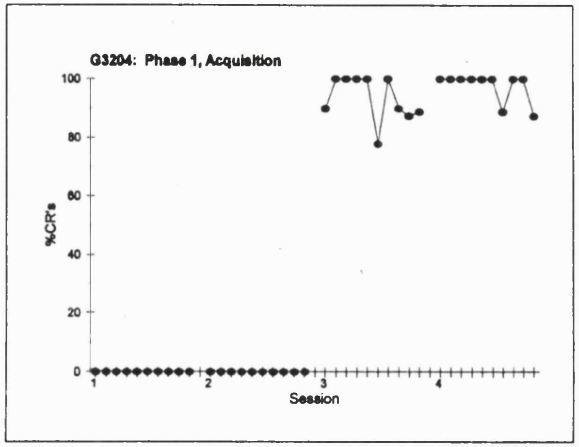




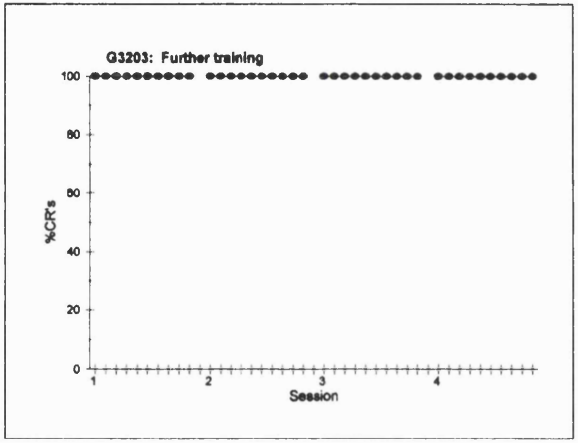
All Low Dose Subjects

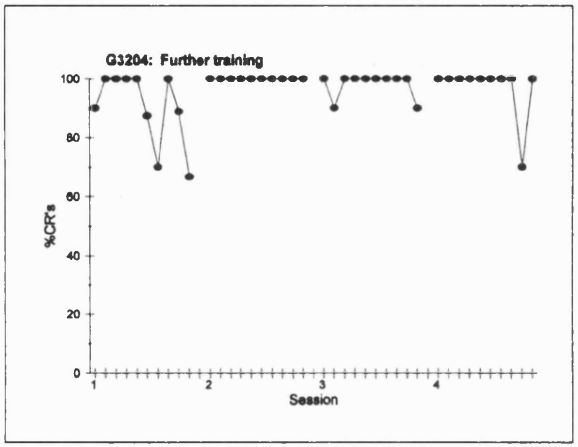
Phase 1
Acquisition



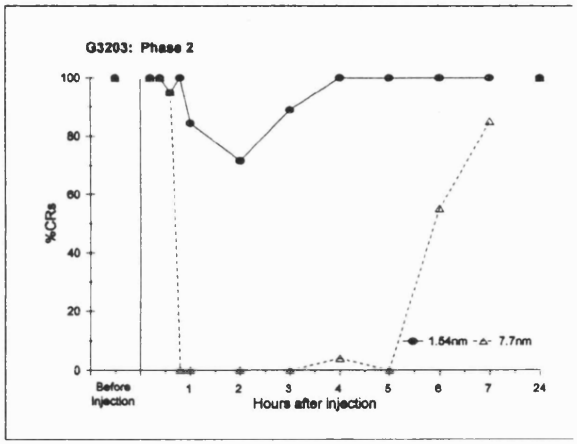


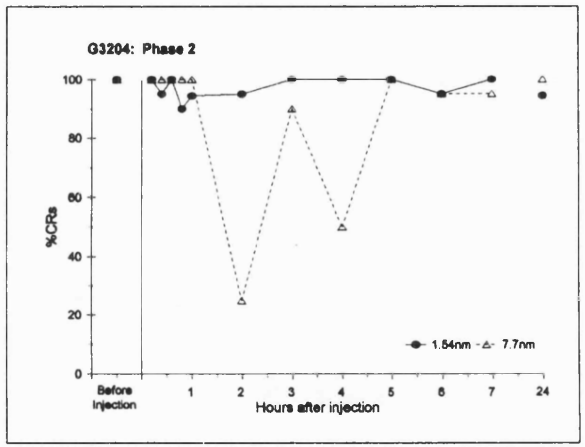
Further training





Phase 2



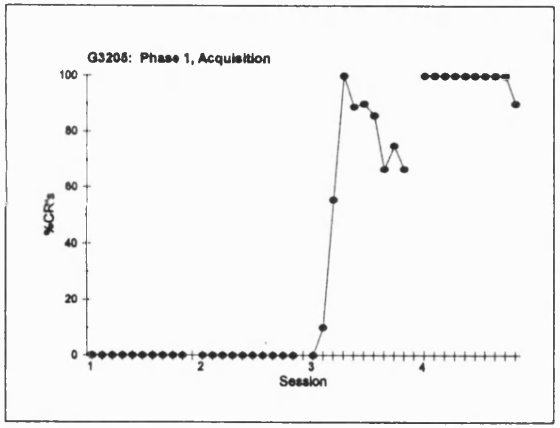


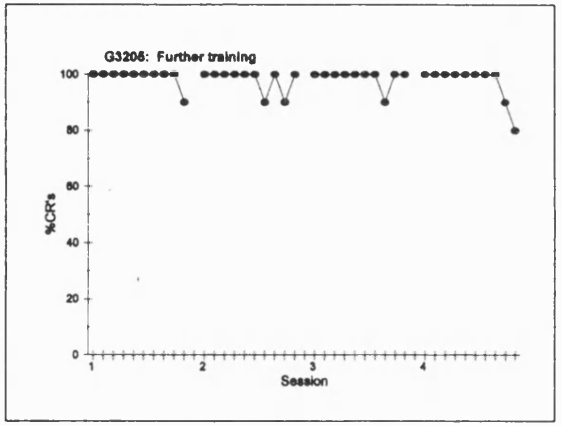
All Low Dose Subjects

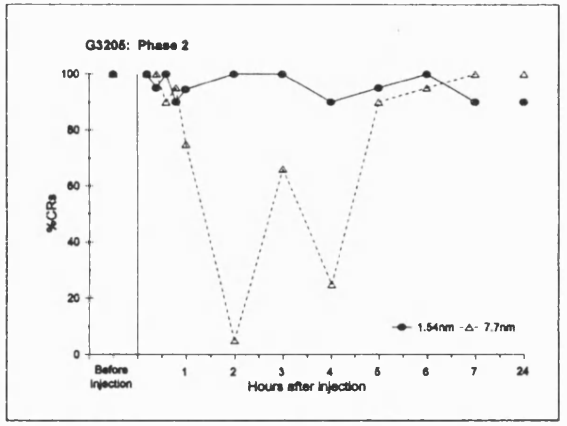
Phase 1
Acquisition

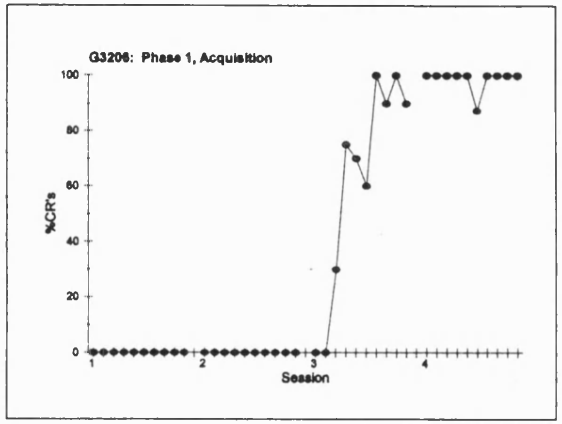
Further training

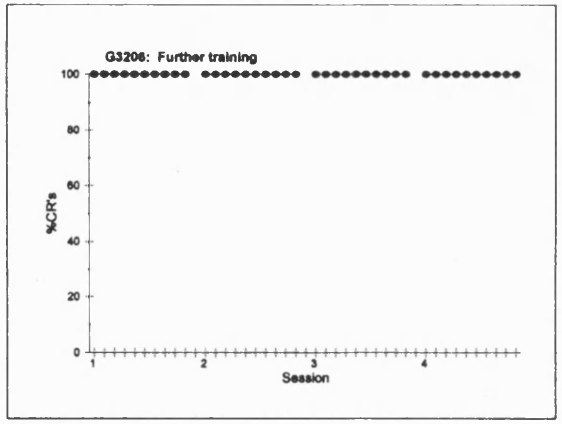
Phase 2

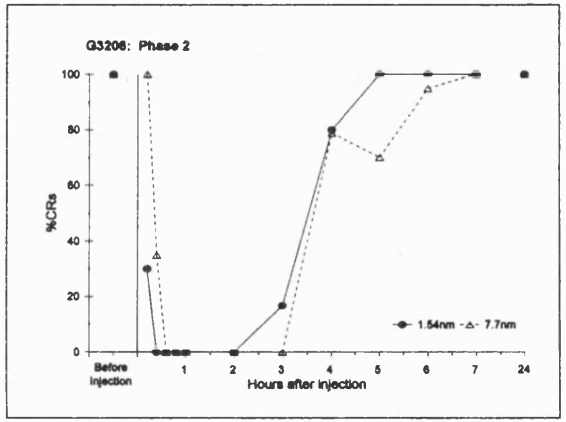






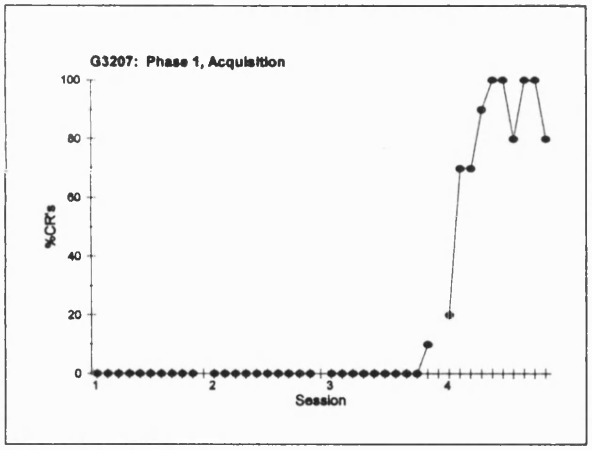


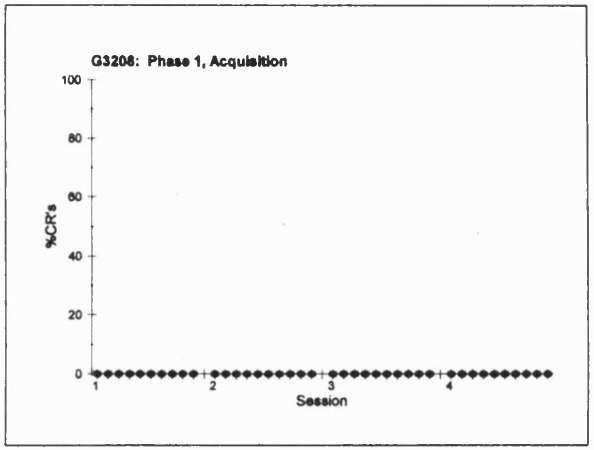




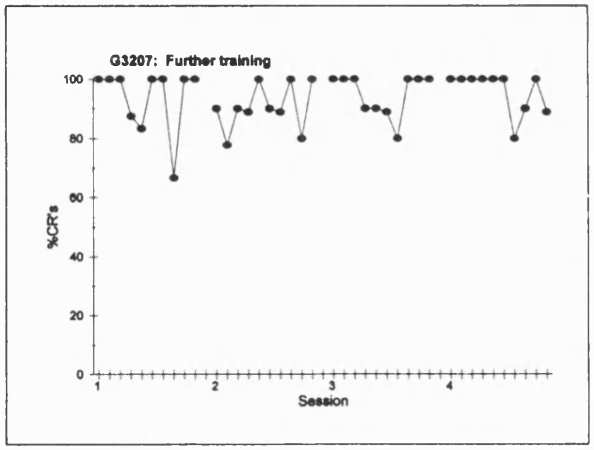
All Low Dose Subjects

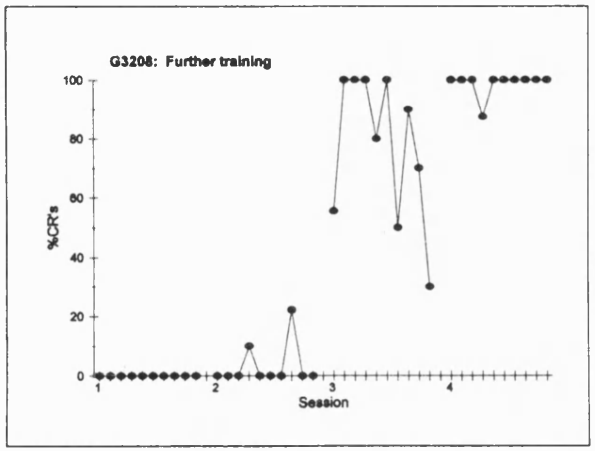
Phase 1
Acquisition



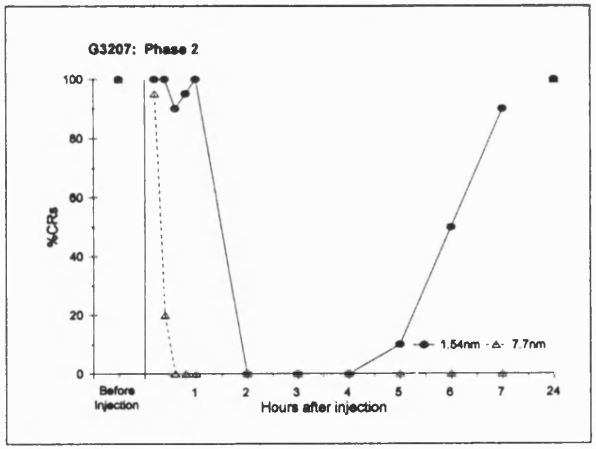


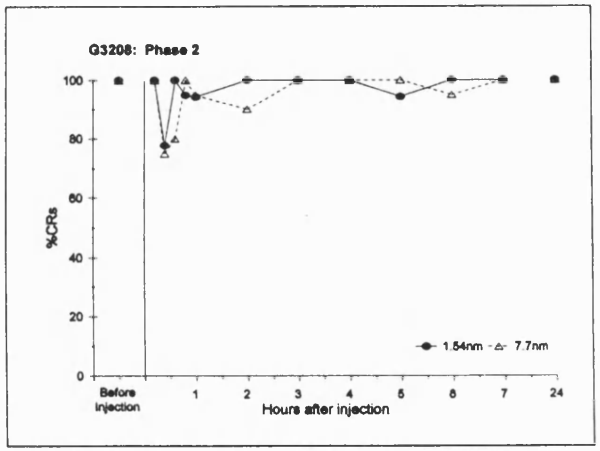
Further training





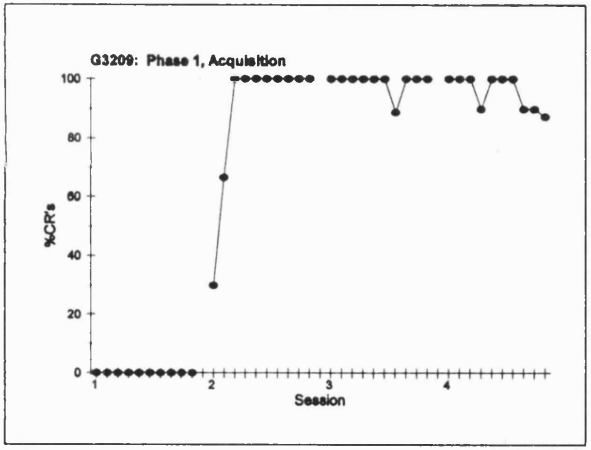
Phase 2

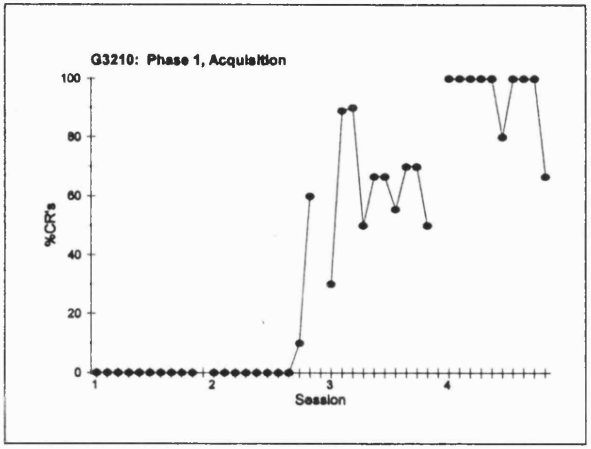




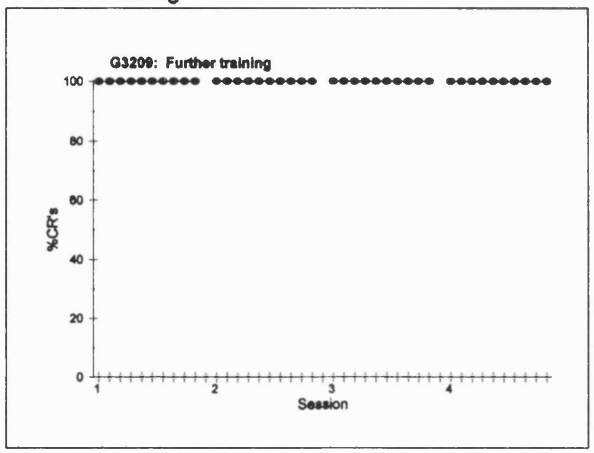
All Low Dose Subjects

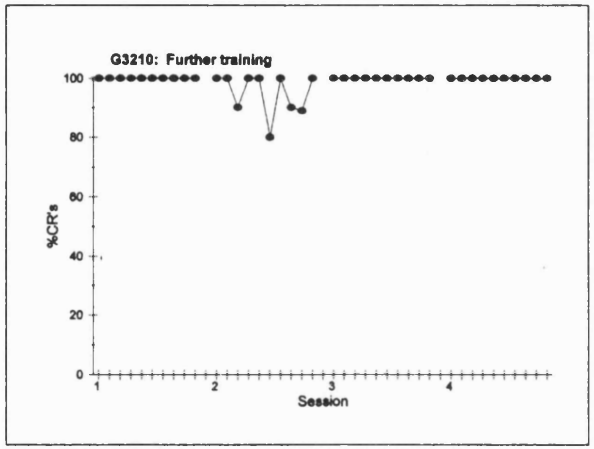
Phase 1
Acquisition





Further training





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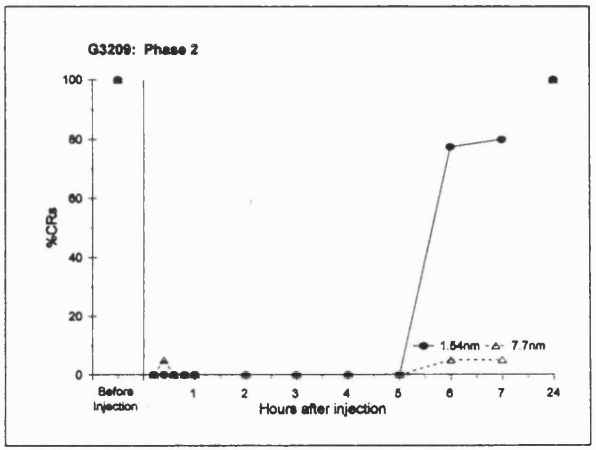
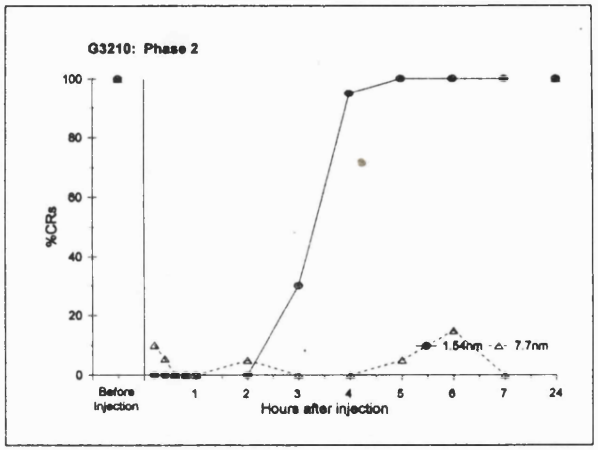
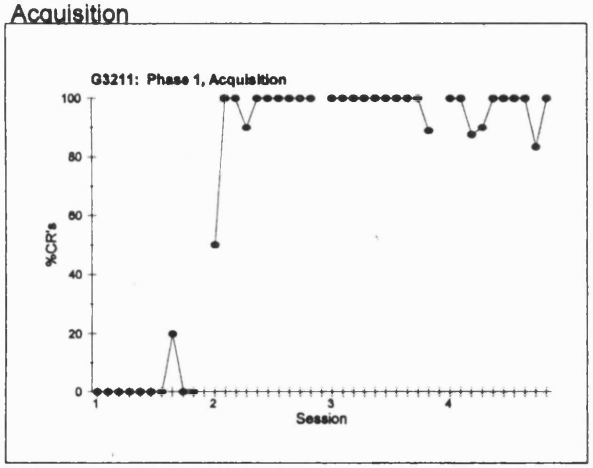
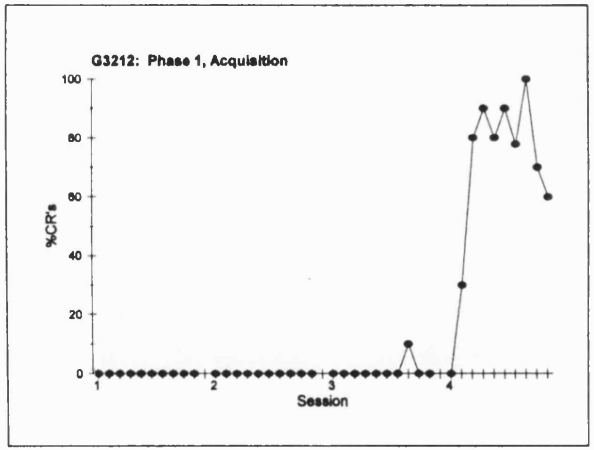




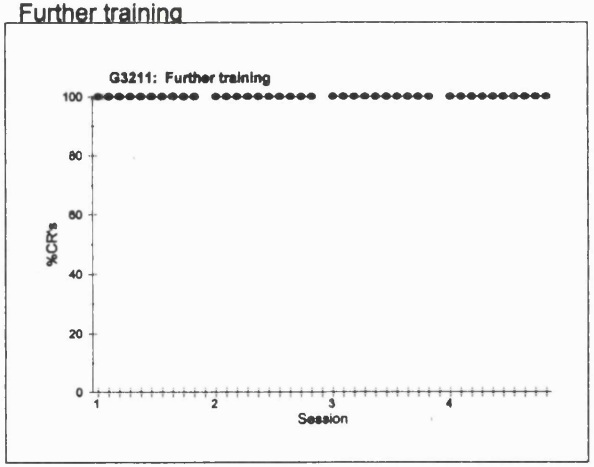
All Low Dose Subjects

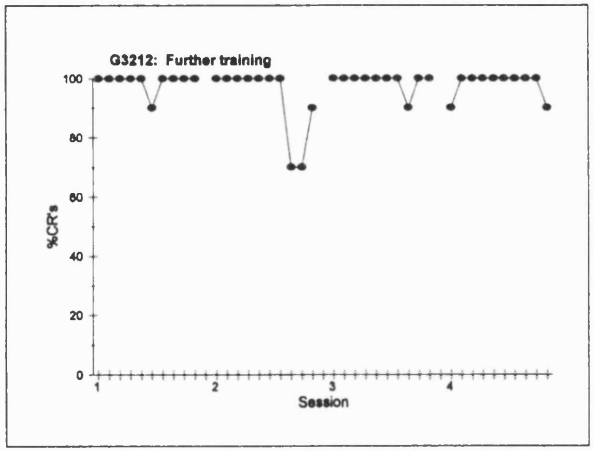
Phase 1
Acquisition



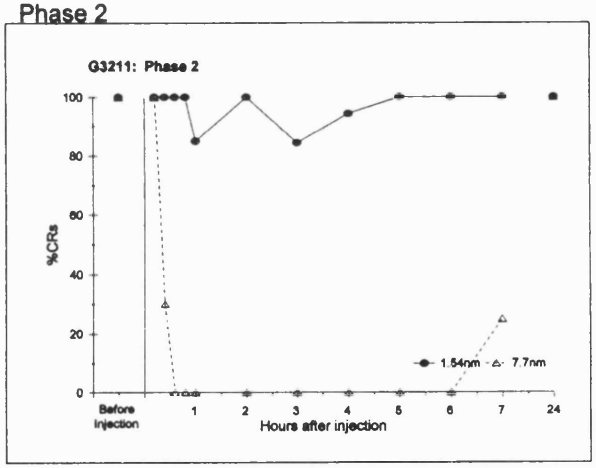


Further training





Phase 2



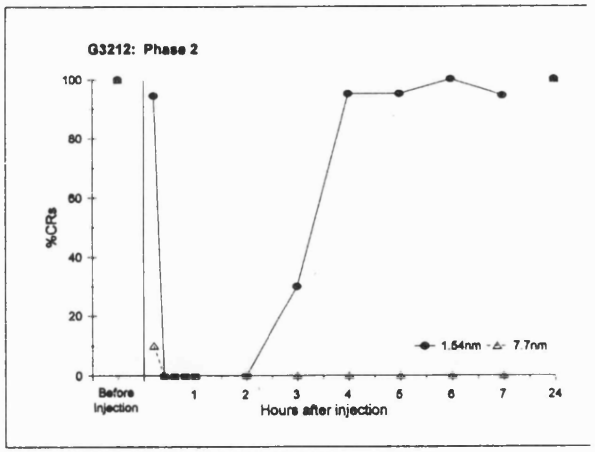
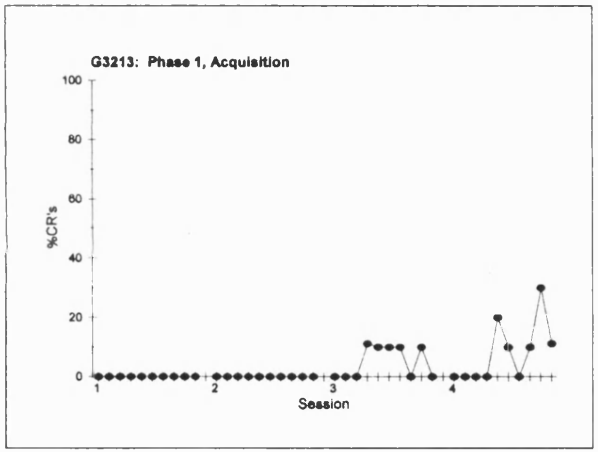


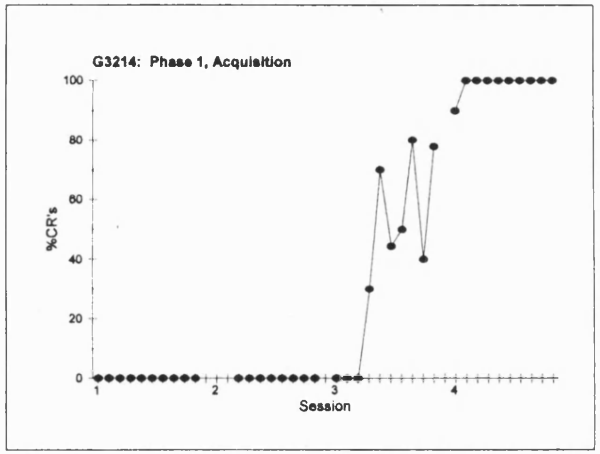
Figure A3.4 :

Phase 1 and 2 data for individual subjects in HD Group.

All High Dose Subjects

Phase 1
Acquisition





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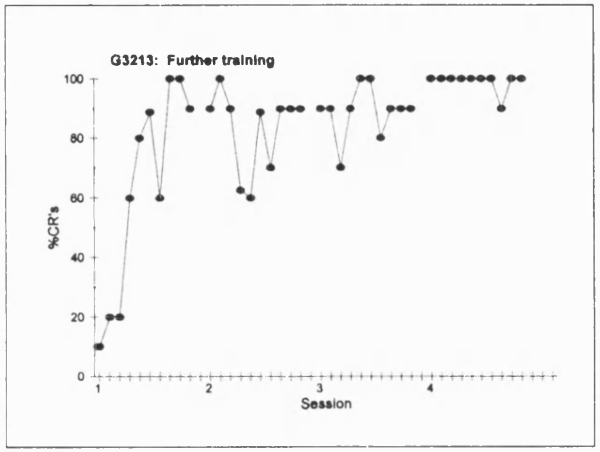

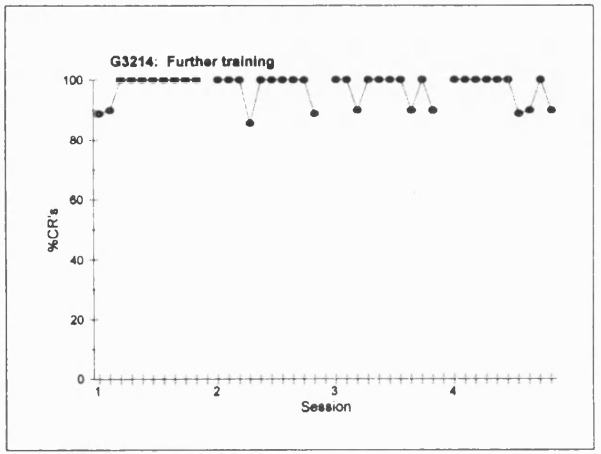

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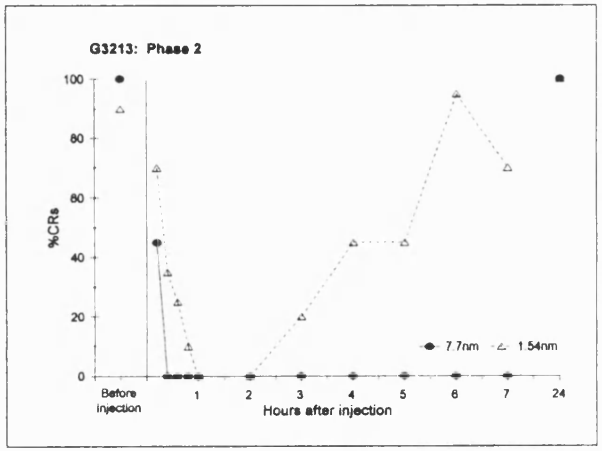

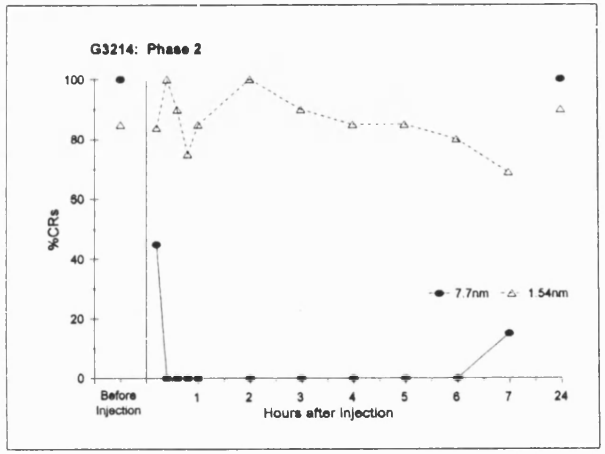

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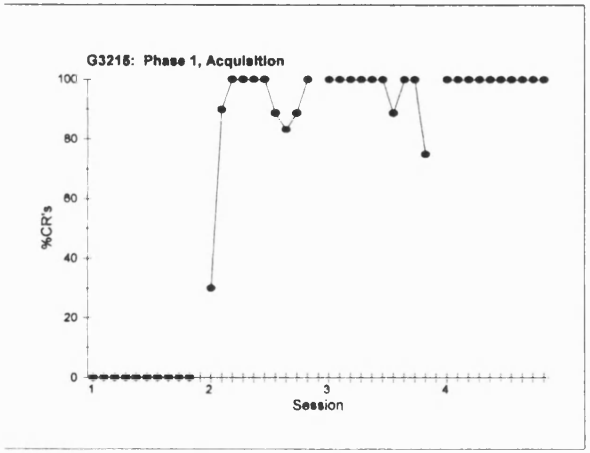

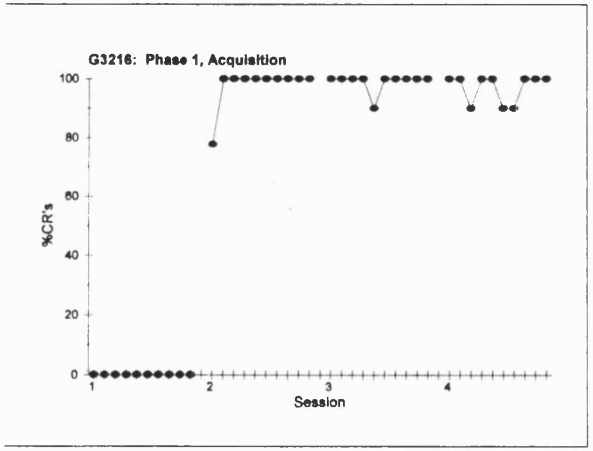

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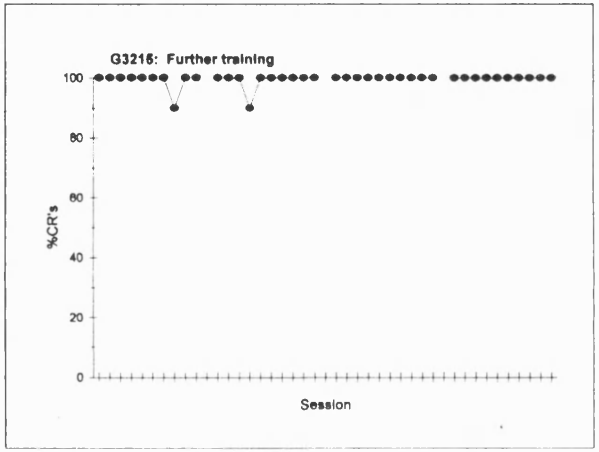

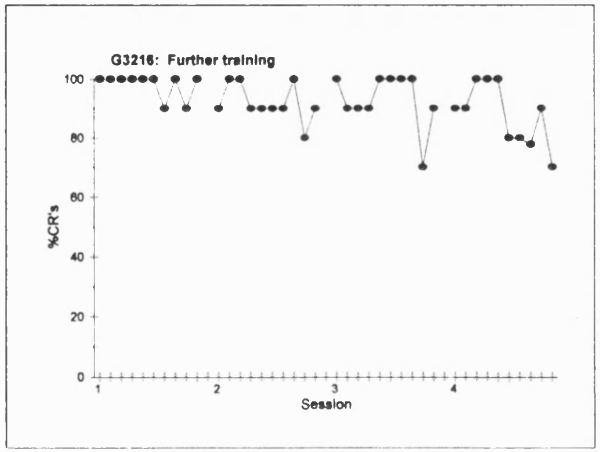

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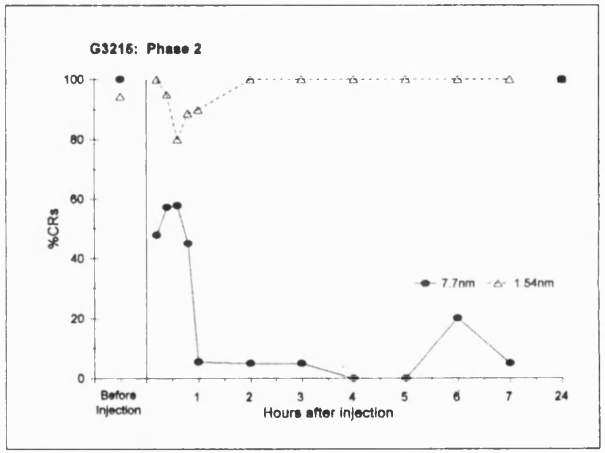

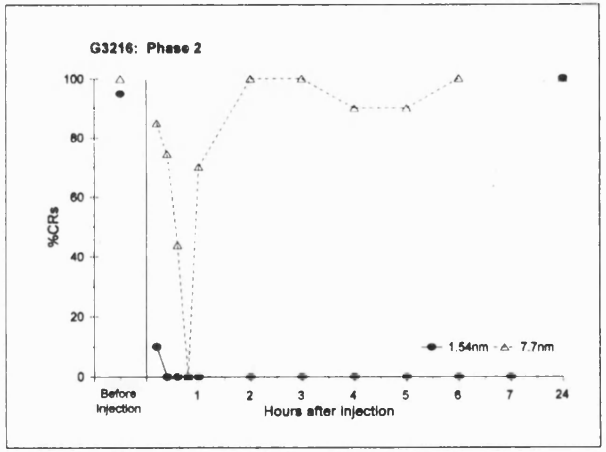

Phase 1
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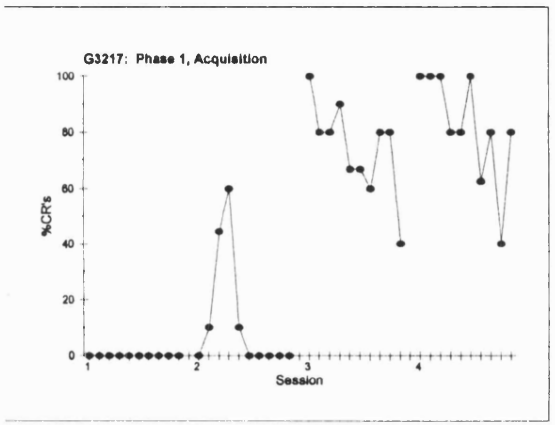
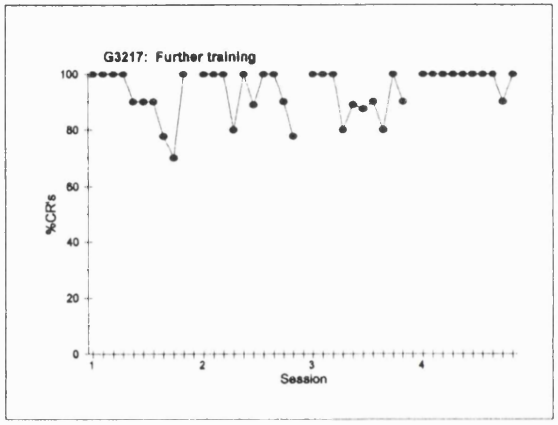
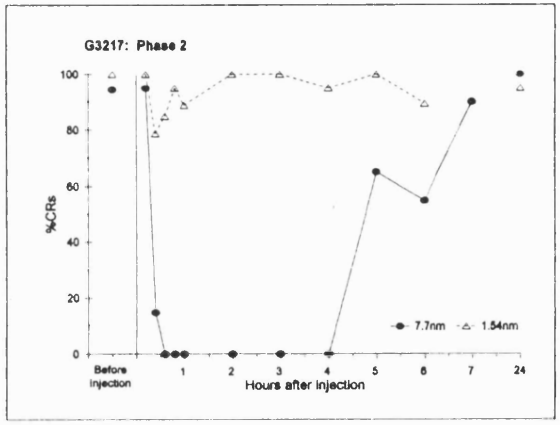
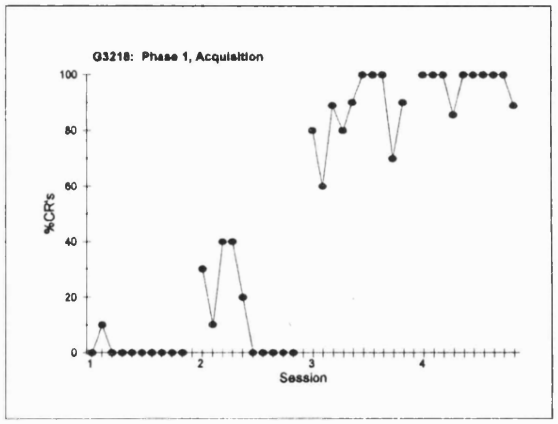
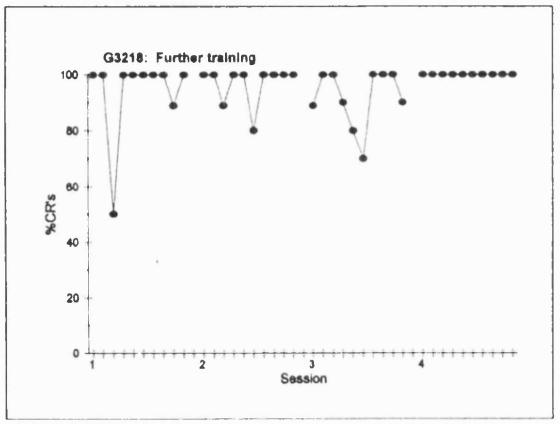
Phase 2

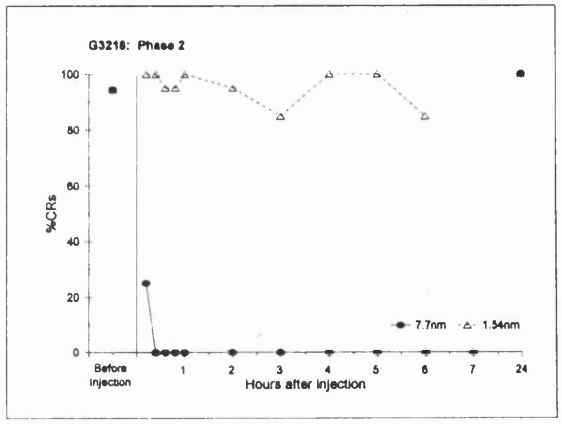












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