THE CEREBELLUM AND EYEBLINK CONDITIONING

PhD Thesis

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ABSTRACT

Lesions of lobule HVI of the cerebellar cortex or of its target in the anterior cerebellar nuclei disrupt the acquisition and retention of conditioned nictitating membrane responses (CRs). The critical locus for the acquisition and retention of CRs remains controversial. Some have argued that the primary site of learning and memory is in the cerebellar nuclei. Others have maintained that the apparent abolition of CRs merely reflects a weakening of all responses or a shift in their timing caused by a motor deficit.

Aspiration lesions of the cerebellar cortex cause retrograde degeneration of precerebellar nuclei. Since the loss of CRs from lesions of the cerebellar cortex might be due to the loss of collateral inputs to the cerebellar nuclei from the precerebellar nuclei, fibre sparing lesions were made which destroyed only cells in the cerebellar cortex. CRs were lost, demonstrating that the cerebellar cortex is required for retention of CRs and that the cerebellar nuclei on their own cannot maintain CRs. Fibre sparing lesions of the anterior cerebellar nuclei prevented the acquisition and abolished retention of CRs. There were no late CRs. Reflex responses remained essentially intact demonstrating that the absence of CRs was not simply due to a motor impairment.

If the cerebellum is involved in conditioning it is likely that an auditory CS would be relayed to the cerebellum from the basilar pontine nuclei (PN). WGA-HRP injections into the inferior colliculus revealed a projection to caudal regions of PN, thus demonstrating a pathway whereby a tone CS can be transmitted to the cerebellum.

Lesions which include the ansiform lobe as well as HVI disrupt CRs more completely than HVI lesions alone. WGA-HRP injections into ansiform lobule revealed connections from the PN and inferior olive appropriate for transmitting CS and US related information. These connections to ansiform lobule were sparse, consistent with the fact that this region is not the primary locus for NMR conditioning.

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THE CEREBELLUM AND EYEBLINK CONDITIONING.

INTRODUCTION

To survive and reproduce animals must learn about the significance of stimuli that make up their environment. One of the central problems in neuroscience is to discover the neural mechanisms by which animals acquire this knowledge, and how it is stored for later recall and expression.

Three fundamental aspects of the environment can be learned. An animal can learn about the existence of the stimuli in its environment, about the relationships between stimuli, and about relationships between its own actions and the stimuli. These three processes can be studied in the laboratory. Sensitisation and habituation reflect the animals knowledge of the existence of stimuli. Pavlovian or classical conditioning reflect the animals knowledge about the relationship between stimuli. Operant conditioning reflects the animals knowledge about the relationship between its own actions and external stimuli. It is not known whether these are independent processes or whether there is a more general unifying learning mechanism which is common to all three.

At the beginning of this century Pavlov developed a powerful tool, the conditioned reflex, to analyse the way in which animals learn about the relationships between stimuli (Pavlov, 1927). Pavlov discovered that an association between certain external events and innate reflexes could be learned and retained for a considerable period of time. The basic procedure for simple Pavlovian conditioning is to present a stimulus that initially fails to evoke a response (the conditioned stimulus - CS) followed by a stimulus that always evokes the reflex response (the unconditioned stimulus - US). After repeated pairings of the CS and US the CS elicits a conditioned response (CR). In one experiment, Pavlov sounded a beating metronome immediately before presenting food to a dog. The sound of the metronome initially elicited no obvious response. On sight of the food the animal would immediately turn towards the food, lick its lips and salivate. This response Pavlov called the alimentary reflex. After several repetitions of sequentially sounding the metronome followed by presenting food, the sound of the metronome alone now evoked the alimentary reflex. In this experiment, food was the US, the alimentary reflex was the UR and the ticking metronome was the CS. The metronome came to elicit the same response as the food, although there were important differences in the magnitude and timing of CRs and URs.

Pavlovian or classical conditioning is a fairly general process whereby associations can be learned. Pavlov focused on the alimentary reflex in his experiments but heart rate, jaw movement, limb flexion, and eyeblinks have all been associatively conditioned. Even stimuli that have no obvious biological significance and elicit no obvious UR can be conditioned. In this procedure, called sensory preconditioning, two stimuli (S1, S2) are paired, much as they would be in ordinary Pavlovian conditioning i.e. S1 is presented immediately followed by S2. No

response is elicited by either stimulus. S2 is then paired with a US until CRs are evoked by S2. If S1 is then presented to the animal on its own, it can elicit CRs, even though it had never been paired with the US.

Pavlovian conditioning may be confounded by 'conscious processes' since there may be voluntary responses elicited by the CS that are non-associative in nature. To avoid contamination of the results by such non-associative processes, Cason (1922) developed a technique for conditioning eyeblinks in humans, in which a sound CS was paired with periorbital electrical stimulation as a US. After repeated pairings the sound would elicit a conditioned eyeblink. This technique allowed precise measurements of the timing of responses to the compound CS+US, to the US alone, and to the CS alone, so helping to distinguish between learned, reflex and voluntary responses.

Eyeblink conditioning can be applied to other mammals. The nictitating membrane is a third eyelid which sweeps horizontally across the eyeball. Gormezano and his colleagues found that blinking of the nictitating membrane, and the external eyelids of rabbits, could be readily conditioned to sounds (Gormezano *et al.,* 1962; Schneiderman *et al.,*1962). The conditioned nictitating membrane response in rabbits is a useful animal model for studies of the mechanisms of simple associative learning (Thompson, 1976). The most important reasons for its usefulness are:

i. The conditioned nictitating membrane (NMR) behaves in a way which is similar to other conditioned responses, and so obeys the principles of classical conditioning described by Pavlov (1927). For example, trace conditioning, higher order conditioning, conditioned inhibition, blocking and discrimination have all been demonstrated using this preparation.

ii. The NMR can be conditioned without contamination by other, non-conditioned responses, for example sensitisation, habituation or pseudoconditioning.

iii. The NMR is a very specific and highly reproducible response. Conditioned and unconditioned responses can be characterised by their latency and amplitude. These factors allow the effects upon learning of manipulation of the conditioning stimuli or the brain, to be accurately determined and to be distinguished from effects upon sensory and motor structures. Thus, for example, a critical brain area can abolish the conditioned response whilst leaving the unconditioned response essentially unchanged.

iv. The conditioned NMR is a simple movement so it should be possible to identify the essential circuits linking the CS to the movement. It is likely that an essential element in the learning circuit is where there is integration of the neuronal representation of the CS and US. If the site or sites of this integration can be determined then the mechanisms underlying associative conditioning can be analysed.

The nictitating membrane response

The nictitating membrane (NM) is a semicircular membrane containing elastic material and cartilage. The NM protrudes from the nasal palpebral angle in birds, fish and several mammals. Bright lights, looming stimuli and stimulation of the cornea or periorbital regions of the face

cause retraction of the eye into the orbit which compresses orbital fat and connective tissue against the base of the NM thus forcing it laterally across the eye. The NM in rabbits retracts mainly by passive movement of the eyeball back to its normal position upon releasing pressure on its base. Contraction of the retractor nictitans muscle aids this retraction (Motais, 1885; Back-y-Rita 1971; Cegavske *et al* ., 1976).

In rabbits, the retractor bulbi is the principal muscle responsible for eyeball retraction and hence the nictitating membrane response (Berthier and Moore 1980, 1983; Mis 1977). The retractor bulbi is activated mainly by way of motorneurons in the accessory abducens nucleus (accVI) (Disterhoft *et al.,*1985; Delgado-Garcia *et al.,*1990; Harvey *et al.,*1984), and to some extent in the abducens nucleus (VI) itself (Powell *et al.,*1979; Cegavske *et al.,*1976; Murphy *et al.,*1986). Eyeball retraction and the accompanying NMR is further facilitated by the simultaneous activation of all the extraocular muscles (Lorente de No, 1933; Berthier and Moore 1980; Disterhoft *et al.,*1985; Evinger *et al.,*1984).

The conditioned NMR is part of a co-ordinated defensive reflex that also involves closure of the external eyelids (McCormick *et al.,*1982) by activation of the orbicularis oculi muscles whose motorneurons are located in the intermediate nucleus of the facial nucleus (VII) (Holstege *et al.,*1986). Conditioned eyeblinks to corneal airpuff are typically accompanied by cheek twitch and neck movements. All of these movements serve to prevent damage to the eyes (McCormick *et al.,*1982).

In this report the term eyeblink is used to describe responses of both the external eyelids and of the nictitating membrane. There are however important differences between these responses. Presentation of tone CSs used in conditioning of the external eyelids in cats, rats, rabbits and humans elicits short latency, or alpha responses. Short latency responses are not elicited by the CS during conditioning of the NMR in rabbits. The difference is due to pathways that can convey CS information to the facial nucleus and abducens nucleus (Cegavske *et al.,*1976) but not to the accessory abducens nucleus since the abducens nucleus in the rabbit does not respond to tones (Disterhoft *et al.,*1985). There are important pathways that influence the external eyeblink circuitry but do not influence so strongly the NMR. For example, the motor cortex is necessary for conditioning of short and long latency responses in cats (Woody et al.,1992) but motor cortex is not necessary for NMR conditioning (Oakley and Russell, 1972).

The basic circuitry of the reflex response has been defined. Eyeblinks can be elicited by a brief electrical stimulus applied close to the eyelids or by a puff of air directed at the cornea and sclera. Periocular and corneal branches of the supraorbital nerve convey this somatosensory information to several regions within the spinal trigeminal nucleus (Harvey *et al.,*1984; Durand *et al.,*1983), although only rostral parts of the trigeminal nuclei are essential for the production of URs (Berthier and Moore, 1983). Monosynaptic and polysynaptic pathways convey this information to motorneurones in the accVI and VII (Berthier and Moore, 1983; Grant and

Horcholle-Bossavit, 1983; Holstege *et al.,*1986). In cats collateral branches of trigeminal cells contact motorneurones both in the accessory abducens and facial nuclei (Grant *et al.,*1979) consistent with the close correspondance between external eyeblinks and NMRs.

The circuitry required for the formation of conditioned reflexes is less well known. Whether there is a particular site or locus of learning, or whether several different brain regions are involved in the formation of CRs is currently not known. CRs are produced by the co-ordinated activation of several motorneuron groups so it is unlikely that there are sites of learning and memory in each of these groups. In principle CRs might be formed within the UR pathway prior to the eyeblink motorneurons, or in other brain regions which are in parallel with the reflex circuit.

The search for the site/s of eyeblink learning

Pavlov 1927, assumed that the process of reflex conditioning must take place within regions of the cerebral cortex specialised for analysing the CS and US. But decerebrate cats, ferrets and rabbits, in which all brain tissue above the level of the red nucleus has been removed, can still acquire and retain eyeblink CRs (Lovick and Zbrozyna, 1975; Mauk and Thompson, 1987; Norman *et al.,*1977). There must be a circuit capable of simple associations in the brainstem. Brain regions above the red nucleus may be involved in conditioning in the intact animal but they are not necessary for conditioning to occur. For example there is evidence that sensory and motor regions of the cerebral cortex, thalamus and hippocampus, if present, play a role in some aspects of eyeblink conditioning.

Cerebrum

Marquis and Hilgard, (1936) demonstrated that the cerebral cortex is not essential for delay conditioning when they conditioned external eyelid responses to a tone CS in decorticate dogs. More complex associations can also be formed without the cerebral cortex. Rabbits can discriminate between two different CSs which have been differentially paired with the US (Yeo *et al.,*1983) and continue to retain CRs acquired prior to complete removal of the neocortex (Oakley and Russell, 1977). Trace conditioning is also unimpaired in neodecorticates (Yeo *et al.,*1982).

Although CRs can be established without the cerebral cortex, this region of the brain is involved in conditioning in the intact animal. During acquisition training in intact rabbits CRs first appear at the time of the US and then grow backwards in time towards the onset of the CS, but CRs in decorticate rabbits do not progress to a shorter latency (Oakley and Russell, 1972). CRs established to a light CS are temporarily abolished by the loss of activity in motor and visual cortex caused by cortical spreading depression (Megirian and Bures, 1970; Gutmann *et al* ., 1972; Papsdorf *et al* . 1965). Lesions of the retrosplenial cortex produce deficits in reversal learning (Berger *et al.,*1986).

It is possible that the results of these experiments are due to the loss of facilitatory inputs to subcortical regions rather than the loss of a critical region of brain but this cannot be the only explanation. CRs can be acquired to a light CS when both the pre-tectal nuclei and superior colliculus are removed, so visual cortex must be actively involved in this process (Koutalidis *et al* ., 1988).

Hippocampus

The hippocampus is not essential for eyeblink conditioning. Neither dorsal hippocampal lesions (Shmaltz and Theios, 1972; Solomon and Moore , 1975), nor complete destruction of the hippocampus prevent delay conditioning (Akase *et al.,*1989), and conditioned discrimination (Berger and Orr, 1983).

Loss of the hippocampus does affect eyeblink learning when more complex conditioning procedures are used. Dorsal hippocampal lesions, which destroy the fimbria-fornix,thus removing the majority of efferents from the hippocampus, prevent latent inhibition, blocking (Solomon and Moore, 1975), and discrimination reversal learning (Berger and Orr, 1983). Similar lesions impede extinction in well conditioned rabbits (Schmaltz and Theos, 1972; McCormick and Thompson, 1982), but enhance extinction in less well conditioned rabbits (Akase *et al.,*1989).

The results of these seemingly disparate studies may be explained by a failure to recognise changes in the CS-US relationship. Thus, even though new relationships can be formed, previously learned responses can not be altered when the CS-US relationship changes. Other possibilities can explain some of these results. Latent inhibition and blocking are impaired by lesions of the hippocampus, suggesting that it may be involved in tuning out unimportant stimuli (Solomon, 1987). Since extinction is rapid in poorly trained rabbits perhaps the hippocampus is important for the incorporation of the CR memory into a long term memory (Akase *et al.,*1989).

The hippocampus is not essential for simple delay conditioning but it is essential if greater demands are made upon the memory of the CS and US. Complete hippocampectomy prevents the acquisition of trace CRs when an interval of 500ms or more separates the CS offset and US onset, but not when the interval is 350ms (Solomon *et al.,*1986; Moyer *et al.,*1990). Partial lesions restricted to dorsal hippocampus (so destroying the fimbria-fornix) had little effect upon trace conditioning when an interval of 500ms was used (James *et al.,*1986; Solomon *et al.*, 1986). Since complete hippocampectomy prevents trace conditioning there must be an essential route for efferent fibres other than through the fornix, possibly through the perforant path and entorhinal cortex.

The role of the hippocampus in trace conditioning is not straightforward. Some authors suggested that the hippocampus is involved in the temporary storage of the neuronal representation of the CS so that the trace interval can be bridged (Carlson 1976; Weisz *et*

glutamate receptor binding (Mamounas *et al.,*1984; Sunayashiki-Kusuzaki *et al.,*1993) in hippocampal neurones similar to those found in long term potentiation.

Although the hippocampus is not necessary for simple eyeblink conditioning, it probably has some role in conditioning. The forebrain also may play a role in conditioning, although it is not clear yet which parts and why. Perhaps, as suggested by Berger *et al.,*(1986), the hippocampus can modify CRs produced in the brainstem via pathways through the subiculum. Since some forms of conditioning are prevented by dorsal hippocampal lesions, whereas trace conditioning is only prevented by complete hippocampectomy, different parts of the forebrain may be engaged in different aspects of eyeblink conditioning.

*al.,*1984). However extensive recordings in the hippocampus during conditioning have failed to show neuronal activity related solely to the CS (Solomon *et al.,*1986). Moyer et al., (1990) suggested that the hippocampus is involved in the short term memory of the CS/US association. This idea is consistent with the finding that lesions made one day after trace conditioning with a 500ms CS-US interval profoundly affect the retention of CRs whereas lesions made one month after training have no effect upon the retention of CRs (Kim et al., 1996). In contrast CRs acquired during delay conditioning are unaffected by lesions made one day or one month after training. These results show that different neural mechanisms may underlie the formation of the CS/US association in trace and delay conditioning.

Neuronal activity in the dentate gyrus (Weisz *et al.,*1984,) and in the CA1 and CA3 regions of the hippocampus, increases significantly during both delay (Thompson 1976; Berger *et al.,*1976,1983; Berger and Thompson, 1978,1981) and trace conditioning (Solomon *et al.,*1986). The increased activity is related exclusively to CRs since there is no increase in activity during unpaired presentations of the CS and US. Thus, hippocampus may be particularly interested in CRs and not simply sensory processing of the CS or US, or in the movement itself.

Thompson, (1976) argued that the hippocampus must be involved in learning since neuronal activity increases very early on in conditioning, well before CRs are produced. Also, when CRs are produced, hippocampal activity increases prior to the onset of CRs. But other studies show that CR related hippocampal activity is dependant upon cerebellar output, which suggests that CRs are neither produced nor stored in hippocampus. Unilateral lesions of the cerebellum abolish both overt CRs and CR related activity in the hippocampus (Clark *et al.,*1984; Sears *et al.,*1990). Following unilateral lesions of the cerebellum training of the intact side leads both to the production of CRs on that side, and to the reinstatement of CR related activity in the hippocampus (Sears et al., 1990).

Although the hippocampus is not the site of the CS/US association it may be important for the short term memory of this association, since conditioning leads to long term physiological and biochemical changes in it. There is an increase in the monosynaptic granule cell population spike to perforant path stimulation similar to that found in long term potentiation (Weisz *et al.,*1984). There is a reduction in after-hyperpolarization in pyramidal cells of CA1 region which may cause an increase in excitability (Disterhoft *et al.,*1986, 1988). There are conditioning specific changes in protein kinase C (Bank *et al.,*1988; Olds *et al.,*1989) and an increase in

cont

Cerebellum

Since decerebrate animals can be conditioned there must be a circuit for conditioned eyeblinks within the brainstem. Evidence from a variety of studies suggest that this circuit is centred upon lobulus simplex, or lobule HVI, of the cerebellar cortex. Lobule HVI is essential for NMR conditioning because it has inputs related to the CS and US and because it has outputs to the eyeblink motor circuitry.

The cerebellar cortex receives auditory and visual CS information from the cerebrum and colliculus indirectly through mossy fibres from the basilar pontine nuclei (Kawamura and Brodal, 1973; Azizi et al., 1985). The most important auditory and visual inputs to the cerebellum are from the colliculus, since neither lesions of the neocortex (Oakley and Russell, 1972) nor decerebration (Mauk and Thompson, 1987) prevent the acquisition and retention of conditioned eyeblinks.

McCormick *et al.*,(1982) were the first to demonstrate the importance of the cerebellum in eyeblink conditioning. These authors found that CRs which had been previously acquired to a tone CS were abolished by large unilateral lesions of the cerebellar nuclei and cortex. Similar lesions of the cerebellum prevented the acquisition of CRs (Clark et al, 1982). The lesions had not simply destroyed sensory circuits engaged in the detection of the CS and US, nor had they destroyed motor circuitry necessary for the production of eyeblinks - the animals were neither deaf nor paralysed - since CRs could be established on the side opposite the lesions when the CS was paired with a US applied to that side and URs were normal after the lesions.

Only a small region of the cerebellum is essential for eyeblink conditioning. Lesions centred upon lobule HVI of the cerebellar cortex severely impaired both the acquisition (Lavond *et al.*,1985) and retention of CRs (Yeo *et al.*,1985b), whereas large lesions that did not include lobule HVI had very little effect upon CRs (Glickstein *et al.*,1983; Yeo *et al.*,1985b). Lesions of the targets of lobule HVI, in anterior parts of the interpositus and dentate nuclei, also prevented the acquisition of CRs (Sears and Steinmetz, 1990) and abolished previously acquired CRs (Lavond *et al.*,1985; Yeo *et al.*,1985a). Lesions of posterior parts of the interpositus nucleus or lesions of lateral parts of the dentate nucleus had little or no effect upon CRs (Yeo *et al.*,1985a).

Lobule HVI receives inputs related to CSs and USs. Information related to a periocular US can reach lobule HVI through mossy fibre and climbing fibre pathways. Stimulation of the face, including the periorbital region, causes an increase in mossy fibre activity (Welker, 1987; Berthier and Moore, 1986) and climbing fibre activity (Akaike, 1989; Cody and Richardson, 1979) within HVI. This lobule receives inputs directly from the trigeminal nuclei through mossy fibres (Ikeda, 1979; Yeo *et al.*, 1985c), and through climbing fibres from face areas (Cook and Wiesendanger, 1976; Gellman *et al.*, 1983) of the dorsal accessory olive (Yeo *et al.*, 1985c; Van Ham and Yeo, 1992; Azizi & Woodward, 1987; Kawamura and Hashikawa, 1979). Lesions of the inferior olive that include rostro-medial parts of the dorsal accessory olive abolish previously learned CRs (McCormick *et al.*, 1985; Yeo *et al.*, 1986). CS-related information is also relayed to lobule HVI during NMR conditioning. Auditory and visual mossy fibre and climbing fibre responses can be recorded within lobule HVI and adjacent lobules in cat (Altman *et al.*, 1976) and monkey (Snider and Eldred, 1952; Mortimer, 1975). Auditory CS-related activity can be recorded in HVI (Berthier and Moore, 1986) during NMR conditioning. In rabbits, there is anatomical evidence for a visual input to HVI. The superior colliculus projects to caudal dorsolateral and lateral pontine nuclei (Wells *et al.*, 1989) which in turn project to HVI (Yeo *et al.*, 1985c).

cont

The idea that there is a convergence of CS and US information within the cerebellar cortex receives some support from the fact that direct electrical stimulation of cerebellar afferents can serve as CSs and USs in conditioning. CRs were formed when the PN were stimulated as a CS paired with an air puff directed at the eye as a US (Steinmetz *et al.*,1986). Conditioning was also

established when an auditory stimulus was paired with stimulation of the IO as a US (Mauk *et al.,*1986) and when the PN was stimulated as a CS paired with stimulation of the IO as a US (Steinmetz *et al.,*1989). However, the mechanism of this learning is controversial. Since stimulation of the IO does not produce movement (^) the mechanism whereby such stimulation serves as a US is not immediately apparent.

There is a pathway from lobule HVI to the eyeblink motorneurons through which it can exert control over eyeblinks. Lobule HVI projects mainly to anterior and lateral parts of the interpositus nucleus and to anterior and medial parts of the dentate nucleus (Yeo *et al.*, 1985c). In cats these nuclei project to a face area in dorsal-lateral magnocellular red nucleus (Daniel *et al.*, 1987; Gibson *et al.,*1987; Robinson *et al.*, 1987). The red nucleus connects directly and indirectly (Holstege *et al.* 1986; Desmond *et al.*, 1983) to eye blink motorneurons. Stimulation of sites within the cerebellar cortex (Hesslow, 1989, 1994a,1994b), cerebellar nuclei (McCormick and Thompson, 1984) and within the red nucleus (Chapman *et al.*, 1988; Haley *et al.,*1988) all elicit eyeblinks. Lesions of any part of this circuit, including the red nucleus (Rosenfield and Moore, 1983, 1985; Haley *et al.,*1988), severely impair CRs but leave URs essentially intact, suggesting that this circuit is particularly involved in the control of conditioned eyeblinks.

These studies demonstrate that the cerebellum is a major component of a circuit concerned with learned eyeblinks. Two controversies have arisen from these studies. The first concerns whether the cerebellar cortex is necessary for eyeblink conditioning. Yeo and his collegues provided evidence that the cerebellar cortex is necessary for NMR conditioning. However, Thompson and his colleges argued that the cerebellar nuclei alone are necessary and sufficient for the acquisition and retention of conditioned eyeblinks. The second controversy concerns the reasons why the cerebellum is important for eyeblink conditioning. Sensory information related to CSs and USs converges upon parts of the cerebellar cortex that can modulate eyeblinks. Lesions of those parts of the cerebellar cortex severely impair the acquisition of CRs and prevent the retention of previously learned CRs. Thus the essential neural mechanisms of learning underlying eyeblink conditioning are within the cerebellum. The suggestion that eyeblinks are learned by the cerebellum is consistent with the idea first put forward by Brindley, (1964) that one function of the cerebellum is to learn movements (Eccles et al., 1967; Ito, 1984;Thach et al., 1992). Based upon the unique neuronal architecture of the cerebellum several mathematical models of cerebellar learning have been proposed in which parallel fibres to Purkinje cell synapses are modified by the activity of climbing fibres (Marr, 1969; Grossberg, 1969; Blomfield and Marr, 1970; Albus, 1971; Gilbert 1974). Consistent with these models is the evidence that during eyeblink conditioning parallel fibres conveying CSs information converge upon Purkinje cells activated by climbing fibres carrying US information. The idea that the cerebellum is actively involved in eyeblink learning has been rejected by some authors (Welsh and Harvey, 1992; Kelly et al., 1990; Bloedel, 1992). These authors claim that the inability to produce learned eyeblinks after cerebellar lesions is simply due to prevention of the expression of CRs produced elsewhere in the brain. Each of these ideas is further explored in the general discussion.

Kainic acid lesions of the cerebellar cortex severely impair or abolish conditioned nictitating membrane responses in the rabbit.

INTRODUCTION

Large unilateral lesions of the cerebellum prevent the acquisition (Lincoln *et al.*, 1982), and impair retention (McCormick *et al.,* 1981) of conditioned nictitating membrane (NM) responses. Lesions of the anterior interpositus and rostral medial parts of the dentate nucleus profoundly impair or abolish previously established conditioned NM responses (Clark *et al.*, 1984; McCormick & Thompson, 1984; Lavond *et al.*, 1985; Yeo *et al.*, 1985a).

Lesions of lobule HVI of the cerebellar cortex also impair or abolish conditioned NM responses (Yeo *et al.*, 1985b). This finding is consistent with the anatomical connections since a major output of lobule HVI is to the anterior interpositus nucleus (Yeo *et al.*, 1985c). Several other studies also demonstrate an important role of the cerebellar cortex in NMR conditioning. Lesions of the cerebellar cortex including HVI severely retard acquisition of conditioned NM responses (Lavond and Steinmetz, 1989). Injection of GABA antagonists into HVI selectively and reversibly abolishes conditioned NM responses (Mamounas *et al.*, 1987). Some Purkinje cells in HVI are active prior to a conditioned NM response and some are driven by the conditioned or unconditioned stimuli (Berthier & Moore, 1986). Multiple-unit recordings from cerebellar cortex adjacent to HVI, in the Ansiform lobule, have revealed activity related to the performance of conditioned NM responses (McCormick and Thompson 1984).

In some studies, lesions of the cerebellar cortex were reported to have little or no effect upon conditioned NM responses (Clark *et al.,* 1984; McCormick & Thompson, 1984). However, in a more recent study, large lesions that included HVI transiently abolished previously established conditioned responses (Lavond *et al.*, 1987).

The cerebellar cortex receives mossy fibre inputs from several nuclei and climbing fibre input from the inferior olive. Some of the nuclei supplying mossy fibres send collaterals to the cerebellar nuclei (Qvist, 1989). Although the dentate and posterior interpositus nuclei receive input from the nucleus reticularis tegmenti pontis together with a much smaller projection from the basilar pontine nuclei (Dietrichs *et al.*, 1983; Gerrits and Voogd, 1987; Shinoda *et al.*, 1992), the anterior interpositus nucleus may only receive mossy fibre collaterals from the lateral reticular nucleus (Russchen *et al.*, 1976) and spinal cord (Matsushita and Ikeda, 1970). All of the cerebellar nuclei receive collaterals from the inferior olive (Van der Want *et al.*, 1989). Aspiration lesions of the cerebellar cortex result in degenerative changes in these precerebellar nuclei. Several sources of mossy fibre inputs, including the nucleus reticularis tegmenti pontis and basilar pontine nuclei degenerate (Brodal and Jansen, 1946). In rabbit, there is a particularly rapid degeneration of cells within the inferior olive (Brodal, 1939). HVI receives olivary input from face areas in the medial part of the dorsal accessory olive (Yeo *et*

al., 1985c). Lesions of this olivary region cause an immediate and enduring loss of conditioned NM responses (Yeo *et al.*, 1986). Thus aspiration lesions of HVI may impair CRs due to the primary loss of cerebellar cortex or due to the loss of collaterals to the cerebellar nuclei from the inferior olive or other precerebellar nuclei. In this study, kainic acid (KA) was used to produce fibre-sparing lesions of the cerebellar cortex. At appropriate concentrations, it has a neurotoxic action on cells of the cerebellar cortex, and does not damage afferent and efferent fibres. The precerebellar nuclei and their collaterals to the deep nuclei should therefore remain intact. Since intracortical collaterals (Armstrong *et al.*, 1973; Hrycyshyn *et al.*, 1989) would also be spared, the location of sites critical for the conditioned NMR could be more accurately defined.

METHODS

Subjects

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

Techniques and equipment

Each animal was trained using techniques and equipment similar to those first developed by Gormezano *et al.* (1962) and described in an earlier paper (Yeo *et al.*, 1985a). Each animal was placed in a Perspex restraining box and a low-torque potentiometer was attached to the head by clips around the ears and muzzle. The shaft of the potentiometer was attached by a lever and thread to a suture placed in the NM. A counterbalance weight provided a restoring force of 1 gm to maintain tension in the thread. The animal was placed in a ventilated sound-attenuating chamber facing a pair of 65mm square translucent plastic panels separated by a loudspeaker mounted centrally.

Conditioning

The behavioural training was Pavlovian delay conditioning using light and white noise as conditioned stimuli. In each daily session of 200 trials, there were 100 presentations of the light CS and 100 of the white noise CS. The CSs were given in a fixed pattern of five light, followed by five white noise, and so on. A daily session consisted of 90 presentations of each CS type paired with the US and ten presentations of each CS type alone. Every tenth presentation of each CS type was unpaired.

The light CS was the illumination of both translucent panels to 6.8 $cd.m^{-2}$, the white noise CS intensity was 90 dB (re $20\mu N.m^{-2}$). The duration of both CSs was 550 msec. The US was a 50 ms, 2.5 mA, 50 Hz AC periorbital electrical stimulus. On paired trials the inter-stimulus interval between the CS and US onsets was 500 ms. The intertrial interval (ITI) was 15 seconds.

Acquisition and retention training

All subjects were initially given five sessions of training with the US and NM transducer on the right side. Surgery was then performed and after a period of recovery all subjects underwent retention testing. There were four experimental groups. (see Table of Subject Histories).

Group A

The experimental procedure in this group was similar to our earlier aspiration lesion study (Yeo *et al.* 1985b). Ten subjects received injections of KA into hemispheral parts of lobules V, VI and VII of the right cerebellar cortex. Animals in this group were then trained for five sessions with the US applied to, and the NMR recorded from, the right side (lesioned). Five sessions were then given with the US and the NM transducer on the left side (not lesioned). The US and NM transducer were then transferred back to the right side for five more sessions.

Pilot studies revealed that there was some recovery of CRs ipsilateral to the lesion during the third post-operative block of five conditioning sessions. This recovery might relate to some transfer of responses from the conditioning sessions to the non-lesioned side during the second post-operative block. To test for this possibility, group B and control groups C and D were tested with three consecutive blocks (15 sessions in total) to the right, lesioned side, before conditioning to the non-lesioned side.

Group B

Sixteen subjects received injections of KA into hemispheral parts of lobules V, VI and VII of the right cerebellar cortex. Animals in this group were post-operatively trained for fifteen sessions with the US applied to, and the NMR recorded on, the right side. The US and NM transducer were then placed on the left side and training given for five sessions. This was followed by a further five sessions with the US and NM transducer on the right side.

Group C

Two subjects received injections of kainic into the Ansiform lobule of the right cerebellar cortex. Post-operative behavioural training was identical to group B.

Group D

Three subjects did not undergo surgery but were retrained for fifteen sessions using the same schedule as for group B. These subjects were conditioned controls for subsequent UR testing (see below) - only their UR data is presented.

UR testing

Following behavioural training the reflex responses of 8 subjects from group B and all from group D, were tested on a series of unconditioned stimuli of varying intensity. Each trial consisted of a 50ms, 50Hz periorbital US followed by a 60s ITI. The US intensity was varied in the fixed sequence 2.5, 2.0, 1.5, 1.0, 0.5, 0.25, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5mA. Five trials at each stimulus intensity were given with the stimulus applied to the right side. After a 30 minutes

rest period the same procedure was repeated with the US applied to the left side. Responses on the right side were compared with responses on the left.

Surgery

Each rabbit was anaesthetised using a fentanyl, fluanisone mixture (Hypnorm, Janssen; 0.1/5.0 $mg.kg^{-1}$, i.m.) and a supplement of benzodiazepam (Valium, Roche; 0.5 $mg.kg^{-1}$, i.v.) was used as a relaxant. Benzodiazepam also protected the animal after the injection of KA from possible damage to structures outside the injection site (Ben-Ari *et al.*, 1979). The rabbit was intubated, placed in a Kopf stereotaxic instrument and connected to a halothane evaporator. Naloxone hydrochloride (Narcan, Du Pont; 3-5 $\mu g.kg^{-1}$, i.v.) was used to antagonise the fentanyl and deep anaesthesia was maintained throughout the operation by 3% halothane in a 30% nitrous oxide/oxygen mixture.

After stable anaesthesia had been attained, mannitol (4 $g.kg^{-1}$, i.v.) was infused at a rate of 3 ml per minute. Mannitol was used to reduce brain volume, the effect of which was to expose rostral areas of the cerebellum, lower intracranial pressure and reduce the possibility of cerebral oedema. The scalp was reflected and bone and dura removed to expose the right cerebellar cortex.

Kainic acid was injected through a micropipette into the hemispheral parts of the right cerebellar cortex. Each animal received between two and eighteen stereotaxic placements of KA (Sigma, 1 $mg.ml^{-1}$ kainic acid in sterile 0.9% NaCl, pH 7.2-7.4). The placements were made on a three dimensional grid that extended from one to four millimetres below the cortical surface. At each placement, an injection of KA (25-250 nl) was made over one to three minutes. A total of 500-1800nl (2.3-8.4 nmol) of KA was injected into each animal.

After the injections had been made the scalp was sutured and 30 ml of glucose/saline was slowly infused intravenously and the animal removed from the stereotaxic. Buprenorphine hydrochloride (Temgesic, Reckitt and Colman; 0.025 $mg.kg^{-1}$, i.m.) was given immediately after surgery and twice daily for 2 days post operation. The subjects were allowed to recover for between 4 and 21 days. There was no evidence either during the operation, or subsequent to it, of epileptiform convulsions as have been described following kainic acid injections into limbic regions of the brain (Ben-Ari *et al.*, 1979).

Histology

At the end of the experiment each rabbit was given an injection of heparin sodium (500 $U.kg^{-1}$, i.v.) and a lethal dose of pentobarbitone sodium (90 $mg.kg^{-1}$, i.v.). The rabbit was perfused through the aorta with 0.9% saline followed by 10% formalin and the brain removed from the skull. The brain was embedded in 10% gelatin and frozen sections were cut at 30 µm in the

coronal plane. Alternate sections were mounted onto gelatinised slides and stained with cresyl violet to reveal Nissl substance.

Data analysis

Control, data acquisition and on line analysis of the experiment was performed on a microcomputer using an analogue to digital conversion rate of 100Hz for the delay conditioning and a rate of 1KHz for the UR testing.

Conditioning

Responses with an amplitude greater than or equal to 0.5mm contributed to the measure of CR frequency. A response was scored on paired trials if it was made during the inter-stimulus interval (500ms from CS onset) and on unpaired trials if it was made during a period of 1000ms starting from the CS onset. For both CS types there were 90 paired CS-US presentations and 10 CS alone presentations within each session. Session CR frequencies for each CS type on paired and unpaired trials were expressed as percentages. The peak amplitude of all responses made during the inter-stimulus interval on paired trials and during 1000ms from CS onset in unpaired trials was measured and presented as the average peak CR amplitude for each CS. The peak amplitude of all responses made from US onset during paired trials was measured and presented as the averaged UR amplitude for both CS trial types. In addition, the frequency, peak amplitude and latency to the peak amplitude of all responses equal to or greater than 0.15mm was also measured during unpaired trials.

UR testing

Signal averaging techniques were used to produce an averaged waveform for the ten responses at each stimulus intensity. The homogeneity of responses was estimated in several subjects by comparing the responses at each stimulus level. There was a very slight or no variation in the response topography at each stimulus level. Various response parameters were obtained from the averaged waveform. The NM response onset latency was measured from the stimulus onset to the first reliable inflexion above baseline noise (0.05mm). The amplitude to the first response peak and the latency from stimulus onset to this response peak were determined. From these values the average velocity to the first response peak was calculated. The amplitude of, and latency to, the maximum NM extension were determined.

Data from four subjects with the largest impairment of CRs and from four subjects with the least impairment of CRs, all from group B, together with data from all subjects of the non-lesioned group D, were examined statistically. Data from these subjects were analysed using a 3-way analysis of variance (ANOVA) with repeated measures on 2 factors, using program 2V from the BMDP statistical suite of programs. The factors were behavioural group, side and stimulus level. There were three levels of behaviour: 'most affected', 'least affected' and 'normal'. The factor side had two levels: left and right. The factor stimulus had six levels: 2.5,

An analysis of variance of the UR amplitude revealed that there was unacceptable variation in the data. One source of variance in the subject means probably was due to differences between subjects in the maximum possible extension of the NM. The variation in means may also have been due to the position of the US electrodes. Although the positioning of the electrodes was approximately the same in all subjects, and the intensity of the US was kept constant the position of the electrodes relative to the facial nerve may have differed and hence some variability in the strength of the US. In order to reduce variation the data was transformed by using the square root of the UR amplitude in the ANOVA, a procedure which reduced the higher amplitude responses and reduced the variation in sphericity. The results of the ANOVA on the peak UR amplitudes, both with and without the square root transformation, are given in Table 6. Differences the results of each ANOVA are small and do not affect the overall result.

Where appropriate, the Greenhouse-Geisser correction for non-sphericity of data within the BMDP program was used to calculate probabilities. The 5% level of significance was used in all statistical tests.

2.0, 1.5, 1.0, 0.5, 0.25. The reciprocal of the UR latency to onset was used to obtain the speed of the response. This transformation makes the data less dependent upon the time window used to detect a response. The data is less biased by long latency responses that may not be true URs.

cont

Histological verification of lesions

The transverse sections were examined microscopically for degeneration of cells within the cerebellar cortex, cerebellar nuclei, the inferior olive (IO), lateral reticular nucleus, basilar pontine nuclei and nucleus reticularis tegmenti pontis. Photographs of individual sections were made at 0.5mm intervals throughout the cerebellum. Drawings of the cerebellum were prepared by tracing these photographs, and regions of degeneration were indicated upon them. From each of the drawings the linear extent of PC loss in each lobule was estimated and is presented in Table 5.

RESULTS

Conditioning

Tables 1, 2 and 3 summarise the behavioural results. In these tables the subjects are arranged in a descending order of behavioural deficit. The order was determined by the sum of the frequency of responses to both CSs on sessions six to ten. Table 1 shows the frequency of CRs during paired trials, table 2 shows the frequency of CRs during unpaired (CS alone) trials and Table 3 shows the average amplitude on unpaired trials. In each of these tables the post-operative data is averaged in blocks of five sessions. The last session of acquisition training (session five) is shown for comparison. Six subjects failed to learn to the white noise CS within the five pre-operative training sessions. Two subjects failed to acquire CRs to the light CS on the left side post-operatively. All eight subjects were rejected from this study.

Acquisition

Paired trials

During the preoperative acquisition phase CRs rapidly increased in amplitude and number from session two onwards. All subjects reached an asymptotic level of greater than 80% CRs to the white noise CS on session three or four, but responses to the light CS were more variable, reaching 50-90% CRs on session five. The conditioned response peak amplitude attained a final level of 4-4.5 mm to the white noise and 1.3-4.5 mm to the light.

Unpaired trials

Tables 1 and 2 show that more conditioned responses were detected during unpaired trials, indicating that on paired trials some CRs were masked by the UR. Overall the subjects had attained a high level of CRs to both CSs by the end of acquisition training.

Both paired and unpaired trials show a higher frequency of responses to white noise than to light at the end of the five sessions of acquisition training.

Retention

Paired trials

Group A

Subjects A1 and A2 showed a total loss of responses for the first five sessions of right (lesion) side training. A4 showed a few responses in sessions six and seven and then none for the next three sessions. A3 showed a complete loss of responses for three sessions and an increase in responses from session four. There was no loss of CRs in either subject A6 or A7. All subjects showed rapid learning to both CSs when trained on the left (non-lesioned) side. Subsequent retraining on the right side revealed an immediate increase in CRs in all subjects. Subjects A2-4 subsequently recovered to preoperative levels of responding, although a comparison of the paired and unpaired data shows that the CRs were slightly retarded in latency. CRs in subject A1 were still impaired at the end of training.

Group B

The subjects in this group showed a range of behavioural deficits, from a complete and permanent loss to a slight and transient loss of CRs. All subjects except B10 showed either a total absence of CRs or a considerable reduction in CR frequency on session six, the first session of post-operative training. Subjects B1-B4 showed a complete loss of CRs for at least six sessions of retraining. At the end of retraining the CRs of B1 and B2 remained severely impaired. B3 and B4 showed a gradual recovery of CR frequency and amplitude although a deficit remained in the frequency of CRs to the light CS in B3 at the end of retraining.

In general where CRs recovered, they did so more rapidly to the white noise CS than the light CS. Overall this pattern of recovery is similar to the initial, preoperative acquisition during which CRs were more rapidly acquired to the white noise CS, although the rate of recovery of CRs was, in general, noticeably impaired compared with the rate of acquisition. Also, where CRs had recovered in frequency to preoperative levels, the amplitude often remained smaller for several additional sessions of retraining.

Training on the non-lesioned side produced very rapid acquisition of CRs to both CSs in all Group B subjects. This is illustrated by the data from paired and unpaired trials in subjects B1-3 (see Figures 6-8) - fully formed CRs were evident on the first unpaired trial in session 21.

Group C

Both subjects showed an impairment of CRs on early sessions of retraining, particularly subject C1, which on the first day of retention training had a frequency score of 10% to the light CS and 30% to the white noise CS.

Unpaired trials

The data from unpaired trials indicates that the CRs of several subjects recovered partially or completely by the end of the 25 sessions of post-operative training. Where there was a complete loss of CRs on paired trials there was also a complete loss on unpaired trials. A recovery of CRs was detected earlier in unpaired trails than paired trials indicating that initially the recovering responses were made later.

The NM response latency and amplitude changes during recovery of subjects B1-B4 were analysed in greater detail as described below.

CS related responses smaller than 0.5 mm

The analysis described above was based upon a criterion for a CR of 0.5 mm movement, a level that is generally used for NMR data (Smith, 1968). The KA lesions primarily produced deficits in the amplitude of the CR. In order to determine whether the CR was present but merely depressed in amplitude all responses greater than or equal to 0.15mm (twice the level of noise measured peak to peak) made by subjects B1-B4 were plotted. The percentage of responses that reached this new criterion during the unpaired trials are given in table 4 and Figures 6C, 7C and 8C show the amplitude and latency to the peak of these responses.

This analysis revealed that during acquisition training there were only a few responses with amplitudes less than 0.5mm. Moreover these small responses were only made during initial training sessions. During post-operative training an increase in small responses was seen but these showed a greater variation in latencies than responses made during the acquisition training. Because cerebellar lesions are classically described to produce deficits in timing (Holmes, 1917) it was expected that responses would be shifted to a longer latency. However many of these small responses were made very soon after the CS onset. In subjects B1 and B3, during post-operative sessions several responses peaked within 100 ms of CS onset, especially those made to the white noise CS. These latencies are short enough for the responses to be either alpha responses or sensitised responses - although original studies on the NMR found no evidence of alpha or sensitised responses to a tone CS in normal, non-lesioned animals (Gormezano *et al.,* 1962). Some responses were made with a latency to peak greater than normal, for example in B2 and B3. Smith (1968) has shown that in the intact animal the CR peaks at about the time of presentation of the US. Many of the small responses shown here do not follow this pattern and may be behaviourally 'maladaptive' (McCormick and Thompson, 1984). Without additional controls for alpha responses, sensitisation and pseudoconditioning in animals lesioned with KA it cannot be determined whether these responses are non-associative in nature or whether they are abnormally small, and incorrectly timed, CRs.

Unconditioned response

Unconditioned responses measured during paired trials showed no consistent effect of the KA lesion, but subjects A1 and B3 showed an increased UR during post-operative training. In subject B3, shown in Figure 8B, the UR remained elevated even after CRs to the white noise CS had fully recovered.

UR testing

Welsh and Harvey (1989) suggested that interpositus lesions do not damage learning but simply impair movement. These authors lesioned unilaterally anterior parts of the cerebellar nuclei in previously trained rabbits. After the lesions, CRs were profoundly deficient in both frequency and timing. URs elicited by very low intensity USs were similarly effected. Welsh and Harvey concluded that the cerebellar lesions had caused a loss of excitation acting upon the eyeblink motor system so preventing CRs produced outside the cerebellum from gaining access through the depressed motor system.

The possibility that lesions of the cerebellar cortex produce UR deficits was tested by comparing the URs elicited by different intensities of US in three groups of rabbits. The Most Affected Group consisted of four subjects from Group B which had the largest impairment of CRs. The Least Affected Group consisted of four subjects from Group B which had the smallest impairment of CRs. The Normal Group consisted of three non-lesioned subjects from Group D.

Subjects from Group B were divided into two groups according to the relationship between behavioural deficit and the amount of PC loss in lobule HVI and the Ansiform lobule. A cluster analysis revealed that the subjects fell into two main groups. Subjects B1-B3 were similar to each other and subjects B5-B10 were similar to each other. Subject B4 lay outside both clusters but was more similar to subjects B1-B3 than to subjects B5-B10. The cluster analysis was carried out using program 2M from the BMDP suite of programs. The centroid method of linkage was used in the analysis. Variables were Subject, PC loss, which was pooled across lobules HVIm, HVII, CrusIm, CrusIL, CrusII (Table 1) and CR frequency, which was the total frequency of CRs measured across all postoperative training sessions on the right hand side (Table 5).

Subjects B1-B4 were designated the Most Affected Group. All of these subjects showed a complete absence of CRs to both light and white noise CSs for at least six sessions of training (Table 5). All of this group except B4 showed deficits in the number and amplitude of CRs at the time of UR testing. These subjects had the most damage to lobule HVI and the Ansiform lobule. The Least Affected Group consisted of subjects B7-B10. In two of these subjects the CR was absent for one session but all had recovered to preoperative levels of responding by session twenty. The Normal Group consisted of subjects D1-D3. Group D subjects did not undergo surgery but received the same conditioning as Group B subjects.

If Welsh and Harvey (1989) are correct in their assumption that CRs are impaired because of a motor deficit, then subjects with the largest impairment of CRs (Most Affected Group) should show the greatest UR deficit. The Least Affected Group was included in the comparison in order to rule out the possibility that any differences between the Most Affected Group and the Normal Group could simply be due to surgical trauma.

The responses to the US alone were tested at six different levels ranging from 0.25 mA to 2.5 mA. At different stimulus levels there was a variation in the response topography. All subjects showed a bimodal or polymodal response to stimuli greater than 1.0 mA. The first peak occurred at 50-70 ms and the second at 90-110 ms. The maximum amplitude generally occurred at the second peak. The response elicited by a 1.0 mA stimulus or less in most subjects had only one peak.

A change in US intensity produced significant changes in all aspects of the reflex response in all groups: frequency ($F=38.09$, df 5,40: $P<0.05$), peak amplitude ($F=162.06$, df 5,40: $P<0.05$), onset latency ($F=57.04$, df 5,40: $P<0.05$), latency to peak ($F=19.87$, df 5,40: $P<0.05$), average velocity ($F=97.67$, df 5,40: $P<0.05$), rise time ($F=4.28$, df 5,40: $P<0.05$).

The UR amplitude was significantly greater on the right side than on the left side in all groups ($F=10.94$, df=1,8; $P<0.05$). Separate ANOVAs using factors side and group were calculated at each level of US. These revealed a side difference at 2.0 mA ($F=11.28$, df=1,8; $P<0.05$), 1.5 mA ($F=6.92$, df=1,8; $P<0.05$), and 1.0 mA ($F=12.50$, df=1,8; $P<0.05$). The amplitude difference between the sides is shown in Figure 1 to be a general trend in all groups. This difference probably relates to the larger number of trials given to the right side during behavioural training.

The frequency and timing of the UR was not significantly different between the groups at any US intensity.

The average velocity of the UR was significantly different across the groups at some or all levels as shown by a significant group/level interaction ($F=3.59$, df=5,40; $P<0.05$). Separate ANOVAs with factors group and side were calculated for each level of US. A significant group

difference was detected only at the 1.5 mA level ($F=6.07$, $df=1,8$; $P<0.05$). Although the average velocities were significantly different only at 1.5 mA, inspection of the graphs of average velocity in Figure 1 reveals that responses in the 'normal' group had a higher velocity at 1 mA and above. This may be due to the unusually short onset latency of these responses. Importantly, at the low stimulus levels this trend is reversed and responses of the lesioned subjects showed higher average velocities.

Except for the consistent differences between left and right side UR amplitudes in all subjects there were no significant differences in URs within any group at any stimulus level. Overall, reflex responses elicited by a wide range of stimulus strengths of the 'most impaired', 'least impaired', and 'normal' subjects were not different.

Histological verification of lesions

The KA injection placements were on a one millimetre three dimensional grid. In some cases there was a complete loss of all cortical cells between placements in this grid. In some cases only the Purkinje cell (PC) layer was absent leaving the granule cell layer intact. In some instances there were patches of total loss of PCs with intact regions of cortex between. At the edge of the injections there was a gradual change in the number and type of cells affected. Dependent upon the distance from the injection site, first the granule cells and then the PCs appeared normal (see Figure 2). The finding that there is less damage to granule cells is in agreement with previous reports (eg. Herndon *et al.*, 1980).

Because KA lesions of the deep cerebellar nuclei cause a loss of the conditioned NMR (Lavond *et al.*, 1985) the cerebellar nuclei were examined in all subjects comparing the lesioned and non-lesioned sides. All subjects that showed any sign of degeneration in the cerebellar nuclei were rejected from this study. Four subjects from Group B had some degree of cell loss within the nuclei. In two subjects from Group B the plane of section of the histological material was not optimal for a comparison between the nuclei of each side. It was therefore not clear whether loss of cells within the nuclei had occurred in these subjects. All six subjects were excluded from this study. Three subjects from group A were found to have damage, from haemorrhage caused during the surgery, to the most rostral dorsal parts of HVI and the paramedian lobule. These subjects were also rejected from this study.

The remaining 19 subjects showed no signs of degeneration within the cerebellar nuclei. Figure 3A shows a section through the right cerebellar nuclei of subject B1, at a rostral level essential for normal CRs. All of the cells appear normal. As a comparison, Figure 3B shows the nuclei of a subject in which KA was injected directly into the cerebellar nuclei. There are clear signs of degeneration and gliosis within the anterior interpositus nucleus/medial dentate nucleus in which neurons are absent.

There was no sign of cell loss or gliosis in the inferior olive (IO), basilar pontine nuclei, nucleus reticularis tegmenti pontis or lateral reticular nuclei. Figure 4A illustrates the left IO, contralateral to the KA injections of subject B1. There was no evidence of cell shrinkage such as is found in the dorsal cap of the IO following KA injections into the flocculus (Ito *et al.*, 1980). For comparison Figure 4B shows the left IO of a subject from an earlier study in which an aspiration lesion of the right cerebellar cortex was made. The lesion was restricted to anterior parts of HVI and adjacent hemispheral cortex. There was cell loss and gliosis within medial, anterior parts of the dorsal accessory olive, medial accessory olive and principal olive.

In all cases the KA injections caused more damage to PCs than to granule cells, and so destroyed output from the cerebellar cortex. The amount of damage to the PC layer in each subject was therefore plotted and the total loss of PCs in each lobule was estimated and presented in Table 5. In this table the subjects are arranged in a descending order according to the severity of behavioural deficit.

Subjects with the largest lesions of HVI and Ansiform lobule showed the greater behavioural deficit eg. subjects B1-B6 in table 5. In subjects B1-B3 shown in Figures 6A, 7A and 8A, the ventral half of HVI and the adjacent folium of crus I was completely devoid of PCs. Only in these subjects was there also considerable loss of PCs in the lateral folium of crus I and in crus II. Subjects with sparing of PCs in the ventral half of the lateral folium of HVI and the adjacent folium of crus I in general showed only a slight and transient loss of CRs eg. A4, B5, B8, B10. The notable exception to this was subject B7 in which all but the dorsal part of the medial folium of HVI, the whole of the lateral folium of HVI and most of the medial folium of crus I were devoid of PCs.

Subjects with damage to comparable amounts of the PC layer within HVI showed a range of CR deficit eg. B4 and B9. Subjects B9 and B10 showed a transient CR deficit and both had extensive loss of PCs in HVI, but in these subjects patches of Purkinje cells and granule cells within HVI were spared.

Subject C1 had extensive loss of PCs within crus I and crus II and subject C2 had extensive damage to crusI. The amount of damage to the Ansiform lobule in these subjects was greater than that of all other subjects except B1. Neither of these subjects showed a large or enduring loss of CRs.

In all subjects there was some damage in HIV/V and the paramedian lobule. These lobules were extensively damaged in subjects A1-A3 but lesions of these lobules in group B subjects did not reveal a consistent behavioural deficit. For example lobule HIV/V showed extensive loss of PCs in subjects A2, A3, B5, B6 and B10, and paramedian lobule in subjects A1, A2, B1, B5 and C2. In subjects A6 and A7 there was only a slight loss of PCs in rostral, dorsal

paramedian lobule, neither of these subjects showed any loss of CRs. Subjects A1-A4 and B4-B8 showed a slight loss of PCs within the right half of vermian lobules II-VI. In subjects A2 and B6 this loss was extensive. There was no loss of PC within the vermis in subjects B1-B3.

DISCUSSION

Unilateral, kainic acid lesions of the cerebellar cortex produced an initial total loss of conditioned NM responses established to light and white noise stimuli. Unconditioned responses to periorbital stimuli appeared normal. Lesions produced by KA were restricted to cells of the cerebellar cortex and did not damage the precerebellar nuclei or efferent cortical targets in the cerebellar nuclei. The behavioural deficits were therefore due to the loss of cerebellar cortical neurons. Extended retraining allowed some recovery of CRs, though only to very low levels in those cases with the largest lesions of lobule HVI and Ansiform lobule. The degree of recovery was related to the location and extent of the cortical lesion.

HVI and Ansiform lobule are essential for normal conditioned responses

Purkinje cells are particularly susceptible to damage by kainic acid although granule cells are also damaged at higher KA concentrations. KA injections spare afferent fibres (Coyle *et al.*, 1978) including climbing fibres (CF) in the cerebellar cortex (Rossi *et al.*, 1991). Aspiration lesions of the cortex produce retrograde degeneration in the inferior olive (Yeo *et al.*, 1985b). In the present study KA lesions produced no retrograde degeneration in the inferior olive or other pre-cerebellar nuclei. Because CFs branch to different cortical areas (Armstrong *et al.*, 1973; Hrycyshyn *et al.*, 1989) aspiration lesions deprive areas outside the primary lesion of CF inputs and so for similar areas of primary damage KA lesions are functionally smaller than aspiration lesions.

In an earlier study, aspiration lesions that included all of HVI caused a profound loss of CRs throughout all five post-operative training sessions (Yeo *et al.*, 1985b). In the present study there were some surviving Purkinje cells in HVI in all subjects, but in all cases in which Purkinje cells in HVI were lost there were deficits in the frequency and amplitude of CRs. The magnitude and duration of the deficit depended upon the amount of damage to HVI and crus I and crus II of the Ansiform lobule. KA lesions of the cerebellar cortex that spared HVI had either no effect, or a slight and transitory effect upon CRs. Six subjects with lesions of HVI lost CRs for five or more sessions of retraining. Subjects with more extensive damage to Ansiform lobule as well as HVI lost CRs for longer than five sessions. It is therefore concluded that the loci essential for normal conditioned NM responses are within lobule HVI but that adjacent regions of the Ansiform lobule can sustain recovery of conditioning. Given that KA lesions of the cerebellar cortex are functionally less extensive than aspiration lesions of similar primary extents these findings are in close agreement with a recent aspiration lesion study (Yeo and Hardiman, 1992).

Cerebellar cortical neurones are essential for normal conditioned responses

CFs and some mossy fibre afferents to the cortex branch to provide an input to the cerebellar nuclei (Qvist, 1989). Because aspiration lesions of the cortex produce retrograde degeneration in the inferior olive and other precerebellar nuclei (Brodal, 1939; Brodal and Janssen, 1946), the loss of CRs following such lesions might relate to a loss of collaterals to the cerebellar nuclei, and not to the loss of an essential input from the cerebellar cortex. If the association critical for conditioning of the NMR is within the cerebellar nuclei, as suggested by McCormick and Thompson (1984), and is independent of cortical mechanisms, then the deep nuclei alone should sustain conditioned responses. The present study demonstrates that, even with their collateral connections intact, the cerebellar nuclei alone are not sufficient to maintain conditioned responses. Deficits in CRs must be due to a loss of input to the cerebellar nuclei from the cortex.

Does training of the intact side aid a recovery of CRs ?

CRs in subjects A1-A3 were severely reduced or absent during the first 5 post operative sessions (sessions 6-10). However, CRs recovered rapidly immediately after training the non-lesioned side (sessions 16-20). Subjects B3 and B4 similarly showed a profound loss of CRs during the first 5 post-op sessions (sessions 6-10) and a recovery on sessions 16-20. In these subjects there was considerable recovery of CRs during sessions 11-15 but not to the same level as seen in sessions 16-20 of subjects A1-3. It is therefore likely that sessions 11-15 were beneficial to the recovery of CRs in subjects A1-3. But training of the non-lesioned side did not enhance CRs produced on the lesion side in subjects B1-3. The rapid acquisition of CRs on the non-lesioned side suggests that CRs are produced bilaterally even though the US is applied unilaterally. The immediate appearance of CRs in group A subjects after training the non-lesioned side (session 16) is likely due to the continued training on the lesioned side through weak collateral input of the US during training of the non-lesioned side (sessions 11-15). However, the data from group B subjects does not allow one to determine whether training, or simply time, aided the recovery of CRs in the group A subjects.

Do KA lesions of the cerebellar cortex produce motor deficits ?

In the present study, KA lesions of the cerebellar cortex produced dissociated effects upon CRs and URs. CR amplitudes were severely impaired but UR amplitudes were not changed by the lesion. Aspiration lesions of HVI and Ansiform lobule (Yeo and Hardiman, 1992) depress the amplitude and frequency of CRs but enhance the amplitude of URs across a range of US intensities. Neither of these studies provides evidence for a motor deficit common to the CR and UR sufficient to abolish CRs. In both studies, loss of the cerebellar cortex exclusively impairs CR amplitude. These findings are consistent with the suggestion that the cerebellar cortex is essential for learned components of the CR.

Table of Subject History

Group	Subject	Acquisition R	KA lesion R	Retention R	Retention L	Retention R	US test	Rejected
A	1-5	5 sessions	HVI-crusII	5 sessions	5 sessions	5 sessions	-	-
	6-7	"	"	"	-	-	-	-
	8-10	"	"	"	"	"	-	✓
B	1-3	"	"	15 sessions	"	"	✓	-
	4-6	"	"	"	"	"	-	-
	7-10	"	"	"	"	"	✓	-
	11-18	"	"	"	"	"	-	✓
C	1-2	"	crusI-crusII	"	"	"	-	-
D	1-3	"	-	"	"	"	✓	-
	34-39	"	-	-	-	-	-	✓

Subjects A8-A10 were rejected because of damage to lobule HVI due to surgery.
Subjects B11-B16 were rejected because of KA damage to the cerebellar nuclei.
Subjects B17-B18 were rejected because they failed to learn to white noise CS when trained on left side, even though they learned well to light CS.
Subjects 34-39 were rejected because they failed to acquire CRs to the white noise CS even though they acquired well to the light CS.

Table 1

The number of responses that reached an amplitude of at least 0.5mm, expressed as a percentage of the number of paired trials within a block of 5 sessions. Session 5 is the final preoperative session and is shown for comparison. LI = light. WN = white noise.

Paired Trials

CR Frequency

Grp	Session	5		6-10		11-15		16-20		21-25		26-30		# Sessions < 10% CRs
	Side	R		R		L		R						
	Stimulus	LI	WN	LI	WN	LI	WN	LI	WN	LI	WN	LI	WN	
	Subject													
A	1	78	98	1	1	78	89	20	42					5
	2	77	96	1	1	78	90	59	70					5
	3	78	98	1	10	26	70	50	64					4
	4	90	93	16	6	58	78	87	93					3
	5	82	97	31	48	28	53	36	86					1
	6	80	100	82	94									0
	7	71	97	87	95									0
	Side	R		R		R		R		L		R		
B	1	50	96	1	0	0	1	0	1	95	99	0	7	25
	2	77	96	0	1	0	3	0	2	89	99	1	4	25
	3	86	98	0	2	1	19	11	72	80	94	25	74	7
	4	73	96	0	1	7	78	31	99	83	99	90	98	6
	5	74	96	10	27	68	92	93	96	80	81	98	99	3
	6	92	99	24	18	86	87	93	97	62	64	96	98	2
	7	63	90	44	50	78	93	81	95	91	95	78	92	1
	8	82	86	32	60	71	97	78	98	76	93	80	94	1
	9	60	89	33	82	60	93	81	92	37	69	80	95	0
	10	90	90	85	96	89	98	98	98	84	85	96	100	0
C	1	52	91	51	71	78	88	81	86	52	70	16	19	0
	2	82	90	69	76	78	96	93	96	60	71	87	98	0

Table 2

The number of reponses that reached an amplitude of at least 0.5mm, expressed as a percentage of the number of unpaired (CS alone) trials within a block of 5 sessions. Session 5 is the final preoperative session and is shown for comparison.

Unpaired Trials CR Frequency

	Session	5		6-10		11-15		16-20		21-25		26-30	
	Side	R		R		L		R					
	Stimulus	LI	WN	LI	WN	LI	WN	LI	WN	LI	WN	LI	WN
Grp	Subject												
A	1	90	100	0	0	94	90	64	64				
	2	60	100	0	4	90	94	84	82				
	3	90	100	8	18	44	80	78	84				
	4	80	100	14	8	70	80	92	92				
	5	90	100	46	50	38	50	66	92				
	6	70	100	94	100								
	7	70	100	90	100								
	Side	R		R		R		R		L		R	
B	1	90	100	0	0	0	2	2	0	100	100	2	8
	2	100	100	0	2	0	12	2	4	96	100	4	36
	3	100	100	2	0	10	40	50	88	92	92	50	96
	4	80	100	0	0	22	84	74	98	86	100	98	100
	5	80	100	22	36	86	94	94	100	96	88	100	100
	6	90	90	32	32	92	86	98	94	76	72	100	98
	7	90	80	62	60	96	94	98	100	100	100	100	100
	8	90	100	38	64	76	98	82	100	90	98	88	98
	9	60	100	54	94	76	98	88	98	46	72	88	100
	10	90	100	98	96	98	100	100	100	94	92	98	100
C	1	100	100	66	82	82	84	78	88	46	70	90	98
	2	90	90	96	92	96	100	98	98	70	84	96	100

Table 3

The amplitude in millimetres of all responses made during 1000 ms from CS onset in unpaired trials. Session 5 is the final preoperative session and is shown for comparison. All other columns show response amplitudes averaged across 5 sessions.

Unpaired Trials NM Peak Amplitude

Grp	Session	5		6-10		11-15		16-20		21-25		26-30	
	Side	R		R		L		R					
	Stimulus	LI	WN	LI	WN	LI	WN	LI	WN	LI	WN	li	wn
	Subject												
A	1	7.1	6.8	0.0	0.0	9.6	9.1	2.9	3.1				
	2	2.6	4.4	0.0	0.0	3.7	3.8	2.3	2.3				
	3	3.8	6.1	0.2	0.4	2.7	5.6	3.7	4.4				
	4	3.9	5.4	0.4	0.2	3.8	4.5	4.1	4.4				
	5	3.9	4.2	2.2	2.3	1.6	2.0	2.0	3.4				
	6	2.5	4.0	4.1	4.2								
	7	2.9	4.3	4.5	5.1								
	Side	R		R		R		R		L		R	
B	1	1.1	5.5	0.0	0.0	0.0	0.0	0.0	0.0	4.2	4.8	0.0	0.2
	2	5.3	5.1	0.0	0.0	0.0	0.2	0.0	0.1	5.6	6.3	0.1	1.3
	3	4.5	4.1	0.0	0.0	0.1	0.5	0.6	1.7	4.3	3.9	2.8	5.0
	4	5.0	6.3	0.0	0.0	0.6	3.8	2.6	4.9	5.2	6.7	5.7	6.1
	5	3.4	4.3	0.3	0.5	2.4	2.9	3.4	3.7	3.7	3.5	5.2	5.4
	6	4.4	4.3	0.5	0.6	3.8	3.9	5.2	5.1	3.5	3.0	5.2	5.7
	7	4.3	3.8	1.1	1.1	2.2	2.3	2.8	2.9	5.3	5.3	4.0	4.0
	8	4.2	5.4	2.0	3.4	4.0	5.1	4.6	5.8	5.4	5.7	5.6	6.6
	9	2.4	4.3	1.4	3.1	2.6	3.7	3.8	4.5	1.1	1.5	4.5	5.3
	10	5.5	5.7	4.7	4.6	4.8	4.7	6.3	6.2	4.8	4.8	4.7	4.8
C	1	2.3	2.6	3.7	4.4	4.9	4.9	3.7	4.0	1.7	2.6	5.2	5.6
	2	3.2	3.3	5.1	4.9	4.8	4.7	5.4	5.7	3.1	3.4	4.9	5.6

Table 4

The number of reponses that reached an amplitude of at least 0.15mm, expressed as a percentage of the number of unpaired trials within a block of 5 sessions. Session 5 is the final preoperative session and is shown for comparison.

Unpaired Trials Frequency of responses >= 0.15mm

	Session	5		6/10		11/15		16/20		21/25		26/30	
	Side	R		R		R		R		L		R	
	Stimulus	LI	WN	LI	WN	LI	WN	LI	WN	LI	WN	LI	WN
Grp	Subject												
B	1	100	100	0	6	8	6	6	34	100	100	8	20
	2	100	100	2	14	4	20	4	10	96	98	10	50
	3	100	100	2	44	12	94	70	94	94	94	54	98
	4	80	100	2	20	28	94	76	98	88	100	96	100

Table 5

The extent of Purkinje cell loss following kainic acid injections in the right cerebellar cortex. The subjects are tabulated in order of the severity of their behavioural deficit. Each cross indicates approximately 10% loss of Purkinje cells.

HVI m - medial folium of hemispheral lobule VI; HVI l - lateral folium of hemispheral lobule VI; Crusl m - medial folium of crus I; Crusl l - lateral folium of crusl.

Grp	Ctx Div / Subject	II-VI	HIV/V	HVI m	HVI l	Crus I m	Crus I l	Crus II	PM
A	1	+	+++++	+++++++	++++++++++	++++++++	++++++++	+++++	+++++++++
	2	++++	+++++++++	++++++++++	++++++	++++	.	.	++++++++
	3	+	++++++++++	+++++++++	+++++++++	++++++++++	+++	.	+++++++
	4	+	+	+++++	++++++	+++	+	.	+++++++
	5	.	.	+++	+	.	.	.	+++
	6	.	.	.	.	.	.	.	++
	7	.	.	.	.	.	.	.	+
B	1	.	++++	+++++	+++++++++	++++++++++	++++++++++	++++++++++	++++++++
	2	.	+++	+++++++++	+++++++++	+++++++++	+++++++	++	++++
	3	.	++++	+++++++	++++++++	++++++++++	+++	+	+++++
	4	+	++	+++++++	+++++++	++++++++	.	.	+++
	5	+	++++++++++	+++++++	+++++	++++	+	.	++++++++
	6	+++	++++++++++	+++++++++	++++++	+++	.	.	+++
	7	+	++++	++++++++	++++++++++	+++++++++	+	.	+++++++
	8	+	+++++	++++++++++	++++	+++++	.	.	.
	9	.	+++++	++++++++	++++++	++++++++	.	.	++++
	10	+	++++++++++	+++++++	+++++	++++++	+	.	+++++
C	1	.	.	.	.	.	+++++++	++++++++	.
	2	.	.	.	.	+	+++++++++	++++	++++++++

Table 6

ANOVA: UR peak amplitude. No transformation

SOURCE	SUM OF SQUARES	D.F.	MEAN SQUARE	F	TAIL PROB.	GREENHOUSE GEISSER PROB.	HUYNH FELDT PROB.
MEAN	3164.185	1	3164.185	173.95	0		
GROUP	39.65562	2	19.82781	1.09	0.3814		
1 ERROR	145.5238	8	18.19047				
SIDE	39.897	1	39.897	7.84	0.0232		
SG	5.11809	2	2.55905	0.5	0.6227		
2 ERROR	40.70719	8	5.0884				
LEVEL	1627.682	5	325.5364	152.28	0	0	0
LG	65.19194	10	6.51919	3.05	0.0058	0.028	0.0058
5 ERROR	85.51239	40	2.13781				
SL	11.41551	5	2.2831	2.71	0.0338	0.0824	0.0399
SLG	10.40734	10	1.04073	1.23	0.3004	0.331	0.306
6 ERROR	33.75766	40	0.84394				

ANOVA: UR peak amplitude. Square root transformation

SOURCE	SUM OF SQUARES	D.F.	MEAN SQUARE	F	TAIL PROB.	GREENHOUSE GEISSER PROB.	HUYNH FELDT PROB.
MEAN	319.3997	1	319.3997	288.38	0		
GROUP	0.84718	2	0.42359	0.38	0.694		
1 ERROR	8.86062	8	1.10758				
SIDE	1.73689	1	1.73689	10.94	0.0107		
SG	0.01551	2	0.00775	0.05	0.9526		
2 ERROR	1.27043	8	0.1588				
LEVEL	108.0559	5	21.61118	162.06	0	0	0
LG	2.77765	10	0.27776	2.08	0.0494	0.099	0.0494
3 ERROR	5.33408	40	0.13335				
SL	0.18864	5	0.03773	1	0.4292	0.4074	0.4292
SLG	0.62556	10	0.06256	1.66	0.1246	0.1773	0.1246
4 ERROR	1.50647	40	0.03766				

Figure 1

A: 'Most affected' Group. B: 'Least affected' Group. C: 'Normal' Group.

Amplitude, average velocity and onset latency of NM responses to periocular stimuli of 2.5mA to 0.25mA intensities. Responses made by the right NM are hatched, responses made by the left NM are solid. Bars indicate standard error of the mean.

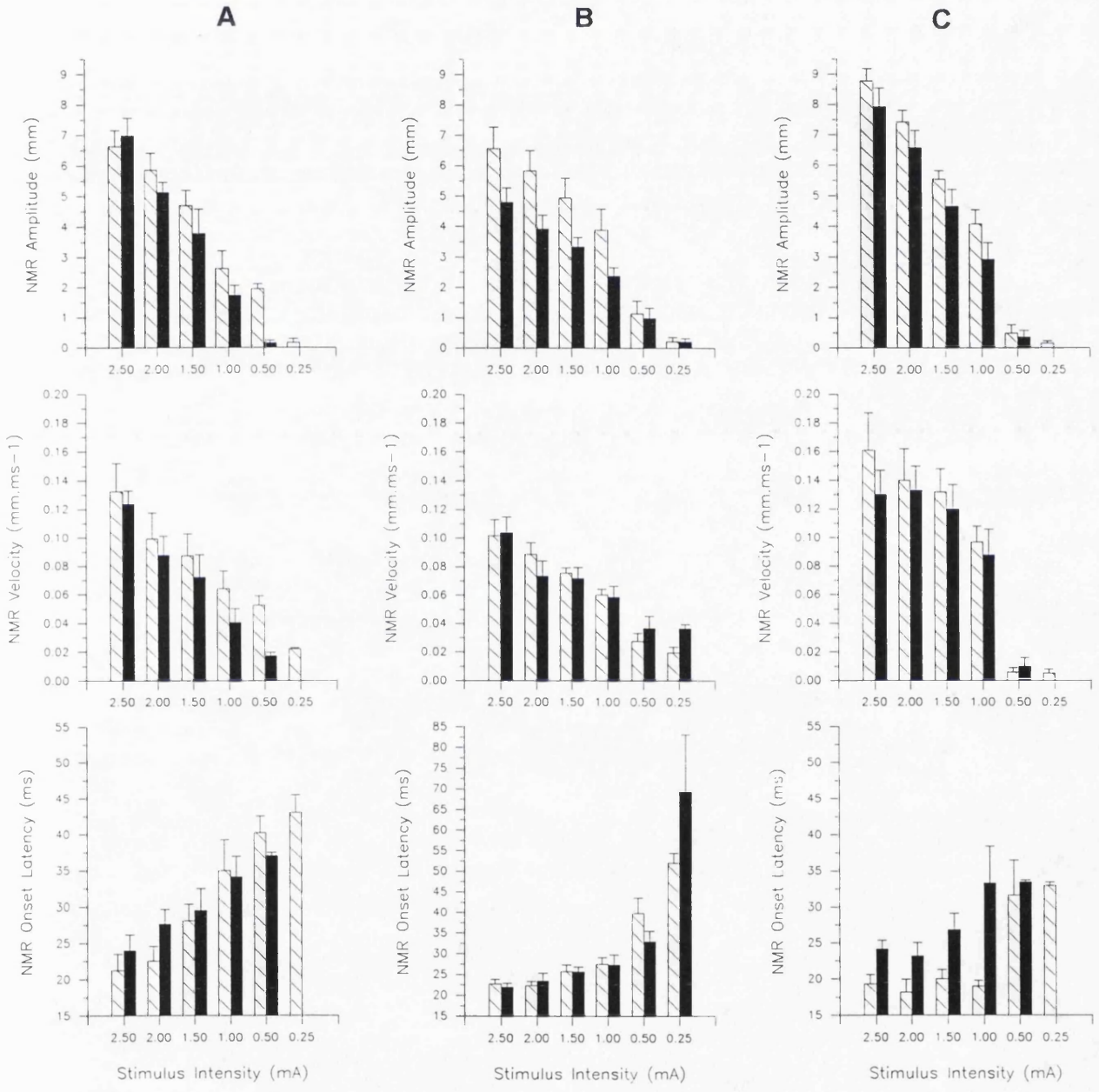
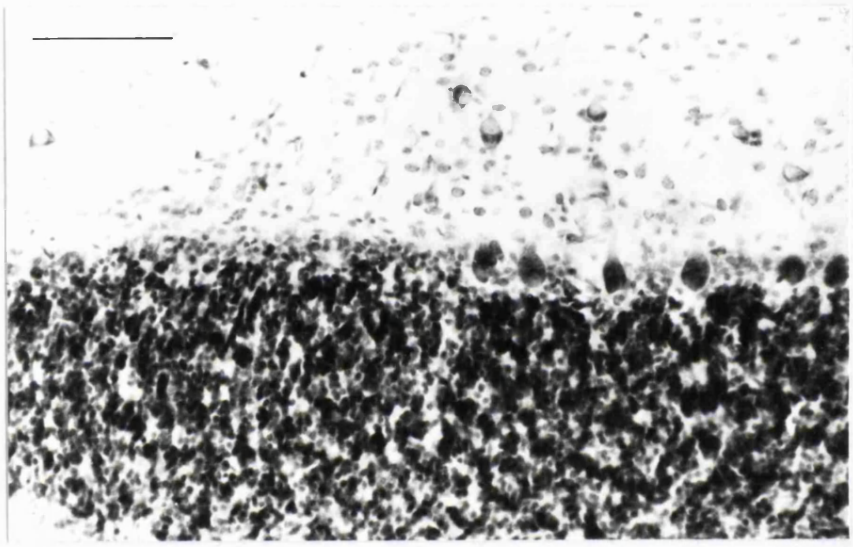
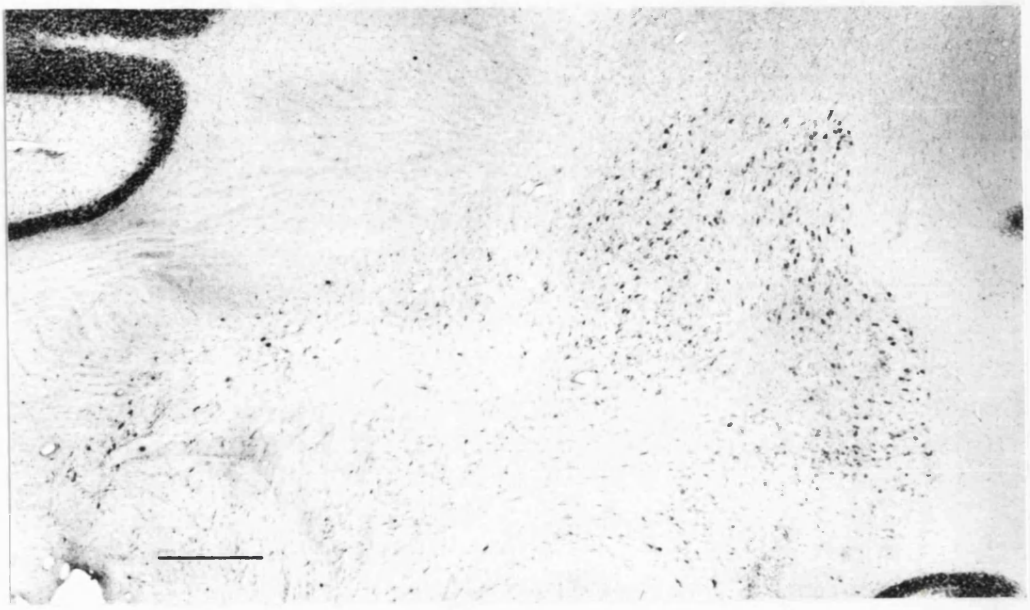

A
B
C
NMR Amplitude (mm)
NMR Velocity (mm.ms-1)
NMR Onset Latency (ms)
2.50 2.00 1.50 1.00 0.50 0.25
Stimulus Intensity (mA)

Figure 2

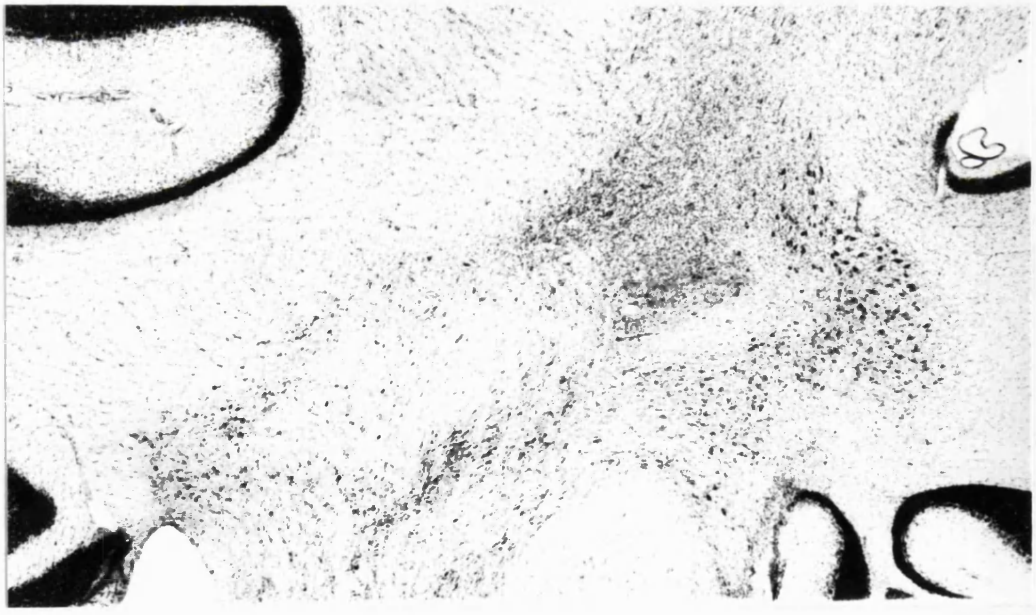


Purkinje cells within the right HVI of subject B1. Changes in concentration of kainic acid have different effects upon the intrinsic cerebellar neurons. At the far left, nearest an injection site, there is a complete loss of PCs, next to this region six normal PCs can be seen. The nucleus is at the bottom of the soma, the normal position in rabbits. In contrast, in cats and monkeys the nucleus is centred within the cell body. Bar = 0.1mm

Figure 3

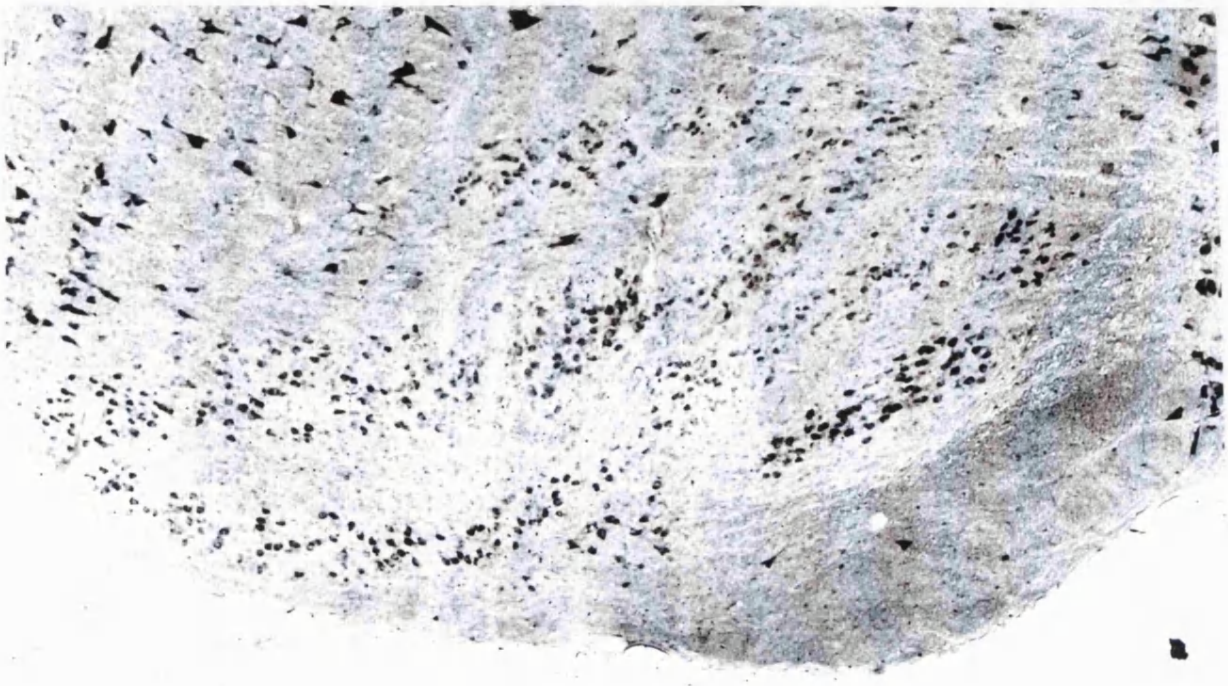


A: Transverse section through the right cerebellar nuclei 3 mm caudal to lambda of subject B1. This subject showed a large loss of Purkinje cells within lobule HIV/V, lobule HVI, ansiform lobe and paramedian lobe. There are no signs of degeneration.



B: Transverse section through the right cerebellar nuclei at the level of lambda in a subject that had an injection of kainic acid directly into the cerebellar nuclei and the overlying cerebellar cortex. There is gliosis and a clear loss of neurons within the crucial region of the anterior interpositus/dentate nuclei.
Bar = 0.5mm.

Figure 4



A: Transverse section through the left inferior olive at Brodal's level XII in subject B1. This subject showed a large loss of Purkinje cells within lobule HIV/V, lobule HVI, ansiform lobe and paramedian lobe. All cells within the inferior olive appear normal.

B: Transverse section through the left inferior olive at Brodal's level XII of a subject that had an aspiration lesion of right lobule HVI, ansiform lobe and paramedian lobe. There is a loss of cells by retrograde degeneration at the junction of the dorsal accessory olive and dorsal principal olive and within the medial part of the ventral principal olive and medial accessory olive.
Bar = 0.5mm

Figure 5

Key to serial sections of cerebellar cortex.

crI and crII	- crus I and crus II of the ansiform lobe
DPFL	- dorsal paraflocculus
FL	- flocculus
HIV/V	- hemispheral lobule IV/V
HVI	- hemispheral lobule VI
ND	- dentate nucleus
NI	- interpositus nucleus
PM	- paramedian lobe
VPFL	- ventral paraflocculus
I-VI	- lobules I-VI of the vermis

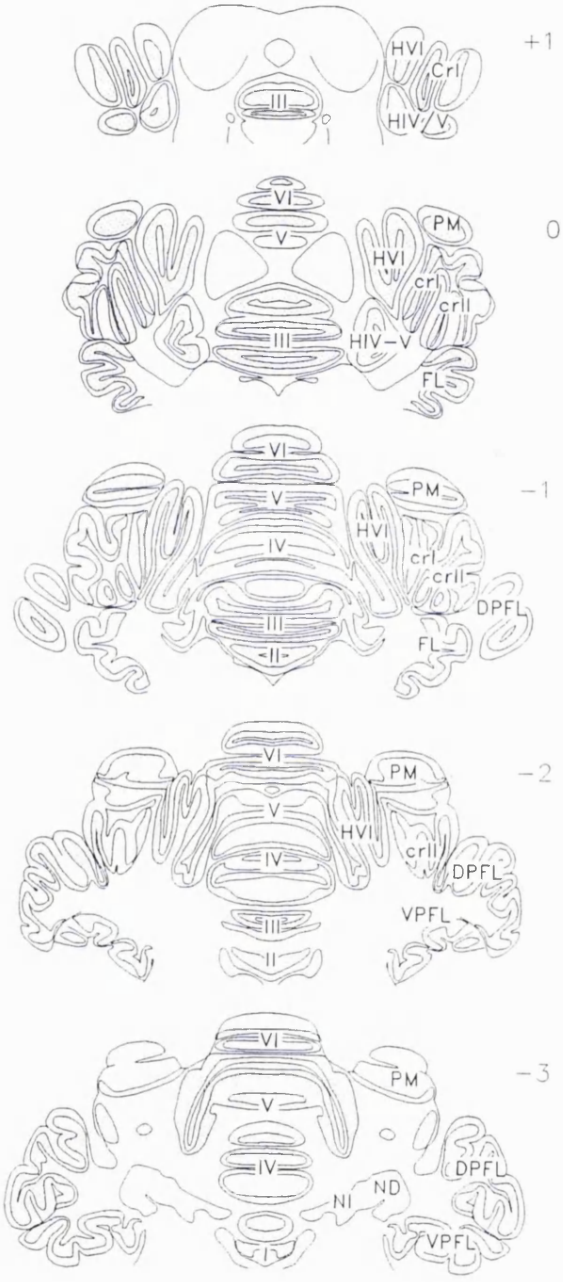

+1
HVI
CrI
III
HIV/V
0
VI
V
PM
HVI
crI
crII
III
HIV–V
FL
–1
VI
V
PM
HVI
IV
crI
crII
DPFL
III
FL
II
–2
VI
PM
V
HVI
IV
crII
DPFL
VPFL
III
II
–3
VI
PM
V
IV
DPFL
NI
ND
VPFL

Figure 6

A: Transverse, serial sections at intervals of 1mm through the right cerebellum of subject B1 showing areas of degeneration following injections of kainic acid. Regions of Purkinge cell degeneration are indicated by a wider line and regions of degeneration of the granule cell layer are indicated by the absence of hatching.

B: NMR peak amplitude and frequency on paired presentations of the CS and US. Open squares - responses measured during the inter-stimulus interval to the light CS. Open triangles - responses measured during the inter-stimulus interval to the white noise CS. Filled symbols - responses measured from onset of the US.

C: Amplitude and latency to the peak response of all NM responses larger than 0.15mm made during unpaired presentations of the US. Open squares - responses to the light CS. Open triangles - responses to the white noise CS.

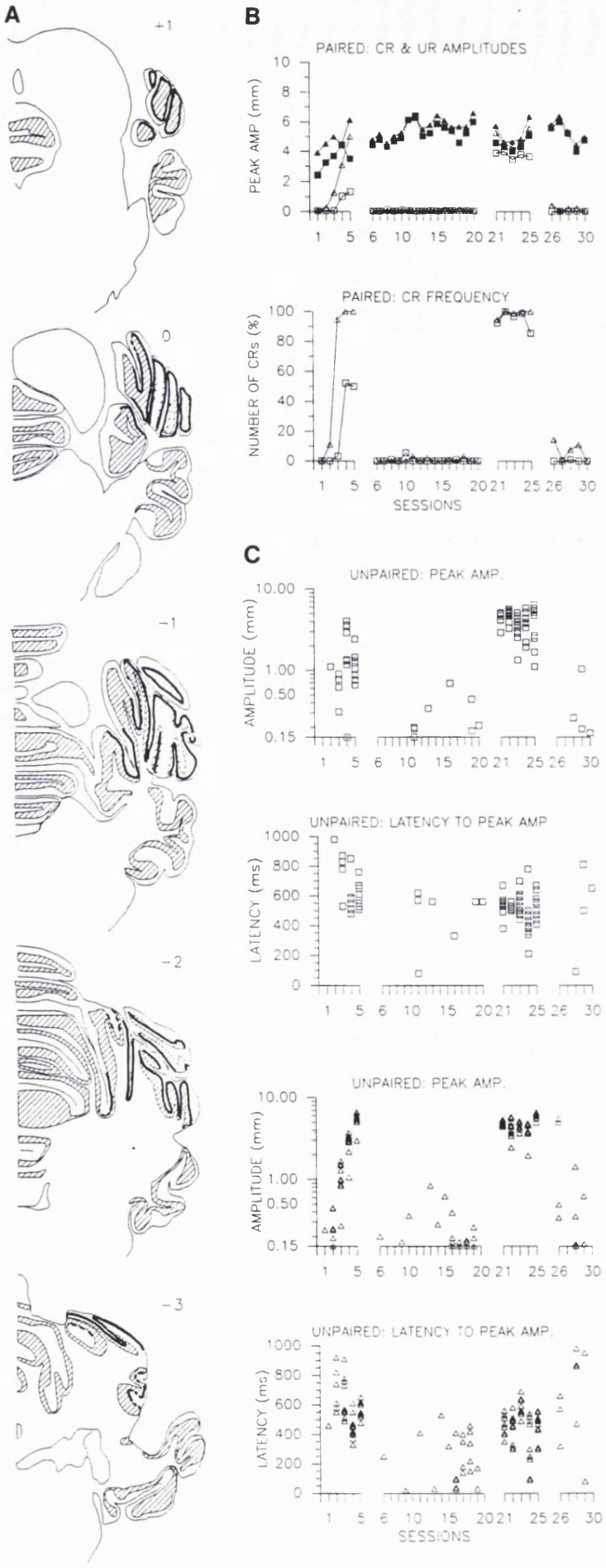

A
+1
0
-1
-2
-3
B
PAIRED: CR & UR AMPLITUDES
PEAK AMP (mm)
PAIRED: CR FREQUENCY
NUMBER OF CRs (%)
SESSIONS
C
UNPAIRED: PEAK AMP.
AMPLITUDE (mm)
UNPAIRED: LATENCY TO PEAK AMP
LATENCY (ms)
UNPAIRED: PEAK AMP.
AMPLITUDE (mm)
UNPAIRED: LATENCY TO PEAK AMP.
LATENCY (ms)
SESSIONS

Figure 7

A: Transverse, serial sections at intervals of 1mm through the right cerebellum of subject B2 showing areas of degeneration following injections of kainic acid. Regions of Purkinge cell degeneration are indicated by a wider line and regions of degeneration of the granule cell layer are indicated by the absence of hatching.

B: NMR peak amplitude and frequency on paired presentations of the CS and US. Open squares - responses measured during the inter-stimulus interval to the light CS. Open triangles - responses measured during the inter-stimulus interval to the white noise CS. Filled symbols - responses measured from onset of the US.

C: Amplitude and latency to the peak response of all NM responses larger than 0.15mm made during unpaired presentations of the US. Open squares - responses to the light CS. Open triangles - responses to the white noise CS.

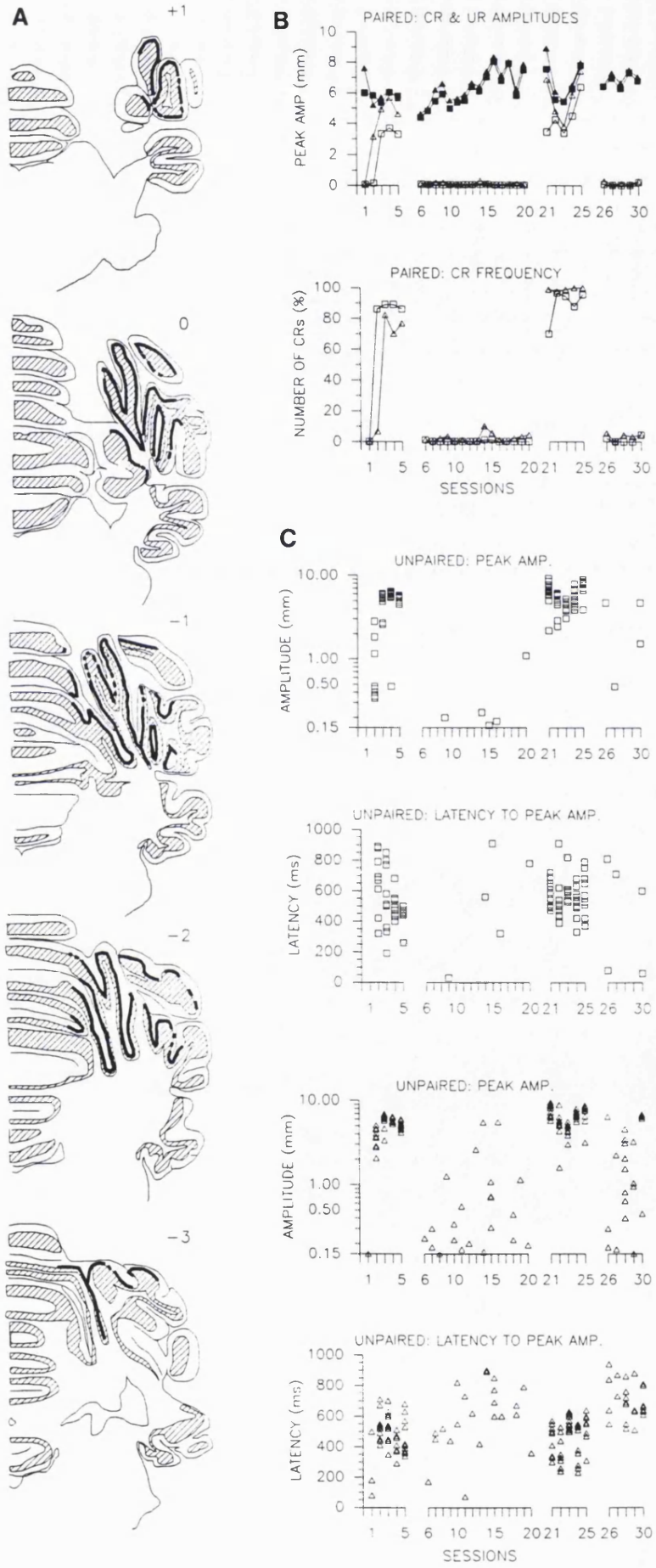
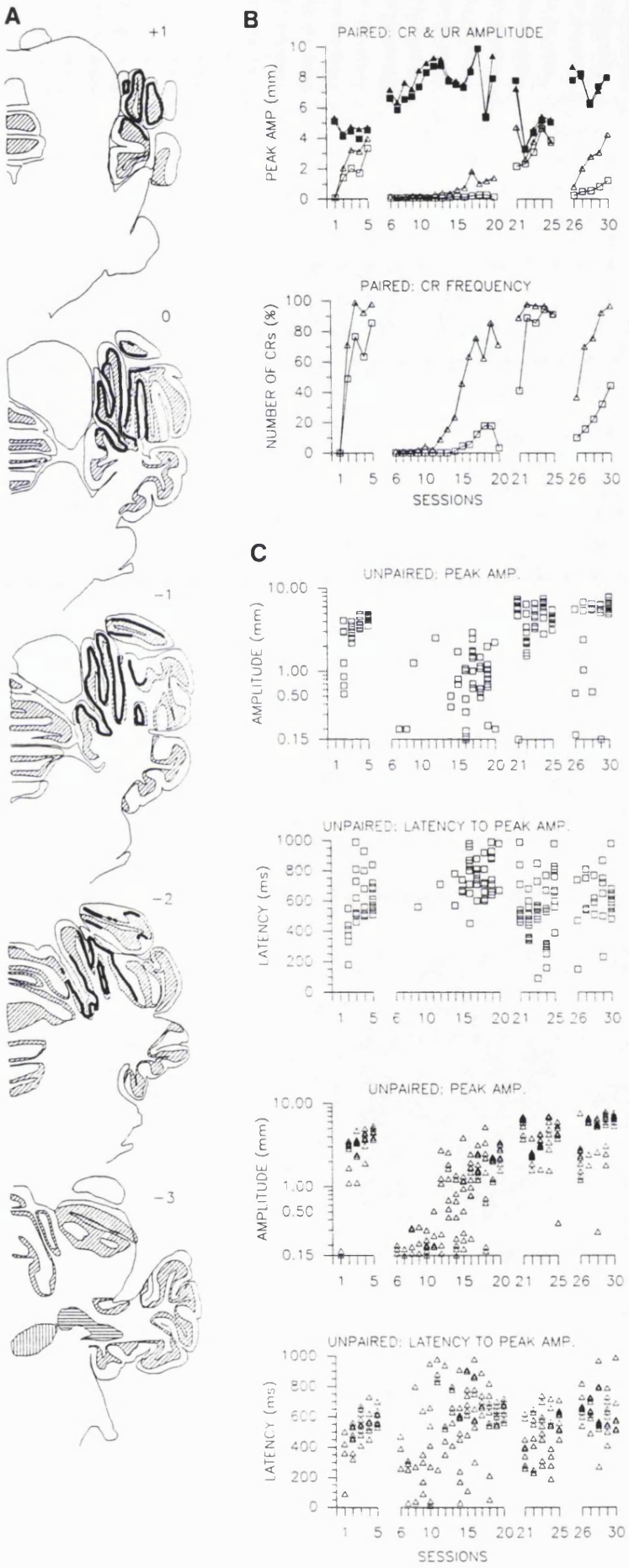

A
+1
0
−1
−2
−3
B
PAIRED: CR & UR AMPLITUDES
PEAK AMP (mm)
PAIRED: CR FREQUENCY
NUMBER OF CRs (%)
SESSIONS
C
UNPAIRED: PEAK AMP.
AMPLITUDE (mm)
UNPAIRED: LATENCY TO PEAK AMP.
LATENCY (ms)
UNPAIRED: PEAK AMP.
AMPLITUDE (mm)
UNPAIRED: LATENCY TO PEAK AMP.
LATENCY (ms)
SESSIONS

Figure 8

A: Transverse, serial sections at intervals of 1mm through the right cerebellum of subject B3 showing areas of degeneration following injections of kainic acid. Regions of Purkinge cell degeneration are indicated by a wider line and regions of degeneration of the granule cell layer are indicated by the absence of hatching.

B: NMR peak amplitude and frequency on paired presentations of the CS and US. Open squares - responses measured during the inter-stimulus interval to the light CS. Open triangles - responses measured during the inter-stimulus interval to the white noise CS. Filled symbols - responses measured from onset of the US.

C: Amplitude and latency to the peak response of all NM responses larger than 0.15mm made during unpaired presentations of the US. Open squares - responses to the light CS. Open triangles - responses to the white noise CS.


A
+1
0
-1
-2
-3
B
PAIRED: CR & UR AMPLITUDE
PEAK AMP (mm)
PAIRED: CR FREQUENCY
NUMBER OF CRs (%)
SESSIONS
C
UNPAIRED: PEAK AMP.
AMPLITUDE (mm)
UNPAIRED: LATENCY TO PEAK AMP.
LATENCY (ms)
UNPAIRED: PEAK AMP.
AMPLITUDE (mm)
UNPAIRED: LATENCY TO PEAK AMP.
LATENCY (ms)
SESSIONS

Efferent and afferent connections of crusI and crusII of the rabbit cerebellar cortex determined by WGA-HRP tracing, and their relevance to eyeblink conditioning.

INTRODUCTION

The cerebellar cortex is essential for normal acquisition (Lavond and Steinmetz, 1989) and retention (Yeo at al., 1984,1985b; Yeo and Hardiman, 1992; Hardiman and Yeo, 1992; Gruart and Yeo, 1995) of conditioned eyeblink responses (CRs) in the rabbit. Lesions centred on lobule HVI cause an immediate, profound and long lasting loss of previously acquired CRs (Yeo at al., 1984,1985b). Lesions restricted to HVI cause a profound loss of CRs but these recover with extensive retraining (Hardiman and Yeo, 1992; Yeo and Hardiman, 1992). Lesions that remove all of HVI and the Ansiform lobule cause a profound loss of CRs and even after extensive retraining there remains a profound deficit in CR amplitude (Hardiman and Yeo, 1992; Yeo and Hardiman, 1992). Lesions restricted to the Ansiform lobule cause only a slight and temporary loss of CRs (Hardiman and Yeo, 1992; Yeo and Hardiman, 1992). Lesions of the vermis and dorsal parts of the cerebellar hemisphere have no effect upon CRs (Glickstein *et al.*, 1983; Yeo *et al.*, 1985b). Therefore the focus essential for normal conditioned eyeblink responses is within lobule HVI but the Ansiform lobule can assist recovery of conditioning.

Anatomical, electrophysiological and behavioural evidence suggests that there is convergence of information related to conditioned (CS) and unconditioned (US) stimuli within lobule HVI, and that this lobule has outputs appropriate for modulating regions of the cerebellar nuclei involved in the control of reflex and conditioned eyeblinks. Lobule HVI receives input from caudal dorsolateral and lateral regions of the basilar pontine nuclei, regions that in monkey and cat and rabbit receive auditory and visual input (Yeo *et al.* 1985c; Wells *et al.*, 1989; Study4 of this thesis). Lobule HVI also receives a major input from rostro-medial dorsal accessory olive (Yeo *et al.*, 1985c), which in rabbit is the primary target of sensory afferents from periorbital regions of the face (Van Ham and Yeo, 1991). Lesion studies indicate that the face regions of the IO but not visual regions are particular important for NMR conditioning (McCormick *et al.*, 1986; Yeo *et al.*, 1986). These data suggest that the cerebellar cortex receives CS related information from the PN and US related information from the IO. Consistent with the idea that there is integration of CS and US information within the cerebellar cortex, the activity of Purkinje cells within HVI during eyeblink conditioning is related to auditory CSs and periorbital USs as well as to conditioned (CRs) and unconditioned (URs) responses (Berthier and Moore, 1986).

The primary target of outputs from lobule HVI is to the border of anterior parts of the interpositus and dentate nuclei (Yeo *et al.*, 1985c). Behavioural studies have shown that not only is this region of the cerebellar nuclei essential for eyeblink conditioning (Lavond *et al.*, 1985;Yeo *et al.*, 1985a) but, during conditioning, the activity of Purkinje cells within this region is related to the CS, US and CR and UR (Berthier and Moore, 1992; McCormick and Thompson, 1984).

In veiw of the data reveiwed above it is hypothesised that the regions of the cerebellar cortex importantly involved in eyeblink conditioning are defined by their connections. They must receive convergent inputs from face parts of the inferior olive, and from auditory and visual parts of the pontine nuclei, and they must project to eyeblink regions of the cerebellar nuclei.

There is some experimental evidence to suggest that parts of the Ansiform lobule receive CS and US related information, and project to eyeblink regions of the cerebellar nuclei. Auditory and visual mossy fibre and climbing fibre responses can be recorded within Ansiform lobe in cat (Altman *et al.*, 1976) and monkey (Snider and Eldred, 1952; Mortimer, 1975). Auditory CS-related activity can be recorded in the Ansiform lobe (McCormick and Thompson, 1984) of rabbits during NMR conditioning. McCormick and Thompon (1984) also recorded activity in this lobule related to airpuff USs, similarly, recordings in rat (Akaike, 1989) and cat (Cody and Richardson, 1979) revealed climbing fibre activity in Ansiform lobule related to stimulation of the face. Parts of the Ansiform lobule in cat and rat project to anterior regions of the interpositus and dentate nuclei (Voogd and Bigaré, 1980; Dietrichs and Walberg, 1980; Brodal and Jansen, 1946).

There is currently little anatomical data from rabbits to support the idea that Ansiform lobule receives information important for eyeblink conditioning. So in this study WGA-HRP was injected into rostral parts of this lobule in rabbits and its afferent and efferent connections described.

METHODS

Subjects

Eight Dutch belted rabbits wheighing from 1.8kg to 2.5kg were used for this experiment. They were housed individually within a temperature controlled colony with a 12hr light/dark cycle of artificial light and allowed ad libitum food and water.

Surgery

Each rabbit was anaesthetised using a fentanyl, fluanisone mixture (Hypnorm, Janssen; 0.1/5.0 $mg.kg^{-1}$, i.m.) and a supplement of benzodiazepam (Valium, Roche; 0.5 $mg.kg^{-1}$, i.v.). The rabbit was intubated, placed in a Kopf stereotaxic instrument and connected to a halothane evaporator. Naloxone hydrochloride (Narcan, Du Pont; 3-5 $\mu g.kg^{-1}$, i.v.) was used to antagonise the fentanyl and deep anaesthesia was maintained throughout the operation by 3% halothane in a 30% nitrous oxide/oxygen mixture.

After stable anaesthesia had been attained, mannitol (4 $g.kg^{-1}$, i.v.) was infused at a rate of 3 ml per minute. Mannitol was used to reduce brain volume, the effect of which was to expose

rostral areas of the cerebellum, lower intracranial pressure and reduce the possibility of cerebral oedema. The scalp was reflected and bone and dura removed to expose the right cerebellum. WGA-HRP was then pressure injected into the Ansiform lobule through a micropipette.

After the injections had been made the scalp was sutured and 30 ml of glucose/saline was slowly infused intravenously and the animal removed from the stereotaxic. Buprenorphine hydrochloride (Temgesic, Reckitt and Colman; 0.025 $mg.kg^{-1}$, i.m.) was given immediately after surgery.

Injection of HRP

Seven subjects received one or more pressure injections of WGA-HRP. Micropipettes with internal tip diameters of 10-15µm were made from 5µl graduated capillary glass (Blaubrand, white). The glass was first pulled to form a microelectrode and the tip broken in Hessop glass using the method of Merril and Ainsworth (1972). The pipette was placed in an electrode holder fitted to a micromanipulator and filled with WGA-HRP under vacuum.

One or more injections of WGA-HRP (4% WGA-HRP in 0.09% saline) were made into the right Ansiform lobule at depths of 0.5mm to 2mm below the pial surface. At each injection site 7.5-20nl of WGA-HRP was expelled by presure over five minutes. The volume of WGA-HRP injected was accurate to 1nl. This was achieved by monitoring, during expulsion, the position of the meniscus of the WGA-HRP through a microscope fitted with a graduation eyepiece.

Iontophoresis

One subject received injections of WGA-HRP by iontophoresis at 3 points within the right Ansiform lobule. Each injection was made 0.5-1mm below pial surface. A micropipette, with an internal diameter of 20µm, was filled with 1% WGA-HRP in Tris buffer pH 8.3. A 1Hz square wave voltage, sufficient to give a maximum of 2µA current, was applied for 20 minutes to eject the WGA-HRP. At each placement the pipette was left in situ for 10 minutes after the injection. During insertion and withdrawal of the micropipette a holding current of -12V was applied to the micropipette prevent leakage of WGA-HRP.

Histology

WGA-HRP

All subjects were perfused 24hrs after the injection of WGA-HRP.

Each rabbit was given heparin sodium (Monoparin, CP Pharmaceutical; 500$U.kg^{-1}$, i.v.) and a lethal dose of pentobarbitone sodium (Saggatal, May & Baker; 90$mg.kg^{-1}$, i.v.). Each rabbit was then perfused through the aorta with 2l of 0.9% saline containing 1000U of heparin over 20mins, followed by 1l of fixative (4% paraformaldehyde, 0.1M phosphate buffer, pH 7.3) over 30mins. The fixative was washed out by 1l of 10% sucrose (at $4^{o}C$, buffered with 0.1M phosphate at pH 7.3) over 20mins and 1l of chilled 20% buffered sucrose over 20mins. The brain was then

removed from the skull and stored overnight in 30% buffered sucrose at 4°C and the next day embeddied in gelatin. The brain was placed in a 5% gelatin/30% sucrose solution and kept at 37°C, in -1atm for 30mins. The brain was then embeddied in 10% gelatin/30% sucrose, and allowed 1 day in chilled 30% buffered sucrose before sectioning frozen at 60μm in the coronal plane. Every 4th section was processed using the chromogen tetramethylbenzidine (TMB) as described by Legg *et al.* (1989). The TMB method described by Legg *et al.* (1989) is a modification of the methods first described by Mesulam (1982) and later by Gibson *et al.* (1984). Every 3rd out of 4 sections throughout the site of injection was processed using diaminobenzidine (DAB). This chromogen is less sensative than TMB but gives a better indication of the site of uptake of WGA-HRP (Legg *et al.*, 1989). The sections were then mounted onto gelatinised slides, allowed to dry, and stained with 1% neutral red (buffered with 0.1M phosphate at pH 7.3) to show cell bodies.

Analysis of histological material

Transverse sections through the brain of each rabbit were examined microscopically, under light and crossed polarised illumination, for transport of WGA-HRP. The primary site of label, as exposed by the TMB and DAB reaction methods, was indicated upon drawings of standard sections at intervals of 0.5mm throughout the cerebellum. Anterograde label transported to the cerebellar nuclei were also indicated upon these sections. The primary site of WGA-HRP injections for each subject is shown in Figures 1A-H. These figures also show anterograde label within the cerebellar nuclei. Table 1 summarises the position of the primary sites. Table 2 summarises the sites of anterograde label within the cerebellar nuclei.

Retrograde transport of label to the inferior olive (IO) in each subject was plotted on standard serial sections of the IO based upon Brodal (1939). These are shown in Figures 4A and 4B. A summary of the label found within each major subdivision of the IO, the principal olive (PO), the dorsal accessory olive (DAO) and the medial accessory olive (MAO) for each subject is presented in Table 3.

Retrograde transport of WGA-HRP to the basilar pontine nuclei (PN) and nucleus reticularis tegmenti pontis (NRTP) was marked onto drawings of standard transverse sections at intervals of 0.5mm. This label is shown in Figure 6A and 6B. For analysis of this data drawings of the PN were subdivided according to the cytoarchitectonic boundries described by Brodal and Jansen, (1946) as shown in Figure 5.

Retrograde transport to other parts of the brainstem was marked onto standard drawings of transverse sections through the brainstem. Figures 7 and 8 show the projections in subjects ANS3, ANS7 and ANS8.

In one subject (ANS2) WGA-HRP was injected into the ansiform lobule by iontophoresis in an attempt to fill CrusII with WGA-HRP and prevent the spread of WGA-HRP to the adjacent cerebellar lobules. CrusII was particularly difficult to inject with WGA-HRP because both the paramedian lobule and the lateral sinus overly it. Mannitol injections usefully shrank the paramedian lobule to reveal sufficient amounts of lobule HVI, and CrusI for direct injections into these lobules, but CrusII was not revealed by this method. Because pressure injections of WGA-HRP always result in spread of WGA-HRP back up the cannula track the alternative strategy of passing the injection cannula through the paramedian lobule to inject the underlying crusII was not used. In one subject (ANS3) WGA-HRP was injected into lobule HVI, CrusI and CrusII by passing the cannula through the tentorium. This method was hazardous in that the right occipital lobe must be held away from the tentorium with a small spatula, and because the tentorium contains venous sinuses.

The iontophoretic label of CrusII with WGA-HRP was entirely successful. CrusII was substantially filled in subject ANS2, only in the most rostral and most caudal parts was there an absence of label (Figure 2B). Figure 2B reveals that there was no spread of label into either the overlying paramedian lobule or the adjacent crusI. Although successful, this technique required a considerable period of time to inject the WGA-HRP (1.5hrs). This technique was not used to label lobule HVI and crusI since these lobules could be injected directly.

Standard drawings of the cerebellum, pontine nuclei and brainstem were produced from sections of a normal rabbit brain that had been counter-stained with cresyl violet.

RESULTS

Primary site

In rabbits the Ansiform lobule, or lobule HVIIA in the terminology of Larsell, comprises of two folia situated immediately lateral to lobule HVI named crus I by Brodal (1940), and three more lateral folia named crusII (see Larsell 1970). In this study I have used the terminology crusI and crus II and the collective Ansiform lobule to describe this region of the cerebellum.

The most complete fill of the Ansiform lobule with WGA-HRP was in subject ANS3. The medial lobule of crusI was almost completely filled in subject ANS3 and dorsal and middle parts partially filled in subject ANS8, rostral parts were partially filled in subjects ANS4 and ANS6, and more caudal parts in subject ANS7. The lateral lobule of crusI was substantially filled in subjects ANS1, ANS3 and ANS5 and crusII was substantially filled in subjects ANS2 and ANS3.

There was spread of primary label into parts of the lateral folium of lobule HVI in subjects ANS3 and ANS8 and in subject ANS3 there was slight spread of primary label into anterior parts of the Paramedian lobule that overly the Ansiform lobule.

cont

Cortico-nuclear connections

In every subject the cerebellar nuclei were labelled by transported WGA-HRP. In each case there was clear evidence of anterograde and retrograde label. In this study only the cortico-nuclear projection is described. Anterograde transport of WGA-HRP to the cerebellar nuclei is shown for each subject in Figures 1A-H and is summarised in Table 2.

The anterior border of the interpositus (NI) and dentate (ND) nuclei is ill defined in rabbits. In horizontal sections of the cerebellum fibres clearly divide anterior parts of the interpositus nucleus and the dentate nucleus, and rostral and caudal parts of the interpositus nucleus (see Figure 3). However these divisions are not clear in in transverse section. Since all brains in this study were sectioned in the transverse plane there was no clear division of NI and ND in rostral sections. I have therefore referred to this anterior region of the cerebellar nuclei as aNI/ND.

In all subjects except ANS2 the major projection was to middle regions of the cerebellar nuclei, on the border of NI and ND. In general, the projections were to more rostral than caudal parts of the nuclei. All subjects except ANS2 projected to the prominant hump of the aNI/ND at 3mm caudal to lambda. Projections to antero-medial parts of NI were seen only in subjects ANS3 and ANS4. In subjects ANS2-ANS6 there was a projection to lateral parts of anterior ND. In subject ANS2 there was a projection to lateral ND only. In subjects ANS3-ANS5 label in ND spread laterally in more caudal sections. Only in subjects ANS1 and ANS8 was there a projection to the

fastigial nucleus (NF). Subject ANS8 was noteworthy in that medial regions of NI, ND and NF throughout the extent of the nuclei were heavily labelled.

Inferior olive

In all subjects, the Ansiform lobule received a projection only from the contralateral IO. In all subjects only rostral pasts of the IO projected to Ansiform lobule (see Figures 4A, B, levels VI-XV).

A projection from the dorsal accessory olive (DAO) was seen only in subjects ANS3, ANS7 and ANS8. In each of these subjects the projection was from the medial bend of the DAO at its border with the dorsal leaf of the principal olive (PO) (see Figures 4A,B) . In subject ANS8 there was a projection from the entire rostral half of the DAO. In this subject WGA-HRP label ended quite abruptly between level IX and X of Brodal, (1940) suggesting that the division between anterior and posterior DAO occured at this point (see Figure 4B, ANS8, level X). There was no projection from caudal parts of the DAO in any subject.

A projection from PO was seen in every subject. In most subjects, there was a prominant projection from intermediate parts of the dorsal (dPO) and ventral (vPO) leaves of the PO. In all subjects but ANS4, ANS5 and ANS7 the dPO was labelled. In subjects ANS1, ANS3 and ANS6 intermediate parts of dPO were labelled. In all subjects except ANS4, ANS6 and ANS8 cells were labelled in lateral parts of the vPO, and in all subjects except ANS2 and ANS8 cells were labelled within intermediate parts of the vPO. No cells were labelled in the caudal half of the PO.

In all subjects except ANS2 and ANS6 the Ansiform lobule received a projection from anterior parts of the medial accessory olive (MAO). In subjects ANS1, ANS3 and ANS7 cells were labelled throughout the anterior MAO. Only in subjects ANS1 and ANS8 was there label in posterior parts of the MAO, and in ANS1 this label was sparse.

Pontine nuclei

There were projections from the basilar pontine nuclei (PN) to the Ansiform lobule in all subjects except ANS1. In all other subjects there was clear retrograde labelling of cells bilaterally within middle to caudal regions of the PN. In general the contralateral PN were more heavily labelled than the ipsilateral nuclei. In all subjects except ANS6 there was a bilateral concentration of retrograde label in a region ventral and medial to the cortico-spinal tract. This label was situated mainly within lateral PN (LPN) but there was some spread into ventral PN (VPN). In subjects ANS2-ANS5 this label had a rod like appearance extending for 1-3 mm through middle to caudal regions of the PN (see Figures 6A,B).

Dorsolateral PN (DLPN) was labelled heavily in subject ANS3 and sparsely in subjects ANS7 and ANS8. Subjects ANS2, ANS5 and ANS6 had no labelled cells in DLPN. In all subjects except ANS2 there was label within the peripeduncular (PPN) region but in subjects ANS4 and ANS5 this label was sparse. In subject ANS6 only cells within contralateral peripeduncular PN and the nucleus reticularis tegmenti pontis (NRTP) were labelled. In all other cases there was bilateral label within NRTP. In subject ANS2 and ANS6 the NRTP was labelled only in the most caudal parts. In subjects ANS4 and ANS8 label in NRTP was sparse. Paramedian PN was labelled only in subject ANS3.

The pontine projection of subject ANS3 was noteworthy. In this subject the Ansiform lobule was substantially filled with WGA-HRP and the PN and NRTP were heavily labelled bilaterally. There was a large continuous band of label from NRTP through PPN and into LPN (see Figure 6A, section at 1mm anterior to Lambda). Only in this subject was DLPN heavily labelled. The label within DLPN was restricted to the most dorsal and lateral parts and was most concentrated in caudal regions (see Figure 6A sections 2.5-1.0mm anterior to Lambda).

Lateral reticular nucleus

The lateral reticular nucleus (LRN) was labelled bilaterally in subjects ANS3 and ANS7 (see Figure 8). The LRN was heavily labelled in subject ANS3. At 6mm posterior to Lambda the label was restricted to dorsal parts of the LRN corresponding to the parvocellular sub-trigeminal region described by Walberg, (1952). At 8mm posterior to Lambda the label was distributed throughout LRN and at 9mm posterior Lambda the label was found only in ventral parts corresponding to the magnocellular division of Walberg, (1952). In subject ANS7 the majority of label was found in dorsal LRN.

Trigeminal nucleus

The ipsilateral spinal trigeminal nucleus (SpV) was retrogradely labelled in subjects ANS3, ANS7 and ANS8. The largest projection was seen in ANS3. Only in this subject were cells retrogradely labelled in the spinal trigeminal nucleus pars oralis (Vo: Meessen and Olszewski, 1949) (see Figure 8, Lambda -4 to -5 mm). In each of these subjects the majority of label was in pars interpolaris (Vi) (see Figure 8, Lambda -6 to -8).

Vestibular nuclei

Both the inferior (VIN) and medial (VMN) vestibular nuclei were labelled bilaterally in ANS3, ANS7 and ANS8. The majority of label was found in caudal parts of these nuclei (see figure 8, Lambda -6 and -7mm).

Reticular formation

Labelled cells were scattered within the bulbar reticular formation at the level of the IO and LRN in subjects ANS3 and ANS7. A large concentration of label within a small group of cells was

seen just below the motor tract of the trigeminal in subjects ANS3 and ANS7. Although this label was bilateral there was an ipsilateral bias (see Figure 8, Lambda 0). This compact group of cells receives a large input from the spinal trigeminal nucleus pars caudalis (Van Ham and Yeo, 1992: Fig.2, subject TR19) and projects to lobule HVI and the Paramedian lobe (Van Ham and Yeo, 1992: Fig. 11).

DISCUSSION

Major findings

Discrete injections of WGA-HRP into crusI and crusII of the Ansiform lobule revealed the afferent and efferent connections of this lobule. A general trend in olivo-cortico-nuclear projections was seen - medial injections of the Ansiform lobule labelled more medial parts of the IO and cerebellar nuclei whilst lateral injections labelled more lateral parts of the IO and cerebellar nuclei. The Ansiform lobule projected primarily to lateral parts of the anterior interpositus nucleus and to adjacent parts of the dentate nucleus. The major projections to Ansiform lobule were from anterior parts of intermediate and lateral regions of the ventral principal olive, from anterior parts of medial regions of the medial accessory olive, and from caudal ventro-medial parts of the basilar pontine nuclei.

Olivo-cortico-nuclear projections

Evidence from electrophysiological (Armstrong *et al.*, 1973; rev: Armstrong, 1974; Oscarsson, 1980) and anatomical studies (cat: Courville *et al.*, 1974; Groenewegen and Voogt, 1977; Groenewegen *et al.*, 1979; Kawamura and Hashikawa, 1979; Brodal and Kawamura 1980; rat: Campbell and Armstrong 1983; Azizi and Woodward, 1987; Buisseret-Delmas and Angaut, 1993; Dietrichs and Walberg, 1979; Voogd, 1969) suggests that climbing fibre inputs to the cerebellar cortex and outputs from the cerebellar cortex to the cerebellar nuclei (cat: Voogd, 1964,1969; Voogd and Bigaré, 1980; rabbit: Van Rossum, 1969), are organised in discrete zones that lie perpendicular to the transverse fissures of the cerebellar cortex and parallel to the Purkinje cell dendritic trees. Although an alternative organisation of olivo-cerebellar connections into somatotopically distinct patches was proposed by Robertson et al (1982) there is so far little evidence to support this idea. The following discussion of the olivo-cortico-nuclear connections of the Ansiform lobule is therefore based upon the idea of a zonal organisation. This organisational principle can explain to a great extent why some areas of the cerebellar cortex, and not others, are important for eyeblink conditioning.

Olivo-cortical projections

The olivary projections to Ansiform lobule described in this study are in good agreement with those described in cats (Kotchabhakdi *et al.*, 1979) and those described for more lateral parts of the Ansiform lobule in rat (Azizi and Woodward, 1987).

In this current study there was little evidence for a projection to Ansiform lobule from caudal MAO, the scource of A zone fibres. The largest projection from caudal MAO was seen in subject ANS8 in which the injection site was restricted primarily to dorsal parts of the medial folium of crusI. There was no projection from caudal DAO the source of B zone fibres. The lack of A and B zone inputs to the Ansiform lobule are consistent with reports in cat (Kotchabhakdi *et al.*, 1979) and rat (Azizi and Woodward, 1987).

A projection from the DAO, the source of zones C1 and C3, was seen in only three subjects. In subjects ANS7 and ANS8 there was a small input from rostral parts of the DAO to the mid-dorso-ventral apect of the medial folium of crusI. Subjects that only received injections into the lateral folium of crusI and crusII did not show an input from DAO. Subject ANS3 also received a projection from DAO but the primary injection site also invaded parts of the lateral folium of HVI. As lobule HVI projects heavily to medial DAO (Yeo *et al.*, 1985c) then the projection from DAO in this subjects may due entirely to the spread of primary label into HVI.

In cat (Kawamura and Hashikawa, 1979; Kotchabahkdi *et al.*, 1978) and rat (Azizi and Woodward, 1987; Buisseret-Delmas and Angaut, 1993) there is anatomical evidence to suggest that zones C1 and C3 are narrow in crusI and crusII. In rat, Buisseret-Delmas and Angaut (1993) described a widening of the A zone within crusI and crusII and a corresponding narrowing of the C1 and C3 zones. Although in the current study the projection from caudal MAO was small, if there is a thinning of the C1 and C3 zones in rabbits similar to that described in cat and rat then it is likely that the injections of WGA-HRP were in general too dorsal to label the C1 zone, which appears to be situated at the depths of lobule HVI and medial Crus1, and too dorsal to heavily label the C3 zone.

Anterior MAO, the source of the C2 zone fibres, projects to medial and lateral parts of crusI but not to crusII. The evidence for this comes from a comparison of subjects ANS1,2,3,5,7. Medial crusI was labelled in subjects ANS1,3 and 5. Lateral parts of crusI and crusII were heavily labelled in subjects ANS1,2,3,5 and lateral crusI slightly labelled in ANS7. A projection from anterior MAO was seen only in subjects ANS1,3,5. A projection from anterior MAO was also seen in subjects ANS8 in which the primary label was restricted entirely to medial crusI. This data suggests that medial crusI receives the major C2 input. This is in agreement with Goenewegen *et al.*, (1979).

The Ansiform lobule receives a large input from the PO, the source of the D zone fibres. The extreme lateral bend of the PO was found to project to crusII. This was clearly shown in subject ANS2 in which the injection of WGA-HRP was restricted to crusII. Intermediate parts of the rostral PO were found to project to lateral crusI.

Cortico-nuclear projections

There is some debate on the organisation of cortical projections to the cerebellar nuclei. In cats, Voogd and others described inputs to anterior interpositus (NIA) exclusively from the C1 and C3 zones (Voogd 1964,1969; Voogd and Bigaré, 1980). But, Dietrichs and Walberg (1980, 1981) suggested that in cats the rostral transition region between NIA and medial ND receives adjacent inputs only from cortical zones C3 and D1, and that lateral ND receives inputs from zone D2. These authors suggested the C1 cortical zone projects to medial NIP immediately ventral to the C2 projection zone. The cortico-nuclear projection in rats has similarities with both of these descriptions. Buisseret-Delmas and Angaut (1993) suggested that NIA receives input from the C1, C3 and C2 zones whereas NIP receives inputs from zones C1 medially and C2 laterally. These authors also suggested that anterior parts of ND receive inputs exclusively from zone D1. More caudal regions of the ND receive inputs from zones D0 medially, D1 laterally and D2 ventrally.

In this study all subjects showed a strong projection to aNI/ND. As NIA in cats and rats receives inputs from the C3 zone, then it is surprising that only subjects ANS3, ANS7 and ANS8 received a projection from anterior DAO. In these subjects there was some spread of primary label into lateral folium of HVI. This lobule receives input from DAO and projects heavily to aNI/ND (Yeo *et al.*, 1985c). So some of the inputs from DAO may have been destined for lobule HVI. This might mean that the region of the cerebellar nuclei that I have labelled aNI/ND comprises solely anterior ND. It is more likely that the zonal organisation in this region is complex and the C and D zones cannot be resolved by this study. Labelled regions of aNI/ND in subjects in which the DAO was not labelled must consist of a D zone whereas labelled regions of aNI/ND in subjects in which DAO was labelled must contain C and D zones.

CFs convey US information to the cerebellar cortex

Behavioral, physiological and anatomical studies indicate that rostral parts of medial DAO are important for eyeblink conditioning. These regions of the IO receive periorbital somatosensory input from the trigeminal nucleus (Gellman *et al.*, 1983,1985; Van Ham and Yeo, 1992), and so potentially convey US information to the cerebellar cortex (Yeo *et al.*, 1985c, 1986). In contrast, mid-rostro-caudal parts of the MAO and the dorsal cap of Cooy of the caudal PO, regions of the IO that receive visual inputs (Saint-Cyr and Courville, 1982; Ito, 1984), and are therefore a possible source of CS information, do not appear necessary for eyeblink conditioning (Yeo *et al.*, 1985c, 1986).

Of all the divisions of the IO the DAO receives the largest input from periorbital regions of the trigeminal nucleus (Van Ham and Yeo, 1992). It is therefore likely that during conditioning US related information is conveyed primarily to the C1 and C3 zones in the cerebellar cortex. Anatomical studies in cat and rat have shown that these C zones lie adjacent to vermis and extend from anterior lobule through most of the posterior lobule ( ). However, behavioural

Van Rossum (1969)

studies have so far found no reliable effect of lesions of lobule HIV/V and paramedian lobule on NMR conditioning, eventhough damage to these regions often accompany behaviourally effective lesions (Hardiman and Yeo 1992; Yeo and Hardiman 1992). It is therefore likely that the C1 and C3 regions of prime importance to eyeblink conditioning lie within lobule HVI and the Ansiform lobule. The idea that only a restricted region of the C1 and C3 zones is important for eyeblink conditioning is consistant with the finding that there are different inputs to these zones allong their rostro-caudal extent (Oscarsson and Sjorlund, 1977ab). Thus only the C1 and C3 regions of cortex that receive inputs from rostro-medial DAO, that in turn receive inputs from the trigeminal nuclei, will be important for eyeblinks.

Several anatomical and physiological studies indicate that Ansiform lobule receives input from DAO (ref[1]). Of particular relevence is an electrophysiological study in the rat in which there was evidence of a C1/C3 region deep within the folia between HVI and crusI (Akaike, 1989). Behavioral studies indicate that in effective cases the depths of lobule HVI and the adjacent lobule of crusI were always lesioned (Yeo and Hardiman 1992; Hardiman and Yeo 1992). In this current study only one case (ANS3) had injections into the depths of crusI. The injection site also spread into lobule HVI, but this case did reveal large inputs from r-m DAO (and from DLPN). There was also evidence of a small projection to the mid-dorso-ventral region of crusI from rostro-medial DAO. A potentially important input from intermediate parts of ventral PO was seen in all subjects except ANS1 and ANS2. This region of PO receives a small trigeminal input (Van Ham and Yeo, 1992).

Cortico-nuclear connections are important for the control of eyeblinks

There is strong evidence from lesion (Yeo et al., 1985a) and stimulation (ref[2]) and recording (ref[3]) studies to suggest that rostro-lateral parts of NIA and adjacent parts of ND (aNI/ND) are importantly involved in the control of eyeblinks. It is thaught that these regions of the cerebellar nuclei connect directly and indirectly via the red nucleus to eyeblink motorneurons (Desmond *et al.*, 1983; Holstege *et al.*, 1986). A major projection from lobule HVI is to aNI/ND (Yeo *et al.*, 1985c). In this current study there was a major projection to the rostral hump of aNI/ND. It therefore seems that quite a large part of the Ansiform lobule can influence this region of the cerebellar nuclei.

One final point of interest is shown by the route of the cortico-nuclear fibres emanating from crusI. Some of these fibres curve under the base of lobule HVI (see Figures 2A,F,H). It is possible that in some cases, lesions of the depths of lobule HVI will also remove inputs to the cerebellar nuclei from parts of crusI. This may explain why it is neccessary to lesion the depths of lobule HVI in order to obtain a large deficit in conditioning (Yeo *et al.*, 1985b; Hardiman and Yeo, 1992; Yeo and Hardiman, 1992).

1	2	3
Azizi and Woodward, 1987. Cook and Wiesendanger, 1983. Gellman et al., 1983.	McCormick and Thompson, 1984.	Steinmetz, 1990.

MFs from the trigeminal convey US information to the cerebellar cortex

The frequency of the olivary input to the cortex is in the order of 1Hz so this input is likely to convey only information about where upon the body and when the US is applied. Information about the strength and duration of USs - which are important parameters in NMR conditioning (Smith, 1968) - is likely to be conveyed to the cerebellum directly and indirectly from the trigeminal complex through mossy fibres. Lobules HVI and Ansiform receive direct input from the trigeminal nucleus (rabbit: Yeo *et al.*, 1985c; Van Ham and Yeo; rat: Shambes *et al.* 1978; cat: Ikeda 1979; Somana *et al.* 1980; Carpenter & Hanna 1961; Brodal 1983), and indirect input through somatosensory cortex, the basilar pontine nuclei (rat: Mihailoff *et al.* 1978), NRTP (Torigoe *et al.* 1986), and LRN (Matsushita and Ikeda, 1976). Recordings within lobule HVI during NMR conditioning have revealed MF activity to the US (Berthier and Moore, 1986). Van Ham and Yeo (1992) provided evidence of a small projection to crusI and crusII from the principal nucleus of the trigeminal and from the region bordering the spinal trigeminal pars oralis and interpolaris. In the current study the largest projection from SpV was seen in subject ANS3, however, much of this projection may pass to lobule HVI, since the primary label spread into this lobule. Thus there was evidence of only a small direct projection from the trigeminal complex to the Ansiform lobule.

MFs from the PN convey CS information to the cerebellar cortex

Electrophysiological studies have shown that HVI (Berthier and Moore, 1986), and the Ansiform lobule (MCormick and Thompson, 1984) recieve auditory information during eyeblink conditioning. The most likely route for this information is through the dorsolateral pontine nuclei and the nucleus reticularis tegmenti pontis (see Wells *et al.*, 1989).

Only in one subject (ANS2) was there a projection from DLPN, the classically described teleceptive region of the PN. However, as there was spread of injected label into parts of lobule HVI in this subject, then it is possible that this projection from DLPN was to lobule HVI (see Yeo *et al.*, 1985c). The major projection to Ansiform lobule was from caudal parts of the ventral lateral and medial pontine nuclei. There is some evidence of auditory and visual inputs to this region of the PN - see Wells *et al.* (1989) and Study4 in this thesis on the projections of IC.

Are the connections of Ansiform lobule appropriate for eyeblink conditioning ?

CFs from the inferior olive convey US information to the cerebellar cortex (Hesslow, 1994a,b; Van Ham and Yeo, 1992; Yeo at al., 1996). MFs from the basilar pontine nuclei convey CS information to the cerebellum.

The Ansiform lobule does not receive a large input from face regions of the IO. The largest input to Ansiform lobule is from PO, including the face region in ventral PO. It receives only minor inputs from the primary face regions in DAO, and MAO. The Ansiform lobule does not receive a large input from CS regions of the PN. The main input to Ansiform lobule is from

VPN, smaller inputs are received from LPN and PPN. Parts of VPN and LPN receive visual and auditory inputs from the colliculus (Wells et al., 1989; Study 4 of this thesis). There are no inputs to the Ansiform lobule from medial DLPN, a region that can receive visual and auditory inputs from the superior colliculus (Study 4).

Although it is possible that the placements of WGA-HRP in this study were too restricted to label all of the relevant parts of CrusI and CrusII, it is clear that CS and US inputs to the Ansiform lobule are minor compared with those to lobule HVI. Lobule HVI receives a large input from the face region of DAO, and CS inputs from LPN and DLPN (Yeo et al., 1985c). The paucity of CS and US inputs to the Ansiform lobule can explain why it has a subsidiary role in eyeblink conditioning (Study 1).

Table 1

Summary of injections of WGA-HRP into the cerebellar cortex

Site	HVI m	HVI l	CrusI m	CrusI l	CrusII	Volume nl
Subj						
ANS1			+	+++	+	100
ANS2					+++	10NTO
ANS3	+	+	+++++	+++++	++++	100
ANS4			++	+		10
ANS5			+	+++	+	7.5
ANS6			++	+		7.5
ANS7			++	+		7.5
ANS8		+	+++			17.5

m=medial

l=lateral

+++++ = lobule completely filled with WGA-HRP

Table 2

Summary of anterograde label within the cerebellar nuclei.

Div	aNI	aND	pNI	pND	pNF
Site	ml	ml			
Subj					
ANS1	- +	+ -	+	+	+
ANS2	- -	- +	-	-	-
ANS3	+ +	+ +	+	+	-
ANS4	+ +	+ +	-	+	-
ANS5	- +	+ +	-	+	-
ANS6	- +	+ +	-	-	-
ANS7	- +	+ -	+	+	-
ANS8	- +	+ -	+	+	+

m=medial

l=lateral

+ = presence of WGA-HRP label

Table 3

Summary of retrograde label in the Inferior Olive

Divi	aDAO	pDAO	dPO	vPO	aMAO	pMAO
Zone	C1/C3	B	D1	D2	C2	A
Site	mil	mil	mil	mil	mil	mil
Subj						
ANS1	- - -	- - -	- + -	- + +	+ + +	+ - -
ANS2	- - -	- - -	- - +	- - +	- - -	- - -
ANS3	+ - -	- - -	- + +	+ + +	+ + +	- - -
ANS4	- - -	- - -	- - -	- + -	+ - -	- - -
ANS5	- - -	- - -	- - -	- + +	+ - -	- - -
ANS6	- - -	- - -	- + +	- + -	- - -	- - -
ANS7	+ - -	- - -	- - -	- + +	+ + +	- - -
ANS8	+ + +	- - -	+ - -	+ - -	+ + -	- + +

m=medial

i=intermediate

l=lateral

+++ = all cells within an olivary division were labelled with WGA-HRP.

Figure 1

Key to standard sections through the cerebellum

crI and crII	crus I and crus II of the ansiform lobe
DPFL	dorsal paraflocculus
FL	flocculus
HIV-V	hemispheral lobule IV/V
HVI	hemispheral lobule VI
ND	dentate nucleus
NI	interpositus nucleus
NF	fastigial nucleus
PM	paramedian lobe
PCS	superior cerebellar peduncle
PFL	paraflocculus
I-VI	lobules I-VI of the vermis

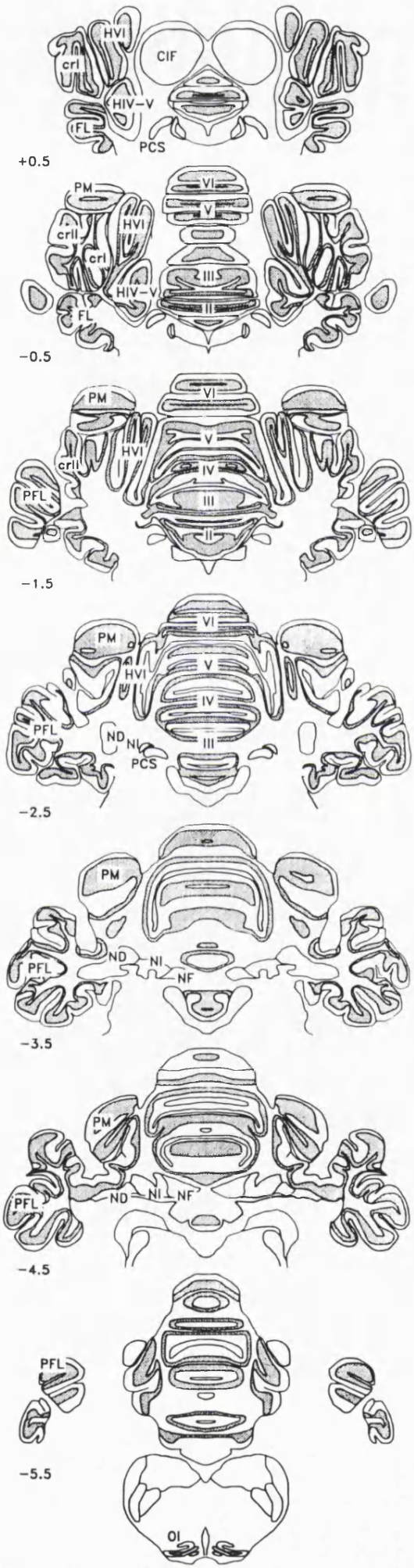

HVI
crI
CIF
HIV–V
FL
PCS
+0.5
PM
VI
V
crII
HVI
crI
III
HIV–V
II
FL
–0.5
PM
VI
V
HVI
crII
IV
PFL
III
II
–1.5
PM
VI
V
HVI
IV
PFL
ND
NI
III
PCS
–2.5
PM
ND
NI
PFL
NF
–3.5
PM
PFL
ND
NI
NF
–4.5
PFL
–5.5
OI

Figure 2A

ANS1

Standard sections through the right cerebellum indicating the site of WGA-HRP injections, the course of labelled fibres connecting the cerebellar cortex and nuclei, and anterograde label within the cerebellar nuclei.

Numbers indicate the distance in mm from the skull suture Lambda.

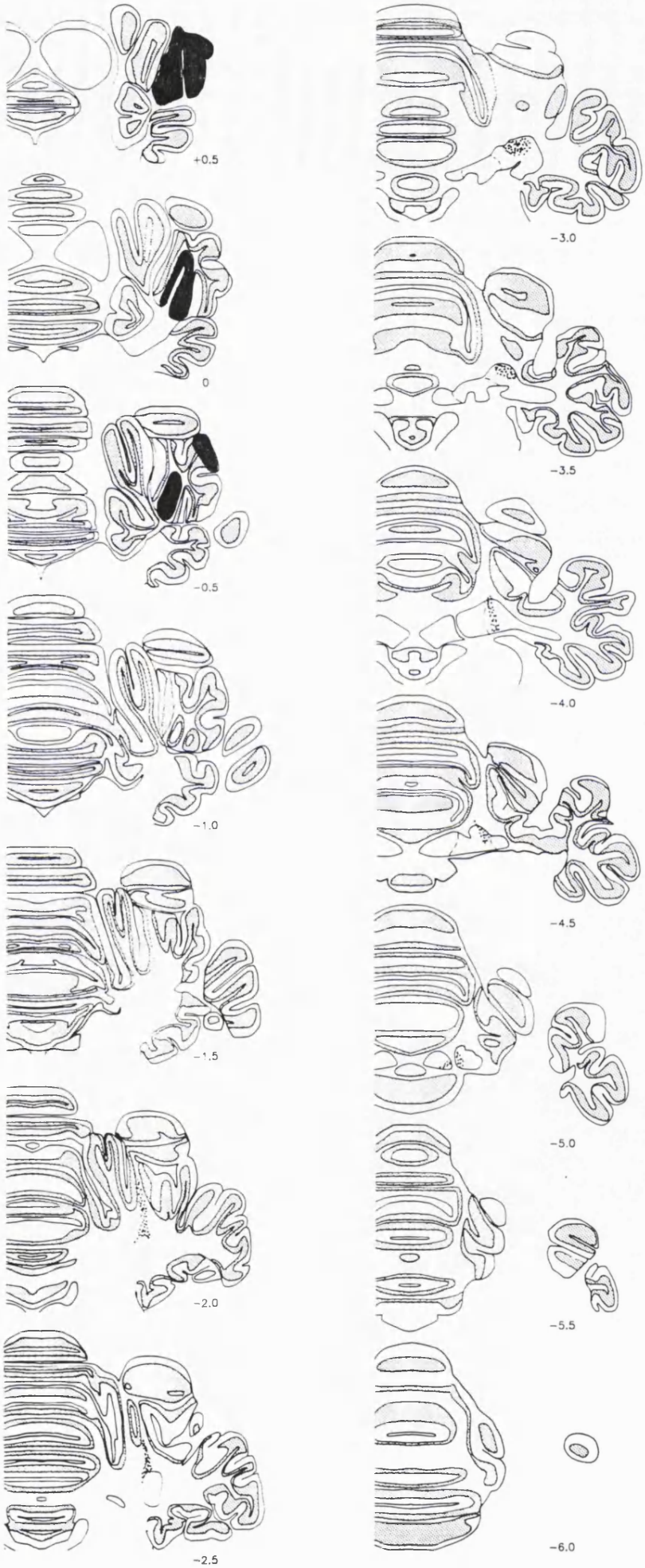

+0.5
0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
-4.0
-4.5
-5.0
-5.5
-6.0

Figure 2B

ANS2

Standard sections through the right cerebellum indicating the site of WGA-HRP injections, the course of labelled fibres connecting the cerebellar cortex and nuclei, and anterograde label within the cerebellar nuclei.

Numbers indicate the distance in mm from the skull suture Lambda.

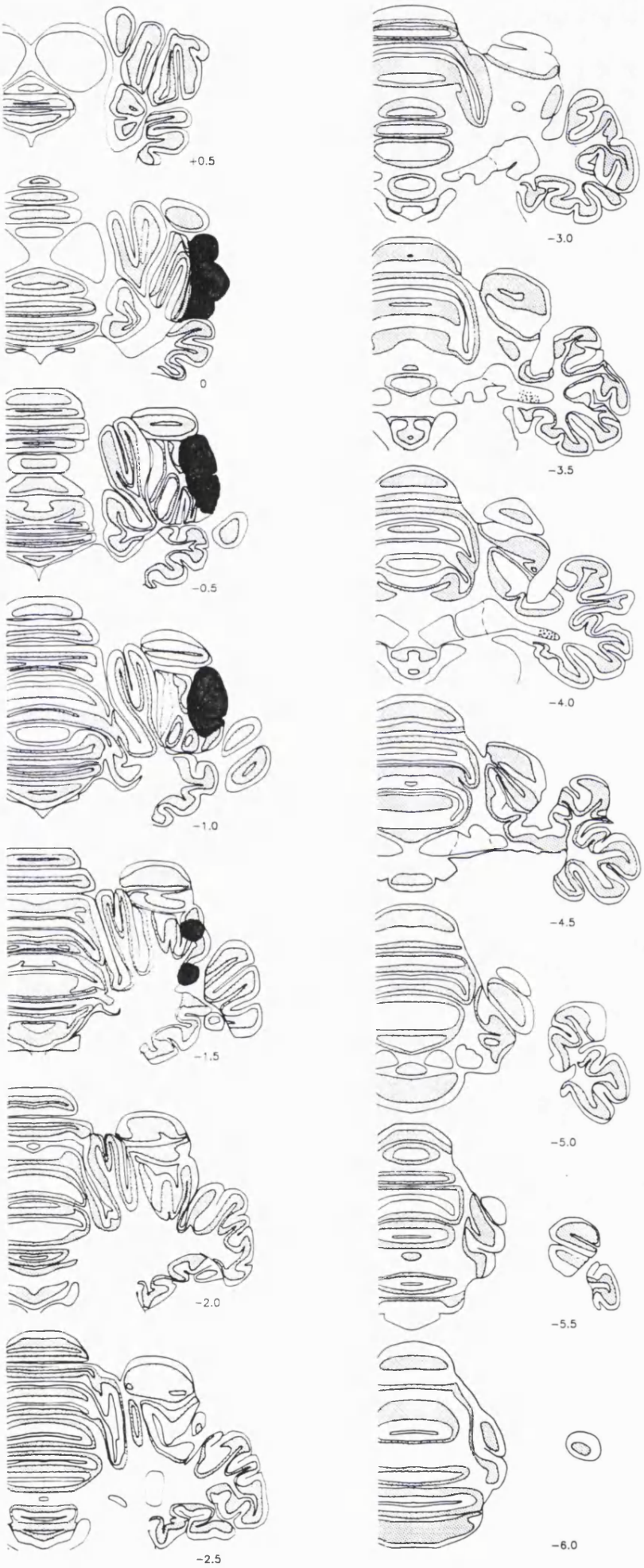

+0.5
0
−0.5
−1.0
−1.5
−2.0
−2.5
−3.0
−3.5
−4.0
−4.5
−5.0
−5.5
−6.0

Figure 2C

ANS3

Standard sections through the right cerebellum indicating the site of WGA-HRP injections, the course of labelled fibres connecting the cerebellar cortex and nuclei, and anterograde label within the cerebellar nuclei.

Numbers indicate the distance in mm from the skull suture Lambda.

+0.5
0
−0.5
−1.0
−1.5
−2.0
−2.5
−3.0
−3.5
−4.0
−4.5
−5.0
−5.5
−6.0

Figure 2D

ANS4

Standard sections through the right cerebellum indicating the site of WGA-HRP injections, the course of labelled fibres connecting the cerebellar cortex and nuclei, and anterograde label within the cerebellar nuclei.

Numbers indicate the distance in mm from the skull suture Lambda.

-0.5
0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
-4.0
-4.5
-5.0
-5.5
-6.0

Figure 2E

ANS5

Standard sections through the right cerebellum indicating the site of WGA-HRP injections, the course of labelled fibres connecting the cerebellar cortex and nuclei, and anterograde label within the cerebellar nuclei.

Numbers indicate the distance in mm from the skull suture Lambda.

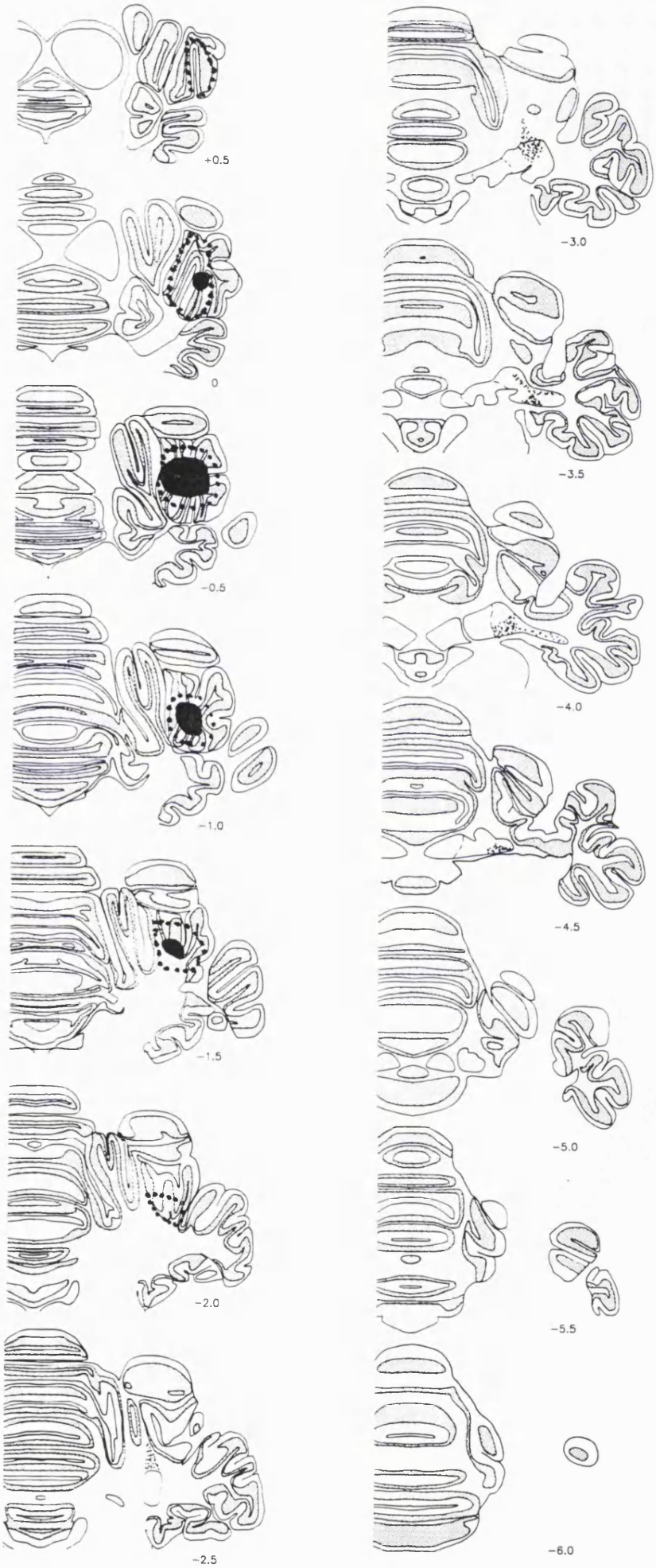

+0.5
0
−0.5
−1.0
−1.5
−2.0
−2.5
−3.0
−3.5
−4.0
−4.5
−5.0
−5.5
−6.0

Figure 2F

ANS6

Standard sections through the right cerebellum indicating the site of WGA-HRP injections, the course of labelled fibres connecting the cerebellar cortex and nuclei, and anterograde label within the cerebellar nuclei.

Numbers indicate the distance in mm from the skull suture Lambda.

+0.5
0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
-4.0
-4.5
-5.0
-5.5
-6.0

Figure 2G

ANS7

Standard sections through the right cerebellum indicating the site of WGA-HRP injections, the course of labelled fibres connecting the cerebellar cortex and nuclei, and anterograde label within the cerebellar nuclei.

Numbers indicate the distance in mm from the skull suture Lambda.

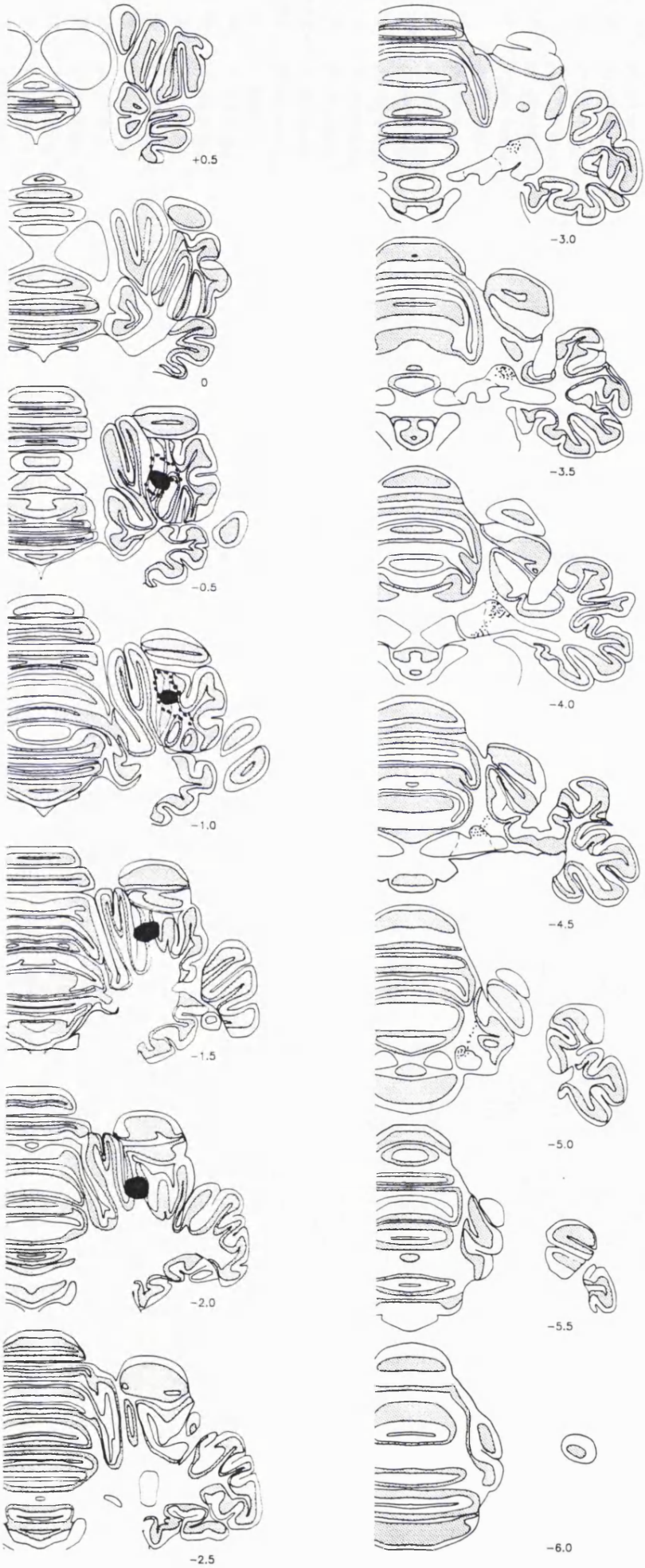

+0.5
0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
-4.0
-4.5
-5.0
-5.5
-6.0

Figure 2H

ANS8

Standard sections through the right cerebellum indicating the site of WGA-HRP injections, the course of labelled fibres connecting the cerebellar cortex and nuclei, and anterograde label within the cerebellar nuclei.

Numbers indicate the distance in mm from the skull suture Lambda.

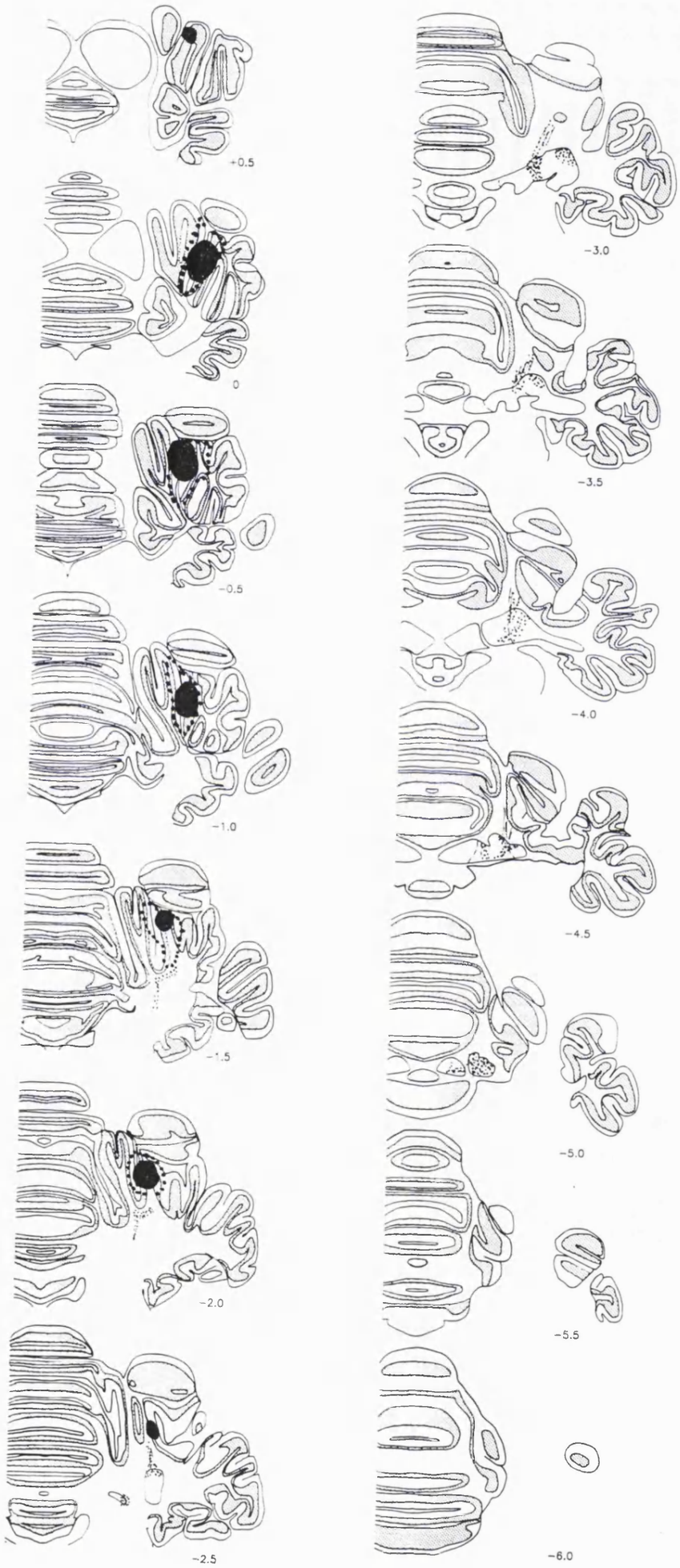
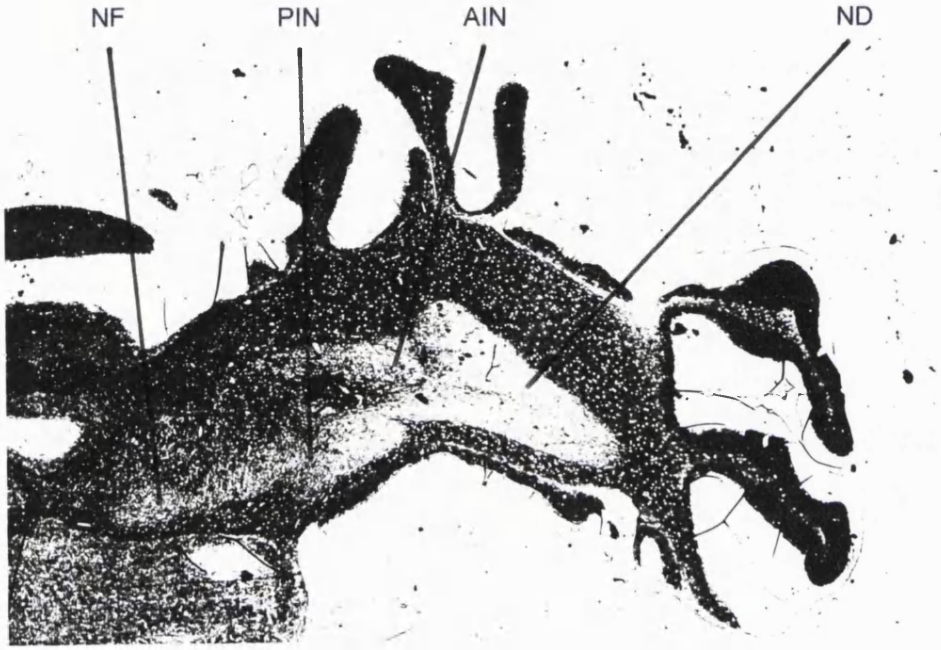

+0.5
0
−0.5
−1.0
−1.5
−2.0
−2.5
−3.0
−3.5
−4.0
−4.5
−5.0
−5.5
−6.0

Figure 3

Consecutive horizontal sections through the right cerebellar nuclei. Top section stained for myelin with luxol blue, bottom section stained for Nissl substance with cresyl violet.

AIN anterior interpositus nucleus
ND dentate nucleus
NF fastigial nucleus
PIN posterior interpositus nucleus

Anterior parts of the interpositus nucleus are clearly separated from more caudal parts of the interpositus nucleus and from the dentate nucelus by fibres.


NF
PIN
AIN
ND

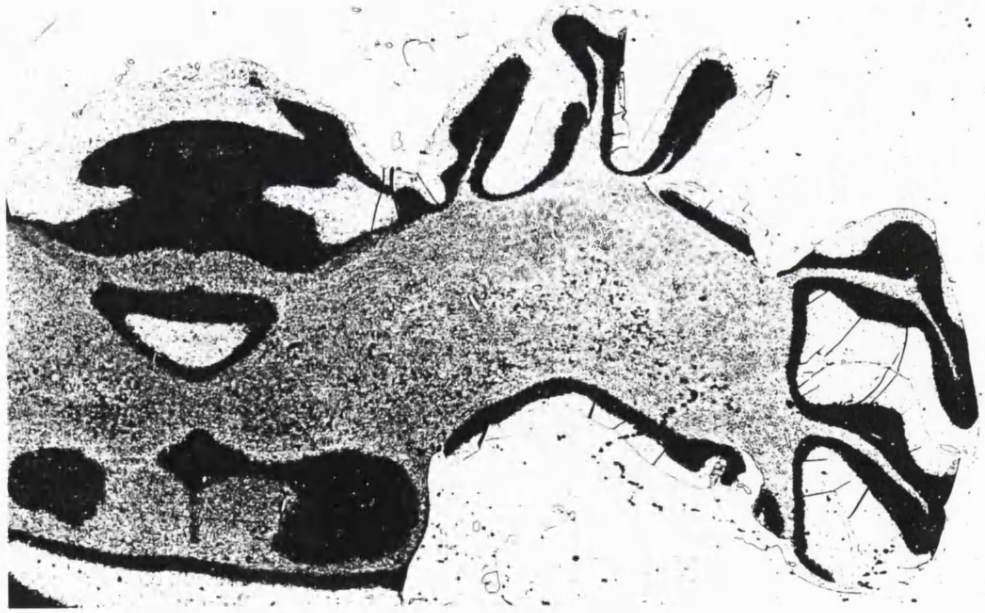


Figure 4A

ANS1, ANS2, ANS3, ANS4

A series of 15 evenly spaced transverse sections through the left inferior olive after Brodal (1940).

Retrograde label is indicated by stippling.

Key to divisions of the inferior olive:

Dorsal accessory olive	- black
Principal olive	- white
Medial accessory olive	- oblique lines
dorsomedial cell collumn	- stipple
dorsal cap of Kooy	- cross hatching
XV	- rostral
I	- caudal

ANS1
ANS2
ANS3
ANS4
KEY
XV
XIV
XIII
XII
XI
X
IX
VIII
VII
VI
V
IV
III
II
I

Figure 4B

ANS5, ANS6, ANS7, ANS8

A series of 15 evenly spaced transverse sections through the left inferior olive after Brodal (1940).

Retrograde label is indicated by stippling.

Key to divisions of the inferior olive:

Dorsal accessory olive	- black
Principal olive	- white
Medial accessory olive	- oblique lines
dorsomedial cell collumn	- stipple
dorsal cap of Kooy	- cross hatching
XV	- rostral
I	- caudal

KEY
ANS5
ANS6
ANS7
ANS8
XV
XIV
XIII
XII
XI
X
IX
VIII
VII
VI
V
IV
III
II
I

Figure 5

Key to divisions of the basilar pontine nuclei based upon Brodal and Jansen (1946)

Dorso-lateral pontine nucleus	- small dots
Lateral pontine nucleus	- short horizontal lines
Paramedian pontine nucleus	- long horizontal lines
Ventral pontine nucleus	- vertical lines
Peripeduncular pontine nucleus	- thick line
Nucleus reticularis tegmenti pontis	- large dots

The top section is rostral.

Numbers indicate the distance in mm from the skull suture lambda.

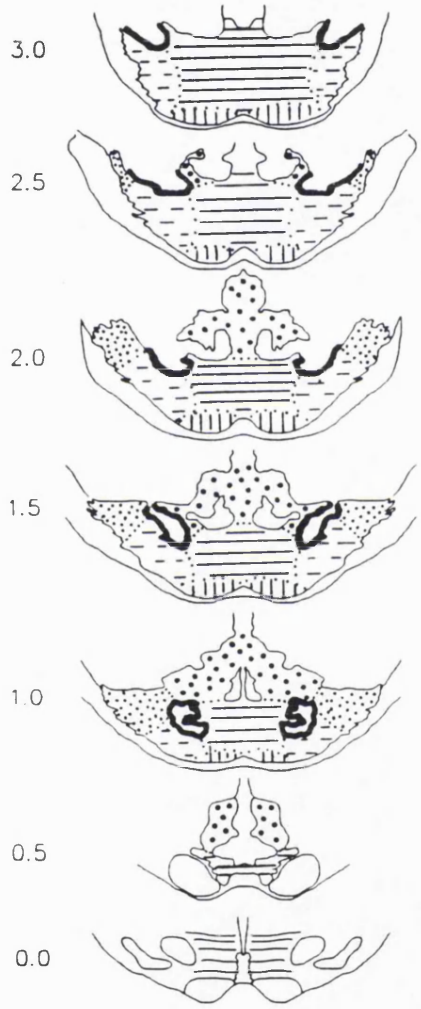

3.0
2.5
2.0
1.5
1.0
0.5
0.0

Figure 6A

ANS2, ANS3, ANS4, ANS5

Standard transverse sections through the basilar pontine nuclei. Retrograde WGA-HRP label is indicated by stippling.

Numbers indicate the distance in mm from the skull suture Lambda.

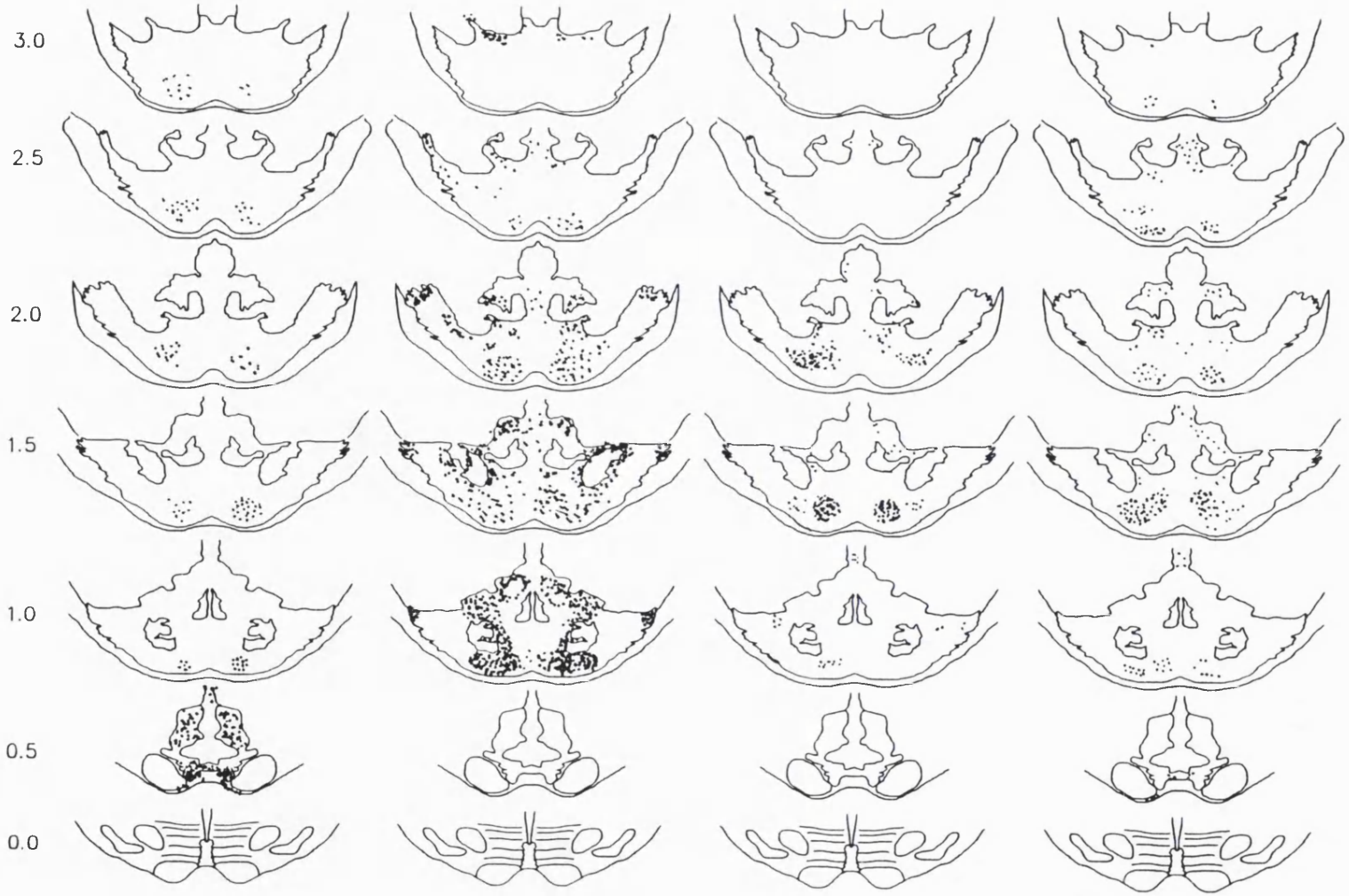
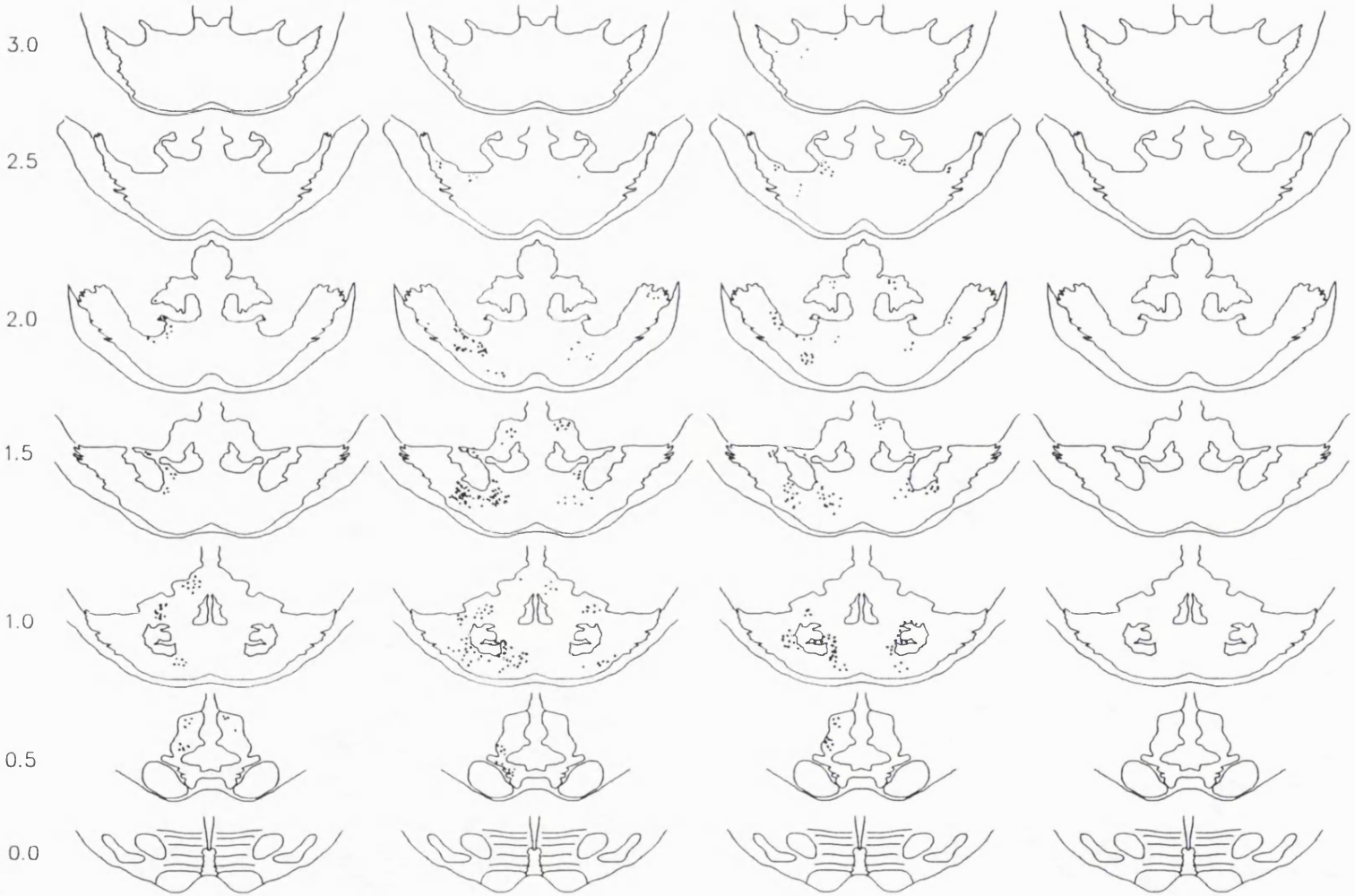

3.0
2.5
2.0
1.5
1.0
0.5
0.0

Figure 6B

ANS6, ANS7, ANS8

Standard transverse sections through the basilar pontine nuclei. Retrograde WGA-HRP label is indicated by stippling.

Numbers indicate the distance in mm from the skull suture Lambda.


3.0
2.5
2.0
1.5
1.0
0.5
0.0

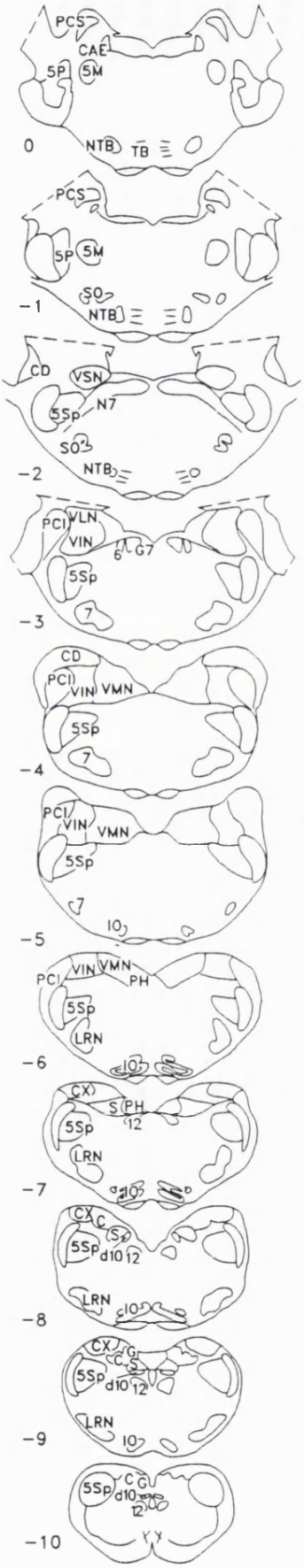


Figure 7

Key to standard sections through the brainstem

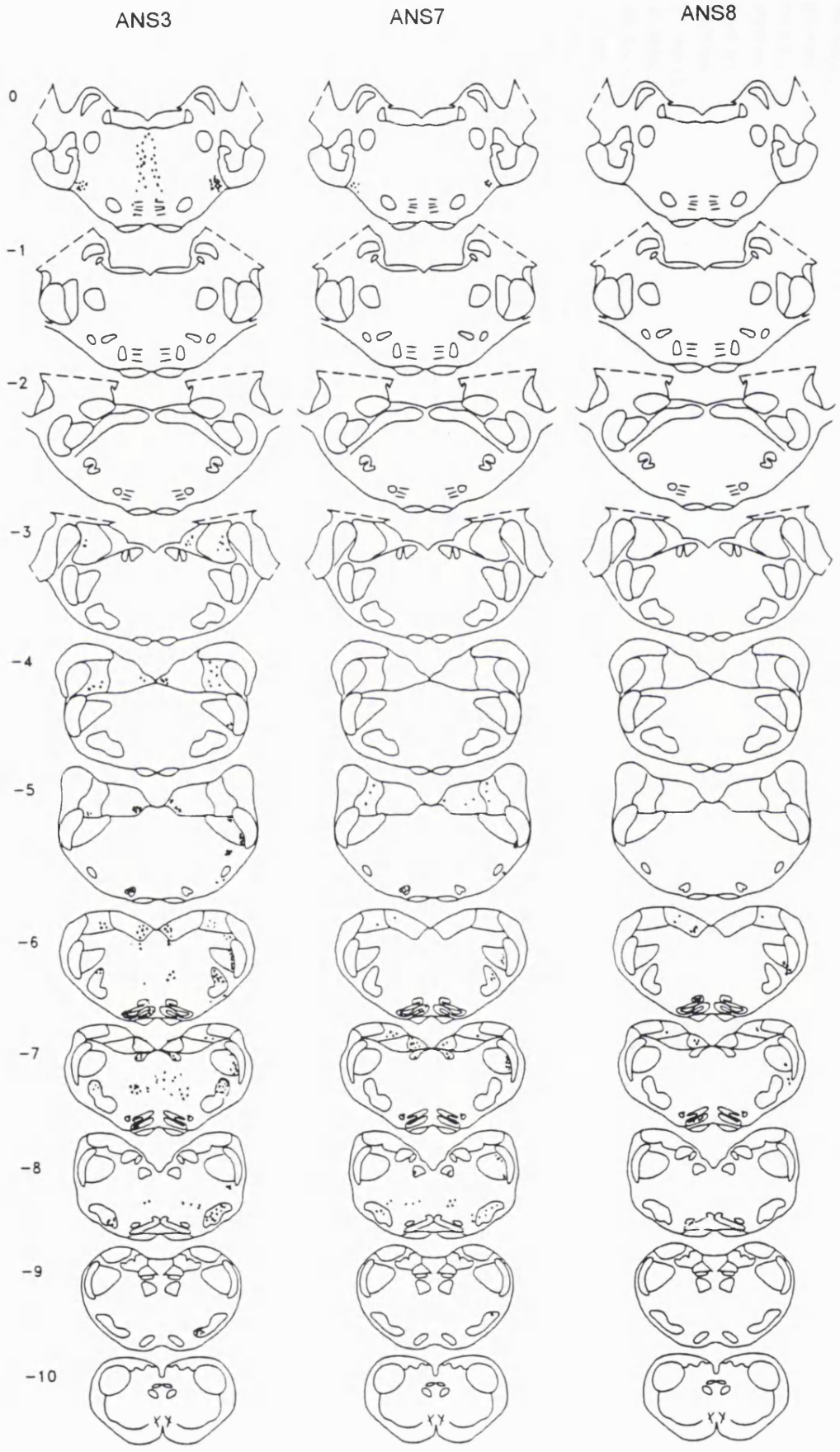
CAE	nucleus coeruleus
CD	dorsal cochlear nucleus
C	nucleus cuneatus
CX	external cuneate nucleus
G	nucleus gracilis
IO	inferior olive
LRN	lateral reticular nucleus
NTB	nucleus of the trapezoid body
PCI	inferior cerebellar peduncle
PCS	superior cerebellar peduncle
PH	perihypoglossal nucleus
SO	superior olivary nucleus
TB	trapezoid body
Vc	spinal trigeminal nucleus pars caudalis
Vi	spinal trigeminal nucleus pars interpolaris
VIN	inferior vestibular nucleus
VMN	medial vestibular nucleus
Vo	spinal trgeminal pars oralis
Vp	spinal trigeminal nucleus pars principalis
VSN	superior vestibular nucleus
5M	motor trigeminal nucleus
5P	principal trigeminal nucleus
5Sp	spinal trigeminal nucleus
6	abducens nucleus
7	facial nucleus
G7	genu of the facial nerve
N7	facial nerve
12	hypoglossal nucleus

Figure 8

ANS3, ANS7, ANS8

Standard series of sections through the brainstem. Retrograde WGA-HRP label is indicated by stippling.

Numbers indicate the distance in mm from the skull suture Lambda.


ANS3
ANS7
ANS8
0
-1
-2
-3
-4
-5
-6
-7
-8
-9
-10

Kainic acid lesions of the cerebellar nuclei and the overlying cortex abolish conditioned eyeblinks in the rabbit.

INTRODUCTION

Unilateral lesions of rostral cerebellar nuclei and overlying cortex prevent the acquisition of classically conditioned nictitating membrane responses on the side of the lesion (Lincoln *et al.*, 1982; Lavond *et al.*, 1984). Similar lesions cause a profound loss of previously acquired conditioned responses (CRs) (McCormick *et al.*, 1982; McCormick & Thompson, 1984; Clark *et al.*, 1984). Unilateral lesions restricted to anterior parts of the cerebellar nuclei prevent the acquisition of CRs (Sears and Steinmetz, 1990) and produce a profound loss of previously acquired CRs (Lavond *et al.*, 1985; Yeo *et al.*, 1985b). Lesions of posterior interpositus nucleus or of posterior dentate nucleus or of the fastigial nucleus do not impair retention of CRs (Yeo *et al.*, 1985). As unconditioned nictitating membrane responses (URs) elicited by the unconditioned stimulus (US) used in conditioning are not affected by the cerebellar lesion so the loss of CRs is not simply due to an impairment of motor output (McCormick and Thompson, 1984; Yeo *et al.*, 1985a). The results of these experiments are consistent with the idea that a major function of the cerebellum is to subserve motor learning (Ito 1984; Marr, 1969; Albus, 1971; Thach 1992).

Welsh and Harvey (1989) questioned this interpretation. They suggested that interpositus lesions do not damage learning but simply impair movement. These authors unilaterally lesioned anterior parts of the cerebellar nuclei in trained rabbits and found that although many subjects failed to produce CRs on paired trials, several of these subjects produced small, late responses on trials in which the conditioned stimulus (CS) was presented alone. CRs were delayed in appearance and so masked on paired trials by the UR, suggesting that the abolition reported in earlier studies (Sears and Steinmetz, 1990; Lavond *et al.*, 1985; Yeo *et al.*, 1985b) may not have been complete, as in these studies CRs were examined only on paired trials. A similar deficit in frequency and timing was shown by URs elicited by very low intensity USs, although URs elicited by the intensity of US used in conditioning were normal. As the few remaining CRs were similar in size and timing to URs produced by the low intensity USs, Welsh and Harvey argued that the cerebellar lesions had caused a loss of excitation acting upon the eyeblink motor system so preventing CRs from fully exciting this system. Moreover, since no relationship was found between either the size or position of the lesions and the severity of CR and UR deficit, Welsh and Harvey concluded that the deficits were caused by a general motor impairment rather than by the loss of a specific, learning related, output from the cerebellum. This hypothesis implies that the cerebellum has no role in eyeblink learning but regulates in some non-specific way the expression of learning formed elsewhere in the brain.

More recently Lavond *et al.*, (1990) made unilateral lesions of the cerebellar nuclei and tested for retention of CRs during paired and CS alone trials. No CRs were found in any trial, even

after twenty sessions of retraining. This result indicates that the abolition of CRs cannot be due simply to a delay in their appearance. However, these authors did not fully test for changes in the reflex response, perhaps their lesions had caused a large motor deficit that prevented the appearance of all CRs.

Two other important features in the study of Welsh and Harvey (1989) that may account for the discrepancies in results were the extensive number of pre- and post-operative training sessions. These authors trained the rabbits for 1056 trials over sixteen sessions before making lesions. The animals were then retrained for a similar number of trials post-operatively. In all previous studies rabbits had been trained pre-operatively for 400-560 trials over five sessions, which is normally sufficient for acquisition of CRs. Extended pre-operative training might lead to strengthening of the CR memory trace so that it is more resistant to restricted lesions of the cerebellar nuclei. The region of ipsilateral cerebellar nuclei engaged in NMR conditioning may become extended, or the contralateral cerebellum may become important for learning or for the expression of CRs on the trained side. The results of unilateral KA lesions of the cerebellar cortex suggested that some recovery of CRs was mediated by the non-lesioned side (Hardiman and Yeo, 1992). Similar results have been found in rats in which only complete lesions of the entire cerebellar nuclei bilaterally produce a permanent loss of previously acquired CRs (Skelton *et al.*, 1988). Finally, a recent report using ferrets has shown that there is bilateral control of eyeblink responses from one cerebellar hemisphere (Ivarsson and Hesslow, 1993).

Extensive post-operative training may also aid recovery of CRs, particularly in subjects well trained prior to lesioning. Cerebellar lesions decrease tonic activation of both alpha and gamma motorneurons (Granit *et al.*, 1955). The consequences of this are a loss of muscle tone (Gilman 1969), a major symptom of cerebellar damage (Holmes 1917). Compensation can occur over several weeks leading to a recovery of muscle tone and some return of co-ordinated muscular activity (Van der Meulen and Gilman, 1965). If lesions of the cerebellum cause a loss of a tonic drive acting upon eyeblink motor pathways, and so prevent the expression of CRs as suggested by Welsh and Harvey (1989), then compensation for such a loss of activity may occur in time or with extensive re-training. This possibility has not been extensively tested. Lavond *et al.* (1984) reported that CRs could not be acquired in subjects with bilateral lesions of the cerebellar nuclei, even after three months of intermittent post-operative training. In addition, these authors found no recovery of CRs during three months of intermittent post-operative training in one subject that had been trained pre-operatively.

This current study was designed to examine four main points. 1. Does extensive pre-operative training make a difference in whether CRs are retained ? 2. Does extensive post-operative-recovery allow a recovery of CRs ? 3. Were the residual CRs seen by Welsh and Harvey (1989) simply due to incomplete lesions of the cerebellar nuclei ? 4. Do lesions of the cerebellar nuclei cause a general depression of the eyeblink system.

Firstly to establish that the cerebellum controls a co-ordinated conditioned response that includes the external eyeblinks as well as the nictitating membrane response (McCormick et al., 1982). Secondly, OO EMG activity is potentially a more sensitive method of recording the effect of lesions of the cerebellar nuclei upon CRs. Welsh and Harvey (1989) suggested that lesions of the cerebellar nuclei cause a motor deficit which prevents the expression of CRs but does not prevent learning. An apparent loss of learning could be due to the fact that the NMR is only indirectly coupled to eyeball retraction. The NMR is a passive movement caused by retraction of the eyeball which displaces orbital fat which in turn pushes the NM across the eyeball. It is possible that a small impairment in eyeball retraction caused by a small decrease in activation of the retractor bulbi - the primary muscle responsible for eyeball retraction - would result in a disproportionately large effect upon the NM response. Thus the NM may not move at all even though the command to make a CR has been sent to the retractor bulbi muscle. Whether or not cerebellar lesions prevent a CR command from activating the retractor bulbi muscles can be tested by recording its EMG activity during training. The presence of EMG activity related to CRs but an absence of NM CRs would strongly support the idea that lesions of the cerebellar nuclei cause a motor deficit and not a learning deficit. However the practical difficulties in attaching EMG electrodes to the retractor bulbi, which consists of four thin strips of muscle surrounding the optic nerve, prohibit such an experiment. Since conditioned external eyeblinks are closely coupled to conditioned NMRs (McCormick et al., 1982) it is more practicable to test for the presence or absence of CRs by recording EMG activity in the orbicularis oculi muscle. Thirdly, OO EMG activity potentially can provide a sensitive method to examine lesion effects upon the eyeblink motor system. In naive rabbits NM URs are larger when a tone CS is paired with an electrical US, than when the US is presented on its own (Ison and Leonard, 1971). If lesions of the cerebellar nuclei decrease the excitability of NM motor pathways as suggested by Welsh and Harvey, (1989), then excitation of the UR by the CS should be prevented. Weisz and LoTurco (1988) reported that lesions of the cerebellar nuclei have no effect upon facilitation by a tone CS of NM URs elicited by an air puff to the eye. It may be that in the study by Weisz and LoTurco there was an underlying motor deficit that was overcome by the use of a strong air puff US. This possibility was examined by recording OO EMG activity elicited by a very weak electrical US.

There were two groups in this study. In the first group large bilateral lesions of the cerebellar nuclei were made in rabbits which had been extensively pre-trained. In the second group large unilateral lesions of the cerebellar nuclei were made in rabbits that had no prior training. Subjects in both groups were allowed an extensive period of postoperative recovery before undergoing an extensive period of training. These groups were chosen in order to test the extremes in lesion size and the amount of pre-operative training.

Fibre-sparing lesions were made by kainic acid (KA) in order to avoid damaging afferent fibres to the cerebellar nuclei thus leaving intact the pre-cerebellar nuclei (Coyle *et al.*, 1978). Unpaired CS probe trials were used to detect any longer latency responses. URs were examined at several intensities of US to fully test for possible motor deficits. During the final session of training orbicularis oculi electromyogram activity (OO EMG) was recorded along with the nictitating membrane response. OO EMG activity was recorded for three reasons.

cont

METHODS

Subjects

Eleven Dutch-belted rabbits weighing from 1.8Kg-2.5Kg were used for this experiment. They were housed individually, allowed ***ad libitum*** food and water and maintained on a 12hr light, 12hr dark cycle.

Techniques and equipment

Each animal was trained using techniques and equipment similar to those first developed by Gormezano *et al.* (1962). Each animal was placed in a Perspex restraining box. A low-torque potentiometer was attached to the head by clips around the ears and muzzle. The shaft of the potentiometer was directly attached by a lever and universal joint to a suture placed in the nictitating membrane. The animal was then placed in a ventilated sound-attenuating chamber facing a loudspeaker mounted centrally.

Conditioning

Training was Pavlovian delay conditioning using white noise as a CS. The CS duration was 310 msec and the intensity was 87 dB (re 20μN.m^{-2}). Background noise produced by ventilation fans was 60 dB (re 20μN.m^{-2}). The US was a 60 ms, 2.0 mA, biphasic (20ms positive, 20ms negative) electrical stimulus. The US was applied through stainless steel wound clips, one placed immediately behind the temporal canthus of the eye and the other placed centrally within the lower eye lid. On paired trials the inter-stimulus-interval between the CS and US onsets was 250 ms. The inter-trial-interval was randomly selected between 25-35 seconds.

The rabbits formed three groups according to lesion size and the amount of pre-operative training. All subjects had a daily session of 100 trials. In each daily session the CS was paired with the US in eighty trials and in twenty trials the CS was presented alone. The CS was presented alone on every fifth trial.

Experimental Groups

There were three experimental groups. Subjects in Group A had bilateral cerebellar lesions, Group AC were un-operated controls for Group A. Subjects in Group B had unilateral lesions of the cerebellum. Groups A and AC were trained differently to group B. The experimental design is summarised in Table1.

Table 1.

Grp	Training sessions	Surgery	Wait weeks	Training sessions		Wait weeks	Training sessions	UR Test session	Wait weeks	Training sessions	OO + NM session
A	15R	bilateral	11	15R		5	1R	1	13	5R	1
AC	15R	none	11	15R		5	1R	1	13	5R	1
B	none	unilateral	11	15R	5L	5	1R	1	13	5R	1

R - US applied to the right side
L - US applied to the left side

Bilateral Lesion Group (A) - C1502,4,5,6,7

Five subjects were trained for fifteen sessions with the US applied to, and the NMR recorded on, the right side. Following bilateral lesions of the cerebellar nuclei the subjects were allowed eleven weeks of recovery and then trained on the right side for fifteen sessions. After a further five weeks of recovery each subject had one session of training on the right side, and then after two days without training URs were tested. The subjects were then allowed thirteen weeks with no training followed by five sessions of training on the right side.

Control Group (AC) - C1515,16,17

Three subjects served as unoperated controls for group A. These subjects underwent the same training as Group A subjects.

Unilateral Lesion Group (B) - C1510,11

Two subjects were not trained pre-operatively. Following unilateral lesions of the cerebellar nuclei and eleven weeks recovery these subjects received fifteen sessions of training to the right side then five sessions of training to the left side. Following five weeks without training one further session of training was given to the right side followed two days later by UR testing. The subjects were then allowed a further thirteen weeks without training followed by five sessions of training on the right side.

NM and OO response testing

On the final day of training, subjects C1502,4,5 and 6 from Group A, subjects C1515 and C1516 from Group AC and subject C1511 from Group B, were implanted with electromyogram (EMG) electrodes into the orbicularis oculi muscle (OO) bilaterally and fitted with NM transducers bilaterally. NM and OO EMG responses were recorded simultaneously.

The EMG electrodes were 30g Teflon coated stainless steel wire, bared 1mm from the end. The electrodes were placed in the OO via a 28g hypodermic needle. The steel wire was threaded through a needle and the bared 1mm bent over the tip of the needle. The needle was inserted into the OO and immediately withdrawn leaving the electrode hooked firmly into the muscle.

The CS was the same as used in the previous conditioning trials. The US was a single biphasic periorbital electrical stimulus presented 250ms after the onset of the CS. The strength and duration of the US was selected to give a UR of about 1mm. This was sufficient to minimise stimulus artefact which enabled accurate recordings of OO EMG activity. The range of US was a current of 5-10mA, and the duration of each phase was 350-500µs. The inter-stimulus-interval was 250ms. The inter-trial-interval was selected randomly between 25-35 seconds.

All subjects were first given 100 normal conditioning trials using the standard US as described above. They were then given a further 20 trials of a mixed trial design using the low intensity US. For all subjects except C1502 the trials were given in the recurring sequence of paired (CS+US), US alone, paired, paired, CS alone. Subject C1502 was given 20 trials in the recurring sequence: paired, paired, paired, paired, US alone.

Because reflex responses may be affected by overlapping CS and US (Sanes and Ison 1979) subjects C1515 and C1516 received a further 100 normal conditioning trials using the standard US but the CS duration was 250ms, and the interstimulus interval was 250ms. Thus the CS terminated immediately before the US. This session was followed by another 20 trials of the mixed trial design. On paired trials the CS terminated immediately prior to the US.

NM UR testing

The nictitating membrane reflex responses of all subjects were tested on a series of unconditioned stimuli of varying intensity at one minute intervals. Each trial consisted of a 60ms, biphasic US. US intensity was varied in the fixed sequence 2.0, 1.0, 0.75, 0.5, 0.375, 0.25, 0.25, 0.375, 0.5, 0.75, 1, 2.0 mA. Five trials at each stimulus intensity were given with the stimulus applied to the right side. After a 30 minute rest period the same procedure was repeated with the US applied to the left side.

Surgery

Each rabbit was anaesthetised using a fentanyl, fluanisone mixture (Hypnorm, Janssen; 0.1/5.0 $mg.kg^{-1}$, i.m.) and a supplement of benzodiazepam (Valium, Roche; 0.5 $mg.kg^{-1}$, i.v.) was

used as a relaxant. Benzodiazepam also protected the animal after the injection of KA from possible damage to structures outside the injection site (Ben-Ari *et al.*, 1979). The rabbit was intubated, placed in a Kopf stereotaxic instrument and connected to a halothane evaporator. Naloxone hydrochloride (Narcan, Du Pont; 3-5 $\mu g.kg^{-1}$, i.v.) was used to antagonise the fentanyl and deep anaesthesia was maintained throughout the operation by 3% halothane in a 30% nitrous oxide/oxygen mixture. After stable anaesthesia had been attained, mannitol (4 $g.kg^{-1}$, i.v.) was infused at a rate of 3 ml per minute. Mannitol was used to reduce brain volume thus exposing rostral areas of the cerebellum, lowering intracranial pressure and reducing the possibility of cerebral oedema during the operation. The scalp was reflected and bone and dura removed to expose the right cerebellar cortex.

Kainic acid was injected through a micropipette into the cerebellar nuclei. Each animal received between eight and fourteen stereotaxic placements of KA (Sigma, 1 $mg.ml^{-1}$ kainic acid in sterile 0.9% NaCl, pH 7.2-7.4) on a 1mm cubic grid. At each site either 25 or 50 nl of KA was injected over 3 minutes. Subjects in Groups A received between 300-550 nl (1.4-2.6 nmol) of KA injected into anterior parts of the cerebellar nuclei on both sides of the cerebellum. Subjects in Group B received a similar quantity of KA injected into anterior parts of the right cerebellar nuclei only.

After the injections had been made the scalp was sutured and 30 ml of glucose/saline was slowly infused intravenously and the animal removed from the stereotaxic. Buprenorphine hydrochloride (Temgesic, Reckitt and Colman; 0.025 $mg.kg^{-1}$, i.m.) was given immediately after surgery and twice daily for 2 days post operation. All operated subjects were allowed to recover for eleven weeks.

Histological processing

At the end of the experiment each rabbit was injected with heparin sodium (500 $U.kg^{-1}$, i.v.) and a lethal dose of pentobarbitone sodium (90 $mg.kg^{-1}$, i.v.). The rabbit was perfused through the aorta with 0.9% saline followed by 10% formalin and the brain removed from the skull. The brain was embedded in 10% gelatin, and frozen sections were cut at 30 µm in the transverse plane. Alternate sections were mounted onto gelatinised slides and Nissl-stained with cresyl violet.

Data analysis

Control, data acquisition and on line analysis was performed on a microcomputer using an analogue to digital conversion rate of 1KHz for the delay conditioning and the UR testing. OO EMG activity was recorded differentially, filtered to pass signals of 300Hz-10KHz and amplified 1000x. Simultaneously, NM movement and OO EMG activity was recorded bilaterally and digitised at a sample rate of 8KHz using a digital interface (CED 1401) for later analysis using a signal averaging program (SIGAVG, CED).

Conditioning

There are two ways in which the presence of a conditioned NMR was measured: frequency and amplitude. Nictitating membrane responses whose amplitude was greater than or equal to 0.5mm contributed to the measure of CR frequency. A response was scored on paired trials if it was made during the inter-stimulus interval (250ms from CS onset) and on CS alone trials if it was made during a period of 1450ms starting from the CS onset. The percentage of CRs in each session was recorded for paired and CS alone trials. For each group the responses for individual subjects were averaged and means of CR frequency and CR amplitude calculated. The percentage of responses on CS alone trials was presented as the CR frequency. The peak amplitude of all responses from CS onset in CS alone trials was presented as the average peak CR amplitude.

NM and OO response testing

NMR data and orbicularis oculi EMG activity from the final session of training was treated differently. The four responses on each paired (CS+US), CS alone and US alone trials were signal averaged separately. The averaged responses from CS alone trials were examined for the presence of responses to the CS. The averaged responses from paired and US alone trials were compared in order to determine whether the presence of the CS had any effect upon URs. The peak amplitude and peak velocity of the averaged responses from CS and US alone trials were compared to determine the relative amount of activity in the eyeblink motor pathways during the production of CRs and URs.

NM UR testing

Signal averaging was used to produce an averaged waveform for the ten responses at each stimulus intensity. A number of response parameters were obtained from the averaged waveform. The nictitating membrane response onset latency was measured from the stimulus onset to the first reliable inflexion above baseline noise (0.05mm). The peak and average velocity of the response were determined. The amplitude and latency of the maximum nictitating membrane extension were also determined.

Histological verification of lesions

Transverse sections of the brain from subjects in Groups A and B were examined microscopically for loss of neurons within the cerebellar cortex, cerebellar nuclei and the inferior olive. Regions of degeneration were indicated upon standard drawings of the cerebellum. Brodal's criteria for retrograde degeneration were used to determine the extent of degeneration (Brodal 1939, 1940).

RESULTS

Conditioning

Group AC

All subjects learned well to the CS and all had reached an asymptotic level of responding by session 6. All subjects showed a high level of CRs throughout retention testing in sessions 16-36. Importantly, all subjects showed CRs on the very first conditioning trial (session 16) after the eleven week period with no training. The group means of CR frequency and amplitude measured on unpaired trials are shown in Figures 1 and 2.

Group A

All subjects in this group learned pre-operatively at a similar rate to those of Group AC. Post-operative data recorded on paired and CS alone trials was identical. All subjects showed an immediate, profound and enduring loss of CRs, with fewer than 5% responses made by any subject in any session of post-operative training. The absence of any response is clearly shown by the group means of frequency and amplitude in Figures 1 and 2. These graphs are calculated from responses made within a period of 1450ms from CS onset, thus if there were any long latency responses similar to those reported by Welsh and Harvey (1989) they have shown up on these graphs. But, there were no long latency responses at all.

Group B

Neither animal in this group showed any signs of learning when trained on the side of the cerebellar lesion. No more than 5% responses were made during the ISI in any session of post-operative training on the lesioned (right) side. However, when trained on the intact (left) side (sessions 16-20) both subjects rapidly acquired CRs. When training was resumed on the lesioned side there were once again no CRs (see Figures 1 and 2).

NM and OO response testing

Groups A and B

CRs

OO EMG activity was recorded to test whether conditioned eyeblink responses were abolished by the lesions or whether there was a motor deficit that simply weakened the contraction of the retractor bulbi muscles so impairing eyeball retraction and movement of the NM.

The results from Group A and B subjects were similar. During this session, as in all of the preceding sessions there was no evidence of NM CRs in any subject. There was also no evidence of OO EMG activity related to the CS on either paired or CS alone trials in any subject. Examples of NM and OO EMG responses recorded in subject C1502 from group A are shown in Figure 3. This figure demonstrates that there were no anticipatory NM or OO EMG responses on the paired trials, and no NM responses at all were made on the CS alone trials. EMG activity was clearly unrelated to the stimulus on this trial. In all subjects the onset of OO

EMG activity to the US preceded the onset of the NM UR. This is shown in Figure 3. On the paired trial (trial 9), OO EMG activity began 5ms after the US onset and the NM response began 17ms after the US onset.

URs

Welsh and Harvey (1989) argued that CRs are impaired by cerebellar lesions because they cause a deficit in eyeblink motor pathways. As a test for possible motor deficits, URs were compared on paired and US alone trials to determine whether they were enhanced in the presence of the CS as has been shown in normal naive rabbits (Ison and Leonard, 1971). If the lesions had caused a motor deficit then the CS would be prevented from exciting the motor pathways.

In order for the test to be sensitive to any motor deficits a very low intensity US was used. The CS was the same as that used in conditioning. Its intensity was low enough to be below threshold for eliciting eyeblinks as shown by the absence of short latency responses when the CS was presented.

The main finding was that NM responses were larger on trials in which the US was paired with the CS than on US alone trials in all lesioned subjects (see Figures 4 and 5). Responses on paired trials (3rd trace in Figures 4,5) immediately following US alone trials (2nd trace) were always smaller than those elicited by paired trials (1st trace) that preceded the US alone trials. So the order of presentation of the trial types affected the response. However, an examination of the averages for every fourth trial (paired) showed that this response was always as large or larger than responses on the first paired trial. Thus it is clear that the enhancement of responses on paired trials was due to presentation of the CS and not simply due to an order effect. It is not clear why responses on paired trials immediately following the US alone trials were smaller.

Differences were found in OO EMG activity on paired and US alone trials. In subjects C1504 (from group A) and C1511 (from group B) OO EMG activity in the period 6.5ms-13ms after US onset was greater on paired trials than on US alone trials. This enhancement of short latency OO EMG activity is illustrated in Figure 6 (at 356.5-363ms). In other subjects these early responses were more variable in size. In subject C1505 they were smaller on paired trials and in subject C1506 there was no difference between responses on paired and US alone trials. Differences were seen in the amplitude of longer latency responses. A group of responses at 15-25ms after US onset were mostly smaller on paired trials (see Figure 6). A longer latency group of responses generally occurred at about 35-60ms and these were enhanced on paired trials. This is illustrated in Figure 6 in which responses with a latency between 35-60ms (385-410ms in Figure 6) on paired trials (1st and 3rd average) were larger than on US alone trials (2nd average). Because the latencies of these responses were more variable than early responses, signal averaging may not accurately reflect this activity. But inspection of individual responses from paired and US alone trials confirmed that these later responses were enhanced on paired

trials in all subjects. This was the case whether the CS was present throughout the period of these responses (terminated 60ms after US onset) or whether the CS was terminated prior to US onset.

Group AC

Throughout the final session, subjects C1515 and C1516 gave vigorous anticipatory NM and external eyelid CRs on both paired and CS alone trials. An example of NM and external eyelid CRs from subject C1516 is shown in Figure 7. Both NM and OO EMG records are taken from the same paired trial. On this trial OO EMG activity preceded the NM CR by 20ms. This result was found in both subjects in all trials in which a CR was given. In subject C1516 there was always a delay of about 20ms between the onset of OO CRs and NM CRs, but in subject C1515 the difference in latencies was often 50ms or more.

In subjects C1515 and C1516 NM CRs were at least twice the size of URs. The peak velocity of CRs and URs were similar. Table 2 shows the average peak amplitude and peak velocity of 5 CRs and 5 URs recorded in these subjects on trials in which the CS and US were presented alone. Figure 8 shows the average of 5 CRs and 5URs from subject C1516.

Table 2.

Subject	Peak amplitude (mm)		Peak velocity (v/s)	
	CR	UR	CR	UR
C1515	3.91	1.19	19.53	17.09
C1516	4.54	1.79	24.4	21.97

On paired trials, when CRs were given, OO EMG responses were severely reduced in the period 6-18ms following the US onset. Another group of responses generally occurred at 20-30ms, these were either unaffected or enhanced. This is illustrated in Figure 9. On paired trials (1st trace), there is a marked decrease in responses in the period 6-18ms after US onset (at 356-368ms). By inspection of the second average, from US alone trials, this period clearly corresponds to the short latency response of the UR. On CS alone trials (3rd trace) during the production of CRs there is no loss of responses occurring at this time (at about 355ms). A similar reduction in OO EMG activity can also be seen in Figure 7 in the period 356-365ms.

NM UR testing

Group A

The reflex response of each subject was tested at different US intensities ranging from 0.25mA to 2.0mA.. There was virtually no variation in the response topography at each stimulus level indicating that the technique of signal averaging would not be inappropriate for the analysis of this data.

The results for Groups A and AC are summarised in Figure 10 which shows the amplitude to the peak response, and in Figure 11 which shows the peak velocity. From these figures it can be seen that in the lesioned subjects responses on the right side were generally poorer than responses on the left side. Responses in the lesioned group were always smaller than responses in the non-lesioned group. Responses in the lesioned group showed an ordered decrease in amplitude and peak velocity as the US intensity was reduced. The non-lesioned group was different in this respect. At 0.75mA a longer latency response was made in addition to the original short latency UR. By 0.375mA this longer latency response had increased dramatically in amplitude whereas the shorter latency response had disappeared.

Histological verification of lesions

Injections of KA into the cerebellar nuclei caused a loss of Purkinje cells in rostral areas of the cerebellar cortex and in rostral parts of the cerebellar nuclei in all subjects. The regions of cell loss in all subjects are shown in Figures 13A-!3E for Group A subjects, and Figures 13F-13G for Group B subjects. All subjects had substantial loss of Purkinje cells in the base of lobule HVI, in the base of the Ansiform lobule and in dorsal parts of lobule HIV/V. There was a complete loss of cells within anterior parts of the interpositus and dentate nuclei in all subjects. The loss of cells was most prominent at the border between lateral parts of the interpositus nucleus and medial parts of the dentate nucleus. In Group A subjects, all except C1506 had lesions in the equivalent parts on both sides of the cerebellum. Lesions in subject C1506 were less extensive on the left side, but the greatest damage was to crucial parts of the dentate/interpositus border at 3mm caudal to lambda. Lesions of the cerebellar nuclei in subject C1510 of Group B were less extensive than those of subject C1511 but in both cases crucial regions of the dentate/interpositus border at 3mm caudal to lambda were lesioned. In no subject was there any evidence of retrograde degeneration in the inferior olive or any other pre-cerebellar nucleus.

DISCUSSION

Unilateral kainic acid lesions of the cerebellar nuclei and overlying cortex prevented the acquisition of CRs on the side of the lesion. Bilateral lesions of the cerebellar nuclei and overlying cortex caused a complete loss of CRs which had been previously established. Neither extended post-operative time nor extensive post-operative training facilitated a recovery of CRs over the 9 months of testing. URs elicited to a range of US intensities were different from those given by non-lesioned control subjects. URs of lesioned subjects were typically smaller and of lower average and peak velocity than in the control subjects. There was no evidence to support the idea that this slight difference in URs was due to a motor deficit.

KA injections removed the output of the cerebellum

There was considerable damage to the cerebellar nuclei and overlying cortex in all subjects. It is possible that some of the results in this study were due to a loss of input from the cerebellar cortex to the remaining nuclear cells. There are two indications that this is not the case. Firstly, lobules HVI and HVIIA, regions of the cerebellar cortex known to be essential for retention of CRs, had some loss of PCs but in no case was the loss of PCs in these lobules complete. Previous studies have shown that only in subjects with a complete loss of PCs in lobules HVI and HVIIA was there an enduring loss of CRs (Hardiman and Yeo, 1992; Yeo and Hardiman, 1992). Secondly, the loss of PCs was confined to the lobules overlying lesioned regions of the cerebellar nuclei, so the cortical damage could have little effect upon cerebellar output as the greater part of the nuclear target of these regions was also destroyed. The primary effect of the lesions was to remove the output of the cerebellum through loss of anterior interpositus and dentate nuclei.

Unilateral lesions of the cerebellar nuclei prevent acquisition of CRs

In two previous studies it has been shown that unilateral lesions of the cerebellar nuclei are sufficient to prevent acquisition of CRs, however in one study (Lavond *et al.*, 1984) the presence of CRs was measured only during paired trials, and in the other study (Sears and Steinmetz, 1990) the subjects were allowed only a short period of post-operative recovery and retraining. In the present study there was no acquisition of CRs on either paired or unpaired trials even after extensive postoperative recovery and training.

Training on the side contralateral to the lesions showed that although CRs were not present on initial trials, they were acquired very rapidly. When training was resumed on the lesioned side there were still no CRs. Training on the contralateral side had not facilitated a recovery of CRs on the lesioned side. There was no evidence for a central site for eyeblink conditioning, either afferent to or efferent from the cerebellum.

Bilateral lesions of the cerebellar nuclei abolish CRs

The loss of CRs described in this study is similar to that found in earlier studies in which unilateral (McCormick *et al.*, 1982; McCormick & Thompson, 1984; Clark *et al.*, 1984; Yeo *et al.*, 1985) lesions of the cerebellar nuclei were made. These data argue against the presence of delayed CRs masked by the UR as suggested by Welsh and Harvey (1989).

All subjects were given a similar amount of pre-lesion training to that used by Welsh and Harvey (1989) in order to see if extended pre-lesion training prevented the loss of CRs or promoted recovery of CRs. In contrast to the results of Welsh and Harvey (1989), none of the subjects in this study gave any CRs after lesions of the cerebellar nuclei.

Because NM movement occurs passively as a consequence of eyeball retraction (Motais,1885) measurements of NMRs may not accurately reflect the effect of brain lesions, particularly if the

motor system has been damaged by the lesions. As external eyeblinks are closely associated with NMRs (McCormick et al., 1982) recordings of OO EMG were made as an additional and potentially more accurate test for the presence of CRs. None of the lesioned subjects had EMG activity related to the CS.

In other studies, lesions restricted primarily to the anterior interpositus nucleus also produced profound losses of CRs, but subjects in these studies were not trained extensively after the lesions (Yeo *et al.*, 1985; Lavond *et al.*, 1985). The most likely explanation for the more complete loss of CRs in this current study than Welsh and Harvey (1989) is because of the greater damage to the cerebellar nuclei. The critical additional damage may have been to the ipsilateral or contralateral cerebellar nuclei, although recently Steinmetz *et al.* (1992) showed that unilateral lesions of the cerebellar nuclei were sufficient to abolish CRs in rabbits trained with the same procedures as Welsh and Harvey (1989). There is now sufficient evidence to suggest that the variable effect upon CRs in the study of Welsh and Harvey (1989) was due to incomplete lesions of the cerebellar nuclei.

Bilateral lesions of the cerebellar nuclei cause a deficit in NM URs

Reflex responses in the non-lesioned group differed from responses in the lesioned group. In intact subjects as the US intensity was reduced the normally evoked short latency response declined but there was a long-latency response to weak stimuli. These long-latency responses were elicited by a US that was sub-threshold for activating short latency blink responses but not for long latency blink responses that are possibly voluntary in origin. This longer latency response was not seen in earlier studies where the reflex responses of normal subjects were tested (Yeo and Hardiman, 1992; Hardiman and Yeo, 1992) and is probably a consequence of the extended training used in this present study. Lesioned subjects did not produce long latency URs. Because of this difference it was not possible to compare accurately URs elicited by low stimulus intensities in lesioned and non-lesioned subjects. However, at higher US intensities the URs in lesioned subjects were smaller and achieved lower average and peak velocities than non-lesioned subjects.

Welsh and Harvey (1989) reported that unilateral lesions of the anterior interpositus nucleus affected the timing of URs, but only at very low US intensities. Steinmetz *et al.* (1992) reported that unilateral lesions of the cerebellar nuclei had no effect upon the URs. In this current study URs of lesioned subjects were different to those of normal subjects, a deficit in URs being apparent at higher US intensities. Both Welsh and Harvey (1989) and Steinmetz *et al.* (1992) used an air puff US whereas an electrical US was used in this study. As the strengths of US cannot be easily equated between the different types of USs the results of these studies cannot be easily compared.

The finding of a small deficit in the UR in this study is consistent with the idea that the cerebellum exerts some control over URs as well as CRs. Evidence for a cerebellar control of URs comes from studies in which stimulation of sites within the cerebellar cortex (Hesslow,

Together with the findings of Weisz and LoTurco, (1988) this study provides evidence for a tone pathway to the eyeblink motorneurons that is independent of the cerebellar circuit. Tones used in conditioning do not produce activation of motorneurons in the VII, VI or accVI nuclei (Cegavske et al., 1979, 1987; Disterhoft et al., 1985) so it is unlikely that there is a direct route from auditory structures to the eyeblink motorneurons. But, a more direct input to motorneurons of tone information than through the cerebellar circuitry could mean that the CS/US association essential for learning is made at the motorneurons. This possibility is unlikely since the same association would need to be made at the nuclei of the VII, VI and accVI, and possibly at the motorneurons of those extraocular muscles that contribute to the conditioned NMR (Murphy et al., 1986). A less complicated mechanism of CR production would involve learning the association before the motorneurons and distributing the CR command to the necessary motorneurons.

1989, 1994a,1994b), cerebellar nuclei (McCormick and Thompson, 1984) and red nucleus (Chapman et al., 1988; Haley et al., 1988) , all cause eyeblinks. Since the cerebellum normally modulates the UR, lesions of the cerebellar nuclei would be expected to decrease this modulation of UR pathways.

Eyeblink motor pathways are excitable after cerebellar lesions

An examination of CS and US driven NM and OO responses provided evidence that the loss of CRs is unlikely to be due entirely to a loss of a general activation of the eyeblink motor pathways as suggested by Welsh and Harvey, (1989) and Kelly *et al.*, (1990).

In naive rabbits paired presentations of a CS and US cause facilitation of NM (Ison and Leonard, 1971; Weisz and LoTurco,1988) and OO (Baker *et al.*, 1992) URs. This facilitation is evident on initial pairings of the CS and US, and is present well before learning has occurred - or at least before there are overt signs of learning (ie. CRs). Weisz and LoTurco (1988) measured URs before and after unilateral lesions of the cerebellar nuclei and found that the lesions did not effect reflex facilitation. In this study, URs were not examined on initial conditioning trials, although subject C1511 from Group B may be considered as naive as this subject did not acquire CRs. When examined on the final session of training all lesioned subjects showed facilitation of NM and OO EMG reflex responses by the CS.

Two hypotheses can account for the facilitation URs in these subjects. Firstly, the UR is enhanced by summation of activity driven by the **CS** and US within the reflex circuit. This hypothesis assumes that there is a CS pathway into the reflex circuit that is independent of the cerebellum and is therefore unaffected by cerebellar lesions. Alternatively, the enhanced response may be due to summation of **CR** and US activity. This hypothesis is based upon the idea that lesions of the cerebellar nuclei cause a loss of excitability in eyeblink motor pathways such that CRs produced in a circuit independent of the cerebellum are prevented from activating this pathway. On paired trials the powerful excitation of this pathway by the US allows the blocked CR to now gain access resulting in an enhanced UR.

There is no firm evidence to support either view. However, there is some indication that the first hypothesis is correct. Firstly, it is likely that blink reflexes are facilitated by the same mechanism in naive and lesioned rabbits. In normal rabbits reflex facilitation can occur even on the first paired trial and is therefore at this time independent of conditioning. Thus the enhancement of URs at this time cannot be caused by summation of CR and US inputs within the reflex circuit. Secondly, the alternative hypothesis assumes that USs activate the eyeblink motor pathways more powerfully than CSs. Welsh and Harvey, (1989) argued that this was the case, as in normal subjects the US always produced larger URs than CRs. Consequently, CRs are more liable to disruption than URs to disturbances of the motor system. This hypothesis was tested by the use of a very low intensity of US, which in normal subjects produced much smaller URs than CRs. By the argument of Welsh and Harvey the motor system should have

been excited more powerfully by CRs than by USs. However, even though all of the lesioned subjects produced URs to the weak US, none produced CRs. Thirdly, if the motor system were depressed by a general loss of activation, then this impairment would be most pronounced at very low intensity USs. But in this study, even though very low US intensities were used in the final session of training, both OO and NM URs were facilitated by the CS. Finally, in normal rabbits the duration of CRs is much longer than URs. Summation of these responses would likely result in a response with a long duration. But responses made during paired trials in lesioned subjects were similar in duration to URs elicited on unpaired US trials.

Eyeblink reflexes in lesioned rabbits are similar to naive rabbits

In normal subjects the short latency component of the OO reflex response was inhibited during the production of CRs. In contrast, lesioned subjects showed no such loss of activity, the R1 component of the UR was actually enhanced in some subjects as it is in naive rabbits.

The loss of OO EMG activity may simply be due to over excitation of neurons within the reflex pathway by temporal summation of CR activity and activity generated by the US, causing a refractory period (Furedy and Klajner, 1974). Ison *et al.*, (1990) argued against this explanation as it was found in humans that pairing tones and periorbital electrical stimuli in a Pavlovian delay conditioning design caused both reflex enhancement of the R1 and a learning related decline in the R2. Hammond and Plant (1993) showed that the R1 is actually enhanced in humans when elicited by closely timed paired periorbital electrical stimuli.

Alternatively, the loss of early responses may be due to an active inhibition of either sensory inputs to the reflex pathway, or motor output, during the production of CRs. It is unlikely that there was inhibition of the final motor elements because the longer latency responses were unaffected. It is more likely that there was an inhibition of sensory inputs. An inhibition of sensory components is consistent with the presence of an inhibitory pathway from the red nucleus to the spinal trigeminal nucleus pars oralis, as shown in cats (Davis and Dostrowski, 1986). A CR command from the cerebellum might activate a similar pathway in the rabbit causing inhibition of the trigeminal sub nucleus oralis, a region of the trigeminal involved in the NM reflex pathway (Richards *et al.*, 1991). This idea suggests that changes in URs following lesions of the cerebellum may be due to alterations in sensory components of the reflex and not, or not only, due to changes in activation of motor pathways (Yeo and Hardiman, 1990).

Thus the suggestion that eyeblinks elicited on paired trials in lesioned rabbits are similar to those in naive rabbits can be explained by the loss of CRs. Lesions of the cerebellum cause the loss of CRs and the loss of a learning related activity acting upon sensory inputs to the reflex pathway. These ideas are summarised in Figure 14.

Do lesions of the cerebellar nuclei cause a loss of learning or performance?

The loss of CRs seen in this study may be due to a permanent learning deficit, or a permanent motor deficit. It has been reported that in time, cats show a recovery of reflex activity and co-

ordinated limb movements after lesions of the cerebellar nuclei (Van der Meulen and Gilman, 1965). But, in this current study there was no recovery of conditioned movements in time or with training. When tested a considerable period of time after lesioning a small deficit was found in the URs. Welsh and Harvey, (1989) argued that an impairment of URs indicates that cerebellar lesions cause a general motor deficit, and not a deficit in learning. But both NM and OO responses to a very weak US were greatly enhanced by the CS. These data demonstrate that neither the NM nor the external eyeblink motor pathways were depressed sufficiently to account for the loss of CRs. These results are consistent with a loss of brain regions engaged in production of the CR and a loss of activity from these regions that normally modulates the UR. These results support the idea that within the cerebellum there are sites of learning for conditioned eyeblink responses, either in the cerebellar nuclei (McCormick and Thompson, 1984) or in the cerebellar cortex (Yeo *et al.*, 1985) or in both.

Figure 1

NMR frequency on CS alone trials.

Bilateral Lesion Group (Group A)

Pre-op training sessions 1-15, then surgery, then 11 weeks wait,

Post-op training sessions 16-30, 5 weeks wait, session 31, UR testing, 13 weeks wait, sessions 32-36.

Control Group (Group AC)

These subjects did not undergo surgery. The training schedule was the same as that of the Bilateral Lesion Group.

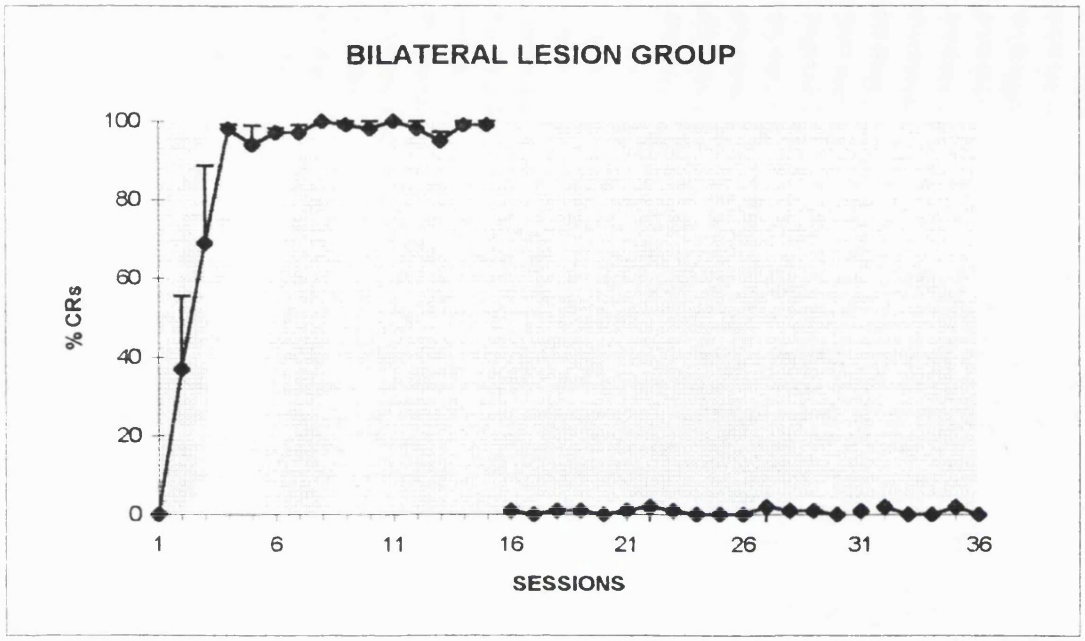
Unilateral Lesion Group (Group B)

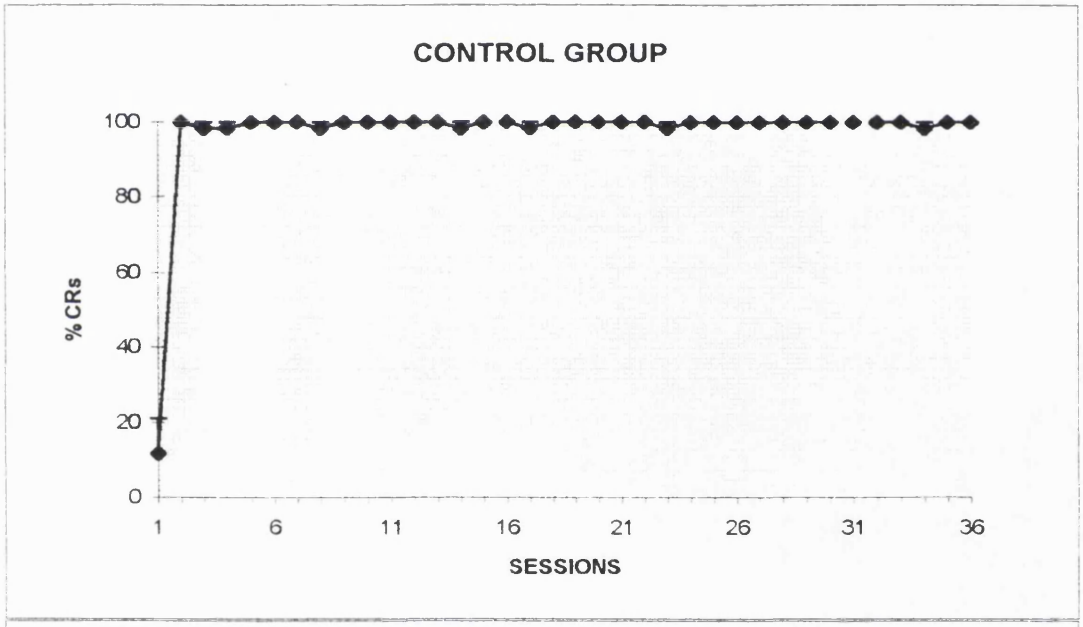
These subjects were not trained before lesioning the cerebellum.

Surgery, 11 weeks wait, sessions 1-15 training on right, sessions 16-20 training on left, 5 weeks wait, session 21, UR testing, 13 weeks wait, sessions 32-36.

The number of CRs for individual subjects in each group were averaged.

Error bars indicate standard error of the mean.


BILATERAL LESION GROUP
% CRs
100
80
60
40
20
0
1
6
11
16
21
26
31
36
SESSIONS


CONTROL GROUP
% CRs
100
80
60
40
20
0
1
6
11
16
21
26
31
36
SESSIONS

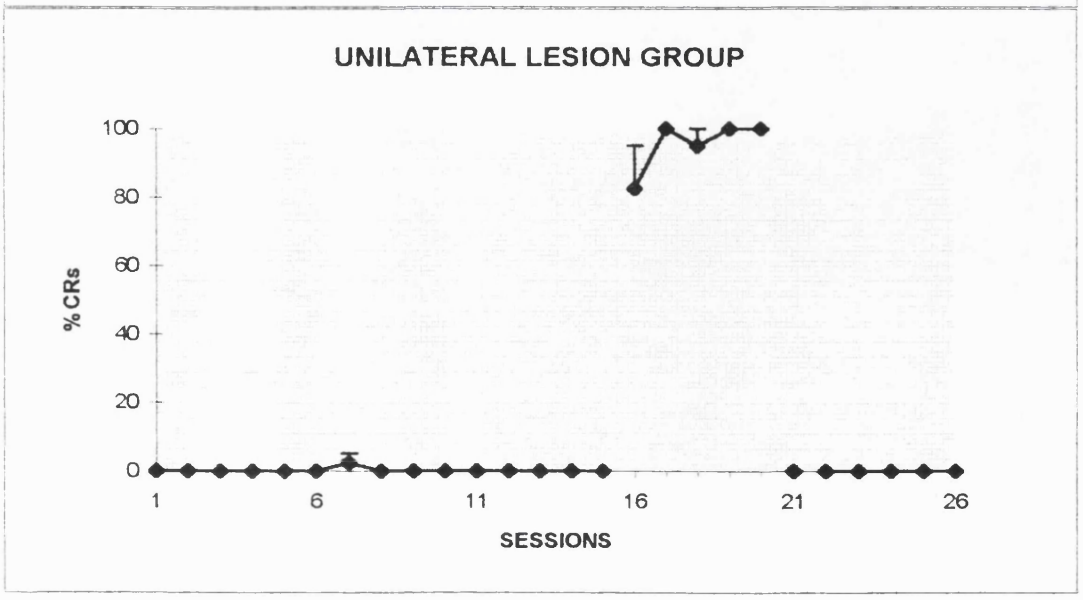

UNILATERAL LESION GROUP
% CRs
100
80
60
40
20
0
1
6
11
16
21
26
SESSIONS

Figure 2

NMR peak amplitude on CS alone trials.

Bilateral Lesion Group (Group A)

Pre-op training sessions 1-15, then surgery, then 11 weeks wait,

Post-op training sessions 16-30, 5 weeks wait, session 31, UR testing, 13 weeks wait, sessions 32-36.

Control Group (Group AC)

These subjects did not undergo surgery. The chronology of training was the same as for the Bilateral Lesion Group.

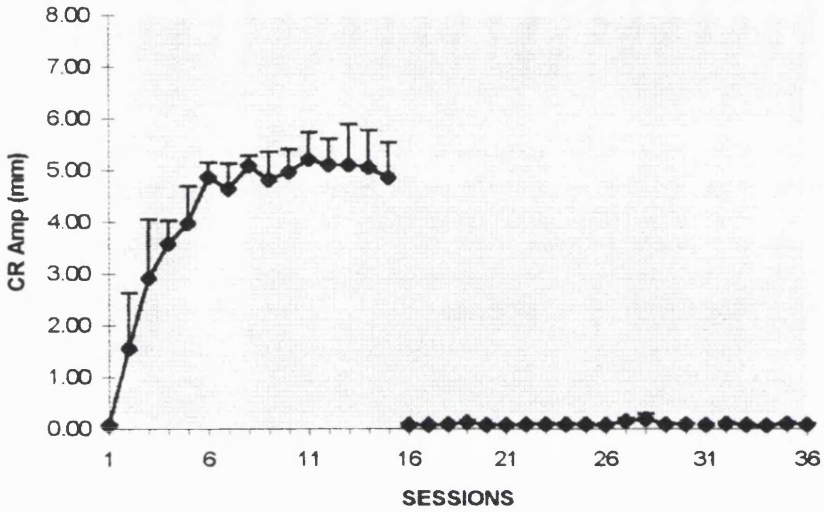
Unilateral Lesion Group (Group B)

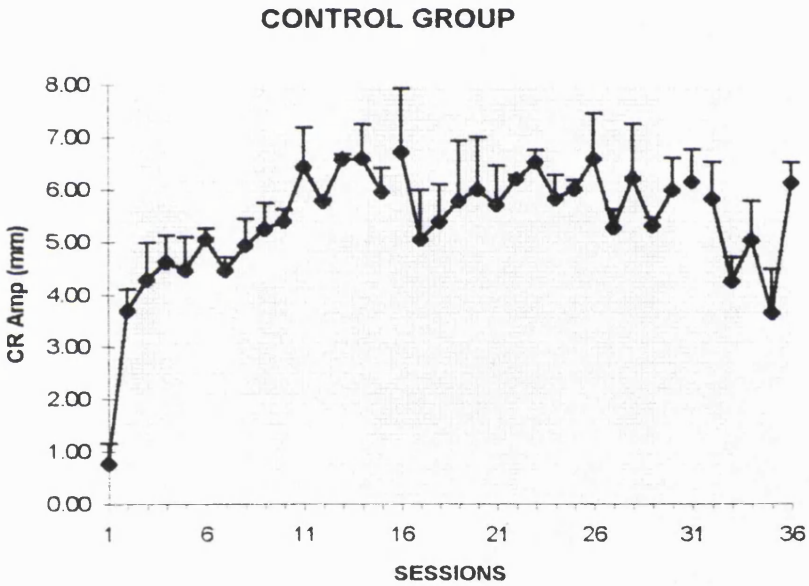
These subjects were not trained before lesioning the cerebellum.

Surgery, 11 weeks wait, sessions 1-15 training on right, sessions 16-20 training on left, 5 weeks wait, session 21, UR testing, 13 weeks wait, sessions 32-36.

The number of CRs for individual subjects in each group were averaged.

Error bars indicate standard error of the mean.


BILATERAL LESION GROUP
CR Amp (mm)
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
1
6
11
16
21
26
31
36
SESSIONS


CONTROL GROUP
CR Amp (mm)
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
1
6
11
16
21
26
31
36
SESSIONS

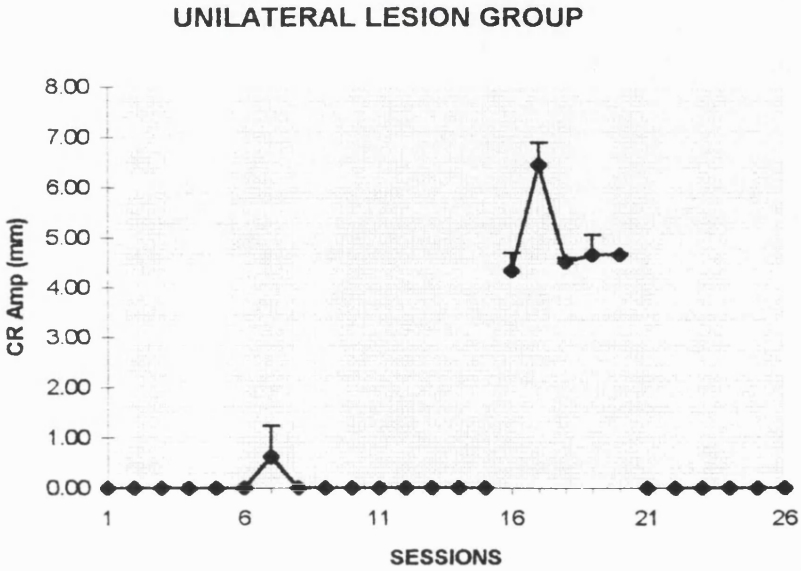

UNILATERAL LESION GROUP
CR Amp (mm)
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
1
6
11
16
21
26
SESSIONS

Figure 3

Bilateral Lesion subject C1502.

The responses shown in this figure were recorded on trial 9 (paired) and 10 (probe) of the final session of training (session 37). OO EMG activity and NMRs were recorded simultaneously. On trial 9 the CS and US were paired. CS onset at 50ms, CS offset at 360ms, US onset at 300ms. On trial 10 only the CS was presented. CS onset at 50ms and CS offset at 360ms.

There were no CRs on either trial. On trial 10 (paired) the OO reflex response preceeded the NM reflex response by 12ms.

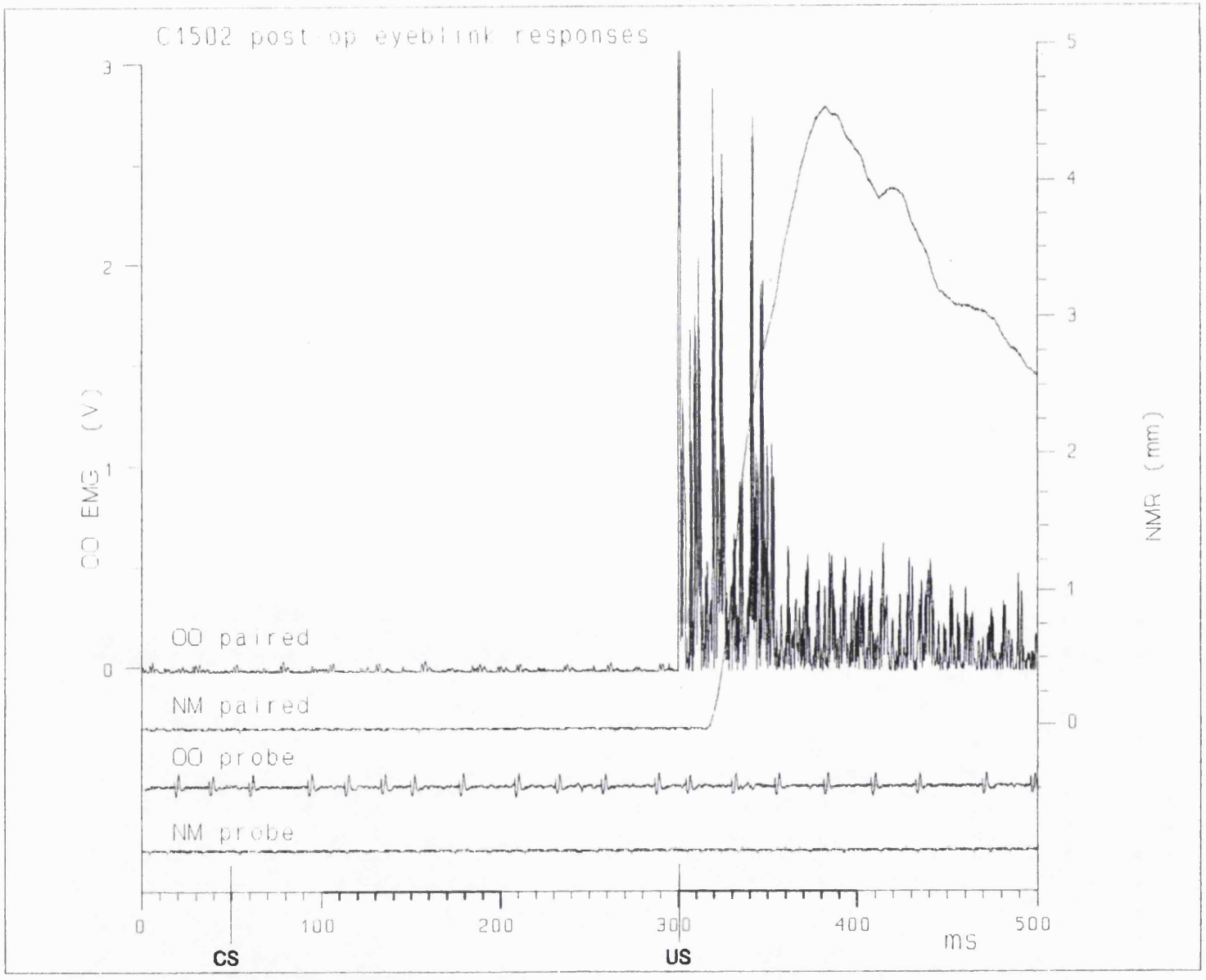

C1502 post-op eyeblink responses
OO EMG (V)
NMR (mm)
OO paired
NM paired
OO probe
NM probe
CS
US
ms
0
100
200
300
400
500
0
1
2
3
0
1
2
3
4
5

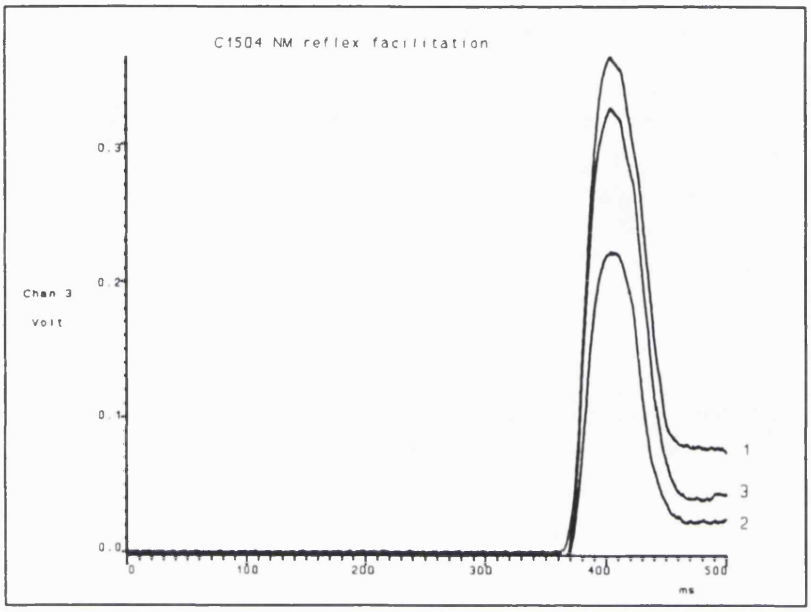
Figure 4

These figures show NM responses from Bilateral Lesion subjects C1504 and C1505 recorded on the final session of training. Trials in this session were presented in a repeating sequence: paired, US alone, paired, paired, CS alone. Each trace is the average response from 4 trials.

Paired - CS onset at 100ms, CS offset at 410ms, US onset at 350ms.
US alone - US onset at 350ms.

Trace 1 - average of the first trial in the sequence ie. paired
Trace 2 - average of the second trial in the sequence ie. US alone
Trace 3 - average of the third trial in the sequence ie. paired

In both subjects the NMRs on paired trials were larger than NMRs on US alone trials.


C1504 NM reflex facilitation
Chan 3
Volt
0.3
0.2
0.1
0.0
0
100
200
300
400
500
ms
1
3
2

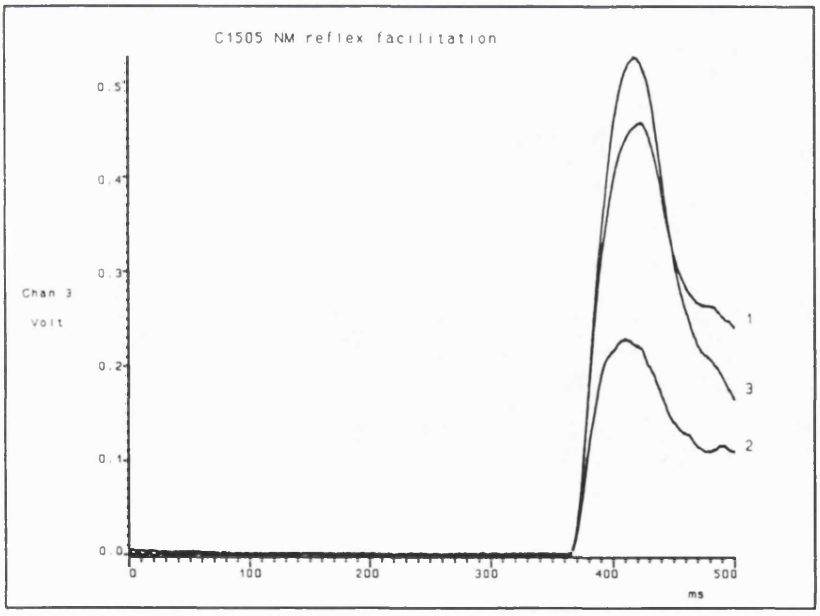

C1505 NM reflex facilitation
Chan 3
Volt
0.5
0.4
0.3
0.2
0.1
0.0
0
100
200
300
400
500
ms
1
3
2

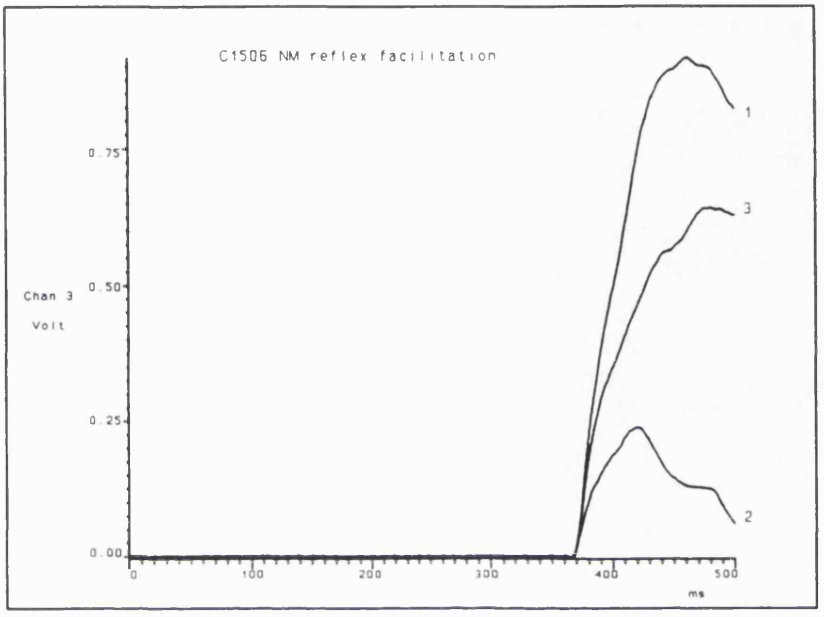
Figure 5

These figures show NM responses from subject C1506 (Bilateral Lesion) and from subject C1511 (Unilateral Lesion) recorded on the final session of training. Trials in this session were presented in a repeating sequence: paired, US alone, paired, paired, CS alone. Each trace is an average of the responses on 4 trials.

Paired - CS onset at 100ms, CS offset at 410ms, US onset at 350ms.
US alone - US onset 350ms.

Trace 1 - average of the first trial in the sequence ie. paired
Trace 2 - average of the second trial in the sequence ie. US alone
Trace 3 - average of the third trial in the sequence ie. paired

In both subjects the NMRs on paired trials were larger than NMRs on US alone trials.


C1506 NM reflex facilitation
Chan 3
Volt
0.75
0.50
0.25
0.00
0
100
200
300
400
500
ms
1
3
2

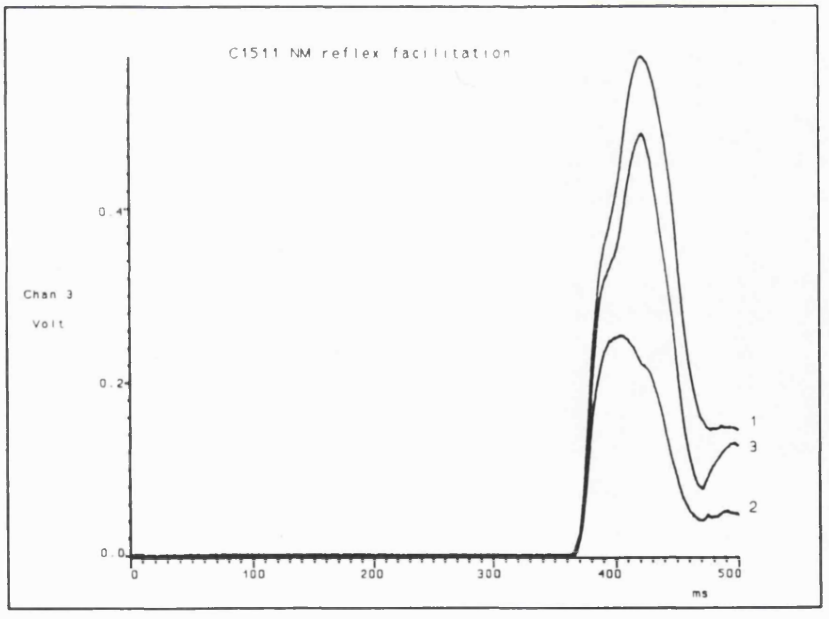

C1511 NM reflex facilitation
Chan 3
Volt
0.4
0.2
0.0
0
100
200
300
400
500
ms
1
3
2

Figure 6

Unilateral Lesion subject C1511.

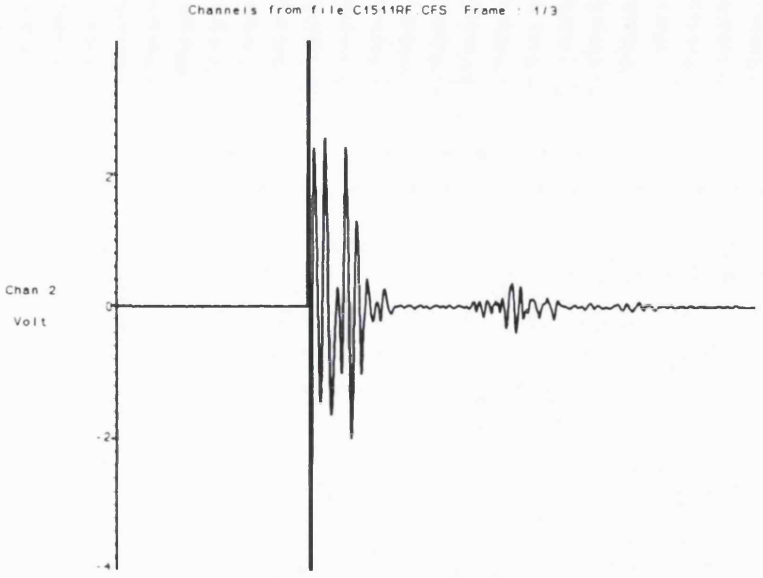
OO EMG response recorded on the final session of training in paired and US alone trials. Trials in this session were presented in a repeating sequence: paired, US alone, paired, paired, CS alone. Each trace is an average of responses on 4 trials.

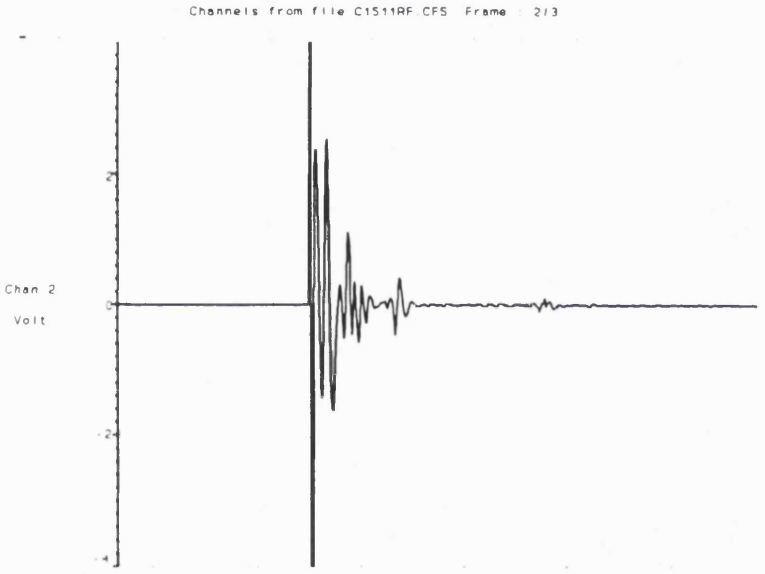
Paired - CS onset at 100ms, CS offset at 410ms, US onset at 350ms.
US alone - US onset at 350ms.

Top trace	- average of the first trial in the sequence ie. paired
Middle trace	- average of the second trial in the sequence ie. US alone
Bottom trace	- average of the third trial in the sequence ie. paired

There were no CS related responses in any paired trial.
Early responses, at 6-12ms after US onset, and late responses, at 35-45ms after US onset, are facilitated on paired trials.

On each trace the large response at US onset is stimulus artefact.


Channels from file C1511RF.CFS Frame : 1/3
Chan 2
Volt
2
0
-2
-4


Channels from file C1511RF.CFS Frame : 2/3
Chan 2
Volt
2
0
-2
-4

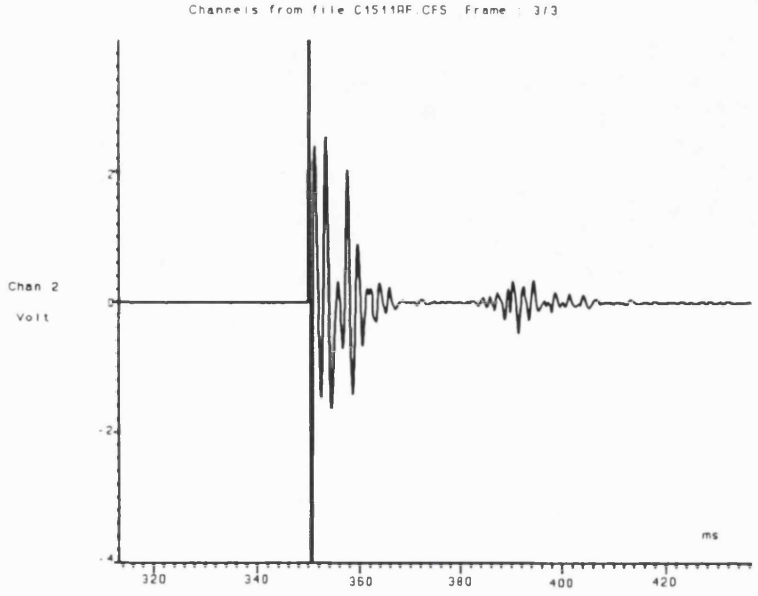

Channels from file C1511RF.CFS Frame : 3/3
Chan 2
Volt
2
0
-2
-4
ms
320
340
360
380
400
420

Figure 7

Control subject C1516.

The responses shown in this figure were recorded on trial 9 of the final session of training. On this trial the CS and US were paired. CS onset at 100ms, CS offset at 410ms, US onset at 350ms. OO EMG activity and the NMR were recorded simultaneously.

Both external eyelid and an NM responses are conditioned responses. The OO response preceeded the NM response by 25ms.

The large response at US onset is stimulus artefact.

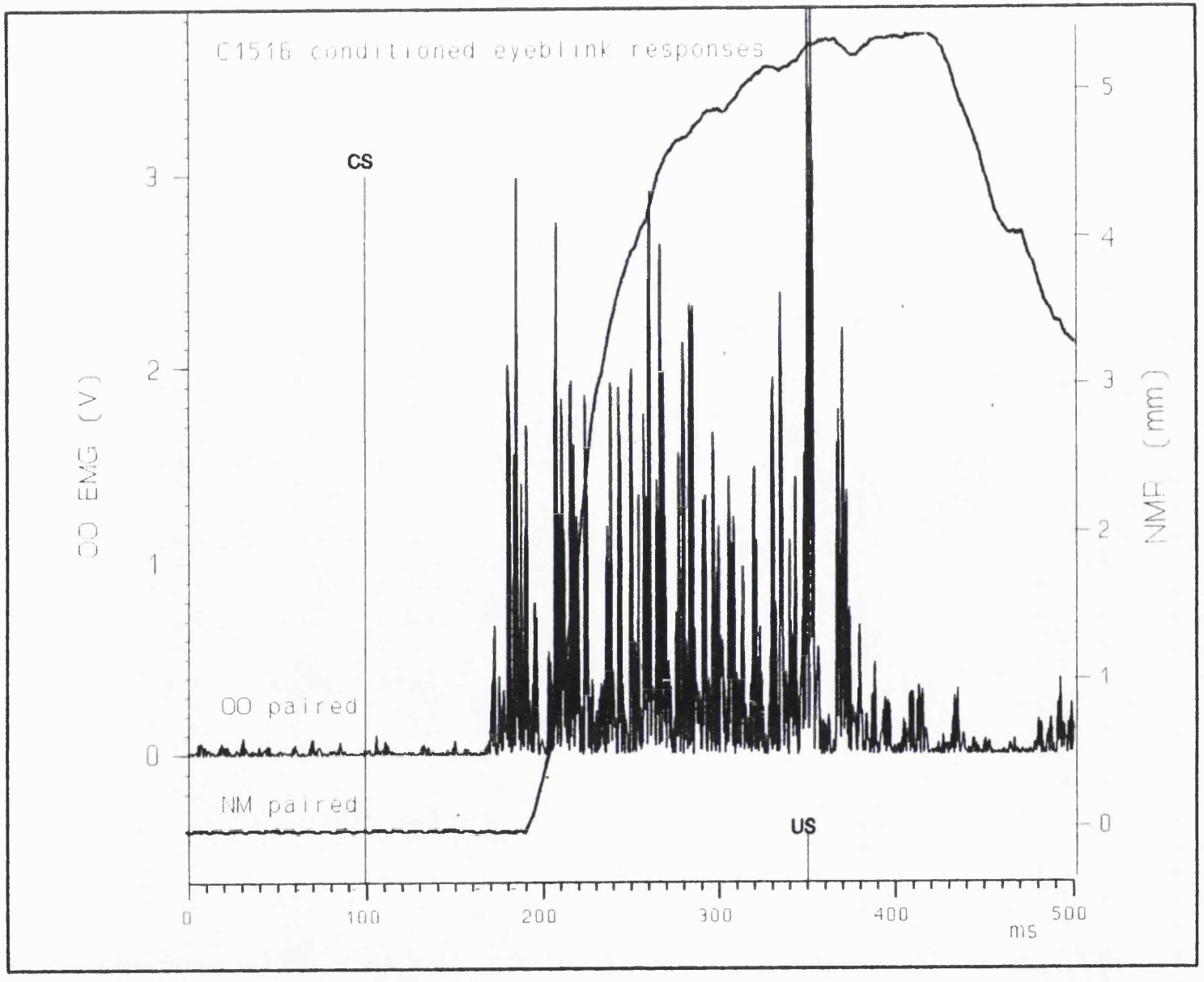

C1516 conditioned eyeblink responses
CS
US
OO paired
NM paired
OO EMG (V)
NMR (mm)
0
1
2
3
4
5
0
100
200
300
400
500
ms

Figure 8

Control subject C1516.

NM responses recorded on the final session of training. CS onset at 100ms, offset at 410ms, intensity 87 dBA, US onset at 2x 350ms, duration 350μs, intensity 5mA. Each trace is the average of responses on 5 CS alone trials (CR) and 5 US alone trials (UR).

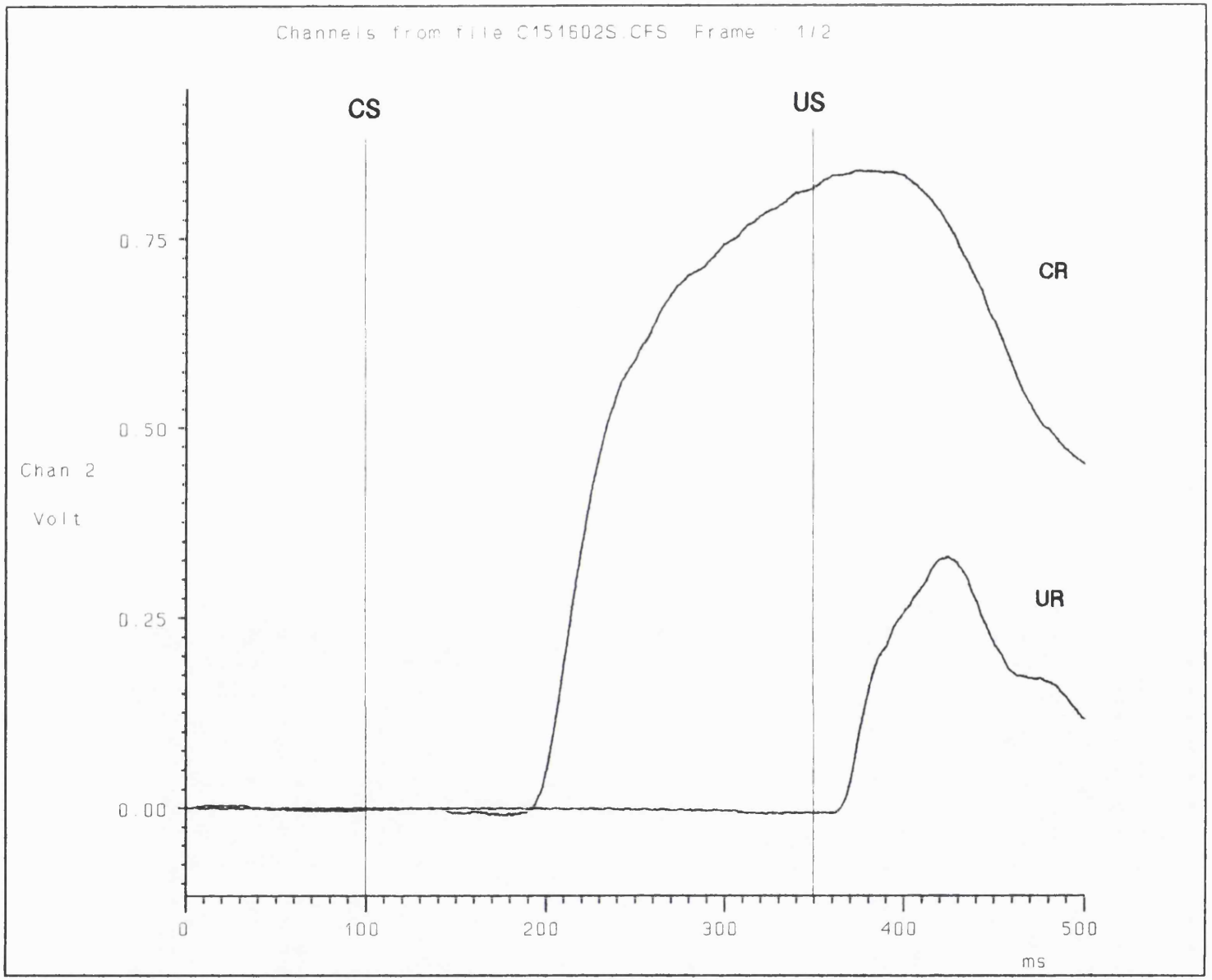

Channels from file C151602S.CFS Frame 1/2
CS
US
CR
UR
0.75
0.50
0.25
0.00
Chan 2
Volt
0
100
200
300
400
500
ms

Figure 9

Control subject C1516.

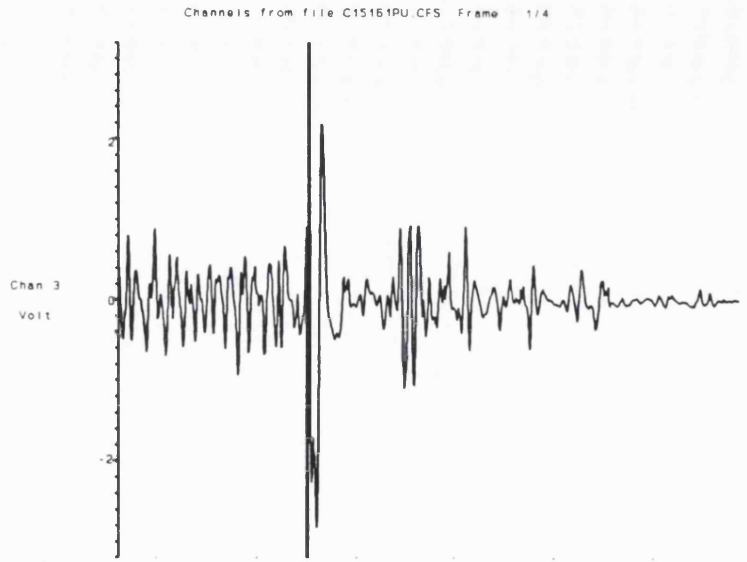
OO EMG responses recorded on the final session of training in paired, US alone and CS alone trials. Each trace is an average of responses on 4 trials.

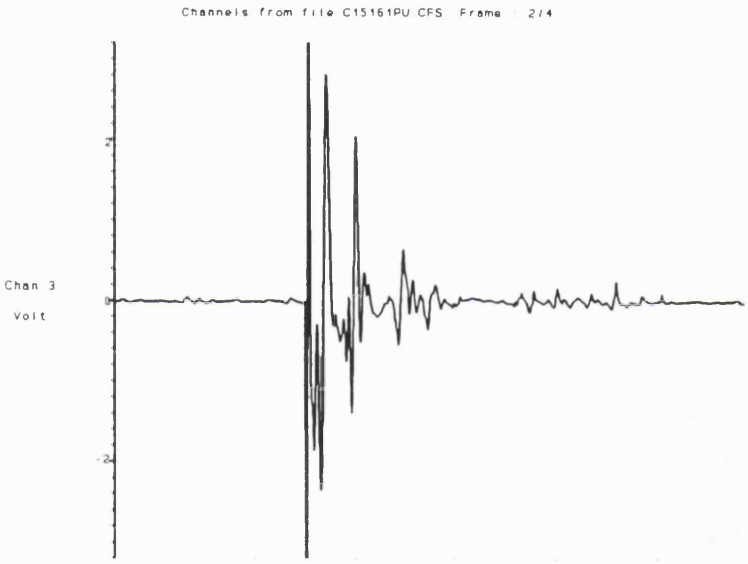
Paired - CS onset at 100ms, CS offset at 410ms, US onset at 350ms.
US alone - US onset at 350ms.
CS alone - CS onset at 100ms, CS offset at 410ms.

Top trace	- average of the first trial in the sequence ie. paired
Middle trace	- average of the second trial in the sequence ie. US alone
Bottom trace	- average of the fifth trial in the sequence ie. CS alone

There is a loss of OO EMG activity during CRs. The top trace shows CR activity preceeding the US. There is no EMG activity between 6 and 14ms after the US onset. As shown by the second trace this loss of activity occurs at the time of the R1 response. There is no corresponding loss of activity in the third trace taken from trials in which the CS was presented alone.

On each trace the large response at US onset is stimulus artefact.


Channels from file C15161PU.CFS Frame 1/4
Chan 3
Volt
2
0
-2


Channels from file C15161PU.CFS Frame 2/4
Chan 3
Volt
2
0
-2

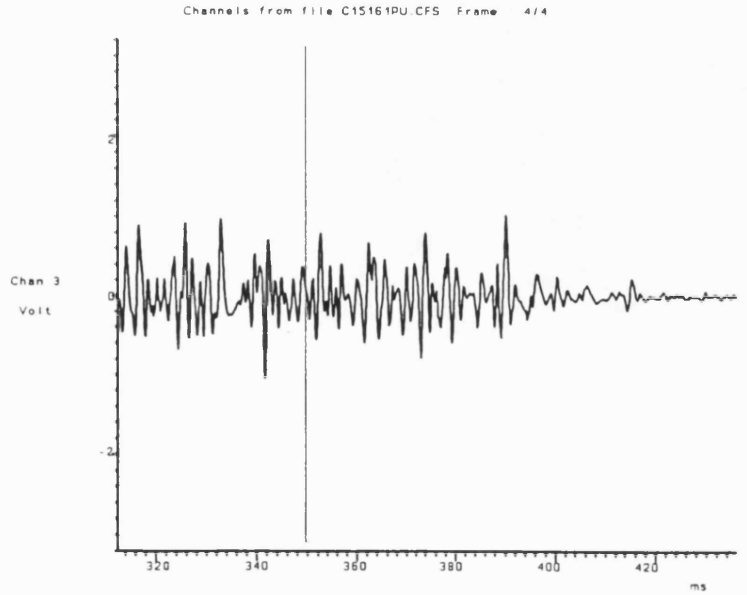

Channels from file C15161PU.CFS Frame 4/4
Chan 3
Volt
2
0
-2
320
340
360
380
400
420
ms

Figure 10

Peak amplitude of reflex responses to US intensities ranging from 2mA-0.25mA.

No symbol :	Bilateral Lesion Group subjects right side
Solid :	Bilateral Lesion Group subjects left side
Diagonals :	Control Group right side
Squares :	Control Group left side

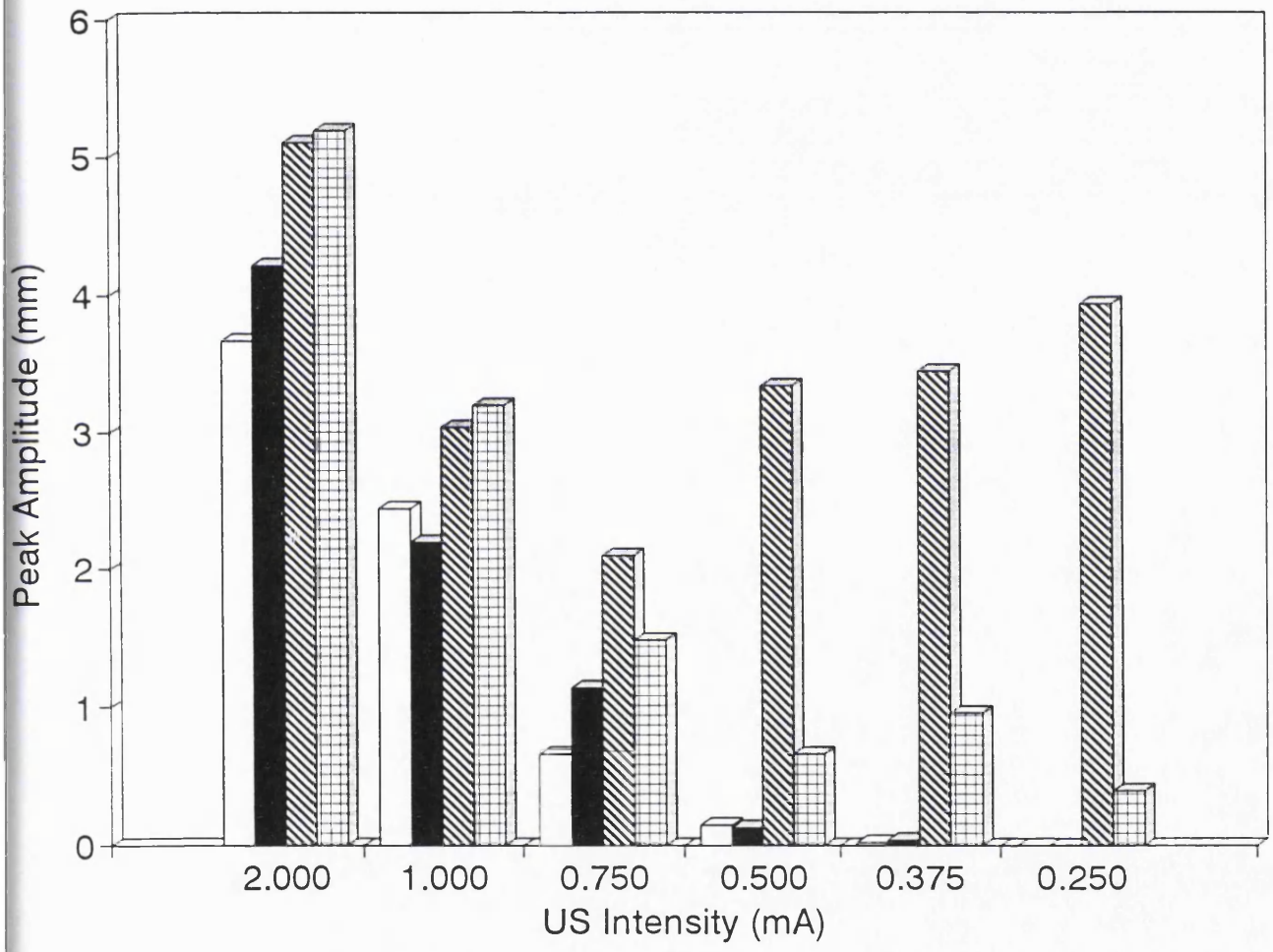

6
5
4
3
2
1
0
Peak Amplitude (mm)
2.000
1.000
0.750
0.500
0.375
0.250
US Intensity (mA)

Figure 11

Peak velocity of reflex responses to US intensities ranging from 2mA-0.25mA.

No symbol :	Bilateral Lesion Group subjects right side
Solid :	Bilateral Lesion Group subjects left side
Diagonals :	Control Group right side
Squares :	Control Group left side

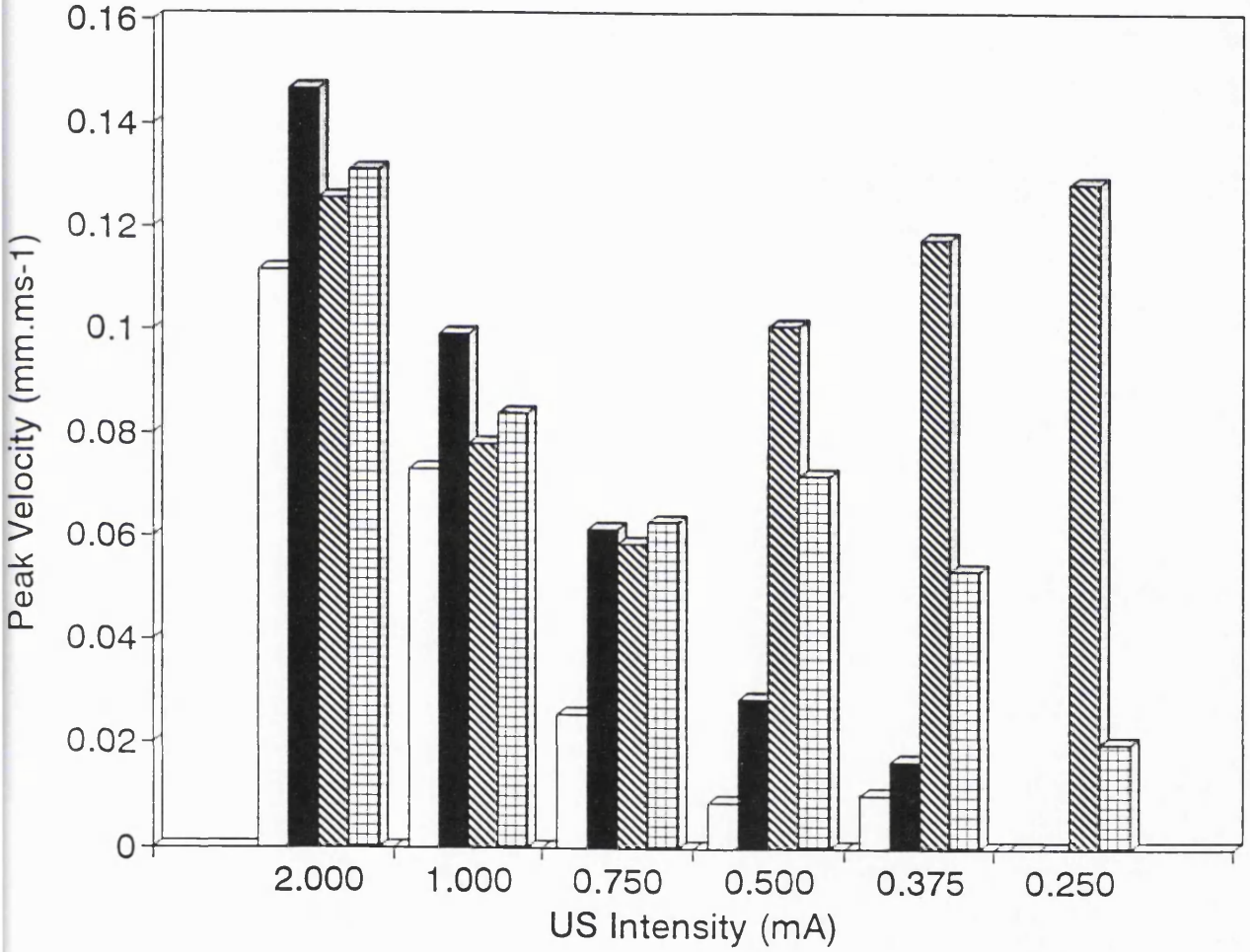

0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Peak Velocity (mm.ms-1)
2.000
1.000
0.750
0.500
0.375
0.250
US Intensity (mA)

Figure 12

Key to standard sections through the cerebellum

crI and crII	crus I and crus II of the ansiform lobe
DPFL	dorsal paraflocculus
FL	flocculus
HIV-V	hemispheral lobule IV/V
HVI	hemispheral lobule VI
ND	dentate nucleus
NI	interpositus nucleus
NF	fastigial nucleus
PM	paramedian lobe
PCS	superior cerebellar peduncle
PFL	paraflocculus
I-VI	lobules I-VI of the vermis

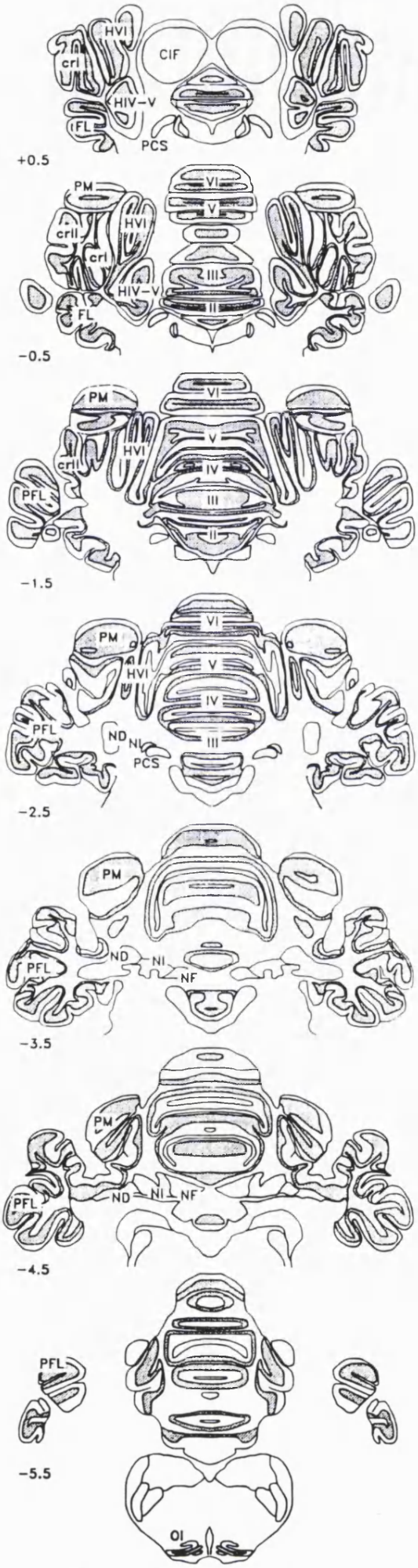
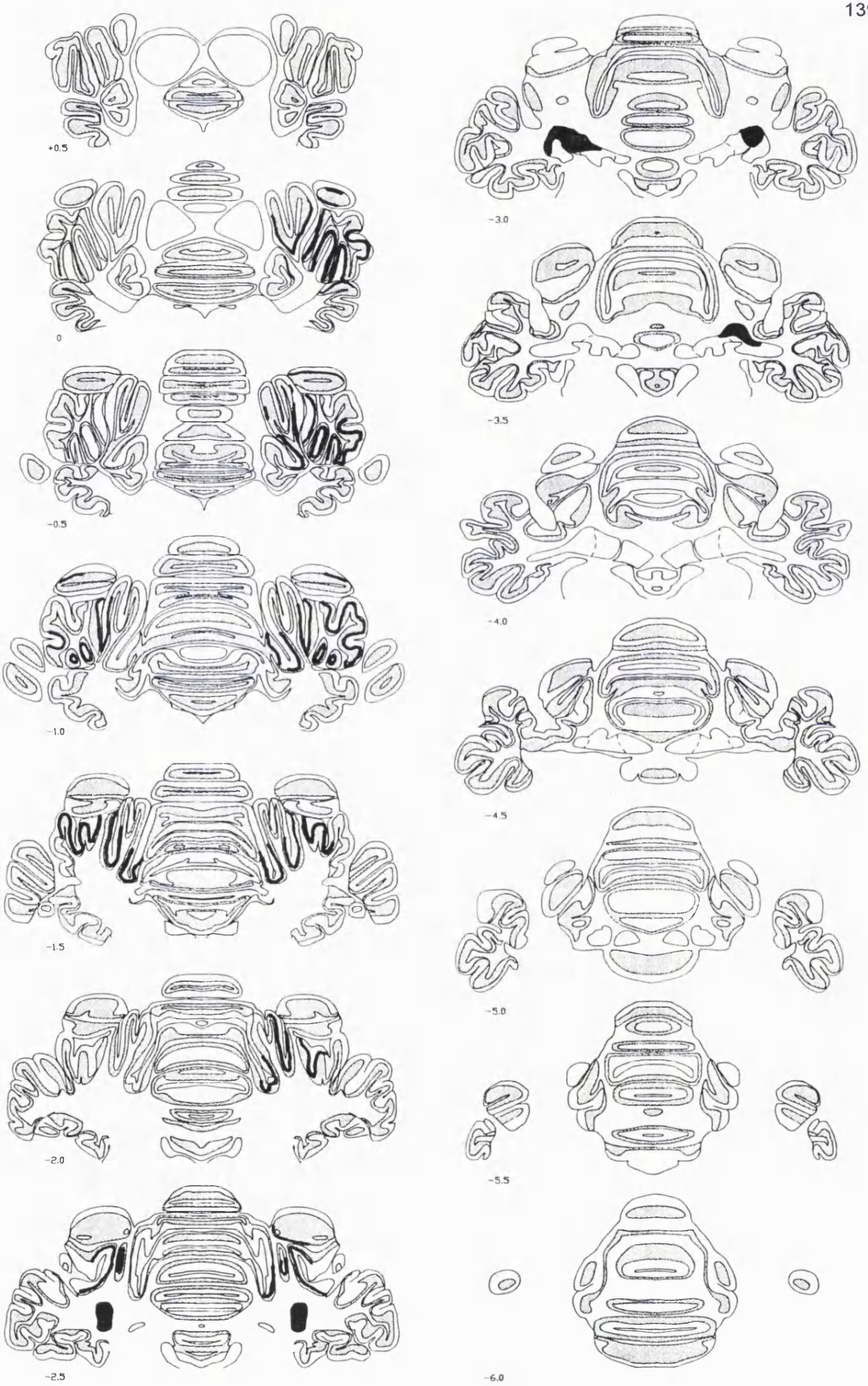

HVI
CIF
crI
HIV–V
IFL
PCS
+0.5
PM
VI
V
crII
HVI
crI
III
HIV–V
II
FL
–0.5
PM
VI
V
HVI
crII
IV
PFL
III
II
–1.5
VI
PM
V
HVI
IV
PFL
ND
NI
III
PCS
–2.5
PM
ND
NI
PFL
NF
–3.5
PM
PFL
ND
NI
NF
–4.5
PFL
–5.5
OI

Figure 13A

Bilateral Lesion Group (Group A)

C1502

Standard sections through the cerebellum showing regions of Purkinje cell loss in the cerebellar cortex and cell loss in the cerebellar nuclei following KA injections into the cerebellar nuclei.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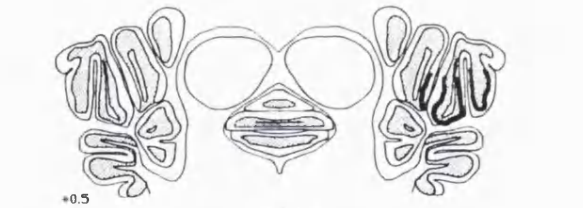
-5.5
-6.0

Figure 13B

Bilateral Lesion Group (Group A)

C1504

Standard sections through the cerebellum showing regions of Purkinje cell loss in the cerebellar cortex and cell loss in the cerebellar nuclei following KA injections into the cerebellar nuclei.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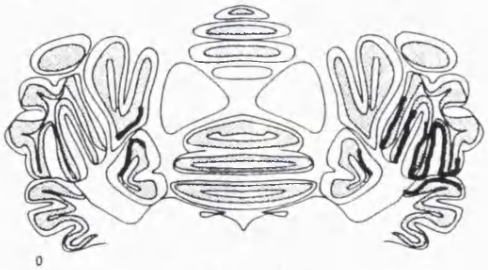
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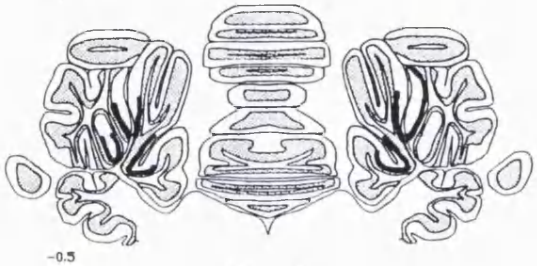
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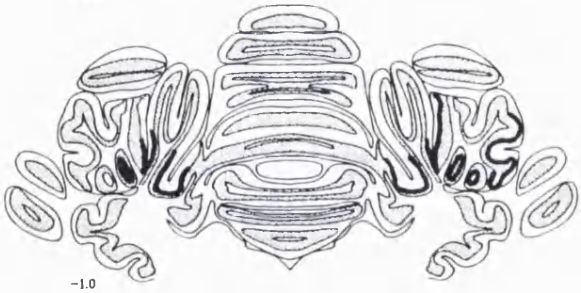
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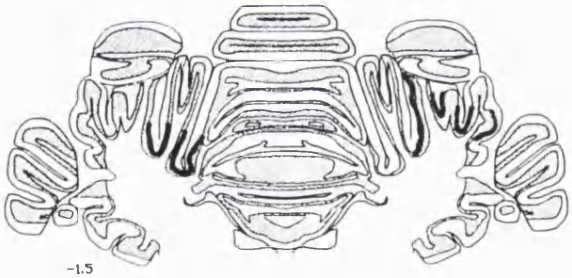
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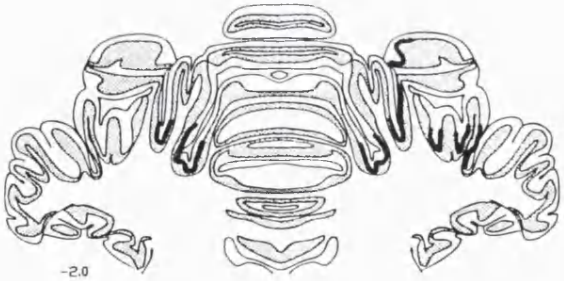
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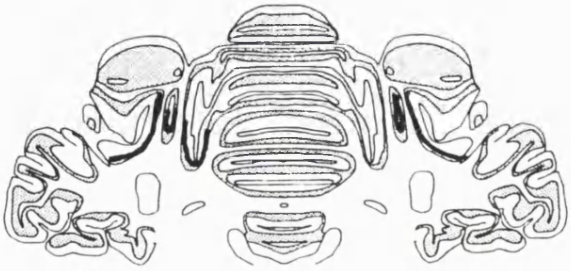
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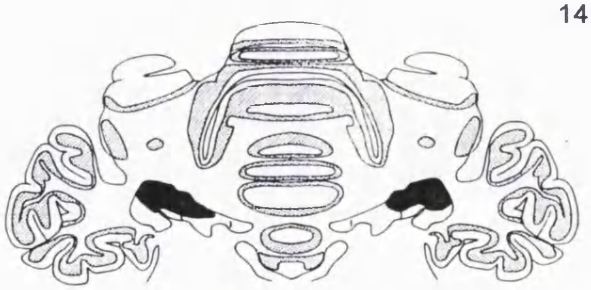
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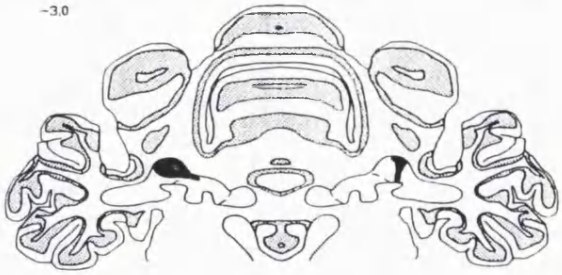
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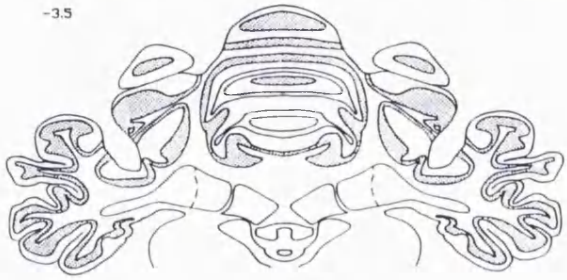
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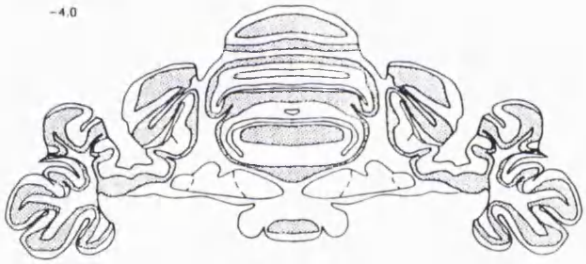
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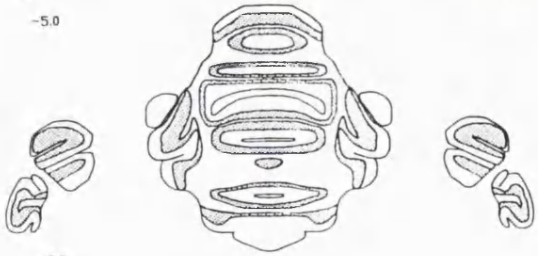
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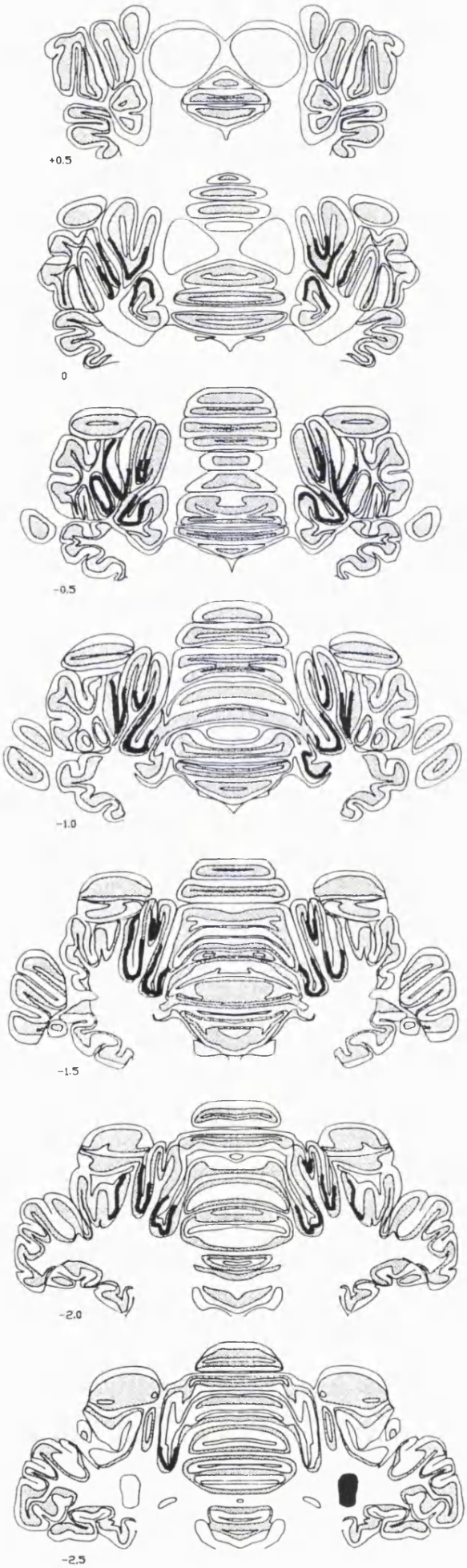
Figure 13C

Bilateral Lesion Group (GroupA)

C1505

Standard sections through the cerebellum showing regions of Purkinje cell loss in the cerebellar cortex and cell loss in the cerebellar nuclei following KA injections into the cerebellar nuclei.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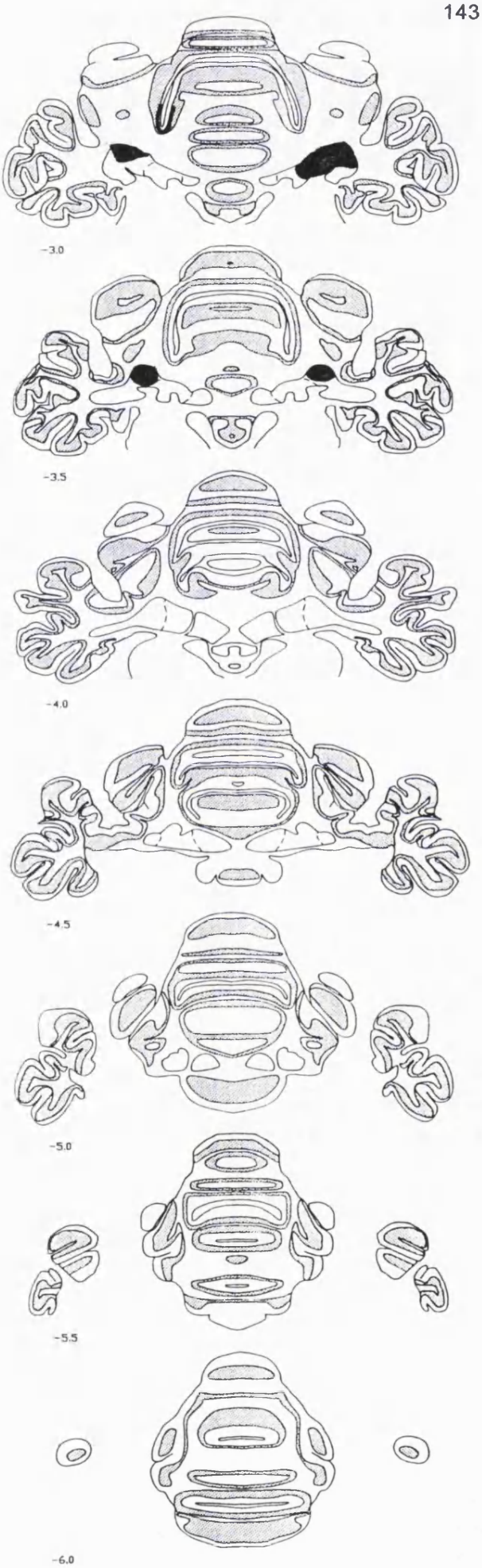
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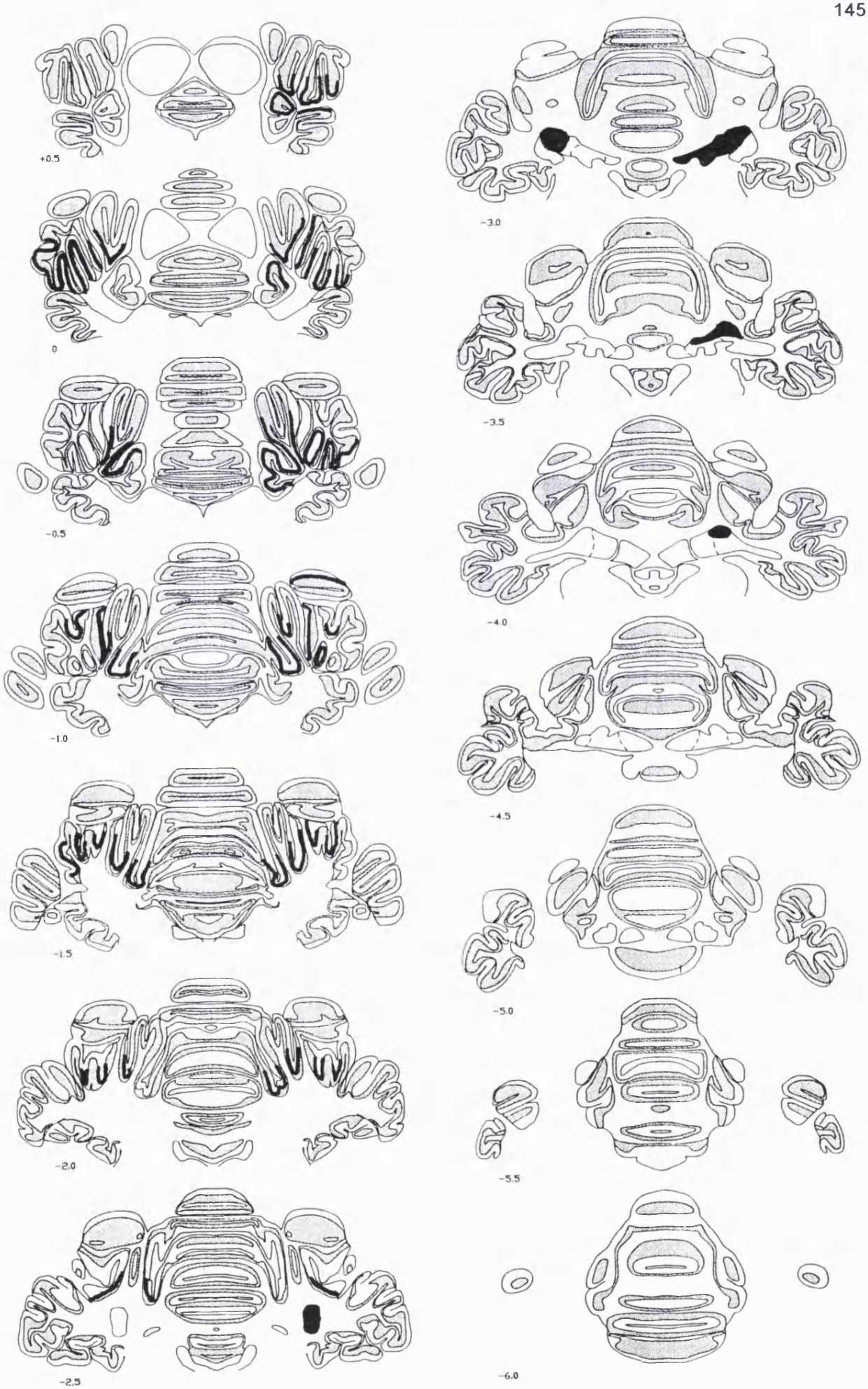
-5.5
-6.0

Figure 13D

Bilateral Lesion Group (GroupA)

C1506

Standard sections through the cerebellum showing regions of Purkinje cell loss in the cerebellar cortex and cell loss in the cerebellar nuclei following KA injections into the cerebellar nuclei.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-5.5
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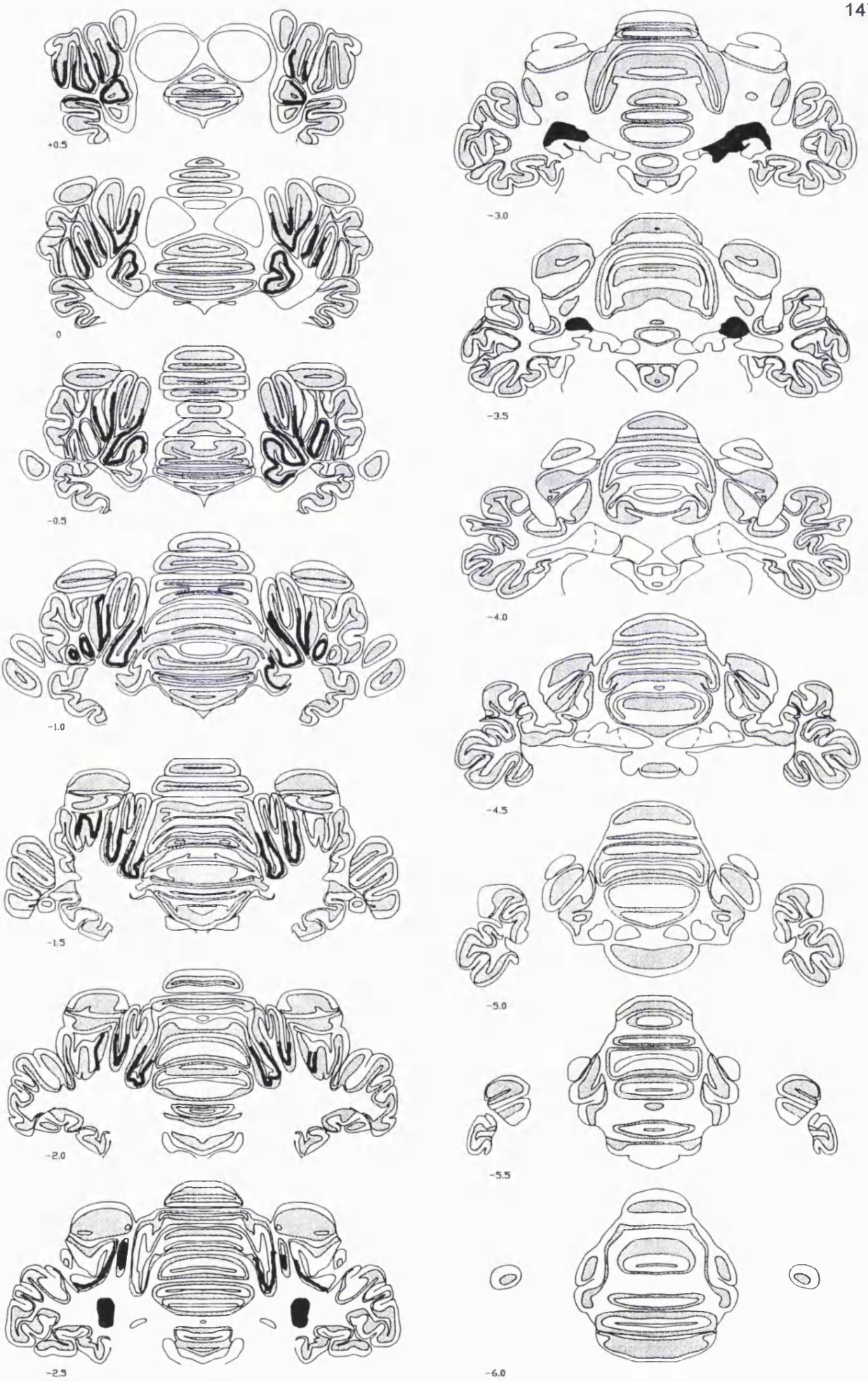
Figure 13E

Bilateral Lesion Group (GroupA)

C1507

Standard sections through the cerebellum showing regions of Purkinje cell loss in the cerebellar cortex and cell loss in the cerebellar nuclei following KA injections into the cerebellar nuclei.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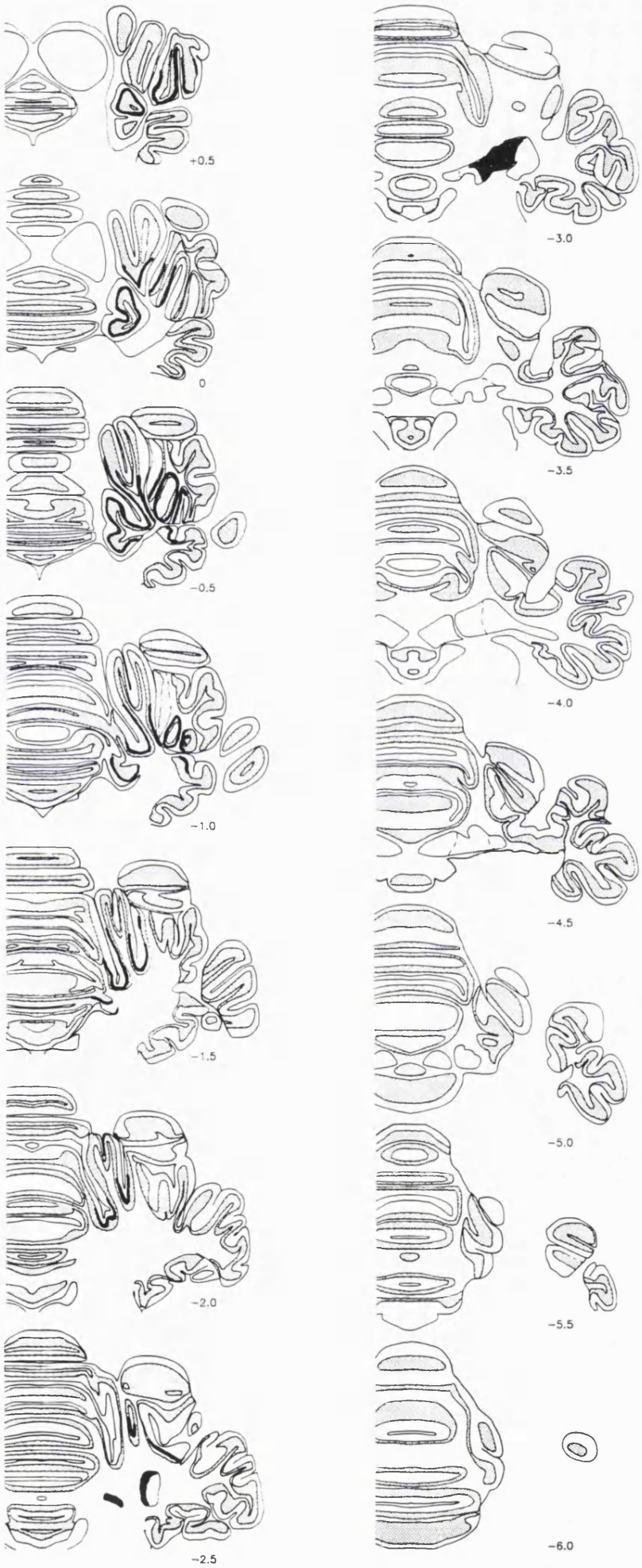
-5.5
-6.0

Figure 13F

Unilateral Lesion Group (GroupB)

C1510

Standard sections through the cerebellum showing regions of Purkinje cell loss in the cerebellar cortex and cell loss in the cerebellar nuclei following KA injections into the cerebellar nuclei.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−5.5
−6.0

Figure 13G

Unilateral Lesion Group (GroupB)

C15011

Standard sections through the cerebellum showing regions of Purkinje cell loss in the cerebellar cortex and cell loss in the cerebellar nuclei following KA injections into the cerebellar nuclei.

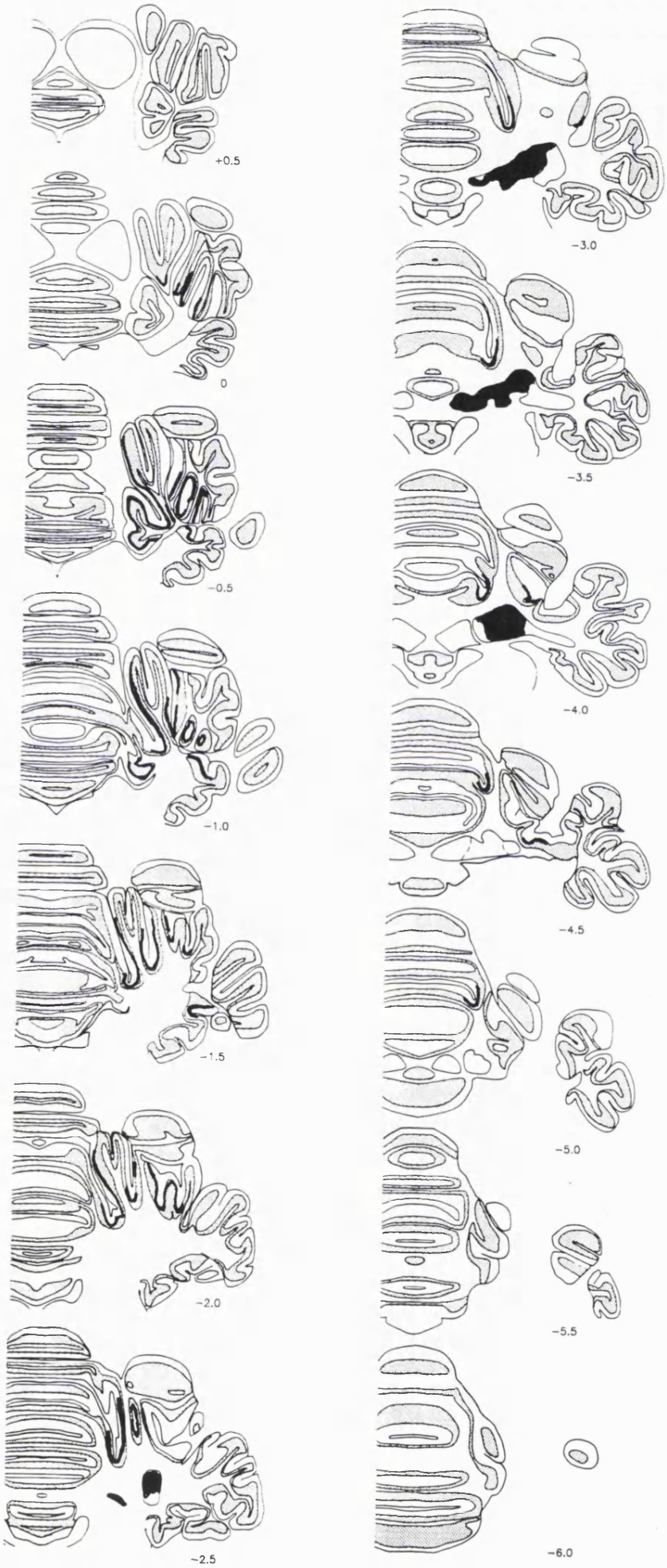
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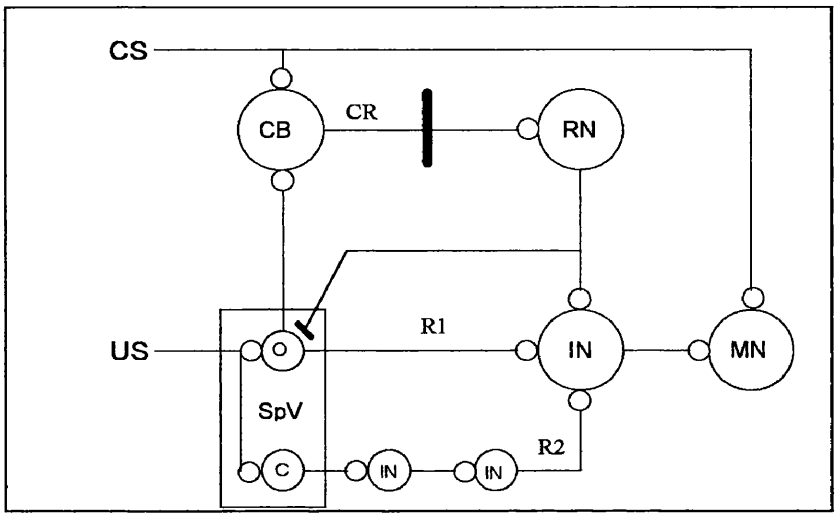
Figure 14

Summary Diagram



C - spinal trigeminal pars caudalis
CB - cerebellum
CS - conditioned stimulus
CR - conditioned response command
IN - interneuron
Bold line- cerebellar lesion
MN - motoneuron
O - spinal trigeminal pars oralis
R1 - short latency (6-18ms) component of the external blink reflex
R2 - longer latency (18-30ms) component of external blink reflex
RN - red nucleus
SpV - spinal trigeminal nucleus
US - unconditioned stimulus

The short latency OO EMG activity described in this report corresponds to the R1 response described in humans, cats and rabbits (Evinger et al., 1984). The R1 response involves very few neurons, possibly only two (Hiraoka and Shimamura, 1977). In this diagram the R1 is shown as a tri-synaptic pathway (Tamai et al., 1986). The spinal trigeminal pars oralis (SpVo) is involved in the R1 response and more caudal parts of SpV, for example the spinal trigeminal pars caudalis (SpVc), are involved in longer latency eyeblink responses (Fanardjian, 1983). Primary afferents from the face project mainly to SpVc but also to SpVo in rabbits (Van Ham and Yeo, 1992). Inhibition of the R1 response and facilitation of longer latency responses during the production of CRs can be explained if a CR related inhibitory output from the RN acts only upon neurons within the SpVo (Davis and Dosrovsky, 1986), whereas both long and short latency pathways are facilitated by CS inputs acting upon eyeblink motorneurons or interneurons. The similarity between eyeblink responses in naive rabbits and those with cerebellar lesions can be explained by the lack of a CR command. Lesions of the output of the cerebellum block CR related inputs to the RN and so prevent inhibition of the R1.

Projections of the inferior colliculus to the pontine nuclei in the rabbit revealed by WGA-HRP and their importance for associative eyeblink conditioning.

INTRODUCTION

In pigmented rabbits, lobule HVI and the adjacent Ansiform lobule of the cerebellar cortex are necessary for the acquisition of conditioned eyeblinks through the association of auditory and visual conditioned stimuli (CSs) to a weak electrical impulse or an airpuff unconditioned stimulus (US) applied close to the eye (Hardiman and Yeo, 1992; Yeo and Hardiman, 1992; Yeo et al, 1995a). These lobules receive climbing fibre inputs from the inferior olive that are related to the unconditioned stimuli used in eyeblink conditioning (Van Ham and Yeo, 1992), and they have access through the cerebellar nuclei to eyeblink motor systems (Hesslow, 1993,1994). Lobule HVI and the Ansiform lobe are probably important for the control of *conditioned* eyeblinks because they also receive mossy fibre information related to auditory and visual conditioned stimuli (Hardiman and Yeo, 1992; Yeo and Hardiman, 1992; Yeo *et al.*, 1985b). Auditory (cat: Mower *et al.*, 1979) and visual (rabbit: Wells *et al.*, 1989) structures project to parts of the basilar pontine nuclei that in turn project to lobule HVI (rabbit: Yeo *et al.*, 1985b). Since this region receives US information through climbing fibres from the inferior olive and CS information through mossy fibres from the pontine nuclei it could well serve as an important site for eyeblink conditioning. Consistent with theoretical studies which suggested that the cerebellar cortex plays a role in motor learning (Marr, 1969; Albus, 1971) such processing may be the basic mechanism underlying the conditioned eyeblink. In order to test these ideas, it is necessary to establish that auditory and visual CS inputs reach the same destinations in the cerebellar cortex and whether any of these lie within cortical regions receiving US related inputs. An initial step in this analysis is to determine the pathways for auditory CS information and their targets in the cerebellar cortex.

Electrophysiological evidence suggests that the cerebellum receives auditory information predominantly through mossy fibres (Shofer and Nahvi, 1969). Auditory evoked mossy fibre responses have been recorded mainly within vermian lobules VI and VII (monkey: Snider and Stowell, 1944; cat: Aitken and Boyd, 1975; rat: Huang and Liu, 1990) as well as the cerebellar hemisphere, in lobule HVI and the Ansiform lobule (monkey: Mortimer, 1975; rat: Huang *et al.*, 1991) and paraflocculus (rat: Azizi *et al.* 1985). Auditory mossy fibre activity related to the conditioned stimuli has been recorded within lobule HVI (Berthier and Moore 1986) and the Ansiform lobule (McCormick and Thompson, 1984).

The cerebellar cortex receives auditory mossy fibre inputs directly from the cochlear nuclei (cat: Huang *et al.*, 1982). All other auditory inputs are received indirectly through the basilar pontine nuclei (cat: Aitken and Boyd, 1978) from the cerebrum (cat: Kawamura and Brodal 1973; rat: Azizi *et al.*, 1985) and inferior colliculus (cat: Kawamura and Brodal, 1973). The most important inputs to the cerebellum are from the inferior colliculus (IC). Snider and Stowell

(1944) abolished cerebellar auditory evoked responses after bilateral destruction of the IC but not after mid-mesencephalic decerebration which would have spared the pathway from the IC to the basilar pontine nuclei (PN). Huang and Liu (1985) found that decerebration behind the IC abolished all but the shortest latency auditory responses of the cerebellar cortex, hence these residual responses were probably relayed directly from the cochlear nuclei. Moreover, auditory responses recorded within the cerebellar cortex are evoked by binaural stimulation (Highstein and Coleman, 1968), they are related to interaural time and intensity (Rose *et al.*, 1966), and they are sensitive to the direction of moving stimuli (Altman *et al.*, 1976). This evidence indicates that a major mossy fibre input is from auditory structures upstream of the cochlear nuclei, probably from the IC (Aitken and Boyd, 1975; Altman *et al.*, 1976).

It is likely that the IC provides the most important auditory inputs to the cerebellum for eyeblink conditioning. Both acquisition and retention of conditioned eyeblinks to tone CSs are largely unaffected by lesions of the neocortex (Oakley and Russell, 1972) or by pre-collicular decerebration (Mauk and Thompson, 1987). Lesions of the brachium pontis severely impair conditioned responses (Yeo, 1987).

Connections from the IC to the cerebellum have been studied most thoroughly in cats. The IC projects to the most lateral and dorsal part of the middle region of the ipsilateral PN (Anderson *et al.*, 1980; Hashikawa, 1983; Kawamura 1975; Kawamura and Brodal, 1973; Mower *et al.* 1979). Cells within this region of the PN project to vermian and paravermian regions of lobules VI, VII and VIII of the cerebellar cortex (cat: Hoddevik *et al.*, 1977; Mower *et al.*, 1979). Connections between IC and PN in other species have not been studied as extensively. Projections from IC to PN have been described in rat (Burne et al, 1981) and monkey (Moore and Goldberg, 1966). Although the overall pattern of the projection is similar to that in cats, its size and site of termination within the PN is disputed. In neither rats (Burne *et al.*, 1981) nor monkeys (Moore and Goldberg, 1966) is there a clear site of termination within the PN. Faye Lund, (1986) could find no connections between the IC and PN in rats. It is possible that the IC does not project to the PN in either of these species. The connections between IC and PN have not been studied in the rabbit. The projections from the superior colliculus to PN of rabbits (Wells *et al.*, 1989) differs from cats, hence it is important to establish the projections from the IC.

WGA-HRP was injected into the IC of pigmented rabbits as an orthograde tracer in order to examine projections from IC to PN as a likely source of auditory information involved in eyeblink conditioning. This is a first step towards learning where auditory mossy fibres terminate within lobule HVI / Ansiform lobule.

METHODS

Subjects

Eleven Dutch belted rabbits weighing from 1.8kg to 2.5kg were used for this experiment. They were housed individually within a temperature controlled colony with a 12hr artificial light/dark cycle and allowed *ad libitum* food and water.

Surgery

Each rabbit was anaesthetised using a fentanyl, fluanisone mixture (Hypnorm, Janssen; 0.1/5.0 $mg.kg^{-1}$, i.m.) and a supplement of benzodiazepam (Valium, Roche; 0.5 $mg.kg^{-1}$, i.v.). Each rabbit was intubated, placed in a Kopf stereotaxic instrument and connected to a halothane evaporator. After the halothane had taken effect, naloxone hydrochloride (Narcan, Du Pont; 3-5 $\mu g.kg^{-1}$, i.v.) was used to antagonise the fentanyl, and deep anaesthesia was maintained throughout the operation with 3% halothane in a 30% nitrous oxide/oxygen mixture via the endotracheal tube. Mannitol (4 $g.kg^{-1}$, i.v.) was then infused at a rate of 3 ml per minute. Mannitol reduced brain volume, thus exposing much of the surface of the IC by shrinking the overlying occipital lobe and cerebellar anterior lobe. The scalp was reflected and bone and dura removed to expose the right inferior colliculus. WGA-HRP was then either pressure-injected or iontophoresed into the inferior colliculus through a micropipette. In some subjects single injections were placed into a specific region of the IC and in other subjects multiple injections were placed throughout the IC.

After the injections had been made the scalp was sutured and 30 ml of glucose/saline was slowly infused intravenously and the animal removed from the stereotaxic instrument. Buprenorphine hydrochloride (Temgesic, Reckitt and Colman; 0.025 $mg.kg^{-1}$, i.m.) was given to provide analgesia immediately after surgery.

Pressure injection of HRP

Micropipettes with internal tip diameters of 10-15µm were made from graduated capillary glass (Blaubrand, white). The glass was first pulled to form a microelectrode and the tip broken in low melting point glass using the method of Merril and Ainsworth (1972). The pipette was placed in an electrode holder fitted to a micromanipulator and filled with WGA-HRP under vacuum.

In nine subjects one or more injections of WGA-HRP (4% WGA-HRP in 0.09% saline) were made into the right inferior colliculus at depths of 0.5-3mm below the pial surface. At each injection site 7.5-20nl of WGA-HRP was expelled by pressure over five minutes.

Iontophoresis

In two subjects WGA-HRP was injected by iontophoresis at several points between 0.5mm and 1mm below pial surface. A micropipette (id 20µm) was filled with 1% WGA-HRP in Tris buffer pH 8.3. A current of 2µA pulsed (0.5s on 0.5s off) for 20mins was used to eject the WGA-HRP. During insertion of the micropipette and after each injection a voltage of -12v was applied to prevent leakage of WGA-HRP.

Histological processing

WGA-HRP

All subjects were perfused 24hrs after the injection of WGA-HRP. Each rabbit was given heparin sodium (Monoparin, CP Pharmaceutical; 500U.kg^{-1}, i.v.) and a lethal injection of pentobarbitone sodium (Sagatal, May & Baker; 90mg.kg^{-1}, i.v.). Each rabbit was then perfused through the aorta with 2l of 0.9% saline containing 1000U of heparin over 20mins, followed by 1l of fixative (4% paraformaldehyde in 0.1M phosphate buffer, pH 7.3) over 30mins. The fixative was washed out by 1l of 10% sucrose (at 4°C, buffered with 0.1M phosphate at pH 7.3) over 20mins and 1l of chilled 20% buffered sucrose over 20mins. The brain was then removed from the skull and stored overnight in 30% buffered sucrose at 4°C and the next day embedded in gelatin. The brain was placed in a 5% gelatin/30% sucrose solution and kept at 37°C under vacuum for 30mins. The brain was then embedded in 10% gelatin/30% sucrose, and allowed 1 day in chilled 30% buffered sucrose before freezing and sectioning at 60µm in the transverse plane. Every 4th section was processed using a tetramethylbenzidine method based on that of Mesulam (1982) and Gibson *et al.* (1984) and described by Legg *et al.* (1989). Every 3rd of 4 sections throughout the injection site was processed using the chromogen diaminobenzidine (Legg *et al.* 1989). The sections were then mounted onto gelatinised slides, allowed to dry, and counter-stained to show cell bodies with 1% neutral red (adjusted to pH 3.3 with acetate buffer).

Cytoarchitectonics of the inferior colliculus

The major sub-divisions of the inferior colliculus were revealed by staining adjacent sets of sections through the inferior colliculus of a rabbit for cytochrome-oxidase (Wong Riley, 1979), or with cresyl violet. The major cytoarchitectonic boundaries were estimated and indicated upon standard drawings of the IC (see Figure 2).

Analysis of WGA-HRP histological material

The transverse sections were examined microscopically under bright field and crossed-polarised illumination. WGA-HRP in the pontine nuclei and superior colliculus were plotted on camera-lucida drawings of sections throughout the pons and colliculus. The injection sites within the IC, as revealed by the TMB and DAB methods, were drawn on standard sections.

RESULTS

This study revealed a projection from all parts of the inferior colliculus to a small region situated in ventral and lateral parts of the basilar pontine nuclei at its most caudal end.

Primary injection site

Like the IC of the cat (Berman, 1968; Morest and Oliver, 1984) and rat (Faye-Lund and Osen, 1985; Herbert *et al.*, 1991) the rabbit has three major cytoarchitectonically distinct regions which can be recognised in sections stained for Nissl substance and cytochrome-oxidase. The central nucleus (ICC), the laterally placed external nucleus (ICX) and the dorsally placed

pericentral nucleus (ICP) are shown in Figure 1. The nomenclature for these divisions is that used by Berman (1968) for the cat.

The position of the border between ICP - called dorsal cortex by Morest and Oliver, (1984) in cats and by Herbert *et al.*, (1991) in rats - and ICC was not obvious either in the Nissl or the cytochrome oxidase stained sections. Although the borders as marked are probably correct, I may have included part of the pericentral cortex (dorsal cortex layer IV in the cat; dorsal cortex layer 3 in the rat) in the central nucleus. In both cats and rats the dorsal cortex may be more extensive medially than in rabbits.

The extent of the primary injection sites of WGA-HRP was assessed with reference to the major divisions shown in this study (ICC, ICX, ICP) and with reference to those described by Morest and Oliver (1984). In Table 1 the site and volume of WGA-HRP injected for each subject are summarised. The injection sites of each subject are shown in Figure 2.

In all cases but two (IC5 and IC7) the injections were entirely confined to the inferior colliculus. The primary injection sites varied in size from quite extensive (IC7 and IC8) to relatively small. Although no injection site was entirely confined to a single subdivision of the IC, subjects IC1, IC2 and IC3 had injections that were predominantly in ICP, ICX and ICC respectively. In subjects IC4, IC5 and IC6 the injections labelled mainly ICC but there was clear spread of label into ICP and ICX. In subjects IC7 and IC8 the majority of ICX was labelled together with much of ICC and some ICP.

In Table 1 is an analysis of the injection sites by subdividing the IC according to the scheme of Morest and Oliver, (1984). If the description of the IC in cats by Morest and Oliver is directly applicable to rabbits then by this scheme label in subjects IC3 - IC6 was restricted mainly to the dorsal and caudal cortex and in subject IC8 the paracentral nucleus was most heavily labelled together with parts of dorsal and caudal cortex.

In subjects IC5 and IC7 the primary injection extended beyond the IC to include a small region of Purkinje cells within rostral parts of lobules HVI and HVIIA of the cerebellar cortex which lie adjacent to the IC. In subject IC7 the primary site also spread medially into the ipsilateral periaqueductal grey. In no subject was there spread of primary label into other auditory centres, for example the nucleus sagulum or dorsal nucleus of the lateral lemniscus. Three cases (not shown) were rejected from this study because of excessive spread of the tracer beyond the IC.

IC projections to the pontine nuclei

A large tract of fibres was seen to descend or ascend from the IC to the superior olivary complex (SOC) at the caudal end of the pons. Some fibres diverged from this tract to the basilar pontine nuclei (PN) where they clearly terminated in fields within its ventral and lateral divisions (see Figure 3 for these divisions). The distribution of WGA-HRP labelled fibres and terminals within the PN was similar for all subjects irrespective of the location within the IC of the site of injection.

Figure 4 shows the tectopontine projection in subjects IC1, IC2 and IC3 in which the injection sites were principally within ICP, ICX and ICC respectively. In no case was there a projection to the nucleus reticularis tegmenti pontis.

Descending fibres from the IC lay adjacent to the PN for most of their route through the pons. The entire course of these fibres, in particular the region in and around the dorsolateral pontine nucleus (DLPN) was examined in order to establish whether any terminated within the PN. The IC projection in subject IC7, illustrated in Figures 5, 6 and 7, was typical of all others in this study hence it is described in detail. A large tract of fibres descended from the IC through the ipsilateral lateral lemniscus to just below the ventral nucleus of the lateral lemniscus and adjacent to the most dorsal aspect of the DLPN. At this position, at the middle rostro-caudal extent of the PN, most of the fibres abruptly turned to course caudally. They formed a horizontal plane lying dorsal to the most dorsolateral part of the DLPN. Additional fibres descending from the IC continued to join this tract along the length of the PN. In subjects IC2, IC3, IC7 and IC8 fibres in this tract were intensely labelled which made it difficult to determine whether any of them terminated immediately below, in the DLPN (see Figures 5A-D, Figure 6A-C and Figures 7B,C). At some rostro-caudal levels the tract was clearly separated from the DLPN by a narrow region of unlabelled fibres which formed part of brachium pontis. This arrangement was especially clear in subject IC5 in which heavily labelled fibres were separated from DLPN throughout its extent by fibres of the brachium pontis. At other levels in IC7, the tract was immediately dorsal to or even within the boundaries of the DLPN and the possibility of fibres terminating there could not be ruled out - although the relatively small number of fibres coursing within the boundaries of the PN would indicate that such a projection was sparse. This possible projection is illustrated in Figure 7B in which a group of cells lying immediately below the projecting fibres of the IC appear to receive sparsely labelled terminals. These cells differ anatomically from cells within the main body of DLPN situated immediately below, hence they may not be true DLPN cells. In all other subjects these fibres were more lightly labelled and there was no evidence of a terminal field in DLPN.

In subject IC7, as in all other subjects, the majority of fibres in this tract continued to course along the dorsal surface of DLPN and to emerge from the caudal end of the PN where they became concentrated parallel to the edge of the brainstem (see Figure 5E). Most fibres were seen to terminate or originate within the superior olivary complex (SOC) (see Figure 5F).

Throughout its course dorsal to lateral DLPN, groups of fibres separated from the most medial part of this tract and entered the medial portion of DLPN (see Figures 5A-5D). A few fibres also separated from more lateral parts of this tract and traversed the lateral DLPN to join fibres in more medial parts of DLPN (see Figure 7A). Labelled fibres passing through medial parts of DLPN formed three main groups.

The largest group of fibres remained within dorso-medial DLPN to the caudal end of the PN where they merged with more lateral fibres on the way to the SOC. All label in this region of the

PN had the appearance of fibres of passage (see Figures 7A, 7B and 7C). In subjects IC7 and IC8 this label was so intense that it could not be determined whether a terminal field was obscured by fibres of passage (see Figures 5C, 7B and 7C). In all other subjects label in this region was less intense and there was no evidence of termination within this region of the PN (see Figure 4).

A second, much smaller group of fibres, passed ventro-medially through DLPN and divided to course on both sides of the cerebral peduncle (see Figure 4C). In subjects IC4, IC5, IC7 and IC8 some fibres left the tecto-pontine tract medially to take a route between the cerebral peduncle and the nucleus reticularis tegmenti pontis. In subjects IC7 and IC8 a few fibres took a route below the cerebral peduncle. Both sets of fibres coursed across the midline through the contralateral DLPN. The final course of these fibres was not clear but they travelled up to the contralateral dorsal nucleus of the lateral lemniscus and possibly continued to the dorsal cochlear nucleus.

A third group of fibres took a ventro-medial route and terminated lateral and ventral to the cerebral peduncle at the most caudal region of the ipsilateral PN. This terminal field was present in all subjects and extended through the most caudal millimetre of the PN as illustrated in Figures 4 and 5D. In subjects IC4, IC5 and IC7 there was also a small amount of terminal label within an identical region on the contralateral side of the PN (see Figure 4A at 1.5mm rostral to lambda). This label may have been from fibres crossing the midline within the PN or from fibres crossing in the commisure of the inferior colliculus and travelling down the contralateral tegmentum as described below.

In subjects IC7 and IC8 labelled fibres projected from the ipsilateral IC through the commisure of the inferior colliculus and contralateral IC, and then descended down the lateral margin of the tegmentum to the ventral nucleus of the lateral lemniscus (where several cell bodies were weakly labelled,) to arrive at the dorsolateral margin of the contralateral DLPN. At this point all fibres turned caudally and formed a horizontal sheet dorsal to DLPN. Because fewer fibres were labelled contralaterally than ipsilaterally they could be visualised more clearly. These fibres passed over and in some places may have terminated on a group of cells located adjacent to the extreme dorsolateral part of DLPN. This is similar to the more intense projection seen on the ipsilateral side. There was no evidence of a terminal projection on cells of the DLPN.

Other projections of the IC

Because there was very little evidence of a projection to the most dorsolateral parts of DLPN, a region that in other animals is reported to receive inputs from the inferior colliculus, other projections of the inferior colliculus were examined in order to compare these projections with those described in other studies and so test the sensitivity of the methods used in this study.

Superior olivary complex

This large auditory centre consists of a group of nuclei situated immediately caudal to the PN. The main nuclei of the superior olivary complex are illustrated in Figure 6C. At the caudal end of the PN the labelled fibres were concentrated parallel to the edge of the brain (see Figures 4 and 5E). Most of these fibres terminated at the superior olivary complex, in the ipsilateral medial preolivary nucleus (see Figure 5F). Weak anterograde label was also found in the ipsilateral medial nucleus of the trapezoid body. Cell bodies in the lateral superior olive were strongly labelled on both sides in subjects IC5, IC6 and IC8 but only on the ipsilateral side in all other subjects except in subject IC4 which showed no labelling in the superior olive. Most of the label in the superior olive was confined to its dorsolateral tip except in subject IC8, in which cells throughout the dorsal half were labelled. Retrograde label was also seen in the ipsilateral retroolivary nucleus and medial superior olive. A few retrogradely labelled cells were seen in the ipsilateral dorsal periolivary nucleus in subjects IC7 and IC8. Cells in the ipsilateral lateral preolivary nucleus were also weakly labelled.

Cochlear nuclei

Subjects IC1, IC2, IC3, IC5 and IC8 all showed a considerable amount of retrograde label throughout the contralateral cochlear nuclei. In subject IC5 some cell bodies in the ipsilateral anteroventral cochlear nucleus were weakly labelled.

Brain stem

In subjects IC1, IC2, IC6 and IC8 a small number of retrogradely labelled cells were found within caudal, dorso-medial parts of the contralateral spinal trigeminal sub nucleus interpolaris, and within the contralateral gracile and cuneate nuclei.

Superior colliculus

A large tract of fibres took a route rostral to the IC. Many of these fibres were seen branching away from the brachium of the inferior colliculus to take a dorsal route through layer V of the superior colliculus (SC) and ending in layer VI of the SC and adjacent parts of dorsolateral periaqueductal grey. Both anterograde and retrograde label were seen within layers IV, V and VI of the ipsilateral SC particularly in subjects IC2, IC7 and IC8, but also in subject IC3. In subjects IC2, IC7 and IC8 there were also several retrogradely filled cells in layers IV and VI of the contralateral SC. There was no transport of WGA-HRP into the SC in subject IC1. Figure 9 illustrates the pattern of label in the SC of subjects IC2 and IC3. As the primary injection site was restricted mainly to ICP in IC1, to ICX in IC2 and ICC in subject IC3 then the majority of connections with SC are to and from ICX. Figure 10 shows anterograde and retrograde label within the SC of subject IC7. This figure illustrates the complete overlap of the reciprocal connections between the IC and SC.

Medial geniculate nucleus

A projection from IC to the ipsilateral medial geniculate nucleus was seen in all subjects. A small projection to the contralateral MGN was also seen, although in subject IC1 this projection

was very sparse. Both ipsilateral and contralateral projections were restricted almost entirely to the ventral division of the medial geniculate. This projection is illustrated in Figure 9.

DISCUSSION

Injections of WGA-HRP centred on either the central nucleus, pericentral nucleus or external nucleus of the inferior colliculus revealed projections to the most caudal parts of the ipsilateral ventral and lateral pontine nuclei and a small symmetrical projection to the contralateral side. Fibres from each division of the IC coursed ventrally through the lateral lemniscus and, when adjacent to the most lateral aspect of the dorsolateral pontine nucleus, turned sharply caudal and continued on to the superior olivary complex and cochlear nuclei. There was no evidence of terminal WGA-HRP label in lateral parts of the dorsolateral pontine nucleus. WGA-HRP label within the dorsolateral nucleus was restricted almost entirely to its medial parts and had the appearance of fibres of passage that traversed the dorsolateral nucleus on the way to more caudal, lateral and ventral regions of the pontine nuclei, the superior olivary complex and the cochlear nuclei.

IC projects directly to PN

There was clear evidence of a projection from IC to the most caudal region of medial and lateral PN. Martin (1969) mentioned a projection to lateral PN in opossum but a similar projection has not been shown anatomically in monkey, rat or cat although auditory evoked responses have been recorded within this pontine region in cat (Aitken and Boyd, 1978).

In cats, the IC projects primarily to the dorsolateral margin of middle parts of the ipsilateral dorsolateral pontine nuclei (Aitken and Boyd, 1978; Anderson *et al.*, 1980; Hashikawa, 1983; Kawamura 1975; Kawamura and Brodal, 1973; Mower *et al.* 1979). It is not clear from which division of the IC this projection arises. Using degeneration techniques Kawamura (1975) described a projection from ICC to the anterior two thirds of DLPN. But Anderson *et al.* (1980) injected HRP and tritiated leucine into ICC and found very little label in DLPN. Mower *et al.* (1979) injected HRP into middle regions of the lateral DLPN and found labelled cells in all parts of the IC. Hashikawa (1983) injected HRP, WGA-HRP and ^{3}H-leucine into the rostral two thirds of DLPN and adjacent regions of the peduncular and lateral pontine nuclei and demonstrated a large projection from caudal ICX, a smaller projection from ICP and no projection from ICC. In the current study the primary injection site was not strictly confined to a single division of the IC in any one subject. Nevertheless, in subjects IC1, IC2 and IC3 the injections were mostly restricted to ICC, ICP and ICX respectively. A projection from IC to ventral and lateral PN was seen in all subjects, hence it is likely that all regions of the IC project to PN.

There was no evidence of a direct projection from the IC to dorsolateral parts of the DLPN as described in cats. There are several points which suggest that this pattern of collicular projections is a valid one. Firstly, even though the IC was not completely labelled in any single

DLPN in Figures 7A,B and C. There is a slight possibility that IC projects to medial parts of DLPN, as in those cases with the largest injections of WGA-HRP there were labelled fibres in this region that may have obscured the presence of a terminal field. It is also possible that there are connections from the IC through extended dendrites of DLPN cells in rabbits, as suggested by Burne et al., (1981) in rats. These possibilities are less likely in view of the clear evidence of termination within more caudal parts of VLPN.

subject, the sum of injections in this study shows that all parts of the IC were filled, except for its extreme caudal pole. Secondly, each major division of the IC projected to the same region of ventral and lateral PN but not to DLPN even though in no case was the primary site limited to a single collicular division. This suggests that no region was likely to have been omitted. Finally, the other connections of the IC were consistent with those seen in other species (Huffman and Henson, 1990) and in other studies in rabbits (Borg, 1973a,b).

The heavily labelled tract of fibres lying above the most dorsolateral aspect of the ipsilateral DLPN throughout the caudal half of the PN is identical to that reported by Moore and Goldberg (1966) in monkeys and by Burne *et al.* (1981) in rats. Both studies showed that fibres from the IC course adjacent to the ipsilateral DLPN and peduncular region. In both of these studies it was difficult to decide if fibres of this tract terminated within PN. Termination seems unlikely as two further studies found no evidence for a direct projection from IC to PN in rats. Faye-Lund (1986) injected HRP into the DLPN of rats and found no evidence of a direct projection from the IC. Mihailoff et al., (1989) found no evidence for a projection from IC to PN after extensive fills of the PN with WGA-HRP. However, Burne *et al.* (1981) suggested that collicular fibres could contact dorsally-extended dendritic branches of the DLPN cells. There was no evidence for a projection from IC to lateral DLPN in this current study. This is clearly illustrated by the absence of anterograde label within lateral parts of the

cont

IC projects indirectly to PN via SC

A large projection from IC to the deep layers of the SC was found, similar to that described in previous studies (cat: Edwards *et al.*, 1979; rat: Coleman and Clerici, 1987; rabbit: Tarlov and Moore, 1966). Retrograde and anterograde WGA-HRP label within layers IV, V and VI of the SC and within the periaqueductal grey was seen to derive mainly from the external nucleus (subject IC2 see Figure 9). Equivalent label detected in these areas following an injection into the central nucleus (subject IC3, see Figure 9) was probably due to spread of the primary site into ICX. ICX was confirmed to be the major source of input from IC to the deep layers of SC by re-examination of cases from the study by Wells *et al.*, (1989) in which injections of WGA-HRP were made in the deep layers of the SC - only cells within ICX were labelled in these cases.

In several studies a projection from the deep layers of the SC to PN has been described (rat: Burne *et al.*, 1981; cat: Kawamura and Brodal, 1973; Mower *et al.*, 1979). In the rabbit, there is a projection from the deep layers of the SC to caudal regions of the ipsilateral PN, including ventral PN, lateral PN and medial DLPN (Wells *et al.*, 1989). Thus in the most caudal regions of ventrolateral PN there is overlap of projections from the IC and deep layers of the SC in the rabbit. Projections from the deep layers of the SC to the PN (Wells *et al.*, 1989) may be particularly important as the direct projection of the IC to PN appears to be much smaller.

the PN of inputs from ICC and ICP (see also Kamada *et al.*, 1992). Secondly, the projection from IC to the deep layers of the SC appears to be much larger than the projection from the IC to PN.

The possibility that the cerebellum receives auditory and visual CS information from the deep layers of the SC has two implications. Firstly, because the deep layers of the IC are involved in movement its output to the cerebellum may be some derivative of auditory and visual CS information. Secondly, the cerebellum can receive auditory information from regions of the PN that receive inputs from the SC including caudal, medial, parts of DLPN (Wells et al., 1989).

Projections to the PN from IC described in this study, and those from SC described by Wells *et al.*, (1989) are similar. The possibility that the projections described here were due to the inadvertent labelling of fibres of passage from SC to PN passing through IC, or due to transsynaptic transport of WGA-HRP through the SC is highly unlikely. There are no fibres of passage form SC that pass through the IC on the way to the PN (Wells *et al.*, 1989) and the route of the superior coliculo-pontine tract described by Wells *et al.*, (1989) was medial to that described in this study. Transsynaptic transport would likely require a longer time to transport from IC to SC and then to PN than the 24 hours survival time.

Auditory inputs to the cerebellar cortex via PN may originate primarily within ICX and the deep layers of the SC. ICX projects to the deep layers of the SC so this may be the primary route for auditory inputs to the PN. Two lines of evidence support this idea. Firstly, like deep layers of the SC and ICX (Aitken *et al.*, 1978)., cells within the cerebellar cortex that are driven by auditory stimuli have poor auditory frequency discrimination (cat: Altman *et al.*, 1976; Huang and Burkard, 1986; but see Wolf, 1972). In contrast, cells within ICC and ICP respond primarily to a narrow range of frequencies and are grouped tonotopically (Aitken *et al.*, 1980). ICC and ICP project directly to the PN. If these regions provide a major input to the cerebellum then it is likely that the tonal information would be preserved in the cerebellum, but there is little evidence for this. Alternatively there may be processing within

cont

Auditory input to the cerebellum and the regulation of reflex and learned behaviours

As suggested by Fadiga and Pupilli (1964) orientation and other movements may be dependant upon auditory as well as other teleceptive inputs to the cerebellum. The IC-cerebellar cortex pathway may be important for spatial location of auditory stimuli (Jenkins and Masterton, 1982), audio-spinal reflexes (Huffman and Henson, 1990), and aspects of the long latency acoustic startle response (Huffman and Henson, 1990; Parnham and Willott, 1990). Auditory inputs to lobule HVI and the Ansiform lobule via PN may also be important for Pavlovian conditioning of eyeblinks in rabbits (Berthier and Moore, 1986; Yeo *et al.*, 1985b).

The most likely route for auditory CS information to the cerebellum during eyeblink conditioning is through the PN. However, current evidence in support of this idea is indirect. During conditioning, multiunit responses related to tone conditioned stimuli and to conditioned responses have been recorded within dorso-lateral and lateral regions of the PN (McCormick *et al.*, 1983), and unilateral lesions of the brachium pontis impair conditioned responses (Yeo, 1987).

The results of other studies are consistent with the idea that the basilar pontine nuclei relay auditory information necessary for eyeblink conditioning to the cerebellum. Lesions of the brachium pontis abolished CRs to tone CSs (Soloman *et al.*, 1986), electrolytic lesions of the PN abolished CRs to tone CSs (Knowlton and Thompson, 1988,1992) but not to light CSs (Steinmetz *et al.*, 1987), and stimulation of sites close to and within dorso-lateral and lateral regions of the PN as a CS, supported eyeblink conditioning (Steinmetz, 1990; Steinmetz *et al.*,

1986). In each of these studies other interpretations can explain the results. The results of the study by Solomon *et al.*, (1986) may be entirely due to cellular damage within lobule HVI of the cerebellar cortex - lesions of this cortical region severely disrupt CRs (Yeo *et al.*, 1984,1985b). In the other studies there were insufficient controls to rule out the possibility that the results were due to lesions, or stimulation, of nuclei within the primary auditory pathways, or of their ascending and descending fibres. The nuclei of the trapezoid body lie immediately behind regions of the PN that receive inputs from the IC, the ventral nucleus of the lateral lemniscus lies immediately above DLPN, and major ascending and descending auditory pathways lie immediately adjacent to DLPN, LPN and VPN - all regions of the PN implicated in the above experiments. Indeed in the study of Steinmetz *et al.*, (1987) their lesions invaded the ventral nucleus of the lateral lemniscus. In addition to direct damage to this major auditory nucleus the lesions would have cut the ascending and descending auditory fibre tracts. Similar damage clearly occurred in the study by Knowlton and Thompson, (1992). That the lesions of Steinmetz *et al.*, (1987) did not affect conditioning to a light CS probably indicates that the primary auditory pathways were damaged. Auditory projections to the PN from IC and visual inputs to the PN (Wells *et al.*, 1987) terminate in similar regions, thus lesions of auditory parts of the PN would be expected to prevent conditioned responses to both lights and tones.

The role of the cerebellum in eyeblink conditioning remains controversial (Llinas and Welsh, 1994) hence it is important to determine whether the PN are a necessary relay for auditory CS information. Attempts to block conditioning by interrupting the CS pathway must ensure that the major auditory pathways are not damaged, otherwise this may block information being conveyed to sites that are important for eyeblink conditioning other than the PN and cerebellum.

This present study provides evidence for a direct projection from IC to caudal regions of the ventral-lateral PN and an indirect pathway from IC to deep layers of the SC which projects to caudal ventral-lateral PN and to caudal and medial parts of DLPN (Wells *et al.*, 1989). Further studies will be necessary to determine whether these regions of the PN project to eyeblink regions of the cerebellar cortex, but both direct and indirect pathways from the IC are potentially important for conveying auditory information to the cerebellar cortex during eyeblink conditioning.

Table 1.

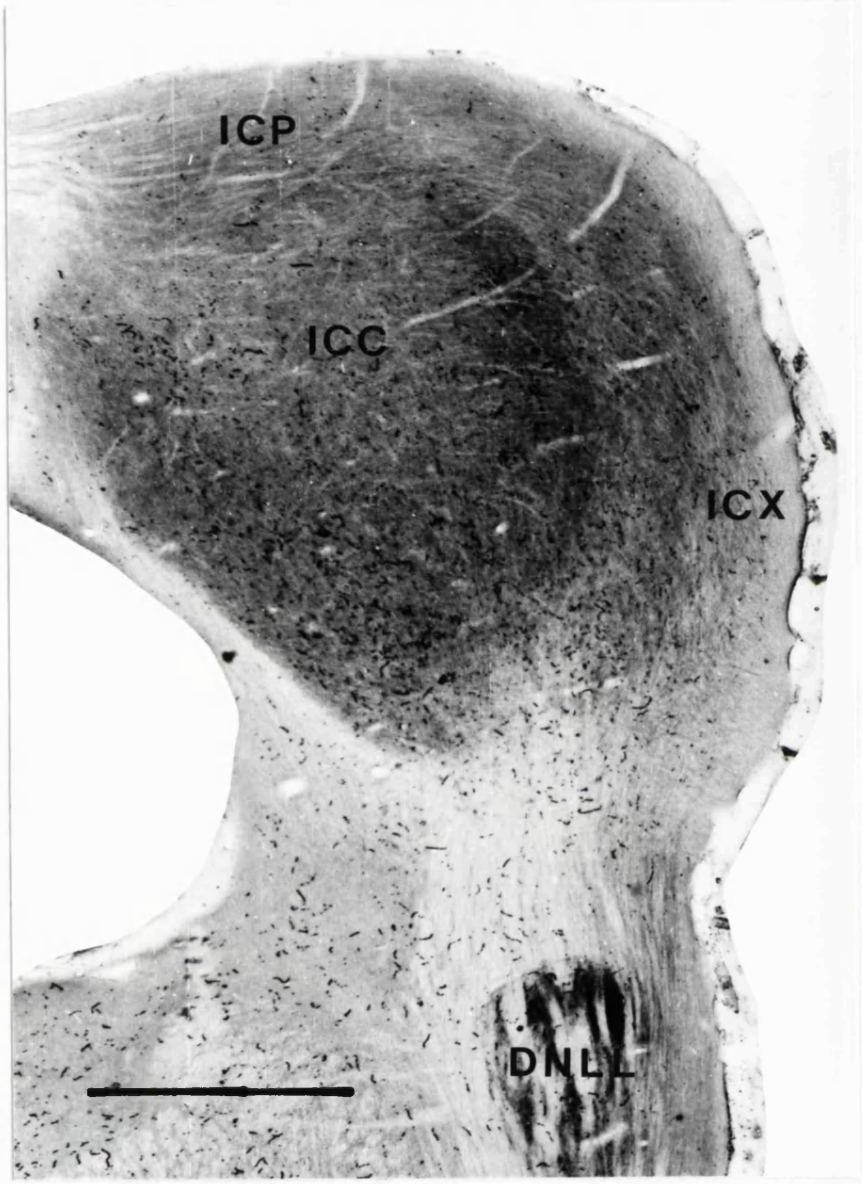
Summary of WGA-HRP injections

SUBJECT	INJECTION SITE						VOLUME (nl)
	Divisions of IC			Morest and Oliver divisions of IC			
	ICC	ICP	ICX	Central nucleus	Cortex	Paracentral nucleus	
IC1		+++	+		+++	+	Iontophoresis
IC2	+	+	+++		+	+++	35
IC3	++++		++	+	+++	++	15
IC4	+++	+	+		+++	+	300
IC5	++	+	+	+	++	+	250
IC6	+	+	+		++	+	38
IC7	++++	+	++++	++++	++	++++	82.5
IC8	+++	++	++++	++	+++	++++	95

+ Estimate of primary site - 100% of a division labelled = +++++

Position of the main injection site of WGA-HRP within IC and the total volume injected for each subject. For each division of the IC an estimate was made of the area labelled by the primary injection site. Each cross indicates label in ~ 20% of a division.

Figure 1



Photomicrograph of a mid-level section through the right inferior colliculus at 1mm rostral to lambda, showing its major divisions as revealed by cytochrome oxidase staining.

ICC = central nucleus

ICP = pericentral nucleus

ICX = external nucleus

DNLL = dorsal nucleus of the lateral lemniscus

Bar = 1mm

Figure 2

Primary injection site within the right IC revealed by DAB (oblique shading) or by TMB (crosses) reaction product. TMB alone was used as the chromogen in subjects IC4 and IC6. Numbers to the left of whole brain sections indicate position of the section in relation to lambda in mm.

Key: Oblique shading = central nucleus of IC (ICC)
Horizontal shading = external nucleus of IC (ICX)
Vertical shading = pericentral nucleus of IC (ICP)

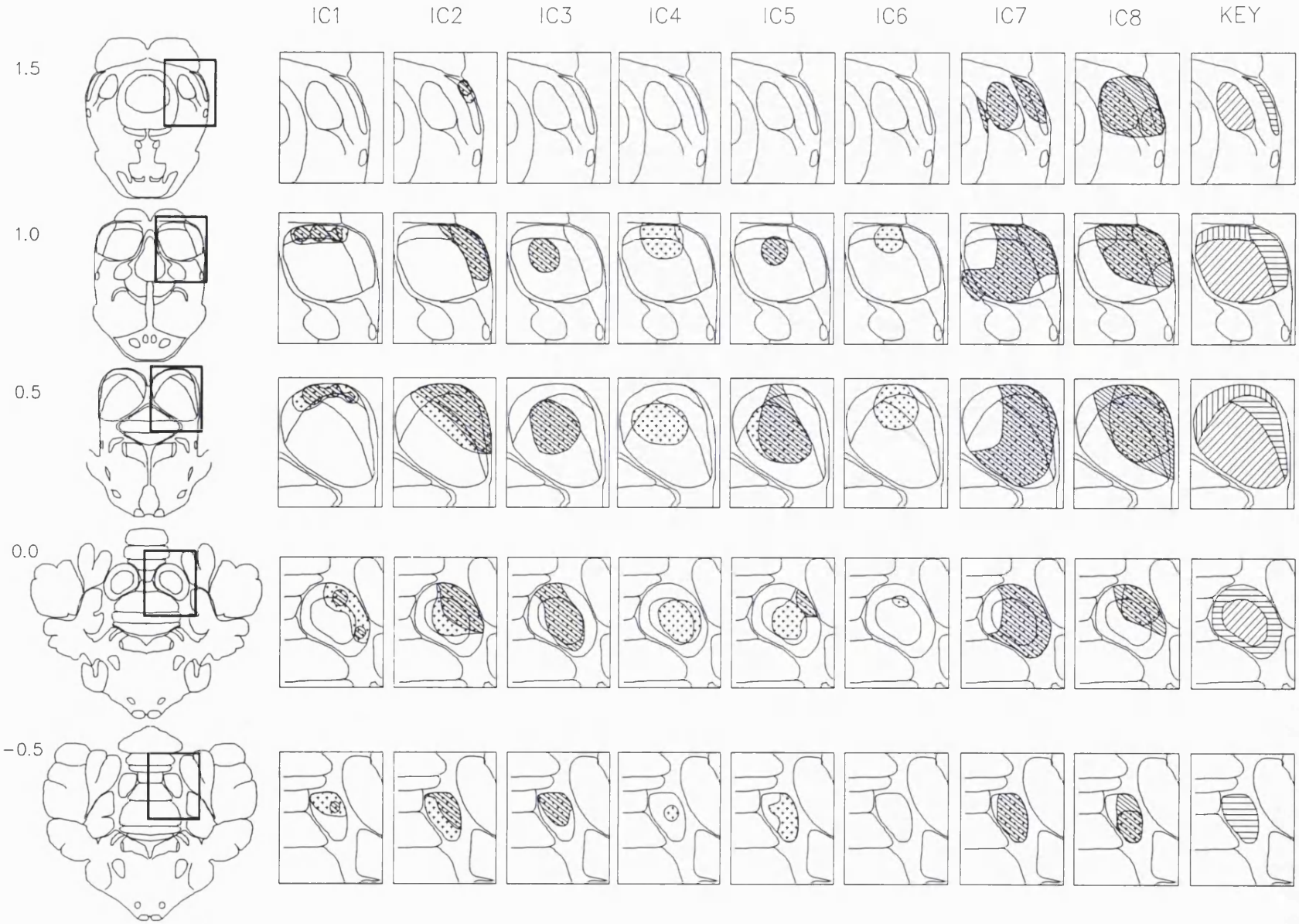

IC1
IC2
IC3
IC4
IC5
IC6
IC7
IC8
KEY
1.5
1.0
0.5
0.0
−0.5

Figure 3

Key to divisions of the pontine nuclei based upon Brodal and Jansen (1946)

Dorso-lateral pontine nucleus	- small dots
Lateral pontine nucleus	- short horizontal lines
Paramedian pontine nucleus	- long horizontal lines
Ventral pontine nucleus	- vertical lines
Peripeduncular pontine nucleus	- thick line
Nucleus reticularis tegmenti pontis	- large dots

Numbers indicate the distance in milimetres from the skull suture lambda. The top section is rostral.

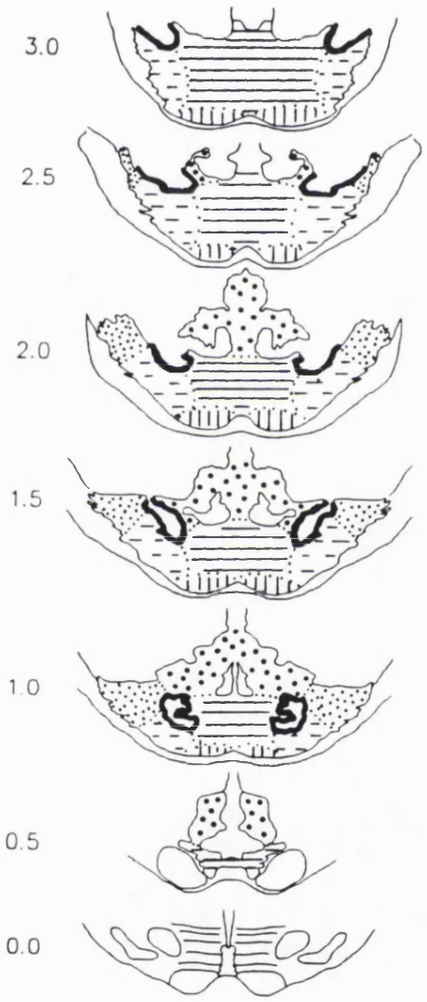

3.0
2.5
2.0
1.5
1.0
0.5
0.0

Figure 4.

Camera lucida drawings of adjacent sections through the caudal 2/3rd of right PN showing the course of afferent and efferent fibres of the right IC in subjects IC1 (A), IC2 (B) and IC3 (C). The top section is rostral. The bottom section in each subject was cut at approximately lambda. Each section is 480μm apart.
Large dots = retrogradely labelled cells within the nuclei of the lateral lemniscus.
Small dots = labelled fibres and terminal field.

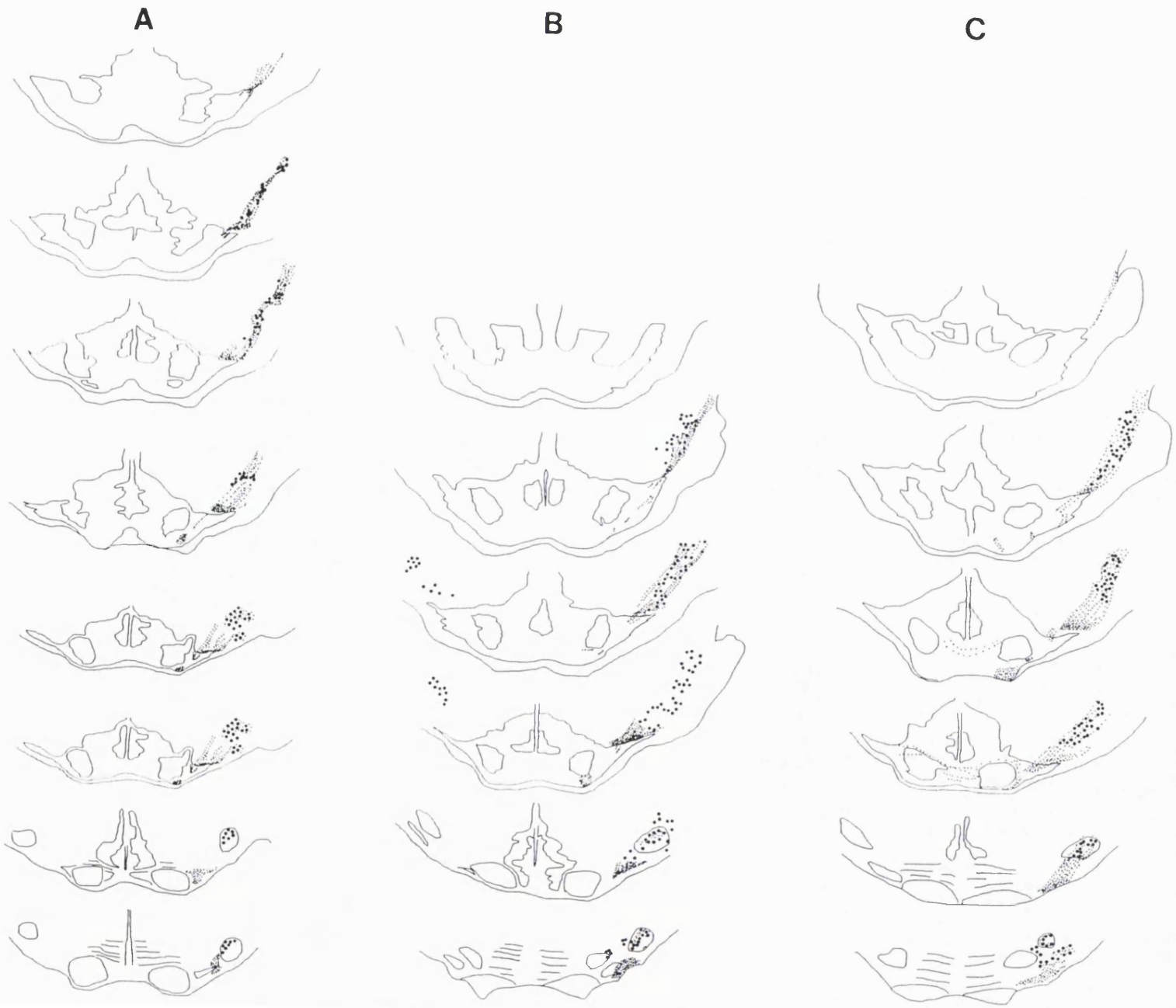

A
B
C

Figure 5.

Subject IC8
Photomicrographs taken under cross-polarised illumination of serial sections through the caudal half of the pons, ipsilateral to WGA-HRP injection in IC.

A, B and C - sections through DLPN at 3.0mm, 2.5mm and 2.0mm rostral to lambda. This series shows the development of the tectopontine tract with clumps of fibres branching off and passing through DLPN. Most fibres traverse DLPN medially, only a few fibres were seen within lateral DLPN.

D - section through PN at 1.5mm rostral to lambda. This photograph shows afferent and efferent fibres of the IC coursing through the lateral lemniscus, and terminal label within ventral and lateral PN.

E - section through pons at 0.5mm rostral to lambda showing terminal site of anterograde label within VNLL and label destined for terminal sites within VNTB, the primary terminal site of descending fibres from the IC.

F - section through the superior olivary complex at 0.5mm caudal to lambda showing anterograde label within MPO and MSO and retrograde labelled cells in DO, LSO, MSO, LPO, NTB and RO.

Bar = 0.25mm for A,B,C and 0.5mm for D,E,F

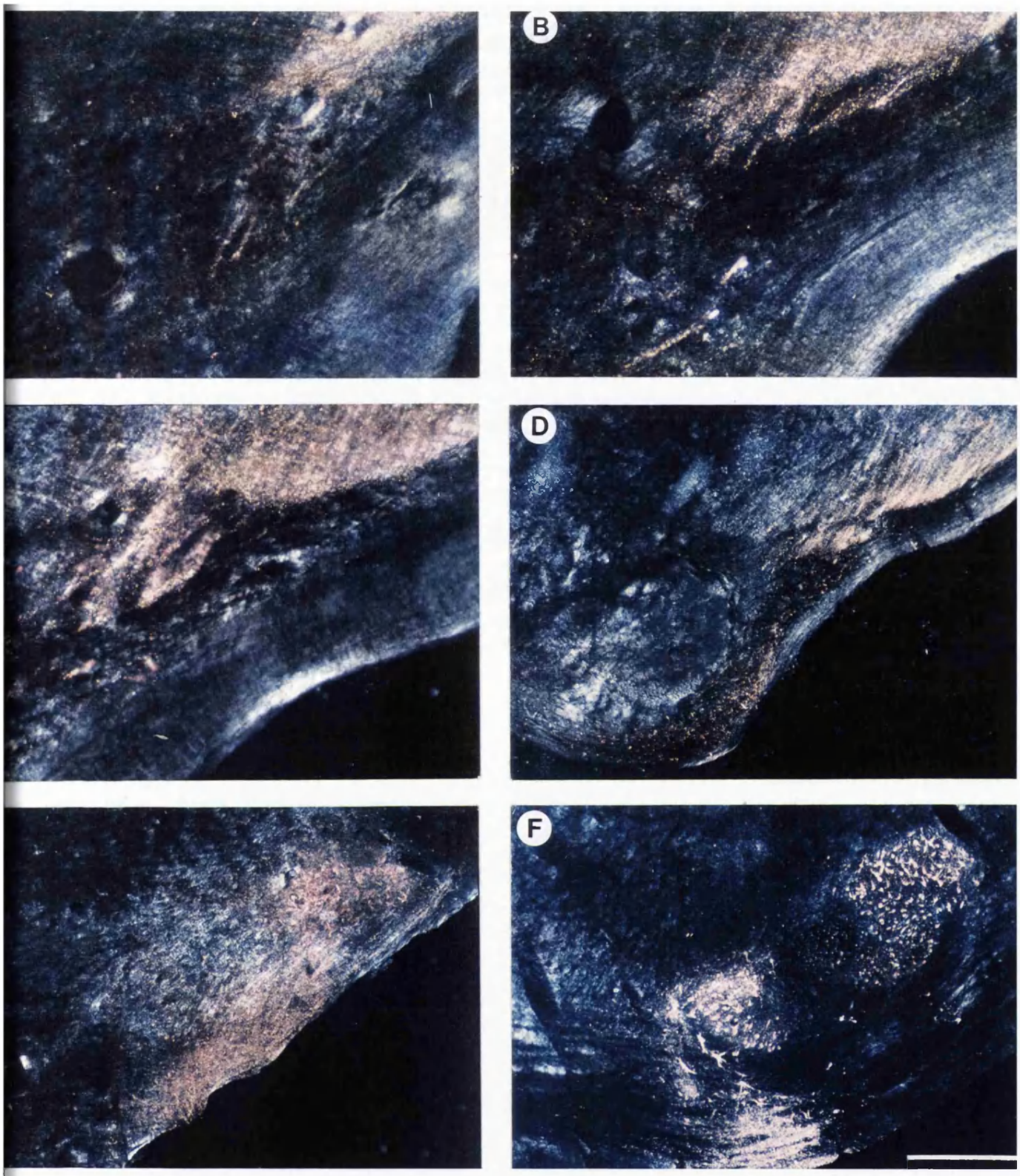

B
D
F

Figure 6

A - Line drawing of Figure 5D.

BP = brachium pontis

CS = cortico-spinal tract

NRTP = nucleus reticularis tegmenti pontis

DLPN = dorso-lateral division of the basilar pontine nuclei

LPN = lateral division of the basilar pontine nuclei

VPN = ventral division of the basilar pontine nuclei

LL = lateral lemniscus

VNLL = ventral nucleus of the lateral lemniscus

B - Line drawing of Figure 5E.

CS = cortico-spinal tract

VNLL = ventral nucleus of the lateral lemniscus

X = afferent and efferent fibres connecting the superior olivary complex and inferior colliculus

C - Line drawing of the superior olivary complex shown in Figure 5F.

DO = dorsal periolivary nucleus

LPO = lateral preolivary nucleus

LSO = lateral superior olive

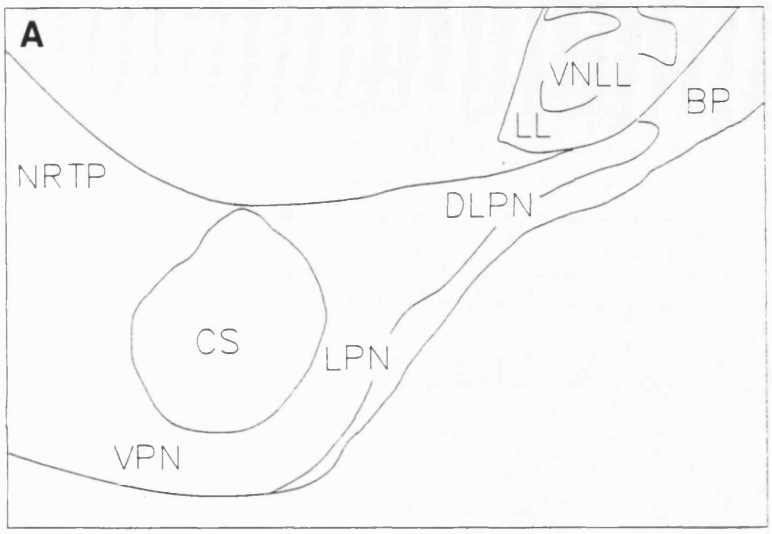
MPO = medial preolivary nucleus

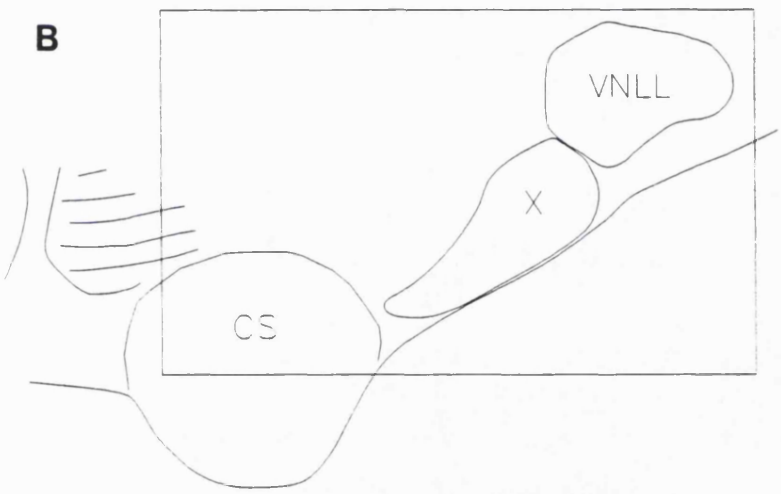
MSO = medial superior olive

NTB = nucleus of the trapezoid body

RO = retroolivary nucleus

Nomenclature taken from Borg (1973a).


A
VNLL
BP
LL
NRTP
DLPN
CS
LPN
VPN


B
VNLL
X
CS

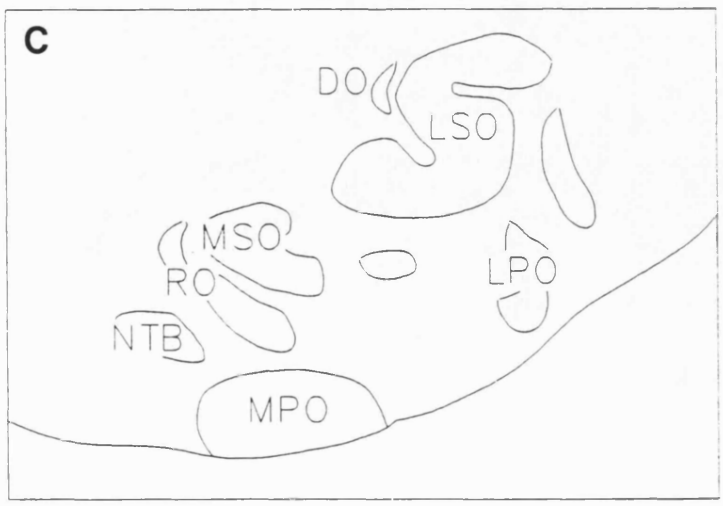
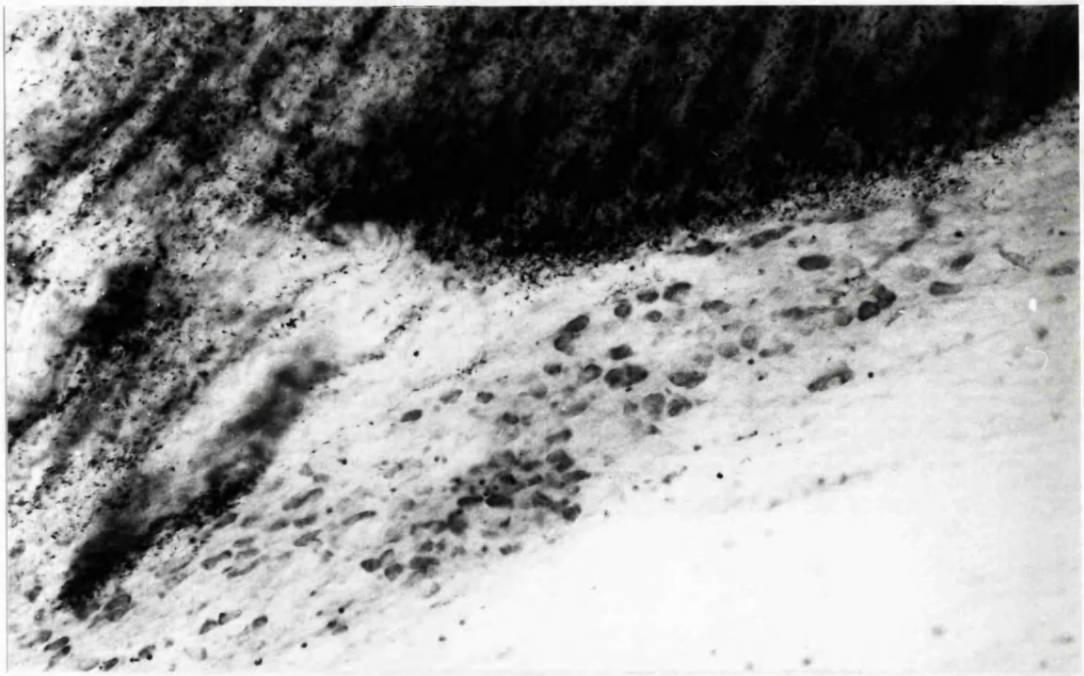

C
DO
LSO
MSO
RO
LPO
NTB
MPO

Figure 7

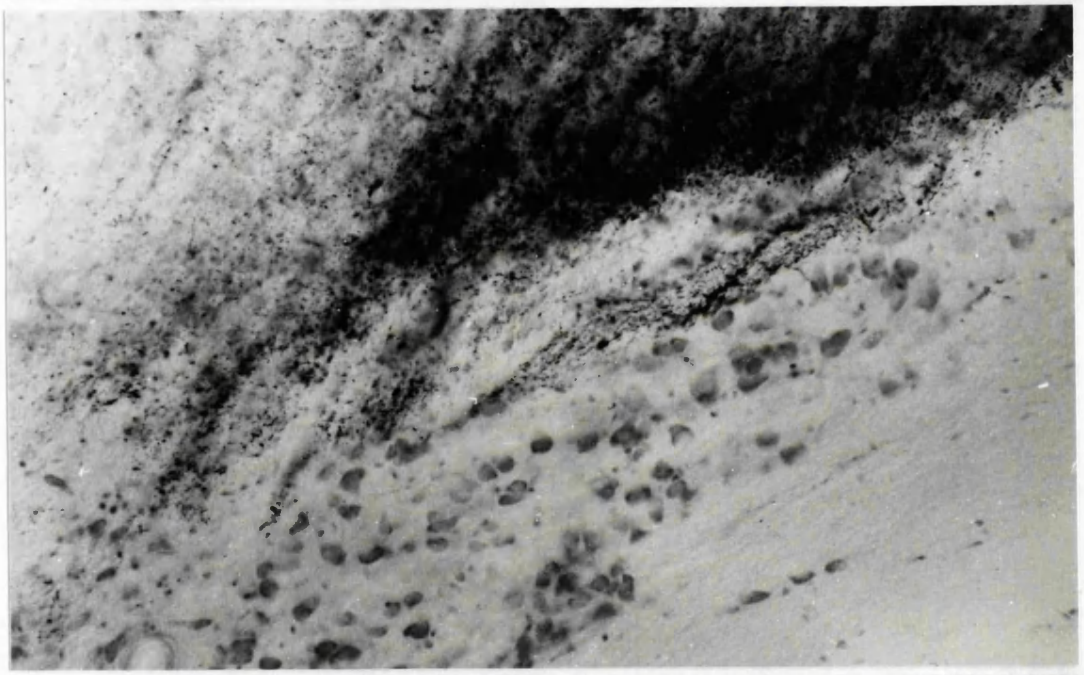
Subject IC8

Photographs taken under bright field illumination. Sections through DLPN at 2.5mm (A), 2.0 (B) and 1.5mm (C) rostral to lambda.

Label within lateral DLPN in 6A has the appearance of fibres traversing this region. There is no label in lateral DLPN in 6B or 6C. In 6B and 6C large amounts of label in medial DLPN appear to be within fibres of passage but a terminal field may be obscured by the density of label.
Bar = 0.25mm



A



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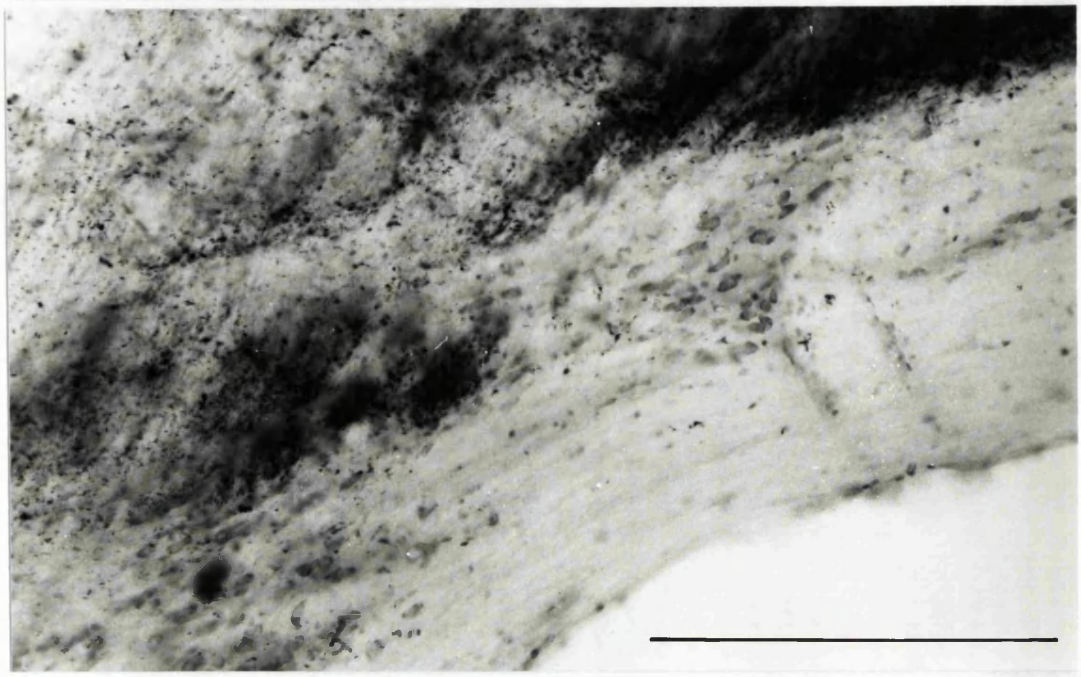
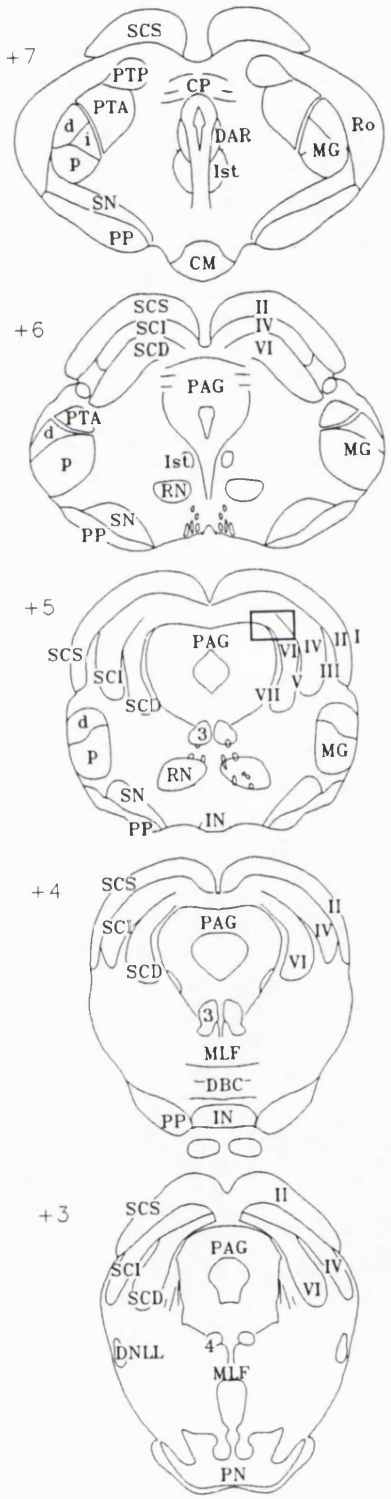


C

Figure 8

Key to divisions of the superior colliculus and medial geniculate body.

CP = commissura posterior
CM = corpora mammillaria
DAR = nucleus of Darkschewitsh
DBC = decussation of the brachium conjunctivum
DNLL = dorsal nucleus of the lateral lemniscus
IN = interpeduncular nucleus
MG = medial geniculate nucleus
d = dorsal sub-nucleus of the medial geniculate nucleus
m = medial sub-nucleus of medial geniculate nucleus
p = principal sub-nucleus of the medial geniculate nucleus
MLF = medial longitudinal fasciculus
PAG = periaqueductal grey
PN = pontine nuclei
PP = pes pedunculus
PTA = anterior pretectal nucleus
PTP = posterior pretectal nucleus
RN = red nucleus
Ro = optic radiation
SCD = deep layers of superior colliculus
SCI = intermediate layers of superior colliculus
SCS = superficial layers of superior colliculus
SN = substantia nigra
I..VII = layers of superior colliculus
3 = oculomotor nucleus
4 = trochlear nucleus
+3..+7 = position in mm of the section in relation to lambda.


+7
SCS
PTP
CP
PTA
d
i
p
DAR
Ist
Ro
MG
SN
PP
CM
+6
SCS
SCI
SCD
II
IV
VI
PAG
PTA
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Ist
RN
MG
SN
PP
+5
SCS
SCI
SCD
PAG
VI
IV
II
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III
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VII
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RN
MG
SN
PP
IN
+4
SCS
SCI
SCD
PAG
II
IV
VI
3
MLF
DBC
PP
IN
+3
SCS
II
PAG
SCI
SCD
IV
VI
DNLL
4
MLF
PN

Figure 9

Standard serial sections through the superior colliculus and medial geniculate nucleus of subjects
IC1 (A), IC2 (B) and IC3 (C) showing anterograde and retrograde WGA-HRP label.
Large dots = retrogradely labelled cells.
Small dots = labelled fibres.

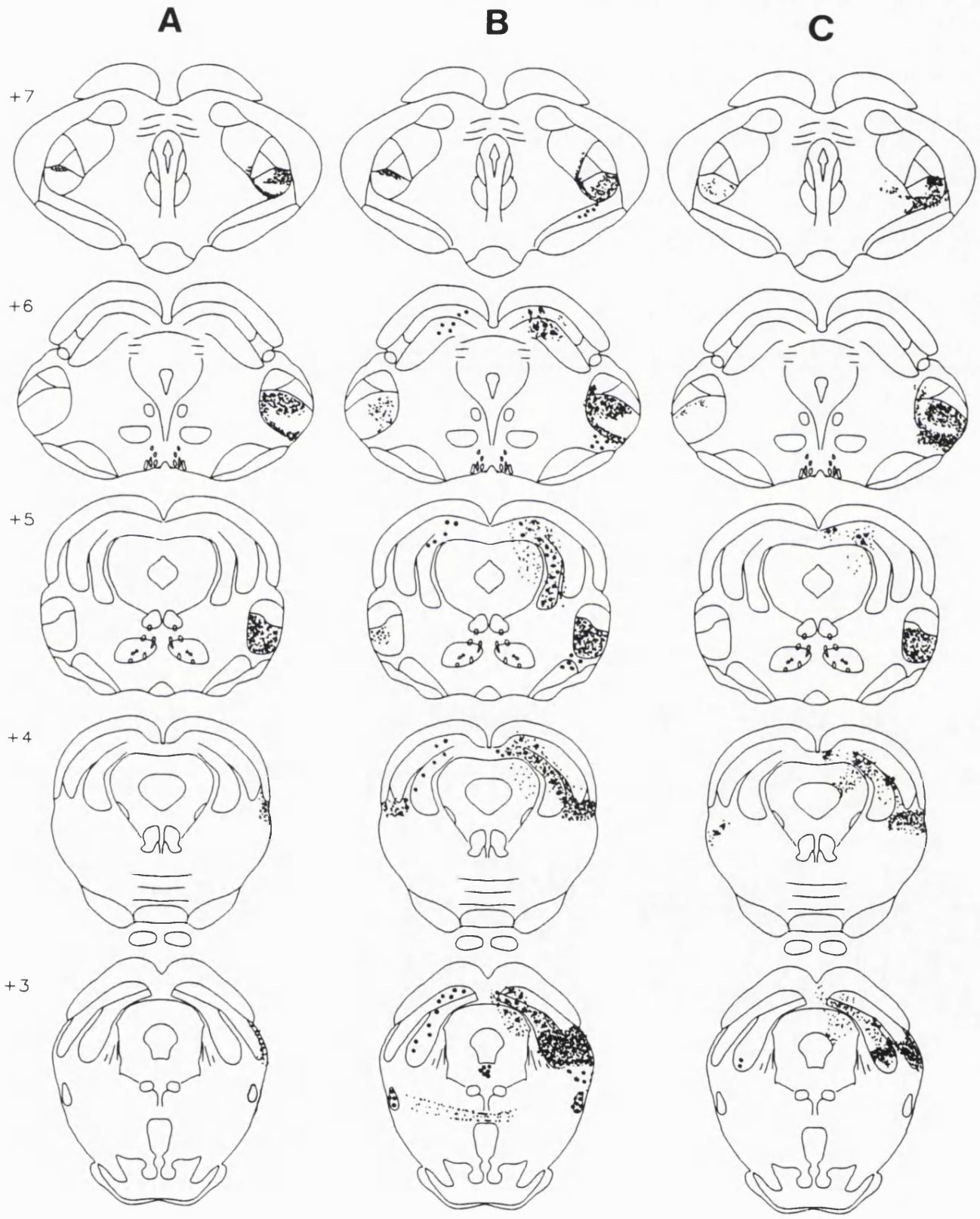
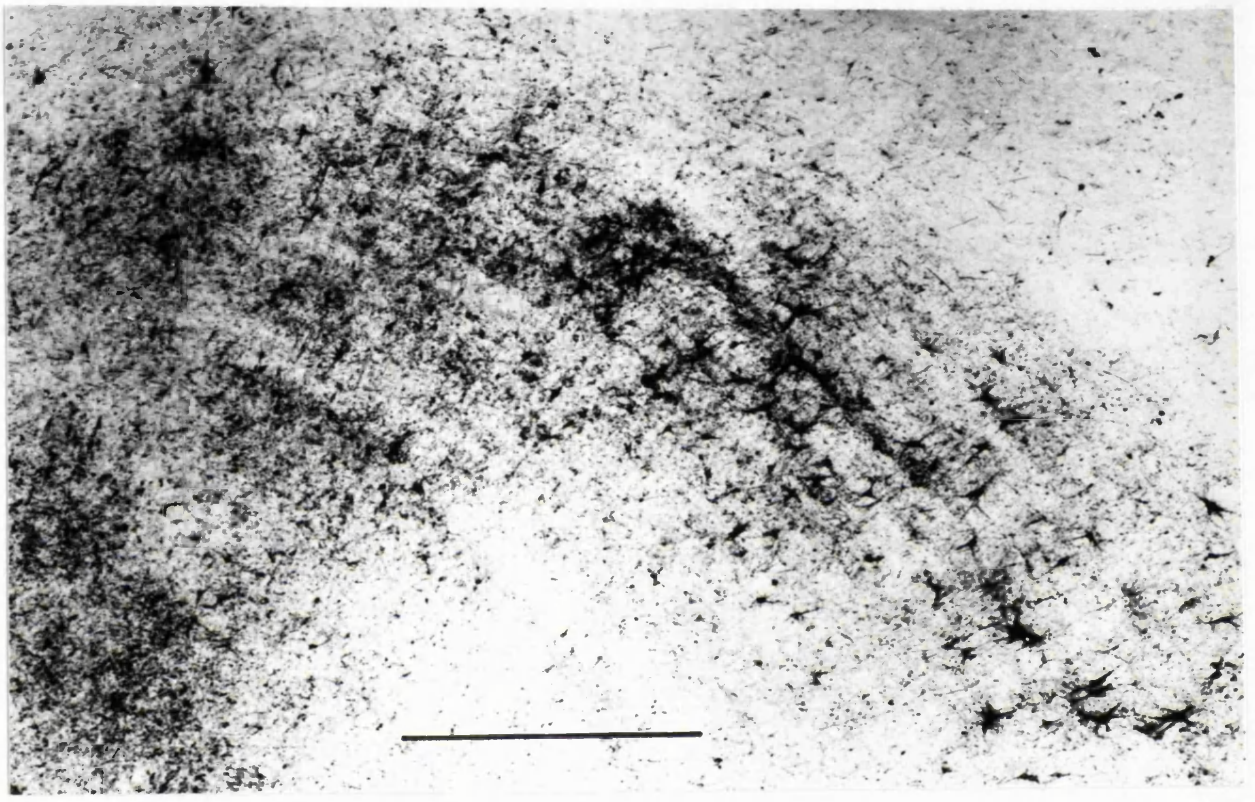

A
B
C
+7
+6
+5
+4
+3

Figure 10



Photomicrograph of superior colliculus at 5mm rostral to lambda in subject IC7 (region enclosed by the rectangle in Figure 8). This shows large amounts of anterograde and retrograde label in layer VI of the ipsilateral superior colliculus and in dorso-lateral periaqueductal grey.
Bar = 0.5mm

DISCUSSION

The studies in this thesis were designed primarily to answer three questions.

1. Is the cerebellar cortex necessary for eyeblink conditioning?

Thompson and his colleges initially reported that lesions of the cerebellar nuclei abolished CRs whereas large lesions of the cerebellar cortex had little effect upon CRs (Clark *et al.,* 1984; McCormick & Thompson, 1984). They concluded that the cerebellar cortex is not important for NMR conditioning, since cerebellectomy abolished CRs the critical site for NMR conditioning must be in the cerebellar nuclei. Others suggested that the cerebellar cortex is necessary for NMR conditioning. Yeo et al., (1984,1985b) showed that lesions centred on lobule HVI of the cerebellar cortex either abolished, or severely impaired CRs. Thompson's initial results may have been due to incomplete lesions of lobule HVI. Thompson's group subsequently reported that large cortical lesions which included lobule HVI did indeed produce profound effects upon both the acquisition and retention of CRs (Lavond and Steinmetz, 1989; Lavond et al., 1987).

Since the lesions would have destroyed afferent fibres as well as cortical tissue it is possible that the behavioural loss was due to retrograde degeneration in the inferior olive, or other precerebellar nuclei, thus depriving the cerebellar nuclei of essential inputs. This possibility was tested in the first study of this thesis. Discrete fibre sparing lesions of the cerebellar cortex were made with kainic acid (KA). The lesions produced profound deficits in CRs even though the cerebellar nuclei, inferior olive, and other precerebellar nuclei were not affected. In all cases there were initial deficits in CRs but in some cases the deficits were permanent. These data confirmed that the cerebellar cortex is essential for NMR conditioning. and showed that the cerebellar nuclei on their own cannot support NMR conditioning.

HVI is not the only cortical region involved in NMR conditioning. Although lesions centred on lobule HVI severely impaired or abolished CRs for 5 post-operative training sessions, with extended training CRs gradually recovered in several subjects. The abolition of CRs following selected ablation of lobule HVI is in agreement with previous studies of Yeo et al., (1984,1985). Two other sites in the cerebellum may have mediated recovery. The degree of recovery of CRs was related to the amount of Ansiform lobule spared by the lesions. Thus there are important but subsidiary sites within the Ansiform lobule. The intact contralateral cerebellum may also mediate in recovery of CRs. After 5 sessions of training on the lesion side some subjects had produced no CRs. The intact side was then trained for 5 sessions. All subjects rapidly learned to produce CRs on this side. When training was resumed on the lesion some subjects that had previously shown no CRs on this side immediately produced CRs. The idea that the contralateral cerebellum may participate in recovery was formally tested by Gruart and Yeo, (1995). Rabbits were first extensively over-trained. Large aspiration lesions of the cerebellar cortex which included lobule HVI and the Ansiform lobule were then made. CRs recovered in rabbits with unilateral lesions but failed to recover in rabbits with bilateral lesions. Thus the contralateral cerebellum can be involved in the production of ipsilateral CRs.

CRs are formed bilaterally even when the US is applied to one side. Bilaterality was clearly demonstrated in Study 1 of this thesis. Unilateral kainic acid lesions of the cerebellar cortex were made. The subjects were then heavily trained with the US applied to the right side. When the US was applied to the left side CRs were produced either immediately, or within very few trials (Study 1: Table 1 and Figures 6C,7C,8C). The same result was seen in the Study 3 of this thesis. In Study 3 Group B subjects received unilateral lesions of the cerebellar nuclei. The subjects were then trained ipsilaterally to the lesion. There were no CRs during this training. However, CRs were rapidly produced on the contralateral side when training commenced with the US applied to this side. Two mechanisms can explain the rapid acquisition of CRs by the contralateral side.

Firstly, US information is conveyed to both sides of the cerebellum and learning occurs in both hemispheres. This pathway can explain the immediate presence of CRs on the intact side when this side was trained after unilateral destruction of the cerebellar nuclei (Study 3). The contralateral cerebellar hemisphere can receive US inputs by two routes. Caudal parts of MAO receive a major bilateral input from face regions of the trigeminal nucleus (Van Ham and Yeo. 1992). Caudal MAO projects strongly to the borders of vermian VI and HVI (Yeo et al., (1985c). Crus I also receives a small projection from caudal MAO (Fig 4B, ANS8). The US may also gain access to the contralateral cerebellum through inter-trigeminal connections. Currently there is no evidence for a contralateral input to DAO. However, although CI only receives ipsilateral inputs, long latency responses can be recorded in C3 that may be contralateral in origin (Hesslow, personal communication). Thus A and C3 zones of the contralateral cerebellar hemisphere potentially can receive US information when the US is applied to the other side.

Secondly, there are pathways from one cerebellar hemisphere to eyeblink motorneurons on both sides. In ferrets, bilateral eyeblinks are produced by stimulation of the cerebellar cortex of one hemisphere (Ivarsson and Hesslow, 1993). A bilateral output from one cerebellar hemisphere can explain the presence of small, late CRs after unilateral destruction of the cerebellar cortex (Study 1, Figure 8C).

Since the Ansiform lobule may be involved in NMR conditioning the afferent and efferent connections of this lobule were studied. WGA-HRP tracing revealed inputs mainly from the ventral leaf of the principal division of the inferior olive and from anterior parts of the medial accessory olive. Ansiform lobule also receives minor inputs from face regions of the dorsal accessory olive and principal olive. Ansiform lobule receives inputs mainly from caudal ventro-lateral parts of the basilar pontine nuclei. The efferent connections of this lobule, particularly the regions of crusI immediately adjacent to lobule HVI, is primarily to rostro-lateral regions of the interpositus nucleus and to adjacent regions of the dentate nucleus. These results help to interpret why some parts of the Ansiform lobule can participate in NMR conditioning. There is a small input from face regions of the inferior olive as well as visual and auditory parts of the pontine nuclei. This region of the cortex projects to parts of the cerebellar nuclei that control eyeblinks.

2. Do lesions of the cerebellar nuclei cause a deficit in timing of CRs and URs?

Welsh and Harvey (1989) suggested that lesions of the cerebellar nuclei impair CRs because they cause a motor and not a learning deficit. This idea was suggested by their evidence that the lesions impaired both CRs and URs. In some subjects cerebellar lesions reduced the response amplitude and increased the onset latency. Responses were slowed so they did not fall within the time window normally used to observe CRs. This observation suggests that in previous studies which reported a complete loss of CRs the responses may have been delayed hence apparently abolished. URs elicited by the strength of US normally used in conditioning were unaffected by the lesions, but URs elicited by very low intensity USs were depressed in frequency and had abnormally long latency-to-peak and rise time. The lesions had depressed the eyeblink pathways. Welsh and Harvey argued that since CRs are normally smaller than URs they must be produced by a weaker drive in eyeblink motorneurons hence cerebellar lesions might appear to abolish CRs whilst leaving URs intact.

CRs might be apparently abolished because they are weakened and delayed by cerebellar lesions. To see if the apparent loss of CRs following cerebellar lesions was simply caused by a delay in CR production, fibre sparing lesions of the cerebellar nuclei were made with KA. KA lesions of the cerebellar nuclei produced a complete and permanent loss of CRs in previously trained rabbits, and prevented the acquisition of CRs in naive subjects. The loss of CRs was not due to a deficit in timing - there were no responses at all. The lesions caused a slight depression of UR amplitudes which was far too weak to account for the profound loss of CRs. There was no evidence to support the idea that CRs are weaker than URs and so are more susceptible to injury from cerebellar lesions. When tested with a very low intensity US CRs of normal subjects were larger and had a higher peak velocity than URs. CRs were produced by a stronger drive in the eyeblink motorneurons. If the lesions caused a reduction in activation of the motorneurons then both CRs and URs should be depressed. But in lesioned subjects CRs were abolished and URs remained normal. In addition, even though there were no CRs, URs were enhanced in the presence of the CS. Thus the eyeblink motor circuit was not depressed sufficiently to prevent

further excitation, even by stimuli as weak as a tone CS. These data demonstrate that the cerebellar nucleus lesions could not have caused a deficit in CRs simply through depression of the eyeblink motor system.

3. Does the inferior colliculus project to the pontine nuclei in rabbits ?

Yeo et al., (1985c) suggested that lobule HVI is important for NMR conditioning because it receives climbing fibre inputs from face regions of the inferior olive that can provide information about periorbital USs and because it receives mossy fibre inputs from auditory and visual regions of the basilar pontine nuclei (PN) that can provide information about auditory and visual CSs.

Because NMR conditioning is largely unaffected by removal of the forebrain, auditory CS information is probably relayed to the cerebellum from the inferior colliculus (IC). In cats the IC projects heavily to middle parts of the dorso-lateral pontine nucleus. There is inconclusive evidence for a direct connection between the IC and PN in rats and monkeys. Projections from the IC to the PN have not been investigated in rabbits. The final study in this thesis was to determine whether the IC projects to the basilar pontine nuclei of rabbits. WGA-HRP was injected into the inferior colliculus which revealed a small direct pathway to the most caudal part of ventral and lateral regions of the pontine nuclei. An alternative route for CS transmission to the pons may be from the superior colliculus. There is a much larger projection from the IC to the deep layers of the superior colliculus. As these regions of the superior colliculus connect to similar regions of the PN as the IC either pathway could relay auditory information to the cerebellum.

General Discussion

A brainstem circuit engaged in eyeblink conditioning

Although the conclusion is still controversial (Llinas and Welsh, 1993), the evidence suggests that the cerebellum and its associated circuitry are essential components of a brainstem circuit engaged in the production of conditioned eyeblinks. The proposed circuit is shown in Figure 1. The cerebellar cortex receives CS information through the PN and US information through the IO. It connects to eyeblink motorneurons by way of the anterior interpositus/dentate nucleus. CS and US information probably converge within the same cortical regions that modulate nuclei controlling eyeblinks.

The regions of cerebellar cortex involved in eyeblink conditioning are defined by their inputs from the inferior olive that are related to the US, and by their outputs to parts of the cerebellar nuclei that control eyeblinks. This idea is based upon the proposal that the cerebellum is organised into discrete functional units or olivo-cerebellar complexes. Each unit receives a

unique set of inputs from the inferior olive and has outputs to specific parts of the cerebellar nuclei which control particular movements (Andersson et al., 1987).

The dendritic tree of each Purkinje cell is flattened in a plane orthogonal to the folial fissures. The axons from Purkinje cells form discrete fibre bundles as they project down to the cerebellar and vestibular nuclei. The fibre bundles are also arranged orthogonal to the folial fissures. These fibre bundles are most apparent in the vermis where they divide the cerebellar cortex into parasagittal zones. In the cerebellar hemispheres the zones remain orthogonal to the folial fissures, but the folia are curved so the zones are no longer arranged in parasagittal strips. The zones are named from medial to lateral: A,X,B,C1,C2,C3,D1,D2. The uniqueness of each zonal projection is essentially maintained in the cerebellar nuclei thus dividing the nuclei into discrete regions. Each cortico-nuclear zone receives climbing fibres from specific regions of the inferior olive. Purkinje cells in each zone receive input from one region of the olive only but some regions of the IO project to two zones through collateral branches of the climbing fibres (Andersson et al., 1987; Armstrong et al., 1973; Hrycyshyn et al., 1989).

The powerful influence of climbing fibre activity upon the development of Purkinje cells suggests that the zonal organisation of the cerebellar cortex is due its inputs from the inferior olive. If this idea is correct then the cortical regions of importance in eyeblink conditioning are those which receive inputs from the inferior olive related to the US. Anatomical and physiological evidence show that rostro-medial parts of the dorsal accessory olive (DAO) receives somatosensory information from the cornea and periorbital regions of the face (Harvey ey ai., 1984; Van Ham and Yeo, 1992; Cook and Wiesendanger, 1976; Gellman et al., 1983). The DAO projects to the C1 and C3 zones of the cerebellar cortex (Groenewegen et al., 1979). These zones lie adjacent to the vermis in a band extending from lobule IV to lobule VIIB (Van Rossum, 1969).

It is iikely that only a subset of the C1 and C3 zones lying mainly within lobule HVI and the Ansiform lobule are involved in eyeblink conditioning. Other regions of the C1 and C3 zones, lying within lobules IV and V of the cerebellar cortex, control fore and hind limb movements (Oscarsson, 1973). Thus specific cerebellar cortical regions that receive input from face somatosensory parts of the IO (Akaike, 1989; Cody and Richardson, 1979; Miles and Wiesendanger, 1975a,b) are uniquely involved in the control of eyeblink muscles (Hesslow, 1994a,b) through modulation of the anterior interpositus nucleus/medial dentate nuclei (McCormick and Thompson, 1984) and their targets in dorsal parts of the magnocellular RN (Chapman et al., 1988; Haley et al., 1988).

The same circuit appears to be involved in human eyeblink learning. Human subjects with large lesions of the hippocampus and thalamus show normal eyeblink conditioning (Weiskrantz and Warrington, 1979). In contrast, subjects with damage to the cerebellum (Lyle et al., 1988) or its associated circuitry (Solomon et al., 1989) are severely impaired in eyeblink conditioning. Eyeblink conditioning in rabbits is a useful model of human associative learning.

Why is the cerebellum necessary for eyeblink conditioning ?

Two main ideas have been put forward on the role of the cerebellum in conditioning.

1. There are sites of eyeblink learning within the cerebellum

The most compelling interpretation is that lesions of the cerebellum damage the neural mechanism of eyeblink learning. Both acquisition (Lincoln et al., 1982) and retention (McCormick et al., 1982; Yeo et al., 1985a,b) of eyeblink CRs are prevented by lesions of the cerebellum so it is likely that the cerebellum is either engaged in, or even a site of, memory formation and storage of eyeblink CRs.

The anatomical connections of HVI and Ansiform lobe are consistent with their role in the formation and execution of CRs. Sensory information related to the CS and US converge upon HVI and Ansiform lobule. These regions of the cerebellar cortex have efferent targets which can modulate the NM and external eyelids. During conditioning, cells within these lobules are activated by both the CS and US. Importantly the CS driven activity can precede overt CRs by up to 200 ms (Berthier and Moore, 1986; McCormick and Thompson, 1984). This pattern of activity would be expected if CRs are formed and initiated within these lobules.

A convergence of climbing fibre US information with mossy/parallel fibre CS information (Yeo *et al.*, 1985c) is consistent with a simple implementation of models of motor learning in the cerebellar cortex (Marr, 1969; Albus, 1971; Gilbert 1974). These models propose that parallel fibres to Purkinje cell synapses are modified by climbing fibre activity. The evidence for such a modification is still controversial but behavioural and physiological studies are in broad agreement with these models. Related changes in simple and complex spike activity of PCs occur during the modification of forelimb movements in monkeys (Gilbert and Thach, 1977). Conjunctive stimulation of parallel and climbing fibres causes a reduction in the efficiency of parallel fibre to Purkinje cell synapses (Ito, 1989).

2. Learning does not occur within the cerebellum

An alternative view is that lesions of the cerebellum do not damage learning but simply impair movement (Welsh and Harvey, 1989; Kelly et al.,1990). The lesions might disrupt the eyeblink motor pathway so that CRs produced outside the cerebellum cannot be properly expressed through the disturbed pathway. This idea is based upon the assumption that the excitability of the eyeblink motor circuitry is impaired by cerebellar lesions. This view is consistent with earlier studies in which lesions of the cerebellum were found to be associated with changes in tonic activity of motorneurons (Gilman, 1969) and limb, head and orbital muscle tone (Chambers and Sprague, 1955a,b).

A simple mechanism could account for a change in excitability within the eyeblink circuit. Lesions of either the cerebellar cortex or the cerebellar nuclei may cause a loss of inputs to eyeblink pathways that ultimately impinge upon the eyeblink motorneurons. Lesions of the cerebellar cortex will disinhibit cells in the cerebellar nuclei which will cause an increase in their firing rate. This will likely cause an increase in the tonic excitation of efferent nuclei. A facilitation of neuronal activity in the red nucleus would lead to an increase in activation of eyeblink motorneurons. However an increase in general excitation of the red nucleus may block inputs from other pathways through to the brainstem (eg. CRs from cerebral cortex). Thus lesions of the cerebellar cortex may cause an over-excitation of the eyeblink motor pathways. Conversely, lesions of the cerebellar nuclei will cause a disfacilitation of RN cells which will result in a decrease of the rate of firing or even a cessation of activity in the RN (Eccles *et al.*, 1967) and the subsequent disfacilitation of eyeblink motorneurons. This may also block inputs from other pathways from gaining access through the brainstem.

Studies which tested for changes in excitability of the eyeblink motor system caused by cerebellar lesions have yielded inconclusive results. Although lesions of the cerebellar cortex can facilitate URs (Yeo and Hardiman, 1992) and lesions of the nuclei produce a small motor deficit (Welsh and Harvey, 1989), other studies show no overall change in URs (Steinmetz et al., 1992). The inconsistency of effects suggest either that there is no correlation between a deficit in CRs and a change in URs or some other factor is involved. The lesions may cause an initial motor deficit which recovers. This idea is consistent with a large body of evidence showing recovery of limb movements following cerebellar lesions (Dow and Moruzzi, 1958; Gilman, 1969; Van der Meulen and Gilman, 1965). Temporary inactivation of the cerebellar nuclei by lidocaine or by muscimol produce immediate deficits in URs (Welsh and Harvey, 1989b; Bracha et al., 1994). Similarly, temporary inactivations of the red nucleus produce immediate effects upon URs (Bracha et al., 1993) whereas permanent lesions do not (Rosenfield and Moore, 1985). The difference in results is likely to be related to the amount of time before testing. Training may also assist in a motor recovery since URs in normal rabbits can be enhanced simply by the amount of behavioural training (see KA lesions of the cerebellar nuclei in this thesis; Gruart and Yeo, 1995).

Independent of their effects upon URs there was always a profound loss of CRs in these studies. There is little evidence for a motor deficit that is common to both CRs and URs (see Yeo and Hardiman, 1992). All the findings are consistent with the idea that the cerebellum is essential for learned components of conditioned eyeblinks, and that it **also has some control over reflex eyeblinks**.

Is eyeblink conditioning possible without the cerebellum ?

Kelly et al. (1990) argued that cerebellar lesions may only prevent the expression of CRs whilst leaving the CR memory intact. These authors found that eyeblinks conditioned in decerebrate rabbits were unimpaired after large lesions of the cerebellum, hence they concluded that the cerebellum was not engaged in eyeblink learning. They suggested that cerebellar lesions

remove tonic drive acting upon motor pathways mediating the NM response. Decerebrations, they argue, would restore the level of this tonic activity thus allowing the expression of CRs formed in circuitry outside the cerebellum.

These results have not been confirmed. In a similar experiment, Yeo (1992) found that complete cerebellectomy in decerebrate rabbits prevented normal eyeblink conditioning. Since the lesions of Kelly et al., (1990) were mostly confined to one half of the cerebellum it seems likely that the CRs were produced by the intact cerebellum (see Gruart and Yeo, 1995). The results of Kelly et al. (1990) were also questioned by Nordholm et al. (1991). They showed that the inter-trial intervals used by Kelly et al. (1990) do not support NMR conditioning in the intact rabbit. Responses driven by the CS in the study by Kelly et al., (1990) may not have been true CRs produced by an associative process, hence this study may not be a proper test of the role of the cerebellum in conditioning.

Although the conclusions of Kelly et al., (1990) are controversial, extra-cerebellar regions may support eyeblink conditioning. There may be parallel brainstem circuits engaged in eyeblink learning. Yeo (1992) suggested that the decerebrations of Kelly et al. (1990) probably damaged the RN, hence CRs had to be acquired in an extra-cerebellar circuit, and so the subsequent cerebellar lesions would be expected to have little effect upon learning. However, it is unlikely that there are important learning circuits that operate in parallel with the cerebellum but without its participation. Norman et al. (1977) found that some cats decerebrated mid-way through the cerebellum could learn to discriminate between two different tones. But since CRs were small and produced intermittently even after several months of training the lesions must have removed a brain region normally engaged in this learning. It is highly likely that the missing brain region was the cerebellum.

Are there sites of eyeblink learning other than the cerebellum ?

Even though some authors are adamant that the cerebellum cannot be involved in learning (Bloedel, 1992; Llinas and Welsh, 1993), very few studies have been aimed at finding an alternative site of learning. In two studies it was suggested that the CS/US association necessary for learning takes place within the spinal trigeminal pars oralis (SpVo) (Nowak and Gormezano, 1990) or in a region of the reticular formation lying adjacent to the SpVo (Bracha *et al.*, 1991).

Nowak and Gormezano, (1990) directly stimulated the reticular formation, the SpVo, the abducens nucleus, and the accessory abducens nucleus. URs elicited by the stimulation did not differ across the sites. A tone CS was then paired with direct stimulation of each site as a US. When the CS was paired with stimulation of the SpVo URs were enhanced and CRs were rapidly acquired as training proceeded. This was the only site at which stimulation produced enhanced URs and CRs. The authors suggested that this was evidence for a locus of learning at the SpVo. In contradiction to these results, Young et al., (1976) reported that direct stimulation of the abducens nucleus as a US together with a tone CS caused facilitation of the

UR and to the production of NM CRs. These authors thought it unlikely that VI nucleus was a locus of learning. The result that direct stimulation of the SpVo and the abducens nucleus paired with a tone CS can lead to NMR conditioning can be readily explained. Firstly, stimulation of the abducens nucleus could lead to back firing of the SpV nucleus. Although the SpVo is not a major site of primary afferent input from the face and cornea (Van Ham and Yeo, 1996) it may receive inputs from other regions of the SpV (Van Ham and Yeo., 1992). Stimulation within SpVo would also activate other trigeminal regions through activation of intra-trigeminal connections and by directly activating the primary afferent axons passing through this region (Van Ham and Yeo., 1992). Thus rapid learning would be expected wherever the locus of learning. Since SpVo and other parts of the SpV project directly to the cerebellum and indirectly via the IO (Van Ham and Yeo., 1992) the simplest explanation for these results is that learning occurred in the cerebellum.

Bracha et al., (1991) injected lidocaine into, and close to, SpVo during NMR conditioning. Injections into SpVo abolished CRs and diminished URs. These injections clearly inactivated the eyeblink reflex pathway. In contrast to Nowak and Gormezano, (1990), the authors rejected SpVo as a locus of eyeblink learning. Injections adjacent to SpVo abolished CRs but had no effect upon URs, hence the authors suggested that the trigeminal nucleus could be a locus of eyeblink learning. Two criticisms can be aimed at this study. Firstly, there was no test of URs elicited by low intensity USs which might have revealed an effect of the inactivations upon URs. Inactivations at this site may have merely prevented the expression of CRs formed elsewhere (see Welsh and Harvey, 1989). Secondly, the results are readily explained by the inactivation of connections between the SpV and IO and possibly by the inactivation of direct connections to the cerebellum (Van Ham and Yeo, 1992). These connections convey US information to the cerebellum that is essential for NMR conditioning (Yeo *et al.*, 1986). The most likely explanation for the loss of CRs but not URs is that the cerebellum was deprived of essential information about the US from the IO.

Other brainstem sites have been implicated in learning. For example the red nucleus has been proposed as a site of learning for classically conditioned limb movements in the cat (Tsukahara *et al*, 1981; Oda *et al.*, 1988). Classical conditioning of forelimb flexion can be produced by pairing electrical stimulation of the cerebral peduncle as a CS, with electrical stimulation of the forelimb as a US. An increased responsiveness of RN cells to the CS is correlated with the acquisition of conditioned forelimb flexion. In contrast, responses of RN cells, as well as the behavioral response, induced by stimulation of the interpositus nucleus of the cerebellum, are not enhanced after paired conditioning. Differences between responses to stimulation of the cerebral peduncle and interpositus nucleus suggest that the primary site of neuronal plasticity is in the RN and not cerebellum. It was suggested that the appearance of new cortico-rubral synapses could underlie the behavioral modification. It is possibile that the RN is a site of

eyeblink learning, however, because lesions of the cerebrum and decerebration do not prevent eyeblink conditioning then cortico-rubral synapses are not neccessary for this learning. It is possible that interposito-rubral synapses are capable of plasticity. The evidence from temporary inactivation experiments discussed below suggest that even if there is plasticity in the RN, it is not essential for eyeblink conditioning.

Llinas and Walton, (1979) suggested that the idea of a single locus of learning (Marr, 1969) is unlikely and that motor skills probably require learning in multiple brain sites. This idea can mean either that there is learning in the cerebellum and learning in parallel elsewhere or that other brain sites are involved together with the cerebellum in a distributed neuronal network that learns. In this idea there is no particular locus of learning but the entire cerebellar circuitry is the learning network.

Evidence from studies on modification of the vestibular ocular reflex (VOR) suggest that there may be parallel sites of learning for this response or that the cerebellum and its brain stem targets are acting as a neuronal network that learns. The VOR serves to stabilise images on the retina during head movements, resulting in eye movements equal in amplitude but opposite in direction to the head movements. This is accomplished by the detection of retinal slip as the image moves across the retina and head velocity by the vestibular hair cells in the semicircular canals and otolith organ. A long term modification of the VOR can be induced by oscillating the subject at a constant frequency whilst the subject views a stationary scene composed of random dots or stripes. The flocculus has an essential role in the modification of the VOR. Ito (1984) maintains that the flocculus is the site of neuronal processes underlying this modification. Others have suggested that the crucial neuronal processes are contained in the vestibular nuclei (Lisberger, 1988). More recent evidence suggests that there are sites of plasticity in the cerebellum and the brainstem (Stone and Lisberger., 1990; Lisberger, 1994) but each site is responsible for different aspects of the VOR, characterised by different latencies. Since the brain stem neurons receive direct inhibitory input from PCs in the flocculus these neurons can be considered equivalent to the deep cerebellar nuclei. Thus if adaptation of the VOR is considered to occur in a distributed neuronal network, the network is equivalent to the cerebellar cortex and cerebellar deep nuclei.

The cerebellum is actively engaged in eyeblink learning.

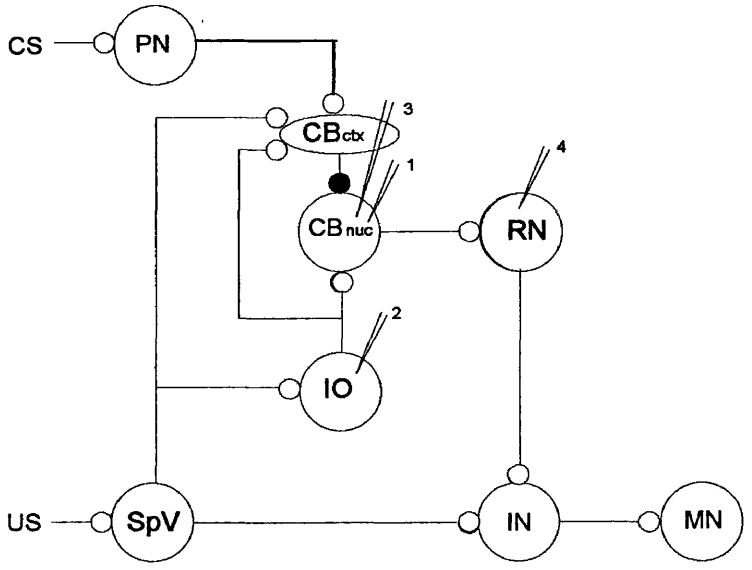
Lesions of the cerebellum prevent the acquisition and retention of eyeblink CRs by a deficit in learning. Neither permanent lesions of the cerebellar cortex (Study 1) nor permanent lesions of the cerebellar nuclei (Study 3) cause a motor deficit sufficient to prevent the expression of CRs learned elsewhere in the brain as has been suggested by some (Bloedel et al., 1991; Kelly et al., 1990; Llinas and Welsh, 1993; Welsh and Harvey, 1989). The cerebellum actively contributes to the acquisition and retention of CRs. An active contribution would mean either

that there are neural mechanisms underlying eyeblink conditioning within the cerebellum - the cerebellum may be the locus of learning or it may be part of a neural network which learns - or that the cerebellum provides an essential input to a site of learning elsewhere. Permanent lesions of the cerebellum provide insufficient data to distinguish between these possibilities.

Temporary inactivation of the cerebellar circuitry can help to dissociate these possibilities. This procedure involves temporarily blocking the neuronal activity of a putative site of plasticity. Training trials are given during the blockade. If the blockade is successful then no CRs will be produced during training. Two possibilities can explain this outcome, either learning is prevented by the blockade, or learning is normal but CRs are prevented from expression by the blockade. The correct possibility can be deduced by allowing sufficient time for the neuronal blockade to dissipate and then retraining. If, during this second training period, CRs are acquired as if the animal is naive, then the temporary inactivation probably prevented learning -

the site of inactivation must be actively engaged in the learning process. But if CRs are produced on first testing, then the inactivation did not prevent learning - the site of inactivation was not actively engaged in NMR conditioning.

Figure 1 Brainstem eyeblink circuit showing sites temporarily inactivated during training.



CBctx - cerebellar cortex
CBnuc - cerebellar nuclei
CS - conditioned stimulus
IN - interneuron
IO - inferior olive
MN - motoneuron
SpV - spinal trigeminal nucleus
US - unconditioned stimulus

1. Welsh and Harvey, 1992.
2. Welsh and Harvey, 1990.
3. Krupa et al., 1993; Clark and Lavond, 1993; Clark et al., 1993
4. Clark and Lavond, 1993; Clark et al., 1993.

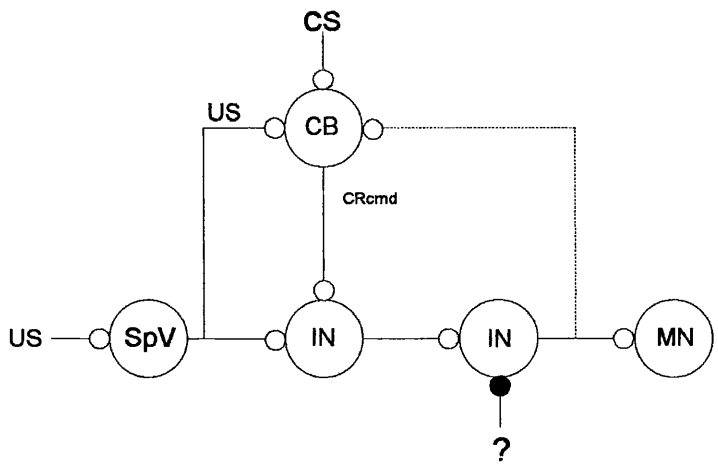
In the first of such studies, Welsh and Harvey (1991) conditioned rabbits to blink to a light CS. They then injected lidocaine into the cerebellar nuclei whilst training to a novel tone CS (see Figure 1). The effectiveness of the lidocaine block was tested by periodically presenting the light CS during the tone training session. There were no CRs to either the light or the tone CS during the application of lidocaine. After recovery from the effects of lidocaine the subjects were tested by presenting the tone CS alone. The subjects immediately produced CRs. The inactivation had not prevented learning. Welsh and Harvey (1991) concluded that the cerebellar nuclei are neither a site of essential plasticity nor do they provide a necessary modulating influence for learning elsewhere.

In other experiments, cooling probes (Clark et al. 1993; Clark and Lavond 1993) and the γ-aminobutyric acid-A ($GABA_A$) agonist muscimol (Krupa et al. 1993) were used to block activity either in the cerebellum, or in the red nucleus (RN), during NMR conditioning (see Figure 1). No CRs were produced during inactivation of either the cerebellum or the RN. When the cerebellar blockade was removed the subjects initially produced no CRs. They subsequently learned at rates which were similar to those of naive subjects. In contrast, CRs were immediately produced after removal of the RN blockade. The animals performance showed that learning had occurred during inactivation of the RN. Although no CRs were apparent during inactivation of the RN, neuronal activity in the nucleus interpositus was similar to that recorded after the subjects had recovered from the neuronal block and were producing overt CRs. Also, when the cerebellum

was blocked, neuronal activity in the RN was not related to CRs. But CR related activity did become manifest during subsequent training sessions without the blockade (Clark and Lavond, 1993). These data show that the cerebellum is actively involved in NMR conditioning and indicate that there are mechanisms of learning within it.

Welsh and Harvey (1991) found that learning proceeded normally when the cerebellar nuclei were blocked, yet Clark et al. (1993), Clark and Lavond (1993) and Krupa et al., (1993) found that the learning was blocked. The results of these studies seem incompatible but they can be explained if there are essential sites of plasticity within the cerebellar cortex. If the lidocaine inactivation's of Welsh and Harvey (1991) were restricted to the nuclei without affecting the cerebellar cortex and the muscimol inactivations in the study by Krupa et al. (1993) or the cooling inactivation by Clark et al. (1993) also invaded the cerebellar cortex then learning must have taken place in a site afferent to the cerebellar nuclei. Since inactivation of the inferior olive prevented the acquisition of CRs (Welsh and Harvey 1990) learning must be either within the IO or efferent from it. Cells of the IO have a very low frequency of firing hence they can provide the cerebellum with a limited amount of information. It is unlikely that a CR motor command is formed within the olive. The most likely site is within the cerebellar cortex (see Figures 1 and 2).

Figure 2 Learning within the cerebellum



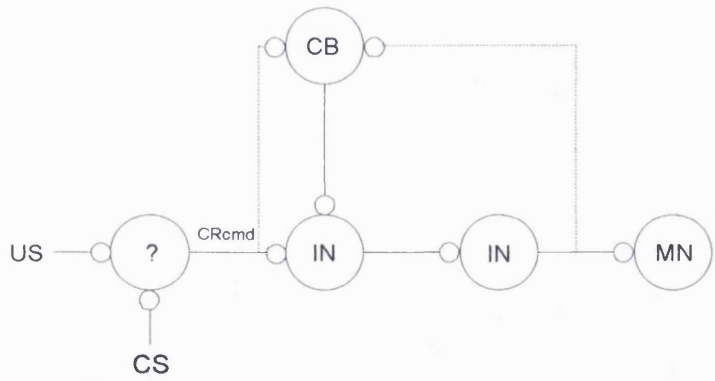
Learning in cerebellum accounts for early, learning related, neuronal activity in cerebellum. There is integration of the cerebellar output with the reflex circuit before the MNs allowing both integration of other inputs (possibly inhibitory) from pathways controlling eye movements, and the distribution of outputs to several motoneurons - in cranial nuclei III, IV, VI, accVI and VII. Dotted line shows feedback from final movement command.

Alternatively the inactivation s of Welsh and Harvey (1991) may have been incomplete. The blockade may have been sufficient to prevent the overt appearance of CRs but insufficient to prevent low levels of conditioning to the second CS. An initial low level of conditioning might allow the rapid transfer of conditioning (Kehoe and Holt, 1984; Kehoe et al., 1984) from the first CS leading to a high level of conditioning to the second CS by the end of training. This would be apparent upon withdrawal of the neuronal block as an immediate appearance of CRs to the second CS. Circuitry supporting these low levels of conditioning might be in the ipsilateral cerebellum or in the contralateral cerebellum. Bilateral inactivation of the inferior olive prevents the acquisition of CRs (Welsh and Harvey 1990) and bilateral lesions of the cerebellar cortex abolish CRs (Gruart and Yeo 1995). Both results suggest that the contralateral cerebellum can

support conditioning. Another possibility is that the lidocaine preferentially affected cerebellar efferent fibres in the brachium conjunctivum leaving the cerebellar nuclei essentially intact. Unless there was complete inactivation of all relevant nuclear sites in the study by Welsh and Harvey (1990), then the possibility put forward by Clark and Lavond (1993) that there are mechanisms of eyeblink learning within the cerebellar nuclei must be allowed.

Whether or not the essential region of the cerebellum is the cortex or the nuclei, the results of three recent studies confirmed that the cerebellum is actively engaged in eyeblink learning. Temporary inactivation of the cerebellum during conditioning prevented both the acquisition and extinction of conditioned NMRs (Hardiman et al., 1995; Ramnani et al., 1995). Temporary inactivation of the cerebellum in pretrained rabbits prevented the acquisition of CRs to a second CS. Both CSs were a 1KHz tone, but they differed in the duration of the tone hence the time interval between the CS and US. (Lobo et al., 1996)

Figure 3 A site of learning afferent to the cerebellum

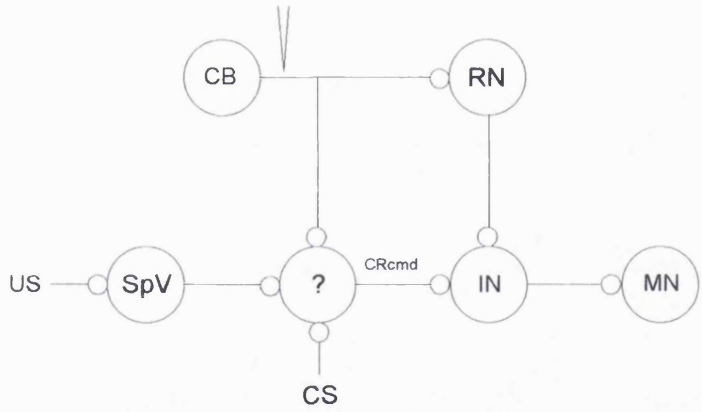


Loss of output from the cerebellum may cause disfacilitation of afferent nuclei thus preventing the expressions of CRs. Dotted lines showing possible feedback and feedforward pathways. Learning before cerebellum allows a feedforward pathway thus accounting for early, learning related, neuronal activity in cerebellum

Temporary inactivation of the cerebellum prevents learning. It is therefore highly unlikely that there is an important site of eyeblink conditioning afferent to the cerebellum (Figure 3). The idea that CRs formed in a brainstem site - for example in interneurones close to the spinal trigeminal nucleus pars oralis as suggested by Bracha et al., (1991) - are prevented from expression through a depressed motor pathway caused by cerebellar lesions (Kelly et al. 1990; Welsh and Harvey 1989, 1991; Llinas and Welsh 1993) cannot be upheld, as this would require that the essential learning is in a site afferent to the cerebellum. It is also unlikely that there is an essential pathway through the cerebellum for the CR command since learning should be unaffected by such inactivations. Equally unlikely is the idea put forward by Marr (1969) and Albus (1971) that motor learning occurs first within the cerebrum and is then transferred to the cerebellum through instruction by the IO since all learning is prevented by inactivation of the cerebellum. This also means that during eyeblink conditioning, the IO is unlikely to act as a comparator between intended and actual movement and signal errors in motor performance, as has been suggested for other movements (Miller and Oscarsson 1970; Ito, 1982).

Because inactivation of the red nucleus during NMR conditioning does not prevent the acquisition of CRs, it was suggested that the essential plasticity must either be within the cerebellum or afferent to it (Kelly et al., 1990; Clark and Lavond, 1993). This can be concluded only if the RN is the sole efferent target of cerebellar output related to conditioned eyeblinks. If thalamic or other brainstem pathways, for example tectal, are involved, there might be mechanisms of learning in a site efferent from the cerebellum (see Figure 4). In this case the role of the cerebellum might be to provide an input necessary for learning elsewhere - the cerebellum either has no capacity for plasticity relating to eyeblink conditioning or it is part of a distributed learning network. Much less likely is the possibility that the cerebellum has no special role in eyeblink learning but is necessary simply to provide a general facilitatory drive. In this case lesions of the cerebellum would not prevent the expression of CRs as proposed by some authors (Welsh and Harvey, Bloedel, Llinas), but the lesions would prevent the learning itself by blocking essential inputs to a site of learning. In addition, lesions of the RN would prevent the expression of CRs through a loss of general facilitation in the eyeblink motor pathway (see Figure 4).

Figure 4 A site of learning efferent from the cerebellum



A brainstem site of learning receives an essential input from the cerebellum through a pathway other than the RN. Inactivation of the CB blocks the integration of CS and US preventing learning. Inactivation of the RN blocks the expression of learning.

Whether or not the RN is the essential target can be tested by training rabbits whilst inactivating the brachium conjunctivum close to the cerebellum, thus blocking all inputs to targets other than the RN as well as the RN (see Figure 4). If learning is prevented, the site of learning would be efferent from the cerebellum. If learning is not prevented, then learning must occur before the site of inactivation - most likely within the cerebellum. Thompson and Krupa (1995) injected tetrodotoxin into the brachium conjunctivum close to its exit from the cerebellum. There were no CRs during acquisition training sessions with the brachium inactivated. CRs were immediately present when the block was removed. If all fibres necessary for eyeblink conditioning were blocked during training then there cannot be essential sites of learning efferent from the cerebellum (see Figure 4).

The simplest explanation for all of these inactivation studies is that there are sites of learning within the cerebellum. The doubts expressed by some authors as to whether the cerebellum is actively involved in eyeblink conditioning (Llinas and Welsh, 1993), or that there are more important parallel pathways through which eyeblink learning can take place (Bloedel et al., 1993), are unfounded. The results from both permanent and temporary lesions studies indicate that the neural mechanisms of eyeblink conditioning are contained solely within the cerebellum. There is no evidence to support the idea that there are other sites of learning or that the cerebellum is part of a distributed neural network.

Marr (1969), Albus (1971) and Gilbert (1974) suggested that there are motor learning mechanisms within the cerebellar cortex. On the basis of its anatomical connections, the effects of lesions and neuronal responses it is clear that HVI and Ansiform lobe can produce conditioned NM responses that are appropriately timed and of the correct topography. Most theories of cerebellar learning propose that there is a long term change in the efficacy of parallel fibre (PF) to Purkinje cell (PC) synaptic activity according to a learning rule provided by climbing fibre (CF) inputs from the inferior olive (IO). Marr suggested two mechanisms of learning, each requiring a different function for the IO. One mechanism requires that movements are first learned in the cerebral cortex. A movement is composed of basic elements each of which is coded in the IO. When the cerebrum produces a movement the appropriate cells in the IO are excited in sequence as the movement progresses. The IO would then teach the cerebellum how to make the same movements. If, during activation of a particular PC by its CF, the pattern of activity in the PFs (or frequency of firing: Gilbert, 1974) produces an output in which the correct muscles for that elemental movement are activated - then the synaptic strength of active PFs will increase (or the synaptic strength of incorrect PFs will decrease: Albus, 1971). The second mechanism can account for situations in which the context of the movement changes slowly such as in maintained learned postural reflexes. In this case the olivary cells would be activated by a signal that occurs just after the movement command signal, for example from proprioceptive receptors. Although either mechanism could account for eyeblink conditioning, the idea that learning first occurs in the cerebral cortex is unlikely. Eyeblink conditioning is largely unaffected by decerebration, whilst all learning is prevented during inactivation of the cerebellum. Conditioned eyeblinks are not maintained reflexes, since they are transient and of varying amplitude.

The evidence suggests a different role of the inferior olive in eyeblink conditioning to that of a teacher (Marr, 1969; Albus, 1971). During eyeblink conditioning the IO provides the cerebellum with information about the US. Regions of the IO that receive face somatosensory input are essential for eyeblink conditioning (Yeo et al., 1985c; Yeo et al., 1986; Van Ham and Yeo, 1992). It is unlikely that IO relays CS information to the cerebellum because eyeblink conditioning is unaffected by lesions of regions of the IO that receive visual inputs (Yeo et al., 1985c), and because there are no known auditory inputs to the IO. Thompson's group have consistently argued that the IO is **the** US channel (Thompson, 1990; Thompson and Krupa,

1995) since they found that lesions of the IO cause extinction of CRs (McCormick *et al.*, 1985), but other studies have shown that lesions of the IO abolish CRs immediately (Welsh and Harvey, 1990; Yeo et al., 1986). If the lesions simply removed the US the CS would initially elicit CRs but CRs would slowly decline despite repeated CS-US pairing. IO cannot only provide US information to the cerebellar cortex. CFs are also essential for tonically regulating the cerebellar cortex, since the absence of this input causes MFs to overexcite the PCs (Colin et al., 1980).

Because olivary neurons fire at a very low frequency (~1Hz) the information content of CFs is severely limited. However, the IO can provide the cerebellum with at least two pieces of information necessary for eyeblink conditioning. Firstly, the presence of a US - from which the timing between the CS and US could be obtained. Secondly, because inputs to the IO, particularly to the DAO, are somatotopically arranged (Gellman et al., 1983) the IO can provide information about the position on the body of the US. The intensity and duration of USs are also important parameters in conditioning. Although it is possible that UR intensity could be coded as an increase in the probability of firing of the IO, the cerebellum can obtain information about both the UR intensity and duration through mossy fibre pathways from the trigeminal nucleus.

Studies of cat limb movement have shown that IO acts as an event detector that signals unexpected external events (Gellman et al., 1985), or unexpected perturbations of the body (Anderson and Armstrong, 1987). If the IO acts in the same way during eyeblink conditioning then it will signal unexpected occurrences of the US. This idea is compatible with Marr's (1969) assumption that once learning is complete, olivary inputs are not required. Thus as CRs are acquired the IO will be inhibited, either by nucleo-olivary pathways (Andersson and Hesslow, 1987) or by inputs from the RN (Weiss et al., 1993). As pointed out by Andersson et al., (1988), such a mechanism can explain blocking since once the CFs carrying US information to the cerebellum are inhibited then further learning cannot occur. The idea that US inputs to the cerebellum are gradually turned off during learning fits well with theoretical studies which propose that associative learning is a function of the novelty or unexpectedness of the US (Rescorla and Wagner, 1972).

During eyeblink conditioning the IO has two functions. Firstly it provides the cerebellum with information about the occurrence of the US. Secondly it tonically regulates PCs (Colin et al., 1980; Yeo et al., 1986). If there is inhibition of the IO during learning there must be some mechanism for maintaining the tonic regulation of PCs. If learning only requires that there is a reduction in the probability of firing of olivary cells then it is possible that the inhibition is not complete. Perhaps only an occasional CF input to the cerebellar cortex is necessary to maintain the regulation of PCs.

At first sight the evidence that the cerebellum is actively involved in the extinction of CRs (Hardiman et al., 1996; Ramnani and Yeo, 1996) does not readily fit with the idea that the IO provides the cerebellum with information about the occurrence of the US. Only the CS is presented in extinction training. Since there is no US, those parts of the IO that normally signal the occurrence of the US will be silent. In order to prevent extinction of CRs during normal learning the cerebellum will need to distinguish between the loss of CF inputs through active inhibition of the IO during learning, and when there is no US. A distinction could be made on the basis of PF activity in the cerebellar cortex since MFs will be carrying US information to the cerebellar cortex during paired presentations of the CS and US.

The neural mechanism of extinction are currently unknown. Although extinction can occur through the destruction of the original memory, a complete loss of the CS-US association does not easily fit with the evidence of spontaneous recovery of CRs between extinction sessions or with the rapid re-acquisition of CRs after extinction, or with the very rapid extinction of CRs during further extinction training (Napier et al., 1992). Another possibility is that extinction occurs through the formation of a new memory that inhibits the original memory. If the ideas discussed above are correct then there will be no olivary input to the cerebellum during extinction training. This does not fit with existing mathematical models of cerebellar learning which require that the cerebellar cortex receives input from the IO during the formation of a new memory (Marr, 1969; Albus, 1972; Gilbert, 1974).

Even though some facts do not fit with the cerebellar learning theories, the neural mechanism of eyeblink learning may be as Marr and Albus suggested, through changes in the efficacy of parallel fibre to Purkinje cell synapses. Albus' proposal that there is a long term depression (LTD) of the parallel fibre to Purkinje cell synapses has received much experimental support from work on learned eye movements by Ito and his colleges (Ekerot and Kano, 1985; Ito, 1989; Nagao and Ito, 1991). LTD is caused by the sustained desensitisation of the ionotropic glutamate receptor subtype AMPA, which is present on Purkinje cells (Hemart et al., 1994; Ito and Karachot 1992; Kano and Kato, 1987). The fundamental biochemical mechanisms of this desensitisation, involves presynaptic and postsynaptic mechanisms (Glaum et al., 1992; Hemart et al., 1995; Linden and Connor, 1993; Shibuki and Okada, 1991) that might lead to an alteration in gene expression (Nakazawa et al., 1993). The exciting possibility that similar neuronal and biochemical mechanisms underlie eyeblink conditioning in rabbits and man remains to be determined.

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GLOSSARY OF CONDITIONING TERMS

Alpha response - Presentation of the CS on its own elicits a response. The latency of this response is usually very short (<30ms) suggesting that it is elicited as soon as the CS is detected.

Blocking - CRs are firmly established to a CS (CS1) and the US. Training is then given in which a second CS (CS2) is presented at the same time as CS1, and both stimuli are paired with the US. CRs are given to the compund stimulus. Training is then given with CS2 on its own. No CRs are elicited by this CS.

Blocking, Prevention of - Prior experience of CS1, which usually prevents conditioning to a second stimulus (CS2) when both are reinforced in a compound (CS1+CS2+US), does not prevent the appearance of CRs when the second stimulus is presented on its own.

Conditioned Discrimination - There are two different trial types. Trials may be alternated between the types. One type consists of a CS, typically a tone paired with the US. The compound stimulus (CS1+US) is called the CS^+. The second trial type consists of a different CS, for example a tone of a different frequency (CS2) presented on its own. During initial stages of conditioning CRs are usually given to both CSs. Gradually, CRs to the CS^- become smaller and less frequent until they are not given at all.

Conditioned Discrimination Reversal - Training is then given in which the meaning of the CSs is reversed. Thus CS^+ becomes CS2+US and CS^- becomes CS1. CRs are continued to be given to original CS^+.

Conditioned Inhibition - There are two different trial types. Trials may be alternated between the types. One type consists of a CS, for example a tone, paired with the US. This compound stimulus is called the CS^+. The second trial type consists of a different CS, for example light, or a tone of a different frequency, presented together with the first CS. This compound is called the CS^-. During initial stages of conditioning CRs are usually given on both trial types. Gradually, CRs to the CS^- are inhibited. The second CS acquires strong inhibitory properties.

CR - conditioned response.

CS - conditioned stimulus.

Delay conditioning - CS and US overlap in time. CS may continue beyond the US offset, or the US may continue beyond CS offset. CS and US are usually terminated at the same time.

Extinction - Conditioning is first established to paired training (CS+US). Training is continued with presentation of the CS alone. On initial trials of CS alone training CRs are normal in size and frequency of responding. After several presentations (about 50-100) both the amplitude and frequency of CRs are reduced. On initial trials of subsequent daily sessions, CRs recover in amplitude and frequency but to a lower level than previous sessions. Eventually, no response will be elicited by the CS.

Extinction, Retardation of - More CS alone trials are required to extinguish CRs than is normally required.

Habituation - A response is initially elicited by a stimulus. With repeated presentations of the stimulus the response diminishes.

Higher order conditioning - Conditioning to a conditioned stimulus (CS1) and US is firmly established. A second stimulus (CS2) is then presented before the CS1. No US is given, but CS1 is presented as if it is a US. With repeated pairings of CS2 and CS1 CRs are elicited by the CS2.

Latent Inhibition - A CS is presented on its own for several trials. The CS is then paired with the US. Significantly more paired trials are required before CRs appear than in control subjects which have no prior knowledge of the CS. The appearance of CRs is retarded by pre-exposure to the CS.

Pseudoconditioning - Responses similar to URs may be elicited by other stimuli. This might be because the UR has generalised to stimuli similar to US. Perhaps repeated exposure to the US causes a change in internal state which causes any external stimulus to elicit escape or avoidance behaviour.

Sensitisation - CS may reflexively elicit a response, which is potentiated by exposure to the US. The response elicited by the CS may even resemble a CR. eg. in human eyeblink conditioning a light CS generally elicits short latency eyeblinks.

Trace conditioning - CS offset and US onset are separated by an interval of time during which no stimulus is given. Eyeblinks can be conditioned with a trace interval up to 1000ms.

UR - unconditioned response.
US - unconditioned stimulus.

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The Effect of Kainic Acid Lesions of the Cerebellar Cortex on the Conditioned Nictitating Membrane Response in the Rabbit

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Key words: cerebellar cortex, classical conditioning, kainic acid, nictitating membrane

Abstract

In previous studies we have shown that aspiration lesions centred on lobule HVI in the cerebellar cortex of rabbits produce a profound loss of conditioned nictitating membrane (NM) responses. Because aspiration lesions of the cerebellar cortex cause retrograde degeneration in precerebellar nuclei we tested in rabbits whether excitotoxic lesions of the cerebellar cortex that spare these precerebellar nuclei also cause a loss of conditioned NM responses. Following discrete injections of kainic acid into HVI and rostral regions of the adjacent folia of crus I and crus II, we observed an immediate loss of conditioned NM responses. Following extensive retraining several subjects showed a gradual recovery of conditioned responses. But subjects with the most complete lesions never recovered more than a few conditioned responses. Kainic acid lesions did not change ipsilateral unconditioned reflex responses to a range of stimulus intensities. The kainic acid injections caused obvious degeneration of Purkinje and granule cells but not of the precerebellar nuclei. We conclude that HVI and parts of crus I and crus II are essential for normal retention of conditioned NM responses.

Introduction

The nictitating membrane response (NMR) of rabbits is a defensive reflex brought about by retraction of the eyeball. The NMR may be associatively conditioned by presenting a conditioned stimulus (CS) that initially does not elicit an NMR, followed by an unconditioned stimulus (US) that always elicits the unconditioned NMR (unconditioned response, UR). After repeated presentations of these paired stimuli a conditioned response (CR) is elicited by the CS.

The classically conditioned NMR of the rabbit is appropriate for the analysis of mechanisms of learning and memory (Thompson, 1976). An important step in this analysis is to determine the essential neural circuitry. Recent lesion and electrophysiological recording studies have implicated the cerebellum in NMR conditioning. In this report we further examine the role of the cerebellar cortex.

Large unilateral lesions of the cerebellum prevent the acquisition (Lincoln *et al.*, 1982) and impair retention (McCormick *et al.*, 1981) of conditioned NM responses. Lesions of the anterior interpositus and rostral medial parts of the dentate nucleus profoundly impair or abolish previously established conditioned NM responses (Clark *et al.*, 1984; McCormick and Thompson, 1984; Lavond *et al.*, 1985; Yeo *et al.*, 1985a).

Lesions of lobule HVI of the cerebellar cortex also impair or abolish conditioned NM responses (Yeo *et al.*, 1985b). This finding is consistent with the anatomical connections since a major output of lobule HVI is to the anterior interpositus nucleus (Yeo *et al.*, 1985c). Several other studies also demonstrate an important role of the cerebellar cortex in NMR conditioning. Lesions of the cerebellar cortex including HVI severely retard acquisition of conditioned NM responses (Lavond and Steinmetz, 1989). Injection of γ-aminobutyric acid antagonists into HVI selectively and reversibly abolishes conditioned NM responses (Mamounas *et al.*, 1987). Some Purkinje cells in HVI are active prior to a conditioned NM response and some are driven by the conditioned or unconditioned stimuli (Berthier and Moore, 1986). Multiple-unit recordings from the cerebellar cortex adjacent to HVI, in the ansiform lobe, have revealed activity related to the performance of conditioned NM responses (McCormick and Thompson, 1984).

In some studies, lesions of the cerebellar cortex were reported to have little or no effect upon conditioned NM responses (Clark *et al.*, 1984; McCormick and Thompson, 1984). However, in a more recent study, large lesions that included HVI transiently abolished previously established conditioned responses (Lavond *et al.*, 1987).

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The cerebellar cortex receives mossy fibre inputs from several nuclei and climbing fibre input from the inferior olive. Some of the nuclei supplying mossy fibres send collaterals to the cerebellar nuclei (Qvist, 1989). Although the dentate and posterior interpositus nuclei receive input from the nucleus reticularis tegmenti pontis together with a much smaller projection from the basilar pontine nuclei (Dietrichs *et al.*, 1983; Gerrits and Voogd, 1987; Shinoda *et al.*, 1992), the anterior interpositus nucleus may only receive mossy fibre collaterals from the lateral reticular nucleus (Russchen *et al.*, 1976) and spinal cord (Matsushita and Ikeda, 1970). All of the cerebellar nuclei receive collaterals from the inferior olive (Van der Want *et al.*, 1989). Aspiration lesions of the cerebellar cortex result in degenerative changes in these precerebellar nuclei. Several sources of mossy fibre inputs, including the nucleus reticularis tegmenti pontis and basilar pontine nuclei, degenerate (Brodal and Jansen, 1946). In rabbits, there is a particularly rapid degeneration of cells within the inferior olive (Brodal, 1939). HVI receives olivary input from face areas in the medial part of the dorsal accessory olive (Yeo *et al.*, 1985c). Lesions of this olivary region cause an immediate and enduring loss of conditioned NM responses (Yeo *et al.*, 1986). Thus aspiration lesions of HVI may impair CRs due to the primary loss of cerebellar cortex or due to the loss of collaterals to the cerebellar nuclei from the inferior olive or other precerebellar nuclei. In this study, we have used kainic acid (KA) to produce fibre-sparing lesions of the cerebellar cortex. At appropriate concentrations, it has a neurotoxic action on cells of the cerebellar cortex, and does not damage afferent and efferent fibres. The precerebellar nuclei and their collaterals to the deep nuclei should therefore remain intact. Since intracortical collaterals (Armstrong *et al.*, 1973; Hrycyshyn *et al.*, 1989) would also be spared, the location of sites critical for the conditioned NMR could be accurately defined.

A short report of some of these findings has appeared previously (Hardiman *et al.*, 1988).

Materials and methods

Subjects

Thirty-nine male Dutch belted rabbits weighing from 1.8 to 2.5 kg were used for this experiment. They were housed individually, allowed *ad libitum* food and water and maintained on a 12-h light and 12-h dark cycle.

Techniques and equipment

Each animal was trained using techniques and equipment similar to those first developed by Gormezano *et al.* (1962) and described in an earlier paper (Yeo *et al.*, 1985a).

Each animal was placed in a Perspex restraining box and a low-torque potentiometer was attached to the head by clips around the ears and muzzle. The shaft of the potentiometer was attached by a lever and thread to a suture placed in the NM. A counterbalance weight provided a restoring force of 1 g to maintain tension in the thread.

The animal was placed in a ventilated sound-attenuating chamber facing a pair of 65 mm square translucent plastic panels separated by a loudspeaker mounted centrally.

Conditioning

The behavioural training was Pavlovian delay conditioning using light and white noise as conditioned stimuli. In each daily session of 200 trials, there were 100 presentations of the light CS and 100 of the white noise CS. The CSs were given in a fixed pattern of five light followed by five white noise, and so on. The inter-trial interval was 15 s. A daily session consisted of 90 presentations of each CS type paired with the US and ten presentations of each CS type alone. Every tenth presentation of each CS type was unpaired.

The light CS was the illumination of both translucent panels to 6.8 cd m^{-2}, the white noise CS intensity was 90 dB (re 20 μN m^{-2}). The duration of both CSs was 550 ms. The US was a 50 ms, 2.5 mA, 50 Hz AC periorbital electrical stimulus. On paired trials the interstimulus interval between the CS and US onsets was 500 ms.

Acquisition and retention training

All subjects were initially given five sessions of training with the US and NM transducer on the right side. Surgery was then performed and after a period of recovery all subjects underwent retention testing. There were four experimental groups.

Group A

The experimental procedure in this group was similar to our earlier aspiration lesion study (Yeo *et al.*, 1985b). Ten subjects received injections of KA into hemispheral parts of lobules V, VI and VII of the right cerebellar cortex. Animals in this group were then trained for five sessions with the US applied to, and the NMR recorded from, the right side (lesioned). Five sessions were then given with the US and the nictitating membrane (NM) transducer on the left side (not lesioned). The US and the NM transducer were then transferred back to the right side for five more sessions.

Pilot studies revealed that there was some recovery of CRs ipsilateral to the lesion during the third postoperative block of five conditioning sessions. This recovery might relate to some transfer of responses from the conditioning sessions to the non-lesioned side during the second postoperative block. To test for this possibility, group B and control groups C and D were tested with three consecutive blocks (15 sessions in total) to the right, lesioned side, before conditioning to the non-lesioned side.

Group B

Sixteen subjects received injections of KA into hemispheral parts of lobules V, VI and VII of the right cerebellar cortex. Animals in this group were postoperatively trained for 15 sessions with the US applied to, and the NMR recorded on, the right side. The US and the NM transducer were then placed on the left side and training was given for five sessions. This was followed by a further five sessions with the US and NM transducer on the right side.

Group C

Two subjects received injections of KA into the ansiform lobe of the right cerebellar cortex. Postoperative behavioural training was identical to group B.

Group D

Three subjects did not undergo surgery but were retrained for 15 sessions using the same schedule as for group B. These subjects were conditioned controls for subsequent UR testing (see below)—only their UR data are presented.

UR testing

Following behavioural training the reflex responses of eight subjects from group B and all from group D were tested on a series of unconditioned stimuli of varying intensity. Each trial consisted of a 50-ms, 50-Hz periorbital US followed by a 60-s inter-trial interval. The US intensity was varied in the fixed sequence 2.5, 2.0, 1.5, 1.0, 0.5, 0.25, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 mA. Five trials at each stimulus intensity were given with the stimulus applied to the right side. After a 30-min rest period the same procedure was repeated with the US applied to the left side. Responses on the right side were compared with responses on the left.

Surgery

Each rabbit was anaesthetized using a fentanyl/fluanisone mixture (Hypnorm, Janssen; 0.1/5.0 mg kg^{-1}, i.m.), and a supplement of benzodiazepam (Valium, Roche; 0.5 mg kg^{-1}, i.v.) was used as a relaxant. Benzodiazepam also protected the animal after the injection of KA from possible damage to structures outside the injection site (Ben-Ari *et al.*, 1979). The rabbit was intubated, placed in a Kopf stereotaxic instrument and connected to a halothane evaporator. Naloxone hydrochloride (Narcan, Du Pont; 3–5 μg kg^{-1}, i.v.) was used to antagonize the fentanyl, and deep anaesthesia was maintained throughout the operation by 3% halothane in a 30% nitrous oxide/oxygen mixture.

After stable anaesthesia had been attained, mannitol (4 g kg^{-1}, i.v.) was infused at a rate of 3 ml per min. Mannitol was used to reduce brain volume, the effect of which was to expose rostral areas of the cerebellum, lower intracranial pressure and reduce the possibility of cerebral oedema. The scalp was reflected and bone and dura removed to expose the right cerebellar cortex.

Kainic acid was injected through a micropipette into the hemispheral parts of the right cerebellar cortex. Each animal received between two and 18 stereotaxic placements of KA (Sigma, 1 mg ml^{-1} kainic acid in sterile 0.9% NaCl, pH 7.2–7.4). The placements were made on a three-dimensional grid that extended from 1 to 4 mm below the cortical surface. At each placement, an injection of KA (25–250 nl) was made over 1–3 min. A total of 500–1800 nl (2.3–8.4 nmol) of KA was injected into each animal.

After the injections had been made the scalp was sutured and 30 ml of glucose/saline was slowly infused intravenously, and the animal was removed from the stereotaxic instrument. Buprenorphine hydrochloride (Temgesic, Reckitt and Colman; 0.25 mg kg^{-1}, i.m.) was given immediately after surgery and twice daily for 2 days post operation. The subjects were allowed to recover for 4–21 days.

There was no evidence either during the operation, or subsequent to it, of epileptiform convulsions as have been described following kainic acid injections into limbic regions of the brain (Ben-Ari *et al.*, 1979).

Histology

At the end of the experiment each rabbit was given an injection of heparin sodium (500 U kg^{-1}, i.v.) and a lethal dose of pentobarbitone sodium (90 mg kg^{-1}, i.v.). The rabbit was perfused through the aorta with 0.9% saline followed by 10% formalin and the brain removed from the skull. The brain was embedded in 10% gelatin and frozen sections were cut at 30 μm in the coronal plane. Alternate sections were mounted onto gelatinized slides and stained with cresyl violet to reveal Nissl substance.

Data analysis

Control, data acquisition and on-line analysis of the experiment were performed on a microcomputer using an analogue-to-digital conversion rate of 100 Hz for the delay conditioning and a rate of 1 kHz for the UR testing.

Conditioning

Responses with an amplitude $\geq$0.5 mm contributed to the measurement of CR frequency. A response was scored on paired trials if it was made during the interstimulus interval (500 ms from CS onset) and on unpaired trials if it was made during a period of 1000 ms starting from the CS onset. For both CS types there were 90 paired CS–US presentations and ten CS alone presentations within each session. Session CR frequencies for each CS type on paired and unpaired trials were expressed as percentages.

The peak amplitude of all responses made during the interstimulus interval on paired trials and during 1000 ms from CS onset on unpaired trials was measured, and is presented as the average peak CR amplitude for each CS. The peak amplitude of all responses made from US onset during paired trials was measured, and is presented as the averaged UR amplitude for both CS trial types.

We also measured the frequency, peak amplitude and latency to the peak amplitude of all responses $\geq$0.15 mm during unpaired trials.

UR testing

We used signal averaging techniques to produce an averaged waveform for the ten responses at each stimulus intensity. The homogeneity of responses was estimated in several subjects by comparing the responses at each stimulus level. There was a very slight or no variation in the response topography at each stimulus level. Various response parameters were obtained from the averaged waveform. The NM response onset latency was measured from the stimulus onset to the first reliable inflexion above baseline noise (0.05 mm). The amplitude to the first response peak and the latency from stimulus onset to this response peak were determined. From these values we calculated the average velocity to the first response peak. The amplitude of, and latency to, the maximum NM extension were determined.

Data from four subjects with the largest impairment of CRs and from four subjects with the least impairment of CRs, all from group B, together with data from all subjects of the non-lesioned group D, were examined statistically. Data from these subjects were analysed using a 3-way analysis of variance (ANOVA) with repeated measures on two factors, using program 2V from the BMDP statistical suite of programs. The factors were behavioural group, side and stimulus level. There were three levels of behaviour: 'most affected', 'least affected' and 'normal'. The factor side had two levels: left and right. The factor stimulus had six levels: 2.5, 2.0, 1.5, 1.0, 0.5 and 0.25. In order to reduce its heterogeneity the amplitude data were transformed by taking the square root value. The reciprocal of latency values was used in the ANOVA. Where appropriate we used the Greenhouse–Geisser correction for non-sphericity of data within the BMDP program. We used the 5% level of significance in all statistical tests.

Histological verification of lesions

The transverse sections were examined microscopically for degeneration of cells within the cerebellar cortex, cerebellar nuclei, the inferior olive (IO), lateral reticular nucleus, basilar pontine nuclei

TABLE 1. The number of responses that reached an amplitude of ≥0.5 mm, expressed as a percentage of the number of paired trials within a block of five sessions. Session 5 is the final preoperative session and is shown for comparison. LI, light; WN, white noise

		CR frequency												
	Session	5		6–10		11–15		16–20		21–25		26–30		Number of sessions
	Side	R		R		L		R						<10% CRS
	Stimulus	LI	WN	LI	WN	LI	WN	LI	WN	LI	WN	LI	WN	
	Subject													
Group A	1	78	98	1	1	78	89	20	42					5
	2	77	96	1	1	78	90	59	70					5
	3	78	98	1	10	26	70	50	64					4
	4	90	93	16	6	58	78	87	93					3
	5	82	97	31	48	28	53	36	86					1
	6	80	100	82	94									0
	7	71	97	87	95									0
	Side	R		R		R		R		L		R		
Group B	1	50	96	1	0	0	1	0	1	95	99	0	7	25
	2	77	96	0	1	0	3	0	2	89	99	1	4	25
	3	86	98	0	2	1	19	11	72	80	94	25	74	7
	4	73	96	0	1	7	78	31	99	83	99	90	98	6
	5	74	96	10	27	68	92	93	96	80	81	98	99	3
	6	92	99	24	18	86	87	93	97	62	64	96	98	2
	7	63	90	44	50	78	93	81	95	91	95	78	92	1
	8	82	86	32	60	71	97	78	98	76	93	80	94	1
	9	60	89	33	82	60	93	81	92	37	69	80	95	0
	10	90	90	85	96	89	98	98	98	84	85	96	100	0
Group C	1	52	91	51	71	78	88	81	86	52	70	16	19	0
	2	82	90	69	76	78	96	93	96	60	71	87	98	0

TABLE 2. The number of responses that reached an amplitude of ≥0.5 mm, expressed as a percentage of the number of unpaired (CS alone) trials within a block of five sessions. Session 5 is the final preoperative session and is shown for comparison

		CR frequency											
	Session	5		6–10		11–15		16–20		21–25		26–30	
	Side	R		R		L		R					
	Stimulus	LI	WN	LI	WN	LI	WN	LI	WN	LI	WN	LI	WI
	Subject												
Group A	1	90	100	0	0	94	90	64	64				
	2	60	100	0	4	90	94	84	82				
	3	90	100	8	18	44	80	78	84				
	4	90	100	14	8	70	80	92	92				
	5	90	100	46	50	38	50	66	92				
	6	70	100	94	100								
	7	70	100	90	100								
	Side	R		R		R		R		L		R	
Group B	1	90	100	0	0	0	2	2	0	100	100	2	8
	2	100	100	0	2	0	12	2	4	96	100	4	36
	3	100	100	2	0	10	40	50	88	92	92	50	96
	4	80	100	0	0	22	84	74	98	86	100	98	100
	5	80	100	22	36	86	94	94	100	96	88	100	100
	6	90	90	32	32	92	86	98	94	76	72	100	98
	7	90	100	38	64	76	98	82	100	90	98	88	98
	8	90	80	62	60	96	94	98	100	100	100	100	100
	9	90	100	54	94	76	98	88	98	46	72	88	100
	10	90	100	98	96	98	100	100	100	94	92	98	100
Group C	1	100	100	66	82	82	84	78	88	46	70	90	98
	2	90	90	96	92	96	100	98	98	70	84	96	100

and nucleus reticularis tegmenti pontis. Photographs of individual sections were made at 0.5 mm intervals throughout the cerebellum. Drawings of the cerebellum were prepared by tracing these photographs, and regions of degeneration were indicated upon them. From each of the drawings the linear extent of PC loss in each lobule was estimated and is presented in Table 5.

TABLE 3. The amplitude in millimetres of all responses made during 1000 ms from CS onset in unpaired trials

		NM peak amplitude											
	Session	5		6–10		11–15		16–20		21–25		26–30	
	Side	R		R		L		R					
	Stimulus	LI	WN	LI	WN	LI	WN	LI	WN	LI	WN	LI	WN
	Subject												
Group A	1	7.1	6.8	0.0	0.0	9.6	9.1	2.9	3.1				
	2	2.6	4.4	0.0	0.0	3.7	3.8	2.3	2.3				
	3	3.8	6.1	0.2	0.4	2.7	5.6	3.7	4.4				
	4	3.9	5.4	0.4	0.2	3.8	4.5	4.1	4.4				
	5	3.9	4.2	2.2	2.3	1.6	2.0	2.0	3.4				
	6	2.5	4.0	4.1	4.2								
	7	2.9	4.3	4.5	5.1								
	Side	R		R		R		R		L		R	
Group B	1	1.1	5.5	0.0	0.0	0.0	0.0	0.0	0.0	4.2	4.8	0.0	0.2
	2	5.3	5.1	0.0	0.0	0.0	0.2	0.0	0.1	5.6	6.3	0.1	1.3
	3	4.5	4.1	0.0	0.0	0.1	0.5	0.6	1.7	4.3	3.9	2.8	5.0
	4	5.0	6.3	0.0	0.0	0.6	3.8	2.6	4.9	5.2	6.7	5.7	6.1
	5	3.4	4.3	3.0	0.5	2.4	2.9	3.4	3.7	3.7	3.5	5.2	5.4
	6	4.4	4.3	0.5	0.6	3.8	3.9	5.2	5.1	3.5	3.0	5.2	5.7
	7	4.2	5.4	2.0	3.4	4.0	5.1	4.6	5.8	5.4	5.7	5.6	6.6
	8	4.3	3.8	1.1	1.1	2.2	2.3	2.8	2.9	5.3	5.3	4.0	4.0
	9	2.4	4.3	1.4	3.1	2.6	3.7	3.8	4.5	1.1	1.5	4.5	5.3
	10	5.5	5.7	4.7	4.6	4.8	4.7	6.3	6.2	4.8	4.8	4.7	4.8
Group C	1	3.2	3.3	5.1	4.9	4.8	4.7	5.4	5.7	3.1	3.4	4.9	5.6
	2	2.3	2.6	3.7	4.4	4.9	4.9	3.7	4.0	1.7	2.6	5.2	5.6

Session 5 is the final preoperative session and is shown for comparison. All other columns show response amplitudes averaged across five sessions.

TABLE 4. The number of responses that reached an amplitude of ≥0.15 mm, expressed as a percentage of the number of unpaired trials within a block of five sessions

		Frequency of responses ≥0.15 mm											
	Session	5		6–10		11–5		16–20		21–25		26–30	
	Side	R		R		R		R		L		R	
	Stimulus	LI	WN	LI	WN	LI	WN	LI	WN	LI	WN	LI	WN
	Subject												
Group B	1	100	100	0	6	8	6	6	34	100	100	8	20
	2	100	100	2	14	4	20	4	10	96	98	10	50
	3	100	100	2	44	12	94	70	94	94	94	54	98
	4	80	100	2	20	28	94	76	98	88	100	96	100

Session 5 is the final preoperative session and is shown for comparison.

Results

Conditioning

Tables 1, 2 and 3 summarize the behavioural results. In these tables the subjects are arranged in descending order of behavioural deficit. The order was determined by the sum of the frequency of responses to both CSs on sessions six to ten. Table 1 shows the frequency of CRs during paired trials, Table 2 shows the frequency of CRs during unpaired trial (CS alone) trials and Table 3 shows the average amplitude on unpaired trials. In each of these tables the postoperative data are averaged in blocks of five sessions. The last session of acquisition training (session five) is shown for comparison.

Six subjects failed to learn to the white noise CS within the five preoperative training sessions. Two subjects failed to acquire CRs to the light CS on the left side postoperatively. All eight subjects were rejected from this study.

Acquisition

Paired trials

During the preoperative acquisition phase CRs rapidly increased in amplitude and number from session two onwards. All subjects reached an asymptotic level of >80% CRs to the white noise CS on session three or four, but responses to the light CS were more variable, reaching 50–90% CRs on session five. The conditioned response peak amplitude attained a final level of 4–4.5 mm to the white noise and 1.3–4.5 mm to the light.

Unpaired trials

Tables 1 and 2 show that more conditioned responses were detected during unpaired trials, indicating that on paired trials some CRs were masked by the UR. Overall the subjects had attained a high level of CRs to both CSs by the end of the acquisition training.

Both paired and unpaired trials show a higher frequency of responses to white noise than to light at the end of the five sessions of acquisition training.

Retention

Paired trials

Group A. Subjects A1 and A2 showed a total loss of responses for the first five sessions of right (lesion) side training. A4 showed a few responses in sessions six and seven and then none for the next three sessions. A3 showed a complete loss of responses for three sessions and an increase in responses from session four. There was no loss of CRs in either subject A6 or A7. All subjects showed rapid learning to both CSs when trained on the left (non-lesioned) side. Subsequent retraining on the right side revealed an immediate increase in CRs in all subjects. Subjects A2–A4 subsequently recovered to preoperative levels of responding, although a comparison of the paired and unpaired data shows that the CRs were slightly retarded in latency. CRs in subject A1 were still impaired at the end of training.

Group B. The subjects in this group showed a range of behavioural deficits, from a complete and permanent loss to a slight and transient loss of CRs. All subjects except B10 showed either a total absence of CRs or a considerable reduction in CR frequency on session six, the first session of postoperative training. Subjects B1–B4 showed a complete loss of CRs for at least six sessions of retraining. At the end of retraining the CRs of B1 and B2 remained severely impaired. B3 and B4 showed a gradual recovery of CR frequency and amplitude, although a deficit remained in the frequency of CRs to the light CS in B3 at the end of retraining.

In general, where CRs recovered they did so more rapidly to the white noise CS than the light CS. Overall this pattern of recovery is similar to the initial, preoperative acquisition during which CRs were more rapidly acquired to the white noise CS, although the rate of recovery of CRs was, in general, noticeably impaired compared with the rate of acquisition. Also, where CRs had recovered in frequency to preoperative levels, the amplitude often remained smaller for several additional sessions of retraining.

Training on the non-lesioned side produced very rapid acquisition of CRs to both CSs in all group B subjects. This is illustrated by the data from paired and unpaired trials in subjects B1–B3 (Figs 6–8)—fully formed CRs were evident on the first unpaired trial in session 21.

Group C. Both subjects showed an impairment of CRs on early sessions of retraining, particularly subject C1, which on the first day of retention training had a frequency score of 10% to the light CS and 30% to the white noise CS.

Unpaired trials

The data from unpaired trials indicate that the CRs of several subjects recovered partially or completely by the end of the 25 sessions of postoperative training. Where there was a complete loss of CRs on paired trials there was also a complete loss on unpaired trials. A recovery of CRs was detected earlier in unpaired trials than in paired trials, indicating that initially the recovering responses were made later.

The NM response latency and amplitude changes during recovery of subjects B1–B4 were analysed in greater detail as described below.

CS-related responses smaller than 0.5 mm

The analysis described above was based upon a criterion for a CR of 0.5 mm movement, a level that is generally used for NMR data (Smith, 1968). The KA lesions primarily produced deficits in the amplitude of the CR. In order to determine whether the CR was present but merely depressed in amplitude we also plotted all responses ≥0.15 mm (twice the level of noise measured peak-to-peak) made by subjects B1–B4. The percentage of responses that reached this new criterion during the unpaired trials are given in Table 4, and Figures 6C, 7C and 8C show the amplitude and latency to the peak of these responses.

This analysis revealed that during acquisition training there were only a few responses with amplitudes <0.5 mm. Moreover, these small responses were only made during initial training sessions. During postoperative training an increase in small responses was seen but these showed a greater variation in latencies than responses made during the acquisition training. Because cerebellar lesions are classically described to produce deficits in timing (Holmes, 1917) we expected to find responses shifted to a longer latency. However, many of these small responses were made very soon after the CS onset. In subjects B1 and B3, during postoperative sessions several responses peaked within 100 ms of CS onset, especially those made to the white noise CS. These latencies are short enough for the responses to be either alpha responses or sensitized responses—although original studies on the NMR found no evidence of alpha or sensitized responses to a tone CS in normal, non-lesioned animals (Gormezano *et al.*, 1962). Some responses were made with a latency to peak greater than normal, for example in B2 and B3. Smith (1968) has shown that in the intact animal the CR peaks at about the time of presentation of the US. Many of the small responses shown here do not follow this pattern and may be behaviourally 'maladaptive' (McCormick and Thompson, 1984). Without additional controls for alpha responses, sensitization and pseudoconditioning in animals lesioned with KA it cannot be determined whether these responses are non-associative in nature or whether they are abnormally small, and incorrectly timed, CRs.

Unconditioned response

Unconditioned responses measured during paired trials showed no consistent effect of the KA lesion, but subjects A1 and B3 showed an increased UR during postoperative training. In subject B3, shown in Figure 8B, the UR remained elevated even after CRs to the white noise CS had fully recovered.

UR testing

The responses to the US alone were tested at six levels ranging from 0.25 to 2.5 mA. At different stimulus levels there was a variation in the response topography. All subjects showed a bimodal or polymodal response to stimuli >1.0 mA. The first peak occurred at 50–70 ms and the second at 90–100 ms. The maximum amplitude generally occurred at the second peak. The response elicited by a stimulus of ≤1.0 mA in most subjects had only one peak.

In order to compare these data with previous studies (Welsh and Harvey, 1989; Yeo and Hardiman, 1992) subjects from group B were divided into two groups according to the degree of CR deficit. The 'most affected' group consisted of subjects B1–B4 who showed a complete absence of CRs to either CS for at least six sessions. All of this group except B4 showed deficits in the number and amplitude of CRs at the time of UR testing. The 'least affected' group consisted of subjects B7–B10. In two of these subjects the CR was absent for one session, but all had recovered to preoperative levels of responding by session twenty. A 'normal' group consisted of subjects D1–D3.

A change in US intensity produced significant changes in all aspects of the reflex response in all groups: frequency ($F = 58$, df = 5,40; $P < 0.05$), peak amplitude ($F = 162$, df = 5,40; $P < 0.05$), onset latency ($F = 38$, df = 5,40; $P < 0.05$), latency to peak ($F = 20$, df = 5,40; $P < 0.05$), average velocity ($F = 98$, df = 5,40; $P < 0.05$) and rise time ($F = 4.28$, df = 5,40; $P < 0.05$).

The UR amplitude was significantly greater on the right side than on the left side in all groups ($F = 10.9$, df = 1,8; $P < 0.05$). Separate ANOVAs using the factors side and group were calculated at each level of US. These revealed a side difference at 2.0 mA ($F = 11.28$, df = 1,8; $P < 0.05$), 1.5 mA ($F = 6.92$, df = 1,8; $P < 0.05$) and 1.0 mA ($F = 12.5$, df = 1,8; $P < 0.05$). The amplitude difference between the sides is shown in Figure 1 to be a general trend in all groups. This difference probably relates to the larger number of trials given to the right side during behavioural training.

The frequency and timing of the UR was not significantly different between the groups at any US intensity.

The average velocity of the UR was significantly different across the groups at some or all levels, as shown by a significant group/level interaction ($F = 3.59$, df = 5,40; $P < 0.05$). Separate ANOVAs with factors group and side were calculated for each level of US. A significant group difference was detected only at the 1.5 mA level ($F = 6.07$, df = 1,8; $P < 0.05$). Although the average velocities were significantly different only at 1.5 mA, inspection of the graphs of average velocity in Figure 1 reveals that responses in the 'normal' group had higher velocities at ≥1 mA. Importantly, at the low stimulus levels this trend is reversed and responses of the lesioned subjects showed higher average velocities.

Except for the consistent differences between left and right side UR amplitudes in all subjects there were no significant differences in URs within any group at any stimulus level. Overall, reflex responses elicited by a wide range of stimulus strengths of the 'most impaired', 'least impaired' and 'normal' subjects were not different.

Histological verification of lesions

The KA injection placements were on a 1-mm three-dimensional grid. In some cases there was a complete loss of all cortical cells between placements in this grid. In some cases only the Purkinje cell (PC) layer was absent, leaving the granule cell layer intact. In some instances there were patches of total loss of PCs with intact regions of cortex between. At the edge of the injections there was a gradual change in the number and type of cells affected. Dependent upon the distance from the injection site, first the granule cells and then the PCs appeared normal (Fig. 2). The finding that there is less damage to granule cells is in agreement with previous reports (e.g. Herndon *et al.*, 1980).

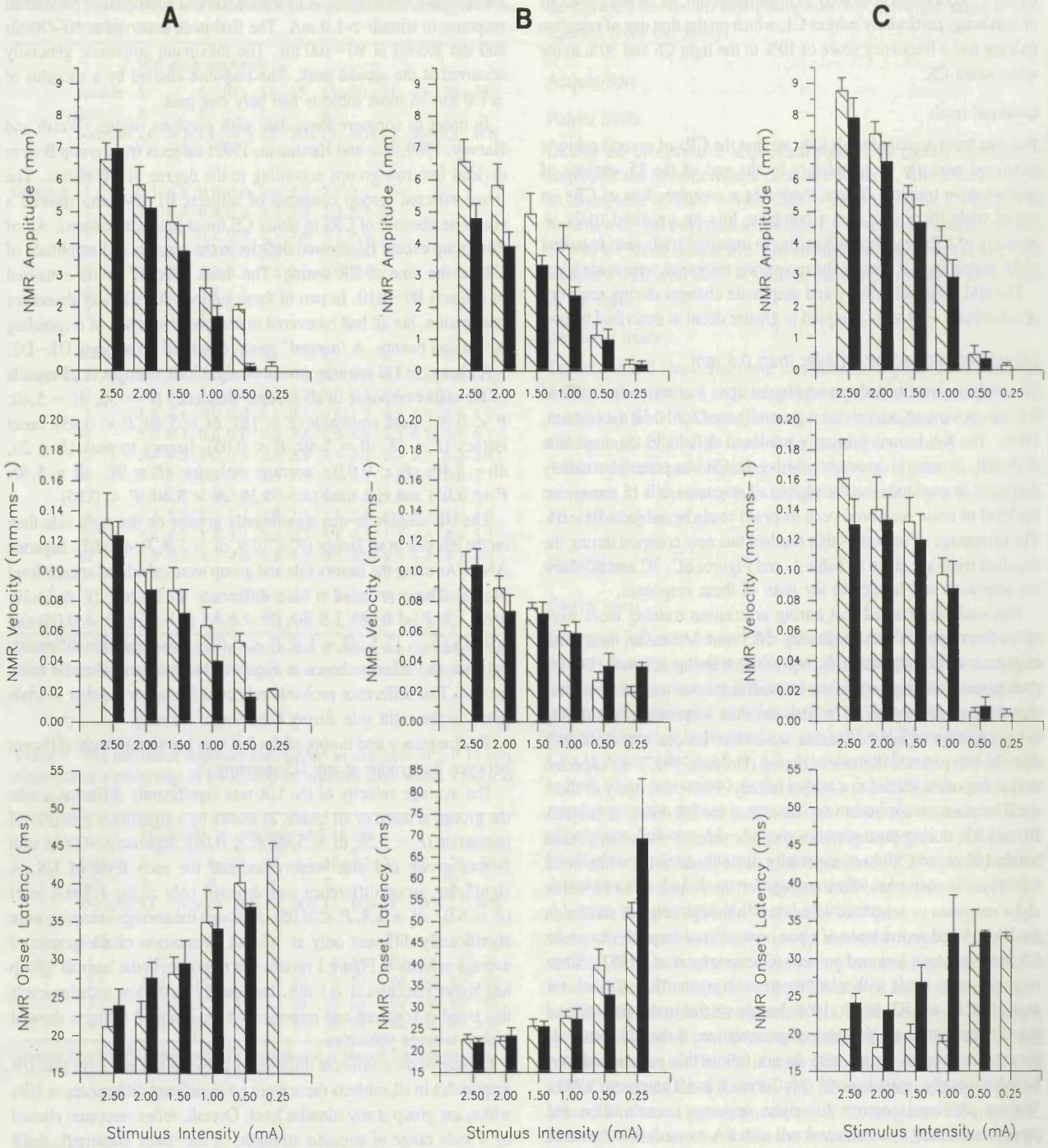

FIG. 1. Amplitude, average velocity and onset latency of NM responses to periocular stimuli of 2.5–0.25 mA intensities. Responses made by the right NM are hatched, responses made by the left NM are solid. Bars indicate standard error of the mean. (A) 'Most affected' group. (B) 'Least affected' group. (C) 'Normal' group.

Because KA lesions of the deep cerebellar nuclei cause a loss of the conditioned NMR (Lavond *et al.*, 1985) we examined the cerebellar nuclei in all subjects, comparing the lesioned and non-lesioned sides. We rejected all subjects that showed any sign of degeneration in the cerebellar nuclei. Four subjects from group B had some degree of cell loss within the nuclei. In two subjects from group B the plane of section of the histological material was not optimal for a comparison between the nuclei of each side. We therefore could not be certain whether loss of cells within the nuclei had occurred. All six subjects were excluded from this study. Three subjects from group A were found to have damage, from haemorrhage caused during the surgery, to the most rostral dorsal parts of HVI and the paramedian lobe. These subjects were also rejected from this study.

The remaining 19 subjects showed no signs of degeneration within

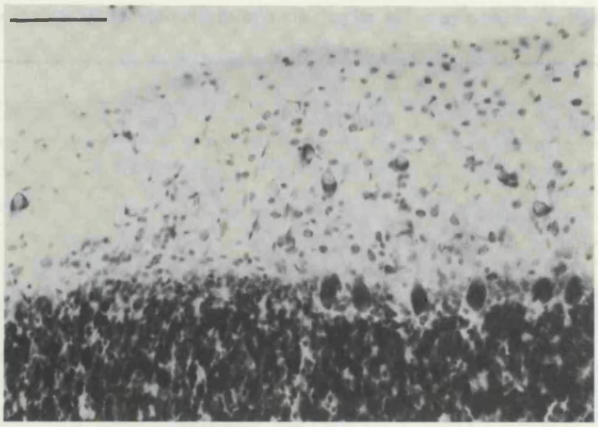

FIG. 2. Purkinje cells within the right HVI of subject B1. Changes in concentration of kainic acid have different effects upon the intrinsic cerebellar neurons. At the far left, nearest an injection site, there is a complete loss of PCs; next to this region six normal PCs can be seen. The nucleus is at the bottom of the soma, the normal position in rabbits. In contrast, in cats and monkeys the nucleus is centred within the cell body. Bar = 0.1 mm.

the cerebellar nuclei. Figure 3A shows a section through the right cerebellar nuclei of subject B1, at a rostral level essential for normal CRs. All of the cells appear normal. As a comparison, Figure 3B shows the nuclei of a subject in which KA was injected directly into the cerebellar nuclei. There are clear signs of degeneration and gliosis within the anterior interpositus nucleus/medial dentate nucleus, in which neurons are absent.

There was no sign of cell loss or gliosis in the inferior olive (IO), basilar pontine nuclei, nucleus reticularis tegmenti pontis or lateral reticular nuclei. Figure 4A illustrates the left IO, contralateral to the KA injections of subject B1. There was no evidence of cell shrinkage such as is found in the dorsal cap of the IO following KA injections into the flocculus (Ito *et al.*, 1980). For comparison Figure 4B shows the left IO of a subject from an earlier study in which an aspiration lesion of the right cerebellar cortex was made. The lesion was restricted to anterior parts of HVI and adjacent hemispheral cortex. There was cell loss and gliosis within medial, anterior parts of the dorsal accessory olive, medial accessory olive and principal olive.

In all cases the KA injections caused more damage to PCs than to

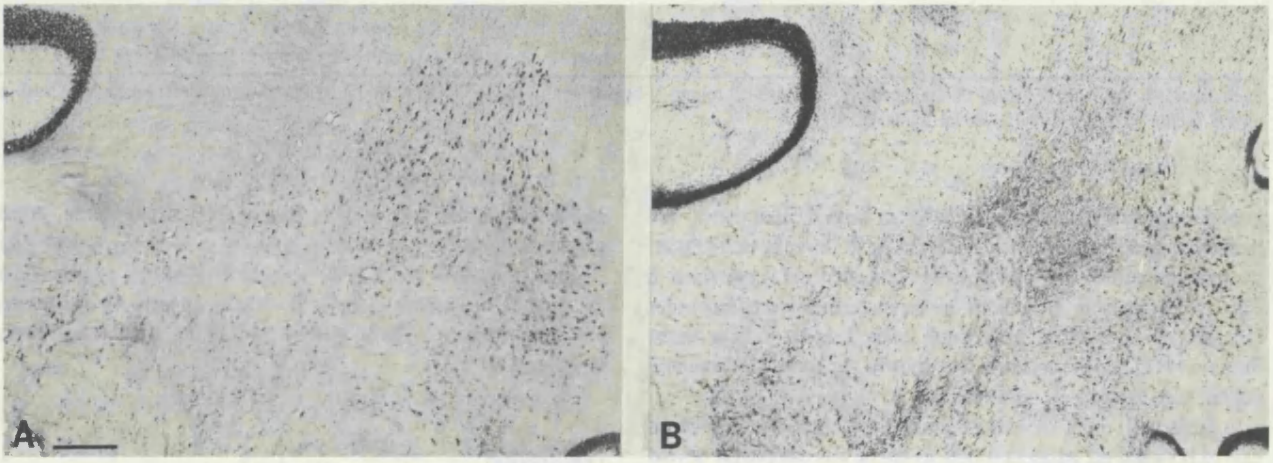

FIG. 3. (A) Transverse section through the right cerebellar nuclei 3 mm caudal to lambda of subject B1. This subject showed a large loss of Purkinje cells within lobule HIV/V, lobule HVI, the ansiform lobe and the paramedian lobe. There are no signs of degeneration. (B) Transverse section through the right cerebellar nuclei at the level of lambda in a subject that had an injection of kainic acid directly into the cerebellar nuclei and the overlying cerebellar cortex. There is gliosis and a clear loss of neurons within the crucial region of the anterior interpositus/dentate nuclei. Bar = 0.5 mm.

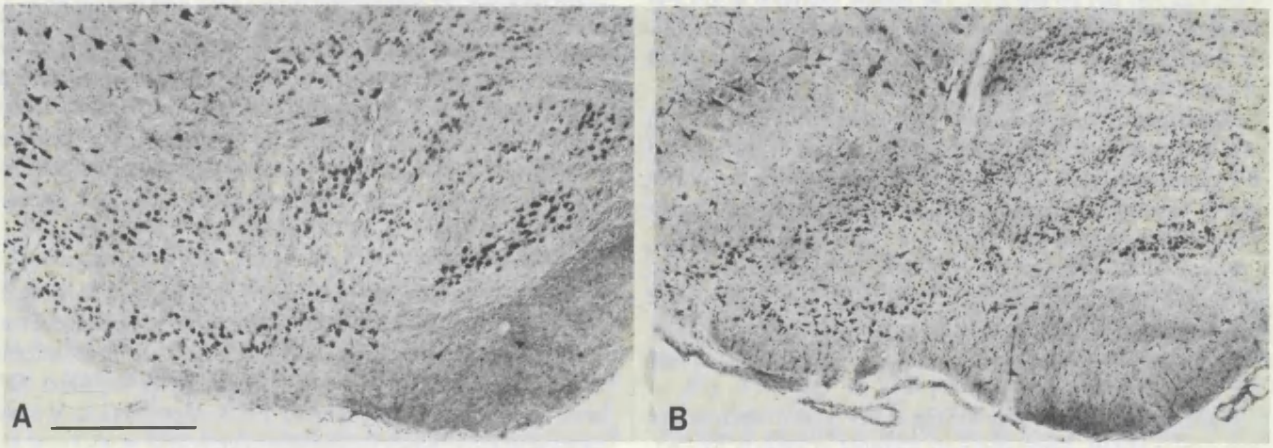

FIG. 4. (A) Transverse section through the left inferior olive at Brodal's level XII in subject B1. This subject showed a large loss of Purkinje cells within lobule HIV/V, lobule HVI, the ansiform lobe and the paramedian lobe. All cells within the inferior olive appear normal. (B) Transverse section through the left inferior olive at Brodal's level XII of a subject that had an aspiration lesion of right lobule HVI, the ansiform lobe and paramedian lobe. There is a loss of cells by retrograde degeneration at the junction of the dorsal accessory olive and dorsal principal olive and within the medial part of the ventral principal olive and medial accessory olive. Bar = 0.5 mm.

TABLE 5. The extent of Purkinje cell loss following kainic acid injections in the right cerebellar cortex. The subjects are tabulated in order of the severity of their behavioural deficit

Group	Subject	Cortex division				
		HVI m	HVI l	CrusI m	CrusI l	Crus II
A	1	+++++++	++++++++++	++++++++	++++++++	+++++
	2	++++++++++	++++++	++++	.	.
	3	+++++++++	+++++++++	++++++++++	+++	.
	4	+++++	++++++	+++	+	.
	5	+++	+	.	.	.
	6	.	.	.	.	.
	7	.	.	.	.	.
B	1	+++++	++++++++++	++++++++++	++++++++++	++++++++++
	2	+++++++++	++++++++++	+++++++++	+++++++	++
	3	+++++++	++++++++	++++++++++	+++	+
	4	+++++++	+++++++	++++++++	.	.
	5	+++++++	+++++	++++	+	.
	6	+++++++++	++++++	+++	.	.
	7	++++++++	++++++++++	+++++++++	+	.
	8	++++++++++	++++	+++++	.	.
	9	++++++++	++++++	++++++++	.	.
	10	+++++++	+++++	++++++	+	.
C	1	.	.	.	+++++++	++++++++
	2	.	.	+	+++++++++	++++

Each cross indicates ~10% loss of Purkinje cells. HVI m, medial folium of hemispheral lobule VI; HVI l, lateral folium of hemispheral lobule VI; CrusI m, medial folium of crus I; CrusI l, lateral folium of crus I.

granule cells, and so destroyed output from the cerebellar cortex. We therefore plotted the amount of damage to the PC layer in each subject; the total loss of PCs in each lobule was estimated and is presented in Table 5. In this table the subjects are arranged in a descending order according to the severity of behavioural deficit. Subjects with the largest lesions of HVI and the ansiform lobe showed the greatest behavioural deficit, e.g. subjects B1–B6 in Table 5.

In subjects B1–B3, shown in Figures 6A, 7A and 8A, the ventral half of HVI and the adjacent folium of crus I was completely devoid of PCs. Only in these subjects was there also considerable loss of PCs in the lateral folium of crus I and in crus II. Subjects with sparing of PCs in the ventral half of the lateral folium of HVI and the adjacent folium of crus I in general showed only a slight and transient loss of CRs, e.g. A4, B5, B8 and B10. The notable exception to this was subject B7, in which all the dorsal part of the medial folium of HVI, the whole of the lateral folium of HVI and most of the medial folium of crus I were devoid of PCs.

Subjects with damage to comparable amounts of the PC layer within HVI showed a range of CR deficit, e.g. B4 and B9. Subjects B9 and B10 showed a transient CR deficit and both had extensive loss of PCs in HVI, but in these subjects patches of Purkinje cells and granule cells within HVI were spared.

Subject C1 had extensive loss of PCs within crus I and crus II and subject C2 had extensive damage to crus I. The amount of damage to the ansiform lobe in these subjects was greater than that of all other subjects except B1. Neither of these subjects showed a large or enduring loss of CRs.

In all subjects there was some damage in HIV/V and the paramedian lobe. These lobules were extensively damaged in subjects A1–A3, but lesions of these lobules in group B subjects did not reveal a consistent behavioural deficit. For example, lobule HIV/V showed extensive loss of PCs in subjects A2, A3, B6 and B10, and the paramedian lobe in subjects A1, A2, B1, B5 and C2. In subjects A6 and A7 there was only a slight loss of PCs in the rostral, dorsal paramedian lobe; neither of these subjects showed any loss of CRs.

Subjects A1–A4 and B4–B8 showed a slight loss of PCs within the right half of vermian lobules II–VI. In subjects A2 and B6 this loss was extensive. There was no loss of PC within the vermis in subjects B1–B3.

Discussion

Unilateral, kainic acid lesions of the cerebellar cortex produced an initial loss of conditioned NM responses established to light and white noise stimuli. Unconditioned responses to periorbital stimuli appeared normal. Lesions produced by KA were restricted to cells of the cerebellar cortex and did not damage the precerebellar nuclei or efferent cortical targets in the cerebellar nuclei. The behavioural deficits were therefore due to the loss of cerebellar cortical neurons. Extended retraining allowed some recovery of CRs though only to very low levels in those cases with the largest lesions of lobule HVI and the ansiform lobe. The degree of recovery was related to the location and extent of the cortical lesion.

HVI and the ansiform lobe are essential for normal conditioned responses

Purkinje cells are particularly susceptible to damage by kainic acid, although granule cells are also damaged at higher KA concentrations. KA injections spare fibres of passage (Coyle *et al.*, 1978) and afferent fibres (Woodward and Coull, 1988), including climbing fibres (CF) in the cerebellar cortex (Rossi *et al.*, 1991). Aspiration lesions of the cortex produce retrograde degeneration in the inferior olive (Yeo *et al.*, 1985b). In the present study we found that KA lesions produced no retrograde degeneration in the inferior olive or other precerebellar nuclei. Because CFs branch to different cortical areas (Armstrong *et al.*, 1973; Hrycyshyn *et al.*, 1989) aspiration lesions deprive areas outside

FIG. 5. Key to serial sections of cerebellar cortex. crI and crII, crus I and crus II of the ansiform lobe; DPFL, dorsal paraflocculus; FL, flocculus; HIV/V, hemispheral lobule IV/V; HVI, hemispheral lobule VI; ND, dentate nucleus; NI, interpositus nucleus; PM, paramedian lobe; VPFL, ventral paraflocculus; I–VI, lobules I–VI of the vermis.

the primary lesion of CF inputs, and so for similar areas of primary damage KA lesions are functionally smaller than aspiration lesions.

In an earlier study, aspiration lesions that included all of HVI caused a profound loss of CRs throughout all five postoperative training sessions (Yeo *et al.*, 1985b). In the present study there were some surviving Purkinje cells in HVI in all subjects, but in all cases in which Purkinje cells in HVI were lost there were deficits in the frequency and amplitude of CRs. The magnitude and duration of the deficit depended upon the amount of damage to HVI and crus I and crus II of the ansiform lobe. KA lesions of the cerebellar cortex that spared HVI had either no effect, or a slight and transitory effect, upon CRs. Six subjects with lesions of HVI lost CRs for five or more sessions of retraining. Subjects with more extensive damage to the ansiform lobe as well as HVI lost CRs for longer than five sessions. Our original

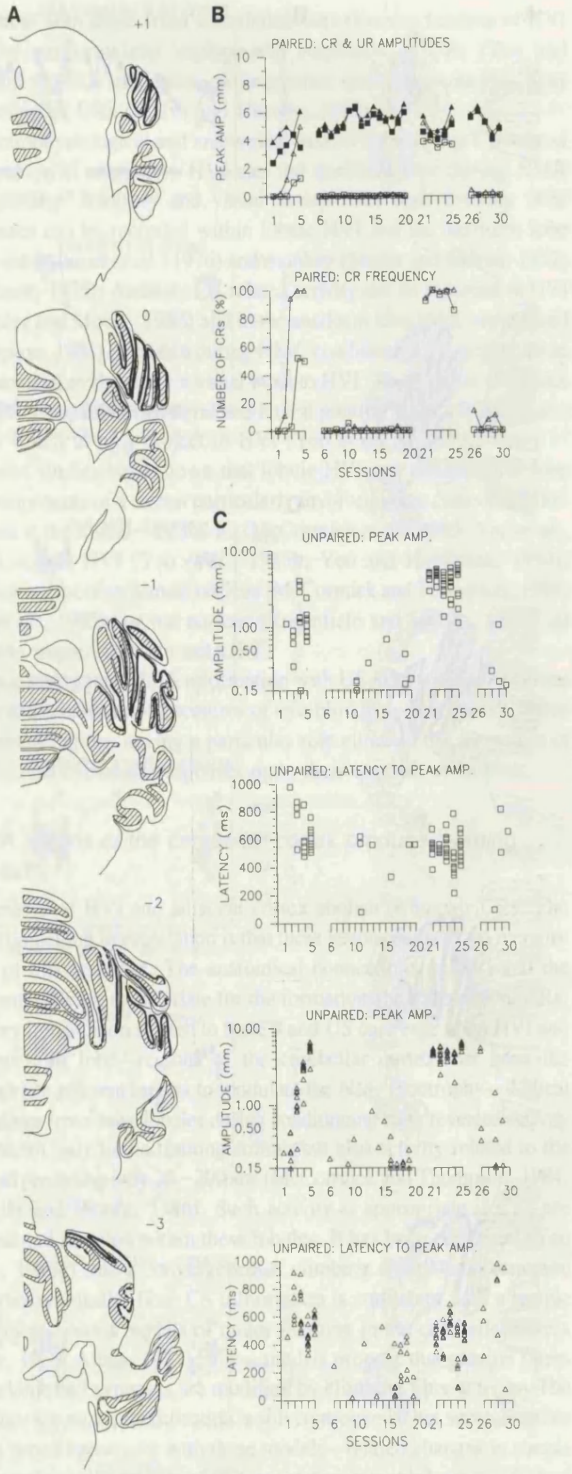

FIG. 6. (A) Transverse, serial sections at intervals of 1 mm through the right cerebellum of subject B1 showing areas of degeneration following injections of kainic acid. Regions of Purkinje cell degeneration are indicated by a wider line and regions of degeneration of the granule cell layer are indicated by the absence of hatching. (B) NMR peak amplitude and frequency on paired presentations of the CS and US. Open squares, responses measured during the interstimulus interval to the light CS. Open triangles, responses measured during the interstimulus interval to the white noise CS. Filled symbols, responses measured from onset of the US. (C) Amplitude and latency to the peak response of all NM responses ≥ 0.15 mm made during unpaired presentations of the CS. Open squares, responses to the light CS. Open triangles, responses to the white noise CS.

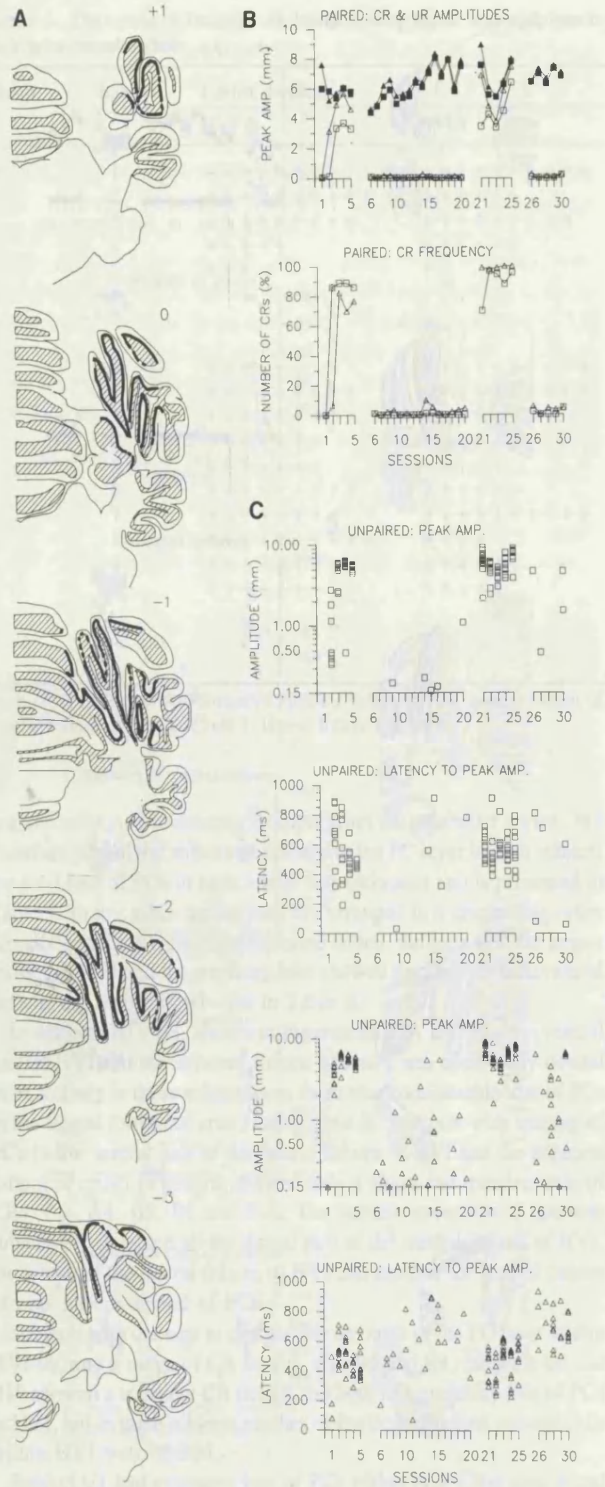

FIG. 7. Subject B2. Reconstruction of lesion (A) and behavioural data (B) and (C). All conventions as in Fig. 6.

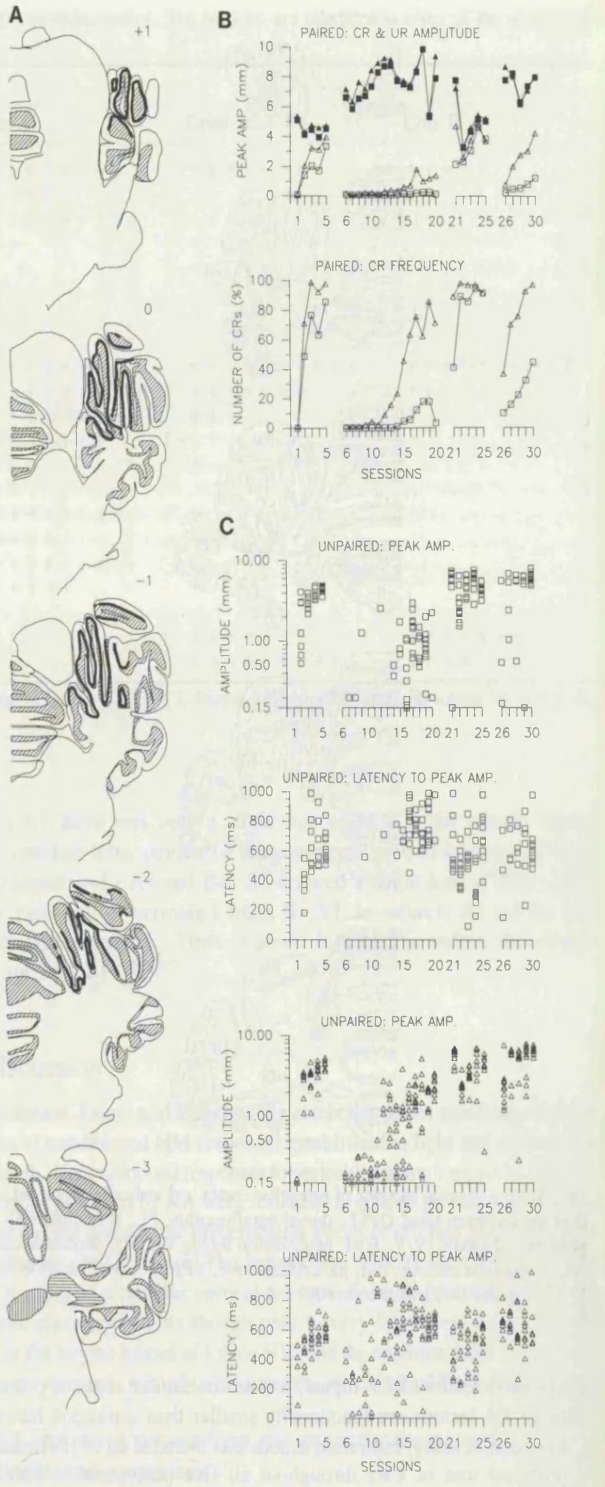

FIG. 8. Subject B3. Reconstruction of lesion (A) and behavioural data (B) and (C). All conventions as in Fig. 6.

idea was that conditioning on the left side, contralateral to the KA cortical lesion, might facilitate the recovery of conditioning on the right side. The interpolation of five postoperative sessions to the left side after five postoperative sessions to the right side (group A) or after 15 postoperative sessions to the right side (group B) was designed to test for contralateral facilitation. Comparison of CR frequencies in sessions 6–10 and 16–20 of group A with those in sessions 6–10 and 11–15 of group B for subjects with comparable initial deficits possibly reveals a small contralateral facilitation for some subjects. But subjects B1 and B2, with the most severe CR deficits, showed

almost no CRs and so no contralateral facilitation over sessions 26–30. Because the loci and extents of the KA lesions are all slightly different, comparisons between subjects in group A and group B must be tentative, and prevent a firm conclusion that some recovery of conditioning on the lesioned side may be facilitated by contralateral conditioning.

We conclude that the loci essential for normal conditioned NM responses are within ipsilateral lobule HVI but that adjacent regions of the ansiform lobe can sustain recovery of conditioning. Given that KA lesions of the cerebellar cortex are functionally less extensive than aspiration lesions of similar primary extents, these findings are in close agreement with a recent aspiration lesion study (Yeo and Hardiman, 1992).

Cerebellar cortical neurons are essential for normal conditioned responses

CFs and some mossy fibre afferents to the cortex branch to provide an input to the cerebellar nuclei (Qvist, 1989). Because aspiration lesions of the cortex produce retrograde degeneration in the inferior olive and other precerebellar nuclei (Brodal, 1939; Brodal and Janssen, 1946), the loss of CRs following such lesions might relate to a loss of collaterals to the cerebellar nuclei, and not to the loss of an essential input from the cerebellar cortex. If the association critical for conditioning of the NMR is within the cerebellar nuclei, as suggested by McCormick and Thompson (1984), and is independent of cortical mechanisms, then the deep nuclei alone should sustain conditioned responses. The present study demonstrates that, even with their collateral connections intact, the cerebellar nuclei alone are not sufficient to maintain conditioned responses. Deficits in CRs must be due to a loss of input to the cerebellar nuclei from the cortex.

Output and input pathways of HVI and the ansiform lobe

Evidence from a variety of studies suggests that regions within HVI and the ansiform lobe receive information related to unconditioned and conditioned stimuli and are involved in control of NM and eye-blink unconditioned and conditioned responses. Stimulation of the face, including the periorbital region, causes an increase in mossy fibre activity (Berthier and Moore, 1986; Welker, 1987) and climbing fibre activity (Cody and Richardson, 1979; Akaike, 1989) within HVI and the ansiform lobe. These lobules receive inputs directly from the trigeminal nuclei through mossy fibres (Ikeda, 1979; Yeo *et al.*, 1985c), and through climbing fibres from face representation areas (Cook and Wiesendanger, 1976; Gellman *et al.*, 1983) of the dorsal accessory olive (Kawamura and Hashikawa, 1979; Yeo *et al.*, 1985c; Azizi and Woodward, 1987). Information related to a periocular US can therefore reach HVI and the ansiform lobe through mossy and climbing fibre pathways.

HVI (Yeo *et al.*, 1985c) and the ansiform lobe (Dietrichs and Walberg, 1980; Voogd and Bigaré, 1980) project to the anterior interpositus/medial dentate nucleus and thence to the dorsal–lateral magnocellular red nucleus (Daniel *et al.*, 1987; Robinson *et al.*, 1987). The red nucleus connects directly and indirectly (Desmond *et al.*, 1983; Holstege *et al.*, 1986) to eye-blink motoneurons. Several studies indicate that lobule HVI and the ansiform lobe and their efferent targets can modulate reflex NM and eye-blink responses. Stimulation of lobule HVI in cats elicits eye blinks to the pulse train offset (Hesslow, 1989). Similarly, stimulation of the anterior interpositus/medial dentate nuclei (McCormick and Thompson, 1984) or their targets in dorsal parts of the magnocellular red nucleus (Chapman *et al.*, 1988; Haley *et al.*, 1988) elicits NM responses in rabbits. Results from lesion studies are consistent with those from stimulation experiments. Lesions of HVI and the ansiform lobe increase the amplitude of URs (Yeo and Hardiman, 1992) and lesions of the anterior interpositus nucleus, alter the timing of URs (Welsh and Harvey, 1989).

Electrophysiological and anatomical studies indicate that CS-related information is relayed to HVI and the ansiform lobe during NMR conditioning. Auditory and visual mossy fibre and climbing fibre responses can be recorded within lobule HVI and the ansiform lobe in the cat (Altman *et al.*, 1976) and monkey (Snider and Eldred, 1952; Mortimer, 1975). Auditory CS-related activity can be recorded in HVI (Berthier and Moore, 1986) and in the ansiform lobe (McCormick and Thompson, 1984) of rabbits during NMC conditioning. In rabbits, there is anatomical evidence for a visual input to HVI. The superior colliculus projects to caudal dorsolateral and lateral pontine nuclei (Wells *et al.*, 1989) which in turn project to HVI (Yeo *et al.*, 1985c).

Lesion studies have shown that lobule HVI and the ansiform lobe are components of a circuit particularly involved in the control of CRs. Lesions at the rostral–medial IO (McCormick *et al.*, 1985; Yeo *et al.*, 1986), lobule HVI (Yeo *et al.*, 1985b; Yeo and Hardiman, 1992), anterior interpositus/dentate nucleus (McCormick and Thompson, 1984; Yeo *et al.*, 1985a) or red nucleus (Rosenfield and Moore, 1983), all severely impair CRs but not URs.

The convergence of CS information with US information in regions of cortex involved in the control of eye-blink responses is consistent with these regions having a particular role either in the formation of conditioned eye-blink responses or in their control, or in both.

Do KA lesions of the cerebellar cortex produce learning deficits?

KA lesions of HVI and adjacent cortex abolish or impair CRs. The most compelling interpretation is that these lesions damage the memory trace of this response. The anatomical connections of HVI and the ansiform lobe are appropriate for the formation and execution of CRs. Sensory information related to the CS and US converge upon HVI and the ansiform lobe—regions of the cerebellar cortex that have the appropriate efferent targets to modulate the NM. Electrophysiological recordings from these lobules during conditioning have revealed activity related not only to the training stimuli but also activity related to the CR and preceding it by 20–200 ms (McCormick and Thompson, 1984; Berthier and Moore, 1986). Such activity is appropriate if CRs are formed and initiated within these lobules. It has been suggested (Yeo *et al.*, 1985c) that a convergence of climbing fibre US information with mossy/parallel fibre CS information is consistent with a simple implementation of models of motor learning in the cerebellar cortex (Marr, 1969; Albus, 1971). These models propose that parallel fibres to Purkinje cell synapses are modified by climbing fibre activity. The evidence for such a modification is still controversial but several studies are in broad agreement with these models—related changes in simple and complex spike activity of PCs occur during motor learning (Gilbert and Thach, 1977), and conjunctive stimulation of parallel and climbing fibres causes a reduction in efficacy of parallel fibre to Purkinje cell synapses (Ito, 1989).

An alternative view is that KA lesions of the cerebellar cortex do not damage learning but simply impair movement. Perhaps the lesions disrupt the NM motor pathway so that CRs produced outside the cerebellum cannot be properly expressed through the disturbed pathway. In previous studies, cerebellar lesions were reported to impair only CRs and not URs (McCormick and Thompson, 1984; Yeo *et al.*, 1985a,b). It was concluded that cerebellar lesions impaired only learned

components of the reflex and did not substantially affect motor expression of CRs and URs. However, these conclusions were based upon measurements of the size of the UR during paired presentations of the CS and US, in which the UR may be facilitated by the CS (Ison and Leonard, 1971) or enhanced by a preceding CR. Such measurements may not be an accurate indication of the effects of lesions upon the UR and so general impairments of movement should not have been ruled out.

In a more recent study, Welsh and Harvey (1989) again found that lesions of the interpositus nucleus severely impaired or abolished CRs but disrupted URs too. Presentation of the US alone at low intensities after interpositus nucleus lesions revealed URs with depressed frequency and extended latency to peak and rise time. The few residual CRs had similar latency deficiencies. Welsh and Harvey argued that because the CR and UR have a common motor pathway the lesions had impaired expression rather than learning of the response. However, the lesions also produced deficits that were exclusive to the learned response. CRs were profoundly depressed in amplitude but URs had normal amplitude. CRs had significantly extended onset latency but UR onset latency was normal. Clearly, the lesions produce some motor deficits, but these deficits probably do not account for the profound depression or complete loss of CR amplitude when there was no corresponding change in UR amplitudes. We suggest that these differential effects upon CRs and URs are due to a learning deficit. This does not imply that the locus of learning is necessarily within the cerebellar nuclei, and a recent study (Welsh and Harvey, 1991) supports this view—reversible inactivation of the interpositus nucleus does not prevent the acquisition of ipsilateral CRs. Assuming that cerebellar cortical function can be relatively normal during inactivation of the nuclei, these findings are consistent with the suggestion that there is important plasticity within the cerebellar cortex.

In the present study, KA lesions of the cerebellar cortex also produced dissociated effects upon CRs and URs. CR amplitudes were severely impaired but UR amplitudes were not changed by the lesion. Aspiration lesions of HVI and the ansiform lobe (Yeo and Hardiman, 1992) depress the amplitude and frequency of CRs but enhance the amplitude of URs across a range of US intensities. Neither of these studies provide evidence for a motor deficit common to the CR and UR sufficient to abolish CRs. In both studies, loss of the cerebellar cortex exclusively impairs CR amplitude. We suggest that these findings are consistent with the suggestion that the cerebellar cortex is essential for learned components of the CR.

Kelly *et al.* (1990) found that decerebrate rabbits could acquire conditioned NM responses after large cerebellar lesions. They suggested that cerebellar lesions remove tonic drive acting upon motor pathways mediating the NM response, and decerebrations restore the level of this tonic activity, thus allowing expression of CRs formed in circuitry outside the cerebellum. But Nordholm *et al.* (1991) recently showed that the intertrial intervals used by Kelly *et al.* (1990) do not support NMR conditioning in the intact rabbit. This finding raises the question whether responses driven by the CS in partially cerebellectomized/decerebrate subjects are true CRs produced by an associative process. If they are not, then the decerebrate experiments of Kelly *et al.* (1990) do not test whether the cerebellum is important for NM conditioning.

Lesions of the cerebellum are known to be associated with changes in tonic activity of motoneurons (Gilman, 1969) and limb, head and orbital muscle tone (Chambers and Sprague, 1955a,b), that may recover in time (Van der Meulen and Gilman, 1965). Kainic acid lesions of the cerebellar cortex presumably cause a loss of inhibitory modulation of the largely tonically active cerebellar nuclei. A change in tonic activity would be reflected in UR changes, particularly at levels of US close to threshold. In the present study there was no evidence of this; at the time of testing URs were normal during maintained depression of CR frequency. As there was no direct evidence of tonic changes after KA lesions of the cortex then the disruption of CRs in this study was more likely to be due to the loss of phasic modulations of the eye-blink system by the cerebellum than to an alteration in tonic drive.

Electrophysiological recordings of neuronal activity during conditioning confirm that the cerebellum is involved in the phasic regulation of the motor system during conditioned and unconditioned responses. Neuronal activity related to the US, UR, CS and CR is found within HVI (Berthier and Moore, 1986), the ansiform lobe (McCormick and Thompson, 1984), anterior interpositus/dentate nuclei (McCormick and Thompson, 1984; Berthier and Moore, 1990) and RN (McCormick *et al.*, 1983; Haley *et al.*, 1988; Desmond and Moore, 1991). Lesion and electrophysiological studies indicate that within HVI and the ansiform lobe there is sensory–motor integration in order to produce conditioned NM responses that are appropriately timed and of the correct topography, consistent with suggestions by Marr (1969) and Albus (1971) that there are motor learning mechanisms within the cerebellar cortex.

Acknowledgements

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Abbreviations

ANOVA	analysis of variance
CF	climbing fibre
CS	conditioned stimulus
CR	conditioned response
IO	inferior olive
KA	kainic acid
NM	nictitating membrane
NMR	nictitating membrane response
PC	Purkinje cell
UR	unconditioned response
US	unconditioned stimulus

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