Higher Order Conditioning of the Eyeblink Reflex

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This dissertation is submitted for the degree of Doctor of Philosophy
This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text. It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. It does not exceed the prescribed word limit for the relevant Degree Committee.
Classical conditioning of the eyeblink response is a well-established model of cerebellar-dependent associative motor learning. In this paradigm an initially neutral conditioned stimulus (CS - e.g. an auditory tone or light) that elicits no response is paired with an aversive unconditioned stimulus (US - e.g. an air puff to the eye) that elicits an innate reflex blink response. A CS paired with the US at a fixed latency comes to elicit a predictive blink, if the CS begins sufficiently in advance of the US. A predictive blink is such that eyelid closure is maximal at the time of expected air puff delivery. Physical and pharmacological manipulation experiments have determined that this behaviour is dependent on the cerebellum, and theoretical accounts based on the structure of the cerebellum have proposed that it represents a mechanism for this type of learning (Marr, 1969; Albus, 1971).

The cerebellum has two distinct afferent pathways. The first are the mossy fibres which have diverse origins from various nuclei, including sensory structures. The second pathway is that of the climbing fibres that originate from the inferior olivary nuclei. In Marr’s proposal, the climbing fibres represent instructive signals for plasticity, and the mossy fibres carry "context" information on which plasticity operates so that the contextual signals can activate learned movements. Studies in the eyeblink paradigm have confirmed that the climbing fibre activity is correlated with the US and that they encode error signals. Unexpected aversive stimuli cause an increase in activity of climbing fibres, whereas the absence of a predicted aversive stimulus causes a decrease in the activity.

Recent evidence has revealed that after training the climbing fibres become responsive to the conditioned stimulus, firing shortly after its onset. This suggests that the conditioned stimulus now has a prediction error value and could itself become a conditioned reinforcer. To test this experiments were devised in both rabbit and human subjects using second-order conditioning schedules, where conventional pairings of a CS1 and the US were interleaved with pairings of a different CS2 and the CS1 (but no US). Consistent results were obtained from both sets of experiments: some subjects learned to respond to the CS2, but more weakly than to the CS1. Strikingly the responses to the CS2 were more closely tied to the onset of CS1 (the time at which climbing fibres respond after training) rather than to the time of expected US. These findings confirm that the CS1 can become a conditioned reinforcer, but have implications for the prevailing view that eyeblink conditioning as a paradigm is primarily about the timing of the response. The relevance of the experimental eyeblink paradigms to natural behaviour is discussed.
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Table of Contents

1 Introduction .............................................................................................................................................. 1
  1.1 The Cerebellum .................................................................................................................................... 1
    1.1.1 Anatomy and structure ..................................................................................................................... 1
    1.1.2 Gross connections ............................................................................................................................. 3
    1.1.3 Cell layers, cells and the microcircuit of the cerebellum ................................................................. 4
    1.1.4 Mossy fibre afferents ....................................................................................................................... 9
    1.1.5 Precerebellar nuclei ......................................................................................................................... 9
    1.1.6 Inferior olivary nuclei and olivocerebellar climbing fibres ......................................................... 10
    1.1.7 Zonation of the cerebellum ........................................................................................................... 12
    1.1.8 Function ....................................................................................................................................... 15
  1.2 Associative cerebellar motor learning ................................................................................................. 16
    1.2.1 Eyeblink conditioning ...................................................................................................................... 17
    1.2.2 Localisation within cerebellum ......................................................................................................... 20
    1.2.3 Activity of cells during conditioning ............................................................................................... 21
    1.2.4 Changes in climbing fibre receptive fields after conditioning .................................................. 23
    1.2.5 Higher-order conditioning ............................................................................................................. 26
    1.2.6 Human eyeblink conditioning ....................................................................................................... 28
  1.3 Experimental aims ............................................................................................................................... 30

2 Statistics ................................................................................................................................................ 32
  2.1 Determination of responses of neurons to stimulation pulses .......................................................... 32
  2.2 Determination of onset and offset latencies ....................................................................................... 38
  2.3 Generalised linear model – binomial logistic regression .................................................................... 39

3 Pontine Neurons: Extracellular Electrophysiology and Responses to Peripheral Somatosensory
  Stimulation ................................................................................................................................................. 41
  3.1 Introduction ....................................................................................................................................... 41
  3.2 Materials and Methods .................................................................................................................... 42
    3.2.1 Animals and anaesthesia ............................................................................................................... 42
    3.2.2 Stimulating and recording electrodes ............................................................................................ 42
    3.2.3 Peripheral somatosensory afferent stimulation ........................................................................... 43
    3.2.4 Surgery ........................................................................................................................................ 44
    3.2.5 Identification of neurons and recording apparatus ........................................................................ 44
    3.2.6 Analysis ....................................................................................................................................... 45
  3.3 Results ............................................................................................................................................... 46
3.3.1 Spontaneous activity of basilar pontine neurons .................................................46
3.3.2 Antidromic latencies and failure of antidromic stimulus ........................................49
3.3.3 Responses to somatosensory peripheral afferent stimulation are often bilateral and sometimes inhibitory .........................................................54
3.4 Discussion .............................................................................................................60
3.4.1 Antidromic failure .................................................................................................60
3.4.2 Bilateral and rostrocaudally receptive pontine neurons ......................................61
3.4.3 Excitatory and inhibitory responses ....................................................................61
4 Response of putative Golgi cells to middle cerebellar peduncle stimulation and peripheral somatosensory stimulation ...........................................63
4.1 Introduction ...........................................................................................................63
4.2 Materials and Methods .........................................................................................65
4.2.1 Animals and anaesthesia ....................................................................................65
4.2.2 Stimulating and recording electrodes .................................................................65
4.2.3 Peripheral somatosensory afferent stimulation ..................................................65
4.2.4 Lesions and histology ........................................................................................66
4.2.5 Golgi cell identification ......................................................................................66
4.2.6 Analysis ................................................................................................................66
4.3 Results ..................................................................................................................67
4.3.1 Histology ............................................................................................................67
4.3.2 Identification of putative Golgi cells and their spontaneous activity ..................69
4.3.3 Response to middle cerebellar stimulation and peripheral somatosensory stimulation differ 71
4.3.4 Response onset and offset times differ between peripheral and MCP stimulation ...77
4.3.5 Latency of cells firing in response to MCP stimulus ...........................................79
4.3.6 Probability of spikes to pulses within the MCP pulse train ..................................80
4.3.7 Short latency depression following MCP stimulation .........................................84
4.4 Discussion .............................................................................................................88
4.4.1 Identification of Golgi cells ..................................................................................88
4.4.2 Golgi cell responses to peripheral afferent stimulation are different to those to MCP stimulation .................................................................88
4.4.3 Putative Golgi cell activation in response to MCP stimulation .............................89
4.4.4 Short lasting depression in response to MCP stimulation ......................................90
5 Second Order Conditioning of the Eyeblink Response in Rabbits ............................92
5.1 Introduction ..........................................................................................................92
5.2 Materials and Methods .........................................................................................93
5.2.1 Animals ..............................................................................................................93
Table of figures

Figure 1-1: Macroscopic Morphology of the Cerebellum in Rats (left) and Humans (right) ................. 2
Figure 1-2: Cytoarchitecture of the cerebellum and its circuitry ................................................................. 4
Figure 1-3: Longitudinal zones of the cerebellum ......................................................................................... 13
Figure 1-4: Transverse zones of the cerebellum ............................................................................................. 14
Fig. 1-5: Delay and Trace Conditioning ...................................................................................................... 18
Fig. 1-6: Simplified circuit diagram of the essential components of the classically conditioned eyeblink response .................................................................................................................. 19
Figure 1-7: Purkinje cell and anterior interpositus cell activity during and after aquisition of conditioned responses ............................................................................................................................ 23
Figure 1-8: Complex spike density in populations of Purkinje cells recorded in eyeblink microzones before and after conditioning .................................................................................................. 24
Figure 1-9: Prediction error encoding by climbing fibres in mice ................................................................. 25
Figure 1-10: The relationship between stimuli in first order (CS1-US) trials and second order (CS2-CS1) trials ........................................................................................................................................... 26
Figure 2-1: Resampling spontaneous data to generate spontaneous PSTHs .............................................. 33
Figure 2-2: Comparative bin values are normally distributed .................................................................... 34
Figure 2-3: Comparison of first and second bin values of stimulation PSTH against same values from the spontaneous PSTHs of the same cell .............................................................................. 36
Figure 2-4: Comparison of multivariate Gaussian analysis to t-test ............................................................ 37
Figure 2-5: Change point detection .................................................................................................................. 38
Figure 3-1: Schematic diagram showing the relation of recording site to the cerebellar white matter stimulating electrodes ................................................................................................................. 43
Figure 3-2: Schematic diagram showing the Expected pathway from peripheral somatosensory stimulation to the pons ....................................................................................................................................... 43
Figure 3-3: Antidromic collisions used to identify the cells as having pontocerebellar afferents ............... 45
Figure 3-4: Example recordings from an antidromically identified pontine neuron ..................................... 46
Figure 3-5: Interspike interval histogram of spontaneous intervals for the cell shown in Fig. 3-4 .......... 47
Figure 3-6: Characteristics of the spontaneous activity of the population of pontine cells ....................... 48
Figure 3-7: Failure of the antidromic stimulus to generate action potentials in cell that has shown what appears to be antidromic collision ......................................................................................... 49
Figure 3-8: Latency to activation following antidromic stimulus ............................................................... 51
Figure 3-9: Adding a delay from spontaneous spike to stimulus activation increases the number of responses seen to first stimulus pulse in the train ...................................................................... 52
Figure 3-10: Probabilities of firing to a given antidromic stimulus pulse (in a train of three) were less than 1 for each pulse for each cell ......................................................................................... 53
Figure 3-11: Inhibitory responses bilaterally to both hindlimb and forelimb stimulation in a single cell .................................................................................................................................................. 55
Figure 3-12: Contra – and ipsilateral forelimb responses with ipsilateral hindlimb responses in the same cell ........................................................................................................................................... 56
Figure 3-13: Strong excitatory responses to the ipsilateral and contralateral forelimb in the same cell .................................................................................................................................................. 57
Figure 3-14: Inhibitory responses to the contralateral and ipsilateral hindlimb stimulation in the same cell ........................................................................................................................................... 58
Figure 3-15: Excitatory contralateral forelimb responses ............................................................................. 59
Figure 4-1: Histology to determine placement of MCP electrodes ............................................................ 68
Figure 4-2: Example of a putative Golgi cell’s spontaneously firing and depression response to peripheral stimulation ................................................................. 69
Figure 4-3: Identification of putative Golgi cells by spontaneous firing activity and coefficient of variation of log ISI .......................................................................................... 70
Figure 4-4: A putative Golgi cell activated at short latency by the middle cerebellar peduncle stimulation ................................................................................................. 72
Figure 4-5: Putative Golgi cell that had significant responses to MCP stimulation and stimulation of the periphery ................................................................................................. 73
Figure 4-6: Putative Golgi that only showed significant responses to stimulation of the middle cerebellar peduncle ................................................................................................. 74
Figure 4-7: Putative Golgi cell with significant response only to the peripheral stimuli ................................................................................................. 75
Figure 4-8: Putative Golgi cell with significant decrease in activity in response to infraorbital nerve stimulation ................................................................................................. 76
Figure 4-9: Distribution of onset latencies by stimulus type as estimated using a change point model ................................................................................................. 77
Figure 4-10: Distribution of offset latencies by stimulus type as estimated using a change point model ................................................................................................. 79
Figure 4-11: Action potential onset latencies following single pulse MCP stimulation ................................................................. 80
Figure 4-12: Observed and predicted probabilities of an action potential following each pulse of the 5 MCP stimulus train ................................................................................................. 81
Figure 4-13: Probability of action potentials following a stimulus pulse if a previous pulse has driven the cell or subsequent a one will drive the cell, or both. ................................................................................................. 83
Figure 4-14: Three 5 MCP sweeps from a putative Golgi cell that still showed short lasting depression from the stimulus, despite not showing evidence of being activated by it ................................................................................................. 84
Figure 4-15: Three putative Golgi cells that show short lasting depression in responses to MCP train stimulation ................................................................................................. 85
Figure 4-16: Separation of 5MCP PSTH into sweeps with short latency excitation and into sweeps without leaves the short lasting depression intact in both ................................................................................................. 86
Figure 4-17: Interspike intervals of the Golgi cells under 3 different conditions ................................................................................................. 87
Figure 5-1: Order of trials in second order conditioning experiments ................................................................................................. 94
Figure 5-2: Second order conditioning in subject 1 ................................................................................................. 98
Figure 5-3: Second order conditioning in subject 2 ................................................................................................. 100
Figure 5-4: Second order conditioning in subject 3 ................................................................................................. 103
Figure 5-5: Change subject 1’s in CS2-CS1 onset latencies as conditioning progresses ................................................................................................. 105
Figure 5-6: Change subject 2’s in CS2-CS1 onset latencies as conditioning progresses ................................................................................................. 107
Figure 5-7: No change in subject 3’s in CS2-CS1 onset latencies as conditioning progresses ................................................................................................. 109
Figure 5-8: Peak latencies of CS2 alone CRs made by subject 1 ................................................................................................. 111
Figure 5-9: Peak latencies of CS2 alone CRs made by subject 2 ................................................................................................. 111
Figure 5-10: Subject 3 made fewer responses to CS2-CS1 trials as training progressed but CS1-US responses were unaffected ................................................................................................. 112
Figure 5-11: Conditioned response amplitudes to CS1-US and CS2-C1 by session number ................................................................................................. 114
Figure 5-12: Percentage of conditioned responses to CS1-US, CS2-CS1 and CS2 alone trials for subjects 1 to 3 – Auditory CS1 ................................................................................................. 117
Figure 5-13: Percentage of conditioned responses to CS1-US, CS2-CS1 and CS2 alone trials for subjects 4 to 6 – Visual CS1 ................................................................................................. 119
Figure 5-14: Session averages of different trial types from two consecutive sessions of training of Subject 4 ................................................................................................. 120
Figure 5-15: Session average of the various trial types from subject 6's responses showing possible conditioned inhibition...
Figure 5-16: Saturation of the signal during a single CS1-US trial ....................................................... 121
Figure 6-1: Experimental procedure ................................................................................................. 130
Figure 6-2: Response windows for CS1-US trial and CS2-CS1 trials .................................................. 131
Figure 6-3: Eyeblink onset detection algorithm .................................................................................. 133
Figure 6-4: Most individuals learned to make responses after first order training, however some did not
.................................................................................................................................................. 134
Figure 6-5: Second order conditioning caused responses to CS2-CS1 to shift earlier than CS1 onset in
the majority of individuals ............................................................................................................... 135
Figure 6-6: An individual who showed first order responses during CS1-US (left) but no response
during CS2-CS1 (right) .................................................................................................................... 136
Figure 6-7: Response onsets times for CS1-US trial averages and CS2-CS1 trial averages ............. 137
Figure 6-8: Average responses to CS2 alone trials in the second order training session by all subjects
.................................................................................................................................................. 138
Figure 7-1: Experimental setup and outline of experimental conditioning group and control
pseudoconditioning groups protocol ................................................................................................. 143
Figure 7-2: Determination of CR ..................................................................................................... 145
Figure 7-3: Difference between conditioning and pseudoconditioning for a number of factors is non
significant ......................................................................................................................................... 146
Figure 7-4: Number of button presses by button and group ............................................................. 147
Figure 7-5: Conditioned responses increase significantly in number as training progresses in the
conditioning group only .................................................................................................................. 148
Figure 7-6: Development of conditioned responses from an individual who was in the conditioning
group ............................................................................................................................................... 148
Figure 7-7: No development of conditioned responses from an individual who was in the
pseudoconditioning group ............................................................................................................. 149
Figure 7-8: The CR incidence for the conditioning group but broken down by reinforced and non
reinforced buttons ......................................................................................................................... 151
Figure 7-9: Average responses made by an individual in the conditioning group to the different
button types during the first 120 trials ......................................................................................... 152
Figure 7-10: Conditioned responses are still made after switching from right to left hands ......... 154
Figure 7-11: Average responses between last block of training (trials 101-120) and a period of
pressing with the opposite hand (trials 121-140) ...................................................................... 155
Figure 7-12: CR incidence when experimenter presses the button .................................................. 156
Figure 7-13: Average responses while the subject presses for themselves compared (trials 181-200)
to when the experimenter presses (trials 201-220) .................................................................. 157
1 Introduction

The work contained within this dissertation largely centres around the cerebellum. The electrophysiology that was carried out involved recording Golgi cells, which reside in the cerebellar cortex; and pontine neurons, which send mossy fibre projections into the cerebellum. The behavioural work follows the conclusions from previous neurophysiological studies and is entirely composed of classical conditioning of the eyeblink reflex. This is a model of associative learning that has been shown to be dependent on the circuits within the cerebellum.

1.1 The Cerebellum

1.1.1 Anatomy and structure

The cerebellum is one of the most studied brain structures in neuroscience. This is partly due to its small number of well-defined cell types and its regular repeating cortical microcircuit structure that has a specific geometrical layout and uniformity across the sheet of the cerebellar cortex. Within the human brain, it occupies 10% of the volume, but it contains 50% of the neurons (Zagon et al. 1977).

The cerebellum comprises a major part of the hindbrain and is situated in the posterior cranial fossa, and sits dorsally to the pontine and medullary brainstem upon three cerebellar peduncles; the inferior, middle and superior peduncles (Brodal 1981). The structure is a feature of all vertebrate nervous systems, from simple cerebellar like structures observed in primitive fish to complex and convoluted structures observed in mammals, and primates especially (Ramnani 2006). The surface area of the cerebellum increases proportionally with the surface area of the neocortex, thus increasing their computational ability linearly and suggesting an evolved functional co-dependence between the two areas (Sultan 2002).

At the macroscopic level, the cerebellum is divided into three major lobes comprising the anterior, posterior and the flocculonodular lobe. The gross anatomy of the cerebellum can be seen in Fig. 1-1 overleaf. The anterior lobe is divided from the posterior by the primary fissure, and the posterior from the flocculonodular lobe by the posterolateral fissure. The lobes are further divided into a total of 10 sections known as lobules I through to X, from anterior to posterior, as designated by Larsell (Larsell 1967). A comparative designation given by Bolk is shown in Fig. 1-1 (Brodal 1981).
Mediolaterally the structure is divided into lateral hemispheres separated by a structure known as the vermis along the midline. The medial area of the anterior and posterior lobes is designated as the spinocerebellum, and the hemispheres called the cerebro-cerebellum. The spinocerebellum is involved in the regulation of postural and limb movements and receives many inputs from the spinocerebellar tracts. The cerebro-cerebellum as the name suggests receives input from the cerebral cortex via the pons and is involved in the planning and initiating motor actions and processing motor and sensory information to maintain internal models. In recent years evidence has emerged for the role of the cerebrocerebellum in cognitive functions as well as motor functions (Schmahmann and Caplan 2006). Although these names do reflect a mediolateral division between input and output connections, they are by no means definitive as the cerebrocerebellum does not just receive inputs derived from the neocortex.

Figure 1-1: Macroscopic Morphology of the Cerebellum in Rats (left) and Humans (right)
Dorsal view of the cerebellum of rat (left) and humans (right). Top left indicates the three mediolateral compartments: the vermis, the paravermis and the hemisphere. Down the left side of the vermis is the designation of the lobules as observed by Larsell. The right side of the vermis shows Bolk’s comparative nomenclature. The primary fissure (pf) for both species is denoted by the bold line and separates the anterior lobe from the posterior lobe. The nomenclature for the hemispheric regions of the rat cerebellum are given on the far left, whilst the nomenclature of the hemispheric regions of the human cerebellum are given on the right. Where regions are comparative the colours match on the two sides. Abbreviations: pf, primary fissure; psf, posterior superior fissure; PF, paraflocculus; FL, flocculus; PML, paramedian lobe; COP, copula pyramidis. Reproduced from Apps & Hawkes, 2009
The flocculonodular lobe is also known as the vestibulocerebellum and is the oldest part of the cerebellum regarding its evolution. It receives input from the vestibular nuclei and the semicircular canals of the ear and regulates balance and eye movements.

The cortex is formed from a small number of cells and is approximately 1mm thick that is extensively folded to form folia which run mediolaterally, the peaks of which create the observable cerebellar surface.

1.1.2 Gross connections

Information is conveyed to and carried away from the cerebellum via the three peduncles. The superior cerebellar peduncle (brachium conjunctivum) connects with the midbrain and contains mainly efferent fibres that originate from the deep cerebellar nuclei. It consists of the cerebellothalamic tract that runs to the contralateral thalamus and cerebellorubral efferents to the red nucleus in the rostral midbrain, and many of these fibres arise from the same nuclear cell. The middle cerebellar peduncle (brachium pontis) largely carries afferent fibres from the pontine nuclei into the cerebellum which branch both at the cortex and the nuclei. The inferior cerebellar peduncle (restiform and juxtarestiform body) carries afferent and efferent fibres and connects to the medulla oblongata. Important afferent fibres arise from the inferior olivary nuclei and the dorsal spinocerebellar tract and pass through the inferior peduncle (Brodal 1981).

The cerebellum is comprised of a sheet of cortex that has a laminar structure and sits over the underlying deep cerebellar nuclei. The two main afferent systems to the structure are the mossy fibres arising from various precerebellar nuclei and the climbing fibres which arise from the inferior olivary nucleus. These afferent systems converge within the cortex and also at the deep nuclei via axon collaterals. The site of convergence within the cortex is at the level of the Purkinje cell, the single output neuron within the cerebellar cortex. Mossy fibres do not synapse directly with Purkinje cells but are linked to them through a disynaptic pathway. All Purkinje cells send their inhibitory axon projections to deep nuclear cells, except a few Purkinje cells in the flocculonodular lobe that project directly to the vestibular nuclei in the brainstem.

The deep cerebellar nuclei are bilaterally symmetrical with four nuclei per hemisphere. Running from the midline to most lateral the nuclei are the fastigial, globose, emboliform and the dentate. The globose and emboliform nuclei are often grouped and known as the posterior and anterior interposed nuclei. The axons of fastigial nuclear cells leave the cerebellum via the inferior peduncle, alongside Purkinje cell axons that project to the vestibular nuclei. The axons of the dentate and the interposed nuclei leave the cerebellum via the superior peduncle (Brodal 1981).
1.1.3 Cell layers, cells and the microcircuit of the cerebellum

The cerebellar cortex is composed of 3 cell layers, which are moving inwards from the most superficial: the molecular layer, the Purkinje cell layer, and the granule cell layer. Underneath these layers is the cerebellar white matter and deep inside of this are the four nuclei of the cerebellum. There are seven defined cell types within the cerebellar cortex: Purkinje cells, Golgi cells, granule cells, unipolar brush cells, Lugaro cells, basket cells and stellate cells. The interconnections of these neurons are shown in Fig. 1-2 below, demonstrating the known cerebellar microcircuit.

Figure 1-2: Cytoarchitecture of the cerebellum and its circuitry

**Brainstem:** contained within the brainstem is the inferior olive, the source of climbing fibre afferents which ascend into the cerebellum and make extensive connections with Purkinje cell dendritic trees (red fibres). Other precerebellar nuclei not shown.

**White matter:** the white matter is composed of the myelinated axon tracts of afferent and efferent fibres. Embedded within the ventral region of the white matter are the deep cerebellar nuclei which both mossy fibres and climbing fibres send collaterals to.

**Granule cell layer:** Main cellular components of this layer are the numerous granule cells, unipolar brush cells (UBCs – pale green), Golgi cell somas (dark grey). Lugaro cell bodies (light purple) are often at the boundary of the Purkinje cell layer. Mossy fibres (dark purple) synapse with granule cells and Golgi cell basal dendrites in large structures known as glomeruli, and onto UBCs brush like dendrioles.

**Purkinje cell layer:** monolayer consisting of Purkinje cell bodies (green)

**Molecular layer:** contains the molecular layer interneurons (MLI - blue) and parallel fibres which are resultant from the bifurcation of granule cell axons which form synapses with the MLIs and Purkinje cells

Reproduced from Cerminara et al., 2015
The cerebellar cortex is often given as an example of ordered cytoarchitecture that consists of repeating microcircuits that are uniform across all regions of the cerebellum in terms of structure and connectivity. However, some regional variations do exist with UBCs distributed differentially and differences in packing density across regions for granule cells and Purkinje cells. Also, Purkinje cells are not uniform in their expression of Zebrin II across the cortex. For further information see Cerminara et al., 2015

1.1.3.1 Granule cells

Granule cells are one of the smallest neurons in the brain, at 5-8µm in diameter, and also the most numerous with an estimated $10^{11}$ in humans (Braitenberg and Atwood 1958; Zagon et al. 1977). The cells are densely packed within the granule cell layer. They receive input from mossy fibres and relay input to the other cells in the cortex by the parallel fibres. Unusually, these cells have only 3 to 5 short distal dendrites, each of which receives excitatory input from a single mossy fibre within a glomerulus structure (Eccles et al. 1967; Palay and Chan-Palay 1974; Chadderton et al. 2004). The small number of connections of mossy fibre afferents to a single granule cell indicates low convergence of disparate signals reaching the cerebellar cortex, and it has been suggested that granule cells employ sparse coding of contextual signals carried by the afferent pathways (Brunel et al. 2004; Philipona and Coenen 2004). In areas that contain UBCs granule cells also receive input from them.

Granule cells give rise to unmyelinated axons that rise into the molecular layer and bifurcate with a T-shape to form parallel fibres. These run along the mediolateral axis of the cerebellum in the same orientation as the folium. Parallel fibre length from the T-branch to the terminal has been estimated to be 4-6mm, but this estimate varies from species to species (Mugnaini 1983; Harvey and Napper 1988; Pichitpornchai et al. 1994). A single parallel fibre passes through the dendrites of 450 Purkinje cells along its length (Eccles et al. 1967). The ascending parallel fibre segment synapses with the smallest diameter distal regions of the Purkinje cell dendrites, while the branched segment synapses with spiny branchlets of the Purkinje cell dendritic arborisation. The ascending segment accounts for an estimated 20% of parallel fibre-Purkinje cell synapses (Gundappa-Sulur et al. 1999). The parallel fibres also make synapses onto Golgi cells and the molecular layer interneurons (basket and stellate cells).

1.1.3.2 Purkinje cells

The Purkinje cell has been the most studied of the cells in the cortex, partly due to its size, which makes it electrophysiologically tractable, and its important position within the circuit. The cell was first described by Johannes Purkinje in 1837 and was the first CNS neuron to be named and
discovered. Purkinje cells are the largest of the cerebellar cortical neurons and are the sole output from the cortex. They reside in a single monolayer, the Purkinje cell layer, which is the middle cell layer of the cortex. The cell body ranges from 25µm in rats to 50µm in diameter in humans. The total area of the monolayer is estimated to 280mm$^2$ with 1200 cells per mm$^2$, in rats (Armstrong and Schild 1970).

The cell has a distinct morphology with the cell body having a teardrop or bulb shape and with an extensively branching dendritic tree, otherwise known as the dendritic arbour. This dendritic arbour is often described as fan-like and is almost flat on a 2D plane, and sagittally orientated such that it is perpendicular to the transverse parallel fibres. This maximises the number of different parallel fibre synapses onto a single dendritic tree. Estimates of the number of parallel fibre synapses onto a single Purkinje cell range from 60,000 to 180,000 in humans (Fox and Barnard 1957; Palay and Chan-Palay 1974), and 175,000 in rats (Napper and Harvey 1988). Whole cell recordings from synaptically connected granule cells and Purkinje cells in *in vitro* slices suggests that 50 simultaneously active granule cells are sufficient to discharge a Purkinje cell; this is on the basis that an impulse from a single granule cell generates EPSC of 2-60 pA (Barbour 1993). Evidence also from *in vitro* electrophysiology suggests that a large number of these synapses are silent with a detectable EPSC in only 7% of Purkinje cells whose dendritic trees are in the path of a single granule cell’s parallel fibre (Isope and Barbour 2002).

The Purkinje cells also have synapses from climbing fibres, with each Purkinje cell innervated by a solitary climbing fibre that makes numerous active synaptic contacts with the dendritic tree (Eccles et al. 1966). Purkinje cells also receive inhibitory GABAergic inputs from the molecular layer interneurons, the stellate and basket cells.

Purkinje cells exhibit two different types of action potentials: simple spikes and complex spikes. Simple spikes are reflective of spontaneous activity or from input via mossy fibre-granule cell pathways, and they tend to occur in the range of 30-100Hz (Armstrong and Rawson 1979). Simple spikes even occur spontaneously at high rates independently of input in *in vitro* slices (Häusser and Clark 1997). Complex spikes are generated in Purkinje cells in response to the powerful glutamatergic input of the climbing fibre. The result of a climbing fibre firing leads to large EPSP in the Purkinje cell which always generates an action potential followed by a variable number of smaller spikelets, caused by Ca$^{2+}$ influx (Eccles et al. 1966). These complex spikes occur at a much lower frequency than the simple spikes, typically at a frequency around 1Hz (Armstrong and Rawson 1979).
Purkinje cell axons arise from the soma and project to the cerebellar nuclei or in some cases directly to the vestibular nuclei in the brainstem. The axons are myelinated, and the synapses they make with their target cells are GABAergic (Ito et al. 1964; Ito and Yoshida 1964). The Purkinje cells are unusual in this respect as they represent the sole output projection neuron from the cortex, but they are inhibitory upon their target cells, not excitatory.

1.1.3.3 Molecular layer interneurons

The parallel fibres of granule cells do not only synapse onto Purkinje cells, but these fibres also make numerous synapses with the inhibitory interneurons in the molecular layer; the basket and stellate cells. These cells are GABAergic and release GABA onto GABA$_A$ receptors found on the postsynaptic Purkinje cell. These cells provide the mechanism for feedforward inhibition onto the Purkinje cells.

The molecular layer interneuron types differ in their point of synaptic contact with Purkinje cells. The basket cells make a synapse on the Purkinje cell body as it constricts towards the axon and stellate cells synapse onto the Purkinje cell dendrites (Ito 2006). Regarding electrophysiology, it is difficult to tell them apart, hence why they are frequently grouped and collectively known as the molecular layer interneurons.

Basket cells send their axons perpendicular to parallel fibres, and it is estimated that one cell contacts 50 surrounding Purkinje cells, and that one Purkinje cell receives inhibitory input from 20-30 baskets cells (Eccles et al. 1967).

The input to stellate and basket cells from parallel fibres is mediated by AMPA, and NMDA receptors in the case of stellate cells. There are also mGluR1α receptors which are activated by glutamate spillover in the synapses (Carter and Regehr 2000; Karakossian and Otis 2004). Activation of the molecular layer interneurons results in IPSPs in Purkinje cells (Eccles et al. 1967). The tonic inhibition provided by the molecular layer interneurons plays a role in the spiking irregularity of Purkinje cells (Häußser and Clark 1997).

Basket cells receive collaterals of climbing fibres and Purkinje cell axons (Jaeger et al. 1988). Climbing fibre responses generate EPSPs in basket cells at the same time as neighbouring Purkinje cells and are shortly followed by IPSPs (O’Donoghue et al. 1989).

1.1.3.4 Golgi cells

Golgi cells are also inhibitory interneurons. However they do not reside in the molecular layer. They are the second largest neurons in the cerebellar cortex and have their cell bodies positioned in the granule cell layer, with their dendrites branching extensively in the molecular layer, but dendrites
also extending into the granule cell layer. The Golgi cells are capable of providing both feedforward and feedback inhibition onto granule cells (Eccles et al. 1964).

A single Golgi cell has roughly 4700 synapses on its molecular layer dendrites from parallel fibres and a smaller number of synapses with mossy fibres, around 200, on its basal dendrites (Pellionisz and Szentágothai 1973).

AMPA and NMDA receptors mediate excitation through parallel fibre-Golgi cell synapses, and through these receptors, an excitatory effect is acted upon the cell (Dieudonne 1998). The Golgi cell has been estimated to make 5700 GABAergic synapses upon granule cells with its axon in glomerulus structures in cats (Palkovits et al. 1971). In vivo, the cells discharge at rates 5-20Hz and provide tonic inhibition to granule cells, as well as mediate inhibition upon non-postsynaptic cells in the glomerulus through GABA spillover (Rossi and Hamann 1998). This suggests that Golgi cells regulate noise in parallel fibre discharge when receiving noisy input from mossy fibres (Philipona and Coenen 2004).

Golgi cells also express mGluR2 receptors at the molecular layer dendrites and also on the soma and axons (Ohishi et al. 1994). Activation of the mGluR2 causes hyperpolarising currents in the Golgi cell which can silence the cell (Watanabe and Nakanishi 2003). Such behaviour has been demonstrated in vivo in response to peripheral somatosensory stimulation (Holtzman et al. 2006b), and this is thought to be mediated by glutamate spillover resulting in activation of extrasynaptic mGluR2 receptors (Holtzman et al. 2011). This suggests that granule cells may provide feedforward inhibition to Golgi cells and prolong their own activity through this mechanism. This has complicated the original view, showing that Golgi cells do not just provide inhibition onto granule cells in the form of feedback inhibition (Houston and Brickley 2011).

1.1.3.5 Lugaro and unipolar brush cells

Lugaro cells and unipolar brush cells are two interneuron cell types that are found in the cerebellar cortex, but are rarer, and less uniformly distributed. The Lugaro cell is an inhibitory interneuron that has its spindle-shaped cell body in or just below the Purkinje cell layer (Sahin and Hockfield 1990). Unipolar brush cells are oval-shaped cells that reside in the molecular layer with brush like dendrites, known as dendrioles (Mugnaini and Floris 1994). Lugaro and unipolar brush cells have been less extensively studied compared to the other cells in the cortex.

Lugaro cells have their axons orientated in the parasagittal plane, but the terminal ends travel transversely for a short distance. The axons synapse onto the apical dendrites of Golgi cells. Lugaro cells remain silent in slice preparations unless serotonin is applied, which when present the cells fire
at 5-15Hz and ISPCs are observable in postsynaptic Golgi cells (Dieudonné and Dumoulin 2000). This suggests that Lugaro cells are the intended recipient of cerebellar serotonergic innervation and it has been suggested that they may account for the role of serotonin in cerebellar ataxias (Trouillas, 1993).

Unipolar brush cells are excitatory local circuit neurons. The dendrioles receive synaptic input from mossy fibres, and their axons target granule cells postsynaptically (Diño et al. 2000). The UBCs are not homogenous in their distribution across the cerebellum, they are found in higher numbers in the vermis and flocculonodular lobe, while they are absent from the lateral cerebellar areas. This suggests their function is an amplification of signals relating to body, head and eye position (Mugnaini et al. 2011).

1.1.4 Mossy fibre afferents

The origin of mossy fibres are diverse, but they make up the most significant afferent pathway by sheer number. Numerous precerebellar nuclei give rise to mossy fibre afferents such as the pons, spinocerebellar pathways and those within the brainstem.

The mossy fibres are large myelinated fibres, 2-10µm in diameter, which bifurcate extensively upon entering the cerebellar white matter and terminate in the granule cell layer of the cortex as a rosette structure within a glomerulus (Eccles et al. 1967). They are described as rosettes due to their twisted and knotted appearance. Within the glomerulus, mossy fibre terminals synapse with granule cells and descending dendrites of Golgi cells. Also contained within the glomerulus are Golgi cell axons (Ito 2006). The afferents are primarily glutamatergic and terminate in lobule specific patterns of ill-defined patches, in which it estimated that single fibre synapses with 400-600 single granule cells in a single folium (Voogd and Glickstein 1998; Ito 2006). Projections from some precerebellar nuclei also send collaterals that synapse at the deep cerebellar nuclei (Gerrits and Voogd 1987; Mihailoff 1993; Matsushita and Yaginuma 1995).

1.1.5 Precerebellar nuclei

Mossy fibres originate from many different precerebellar nuclei found in various areas. Two of these areas are important for the subsequently presented work; the basilar pontine nuclei and the lateral reticular nuclei.

The basilar pontine nuclei (BPN) is a substantial mossy fibre source in most mammals within the brainstem that contains projection neurons and small local interneurons (Brodal and Bjaalie 1992). The pons is largely seen as a functional relay receiving a large number of fibres from all areas of the cerebral cortex via cortico-pontine projection of the cerebral peduncle (Brodal 1978). The pons is
crucial for the relay of auditory stimulus in delay eyeblink conditioning (Knowlton and Thompson 1988; Christian and Thompson 2003; Halverson and Freeman 2010), which is discussed subsequently. Anterograde neuroanatomical tracing has shown that input from areas of sensorimotor cortex in rat results in terminals that are neither confined to one area nor diffusely distributed across the pontine nuclei, suggesting a reshaping of neocortical maps within the pons (Brodal 1978; Schwarz and Möck 2001). Autoradiographic tracing from forelimb and hindlimb areas of one neocortical hemisphere show a small number of fibres that cross the midline and terminate in the contralateral pons (Mihailoff et al. 1978).

GABAergic terminals have been found in the pontine nuclei (Border and Mihailoff 1990), and inhibitory responses have been reported in response to stimulation of cerebellar nuclei that project back to the pons, although stimulus intensity was not well controlled (Berretta et al. 1991). GABAergic afferents that project to the pons have been described from various brain regions, including the zona incerta, anterior pretectal nucleus, lateral cerebellar nucleus and perirubral area (Border et al. 1986). This suggests the possibility of neural responses in the BPN that are more complex than that of it just being a simple relay.

The pontocerebellar fibres transverse the midline and enter the cerebellum through the middle cerebellar peduncle of the opposite hemisphere. The fibres branch extensively in the lateral areas of the cerebellum and also some project bilaterally (Rosina and Provini 1984; Holtzman et al. 2009). Projections are also sent to the cerebellar nuclei, and there is electrophysiological evidence of a communication loop between the two nuclei (Berretta et al. 1991; Möck et al. 2006).

The lateral reticular nucleus (LRN) is another brainstem precerebellar nucleus that contributes a diffuse and widely projecting network of mossy fibres bilaterally to the cerebellum (Clendenin et al. 1974; Wu et al. 1999). It receives inputs from a wide number of areas, including the cerebral cortex and ascending spinal fibres. These spinal inputs are multisensory bilateral inputs which cause LRN neurons to have wide sensory receptive fields (Rosén and Scheid 1973; Menétrey et al. 1983; Ness et al. 1998; Robbins et al. 2005; Xu et al. 2013). The lateral reticular nucleus is implicated in relaying peripheral somatosensory stimulus to the cerebellar cortex and as such generating long depressions of firing in Golgi cells in response to such a stimulus (Holtzman et al. 2006a; Xu and Edgley 2010).

1.1.6 Inferior olivary nuclei and olivocerebellar climbing fibres

A single pair of brainstem nuclei gives rise to the other major afferent system to the cerebellum, the inferior olivary nuclei. The nuclei reside in the medulla of the brainstem and are a bilateral structure. Each nucleus can be divided into three subnuclei: the dorsal accessory olive (DAO), medial accessory olive (MAO) and the principal olive. Inferior olivary nuclei send their projections through the
contralateral inferior cerebellar peduncle to the contralateral cerebellar hemisphere. These projections are of a small diameter and have a thin myelin sheath (Palay and Chan-Palay 1974).

Within the cerebellum, the inferior olivary projections form the unique structures known as climbing fibres. An olivary cell axon has on average six branches, but can be 7-10 depending on species, that terminate as climbing fibres that synapse with Purkinje cells in a one-to-one manner (Sugihara et al. 1999). Each climbing fibre makes numerous contacts with the Purkinje cell dendritic tree with an estimated 500 in rats (Palay and Chan-Palay 1974). It is this extensive connection of a single climbing fibre to a single Purkinje cell that allows it to exert such a powerful effect on the cells. The activation of the climbing fibre results in large EPSPs in the Purkinje cell which generates a spike followed by smaller Na/Ca\(^{2+}\) spikelets which can vary in number from one complex to another (Eccles et al. 1966; Thach 1967). The climbing fibre-Purkinje cell synapse has been shown to be plastic in vitro with 5Hz repetitive stimulation causing a weak LTD of climbing fibre EPSCs, the relevance of this in behaviour though is not known (Hansel and Linden 2000). Individual Purkinje cells generate complex spikes at a frequency of 1-3Hz, but inferior olivary neurons show subthreshold membrane oscillations at about 10 Hz, and it has been suggested that populations of Purkinje cells show correlated complex spike activity about this frequency (Sasaki et al. 1989). It has been proposed that such oscillatory behaviour act as a clock for motor coordination and timing (Kazantsev et al. 2004).

The inferior olivary neurons also send collaterals to cells of the deep cerebellar nuclei. The cerebellar nuclei, in turn, send projections back to the inferior olivary nuclei (Dietrichs and Walberg 1981). This reciprocal connection has been shown to be mediated by GABA and as such has an inhibitory influence on the IO cells (Andersson et al. 1988). This reciprocal loop has been hypothesised to allow feedback control of Purkinje cell activity with a long time course of seconds, feedback control of cerebellar learning and/or gating of input through the olivary nucleus (Bengtsson and Hesslow 2006). Disruption of inhibition to the inferior olivary nucleus prevents Kamin blocking that can be observed in cerebellar learning (Kim et al. 1998). Kamin blocking stops learning to a novel stimulus if it presented as a compound with a stimulus that has already been learnt in a classical conditioning task.

The inferior olivary nuclei receive input from multiple areas. There are direct spino-olivary pathways that carry signals from muscle, cutaneous and C-fibre afferents mostly from the contralateral spinal cord (Armstrong 1974). Direct projections have been demonstrated from the trigeminal nuclei to the contralateral nuclei mediating a pathway for signals from cranial nerves V, VII, IX, and X to olivo-cerebellar pathways (Armstrong 1974; Andersson and Eriksson 1981).
There are also descending cortical pathways to the inferior olive. Direct input has been demonstrated from the motor cortex of cats (Sousa-Pinto and Brodal 1969) and sensorimotor cortex of rats (Swenson et al. 1989), projecting to the ipsilateral IO. However, other studies have suggested there is a midbrain relay from descending cortical projections (Saint-Cyr 1987; Rutherford et al. 1989). Pharmacological inactivation of the cuneate nucleus with lidocaine in cats has shown the reduction of climbing fibre responses recorded in C1 and C3 zones (see section 1.1.7) in response to stimulation of the pericruciate cortex in cats, suggesting a relay role for this brainstem nucleus (Andersson 1984). Similarly, anatomical tracing in rats has shown the absence of labelled terminals in the inferior olive when injections are made into the forelimb regions of the motor cortex of rats, but terminals were observed in the dorsomedial medulla (Ackerley et al. 2006). Pharmacological inactivation of this site reduced transmission in the cerebro-olivocerebellar paths targeting forelimb or hindlimb-receiving parts of the C1 zone. In comparison to the spinal pathways, it seems that the picture is much less clear for the descending cortical pathway, with evidence that some pathways are relayed through other structures before reaching the olive. Spinocerebellar pathways appear to show movement related gating of transmission of signals, demonstrated through recordings of climbing fibre activity during a forelimb reaching task in cats. Active movement reduced the ability of electrical stimulation of the superficial radial nerve to evoke large field potentials in C1, 2 and 3 paravermal zones. Such gating is hypothesised to prevent self-generated irrelevant sensory signals inputs to the cerebellum in a task-dependent manner (Apps 1999).

1.1.7 Zonation of the cerebellum

Beyond the observations of gross anatomy, further divisions can be made by examination of anatomy, physiology and gene expression. The different maps and terminology associated with them can be quite complex, and it is beyond the scope of this dissertation to review in full, so this section represents a short overview to provide clarity on localisations described subsequently. For further information see Apps and Garwicz 2005; Apps and Hawkes 2009; Cerminara and Apps 2011; Cerminara et al. 2015.

Beginning with anatomical and physiological observations longitudinal zones are observed that run perpendicular to the transverse axis of the lobules along the rostrocaudal length. These zones were originally deduced from tracing and grouping Purkinje cell output, but such zonation has been shown to be consistent in the organisation of the nuclei and the inferior olivary nuclei (Groenewegen and Voogd 1977; Oscarsson 1979; Trott and Armstrong 1987; Voogd and Ruigrok 2004). Running medially from the vermis to lateral hemispheres the zones are A, X, B, C1, C2, C3, D1 and D2, and are depicted in Fig 1-3 overleaf.
Modules are also longitudinally designated regions that represent a grouping of a layer of Purkinje cells with its associated olivocerebellar afferents and corticonuclear links, as well as consistent reciprocal connections between the IO and the cerebellar nuclei. (Pijpers et al. 2008). They are thought to represent the basic functional unit of the cerebellum, in much the same role that columns of the neocortex have been hypothesised to fulfil.

Microzones also represent longitudinal zones of Purkinje cells that are defined by fine-scale electrophysiological mapping. All Purkinje cells within a microzone have distinct climbing fibre receptive fields that correspond to the innervation of different areas of the body. Each microzone
runs rostrocaudally and is roughly 300µm wide and resides in the larger longitudinal zones (Oscarsson 1979; Garwicz et al. 1998). In the B zones of the cat, microzones extend the whole length of the zone, whereas in C3 they tend only to cover several folia (Apps and Hawkes 2009).

Studies of gene expression reveal transverse zones that subdivide the cerebellum. These zones are anterior (lobules I-V), central (lobules VI and VII), posterior (lobules VIII to partial IX) and the nodular zone (lobules IX and X). This zonation is based on the expression of molecular marker zebrin II, which is the metabolic enzyme aldolase C (Brochu et al. 1990; Ahn et al. 1994; Hawkes and Herrup 1995). The zebrin II marker also creates stripes of expression that run rostrocaudally and are consistent within zones. These stripes are caused by groups of Purkinje cells that express zebrin II neighbouring groups of Purkinje cells that do not. The functional significance of these zones is not known, but recent studies have shown that Purkinje cells in Z- areas show higher rates of spontaneous simple spikes in comparison to those of Z+ areas (Xiao et al. 2014; Zhou et al. 2014). The organisation of these zones is detailed below in Fig. 1-4.

Figure 1-4: Transverse zones of the cerebellum
Depiction of the transverse zones of the mouse cerebellum as revealed by Zebrin II immunoreactivity. (A) Anterior zone, AZ, that has common transverse zones across lobules I – V, as viewed from the anterior. (B) Central zone, CZ, that has Zebrin II pattern between lobules VI and VII, as viewed dorsally. (C) Posterior, PZ, and nodular zones, NZ, as viewed from the posterior. Posterior zone has common pattern between dorsal VII, VIII and anterior XI. Nodular zone has common patterns between caudal XI and X. Adapted from from Apps & Hawkes, 2009
Mossy fibre afferents have also been studied for maps onto the cerebellar cortex. The fine electrophysiological analysis reveals a fractured somatotopic map of inputs. This fractured somatotopy is made up of individual patches with accurate somatotopic representation, but these patches are further mosaicked across the cortex resulting in disparate representation across the cortex (Shambes et al. 1978; Manni and Petrosini 2004).

1.1.8 Function

The function of the cerebellum is revealed by damage to it and its associated structures. The importance of the structure in the performance of motor skills was apparent to scholars in the early 19th century. Luigi Rolando in 1809 identified the motor symptoms that followed cerebellar lesions in animals, identifying the deficits in motor skills but not in sensory or cognitive functions. Rolando concluded that the cerebellum was required for initiating movement. Flourens (1824) observed that movements were still made after cerebellar ablation but that they were uncoordinated. This led him to conclude that initiation and production of movement were separate processes from coordination of movement and that the cerebellum must play a role in this coordination process.

Luigi Luciani (1891) used improved surgical technique in animals to finely study the effects of cerebellar damage. He came to describe three persistent symptoms of cerebellar damage in contrast to those effects that he saw as transient; these were asthenia (physical weakness), atonia (lack of muscle tone) and astasia (unstable muscle contractions).

Contributions shortly followed by Joseph Babinski (1902) and Gordon Holmes (1917) in the study of cerebellar function by observing clinical symptoms in patients who had received damage to their cerebellums. Their work further confirmed that which had preceded it and by the 20th century it was accepted that damage to the cerebellum resulted in deficits of motor performance and coordination but not initiation (Glickstein et al. 2009).

Increased knowledge of the fine anatomical structure and physiology of the cerebellum has led to the postulation of its role in sensory-based motor learning, discussed subsequently. Today the complexity of behaviours of which the cerebellum can modify or adapt has increased. The cerebellum is implicated in modification of the vestibular ocular reflex (Blazquez et al. 2004), saccadic adaption tasks (Prsa and Thier 2011), eyeblink conditioning (Thompson and Steinmetz 2009), long-term habituation of startle reflexes (Leaton and Supple 1986) and optical smooth pursuit tasks (Yang and Lisberger 2014, 2014). There is even evidence to suggest the cerebellum may have a role in cognitive tasks (Schmahmann and Caplan 2006).
It has been postulated the cerebellum performs a single computation rather than several functions distributed across the cortical regions, and that it is the inputs via the mossy fibre and climbing fibre to the modular units of the cerebellum that determine its functional output (Jaeger et al. 2013). If that computation exists, and what it is remains to be determined.

1.2 Associative cerebellar motor learning

It was not until 1964 that Giles Brindley suggested that cerebellum may have a role in associative learning. Brindley’s graduate student David Marr followed up this line of enquiry and formulated a theoretical account of how such learning could occur based on the microcircuitry that had been revealed by his contemporaries (Eccles et al. 1967; Marr 1969).

Marr’s theory relies on the convergence of the two afferent systems at the Purkinje cell and the modification of synaptic weights by experience. Marr assigned the climbing fibre system the role of instruction, and thus it sends a ‘teaching’ signal indicating when a movement is to be learned. Marr accounted for a role of the cerebral cortex in activating the inferior olive in generating this signal, but also for ascending systems doing the same when conditioned reflexes were to be learnt. The massively divergent and convergent parallel fibre system was allocated the role of carrying the ‘context’ signal, i.e. the context under which the learned movement was to be performed.

Concurrent activation of the parallel fibres and climbing fibres at Purkinje cells was hypothesised to lead to a strengthening of the active parallel fibre-Purkinje cell synapse, such that in the future activation of the parallel fibres would be sufficient to elicit the movement (Marr 1969).

Subsequently, James Albus arrived at a similar theoretical account of how the cerebellum could mediate motor learning and extended Marr’s theory to include climbing fibre error detection (Albus 1971). Albus proposed an error-detection role for the climbing fibres and modification of parallel fibre-Purkinje cell synaptic weights following learning, in agreement with Marr’s theory. However, Albus proposed that, rather than strengthening of the synapses, weakening would occur in such a way that subsequent activity at the parallel fibre-Purkinje cell synapse would now mimic the actions of climbing fibre activity and complex spikes on simple spike output of the Purkinje cell; that is decreasing simple spike activity. In his proposed system the climbing fibre signal signifies an error and a change of behaviour that removes the error ‘nulls’ the climbing fibre signal.

Subsequent experimental work supported the theories of the mechanism of motor learning in the cerebellum. Long-term depression (LTD) was identified at parallel fibre-Purkinje cell synapses that were concurrently active with climbing fibre synapses, occurring both in vivo and in vitro in slices (Ito and Kano 1982; Ekerot and Kano 1985; Ito 1986; Karachot et al. 2001).
Several molecular events are required for the induction of LTD, including activation of mGluR1 and AMPA receptors at PF-PC synapses and AMPAR at CF-PC synapses which leads to an increase in Ca\(^{2+}\) by voltage-gated calcium channels. Subsequent second messenger signalling via protein kinase G and protein kinase C results in the inactivation of postsynaptic AMPARs ultimately leading to their internalisation (Ito 2001).

Although LTD at PF-PC synapses bolsters the theories of Marr and Albus, and suggests that learning-related changes may occur in the cerebellar cortex, it is important to note that bidirectional plasticity has been seen at other synapses within the cerebellar cortex and even within the deep nuclei (Jörntell and Ekerot 2003; Zheng and Raman 2010; Carey 2011; Gao et al. 2012). However, all of these are only shown mechanistically in vitro. Studies of mGluR1 deficient mice that exhibit a deficiency in LTD and show ataxic gait are impaired in eyeblink conditioning (Aiba et al. 1994). However, mutant mice in which the mutations affect the internalisation of AMPA receptors appear not to be impaired in several cerebellar motor learning tasks, including eyeblink conditioning (Schonewille et al. 2011). The original theories of Marr and Albus although giving the impetus to study synaptic weights in critical circuits are unlikely to be wholly complete, and in some cases, the minutiae of the theories appear inconsistent with the experimental evidence.

1.2.1 Eyeblink conditioning

The classically conditioned eyeblink response is the most studied example of cerebellar-dependent associative motor learning. In this paradigm, a neutral conditional stimulus (CS, usually auditory or visual) is paired with an aversive periocular unconditional stimulus (US, usually an air puff or electrical stimulus). The US generates a reflexive blink by itself. However, after enough training, the presence of the CS begins to elicit a conditioned reflex that generates an anticipatory blink such that the eye is closed at the time of US arrival. The nictitating membrane response (NMR) in rabbits involves the extension of the third eyelid in response to an unconditional stimulus. This response is advantageous in the study of motor learning as it is an event that rarely occurs spontaneously, and as such has been used to explore the properties of classical conditioning since the 1960s (Gormezano et al. 1962). The external eyelid response is also conditionable, but the recording of NMR is favourable in animals that possess the nictitating membrane. The conditioning has largely been studied in two forms, delay conditioning and trace conditioning, presented in Fig. 1-5, overleaf.

Delay conditioning is the form in which the conditional stimulus begins before the unconditional stimulus and is continually present up until the US is given. This type of conditioning has been shown to be dependent on an intact cerebellum and its associated circuitry. Electrophysiology shows cellular activity related to CS and US in Purkinje cells and lesions ipsilateral to the trained eye
abolished the expression of CRs and any relearning but still allowed the contralateral eye to be trained (A McCormick 1981; McCormick et al. 1982a; Bracha 2004; Thompson and Steinmetz 2009). Lesions of the ipsilateral dentate-interpositus nuclei also abolish expression of CRs, as do lesions of the superior peduncle (McCormick et al. 1982b; McCormick and Thompson 1984). Lesions of the inferior olivary nuclei lead to extinction even when the trials are reinforced with a US, suggesting that climbing fibres have a role in signalling the US (McCormick et al. 1985).

Trace conditioning involves a CS that terminates and is then followed by a gap before the US onsets and relies on the hippocampus/medial temporal lobe as revealed by lesion and decerebration studies (Solomon et al. 1989; Moyer et al. 1990). Trace conditioning is slow to learn and can be unreliable. Due to this delay conditioning has largely been the paradigm of choice for eyeblink motor learning.

The behaviour of the nictitating membrane during delay conditioning consists of two elements, the unconditioned response (UR) and the learned conditioned response (CR). Reflex eyeblinks can be elicited by periocular stimulation or corneal air puff, as the unconditional stimulus (US), and involves the closure of the eyelid, retraction of the eyeball and extension of the nictitating membrane in a coordinated response. The nictitating membrane response is controlled largely by the actions of the sixth cranial nerve and the retractor bulbi muscle (Cegavske and Thompson 1976). The conditioned responses are effected through the same nerve and muscle efferent pathways but differ on temporal dynamics; firstly the conditioned response is anticipatory and NM extension peaks at the point at which the US would normally be delivered, even over a range of CS stimulus lengths from 100-4000ms. Secondly, the UR is a reactive NMR at a minimum onset of around 25-40ms, but the CR
has an onset latency of 90-100ms from the onset of the stimuli (Coleman and Gormezano 1971; Steinmetz 1990).

The neural pathways of classical conditioning have been explored; a simplified circuit diagram is illustrated in Fig. 1-6, below. The unconditioned reflex is believed to be relayed by the trigeminal nucleus by direct projections to the accessory abducens and facial nucleus, and indirect projections to the facial nucleus (Cegavske et al. 1979). Considerable evidence suggests that the US signal is carried from the trigeminal nuclei to the contralateral dorsal accessory olive of the inferior olive. It is relayed to the cerebellar cortex and also the anterior interpositus nucleus via climbing fibres (Van Ham and Yeo 1992; Thompson and Steinmetz 2009).

Fig. 1-6: Simplified circuit diagram of the essential components of the classically conditioned eyeblink response

The air puff or electrical periocular stimulus (US) causes an unconditioned response (UR), by direct reflex pathways. It also activates the olivocerebellar climbing fibres in the dorsal accessory olive (DAO), a nucleus of the inferior olive, these project to Purkinje cells in an eyeblink controlling microzone in the hemisphere of lobule VI.

The CS is relayed via auditory nuclei to pontine nuclei. Pontine mossy fibres reach the cerebellum through the middle cerebellar peduncle on the contralateral side. These synapse with granule cells whose parallel fibres converge with the same Purkinje cells innervated by the DAO in the eyeblink controlling microzone.

The CR is preceded by the increased excitation of anterior interpositus neurons (NIA) which project to the contralateral red nucleus, which in turn projects to the bulbar motor nuclei. Solid arrowheads represent excitatory connections and open circle arrowheads represent inhibitory connections.

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According to theory, any mossy fibre activation that predicts the US can be a CS, but in the most commonly used paradigms, an auditory tone or light stimulus are used. In these cases the CS is presumed to be relayed via the pontine nuclei, which projects mossy fibres widely to the cerebellum (Kawamura and Hashikawa 1981; Brodal 1982; Serapide et al. 2002), has afferents from visual and auditory cortex (Glickstein et al. 1985; Leergaard and Bjaalie 2007) and has cells receptive to both modalities (Baker et al. 1976). Lesions of the middle cerebellar peduncle, which carry pontine mossy fibres to the cerebellar cortex and anterior interpositus nucleus, abolish conditioning to both modalities (Lewis et al. 1987).

The anterior interpositus nucleus is important for eyeblink conditioning on the ipsilateral side shown by the fact that stimulation of it before training causes an eyeblink (McCormick and Thompson 1984); suggesting that the output is hardwired and becomes associated with a contextual stimulus in learning. Lesions before training prevent acquisition (Lincoln et al. 1982), and post-training lesions abolish learned responses (McCormick and Thompson 1984; Yeo et al. 1985a). Similarly, reversible inactivation prevents acquisition such that when inactivation is reversed animals behave as if naïve (Clark et al. 1992), although this inactivation was done by cooling so the extent to which it was confined to the nuclei is unknown.

The conditioned response is generated by the increased firing of the anterior interpositus nuclear neurons which in turn excite the red nucleus on the contralateral side (see Fig 1-6, previous page). Lesions of the superior cerebellar peduncle, which carry efferents from the anterior interpositus nucleus to the contralateral red nucleus, prevent expression of the conditioned response (McCormick and Thompson 1984). Crucially, reversible inactivation of the red nucleus prevents the performance of the conditioned response during acquisition, yet upon lifting of inactivation the animals behave as if trained (Clark and Lavond 1993). The red nucleus has been shown to be anatomically connected to motor nuclei involved in the reflex pathway, and the conditioned response is presumed to be driven through this nucleus (Morcuende et al. 2002).

1.2.2 Localisation within cerebellum

A region of cortex that appears to be important in eyeblink delay conditioning is lobule HVI (hemisphere of lobule VI), an area which receives overlapping CS and US inputs (Yeo et al. 1986). Lesion studies have had inconsistent results that may be dependent on the parameters and the extent to which affect the region. Yeo and colleagues have reported that lesions in HVI after five days training abolish conditioning on the ipsilateral side in studies that used auditory and visual CSs (Yeo et al. 1985b; Hardiman and Yeo 1992). However, others have found that under the same
conditions but with post-lesion training extended from 5 to 10 days only a temporary abolition was observed (Lavond et al. 1987). In some experiments, lesions have revealed a change in conditioned response timing rather than abolition such that onset and offset were now before where the US would be in time (McCormick and Thompson 1984).

Work conducted in our lab has however identified microzones in lobule HVI, which have periocular driven climbing fibre receptive fields that only relate to the ipsilateral eye. Infusions of small amounts of CNQX (0.2-0.8µl; 3mM in PBS) into these areas blocks expression of conditioned responses in trained animals rapidly and reversibly (Mostofi et al. 2010). This area has been highly controversial largely because of the complex structure of the cerebellar cortex with a small but deeply located area of interest below the surface. A lesion that misses this area will give an impression that there is no role of the cortex in conditional learning, a lesion that partly damages it may change the size and shape of the responses, giving the idea that the cerebellar cortex shapes, but does not generate the responses.

Similarly, there have been debates about whether lobule HVI or anterior lobe is crucial for eyeblink conditioning. Cortical lesions of anterior lobe in rabbits have been reported to affect CR timing when lesions are created in after training, causing responses to become maximal much earlier than the time the US would be present (Perrett et al. 1993). Similar experiments show that after anterior lobe lesions subjects cannot acquire conditioned responses to a new CS, even after a significant amount of training (Garcia et al. 1999). Electrophysiological studies, discussed subsequently, show eyeblink related Purkinje cell activity in the anterior lobe (Green and Steinmetz 2005).

However, there is no reason that these results in HVI and anterior lobe are inconsistent with each other. The primary fissure separates the two areas but this is just a visual separation, in reality, the Purkinje cells between the two areas are continuous. Mostofi et al., 2010 found microzones that were deep in the base of lobule HVI and that were near the base of the primary fissure. A major problem here is that no two lesions are the same, and the cerebellum has a complex lobular structure. Superimposed upon this, is the notion that sagittally orientated strips of cortex – microzones – mediate function, but have a complex three-dimensional orientation within a folded cortex, means that different lesions may affect more or less of a microzone giving inconsistent results.

1.2.3 Activity of cells during conditioning

One of the major goals of modern neuroscience is to relate observable and repeatable behaviours to the activity of individual or small populations of neurons. With the identification of areas related eyeblink conditioning within the cerebellum and the small number of cell types, this model provides
an excellent opportunity to study the neurophysiology of a behaviour that has aspects of timing, acquisition, consolidation and retention of an associative and motor learning task.

Within the cerebellar cortex, the activity of Purkinje cells has been recorded during the performance of eyeblink conditioning, in part because they are a site of convergence for the two afferent systems parallel fibres carrying input from mossy fibres and climbing fibres bringing input from the dorsal accessory olivary nuclei. They have the additional benefit of being large cells with high regular firing rates and easily identifiable by the presence of complex spikes in recording traces.

Two aspects of Purkinje cell firing are often examined during behaviour; the simple spike firing which is modulated via parallel fibres; complex spike firing which is modulated by climbing fibres. In untrained animals Purkinje cell simple spike activity is either largely unmodulated by the conditional stimulus or shows a weak increase in the simple spike firing rate following stimulus presentation. Jirenhed et al. 2007 show moderate 120% increase in simple spikes in Purkinje cells in decerebrate ferrets during acquisition training; this, however, was in C3 zone of lobule VI. Similar responses to a CS- (a CS that is never paired with the US) in trained rabbits have been observed in Purkinje cells in lobule HVI (Berthier and Moore 1986). In accordance with the Marr-Albus theory, the periocular electrical stimulus or air puff generate complex spikes in the Purkinje cells of naïve animals (Berthier and Moore 1986; Jirenhed et al. 2007). In fact, the specificity of the cells receptive field for this aversive stimulus is the criteria for establishing the area as an eyeblink related zone of the cerebellum (Mostofi et al. 2010).

After receiving training, the Purkinje cells change their pattern of activity in response to the CS in contrast to their naïve state. Purkinje cells now show a significant decrease in firing that begins shortly after conditional stimulus presentation. This has been demonstrated in cells in the anterior lobe which were recorded in rabbits after learning (Green and Steinmetz 2005) and shown to develop during acquisition in C3 zone of decerebrate ferrets (Jirenhed et al. 2007). It has also been shown in a small number of cells recorded in lobule HVI, but this study was limited as cells were not identified by the climbing fibre receptivity to periocular stimulation (Berthier and Moore 1986). The loss of Purkinje cell excitation leads disinhibition of neuron within the interpositus nucleus.

Firing increases in frequency in a population of neurons within the anterior interpositus nucleus before the execution of the conditioned response (Berthier & Moore, 1990). The red nucleus also develops a neuronal model of the learned response, but this is dependent on the integrity of the AIP (Chapman et al., 1990).
Fig. 1-7 below shows examples of Purkinje cell activity (top row) that have been recorded in these studies after the acquisition, showing the Purkinje cell pause. The activity of anterior interpositus cells during acquisition are shown in the bottom row and shows the development of increased firing rate during CS-US intervals as the number of days trained progress.

In decerebrate ferrets Golgi cells have also been examined during conditioning and while they do show responses related to the conditional stimulus they do not appear to be modulated by learning (Rasmussen et al. 2014), consistent with theoretical considerations that state that the parallel fibre – Purkinje cell synapse is the site of plasticity.

1.2.4 Changes in climbing fibre receptive fields after conditioning
Recent findings have shown that climbing fibre responsiveness is altered after learning. Work carried out on awake behaving rabbits in this laboratory has shown that after acquisition and retention of conditioned responses that Purkinje cells fire complex spikes in response to the conditional stimulus, in essence, the climbing fibre system is now responsive to the conditional stimulus. 20 cells were recorded in eyeblink microzones before (n=8) and after (n=12) conditioning and the complex spike density Purkinje cells were compared (Mostofi et al., Unpublished work), the results of this
experiment are shown in Fig. 1-8, underneath. In this study eyeblink microzones are defined as small regions of lobule HVI that receive olivocerebellar climbing fibres from ipsilateral periocular receptive fields (consistent with C3 zone). These project to the anterior interpositus nuclei which is known to be involved in conditioning (Mostofi et al. 2010). The Purkinje cells show an increased complex spike density that begins ~60ms following the onset of the CS that was not observed before conditioning.

This has been replicated in eyeblink microzones in mice using an air puff aversive stimulus (Ohmae and Medina 2015), in comparison to the electrical stimulus used by Mostofi et al. Fig. 1-9, overleaf, shows the complex spike firing rate in relation to three stimuli conditions: an unpaired air puff; air puff paired with LED conditional stimulus; and LED conditional stimulus with the air puff omitted. Whether the air puff is presented or not there is a clear increase in complex spike rate related to the LED conditional stimulus. These neurons seem to show firing patterns related to prediction error remarkably similar to dopamine in the primate reward system (Romo and Schultz 1990; Schultz et al. 1997; Schultz and Dickinson 2000). In this system neurons greatly increase their firing to an unexpected reward, however, if this reward can be reliably predicted by a CS this increase in firing is transferred to the predictor. Additionally, if a CS predicts a reward but that reward is not received
the cells decrease their firing at the time of the expected reward. This difference between expected and received is what the neuron is modelling with its prediction error (Schultz 2016).

The fact that the climbing fibres change their responsiveness in this same manner suggest that the CS in a trained animal now generates an intrinsic unexpected error signal that may itself also become a conditional reinforcer that can be anticipated by a novel predictor that precedes it.

Figure 1-9: Prediction error encoding by climbing fibres in mice

All graphs except (D) consist of the same three elements: (top) an average measure of eyelid closure recorded, and one individual trial in black for each trial type, (middle) example of Purkinje cell firing during one trial, (bottom) complex spike raster

(A) Unpaired US trials, showing a complex spike following an unexpected airpuff

(B) Paired CS-US trials, showing complex spikes shortly after CS stimulus onset and to the US component of the trial

(C) CS alone trials, showing complex spikes shortly after CS stimulus onset. No US is given in these trials and there is a decrease in complex spikes at this point of the trial.

(D) Peristimulus time histogram showing complex spike rates for the three trial types

Reproduced from Ohmae and Medina, 2015
This is one of the major predictions of this dissertation and is the focus of large part of the work subsequently presented.

### 1.2.5 Higher-order conditioning

The change mentioned previously in responsiveness of climbing fibres to the conditional stimulus leads directly to the question of whether higher-order conditioning is possible within the eyelblink conditioning system. The CS after conditioning generates climbing fibre responses, and the question remains as to what extent the CS can now be a conditional reinforcer itself.

Second order conditioning is an example of higher order conditioning that occurs when an individual who has learned to respond to a conditional stimulus then has that stimulus preceded by a novel conditional stimulus. In this form, training consists of a CS1 that predicts the US, and the new CS2 predicts the CS1 but in such a way that the CS2 is never paired with the US. The layout of these stimuli is shown in Fig. 1-10 below.

![Diagram of CS1-US and CS2-CS1 trials](image)

**Figure 1-10: The relationship between stimuli in first order (CS1-US) trials and second order (CS2-CS1) trials**

Second order conditioning involves using the first order conditional stimulus (CS1), as the instructional signal for the second conditional stimulus. Crucially, the US is the only ever paired with the CS1 so if the CS2 on its own, after sufficient training, elicits a conditioned responses there must have been an association made to the UR by the CS2 but it was made indirectly via transference of information from the first order conditional stimulus (CS1), acting as a conditional reinforcer, to the second order conditional stimulus. To be clear, a CS2-CS1 and CS1-US trial are not given at the same time, and are usually interspersed randomly with intertrial intervals of >20s. Figure by author.

In reality, perfect second order relationships are probably unlikely to exist. A CS2 that predicts a CS1, and a CS1 that predicts a US are likely on some occasions to have a CS2 paired with the US either as serial compound stimulus (CS2-CS1-US) or as trace conditioning stimulus (CS2-gap-US). As such...
second order conditioning may represent an unnatural experiment, but it is one that allows us to test whether CS1 can act as a conditional reinforcer.

Second order conditioning has been observed in several different classical conditioning tasks. The gill-withdrawal reflex in *Aplysia* has been shown to be able to respond to a second order conditional stimulus after pairing with CS1 (Hawkins et al. 1998). Similarly, *Drosophila melanogaster* show conditioned avoidance responses to an odour CS2 when it is paired with an odour CS1 that has previously predicted electric shocks (Tabone and de Belle 2011). These represent examples of higher order classical conditioning in simple invertebrate systems, but there are many examples in vertebrate model systems. These include conditioned analgesia whereby the response to thermal stimulation is reduced in the presence of a CS2 that has been paired with a CS1; the CS1 was previously associated with analgesia (Ross 1986). Fear conditioning in rats as measured by lick suppression has also been shown to pliable to second order conditioning (Cicala et al. 1990).

Indeed, second order conditioning of the eyeblink response has been examined in the rabbits. Two studies have reported positive results in attempting second order conditioning (Kehoe et al. 1981; Gibbs et al. 1991), but earlier reports suggest that conditioning of the rabbit eyeblink reflex is not conducive to second order conditioning. Maisiak & Frey, 1977 examined second order conditioning in the rabbit eyeblink model but found very few responses (2%) to the CS2 alone. However, their animals received six days of initial training of CS1-US pairs before having ten days which included second order training. They received 24 trials a day, only 8 of which were second order CS2-CS1 trials. In comparison (Gibbs et al. 1991) gave 30 CS2-CS1 trials per day for 16 days.

The experimental design in the two experiments that reported successful second order conditioning was devised mainly to examine the effect of ‘trace ‘intervals between CS2 and CS1, but in some of the experimental groups, CS2 terminated at the same time that CS1 began. The experiments found that the rate of response to a CS2 stimulus was inversely related the interval between CS2 and CS1. A CS2-CS1 trial that has no interval between the offset of CS2 and the onset of CS1 (delay conditioning) produces the highest percentage of responses, at 64%, following 16 days of training.

While the studies provide evidence that second order conditioning of the eyeblink response is possible they provided no details on one of the most crucial factors related to the eyeblink conditioning, response timing. The first order conditioned response is precisely timed to be maximal at the time of the air puff or periocular electrical stimulus, an event which is signalled to Purkinje cells via olivocerebellar climbing fibres and which has been seen to be the critical thing learned in eyeblink conditioning (Ohyama et al. 2003). However, these papers report nothing related to the response timing other than that a CR occurred in an 8-second long response window. In light of the
recent finding that changes in climbing fibre activity are related to learning, a complete description of the time course of any second order responses is necessary to further explore the relationship between climbing fibre activity and the temporally precise conditioned responses.

1.2.6 Human eyeblink conditioning

One of the advantages of animal models is the level with which manipulations can be performed to perturb behaviour and cellular activity, while simultaneously recording neural activity. Obviously much evidence that delay eyeblink conditioning is dependent on a functioning cerebellum and the implication of several cerebellar areas’ involvement in the behaviour has been deduced from animal studies. However, there is considerable evidence that eyeblink conditioning in humans is dependent on the same structures (Woodruff-Pakand and Steinmetz 2002; Gerwig et al. 2007).

The human eyeblink conditioning method is largely analogous to those used in animals. An air puff is more commonly used as the US than periocular electrical stimulus, and the eyeblink is recorded by a variety of means ranging from EMG to infrared sensors which measure external eyelid. The method presents some challenges with humans blinking at a high spontaneous rate and having an awareness of the stimulus contingencies.

The use of MRI to define focal lesions and fMRI in healthy patients has helped to examine the neural correlates related to aspects of eyeblink conditioning in humans. Conditioned response acquisition has been shown to be sensitive to cerebellar damage (Daum et al. 1993). A 54-year-old woman who presented with damage to cerebellar afferents after a cerebrovascular incident was demonstrated to show a low level of responding after conditioning. She emitted just 6 CRs in 100 trials, compared to an average of 56.7 out of 70 from 5 age-matched control individuals (Solomon et al. 1989). A study examining acquisition in 14 patients with cerebellar lesions (7 bilateral, 7 unilateral) in comparison to 20 control participants found that patients with lesions ipsilateral to the tested eye performed significantly worse than those that had lesions that were contralesional, or to controls (Woodruff-Pak et al. 1996). This is consistent with animal models where the ipsilateral cerebellum is integral for CR acquisition and performance.

Functional imaging studies have provided evidence of cerebellar activity during CR acquisition that is also consistent with animal studies. Mapping of areas with activity related to the US have shown that in the anterior region activation maxima is recorded in hemispheral lobule VI and Crus I (Dimitrova et al. 2002). Similarly, hemodynamic responses to CS presentation are shown to evolve slowly in the same areas whilst the association is being learned (Ramnani et al. 2000). More recent evidence has shown concomitant activity in lobule HVI and at the level of cerebellar nuclei while the conditioned response is acquired (Thürling et al. 2015).
An area in humans which has not received as much study as in animals is the differing contributions of the hippocampus and the cerebellum to trace conditioning versus delay conditioning. One study suggested that both trace and delay conditioning were intact in a patient with cerebellar cortical degeneration (Fortier et al. 2000), implicating the cerebellar nuclei as important for both behaviours. However, this would contradict the several lesion studies showing a deficiency in delay conditioning. Another study assessed trace conditioning in patients who have already shown deficits in acquiring responses in delay conditioning and determined that patients with only cortical lesions show no deficit in learning trace conditioning (Gerwig et al. 2006). An imaging study using fMRI reveal activations of the cerebellum during both trace and delay conditioning, but hippocampal activation is much greater in trace condition relative to delay (Cheng et al. 2008).

A limitation in human studies is the amount of awareness of the stimulus contingencies and conscious effort to avoid the aversive US plays in the performance of conditioned responses. Amnesiac patients can acquire conditioned responses to delay eyeblink conditioning suggesting that responses are independent of intact hippocampal formation (Gabrieli et al. 1995; Clark and Squire 1998), but the evidence regarding the role of awareness is mixed. Self-reported awareness of stimulus contingency shows no difference in rates of response for delay conditioning between those who were aware and those who were not, but it does affect the percentage of responses for trace conditioning (Manns et al. 2000, 2001; Smith et al. 2005).

Recently it has been shown that individuals can learn to make precisely timed blinks in response to a stimulus in the absence of the US if they are instructed to do and given 40 training trials (Rasmussen and Jirenhed 2017). In this experiment participants (n=21) were told to respond to a stimulus at least 150ms after it began and make blinks that were maximal at intervals of 300ms following stimulus onset for 40 trials. During these 40 trials, they were either given offline feedback, where after each attempt trial they were shown their eyeblink trace (n=13); or they had an online indicator of when to blink in the form of an auditory click at 300ms (n=8). Subsequently, these individuals were told to continue to respond to 20 more trials where there were no more indicators or feedback, during this the subjects made a high percentage (~100%) of eyeblink movements that would be considered conditioned responses if they were eyeblink conditioned. Following this, the subjects were told to forcibly not respond to the stimulus while it was used in a delay eyeblink conditioning task with a 300ms CS-US interval. Initially, the responses to the CS dropped but not to levels that would be expected if they were naïve to conditioning. The response timing of the voluntary movements to those that were produced after conditioning were very similar. The interpretation of these results is difficult. Obviously, blinks can be voluntarily controlled in humans, and this shows that they can be trained to display a precisely timed movement something which is in the domain of cerebellar
function. This however does not prove that volition is the sole driver of eyeblink conditioning in humans, but it reminds us that we must be careful in our interpretation of human eyeblink studies especially where there is no attempt at masking the stimulus contingencies.

Although there are limitations of human eyeblink conditioning, it still serves as a useful model of associative learning in primates and as a comparative approach to those undertaken in animal subjects.

1.3 Experimental aims

The experiments described in the following chapters can be broadly split into two categories: extracellular in vivo electrophysiology, and behavioural experiments.

The extracellular in vivo electrophysiology is mainly concerned with the behaviour of Golgi cells within the cerebellum in response to different stimuli that either directly or indirectly activates different cerebellar afferents; namely pontocerebellar afferents and lateral reticular nucleus afferents. These experiments aimed to test the same Golgi cells to stimulation of two different mossy fibre sources and to see if these produced similar or different patterns in the response. These experiments are not directly related to learning within the cerebellum but examine the responses of an important interneuron within the cerebellar microcircuit. This gives us a basic understanding of the behaviour of the cells in the circuit and to allow the application of this knowledge to comprehend how the cell might behave while carrying out behaviour.

The responses of cells in the pontine nuclei to peripheral stimulation are examined. We give an account of the cells under spontaneous conditions and report the failure of antidromic invasion recorded in these cells under our conditions. The cells show unexpected bilateral responses to peripheral stimulation and also show depressions in firing without any excitation. These experiments aimed to characterise in vivo extracellular electrophysiology of the hard to reach pons cells and to develop these experiments for further work. However due to the outcomes presented the experiments did not progress much further.

The behavioural element of this dissertation largely applies to second order conditioning using eyeblink conditioning. This follows from the recent findings relating to changes in climbing fibre activity before and after ordinary delay conditioning in eyeblink related microzones (Ohmae and Medina 2015). The fact that climbing fibres become responsive to the conditional stimulus after an animal has been well trained suggests, in accordance with the Marr-Albus theory, that the conditional stimulus now has predictive value. This prediction error value of the climbing fibre suggests that CS is now the ‘unexpected’ event and perhaps that it can be predicted with a reliable
temporal predictor preceding it; the above mentioned second order study of the nictitating membrane response suggests this is the case, but they do not describe the timing of responses. The shift in timing of climbing fibre responsiveness allows the exploration of the relation of climbing fibre activity to the temporally defined eyeblink movement. It allows us to ask does a second order stimulus predict the US or does it predict the unexpectedness of the first order stimulus? The second order conditioning experiments attempt to demonstrate this behaviour in both rabbits and humans and to show the time course of such learned movements.

The final part is concerned with using a more complex stimulus to condition eyeblink responses in humans. We wanted to see if it was possible to use ones’ motor action to predict an aversive air puff and ultimately tie motor sequences together. To our knowledge, such work has not been carried out, and conditioning of this sort has not been demonstrated.
2 Statistics

Throughout the analysis of work contained within this dissertation, two statistical techniques have been commonly used. Firstly, to determine if the responses of neurons differed from their spontaneous firing when a stimulus was applied a Gaussian multivariate analysis on the bin counts from peri-stimulus time histograms was conducted. Secondly, where responses, such as conditioned responses in eyeblink conditioning, or the firing of the cell in response to a stimulus pulse can be categorised as 1 or 0 for presence or absence, a binomial logistic regression model has been used to determine the influence of predictors on the presence of response. These processes are described subsequently.

2.1 Determination of responses of neurons to stimulation pulses

To determine if neurons reacted to stimulation of various afferents, their pattern of spontaneous firing was compared to responses observed after stimulation. This comparison was mainly facilitated by the use of peristimulus time histograms (PSTH) and an anomaly detection algorithm. The process is outlined in the following paragraphs.

A peristimulus time histogram (PSTH) consists of recording the time of action potentials relative to stimulus onset in a small window around the stimulus. Usually a small period before the stimulus is taken as a baseline period, 300ms for example, and then a short period after to determine if changes in firing occur following stimulation. The stimulus is presented a number of times, each single presentation being known as a sweep, and these are collated together into a single histogram whereby the occurrence of each action potential relative to the stimulus onset time is binned into histogram bins of a width selected by the experimenter.

To determine the distribution of bin counts that would be observed if no stimulus was given, the recordings of spontaneous activity were resampled. The process is outlined overleaf in Fig. 2.1. For a single sweep a randomly selected time point in the spontaneous data was chosen to act as the time of pseudo stimulus onset and the time of action potentials relative to it were logged, this was repeated a number of times to build a number of sweeps into a single histogram. The number of sweeps chosen to build a single histogram was set at the number used in creating stimulation histograms, to which the spontaneous histograms would later be compared. Commonly this was around 100 sweeps but in some cases increased to 500 sweeps. This process was repeated to build up a set of 500 peristimulus histograms that were generated from the spontaneous data recorded for each cell. Each histogram had its counts normalised to the average bin count observed in the baseline period and was expressed as a percentage of baseline activity across the whole histogram.
To detect effects of stimulation protocols on neurons firing rate the resultant PSTHs were compared to PSTHs that were sampled from the spontaneous firing data using the same parameters as the stimulation PSTHs (bin width, number of sweeps). The process was as follows: 1) Randomly select a time to act as the pseudostimulus onset and bin the spikes in the stimulation window into an individual sweep of the PSTH. 2) Repeat until enough sweeps have been collected into a single spontaneous histogram, which has a comparable number of sweeps as the stimulation histogram. 3) Repeat the whole process to make 500 spontaneous histograms for each individual cell.

Figure 2-1: Resampling spontaneous data to generate spontaneous PSTHs
The generation of multiple spontaneous histograms for each individual cell leads to a normal distribution of values at each bin, with 500 observations per bin width per cell. This is demonstrated in Fig 2.2 shown above, where the values of the first bin following the onset of the pseudo stimulus have been combined from multiple PSTHs to form their own normally distributed histogram. In this manner it is possible to perform a multivariate Gaussian analysis, whereby the values obtained to genuine stimulation are compared to the spontaneous distributions at each bin, and if it falls outside a given probability distribution across the whole range it is classified as having deviated from spontaneous firing. The stimulation PSTHs are constructed with the same bin width and the number

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*Figure 2-2: Comparative bin values are normally distributed*

The first bin values from the 500 spontaneous PSTHs are collated together and we see that they form a normal distribution as shown in the ordinary histogram on the right. This process is repeated for all comparative bin values across the spontaneous PSTHs.
of sweeps collected during stimulation is closely matched by the number used in generating a single spontaneous PSTH.

The process is difficult to visualise in an n-dimensional space above two dimensions but the result of a two dimensional analysis is outlined in Fig. 2.3 overleaf. The upper PSTH (Fig. 2.3A) shows the result of stimulation of middle cerebellar peduncle stimulation in a putative Golgi cell, the first two bins post stimulus onset are highlighted in red. In the lower panel (Fig. 2.3B) the distribution of spontaneous values for the first bin following the pseudo stimulus are plotted against the second bin values (black dots and marginal histograms). The inner concentric rings represent confidence interval ellipses of 20, 40, 60 and 80%, and the dotted red ellipse represents the 99.99% confidence interval for the two dimensional distributions. Here we can see that the two bin values for the stimulation PSTH (red dot) fall outside of this interval and we can consider that these values would only occur by chance at a rate of 1 in 10000. The method has been applied at this level of confidence to remain somewhat conservative to avoid false positives, but at this level we can be fairly confident that there are differences between the spontaneous histograms and the stimulation histograms that may truly reflect the effect of the stimulus on the cell.

To extend this approach to an n-dimensional case the following inequality is tested:

\[(x - \mu)^T \Sigma^{-1} (x - \mu) \leq \chi^2_k(p)\]

where \(x\) is the values of the stimulation histogram bins, and the \(\mu\) parameter vector represents the mean values of the spontaneous histogram bins (Johnson and Wichern 2007). \(\Sigma^{-1}\) denotes the inverse of the covariance matrix of the spontaneous bin values, and \(\chi^2_k(p)\) denotes the quantile function for probability \(p\) of the chi-squared distribution with \(k\) degrees of freedom, where \(k\) is the number of bins over which the comparison is being made. The formula is only true for an \(x\) vector that falls within a given confidence interval. In this case \(p\) was chosen to be 0.0001 such that if \(x\) did not satisfy the above equation the values of the stimulation histogram fell outside the 99.99% confidence interval.
Figure 2-3: Comparison of first and second bin values of stimulation PSTH against same values from the spontaneous PSTHs of the same cell
A) PSTH of putative Golgi cell response to middle cerebellar peduncle stimulation. Red highlighted bins are the first and second of the PSTH.
B) Comparison of these values (red dot) to 500 spontaneous PSTH values (black dots). Coloured inside rings are confidence intervals of 20, 40, 60 and 80%, and the dotted red ellipse represents the 99.99% confidence interval. In this case the results of stimulation cause the cell to fire in a pattern that is far from the distribution that would be expected under spontaneous conditions. Marginal histograms represent the distributions of the first and second bin values following pseudo stimulus onset in spontaneous PSTHs. For visualisation purposes this has only been shown on 2 values but the process can be applied to any number of bins post stimulus onset.
Previously to determine responses to stimulation, it has been common to use a t-test comparing values in a baseline period of a PSTH to a window following stimulation. This has been applied in the determination of effect of somatosensory stimulation on putative Golgi cells in our lab, see Holtzman et al. 2006, but this effect is a sustained and substantial depression. The multivariate Gaussian analysis has been applied over a t-test here because it provides several advantages. Firstly, there is no need to adjust window sizes, the method can be applied indiscriminately to different stimulation protocol that might produce differently lasting changes in firing. Secondly, it is sensitive to dramatic changes in small number of bins but similarly is able to detect small but prolonged changes that would be unexpected considering the cell’s normal range of firing. Fig. 2.4 below shows an example of a rat lateral reticular neuron showing a response to peripheral somatosensory stimulation, data obtained in the Edgley Lab. In this case, the stimulus causes a marked effect on the cell by generating a phase resetting response. If we perform a simple t-test, taking the sample of bin values in the baseline and comparing it to the sample of bin values post stimulus, we find a non-significant effect (p > 0.9). However, if we apply the multivariate Gaussian analysis with the threshold as specified above we find that this pattern of firing falls outside the range which is covered by resampling the spontaneous firing with the same PSTH parameters.

Figure 2-4: Comparison of multivariate Gaussian analysis to t-test
The above PSTH shows the results of peripheral somatosensory afferent stimulation on a lateral reticular nuclei neuron (data from the Edgley lab) where it exhibits a phase resetting response. Typically, t-tests comparing baseline and post stimulus values have been used to determine if statistically significant effects occur due to stimulation. However, for this cell a t-test is highly non-significant as the effect causes a change in variance but the mean activity over the post stimulus period, $\bar{X}_2$, remains the similar to the baseline mean, $\bar{X}_1$. The multivariate Gaussian analysis finds that this firing falls outside the 99.99% confidence interval when compared to the spontaneous bin value distributions over the whole post stimulus window.
2.2 Determination of onset and offset latencies

To determine onset of responses in a number of cells a change point detection algorithm was applied to the sequence of bin values but only if an initial multivariate Gaussian analysis determined that responses differed from spontaneous firing. The change point algorithm is described in Ross 2015, but is applied as follows.

If we assume that there exists a change point in a sequence of values, then there exists a moment at which values change from being from sampled from one distribution, $F_0$, to coming from another distribution of values, $F_1$. To determine at which point this change occurs we apply a two sample statistical test at each possible change point, choosing a test that is appropriate for the assumptions about the underlying distribution in values. In this dissertation the Mann-Whitney U-test has been applied to avoid making distribution assumptions. The change point that is chosen is the point at which the test statistic (D) is maximised, and only if the test statistic crosses an appropriate threshold, otherwise no change is found. The threshold is chosen by the CPM package to match a type I error rate ($\alpha$) of 0.05.

![Figure 2-5: Change point detection](image)

Left) Sequence of PSTH bin values derived from putative Golgi cell response to peripheral somatosensory afferent stimulation. The detected change point is identified by the vertical dotted line.

Right) Test statistic derived from Mann-Whitney U test at each point of splitting the sequence in to two samples. The identified change point is that which maximises the test statistic and is identified by the vertical dotted line. The horizontal dotted line represents the threshold that must be crossed for a change point to be identified. The CPM package applies correction to account for multiple testing and computes this on the basis of maintaining a type I error rate of 0.05.
Fig. 2.5 (left), previous page, demonstrates the method on a sequence of PSTH bin values generated by a putative Golgi cell in response to peripheral somatosensory afferent stimulation. The change point that is selected follows stimulation at around 30ms, and is denoted by the vertical jagged line. Fig. 2.5 (right) shows the test statistic generated by the test at each possible split point, the horizontal jagged line shows the threshold that is necessary to cross to determine that a change point has occurred. The vertical jagged line shows the time chosen as the change point where the test statistic is maximised.

To extend the method to find multiple change points the values are considered sequentially, checking for a change point in a sequence of fixed length at each point and then adding an additional value to the sequence if no change point is found. If a change point is detected the sequence then grows out from this new starting point. Where this has been applied in Chapter 4, the first change point is taken as the onset time, but offset time is selected by hand from the remaining change points detected, where there were more than two change points identified in total.

2.3 Generalised linear model – binomial logistic regression

In the current literature, it is common to analyse conditioning data using ANOVAs on the percentage. This is problematic as the underlying data with which the percentages are created are discrete categorical data (1=success, 0=failure on a given trial), and the values are constrained to the range 0 – 100 (Dixon 2008). In learning experiments it is common to have percentages fall very close to these bounds which can affect the assumption of normality of the residuals and homogeneity of variances. Logistic regression models provide a more appropriate way for analysing trials with a binomial outcome (Jaeger 2008).

Logistic regression is a generalised extension of linear regression models and is used when the dependent variable is a binomial categorical variable taking the value of either 0 or 1. The standard linear regression model with a single independent variable (X) takes the form of:

\[ y = \beta_0 + \beta_1 \cdot X \]

which denotes the standard equation for a straight line; this states that the predicted value of \( y \) at a given value of \( X \) is equivalent to the intercept (\( \beta_0 \)) plus the coefficient (\( \beta_1 \)) times \( X \). The model is fit in such a way that it minimises the residual sum of squares (RSS), otherwise known as the distance of observed points from their estimated point on the line. The logistic regression model takes the same form except that the \( y \) is the estimate of the log odds of success given \( X \) (success defined as the dependent variable = 1).
The logistic regression model begins with probability and performs a logit transformation otherwise known as the log transformation of the odds of success. If the probability of success is 0.6, then the probability of failure is 0.4. In this case, the odds of success are 1.5, as odds are equivalent to the probability of success / 1 – probability of success. The log transformation of these odds, known as the logit, allows us to take a dependent variable with a limited range of 0-1 in the probability space to a range of negative infinity to positive infinity. In doing this the logistic regression allows us to model the logit transformed probability of success as a linear relationship with independent variables and to access their significance, i.e. are the coefficients of change in log odds associated with the independent variables non-zero. It is common to report the coefficients as the odds ratio, which for categorical independent variables represents the increase in odds over those expressed by the intercept term. For continuous independent variables the odds ratio denotes the increase in odds for a one unit increase in that independent variable. For further clarification sees Bland and Altman 2000 and Sperandei 2014.

Where it was necessary the logistic regression is applied as a mixed model, with random effects of subject accounted for. Random effects account for the repeated measurements as each subject repeats the trials a number of times within a session and therefore an individual subjects trials are not independent of each other. When random effects of the subject have been applied a random intercept model for subject has been fit which allows for an individual intercept for each subject while still estimating the global fixed effects of the independent variables of which we are interested in (Jaeger 2008).
3 Pontine Neurons: Extracellular Electrophysiology and Responses to Peripheral Somatosensory Stimulation

3.1 Introduction
The pons is a vital relay structure for signals passing to the cerebellum from the cerebral cortex. As detailed in Chapter 1, the pons receives input primarily from the neocortex, including sensorimotor areas in rats (Schwarz and Möck 2001; Leergaard et al. 2006; Leergaard and Bjaalie 2007). The properties and responses of pontine neurons are poorly studied - mainly because of their location deep in the brainstem, close to the ventral surface of the skull.

In hypotheses about eyeblink conditioning, it is assumed that the pontocerebellar pathway carries the CS signal and several groups have used stimulation of the pontine nuclei (Rosen et al. 1989; Freeman and Rabinak 2004) or the middle cerebellar peduncle (Svensson et al. 1997) as the conditional stimulus. Damage to the middle cerebellar peduncle abolishes learning for auditory delay conditioning (Solomon et al. 1986).

Given the location of the pontine nuclei in the brainstem, it presents a challenge for in vivo electrophysiological studies, requiring either a progression deep from a dorsal entry or progressing ventrally after exposure of the base of the skull through the oesophagus and larynx. The results presented in this chapter represent early work to examine pontine neurons through extracellular in vivo recordings. The aim of this was to characterise their spontaneous activity and to examine their responses to bilateral peripheral somatosensory stimulation of the fore and hindlimb.

Given that corticopontine projections are excitatory we would expect to see excitatory responses if the somatosensory stimulation is relayed via the cortex, as excitatory responses have been seen to cerebral peduncle stimulation (Möck et al. 2006). However, some in vivo electrophysiological studies have characterised the interplay between cerebellar nuclei and pontine neurons and shown the existence of excitatory and inhibitory responses to stimulation of the cerebellar nuclei (Berretta et al. 1991). Several labelling studies have revealed the presence of GABA-ergic inhibitory terminals and inhibitory interneurons within the pons (Border and Mihailoff 1990; Mihailoff et al. 1992; Möck et al. 1999).

The experiment presented some complications and as such only partial results were obtained. Predominantly slow and erratic firing of the cells made collecting enough data to resolve PSTHs a
time-consuming task. These results nevertheless provide evidence that pontine neurons respond to bilateral stimulation sites and some cells show a range of inhibitory and excitatory responses.

3.2 Materials and Methods

3.2.1 Animals and anaesthesia

All experimental procedures were approved by the Local Ethical Committee of the University of Cambridge and by UK Home Office regulations. Experiments were carried out on male adult Wistar rats (250–350g).

Surgery was performed under general urethane anaesthesia (1-1.2 g kg⁻¹) via intraperitoneal injection. This was supplemented with single intraperitoneal doses of fentanyl-fluanisone (Hypnorm, Vetpharma, UK, 0.015 ml kg⁻¹) or ketamine (single dose, 0.3ml of 10mg/ml solution) whenever required to abolish limb withdrawal reflexes.

3.2.2 Stimulating and recording electrodes

Varnish-insulated stainless steel stimulating electrodes (impedance 0.1-0.2 MΩ) were implanted into the cerebellar cortex to allow antidromic activation of cerebellar projecting pons neurons. 2 or 3 electrodes, spaced by ~1.5 mm mediolaterally were inserted between 2 and 5 mm lateral to the midline to a depth of 1.5 - 2mm. Current pulses of up to 100µA, with durations of 100-200µs, were used. Between one and three pulses were delivered with a 3.3ms interpulse period at a frequency of 0.32Hz. Pontine neurons give rise to the transverse fibres of the pons which pass into the cerebellum through the middle cerebellar peduncle of the opposite hemisphere, so the stimulation electrodes were placed into the white matter of the cerebellar cortex in the hemisphere opposite to the recording electrodes to antidromically activate pontocerebellar fibres, Fig. 3-1, overleaf, shows the relation of recording electrodes to stimulating electrodes in the experimental setup.

Single unit recordings were made using an Eckhorn 7 multi-electrode array setup, each of which is independently moveable (Eckhorn & Thomas, 1993). Recording microelectrodes were platinum-tungsten coated with quartz glass (2-5MΩ). In some experiments glass microelectrodes filled with 2mM saline were used for recordings. Each electrode was advanced to ~3mm, but most recordings were taken <2mm deep. The microelectrode signals were amplified (gain, ×10,000), filtered (bandpass, 0.3–10 kHz) and digitised at 25 kHz using a Micro 1401 interface and Spike 2 software from Cambridge Electronic Design. Pontine neurons were recorded after a ventral surgery exposed the pons recording on the opposite side of the basilar artery to that which had the stimulating electrodes in the cerebellar cortex.
3.2.3 Peripheral somatosensory afferent stimulation

Peripheral somatosensory afferents were activated using percutaneous pins in the footpads of the hindlimb and forelimb bilaterally. Stimulus intensity ranged from 0.5-2 times the threshold required for local reflex activation and was given as 1 or 2 biphasic pulses lasting 200µs. Fig. 3-2, below, shows the expected path of activation through to the pontine neuron.

Figure 3-2: Schematic diagram showing the expected pathway from peripheral somatosensory stimulation to the pons. Two percutaneous pins were placed in the pads of each limb, separated by a small 2-3mm distance from each other. Activation of the peripheral somatosensory afferents expected to transfer the signal through the spinal cord to the cuneate nucleus, the axons of which decussate and project to the contralateral thalamus (Th), neurons of which in turn project to somatosensory cortical areas, (such as S1). Layer 5 corticopontine projections would be expected to transfer the signal to the pons contralateral to the stimulated limb.

Figure created by author
3.2.4 Surgery

To access the basilar pontine nuclei (BPN) a ventral approach was taken that involved intubation of the trachea with the removal of the larynx, and retraction of the neck muscles laterally to allow access to the base of the skull. A craniotomy was performed through the basal occipital bone to expose brain stem. Two landmarks allowed us to target the pons, the basilar artery in the midline and the caudal margin of the posterior pituitary rostrally. Recordings were made in the pontine nuclei to the left of the basilar artery immediately behind the posterior pituitary. The recording electrodes were tilted 30° to the posterior to maximise the length of the BPN that could be recorded from, and the electrodes were advanced to a maximum depth of 3mm.

3.2.5 Identification of neurons and recording apparatus

Antidromic activation allowed the identification of pontine neurons that were projecting their axons into the cerebellum by activating their fibres and causing an action potential to travel down the axon towards the cell body. Antidromic activation is indicated by the reliable appearance of an action potential at a fixed latency after a stimulus; the absence of a synaptic relay gives rise to the fixed latency. The antidromic collision is used to verify antidromic activation: this involves the triggering of the stimulus briefly after a spontaneous action potential. If the delay is short enough, the spike travelling orthodromically from the cell soma towards the terminal meets the spike travelling from the terminal towards the soma, and the two collide leaving an absence of an observable action potential following the stimulus pulse. If a delay of about 0.5ms or greater is applied, the antidromic action potential can be seen following the stimulus pulse. Fig. 3-3 overleaf shows the logic behind the antidromic identification of neurons.

Pontine cells are densely packed, and the cerebellar stimuli evoked large local antidromic field potentials. As such, it was not always possible to see individual spikes in the short intervals following the stimulus. Only cells which were antidromically identified or showed large field potentials on the recording trace following cerebellar stimulation were included in analyses. Large field potentials suggested stimulation acted very strongly upon neighbouring cells.
3.2.6 Analysis

Determination of significant responses to stimulation was carried out with the statistical methods described in Chapter 2. Statistical analysis was carried out using the R programming language for statistical computing.

*Figure 3-3: Antidromic collisions used to identify the cells as having pontocerebellar afferents*

(A) Spontaneous action potential initiated at the soma travels orthodromically down the axon.

(B) Electrical stimulation along the axon or at the terminal generates an action potential that travels antidromically towards the cell body and is seen following the stimulus pulse in recordings that soma.

(C) Triggering an axon/terminal stimulus immediately after a somatic action potential generates a situation where orthodromic and antidromic action potentials collide. This is seen by a disappearance of the antidromic action potential. Figure author’s own work.
3.3 Results

3.3.1 Spontaneous activity of basilar pontine neurons

The spontaneous firing of 55 cells from the pons was sampled. Of the 55 cells, 32 were shown directly to be antidromically activated, and antidromic collision could be demonstrated - with some caveats, see section 3.3.2. The remaining 23 cells were recorded in the region of large local antidromic field potentials. The firing pattern of the cells under spontaneous conditions was slow often with silent periods punctuated by high-frequency bursts. Fig. 3-4 below shows an example of spontaneous firing activity of an antidromically identified pontine neuron (Fig. 3-4A and B). The cell had a mean firing rate of 2.19Hz, and its firing pattern was characterised with bursts of firing followed by periods absent of spontaneous action potentials (Fig. 3-4C).

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Figure 3-4: Example recordings from an antidromically identified pontine neuron
(A) Spontaneous action potential (*) which triggered the antidromic stimulus (greyed area) with a delay which results in an antidromic action potential (blue circle) shortly after. Time scale bar = 1ms, amplitude scale bar = 0.1mV
(B) Spontaneous action potential (*) which triggered the antidromic stimulus at very short latency (greyed area on the left) and subsequent absence of antidromic action potential following stimulus pulse due to collision. An antidromic action potential (blue dot) was seen to the second stimulus delivered 3ms later. Time scale bar = 1ms, amplitude scale bar =0.1mV
(C) Example of the recording trace showing periods of burst firing permeated with long absences of spontaneous action potentials. Blue traces inset in the centre shows the spikes collected by spike templating and which were used to isolate the cell in the recording channel
The distribution of interspike intervals for this cell is shown in Fig. 3-5 below. The distribution reflects the low firing rate and burst pattern with many short intervals, 20-80ms, permeated with much longer intervals when there is a cessation in firing. The maximum interval for this cell was 16.99 seconds (not shown in Fig. 3-5 to allow clarity at the lower end of the interval scale.

![Interspike interval histogram of spontaneous intervals for the cell shown in Fig. 3-4](image)

*Figure 3-5: Interspike interval histogram of spontaneous intervals for the cell shown in Fig. 3-4
Burst patterns in firing resulted in many short intervals. This cell has a peak in the distribution at 80-90ms. Extremely long intervals were seen (off the x-axis) with the longest period of silence being 17 seconds. Bin width = 10ms*

The range of firing rates is shown in the histogram in Fig. 3-6A, overleaf, with a median firing rate of 2.71Hz and a range of 0.12 to 25.26Hz. The majority of the cells (73%) had a spontaneous activity of less than 5Hz. The distributions of the minimum interspike interval for all cells are shown in Fig. 3-6B and the distribution for the maximum interspike intervals are shown in Fig. 3-6C. The minimum intervals were in the range 1.20 – 74.52ms and the maximum intervals were in the range from 728.12ms up to 38.71 seconds, reflecting the short intervals in burst firing permeated by long periods with no firing.
Figure 3-6: Characteristics of the spontaneous activity of the population of pontine cells

(A) The range of spontaneous firing frequencies for all pontine neurons recorded. Bin width = 1Hz

(B) The counts of the minimum spontaneous interspike intervals from the population of cells. The most frequently observed minimum interval was in the range 10-20ms. Bin width = 0.01s

(C) The range of maximum spontaneous interspike intervals. This range was more dispersed than the minimum interval and reflects the long periods that were absent of any spontaneous activity. Bin width = 0.5s
3.3.2 Antidromic latencies and failure of antidromic stimulus

Antidromic collision identified 32 cells, but this was not straightforward. While cerebellar stimuli evoked spikes with fixed latency, and we were able to demonstrate what appeared to be antidromic activation, sometimes even at suprathreshold intensities the cells failed to fire to the antidromic stimulus.

Shown below in Fig. 3-7 is an example of a cell that failed to the antidromic stimulus on occasion despite displaying behaviour that would suggest it to be antidromically activated. Fig. 3-7A shows the cell firing in response to the antidromic stimulus pulse with a very short latency when there is a sufficient delay from the spontaneous spike, in contrast, Fig. 3-7B shows that a pulse following

![Figure 3-7: Failure of the antidromic stimulus to generate action potentials in cell that has shown what appears to be antidromic collision](image)

Spontaneous actions potential (blue dot), stimulus related action potential (red star), stimulus (blue area)

(A) When the antidromic stimulus is triggered by the spontaneous action potential but with a delay an action potential is seen following the stimulus. Time scale bar = 1ms, amplitude scale bar = 0.2mV

(B) When there is no delay from the spontaneous spike to the stimulus no spike is seen following the stimulus suggesting collision. Time scale bar = 1ms, amplitude scale bar = 0.2mV

(C) Train of 3 antidromic stimuli, stimulus-related action potentials to the first two pulses, but an absence reveal field potentials after the third pulse (this is actually noticeable behind the second stimulus related action potential on the previous pulse). This represents a failure after the third pulse despite the stimulus being sufficient to drive the cell at a fixed short latency before. Time scale bar = 1ms, amplitude scale bar = 0.2mV. Stimulation rate 333Hz, 3 pulses in the train.
almost immediately after the spontaneous action potential results in an absence of the stimulus related spike. Fig. 3-7C shows a single stimulus trial when the stimulus was given repetitively, to the first two pulses a stimulus-related spike is seen, denoted by the red star, however after the third stimulus pulse in the train there is an absence of the spike – although a small field potential, or similar, is now revealed. This could potentially be a more distance cell or cells that are obscured when the other cell fires. Alternatively it could represent EPSPs, similar to those observed in giant auditory synapses (Typlt et al. 2010), with the response observed in Fig. 3-7C, after the second stimulus, having a very similar profile to those seen in auditory synapses.

It was common to give a small number of antidromic stimulus trials (2 – 3) with no delay from a spontaneous spike, and then a small number of stimulus trials with the delay (2 -3) to quickly confirm that the cell was antidromic before moving on to stimulation of the periphery. However, this was short-sighted, as mentioned above, the stimulus sometimes failed to produce action potentials seemingly for no reason. This called into question whether the cells were being antidromically activated or were driven through other means.

Of the population of these putative antidromically activated cells, 18 were recorded under three different stimulus regimes for a sufficient number of trials to perform statistical analyses. These three regimes were: i) with the antidromic stimulus free running at 3 Hz, ii) with the antidromic stimulus triggered immediately from a spontaneous spike, iii) with the antidromic stimulus triggered off spontaneous spikes with a delay added beyond what we judged to be the collision interval (0.5-3 ms).

The first line of evidence that these were antidromic activations were the latencies at which the cells were activated in response to the free-running stimulus. The latencies for the 18 cells are shown in Fig. 3-8 overleaf. These range from 0.3ms to 4.4ms with an average latency of 1.3ms (SD: 0.7ms). The bin width of these histograms is 0.1ms, and for most cells, the spikes fall into 2 – 4 prominent bins. The response was thus tightly timed to a 0.2-0.4ms window; the small jitter was most likely due to the small size of the spikes on inevitable noise.
Figure 3-8: Latency to activation following antidromic stimulus

Histograms showing the counts of activation latencies for 18 cells recorded in the pons in response to cerebellar white matter antidromic stimulus. Most cells have two or four prominent bins, with a bin width of 0.1 ms, suggesting only a little scatter of the responses in a 0.2-0.4 ms window. Such reproducible short latency activation would suggest direct activation of the axon rather than activation transynaptically.
We sought to determine if antidromic failure happened at a different rate to what we believed was antidromic collision. The number of responses to the first stimulus pulse when there was a triggered delay from a spontaneous spike (regime iii, above) were compared to the number after the first pulse when there was no delay (ii, above) for each cell. This was done in a 2x2 contingency table (No delay -> response?: yes/no count; Delay -> response?: yes/no count). The counts of responses for each condition (representing just the yes row counts) are shown in Fig. 3-9 below. It shows that when there is no delay from the spontaneous spike to an antidromic stimulus, all 18 cells showed zero responses to the first pulse of the train. When a delay is added, each cell increased their number of responses to the first pulse of the stimulus train. A $\chi^2$ test was applied to each cell individually, and all differences in the proportion of responses were found to be significant ($p < 0.05$). The fact that cells made no responses at all when there was no delay is highly suggestive that action potentials were indeed being antidromically collided. The fact that applying a short delay

Figure 3-9: Adding a delay from spontaneous spike to stimulus activation increases the number of responses seen to first stimulus pulse in the train. Counts shown are to the first pulse in the stimulus train for 18 cells, under the conditions of stimulation triggered with no delay from a spontaneous and triggered with a delay. This delay varied for cells but was started at 0.3ms and increased up to 3ms. Normally <1ms was sufficient to show appearance of spike following the stimulus pulse. Colour represents individual cell.
from a spontaneous spike to stimulus activation, in some cases just 0.3ms, significantly increased the numbers of responses is also suggestive of antidromic activation.

The use of antidromic activation was therefore problematic. We would expect antidromic stimulation to activate the cells after every pulse at suprathreshold intensities, provided there was enough of a delay from a spontaneously generated spike. Fig. 3-10 below shows the proportion of responses to each pulse in the stimulus train (consisting of 3 pulses) for the 18 cells when the antidromic stimulus train was given repetitively at 3Hz. All cells had probabilities of responding that

Figure 3-10: Probabilities of firing to a given antidromic stimulus pulse (in a train of three) were less than 1 for each pulse for each cell. The probability of response to the pulses of the stimulus train were deduced from the free running regime, where a train of 3 pulses were given at 3 Hz. There was no consistent pattern among the cells but they frequently did not respond, some showing increases from pulse to pulse, others showing decreases in the proportion of responses.

MCP, middle cerebellar peduncle
were less than 1 to each individual pulse of the train, but there does not appear to be a consistent pattern among the cells about which pulse to respond to and at what proportion.

These results show that antidromic invasion failed frequently, but without a pattern, complicating the identification of neurons. The absence of any action potentials when the cerebellar stimulus was triggered with no delay from a spontaneous spike suggests that the collision was genuine. The latencies of spikes following stimulus pulses are also suggestive that they were antidromically activated. The failure of the antidromic stimulus in a large number of cases suggests that there was some process which caused the backpropagated action potential to fail to invade the soma of the cell.

3.3.3 Responses to somatosensory peripheral afferent stimulation are often bilateral and sometimes inhibitory

The responses of 25 pontine neurons to peripheral somatosensory neurons were tested. 14 were antidromically activated and collided. The remaining 11 showed large field potentials and were recorded on a track that had shown antidromically activated cells, and was within a 3mm penetration into the pons.

The responses to stimulation were varied; Table 3-1 below shows the number of cells showing significant responses to various limbs. These were detected using the Gaussian multivariate analysis on the bin counts of the PSTH as described in Chapter 2.

<table>
<thead>
<tr>
<th>RESPONSES</th>
<th>COUNTS OF CELLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contralateral forelimb</td>
<td>3</td>
</tr>
<tr>
<td>Contra and ipsilateral forelimb</td>
<td>9</td>
</tr>
<tr>
<td>Contra and ipsilateral hindlimb</td>
<td>1</td>
</tr>
<tr>
<td>Contra and ipsilateral forelimb + Ipsilateral hindlimb</td>
<td>3</td>
</tr>
<tr>
<td>All limbs</td>
<td>2</td>
</tr>
<tr>
<td>No response</td>
<td>7</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>25</strong></td>
</tr>
</tbody>
</table>

*Table 3-1: Counts of significant response combinations in cells to peripheral somatosensory stimulation*

15 cells showed bilateral responses to at least one limb group. Contralateral and ipsilateral forelimb stimulation most commonly evoked a response in the cells tested (n=9). Of the cells that showed responses, 8 showed an inhibitory response to stimulation without excitation, or inhibition that preceded excitation. Table 3-2 (top, overleaf) shows a breakdown of the number of stimulus conditions that showed significant inhibitory responses within cells.
Fig. 3-11 shows an example of a cell that showed responses to all stimulus types. In each case, the cell exhibited short latency inhibitory responses to somatosensory stimulation, but the contralateral responses were more pronounced than that of the ipsilateral limb responses.

Table 3-2: Counts of significant inhibitory response combinations in cells to peripheral somatosensory stimulation

<table>
<thead>
<tr>
<th>RESPONSES</th>
<th>COUNTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contra and ipsilateral forelimb</td>
<td>1</td>
</tr>
<tr>
<td>Contra and ipsilateral hindlimb</td>
<td>1</td>
</tr>
<tr>
<td>Contra and ipsilateral forelimb + Ipsilateral hindlimb</td>
<td>1</td>
</tr>
<tr>
<td>Ipsilateral forelimb</td>
<td>3</td>
</tr>
<tr>
<td>Ipsilateral hindlimb</td>
<td>1</td>
</tr>
<tr>
<td>All limbs</td>
<td>1</td>
</tr>
<tr>
<td>TOTAL</td>
<td>8</td>
</tr>
</tbody>
</table>

Figure 3-11: Inhibitory responses bilaterally to both hindlimb and forelimb stimulation in a single cell
Peristimulus time histogram responses. Time, in seconds, is relative to stimulus onset and counts are converted to a frequency of firing in Hertz (the count of action potentials in a bin divided by bin width x trials). Black arrows denote the limb and laterality. The cell shows qualitatively stronger inhibitory response to contralateral limbs Bin width = 0.005s
For both forelimb responses (Fig. 3-11, previous page) there appears to be a later slow increase in firing starting 0.2s after the stimulus pulse. The hindlimb responses on both sides show an inhibitory decrease in firing within the first 0.05s, but there wasn’t the increase in firing seen later in contrast to forelimb stimulation. Completely inhibitory responses were unexpected in pontine neurons.

Fig. 3-12 below shows the peristimulus time histograms for a cell that showed responses to contra and ipsilateral forelimb plus ipsilateral hindlimb but not contralateral hindlimb. The cell shows short latency excitation to ipsilateral forelimb stimulation, but contralateral forelimb and ipsilateral hindlimb show short latency inhibition in contrast. The inhibition lasts for 0.01s with two consecutive low firing rate bins.

Figure 3-12: Contra – and ipsilateral forelimb responses with ipsilateral hindlimb responses in the same cell Peristimulus time histogram responses. Time, in seconds, is relative to stimulus onset and counts are converted to a frequency of firing in Hertz (the count of action potentials in a bin divided by bin width x trials). Black arrows denote the limb and laterality. The cell shows a mix of responses to the different stimuli, ipsilateral forelimb is excitatory, whilst contralateral forelimb and ipsilateral forelimbs show inhibitory responses mainly. Bin width = 0.005s
Fig. 3-13 show the peristimulus histograms for a cell that responded to contra and ipsilateral forelimb stimulation. This was the most common stimulus combination for the cells to respond to, as shown in Table 3-1. This cell shows strong short latency excitatory responses to the stimulus with the contralateral stimulus generating a larger modulation than ipsilateral, an increase to an instantaneous firing rate of 25Hz versus a 10Hz firing rate respectively. The excitation is followed by a period of depression in which the cell fires very few spontaneous action potentials; this lasted for 180ms following the excitation.

**Figure 3-13:** Strong excitatory responses to the ipsilateral and contralateral forelimb in the same cell
Peristimulus time histogram responses. Time, in seconds, is relative to stimulus onset and counts are converted to a frequency of firing in Hertz (the count of action potentials in a bin divided by bin width x trials). Black arrows denote the limb and laterality. This cell shows strong excitation (qualitatively strongest to contralateral forelimb) with a long silent period following activation that lasts in both cases for 0.2 seconds. Bin width = 0.005s.
Fig. 3-14 shows a cell that only showed a response to both contralateral and ipsilateral hindlimb. In contrast to the forelimb responses, the hindlimb responses are a weaker inhibitory response. The responses start 50ms post-stimulus and last roughly 50ms. Again solely inhibitory responses and bilateral receptive fields were unexpected.

Figure 3-14: Inhibitory responses to the contralateral and ipsilateral hindlimb stimulation in the same cell
Peristimulus time histogram responses. Time, in seconds, is relative to stimulus onset and counts are converted to a frequency of firing in Hertz (the count of action potentials in a bin divided by bin width x trials). Black arrows denote the limb and laterality. This cell shows inhibitory responses begin at roughly 0.05s and last for a similar amount of time. Bin width = 0.005s
Fig. 3-15 show the responses of a cell that was only modulated by contralateral forelimb but no other stimulus conditions. This exhibited a short latency excitation response to the contralateral forelimb stimulus similar to the example shown in Fig. 3-13 but shows less of a following inhibitory response. This response was the one that would have been most often expected to occur, an excitatory response to a single limb on one side, but in reality, this only occurred in 3 of the cells sampled.

![Peristimulus time histogram responses](image)

*Figure 3-15 Excitatory contralateral forelimb responses*

Peristimulus time histogram responses. Time, in seconds, is relative to stimulus onset and counts are converted to a frequency of firing in Hertz (the count of action potentials in a bin divided by bin width x trials). Black arrows denote the limb and laterality. This cell displayed only responses to contralateral forelimb stimulation, exhibiting strong excitatory responses. Bin width = 0.005s.
3.4 Discussion

The results presented here represent an early attempt at the characterisation of pontine neurons and present some interesting findings. However, the work was not continued as the experiment presented several problems such as the low firing rate of the cells, leading to difficult resolution of responses to stimulation and often required a cell to be held over a long periods, which was not consistently possible. Similarly, their resistance to antidromic stimulation made it hard to be certain about identification of the cells as pontine neurons. The limited results presented are thus discussed and what could be done to overcome some of the limitations of the study. The slow but bursty activity of the cells appeared to be common across the pontine neurons. Whether this activity relates to anaesthesia would need to be tested by comparing firing patterns while under a different anaesthetic regime, or preferably without anaesthesia. The low level of firing posed a problem for collecting data in response to peripheral stimulation. To obtain enough data for resolution of PSTHs with reasonably sized bin widths took a considerable amount of time (often >45 minutes per cell), especially when an inhibitory process only weakly modulated the cell. Many bin counts in the PSTH would take a long while to collect any action potentials due to the burst of firing followed by long periods of silence. Holding stable recordings over such periods was also problematic. One question that could be addressed in the future is what influence if any, that inhibition has upon the spontaneous activity of these cells. This could be addressed by infusion of GABA antagonists into the nuclei while spontaneous activity is observed.

3.4.1 Antidromic failure

There are three key features of antidromic activation in neurons: the neuron should fire consistently at suprathreshold stimulation, they should respond at a fixed latency to stimulation, and they should show collision when the stimulus was given with no delay following a spontaneous spike. The ‘antidromic’ neurons identified in this study showed 2 of these criteria consistently. A spike was not observable after the first stimulus pulse following a spontaneous spike if there was no delay but the numbers increased for each cell where a short delay was introduced, consistent with a collision. Similarly, the latencies were tightly timed most only showing a range of 0.2-0.4ms. However many cells where the same cerebellar stimulus was repeatedly given during spontaneous firing did not fire consistently with the stimulus even though the stimulus intensity was suprathreshold. For this reason, we cannot be entirely confident that the cells were antidromic.

The simplest explanation of this phenomenon would be that antidromic spikes in the axon sometimes fail to invade the soma. This might occur in the presence of powerful inhibition, where shunting may prevent currents generated in the axon from invading the cell soma. The pons is
known to contain dense GABAergic terminals, making this a possibility. Whether this phenomenon is related to pontine inhibition is unclear, and examination of any changes in the level of responsiveness to antidromic stimulation while applying a GABA antagonist could be developed as future work.

3.4.2 Bilateral and rostrocaudally receptive pontine neurons

The frequent bilateral responses that were observed were perhaps not that surprising given the evidence that some corticopontine projections cross the midline at the level of the pontine nuclei. However, these bilateral responses were observed perhaps more frequently than suggested by the sparse numbers of crossing fibres (Mihailoff et al. 1978). Responses in individual neurons across all limbs are a bit more surprising, as the pons is suggested to have retain but restructure somatotopic maps in the projection from cortex to the nuclei (Schwarz and Möck 2001). This data suggests that some cells have a wide representation of peripheral somatosensation from all over the body and that these cells are responsible for relaying these signals to the cerebellum as mossy fibre inputs.

Bilaterally responsive neurons have been observed in cortical neurons of rats before in response to vibrissal and limb stimulation, with one study showing that 5% of neurons examined responded to stimulation of all four paws (Zarei and Stephenson 1996; Shuler et al. 2001; Moxon et al. 2008). However individual neurons in our experiment showed disparate responses to contralateral limbs and to separate limbs suggesting that a separate population of upstream neurons were affecting the pontine cell. To further deduce the pathways responsible for these responses it would be useful to record pontine neurons while stimulating the cerebral peduncle, which carries corticopontine fibres, on both sides to determine if the response can be generated from either hemisphere of the cortex.

Alternatively, it is possible that such modulation may come from direct spino-pontine pathways. Even if stimulation of the cerebral peduncle reproduced effects that is seen with peripheral somatosensory afferent stimulation, this does not preclude effects by a direct spino-pontine pathway. To determine if any effects could be mediated by these pathways the cells could be recorded in decerebrate animals whilst the stimulation protocol is carried out, if responses where observed it would suggest that modulation is possible by a non-cortical route.

3.4.3 Excitatory and inhibitory responses

Through stimulation of peripheral somatosensory afferents we would expect excitatory responses in pontine neurons as the corticopontine projection is expected to be excitatory. However, in a small number of cells, inhibitory responses without excitation or preceding excitation were observed. Observations of inhibitory responses have been made from stimulation of cerebellar nuclei that project to the back to the pons but not observed from cerebral peduncle stimulation (Berretta et al. 2008).
1991; Möck et al. 2006). The origin of the inhibitory responses we have shown is unknown, but some areas have GABAergic projections to the pontine nuclei including the zona incerta, anterior pretectal nucleus, lateral cerebellar nucleus and perirubral area (Border et al. 1986). An exploration of the responses observed after stimulation of these areas would be a worthwhile pursuit, as would an infusion of bicuculline or other GABA antagonists to determine their effects on the observed inhibitory responses. The observation of inhibitory responses suggests that there may be more integration and processing of signals at the level of the pons than its role as a relay suggests.
4 Response of putative Golgi cells to middle cerebellar peduncle stimulation and peripheral somatosensory stimulation

4.1 Introduction

The Golgi cell holds an important position in the cerebellar cortex, receiving direct input from mossy fibres, and via parallel fibres it provides the mechanism for both feedback inhibition and feedforward inhibition onto granule cells (Eccles et al. 1964, 1967; Eccles 1967; Kanichay and Silver 2008). Using both synaptic and extrasynaptic mechanisms, Golgi cells can modulate granule cell excitatory input to the cerebellar cortex. This inhibition can be both phasic and tonic inhibition depending on whether the activated receptor is synaptic or extrasynaptic (Brickley et al. 1996; Mapelli et al. 2014). In this manner, it is believed that Golgi cells function as a gain control on the excitability of granule cells. The prevailing view is that Golgi cells provide temporally precise inhibition onto granule cells that narrow the window for the temporal summation of discrete mossy fibre inputs.

However, in vivo recordings of Golgi cells call this into question. In response to peripheral sensory stimulation show that the most frequently observed response is a substantial suppression in firing lasting several hundred milliseconds in comparison to their tonically active spontaneous states (Holtzman et al. 2006). This suppression is shown to be mediated by the extrasynaptic spillover of glutamate derived from granule cells acting on inhibitory mGluR2 (Holtzman et al. 2011). Reduction in the firing of Golgi cells appears to have some physiological relevance as it has also been observed in behaving animals (Edgley and Lidierth 1987; Prsa et al. 2009).

The long-lasting suppression of Golgi cell firing evoked by peripheral afferent stimulation in rats was generated from a very broad receptive field on the body. The lateral reticular nucleus (LRN) is a brainstem nucleus that has convergent afferent inputs from the spinal cord that are multisensory and bilateral (Clendenin et al. 1974; Menétrey et al. 1983; Robbins et al. 2005), and sends mossy fibre projections to a substantial area of the cerebellum (Wu et al. 1999), so it was an obvious candidate to mediate these responses. It was subsequently shown that single pulse stimulation of the LRN could induce the long suppression of firing of Golgi cells. Pharmacological inhibition of the LRN blocks the suppression usually caused by peripheral sensory stimulation, suggesting that the peripheral afferent information that causes the Golgi cells to pause is relayed via the LRN (Xu and Edgley 2010).
In the context of this previous research, the question arises; do mossy fibres from the lateral reticular nucleus have a particular relationship to Golgi cells that generate such a response, or does this response represent a general principle for response to mossy fibre inputs. This project set out to test whether Golgi cells respond in the same manner as they do to the LRN from another major mossy fibre source – the pontocerebellar projection. The pontine nuclei act as the principal relay in the cerebro-cerebellar pathway receiving many afferents from different areas of the cerebral cortex. These include the prefrontal cortex, association areas, motor cortex, somatosensory cortex, and also visual and auditory areas on the ipsilateral side (Baker et al. 1976; Glickstein et al. 1985; Leergaard and Bjaalie 2007). The pathway from the pons to the cerebellum is important for eyeblink conditioning and is necessary for the transfer of the contextual signal to the cerebellum (Thompson and Steinmetz 2009; Halverson and Freeman 2010; Freeman and Steinmetz 2011). The pontocerebellar projection traverses to the contralateral side and enters the cerebellum via the middle cerebellar peduncle (MCP), where it terminates as the usual mossy fibre rosettes in many different areas as shown in studies of cat and non-human primates (Kawamura and Hashikawa 1981; Kelly and Strick 2003). A study of stimulation of the white matter of the contralateral hemisphere evoked short latency activation responses in 18 putative Golgi cells but with no observable long latency depression, consistent with monosynaptic activation. These are proposed to be pontocerebellar axons that project across the midline, but they were not characterised fully (Holtzman et al. 2009). The pontocerebellar fibres were not activated directly but were targeted through the white matter, leaving open the possibility of activation of other mossy fibre sources and other collaterals in the white matter.

This experiment aimed to activate this extensive mossy fibre input directly via the middle cerebellar peduncle (MCP), while recording putative Golgi cell responses in crus I and II of the cerebellum, to examine whether the prolonged suppression in firing is a response unique to the LRN. The responses of putative Golgi cells to stimulation of the pontocerebellar pathway are fully characterised showing a difference in response between this pathway and the LRN. Limited evidence is presented that inhibitory responses are still present in response to MCP stimulation but are much shorter lasting.
4.2 Materials and Methods

4.2.1 Animals and anaesthesia

All experimental procedures were approved by the Local Ethical Committee of the University of Cambridge and by UK Home Office regulations. The data presented here were drawn from experiments carried out on 13 male adult Wistar rats (250-350g).

Surgery was performed under general urethane anaesthesia (1-1.2 g kg\(^{-1}\)) induced via intraperitoneal injection. This was supplemented with single intraperitoneal doses of fentanyl-fluanisone (Hypnorm, Vetpharma, UK, 0.015 ml kg\(^{-1}\)) or ketamine (single dose, 0.3ml of 10mg/ml solution) whenever required to abolish limb withdrawal reflexes.

4.2.2 Stimulating and recording electrodes

Insulated stainless steel stimulating electrodes (0.1-0.2 MΩ) were used for stimulation of the MCP. Single 50-100µA current pulses with durations of 100-200µs were used and given in single or train stimuli. The trains had five pulses with a 3.3ms interpulse period at a frequency of 0.32Hz.

Stimulating electrodes were placed using coordinates from ‘The Rat Brain in Stereotaxic Coordinates - 7th Edition’, Paxinos. To target the MCP a craniotomy was made at 9.3mm caudal to bregma and 3.5mm on the left lateral side relative to the midline; this represented a large area where the MCP fibres are closely bundled together as they enter the cerebellum. The electrodes were initially advanced to a depth of 7-7.5mm. Local field potential recordings were taken from the surface while stimulating and the electrode depth was adjusted in a ±1mm range to find the region that induced the largest field potentials at short latency. The electrode was then fixed in place at this depth.

Single unit recordings were made using an Eckhorn seven electrode array setup, each electrode being independently moveable (Eckhorn & Thomas, 1993). Each electrode was advanced to ~3mm, but most recordings were taken <2mm deep. Recording microelectrodes were platinum-tungsten coated with quartz glass (2-5MΩ), with an outside diameter of 80µm. The microelectrode signals were amplified (gain, ×10,000), filtered (band-pass, 0.3–10 kHz) and digitised at 25 kHz using a Micro 1401 interface and Spike 2 software (Cambridge Electronic Design). Putative Golgi cells were recorded in the left lateral hemisphere of the cerebellum, specifically in Crus I and Crus II of the cerebellum lateral to the paravermal vein.

4.2.3 Peripheral somatosensory afferent stimulation

Peripheral somatosensory afferents were activated using percutaneous pins in the footpads of the hindlimb, forelimb and the vibrissal skin ipsilateral to both stimulation and recording electrodes when recording from putative Golgi cells. The method was as described in the previous Chapter.
Stimulus intensity ranged from 0.5-2 times the threshold required for local reflex activation and was given as one or more biphasic pulses lasting 200µs. On occasion stimulation of the infraorbital nerve was also used as a peripheral stimulus by placing the pins in the mystacial skin underneath the vibrissae.

4.2.4 Lesions and histology

Before termination of the experiment, a 20 s duration current pulse of 80µA intensity was passed through the MCP electrode to create a lesion. Animals were then transcardially perfused with 0.1M phosphate buffer solution (PBS), subsequently followed by 4% paraformaldehyde (PFA) in 0.1M PBS. After fixation brains were embedded in 10% gelatine and postfixed in PFA. Brains were then sectioned using a vibratome in a coronal plane at a thickness of 80-100µm. Sections were then Nissl stained using cresyl violet and examined for a lesion in the area of the interest.

4.2.5 Golgi cell identification

Golgi cells have a ‘signature’ firing pattern. Previous studies have shown that Golgi cells fire in the range of 2-25Hz and have broad peak interspike interval (ISI) histograms with tails skewed to the right and few intervals under 30ms (Simpson et al. 2005; Holtzman et al. 2006; Ruigrok et al. 2011; Dijck et al. 2013).

Cells included as putative Golgi cells exhibited firing patterns with the statistics described by (Dijck et al. 2013). Additionally, if the cell exhibited a long depression in firing in response to the peripheral somatosensory stimulation, it was further evidence that it was a Golgi cell. All of the juxtacellularly filled Golgi cells in Holtzman et al., 2006 exhibited these responses. To be included in the analyses, the putative Golgi cell had to have been recorded while stimulating at least one of the peripheral afferent sites and also to have a recording while the MCP was being stimulated.

4.2.6 Analysis

Significant responses to stimulation were determined by performing a multivariate Gaussian analysis on the bins of the PSTHs, as described in Chapter 2. All statistics were done on the underlying bin counts but for the purposes of plotting a smooth spline was fit to the count data to generate smooth line graphs of activity in relation stimulus onset. The level of smoothing was chosen by leave-one-out cross-validation whereby the smoothing spline is repeatedly fit with the absence of one data point which is then compared to its predicted value to calculate the residual error. This runs through all the points in the set, and the level of smoothing that is chosen minimises the average residual distance from these data points. In some cases, the smoothing appeared overzealous especially where there were fast changes that only lasted for several bins, and the smooth value was chosen by eye to represent the underlying count data better graphically.
4.3 Results

4.3.1 Histology

The stimulating electrodes were initially targeted to a location 9.3-9.8mm caudal to bregma, and 3.5mm lateral to the midline on the left side and were advanced to a depth of 7 – 7.5mm deep to target the middle cerebellar peduncle. To aid in localisation, local evoked field potentials were recorded from ball electrodes placed on the cerebellar surface at crus II. MCP stimulation evoked a strong short-latency potential, and the electrode was positioned to maximise this potential, then cemented in place. Fig. 4-1A shows an example of the averaged local field potentials from stimulation of the MCP in a single experiment. The lesion created at the end of this experiment is shown in Fig. 4-1B with electrolytic burns marking the lesion site.

Fig. 4-1C shows the results of histology to determine the location of lesion sites after the experiments. 11 lesions were found to be in the region of the MCP (red dot), but two were found to be off target (blue dot). As such any cells that were recorded during the off-target experiments were discarded from analyses.
Figure 4-1: Histology to determine placement of MCP electrodes

(A) Local field potentials recorded from the surface of the cerebellum while MCP stimulating electrode was activated, short latency field potentials from activation of a large collection of mossy fibres (*), which were greatest at this position. Electrode was subsequently fixed at this position for the experiment

(B) Electrolytic lesion generated at the end of the experiment by 80µA current residing in the middle cerebellar peduncle (MCP)

(C) Reconstruction of lesion at positions -9.24 and -9.84mm from bregma shown in the coronal plane of the left side of the cerebellum
4.3.2 Identification of putative Golgi cells and their spontaneous activity

Putative Golgi cells were identified by their spontaneous firing patterns and their response to peripheral somatosensory stimulation and the absence of complex spikes in the recording. Previous studies have shown that Golgi cells fire in the range of 2-25Hz and have broad peak interspike interval (ISI) histograms with tails skewed to the right and few intervals under 30ms (Simpson et al. 2005; Holtzman et al. 2006; Ruigrok et al. 2011; Dijck et al. 2013).

Fig 4-2A shows an example of a putative Golgi cell recording from this experiment that displays the fairly regular slow firing rate characteristic of Golgi cells. This cell has a firing frequency of 3.6Hz and an average interspike interval of 280ms. The ISI histogram is shown fig. 4-2B showing the broad distribution of ISI but no intervals less than 80ms.

**Figure 4-2**: Example of a putative Golgi cell’s spontaneously firing and depression response to peripheral stimulation
(A) Example of a putative Golgi cell spontaneously firing, fairly regular intervals but very few short intervals and absence of complex spikes. Time scale bar = 2s, Voltage scale bar = 0.5mV
(B) Spike template for the same cell. Scale bar = 1ms
(C) Interspike interval histogram, most intervals were greater than 100ms and the distribution shows the long tail that is characteristic of Golgi cells
(D) Depression in firing in response to ipsilateral hindlimb stimulation. Characteristic response of Golgi cells. Hindlimb stimulus related activity derived from 100 sweeps of stimulus presentation. Spontaneous activity was generated by resampling spontaneous recordings; 500 histograms were made consisting of 100 sweeps with random times used as a pseudo stimulus time. Only activity from a random 25 spontaneous histograms are plotted
In total 36 cells were identified as putative Golgi cells by their spontaneous firing patterns, of which only 2 of these did not show long latency depression in firing in response to contralateral peripheral somatosensory stimulation. The cells had an average firing frequency of 6.8Hz (SD: 4.3) with a range from 1.2 to 19.5Hz.

Fig. 4-3 shows the range of firing rates plotted against the coefficient of variation of the log ISIs. This statistic was used by (Dijck et al. 2013) for clustering identified Golgi cells. The cells found in our experiment show a similar distribution to previously identified Golgi cells (inset Fig. 4-3, blue circles).

Figure 4-3: Identification of putative Golgi cells by spontaneous firing activity and coefficient of variation of log ISI
Spontaneous activity derived from > 60 second of spontaneous activity recordings plotted against coefficient of variation of log interspike intervals (ISI), also from spontaneous recordings. Comparable to results obtained by Dijck et al. 2013 shown inset in top right, Golgi cells are represented by the blue circles. Note, to clarify the authors refer to the coefficient of variation of log ISI in the method text but it is labelled LCV on the figure giving the erroneous impression from the figure that it could be log of the CV ISI.
4.3.3 Response to middle cerebellar stimulation and peripheral somatosensory stimulation differ

The activity of the 36 putative Golgi cells was recorded while different stimuli were applied to the animal. Not all cells remained stable during the complete battery of stimuli, but in each case, the cell’s activity was recorded under at least forelimb and/or hindlimb stimulation to test responses to peripheral somatosensory stimulation and subsequently to single MCP stimulation and/or multiple MCP stimulation (5 pulses).

The numbers of cells that responded with significant modification to different combinations of stimuli are shown below in Table 4-1. The cells most commonly (n=30) had a response to at least one of the peripheral stimuli and at least one of the MCP stimuli. Four cells only showed a response to peripheral stimulation whereas 2 showed only responses to stimulation of the middle cerebellar peduncle; these were included because of their congruence of their firing characteristics with cells that showed depression to peripheral stimulation. The forelimb and hindlimb stimuli had comparable rates of responses indicating that it was likely where tested they would both generate a significant response. Stimulation of the interorbital nerve (denoted as vibrissae) was also used as a sensory stimulus in a smaller number of cells (n=17) and had a lower response rate. The percentage

<table>
<thead>
<tr>
<th>Responses</th>
<th>Counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Response to both peripheral and MCP</td>
<td>30</td>
</tr>
<tr>
<td>Response to peripheral</td>
<td>4</td>
</tr>
<tr>
<td>Response to MCP</td>
<td>2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stimulus type</th>
<th>Percentage of responses</th>
<th>Total cells tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forelimb</td>
<td>93.9</td>
<td>33</td>
</tr>
<tr>
<td>Hindlimb</td>
<td>93.5</td>
<td>31</td>
</tr>
<tr>
<td>Vibrissae</td>
<td>82.4</td>
<td>17</td>
</tr>
<tr>
<td>Single MCP</td>
<td>81.8</td>
<td>22</td>
</tr>
<tr>
<td>Multiple MCP</td>
<td>87.9</td>
<td>33</td>
</tr>
</tbody>
</table>

Table 4-1 (Top) Significant responses from 36 putative Golgi cells. (Bottom) The percentage of significant responses seen to the various stimuli types

Note vibrissae refers to stimulation of the infraorbital nerve via the tissue under the vibrissae.
of cells that responded to the single and trains of MCP stimuli are shown in Table 4-1, there is a slight difference in the percentage of responses between the two, this is attributable to two cells in which a single MCP pulse did not produce a significant response whereas a train of 5 pulses did. A 5x2 Chi-square test reveals that the difference in the proportion of responses to the various stimuli is non-significant (p > 0.05).

The significant responses to middle cerebellar peduncle stimulation were examined. Fig. 4-4 shows two example traces (top and bottom) from two 5MCP stimulation sweeps in a cell significant responses to the MCP stimuli. The putative Golgi cell was brought to threshold by the first pulse and again shortly after the fourth pulse. When the cells were activated by the middle cerebellar peduncle stimulus it was often found that a single pulse was sufficient to drive the cell, such as the case for the cell in Fig. 4-4, were it was activated after the first stimulus pulse in the train.

![Figure 4-4: A putative Golgi cell activated at short latency by the middle cerebellar peduncle stimulation](image)

*Individual putative Golgi cell activation to a train of 5 MCP stimulation pulses. Stimulus artefact (grey bars), Golgi cell action potential (blue star). On two separate sweeps (top and bottom) the Golgi cell discharged following the first and fourth stimulus. Scale bar = 3ms*

Fig 4-5, overleaf shows the activity of the same putative Golgi cell verifying that it responded to both peripheral stimulation and MCP stimulation. The cell shows marked and long-lasting depression (~1000ms) in response to forelimb and hindlimb stimulation (top row). The response to single MCP (bottom left) and to a train of 5 pulses (bottom right) exhibited a different response profile. The cell
was strongly driven by the stimulation of the MCP fibres firing in response to stimulation at short latency which was followed by a short latency depression (~100ms). Excitation was the most common if there was a significant response to the MCP stimuli, but a small number of cells (n=3) showed inhibitory responses; presented in section 4.3.7.

Figure 4-5: Putative Golgi cell that had significant responses to MCP stimulation and stimulation of the periphery
Top Left: Significant responses to forelimb stimulation, showing the long lasting depression in firing that is characteristic of Golgi cells
Top Right: Similar significant response to hindlimb stimulation
Bottom Left: Significant response to single stimulus pulse to the middle cerebellar peduncle. Cell exhibited a short latency activation followed by a short depression
Bottom Right: Significant response to train of 5 middle cerebellar pulses, again with short latency activation but a stronger excitation, and short lasting depression in firing following stimulation.
Each figure is the result of 100 sweeps of stimulus presentation
Only 2 of the 36 cells tested responded only to the MCP stimuli – these were included in the analyses despite not showing long-lasting depressions to peripheral stimulation, as they clustered with other putative Golgi cells, which did show depression, in the firing rate by CV log ISI plot. Fig. 4-6 below shows the firing activity in relation to the stimuli of cell that only responded to the MCP stimuli. Fig. 4-6 (top row) shows the responses to peripheral stimulation, and it is clear that the stimuli have no effect on the firing activity, and it remains well within the confines of spontaneous activity. The MCP stimuli (bottom row) again reliably caused the cell to fire at a short latency following the stimulus, generating an increase in the firing rate of the cell. This was also followed by short-lasting depression in firing (~100ms).

Figure 4-6: Putative Golgi that only showed significant responses to stimulation of the middle cerebellar peduncle
Top left: Stimulation of forelimb did not produce deviations from spontaneous activity as deemed by Gaussian analysis
Top right: Similarly stimulation of the hindlimb did not produce deviations from spontaneous activity.
Bottom left: Single MCP stimulation pulses were sufficient to excite the cell which was followed by a short lasting depression in firing
Bottom right: Train of 5 MCP stimulation pulses greatly increase the activity of the cell in comparison to spontaneous and also caused short lasting depression in firing. Each figure is the result of 100 sweeps of stimulus presentation.
4 of the 36 cells tested failed to respond to peripheral stimulation. Fig. 4-7 shows an example cell in which the long depression in firing is exhibited in response to forelimb stimulation (top left) and hindlimb stimulation (top right) and is consistent with the behaviour of other cells in response to peripheral stimulation. The MCP stimuli did not produce any significant responses in this individual cell as shown in Fig. 4-7, bottom row.

Figure 4-7: Putative Golgi cell with significant response only to the peripheral stimuli
Top left: Significant depression in activity in response to forelimb stimulation
Top right: Similar significant depression in activity after hindlimb stimulation
Bottom left: No change in activity in response to a single MCP pulse
Bottom right: No change in activity in response to 5 MCP pulses. The small dip around time 0 may reflect the blocking of templating spontaneous action potentials by the stimulus artefact. It however did not fall outside the range of spontaneous activity in the multivariate Gaussian analysis. Each figure is the result of 100 sweeps of stimulus presentation.
17 cells were tested with infraorbital nerve stimulation with pins through the vibrissal skin. When a significant response occurred, it did not differ qualitatively in shape from those observed when peripheral stimulation of the limbs produced significant responses. In contrast to previously recorded results, the infraorbital stimulation protocol did not produce any short latency activations of the putative Golgi cells (Holtzman et al. 2006). Fig. 4-8 below shows an example of a putative Golgi cell that significantly responded to infraorbital stimulation with the long-lasting depression, this cell also responded to hindlimb and forelimb in this manner, not shown.

Figure 4-8: Putative Golgi cell with significant decrease in activity in response to infraorbital nerve stimulation
Responses to infraorbital stimulation showed depression in firing similar to other peripheral somatosensory stimulation. Significant response revealed by multivariate Gaussian analysis. Figure is the result of 100 sweeps of stimulus presentation.
4.3.4 Response onset and offset times differ between peripheral and MCP stimulation

The onset and offset times of the cells that showed significant responses to stimuli were determined by using a change point model as described in the Materials and Method section. This change point method was applied to the 10ms wide bins of the PSTH to determine the bin at which a significant change had occurred in the counts of action potentials. This quantizes the resolution of onsets and offsets to 10ms steps.

The onset times are shown in below Fig. 4-9 for the response to the various stimuli. A single MCP pulse was found to have a median onset latency of 5ms while the train of 5 pulses had a median onset latency of 15ms. This difference is likely because the change point model is determining the major onset changepoint as the change from the high activity generated by the stimulus to the subsequent depression in firing. In reality, the train of 5 MCP pulses reliably drives the cell from the first pulse, but it also sustains the higher activity with later pulses also causing the cell to fire.

![Figure 4-9: Distribution of onset latencies by stimulus type as estimated using a change point model](image)

Latencies to onset as determined by a change point model, see Methods. The distribution of latencies differed significantly across the different stimulation types. Typically the MCP stimuli caused an onset that was much earlier than the responses that were caused by stimulation of the periphery.
The median onset latency for the infraorbital-vibrissal pad stimulation was found to be 10ms, for forelimb response it was 25ms, and for hindlimb responses it was found to be 40ms, but the ranges were much more variable for the peripheral stimuli than those to MCP stimulation response onsets. A Kruskal-Wallis rank sum test revealed the differences in onset times to be significantly different for the different stimuli ($H(4) = 33.60, p < 0.001$). A post hoc Dunn’s test on the pairwise differences was carried out. The p-values for the pairwise comparisons are shown in Table 4-3 below. MCP stimuli onsets did not differ significantly from each other or infraorbital-vibrissal pad stimulation onset, but they were significant differences in onsets between MCP stimuli and forelimb/hindlimb onsets ($p < 0.05$).

<table>
<thead>
<tr>
<th></th>
<th>1MCP</th>
<th>5MCP</th>
<th>FORELIMB</th>
<th>HINDLIMB</th>
</tr>
</thead>
<tbody>
<tr>
<td>5MCP</td>
<td>0.523</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FORELIMB</td>
<td>0.004</td>
<td>0.021</td>
<td></td>
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</tr>
<tr>
<td>HINDLIMB</td>
<td>0.000</td>
<td>0.000</td>
<td>0.523</td>
<td>0.122</td>
</tr>
<tr>
<td>VIB</td>
<td>0.083</td>
<td>0.523</td>
<td>0.523</td>
<td>0.122</td>
</tr>
</tbody>
</table>

Table 4-2: p-values of post-hoc Dunn’s test on response onset latencies
Green highlighted cells show significant differences in onset latencies in the pairwise comparisons

The analysis of offset times revealed further differences between the different responses to stimuli. The response offset times are shown overleaf in Fig. 4-10. A single MCP pulse gave median response offset of 80ms whereas a train of 5 pulses resulted in a median offset time of 125ms. The infraorbital-vibrissae stimuli had a median response offset of 445ms, the forelimb responses had a median offset time of 580ms, and hindlimb stimulus had a median offset time of 685ms. A Kruskal-Wallis rank sum test showed that the difference in offset times was also significantly different between the stimuli ($H(4) = 47.96, p < 0.001$). A post-hoc Dunn’s test revealed significant differences in offset latencies in a pairwise manner. MCP stimuli offset latencies differed significantly in comparison with all other stimulation response offsets.

<table>
<thead>
<tr>
<th></th>
<th>1MCP</th>
<th>5MCP</th>
<th>FORELIMB</th>
<th>HINDLIMB</th>
</tr>
</thead>
<tbody>
<tr>
<td>5MCP</td>
<td>0.835</td>
<td></td>
<td></td>
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<tr>
<td>FORELIMB</td>
<td>0.000</td>
<td>0.000</td>
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</tr>
<tr>
<td>HINDLIMB</td>
<td>0.000</td>
<td>0.000</td>
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</tr>
<tr>
<td>VIB</td>
<td>0.017</td>
<td>0.025</td>
<td>0.835</td>
<td>0.272</td>
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</table>

Table 4-3: p-values of post-hoc Dunn’s test on response offset latencies
Green highlighted cells show significant differences in offset latencies in the pairwise comparisons
4.3.5 Latency of cells firing in response to MCP stimulus

The latency of 12 cells that fired in response to single MCP stimulus pulse was analysed in detail. This subset of cells were chosen as the spike post stimulus could be clearly distinguished from the stimulus artefact removing ambiguity about the onset of the action potential. Fig. 4-11, overleaf, shows the latencies of these responses. The average response latency was 1.5ms with a range from 0.6 to 2.42ms. The lower end of this range was mainly caused by cell 22 which fired at short latency very consistently and with little variation in latency, a possible outlier as discussed in section 4.3.6. The majority of the cells displayed latencies which were greater than 1ms but shorter than 2ms. This is consistent with activation through a monosynaptic route from the middle cerebellar peduncle.
4.3.6 Probability of spikes to pulses within the MCP pulse train

The probability that cells fired to each pulse in the train of 5 was analysed for 28 of the 29 cells that responded to MCP train stimulation. Cell 22, the cell above that responded very consistently at a fixed short latency, was found to be an outlier in behaviour. It discharged to 100% of the third and fourth pulses in the train, and near 100% for the other pulses, in contrast, the other cells often fired to 50% or less of the pulse presentations and much less to the later pulses in the train. Cell 22 was dropped from this analyses for this reason.

The observed probabilities of a cell firing in response to pulses within the stimulus train are shown in Fig. 4-12, overleaf, (blue dots, observed probabilities). Response probabilities span a much higher range to the first stimulus and decrease with subsequent pulses in the train with the maximum observed probabilities following 0.72, 0.48, 0.47, 0.36 and 0.28 for stimulus pulses one to five.
respectively. A similar pattern is seen for the average probabilities following 0.17, 0.14, 0.09, 0.07 and 0.06 for stimulus pulses 1 to 5 respectively.

**Figure 4-12: Observed and predicted probabilities of an action potential following each pulse of the 5 MCP stimulus train**

Analysis of 28 putative Golgi cells that responded to MCP stimulation with a short latency activation of the cell. The observed probabilities that the cell responded following each stimulus pulse are shown as blue dots. A logistic regression model was fit to the data with each stimulus pulse number as a predictor, the predicted probabilities from the model are shown as grey bars.

A logistic regression model was constructed to examine the effect of stimulus pulse number on the probability of the cell firing following a stimulus pulse. Stimulus pulse was coded as a discrete factor rather than as a continuous measure to account for the fact that it is not a reasonable assumption to expect the probability of the cell firing to be linearly related to stimulus number. The predicted probabilities of the model are shown in Fig 4.12 (grey bars), and these reflect the decreasing probabilities of a cell firing to subsequent stimulus pulses. The predicted probabilities from the model are 0.15, 0.12, 0.09, 0.06 and 0.05 for stimulus pulses 1 to 5 respectively. All stimulus pulse numbers were found to have a significant negative effect on the odds of the cell firing relative to the first stimulus pulse (p < 0.01). The associated coefficients and odd ratios are shown in Table 4-4 below. The coefficient for pulse 2 (β = -0.20) represents an 18% reduction in the odds of firing in response to the stimulus relative to the odds for responses to the first pulse. This reduction
increases monotonically with increasing pulse number and represents a 66% reduction in the odds for the fifth pulse relative to the first.

It was commonly observed, such as in Fig. 4-4, that a cell would often respond to the first pulse but not the second but then again on later pulses suggesting that they could not follow high-frequency stimulation. The response probabilities to pulse 2, 3 and 4 were examined while dividing each pulse into four categories depending on whether the previous or subsequent pulse in the stimulus train had generated a response. These four categories were:

- no spike evoked to the previous and subsequent pulse
- spike evoked to the previous pulse but no response to subsequent
- no response to the previous pulse but a spike response to subsequent pulses
- spike responses to both

Fig. 4-13 shows the observed probabilities for the four categories for pulses 2, 3 and 4 (blue dots). The distribution of probability of responses is much greater on each stimulus pulse where there was no response to either the previous or subsequent pulse. The probabilities are lower for each stimulus pulse when there is a response to either the previous or subsequent stimulus or both. A logistic regression model was constructed to determine the effects of pulse number and responses to the previous and subsequent pulse on the odds of a response to a given pulse. The predicted probabilities from the model are shown in Fig. 4-13 (grey bars) and reflect the decreasing probability of a response to a given stimulus pulse if either previous or subsequent pulse in the stimulus train generate responses. The associated coefficients and odds ratios for the predictors are shown in Table 4-4 below. All predictors showed significant effects on the odds of firing in response to a stimulus pulse (p < 0.001). If the cell had responded to the previous pulse the odds of a response to the given pulse decreased by 84% if all else was held constant. If the cell responded to the subsequent pulse the odds of it making a response to the given pulse decreased by 92% if all else

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient (SE)</th>
<th>Lower</th>
<th>Odds Ratio</th>
<th>Upper</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Intercept)</td>
<td>-1.72 (0.05)</td>
<td>0.71</td>
<td>0.94</td>
<td>0.34</td>
</tr>
<tr>
<td>Pulse 2</td>
<td>-0.20 (0.07)</td>
<td>0.48</td>
<td>0.66</td>
<td>0.40</td>
</tr>
<tr>
<td>Pulse 3</td>
<td>-0.57 (0.08)</td>
<td>0.34</td>
<td>0.47</td>
<td>0.40</td>
</tr>
<tr>
<td>Pulse 4</td>
<td>-0.92 (0.09)</td>
<td>0.28</td>
<td>0.40</td>
<td>0.47</td>
</tr>
<tr>
<td>Pulse 5</td>
<td>-1.08 (0.09)</td>
<td>0.28</td>
<td>0.40</td>
<td>0.47</td>
</tr>
</tbody>
</table>

Table 4-4: Output of binomial logistic regression model. All predictors were found to be negatively associated with the odds of the putative Golgi cells firing an action potential relative to the odds for the first stimulus pulse (contained with the intercept). Significance: p < 0.05 *; p < 0.01 **; p < 0.001 ***
was held equal. The results of the model reflect that it was common for the cells to be unable to follow high-frequency stimulation.

### Table 4-5: Output of binomial logistic regression mode. This model uses information about whether the cell has fired to the previous pulse and whether it fires to the subsequent pulse to estimate the odds of firing on the current pulse. All predictors were found to be negatively associated with the odds of the putative Golgi cells firing an action potential relative to the odds for the second stimulus pulse with no response or previous or subsequent pulse (contained with the intercept). Significance: $p < 0.05 \, \, \ast; \, p < 0.01 \, \, \ast\ast; \, p < 0.001 \, \, \ast\ast\ast$

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient (SE)</th>
<th>Lower</th>
<th>Odds Ratio</th>
<th>Upper</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Intercept)</td>
<td>-1.67 (0.05)</td>
<td>0.56</td>
<td>0.65</td>
<td>0.77</td>
</tr>
<tr>
<td>Pulse 3</td>
<td>-0.43 (0.08)</td>
<td>0.37</td>
<td>0.44</td>
<td>0.52</td>
</tr>
<tr>
<td>Pulse 4</td>
<td>-0.83 (0.09)</td>
<td>0.10</td>
<td>0.16</td>
<td>0.24</td>
</tr>
<tr>
<td>Response to previous pulse</td>
<td>-1.83 (0.21)</td>
<td>0.04</td>
<td>0.08</td>
<td>0.18</td>
</tr>
<tr>
<td>Response to subsequent pulse</td>
<td>-2.49 (0.38)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.3.7 Short latency depression following MCP stimulation

The responses of the cells to MCP stimulation differed from those to peripheral stimulation with MCP stimulation more commonly driving the cell to discharge and resulting in a much shorter depression in firing than that generated by peripheral stimulation, see Fig. 4-6.

The question remained whether this short-lasting depression is an inhibitory response or due to a refractory period caused by the cell firing in which it does not fire again. Three cells were identified which had significant responses to the train of 5 MCP pulses but did not appear to show short latency activation. Fig. 4-14 shows the recording trace during MCP stimulation for an example cell in which no short latency activation was observed. It is possible that the stimulus artefact obscures the action potential but given the latencies observed in Fig. 4-11 this seems unlikely.

Figure 4-14: Three 5 MCP sweeps from a putative Golgi cell that still showed short lasting depression from the stimulus, despite not showing evidence of being activated by it. Top row: blue dot show the cell’s spontaneous spike, this occurred just before a delivery of the stimulus – artefact in greyed out zone. Second to fourth row: three sweep of the MCP stimulus train showing the absence of any short latency activation of the cell in response to the stimulus. Stimulus artefact in greyed out zone.
The activity of these three cells in response to 5MCP stimulation are shown in Fig. 4-15 below. The cells all show a similar length depression in their activity to other cells that responded to MCP stimulation but with the absence of increase related to the stimulus. This suggests that the inhibitory responses seen are independent of the short latency activation caused by the MCP stimulation.

As many cells were not reliably driven in response to 100% of the MCP stimulus presentations, it was possible to separate the presentations in which the cell fired from those that which didn’t. Fig. 4-16 shows example PSTHs that result from the splitting of the sweeps. Fig. 4-16A shows the PSTH of the cell including all sweeps and demonstrates that it is driven in response to the MCP stimulus train, but the response is also followed by a short lasting depression in firing. Fig. 4-16B shows the trace from a stimulus presentations in which the cell fired in response, the collated sweeps, when combined into a PSTH show a similar response as to before. In contrast Fig. 4-16C shows an individual trace from a sweep in which the cell did not fire, these when collated into a PSTH still show the short lasting depression in firing, in the absence of short latency activation.
Figure 4-16: Separation of 5MCP PSTH into sweeps with short latency excitation and into sweeps without leaves the short lasting depression intact in both
(A) The full PSTH, shows a putative Golgi cell with significant responses to the MCP stimulus train, where it is activated at short latency, generating a high bin immediately after 0ms. Red dotted lines denote mean firing frequency for those sections.
(B) In many stimulus sweeps, the cell is excited by the stimulus pulses (*) if these are collated together we see a similar PSTH to the original with short latency activation and short lasting depression.
(C) Separation out of the sweeps where there is no excitation in response to the stimulus pulse results in a PSTH that shows a short lasting depression which remains even in the absence of excitation. The lower count/bin numbers reflects that fewer sweeps have no excitation that those that do. Bin widths = 10ms for all PSTHs, 100 sweeps in original PSTH
To further determine if the depression following short latency activation as a result of MCP stimulation was an inhibitory process or simply a refractory period after firing the interspike intervals from the driven spike to the next spontaneous spike were compared against spontaneous interspike intervals. Fig. 4-17A shows the process of selecting the interspike intervals, in the case of spontaneous all intervals from the spontaneous recording of the cell are included. The stimulus conditions are divided into two, in the case where there is excitation the interval is taken from the last driven spike to the next spontaneous spike following the stimulus pulses. If there is no excitation to the stimulus presentation, then the interval is taken from the previous spike before stimulus presentation to the following. The intervals were normalised to the median spontaneous value for each cell that responded to MCP stimulation and collated together. Fig. 4-17B shows the distribution of these intervals in relation to the stimulus presented, there is an upward location shift to the intervals that result from stimulation, both for sweeps where there was excitation and those where there was no excitation. A Kruskal-Wallis rank sum test reveals the differences in the intervals for the given conditions to be significantly different ($H(4) = 1022, p < 0.001$). This indicates that the latency to next spike following stimulation is governed by processes separate from that which governs

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**Figure 4-17:** Interspike intervals of the Golgi cells under 3 different conditions
(A) The three different conditions of interspike interval. Top, excitation: defined as the interval from the last driven spike to the first spontaneous. Middle, no excitation: defined as the interval from the last spike before stimulation to the first spike following it, on trials where the cell did not discharge in response to stimulation. Bottom, spontaneous interspike intervals as recorded with no stimulation applied to the cell
(B) The distribution of intervals for the population of Golgi cells that had significant MCP responses (n=32). Each cells ISIs were divided by the median value of their spontaneous ISI
the spontaneous interspike intervals and is independent of whether the stimulation caused the cell to fire or not.

4.4 Discussion

The responses of the putative Golgi cells to peripheral afferent stimulation and MCP stimulation differed in both their response profile and time course. The results presented from this experiment show that individual Golgi cells can have divergent responses to inputs from different precerebellar nuclei; namely the pontine nucleus and the lateral reticular nucleus. The responses to peripheral stimulation have been shown to be affected by pharmacological manipulation of the lateral reticular nucleus and can be generated directly by stimulation of the nucleus itself. Stimulation of the MCP fibres results in most commonly a short latency activation of the Golgi cell followed by short-lasting depression of 100ms, in contrast to the long latency depression responses shown after peripheral limb stimulation last around 600-1000ms.

4.4.1 Identification of Golgi cells

One of the limitations in this experiment is the identification of Golgi cells. The gold standard method to identify cells of a particular type involves direct morphological identification, such as juxtacellularly filling cells after recording. This procedure is intensive and generates low yield in the cell number. It has been well established that Golgi cells have firing characteristics distinct from other cerebellar cortical neurons (Simpson et al. 2005; Holtzman et al. 2006; Ruigrok et al. 2011; Dijck et al. 2013) and their response to peripheral stimulation (Holtzman et al., 2006a). Using these characteristics for identification increases the yield in a given experiment. The depression in firing in response to peripheral stimulation is a likely indicator of the cell being a Golgi cell as only juxtacellularly filled Golgi cells have shown this response and at a high proportion in previous studies (Holtzman et al. 2006). The interspike intervals, firing rates and the log of the coefficient of variation of the ISIs in the cells identified are all comparable to those from morphologically identified Golgi cells in other studies (Simpson et al. 2005; Ruigrok et al. 2011; Dijck et al. 2013; Rasmussen et al. 2014). The study by Dijck et al. included data from different species, both decerebrate and anaesthetised. Although we can’t be absolutely certain this should allow us to infer that we have identified Golgi cells.

4.4.2 Golgi cell responses to peripheral afferent stimulation are different to those to MCP stimulation

The responses of Golgi cells to the two different stimuli protocol were markedly different. The response to peripheral stimulation has been well characterised before. In this group of Golgi cells we showed that onset time of responses occurred 20-40ms after the stimulus and that the response
lasted till 500 – 700ms after the stimulus pulse, as seen in previous studies. The MCP stimuli evoked sharp onset and shorter duration excitatory responses. In all cases where there was a significant response to hindlimb or forelimb stimulation a depression in firing occurred with no short latency activation, but in response to MCP stimulation, the most common response was a short latency activation followed by a shorter depression (~100ms). This suggests that the two mossy fibre pathways can target the same Golgi cell but produce different responses. Pontocerebellar fibres could act by a monosynaptic mossy fibre – Golgi cell synapse on the basal dendrites (discussed below), whilst LRN mossy fibres generate depression in the Golgi cells which has been hypothesised to be mediated by extrasynaptic spillover of glutamate that activating mGluR2 channels (Watanabe and Nakanishi 2003; Holtzman et al. 2011).

It was observed that a great number of cells showed responses to both stimuli at varying lateral distances in Crus II. This response is certainly more common than tracing of LRN mossy fibres would suggest, where fibres are seen to terminate more medially in the vermis and intermediate zones and much less frequently in the hemispheres (Wu et al. 1999). Although LRN projections do extend as far lateral as the Do/DS1 zones (Pijpers et al. 2006). Although in this experiment we usually searched for cells that showed a pause response to peripheral stimulation before giving the battery of stimuli, such that it may artificially increase their number, it was certainly not difficult to find cells that responded to both MCP and peripheral afferent stimulation.

One thing to note in light of the previous Chapter is that pontine neurons exhibit responses to forelimb and hindlimb afferent stimulation. However, in this study we saw no examples of Golgi cells that showed an excitatory response to limb stimulation and no cells where there was congruence between the responses to peripheral somatosensory stimulation and MCP stimulation. This suggests that the cells identified in this study were not receiving pontocerebellar mossy fibres that were receptive to somatosensory stimulation and that the effects of such stimulation were mediated through another pathway, consistent with activation of LRN mossy fibres. The absence of short latency activations to peripheral somatosensory stimulation differs to the study by Holtzman et al., 2006 where some were described in Golgi cells, and this probably reflects the recording locations – which in this study are more lateral in this study.

4.4.3 Putative Golgi cell activation in response to MCP stimulation

This experiment has demonstrated that the putative Golgi cells can be activated by MCP stimulation often requiring only a single pulse to bring the cell to threshold and generate an action potential. These cells exhibit a latency (~1.6ms) that is consistent with activation through a single synapse suggesting that pontocerebellar mossy fibres synapse directly upon Golgi cell dendrites and provide
direct inhibition on to granule cells from this mossy fibre source. Accepting that synaptic delay occurs in the range of 0.4 – 0.7ms (Eccles 1946; Jankowska and Roberts 1972), then this latency to activation of the Golgi cell would be too short to account for the mossy fibre–granule cell synapse and the parallel fibre–Golgi cell synapse, as well as taking into account the conduction along axons. These results are consistent with those of another study that examined Golgi cell responses evoked by stimulation within the cerebellar white matter and in which the responses were attributed to pontocerebellar stimulation (Holtzman et al. 2009).

The putative Golgi cells were unable to follow high-frequency stimulation in response to a train of 5 MCP pulses, often firing in response to the first pulse but not again till the fourth or fifth pulse. Indeed the logistic regression model constructed showed a spike in response to previous or subsequent pulses to be a significant negative predictor of the probability of firing to current given stimulus pulse. This inability to fire to consecutive stimuli may reflect the strong afterhyperpolarising (AHP) currents that Golgi cells have that generate the long interstimulus intervals in spontaneous firing (Forti et al. 2006). However, subsequent pulses appear to have the ability to overcome this and bring the cell to threshold again in the same stimulus train. This suggests that input via the pontocerebellar mossy fibres to granule cells is moderated by Golgi cell inhibition onto the synapses, with Golgi cells often activated by one pulse, but this inhibition does not increase linearly with pulse numbers in the train. This is in contrast to the effects of peripheral somatosensory stimulation, presumed to be mediated by LRN mossy fibres, where the same Golgi cells show no activation and a silence lasting for ~500-600ms. This suggests the same Golgi cell is providing different functions in the circuit depending on the input source.

4.4.4 Short lasting depression in response to MCP stimulation

The short-lasting depression (~100ms) observed following MCP related responses could have been generated by two means; either it represents a refractory period due to the cell firing and a subsequent AHP or it is due to inhibitory inputs to the Golgi cell. We have presented some limited evidence that the depression in firing may be caused by more than just a refractory period but it is by no means conclusive. This evidence is threefold: (i) that some cells (n=3) appear to have a depression in firing with an absence of excitation, (ii) the separation of PSTHs into sweeps where the MCP stimulus evoked excitation evoked firing occurs and those where it does not shows in the non-excited group a depression still remained, and (iii) the latency to next spike after stimulation pulse, whether the cell was excited or not, is significantly longer than the spontaneous interspike interval.

In the small number of cells (3) which showed depression in the absence of short latency excitation, we cannot be completely confident that the cells have not been activated within the duration of the
stimulus artefact. This is less likely in response to the single MCP pulse unless the cell is firing at extremely short latencies, which would be inconsistent with the latencies observed in the experiment. This suggests that longer time to spike regeneration in single pulse group (with no excitation) may reflect inhibitory processes acting upon the putative Golgi cell.

If the putative Golgi cell is being inhibited the question remains where this inhibition is comes from. The brief depressions evoked by MCP stimuli might be generated by mGluR2 receptors, if they are briefly activated. If so, this begs the question of why they differ from the (presumed) LRN – generated long-lasting depressions. It may be that it is the long-lasting changes in LRN firing evoked by peripheral stimuli (Xu et al. 2013) sum to prolong mGluR2 evoked inhibition – that it is a changed pattern of LRN activity, with more summation of activity that prolongs the depressions. Single or brief trains of MCP stimuli with a duration of 15ms may produce only brief glutamate release.

Other alternatives are possible. In vitro investigation has shown Golgi cells to have inhibitory input from Lugaro cells (GABAergic/Glycinergic) and stellate/basket cell synapses (GABAergic) (Dumoulin et al. 2001). Hull and Regehr 2012 suggest, however, that molecular layer interneurons do not inhibit Golgi cells, but that Golgi-Golgi cell synapses provide an inhibitory input to each other. They provide evidence for this in the form of in vitro optogenetic stimulation of mossy fibres showing that IPSCs occur 2ms earlier in Golgi cells than they do in Purkinje cells, which are inhibited by molecular layer interneurons. Paired recordings of Golgi cells similarly suggest that there is an inhibitory interaction between them (Hull & Regehr, 2012). This would provide an explanation for the three cell responses observed by MCP stimulation, in the case of cells with a pause alone, the Golgi cell may not receive input from the MCP but inhibitory input from a cell that does, where as a cell that discharges and suppresses may receive both.

To determine which of these possibilities operate is difficult to achieve in vivo as manipulation of inhibitory agonists would not only effect Golgi cell inhibition but also perturb the wider role that inhibition plays at many sites in the cerebellar microcircuit. Targeted cell-specific manipulations may provide a way to address this issue, but were not available to the lab during these experiments.
5 Second Order Conditioning of the Eyeblink Response in Rabbits

5.1 Introduction

Chapter 1 introduced the experimental finding of changes in climbing fibre responsiveness after the acquisition of the conditioned eyeblink response. In these experiments by Mostofi et al. (unpublished work, Fig. 1-8) and Ohmae and Medina, 2015 (Fig. 1-9) climbing fibres that were initially receptive to periocular aversive stimulation of the ipsilateral eye (electrical and air puff in the experiments, respectively) began to show responses to the CS1 (auditory and visual in the respective experiments) after classical conditioning of the eyeblink response. This climbing activity was seen only in the eyeblink controlling regions of the cerebellum, and starts shortly after CS1 onset, and appears to show temporal difference prediction error very similar to that seen in dopamine neurons during reinforcement learning (Ohmae and Medina 2015; Schultz 2016).

Given that the climbing fibres have an instructive role that has been hypothesised by the Marr-Albus theory, an idea supported by the responsiveness to the US in naïve animals, the new responsiveness leads to the question of what they are now signalling? An attractive idea is that if the CS (which we can call CS1) now has an instructive value does this mean that with a sufficient predictor (CS2) preceding it, that the CS2 can be learnt to generate conditioned responses. In this way, if a sequence of events were to precede the need for action then the predictive value may move to the earliest predictor. This idea cannot be addressed straightforwardly by simply adding a CS2 stimulus that precedes the CS1 stimulus (a series that would be called compound or serial conditioning, Mackintosh 1983) since the US could reinforce the new CS2. To address this issue experimentally requires a different experimental paradigm – second order conditioning. In this paradigm, the additional CS (CS2) is delivered to precede CS1, but only in trials with no US, such that CS2 is never paired with the US. This represents an unnatural situation in life but avoids the complication of direct reinforcement of CS2.

This has been tested, some studies failed to see second order conditioning, but Gibbs et al., 1991 showed that second order conditioning of the rabbit nictitating membrane (NMR) response was indeed possible. However, this study provided no information on the timing of responses but simply counted a CR as a greater than 0.5mm NMR deflection in an 8-second response window. The study did not report onset and peak latencies and no detailed topography of the blink response.

In light of the recent findings, a detailed study of second order conditioning of the eyeblink response was necessary. We sought to answer several questions by undertaking this experiment in rabbits: (i)
is second order conditioning possible; (ii) if so, does this second order association grow with increased training; (iii) how strong is the association between CS2 and CS1 and how often does CS2 generate conditioned responses in a well-trained animal; (iv) what is the relationship between the CS2 response timing to predicted US time, does the response appear maximal at expected time of US?; (v) what is the activity profile of cells in lobule HVI that are responsible for this behaviour?

The work presented in this chapter shows the attempts to define the behavioural aspect of second order eyeblink conditioning in rabbits, and as such answers i-iv above. Initially, a technique utilising restraint was used in a small number of animals, however, due to changes in the regulatory process we were no longer able to use restraint. A freely moving technique was attempted, but this led to several difficulties, discussed later. As such the goal of conducting neurophysiological experiments while training was not achieved so (v) above cannot be addressed here.

5.2 Materials and Methods

5.2.1 Animals

Male New Zealand White rabbits (2.5-3kg) were used in these experiments. Three animals were used in the restrained conditioning chamber experiments. Two of these animals (designated subject 1 and 3 in results text) had taken part in a previous experiment and had been implanted with guide cannulas to target the eyeblink region of lobule HVI of the cerebellum. In this previous experiment the animals were used as learning controls, receiving delivery of saline and CNQX to test cannula location prior to the second order experiments. Previous conditioning sessions meant that these subjects had reached asymptote for primary learning to the CS1. The third animal (subject 2) was naïve to conditioning from the beginning of the experiment.

Six animals were used for the unrestrained conditioning experiments. These animals were naïve to the experiments but had been implanted with guide cannulas for use in previous experiments, although infusions were never made.

5.2.2 Conditioning chamber and mechanical recording apparatus – restrained animals

Conditioning experiments took place in a sound-attenuated chamber while the animals were restrained in a Perspex stock. The stock was snug-fitting around the body, and the head was held in place by soft clamps that held the ears against a foam support. Ambient background noise produced by ventilation fans was set at 61dB. To transduce movement of the nictitating membrane a fine nylon loop was sutured into the right nictitating membrane after application of a local anaesthetic agent (proxymetacaine hydrochloride, 0.5% w/v). This allowed connection of the membrane to a low-torque potentiometer that was fastened onto the head via an attachment. Movements of the
potentiometer generated a change of voltage that was calibrated to determine movements of the membrane with a resolution of less than 100 microns. To allow the delivery of an electrical periorcular stimulus two stainless steel clips were attached to the skin; one adjacent to the temporal canthus of the eye, the other immediately below the centre of the lower eyelid.

The primary conditional stimulus (CS1) was a 1kHz sine wave tone of 410ms duration and delivered at an intensity of 81dB. In the first order conditioning trials the CS1 was paired with a periorcular unconditioned electrical stimulus (US) that was a 60ms train of three 20ms biphasic pulses, which began 350ms after the CS such that the stimuli were co-terminating. The intensity of the US was set at that which caused a ≥4mm reflex movement (UR) of the nictitating membrane (1.5-2.5mA).

In second order conditioning trials, the CS1 was preceded by a secondary conditional stimulus (CS2) that consisted of a three flashing white LED arrays that flashed with a pulse duration of 20ms with a total of 11 pulses at 25Hz, which generated a stimulus length of 420ms. The CS2 to CS1 inter-stimulus interval was set at 350ms such that CS1 onset began 350ms after CS2 onset, so they overlapped by 60ms. The trials were given in a sequential pattern of a CS1-US trial, a CS2-CS1 trial then repeated for five times followed by a single CS2 alone test trial, such that every 11th trial was a CS2 alone test trial (Fig. 5-1, below). The intertrial interval was set to 25s with an additional random delay of 0-5s, giving intervals in the range of 25-30s. A session consisted of 25 CS1-US trials, 25 CS2-CS1 trials, 5 CS2 alone trials for subjects 1 and 3. Subject 2 received another full sequence bringing

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**Figure 5-1:** Order of trials in second order conditioning experiments

The sequence consisted of a CS1-US trial followed by a CS2-CS1 trial, this was repeated 5 times. Then a CS2 alone trial was given. The interval trials was set to be random but constrained between 25-30 seconds. CS1 to US interval = 350ms, CS2 to CS1 interval = 350ms. Total number of trials in a session differed by subject, refer to text.
the total trial numbers to 30, 30 and 6 respectively. This represents one session of conditioning that lasted 45 minutes and was given once a day.

In the presentation of the results, these sessions have been grouped together into blocks of 3 to condense down the large number of sessions given and to provide a level of smoothing to the response percentages presented.

5.2.3 Unrestrained conditioning and infrared recording apparatus

Six animals were conditioned in an unrestrained manner. The transducer mechanisms used in restrained animals was not suitable for unrestrained animals, so a mechanism to monitor external eyelid blinks was developed. This setup consisted of placing the subject in a roughly 1m³ chamber with an open front facing towards the experimenter. The rabbit was allowed to move freely within this chamber. The rabbit had a custom-made fabric cap placed over the head and fastened around the ears and lower jaw. Stabilised on top of this cap was an apparatus both for transducing external eyelid position and a separate tube to deliver air puffs to the cornea. Eyelid position was transduced by monitoring the voltage across an infrared photodiode that was aimed at the cornea which was flanked on either side by infrared emitting LEDs. As the eyelid closes the amount of infrared reflectance reaching the photodiode increases thus lowering resistance and the voltage drop across the photodiode.

Conditioning consisted of primary CS1-US trials with an interstimulus interval of 350ms and second order conditioning trial consisting of a CS2-CS1 pairing with an interstimulus interval of 450ms. Interleaved with these were single unpaired CS1 and CS2 test trials. For three animals CS1 consisted of a 1kHz tone set at 81dB, whilst for the remaining three this stimulus was the CS2. For three animals the CS2 consisted of a flashing LED array distributed around the chamber, whilst for the remaining three this stimulus was the CS1. The LED flashed at 10Hz with a 50% duty cycle. The US was composed of an air puff (2-3 bar) to the cornea from a pressured air source and was gated by a solenoid valve. The valve opened in response to a 5V pulse that lasted 100ms to deliver the air puff through a 1mm diameter tube positioned 1-2 cm from the eye.

A CS1-US trial was followed by a CS2-CS1 trial, and this was repeated five times and then followed by a CS2 alone trial and then a CS1 alone trial. A session consisted of 30 CS1-US pairings, 30 CS2-CS1 pairings, 6 CS2 alone trials and 6 CS1 alone trials. A foot switch was placed under the conditioning chamber that required the experimenter to press at 10-second intervals to proceed with conditioning; this allowed the opportunity to pause to allow the animal to settle if excessive movement about the chamber occurred. A session was given once a day.
After 18 sessions of second order conditioning 4 session of solely first order conditioning were given in an attempt to strengthen the association between the CS1 and the US. These consisted of 90 CS1-US trials and 10 CS1 alone trials per session. After these 4 sessions second order conditioning was resumed as normal.

5.2.4 Conditioned response (CR) definition

For the restrained animals, a CR was defined as a movement of the nictitating membrane with an amplitude ≥0.5 mm and with onset latency >35ms from CS onset. Onset latencies of <35ms caused the trial to be rejected as they represent startle alpha response and not conditioned blinks. These had to occur within the CS1–US interval (350ms) or CS2 to expected US time (700 ms for both CS2-CS1 and CS2 alone trials) Onset latency was taken as the point at which the movement crossed the 0.5mm threshold. CR frequency (% CRs) and onset latency were calculated for all trials (CS2-CS1, CS1-US and CS2 alone) throughout the conditioning sessions. Peak latencies were calculated for unpaired (CS2 alone) trials only.

In the freely moving animals, the infrared recording system was more complex to use as the external eyelid was represented by an arbitrary voltage rather than absolute closure in distance or area measurements. The CRs were defined by taking a 400ms period prestimulus onset (CS1 in CS1-US, and CS2 in CS2-CS1 or CS2 alone) and determining an average value and standard deviation of changes in the infrared signal during this prestimulus period. If at any point after stimulus onset, up to 350ms following a CS1 and 900ms following a CS2, the waveform crossed a threshold of the average value plus 5 standard deviations a CR was deemed to have taken place. Onset time was recorded as the time the threshold was crossed. Onset latencies of <35ms caused the trial to be rejected as they represent startle alpha response and not conditioned blinks.

All average CRs presented subsequently are aligned relative to the CS1 onset time (given as time 0) and are plotted with ± SEM.
5.3 Results – Restrained animal experiments

The results for the three restrained animals are presented below in subject by subject manner. The percentages of responses to the various trial types are presented by blocks, each consisting of the average percentage of responses from 3 separate sessions of conditioning. The grouping of sessions into blocks aides in the presentation of the results graphically as it condenses a large number of sessions down into a more presentable form with an estimation of the standard error around the mean percentage of responses. The statistics - largely binomial logistic regression models that are described in Chapter 2 are all done on the data from individual sessions. Where the statistics have made use of the trial number, this refers to the number of the trial within a given session, with respect to the total number of trials given for that stimulus type in a session. To clarify, for subject 1 and 3 there were 25 CS1-US trials and 25 CS2-CS1 trials given in a session with 5 CS2 alone trials. For subject 2, there were 30 CS1-US trials and 30 CS2-CS1 trials with 6 CS2 alone trials.

5.3.1 Conditioned responses are made to CS2, but responses are variable

5.3.1.1 Subject 1

The results of second order conditioning were varied among the three subjects. Fig. 5-2A shows the percentage of responses to the different stimulus types for subject 1. The responses to the CS1-US and CS2-CS1 started off at the upper 100% boundary due to reaching an asymptote in previous experiments. The subject showed no conditioned responses to the CS2 alone stimulus in the first block of 3 sessions but showed an increase in conditioned responses across blocks in a non-linear manner. The subject made conditioned responses at 100% level to the CS2 alone stimulus by the final block. This demonstrates achievable second order conditioning of the eyeblink response.

The averaged responses to the different stimulus types are shown in Fig. 5-2B and C, the responses that are made to CS1-US and CS2-CS1 in the first session of block 1 are shown in the top row and demonstrate that the responses are closely tied in shape, and start within the CS1 to US interval, consistent with the response being driven by the presentation of the CS1 stimulus. CS2 alone clearly shows a flat averaged response. In Fig. 5-2C the averaged responses are from the last session in block 8. There is a clear demarcation between the responses to CS1-US and CS2-CS1, with the later making significant deviations from baseline during the CS2 to CS1 interval. The CS2 alone stimulus also now leads to a blink response that is similar in shape and onset to the CS2-CS1 responses, this is consistent with the CS2 alone being sufficient for generating conditioned responses through the association with the CS1 stimulus. The CS2 alone response peaks at the time of onset of the CS1 stimulus.
Figure 5-2: Second order conditioning in subject 1
(A) Percentage of CRs made to each respective trial type across blocks (consists of 3 sessions). Colour key at top of the page. CS1-US (red) and CS2-CS1 (blue) were at asymptote due to first order training in a previous experiment. The number of responses made to CS2 alone (green) increased as training progresses. Error bars ± SEM
(B) Average responses to trial types in the very first session. Responses to CS1-US trials and CS2-CS1 trials are consistent with being related to the CS1 stimulus. Grey area ± SEM
(C) Average responses from session 24, the final session of block 8. CRs to CS1-US and CS2-CS1 are now different with the later starting before CS1 starts. CS2 alone responses are large and now peak shortly after where CS1 would onset. Grey area ± SEM
(D) The observed percentage of responses to CS2 alone by session (green dots) plotted with the predicted percentages from a binomial logistic regression model (green line)
(E) The observed percentage of responses to CS2 alone by trial number within sessions (green dots) plotted with the predicted percentages from a binomial logistic regression model. This all trial numbers independent of session (green line)
A logistic regression model (see Chapter 2) was fit to the data in a backwards stepwise manner to determine the influence of session number and trial number on the probability of success in the CS2 alone trials. Both session and trial number were modelled as continuous variables, as it is a learning task and we would expect improvements in performance to increase with the amount of practice. The trial number was included as a predictor as it was noticed that some subject’s conditioned responses to CS2 alone seemed to be made to the first few test trials and decreased in number throughout the session. The interaction term of session number x trial number was found to be insignificant and was dropped from the model (p > 0.05). Table 5-1 shows the estimated coefficients and associated odds ratio, only session number exhibited a significant effect upon making CR in response to CS2 alone stimulus presentation (β = 0.12, Z = 4.08, OR = 1.13, p < 0.001). This represents a 13% increase in the odds of making a CR for each 1 unit increase in session number. The effect of the trial number within a session was found to be insignificant (p > 0.05).

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Table 5-1: Coefficients and odds ratio associated with the session and trial number predictors from a logistic regression model fit to subjects 1’s observed CS2 alone responses. Significance: * p < 0.05, ** p < 0.01, *** p < 0.001

Fig. 5-2D and E show the logistic regression model’s estimated percentage responses against those observed for session (D) and trial number within a session (E). It can be seen that observed values show a general increase towards 100% as session number increases, which are reflected in the estimated percentages but the observed percentages show some non-linearity with increases in percentage fall backing to 0% for some sessions. The predicted values for trial number percentages are more closely matched to the observed, but these only show a slight decrease as trial number increases which was found to be insignificant.

5.3.1.2 Subject 2

The results of second order conditioning for subject 2 are shown in Fig. 5-3A, overleaf. This subject had not undergone any first order conditioning before the experiment in contrast to the other subjects. The subject only makes conditioned responses to 4.1% of CS1-US trials in the first block and to 4.2% of the CS2-CS1 trials, in both cases the subject showed 0% of responses to either trial type in the very first session of the block. The progressive increase in conditioned responses to the CS1-US trial are consistent.
Figure 5-3: Second order conditioning in subject 2
(A) Percentage of CRs made to each respective trial type across blocks consisting of 3 sessions. Colour key at top of the page. CS1-US (red) and CS2-CS1 (blue) began at 0% as this animal was naïve to training. The number of responses made to CS2 alone (green) increased after block 8. Error bars ± SEM
(B) Average responses to trial types in the fourth session. Responses to CS1-US trials and CS2-CS1 trials are consistent with being related to the CS1 stimulus. Grey area ± SEM
(C) Average responses from session 27, in final block, CRs to CS1-US and CS2-CS1 are now different with the latter starting before CS1 onsets. CS2 alone responses are relatively small but they peak shortly after where CS1 would onset. Grey area ± SEM
(D) The observed percentage of responses to CS2 alone by session (green dots) plotted with the predicted percentages from a binomial logistic regression model (green line)
(E) The observed percentage of responses to CS2 alone by trial number within the sessions (green dots) plotted with the predicted percentages from a binomial logistic regression model (green line)
with normal learning and approach asymptote at block 3. The responses to CS1-US and CS2-CS1 trials are similar in onset and morphology, as shown in the blink response averages from session 4 from block 2 in Fig. 5-3B. The responses are initiated in the CS1 to US interval for both stimulus types which is consistent with the subject learning the first order association. The CS2 alone stimulus shows no responses in the average and as shown in Fig. 5-3A remains at 0% response rate for the first few blocks, only increasing after block 5. Blocks 9 and 10 have response rates of 50 and 44.4%, respectively, to the CS2 alone trials.

The averaged response by trial type shown in Fig. 5-3C takes responses from session 27 in the final block and show a clear response to CS2 alone that begins in the CS2 to CS1 interval. This CS2 alone response now peaks shortly after the time where the CS1 stimulus would onset if it were presented as a pair. Similarly, the CS2-CS1 trial response has shifted in onset, and an initial part is driven by the CS2 element, separating it from the response to CS1-US.

A logistic regression model was fit to subject 2’s observed data in a backwards stepwise manner using the session number and trial number as a predictor of making a CR in the CS2 alone trials. The interaction term session x trial number was found to be insignificant (p > 0.05) so it was dropped from the model. Table 5-2 shows the coefficients and associated odds ratio for the predictors. Session number was found to be significant ($\beta = 0.19, Z = 4.65, OR = 1.21, p < 0.001$) and associated with a 21% increase in the odds of making a conditional response to CS2 alone trials for each single unit increase. Trial number was also found to have a significant effect on the probability of making a CR in response to CS2 alone trials ($\beta = -0.41, Z = -2.60, OR = 0.71, p < 0.01$) representing a 29% decrease in the odds of making a conditional response with each one unit increase in trial number. For subject 2 it was more likely that a conditional response would be made to the first CS2 alone trial than to the last trial within an individual session. This perhaps reflects a consolidation effect between sessions but an antagonistic process during the sessions that make CS2 alone CRs less likely.

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Table 5-2: Coefficients and odds ratio associated with the session and trial number predictors from a logistic regression model fit to subjects 2’s observed CS2 alone responses. Significance: *p < 0.05, **p < 0.01, ***p < 0.001
Fig. 5-3D and E show the observed percentage of responses to CS2 alone trials against those predicted by the model for session number (D) and trial number (E). The percentage of responses increase as session number increases and is consistent with the subject making a learned association between CS2 and CS1, and subsequently with CS2 alone becoming sufficient to drive a response. The response may not have reached horizontal asymptote by session 30 and may have continued to rise. The observed response percentages with trial number show a clear decreasing trend that is captured well by the predicted values.

5.3.1.3 Subject 3

Subject 3 exhibited a different pattern of responses to second order conditioning in contrast with the other two subjects. Fig. 5-4A, overleaf, shows the progression of response rates across blocks of sessions for subject 3. This subject had been previously trained under a first order protocol and had reached asymptote for CS1-US trials with a 100% response rate. The CS1-US response rate remains high throughout the blocks of training sessions. However, the response rate in CS2-CS1 while at the same level initially decreases after block 4 and continues to decrease to 50.3% at block 8. The response rate to CS2 alone was initially low, some responses appeared, but then disappeared with further training.

The average responses to each stimulus type are shown in Fig. 5-4B and C, with responses from a session in the first block (B) showing that both CS1-US and CS2-CS1 stimuli produce a similar blink response in latency and shape, consistent with CS1 evoked responses. CS2 alone does not produce any deviations of the nictitating membrane. The responses from a session in the last block are shown in the bottom row of Fig. 5-3C, they show a clear difference between the response to CS1-US trials and CS2-CS1 trials with the latter showing a response much reduced in amplitude compared to the CS1-US trials. CS2 alone trials once again do not produce deviations in the nictitating membrane as recorded by the mechanical transducer.

A logistic regression model was fit in a backward stepwise manner to subjects 3’s observed data for making conditioned responses to CS2 alone trials. The interaction between session number and trial number was found to be insignificant (p > 0.05) and as such was dropped from the model. Table 5-3, shows the estimated coefficients and associated odds ratio for the session number and trial number predictors. For subject 3 any changes in session number and trial number were not found to significantly affect the probability of making a conditioned response to CS2 alone trials (p > 0.05).

Fig. 5-4D and E show the observed percentage response rates against those estimated by the model for session number (D) and trial number (E). These show no significant change with increasing session or trial number.
Figure 5-4: Second order conditioning in subject 3
(A) Percentage of CRs made to each respective trial type across blocks consisting of 3 sessions. Colour key at top of the page. CS1-US (red) and CS2-CS1 (blue) began at 100% as this animal had already been trained to CS1. The number of responses made to CS2 alone (green) only increased marginally in block 3 but fell after. Error bars ± SEM
(B) Average responses to trial types in the fourth session. Responses to CS1-US trials and CS2-CS1 trials are consistent with being related to the CS1 stimulus. Grey area ± SEM
(C) Average responses from session 24, in final block, CRs to CS1-US and CS2-CS1 are now different with a greatly reduced response to CS2-CS1 trials. CS2 alone responses are absent Grey area. ± SEM
(D) The observed percentage of responses to CS2 alone by session (green dots) plotted with the predicted percentages from a binomial logistic regression model (green line)
(E) The observed percentage of responses to CS2 alone by trial number within the sessions (green dots) plotted with the predicted percentages from a binomial logistic regression model (green line)
5.3.2 Development of early responses to CS2-CS1 trials

The averaged responses to CS2-CS1 stimulus trials were found to onset earlier in later sessions for subjects 1 and 2. It was common for many CS2-CS1 responses to begin in the CS2 to CS1 interval, suggesting that the CS2 by itself was sufficient to generate a conditioned response. Indeed the CS2 alone response in subject 1 and 2 suggest that this is the case. The onset times for responses to the CS1-US and CS2-CS1 trials were examined to determine if this shift in onset developed with increased training, as would be expected by a learned association.

5.3.2.1 Subject 1’s onset latencies to CS2-CS1

Subject 1’s onset times relative to session number are shown in Fig. 5-5, the CS1-US (A) onset times remain relatively consistent but the CS2-CS1 (B) onset times show a separation after session 3-4. This response is bimodal, either having an onset consistent with CS1-US times or starting much earlier in the CS2 period for most of the sessions.

The onset times relative to the trial number are shown in Fig. 5-D and E for CS1-US trial and CS2-CS1 trials respectively. The increasing trial number shows no effect on CS1-US onset times. The CS2-CS1 onsets, however, show a consistent bimodal response latency across trial numbers, either being comparable to CS1-US latency onset or starting much before 0ms relative to CS1 stimulus onset.

To determine the effects of session number and trial number on onset times in CS2-CS1 trials a logistic regression model was constructed. To create a logistic regression model in this instance the trials were categorised as early (before CS1 onset < 0ms = 1) or not early (after CS1 onset ≥ 0ms = 0). A simple linear model on the continuous onset times could have been constructed, but the onsets show a bimodal distribution in relation to the session number so investigating the relationship between the predictors and the probability that the response comes early seemed more appropriate. Similarly, we can be certain that CS2 must initiate a response that starts before 0ms. A backwards stepwise approach was taken to fitting the model with all predictors, assessing the significance of the interaction term session number x trial number before including it in the model or not. The coefficients and associated odds ratios are shown in Table 5-4, overleaf. The effect of session number by itself

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Table 5-3: Coefficients and odds ratio associated with the session and trial number predictors from a logistic regression model fit to subjects 3’s observed CS2 alone responses. Significance: * p < 0.05, ** p < 0.01, *** p < 0.001
Figure 5-5: Change subject 1’s in CS2-CS1 onset latencies as conditioning progresses
(A) CS1-US onset latencies by session number. These show very little change as session number increases. Dotted line denotes CS1 stimulus onset time. It would not be possible for a CR to begin before this time for a CS1-US trial.
(B) CS2-CS1 onset latencies by session number. These show a sudden appearance of onset values before the CS1 stimulus onset time, which increase in number as conditioning progresses. Dotted line denotes CS1 stimulus onset time.
(C) Probabilities of making an early onset CR (<0ms) in CS2-CS1 trials by session number. Blue dots represent the observed probabilities and the blue line shows the predicted probabilities from a logistic regression model fit to subject 1’s observed data
(D) CS1-US onset latencies by trial number within a session. Dotted line denotes CS1 stimulus onset time.
(E) CS2-CS1 onset latencies by trial number within a session. These show a subtle decline in the number of early onsets with higher trial numbers within sessions. Dotted line denotes CS1 stimulus onset time.
(F) Probabilities of making an early onset CR (<0ms) by trial numbers within sessions. Blue dots show observed probabilities and blue line shows predicted probability from a logistic regression model.
was not found to be significant (p > 0.05), but was significant in an interaction with trial number (β = 0.005, Z = 3.21, OR = 1.005, p < 0.01). This represents a small 0.5% increase in the odds of being early for each one unit increase in session x trial number but because this a multiplicative interaction this suggests that later sessions, and later trials within those sessions, were more likely to have an early onset before 0ms. The effect of trial number alone was negative on the odds of making an early response (β = -0.09, Z = -3.40, OR = 0.91, p < 0.001), representing a 9% decrease in the odds of early response for each one unit increase in trial number. Fig. 5-5C and F show the predicted probability of making an early response against the observed, for session number trial number is held at 1 and vice versa for trial number plot. The outcomes of the model for early CS2-CS1 probabilities are similar to the model for the probability of making a conditioned response to CS2 alone, showing effective second order conditioning in subject 1.

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Table 5-4: Coefficients and odds ratio associated with the session and trial number predictors from a logistic regression model fit to subjects 1’s observed probabilities of making early CRs during CS2-CS1 trials. Significance: * p < 0.05, ** p < 0.01, *** p < 0.001

5.3.2.2 Subject 2’s onset latencies to CS2-CS1 trials

Subject 2’s onset times by trial type, as shown in Fig. 5-6 were examined. CS1-US trial onset times (Fig. 5-6A) show a general decrease as the number of sessions increase until they stabilise around session 15. This is consistent with subject 2 learning the association between the CS1 and the US as this animal was naive to all training. The CS2-CS1 onset times show a similar trend (Fig. 5-6B), but after session 15 there is the emergence of response onsets that begin before the CS1 portion of the stimulus. These early responses are not as demarcated as subject 1 but there is a clear separation on several sessions. The onset of CS2-CS1 trials relative to trial number show a decreasing number of early responses as trial number progresses (Fig. 5-6E), and a reversion to onset times similar to those in response to CS1-US trials (Fig. 5-6D). This reflects that subject 2 was more likely to make earlier responses at the beginning of sessions.

The relationship between the probability of making an early response during CS2-CS1 trials and session number and trial number was explored with a logistic regression model. A full model was fit
Figure 5-6: Change subject 2’s in CS2-CS1 onset latencies as conditioning progresses
(A) CS1-US onset latencies by session number. These show changes as subject 2 begins to learn the first order association and shift earlier in time as session number progresses before they settle at around session 15. Dotted line denotes CS1 stimulus onset time. It would not be possible for a CR to begin before this time for a CS1-US trial.
(B) CS2-CS1 onset latencies by session number. These show a gradual shift to earlier onset values, which increases as conditioning progresses. Eventually more and more CRs onset before the CS1 stimulus begins. Dotted line denotes CS1 stimulus onset time.
(C) Probabilities of making an early onset CR (<0ms) in CS2-CS1 trials by session number. Blue dots represent the observed probabilities and the blue line shows the predicted probabilities from a logistic regression model fit to subject 2’s observed data.
(D) CS1-US onset latencies by trial number within a session. Dotted line denotes CS1 stimulus onset time.
(E) CS2-CS1 onset latencies by trial number within a session. These show an obvious decline in the number of early onset responses with higher trial numbers within sessions. Dotted line denotes CS1 stimulus onset time.
(F) Probabilities of making an early onset CR (<0ms) by trial numbers within sessions. Blue dots show observed probabilities and blue line shows predicted probability from a logistic regression model.
first with an interaction term between session number and trial number but this interaction was dropped after it was found to be insignificant (p > 0.05). The coefficients and associated odds ratio with the predictors are shown in Table 5-5, below. Session number was found to be a significant predictor of making an early response in CS2-CS1 trials (β = 0.21, Z = 8.92, OR = 1.23, p < 0.001) and was associated with a 23% increase in the odds of making an early response for each single unit increase in session number. Trial number was also found to be a significant predictor of making an early response to CS2-CS1 stimulus (β = -0.10, Z = -6.23, OR = 0.90, p < 0.001), but in this case increasing trial number is negatively associated with making an early response with a single unit increase in trial number leading to a 10% decrease in the odds of an early response.

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</table>

Table 5-5: Coefficients and odds ratio associated with the session and trial number predictors from a logistic regression model fit to subjects 2's observed probabilities of making early CRs during CS2-CS1 trials. Significance: * p < 0.05, ** p < 0.01, *** p < 0.001

The expected and observed probabilities for response onset to CS2-CS1 are shown for session numbers and trial numbers (Fig. 5-6C and F). The effects of session number and trial number on the probability of making an early response to CS2-CS1 trials are similar to those observed for making a CR to CS2 alone trials for subject 2, indicating that the early onsets to CS2-CS1 are likely generated by the same process as responses to CS2 alone.

5.3.2.3 Subject 3's onset latencies to CS2-CS1 trials

Subject 3’s onset latencies in response to CS1-US and CS2-CS1 trials are shown in Fig. 5-7. The relationship between session number and latency is shown for CS1-US (Fig. 5-7A) and CS2-CS1 (Fig. 5-7B). The CS2-CS1 onsets are mainly consistent with the onset times shown for the CS1-US trials. However, there are several sessions, such as 7-14, where there a few trials where the response begins before 0ms, but this does not progress with increased training. The response onsets by trial number (Fig. 5-7D and E) show that for CS2-CS1 earlier trial numbers have a higher number of early responses than later trial numbers which mainly appear similar in latency to CS1-US trials.
Figure 5-7: No change in subject 3’s CS2-CS1 onset latencies as conditioning progresses

(A) CS1-US onset latencies by session number. These show only the day-to-day variation in CS1 CR onset latency. Dotted line denotes CS1 stimulus onset time. It would not be possible for a CR to begin before this time for a CS1-US trial.

(B) CS2-CS1 onset latencies by session number. These show a gradual shift to earlier onset values, with a small number of trials being made early. This effect seems to fade and trial onsets mostly move back to > 0ms. Dotted line denotes CS1 stimulus onset time.

(C) Probabilities of making an early onset CR (<0ms) in CS2-CS1 trials by session number. Blue dots represent the observed probabilities and the blue line shows the predicted probabilities from a logistic regression model fit to subject 3’s observed data.

(D) CS1-US onset latencies by trial number within a session. Dotted line denotes CS1 stimulus onset time.

(E) CS2-CS1 onset latencies by trial number within a session. These show a decline in the small number of early onset responses made during earlier trials. Dotted line denotes CS1 stimulus onset time.

(F) Probabilities of making an early onset CR (<0ms) by trial numbers within sessions. Blue dots show observed probabilities and blue line shows predicted probability from a logistic regression model.
A logistic regression model was fit to the data to determine the influence of predictors on making an early response during CS2-CS1 trials in a backwards stepwise manner. The interaction term between session number and trial number was found to be insignificant (p > 0.05) and was dropped from the model. Table 5-6 shows the coefficients and associated odds ratios with the predictors included in the model. Session number showed non-significant effects on the odds of making an early response in CS2-CS1 trials (p > 0.05), suggesting that increasing number of training sessions were not associated with earlier responses. The trial number showed a significant effect on the odds of making an early response ($\beta = -0.08$, $Z = -2.76$, OR = 0.93, $p < 0.01$) and was associated with a 7% decrease in the odds of making an early response with each one unit increase in trial number.

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*Table 5-6: Coefficients and odds ratio associated with the session and trial number predictors from a logistic regression model fit to subjects 3’s observed probabilities of making early CRs during CS2-CS1 trials. Significance: * p < 0.05, ** p < 0.01, *** p < 0.001*

Fig. 6C and F show the observed session number and trial number probabilities of making an early conditioned response for subject 3. The session numbers show that in a few observed sessions there were some early responses and these are consistent with the sessions in which conditioned responses are made to CS2 alone trial in Fig. 7.3, suggesting that responses may have been made to the CS2 but this did not progress further with training, possibly as competing processes took over to dampen the response, as discussed in 5.3.4.

5.3.3 Peak latencies for CS2 alone responses for Subject 1 and 2

The shift in onset latency of CS2-CS1 trials for subject 1 and 2 lead to the examination of peak latencies of CS2 alone trials as these would show peaks without further CS1 related responses. If second order conditioning were to be protective against the original US, such that the CS2 could predict the US even though it had not been paired with it, we would expect the blink response to be maximal at the time of the expected US.

Fig. 5-8 shows the distribution of the peak latencies in response to CS2 alone trials for subject 1. The majority of the responses peak much earlier than 350ms, the time that an expected US would appear. There appears to be a bimodal distribution with the bins centred on -100 to -80 and 60 to
80ms having the highest counts. The median peak response latency is 20ms with a range from -162 to 335ms.

Subject 2’s peak latencies in response to CS2 alone are shown in Fig. 5-9. There are fewer responses as subject 2 had not achieved a response rate as high as subject 1. The majority of the latencies are

Subject 1 made a large number of responses to CS2 alone, by the final sessions they were responding to 100% of trials. The majority of these responses peaked just shortly after the time that the CS1 would begin, if it were to be presented with the CS2. Bin width = 20ms.

Subject 2 made fewer CRs to CS2 alone trials than subject 1 but the majority of these responses still peaked far earlier than the time of the expected US. Most peaked shortly after the time where a CS1 would begin if it was presented together with the CS2. Bin width = 20ms.
much earlier than 350ms showing that CS2 alone responses peak much earlier than the time of expected US. The distribution again shows two modes with the bins centred on 80 and 180ms having the highest counts. The median response peak latency is 105ms with a range from -49 to 347ms.

5.3.4 Subject 3’s response amplitude and response rate declined in CS2-CS1 trials

Subject 3 exhibited a different effect of the second order conditioning from that of the other subjects. Subject 3 showed a noticeable decline in response rate to CS2-CS1 as the conditioning continued and also showed a greatly reduced amplitude in CS2-CS1 trials in later conditioning sessions.

![Graph showing response rate and amplitude over sessions](image)

**Figure 5-10:** Subject 3 made fewer responses to CS2-CS1 trials as training progressed but CS1-US responses were unaffected

Dots are the observed data points for each session and trial type and the lines are the predicted values from a binomial logistic regression model with session number, trial type and an interaction term between the two. Subject 3 started making fewer conditional responses in CS2-CS1 trials as training progressed.

The responses to the CS1-US and CS2-CS1 trials by session number were explored with a logistic regression model. The regression model was constructed with the predictors of session number and trial type (CS1-US or CS2-CS1) to determine their effect on making conditional responses. The coefficients and associated odds ratio of the model are shown in Table 5-7, overleaf. A significant interaction was found between the CS2-CS1 indicator and session number ($\beta = -0.19$, $Z = -4.08$, OR = 0.82, $p < 0.001$) which represents an 18% decrease in the odds of making a conditional response for
each single unit increase. Session number by itself does not have a significant effect (p > 0.05) on the odds of making a conditioned response, and neither does CS2-CS1 trial type indicator (p > 0.05). The observed values and those predicted by the model are shown in Fig. 5-10 above. It is clear that there is a decrease in the number of conditional responses being made to CS2-CS1 in much later sessions, but at the beginning of training, this difference between CS1-US and CS2-CS1 response rates was absent.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient (SE)</th>
<th>95% CI for Odds Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Intercept)</td>
<td>4.19 (0.62) ***</td>
<td></td>
</tr>
<tr>
<td>Trial type (CS2CS1)</td>
<td>1.05 (0.78)</td>
<td>0.62 - 2.87</td>
</tr>
<tr>
<td>Session</td>
<td>-0.02 (0.04)</td>
<td>0.90 - 0.98</td>
</tr>
<tr>
<td>Trial type X Session</td>
<td>-0.19 (0.05) ***</td>
<td>0.75 - 0.82</td>
</tr>
</tbody>
</table>

Table 5-7: Coefficients and odds ratio associated with the session and trial type predictors from a logistic regression model which was fit to subjects 3’s observed probabilities of making a CRs during CS2-CS1 and CS1-US trials. Significance: * p < 0.05, ** p < 0.01, *** p < 0.001

The second order conditioning had a similar effect on the amplitude of CS2-CS1 responses. Shown in Fig. 5-11 are the response amplitudes for CS1-US (left) and CS2-CS1 trials (right) relative to session number. A multiple linear regression model was calculated to predict blink amplitude based on the trial type and session number. An interaction term was also included in the model. A significant regression equation was found (F(3, 1263) = 106.2, p < 0.001) with an R² of 0.20. Subject 3’s predicted blink amplitude is equal to 3.99 + trial type x 1.67 + session number x 0.02 – (trial type x session number x 0.19), where trial type is coded as 1 for CS2-CS1 and 0 for CS1-US trials and session number is a continuous variable. All independent variables were found to be significant (p < 0.05). Both the observed data and model prediction show a clear decline in amplitude of the blink response to CS2-CS1 trial as the training progressed. This decrease in amplitude in conjunction with the decreased response rate to CS2-CS1 trials suggest that subject 3 learned to associate the CS2 with an absence of US following CS1 rather than an association between CS2 and CS1. Such a process is known to exist in the form of conditioned inhibition and is discussed in relation to these results later.
Figure 5.11: Conditioned response amplitudes to CS1-US and CS2-C1 by session number. Dots are the observed amplitudes, in millimetres, of movements of the nictitating membrane when making a conditioned response. The lines are the predicted values given by a linear regression model (see text for details of the coefficients).
5.4 Results - Unrestrained second order conditioning experiments

Due to regulatory limitations it became impossible to restrain the rabbits during conditioning. An attempt was made to conduct second order conditioning in a group of 6 rabbits that were able to move during the experiment freely. This experiment was very similar to that used the restrained rabbits regarding the order of trial sequence except that the after the CS2 alone trial a CS1 alone trial was given to test the emergence of first order responses as all animal were naïve. There were 30 trials of CS1-US, 30 CS2-CS1 per session and 6 of the two of the CS alone stimuli. As before, to represent the results graphically, sessions have been grouped into blocks containing 3 sessions, except blocks 7 and 8 that represent 2 sessions each of only first order conditioning. This was given in an attempt to strengthen first order associations. The CS2-CS1 interval is now 450ms, and CS1-US remains at a 350ms interval.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient (SE)</th>
<th>95% CI for Odds Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lower</td>
</tr>
<tr>
<td><strong>SOUND PRIMARY</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Intercept)</td>
<td>-1.62 (1.00)</td>
<td></td>
</tr>
<tr>
<td>Session number</td>
<td>-0.13 (0.06) *</td>
<td>0.78</td>
</tr>
<tr>
<td>Trial number</td>
<td>-0.03 (0.22)</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Intercept)</td>
<td>-2.66 (7.25 x 10^4)</td>
<td></td>
</tr>
<tr>
<td>Session number</td>
<td>-1.43 x 10^-15 (2.49 x 10^3)</td>
<td>0.00</td>
</tr>
<tr>
<td>Trial number</td>
<td>-7.71 x 10^-15 (1.48 x 10^4)</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Intercept)</td>
<td>-2.07 (0.83) *</td>
<td></td>
</tr>
<tr>
<td>Session number</td>
<td>-0.05 (0.03)</td>
<td>0.90</td>
</tr>
<tr>
<td>Trial number</td>
<td>-0.02 (0.18)</td>
<td>0.69</td>
</tr>
<tr>
<td><strong>LIGHT PRIMARY</strong></td>
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<td></td>
</tr>
<tr>
<td>(Intercept)</td>
<td>-5.80 (1.24) ***</td>
<td></td>
</tr>
<tr>
<td>Session number</td>
<td>0.07 (0.03) *</td>
<td>1.002</td>
</tr>
<tr>
<td>Trial number</td>
<td>0.39 (0.19) *</td>
<td>1.002</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Intercept)</td>
<td>-2.31 (0.95) *</td>
<td></td>
</tr>
<tr>
<td>Session number</td>
<td>-0.10 (0.04) *</td>
<td>0.83</td>
</tr>
<tr>
<td>Trial number</td>
<td>0.12 (0.21)</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Intercept)</td>
<td>-4.44 (1.08) ***</td>
<td></td>
</tr>
<tr>
<td>Session number</td>
<td>0.04 (0.03)</td>
<td>0.98</td>
</tr>
<tr>
<td>Trial number</td>
<td>0.22 (0.19)</td>
<td>0.85</td>
</tr>
</tbody>
</table>

Table 5-8: Coefficients and odds ratio associated with making a conditional response to CS2 alone trials for 6 subjects. Broadly there were two group, 3 animals (subjects 1-3) who had the sound as the primary CS1 and 3 (subjects 4-6) who had a visual light stimulus as their primary CS1. No subjects had significant positive coefficients with the exception of subject 4, which is discussed further in the text. Significance: * p < 0.05, ** p < 0.01, *** p < 0.001
Six animals were trained under a second order conditioning protocol, for 3 of which the CS1 was an auditory sound stimulus, and the CS2 was a visual light stimulus and 3 of which had the reverse. The results of the conditioning across blocks of sessions for the group (subjects 1-3) with the sound stimulus as CS1 are shown in Fig. 5-12 overleaf. The subjects 1-3 showed increases in their response rates CS1-US trials and CS2-CS1 but no increase in response to CS2 alone trials. Individual logistic regression models were fit to each participant’s data to determine if session number or trial number within a session had any effect on the odds of making a conditional response to CS2 alone trials. The coefficients and associated odds ratios are shown in Table 5-8, previous page. Subject 1 did show a significant relationship between session number and making a conditioned response to CS2 alone (β = -0.13, Z = -2.30, OR = 0.88, p < 0.05), but this is a negative effect with a 12% decrease in the odds in with one unit increase in session number. This reflects the higher number of baseline responses in the first sessions that then declined to zero as training progressed. The predictors for all the other subjects were found to be non-significant (p > 0.05).
Chapter 5 | Second order conditioning in rabbits

Figure 5-12: Percentage of conditioned responses to CS1-US, CS2-CS1 and CS2 alone trials for subjects 1 to 3 – Auditory CS1
CS1 alone responses are contained with the CS1-US percentages (blue). Blocks represent 3 sessions of conditioning, except for first order conditioning only in blocks 7 and 8, which represents 2 sessions per block. Each first order session consisted of 90 CS1-US trials and 10 CS1 alone. Mean percentage ± SEM error bars.
TOP: Subject 1’s percentage of conditioned responses to the trial types. Inset top left, colour key: CS1-US trials (red), CS2-CS1 trials (blue), CS2 alone (green). Subject shows a general increase in responses to CS1-US and CS2-CS1 trials that reaches an asymptote after block 4. No increase in CS2 CRs as training progresses.
MIDDLE: Subject 2’s percentage of CRs to the different trial types. Increase in CS1-US and CS2-CS1 responses, no responses at all to CS2 alone.
Bottom: Subject 3’s percentage of CRs to the different trial types. Increase in CS1-US and CS2-CS1 responses, some responses to CS2 alone but no increase with progression of training.
The response rates for the different trial types for the group that had the light stimulus as CS1 (subjects 4-6) are shown in Fig 5-13, overleaf. Again these subjects showed an increase in the number of responses to the CS1-US and CS2-CS1 trials, but there isn’t a noticeable increase in the CS2 alone responses. Table 5-8 shows the coefficients for the logistic regression models fit to individual subject’s data. Subject 4 showed a significant positive effect of session number ($\beta = 0.07$, $Z = 2.03$, OR = 1.07, $p < 0.05$) and trial number ($\beta = 0.40$, $Z = 1.97$, OR = 1.48, $p < 0.05$) on the odds of making a CR to CS2 alone trials, but this effect is not pronounced – representing a modest 7% increase in the odds of making a CR to CS2 alone with each increasing session number. This is largely inconsistent with the behaviour of other subjects where there is either a significant negative or no effect of session numbers. Subject 5 showed a significant effect of session number ($\beta = -0.10$, $Z = -2.35$, OR = 0.90, $p < 0.05$), but this was associated with a decrease in the odds as session number progresses. Subject 6 showed no signs of second order conditioning, but there is a divergence in the percentage of responses to CS1-US and CS2-CS1 response in later blocks. This may reflect a conditioned inhibition response similar to subject 3 of the restrained rabbit group.
Figure 5-13: Percentage of conditioned responses to CS1-US, CS2-CS1 and CS2 alone trials for subjects 4 to 6 – Visual CS1
CS1 alone responses are contained with the CS1-US percentages (blue). Blocks represent 3 sessions of conditioning, except for first order conditioning only in blocks 7 and 8, which represents 2 sessions per block. Each first order session consisted of 90 CS1-US trials and 10 CS1 alone. Mean percentage ± SEM error bars.
TOP: Subject 4’s percentage of conditioned responses to the trial types. Inset top left, colour key: CS1-US trials (red), CS2-CS1 trials (blue), CS2 alone (green). Subject shows a general increase in responses to CS1-US and CS2-CS1 trials.. Possible small increase in CS2 responses in later blocks.
MIDDLE: Subject 2’s percentage of CRs to the different trial types. Increase in CS1-US and CS2-CS1 responses, no increase responses to CS2 alone.
Bottom: Subject 3’s percentage of CRs to the different trial types. Increase in CS1-US and CS2-CS1 responses, some responses to CS2 alone but no increase with progression of training.
Fig. 5-14 shows the average responses from the 37th session and session 38 for subject 4 (top and bottom, respectively), in this, we can see that there were early responses to CS2-CS1 that was consistent with previous experiments but this represents only a single session. The bottom figure shows a deflection of the average for the CS2 alone trials (caused by 1 CR out of 6 trials), but this may reflect an opportune spontaneous blink, which without sufficient and consistent increases in responses it is hard to differentiate between them. In contrast, the CS2-CS1 response in this trace also appears to be greatly reduced in size in comparison to the CS1-US response, but this is

![Figure 5-14](image-url)

**Figure 5-14: Session averages of different trial types from two consecutive sessions of training of Subject 4**

**Colour key inset top left.**

Averages show the mean value at each time point (arbitrary voltage signal, normalised to -650 to -450 baseline) ± SEM.

**TOP: Early CS2-CS1 responses revealed in the average profile for that session. Shows a peak at time of CS1 onset. These are from session 37 of subject 4’s training.**

**Bottom: Trial response averages for session 38 for subject 4. Now no early response to CS2-CS1 and reduced size in comparison to CS1-US. CS1 alone has an earlier onset and larger size than CS1-US, showing inconsistency in the responses between the two trials but the SEMs overlap. CS2 alone response shows deviations from baseline about the time where CS1 would be expected to occur. The deviation reflects just one CR occurred from the total of 6 CS2 alone trials.**
inconsistent with the previous day's averages and would be inconsistent with CS2 alone responses.

Fig. 5-15 shows the averaged responses from session 35 for subject 6 who appeared to respond less to the CS2-CS1 stimulus as conditioning progressed (see bottom graph Fig. 5-13). This may be consistent with the CS2 generating a conditioned inhibition response, and it does frequently appear in subject 5's responses. However, the results from these experiments made that difficult to analyse as amplitudes are difficult to compare due to the arbitrary voltage value of the signal, which was affected by the distance of the sensor from the eye. This could be varied by the experimenter or the rabbit itself during one session. Similarly to pick up small movements the sensor had to be moved closer or the signal amplified but this lead to saturation of the signal before complete movement.

Figure 5-15: Session average of the various trial types from subject 6's responses showing possible conditioned inhibition. Inset top left is the colour key. Subject 6 showed reduced number of responses to CS2-CS1 trials in comparison to CS1-US response rates. The trial averages for sessions show smaller responses to the CS2-CS1 trials in comparison to CS1-US trials, such an example is presented here. This is possibly consistent with conditioned inhibition but the analysis of such a conditioned effect is difficult in this experiments. The results presented here are descriptively comparable with conditioned inhibition but a quantification of this effect has not been attempted due to the reasons laid out in the text.
Fig. 5-16 shows an example of a single CS1-US trial showing saturation of the signal just as the US onsets. The animals were often observed to make small movements of their eyelids and made movements that saturated the signal shortly before or just after the US. As such is hard to derive blink amplitudes and whether the predictive blink was maximal before the US, as would be expected from classical conditioning of the eyeblink response. The saturation proved a great technical problem with the infrared method of detecting eyeblinks and makes the results more difficult to interpret. The signal had to be finely balanced between being over-amplified to detect small movements but leading to saturation in larger movements, or minimising the saturation problem to show the topography of complete blink movements but risk missing smaller movements.

The unrestrained conditioning protocol did not produce robust second order conditioning in the majority of the animals. The method was associated with several issues that made the reliability of the results questionable. The data was not further analysed for this reason so as not draw spurious conclusions from it, but several considerations are summarised in the Discussion below.

Figure 5-16: Saturation of the signal during a single CS1-US trial
Timing of stimuli shown at top. Y axis denotes arbitrary voltage values that ranged between -5 and +5V, with increases in voltage indicating greater closure of eyelid. Saturation of the signal can be seen shortly before the US (airpuff) and makes it difficult to discern the size of the movement before this period, and the total size of movement that the airpuff generates.
5.5 Discussion

In the present Chapter, evidence has been presented second order conditioning of the NMR response is possible but that the behaviour is very labile and weak in comparison to first order conditioning. The topography of the second order responses suggests that their onset and peak latencies may be more related to the CS1 driven climbing fibre activity, rather than the time of the predicted but absent US. Although the assumption was not directly tested here, Mostofi et al. reported that in trained rabbits Purkinje cells in eyeblink microzones showed a higher complex spike firing rate roughly 60ms following the onset of the CS1, and consistent with that in these experiments we see that animals with robust second order conditioning make a movement that is shifted forward in time, where it onsets in advance of the CS1 stimulus and peaks close to the time of CS1 onset, or expected CS1 onset. This would be consistent with the current hypothesis that climbing fibre activity signals the time at which future conditioned responses should be maximal, however in the case of second order conditioning the response generated is not involved in the avoidance of an aversive stimuli.

The freely moving conditioning experiments, in contrast to the restrained animal experiments, did not produce much robust conditioning related to the second order stimulus. The results from the two different experiment types will now be discussed in the context of previous studies, in turn, with an emphasis on the limitations for the freely moving experiment.

5.5.1 Restrained second order eyeblink conditioning

5.5.1.1 Conditioned responses in subject 1 and 2

Subject 1 was already well conditioned to CS1-US trials before the experiment began and as such the starting point was different in comparison to subject 2. Nonetheless, both came to make conditioned responses to the CS2 alone with increased frequency as training progressed.

Subject 1 displayed a non-linear but general increase in CS2 response rate across the sessions of training. By the end of the experiment subject 1 was responding very consistently and with large responses to the CS2. This initial increase and decrease in responses may reflect two processes that are underway, a conditioned inhibition which was seen in subject 3 (discussed subsequently) and associative learning of the CS2-CS1 relationship.

Subject 2 took much longer to show responses requiring 18 sessions before responses began to appear, and these only occurred in 50% of the trials in the final blocks. The averaged responses were also smaller than those seen in subject 1. This may have been caused by the fact that the animal was naïve and we can infer that the animal had not established climbing fibre receptiveness to CS1 till some time into the experiment. We do not know at what point climbing fibres become responsive to
the CS1 in eyeblink microzones, and the extent to which starting from naïve with CS2-CS1 trials intermixed with first order trials affects this. Mostofi et al. showed changes before and after conditioning in a population of cells which had climbing fibre responses to only ipsilateral periocular electrical. As the electrodes were removed after each experiment, it was likely that these were not the same cells recorded before and after but 0 out 8 cells showed responses to CS1 initially, with 12 out of 12 showing climbing fibre responses tied to CS1 onset after learning. Ohmae and Medina 2015 showed responses in Purkinje cells in trained mice. Indeed it is difficult to record cells over a long period so such an analysis of changes of climbing fibre responsiveness may prove problematic.

Nonetheless, subject 1 and 2 demonstrate that second order conditioning is possible both starting from naïve or post-primary learning. The rate of learning from subject 1 is more consistent with that observed in Gibbs et al., 1991 but in this study, the rabbits were naïve before beginning intermixed second order training. In this case, the learning rates may be more representative of the rate as their sample size was much larger (n=12).

### 5.5.1.2 Onset to CS2 and CS2-CS1 trials and CS2 alone peak latencies in subjects 1 and 2

The onset of responses before the presentation of CS1 in the CS2-CS1 trials shows that the conditioned response must have been driven, at least initially, by the CS2 component of the stimulus. This shift forward in CS2-CS1 onset began fairly early in subject 1 and was demarcated into bimodal distributions either beginning before CS1 or showing an onset consistent with CS1-US trials, very few responses occurred around the 0ms relative to CS1 onset. This suggests that there were two separate processes in generating these responses, rather than a slow bringing forward in time process, which would be consistent with climbing fibres signalling the time that conditioned blinks should be maximal. This is further evidenced by the peak latencies of CS2 alone responses with a median peak latency of 20ms relative to CS1 for subject 1. Although we do not know the underlying climbing fibre behaviour the timing of these responses to CS2 alone stimuli suggest that they may be related to changes in climbing fibre activity to CS1 after training, i.e. that the responses are now maximal earlier in time and at a time that climbing fibres increased their firing rate to CS1. Ideally, to confirm this it would be necessary to correlate climbing fibre responses in a given individual with their response latencies, but regulatory processes preclude us from doing this. If this could be confirmed it would be consistent with, and further evidence, that the climbing fibre responses are the key mechanism in signalling the timing of the conditioning eyeblink response.

Subject 2 showed similar responses in their CS2-CS1 onsets, although the demarcation between obviously early and ordinary CS1 driven responses was less dramatic, but did become more apparent
in later sessions. The peak latencies to CS2 alone are also consistent with an earlier shifted response that is maximal after CS1 onset but before the expected time of US delivery.

If the purpose of second order conditioning was to associate information about the US with CS2 through a pairing with CS1 (and never with the US), then second order conditioning appears to be a maladaptive process, as responses would be made too early to avoid the US. This may be because the experimental procedure is an unnatural construct, in natural situations such perfect predictors (CS1 predicts only the US; CS2 predicts only the CS1) probably wouldn’t exist, in reality, the US would occur following the CS2 (trace conditioning), or following the CS2-CS1 (serial conditioning). What role second order conditioning plays in strengthening these associations remains to be seen.

Alternatively, it may be that in this behavioural task the climbing fibre function is being co-opted to perform a maladaptive task, but it may not be maladaptive in other motor learning tasks. In the restraint procedure, the animals cannot avoid the periocular electrical stimulus, even by making conditioned responses. However in the real world avoiding the aversive stimuli to the cornea of the eye may not just consist of a discrete eyeblink, a movement of the head or body could aid in avoidance. Then we might expect behaviour to begin with the sign of the earliest possible reliable predictor. If this is due to changes in climbing fibre responsiveness, which we infer are underlying this behaviour, then they are serving an adaptive function in the avoidance of aversive stimuli.

Even though the response onsets to CS2-CS1 are bimodal seemingly showing either a second order response to the CS2 element or a normal CS1 onset response it is hypothesised that the same set of Purkinje cells in eyeblink controlling microzones are responsible for this action. Separate responses from the same Purkinje cells is not at odd with behavioural and neurophysiological data, double eyeblink response have been seen to the same stimulus when it is trained with two distinct interstimulus intervals to the US (Hoehler and Leonard 1976; Millenson et al. 1977). Purkinje cells have been shown to show two separate CR pauses in their firing to a single stimulus that has been trained to predict the US at two distinct intervals, 150ms and 450ms (Jirenhed et al. 2017). How this relates to timing mechanism at the molecular level remains to be seen. The relation of Purkinje cell pauses to second order responses in neurophysiological experiments would determine if these responses are mediated by the same cells.

5.5.1.3 Subject 3 showed conditioned inhibition in response to second order conditioning

Subject 3 did not show consistent responses to CS2 alone stimuli presentations. A small number of responses were made, but the number of responses did consistently grow over time. Subject 3 showed a separate phenomenon of conditioned inhibition. Responses to CS2-CS1 sometimes had their onset before the CS1 but this waxed and waned after session 15. At a similar time after session
12, the level of responses to CS2-CS1 began to significantly drop below that of CS1-US, even though the former stimulus contains the primary predictor CS1. This suggests that the subject had learnt that CS1, when following the CS2, would not be associated with the US and no conditioned responses was necessary. This conditioned inhibition has been described before in a second order eyeblink study in which very few second order responses were observed (Popik et al. 1979). The effect has also been observed when a compound stimulus of light and sound indicates the absence of the US in comparison to a normal light-US pairing, and the effect is observed in decerebrate animals (Moore et al. 1980; Yeo et al. 1983).

How such a mechanism of inhibition is mediated is not known. The inhibition could be acted upon the afferent structures to prevent the relay of signals relating to CS1 to prevent conditioned response expression. Alternatively, it could be acted upon efferent structures related to eyeblink, but as it happens with second order stimuli as well as compound stimuli, this suggests that this inhibition outlasts the CS2 stimulus length, as it acts on a CR that starts 200ms after the offset of the CS2. Given that it is hypothesised that a singular region of cerebellar cortex is responsible for delay eyeblink conditioning it seems unlikely that such inhibition is imposed on outside structures by a separate system. It would be more congruent for this inhibition to act at the same regions. Neurophysiological data in rats show that the activity of the anterior interpositus nucleus is correlated with behavioural responses during conditioned inhibition. Specifically, that trials with a CR showed increased anterior interpositus activity but that conditionally inhibited trials showed no increased firing in anterior interpositus neurons. This suggests that inhibition is mediated within the cerebellum or before, not after (Nicholson and Freeman 2002).

The reduction in amplitude for subject 3 suggests that inhibition did not produce an all-or-none response of the CR and that the inhibition has a varying efficacy to affect the CR expression.

5.5.2 Freely moving second order eyeblink conditioning

The freely moving eyeblink conditioning experiments were a test of the procedure with the hopes of refining the method for future use. Allowing the animals to move did not prevent the expression of conditioned eyeblink responses, but the method proved problematic.

Firstly, allowing movement meant the use of infrared sensors to monitor external eyelid closure rather than transducing the nictitating membrane as was the case in previous experiments. External eyelid movements are under voluntary control and happen spontaneously. This led to significantly more noise in the signal and arbitrary voltage values that were difficult to calibrate to eyelid closure measures, as the distance from the eye affected the signal. This meant that simple thresholds of movement were not reliable for eyeblink CR definition and a method of using standard deviations of
a baseline to determine CR was used. Saturations of the signal were often common before maximal eyeblink, but the sensor had to be close to detect small movements.

The nature of the experiment meant that the use of an aversive air puff US was more suitable than the periocular electrical stimulus due to the animals removing the periocular clips necessary for the electrical stimulus. This represents a change from the electrical stimulation used in the restrained animals. Electrical stimulation is preferable as it is immediate and the effect of it is unchanged by learning; closing your eye does not prevent the stimulation. In contrast, air puffs have a delay to onset as valves are triggered to open to the pressurised air source, and by closing your eye, the air puff now stimulates the eyelid, not the cornea. Electrical stimulation in the unrestrained animals may have provided stronger conditioning, but it was unfeasible to place metal clips around the eye that the animal could access while freely moving. Indeed such an attempt merely aggravated the animal to groom and made conditioning impossible.

The method required each session to be run for each animal separately and could in some cases take an hour for one session as animals needed time to settle. Due to this, there was no consistent inter-trial interval as trials were given if the animal was settled, adding an uneven variable between animals.

Despite this first order conditioning did appear possible, but it progressed more slowly than would normally be expected taking several blocks (each consisting of 4 sessions) before any responses were seen. Usually, in eyeblink conditioning responses would be expected in the 2nd and 3rd sessions and progress in number as training proceeds. However the data generated from these experiments was severely limited by the problems above, and I did not feel confident in analysing it further. The method would have benefitted from refinement, but this may have taken some time and was not guaranteed to produce better results. In light of this, the decision was made to explore second order conditioning in humans to see if data comparable to the original rabbit experiments could be obtained.
6 Second Order Conditioning of the Eyeblink Response in Human Subjects

6.1 Introduction

Due to difficulties encountered in carrying out rabbit eyeblink conditioning efforts were made to replicate the study in human subjects. Eyeblink conditioning in humans provides a comparative approach to the studies undertaken in animal subjects. Conditioning has been shown to be affected by lesions within the cerebellum, and lesion studies implicate the importance of ipsilateral cortical regions in the acquisition and timing of responses (Gerwig et al. 2007). Brain imagining studies using fMRI have revealed significant activity within the cerebellar cortex, and the deep cerebellar nuclei while participants undergo conditioning (Cheng et al. 2014; Thürling et al. 2015). For further reference see ‘Human eyeblink’ section in Chapter 1.

To our knowledge, there are no known studies of second order delay eyeblink conditioning in humans. A study of musicians and non-musicians claimed to show second order responses in a delay conditioning task where an auditory tone from a piano was used as the CS, but in reality, they showed evidence of sensory preconditioning (Bangert et al. 2006). In this study, the two groups were trained to associate a tone at a certain pitch with an air puff. After this session, the two groups were asked to perform keystrokes on a silent piano, and it was shown that the group of musicians blinked at a significantly higher rate in response to the key corresponding to the tone associated with the US. It was hypothesised that musicians had made associations between the pressing of the key and the tone evoked by it through their previous practice of piano and that this association was sufficient to generate a conditioned response.

In this Chapter, we provide evidence of second order conditioned responses in human subjects. The results serve as a comparative study to the similar experiments performed in rabbits and show that the outcome of second order conditioning, in this case, is analogous to those obtained in the restrained rabbit experiments. It should be acknowledged that these experiments were carried out in collaboration with undergraduate students as part of their Part II projects here at the University. Students aided in the recruitment of participants and the collection of data, but all subsequent analyses presented below are my own.
6.2 Materials and Methods

6.2.1 Participants and second order conditioning protocol

16 undergraduate students were recruited to undergo a second order conditioning session. Participants were asked to sit in a chair and watch a silent movie as a distraction while the conditioning procedure was carried out.

The procedure consisted of receiving a first order session made up of CS1-US trials and CS1 alone test trials in a 4:1 sequence respectively. A total of 250 trials were delivered. CS1 consisted of a small 5V vibrating motor strapped to the participant’s finger. It preceded the US, an air puff (2-3 bar), at an interval of 450ms. Fig. 6-1A, overleaf, shows the first order conditioning procedure.

Participants then underwent a second order conditioning procedure, which is detailed in Fig. 6-1B. This was composed of 100 CS1-US trials, 100 CS2-CS1 trials and 50 CS2 alone test trials. A CS1-US trial was followed by a CS2-CS1 trial, with this order repeated twice before a CS2 alone trial was given as the 5th trial in the sequence. The CS2 was an auditory 1kHz tone which had an onset of 550ms before the onset of CS1. The interval between trials was set to a random delay of 8-10 seconds for both first order and second order sessions.

To reiterate the conditional stimuli (CS) were:

- CS1 - tactile vibrating motor stimulus – precedes air puff (US) by 450ms
- CS2 – 1kHz auditory tone – precedes CS1 by 550ms

6.2.2 Infrared recording apparatus

The eyeblinks were detected using an infrared sensor that consisted of an IR photodiode and IR LED. The amount of infrared reflectance affected the amount of current flowing through the diode, this reflectance changes as the eyelid closes. The sensor was mounted to a headpiece that could be placed just below the eye of interest at a distance of 1-1.5cm and angled to direct the infrared beams to the centre of the cornea. Additionally attached to the headpiece was the tubing for the air puff delivery which was directed into the corner of the eye on the temporal canthus side at about a distance of 1-2 cm. The tubing for the air puff was 2m in length and ran through to the adjacent room where a solenoid valve was gated by 5V pulses of 100ms duration to deliver the air puff. The solenoid valve was positioned in the adjacent room as the opening and closure of the valve produced audible clicks and we would not want these to be perceptible by the participants in the experiment, otherwise they may have acted as a conditional stimulus. Signals from the IR blink sensor were fed into a CED Micro 1401 and digitised at a sampling rate of 1kHz.
6.2.3 Detection of conditioned responses in response averages

The optical method of transducing blinks results in noisy individual traces that are sometimes contaminated with eye movement in the signal and spontaneous blinks; for this reason, the responses to trials were averaged for each stimulus type in each session. Thus each subject had four averaged response traces: a CS1-US trial averaged response, and a CS1 alone trial averaged response from the first order session, and a CS2-CS1 trial averaged response, and a CS2 alone trial averaged response from the second order conditioning session.

Figure 6-1: Experimental procedure
(A) First order conditioning sessions consisted of 200 CS1-US trials and 50 CS1 alone trials. Timing of CS one is shown by the blue bar, the US shown by the red bar. These were given in a sequence that consisted of 4 CS1-US trials followed by a single CS1 alone trial. The interval was set to be random but within 8-10 seconds. (B) Second order sessions consisted of 100 CS2-CS1 trials, 100 CS1-US trials and 50 CS2 alone trials. The timing of CS one is shown by the blue bar, of the US by the red bar and of CS to buy the green bar. The sequence consisted of CS1-US trial followed by a CS2-CS1 trial, repeated twice. Then a single CS2 alone trial before beginning the sequence again.
The trial averages were analysed for the presence of a conditioned response and the onset times from the CS2-CS1 and CS1-US trials were determined. To determine if there was a conditioned response, a baseline period was taken, and a mean and standard deviation value were derived from it. For CS1-US trials the baseline period was the -200 to 0 ms relative to CS1 onset, as shown in Fig. 5-1A. For CS2CS1/CS2 alone trials, the baseline period corresponds to -750 to -550 ms in Fig. 6-1B.

A response window (shown in Fig. 6-2) was set that corresponded to the time of stimulus onset (CS1-US trials = 0 ms; CS-CS1 and CS2 alone = -550 ms) up to the time of the US or the expected US onset (450ms). A conditioned response was determined to be present in the averaged responses if during this response window the waveform values crossed a threshold that corresponded to the mean baseline value plus 15 standard deviations of the baseline. This threshold seems conservative, but where blinks occurred consistently, and then averaged, the response was much bigger than the noise. This stringent threshold also prevented false positives.

For CS2CS1 and CS2 trials this response window was broken into two. The process was first applied to the -550 to 0ms period, relative to the timeline in Fig. 6-1B, and if no CR was detected it was then applied to the 0 to 450ms period. If there was a CR in the first period, the process stopped there, and it was recorded that the waveform crossed the threshold in this CS2 to CS1 period.

Figure 6-2: Response windows for CS1-US trial and CS2-CS1 trials
The response windows for the two trial types differ. The presence of CRs, as defined in the text, was checked for in the CS1 onset to US onset window in CS1-US trial averages. For CS2-CS1 trial averages the response window was extended back in time to cover a period for CS2 onset to the expected time of the US (never given with CS2). For CS2-CS1 averages the process of determining if CRs occurred was broken into two.
6.2.4 Determination of onset times

To deduce response onsets, an algorithm was applied that calculated the second derivative, the change in slope, between each 10ms point of the waveform. Fig. 6-3, overleaf, outlines the process. The distance of 10ms was chosen, so that general changes in slope were detected rather than point to point variations. As the blink response begins it changes from a flat baseline to a sharp rise in the voltage signal, given this we should expect the onset to begin at or near the largest second derivative. This process was repeated nine times by rolling the window forward 1ms a time to make sure that elbow of the blink onset was not missed in the first instance. The midpoint of the 10ms window that contained the highest second derivative the greatest number times was chosen as the onset.

For CS2-CS1 response averages, the onset algorithm was applied to only the first section (CS2 onset to CS1 onset) if the previous CR detection had determined that the waveform crossed the threshold of CR definition in this period. In the case of CS2-CS1 averages where CR threshold crossing was detected in CS1 regions of the CS2-CS1 then the onset algorithm was applied to the whole response window, as defined in Fig. 6-2.
To determine the onsets an algorithm was applied to the data which worked as follows:

**TOP:** Typical eyeblink waveform from a single trial

**SECOND ROW:** Down sampling from 1kHz to 100Hz. The time resolution goes from 1ms to 10ms points

**THIRD ROW:** Determination of the value of the slope, the first derivative, i.e. the difference between two consecutive down sampled points

**LAST ROW:** Determination of the change in slope from point to point, the second derivative. Arrow indicates point with largest second derivative and the dotted line shows the corresponding position in the previous plots. Repeated 9 times by moving the point at which reduced sampling begins by 1ms. The midpoint of the 10ms window that had the largest second derivative the greatest number of times.

**Figure 6-3: Eyeblink onset detection algorithm**
6.3 Results

6.3.1 First order learning

Of the 16 participants, 14 showed conditional responses to CS1-US trials in the first session on the basis of their averaged responses. However, two individuals did not develop any learning. Fig. 6-5 below shows example traces from one individual who showed learning responses (A) and one

**A: Individual with first order CRs**

![Graph A: Individual with first order CRs](image1)

**B: Individual with no first order CRs**

![Graph B: Individual with no first order CRs](image2)

Figure 6-4: Most individuals learned to make responses after first order training, however some did not

(A) Average responses plus standard error of the mean from one subject who learned to make CRs in response to the CS1 after first order training. Left, CS1-US responses; Right, CS1 alone response. Orange dot shows estimated onset time.

(B) Average responses from one subject who did not learn to make CRs in response to the CS1 after first order training. Left, CS1-US responses; Right, CS1 alone response
individual who did not (B). It is possible that the individuals who did not learn may have benefitted from longer training.

6.3.2 Second order learning and response onset to CS2-CS1

The 14 individuals who had significant conditioned responses after the first order session continued to the second order session. The onset time from the averages for the CS1-US trials and the CS2-CS1

A: Early onset CS2-CS1 response

B: No early onset CS2-CS1

Figure 6-5: Second order conditioning caused responses to CS2-CS1 to shift earlier than CS1 onset in the majority of individuals
(A) Average responses from one subject who acquired earlier CRs in response to the CS2-CS1 second order training. Left, CS1-US responses; Right, CS1 alone response. Orange dot shows estimated onset time. Grey ribbon ±SEM
(B) Average responses from one subject who did not make earlier CRs in response to the CS2-CS1 after second order training. Left, CS1-US responses; Right, CS1 alone response. Orange dot shows estimated onset time. Grey ribbon ±SEM
trials were compared. Of the 14 individuals, 9 showed responses to CS2-CS1 that began in the CS2 to the CS1 interval (-550 to 0ms relative to the CS1 onset). Shown in Fig. 6-5A is an individual whose responses shifted early in CS2-CS1 trials in comparison to their response in CS1-US trials, going from an onset of 305ms for the first order trials to an onset of -335ms relative to the CS1 onset. Fig. 6-5B shows the averaged trials of a subject where the second order conditioning did not affect the onset of responses, changing from 355ms to 375ms for CS1-US and CS2-CS1 trials respectively.

Three individuals made conditional responses to the CS1-US trials but showed no conditional responses to CS2-CS1 trials. This appears remarkably similar to the conditioned inhibition that was shown in Chapter 5. The averaged responses from an individual who exhibited these responses are shown in Fig. 6-6 The conditioned response to CS1-US was very small on the average trace in comparison to the unconditioned response following US onset, this may indicate that the conditioning was weak for this individual, and an inhibitory influence would have a large effect.

Figure 6-6: An individual who showed first order responses during CS1-US (left) but no response during CS2-CS1 (right). CS1 stimulus is given in CS2-CS1 so this represents conditioned inhibition. Orange dot shows estimated onset time. Grey ribbon ±SEM

The onset of responses in CS1-US trials in comparison with CS2-CS1 trial onset are shown for the remaining 11 individuals in Fig. 6-7. Three subjects did not make earlier responses going from median response onset of 355ms to median response onset of 375ms for CS1-US and CS2-CS1 trials, respectively. The remaining nine subjects had a median onset to CS1-US of 345ms and a median onset of -240ms for CS2-CS1 trials. A paired Wilcoxon signed rank sum test applied to all participants
reveals a significant difference in onset latencies between the CS1-US response averages and the CS2-CS1 response averages (V=64, p < 0.01).

The response averages to CS2 alone trials showed comparable results but not identical to the analysis of the response onsets. The analysis found that seven individuals had significant responses in the averages of the CS2 alone trials, and in the majority of these, they onset before the expected CS1 time and peak just before the expected CS1 onset time, qualitatively. The average responses are shown in Fig. 6-8. These seven individuals had shown earlier onsets (<0ms) in their CS2-CS1 response averages. 2 individuals who made early CS2-CS1 responses, however, did not make significant CS2 alone responses, the reason for this is unknown. It seems unlikely that individuals would preferentially make early-onset responses but only in CS2-CS1 trials. One potential reason is that the smaller number of CS2 alone trials were not sufficient to resolve small responses being made, but
averaging 100 CS2-CS1 trials allowed smaller but consistent responses to be drawn out of the traces. This would make the average baseline value plus 15 standard deviations threshold smaller. Nonetheless, the CS2 alone trial averages from the seven individuals who did show results are consistent with previous results reported in this dissertation. The remaining five individuals that are shown in Fig. 6-8 are those that did not make early CS2-CS1 responses (n=3) and those who made no responses to CS2-CS1 in the trial averages (n=2).

Figure 6-8: Average responses to CS2 alone trials in the second order training session by all subjects. 7 individuals were deemed to have shown significant responses by the CR detection algorithm, denoted by the cross. All 7 individuals had made early onset CS2-CS1 responses, that began before CS1 onset. 2 individuals who did make earlier responses in CS2-CS1 trials, however did not show significant responses in their CS2 alone responses. The 5 remaining individuals were those who didn’t make early responses and those who showed no response in the trial averages to CS2-CS1 trials.
6.4 Discussion

The results in this chapter present evidence for second order conditioning of the eyeblink response in humans, showing that responses are made to the CS2-CS1 stimulus that start during the CS2 to CS1 interval. This shows that the CS2 is sufficient by itself to generate a blink response despite never being paired with the instructive US explicitly. As far as we are aware, this has not been demonstrated in the human eyeblink literature before.

The timing of the second order responses is very similar to that which was seen in the rabbit second order responses presented in Chapter 5. This suggests that there may be a common mechanism by which these responses are generated, and that this method is utilised in both rabbits and humans. The change in climbing fibre responses to CS1, observed in eyeblink microzones in the rabbits, may represent such a mechanism, and it is this that causes conditioned eyeblink responses to be made early when a CS2 is paired with a CS1. In this manner the climbing fibre provides an instructive signal about the timing at which to make future conditioned eyeblinks. By associating the CS2 with the CS1, where there is now an increase in climbing fibre activity at roughly 60ms into the CS1 stimulus, the CS2 contextual signal by association with the CS1 generates an eyelid closure that begins before the onset of CS1 and is maximal shortly after. To determine if this is the case, and to determine what is happening at the level of the Purkinje cell, the necessary neurophysiological experiments would need to be carried out in animals.

This present study does have some limitations. The use of an optical method to transduce the eyelid movements resulted in a noisy signal that can be contaminated with eye movements and spontaneous blinks. This prevented the use of an algorithm to classify the responses to individual trials simply. The use of averages attempted to minimise the noise and draw out the signal, but in doing so, we lose information about the development of responses over time. To counteract this in future experiments, an effort was made to transduce the eyelid movement through mechanical means in future human experiments, in much the same way as the transducer used in restrained rabbits and as detailed in Chapter 7.

6.4.1 First order learning

The fact that some individuals did not learn after first order conditioning could be explained by a number of factors. It seems unlikely that certain individuals in our experiment were unable to learn completely unless they had undiagnosed cerebellar defects. The use of a vibrating tactile stimulus as the CS1 over a visual stimulus allowed the participants to sit and watch a silent movie as a distraction while undergoing conditioning. However, a vibrating tactile stimulus has not been used before in the human eyeblink literature, although a vibrating whisker stimulus has proved effective
in animals (Das et al. 2001; Galvez et al. 2006). It may not have been perceived or recognised as particularly salient by some individuals. Additionally learning rates seem to differ between individuals, and those who did not show conditioned responses may have done so with further training, but these experiments were designed to fit the first and second order conditioning into one session.

To address this and determine the effectiveness of the vibrating tactile stimulus as a conditional stimulus first order conditioning could be undertaken again but this time the number of trials received increased. Similarly, it may prove useful to split the first order and second order conditioning into two separate sessions to increase the number of trials given but to also allow for a consolidation period between the two.

6.4.2 Second order conditioned responses

Despite the limitations, the results show that a large proportion of the subjects (9/14) showed earlier averaged responses to CS2-CS1 trials in comparison to CS1-US trials, with these responses having onsets before the presence of the CS1 stimulus. These results are consistent with those obtained from the restrained second order rabbit experiments detailed in Chapter 4. If the experiment were to be repeated, it would be beneficial to use the mechanical transduction of the human eyelid, described subsequently, to obtain cleaner traces that allow the analysis of the data on a trial-by-trial basis. This would allow us to validate that the responses develop over time which would be consistent with learning.

The fact that some individuals did not show robust CS2 alone responses yet made earlier onset responses in the CS2-CS1 trials is odd. It may be that individuals started to respond late in the session and the extra numbers of CS2-CS1 trials enabled those responses to make an impact on the average, whereas the smaller number of CS2 alone responses are averaged over a greater proportion of trials before learning occurred.

Nevertheless, these results provide complementary evidence that second order conditioning is possible but variable, and that second order responses are shifted earlier, and would not be protective against an aversive stimulus applied to the eye.
7 Self-generated signals as the conditional stimulus

7.1 Introduction

The cerebellum receives a large number of inputs from the motor as well as sensory regions in the cerebral cortex. The corticopontine tract is the largest white matter tract in the primate brain (Rea 2015), and pontocerebellar mossy fibres make up the largest mossy fibre input to the cerebellum. It is substantial in both rabbits and rats with wide projections originating from wide areas of the cortex (Abdel-Kader 1968; Legg et al. 1989).

Hypotheses of cerebellar function include feedforward models in which the cerebellum creates an internal model using both sensory and motor domains to generate a predicted sensory outcome of actions. Such a system has been demonstrated in the electrosensory lobe, a cerebellar like structure in electrosensitive fish, whereby corollary discharge is used to distinguish the sensory consequences of self-movement from that of other relevant sensory information (Requarth et al. 2014; Sawtell 2017). Internal representations are believed to explain the phenomenon of hand displacement in the cup lifting task. In the task lifting a cup off of the palm of your other hand results in little displacement of that hand, however, if lifting is done externally the hand displacement is much greater (Diedrichsen et al. 2003). It has been shown through the use of a robotic lifting mechanism that if the triggering is done by the individual bearing the load that this is enough sensory information to reduce subsequent displacement. Similarly, internal model representations within the cerebellum are hypothesised to be the reason that self-generated tactile stimuli are deemed less ticklish than those generated externally (Blakemore et al. 1998, 1999a, 1999b).

Many eyeblink conditioning experiments have been carried out using different modalities of stimuli. The overriding idea in eyeblink classical conditioning is that if temporally related sensory events predict the need for the motor response, then through repeated associations this sensory event will come to elicit that motor response. It is known that it is possible to condition using sound, light and various other sensory modalities including tactile and vibrissal stimulation. However, to our knowledge, no experiments have attempted to use one’s motor actions and associated sensory stimuli as the conditional stimulus. The experiment described in this chapter attempt to address whether self-initiated movements can serve as a reliable enough stimulus to learn from and whether a voluntary motor action can be tied to a reflexive action in a motor sequence. Given the plethora of modalities that are capable of acting as conditional stimuli and the evidence of internal representation in the cerebellum, we hypothesised that using a reaching task as conditional stimulus
would produce robust conditioning. Though evidence is provided that such is possible, it is difficult to discern whether responses are tied to motor action or sensory reafferences associated with it; this is discussed later.

This experiment was carried out with the aid of a Part II student, as part of their undergraduate project. They aided in the recruitment of participants and the collection of data, but all analyses presented subsequently are my own.

7.2 Materials and Methods

7.2.1 Self-generated conditional stimuli conditioning

Undergraduate students were asked to come to the lab to partake in an experiment looking at reaction time in a quiz task while receiving distractions. The real purpose of the experiment was to see if one’s motor actions could be used as a conditional stimulus that reliably predicts the US and as such be used to generate conditioned responses. Participants were sat in front of a computer screen that presented them with a question with four multiple choice answers of which only one was correct. In front of the screen were four 10cm diameter buttons which corresponded to the layout of the answers shown on the screen (A, B, C and D). Participants had their hand stationary in a homepad which consisted of an infrared sensor beam such that while the hand was at the homepad the beam of light was broken, but when the hand moved away the beam was complete. This allowed the monitoring of the leaving of the hand from the pad (shown in Fig. 7-1A).

17 subjects were recruited in total and divided into a conditioning group (n=11) and a pseudo-conditioning group (n=6), as shown in Fig. 7-1B and C. Each answered 220 questions, trials 1-20 were absent of any aversive air puff for both groups, but 21-120 consisted of an air puff on immediate switch closure for buttons B and C for the conditioning group at an 80% probability. For the control pseudo-conditioning participants trials 21-120 were linked with an air puff (2-3 bar) from buttons B and C at 80% probability but with an additional 0.5 – 2-second random delay such that pressing the button was not a temporally reliable predictor of the aversive US. Following 120 trials both groups were asked to switch to their right hand for trials 121-140 during which both received the pseudo-conditioning protocol. This served as a test of limb specificity for the conditioning. A retraining period was given for trials 141-200 in which the conditioning group once again received air puffs immediately on pressing of buttons B and C to counteract any extinction that had occurred during the switching of the hands. Following this, trials 201-220 were conducted by the participant calling out their chosen and answer and watching the experimenter press the button for them. This period
Figure 7-1: Experimental setup and outline of experimental conditioning group and control pseudoconditioning groups protocol

(A) The setup of the experiment. Participants rested their hand on a homepad. An infrared beam was made when it left this position, recording the time of hand movement onset. 4 answers to multiple choice questions displayed sequentially on a screen in front of the participants corresponded to 4 buttons lying flat on a surface in front of the participant. When the subject chose an answer they were asked to press the button as quickly as possible. Two of the buttons were linked to airpuff delivery, B and C, either with no delay (conditioning group) or with a random delay of 0.5 – 2 seconds (control pseudoconditioning group).

(B) The Conditioning group received airpuffs paired to button pressing on B and C switch closure with no delay. After an initial Baseline period of 20 trials with no airpuff; Training 21-120 trials where airpuffs are present at 80% probability to B and C buttons; Switch hands – 121-140 trial had a pseudoconditioning protocol so there was no reinforcement at fixed latency when using the opposite hand; Training again for 60 trials; Final part experimenter presses for the participant, again there was pseudoconditioning in this part where the airpuff was given but at a 0.5 – 2s random delay after switch closure on B and C buttons, 80% probability.

(C) Control pseudoconditioning group had the same outline to their training as the experimental conditioning group but after the baseline period, 1-20 trials, all subsequent trials were pseudoconditioning where button press was related to airpuff but with an additional 0.5-2s delay for button B and C, 80% probability.
served as a control for both auditory and visual signals that may have been used as the conditional stimuli during learning and allowed us to access the specificity of self-pressing for the making of conditioned responses. Both groups received pseudo-conditioning protocols during this period to prevent any quick learning affecting the results.

The quiz was run by a custom written program in Python on a Raspberry Pi microcomputer. The buttons were linked to both the Raspberry Pi and to a CED Micro 1401, as shown in Fig. 7-1A, which upon switch closure triggered an air puff from buttons B and C with an 80% probability during active conditioning periods.

7.2.2 Mechanical recording apparatus

Eyelid position was transduced by placing a headset on the participant. Attached to this headset was a low torque potentiometer which had an arm attached to the centre wheel which made a right angle bend down towards the eyelid. A small pad was fixed to the end of this arm which was stuck to the eyelid with eyelash glue. Movements of the eyelid rotated the centre wheel, and this was transduced into a voltage change that was recorded via a CED Micro 1401 at a 1kHz sampling rate. This mechanical method of transducing eye movement was introduced as an improvement over the optical methods used in the proceeding Chapter. It is similar to the method used when measuring the rabbits’ nictitating membrane response (see Chapter 5, Materials and methods). Data were recorded using the Spike2 software.

7.2.3 Definition of CRs

To classify responses as either conditioned response or no response, an algorithm was applied to the data using R programming scripts; this process is outlined in Fig. 7-2. This consisted of the following:

- An average unconditioned response (UR) size was determined for each participant, by comparing the maximum waveform value with the minimum waveform value in a 500ms window that began at the time of air puff delivery. Each air puff delivery marker was iterated through, and the differences between the maximum and minimum averaged.
- For each trial, a period of 500ms before the button press was taken. The difference between the maximum and minimum of the first 100ms of this period was determined. If this difference was larger than 10% of the average UR size, then the trial was rejected, as it was likely not to have a flat baseline before button pressing.
- If it was flat, then the first 200ms of this 500ms period were taken as a baseline from which an average value and standard deviation were calculated.
The following period from -300 to +500ms relative to the button press was checked to see if any point crossed the threshold of the baseline average plus 5% of the UR size. If this threshold was crossed onset was derived by determining the point at which the eyelid trace crossed the baseline average plus ten standard deviations of the baseline.

If the onset was determined to be less than 0ms, corresponding to an onset before button press, then the trial response was labelled as a conditioned response. This conservative threshold could be applied as the signal of the eyeblink was much greater than the baseline noise, and it also prevented inclusion of spurious onset values. A visual inspection of 200 trials confirmed that onset was close to that would be determined by eye.

![Diagram of eyeblink response](image)

Figure 7-2: Determination of CR
Top trace: a single UR. For all participants their average UR size was determined by taking the maximum value following US presentation and subtracting the minimum value in a 500ms window after US onset. An average was taken by dividing the sum of the differences by the number of US presentations.

Bottom trace: single trial. Baseline average value plus standard deviation was determined from -500 to -300ms before button press, if the initial 100ms period of this region was flat the trial was accepted for further classification of response, see text for full description. Next -300 to +500ms relative to button press was checked to see if it crosses average baseline value + 5% of UR size. If so onset then determined by the point at which waveform crossed the average baseline value plus 10 standard deviations. If onset began <0ms then the response was classified as a CR. Note that responses beginning very shortly after zero might also represent conditional responses, but we set a conservative threshold for inclusion.
7.3 Results

7.3.1 Comparison of Control Group with Experimental Group

The outcomes of both the control pseudo-conditioning group (n=6) and experimental group (n=11) were compared by the number of button presses, number of air puffs received, the inter-trial time between each button press and the average reaction time (measured as leaving the homepad to button pressing), Fig. 7-3 and 4 show the differences between groups for these factors. Wilcoxon rank sum tests showed that the groups did not differ significantly by the average reaction time (W = 34, p = 0.96), the average inter-trial intervals (W = 33, p = 1) and of air puffs received (W = 52, p = 0.06).

Figure 7-3: Difference between conditioning and pseudoconditioning for a number of factors is non-significant
Boxplots show the median value with the central line, the box runs from the first quartile (25%) to the third quartile (75%), the whiskers extend to the maximum and minimum points but no more than 1.5 x Interquartile range. Points outside this range are plotted as individual points.
(A) Average reaction time, defined as time from leaving homepad to button press. Not significantly different.
(B) Average inter-trial interval, defined as time between one button press to the next. Not significantly different.
(C) Number of airpuffs received during the experiment, as airpuffs were linked to the B and C buttons with 80% probability the exact same number of airpuffs were dependent on this, and how many times the participant chose B
The number of button presses by broken down by button (A to D) and by group are shown in Fig. 7-4. A two-way analysis of variance revealed that there was no significant main effect of group (F(1, 60) = 0.08, p = 0.77) but there was a significant main effect observed for the button letter (F(3, 60) = 65.79, p < 0.01). The interaction between button and group was found to be non-significant (F(3, 60) = 1.57, p = 0.20).

7.3.2 Learning to make predictive blinks following one’s motor actions

The effects of the first 120 training trials on the conditional blink response incidence were examined. The experimental conditioning group were observed to increase the mean incidence as the trial number increased, whereas the control group did not increase above baseline levels of responding, Fig. 7-5 overleaf.
Shown in Fig. 7-6 is an individual in the conditioning group; it can clearly be seen that this individual is making blinks that begin before the time of button press from trial 60 onwards.

Figure 7-6: Development of conditioned responses from an individual who was in the conditioning group. Blocks are colour coded and as at the right. The lines represent the average response ± SEM in grey. Responses preceding the button press first appear in blocks 60 to 80.
In contrast, an individual from the pseudo-conditioning control group is shown in Fig. 7-7 in which there is no effect of increasing trial number on learning to make anticipatory blinks in response to the button press.

A logistic regression model with random effects of subject id was fit to the data, details in Chapter 2; the results are reported in Table 5-1, overleaf. The effect of group by itself was not significant on the odds of making a conditioned response to a button press; the model shows a 14% decrease in odds of making a conditioned response if the subject is in the experimental group. However, this effect was associated with a wide standard error giving a lower 95% CI of a 61% decrease to an upper CI of a 86% increase in the odds of making a conditioned response. Similarly, trial number by itself does not show a significant effect on the odds of making a conditioned response. However, the interaction between experimental conditioning group and trial number showed a significant response ($\beta = 3.07$, $Z = 5.72$, OR = 21.49, $p < 0.001$), this represents a 2149% increase in the odds of making a conditioned response for one unit increase of experimental x trial number. Note, as the trial number is scaled by the division of the maximum number (120) to aid model convergence this one unit increase represents a step from trial 1 to trial 120 for the experimental group.
Table 7-1: Logistic regression coefficients of change in log odds and associated odds ratios for the first 120 trials by group. Note the interaction term is change in odds stepping from trial 1 to 120 for the experimental group, as the trial number fed into the model is divided by its maximum. This is to speed up convergence of the model parameters. Significance: * p < 0.05; ** p < 0.01; *** p < 0.001

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7.3.3 Generalisation of responses to all buttons

Given that only two of the four buttons were linked to air puff generation the question remained if predictive blinks would be generated to all four buttons through a process of generalisation or whether responses would be specific to buttons B and C. Fig. 7-8 below shows the conditioned response incidence for the conditioning group broken down by reinforced buttons (B and C) and non-reinforced buttons (A and D). Responses are made to the non-reinforced buttons and increase in likelihood with the increasing trial number. Fig. 7-9, overleaf, shows the averaged response to each button for a single conditioning individual, it is clear from these averages that a response is being made to each button and that its onset begins before button pressing.

![Graph showing conditioned response incidence for conditioning group broken down by reinforced and non-reinforced buttons]

*Figure 7-8: The CR incidence for the conditioning group but broken down by reinforced and non-reinforced buttons. Responses are generated to all 4 buttons and responses increase in frequency for the conditioning independent of whether the button press was reinforced or not. The responses have generalised.*
A logistic regression model, with random effects for subject, was fit to the data with an indicator of reinforced buttons as a predictor of making a conditioned response, detailed in Table 2. The main effect of reinforced buttons was non-significant. Similarly, any interaction effect of reinforced buttons with other variables was found to be non-significant. The only significant predictor of making a conditioned response was again the interaction between trial number and the indicator of being in the conditioning group ($\beta = 2.87$, $Z = 3.30$, OR = 17.67, $p < 0.001$). This indicates that the reinforced button indicator is not significant in the prediction of making a conditioned response and that the response has generalised to all buttons.

**Figure 7-9:** Average responses made by an individual in the conditioning group to the different button types during the first 120 trials

*Button is given above the individual trace and in the colour key, right. B/C test shows responses to B and C buttons in the absence of airpuff, i.e. the 20% of times that there was no airpuff. A and D were never reinforced. For this individual there was only one B test so there is no associated SEM. The traces represent the average value at each time point ± SEM*
Table 7-2: Logistic regression model based on the first 120 trials, including a reinforced button indicator. The effect of reinforced buttons is non-significant on the odds of making conditional responses. Again the trial number is scaled by maximum, so the significant interaction term between the trial number and the indicator of the conditioning group represents the change from trial 1 to trial 2. Significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$
7.3.4 Generalisation of responses to opposite limbs

The participants of the experiment were asked to switch to their left hand after being trained using their right hand for the first 120 trials. This provided us with the opportunity to see if responses generalised to either hand or were limb-specific. Shown in Fig. 7-10 are the incidences of conditioned responses both before and after switching hands for the conditioning and control group. The conditioning group shows a decline in the proportion of responses after switching hands, but this remains markedly above that of the control group. A simple logistic regression model looking at the effect of group on the likelihood of making a conditioned response during the opposite hand section reveals a significant difference between control pseudo-conditioning and experimental conditioning groups (β = 2.88, Z = 4.33, OR = 17.81, p < 0.001). Coefficients are shown in Table 7-3.

![Graph showing conditioned responses](image)

**Figure 7-10: Conditioned responses are still made after switching from right to left hands**

Trials 121-140 were performed with the opposite hand to training. Here the training section (trials 101-120) CR incidence is compared to the switched hand (trials 121-140). During the switching to left hand period both groups received pseudoconditioning. This was to prevent any quick acquisition of CRs to the opposite hand in the conditioning group. The fact that the response remain high for the conditioning group suggests that conditional response is not limb specific and has generalised to either limb.
Fig 7-11A shows the average responses of an individual in the conditioning group both with the original right hand and the left hand which shows that conditioned responses remained after switching hands and that the blinks were initiated before the pressing of the button. In contrast Fig. 7-11B shows the averaged responses of an individual in the control group, demonstrating no responses from either hand.

Figure 7-11: Average responses between last block of training (trials 101-120) and a period of pressing with the opposite hand (trials 121-140)
(A) Individual from the conditioning group. Large CRs are made with that begin before the button is pressed (left), a movement still remains after switching to their left hand (right). Line represents average and the grey area is ± SEM
(B) Individual from the control group who makes no movement of their eyelid in response button pressing also shows no responses after switching their hands. Line represents average and the grey area is ± SEM
During the period with the left hand both groups received pseudoconditioning.
7.3.5 No conditioned responses when an outsider presses the button

The final part of the experiment consisted of the participants calling out their answers to the experimenter in order for them to press the buttons. This served as a control for any audio or visual aspect of button pressing that may have served as a traditional CS. Fig. 7-12 shows the level of responding after transitioning from self-pressing to an outsider pressing the button. The incidence of conditional responses fall sharply back to the same level as the pseudo-conditioning group when an outsider presses the buttons. A simple logistic regression model fit to the data for the outsider pressing section reveals there is no statistical difference between the incidence of responses between the conditioning group and the control group, details shown in Table 7-4.

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*Table 7-3: Logistic regression showing coefficients of log odds and associated odds ratio with making a CR during the switching of hands period. The conditioning group is associated with much higher odds of making a CR during this period. Significance: * p < 0.05; ** p < 0.01; *** p < 0.001*

**Figure 7-12: CR incidence when experimenter presses the button**

*During this portion of the experiment (trials 201-220) the experimenter pressed the buttons for the participant whilst they called the answers to the quiz questions. Both groups received pseudoconditioning during this period, so they couldn’t be any quick acquisition of CRs to the experimenter pressing. The conditioning groups’ incidence of CRs fell back to the levels exhibited by the control group during this period.*
Fig. 7-13A shows the averaged responses from an individual in the conditioning group. These show a marked difference between themselves pressing the button and an outsider pressing; it can be seen that there are no longer anticipatory blink responses in response to the experimenter pressing the buttons. Fig. 7-13B shows that the averaged responses for an individual in the control group and that the switching to an outsider did not influence the level of responses.

**Figure 7-13**: Average responses while the subject presses for themselves compared (trials 181-200) to when the experimenter presses (trials 201-220).

(A) Individual from the conditioning group. Large CRs are made that begin before the button is pressed by themselves (left), no movement is made when experimenter presses (right). Line represents average and the grey area is ± SEM

(B) Individual from the control group who makes no movement of their eyelid in response to button pressing (left) also shows no responses when the experimenter presses (right). Line represents average and the grey area is ± SEM

During the period where the experimenter presses both groups received pseudoconditioning.
Table 7-4: Logistic regression model showing the coefficient of the log odds of making a CR and associated odds ratio during the period which the experimenter presses. There is no significance of the conditioning group over that of the control (contained within the intercept). Significance: * p < 0.05; ** p < 0.01; *** p < 0.001

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7.4 Discussion

The results presented in this chapter represent to our knowledge the first evidence of conditioning using one’s action as the conditional stimulus. The conditioned responses that appear in due to an association between the air puff and button pressing are robust and increase in frequency with learning. The dynamics of the response and learning appear to similar to eyeblink conditioning studies conducted in humans in other studies (Gabrieli et al. 1995; Cheng et al. 2008; Thürling et al. 2015).

7.4.1 Cerebellar involvement

Although we cannot say with certainty that this learning involves the cerebellum the evidence of cerebellar involvement in eyeblink conditioning (Gerwig et al. 2007), and the detrimental impact of lesions on the learning (Daum et al. 1993) show that the cerebellum is involved in normal delay eyeblink conditioning. Whether the conditional stimulus in our experiment generates an internal representation or is simply sensory reaference needs to be investigated further. A study using tDCS as a manipulation could provide evidence if cerebellar involvement is required in this learning. tDCS studies have been carried out on humans during eyeblink conditioning with cathodal stimulation leading to reduced CR incidence (Zuchowski et al. 2014); however, the efficacy of such modulations and the correct procedure in which to apply them is not currently clear (Beyer et al. 2017).

7.4.2 Volitional blinking in response to button pressing

One issue that remains to be determined is the influence of any conscious decision to blink in response to the pressing of the button. It has been shown that precisely timed conscious eyelid movements can be learned in response to a stimulus in the absence of an aversive US (Rasmussen and Jirenhed 2017). Individuals reported that they were not entirely aware of the association of the button pressing and the air puff. Most individuals believed the air puff was a distraction that was attempting to influence their choice of answers on the quiz, as they were told at the start. Only one subject reported that they knew that the air puff was associated with the B and C buttons, but not that the buttons immediately triggered it. The fact that individuals did not respond to experimenters pressing the buttons suggests that they may not be consciously blinking in response to the button press.

7.4.3 Visual responses driving the conditional response

One thing we cannot account for in this experiment is the influence of the participants seeing themselves pressing the button as a conditional stimulus. It was clear that they responded with both arms but not when the experimenter pressed, so a very specific visual stimulus of themselves
pressing the button could have reliably predicted the air puff for them. One way to tackle this with future work would be to minimise the amount of which they could see their arms perhaps requiring the buttons to be pressed under a cover that prevents the participants from seeing their hands.

7.4.4 Motor or associated sensory signals as the conditional stimulus

We cannot determine from this data whether the conditional stimulus in this experiment represents motor information reaching the cerebellum or whether it could simply be the sensory consequences of the motor reaching task that provide enough contextual information to condition the eyeblink response. One way to address this would be to attempt a similar experiment but have movements of the subjects arm made by an outside influence while the subject remains passive, in such a way that the movement generated by an outsider is a predictor of an aversive air puff. If conditioning is produced, it is possible that sensory reafference of movement could serve as a sufficient predictor in eyeblink conditioning.

Alternatively, we could change the relation of the air puff to leaving the homepad, rather than to button pressing. In this case, this adds the element of motor planning and removes sensory reafference as the relationship is now between the movement beginning and US delivery. Any sensory signals while sitting still before the movement would likely be unchanging and be an unreliable predictor of the air puff.

Despite these limitations, the results in this chapter present an eyeblink conditioning paradigm that has not been described before and has given the impetus to attempt future work to untangle the relationship between the voluntary movement and reflex eyeblink.
8 Conclusion

8.1 Summary

This dissertation outlines the results produced from several distinct areas, but they can broadly be collated under the headings of electrophysiology and behavioural experiments.

The electrophysiology examined the activity of neurons in the cerebellum and a major source of afferent mossy fibres, the pons. The neurons were tested for their response to relatively simple stimuli, and this was compared to their spontaneous activity. Several key findings were reported from this electrophysiological analysis. Firstly, that pontine neurons have bilateral receptive fields, which may have been hypothesised based on anatomical studies, but nevertheless has not been demonstrated before. The source of these bilateral responses - whether mediated by bilaterally responsive neocortical neurons, or by input from both hemispheres of the neocortex - remains to be seen. Another key finding from the electrophysiological analysis of pontine neurons was the existence of wholly inhibitory responses to peripheral somatosensory input. Again, this was a finding that should perhaps be predicted by an examination of the anatomy and the biochemistry of the region, but one that suggests the pons plays a more complex role in the integration of signals than just that of a simple relay.

The results from experiments involving Golgi cells within Crus II of the cerebellar cortex reveal that the individual Golgi cells can make disparate responses to activation of different afferent mossy fibre source, namely that of the pons and to those of the LRN. These results suggest that the classical interpretation of the Golgi cell as a feedback inhibitor of granule cell transmission may be too simplistic, and any explanation of their role within the cerebellar cortex may have to take into account the various mossy fibre inputs that they receive.

The behavioural part of this work followed logically from neurophysiological analyses carried out previously in our lab which revealed changes in climbing fibre receptive fields after learning conditioned eyeblinks in the delay eyeblink classical conditioning task. The results presented demonstrate that eyeblink conditioning can be second order conditioned and implicate a role of the changed climbing fibre receptiveness in that, on the basis of the response timing.

The rabbit and human second order experiments display remarkable congruity between the responses exhibited by the participants, despite the fact that human and rabbit eyeblink conditioning has some obvious differences. Humans tend to develop responses much more quickly than rabbits, and this appears to be the case for second order conditioning where human participants were making what appeared to be second order responses after a single session of
conditioning consisting of only several hundred trials. The rabbits in contrast took many days, even weeks, before the level at which conditioned responses were made to a CS2 alone stimulus were significantly different from their baseline. One of the problems with the analysis of this behaviour is the variability with which it occurs in a population, both for rabbits and for humans. In both species, several subjects showed robust second order conditioned responses, but evidence of conditioned inhibition also occurred in a number of cases. The factors that determine whether an individual demonstrates conditioned inhibition or robust second order conditioning, or neither, are unknown, but the comparative evidence from both species suggest that it operates variably in both.

Second order conditioning likely represents an unnatural phenomenon in classical conditioning of the eyeblink reflex. A CS2 that is never associated with the US is not a useful predictor of an aversive air puff and as such conditioned inhibition represents the correct response to the CS2. However, we do not know what role second order conditioning might play when intermixed with a serial conditional stimuli (CS2-CS1-US). Here the elements may play a role in making faster associations between the stimuli, but to examine this is difficult because any serial conditional stimulus contains a trace conditioning element of the CS2-US.

The final experiment attempted to address whether a more complex conditional stimulus could be used as a predictor of aversive air puffs. The goal of these experiments was to demonstrate a more naturalistic eyeblink conditioning task and show the tying together of a voluntary motor action with a conditioned reflexive action. This experiment demonstrated that a self-initiated reaching task was a reliable predictor and allowed participants to make conditioned responses to avoid the air puff. Whether the reaching stimulus represents motor planning or sensory reafference being provided to the cerebellum as the contextual signal remains to be elucidated.

8.2 Further work

The unreached goal of this doctoral work was to carry out second order conditioning while recording the activity of neurons that underlie the behaviour of learning first order associations. Unfortunately, this became unfeasible with the loss of restraint as a technique for stable preparations. Although we have not carried out neurophysiological experiments, the prospect of doing them remains interesting. Two aspects of the second order conditioning behaviour would benefit from the exploration of neural activity and the linking of this to the behaviour. Firstly, the timing of responses in individuals in relation to their climbing fibre activity would help us to infer the mechanism by which these responses shift forward in latency. Secondly, an examination of the activity of Purkinje cells in animals who show conditioned inhibition responses would demonstrate whether there is cerebellar involvement in the expression of this behaviour. Similarly, an analysis of
simple spike activity during behaviour and microinfusions of CNQX would demonstrate whether this second order behaviour or conditioned inhibition, is driven by the same cells and in the same areas.

Although the rabbit has been the model organism for delay eyeblink conditioning since the 1960s, several other organisms have seen increased use in recent years including mice, rats and ferrets. The rabbit poses several advantages with an easily measurable and quantifiable nictitating membrane response, a larger cerebellum and with the backing of almost 60 years of study locating and manipulating the behaviour at the level of the cerebellum. However, mice and rats have the advantage of the tractable application of new techniques involving genetic manipulation and expression of optogenetic channels. Such techniques make them attractive options to carry out the aforementioned neurophysiological experiments.

Finally, the cerebellum remains an attractive structure to study to relate behaviour to systems and cells. The work presented in this dissertation reveals that the simplified textbook interpretations are perhaps more complex than they seem, as is often the case. However, the cerebellum still possesses many advantages that make it one of the best structures to study to achieve the ultimate goal of neuroscience, understanding the processes and mechanisms by which the nervous system produces so many complex and varied behaviours.
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