Understanding the Mechanisms of Motor Learning in the Vestibulo-ocular Reflex

by

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for the degree of Doctor of Philosophy

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Abstract

The vestibulo-ocular reflex (VOR) is a simple reflex that stabilizes gaze by moving the eyes in the opposite direction to the head. The gain of the VOR (ratio of head to eye velocity) can be increased or decreased during motor learning. It is thought that the memory for learned changes in the VOR gain is initially encoded within the cerebellar flocculus. Furthermore, these learned gain changes can be disrupted or consolidated into a long-term memory. In this thesis we describe novel results that show that consolidation of the VOR can take place rapidly, within 1 hour after learning has stopped. Furthermore, we demonstrated that unlike learning, which has been shown to have frequency selectivity, disruption and rapid consolidation generalize across the range of frequencies. We suggest that disruption and rapid consolidation in the VOR are local mechanisms within the cerebellar cortex, and do not require new learning. This thesis also provides additional evidence for the idea that learned gain increases and decreases are the result of separate mechanisms, most likely long-term depression and potentiation respectively, at the parallel fibre-Purkinje cell synapses. We demonstrate that learned gain increases, but not decreases, require the activation of type 1 metabotropic glutamate receptors (mGluR1) and B type γ-aminobutyric acid ($GABA_B$) receptors. Blocking one or both of these receptors with an

antagonist inverts gain-up learning, while the agonist augments gain-up learning. Furthermore, we provide novel evidence that these receptors are co-activated during gain-up learning.

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Table of Contents

List of Tables

List of Figures

List of Appendices

List of Abbreviations

2-AG	2-Arachidonoylglycerol
AA	Arachidonic acid
ACSF	Artificial cerebro-spinal fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
BC	Basket cell
BCM	Bienenstock, Cooper, Munro
Ca^{+2}	Calcium
CAMK	Ca^{2+}/calmodulin-dependent protein kinases
cAMP	Cyclic adenosine monophosphate
CB1	Cannabinoid receptor type 1
CF	Climbing fibre
cGMP	Cyclic guanosine monophosphate
CGP 52432	3-[[(3,4-Dichlorophenyl)methyl]amino]propyl]diethoxymethyl)phosphinic acid
CHO	Chinease hamster ovary
CN	Cranial nerve
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CR	Conditioned response
CREB	cAMP response element-binding

CS	Conditioned stimulus
DAG	Diacylglycerol
DHPG	3,5-Dihydroxyphenylglycine hydrate
DVN	Descending vestibular nucleus
EPSC	Excitatory post-synaptic current
FTN	Flocculus target neuron
GABA	γ-aminobutyric acid
$GABA_B$	B-type γ-aminobutyric acid receptor
GC	Granule cell
GO	Golgi cell
GRIP1	Glucocorticoid receptor interacting protein 1
HSCC	Horizontal semi-circular canal
IPSC	Inhibitory post-synaptic current
IO	Inferior olive
IP3	Inositol triphosphate
LTD	Long-term depression
LTP	Long-term potentiation
LVN	Lateral vestibular nucleus
MAP	Mitogen-activated protein
MAPK	MAP kinase

MF	Mossy fibre
mGluR	Metabotropic glutamate receptor
mGluR1	Type 1 metabotropic glutamate receptor
MN	Motoneuron
MVN	Medial vestibular nucleus
NMDA	N-Methyl-D-aspartate
NMR	Nictitating membrane response
NO	Nitric oxide
NOS	Nitric oxide synthase
OKR	Optokinetic reflex
PBS	Phosphate buffered saline
PC	Purkinje cell
PF	Parallel fibre
PFA	Paraformaldehyde
PICK	Protein interacting with PRKCA 1
PIP_2	Phosphatidylinositol 4, 5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PLA_2	Phospholipase A2

PLC	Phospholipase C
PTX	Pertussis toxin
PVP	Position vestibular pause
SC	Stellate cell
SOS	Sum-of-sines
SVN	Superior vestibular nucleus
UR	Unconditioned response
US	Unconditioned stimulus
VN	Vestibular nucleus
VOR	Vestibulo-ocular reflex
YM 298198	6-Amino-N-cyclohexyl-N,3-dimethylthiazolo[3,2-a]benzimidazole-2-carboxamide hydrochloride

Chapter 1

1 General Introduction

In this thesis we will discuss the horizontal vestibulo-ocular reflex (VOR), and the mechanisms involved in motor learning and consolidation. To better understand these mechanisms, we will first review the vestibular system which drives the VOR, as well as the basic components of the VOR. We will then review the basic anatomy of the cerebellum, and its contribution to motor learning. We will review the previous research on the sites and theories on motor learning, and outline the current ideas and theories. We will discuss the characteristics of VOR learning and consolidation, and review the mechanisms of synaptic plasticity thought to underlie learning in the VOR.

1.1 The vestibular system

At the heart of the vestibulo-ocular reflex (VOR) are the vestibular signals that drive it. Therefore we will start with a review of the vestibular system, and how the signals that drive the VOR are created.

1.1.1 The semicircular canals

The vestibular labyrinth is composed of two otolith organs (utricle and saccule), and three semicircular canals (horizontal, anterior and posterior). The otolith organs respond to translational, or linear, head movement and gravity. The utricle and saccule are roughly perpendicular to each other, and respond to horizontal and vertical acceleration, respectively. The semicircular canals respond to angular velocity and are roughly orthogonal to each other. The horizontal semicircular canals respond best to rotation around the earth-vertical axis, while the two vertical canals (anterior and posterior) respond to rotation around the earth-horizontal axes.

Each semicircular canal contains a bulbous area at its base called the ampulla. The canal is filled with fluid called endolymph. Within the ampulla, the sensory epithelium or crista contains sensory hair cells. The hair cells extend out of the crista into a gelatinous mass called the cupula. The cupula spans the ampulla and acts as a barrier to the endolymph fluid. As the head moves in the plane of the semicircular canal, the inertia of the endolymph fluid pushes the cupula in the opposite direction to the head movement. The distortion of the cupula causes a distortion of the hair cells within the crista. Each hair cell contains many rod-like projections called "cilia". The many smaller stereocilia are arranged next to a much taller and thicker kinocilium. Deflection of the stereocilia toward the kinocilium causes a depolarization of the hair cells in the ampulla, while deflection of the stereocilia away from the kinocilium results in a hyperpolarization. Thus, angular acceleration of the head in one direction will depolarize the hair cells and excite the afferent axons in one canal, while rotation in the opposite direction will hyperpolarize the hair cells and diminish their spontaneous activity. Furthermore, the magnitude of the response of the hair cells is graded with the amplitude of the stimulation. Greater head acceleration will cause more deflection of the cupula and thus a greater response from the hair cells.

Angular acceleration is encoded bidirectionally. Each semicircular canal is functionally paired with another on the opposite side of the head in the same plane of orientation. Because of the mirror arrangement of the semicircular canals, acceleration in a certain direction will depolarize one of the canals, while the opposite canal will be hyperpolarized. For instance, in the horizontal semicircular canals, a head rotation toward the left side will cause depolarization of the left semicircular canal and hyperpolarize the right semicircular canal. Thus, each functional pair of canals provides a bilateral indication of head movement.

Primary afferent fibres from the semicircular canals provide the brainstem with a head velocity signal. Together, the vestibular nerve and the cochlear nerve make up the eighth cranial nerve (CN VIII). The neurons of the vestibular afferent fibres are spontaneously active with a variable baseline activity of approximately 10-100 spikes/sec (Löwenstein and Sand, 1940; Boyle and Highstein, 1990). They can increase or decrease their firing rates according to the direction of the cupula deflection. The primary afferent neurons are bipolar, so that the dendrites of the neurons

innervate the hair cell and the axon projects to the brainstem. The cell bodies lie within the ganglion of Scarpa.

1.1.2 Secondary vestibular nuclei

Primary afferents terminate heavily within the vestibular nuclei. The function of the vestibular nuclei is to integrate sensory information from the semicircular canals with inputs from the visual system, spinal cord, and cerebellum. The primary vestibular afferents enter the brainstem at the medulla at the level of the lateral vestibular nucleus (LVN), where it divides into the ascending and descending branches. Branches from the afferent fibres in the ascending tract terminate in the superior vestibular nucleus (SVN) or project directly to the cerebellum (the nodulus and uvula) passing through the SVN. Branches from the descending tract end in the medial vestibular nucleus (MVN) and the descending vestibular nucleus (DVN). The interstitial nucleus of the vestibular nerve, which lies within the CN VIII, also receives afferent terminals and sends projections to the flocculus of the cerebellum (Carleton and Carpenter, 1984; Burian et al., 1990).

The secondary vestibular neurons integrate the input from the semicircular canals and project to the motoneurons, cerebellum, and other areas of the brainstem. Many different types of neurons in the vestibular nuclei have been identified based on their behavioural responses and their synaptic inputs. Here, we will discuss two types of these neurons.

The first type of neuron, the position-vestibular-pause neuron (PVP) encodes a combination of head velocity and eye position. PVPs are so named because they have been shown to pause during saccades (Scudder and Fuchs, 1992; Lisberger et al., 1994b). PVPs receive input from the ipsilateral (on the same side) vestibular nerve at monosynaptic latencies and project directly to and excite the contralateral (on the opposite side) abducens nucleus (McCrea et al., 1987; Broussard et al., 1995).

A second type of neuron is the flocculus target neuron (FTN). FTNs are so named because they are directly inhibited by Purkinje cells from the flocculus and parafloccular region (Langer et al., 1985b; Lisberger and Pavelko, 1988; Sato et al., 1988). In addition, FTNs receive excitatory input from both the ipsilateral and contralateral vestibular canals (Broussard and Lisberger, 1992). FTNs project to and inhibit the ipsilateral abducens nucleus (Sato et al., 1988). The synaptic connections of the FTNs make them likely candidates for plastic changes during VOR motor learning, as we will describe in a later section.

1.1.3 Extraocular motor neurons

The head velocity signal is conveyed to motor neurons in the brainstem in order to elicit a compensatory eye movement. There are three pairs of motor nuclei which innervate the extraocular muscles of the eye: the abducens nuclei (CN VI), the oculomotor nuclei (CN III) and the trochlear nuclei (CN IV). Each eye is controlled by six extraocular muscles that form three complementary pairs. The lateral and medial rectus muscles abduct and adduct the eye respectively, and are the muscles involved with the horizontal movement of the eye, such as during the horizontal VOR. The four remaining muscles are involved in vertical and torsional eye movements and are the superior and inferior rectus muscles and the superior and inferior oblique muscles. The motor neurons of the abducens nuclei innervate the lateral rectus muscles. The superior oblique muscles are innervated by the trochlear nuclei. The remaining four muscles, the superior and inferior recti, the inferior oblique, and the medial rectus muscles are innervated by the oculomotor nuclei.

1.2 The Vestibulo-ocular reflex

The VOR is a simple reflex that uses information from the vestibular labyrinth to generate eye movements to stabilize gaze during head movements. When the head moves in one direction, the VOR will move the eyes in the opposite direction with roughly equal amplitude and velocity to maintain a stable retinal image. The VOR is important to stabilize images on the retina during

head movements. Thus, one is able to see and have clear vision during everyday movements such as walking (see: J.C., 1952).

The performance of the VOR is measured in terms of its gain (ratio of eye to head speed) and phase angle (timing between the head and eye movement). Thus, a perfect compensatory VOR would have a gain of 1.0 and a phase of 180 degrees under normal conditions. The gain of the VOR is usually measured in the dark since visual following mechanisms (i.e. smooth pursuit and optokinetic reflex) can also contribute to gaze stabilization. However, at frequencies above 0.5 Hz, these visual pathways contribute only slightly to the overall gain value, as the VOR serves as the main process for gaze stabilization during head movements. The VOR operates effectively in the dark and can respond to head movements with angular velocities up to 360 deg/s (Paige, 1983) and with frequencies as high as 25 Hz (Huterer and Cullen, 2002; Ramachandran and Lisberger, 2005). This rapid response is possible because the VOR pathway is short, and requires only vestibular signals to activate the motor neurons. During low velocity rotation (10-20 deg/s), eye movements were found to be compensatory up to 2 Hz in humans (Tabak and Collewijn, 1994), and up to 5 Hz in cats (Broussard et al., 1999b). This means that the VOR ensures that the eye velocity generated is appropriate with a gain of around 1, and a phase 180 deg to the head movement.

In its simplest form the VOR is a three-neuron arc (Szentagothai, 1950). Primary afferents from the horizontal semicircular canals project to the ipsilateral MVN in the brainstem. Secondary neurons in the MVN project to the contralateral abducens nucleus which activates the lateral rectus muscle innervating the contralateral eye. The contralateral abducens also projects to the ipsilateral oculomotor neurons which innervate the ipsilateral medial rectus muscle. The MVN also inhibits neurons in the ipsilateral abducens which project to the ipsilateral lateral rectus muscle and the contralateral oculomotor nucleus which project to contralateral medial rectus muscle. This inhibitory pathway relaxes the antagonist muscles as the agonist muscles pull the eye. The VOR pathway is shown in Figure 1-1. Leftward head rotation excites and increases the discharge rate of the left vestibular nerve, and decreases the firing rate of the right vestibular nerve in an amount proportional to the head velocity. Through increased excitation of the left

vestibular nucleus, and the decreased excitation of the right vestibular nucleus, the right lateral rectus and left medial rectus muscles are activated, while the right medial rectus and left lateral rectus muscles are inhibited (Fig 1-1, only left pathway is shown). Thus, as the head moves to the left, the eyes move to the right at roughly the same amplitude and velocity.

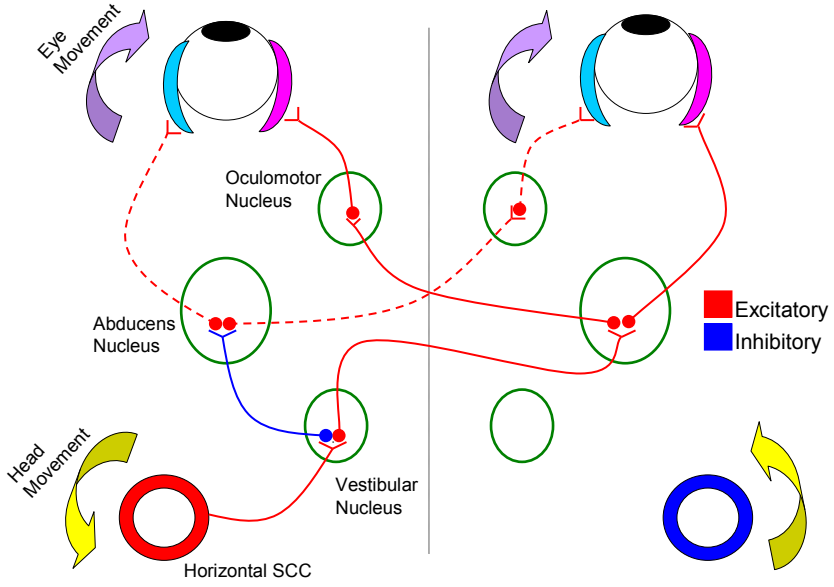

Figure 1-1. The vestibulo-ocular reflex. Head movement to the left (yellow arrows) will excite the left horizontal semicircular canal (SCC) (red), while inhibiting the right canal (blue). Red lines: Excitatory neurons in the vestibular, abducens and oculomotor nuclei (green ovals). Blue lines: Inhibitory interneuron in the vestibular nucleus. Dashed red lines: The excitatory neurons downstream of the inhibitory interneuron will have a decreased excitability. The result of the leftward head movement is the rightward movement of the eyes (purple arrows). The right lateral rectus and left medial rectus muscles are excited and contract (pink), while the right medial rectus and left lateral rectus muscles are inhibited (light blue). Note that only the pathway from the leftward canal is shown, the complementary pathway from the rightward canal is not depicted here.

1.3 The Cerebellum

Although the VOR pathway does not involve the cerebellum, the cerebellum is required to adjust the reflex during motor learning. The cerebellum is known to be critical for motor learning in many processes including the VOR (Robinson, 1976; Lisberger et al., 1984), the eye blink and nictitating membrane (NMR) responses (Yeo et al., 1985a, 1985b), the optokinetic reflex (OKR) (Van Alphen et al., 2002), saccades (Takagi et al., 1998; Barash et al., 1999), smooth pursuit

(Takagi et al., 2000), and more complex limb movements (Andersson and Armstrong, 1987; Martin et al., 1996; Kitazawa et al., 1998). The neurons in the cerebellum are highly organized, and arranged as a basic repeating unit. The conservative nature and repetitive design of the cerebellum allow us to apply knowledge from one area to make suggestions in another.

The cerebellum can be divided into three functional regions: the cerebrocerebellum, the spinocerebellum, and the vestibulocerebellum. Each division receives input from different extra-cerebellar areas and is primarily involved with different motor control functions. The cerebrocerebellum consists of the lateral cerebellar hemispheres. This region receives input from the cerebral cortex, and projects back to the motor, premotor and prefrontal cortices. The cerebrocerebellum is thought to be involved in planning and the mental rehearsal of complex motor actions. The spinocerebellum, composed of the vermis and intermediate hemispheres near the midline, receives information from and projects back to the spinal cord, to help coordinate stability and gait. The vestibulocerebellum is the phylogenetically oldest of the cerebellar regions and contains the floccular region. The flocculus receives input from the vestibular afferents, and unlike other regions of the cerebellar cortex which relay their output via the deep cerebellar nuclei, the flocculus and paraflocculus send their output directly to the vestibular nuclei in the brainstem.

The basic repeating microcircuit of the cerebellum was first described in detail in 1967 (Eccles et al., 1967). The cerebellar cortex is anatomically organized into three distinct layers: the molecular layer, the Purkinje cell layer, and the granular layer (see Figure 1-2). In the flocculus, mossy fibres are thought to convey visual and vestibular information from many different areas. Mossy fibres synapse on to granule cells in the granular layer. The granule cell axons project to the molecular layer where they branch off and form parallel fibres. The parallel fibres travel across the molecular layer where they synapse with the dendrites of the Purkinje cells. Each parallel fibre excites large numbers of Purkinje cells, and each Purkinje cell is contacted by many different parallel fibres. Climbing fibres from the dorsal inferior olive also synapse on the Purkinje cell. In the flocculus, climbing fibres have been shown to carry information on retinal slip (image motion signal) (Graf et al., 1988), vestibular (Simpson et al., 2002) and oculomotor

signals (Winkelman and Frens, 2006). Climbing fibres wrap around the Purkinje cell dendrites and make numerous excitatory synapses. Each Purkinje cell is only contacted by one climbing fibre.

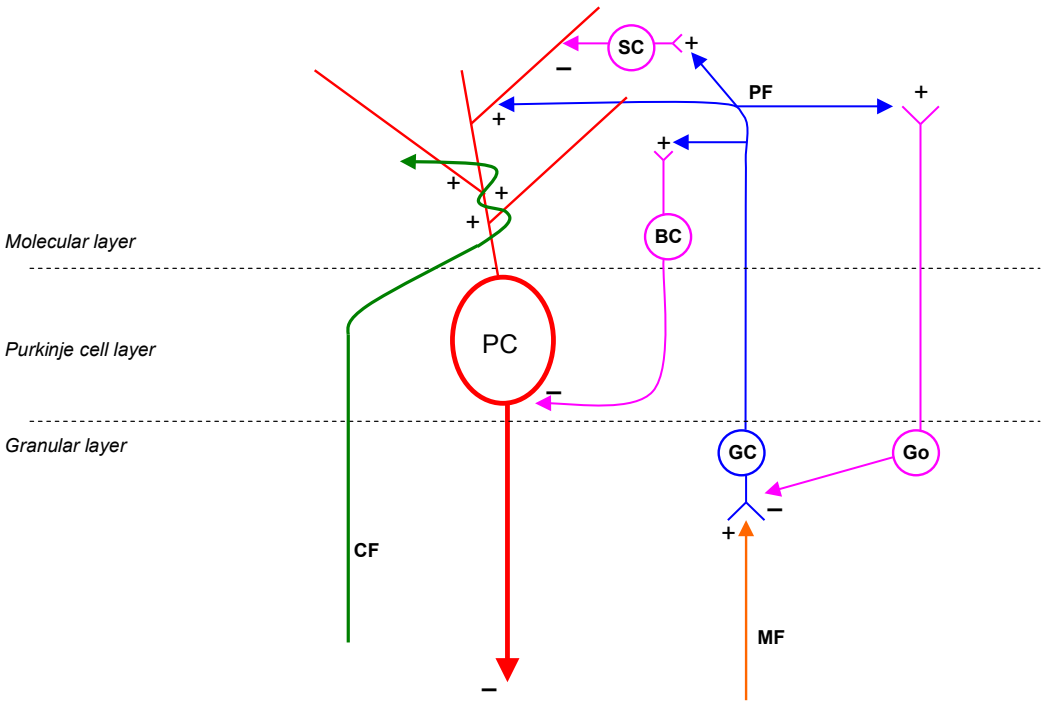

Figure 1-2. The cerebellum. Dashed lines identify the Molecular layer, Purkinje cell layer, and Granular cell layer. Mossy fibres (MF, orange) synapse onto granule cells (GC, blue) which give rise to parallel fibres (PF, blue) that synapse on the Purkinje cells (PC, red). Climbing fibres (CF, green) also form many contacts with a Purkinje cells. Purkinje cells provide the sole output of the circuit and inhibit the deep cerebellar nuclei or vestibular nuclei. Inhibitory neurons (pink; Go: Golgi cells, BC: Basket cells, SC: Stellate cells) feedback onto this circuit. Positive signs (+) indicate an excitatory connection. Negative sign (-) indicate an inhibitory connection.

In addition to these excitatory projections, the cerebellum also contains a number of inhibitory interneurons, which also receive projections from the parallel fibres. Stellate cells and basket cells project to the Purkinje cell dendrites and soma respectively. Golgi cells feed back to and inhibit the mossy fibre-granule cell synapses. This basic cerebellar unit shown in Figure 1-2 is repeated throughout the cerebellum. The uniform architecture of the cerebellum suggests that although different parts of the cerebellum receive and project to different areas, these modules

process information in a similar manner. Thus, to study how the cerebellum functions in one learning task may reveal general principles that can be applied to many different cerebellar-dependent tasks.

Purkinje cells are the only cells that send information out of the cerebellum, and project to the deep cerebellar nuclei or vestibular nuclei. Purkinje cells are known to fire two distinct types of spikes, simple spikes and complex spikes. Complex spikes in a Purkinje cell are thought to be due to the unusually strong connections from a climbing fibre, producing a prolonged depolarization. This results in a large amplitude spike, followed by a burst of smaller amplitude action potentials. Activation of the climbing fibre will always produce a complex spike in a Purkinje cell. The frequency of complex spike firing Purkinje cells is very low, only about 1-5 spikes per second. Simple spikes are the most prominent output from the Purkinje cell, and are small single action potentials. Purkinje cells can fire simple spikes spontaneously, but simple spikes are also thought to result from parallel fibre input. Purkinje cells can fire around 30-80 simple spikes per second (Simpson et al., 1996). Climbing fibres are thought to modulate the simple spike output from Purkinje cells (Barmack and Yakhnitsa, 2003). Indeed, the simple spike firing of a Purkinje cell is known to be depressed immediately after a complex spike fires. This indicates that the climbing fibre can modulate the firing of the Purkinje cell, and control the output of the cerebellum. The climbing fibre is thought to play a role in motor learning, as we will describe in a later section.

1.4 Motor learning

Motor learning can be defined as a process associated with practice or experience leading to a long-term change in a motor movement. Some researchers may also use the term “motor adaptation”. However, as “adaptation” can also refer to short-term neuronal changes, we will use the term “motor learning”. The VOR operates without visual feedback. As such, it requires precise calibration through motor learning to maintain its accuracy. Motor learning in the VOR is known to depend on an important side pathway through the cerebellar flocculus. Many lines of experimental evidence suggest that the flocculus and parafloccular regions are specifically

critical for motor learning involving the angular VOR. Lesion (Robinson, 1976; Nagao, 1983; Lisberger et al., 1984) and single unit recordings studies (Ghelarducci et al., 1975; Dufossé et al., 1978; Watanabe, 1985; Raymond and Lisberger, 1998) suggest that the flocculus plays an important role in VOR motor learning. In the light, if the VOR is poorly calibrated, head movement will cause image motion on the retina (retinal slip) which results in blurry vision. Motor learning improves the accuracy of the eye movements by either increasing or decreasing the gain of the VOR to minimize retinal slip.

In the laboratory setting, we can induce motor learning by pairing visual and vestibular signals to bring about a long-term change in the gain of the VOR. Experimentally, learning can be invoked in the VOR by manipulating the visual image to induce errors in the stabilization of gaze. VOR motor learning requires a visual signal combined with either head or eye movement (Collewijn and Grootendorst, 1979; Lisberger et al., 1984; Shelhamer et al., 1994). For example, if the retinal image appears to move more slowly relative to that of the head, the gain of the VOR will decrease (gain-down learning). This can be achieved by rotating the head while viewing the world through miniaturizing lenses or by moving the visual field in the same direction as the head by using an optokinetic drum. Wearing magnifying lenses or moving the visual field in the opposite direction of the head has the opposite effect. When the velocity of the retinal image is increased relative to the velocity of the head, the gain of the VOR will increase (gain-up learning).

In addition to changing its gain, the VOR is also capable of changing its phase (relative timing of head and eye velocity). In the perfect compensatory VOR, eye velocity and head velocity are exactly 180 degrees out of phase. If however, the eye velocity tends to follow (lag behind) head velocity, this is called a phase lag. A phase lead is also possible if the eye velocity starts to lead the head velocity. During gain-up or gain-down learning, small changes in phase are also commonly seen. It is also possible to change the phase of the VOR during phase training. In the lab, during phase reversal training, an animal is rotated, while the visual world is rotated (using an optokinetic drum) at the same frequency, but at a higher velocity. This kind of training is usually done gradually over a couple of days, and also causes a decrease in the VOR gain.

In 1980, Miles and Eighmy (Miles and Eighmy, 1980) performed an in-depth study of eight rhesus monkeys wearing various optical devices. With long-term use of magnifying and miniaturizing telescopes (months in some cases), they found that learning was initially induced with an exponential time course, and then seemed to plateau over time. Although the learning process never fully compensated for the change in magnification, the monkeys could achieve up to 75% compensation even after weeks of wearing telescopes and after active and passive rotation. When the telescopes were removed, recovery towards the pre-learning state also followed an exponential time course, although it was generally faster than the original learning process, especially after gain-up learning. If, after gain-down learning, the monkey's head was kept immobilized, a low-gain state was maintained for up to a week. However, it was found that after gain-up learning, the high-gain state was prone to decay. Furthermore, they showed that repeated learning and wearing of telescopes had no effect on the time course of learning or recovery. When monkeys wore dove (left-right reversing) prisms, it was found that not only did the gain of the VOR decrease, but the monkeys are also able to change the phase of their VOR. Although Miles and Eighmy did not show any phase changes with magnifying or miniaturizing telescopes, this may have been a result of the limited and low frequency range they used (0.1-1 Hz). Overall, this was the first in-depth study to investigate the long-term use and repeated use of optical devices on primates. From their results it can be seen that repeated learned changes can occur in both the gain and phase of the VOR. It was concluded that the VOR is a plastic system, which is subject to visually mediated, long-term adaptive regulation. VOR motor learning has been shown to exist in and operate over similar time courses in many species, such as humans (Gonshor and Melvill-Jones, 1976b), squirrel monkeys (Clendaniel et al., 2002), cats (Robinson, 1976), rabbits (Ito et al., 1974b), chickens (Wallman et al., 1982), mice (De Zeeuw et al., 1998), goldfish (Schairer and Bennett, 1986), and others.

1.4.1 Smooth pursuit and cancellation

The cerebellar flocculus is also important for the control of smooth pursuit, which keeps the image of a moving target on the fovea. Purkinje cells in the flocculus and paraflocculus have

been shown to modulate their discharge during smooth pursuit paradigms (Miles and Fuller, 1975; Lisberger and Fuchs, 1978b). Some of these neurons show transient overshoots of activity when pursuit is initiated (Krauzlis and Lisberger, 1994). Indeed, bilateral lesions of the flocculus or total cerebellectomy greatly impair smooth pursuit (Westheimer and Blair, 1974; Zee et al., 1981).

Interestingly, the visual stimulus required for VOR motor learning need not be full field retinal slip. It has been shown that smooth pursuit of a target against a dark background can change the gain of the VOR (Shelhamer et al., 1994).

When a visual target moves with the head in the same direction and speed, the VOR is cancelled in order for gaze to smoothly follow the movement of the target (Lanman et al., 1978). Negation of the VOR during eye-head tracking is known as VOR cancellation. The cerebellar flocculus is also believed to be associated with the cancellation of the VOR, as deficits in VOR cancellation have been shown in studies after floccular lesions or inactivation (Belton and McCrea, 2000; Rambold et al., 2002; Kassardjian et al., 2005).

1.5 Sites of learning

Although the cerebellar flocculus is necessary for motor learning in the VOR, for many decades the actual site of learning remained controversial. It was not clear whether the cerebellum was required to provide an instructive signal for learning or was the actual site of learning. A site of learning is thought to have three requirements. First, it must receive vestibular input. Second, it must receive some kind of error or teacher signal to modify the motor response. Finally, this site must be capable of reversible synaptic changes. The last of these requirements will be addressed in a later section. Historically, two such places have been implicated in VOR motor learning; the floccular region (flocculus and paraflocculus) of the cerebellum and the vestibular nucleus in the brainstem (see Fig 1-3).

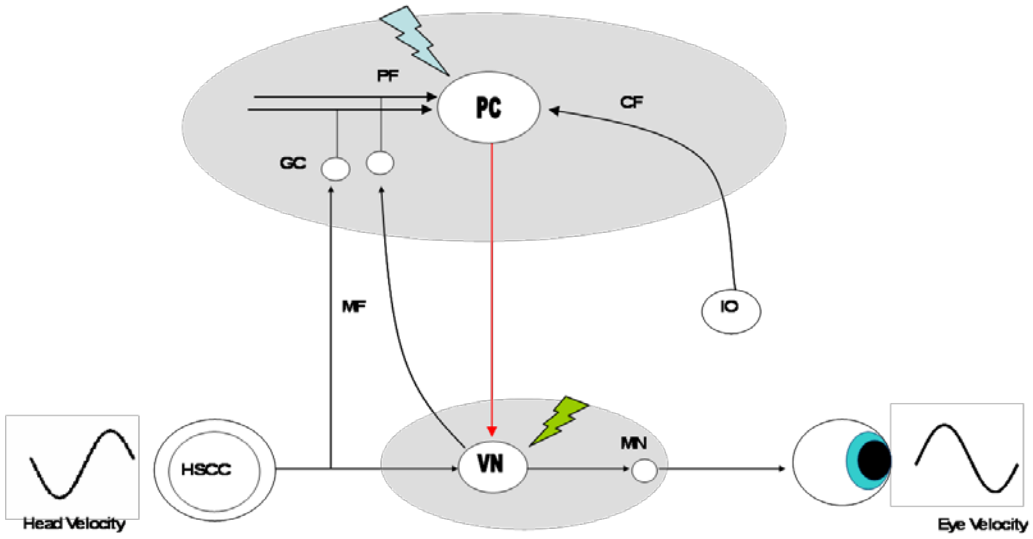

Figure 1-3. Proposed sites of learning for the VOR. Bottom portion outlines the basic circuit of the VOR. Head velocity is detected by the horizontal semicircular canals (HSCC) and projects to the vestibular nucleus (VN). The VN projects to the motor neuron (MN) to move the eyes. Note that the velocity traces show that the eyes move in the opposite direction as the head. Upper portion shows the contribution of the cerebellum. MF: Mossy fibres. GC: Granule cells. PF: Parallel fibres. CF: Climbing fibres. IO: Inferior olive. Purkinje cells (PC) in the cerebellum project to and inhibit (red arrow) the flocculus target neurons (FTNs not shown) in the VN. Small grey oval represents the brainstem. Larger grey oval represents the cerebellum. Blue lightning bolt: Marr-Albus-Ito model in which learning occurs at the PF-PC synapses. Green lightning bolt: Miles-Lisberger model in which learning occurs in the vestibular nucleus (primary afferent to FTN synapses not shown).

1.5.1 The many theories of learning

Early ideas of learning are based on the concepts of Hebbian plasticity (Hebb, 1949). It was proposed that the strength of the synaptic connection between two neurons depends on the timing of the pre- and post-synaptic spikes. When two neurons are activated synchronously, their connection is strengthened. When the neurons fire asynchronously, the connection is weakened. The altered synaptic connection results in a change in the post-synaptic activity, leading to a change in the input-output relationship of the neuronal circuitry. This idea would be the basis of many theories of learning, and inspired many to determine the sites and mechanisms of learning in the cerebral cortex and cerebellum.

Historically, one of the earliest theories of motor learning was proposed by David Marr and James Albus. Based on the remarkable organization of the cerebellum, they proposed that the cerebellum acts as a pattern classification device that can be taught to generate an appropriate output in response to a certain input (Marr, 1969; Albus, 1971). In this model, a certain stimulus would activate a precise population of mossy fibres, and lead to a specific motor output. Activation of a different population of mossy fibres would lead to another different output. This would enable the cerebellum to encode precise stimulus-response mappings. According to the Marr-Albus theory, the climbing fibre provides an instructive signal that regulates the strength of the parallel fibre-Purkinje cell synapses, and guides the encoding of new stimulus-response associations. However, the views of Marr and Albus differed as to the nature of this instructive signal. Marr assumed that the climbing fibre input would cause synchronously activated parallel fibre inputs to be strengthened (based on Hebb's theory), positively reinforcing correct associations (Marr, 1969). Albus, however, believed that the climbing fibre provided an error signal, serving to weaken synapses when the output was incorrect (Albus, 1971). The climbing fibre is thought to report retinal slip, and therefore an error in the function of the VOR. History would later come to agree with Albus.

In agreement with Marr and Albus, Masao Ito proposed that the cerebellar side loop was the site for changes underlying the modification of the VOR (Ito, 1972; 1982). More specifically, Ito predicted that during learning the coincident pairing of vestibular signals via the parallel fibre activity and a visual image motion signal from the climbing fibres would result in the long-term depression (LTD) of the active parallel fibres (Ito, 1982). According to Ito's theory, during gain-up learning, vestibular inputs to Purkinje cells originating from the ipsilateral vestibular labyrinth would fire in phase with complex spike activity and would undergo LTD (Ito, 1982). Under gain-down learning conditions, vestibular inputs originating from the contralateral labyrinth would be more active during complex spikes and would undergo LTD (Ito, 1993). Indeed in 1982, Ito succeeded in demonstrating LTD in the parallel-fibre Purkinje cell synapses after coincident climbing fibre and parallel fibre activation (Ito and Kano, 1982; Ito et al., 1982). Ito originally proposed that the LTD at the parallel fibre-Purkinje cell synapse is paramount, and the

counterpart, long-term potentiation (LTP) is used strictly to reverse the depressed synapses. However, more recent ideas will be discussed later.

In the Marr-Albus-Ito model, the parallel fibre-Purkinje cell synapse is the site of learning (see Fig 1-3, blue symbol), and the climbing fibres from the inferior olive act as a teacher signal, relaying an error signal of visual motion. In agreement with this theory, Maekawa and Simpson found that the climbing fibre signals originate from the retinas. By using a series of electrodes, they stimulated the optic disc of anaesthetized rabbits and recorded in the flocculus of the cerebellum. They found that the climbing fibre signal originates in the retina, via the inferior olive (Maekawa and Simpson, 1972; 1973). Moreover, the dorsal cap of the inferior olive, which projects to the flocculus, receives direct projections from the accessory optic system and the nucleus of the optic tract (Takeda and Maekawa, 1976; Maekawa and Takeda, 1979; Giolli et al., 1985). Subsequently, it has been found that the climbing fibre inputs to the flocculus and ventral paraflocculus fire in response to image motion and can indicate errors in VOR performance when the reflex fails to stabilize images on the retina (Simpson and Alley, 1974; Ghelarducci et al., 1975; Graf et al., 1988; Stone and Lisberger, 1990). Similarly, destruction of the inferior olive or the optic tract alters visual tracking performance and abolishes the ability to change the gain of the VOR (Ito and Miyashita, 1975; Haddad et al., 1980; Tempia et al., 1991; Yakushin et al., 2000).

Shortly after Ito proposed that the cerebellum was the site of motor learning, many investigators were eager to show that the gain of the VOR could be modified and that this learning could be attributed to the cerebellum. Gonshor and Melvill-Jones successfully decreased the gain of human subjects' VOR using dove (left-right reversing) prisms. They found that learning required a visual error signal, as rotation in the dark by itself could not change the gain of the VOR (Gonshor and Melvill-Jones, 1976a). Furthermore, they found that learning is long-lasting and surprisingly robust (Gonshor and Melvill-Jones, 1976b). They attributed the learning they saw to changes taking place in the cerebellum. However, as interesting as these results were at the time, they did not provide evidence for the site of learning. It became clear then, that an animal model would be needed to show a causal relationship between learning and the cerebellum.

At around the same time however, Robinson began training cats to change the gain of their VOR using dove prisms. It was found that the removal of the vestibulocerebellum after training with the prisms abolished the memory of the learning (Robinson, 1976). Similarly, Ito further found in rabbits, that pairing vestibular rotation with a moving slit light illuminated in a dark room could induce changes in the rabbits' VOR gain. Furthermore, these changes could be prevented if the rabbit's flocculus was removed before training (Ito et al., 1974b, 1974a). Similar results were later obtained in monkeys (Lisberger et al., 1984), and cats (Torte et al., 1994). Together, these results suggest that the site of learning is in the flocculus of the cerebellum, and that climbing fibres from the inferior olive act as a teacher signal guiding learning.

However, lesion studies are never exact, and often results in damage to other brain structures and other secondary defects. Lesions to the flocculus can produce deficits in ocular stability and pursuit which could affect and prevent accurate VOR performance and learning. Studies have found that a flocculectomy results in small changes to VOR gain. Some studies found that the gain increased (Robinson, 1976), while others found that the gain decreased (Ito et al., 1980). Furthermore, specific lesions are hard to accomplish and often result in the destruction of surrounding structures. It has been found that the ablation of the flocculus may result in retrograde degeneration in the olivocerebellar neurons in the dorsal cap of the inferior olive (Barmack and Simpson, 1980; Ito et al., 1980). In order to avoid this confound, some investigators used kainic acid to destroy the flocculus. With kainic acid, no retrograde degeneration occurred in the inferior olive, yet motor learning in the VOR was completely abolished following the lesion (Ito et al., 1980; Nagao, 1983).

A different view of motor learning in the VOR was proposed by Miles and Lisberger. Miles and co-workers used monkeys to record the extracellular potentials from Purkinje cells in the ventral paraflocculus elicited by vestibular signals. In order to isolate the responses of Purkinje cells to only their vestibular inputs, the activity of Purkinje cells was recorded during VOR cancellation. Another way to isolate this response is to record from Purkinje cells during smooth pursuit eye

movements and subtract this response from the activity of the cells recorded during the VOR. Using these techniques, they found that the Purkinje cells modify their firing rate during motor learning (Miles et al., 1980b). However, they concluded that it changed in the wrong direction to support the correct direction of learning (Miles et al., 1980a). After gain-up learning, Purkinje cells were significantly more sensitive to head velocity signals than normal, and after gain-down learning using dove prisms Purkinje cells were significantly less sensitive to head velocity and more sensitive to eye velocity than normal (Miles et al., 1980a). According to Ito's model, when Purkinje cells decrease their sensitivity to head velocity, this should increase the sensitivity of the FTNs which would lead to an increase in the VOR gain. When the sensitivity of the Purkinje cells increase the gain of the VOR should decrease.

These findings led to the proposal that the vestibular neurons themselves may be the site in which VOR motor learning is stored (Miles and Lisberger, 1981). In the Miles and Lisberger hypothesis, the Purkinje cell output to the FTNs acts as the teacher signal and modifies the input from the vestibular afferents. Thus, according to this theory, the flocculus is still important for motor learning, to provide the teaching signal; however, the vestibular nucleus is the site at which learning takes place (see Fig 1-3, green symbol). In support of this hypothesis, direct transmission by the 3-neuron arc was shown to be modified slightly during motor learning (Broussard et al., 1992; Khater et al., 1993).

Purkinje cells project to the MVN (Langer et al., 1985b), and inhibit the ipsilateral FTNs (Lisberger and Pavelko, 1988; Lisberger et al., 1994b; Broussard et al., 1995). FTNs are second order vestibular neurons that inhibit the ipsilateral abducens nucleus (Sato et al., 1988; Lisberger et al., 1994a; Broussard et al., 1995), and can therefore drive the VOR pathway. Indeed, FTNs change their activity after long-term changes in VOR gain (Lisberger et al., 1994b). Since the FTNs receive both vestibular and Purkinje cell inputs directly, plasticity could be induced at the primary afferent-FTN synapses, guided by Purkinje cell inputs (Broussard and Lisberger, 1992). The PVPs, the other secondary vestibular neurons, are believed to be unmodified during motor learning (Lisberger and Miles, 1980).

The biggest challenge to the flocculus being the sole site of motor learning is that during rotation the Purkinje cells modify their firing rate in the wrong direction to drive motor learning (Miles et al., 1980a). In response to these results, Ito and his supporters recorded from floccular Purkinje cells themselves. Watanabe recorded from Purkinje cells in monkeys before and after learning (Watanabe, 1984; 1985). The monkeys learned to change the gain of their VOR during passive rotation with a visual scene that moved exactly with or opposite to the monkey to either decrease or increase the gain of the VOR respectively. After learning, Purkinje cells were recorded in the dark during the VOR. They found that after learning, the response of the Purkinje cells were appropriate to support the VOR. Similar results were found in rabbits (Nagao, 1989). Thus, they concluded that the Purkinje cell responses do support the Marr-Albus-Ito model, and are consistent with learned changes taking place in the cerebellar flocculus.

The major differences between the experiments of Miles and colleagues (Miles et al., 1980a) and Watanabe (Watanabe, 1984; 1985) is that while Watanabe measured the response of the Purkinje cells during the VOR in the dark, Miles based his conclusions on the recording of Purkinje cells during VOR cancellation. Indeed, when Miles and co-workers did record Purkinje cells in the dark, a week after VOR learning, they obtained similar results to those of Watanabe (Miles et al., 1980a), at least for gain decreases.

This left a major issue: Should the responses of Purkinje cells be recorded during the VOR in the dark, or during the cancellation of the VOR? Supporters of Ito argued that Purkinje cells measured during VOR cancellation and smooth pursuit are not a valid measurement of the signals present during the VOR. However, one of the major flaws of Ito's "flocculus hypothesis" is that the cerebellum is not a feed-forward arrangement as he originally postulated (Ito, 1972; 1982). In fact, the flocculus receives feedback from the brainstem as an efferent copy of eye velocity signals (Lisberger and Fuchs, 1978a; Langer et al., 1985a). Indeed, mossy fibres transmit information to the cerebellar Purkinje cells about eye position and eye velocity both during pursuit and during the VOR (Lisberger and Fuchs, 1978a; Miles et al., 1980a; Noda,

1986). Therefore, the question remained whether the changes in the VOR caused changes in the response of the Purkinje cells, or whether the changes in the Purkinje cells drive the changes in the VOR as Ito and his supporters maintained.

To reconcile these results and the opposing conclusions, Lisberger and colleagues spent almost a decade recording from different cells in the primate cerebellar cortex and brainstem under different conditions. They recorded from FTNs in the brainstem, during the VOR evoked by rapid changes in head velocity. They first showed that motor learning causes profound changes in the firing of the FTNs that are expressed at a latency of about 12.9 ms after the onset of head motion (Lisberger et al., 1994b). However, when recording from Purkinje cells in the flocculus and ventral paraflocculus, Lisberger and colleagues found that the latency of changes in the Purkinje cells was too long to cause the changes in the FTNs (Lisberger et al., 1994c). Lisberger and co-workers argued that the Purkinje cell response measured in the dark after learning is a result of the efference-copy eye velocity signal. They found that the latency of this response suggested that the signals actually arose from the brainstem by way of the feedback loop to the cerebellum (Lisberger et al., 1994b); as neurons from the medial vestibular nucleus are known to project back to the flocculus (Langer et al., 1985a).

Lisberger and colleagues also used velocity pulses as the vestibular stimulus (as opposed to sinusoidal rotation used by Miles and Watanabe) to measure the gain of the VOR after gain-up and gain-down learning. They found that motor learning causes the responses of the Purkinje cells during the VOR to change in a direction that is appropriate to support the associated changes in the VOR. Furthermore these results are consistent with the findings of both Miles (Miles et al., 1980a), and Watanabe (Watanabe, 1984; 1985), when recording from Purkinje cells in the dark following motor learning.

Lisberger et al. suggested, in agreement with the previous ideas of Ito (Ito, 1972; 1982), that the gain of the VOR would be reduced if the discharges of the Purkinje cells were modulated and

fired in phase with the vestibular inputs from the ipsilateral canal. This would reduce the vestibular drive to the motoneurons by providing cerebellar inhibition to counteract the excitatory inputs from the vestibular nerve. Indeed, they found that firing rate of Purkinje cells recorded in the dark is in phase with the discharge from the ipsilateral vestibular nerve (Lisberger et al., 1994c). Furthermore, when the gain of the VOR is high, the firing of the Purkinje cells is out of phase with the firing of the ipsilateral vestibular nerve. This will increase the modulation of the FTNs, since the inhibition from the Purkinje cells is no longer firing in phase with the excitatory input from the vestibular nerve, and increase the vestibular drive to the motoneurons (Lisberger et al., 1994c). This latter finding disagrees slightly with that of Miles (Miles et al., 1980a), who did not find that increases in the gain of the VOR caused changes in the responses of the Purkinje cells during the VOR. However, Lisberger and colleagues concluded that the Purkinje cell output from the flocculus and paraflocculus provide at least some of the signals that drive the VOR during motor learning.

Finally, Lisberger modelled the discharges of the brainstem interneurons and Purkinje cells during motor learning to make a computer simulation of learning based on a previous model (Lisberger and Sejnowski, 1992). First using the hypothesis of Ito (Ito, 1972, , 1982), he ran a simulation which allowed the only modifiable changes to occur at the vestibular inputs to the Purkinje cells (i.e. the parallel fibre-Purkinje cell synapse). As Ito predicted, with the cerebellar site of learning, under gain-up and gain-down learning conditions, the gain of the VOR increased or decreased in the appropriate manner. However, this model involved a positive feedback loop, represented by the eye velocity signal originating from the brainstem. With this feedback, the eye velocity became unstable and continued to increase or decrease in a “run-away” manner as long as the stimulation was allowed to run. Next, Lisberger modelled the brainstem theory proposed by Miles and Lisberger (Miles and Lisberger, 1981). In this model, which allowed changes only at the vestibular inputs to the FTNs, the gain of the VOR failed to reach its goal. The gain changes simulated in this model were too small compared to real data, and the responses of the simulated Purkinje cells did not reproduce the responses of real Purkinje cells during the VOR. Lastly, Lisberger attempted to reconcile the two disparate views of Miles and Ito, by combining the two ideas in a final model. He found that only when changes were allowed to happen at both the flocculus and FTN sites was learning stable and able to replicate actual results (Lisberger,

1994). With this, Lisberger proposed a new theory of motor learning in the VOR. Lisberger hypothesized that changes in the FTNs cause motor learning, while parallel changes in the Purkinje cells are required to compensate for changes in the brainstem and maintain a stable VOR. Thus, he suggested that the sites of learning during VOR motor learning lie in both the flocculus as Ito originally proposed, as well as the brainstem as proposed by Miles and Lisberger (Lisberger, 1994).

Similar results have been obtained in the vertical VOR. Using squirrel monkeys, Partsalis and colleagues (Partsalis et al., 1995a; Partsalis et al., 1995b) used micro-stimulation in the flocculus, while recording from Y cells in the brainstem. Y cells are thought to be vertical FTN cells, and are located in the dorsal Y group above the vestibular nuclei. The monkeys were trained to increase or decrease the vertical gains of their VOR for long periods of time (up to 2 weeks). After this long-term adaptation, a needle and syringe containing muscimol, a $GABA_A$ receptor agonist, replaced the flocculus electrode and the muscimol was unilaterally injected to inactivate one of the flocculi. In this way, the authors were able to record from Y cells before and after learning, and after unilateral floccular inactivation. Y cells were shown to increase their response in-phase with head velocity during gain decreases, and out-of-phase with head velocity during gain increases (Partsalis et al., 1995a). After the injection of muscimol, the Y cells changed their responses. In normal animals, without learning, musicmol injection caused an increase in the firing rate of Y cells, and the loss of modulation with eye velocity. In animals adapted to gain increases, muscimol resulted in a mean loss of 40% of the cell response to learning. In two animals after prolonged gain-down learning, muscimol resulted in a reduction of 35% of the learned response in one animal and 100% of Y cell responses in the other animal (Partsalis et al., 1995b). Injection sites were later confirmed to be mostly contained within the Y group. Similar to Lisberger, Partsalis and colleagues also suggested the existence of two learning sites in the vertical VOR pathway, one in the flocculus, and the other in the Y cells. However, more current ideas and theories will be discussed in a later section.

1.5.2 The flocculus vs. paraflocculus

Another criticism from Ito and his supporters was related to the role of the flocculus versus the paraflocculus. Many of the extracellular recordings by Lisberger were actually from the Purkinje cells in the ventral paraflocculus (folia 5-10), not the proper flocculus (folia 1-5). It was found that Lisberger used inaccurate coordinates from Madigan and Carpenter (Madigan and Carpenter, 1971). More recent anatomical studies have revealed that the coordinates of Larsell (Larsell, 1970) were more accurate (Gerrits and Voogd, 1989).

This led to the question of whether there is an actual functional difference between the flocculus and its surrounding parafloccular region. Nagao recorded from Purkinje cells in both the floccular and parafloccular regions. He found that Purkinje cells in the flocculus modulate their simple spike activity during head velocity, while Purkinje cells in the paraflocculus show preferential responses to tracking targets and VOR cancellation (Nagao, 1992). However, Lisberger and colleagues recorded from Purkinje cells in both the flocculus and parafloccular regions in their 1994 publications, and found no such differences (Lisberger et al., 1994c; Raymond and Lisberger, 1997).

Supporters of Ito have argued that the floccular and parafloccular regions differ in both the organization of their inputs (Nagao et al., 1997b) and where they project to (Nagao et al., 1997a). They suggested that the flocculus and paraflocculus may mediate different functional roles. They found that the primate flocculus receives mossy fibre input from the primary vestibular afferents, vestibular nuclei and parts of the reticular formation, while the paraflocculus receives major mossy fibre input from the pontine nucleus. They also found that Purkinje cells in the flocculus project mainly to the medial and superior vestibular nuclei as well as the dorsal Y nucleus, whereas the ventral paraflocculus also projects to posterior interpositus and dentate nuclei. They hypothesized that while the flocculus may be involved with the control of the VOR, the ventral paraflocculus may be involved with the control of smooth pursuit eye movements (Nagao, 1992; Nagao et al., 1997b).

However, in a more recent study, Rambold and colleagues ablated regions of the flocculus and ventral paraflocculus, or both, in monkeys in order to determine which of the two regions contributed the most to motor learning (Rambold et al., 2002). They found that the severity of the motor learning deficit was proportional to the amount of tissue damage in the floccular regions. In fact, they found that the greatest deficits were following damage to the paraflocculus, not the flocculus itself. Together, these results suggest that the functionality of the flocculus and paraflocculus greatly overlap, and are thus more similar than different.

1.5.3 The signals that guide learning

In order for learning to be possible, the instructive signals that each site of learning receives must be able to distinguish between opposing stimuli. For example, the signals guiding learning should be able to discriminate between gain-up learning and gain-down learning conditions, as well as high versus low rotational frequencies. In 1998, Raymond and Lisberger recorded neuronal signals from Purkinje cells in awake and behaving animals. By comparing the simple spike activity from Purkinje cells and the vestibular signals during rotation they could analyze the signals that would be present at the brainstem site during different learning conditions. Similarly, the signals at the cerebellar site of learning could be analyzed by comparing complex spikes in the Purkinje cells which indicates climbing fibre activity to the vestibular signals during rotation. Interestingly, they found that while a comparison of simple spike activity and vestibular signals could discriminate between gain-up and gain-down learning conditions at low frequencies, it could not discriminate between these conditions at high rotational frequencies (above 5 Hz) (Raymond and Lisberger, 1998). Thus, at high frequencies the simple spike firing of the Purkinje cells would not be able to guide learning, making the brainstem an unlikely site of learning. While recording complex spike activity, they found that gain-up and gain-down conditions could be discriminated at low frequencies (0.5 Hz), but at higher frequencies the conditions could only be distinguished by comparing the complex spike activity to the vestibular signals present around 100 ms earlier (Raymond and Lisberger, 1998). This requirement is similar to that of classical conditioning paradigms, where learning is most effective when stimuli are separated by an interval of time. Indeed, in the VOR, even though head motion is paired with simultaneous image motion, visual processing causes about a 100 ms delay between image

motion on the retina and representation of that motion in the climbing fibre activity (Stone and Lisberger, 1990). Thus, the cerebellar flocculus receives the signals that are capable of distinguishing between all learning conditions, if there is a delay between climbing fibre and vestibular signals. This delay is thought to be a requirement for other cerebellar dependent forms of learning.

1.5.4 Evidence from eye-blink conditioning

As mentioned, learning in the VOR can also be compared to other cerebellar-dependent learning tasks, such as eye-blink conditioning. In rabbits the third eyelid or nictitating membrane response (NMR) is controlled by the sixth cranial nerve with little or no voluntary component. Most other species lack a nictitating membrane, and so the external eye-blink response, which is controlled by the seventh cranial nerve, and which has a strong voluntary component, is studied instead.

In classical conditioning of the eye-blink response, an unconditioned stimulus (US) such as an air puff or peri-orbital shock to the eye is paired with a conditioned stimulus (CS), a neutral stimulus such as a tone, after a short delay. After many pairings of the CS and the US, a conditioned response (CR) is gradually developed, the eye closing in response to the tone by itself. This learned association is known to depend on the cerebellar lobule HV1 (Attwell et al., 2001), and can be compared to learning in the VOR. The CS, like the vestibular stimulus in the VOR is thought to be provided by mossy fibres, while the US is thought to be provided by climbing fibres, like a retinal error signal (Mauk et al., 1986).

An early study of cerebellar inactivation used muscimol injections in rabbits to temporarily inhibit the cerebellar nuclei while studying the NMR (Hardiman et al., 1996). When muscimol was injected near the interpositus nucleus of the cerebellum one hour before the start of the learning sessions, acquisition of the learned association was significantly impaired. When the learning sessions were then repeated without a prior injection of muscimol, learning occurred normally as if the animal was naïve to the previous conditioning. Extinction of eye-lid

conditioning is accomplished by repeatedly presenting the CS alone. After repeated training sessions to learn a response, muscimol injections in the interpositus nucleus of rabbits prevented the extinction of the learned association. When extinction training was repeated without muscimol, extinction proceeded normally, as if no prior extinction had occurred (Hardiman et al., 1996).

While this study suggests that the learning occurs in the cerebellar nuclei, it has been suggested that inactivation of the cerebellar nuclei breaks the circuit of connections between the inferior olive, the cerebellar cortex and nuclei (olivo-cortico-nuclear loop). Inactivation of the cerebellar nucleus by muscimol can inhibit the inhibitory neurons projecting to the inferior olive. Disturbance of the inferior olive can affect the subsequent projections to the cerebellar cortex and nuclei. Therefore, inactivation of the cerebellar nuclei does not directly confirm that they are the site of learning (Ramnani and Yeo, 1986). A better option than such inhibition experiments in the cerebellar nuclei could be the inactivation of the excitatory transmission using a glutamate antagonist.

Attwell and colleagues (Attwell et al., 1999; Attwell et al., 2001) have addressed this issue using unilateral localized injections of the AMPA/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). The injection of CNQX in the cerebellar cortex is thought to maintain at least some baseline firing, and not block the inhibitory connection of the Purkinje cells to the cerebellar nuclei. It was assumed that the olivo-cortico-nuclear loop would remain intact.

In their first study, rabbits were trained to produce conditioned responses over several training sessions. CNQX or a vehicle was then injected into the right HV1 cerebellar region. Injections of the vehicle had no effect on the performance of conditioned responses. However, after injection of CNQX, CRs were reversibly abolished. The percentage of CRs measured after CNQX was drastically reduced for up to an hour depending on the concentration of CNQX injected. When

the effects of CNQX wore off, rabbits were again able to show normal learned CR responses (Attwell et al., 1999).

In a second study, Attwell and colleagues reversed the order of learning and injections. In this study, they first made a unilateral injection, into the cerebellar cortex (area HV1), of either the vehicle or CNQX. Shortly after the injection, the rabbits underwent a training paradigm pairing the CS (tone) with the US (periorbital shock). During this training session, rabbits that received a vehicle injection learned to perform conditioned responses, however those that received CNQX failed to show any learned response. The CNQX rabbits then underwent a second training session without any prior injection, and showed normal learning progression, as if they were naïve to the training paradigm (Attwell et al., 2001). This suggests that the blockade of the excitatory transmission in the HV1 region of the cerebellar cortex prevented learning.

Together the results from Attwell and colleagues are consistent with the Marr-Albus-Ito theory of the site of learning being the cerebellar cortex. However, these results may also just suggest the importance of the olivo-cortico-nuclear loop. While the findings of Attwell and colleagues (Attwell et al., 1999; Attwell et al., 2001) do not directly address this issue, they do however provide evidence for the importance of the cerebellar cortex for learning and storage of motor learning.

1.5.5 Current theories of VOR motor learning

Over the years, while investigators have argued where exactly the site of motor learning was, technology has advanced to help us better discover the answer. Recently, advances in pharmacology and genetics have provided interesting tools that have allowed investigators to better understand learning and memory in the VOR. It has become clear to many investigators that learning initially takes place in the cerebellum as the Marr-Albus-Ito model predicted, but is more complicated as Lisberger suggested. The best evidence for this is from pharmacological

and genetic manipulation of LTD and synaptic plasticity in the cerebellum which will be discussed in detail in a later section (see: Chapter 1.8).

Using a novel technique developed by Demer et al. (1985), Luebke and Robinson electrically stimulated the climbing fibres leaving the inferior olive in cats in order to examine the role of the flocculus in motor learning. By using the inverse relationship found between simple spikes and complex spikes, Demer and colleagues showed that as stimulation of the climbing fibres increase from 1 to about 10 Hz, the rate of simple spike firing in the Purkinje cells decreased. At a stimulation rate of about 7 Hz, the Purkinje cells were effectively silenced (Demer et al., 1985). Luebke and Robinson used this method to test learning in the VOR, as they theorized that the Purkinje cells in the flocculus under this stimulation clamp would yield no useful output.

Luebke and Robinson showed that while the flocculus was silenced, the gain of the VOR could not return back to normal after a period of learning. Using 4 cats, they first increased or decreased the gain of the VOR using an optokinetic drum. Between learning periods the cats wore either magnifying telescopes, or goggles showing a stationary visual scene to keep the VOR gain increased or decreased respectively. After 3 days of learning the cats VOR gain was twice (gain-up) or half (gain-down) that of their normal value. On the fourth day the cats were rotated for 30 minutes with the opposite learning stimulus to return the gain to normal. This rotation occurred either alone or in combination with flocculus being silenced using the 7 Hz climbing fibre stimulation. Silencing the flocculus prevented the gain of the VOR from returning to its pre-adaptive value (Luebke and Robinson, 1992; 1994). Although the investigators concluded that the site of learning must be the brainstem, all this study really shows is that after 3 days of motor learning the memory was not stored in the flocculus. Finally, Luebke and Robinson tried to test a shorter period before inactivation. After 3 days of learning, the cats were immediately rotated for 30 min using the opposite learning stimulus to return the gain to normal, and after this time the flocculus was silenced. They hypothesized that the memory in the flocculus would be immediately reversed by the silencing; however the gain of VOR did not change back to its learned value (Luebke and Robinson, 1994).

However, there are some criticisms of Luebke and Robinson's work. First, they focused on disrupting the reversal of learning or "de-adaptation", which may not be the same as initial learning period. Also, in both of their experiments the testing followed 3 days of learning. Three days is a long time for learning and, as will be discussed shortly, can affect the site of memory storage. More recent ideas about the long-term storage of memory will be discussed in a later section.

In 1998, a ground-breaking experiment by McElligott and colleagues provided convincing evidence that motor learning initially takes place in the cerebellar cortex (McElligott et al., 1998). By dialyzing lidocaine, a sodium channel blocker, into the vestibulo-cerebellum in goldfish, they were able to produce effective and reversible chemical lesions. In their first experiment, they showed that goldfish can effectively learn to increase or decrease their VOR gain during a 3-hour learning period when artificial cerebrospinal fluid (ACSF) was continually infused in the vestibulo-cerebellum using microdialysis. When, lidocaine was added to the ACSF, the goldfish were unable to change the gain of the VOR during the learning period.

In separate experiments to measure retention of memory, goldfish were able to learn to increase or decrease the gain of the VOR for the 3-hour learning period. After this time a micro-injection of ACSF or lidocaine was made into the fish's vestibulo-cerebellum, and the animal was kept immobilized in the dark for a further 3 hours (retention period). After gain-down learning, an injection of ACSF caused no appreciable change in the VOR gain. The gain remained low for the duration of the retention period. However, the lidocaine-injected animals showed an immediate loss of the adapted VOR gain after the injection. The gain increased to the pre-learned value. In the following 3 hours the gain of the VOR gradually decreased again to the post-learning state. After gain-up learning, retention was not as successful. Immediately after ACSF injection the gain remained high, but over 3 hours the gain gradually decreased back toward the pre-learning value. When lidocaine was injected after learning the gain immediately decreased back to the pre-learning value. During the 3-hour retention period, the gain remained at pre-learning value,

and did not return towards the post-learning value (McElligott et al., 1998). The authors concluded that learning takes place in the cerebellum, as the inactivation of the cerebellum was able to prevent learning. They also concluded that the cerebellum stores the memory of a VOR gain change, as the lidocaine injection was able to block the expression of the memory temporarily (at least for gain decreases).

In a similar study by Nagao and Kitazawa (2003), monkeys wore X2.2 lenses and rotated for 2 hours to increase the gains of their VOR. After the 2-hr learning period each monkey received a bilateral micro-injection into its flocculi, and was left in the dark with its head fixed. If Ringer's solution was injected in the flocculi after learning, the VOR gain remained increased for the duration of the hour. If however, the monkey was injected with lidocaine the gain of the VOR decreased immediately after the injection, and remained around the pre-learning value for the duration of the hour (Nagao and Kitazawa, 2003). Similar to McElligott, Nagao and Kitazawa concluded that inactivation of the flocculus abolishes the VOR motor memory after short-term training (2 hours), and thus the memory of the learning resides in the flocculus.

Together, these studies all suggest the learning occurs in the cerebellar cortex, consistent with the Marr-Albus-Ito hypothesis. However, they also suggest that in the long term, the memory may also be represented at another site outside of the cerebellum, most likely in the brainstem. This theory will be addressed in a later section.

1.5.6 An alternative theory

Over the years, the Marr-Albus-Ito theory has become an accepted theory of motor learning in the cerebellum. More specifically, learning is thought to take place in the cerebellar cortex, as the result of synaptic plasticity of the parallel fibre inputs to the Purkinje cell. This change in synaptic efficacy would result in changes in the Purkinje cell output, or more specifically, changes in the simple spike discharge. The climbing fibre from the inferior olive is thought to act as a "teacher" signal and convey an error signal to the Purkinje cells.

A direct assumption of this theory is that if the climbing fibre represents an error signal, one would expect the modulation of the climbing fibre to peak at the beginning of a learning paradigm when the error would be greatest. To test this, Thier and colleagues used a saccadic motor learning task and recorded from Purkinje cells in the oculomotor vermis during learning. During saccadic motor learning, monkeys are trained to increase or decrease the amplitude of a saccade by consistently having the saccadic target jump inward or outward while the monkey's eyes move toward the target. Over hundreds of trials, the amplitude of the initial saccade made by the monkey will increase or decrease to eventually land near the final target position (McLaughlin, 1967). Thier and colleagues isolated Purkinje cells in the oculomotor vermis and recorded the complex spikes during a saccadic motor learning paradigm. They found that the complex spike activity was random and uncorrelated to the performance error at the beginning of the learning task, i.e., when the error was greatest, and built up a saccade-related discharge pattern during the course of learning. This saccade-related discharge was most pronounced at the end of learning, i.e., when the error was minimal (Catz et al., 2005). These results are not congruent with the idea of climbing fibres signalling an error. The investigators suggest that complex spike firing may underlie the stabilization of learning rather than provide a visual error signal.

To see if this result is applicable to other types of motor learning or just the result of saccadic motor learning, Thier and colleagues recorded the complex spikes during another cerebellum-dependent learning task. In smooth pursuit learning, monkeys are trained to pursue a target moving at a constant velocity. The latency of eye movement to pursuit is about 100-150 ms. If during this time the velocity of the target changes (increase or decrease in velocity) the resultant pursuit will be off target and cause an error (Rashbass, 1961). Over hundreds of trials with the target velocity increasing or decreasing, the monkey will learn to increase or decrease their pursuit velocity (see: Ilg and Thier, 2008). While recording complex spikes from isolated Purkinje cells, Thier and others found that the probability of complex spike discharge changed during learning. As a monkey learned to increase its eye velocity during pursuit, the probability of complex spike firing decreased. As the monkey learned to decrease its pursuit eye velocity,

the probability of complex spike firing increased. The increase in complex spike firing after the monkey learned to decrease pursuit velocity might be due to a preference for low amplitude errors. However, the change in complex spike firing was not correlated with the size of the retinal error, which was extremely variable, but was only correlated with the time course of the experiment (Dash et al., 2010). The investigators suggested that this change in complex spike probability reflected changes in the adaptive state, rather than an error signal. To summarize their findings, Thier and colleagues suggest that the climbing fibre input to the Purkinje cell fires to support learned changes in gain, and do not provide an error signal that is consistent with the Marr-Albus-Ito theory.

A criticism of the studies by Thier and co-workers is that they did not preferentially select Purkinje cells based on their directional sensitivity. In their studies Purkinje cells were not screened based on directional preference, and all learning studies were performed in the same direction. Therefore the absence of correlation between complex spike activity and the retinal slip error may be a population analysis artifact, and the individual preferences of a cells retinal slip direction may have averaged out in the population.

In contrast to Thier, similar studies by Soetedjo and Fuchs found that during saccade motor learning the discharge pattern of complex spikes changed during the error interval in each trial just before the monkey makes corrective saccade back to the target (Soetedjo and Fuchs, 2006; Soetedjo et al., 2008). Furthermore, Soetedjo and colleagues found the probability of complex spike activity to be dependent on both the visual error and size, and found no correlative changes with the progress of learning (Soetedjo et al., 2008). In another study, Medina and Lisberger found during smooth pursuit learning that the presence of a complex spike in one trial was linked to a depression of the simple spike response on the following trial when learning was being expressed (Medina and Lisberger, 2009).

The discharge of a complex spike is known to influence the firing of simple spikes. Therefore, Thier and colleagues also documented the response of simple spikes during saccadic motor learning. While recording Purkinje cell simple spikes during saccadic motor learning, they found that while the discharge of an individual Purkinje cell could not account for the behaviour changes, the dynamics of the simple spike burst from the overall population of Purkinje cells varied with gain increases and decreases. While saccade gain increases were associated with a later onset and peak of the simple spike population burst, saccade gain decreases were associated with an earlier onset and peak of the population burst (Catz et al., 2008). This suggests that motor learning is based on the shape of the overall discharge pattern of simple spikes from the population of Purkinje cells. These results are consistent with the simple spike recordings from Soetedjo and Fuchs, who found that in the majority of Purkinje cells, the change in simple spike activity was in the proper direction for saccade motor learning (Kojima et al., 2010).

The complex spike data from Thier's group in both saccadic (Catz et al., 2005) and smooth pursuit (Dash et al., 2010) learning tasks suggest that climbing fibre activity supports learned changes after motor learning, and does not provide a visual error signal. This idea is not consistent with the theory proposed by Marr, Albus and Ito, and is not supported by similar studies by Soetedjo and Fuchs (Soetedjo and Fuchs, 2006; Soetedjo et al., 2008). Although none of these studies involve VOR motor learning, both saccades and smooth pursuit, like the VOR, are dependent on the cerebellum during learning. Clearly, further research is required. However, the simple spike data obtained from Thier an co-workers (Catz et al., 2008) during saccadic motor learning are consistent with the simple spike data from Soetedjo and Fuchs (Kojima et al., 2010), and are consistent with the cerebellum being the site of learning, proposed by the Marr-Albus-Ito theory.

1.6 Consolidation of learning

1.6.1 Disruption

In many learning systems, a new memory of a specific stimulus can be extinguished or disrupted by the presentation of another stimulus. In motor learning, the skill learned in one motor task is greatly impaired if the subject is forced to learn a second motor task immediately afterwards (Brashers-Krug et al., 1996). In a classical conditioning paradigm, such as NMR or eye-blink conditioning, the expression of the learned conditioned response can be extinguished by the presentation of the conditioned stimulus alone (see: Medina et al., 2002a).

In the VOR, it has been shown that after a short-term learning paradigm, the memory of the learned gain change can be disrupted by rotation in darkness, without a visual stimulus (Cohen et al., 2004; Kassardjian et al., 2005). In cats, during one hour of gain-down learning, the gain of the VOR significantly decreased. Immediately after learning, if the cats were rotated in complete darkness, the gain of the VOR increased back toward the pre-learning value. This indicated that the memory was labile enough to be successfully disrupted. After 72 hours of wearing miniaturizing lenses, rotation in darkness had no effect on the VOR gain. This indicated that the long-term memory was more stable, and not susceptible to the disruption stimulus (Kassardjian et al., 2005).

1.6.2 Consolidation

If a memory is not disrupted, over time it may become more stable and more resistant to disruption. The process by which labile short-term memories become more stable (long-term memory) is known as consolidation. However, while some types of motor systems have been shown to be capable of consolidation (Scavio et al., 1992; Brashers-Krug et al., 1996; Shadmehr and Brashers-Krug, 1997), others do not show consolidation (Goedert and Willingham, 2002). There has been some debate as to whether the simple motor system of the VOR undergoes consolidation.

In some memory systems, consolidation may involve a shift in the site of memory storage (Attwell et al., 2002; Doyon et al., 2002; Ungerleider et al., 2002; Christian and Thompson, 2003; Doyon et al., 2003). For declarative memories, short-term memory is thought to be stored in the hippocampus, and after consolidation, long-term memory is thought to be stored in the neocortex (see: Frankland and Bontempi, 2005). A shift in memory location has also been reported for motor skills learning (Shadmehr and Holcomb, 1997), conditioned eye-blinks (Kim et al., 1995; Medina et al., 2002b), and fear conditioning (Medina et al., 2002b). However, in some memory systems, consolidation is not thought to involve a shift in location (Nadel and Moscovitch, 1997; Doyon et al., 2003).

Consolidation has been reported in other cerebellum-dependent learning tasks such as the eye-blink and NMR conditioning (Attwell et al., 2002; Christian and Thompson, 2003; Inda et al., 2005; Takehara-Nishiuchi et al., 2006). In an effort to discover the site of consolidation, Attwell and colleagues injected muscimol, during the consolidation period, into either the cerebellar cortex or nuclei. Rabbits were trained to produce conditioned responses over repeated training sessions. Muscimol or a saline vehicle was injected after each learning session, at the beginning of consolidation. When muscimol was injected in the cerebellar cortex area HV1, consolidation was prevented. Injections of muscimol or saline in the cerebellar nucleus (anterior interpositus nucleus) had no effect. However, after many learning sessions and over a week later, injections of muscimol in the cerebellar cortex had no effect (Attwell et al., 2002). This study indicates that the cerebellar cortex is involved with consolidation immediately after learning, but in the long-term, the consolidation of a motor memory may depend on another structure.

In the VOR, it has been shown that after weeks of learning, inactivation of the flocculus has little effect on the gain of the VOR (Luebke and Robinson, 1994; Pastor et al., 1994; Partsalis et al., 1995b). If however, the flocculus was inactivated after only 2-3 hours of learning, there is a profound effect, and the memory of the previous learning is abolished (McElligott et al., 1998; Nagao and Kitazawa, 2003). This led to the proposal that memory is first encoded in the flocculus and then transferred to the vestibular nuclei (“transfer hypothesis”) (Galiana, 1986; Peterson et al., 1991; Raymond et al., 1996; Broussard and Kassardjian, 2004).

To test this hypothesis, Kassardjian and colleagues disrupted memory of the VOR and inactivated the cerebellar flocculus after both short-term and long-term learning periods (Kassardjian et al., 2005). After one hour of gain-down learning cats significantly decreased the gains of their VOR. When CNQX was injected bilaterally into each flocculus the cat's ability to cancel it's VOR was significantly reduced, indicating that the signalling in the flocculi was successfully impaired. After 1 hour of learning, a bilateral injection of CNQX eliminated the change in gain, and the gain returned to its pre-learning value. If CNQX was injected after the cats wore the miniaturizing lenses for 72 hours, the gain of the VOR increased, but the memory was not totally eliminated. The gain was still significantly different from the pre-learning value, and thus some memory remained. An injection of a PBS vehicle had no effect on the VOR gain after either short or long-term learning sessions (Kassardjian et al., 2005). These experiments indicate that in the short-term the VOR memory is located in the cerebellar cortex, but in the long-term (over 3 days) the memory is distributed and includes another location, most likely the vestibular nucleus.

These results were recently confirmed and extended to gain-up learning in monkeys (Anzai et al., 2010). Using macaque monkeys, Anzai and colleagues trained the monkeys to decrease or increase the gain of their VOR using dove prisms or magnifying lenses, respectively. If after two hours of learning, a bilateral injection of lidocaine was made in the cerebellar flocculi, the learned change in gain was abolished, and the gain reversed back toward the pre-learning value. An injection of Ringer's solution had no effect on the VOR gain when injected after learning. In a second set of experiments, the monkeys continuously wore the dove prisms (gain-down learning) or magnifying lenses (gain-up learning) for 3 days, and were rotated for 2 hours in the light each day to ensure learning. On the third day, if lidocaine was injected immediately after the learning period, the gain change that occurred during that day was abolished, and the gain returned a value similar to the previous day. If there was no learning period on the third day, the lidocaine had no effect on the gain of the VOR (Anzai et al., 2010). As lidocaine effectively silences neurons, this study illustrates the importance of the olivo-cortico-nuclear loop during consolidation. However, these results are consistent with those found in cats (Kassardjian et al.,

2005), and together indicate the immediate site of memory storage for the VOR is in the flocculus of the cerebellum, but after 3 days the location of the memory is more distributed and may include a site outside of the cerebellum, most likely the vestibular nuclei.

Similar results were also reported in mice after learned changes in the horizontal OKR (Shutoh et al., 2006). After 1 hour of OKR training, a bilateral injection of lidocaine in the cerebellar flocculi returned the gain of the OKR back to the pre-learning value. If however, the mice were trained for 4 days, bilateral inactivation of the flocculi had no effect on the OKR gain, and remained at the post-learning value. Furthermore, Shutoh and colleagues analyzed monosynaptic field responses in the medial vestibular nucleus. Mice that were trained for 4 days showed larger amplitude responses to vestibular nerve stimulation than mice that were naïve to learning or mice that were trained for only 1 hour. This indicates that long-term learning increased the excitability of vestibular neurons that respond to monosynaptic vestibular inputs (Shutoh et al., 2006). Together, these results suggest that the short-term memory of OKR training resides in the cerebellar flocculus, but in the long term is transferred to the vestibular nucleus.

Consolidation and transfer of memory in the VOR is an enticing theory. More recently, a similar model has been proposed to explain the temporary dependence the VOR has within the cerebellum. Dean and colleagues have proposed a “cerebellum-first” model. In this model, VOR learning initially takes place in the cerebellar flocculus, and only after a certain time does the learning in the cortex drive another process in the brainstem to enable learning to occur there as well. In this model, the cerebellum and the brainstem are both sites of learning, but with the learning occurring at different rates (Porrill and Dean, 2007).

1.6.3 Rapid consolidation

The type of consolidation (and transfer) process outlined above was measured over a time course of days and weeks. Recently, Dudai and colleagues have distinguished between two types of consolidation processes (see: Dudai, 2004), systems consolidation and synaptic consolidation.

Systems consolidation is thought to have a time scale of days to weeks, and involves a shift in memory location. Synaptic consolidation is thought to have a shorter time scale of hours to days, and is thought to reinforce learning at the synapses modified during learning. This type of synaptic consolidation could be analogous to the rapid consolidation that has been recently seen in other motor systems.

In an effort to determine the time course of consolidation, Cooke and colleagues expanded on the work of Attwell (Attwell et al., 2002) described earlier. In these experiments, injections of muscimol were made after US-CS learning sessions, either 45 or 90 minutes after a learning session has ended. When muscimol was injected in the cerebellar cortex after 45 minutes, consolidation was prevented. If however, the injection was 90 minutes after each learning session there was no effect, and the learning was consolidated. Therefore, in NMR conditioning a time window of memory transfer was determined. The site of memory was shown to be in the cerebellar cortex for 1 hour after learning, and transferred to another location 1 hour after that (Cooke et al., 2004). This leaves the transfer of memory with a relatively short time window of about two hours. This process could be described as “rapid consolidation” and maybe analogous to the synaptic consolidation described by Dudai.

A similar time window for consolidation has been identified using protein synthesis inhibitors in fear conditioning memory in rats (Schafe and LeDoux, 2000), as well as taste aversion memory in day old chicks (Freeman et al., 1995). This leaves the question open as to whether the simple reflex of the VOR is also capable of undergoing such rapid consolidation. ***We hypothesize that that the VOR is capable of consolidating rapidly, if learning is not disrupted.***

1.7 Characteristics of learning

1.7.1 Frequency selectivity in VOR learning

Learning in the VOR shows specific characteristics. The amount of learning is known to depend on the rotational frequency. Learning occurs best at low frequencies, with less learning occurring at higher frequencies. Learning has been shown to be more effective at frequencies below 4 Hz for both gain decreases (Raymond and Lisberger, 1996; Broussard et al., 1999a), and gain increases (Broussard et al., 1999a). Furthermore, learning in the VOR shows context selectivity. After either gain-up or gain-down learning, the learned change in gain is always greatest when measured at the frequency at which training occurred (Robinson, 1976; Lisberger et al., 1983; Raymond and Lisberger, 1996; De Zeeuw et al., 1998; Kimpo et al., 2005). If the gain of VOR is tested at another frequency, the amount of learning measured will be less than that measured at training frequency. As the training and testing frequencies diverge, the amount of learning measured will decrease, showing "generalization". Generalization is the ability of learning to transfer to a slightly different context. Assuming the training frequency is a context, learning in the VOR can generalize, and be expressed at other frequencies (Kimpo et al., 2005).

In 2005, Kimpo and colleagues described how generalization applied to both gain increases and decreases. The authors quantified the amount of generalization after gain increases and decreases in both mice and monkeys. They found that while both increases and decreases show learning at frequencies other than the training frequency, the amount of generalization was significantly less after gain-up learning. Learned gain increases showed a much sharper tuning curve for the frequency at which training occurred (Kimpo et al., 2005). This study implies that learned gain increases and decreases cannot equally reverse each other with different patterns of generalization.

In 1983, Lisberger theorized that VOR contained frequency channels that allowed preferential learning at the training frequency. Lisberger proposed a model in which the VOR operates as a series of parallel channels, each tuned to a specific frequency, and capable of independently adjusting the VOR gain. These channels most likely overlap partially, allowing learned gain changes at one frequency to generalize to the adjacent frequencies (Lisberger et al., 1983). We

further proposed that the cerebellum also contained frequency specific channels (Broussard et al., 2011). Support for the theory of frequency channels may come from the idea of microzones in the cerebellar circuitry. A microzone in the cerebellum may receive input from the same modality, and the Purkinje cells within the microzone may project to and function in a similar manner (see: Dean et al., 2010), for example they may respond to a similar frequency. In the flocculus, we suggest that synapses are selectively tuned or recruited for certain frequencies of rotation.

Another characteristic of frequency selectivity is shown in the temporal aspect of the VOR, in the measurement of its phase. Many researchers have found that when phase is measured at the frequency of training, the changes in phase are minimal. However, after gain down learning a distinct phase lead is seen at frequencies higher than the training frequency, while a phase lag is observed at frequencies below the training frequency. The opposite is seen after increases, with a phase lag at lower frequencies and a phase lead at higher frequencies. This pattern is known as "phase crossover" and has been well documented (Lisberger et al., 1983; Raymond and Lisberger, 1996; Kramer et al., 1998; Iwashita et al., 2001; Kimpo et al., 2005). Phase crossover can be explained by the idea of frequency channels. To create phase crossover, frequency channels above and below the training frequency would express learning in gain and phase. Channels above the training frequency (higher frequencies) would contribute to a phase lead, while the lower frequencies would contribute to a phase lag.

We describe how learning in the VOR shows frequency selectivity in both the gain and phase. Here, we define all "new learning" in the VOR as showing these frequency selective characteristics. It is thought that the transfer of memory during consolidation would involve new learning. However, it remains unknown whether rapid consolidation or disruption in the VOR are products of new learning, or whether they show frequency selectivity. ***We hypothesize that if rapid consolidation is a mechanism of "new learning" then it too will show frequency selectivity.***

1.8 Synaptic plasticity

Giles Brindley was the first to propose that the cerebellar cortex represents an ideal place for plasticity (Brindley, 1964). Brindley proposed that a Hebbian type of plasticity would be induced at the parallel fibre-Purkinje cell (PF-PC) synapses when parallel fibres (PF) and climbing fibres (CF) fire synchronously. This was later shown to be incorrect. In 1982, Ito was the first to observe long-term depression (LTD) at the PF-PC synapse (Ito and Kano, 1982; Ito et al., 1982). In decerebrate rabbits, Purkinje cells were shown to undergo LTD after vestibular nerve stimulation was applied conjunctively with contralateral inferior olive stimulation (Ito et al., 1982). Effectively, when climbing fibres, thought to be signalling an error, fire synchronously with parallel fibre inputs, the PF-PC synapse is depressed, changing the output of the Purkinje cell. Since then, it has been shown that LTD can been reliably induced in both slice (Sakurai, 1987), and culture preparations (Hirano, 1990a).

More recently, the PF-PC synapses have been shown to be capable of both LTD and long-term potentiation (LTP). However, while LTP has been shown to be both pre- and post-synaptic (Salin et al., 1996; Lev-Ram et al., 2002), it is believed that LTD is only post-synaptic (Sakurai, 1987). Although recently, a new pre-synaptic form of LTD has been described (Qiu and Knöpfel, 2009), this form has not yet been shown to be expressed under *in vivo* conditions. In fact, this form of pre-synaptic LTD can only be expressed when pre-synaptic LTP is pharmacologically blocked.

Decades of research studying LTD at the PF-PC synapse (PF-LTD), has yielded a good understanding of its mechanisms (for reviews see: Daniel et al., 1998; Ito, 2001). However, few studies provide direct evidence for the link between LTD and changes in motor learning. Historically, Ito envisioned LTD to be the sole mechanism of plasticity at the PF-PC synapse, while LTP existed only to re-potentiate the depressed synapses (Ito, 1982). However, more recently it has been proposed that while LTD may be involved with learned gain increases (Boyden et al., 2006; Hansel et al., 2006), LTP may be involved with learned gain decreases (Boyden and Raymond, 2003). Here, we will discuss the mechanisms of PF-LTD and LTP and the dynamic relationship between these two processes. We will discuss and provide evidence for

how these plasticity mechanisms may influence learning in the VOR. Finally, we will look at other forms of plasticity that may also influence learning.

1.8.1 Calcium dependency

The mechanisms of synaptic plasticity in the hippocampus and neocortex are better known than those in the cerebellum. In these systems, early studies suggested that the direction of the post-synaptic plasticity was determined by a calcium threshold. In the post-synaptic cell, LTP is thought to have a high calcium threshold, while a lower threshold determined the induction of LTD (Bienenstock et al., 1982; Bear et al., 1987; Lisman, 1989). This hypothesis is better known as the Bienenstock, Cooper, and Munro (BCM) model (Bienenstock et al., 1982), and has been verified experimentally in the hippocampus and visual cortex (Cummings et al., 1996; Hansel et al., 1996; Hansel et al., 1997). According to the BCM model, an increase in the post-synaptic calcium level will activate LTD at a certain threshold, while a greater concentration of calcium is needed to meet the higher calcium threshold required for LTP induction.

Interestingly, there seem to be different signalling requirements in the cerebellum. While it has long been known that the induction of PF-LTD requires post-synaptic calcium transients (Sakurai, 1990; Konnerth et al., 1992; Augustine et al., 2003), it was mistakenly concluded that post-synaptic LTP was not calcium dependent (Lev-Ram et al., 2002). More recently however, it was shown that the induction of both PF-LTD and LTP depend on the post-synaptic calcium levels. Furthermore, it has been shown that bidirectional synaptic plasticity at the PF-PC synapse may depend on a “reverse” BCM model (Fig 1-4), in which the calcium threshold for LTD is higher than that for LTP (Coesmans et al., 2004; Jörntell and Hansel, 2006; however see: Vogt and Canepari, 2010).

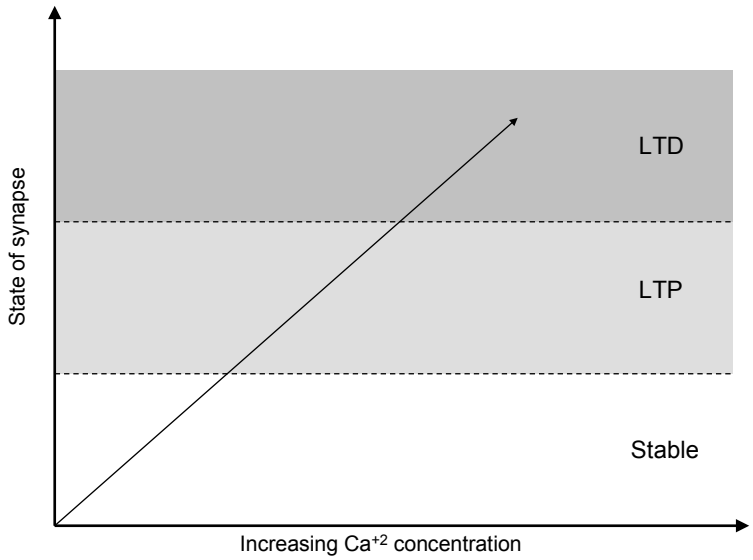

Figure 1-4. Calcium threshold model for LTP and LTD induction in the cerebellum. Bidirectional plasticity at the PF-PC synapse (state of synapse) is determined by calcium thresholds. As the calcium concentration increases within the Purkinje cell dendrite (abscissa), the state of plasticity of the synapse will change. With little or no calcium increase, the synapse is stable. As the amount of calcium increases the thresholds for LTP and LTD will be met. The threshold for LTD induction is higher than that of LTP.

In 2004, Coesmans and colleagues investigated the post-synaptic calcium requirements of PF-LTD and LTP. Using whole-cell recording in cerebellar slices, the authors induced PF-LTD by combined PF and CF stimulation, and induced PF-LTP by PF stimulation alone. They first determined that both LTD and LTP were post-synaptically expressed, and that the LTP induced could be reversed by LTD. The authors found that if the calcium chelator BAPTA, used to lower the internal calcium, was present during the LTD induction protocol, LTP was expressed instead of LTD. In another experiment, during LTP induction, they photolytically released caged calcium from inside the cell, thus raising the internal calcium concentration; LTD was expressed instead of LTP. To show that post-synaptic LTP is calcium dependent, they found that LTP was blocked in the presence of the calcium chelator BAPTA during the LTP induction protocol (Coesmans et al., 2004). This study confirms that both post-synaptic LTD and LTP are calcium dependent. Furthermore, the direction of synaptic plasticity induced in the cerebellum seems to

be the inverse to the BCM model (see Fig 1-4). The induction of PF-LTP requires a small increase in the post-synaptic PC, which is consistent with PF-LTP induction requiring only PF stimulation. The induction of LTD requires a higher calcium concentration than LTP, consistent with LTD induction requiring PF and CF input, a rather large depolarization, or high frequency PF stimulation. This is also consistent with LTD requiring a larger glutamatergic stimulus to activate voltage-gated Ca^{2+} channels and mGluR1 receptors, leading to a larger calcium transient.

1.8.2 Mechanisms of post-synaptic LTD at the PF-PC synapses

Plasticity at PF-PC synapses is thought to be mediated by conductance changes of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor gated ion channels and/or changes in the synaptic density of AMPA receptors (for reviews see: Daniel et al., 1998; Ito, 2001; Jörntell and Hansel, 2006; Kano et al., 2008). AMPA receptors are heteromeric complexes of four homologous subunits, GluR1 to GluR4. In Purkinje cells, GluR2/GluR3 heteromers constitute the majority of AMPA receptors, with GluR2 being the most abundant subunit in the cerebellum (Lambolez et al., 1992). Post-synaptic LTD and LTP can be expressed in the short term by the removal or insertion of AMPA receptors from the post-synaptic density, respectively. Insertion of more AMPA receptors will increase the PC response, while the removal of AMPA receptors will decrease the subsequent response of the PC cell. The insertion and removal of AMPA receptors is thought to be mediated by protein phosphatases and kinases respectively. Protein kinases phosphorylate AMPA receptors which lead to their internalization, while protein phosphatases de-phosphorylate AMPA receptors which leads to their insertion in the cellular membrane. At the PF-PC synapse, during LTD protein kinases are activated, while protein phosphatases are inhibited. During LTP, the opposite occurs, and protein phosphatases are activated while protein kinases are inhibited. This protein kinase-phosphatase switch is the opposite of that in the hippocampus. At hippocampal Schaffer collateral-CA1 pyramidal cell synapses, LTD is controlled by protein phosphatases, while LTP requires protein kinases (see: Jörntell and Hansel, 2006). Thus, just like the calcium requirements of induction relating to the BCM model, the requirements of synaptic plasticity in the cerebellum appears to be the opposite of those in the hippocampus.

The cellular mechanism of PF-LTD is complicated and involves many different proteins, kinases, receptors, and second messengers. Here we will describe the basic requirements of LTD and highlight the important steps that are thought to occur in the signalling pathway. The basic mechanism of PF-LTD is described in Figure 1-5. The induction of LTD requires a strong depolarizing stimulus and large amount of glutamate release from the pre-synaptic neurons. This can be accomplished by the co-activation of parallel fibres and climbing fibres. Climbing fibres enable LTD by causing sufficient post-synaptic depolarization to strongly activate voltage-gated calcium channel dendrites, causing a massive action potential (Eccles et al., 1966), and a large calcium influx (Konnerth et al., 1992). However, climbing fibre activation may be replaced by direct depolarization of the post-synaptic neuron in both slice (Crépel and Krupa, 1988) and culture preparations (Hirano, 1990b; Linden et al., 1991).

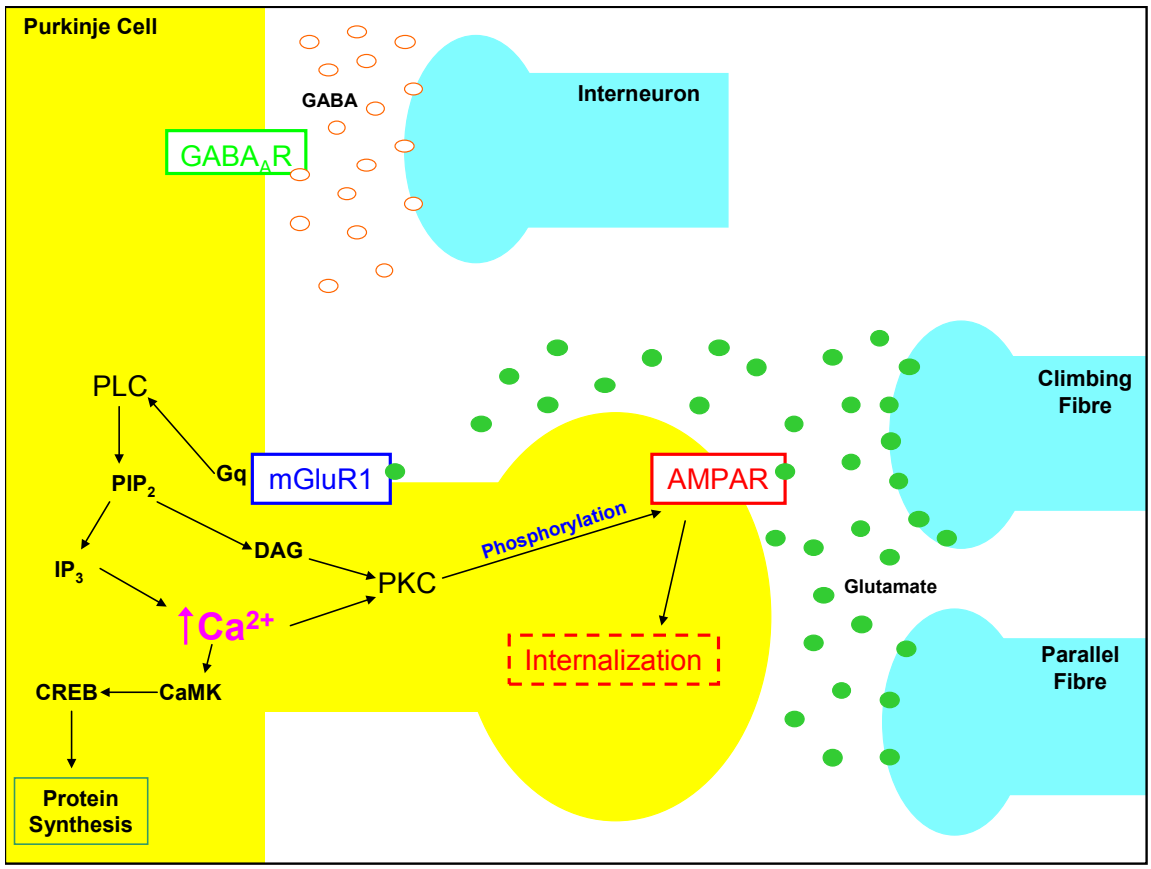

Figure 1-5. Basic mechanism of LTD induction involving mGluR1. During LTD, the combined activation of the parallel fibres and climbing fibres allows glutamate to bind to the type 1 metabotropic glutamate receptor (mGluR1). The Gq protein coupled to mGluR1 will activate phospholipase C (PLC). PLC will split the substrate phosphatidylinositol 4, 5-bisphosphate (PIP_2) into the products inositol 1, 4, 5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 releases calcium from the internal stores, raising the concentration of calcium within the Purkinje cell. The increase in calcium, as well as the product DAG, activates protein kinase C (PKC). PKC phosphorylates AMPA receptors, and internalizes them from the cellular membrane. The increase in calcium also activates calcium/calmodulin-dependent protein kinases (CaMK). CaMK activates the nuclear protein cAMP response element-binding protein (CREB), which leads to protein synthesis and the long-term effects of LTD.

Glutamate, released from parallel and climbing fibres, activates AMPA receptors as well as the type 1 metabotropic glutamate receptors (mGluR1). mGluR1 receptors are coupled to Gq proteins which activate the membrane bound enzyme phospholipase C (PLC). PLC hydrolyzes the substrate phosphatidylinositol 4, 5-bisphosphate (PIP_2) into the products inositol 1, 4, 5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 activates IP_3 receptors and ryanodine receptors on the endoplasmic reticulum facilitated by a protein called HOMER, which cross-links mGluR1 with the IP_3 receptor, to release calcium from the cell's internal stores. The strong depolarization required for LTD induction activates voltage-gated calcium channels in the Purkinje cell, which

also increases the internal calcium concentration (Konnerth et al., 1992; Miyakawa et al., 1992). This increase in calcium, as well as the product DAG, activates protein kinase C (PKC). PF-LTD is mediated by the endocytosis of the GluR2 subunit of AMPA receptors (Wang and Linden, 2000). PKC phosphorylates the GluR2 subunit at the serine 880 residue (Chung et al., 2003), and disrupts the binding of GluR2 to GRIP1 (glutamate-receptor-interacting protein 1), which usually anchors the AMPA receptors to the cytoskeleton lining of the post-synaptic membrane. The endocytosis of the AMPA receptors involves the unbinding of GluR2 from the protein GRIP1 (Dong et al., 1997) and its subsequent binding to PICK1 (protein interacting with C kinase 1) (Xia et al., 1999; Chung et al., 2000). To promote the long-term effects of LTD, the high internal calcium level is also thought to activate calcium/calmodulin-dependent protein kinases (CaMKII or CaMKIV), which activates the nuclear protein cAMP response element-binding protein (CREB), resulting in the transcription of genes (Ahn et al., 1999; Ho et al., 2000; Boyden et al., 2006; Hansel et al., 2006).

Other mechanisms are also thought to be involved with PF-LTD. The ability of PKC to sustain LTD for long-term effects could be prolonged by a positive feedback loop involving the mitogen activating protein (MAP) kinase (MAPK) cascade (see: Ogasawara and Kawato, 2009). The activation of PKC activates MAPK-kinase-kinase, which phosphorylates and activates MAPK-kinase, and finally MAPK. MAPK activates PLA_2 produced the substrate arachidonic acid (AA). It is thought that AA production activates PKC, to keep the pathway of LTD active.

Moreover, the gaseous intercellular messenger nitric oxide (NO) is thought to play a role in the induction of LTD. NO is produced from L-arginine by the enzyme NO synthase (NOS) in the pre-synaptic cell. It diffuses across the synapse to PC neurons, activates gaunylyl cyclase and leads to cGMP and PKG activation. PKG is also thought to be involved with the LTD signalling pathway (see: Ito, 2001). Other intercellular products that may contribute to the induction of LTD are endocannabinoids. The activation of mGluR1 leads to the production of DAG from PLC. DAG is converted to the endocannabinoid 2-AG by DAG lipase. 2-AG is released from the post-synaptic membrane and diffuses, in a retrograde manner, to reach CB1 receptors on the PF terminals. Activated CB1 receptors suppress the release of glutamate and the release machinery

in the pre-synaptic terminals. Thus, endocannabinoid-mediated retrograde suppression is thought to be dependent on both mGluR1 and calcium, and has been thought to represent a local negative feedback loop to prevent the hyper-excitation of Purkinje cells (see: Safo et al., 2006; Kano et al., 2008).

1.8.3 Requirement of LTD for motor learning

As suggested by the Marr-Albus-Ito hypothesis, PF-LTD is probably required for motor learning. Numerous studies have shown that blocking receptors or the downstream mechanisms required for LTD can cause impairments in cerebellar-dependent motor learning tasks (Nagao and Ito, 1991; Li et al., 1995; Shibuki et al., 1996; De Zeeuw et al., 1998; van Alphen and De Zeeuw, 2002; Feil et al., 2003; Koekkoek et al., 2003; Welsh et al., 2005; Boyden et al., 2006; Hansel et al., 2006).

De Zeeuw and colleagues showed that the disruption of LTD impaired VOR motor learning (De Zeeuw et al., 1998). The authors created mutant mice that selectively over-expressed a potent PKC inhibitor only in the cerebellar PCs. It was thought by inhibiting PKC, LTD would be impaired. These mice appeared to have normal PC morphology and spines, including normal innervation by parallel fibres and GABAergic interneurons. The PCs had normal basal electrophysiological conditions, including normal responses from the voltage-gated Ca^{2+} channels and mGluR1 receptors. However, in cultured preparations of PCs from the mutant mice, an LTD induction protocol failed to induce LTD. This suggested that the expression of the PKC inhibitor in the mutant mice prevented the normal induction of LTD. The eye movements measured during OKR or during the VOR in the light were not significantly different from wild-type controls. However, in the VOR, motor learning was found to be impaired during both gain-up and gain-down learning protocols. One hour of vestibular training with an optokinetic drum increased or decreased the VOR gain in wild-type mice; however no significant gain changes were found in mice expressing the PKC inhibitor (De Zeeuw et al., 1998). This study suggests the importance of cerebellar LTD to VOR motor learning.

However, this study was not without its limitations. Like other mutant mice lacking proteins or receptors during development that are required for LTD, these mice fail to undergo normal CF elimination. As a result, these adult mice showed multiple CF innervations to PCs. The authors however, argue that this is not a likely cause of the motor learning deficit. Multiple CF innervations are common in other strains of global knock-out mice, including PKC (Chen et al., 1995; Kano et al., 1995), and have been linked to severe motor ataxia and impaired coordination (Chen et al., 1995). The mice described by De Zeeuw and colleagues (De Zeeuw et al., 1998) did not appear ataxic, and did not show abnormal motor coordination on a rotorod motor test. However multiple CF to PC innervations have been shown to cause impaired motor learning (Kimpo and Raymond, 2007).

The study from De Zeeuw (De Zeeuw et al., 1998) suggested that inhibiting PKC prevented LTD as well as both gain-up and gain-down learning in the VOR. Subsequent studies however, have indicated a more selective role for LTD during learning. More specifically, it has been suggested that LTD is required for learned gain increases in the VOR, but not gain decreases (Boyden and Raymond, 2003).

In 1995, Li and colleagues showed that disruption of NO production can selectively impair gain-up learning in the VOR (Li et al., 1995). Using goldfish, an injection of the NOS blocker L-NMMA bilaterally into the vestibulocerebellum severely impaired subsequent 3 hours of gain-up learning using an optokinetic drum. If goldfish received an injection of the vehicle PBS or the inactive isomer D-NMMA, the gain of the VOR increased normally during learning. Furthermore, injection of L-NMMA after learning had no effect on the retention of the learned gain increases, suggesting that NO is required for the induction of learning but not the expression of the learned changes. Interestingly, the blockade of NO had no effect on the learning of gain decreases. After the bilateral injection of L-NMMA, the goldfish were able to successfully decrease the gains of their VOR during gain-down learning to levels comparable to controls (Li

et al., 1995). This study shows that LTD may have a selective role, and may be required for gain increases, but not decreases in the VOR.

In 2006, Hansel and colleagues also showed a more selective role for LTD during learned gain increases, using knock-out mice lacking αCaMKII (Hansel et al., 2006). Mice lacking αCaMKII were shown to have normal cellular morphology, synapse structure, electrophysiological properties, and synaptic transmission. Interestingly, these knock-out mice were only shown to have delayed CF elimination, unlike other knock-out mice who showed persistent multiple CF innervations (e.g. Chen et al., 1995; Kano et al., 1995; Levenes et al., 1997). In juvenile knock-out mice, 49% of PC neurons had multiple CF innervations, while adult mutant mice had normal single CF innervations. First, the authors showed that LTD induced by PF and CF stimulation was impaired in cerebellar slices from juvenile knock-out mice. This impairment was specific for LTD, as PF stimulation-induced LTP was normal in knock-out mice. The authors then wanted to show that the LTD deficit was due to lack of αCaMKII and not due to the multiple CF innervations. To show this they used the CaMKII inhibitor KN-93. With the KN-93, the LTD induction protocol resulted in LTP in juvenile wild-type mice. Similarly, slices in adult knock-mice showed LTP during the LTD induction protocol (Hansel et al., 2006). These results show that αCaMKII is required for LTD but not LTP. Furthermore, blocking CaMKII (and thus LTD) can result in LTP during the LTD induction protocol.

To see how this specific LTD impairment affects motor learning Hansel and colleagues tested the VOR and OKR in the αCaMKII knock-out mice. Although the mutant mice showed normal baseline performance in both the VOR and OKR at various frequencies, they showed specific impairments during motor learning. During gain-up learning conditions, mutant mice were unable to increase the gains of either their VOR or OKR. Interestingly, the αCaMKII knock-out mice showed no learning deficits during gain-down learning (Hansel et al., 2006). The specific impairments in both LTD and gain-up learning supports the hypothesis proposed by Raymond and colleagues that while gain-up learning depends on LTD, LTP might be responsible for gain-down learning (Boyden and Raymond, 2003).

The inhibition or knock-out of CaMKII during LTD induction led to the production of LTP instead (Hansel et al., 2006). This is compatible with the idea of LTD requiring higher calcium levels than LTP (Coesmans et al., 2004). If the LTD signalling cascade is blocked, the calcium levels required to express LTD might not be reached. Since LTP is thought to have a lower calcium threshold than LTD, LTP might be expressed during the LTD induction paradigm. Indeed, this switch from LTD to LTP has been seen in other studies inhibiting the mechanisms of LTD (Sakurai, 1990; Belmeguenai and Hansel, 2005; Hansel et al., 2006; van Woerden et al., 2009).

Although we have outlined many studies that implicate a role for LTD during motor learning, a recent study has provided some evidence that LTD is not required for cerebellar motor learning, and learning may be due to other not yet known mechanisms. Schonewille and colleagues studied strains of mutant mice that have selective impairments in the internalization of AMPA receptors, such as the PICK1 knock-out mice, and mice with specific deletions in the GluR2 AMPA receptor subunit. These mutant mice showed specific impairments in post-synaptic LTD, while LTP remained unaffected. Surprisingly, these mutant mice were able to learn to both increase and decrease the gains of both their VOR and OKR (Schonewille et al., 2011). Although, this study may cast doubt on the role of LTD in VOR motor learning, it is not without its limitations. This study does not show the protein or expression levels of GluR2 or PICK1 compared to control mice. The mutant mice in this study could have compensated for the impairment of GluR2 internalization, and utilized other mechanisms. Also this study does not address the role of long-term LTD through CaMKII or CaMKIV. Clearly, further research is required.

1.8.4 mGluR1 receptors

It has long been known that ionotropic glutamate receptors are involved in rapid excitatory synaptic transmission (Hayashi, 1952; Curtis et al., 1959). However, over 30 years ago, it was shown that glutamate can also stimulate the production of IP_3 in the cytosol of the post-synaptic

cell, in a G-protein dependent manner (Sladeczek et al., 1985; Nicoletti et al., 1986; Sugiyama et al., 1987). This led to the discovery of the first metabotropic glutamate receptor, mGluR1 (Masu et al., 1991). Metabotropic glutamate receptors (mGluRs) are a family of proteins that have seven trans-membrane domains and affect intracellular chemical messenger systems through their interaction with G-proteins (Houamed et al., 1991; Masu et al., 1991). The family of mGluRs consist of eight different receptors, mGluR1-mGluR8 (Conn and Pin, 1997). Based on their amino acid sequence homology, downstream signal transduction pathways and pharmacological properties, they have been classified into three different groups (Nakanishi, 1992). Group I consists of mGluR1 and mGluR5, which positively couple to PLC through Gq proteins and affect the IP_3/Ca^{2+} signalling pathway. Group II (mGluR2 and 3) and group III (mGluR4, 6, 7, and 8) inhibit adenylyl cyclase through Gi/o proteins and decrease cAMP production.

The expression of mGluR1 is exceptionally high in cerebellar Purkinje cells (Shigemoto et al., 1992; Hampson et al., 1994; Lein et al., 2007). mGluR1 is strongly expressed on the dendrites and soma of PC neurons (Hampson et al., 1994). In general, mGluR1 is expressed post-synaptically at both parallel and climbing fibre synapses (Nusser et al., 1994). More specifically, mGluR1 is thought to be localized perisynaptically and extrasynaptically with respect to these synapses (Grandes et al., 1994; Mateos et al., 2000). Interestingly, there appears to be a spatial segregation between ionotropic and metabotropic glutamate receptors. AMPA receptors are predominantly found opposite to the release site on the post-synaptic membrane, whereas the mGluRs are located at the periphery of the same synapses (Baude et al., 1993; Nusser et al., 1994). The location of mGluR1 receptors may suggest that they are activated during combined PF and CF stimulation, or prolonged PF stimulation, when glutamate release is high and can reach the perisynaptic or extrasynaptic locations. Interestingly, inhibitory neurons in the cerebellum do not express nearly as much mGluR1 (Hampson et al., 1994; Nusser et al., 1994).

1.8.4.1 mGluR1 is required for LTD and motor learning

The role of mGluR1 in PF-LTD is well documented (see: Kano et al., 2008). The activation of mGluR1 activates PLC and leads to the release of calcium from the internal stores, as well as the activation of PKC (see Fig 1-5). Recent evidence has suggested that mGluR1 is essential for cerebellar motor learning tasks.

In 1994, Aiba and colleagues created mutant mice that lack mGluR1 receptors (Aiba et al., 1994b). Using two different protocols to induce LTD in cerebellar slices, it was found that the knock-out mice did not show LTD after depolarization with either a 4 Hz stimulation protocol or pulse trains. Indeed after LTD induction, wild-type PF-EPSCs were depressed, while mGluR1 knock-out mice showed slightly potentiated responses. To see if the impairment in LTD affected cerebellar motor learning, the authors tested eye-blink conditioning in the mutant mice. Although the mutant mice showed normal responses to both the CS (tone) and US (shock), they showed impaired learning of the CR. In wild-type mice, during multiple training sessions the normalized CR response seemed to increase over the entire period of training sessions. Mutant mice seemed to reach a plateau of learning after the second session, after which no further learning occurred (Aiba et al., 1994b). Therefore, while mGluR1 deficient mice could demonstrate some degree of learning, they were significantly impaired in the cerebellum-dependent eye-blink conditioning task. Since these mice were also deficient in PF-LTD, these results would tend to agree with the Marr-Albus-Ito theory, in that deficient cerebellar LTD would cause significant learning impairments. These results also demonstrate the importance of mGluR1 to both LTD and motor learning.

Deficits in PF-LTD were also reported in mGluR1 knock-out mice by Conquet and colleagues (Conquet et al., 1994). Although in normal wild-type mice the LTD induction protocol consistently induced a significant depression in PC neurons, mGluR1 knock-out mice showed significantly reduced PF-LTD. mGluR1 is also required for mossy fibre LTP in the hippocampus. In mGluR1 knock-out mice hippocampal mossy fibre LTP was also significantly reduced. Furthermore, these mice showed deficits in the hippocampal dependent Morris water

maze task testing spatial learning (Conquet et al., 1994). Similar deficits in mossy fibre-LTP and hippocampal-dependent learning tasks were also found by Aiba and colleagues (Aiba et al., 1994a).

In 2002, Shutoh and colleagues tested the ability of mGluR1 knock-out mice to adapt an ocular reflex. Before learning, the OKR gain of mutant mice was found to be significantly lower than the wild-type controls, while the phase of the OKR was found to have a significant lag. The VOR gains of mutant mice were not significantly different from controls; however the phase of the VOR was significantly advanced compared to the normal controls. The authors then tested the OKR during motor learning. After 60 minutes of sustained screen oscillation, control mice had significantly increased the gains of their OKR. However, mGluR1 knock-out mice were unable to change the gains of their OKR (Shutoh et al., 2002). Thus this study demonstrates the importance of mGluR1 to OKR motor learning.

Because the mGluR1 knock-out mice described above were global knock-outs, and mGluR1 is expressed in other areas besides the cerebellum (such as the hippocampus), any deficits cannot be solely linked to the deficits in cerebellar LTD. Furthermore, these knock-out mice were shown to have impaired CF elimination during development (Levenes et al., 1997), which is thought to cause motor learning impairments (Kimpo and Raymond, 2007). In 2000, Ichise and colleagues developed a “rescue” mouse in which the expression of mGluR1 could be controlled by a PC-specific promoter. These mice were deficient in mGluR1 in all areas except for the cerebellum, in which the expression of mGluR1 was 80% of normal. Ichise and colleagues showed that these mice had normal single CF innervations to PC neurons. The authors demonstrated again that while the global mGluR1 knock-out mice were deficient in PF-LTD, the cerebellar rescue mice showed normal LTD (Ichise et al., 2000). However, the question remains of whether the deficit in LTD and therefore cerebellar motor learning in the global knock-out mice could be due to the multiple CF innervations because of the lack of mGluR1 during development, and not due to the missing mGluR1 on the PC neurons.

In a more specific study, it was found that the reduction of mGluR1 can impair cerebellar LTD. In 1994, Shigemoto and colleagues developed two different antibodies that can specifically block mGluR1 receptors and their effects. In CHO cells expressing rat mGluR1 receptors, application of glutamate increased production of measured of inositol phosphate formation, as previously reported (Aramori and Nakanishi, 1992). In the presence of the mGluR1 antibodies, inositol phosphate production was reduced 17-46%. Under whole-cell voltage clamped conditions, PC cells were depolarized while glutamate was ionophoretically applied to induce LTD. After incubation with the mGluR1 antibodies, LTD was significantly impaired. Indeed, after the LTD induction protocol with the antibodies, the cells were slightly potentiated rather than depressed (Shigemoto et al., 1994). This study effectively demonstrates that mGluR1 is required for LTD induction. This requirement could be due to the production of IP_3 and the subsequent increase in calcium. In a similar study, Hartell found using cerebellar slices that the pre-application of the mGluR antagonist MCPG could block LTD induction. In fact, blocking mGluR receptors during LTD induction, brought about LTP instead (Hartell, 1994). However MCPG is not specific for mGluR1, and could affect any mGluR receptor.

Together, these studies indicate a role for mGluR1 in cerebellar LTD and motor learning. Conveniently, it was discovered that mGluR1 is not required for PF-LTP. In cerebellar slices, PF-LTP could readily be induced in the presence of the group I mGluR antagonist AIDA or the mGluR1 antagonist LY 367385 (Belmeguenai et al., 2008). This indicates that mGluR1 is specifically required for LTD, and not involved in LTP. During PF-LTD, activation of mGluR1 receptors *in vitro* causes a large calcium influx into Purkinje cells (Tempia et al., 2001). This suggests that blocking mGluR1 might reduce the post-synaptic calcium levels enough to achieve LTP during LTD induction. As suggested above, a small potentiation was also found in other studies that blocked mGluR1 during LTD induction (Aiba et al., 1994b; Shigemoto et al., 1994). It was also shown that disruption of mGluR1 can impair cerebellar motor learning. In VOR motor learning it was suggested that PF-LTD may mediate gain-up learning, but gain-down learning is dependent on LTP (Boyden and Raymond, 2003). ***We hypothesize that blocking mGluR1 receptors will selectively impair gain-up learning in the VOR.***

1.8.5 $GABA_B$ receptors

GABA (γ-aminobutyric acid) is the major inhibitory neurotransmitter in the central nervous system, and plays a key role in the modulation of neuronal activity. GABA mediates its actions through distinct receptor systems. $GABA_A$ and $GABA_C$ receptors are ionotropic, and initiate a fast acting inhibitory response (early IPSC). $GABA_B$ receptors are metabotropic G-protein coupled receptors that initiate a slow inhibitory response via second messengers (late IPSC). $GABA_B$ receptors are heteromeric, and both the $GABA_{B1}$ and $GABA_{B2}$ subunits are required to assemble together to form a functional receptor. $GABA_B$ receptors share a similar homology to mGluR1 receptors, however they are predominantly coupled to the inhibitory Gi/o protein to mediate their effects (for reviews see: Bettler et al., 2004; Tabata and Kano, 2006; 2010)

In the cerebellum, $GABA_B$ receptors are found at both pre- and post-synaptic sites, but have been suggested to be mostly post-synaptic at the PF-PC synapses (Kulik et al., 2002). It is thought that the splice variants $GABA_{B1a}$ and $GABA_{B1b}$ are mostly pre- and post-synaptically located, respectively. Pre-synaptic $GABA_B$ receptors are thought to inhibit adenylyl cyclase, and the neurotransmitter release machinery. On the post-synaptic membrane, $GABA_B$ receptors are linked to inwardly rectifying K^+ channels (GIRK or Kir3). Upon activation, these channels induce a slow inhibitory post-synaptic current.

In the cerebellum, $GABA_B$ receptors are found primarily found on the PC neurons (Fritschy et al., 1999), and surprisingly, they are most abundant on the periphery of excitatory synapses and are sparsely present at the inhibitory synapses (Fritschy et al., 1999; Ige et al., 2000; Kulik et al., 2002). $GABA_B$ receptors are found on the periphery of the spines or at extra-synaptic sites on the PC dendrites across from parallel fibre synapses. $GABA_B$ receptors are insensitive to the excitatory neurotransmitter glutamate; the GABA required for their activation is thought to result from spill-over from neighbouring inhibitory synapses (Dittman and Regehr, 1997; Hirono et al., 2001). The location of $GABA_B$ receptors is similar to that of mGluR1. mGluR1 and $GABA_B$ receptors have been assumed to be co-localized on the same dendritic spines (Kamikubo et al., 2007; Rives et al., 2009), as they are both present on the PC dendrites (Luján et al., 1997; Kulik

et al., 2002). Recently, an excitatory role for $GABA_B$ receptors has been shown, and is thought to depend on mGluR1. Historically, $GABA_B$ receptors have been suggested to interact with mGluR1 both directly, through receptor to receptor interactions, as well as indirectly via a common downstream mechanism.

1.8.5.1 $GABA_B$ receptors enhance mGluR1 signalling

$GABA_B$ receptors have been implicated in the sensitization of mGluR1 in a calcium-dependent manner through direct interaction of the receptors. Tabata and colleagues (2004) proposed that external Ca^{2+} concentration modulates the glutamate affinity of mGluR1, and that this change in sensitivity was dependent on $GABA_B$ receptors. Using whole-cell recordings in cultured Purkinje cells, in the presence of calcium, low concentrations of DHPG could induce a mGluR1-mediated current. However a higher concentration of DHPG was required when calcium was not present. This indicates that calcium is somehow responsible for the difference in mGluR1 sensitivity. Although a calcium binding site is not present on mGluR1, $GABA_B$ receptors have been shown to bind to calcium, and interact with other receptors independently of its G-proteins (Wise et al., 1999; Galvez et al., 2000; Brown and MacLeod, 2001). Tabata and colleagues found that in the presence of a $GABA_B$ receptor antagonist the sensitivity of the mGluR1 response was abolished, and the low concentration of DHPG failed to produce an mGluR1-dependent current. Furthermore, this calcium-mediated mGluR1 sensitivity was abolished in $GABA_B$ knock-out mice, suggesting that the sensitivity was dependent on $GABA_B$ receptors. The sensitivity was not dependent on G-proteins, as pre-treatment of the cells with pertussis toxin (PTX), which is thought to uncouple $G_{i/o}$ proteins, failed to abolish the response. Finally, using co-immunoprecipitation, the authors show that mGluR1 and $GABA_B$ receptors interact directly and form complexes on PC neurons (Tabata et al., 2004).

The calcium dependent sensitization of the mGluR1 receptors is thought to involve the direct interaction of mGluR1 and $GABA_B$ receptors. This suggests that mGluR1 and $GABA_B$ have to be located on the same dendritic spines. Indeed, mGluR1 and $GABA_B$ receptors have been assumed to be co-localized (Kamikubo et al., 2007). However, our lab has recently shown in

both the mouse cerebellum (Broussard et al., 2011) and in the flocculus of cats (see Fig 1-6), that these receptors do not co-localize to the same dendritic spines. Indeed, we found using immunohistochemistry and confocal microscopy that mGluR1 and $GABA_B$ receptors were rarely present on the same spines. This indicates that a direct interaction, although still possible, would not likely have a major impact on mGluR1 functioning. However, an indirect mechanism effecting mGluR1 would still be likely to occur.

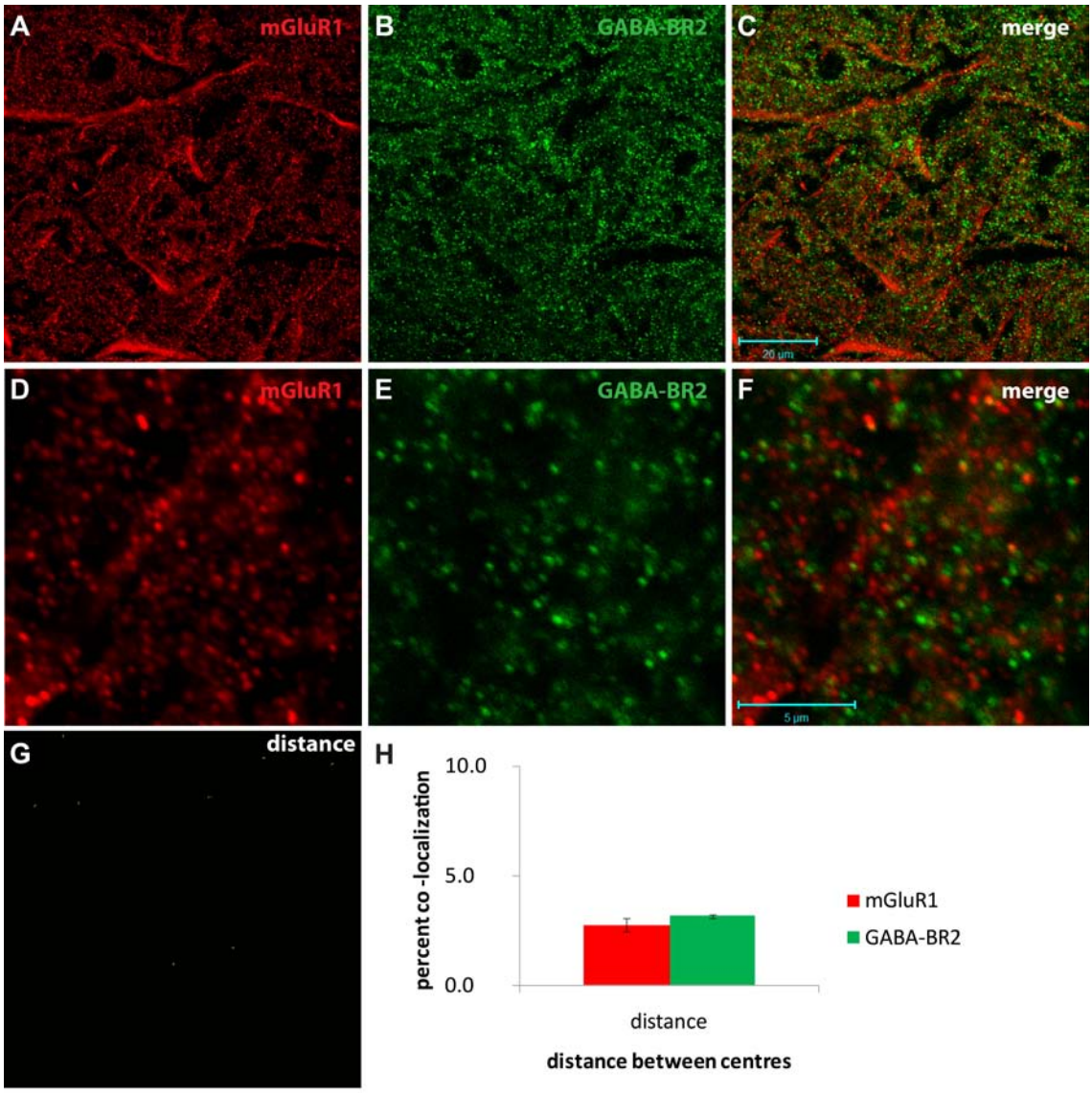

Figure 1-6. Very little co-localization between mGluR1 and $GABA_B$ receptors. Co-localization of the $GABA_B$ receptor antibody (GABA-BR2) and the mGluR1 antibody (mGluR1a) in the molecular layer of the cat flocculus. Expression of mGluR1a (A and D), GABA-BR2 (B and E) and the merged composite images (C and F). Image mask applied to panel F, identifying co-localized immunoreactive puncta based on distance between puncta centres (G). H: A histogram summarizing the mean proportion of co-localizing immunoreactive puncta for mGluR1a – GABA-BR2 combined for cats C, D and E. Scale bars in A-C represent 20 µm; D-F, 5 µm.

In 2001, Hirono and colleagues found that the mGluR1-mediated EPSC could be enhanced by $GABA_B$ receptor activation (Hirono et al., 2001). In voltage-clamped cerebellar slices,

ionophoretic application of the mGluR agonist ACPD produced an inward current in PCs that was mGluR1-dependent. Application of GABA or the $GABA_B$ receptor agonist baclofen significantly enhanced the ACPD produced current. This current enhancement was abolished by the $GABA_B$ receptor antagonist CGP 62349. Furthermore, this $GABA_B$ receptor effect was not dependent on GIRK channels as the current enhancement in the presence of baclofen was not affected by a GIRK channel inhibitor (Hirono et al., 2001).

Hirono and colleagues also investigated the effects of $GABA_B$ on mGluR1- mediated calcium transients in PC neurons. Internal calcium transients were measured using whole-cell recordings and intracellular calcium imaging by a calcium indicator. Application of the mGluR agonist, ACPD, induced an internal calcium transient in PCs. Baclofen enhanced not only the inward current but also the internal calcium transient produced by ACPD. This calcium enhancement was not affected by voltage-gated calcium channel blockers, but was blocked by a Gi/o inhibitor, suggesting the involvement of the G-proteins linked to $GABA_B$ receptors. The $GABA_B$ enhancement was also suppressed by inhibitors of PLC and IP_3 receptors, and the calcium chelator BAPTA (Hirono et al., 2001). This suggests that $GABA_B$ receptors can enhance the mGluR1- mediated currents and calcium transients through its interaction with Gi/o. Furthermore this implicates the involvement of Gi/o with the mGluR1 pathway. Indeed, it has been suggested that the Gi/o proteins can weakly activate PLC (Hahner et al., 1991; Jin et al., 1994; Selbie and Hill, 1998), which is known to be involved in the mGluR1 pathway.

To see if this $GABA_B$ enhancement of mGluR1 signalling extends to LTD induction, which is known to require mGluR1, Kamikubo and colleagues (2007) tested cultured Purkinje cells. First, they induced LTD by ionophoretically applying glutamate to a depolarized PC dendrite held in voltage-clamp. Application of baclofen or GABA to this preparation enhanced LTD, causing an immediate further depression. To see if the $GABA_B$ receptor involvement was dependent on the Gi/o protein, the Purkinje cells were pre-treated with the Gi/o protein inhibitor PTX. With PTX, baclofen had no effect on LTD, meaning the LTD was no longer enhanced. Furthermore, the addition of mastoparan, a Gi/o agonist, by itself enhanced LTD, suggesting that the $GABA_B$ receptor enhancement is via the Gi/o protein. Upon activation, Gi/o is cleaved into α and $\beta\gamma$

subunits. Gi/oα is known to result in the inhibition of adenylyl cyclase. The authors sought to determine which subunit of Gi/o mediated the enhancement of LTD by adding forskolin, an adenylyl cyclase activator. With the addition of forskolin, baclofen was still able to enhance LTD, suggesting it is the βγ subunit of the Gi/o protein that mediates the $GABA_B$ enhancement of mGluR1-dependent LTD. These results were confirmed by Rives and colleagues (Rives et al., 2009). Such early enhancement of LTD suggested that $GABA_B$ receptors were involved with the induction of LTD, not the maintenance or late phase of LTD. This was confirmed, as the application of baclofen before the conjunctive stimuli enhanced LTD, and the application of baclofen after the conjunctive stimuli failed to enhance LTD (Kamikubo et al., 2007).

Using calcium imaging, the authors were able to show that the addition of baclofen further increased the rise in Ca^{2+} normally produced by mGluR1 signalling. This increase in calcium was abolished with the pre-treatment of PTX, again showing that the effect is dependent on the Gi/o protein of the $GABA_B$ receptor. Furthermore, the baclofen induced enhancement of the increase in calcium was still present after the pre-treatment with SKF96365, an antagonist for receptor operated Ca^{2+} channels. This suggests that the $GABA_B$ Gi/o-dependent enhancement is mediated by the increase of calcium from the internal stores. This was confirmed by the addition of thapsigargin, an endoplasmic reticular Ca^{2+}-ATPase inhibitor. By depleting the Ca^{2+} in the internal stores, using thapsigargin, LTD failed to occur at all (Kamikubo et al., 2007). This study shows the $GABA_B$ activation enhances LTD, increasing the level of depression. It is suggested that $GABA_B$ signalling modulates the mechanisms underlying LTD induction. The contribution of the internal calcium stores indicates its involvement in the mGluR1 signalling pathway. It is thought that the activation of the $GABA_B$ receptor causes the βγ subunit, of the Gi/o protein, to interact and enhance PLC (Quitterer and Lohse, 1999); thus increasing the signalling pathway of mGluR1. Although LTD has been shown to be crucial for cerebellar motor learning, an involvement of $GABA_B$ receptors in motor learning has yet to be shown.

In summary, $GABA_B$ receptors have been shown to enhance both mGluR1 dependent currents and LTD. This interaction is most likely via the Gi/o protein and its subsequent activation of PLC. Indeed this $GABA_B$ receptor enhancement has been shown to increase calcium levels,

which is thought to control the induction and the direction of plasticity. Higher levels of calcium are required for the induction of LTD (Coesmans et al., 2004). In the VOR, gain-up learning is thought to depend on LTD (Boyden et al., 2006; Hansel et al., 2006). ***We hypothesize that $GABA_B$ receptors are required for gain-up learning in the VOR.***

1.8.6 Long-term potentiation

The mechanisms involved in cerebellar LTP at the PF-PC synapses are not as well known as that of LTD. The first form of LTP discovered at the PF-PC synapse was pre-synaptic, and increased the amount of neurotransmitter released from the pre-synaptic terminals (for review see: Hartell, 2002). Briefly, pre-synaptic LTP can be induced by high frequency (4-8 Hz) PF stimulation, and involves the activation of adenylyl cyclase, cAMP, PKA, and is triggered by the increase of the pre-synaptic calcium levels. However, since this form of LTP is expressed pre-synaptically, it cannot reverse the post-synaptic LTD described above.

More recently, a post-synaptic form of LTP has been discovered (Lev-Ram et al., 2002), and has been shown to reverse post-synaptic LTD (Lev-Ram et al., 2003). This form of LTP can be induced by low frequency (1 Hz) PF stimulation. Post-synaptic LTP involves the activation of NMDA receptors located on nearby interneurons. The subsequent increase in calcium in these cells activates NOS and produces NO. NO diffuses across the synapse to the post-synaptic Purkinje cell where it leads to the activation of protein phosphatases (e.g. PP1, PP2A, PP2B). Protein phosphatases dephosphorylate the GluR2 subunits in AMPA receptors and lead to their insertion in the post-synaptic membrane (for review see: Hartell, 2002)

PKC and protein phosphatases act as a switch, and are competing processes phosphorylating and dephosphorylating AMPA receptors. During LTD, protein phosphatases are inhibited leaving PKC free to phosphorylate and internalize AMPA receptors. In 2005, Belmeguenai and Hansel showed the delicate balance between post-synaptic LTP and LTD, as well as the role of protein phosphatases in synaptic plasticity (Belmeguenai and Hansel, 2005). Using whole-cell patch-

clamp recordings from PCs in rat cerebellar slices, they first induced PF-LTP using PF stimulation at 1 Hz. Testing the paired pulse facilitation ratio, they showed that this LTP was indeed post-synaptic. They showed that PF-LTP was independent of PKC by inducing LTP in the presence of chelerythrine, a PKC inhibitor. Furthermore, using a general protein kinase inhibitor they showed that PF-LTP is not dependent on any protein kinases, including PKA and PKG. However, PF-LTP is dependent on protein phosphatases. In the presence of okadaic acid or microcystin LR, both of which inhibit PP1 and PP2A, LTP induction resulted in LTD. This was again confirmed with the specific PP1 inhibitor I-2, the PP2A inhibitor fostriecin, and the PP2B inhibitor cyclosporin A. In the presence of these inhibitors, LTP induction again leads to a depression instead of a potentiation. During LTD induction, the presence of microcystin LR, enhanced LTD, leading to a greater depression (Belmeguenai and Hansel, 2005). This shows the importance of protein phosphatases in LTP, and their reciprocal inhibition during LTD.

More recently, these authors have shown the role of protein phosphatases during VOR motor learning (Schonewille et al., 2010). Schonewille and colleagues created mutant mice specifically lacking PP2B in the cerebellar Purkinje cells. These mice were shown to specifically lack LTP, while having normal LTD. Furthermore, these mice showed pronounced defects in cerebellar motor learning. During VOR learning, under gain-up learning conditions, instead of increasing their VOR gain, mutant mice decreased their gain. PP2B knock-out mice also showed a significant reduction in the amount of gain-down learning. These mice were also incapable of learning to change the phase of their VOR. During eye-blink conditioning, the PP2B lacking mice showed specific impairments in their ability to learn conditioned responses (Schonewille et al., 2010). Together, these results show that protein phosphatases (such as PP2B) play a significant role in motor learning. Indeed, this study implies that protein phosphatases and thus LTP are required for both gain-up and gain-down learning in the VOR.

However, like all studies using knock-out mice, this study is not without its complications. The Purkinje cells in the PP2B lacking mice showed severe deficits in their ability to potentiate their intrinsic excitability. These cells were also showed to have a lower firing frequency and an abnormal interspike interval of the simple spike firing. Together, these baseline abnormalities

could account for the extreme learning deficits, especially since the baseline VOR and OKR were also found to be abnormal in the mutant mice (Schonewille et al., 2010). Thus, while protein phosphatases are shown to be required for post-synaptic PF-LTP, and are likely to play a role in motor learning, more research is required to determine its exact role.

1.8.7 Other types of cerebellar plasticity

Although LTD and LTP at the PF-PC synapses have been shown to be required for motor learning, these are not the only synapses in the cerebellum that are capable of synaptic plasticity. Synaptic plasticity has been shown to exist in many synapses in the cerebellum including the CF-PC synapses, the mossy fibre-granule cell synapses, and the inhibitory synapses of the interneurons (for review see: Hansel et al., 2001). Plasticity at any of these cerebellar sites can change the properties and output of the Purkinje cells, and can therefore influence and contribute to motor learning in the cerebellum.

It been shown that synaptic changes at the CF-PC synapses can effect subsequent changes at the PF-PC synapse. PF-LTD is thought to be dependent on the activation of the climbing fibre. LTD at the CF-PC synapse (CF-LTD) is associated with a reduction in the amplitude of climbing fibre evoked calcium transients (Weber et al., 2003). Indeed, in cerebellar slices after the induction of CF-LTD, conditions that normally induce PF-LTD resulted in PF-LTP instead (Coesmans et al., 2004). In addition, the NMDA receptors at the CF-PC synapses have been implicated in the induction of PF-LTD (Piochon et al., 2010). This clearly shows that CF-PC synapses may be directly involved with the plasticity at the PF-PC synapses.

The excitatory inputs onto stellate and basket cells are capable of synaptic changes (Rancillac and Crépel, 2004; Jörntell et al., 2010), as are the inhibitory synapses on the Purkinje cells (Kano et al., 1992; Mittmann and Häusser, 2007). Synaptic plasticity at any of these synapses could affect the firing of Purkinje cells, and thus motor learning. Indeed, it has been suggested that the role of the inhibitory interneurons may be to sharpen the population response of the Purkinje

cells (see: Jörntell et al., 2010). Mutant mice lacking $GABA_A$ receptors on Purkinje cells receive no inhibition from the interneurons (basket cells, stellate cells and Golgi cells). The Purkinje cells in these mice have abnormal simple spike firing, and show specific impairments in the consolidation of learned gain changes, and in the learning of VOR phase changes (Wulff et al., 2009).

Similarly, the NMDA receptor-dependent LTP at the mossy fibre-granule cell synapses have been shown to be involved in motor learning. Mutant mice lacking NMDA receptors were shown to have impaired mossy fibre-granule cell LTP and synaptic excitation. While these mice have normal basic eye movements can successfully decrease their VOR gain, they were shown to have impairments in learning and consolidation of VOR phase changes (Andreescu et al., 2011). However, while some NMDA receptors have recently been shown to present on Purkinje cells, the majority of these receptors are thought to be on the granule cells. Together, these studies suggest that other synapses, besides the PF-PC synapses, are capable of synaptic plasticity and can contribute to motor learning within the cerebellum.

1.8.8 Brainstem plasticity

Although the brainstem and vestibular nucleus has long been theorized as a site of learning (Miles and Lisberger, 1981), the mechanisms of plasticity in this area are not as well known as in the cerebellum (however see: Grassi and Pettorossi, 2001; Gittis and du Lac, 2006). Recently, McElvain and colleagues studied synaptic plasticity at the vestibular nerve synapse onto secondary neurons in the vestibular nucleus. Projection neurons in the vestibular nucleus were shown to be capable of LTP, which required calcium permeable AMPA receptors and post-synaptic hyperpolarization, as well as LTD, which required NMDA receptors and post-synaptic depolarization. Inhibitory interneurons in the vestibular nucleus were also shown to be capable of LTD which was shown to be NMDA receptor-dependent. LTP was also shown to be "unmasked" at these synapses, when LTD was blocked. Interestingly, plasticity in the vestibular nucleus seems to follow the BCM model, with LTP requiring a higher calcium threshold than LTD (McElvain et al., 2010). Synaptic plasticity in the vestibular nucleus was soon confirmed by

Menzies and colleagues, who also proposed that learning in the brainstem may occur along with the cerebellum, but at a slower rate (Menzies et al., 2010). Synaptic plasticity in the vestibular nucleus could likely contribute to learning such as in the VOR. During long-term consolidation, memory for learned gain changes in the VOR is thought to be eventually distributed from the cerebellar cortex to the vestibular nuclei. Thus, this form of plasticity could play a role in the transfer of the memory from the cerebellum to the brainstem.

1.9 List of hypotheses

1) Motor learning in some ocular reflexes is capable of rapid consolidation that is resistant to disruption stimuli. We hypothesise that learning in the VOR will also consolidate rapidly.

2) All learning in the VOR is frequency selective. If rapid consolidation and disruption represent new learning then they will also show frequency selectivity.

3) Since mGluR1 is essential for LTD and not LTP, we hypothesise that an mGluR1 antagonist will selectively inhibit gain-up learning.

4) A $GABA_B$ receptor antagonist will inhibit gain-up learning via its interaction with the mGluR1 signalling pathway.

Chapter 2

2 General methods

A total of 10 cats were used in the studies included in this thesis. Table 2-1 shows the studies in which each cat participated. In all chapters of this thesis, H.K.T. performed all experiments and analyzed all data. As experiments with cats required two people, for safety, lab member Raquel Heskin-Sweezie assisted in the data acquisition, and with handling the cats. Data from two cats (L and Q), in Chapter 3, were collected by R. H.-S. and previous students in the lab (Charles Kassardjian and Ji Yeon Jenni Chung) and were included in this thesis to facilitate statistical analysis.

Cat	Chapter 3	Chapter 4	Chapter 5	Chapter 6	Appendix 1	Appendix 2
L	√					
Q	√					
R	√				√	√
S	√		√	√	√	√
T	√	√			√	√
V		√	√	√	√	√
A		√			√	√
B			√	√	√	√
C			√	√	√	√
E			√	√	√	√

Table 2-1. List of cats included each chapter. Ten cats were used in following projects included in this thesis. Data from cats L and Q were obtained from previous students (see methods).

2.1 Surgery methods

All surgeries were performed by Dr. Dianne Broussard and H.K.T. and assisted by R.H-S. After an initial training period to accustom the cats to restraint and the recording apparatus, all cats were implanted with a head holder to allow fixation of the head and a scleral search coil to record eye movements. Cats S, V, B, C, and E also had bilateral guide cylinders implanted in additional surgeries to allow access to the cerebellar flocculi. A minimum period of 7 days was

allowed for the cats to recover from each surgery. A minimum of 14 days was allowed between surgeries.

2.1.1 Anaesthesia

All surgical procedures were conducted under sterile conditions under isoflurane anaesthesia. Cats were pre-medicated with a mixture of acepromazine (0.1 mg/kg) and atropine (0.04 mg/kg) given subcutaneously. Some cats were also given ketamine (20mg/kg) intramuscularly. The cat was anesthetised with isoflurane, intubated, ventilated and placed in a stereotaxic apparatus. Heart rate, blood pressure, oxygen saturation, end-tidal carbon dioxide, and breathing rate were monitored throughout. Saline (0.9%) or lactated ringers solution was given intravenously (5-10 mg/kg/hr) throughout anaesthesia, which was maintained with 1.5-3% isoflurane.

2.1.2 Analgesia and antibiotics

Prior to the start of all surgeries the cats were given subcutaneous buprenorphine (0.01 mg/kg) or butorphanol (0.2 mg/kg) as an analgesic. A non-steroidal anti-inflammatory drug (ketoprofen (1 mg/kg) or meloxicam (0.2 mg/kg) were given before the end of surgery. Buprenorphine (0.005 mg/kg) was given 10 minutes before the end of surgery, and 6 hours afterward as post-operative analgesia. On the second and third day after surgery the cats were given ketoprofen tablets (5 mg/kg) or an oral suspension of meloxicam (0.1 mg/kg) as an anti-inflammatory and analgesia. Cats received the antibiotic cefazolin (22 mg/kg) IV before the start of surgery or 0.5 ml duplocillin before the end of surgery. Post-operative antibiotic treatment was continued with orbax (2.5 mg/kg) for 7 days.

2.1.3 Head holder surgery

With the cat placed in a stereotaxic apparatus, a midline incision was made and the skin and muscle were retracted. The periosteum was left intact to the extent possible. Four veterinary fixation plates (Synthes, Canada) were placed close to ear bar zero at 90 deg angles to one

another, with the ends of the plates extending upward at right angles to the skull. The fixation plates were attached to the skull by stainless steel self-tapping cortical screws (Synthes, Canada). The head holder, a stainless steel cylinder, was then cemented vertically at the midline, to the plates using dental acrylic. The skin was repositioned around the head holder and sutured using 3-0 prolene sutures.

2.1.4 Eye coil surgery

Eye movements were recorded using a magnetic search coil technique. The surgery to implant the scleral eye coil was done immediately following the head holder surgery. To implant the eye coil, drops of 1% Mydriacyl and 2.5% Mydfrin were applied in the eye to prevent the conjunctiva from swelling. The other eye was protected with ophthalmic ointment. The conjunctiva was blunt dissected away from the globe and four fine sutures (6-0 Mersilene) were attached to the sclera to allow manipulation of the globe. A pre-fabricated eye coil (Alan Baird Ind.), 18-20 mm in diameter, was fitted to the eye and attached using Mersilene sutures. The cornea was irrigated regularly with saline throughout the surgery. The leads from the coil were threaded subcutaneously and soldered to a two-pin connector (Powell Electronics) that was attached to the skull, rostral to the head holder, using cortical screws. The connector was cemented in place using dental acrylic.

2.1.5 Cylinder surgery

Guide cylinders were surgically implanted for access to both flocculi. In two separate surgeries, bilateral guide cylinders (Frederick Haer) were implanted (9 mm lateral and 1.7 mm caudal to ear bar zero) at a 40° angle from the vertical in parasagittal planes, directed at each flocculus. To implant each cylinder, an incision was made caudal to the head holder apparatus, and the muscle and skin were retracted. To expose the dura, a 12 mm diameter hole was drilled in the skull. The cylinders were secured to cortical screws and to the head holder apparatus by dental acrylic.

2.1.6 Maintenance and cleaning of implants

The area around the head holder and eye coil connector was cleaned 5 times per week with hydrogen peroxide. To prevent infection, cylinders were rinsed with dilute hydrogen peroxide in saline 5 times per week and filled with a solution of cefazolin in saline. In some cats, cylinders were also flushed 3 times per week with 5-fluorouracil (25 mg/ml) to inhibit scarring. When scar tissue growth interfered with electrode or needle insertion, the tissue was carefully scrapped from the dura mater. This procedure was performed under isoflurane anaesthesia.

2.2 Recording eye movements

Eye position was monitored using a magnetic phase-detection system with 17" field coils (CNC Engineering). During recording, horizontal and vertical field coils generated alternating magnetic fields at 40 KHz. The signal induced in the eye coil was detected as horizontal and vertical position components. Signals were sampled at 4 KHz using Labview software (National Instruments). Horizontal and vertical eye velocity was calculated using a five-point differentiation algorithm. Eye and head velocity signals were then low-passed filtered using a second-order virtual Butterworth filter (National Instruments) with a roll-off of 55 Hz.

Figure 2-1 illustrates a cat within the recording apparatus. The field coils and cat sat on a velocity-servo turntable (Neurokenetics) and were rotated around an earth-vertical axis. During recording the cat sat upright, restrained in a loose drawstring bag, and in a close-fitting box. The cat's head was positioned 22° nose-down from the horizontal stereotaxic plane, so that the horizontal semicircular canals were in the plane of rotation. The approximate centre of gaze was determined before each experiment. VOR recordings took place in complete darkness. The gain of the VOR was measured during sinusoidal rotation at 0.5, 2 and 8 Hz, at a peak velocity of 10 deg/s. At each frequency, around 60 cycles of rotation were acquired. During 8-Hz rotation, vibration artifacts were prevented by keeping the eye coil leads under tension during recording. We ensured that the cat remained alert by monitoring the eye movements on an oscilloscope. An assistant (H.K.T. or R.H-S.) remained in the recording room with the cat and provided a variety of interesting sounds or tactile stimuli to keep the cat awake.

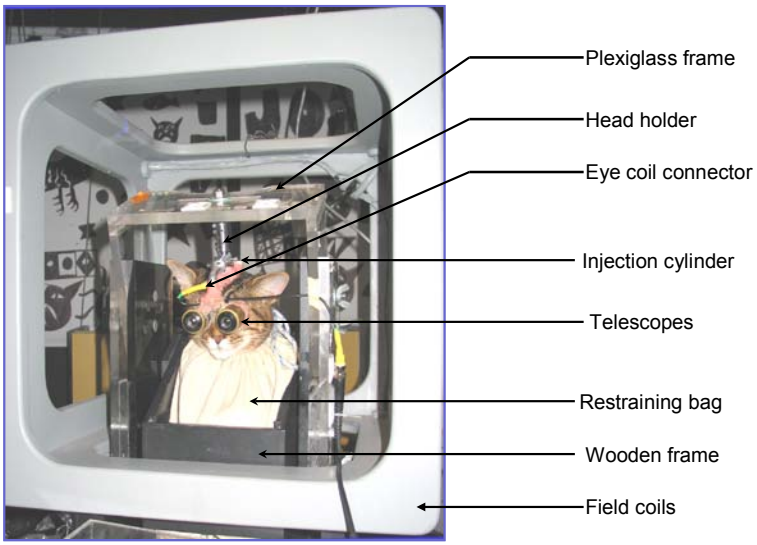

Figure 2-1. Illustration of cat within the recording apparatus. During experiments, the cat was restrained in a loose drawstring bag (restraining bag), and sat within a close fitting box (wooden frame). A head holder, attached to head holder cylinder, was secured to a Plexiglass frame that was attached to the wooden frame. The cat sat within the magnetic field coils to record eye movement signals sent via the eye coil connector, which connected to the surgically implanted eye coil. During learning, the cat wore spectacles that contained telescopes, which were attached to the head holder. Some cats also had bilateral guide cylinders to allow electrode and needle access to the cerebellar flocculi.

2.2.1 Calibration of eye coil

The magnetic coil system was calibrated weekly, using an eye coil identical to those implanted in the cats, attached to a protractor. In addition, the eye coil worn by each cat was calibrated after implantation. The cat (and field coils) was rotated in the light at a constant velocity for 300 ms. The sampling rate during these velocity pulses was 1000 Hz. Eye velocity traces were averaged in both directions for an average of at least 10 traces. Eye velocity was assumed to equal head velocity during the time of constant velocity, and a calibration factor for each cat was determined by calculating the ratio of the eye to head velocity.

2.3 Data analysis

Data were analyzed offline using custom written programs in Labview (National Instruments). To measure the gain of the VOR (ratio of eye to head velocity), cycles of eye and head velocity were averaged together. For 2 and 8 Hz data, any cycles containing quick phases or saccades were omitted. The 0.5 Hz data were desaccaded before averaging. Cycles where the horizontal or vertical eye position deviated from the centre of gaze by more than 15 deg were omitted. In most cases, averages contained at least 20 cycles (minimum of 10). The average eye velocity was plotted against head velocity and corrected for any phase lag or lead. Gain was defined as the average of the slopes of the lines that fit leftward and rightward half-cycles of rotation, minimizing the mean squared error. See Figure 3-1A and B for example traces of the head and eye velocity measured. The VOR gain in each experiment was normalized with respect to the mean baseline gain for that subject.

2.4 Optically-induced learning

Motor learning can increase or decrease the gain of the VOR. In our lab we use telescopes (Designs for Vision) that either magnify or miniaturize the visual world. Spectacles contained X0.25 telescopic lenses to decrease the gain or X2 telescopic lenses to increase the VOR gain. Spectacles were custom fitted to each cat using opaque frames in dental acrylic, so that the cat was only able to see through the lenses. The spectacles were attached to the head holder of each cat by an aluminum bracket (see Fig 2-1).

2.5 Histology

All cats were perfused under deep pentobarbital anaesthesia (>120 mg/kg). In cats S, V, and B the cat was intracardially perfused with 2 L of 0.9% saline followed by 1 L of 10% formalin. The brain of each cat was removed and placed in 10% formalin solution. Before histology, the brain was placed in a 30% sucrose-formalin solution. The cerebellum was cut in half, and cut into a block. The tissue was imbedded in egg yolk, and allowed to fix in a dish lined with a paper towel soaked in 100 % formalin overnight. Sections were cut at 50 μm intervals in the parasagital plane

using a vibrotome or microtome. The sections were mounted on slides and stained using cresyl violet.

Cats C, D and E were used for immunohistochemistry (see Fig 1-6). Cat C was perfused with cold PBS and 4% paraformaldehyde (PFA). Cats D and E were perfused with PBS and a 4% PFA/15% picric acid solution. The brains were embedded in an OCT medium and 50 μm sections were obtained using a cryostat. The immunohistochemistry was done in collaboration with Dr. David Hampson (University of Toronto), and the methods will not be described here.

A 1 μl injection of neutral red dye (2% in PBS) was used to mark the site where drugs were injected in the flocculi of cats S, V, B, and in 1 flocculus of cats C, and E. In these cats we were able to confirm that that the injection was made in the flocculus. Although no dye mark was made in one flocculus of cats C and E, we were able to visualize the track marks of the needle entering the flocculus.

Chapter 3

3 Rapid consolidation of gain changes in the VOR

3.1 Introduction

Consolidation is the process that converts a short-term memory into a long-term memory. While short-term memories are labile and easily disrupted, long-term memories are more durable. Rotation in darkness has been previously shown to disrupt newly learned memories, but not long-term memories in the VOR (Cohen et al., 2004; Kassardjian et al., 2005). In the VOR, short-term learning studies have consistently suggested that the cerebellar cortex is the initial site of learning (McElligott et al., 1998; Nagao and Kitazawa, 2003; Kassardjian et al., 2005; Anzai et al., 2010). However, after weeks of learning, studies suggested that the memory resided in both the cerebellar cortex and brainstem (Luebke and Robinson, 1994; Pastor et al., 1994; Partsalis et al., 1995b). In 2004, Dudai classified two general kinds of consolidation processes (Dudai, 2004). Systems consolidation is thought to have a time scale of days, and may involve a shift in the memory location. After 3 days, learned gain decreases in the VOR gain were found to be less resistant to a disruption stimulus. Furthermore, it was suggested that after this time the memory for the learned change in gain was shifted to include the vestibular nucleus in the brainstem (Kassardjian et al., 2005; Anzai et al., 2010). Synaptic consolidation is thought to have a much shorter time scale, and is thought to reinforce learning. Similarly, a form of rapid consolidation has been shown for NMR conditioning requiring only 1-2 hours (Cooke et al., 2004). Similarly, evidence for rapid consolidation has been identified in saccadic motor learning paradigms as well (Aboukhalil et al., 2003; Shelhamer et al., 2005).

The purpose of this study was to determine the time course of consolidation in the VOR. We hypothesized that like other types of motor learning the VOR would be capable of rapid consolidation. Here, we provide evidence that the VOR is also capable of consolidating rapidly within 1-2 hours after learning has stopped. The results from this study have been published (Titley et al., 2007).

3.2 Methods

Five adult male cats (L, Q, R, S and T), aged 11- 24 months were used in this study.

3.2.1 Experimental protocol

Gain increases (gain-up learning) and gain decreases (gain-down learning) were induced using X2 and X0.25 telescopes respectively. The learning period consisted of 60 minutes of sum-of-sines (SOS) rotation that alternated (every 5 sec) between two waveforms, each waveform consisted of three component frequencies: either 0.5, 2 and 10 Hz or 0.2, 1 and 5 Hz. Each component had peak velocity of 10 deg/s.

Results from three experimental protocols are reported. In the first protocol, a 60 minute learning period was followed by 60 minutes of rotation in complete darkness using the same rotational stimulus. This rotation in darkness was defined as the "disruption period". During the disruption period the cats wore the same spectacles they wore during the learning period, to ensure that any incidental light leak would drive the VOR to show more learning. The cats were actively kept awake during both the learning and disruption periods.

In order to determine the time course of consolidation in the VOR, we inserted "neutral periods". It was hoped that the learned changes in the VOR gain could consolidate during this time. During the neutral periods, the cat was stationary in the light with opaque paper covering the lenses. In the second protocol, a 30 minute neutral period was inserted between the learning and disruption periods. In the third protocol, a 60 minute neutral period was used. In the majority of these trials, the cat was in the light during the neutral period with the lenses covered with paper (as in protocol 2). However, in four sessions, with two subjects, the cats were in complete darkness during the 60 minute neutral period. During the neutral periods the cats were not actively kept awake. As a control for possible changes in gain during the rotation in darkness, the

disruption stimulus was also presented alone in three cats. The number of trials performed in each cat is shown in Table 3-1. In each cat the protocols presented were interspersed in a random fashion.

Cat	Frequency (Hz)	No Learning	Gain-down Learning				Gain-up Learning	
			0 min	30 min	60 min	Dark	0 min	60 min
L	0.5	-	4	-	4	-	-	-
	2	-	4	-	4	-	-	-
Q	2	4	4	5	4	-	-	-
R	0.5	4	4	2	3	-	-	-
	2	4	4	2	3	-	-	-
S	0.5	4	3	2	3	2	1	1
	2	4	3	2	3	2	1	1
T	0.5	-	-	-	-	2	2	3
	2	-	-	-	-	2	2	3

Table 3-1. The number of successful trials performed in each cat in Chapter 3. Each protocol was performed in at least 2 cats. The VOR was not measured at 0.5 Hz in cat Q. A dash represents that a cat did not participate in that protocol.

The gain of the VOR was measured before and after the learning and neutral periods, and every 15 minutes during the disruption period. The gain was measured at 0.5 and 2 Hz in cats L, R, S and T. In Cat Q the gain was only measured at 2 Hz. In some cats the gain was also measured at 8 Hz. However, consistent and accurate recordings were not obtained at 8 Hz in this chapter, and these results are not reported.

At the end of each trial with gain-down learning, the cat was rotated for 30 minutes without telescopes to help return the VOR to the pre-learning value. After the trials involving gain-up learning, the cats were not rotated without lenses but waited ≥ 7 days between trials.

3.2.2 Data analysis

The methods used to measure and calculate VOR gain were described earlier (see: Chapter 2). We determined the amount learned (percent gain change) during the learning period in each trial. The amount learned was calculated as follows:

$$\text{Amount learned} = ((G_{base}-G_{learn})/G_{base}) \times 100$$

Where G_{base} is the pre-learning gain and G_{learn} is the gain measured after the learning period. In each learning trial, a minimum of 12% change in gain from the pre-learning value was required for inclusion in this study. If this did not occur during the learning period, the session was stopped (n=9) and the trial was excluded from the study. To quantify the amount of disruption in each trial, we calculated the slope to the line of best fit through the five gain measurements encompassing the disruption period (see inset in Fig 3-3). Unpaired t-tests were then used to compare the amount of disruption between the different neutral period groups (0, 30 or 60 mins). The normalized pooled VOR gain values were also analyzed using one-way repeated measures ANOVAs across the time points in the disruption periods for each group. Paired Student's t-tests were also used to compare pre-and post- disruption and learning values. In all cases significance was assumed at the 95% level ($P<0.05$).

3.3 Results

3.3.1 Disruption reverses learned changes in gain

One hour of rotation wearing either X2 or X0.25 lenses reliably increased or decreased the gain of the VOR respectively. Figure 3-1A shows examples of the VOR responses at 2 Hz rotation before and after learning. Similar results were seen at 0.5 Hz (not shown). Figures 3-1C and D summarizes the time course of the normalized VOR gain during protocol 1 (no neutral period) for gain-up and gain-down learning at 0.5 and 2 Hz. The gain of the VOR increased significantly during gain-up learning protocol ($P<0.02$ for 0.5 and 2 Hz, paired t-tests) and decreased significantly during gain-down learning ($P<0.000001$ for 0.5 and 2 Hz, paired t-tests).

The change in VOR gain induced during the one hour learning period was gradually reversed by rotation in darkness that immediately followed learning (protocol 1). Figure 3-1B shows example plots of eye versus head velocity for both gain increases and decreases before and after learning, and after disruption. A repeated measures ANOVA during the disruption period

showed a significant trend in the VOR gain towards the pre-learning value (gain up: $P<0.004$ for 0.5 and 2 Hz; gain down: $P<0.0001$ for 0.5 and 2 Hz). The change in gain from the post-learning to the post-disruption was significant after both gain-up learning (0.5 Hz, $P<0.02$; 2 Hz, $P<0.03$, paired t-tests) and highly significant after gain-down learning (0.5 Hz, $P<0.002$; 2 Hz, $P<0.00001$, paired t-tests). The phase angle between head and eye velocity did not significantly change during learning or disruption (not shown).

The disruption stimulus without prior learning (open circles, Fig 3-1C, D) did not cause a significant change in the VOR gain at any frequency ($P>0.05$, ANOVA). The pre-learning VOR gain was not affected by repeated learning sessions nor did the amount of learning show any consistent effects of repeated training sessions (see Appendix 2).

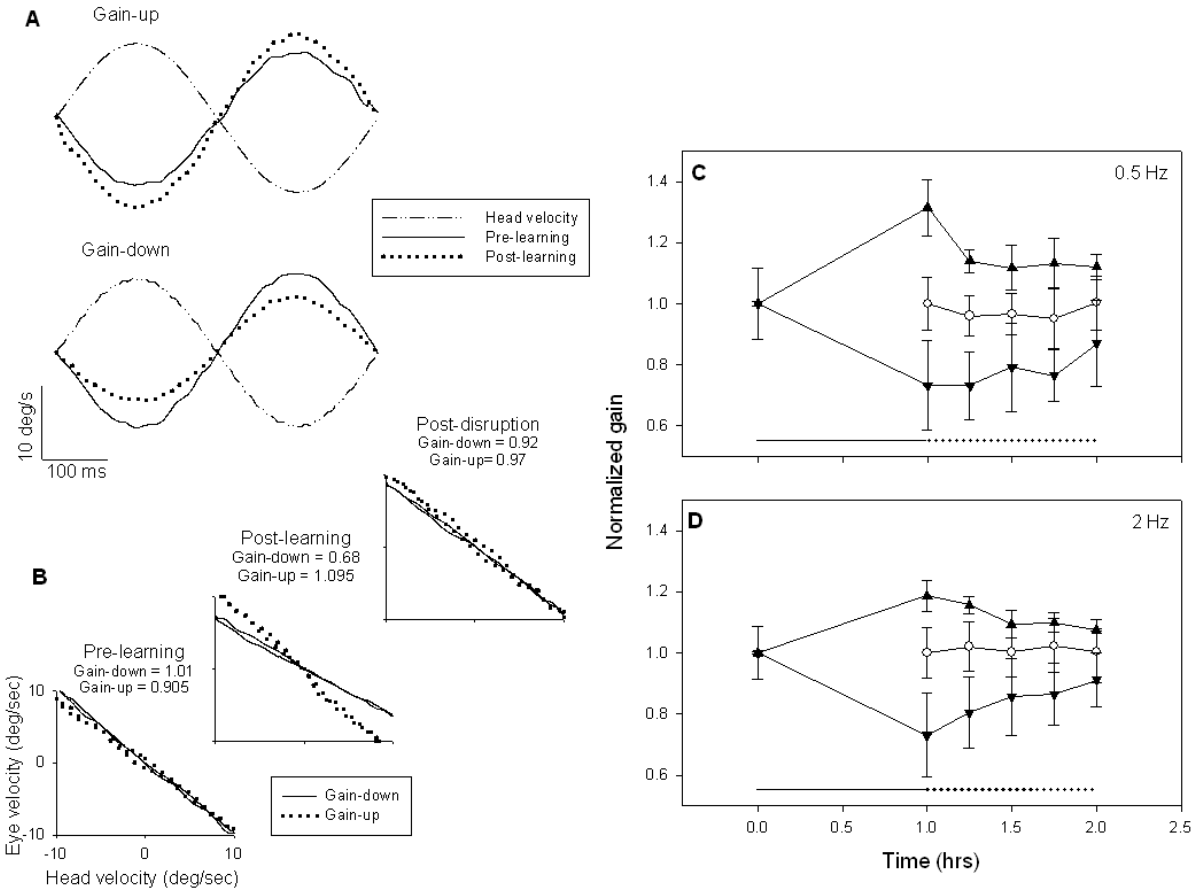

Figure 3-1. Rotation in darkness disrupts learning. Rotation in darkness immediately following learning disrupted learned changes in the VOR gain. A: Sample traces of head and eye velocity plotted over time during 2 Hz rotation before and after gain-up (top) and gain-down (bottom) learning. Traces are averages of 10 or more cycles. Dash-dotted line: Head (turntable) velocity. Solid line: Eye velocity before learning. Dotted line: Eye velocity after 60 minutes of learning with either X2 or X0.25 lenses. B: Eye velocity plotted against head velocity during the VOR before (pre-learning) and after learning (post-learning) and after disruption (post-disruption). Gain values represent the non-normalized gain in each condition. Solid line: gain-down learning. Dotted line: gain-up learning. Examples are from cat S. C-D: Time courses of normalized VOR gain pooled across all subjects at 0.5 Hz (C) and 2 Hz (D). The learning period is indicated by the solid line. The dotted line indicates the disruption period. Open circles: Disruption period without prior learning. Triangles: gain-up learning. Inverted triangles: gain-down learning. For all figures error bars represent standard deviation.

3.3.2 Gain decreases consolidate within 1 hour

We hypothesized that a new memory would consolidate completely if learning was stopped during a neutral period. This prediction was upheld for gain decreases after a 60 minute neutral period (protocol 3), but not after a 30 minute neutral period (protocol 2). Figure 3-2 illustrates the time course of the VOR gain in protocols 2 and 3 (30 and 60 minute neutral periods) at 0.5

and 2 Hz. After a 30 minute neutral period, a learned gain decrease did not show a significant increasing trend during the disruption period at any frequency (0.5 Hz, P=0.072; 2 Hz, P=0.20, ANOVA). However, at 2 Hz, the gain of the VOR after the neutral period was significantly less than the VOR gain measured after the disruption period (P<0.05, paired t-test), indicating that some disruption did occur. After a 60 minute neutral period, a learned gain decrease was completely resistant to the 60 minute disruption period. If the cat was in complete darkness during the 60 minute neutral period, this did not change the result at any frequency; the memory was still resistant to disruption. Figures 3-2C and D show the time course of experiments with a 60 minute neutral period. In all gain-down conditions, after the 60 minute neutral period, the ANOVAs failed to show a significant trend during the disruption period at any frequency (P>0.31).

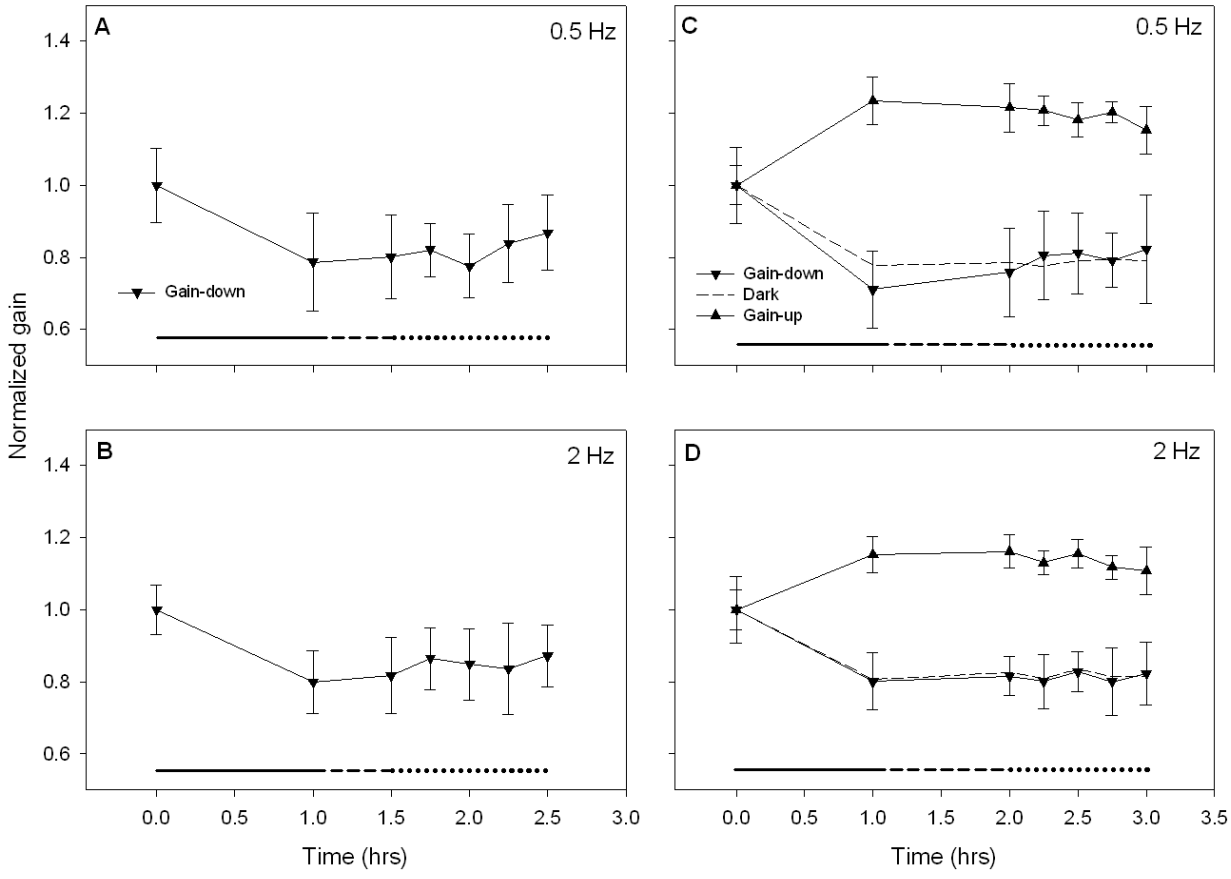

Figure 3-2. Disruption was less effective after a neutral period. Rotation in darkness was less effective when a neutral period immediately followed learning. A and B: Time course of VOR learning with a 30 minute neutral period. C and D: Time course of VOR learning with a 60 minute neutral period. Lines along the abscissa indicate the learning period (solid line), neutral period (dashed line) and disruption period (dotted line). Triangles: gain-up

learning. Inverted triangles: gain-down learning. Dashed line: Gain-down learning followed by a 60 min learning period in the dark. A and C: 0.5 Hz. B and D: 2 Hz.

To quantify the effect of the neutral period on disruption, we calculated the slope of the best-fit line of the gain values encompassing the time during the disruption period. The slope of this line represents the amount of reversal during the disruption period. The results of the quantified reversal after gain-down learning are summarized in Fig 3-3. Figures 3-3 A and B show the pooled results across subjects at 0.5 and 2 Hz. The amount of reversal after a 60 minute neutral period was significantly less than that without a neutral period at both frequencies (0.5 Hz: $P<0.05$; 2 Hz: $P<0.00001$, unpaired t-tests). After a 30 minute neutral period the amount of reversal was significantly lower without a neutral period at 2 Hz ($P<0.003$, unpaired t-test) but not at 0.5 Hz. Figures 3-3B and D show the amount of reversal for each of the cats after gain down learning. In all cats a 60 minute neutral period consistently prevented disruption; however the 30 minute neutral period led to inconsistent results among subjects.

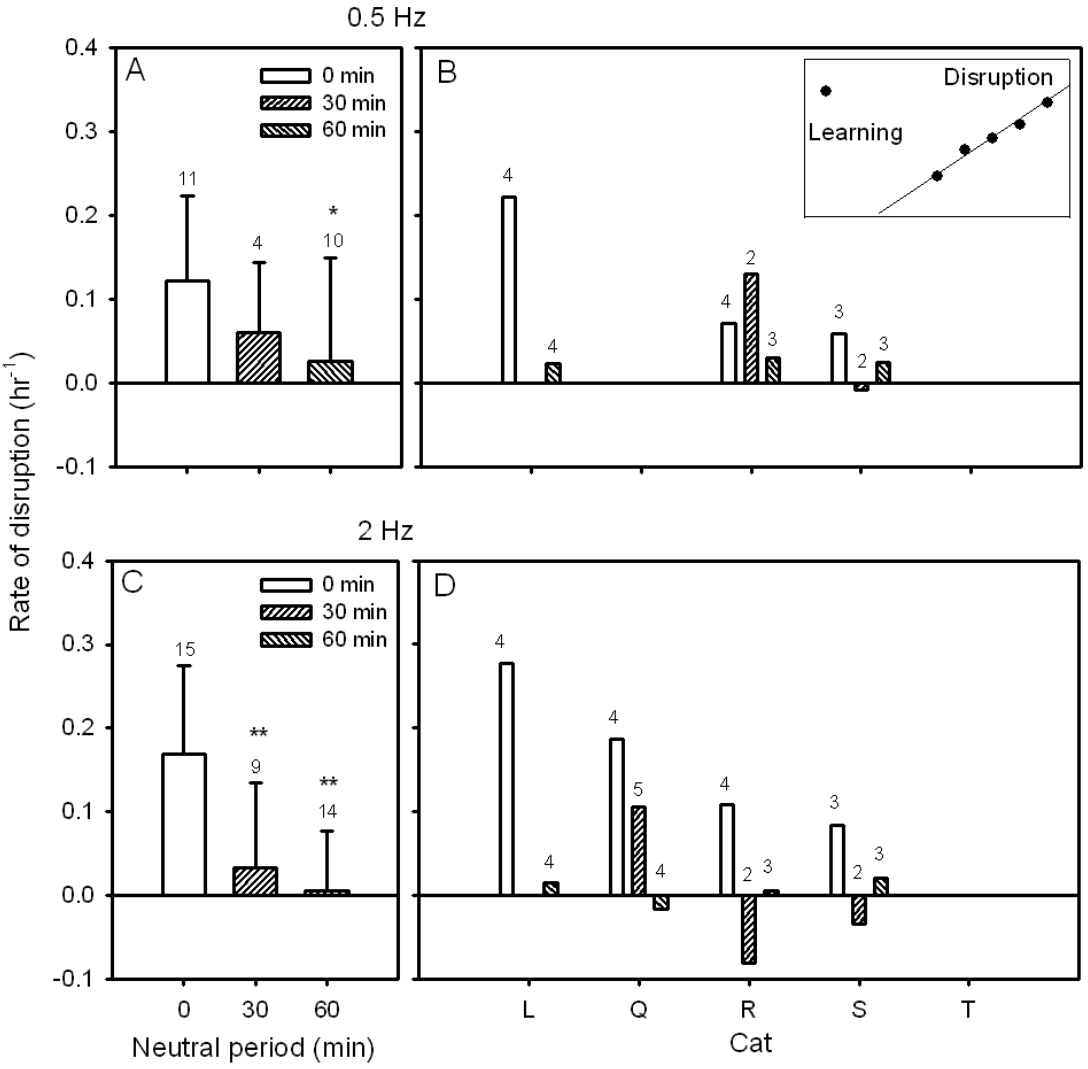

Figure 3-3. Quantification of disruption after gain-down learning. Slopes of the lines of best-fit measured from the gain values encompassing the disruption periods following gain-down learning. The rate of disruption was significantly less when a neutral period was present. A and C: Mean slope values pooled across cats. B and D: Average disruption for individual cats. Error bars represent standard deviation for the pooled data. A and B: 0.5 Hz. C and D: 2 Hz. *** P<0.0001, ** P<0.01, * P<0.05. Inset: Plot of VOR gain vs. time, as in Figure 3-1. Disruption was measured by fitting a line of best fit (diagonal line in plot) through the five gain measurements encompassing the disruption period.

3.3.3 Less consolidation after gain increases

Gain-up learning appeared to consolidate less than gain-down learning. Learned gain increases appeared to be less resistant to disruption after a neutral period. Figure 3-2 (C and D) shows the time course of the 60 minute neutral period protocol at 0.5 and 2 Hz (30 minutes was not used for gain-up). At both frequencies some reversal occurred; the post-neutral period gain was

significantly greater than the post-disruption gain ($P<0.03$, paired t-test). However, unlike after gain-down learning, the trends toward the pre-learning value were not significant at either frequency ($P>0.14$, ANOVAs).

The amount of disruption after gain-up learning is quantified in Figure 3-4. Although the results were not as clear as after gain-decreases, learned gain increases did partially consolidate after a 60 minute neutral period. In Figure 3-4, A and B show the pooled results of the two subjects after gain up learning at each frequency, while C and D show the individual results for each subject. At both frequencies the slope of the disruption period was significantly less than that without a neutral period ($P<0.05$, unpaired t-tests).

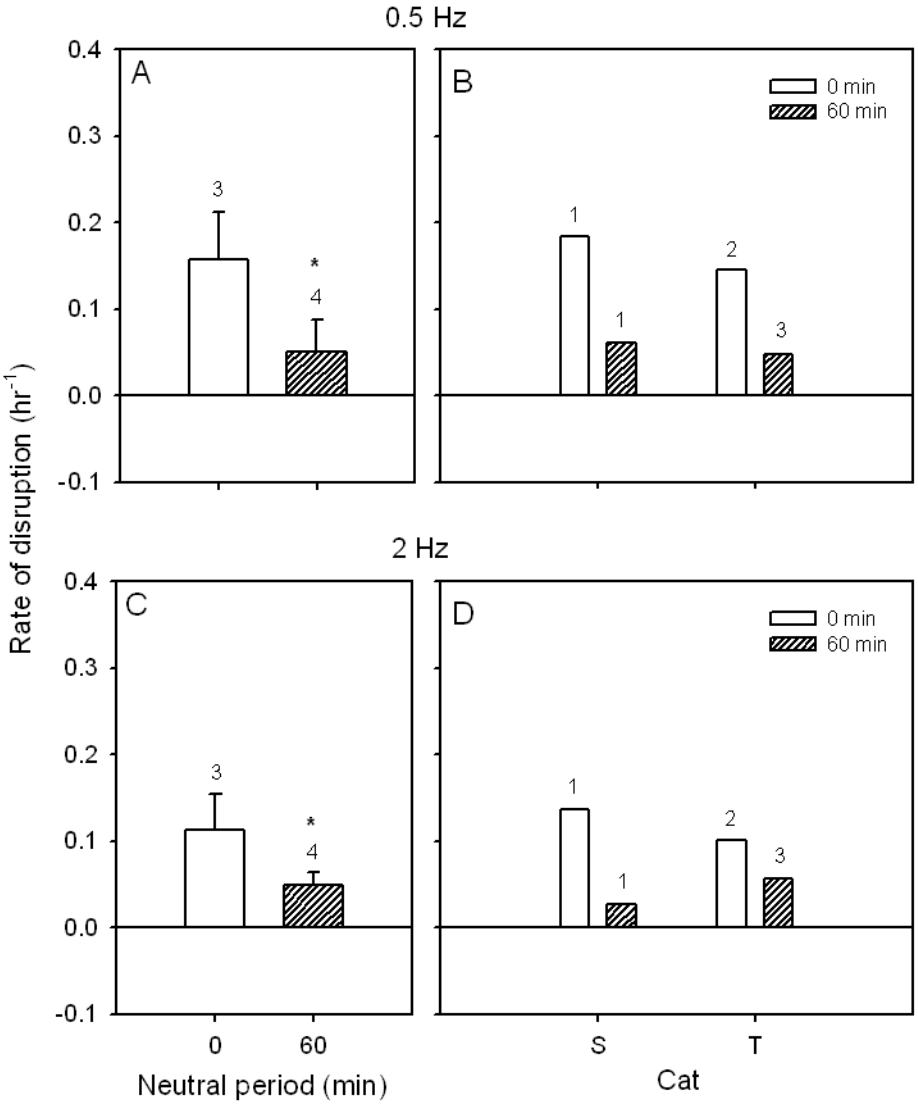

Figure 3-4. Quantification of learning after gain-up learning. Slopes of the lines of best-fit following gain-up learning for 0.5 Hz (A and C) and 2 Hz data (B and D). A and B: Mean slope values pooled across cats. C and D: Average disruption for individual cats. Error bars present standard deviation. * P<0.05.

When comparing the amount of disruption after gain-up and gain-down learning, we found that gain increases did not consolidate as much as gain decreases (compare Figs 3-3 to 3-4). When comparing the amount of disruption after a 60 minute neutral period, the amount of disruption was found to be significantly greater after gain-up learning than after gain-down learning at 2 Hz ($P<0.03$, unpaired t-test), but not at 0.5 Hz. There was no significant difference in amount of disruption at any frequency after gain increases and decreases without a neutral period ($P>0.05$, unpaired t-tests).

3.4 Discussion

Here we show that the VOR is capable of consolidating learned gain changes within 1 hour after learning has stopped. A similar time course for rapid consolidation has been described for NMR conditioning. In that study, using muscimol injections to inactivate the cerebellar cortex, the consolidation of conditioned responses was shown to require only 1-2 hours (Cooke et al., 2004).

In this study, we measured consolidation in the VOR at 0.5 and 2 Hz. Although we found that consolidation was more robust at 2 Hz, consolidation was also shown at 0.5 Hz after a 60 minute neutral period. We suggest that consolidation is frequency independent, as these results show that the VOR is capable of consolidation at both frequencies. However, this idea will be better explored in Chapter 4.

3.4.1 Learned gain changes can consolidate

During a neutral period, the gain of the VOR remained stable after either a learned gain increase or decrease. During this time, the gain of the VOR did not change but remained at the post-learning value. Our results are in agreement with previous studies which found that learned gain increases (Nagao and Kitazawa, 2003) or decreases (Cohen et al., 2004) do not spontaneously decay after learning. Other studies have found that while learned gain decreases do not decay, learned gain increases do (Miles and Eighmy, 1980; Cohen et al., 2004); indicating that gain-up learning is inherently more labile than gain-down learning. In our study, we found that consolidation was more obvious after gain-down than after gain-up learning, perhaps supporting the idea that gain-up learning requires more time to fully consolidate.

In contrast to our results, Kuki and colleagues found decay of both gain increases and decreases. Although they did find that learning becomes less labile with increasing periods of learning, they found that both gain-up and gain-down memory decayed back toward the pre-learning value

when the animal was left stationary in the dark (Kuki et al., 2004). However, the authors of that study tested the VOR every 10-15 minutes. By stopping learning and rotating the animal in darkness to measure the VOR, the learned change in gain could have been disrupted. Also, the squirrel monkeys in this study were given amphetamines to maintain alertness. Reports have shown amphetamines to have variable effects on memory; with some studies reporting memory enhancement (Weitzner, 1965), impairment (Burns et al., 1967) or no real effect (Kornetsky, 1958; Hurst et al., 1969). We suggest that in the study by Kuki and colleagues the memory may have been disrupted by the rotation in darkness, and may have not decayed passively. In our study, our results suggest that learned gain changes do not decay toward the pre-learning value, but consolidate during a 60 minute neutral period.

A previous study in monkeys showed that after 24 hours of learning, rotation in darkness could still disrupt learned gain changes (Cohen et al., 2004). However, in Cohen et al.'s study the monkeys wore lenses and were permitted to move their heads right up until testing. At no point was learning stopped during the trial. In our experiments, we stopped learning, and included a neutral period. Therefore, in the study by Cohen and colleagues, new labile memory could still have been present even after 24 hours, and thus, still show disruption.

It has previously been suggested that while learning may first take place in the cerebellar cortex, over time the sites of learning may become more distributed (Galiana, 1986; Peterson et al., 1991; Raymond et al., 1996; Broussard and Kassardjian, 2004). This shift in memory location has been suggested to take place during systems consolidation, which occurs over days. However rapid consolidation in NMR conditioning is thought to occur in the cerebellar cortex (Attwell et al., 2002), which occurs over few hours. This could also be the case for rapid consolidation in the VOR.

3.4.2 Rotation in darkness as a disruption stimulus

Rotation in darkness has been previously shown to disrupt learned gain changes in the VOR (Cohen et al., 2004; Kassardjian et al., 2005). Here we confirm that rotation in darkness is an effective means of disrupting newly-learned changes in gain. However, the mechanism behind this disruption stimulus remains unknown. In classical conditioning, the presentation of a conditioned stimulus by itself is enough to extinguish a learned response. A similar process in the VOR is head movement in the absence of image motion. Although the mechanism for disruption remains unknown, climbing fibres have been shown to respond to vestibular input (at higher velocities) in total darkness (Simpson et al., 2002). As climbing fibres are thought to control motor learning (Coesmans et al., 2004), this could imply an active reversal of the gain by synaptic plasticity.

We tested the effects of rotation in darkness on normal VOR function. With no prior learning, the disruption stimulus had no effect on VOR gain. This indicates that rotation in darkness serves to disrupt memory only, and does not cause any change in gain by itself. Furthermore, in our experiments the cats wore spectacles throughout the disruption period to ensure that any incidental light leak would drive the VOR to show more learning. As our results clearly show, the disruption period did not result in any further learning. This suggests that disruption is not an active process of new learning, as extinction is thought to be (Robleto et al., 2004). We suggest disruption may be a more passive process.

3.4.3 Conclusion

In conclusion, our results suggest that learned gain decreases and increases in the VOR can consolidate rapidly, within 1 hour after learning has stopped.

Chapter 4

4 Disruption and consolidation generalize across frequencies

4.1 Introduction

It has been suggested that while learned gain changes initially take place in the cerebellar cortex, during long-term consolidation (a couple of days) the locus of the memory is shifted to include the brainstem (Kassardjian et al., 2005; Shutoh et al., 2006; Anzai et al., 2010). However, we have previously shown that learned gain decreases can consolidate rapidly, if a 60 minute neutral period immediately follows learning (Chapter 3). It is not known whether this rapid consolidation involves a shift in the memory, which may involve new learning.

Learned changes in the VOR gain have been shown to be greater for frequencies lower than 4 Hz for both gain decreases (Raymond and Lisberger, 1996; Broussard et al., 1999a) and gain increases (Broussard et al., 1999a). Furthermore, new learned changes in gain are known to be frequency selective. Learning is greatest when measured at the frequency at which training occurred (Robinson, 1976; Lisberger et al., 1983; Raymond and Lisberger, 1996; De Zeeuw et al., 1998; Kimpo et al., 2005), and declines as the training and testing frequencies diverge (Kimpo et al., 2005).

Here we define “new learning” as showing frequency selectivity. It is thought new learning might be involved in the transfer of memory, known to occur with some types of consolidation. We suggest that if rapid consolidation is a mechanism of “new learning” then it too will show frequency selectivity. If however, rapid consolidation is reinforcing the original site of memory, and does not involve new learning then it will not show frequency selectivity. Here we show that disruption and rapid consolidation do not show frequency selectivity, but generalize across the

frequency range. The frequency tuning that appeared during learning was retained during the post-learning processing. The results from this study have been published (Titley et al., 2009).

4.2 Methods

Three cats (2 male, 1 female), aged 7-17 months, were used in this study.

4.2.1 Experimental protocols

Learned gain decreases were induced by having the cats wear spectacles with X0.25 telescopes set within opaque frames. Gain increases were not tested in this study, as we have previously shown that gain decreases consolidated better than increases (see: Chapter 3). Results from two experimental protocols are reported. In the first protocol, the cat was subjected to 60 minutes of rotation at a single frequency in the light, viewing the lab through the open door of the recording room while wearing the telescopes (learning period). This was followed by 60 minutes of rotation in complete darkness with the same rotational stimulus (disruption period). In the second protocol, a 60 minute neutral period was inserted between the learning and disruption periods. During the neutral period the cat was stationary in the light, with the telescopes covered by paper.

In each protocol the cat was rotated at a single frequency (training frequency) during both the learning and disruption periods. The training frequency was either 0.5, 2 or 8 Hz with a peak velocity of 10 deg/s. The gain of the VOR was tested at all three frequencies (testing frequencies) before and after the learning and neutral period (if any), and every 15 minutes during the disruption period. For each cat, the training frequency used in each trial was varied. After each trial, the cat was rotated for 30 minutes in the light at the training frequency without telescopes. Trials in each cat were separated by an average of 7 days (minimum of 5 days).

4.2.2 Data analysis

The change in VOR gain during the learning period was calculated as a percentage of the initial gain. See chapter 3.2.1 for the calculation of amount learned. A minimum of 12% decrease from the pre-learning gain in each trial was required for inclusion in this study. If the learned decrease was less than 12% (n=4) or if the cat was unable to finish the entire protocol (n=5), the disruption data were not used. See Table 4-1 for the number of successful trials in each cat. To summarize the amount of learning across frequencies, we reported all learning data, including the trials that did not meet the 12% learning criterion (n=4, see above). The amount of disruption measured at the end of the disruption period proved to be extremely variable. Therefore, to measure the amount of disruption, we generated a regression line using the 5 measurements encompassing the disruption period for both the gain and phase. To account for differences in the amount of learning, that value was then normalized by dividing by the change in gain or phase during the learning period for that particular trial. Thus, normalized disruption was measured in proportion to the amount of learning. Pooled, normalized VOR gain values during the disruption period were also analyzed using repeated measures ANOVAs. To determine significance, post-hoc paired t-tests were also used to compare the pre- and post-learning and disruption values. To analyze the amount of learning and disruption at the different training and testing frequencies, one-way ANOVAs and unpaired t-tests were used to compare values across the testing and training frequency ratios. In all cases, we assumed significance at a level of $P<0.05$.

Cat	Training frequency (Hz)	Learning period only	Successful trials	
			No consolidation period	60 min consolidation period
T	**0.5**	0	1	2
	2	0	1	1
	8	0	1	1
V	**0.5**	2	2	1
	2	2	2	1
	8	4	2	0
A	**0.5**	0	2	2
	2	0	2	3
	8	1	2	4
Total	**0.5**	2	5	5
	2	2	5	5
	8	5	5	5

Table 4-1. Number of trials for each protocol and cat at each training frequency. The learning period only indicates the number of unsuccessful trials that did not meet the 12 % learning criterion or could not complete the whole protocol.

4.3 Results

4.3.1 Consolidation of learning

In this study, we used sinusoidal rotation to induce learning, and then measured the effect of disruption at the same frequency. The VOR gain was measured at a range of frequencies. One hour of gain-down learning significantly decreased the gain of the VOR from the pre-learning value ($P<0.01$, paired t-tests) at all 3 training frequencies (Fig 4-1). The decrease in the VOR gain during learning depended on the training frequency. Learning was greatest at the lower training frequencies. The normalized pooled pre-learning gains were 0.97, 0.90 and 1.07, and the mean decrease in the gain was 0.24, 0.24 and 0.15 at 0.5, 2 and 8 Hz respectively when tested at the training frequency. Table 4-2 shows the percent changes in the gain for each cat.

Cat	T									V									A								
Training frequency	0.5 Hz			2 Hz			8 Hz			0.5 Hz			2 Hz			8 Hz			0.5 Hz			2 Hz			8 Hz		
Test frequency	0.5	2	8	0.5	2	8	0.5	2	8	0.5	2	8	0.5	2	8	0.5	2	8	0.5	2	8	0.5	2	8	0.5	2	8
Mean amount learned (%)	24	9.8	16	18	31	21	13	11	18	24	17	5.2	9.0	27	20	1.9	6.9	14	28	17	8.5	18	26	9.8	4.2	12	13
Standard deviation	1.8	3.1	13	0.8	0.6	8.7	0.4	8.7	0.6	6.6	4.2	5.1	3.7	3.2	9.8	5.3	7.6	7.5	3.4	4	3.2	7.1	5.8	3.2	3.7	9.8	3.7

Table 4-2. The amount of learning in each condition and subject. Averages of the amount learned at each testing frequency, across training frequencies for each subject. Learned gain changes were greatest when measured at the training frequency. Learning was also greater when measured at 0.5 or 2 Hz as compared to 8 Hz.

Figure 4-1(A-C) shows the time course of the normalized VOR gain measured at the training frequencies of 0.5, 2 and 8 Hz. In half of the trials, rotation in darkness at the training frequency (disruption period) immediately followed learning. In the other half, learning was followed by a neutral period, during which the subject was stationary without form vision. If there was no neutral period, the VOR gain increased back towards the pre-learning value during the disruption period (See Chapter 3). Repeated measures ANOVAs for each training frequency showed that the gain significantly increased during the disruption period at 0.5 and 2 Hz (0.5 Hz: $P<0.005$; 2 Hz: $P<0.001$). Although the increase during the disruption period was not significant at 8 Hz ($P=0.06$, repeated measures ANOVA), the post-disruption gain value was significantly greater than the post-learning value ($P<0.005$, paired t-test).

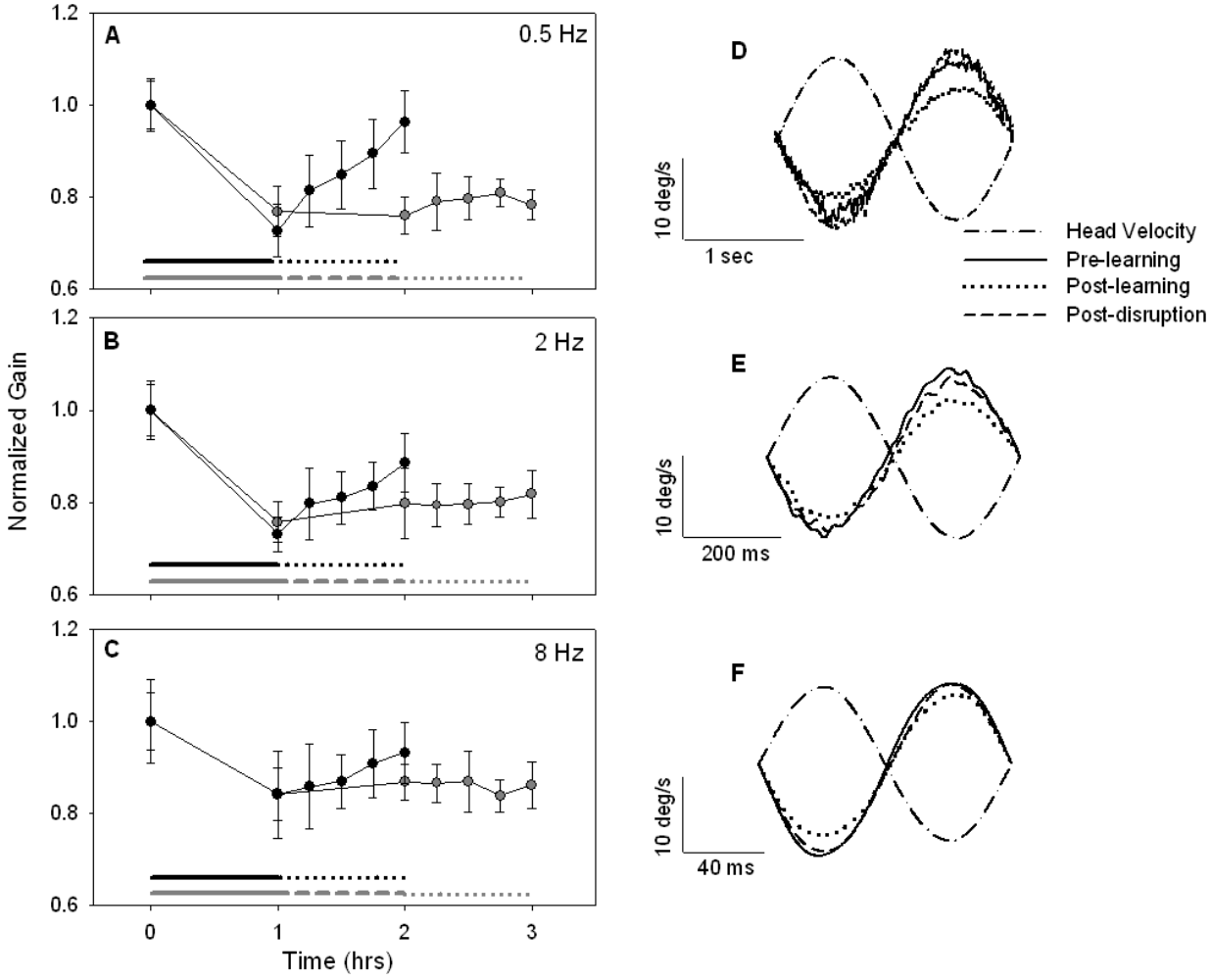

Figure 4-1. A neutral period prevents the disruption of learned decreases at all frequencies. Learned gain decreases in the VOR gain could be disrupted by rotation in darkness, unless a neutral period immediately followed learning. A-C: Normalized gains of the VOR pooled across subjects and training sessions as a function of time. When rotation in darkness immediately followed learning (black symbols) the gain increased back toward the pre-learning value. When a neutral period followed learning (grey symbols) disruption had little effect on the VOR gain. For all figures error bars indicate standard deviation. Only data gathered when the training and testing frequencies matched are shown. Lines across the abscissa indicate the learning periods (solid line), neutral periods (dashed line) and disruption periods (dotted line). D-F: Sample traces of head (dash-dotted line) and eye velocity (solid line: pre-learning; dotted line: post-learning; dashed line: post-disruption) of trials with no neutral period plotted against time. Examples are from cat A.

Figure 4-1(A-C) also shows the effect that a neutral period had on disruption. Although the gain of the VOR did not change during the neutral period, the neutral period had a profound effect on the success of disruption. Specifically, it prevented the gain of the VOR from reverting back toward the pre-learning value during the disruption period. Repeated measures ANOVAs showed

no significant change in the VOR gain during the disruption periods when they followed a neutral period ($P>0.05$, at all 3 frequencies).

4.3.2 Learning across frequencies

The amount of learning depended on both the training and testing frequencies. The percentage change in the VOR gain was always greatest when measured at the training frequency (i.e. when the training and testing frequency matched). Each training frequency showed significantly more learning when tested at that particular frequency when compared to the other two ($P<0.005$, unpaired t-tests). The VOR gain is represented by the slope of eye velocity vs. head velocity. Figure 4-2(A-C) shows example plots of eye vs. head velocity, before and after learning at each training frequency. Figure 4-2D shows the percentage change in gain at all of the training and testing frequencies. Each group of three bars (testing frequencies) represents the amount of learning induced by rotation, wearing the X0.25 telescopes, at the training frequencies marked on the abscissa. Arrows indicate where the training frequency and testing frequency matches. Figure 4-2E shows the amount learned for each cat at the training frequency. When measured at the training frequency, learning was significantly less effective at 8 Hz than at 0.5 and 2 Hz. When looking across all of the learning trials, there was a significant trend toward lower percentage changes in the higher training frequencies ($P=4.11 \times 10^{-8}$, one-way ANOVA). This was consistent across the three subjects (Table 4-2, Fig 4-2E). The distribution of the amount learned for all three training frequencies is shown in Figure 4-2 (F-H). The training frequency of 8 Hz had smaller values of learned changes when compared with the lower training frequencies of 0.5 and 2 Hz.

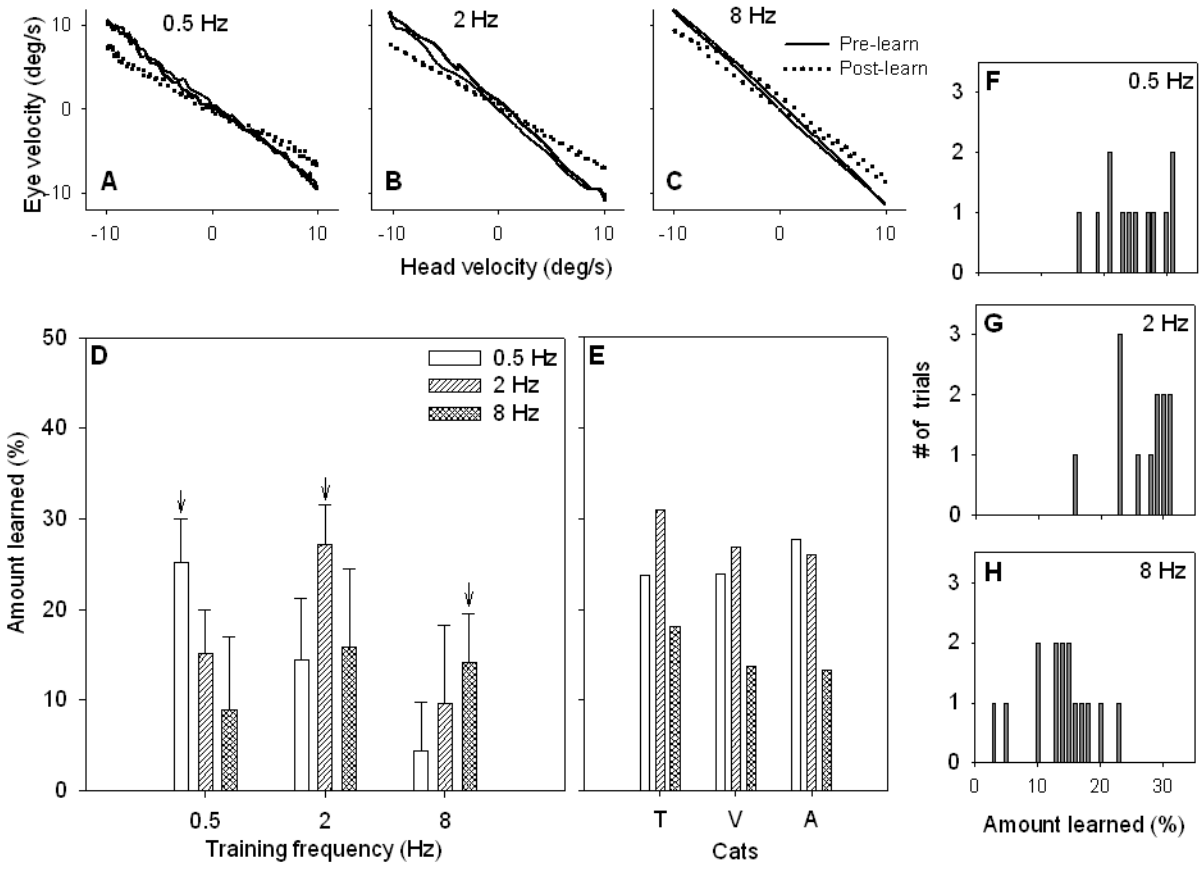

Figure 4-2. Learning shows frequency selectivity. Learned gain changes were greater for lower frequencies, and showed selectivity for the training frequency. A-C: Eye velocity plotted against head velocity when training and testing frequencies matched. Solid line: pre-learning value. Dotted line: Post-learning value. Examples are from cat A. D: Summary of the amount learned as a percentage of the pre-learning gain. Training frequencies are indicated along the abscissa. Bars indicated testing frequency. Arrows indicate when training and testing frequencies matched. E: Averages of the amount learned measured at the training frequency for each cat (abscissa). F-H: The distribution of learned gain decreases, pooled across trials, for each training frequency.

4.3.3 Disruption and consolidation across frequencies

Disruption was measured as the slope of the best-fit line through the points encompassing the disruption period. Similar to what we found in Chapter 3, disruption was less effective when a neutral period immediately followed learning. This was consistent at all testing and training frequencies. Interestingly, we found that without a neutral period, the averaged slopes of the disruption period retained the frequency dependence of the learning period (not shown). However, as it is reasonable to assume that a greater amount of learning would result in a greater

amount of disruption, we normalized disruption based on the amount of learning. Figures 4-3A and 4-3B summarizes the amount of normalized disruption at each test frequency, for the neutral periods of 0 and 60 minutes respectively. Although learning had been frequency selective, normalized disruption was not selective for the disruption frequency (same as training frequency). Instead the amount of normalized disruption was always largest for a frequency other than the disruption frequency and was relatively small when the testing and training frequencies matched (arrows).

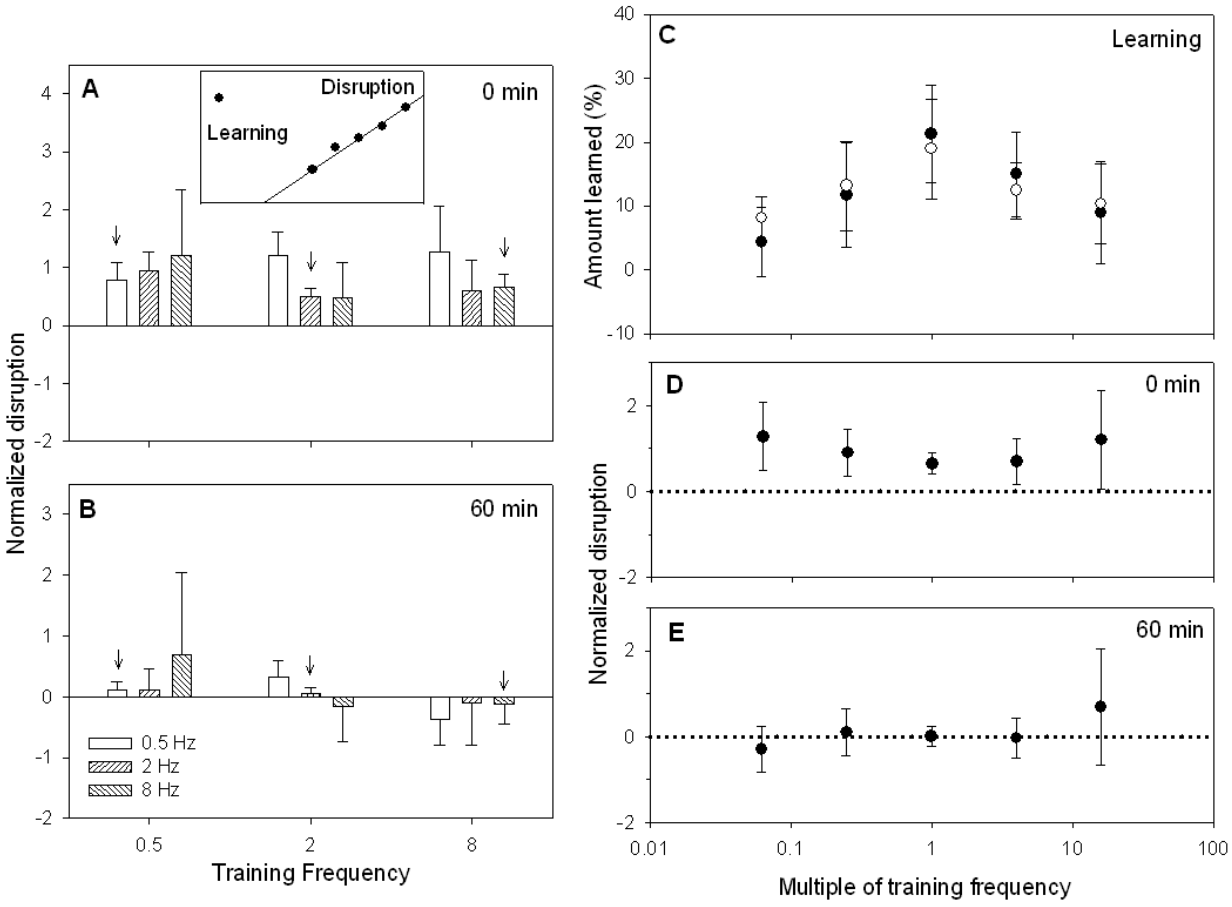

Figure 4-3. Disruption and consolidation were not frequency selective. The amount of disruption increased as the training and testing frequencies diverged. A: Normalized disruption when there was no neutral period at the different training frequencies (abscissa), measured at the different testing frequencies (bars). Arrows indicate when the testing and training frequencies matched. Inset in A: Plot of VOR gain vs. time, as in Fig 4-1. Disruption was measured by fitting a line of best fit through the five gain measurements surrounding the disruption period, and normalized to the amount of learning during that trial. B: Normalized disruption after a 60 minute neutral period. C: The amount of learning summarized as a function of the test frequency to the training frequency. D: When immediately following learning, disruption increased at frequencies different from training frequency. E: With a 60 minute neutral period disruption was not significant at any testing ratio.

When training is carried out at one frequency and learning was measured at another frequency, any learned change is taken to represent "generalization", or the ability of a learned change to transfer to a slightly different context (see: Kimpo et al., 2005). To summarize generalization in our study, we plotted the amount of learning and normalized disruption as a function of the ratio of the test frequency to the training frequency. For example, if the training frequency was 8 Hz and the testing frequency was 2 Hz, the ratio of the training frequency could be represented as the fraction (or multiple) 1/4. Figure 4-3C (filled symbols) shows that the amount of learning tended to be greater at test frequencies closer to the training frequency. At testing frequencies further away from the training frequency, the amount of learning was less. At multiples of 1/16 and 16, the amount learned was significantly less than when the multiple was 1 ($P<0.01$, unpaired t-test). In cases where there was a 60 minute neutral period after learning, the memory of the gain change remained frequency dependent (Fig 4-3C, open symbols). The most divergent ratios (1/16 and 16) still showed significantly less learning compared to the multiple of 1 ($P<0.05$, unpaired t-test).

Figures 4-3D and 4-3E illustrate the generalization of disruption after the 0 minute and 60 minute neutral periods, respectively. With no neutral period, normalized disruption tended to be more successful as the disruption and testing frequencies diverged. When combining the multiples of 1/16 and 16 together, the most divergent groups showed significantly more disruption than when the training and testing frequencies matched ($P<0.05$, unpaired t-test). If a neutral period followed learning, normalized disruption appeared more successful when the multiple of learning was 16, however this was not significant ($P=0.16$, unpaired t-test). To summarize, we found that without a neutral period, although the disruption of learned gain decreases depended weakly on the ratio of the training and testing frequency, it was not selective for the disruption frequency. Disruption after a 60 minute neutral period did not show any frequency selectivity or dependence.

4.3.4 Phase changes depended on frequency

Figure 4-4 shows the effect of learning and the subsequent disruption on the phase angle between eye and head velocity during the VOR. Figures 4-4A and 4-4B show the changes during the learning period. A positive value of the phase difference indicates a phase lead, meaning that eye velocity became less delayed relative to head velocity. Negative values indicate a phase lag, or that the eye velocity became more delayed relative to the head velocity. Although shifts in phase were quite small, the phase did show significant learning related changes at some test frequencies. Figure 4-4B illustrates the generalization of phase changes during learning. At frequencies above the training frequency, the average effect of learning was a phase lead, and at frequencies below the training frequency, the mean effect was a phase lag. These differences were found to be significant for the ratios above and below 1 ($P<0.05$, unpaired t-tests). This effect is known as "phase cross-over" and has been described by previous studies (Lisberger et al., 1983; Raymond and Lisberger, 1996; Kramer et al., 1998; Kimpo et al., 2005). Figures 4-4C and 4-4D show the effects of disruption on phase when a neutral period was present, and without a neutral period. Without a neutral period (Fig 4-4C), significant changes in phase occurred during the disruption period at frequencies above the training frequency, reversing the phase lead that had been induced by learning. The change in phase when the multiple was 16 was found to be significantly less than the change in phase when the multiple was 1 ($P<0.05$, unpaired t-test). At the training frequency and lower frequencies, there were no significant changes in phase during disruption. With a neutral period (Fig 4-4D) there were no significant changes in phase during disruption.

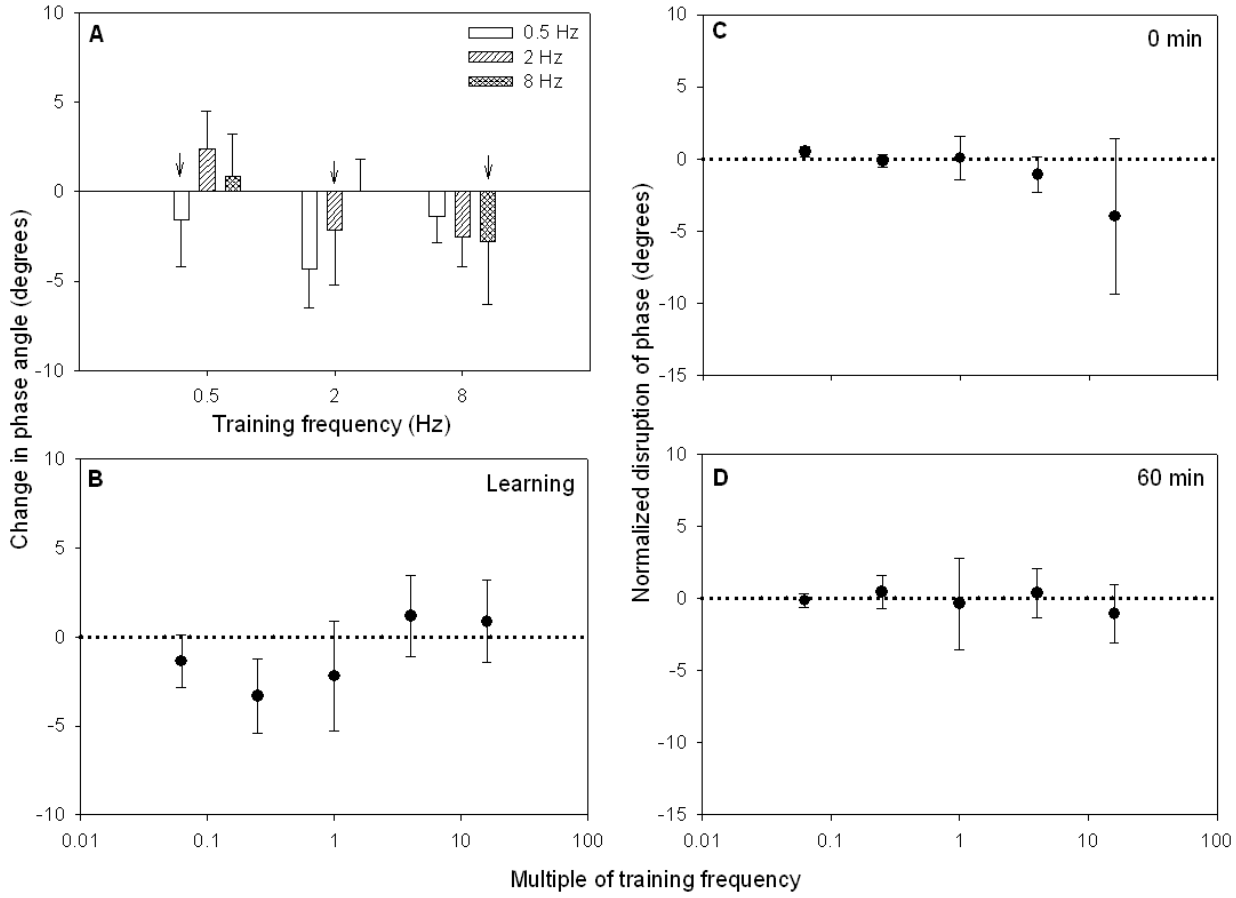

Figure 4-4. Changes in phase during learning and disruption. Learning induced small phase leads at frequencies above the training frequency, and small phase lags at the frequencies below. A: Change in phase angle between head and eye velocities during learning at the different training and test frequencies. Arrows indicate when training and testing frequencies matched. B: Phase changes that occurred during learning as a function of the ratio of the test and training frequencies. C: Disruption that took place immediately after learning depended on the multiple of the training frequency. Changes in phase during disruption were normalized for the changes that occurred during learning at the same test frequency. D: Normalized changes in phase during disruption that occurred following a 60 minute neutral period.

4.4 Discussion

In this study we investigated the generalization of disruption and consolidation in an effort to determine if they show frequency selectivity that would suggest new learning. The basis for our investigation stems from the well established frequency selectivity in VOR motor learning. Our results of learning in the VOR confirm previous observations that learning is greatest when the conditions of testing match the conditions present during learning (Robinson, 1976; Lisberger et al., 1983; Raymond and Lisberger, 1996; De Zeeuw et al., 1998; Kimpo et al., 2005), and that

the amount of learning decreases as the these conditions diverge (Kimpo et al., 2005). Furthermore, when the testing and training frequencies matched, learning was always greater at the lower frequencies of 0.5 and 2 Hz compared to 8 Hz (Raymond and Lisberger, 1996; Broussard et al., 1999a). During gain-down learning, the phase of the VOR showed a phase lead or lag if measured at a frequency above or below the training frequency, respectively; this is also consistent with previous observations (Lisberger et al., 1983; Raymond and Lisberger, 1996; Kramer et al., 1998; Kimpo et al., 2005).

Learning in the VOR is thought to occur in the cerebellar cortex (Ito, 1972; De Zeeuw et al., 1998; McElligott et al., 1998; Nagao and Kitazawa, 2003; Kassardjian et al., 2005), and could be the result of synaptic changes such as LTD (Sakurai, 1987) or LTP (Salin et al., 1996; Lev-Ram et al., 2002) at the parallel fibre-Purkinje cell synapses. Learned gain decreases in particular are thought to be the result of LTP (Boyden and Raymond, 2003). The selectivity of learning for the training frequency could be the result of changes localized to a particular group of synapses that are functionally tuned to that particular frequency (see: Lisberger et al., 1983; Dean et al., 2010; Broussard et al., 2011).

4.4.1 Rapid consolidation does not involve new learning

Unlike learning, the processes underlying consolidation are largely unknown, and are not well understood. When measured over many days, consolidation is thought to involve a shift in the memory location from the cerebellar cortex to the brainstem (Kassardjian et al., 2005; Shutoh et al., 2006; Anzai et al., 2010). This memory transfer suggests the involvement of “new learning” and the modification of new synapses. However, rapid consolidation has been shown to occur within 1 hour after learning has stopped (Cooke et al., 2004). It is not known whether this type of consolidation involves a shift in memory location. In other memory systems it is believed that rapid consolidation occurs locally, while long-term consolidation involves a shift in the memory location (Dudai, 2004). Here we show that unlike learning, consolidation did not show any frequency selectivity in the gain or phase, and generalized completely across the range of frequencies. Frequency selectivity suggests new learning; we found that no frequency selectivity

was added by the disruption or consolidation processes. Indeed, consolidation maintained the frequency selectivity seen during learning. We suggest that rapid consolidation in the VOR takes place at the same set of synapses that were initially modified during learning. However, since we did not record from these or any other synapses, we cannot rule out the involvement of non-synaptic plasticity or learning in another area such as the brainstem, which may not show frequency selectivity.

4.4.2 Possible mechanisms of disruption

Disruption of newly learned VOR memories by rotation in darkness has been previously reported in monkeys (Cohen et al., 2004) and cats (Kassardjian et al., 2005; Chapter 3), but the mechanisms involved remain unknown. Here we show that the amount of disruption, following gain-down learning, generalized over the range of frequencies, and showed a slight increase as the training and testing frequencies diverge. Gain-up learning has been shown to be highly selective for the training frequency and shows phase cross-over (Kimpo et al., 2005), these characteristics were not seen during disruption. Therefore, gain-up learning is not likely the mechanism behind the disruption of learned gain decreases. The broad generalization of disruption suggests that disruption may reverse any synaptic changes that might have occurred, but have not yet been stabilized. This process could be analogous to the de-potentiation of recently potentiated synapses that occurs during the extinction of fear memories (Myers et al., 2006). We propose that disruption in the VOR involves a similar reversal of the recent changes made at synapses during learning.

It has been suggested that VOR signals are transmitted by an array of frequency filters or "channels" (Lisberger et al., 1983). A particular set of synapses encoding a certain rotational frequency could be considered a frequency channel. This is furthered by evidence of functional microzones within the cerebellar cortex (Dean et al., 2010). These frequency channels may not be completely separate, but may be broadly tuned, with neighbouring channels participating in the transmission of a particular frequency (Broussard et al., 2011). This theory is useful in explaining the phase crossover shown in this study and by many others (Lisberger et al., 1983;

Raymond and Lisberger, 1996; Kramer et al., 1998; Kimpo et al., 2005). However, as this is just one theory, we do not have any direct evidence to support the idea of frequency channels.

Disruption was most effective at frequencies that were furthest away from the training frequency. These frequencies also showed the least amount of learning. This could suggest that fewer synapses encode the learned change in gain as the training and testing frequencies diverge (Titley et al., 2009). These fewer synapses might be slower to stabilize, and thus more susceptible to a disruption stimulus. The synapses within the channel encoding the training frequency would have been preferentially modified and stabilized. A possible method for this preferential selection of synapses has been described in the hippocampus as "synaptic tagging"(Frey and Morris, 1997; Redondo and Morris, 2011). Tagging the synapses within the training frequency channel could lead to the selective stabilization of the synapses encoding that frequency, while the other synapses could be disrupted.

4.4.3 Conclusions

We conclude that unlike learning which shows frequency selectivity, disruption and rapid consolidation generalize across the frequency range. We therefore suggest that disruption and rapid consolidation does not involve learning, and most likely does not involve a shift in memory location.

Chapter 5

5 Motor learning in the VOR requires cerebellar mGluR1 receptors

5.1 Introduction

As the previous chapter has shown, learning shows frequency dependence. The amount of learning has been shown to be greater at lower frequencies (Raymond and Lisberger, 1996; Broussard et al., 1999a; Chapter 4). Here, we found that this frequency dependence is reversed, when learning is inverted.

The parallel fibre – Purkinje cell synapses are capable of bidirectional synaptic changes, LTP (Salin et al., 1996; Lev-Ram et al., 2002) and LTD (Sakurai, 1987; Qiu and Knöpfel, 2009). In the post-synaptic neuron, the direction of this plasticity is thought to depend on the calcium concentration (Jörntell and Hansel, 2006). A small increase in calcium will trigger LTP, while a larger increase in calcium is required for LTD (Coesmans et al., 2004). This suggests that at the PF-PC synapse, LTD has a higher calcium threshold than LTP. Indeed, blocking a large increase in calcium may prevent LTD and induce LTP instead.

The type 1 metabotropic glutamate receptor (mGluR1) has been shown to be required for cerebellar motor learning. Mutant mice lacking mGluR1 are known to have impairments in the optokinetic and eye blink reflexes (Aiba et al., 1994b; Shutoh et al., 2002). These receptors are known to be required for post-synaptic LTD (see: Kano et al., 2008), but not for LTP (Belmeguenai et al., 2008). In cerebellar slices, blocking mGluR1 abolishes LTD, and results in LTP (Hartell, 1994). In the VOR, learned gain increases are thought to depend on LTD (Hansel et al., 2006), while gain decreases are thought to be the result of LTP (Boyden and Raymond, 2003). We hypothesize that mGluR1 is required for learned gain increases, but not learned gain decreases. The results from this study have been previously published (Titley et al., 2010).

5.2 Methods

Data from 5 male cats (S, V, B, C and E) aged 8-27 months were included in this study.

5.2.1 Drug injections

In order to allow access to the cerebellar flocculi, bilateral injection cylinders were surgically placed over each flocculus (see Chapter 2). To position the injection needle, a grid was fitted inside each injection cylinder. We mapped each flocculus using trains of biphasic current pulses, delivered through bipolar concentric stimulating electrodes (Rhodes Biomedical), to find the optimal location for the drug injection. We defined the best injection site as the site at which stimulation produced the most robust horizontal smooth eye movements.

Before each experimental trial, the cylinders were cleaned, and for the animal's comfort, the cylinders were filled with either 1% lidocaine solution or 2% lidocaine jelly for at least 20 minutes. Cylinders were then rinsed with saline before the stimulating electrode or needle was inserted. For injections, a Hamilton syringe containing the drug was attached to a 24-gauge needle. The syringe was secured to an electrode carrier (David Kopf) and positioned identically to the stimulating electrode. The tip of the needle was advanced slowly to the depth of the best stimulation site, and 1 μl of a drug or vehicle (phosphate buffered saline, PBS) was pressure injected at a rate of 1 μl/min. In cat S, some injections of PBS were 5 μl (see Table 5-1). After the injection, the needle was left in position for 3 min to allow diffusion of the drug away from the needle tip, and then slowly withdrawn. In all experiments, injections were made first in the left flocculus and then in the right flocculus. The minimum time between the end of the second injection and the post-injection VOR measurement was 5 minutes.

Most cats appeared to be unaffected by the passage of the injection needle through the dura after the lidocaine was given. However, cat V appeared to be sensitive to the needle. In cat V, we

therefore administered butorphanol, an opioid analgesic, 0.2 mg/kg (s.c.) 30 minutes before each experiment for comfort. We controlled for the effects of the butorphanol by comparing learning in experiments with and without injections and vehicles injections with and without butorphanol (see: Appendix 1).

5.2.2 Experimental protocol

Learned gain increases and decreases were induced by having the cat wear X2 (gain-up learning) or X0.25 telescopes (gain-down learning) set in opaque frames closely fitted to the cat's head. The learning period consisted of 60 minutes of SOS rotation in the light, alternating between two waveforms. Each waveform consisted of three frequencies: 0.5, 2 and 8 Hz or 0.2, 1 and 5 Hz. Each component had a peak velocity of 10 deg/s.

During the experimental protocol, we injected 1 μl of either the mGluR1 antagonist, YM 298198 (50 μM), the agonist, (S)-DHPG (1 μM), or the vehicle alone, PBS, into the left and right flocculi of the cerebellum. The cats were then subjected to a 60 minute learning period to increase or decrease the VOR gain. The gain of the VOR was measured at 0.5, 2 and 8 Hz in complete darkness before and after the injection, and after 30 and 60 minutes of the learning period. The change in gain was calculated as follows:

$$\text{Gain change} = [(G_{60\text{min}} - G_{\text{post-inj}})/G_{\text{base}}]*100$$

Where $G_{60\text{min}}$ is the VOR gain at the end of the 60 min learning period, $G_{\text{post-inj}}$ is the gain measured after the drug injection, and G_{base} is the baseline gain of the VOR measured before the injections. After each learning trial, the cat was rotated in the light for 30 minutes without telescopes to help return the VOR gain back to baseline levels. Trials that involved learning were separated by at least 6 days and in some cats as much as 14 days.

We also performed separate control trials to measure the effects of the drugs without learning. During these control trials the cats did not wear telescopes during the learning period, and were

either rotated in complete darkness or in the light (see Table 5-1 for the number of trials for each cat). We calculated the average change in VOR cancellation over the course of each control trial to determine the effects of the drugs on other gaze stabilizing mechanisms (i.e. smooth pursuit). VOR cancellation was measured at 0.2 Hz rotation. A black and white patterned screen with a central fixation point was attached to the turntable that enclosed the cat's field of vision. The percentage of VOR cancellation was calculated as follows:

$$\text{Cancellation} = [(G_{vor} - G_{cancel})/G_{vor}]*100$$

Where G_{vor} is the gain in darkness, and G_{cancel} is the gain of the VOR during the cancellation protocol (Zee et al., 1981; Kassardjian et al., 2005).

Cat	Antagonist – YM 298198			Agonist – DHPG			Vehicle – PBS		
	Gain-up	Gain-down	No lenses	Gain-up	Gain-down	No lenses	Gain-up	Gain-down	No lenses
S	1	1	2	0	0	0	1 †	1 †	2 †
V	2	2	2	0	0	0	2	2	2
B	2	1	2	3 ‡	3	2	1	1	1
C	1	1	0	1	1	2	1	1	1
E	0	0	0	1	1	0	1	0	0
TOTAL	**6**	**5**	**6**	**5**	**5**	**4**	**6**	**5**	**6**

Table 5-1. The number of learning trials in each cat and total sample sizes. † indicates an injection volume of 5 µl. Other injections had a volume of 1 µl. ‡ indicates that one data point was missing from one frequency; that set was excluded from the statistical analysis, but was included in the figures.

5.2.3 Data analysis

We used mixed-model repeated measures ANOVAs to look for effects of time (learning period), test frequency (0.5, 2, or 8 Hz), and drug group (vehicle, YM 298198, and DHPG) for each protocol. A repeated-measures ANOVA was also used to compare the percent cancellation of the VOR among drug groups. A Bonferroni correction was used for multiple comparisons, and the Greenhouse-Geisser sphericity correction was used when variances were unequal. Because we found significant interactions between factors, we used post-hoc paired Student's t-tests to compare the pre- and post-learning gains within each group, and unpaired t-tests to compare the

post-learning gain and the learned changes between groups at each frequency. Finally, we compared the amount learned at different frequencies using paired t-tests. In all cases we assumed significance at the 95% level ($P<0.05$). Data was analyzed using SPSS 18 software.

5.3 Results

5.3.1 Histology and controls

The injection sites in each cat were located within the flocculus of the cerebellum (Fig 5-1). The same regions of the flocculus that are concerned in motor learning are known to take part in the immediate cancellation of the VOR (Rambold et al., 2002; Kassardjian et al., 2005).

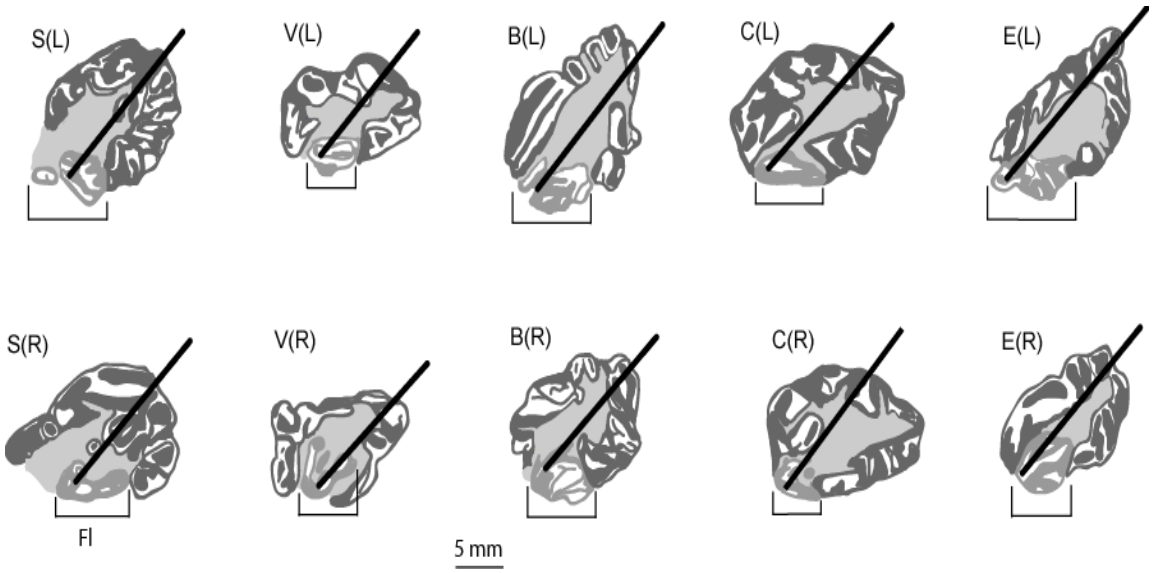

Figure 5-1. Location of injection sites in each cat. In all cats used in the injection experiments (cats S, V, B, C and E), the injection sites were located in the cerebellar flocculi. Parasagittal sections through the flocculus (light grey) and the adjacent cerebellar lobes (darker grey) are illustrated. Cannula tracks of the needle (black line) are shown for both the left (L) and right (R) sides. The borders of the flocculi (Fl) are indicated by brackets. Rostral is leftward for all sections.

We asked if the injection of a group I mGluR agonist (S)-DHPG, or a specific mGluR1 antagonist YM 298198, into the flocculus had any effect on the cat's normal VOR or their ability to cancel the VOR when looking at a full-field visual pattern. We measured the gain and the cancellation of the VOR before and after the injection, as well as 30 and 60 minutes after the injection. The change in cancellation was normalized to the pre-injection value which was set at a value of 100%. Neither drug had any effect on the gain of the VOR before learning (Fig 5-2A). A repeated measures ANOVA found no significant effect of drug group ($F(2,12)=0.21$, $P=0.98$), time ($F(2,24)=0.74$, $P=0.49$), or frequency ($F(2,24)=0.31$, $P=0.74$) on the VOR gain. Similarly, we found no significant difference in the percent gain change (amount learned) between the drugs and vehicle (Fig 5-2 C, E; $P>0.05$, unpaired t-tests). We also found no effects of either drug on VOR cancellation (Fig. 5-2 B); the ANOVA again showed no significant effect of drug treatment group ($F(2,11)=0.87$, $P=0.45$) or time ($F(2,22)=1.1$, $P=0.35$). There was no significant difference between any of the drug groups on the change in percent cancellation (Fig 5-2 D, E; $P>0.05$, unpaired t-tests).

In summary, the mGluR1 drugs did not have any effect without learning. These results suggest that mGluR1 is not involved in the normal signal processing in the flocculus.

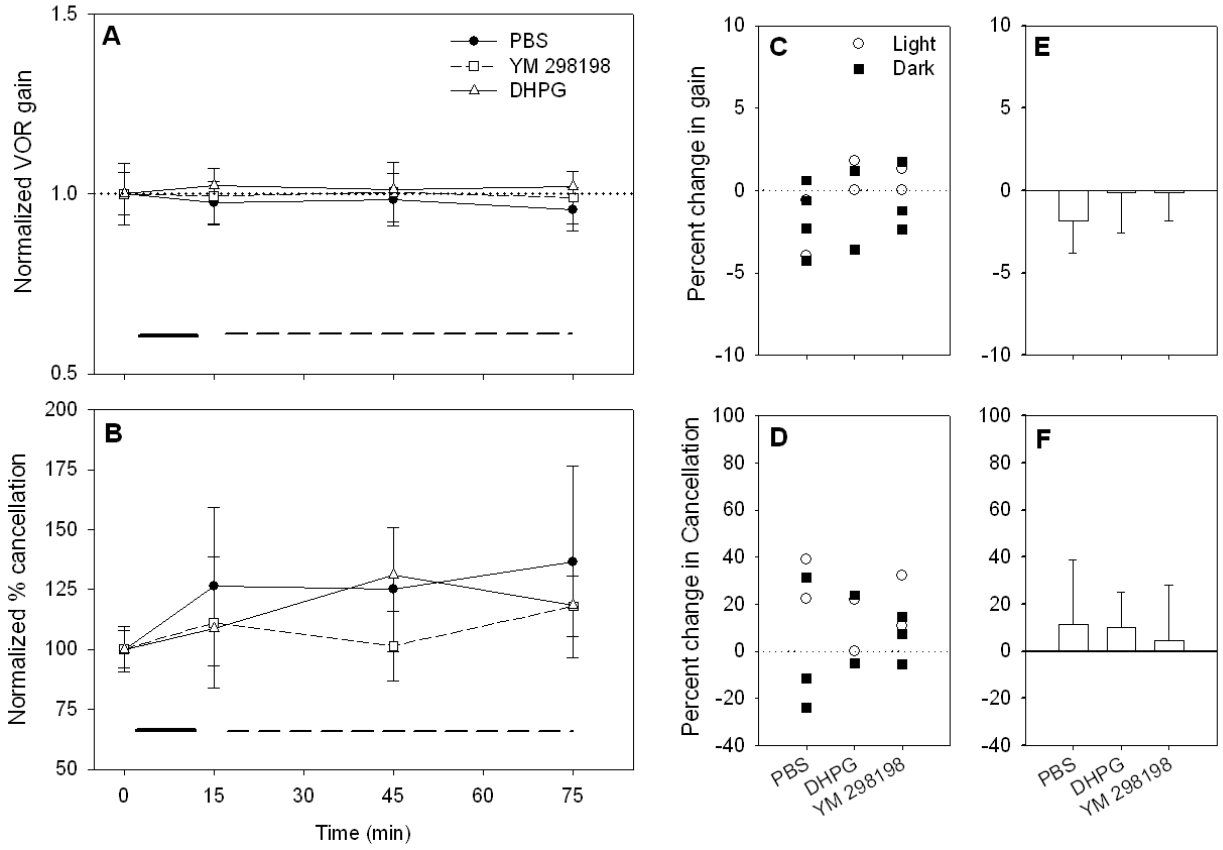

Figure 5-2. mGluR1 had no effect on the VOR or VOR cancellation without learning. In control protocols that did not involve learning, the drugs had no effect on the VOR gain or the cancellation of the VOR. A: Time course of the VOR at 2 Hz gain during the control protocols. Following the injection of the drug (solid line) the cat was rotated in the dark or light for 60 minutes without telescopes (dashed line). Solid circles: vehicle (PBS) injections, n=6. Open squares: YM 298198, n=6. Open triangles: (S)-DHPG, n=4. B: Time course of the percentage change in gain measured at 0.2 Hz during the same trials as in A. Drugs had no effect on the VOR cancellation. C: The percentage change in gain during the period of rotation of trials either in the light (open circles) or in dark (closed circles). D: Percent change in cancellation during rotation in the light or dark. Symbols same as C. E: Mean percent change in gain of data in panel C. F: Mean percent change cancellation of data in panel D. Error bars represent S.D. for this and all figures.

5.3.2 YM 298198 reversed gain-up learning

During the gain-up protocol the mGluR1 antagonist, YM 298198 (1 µl, 50 µM), changed the direction of learning, and resulted in a learned decrease in the VOR gain instead of an increase. Figure 5-3A shows examples of the VOR gain at 2 Hz before and after the gain-up learning protocol following an injection of either PBS or YM 298198. The VOR maintained a linear relationship between the head and eye velocity at all three test frequencies (0.5, 2 and 8 Hz),

regardless of the drug injected (Fig 5-3B). The linear relationship and the slope of these plots suggest that the drugs did not affect the normal functioning of the VOR. However, the direction of the gain change differed after the gain-up learning period depending on whether YM 298198 or PBS was injected.

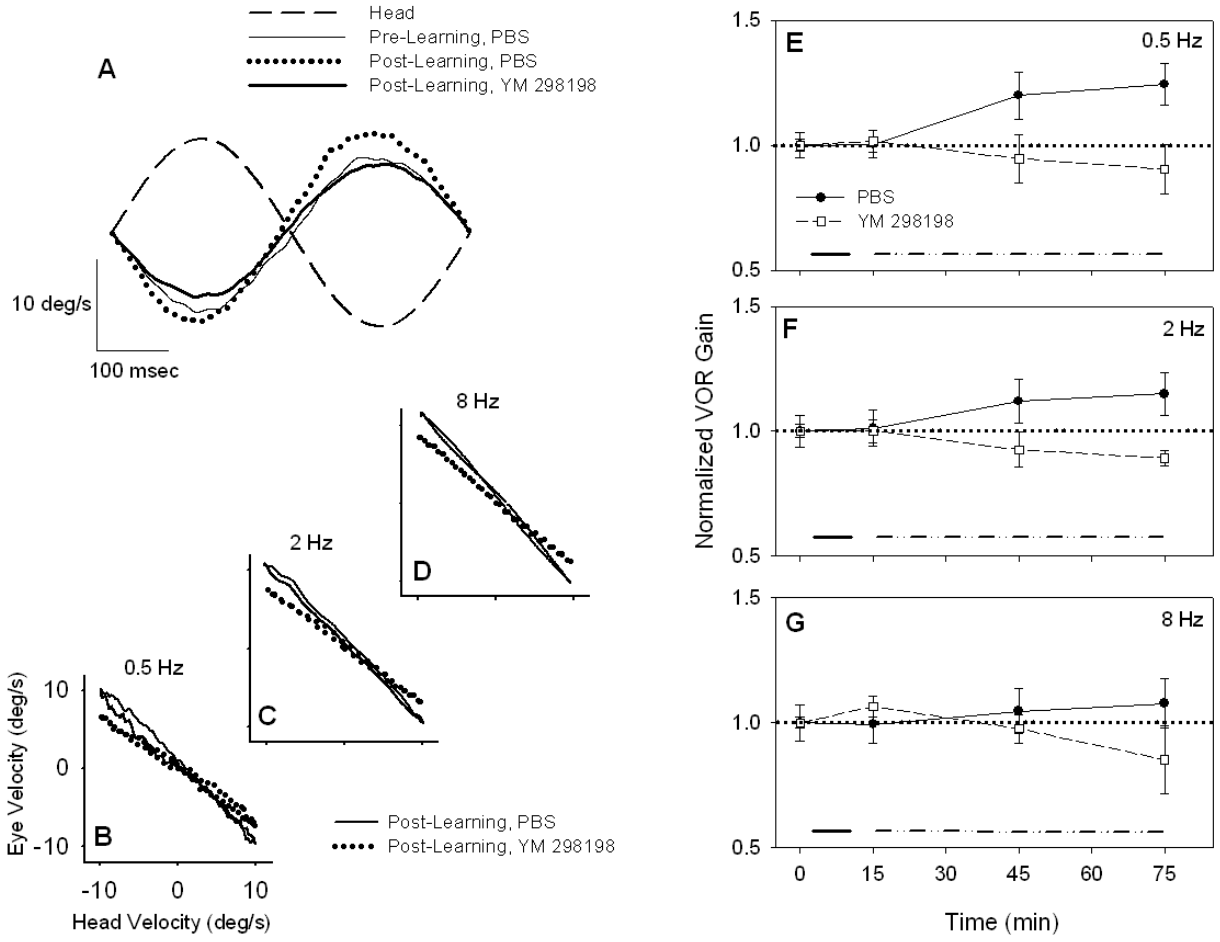

Figure 5-3. mGluR1 antagonist inverted gain-up learning. After injection of YM 298198 into both flocculi, the gain-up protocol results in a learned gain decrease. A: Typical examples of angular head rotation measured at 2 Hz. All traces are averages of≥ 10 cycles. Dashed line: Head (turntable) velocity. Thin solid line: Eye velocity before gain-up learning protocol. Dotted line: After 60 minutes of gain-up learning the eye velocity showed a greater excursion, indicating a higher VOR gain. Thick solid line: In the presence of YM 298198, after gain-up learning the eye velocity showed a reduced excursion, indicating a reduced VOR gain. B-D: Eye velocity as a function of head velocity, at all three test frequencies, after gain-up learning in the presence of either the vehicle (PBS) (solid line) or YM 298198 (dotted line). Examples from cat V. E-G: Time courses of the VOR gain at the different test frequencies during the gain-up learning protocol. Solid circles: Vehicle injections. Open squares: YM 298198 injections. Heavy solid line: Injection period. Dash-dotted line: learning period.

The injection of YM 298198 resulted in a gain decrease during the gain-up protocol. Figure 5-3(C-E) shows the gain of the VOR during the time course of the gain-up learning protocol. A repeated measures ANOVA for the gain-up learning period showed significant effects of time ($F(2,28)=40.5$, $P<0.001$) and drug group ($F(2,14)=13.6$, $P<0.001$) on the VOR gain as well as a significant interaction between time and drug group ($F(4,28)=68.7$, $P<0.001$). At all 3 frequencies the gain of the VOR increased during the learning period for the PBS injections, but decreased throughout the learning period for the YM 298198 injections.

The outcome of the ANOVA was confirmed with post-hoc comparisons. In the presence of PBS, the increase in the VOR gain after learning was significantly greater than the pre-learning value at all 3 frequencies ($P<0.01$, paired t-tests). After injection of YM 298198, the decrease in gain after the gain-up learning period was significantly less than the pre-learning gain at all 3 frequencies ($P<0.02$, paired t-tests). The post-learning VOR gains and the amount learned after the learning period differed significantly between PBS and YM 298198 injections at all frequencies ($P<0.01$, unpaired t-tests). The results of the t-tests are shown in more detail in Table 5-2. Furthermore, this gain decrease was consistent among cats. Table 5-3 shows the mean gain change in each cat for the different learning conditions.

		PBS			YM 298198			DHPG		
	Comparison Sample	**0.5 Hz**	**2 Hz**	**8 Hz**	**0.5 Hz**	**2 Hz**	**8 Hz**	**0.5 Hz**	**2 Hz**	**8 Hz**
Pre-Learning										
Drug effect	PBS alone	-	-	-	0.3180	0.3027	0.3045	0.2899	0.3028	0.0855
Gain-up learning										
Learned Changes	Pre-learning gain	**3.42E-05**	**0.0002**	**0.0034**	**0.0111**	**0.0008**	**0.0054**	**7.72E-05**	**0.0006**	**0.0009**
Drug effect on final gain	PBS alone	-	-	-	**3.91E-05**	**2.03E-05**	**0.0042**	0.0645	**0.0486**	0.0748
Drug effect on % change	PBS alone	-	-	-	**1.93E-06**	**5.09E-07**	**0.0002**	**0.0026**	**0.0016**	**0.0170**
Test frequency	0.5 Hz	-	**0.0002**	**0.0004**	-	0.4729	**0.0093**	-	0.0778	**9.14E-05**
Test frequency	2 Hz	**0.0002**	-	**0.0035**	0.4729	-	0.0852	0.0778	-	**0.0054**
Test frequency	8 Hz	**0.0004**	**0.0035**	-	**0.0093**	0.0852	-	**9.14E-05**	**0.0054**	-
Gain-down learning										
Learned Changes	Pre-learning gain	**0.0005**	**0.0010**	**0.0046**	**0.0001**	**0.0024**	**0.0449**	**8.90E-05**	**0.0008**	**0.0011**
Drug effect on final gain	PBS alone	-	-	-	**0.0052**	0.0501	0.1736	0.3663	0.0729	0.2840
Drug effect on % change	PBS alone	-	-	-	0.1901	0.2279	0.1061	0.4231	0.2427	0.1254
Test frequency	0.5 Hz	-	0.0587	**0.036**	-	**0.0333**	**0.0033**	-	**0.0039**	**0.0007**
Test frequency	2 Hz	0.0587	-	0.0974	**0.0333**	-	**0.0141**	**0.0039**	-	**0.0286**
Test frequency	8 Hz	**0.036**	0.0974	-	**0.0033**	**0.0141**	-	**0.0007**	**0.0286**	-

Table 5-2. Results of post-hoc t-tests with mGluR1 drugs. Post-hoc Student's t-tests for individual comparisons. The first column indicates what factor was tested. Each number indicates the result (P value) of a 1-tailed comparison to the sample listed in the second column. Boldface numbers indicate significance at P<0.05. Pre-learning: The gain of the VOR after drug injection, but before learning was compared to the gain after injection of PBS alone. Learned changes: The pre- and post-learning gain values are compared to verify that learning caused a significant change. Drug effect on final gain: The post-learning gain values after drug injection are compared to that of PBS. Drug effect on % change: The learned percent changes in gain are compared among drug groups. Test frequency: The post-learning gains are compared among frequencies.

		Gain-up protocol				Gain-down protocol			
Drug	**Cat**	**Trials**	**0.5 Hz**	**2 Hz**	**8 Hz**	**Trials**	**0.5 Hz**	**2 Hz**	**8 Hz**
PBS	S	1	32	19	10	1	-23	-25	-26
	V	2	22	15	13	2	-33	-30	-9.2
	B	1	25	15	6.5	1	-31	-21	-20
	C	1	23	11	7.2	1	-26	-16	-17
	E	1	19	7.1	1.1	0	-	-	-
YM 298198	S	1	-17	-16	-18	1	-28	-20	-17
	V	2	-19	-7.2	-36	2	-26	-20	-2.7
	B	2	-3.4	-10	-13	1	-25	-22	-17
	C	1	-5.1	-14	-13	1	-24	-21	-5
DHPG	B	3	36	26	17	3	-27	-18	-12
	C	1	30	25	15	1	-31	-22	-12
	E	1	33	38	15	1	-38	-31	-11

Table 5-3. Mean percent gain change for each protocol. Mean percent gain changes in the VOR for the individual cats during the learning period in the presence of the different drugs. The change in gain is given separately for both the gain-up and gain-down protocols. The number of trials is given for each case. Negative numbers indicate learned gain decreases.

5.3.3 DHPG increased gain-up learning

The group 1 mGluR agonist (DHPG) augmented gain-up learning. Figure 5-4(C-E) illustrates the time courses for gain-up learning for PBS and (S)-DHPG (1 μl, 1 μM) injections. During gain-up learning, after injection of DHPG, the gain of the VOR was significantly greater than the pre-learning gains at all 3 frequencies ($P<0.01$, paired t-tests). Although the post-learning gains after DHPG injection were greater than after the PBS injection, this was only found to be significant at 2 Hz ($P<0.05$, unpaired t-test). However, the change in gain in the presence of DHPG was significantly greater than in the presence of PBS, at all 3 frequencies ($P<0.02$, unpaired t-tests). This result was also consistent among cats, with the amount learned being greater with DHPG than in the presence of PBS (Table 5-3). Typical averaged responses before and after learning are shown in Fig 5-4B. The response of the VOR was linear in all cases, indicating that the drug did not effect normal VOR functioning.

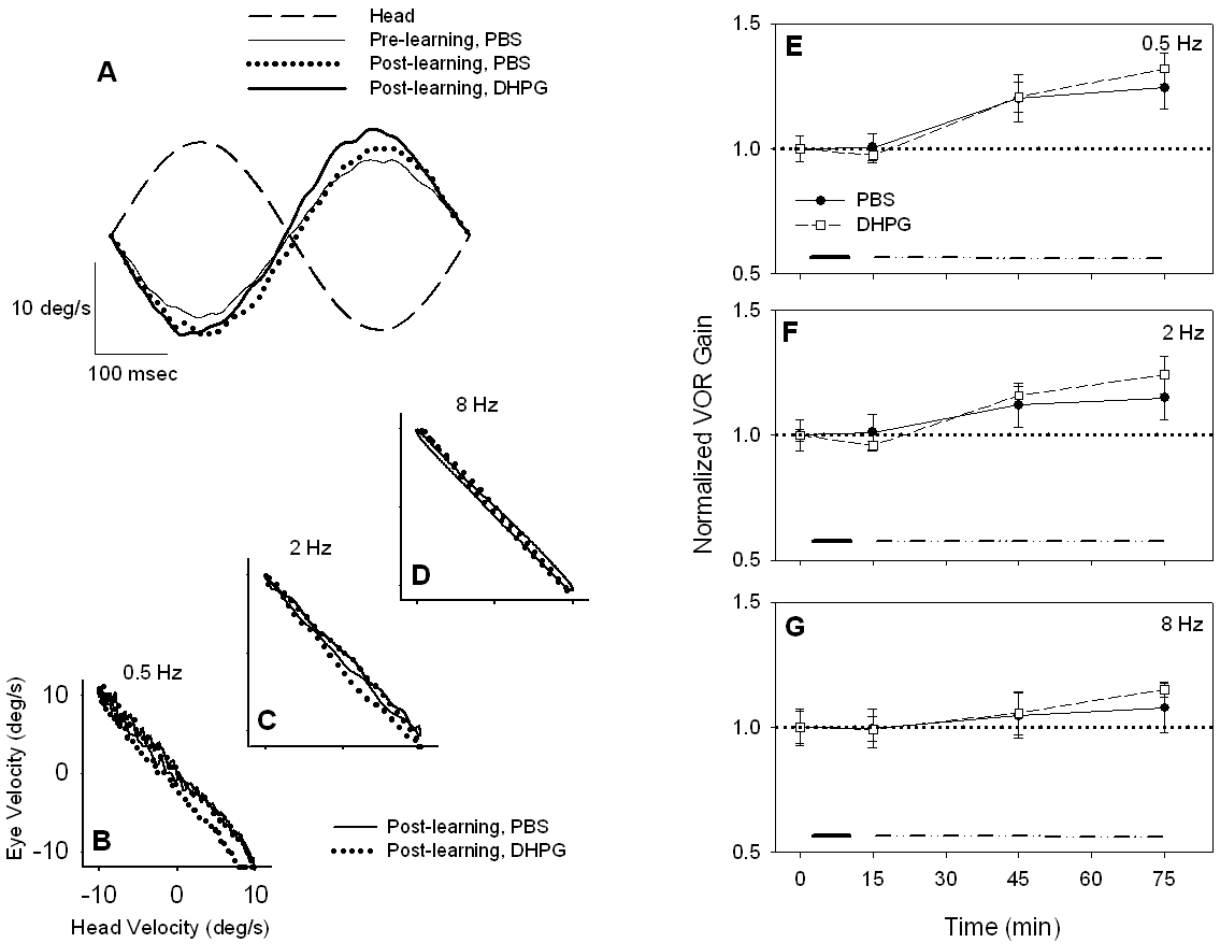

Figure 5-4. mGluR1 agonist augmented gain-up learning. After injection of DHPG, gain-up learning was augmented. A: Sample traces of head and eye velocity at 2 Hz plotted against time. Head velocity (dashed line) and pre- (thin solid line) and post-learning (dotted line) eye velocity in the presence of a vehicle are same as Fig 5-3. In the presence of DHPG (thick solid line), the gain of the VOR was slightly augmented after gain-up learning. B-D: Eye velocity plotted against head velocity after gain-up learning at all three frequencies in the presence of the vehicle (solid line) or DHPG (dotted line). Examples from cat B. E-G: Time courses of normalized VOR gain at the three frequencies during gain-up learning after injections of vehicle (solid circle) or DHPG (open square). Horizontal lines are same as Fig 5-3.

5.3.4 Gain-down learning was not affected

During gain-down learning, the gain of the VOR decreased in all conditions. There was no significant effect of YM 298198 or DHPG on gain-down learning. A repeated measures ANOVA during the gain-down learning period showed a significant effect of time ($F(2,24)=203.6$, $P<0.001$), but no effect of drug group ($F(2,12)=1.69$, $P=0.23$). Figure 5-5 shows the time course of the VOR gain during the gain-down protocol. The amount learned in the presence of YM

298198 or DHPG was not significantly different than the amount learned in the presence of the vehicle at any frequency (P>0.05, unpaired t-tests).

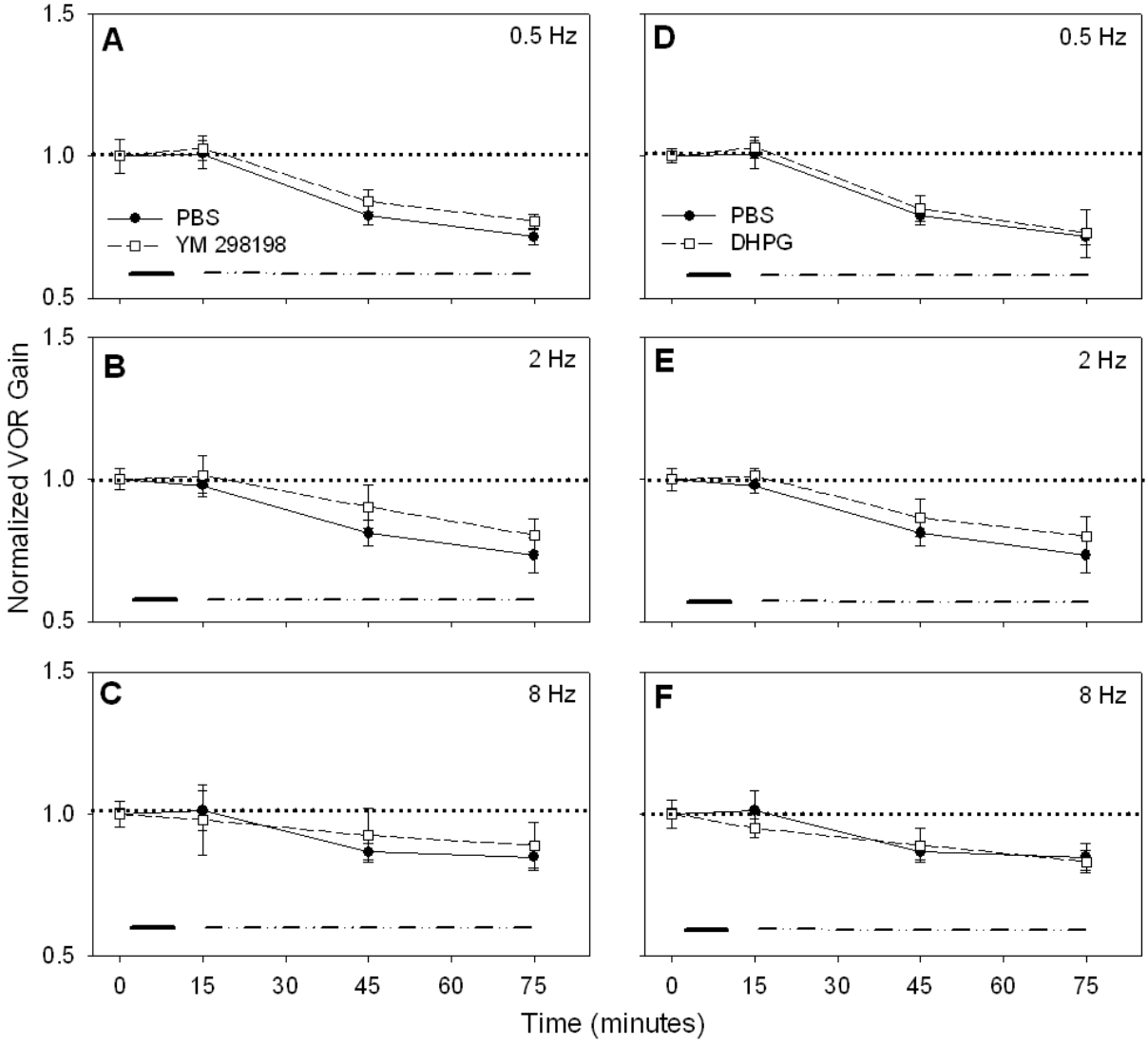

Figure 5-5. mGluR1 had no effect on gain-down learning. Gain-down learning was not affected after injection of YM 298198 or DHPG. Time courses of the gain-down learning protocol at the different frequencies. A-C: In the presence of YM 298198 (open squares), gain-down learning was not different from vehicle controls (solid circles). D-F: Following injection of DHPG (open squares) gain-down learning was not significantly different from controls (solid circles; re-plotted from A-C). Horizontal lines same as in Fig 5-3.

5.3.5 Inversion of frequency dependence

Learning in the VOR is frequency dependent. The amount learned is greater when measured at a lower frequency (Chapter 4; Raymond and Lisberger, 1996; Broussard et al., 1999a). The repeated measures ANOVAs revealed a significant effect of test frequency for both the gain-up ($F(2,28)=16.5$, $P<0.001$) and gain-down ($F(1.4,16.3)=6.82$, $P<0.02$) learning conditions. In both learning protocols there was also a significant interaction between frequency and time (gain-up: $F(2.3,32.0)=16.3$, $P<0.001$; gain-down: $F(2.3,27.4)=22.6$, $P<0.001$). This interaction is thought to reflect a change in the dynamics of the VOR associated with learning. Figures 5-6 A and B show the amount learned during the learning period for each drug at a given frequency. For gain-up learning, when learned changes were in the "correct" direction, as they were for injections of PBS and DHPG, the amount learned was greater at the lower frequency and less at the higher frequency (Fig 5-6A). The amount learned at 0.5 Hz was greater than 8 Hz after both PBS ($P<0.01$, paired t-test) and DHPG injections ($P<0.01$, paired t-test). Interestingly, the frequency dependence as well as the direction of learning was altered by blocking the mGluR1 receptor (Fig 5-6A). During gain-up learning, the repeated measures ANOVA showed a significant interaction between frequency and drug group ($F(4,28)=5.5$, $P<0.002$). This interaction was made clear; as we found that in the presence of YM 298198 the frequency dependence was reversed after gain-up learning. The amount learned at the 8 Hz frequency was significantly greater than that at the 0.5 Hz frequency ($P<0.01$, paired t-test).

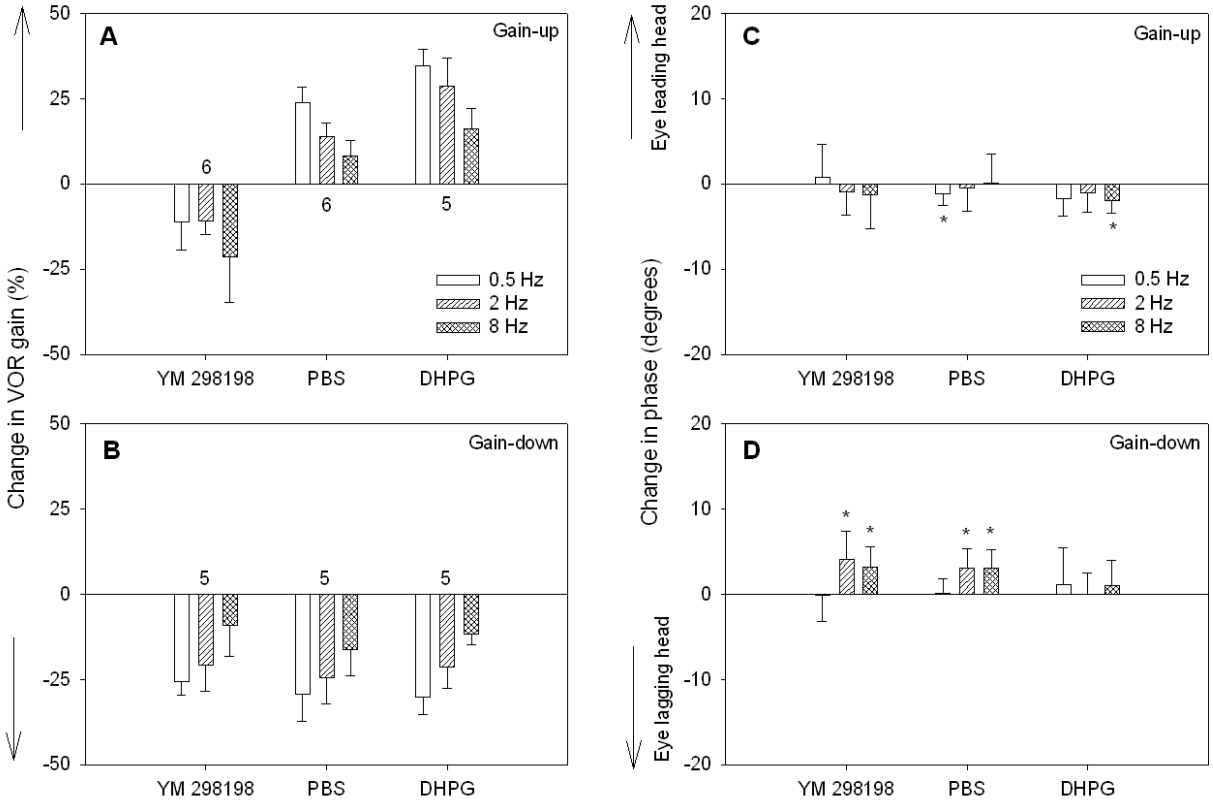

Figure 5-6. Summary of changes on VOR gain and phase with mGluR1 drugs. Summary of changes in VOR gain and phase between the post-injection time point and the end of learning. A: The change in gain during the gain-up learning protocol as a percentage of the pre-injection gain. Each group of bars represents the change in gain measured at the three frequencies for each drug marked on the abscissa. The numbers on the plot represent sample sizes for each condition. B: The change in gain during the gain-down learning protocol. C: The change in phase, in degrees, during the gain-up protocol. The polarity of phase lag and lead are indicated along the ordinate. Data are from the same trials in panel A. D: The change in phase during the gain-down protocol. Data are from the same trials as in B. Asterisks (in C and D) indicate a significant change in phase during the learning period ($P<0.05$).

In the gain-down protocol, there was no effect of any drug on VOR dynamics. Learning was always in the correct direction and the amount learned decreased as the frequency increased (Fig 5-6B). The amount learned at 0.5 Hz was significantly greater than 8 Hz for all drug injections ($P<0.05$, paired t-tests).

5.3.6 mGluR1 had little effect on VOR phase

Small changes with phase angle of the VOR were found during the learning periods. A repeated measures ANOVA of the phase angle revealed a significant effect of time for both learning protocols (gain-up: $F(2,28)=4.0$, $P<0.05$; gain-down: $F(2,24)=6.65$, $P<0.005$). During gain-down learning there was also a significant interaction between frequency and time ($F(4,48)=3.81$, $P<0.01$). Figures 5-6C and D show the change in the phase angle measured during the gain-up and gain-down learning periods. During gain-up learning there was a significant increase in phase lag at 0.5 Hz in the PBS group and at 8 Hz in the DHPG group ($P<0.05$, paired t-tests). During gain-down learning, there was a significant increase in the phase lead of the VOR at 2 and 8 Hz, for both the PBS and YM 298198 groups ($P<0.05$, paired t-tests). These results are consistent with gain-up learning causing a small phase lag, while gain-down learning caused a small phase lead. There was no effect of frequency or drug group on the phase angle during gain-up or gain-down learning, indicating that the mGluR1 drugs did not cause any significant changes in the VOR phase.

5.4 Discussion

5.4.1 The flocculus is the site of motor learning

We found that an mGluR1 antagonist injected directly into the cerebellar flocculi can preferentially inhibit and reverse gain-up learning. These results directly confirm earlier studies establishing the cerebellar flocculus as the initial site of motor learning (Ito, 1972; McElligott et al., 1998; Nagao and Kitazawa, 2003; Kassardjian et al., 2005). Here, we further support the idea that learned gain increases and decreases are the result of separate mechanisms, most likely LTD and LTP respectively (Boyden and Raymond, 2003).

5.4.2 mGluR1 receptors are required for gain-up learning

The requirement of mGluR1 for certain types of motor learning has been demonstrated using knock-out mice. Mice lacking mGluR1 have been shown to have impaired eye-blink (Aiba et al., 1994b) and optokinetic learning (Shutoh et al., 2002). Here we show that mGluR1 receptors are also required for motor learning in the VOR.

In the VOR, the gain-up learning protocol results in the perception of the visual world moving faster during a head movement. This results in the near coincidental arrival of vestibular and retinal error signals (climbing fibres) at the Purkinje cell, with a short delay (Raymond and Lisberger, 1998). The brief delay between the climbing fibre and parallel fibre arrival is believed to be necessary to maximize the level of calcium within the Purkinje cell (Wang et al., 2000). mGluR1 is present at both climbing fibre and parallel fibre synapses (Nusser et al., 1994), and could be activated by both types of input. The activation of mGluR1 and its signalling pathway leads to the release of calcium from the internal stores, and has been suggested to be important for the coincidence detection of parallel fibres and climbing fibres onto Purkinje cells (Wang et al., 2000; Kano et al., 2008). Thus, the near coincidence of the vestibular and retinal slip signals onto Purkinje cells during gain-up learning results in PF-LTD, and is dependent on mGluR1.

5.4.3 Gain-up learning may require higher calcium levels

The blockade or inactivation of mGluR1 has been shown to impair LTD (Aiba et al., 1994b; Shigemoto et al., 1994; Ichise et al., 2000). Activation of the mGluR1 receptors *in vitro* is also thought to cause a large influx of calcium through the plasma membrane (Tempia et al., 2001). It has been suggested that the induction of LTD may have a higher calcium threshold than LTP (Coesmans et al., 2004). In our study, by blocking mGluR1 with the antagonist YM 298198, we may have limited the calcium, and prevented the calcium increase needed for LTD, thus selectively blocking gain-up learning. However, additional studies are needed to show that YM 298198 can effectively block LTD.

Unlike PF-LTD, LTP is thought to have a lower calcium threshold than LTD (Coesmans et al., 2004). As a result, LTP can be induced by the stimulation of parallel fibres alone (Salin et al., 1996; Lev-Ram et al., 2002). It is thought that PF-LTP does not require mGluR1 activation (Belmeguenai et al., 2008). In our study, we found that neither the mGluR1 antagonist nor agonist had any appreciable effect during gain-down learning. This is consistent with the idea of PF-LTP being the mechanism of gain-down learning (Boyden and Raymond, 2003).

5.4.4 Requirements of bidirectional learning

Here, we show that in the presence of an mGluR1 antagonist, learning was inverted during the gain-up protocol. This suggests that by blocking mGluR1, we prevented gain-up learning, and caused gain-down learning instead. Similar conclusions were drawn in slice experiments, where it was found that after blocking mGluR1 the LTD induction protocol results in potentiation (Hartell, 1994). Indeed "inversions" of plasticity were shown after blocking other mechanisms of LTD downstream to mGluR1 (Sakurai, 1990; Belmeguenai and Hansel, 2005; Hansel et al., 2006; van Woerden et al., 2009). A possible explanation for these inversions of plasticity comes from the idea of a "calcium switch", involving two thresholds of calcium (see Fig 1-4). LTP has a lower calcium threshold, while LTD has a higher threshold (Coesmans et al., 2004; Jörntell and Hansel, 2006). If the LTD signalling cascade is blocked, the calcium levels required to express LTD might not be reached, and as a result LTP would be expressed during the LTD induction paradigm. Blocking mGluR1 with YM 298198 could have interfered with the release of intracellular calcium, preventing LTD but not LTP. Accordingly, learning in the VOR was inverted during the gain-up protocol.

By a similar argument, we suggest that the augmented gain-up learning seen in the presence of DHPG could have been due to higher than normal calcium levels. (S)-DHPG is a group I mGluR agonist, which affects both mGluR1 and mGluR5 receptors. mGluR5 is expressed in cerebellar Golgi cells, but not in Purkinje cells (Neki et al., 1996), whereas mGluR1 is highly expressed in cerebellar Purkinje cells (Shigemoto et al., 1992; Hampson et al., 1994). Therefore, we suggest that the augmented learning seen in our study was most likely the result of mGluR1. However,

we cannot be certain if the DHPG affected mGluR5 receptors as well, and what effects mGluR5 receptors would have on VOR gain learning.

5.4.5 The frequency selectivity of learning

In our study, we showed that after both gain-up and gain-down learning, the amount learned was greatest when measured at the lowest frequency (0.5 Hz) than the highest frequency (8 Hz). This is consistent with the observations from previous studies (Raymond and Lisberger, 1996; Broussard et al., 1999a; Kimpo et al., 2005; Chapter 4). Interestingly, in the presence of YM 298198 gain-up learning was inverted, along with the frequency dependence normally seen during learning. The reasons for this frequency selectivity are not well understood. However, it was previously suggested that frequencies are transmitted by frequency channels within the cerebellar flocculus (Lisberger et al., 1983). This idea is supported by the finding of spatial microzones within the cerebellar cortex (Dean et al., 2010). We propose that frequency channels might have a physical basis within the cerebellar flocculus (Broussard et al., 2011).

We further propose that the area representing the frequency of 0.5 Hz might be greater than the area representing the 8 Hz frequency. Furthermore, we assume that both LTP and LTD might be happening at the same time, but at different synapses. An injection of a drug within the cerebellar cortex would create a concentration gradient, with a higher drug concentration closer to the injection site and smaller concentration further away from the injection site. Assuming that the YM 298198 interferes with the calcium increase needed for LTD, we suggest that the inverted frequency dependence could be explained by the concentration gradient of the mGluR1 antagonist. After the YM 298198 injection, PF-PC synapses closer to the injection site would be more effected by the antagonist resulting in LTP (or no plasticity if no threshold is met), while LTD would predominate at synapses further away from the injection, where the drug would have less of an effect. We predict that 8 Hz would be most affected by the drug as most of its synapses would undergo LTP. Whereas, the larger area of the 0.5 Hz synapses would mean a more equal balance of LTP and LTD, leading to a smaller gain decrease (Broussard et al., 2011). However, this explanation is speculative, and further investigation is required.

5.4.6 Conclusions

We conclude that VOR motor learning requires cerebellar mGluR1 receptors to be bidirectional. In the absence of mGluR1, gain-up learning is inverted, and only gain decreases are possible. We suggest that this may be a consequence of calcium thresholds required for PF- LTD and LTP.

Chapter 6

6 $GABA_B$ receptors are required for VOR motor learning

6.1 Introduction

The type 1 metabotropic glutamate receptor (mGluR1) has been shown to be required for PF-LTD (see: Kano et al., 2008). In the preceding chapter we have shown that an mGluR1 antagonist, injected into the cerebellar flocculi, causes a learned gain decrease during the gain-up learning protocol, while an mGluR1 agonist enhances gain-up learning (Chapter 5; Titley et al., 2010). Another G-protein coupled receptor, the B-type γ-aminobutyric acid ($GABA_B$) receptor, has also been shown to facilitate PF-LTD (Kamikubo et al., 2007). Both mGluR1 and $GABA_B$ receptors are present on the dendrites of the Purkinje cells (Luján et al., 1997; Kulik et al., 2002; Luján and Shigemoto, 2006; Rives et al., 2009), and have been shown to be present together on at least some of the same dendritic spines (Kamikubo et al., 2007; Rives et al., 2009). It is thought that $GABA_B$ receptors can interact with mGluR1 receptors either directly through receptor-receptor interactions (Tabata et al., 2004), or indirectly via phospholipase C and intracellular calcium release (Kamikubo et al., 2007; Rives et al., 2009).

We asked if $GABA_B$ receptors are required for VOR motor learning, and if they interact with mGluR1 receptors. We found that like mGluR1, $GABA_B$ receptor activity is necessary for learned gain increases, but not decreases. Furthermore, we found that the co-activation of the mGluR1 and $GABA_B$ receptors is required for gain-up learning. Finally, we present a simple model that suggests that the two receptors control signalling on different spines and that learning is determined by a summation of these spines. Preliminary results of this study have been published (Broussard et al., 2011).

6.2 Methods

Data from 5 alert male cats (S, V, B, C and E) aged 14-27 months were included in this study. Methods for head holder, eye coil and injection cylinder placement, as well as methods for recording eye movements were described earlier (Chapter 2).

In each cat, drugs were bilaterally injected into the cerebellar flocculi. Injections were placed using maps based on microstimulation. The positioning and penetration of the needle and electrodes were described earlier (Chapter 5.2.1). Briefly, drugs were injected using a Hamilton syringe attached to a 24 gauge needle that was slowly lowered into position. After each injection the needle was left in place for 3 minutes to allow diffusion of the drug, before the needle was slowly withdrawn.

6.2.1 Experimental methods

Experimental methods are similar to those described in the previous chapter (Chapter 5.2.2). Briefly, gain-up and gain-down learning were induced using X2 and X0.25 telescopes, respectively. Telescopes were worn during the "learning period" (60 minutes of SOS rotation in the light). After each learning period (defined as a "trial"), the cat was rotated for an additional 30 minutes in the light without telescopes, to return the gain to the pre-learning value. Each trial was separated by at least 6 days. Table 6-1 illustrates the cats that were used in each learning protocol.

Drug	Cat	Gain-up protocol				Gain-down protocol			
		Trials	0.5 Hz	2 Hz	8 Hz	Trials	0.5 Hz	2 Hz	8 Hz
PBS	S	1	32	19	10	1	-23	-25	-26
	V	2	22	15	13	2	-33	-30	-9.2
	B	1	25	15	6.5	1	-31	-21	-20
	C	1	23	11	7.2	1	-26	-16	-17
	E	1	19	7.1	1.1	0	-	-	-
YM 298198	S	1	-17	-16	-18	1	-28	-20	-17
	V	2	-19	-7.2	-36	2	-26	-20	-2.7
	B	2	-3.4	-10	-13	1	-25	-22	-17
	C	1	-5.1	-14	-13	1	-24	-21	-5
CGP 52432	B	1	-9.6	-21	-23	1	-30	-24	-19
	C	1	-15	-20	-19	1	-21	-21	-27
	E	2	-23	-15	-16	2	-34	-21	-16
Baclofen	B	1	46	31	17	0	-	-	-
	C	1	29	39	23	1	-23	-16	-13
	E	2	34	24	19	3	-34	-24	-19
YM 298198 + Baclofen	C	1	-14	-14	-20	1	-17	-16	-24
	E	3	-19	-17	-17	3	-24	-25	-27
YM 298198 + CGP 52432	C	1	-18	-14	-14	0	-	-	-
	E	1	-19	-9	-12	2	-29	-25	-15

Table 6-1. Mean change in gain for each protocol and cat in Chapter 6. Percent gain changes in VOR gain during the learning period for each subject in the presence of the different drugs. Results were consistent across subjects. The mean change in gain is given separately for each test frequency, for both the gain-up and gain-down learning protocols. The number of trials per subject is given for each case. Negative numbers indicate gain decreases.

Results from two experimental protocols are presented. In the first protocol, we sought to determine what effects a $GABA_B$ receptor antagonist and agonist would have on learning. Before each trial, a 1 μl injection of either the $GABA_B$ receptor antagonist CGP 52432 (10 μM) or the agonist (R)-baclofen (2.5 mM) was made bilaterally into each flocculus. All drugs were dissolved in PBS. In a few preliminary experiments, cat V received a lower concentration, 1 μM, of CGP 52432. However, these injections were considered less effective and the data are not presented.

In the second protocol, we asked how the $GABA_B$ receptor interacts with mGluR1. To test this relationship, before each trial we injected the mGluR1 antagonist YM 298198 in combination with either CGP 52432 or (R)-baclofen. The injections that contained two drugs were 2 µl in volume, and the concentrations were halved, i.e., 25 µM YM 298198 combined with either 5 µM CGP 52432 or with 1.25 mM (R)-baclofen.

During each trial, the gain of the VOR was measured at 0.5, 2 and 8 Hz before and after the injection, and every 30 minutes during the learning period. The learned change in gain was calculated as a percentage of the pre-learning gain.

To test what effect each drug or drug combination might have on the normal VOR function, we performed control trials. In the control trials, the cats did not wear telescopes during the learning period. The gain of the VOR was measured at 0.2, 0.5, 2 and 8 Hz, and the ability of the cat to cancel the VOR was measured at 0.2 Hz. Cancellation of the VOR was calculated as described earlier (Chapter 5.2.2).

6.2.2 Data analysis

The effectiveness of the CGP 52432 or baclofen alone was compared to the vehicle, PBS (presented earlier in Chapter 5). In addition, the combination of these drugs with the mGluR1 antagonist was compared to injections of the YM 298198 alone, the results of which were presented in Chapter 5.

A mixed-model repeated measures ANOVA was performed to look for effects of time (over the learning period), test frequency, and drug group (groups of trials). Only the $GABA_B$ receptor drugs (CGP 52432 and baclofen) and the PBS group were included in the ANOVA. The effects of the YM 298198 were reported previously (see Chapter 5), and were not included in the ANOVA. A Bonferroni correction was used for multiple comparisons, and the Greenhouse-

Geisser sphericity correction was used when variances were unequal. A repeated measures ANOVA was also used to compare the percent cancellation of the VOR among drug groups.

Because we found significant interactions between the factors, we also used post-hoc paired t-tests to compare the pre- and post-learning gain values within each drug group, and unpaired t-tests to compare the post-learning gains and learned changes between groups at each frequency. Finally, we compared the amount learned at different frequencies using paired t-tests. The statistical outcomes are summarized in the text, and the results of the 1-tailed t-tests are given in Table 6-2. The YM 298198 and CGP 52432 combination group, as well as both combination groups during control trials, were not included in any statistical analysis because of the low sample size.

		CGP 52432			Baclofen			YM 298198 + Baclofen		
	Comparison Sample	**0.5 Hz**	**2 Hz**	**8 Hz**	**0.5 Hz**	**2 Hz**	**8 Hz**	**0.5 Hz**	**2 Hz**	**8 Hz**
Before Learning										
Drug effect	PBS	0.27	0.20	0.37	0.070	0.27	0.17	0.077	0.061	0.37
Gain-up learning										
Learned Changes	Pre-learning gain	**0.0057**	**0.0020**	**0.0022**	**0.0017**	**0.0018**	**0.0012**	**0.0036**	**0.0032**	**0.0007**
Drug effect on final gain	PBS	**2.5E-05**	**5.5E-05**	**0.0010**	0.11	**0.0127**	0.0612	**6.33E-06**	**4.77E-05**	**0.0005**
Drug effect on % change	PBS	**1.0E-06**	**1.3E-06**	**9.9E-06**	**0.011**	**0.0010**	**0.0017**	**3.6E-07**	**1.9E-06**	**4.3E-06**
Drug effect on final gain	YM 298198	-	-	-	-	-	-	**0.044**	**0.0038**	0.18
Drug effect on % change	YM 298198	-	-	-	-	-	-	0.093	**0.046**	0.31
Test frequency	0.5 Hz	-	0.49	0.44	-	0.18	**0.035**	-	0.077	0.49
Test frequency	2 Hz	0.49	-	0.41	0.18	-	**0.024**	0.077	-	0.18
Test frequency	8 Hz	0.44	0.41	-	**0.03**	**0.02**	-	0.49	0.18	-
Gain-down learning										
Learned Changes	Pre-learning gain	**0.0017**	**0.0007**	**0.0023**	**0.0017**	**0.0018**	**0.0012**	**0.009**	**0.015**	**0.011**
Drug effect on final gain	PBS	0.48	0.11	0.12	**0.047**	0.14	**0.03**	0.22	0.063	**0.032**
Drug effect on % change	PBS	0.48	0.25	0.25	0.33	0.28	0.40	0.08	0.34	**0.032**
Drug effect on final gain	YM 298198	-	-	-	-	-	-	**0.02**	0.45	**0.021**
Drug effect on % change	YM 298198	-	-	-	-	-	-	0.14	0.36	**0.0065**
Test frequency	0.5 Hz	-	**0.046**	0.083	-	**0.0030**	**0.0045**	-	0.49	0.12
Test frequency	2 Hz	**0.046**	-	0.26	**0.0030**	-	**0.017**	0.49	-	0.22
Test frequency	8 Hz	0.083	0.26	-	**0.0045**	**0.017**	-	0.12	0.22	-

Table 6-2. Results of post-hoc t-tests with GABAB drugs. P values of 1-tailed Student's t-tests comparing normalized values of VOR gain. The comparisons showed significant effects of all drugs on gain-up learning at all test frequencies, but no effects on the VOR before learning. Significance of PBS and YM 298198 injections are described earlier (see: Table 5-2). The first column indicates what factor was tested and the second column indicates what sample was used in the comparison. Pre-learning: The gain of the VOR after drug injection but before learning is compared with the gain after PBS injections, to verify that there were no pre-learning effects of any of the drugs.

Learned changes: Pre- and post-learning gains are compared to verify that learning caused a significant change. Drug effect on final gain: Post-learning gains are compared among the drug groups. Drug effect on % change: The learned percentage change in gain is compared among drug groups. Test frequency: The post-learning gains are compared among frequencies. The boldface type highlights significance at $P<0.05$.

6.3 Results

6.3.1 Histology and controls

Locations of the injection sites in each cat were confirmed using cresyl violet staining, and were shown in the previous chapter to be located within the cerebellar flocculi (see Fig 5-1). The flocculus is known to be involved in VOR cancellation (Rambold et al., 2002; Kassardjian et al., 2005). We confirmed that the $GABA_B$ receptor agonist (baclofen) and antagonist (CGP 52432) either alone, or in combination with the mGluR1 antagonist (YM 298198) had no effect on the cat's VOR or its ability to cancel the VOR while looking at a full field visual pattern. During control trials, we measured the gain and cancellation of the VOR before and after the injection, and up to 60 minutes after the injection. Figure 6-1A shows that the drugs did not change the gain of the VOR. A repeated measures ANOVA showed no significant effect of drug group ($F(2,11)=0.42$, $P=0.67$), time ($F(2,22)=1.17$, $P=0.33$) or frequency ($F(2,22)=1.02$, $P=0.38$) on the VOR gain. We found no significant difference in the percent gain change during the control trials between any of the drug groups (Fig 6-1B; $P=0.25$, single factor ANOVA). In addition, during the learning experiments, neither the $GABA_B$ receptor agonist nor the antagonist had any effect on the VOR gain before learning ($P>0.2$, single factor ANOVA).

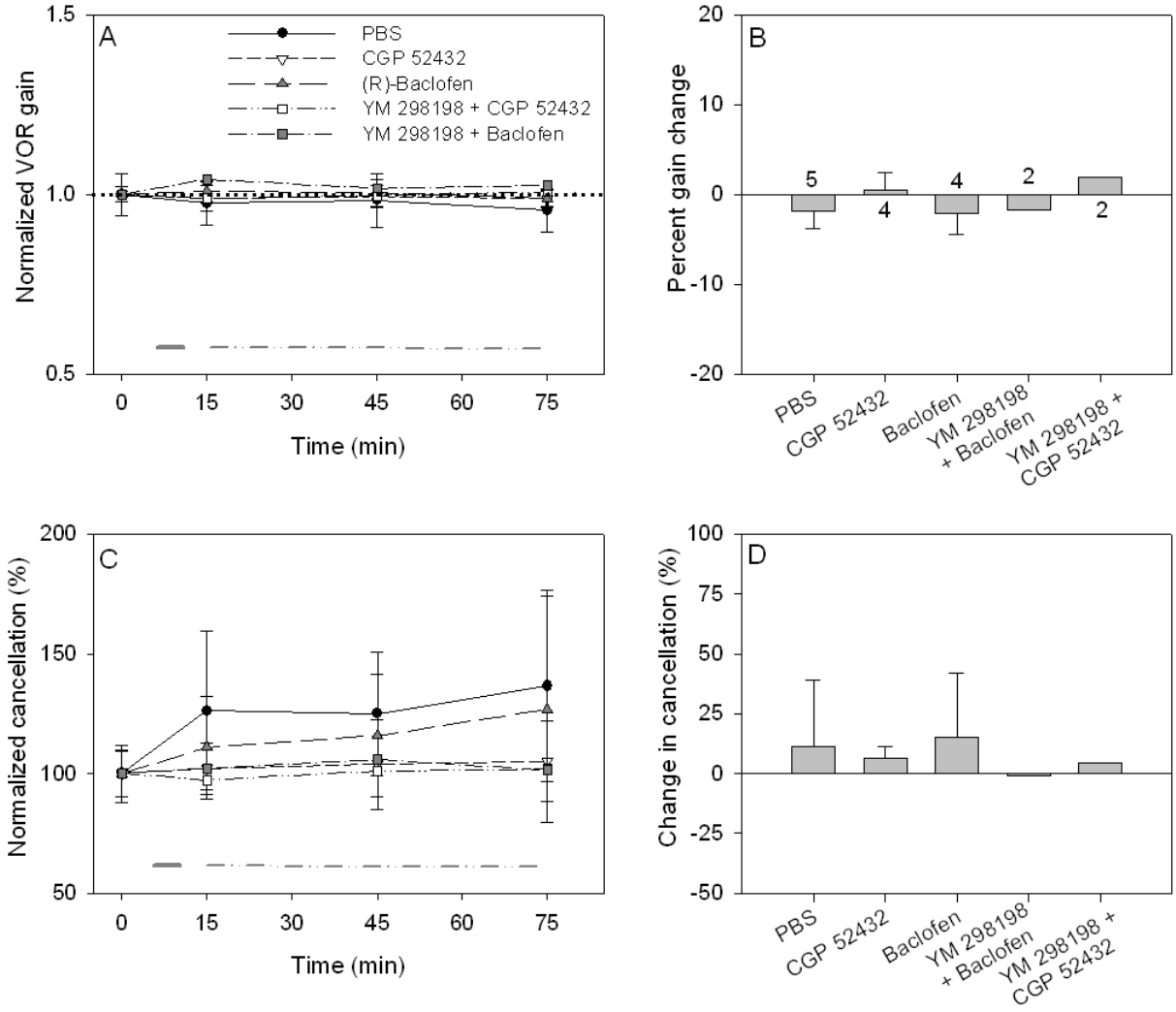

Figure 6-1. $GABA_B$ had no effect on the VOR or VOR cancellation without learning. In control protocols that did not involve learning, neither the VOR gain nor VOR cancellation was affected by injections. A: The time course of VOR gain at 2 Hz during the control protocol. The grey solid line indicates the time of injection. Following the injection, cats were rotated for 60 minutes without telescopes (grey dashed-dotted line). The normalized VOR gain is plotted following injections of PBS alone (filled circles), CGP 52432, (open inverted triangles), baclofen (grey triangles), YM 298198 and CGP 52432 together (open squares), and YM 298198 and baclofen together (grey squares). B: The percentage change in gain during the control protocol. Numbers above bars indicate sample sizes. C: VOR cancellation, monitored at 0.2 Hz during the same trials shown in A, became slightly more effective within some trials. This effect was not significant. Cancellation was not affected by any of the substances. D: The percentage change in cancellation. Error bars indicate standard deviation. Sample sizes as in B.

Figure 6-1C shows that the drugs had no effect on the cancellation of the VOR. The change in cancellation was normalized to the pre-injection value which was set at a value of 100%. A

repeated measures ANOVA showed no effect of drug group ($F(2,10)=0.97$, $P=0.41$) or time ($F(1.3, 12.9)=2.18$, $P=0.16$) on the percent cancellation. There was no significant difference between any of the drug groups in the change in percent cancellation of the VOR during the learning period without telescopes (Fig 6-1D; $P=0.86$, single factor ANOVA).

6.3.2 CGP 52432 reversed gain-up learning

During the gain-up learning protocol, the $GABA_B$ receptor antagonist CGP 52432 (1 µl, 10 µM), reversed the direction of learning. Figure 6-2A shows examples of the VOR at 2 Hz before and after the gain-up protocol in the presence of CGP 52432 or the PBS vehicle. The VOR maintained a linear relationship between the head and eye velocity in the presence of CGP 52432 throughout the experiment at all three frequencies (Fig 6-2 C-E). This suggests that during learning, the CGP 52432 only affected the gain of the VOR.

During gain-up learning, the gain of the VOR increased after a PBS injection; however in the presence of CGP 52432 the VOR gain decreased. Figure 6-2 (F-H) shows the time course of the gain-up protocol in the presence of CGP 52432 and the PBS vehicle. A repeated measures ANOVA during the gain-up learning period showed a significant effect of drug group ($F(2,11)=20.5$, $P<0.001$), and time ($F(2,22)=41.7$, $P<0.001$) on normalized VOR gain. Significant interactions were found between time and drug ($F(4,22)=98.3$, $P<0.001$), and between time, drug and frequency ($F(5.2, 28.6)=2.16$, $P=0.05$).

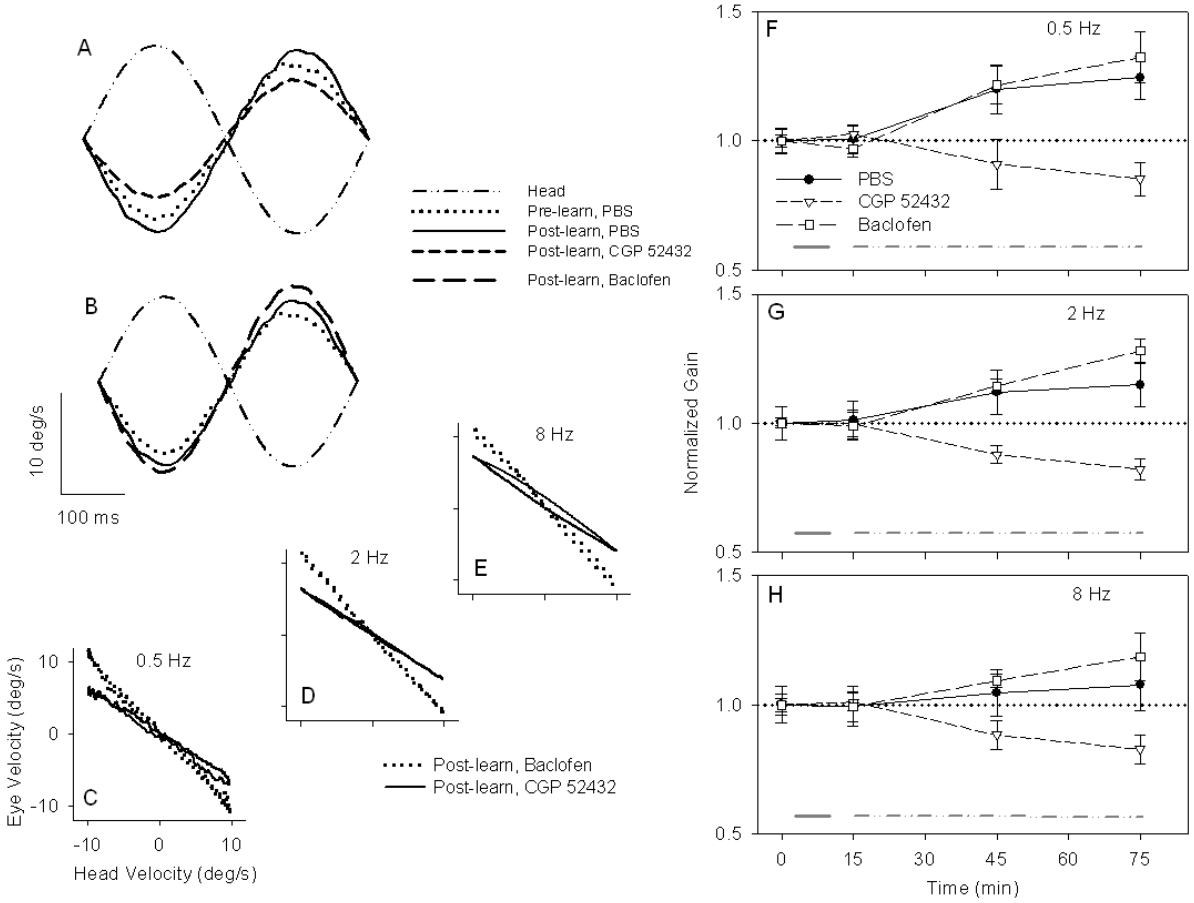

Figure 6-2. Gain-up learning was inverted with $GABA_B$ antagonist and augmented with agonist. During the gain-up learning protocol, the $GABA_B$ receptor antagonist CGP 52432 inverted gain-up learning, while the agonist, (R)-baclofen, augmented gain-up learning. A: Typical examples of angular head and eye velocity at 2 Hz rotation. Averages of 10 or more cycles are shown. Dashed-dotted line: Head (turntable) velocity. Dotted line: Eye velocity before learning. Solid line: Eye velocity after 60 minutes of gain-up learning. Dashed line: In the presence of CGP 52432, the gain-up protocol resulted in a reduction in eye velocity. B: Typical examples of head and eye velocity in the presence of baclofen. PBS traces are the same as in A. Dashed line: In the presence of baclofen, the gain-up protocol resulted in a greater increase in eye velocity. C-E: Eye velocity as a function of head velocity, at all 3 test frequencies, after gain-up learning in the presence of baclofen (dotted lines) or CGP 52432 (solid lines). Examples in A-E are from cat E. F-H: Time courses of the VOR gain at the different test frequencies. The injection period is indicated by heavy grey lines. The learning period is indicated by the dashed-dotted grey lines. Filled circles: PBS injections (re-plotted from Chapter 5). Open inverted triangles: CGP 52432 injections. Open squares: Baclofen injections. All gain values are normalized. Error bases are S.D. in this and all figures.

We confirmed the results of the ANOVA with post-hoc comparisons. After injection of CGP 52432, the gain after the gain-up learning period was significantly lower than the pre-learning value at all 3 frequencies ($P<0.01$, paired t-tests). Both the post-learning values and the amount learned were significantly different from the vehicle PBS ($P<0.001$, unpaired t-tests, at all

frequencies). Table 6-2 shows the results of the post-hoc t-tests, and Table 6-1 shows that these results were consistent across subjects.

6.3.3 (R)-baclofen augmented gain-up learning

We previously found that an mGluR1 antagonist prevented gain-up learning, while the mGluR1 agonist augmented gain-up learning (Chapter 5). Since blocking the $GABA_B$ receptor also prevented gain-up learning, we asked whether the tonic activation of the $GABA_B$ receptor would enhance gain-up learning. Indeed, in the presence of the $GABA_B$ receptor agonist, (R)-baclofen (1 µl, 2.5 mM), gain-up learning was significantly enhanced at one of the test frequencies. Figure 6-2 B shows an example of the VOR at 2 Hz before and after learning in the presence of baclofen. Figure 6-2 C-E shows that the VOR remained linear in the presence of baclofen, showing that the $GABA_B$ agonist did not affect the normal linear relationship between head and eye velocity. The time courses for gain-up learning following an injection (R)-baclofen (open squares) is shown in Figure 6-2 F-H at all 3 frequencies. After gain-up learning, the gain of the VOR was significantly increased in the presence of baclofen at all 3 frequencies ($P<0.002$, paired t-tests). When comparing the post-learning gain values, baclofen was found to be significantly greater than PBS at the 2 Hz frequency ($P<0.02$, unpaired t-test), but the difference did not reach significance at the 0.5 and 8 Hz frequencies ($0.11<P<0.062$, unpaired t-tests). The amount of gain-up learning in the presence of baclofen was also found to be significantly greater than the amount of learning in the presence of vehicle at all 3 frequencies ($P<0.02$, unpaired t-tests).

6.3.4 mGluR1 alters the $GABA_B$ receptor contribution to gain-up learning

It was thought that if $GABA_B$ and mGluR1 receptors interact via a common pathway, a co-injection of YM 298198 with baclofen might be expected to prevent the effect that baclofen had on gain-up learning. However, our results do not completely support this prediction. We found that the effect of baclofen was not prevented, but was inverted (like learning itself) when YM 298198 was included in the injection. Figure 6-3 (A-C) summarizes the time courses during the gain-up learning experiments when YM 298198 was injected in combination with baclofen. During the gain-up protocol, after the injection of YM 298198 (25 µM) and baclofen (1.25 mM),

the gain of the VOR decreased significantly at all 3 frequencies ($P<0.004$, paired t-tests). The post-learning gain values and the percent change in gain after the YM 298198 and baclofen injection were significantly less than after an injection of PBS ($P<0.001$, unpaired t-tests, at all frequencies). The YM 298198 data, which was presented earlier (see Fig 5-3), are shown again in Figure 6-3 (A-C). We compared learning in the presence YM 298198 and baclofen together to the learning seen with YM 298198 alone, and observed an effect of baclofen, which was probably independent of the mGluR1 receptor. The decrease in the VOR gain seen with the combination of YM 298198 and baclofen was greater (indicating more learning) than the decrease seen with YM 298198 alone. With baclofen added, the post-learning gain was significantly lower than the post-learning gain with YM 298198 alone at 0.5 and 2 Hz ($P<0.05$, unpaired t-tests). At 8 Hz the difference was not significant ($P>0.18$, unpaired t-test). The percentage change in gain at 2 Hz was significantly greater with YM 298198 plus baclofen than it was with YM 298198 alone ($P<0.05$, unpaired t-test).

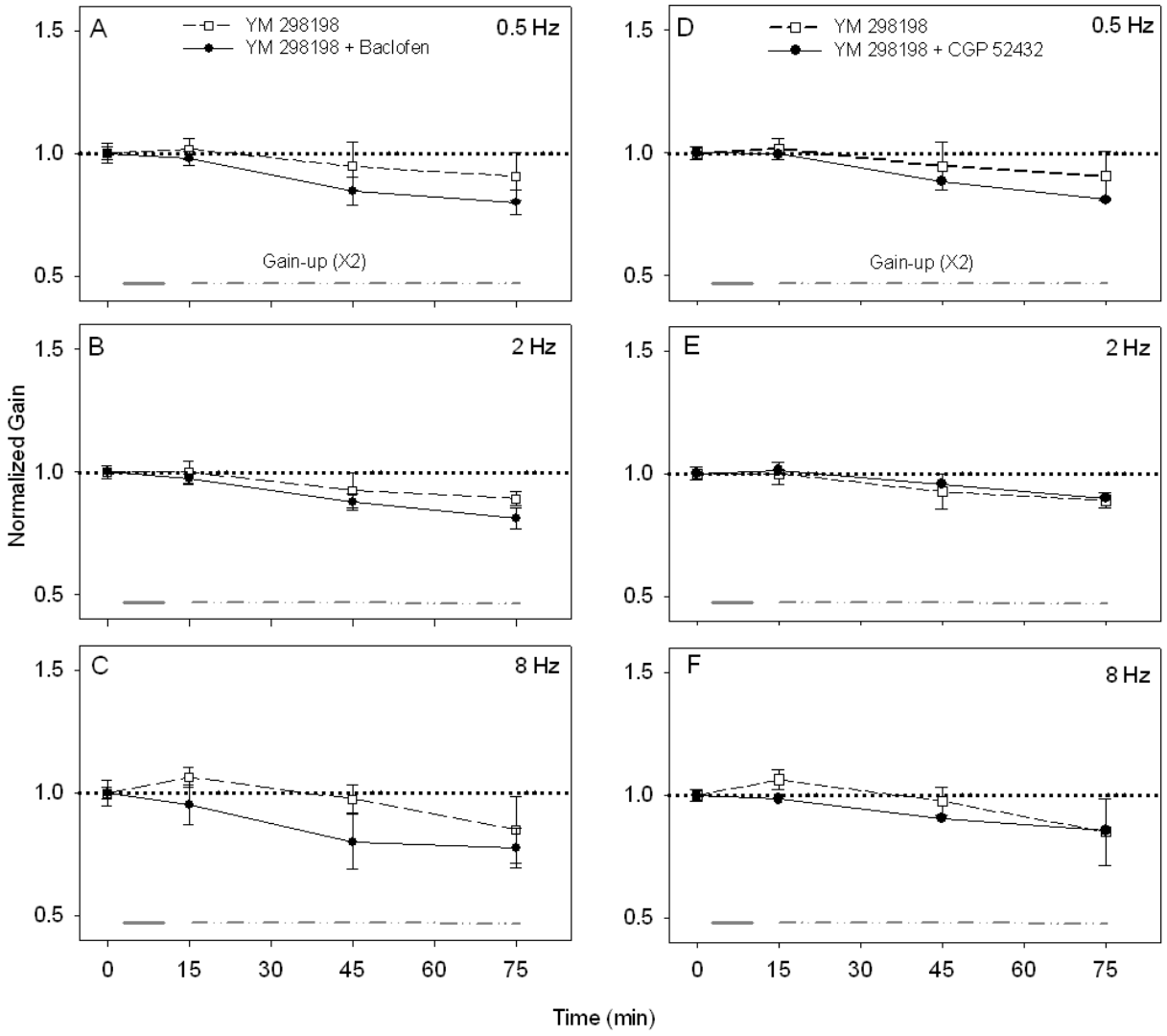

Figure 6-3. Blocking mGluR1 altered the effects of $GABA_B$ on gain-up learning. During gain-up learning, the effects of the $GABA_B$ receptor agonist and antagonist were altered when mGluR1 was blocked. A-C: Baclofen slightly augmented the inverted learning in the presence of YM 298198. Filled circles: YM 298198 injections (re-plotted from Chapter 5). Open squares: Combined injections of YM 298198 and baclofen. D-F: CGP 52432 had no effect in the presence of YM 298198. Open squares: Combined injections of YM 298198 and CGP 52432. Horizontal grey lines as in Figure 6-2.

If mGluR1 activation is necessary for the contribution of the $GABA_B$ receptor to gain-up learning, then blocking both receptors should not be different than blocking mGluR1 alone. We injected a combination of YM 298198 (25 μM) and CGP 52432 (5 μM) and tested the effects on gain-up learning in 2 trials. The gain of the VOR decreased during the gain-up learning protocol, after both antagonists were injected together (Fig 6-3 D-F). Consistent with this hypothesis, the

gain decrease during gain-up learning did not appear different from the decrease caused by YM 298198 alone. However, because the combined antagonists were only tested twice, these data were not included in the statistical analysis. It should also be noted that the effects of the CGP 52432 alone during gain-up learning were similar to the effects of the two antagonists together. These results may suggest that the activation of the $GABA_B$ receptor is necessary for the contribution of mGluR1 to gain-up learning. In other words, both receptors must be active concurrently for gain-up learning.

6.3.5 Gain-down learning was not affected

During gain-down learning, the gain of the VOR decreased significantly ($P<0.02$, paired t-tests) in all drug groups. The $GABA_B$ receptor antagonist had no apparent effect on gain-down learning. Figure 6-4 (A-C) shows the time courses of the gain-down learning protocol in the presence of CGP 52432 and PBS. CGP 52432 had no effect on gain-down learning. We have previously shown that YM 298198 had no effect during gain-down learning (Chapter 5, re-plotted in Fig 6-4 D-F). When combined, YM 298198 and CGP 52432 still had no effect during the gain-down learning protocol (Fig 6-4 D-F).

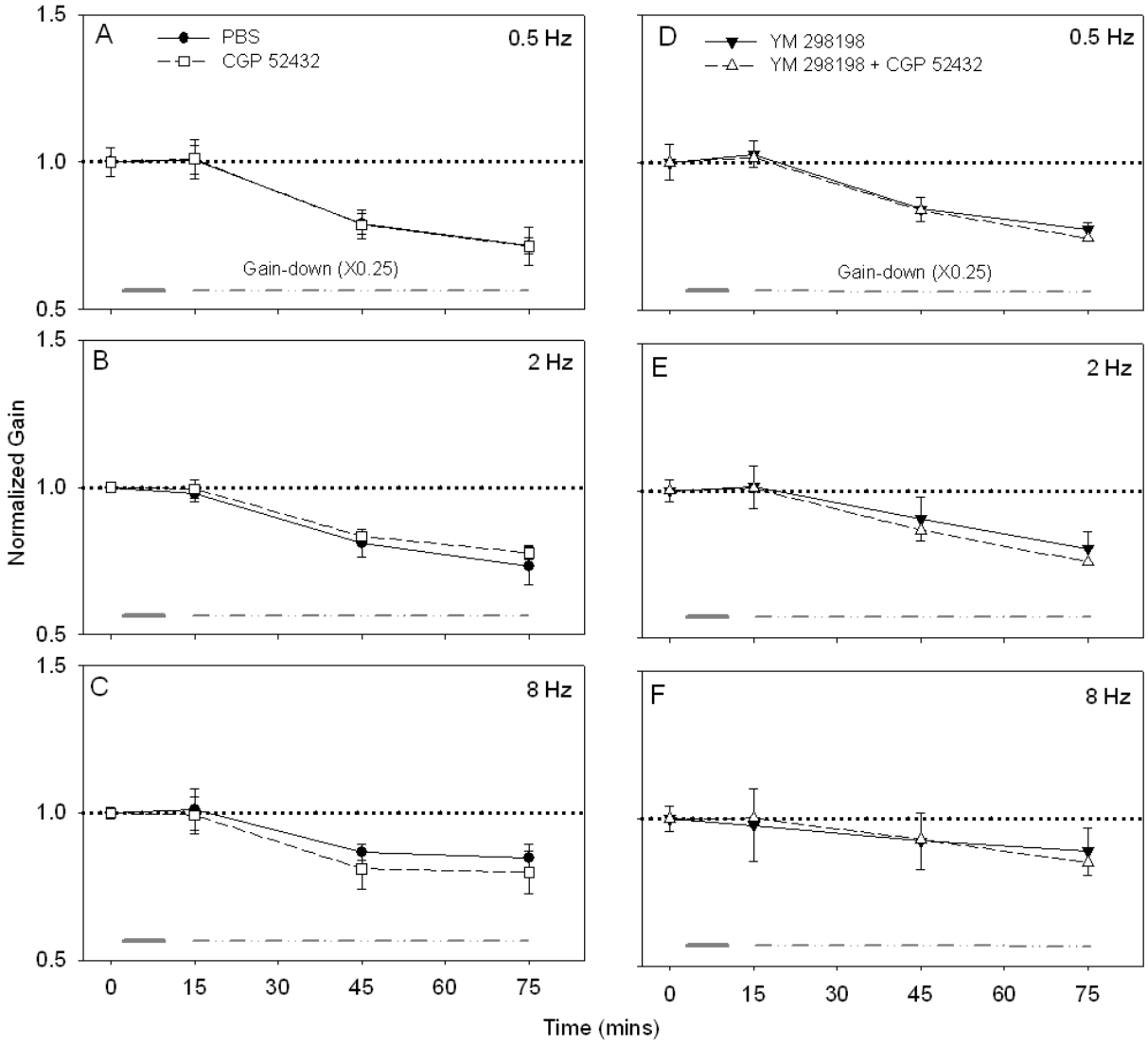

Figure 6-4. $GABA_B$ antagonist had little effect on gain-down learning. In the gain-down protocol, CGP 52432 had no effect, either alone or in combination with YM 298198. A-C: The time courses of gain-down learning, at the different test frequencies, following injection of PBS (filled circles) or CGP 52432 (open squares). D-F: Time courses at the different test frequencies following injection of either YM 298198 alone (filled inverted triangles) or in combination with CGP 52432 (open triangles).

Consistent with the hypothesis that the effects of the $GABA_B$ receptors are selective for gain increases, baclofen alone had little effect during gain-down learning when compared to PBS (Fig 6-5 A-C). However, the effects of the combination of YM 298198 with baclofen on gain-down learning were ambiguous (Fig 6-5 D-F). When comparing final gain values, the effect of baclofen with YM 298198 was significantly lower than YM 298198 alone at 0.5 and 8 Hz

(P<0.05, unpaired t-tests). The increase in the amount of gain-down learning was significant only at 8 Hz (P<0.01, unpaired t-test).

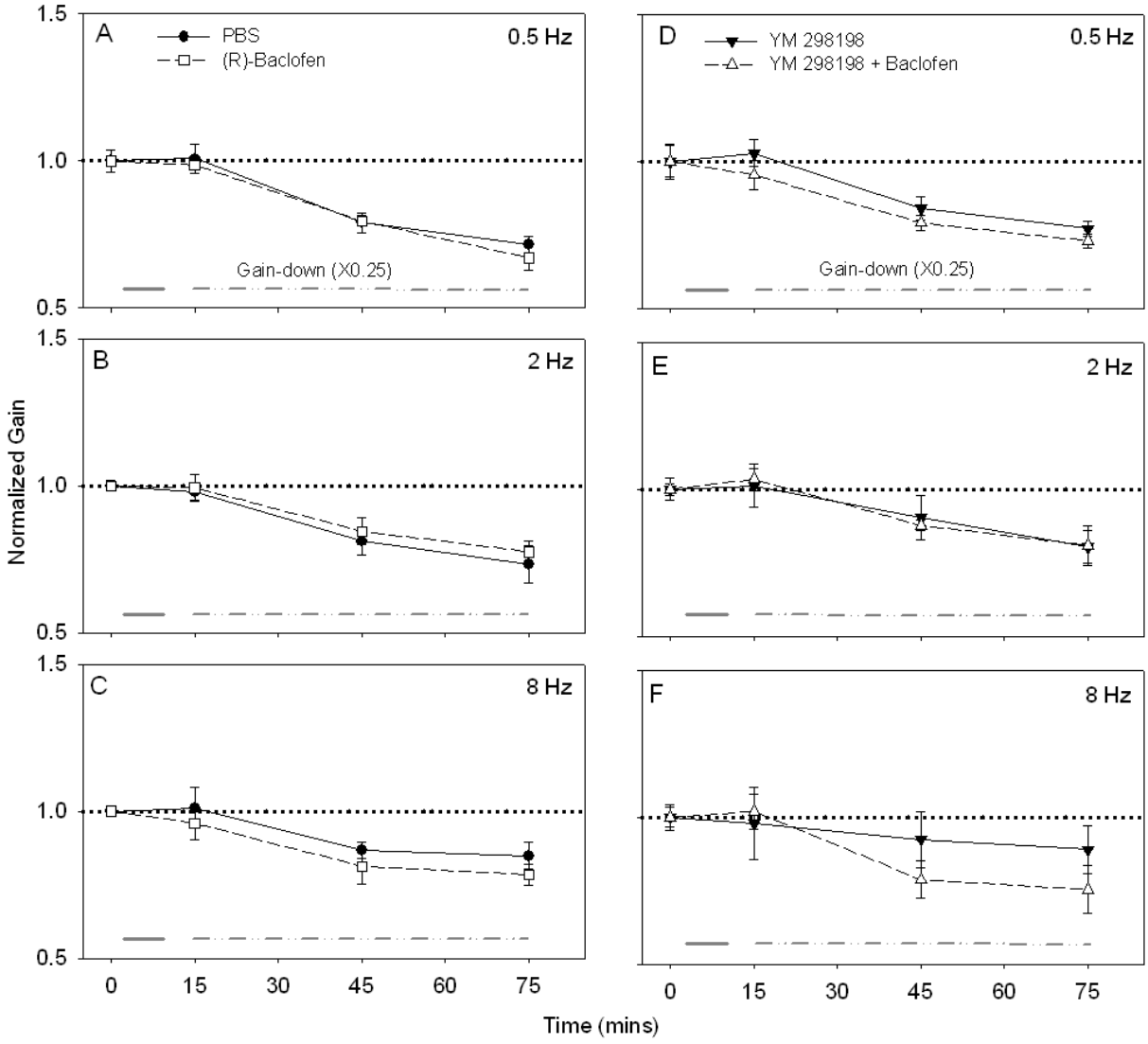

Figure 6-5. $GABA_B$ agonist had little effect on gain-down learning. In the gain-down protocol, baclofen enhanced learning at the 8 Hz frequency both alone, and in the presence of YM 298198. A-C: The time courses of gain-down learning, at the different test frequencies, following injection of PBS (filled circles) or baclofen (open squares). D-F: Time courses at the different test frequencies following injection of either YM 298198 alone (filled inverted triangles) or in combination with baclofen (open triangles).

A repeated measures ANOVA on the PBS, CGP 52432, and baclofen groups during gain-down learning showed no significant effect of drug group ($F(2,10)=0.58$, $P=0.56$). The ANOVA revealed a significant effect of time ($F(2,20)=324.7$, $P<0.001$), which reflected the learning that

occurred during this protocol. Overall, these results confirm that the effects of the $GABA_B$ receptor drugs are selective for gain-up learning, and had no consistent effect during gain-down learning.

6.3.6 Changes in frequency dependence

In the previous chapter, we found that the frequency selectivity of learning could be inverted when learning was reversed in the presence of the mGluR1 antagonist, YM 298198 (see Fig 5-6). Here we report a similar finding when learning is reversed with the $GABA_B$ antagonist or when the $GABA_B$ agonist or antagonist was injected in combination with the mGluR1 antagonist. The repeated measures ANOVAs for both the gain-up and gain-down learning protocols revealed a significant effect of test frequency ($F(2,22)=8.56$, $P<0.003$, for gain-up; $F(2,20)=5.18$, $P<0.02$, for gain-down). In both protocols there was also a significant interaction between frequency and time (gain-up: $F(2.6,28.6)=10.0$, $P<0.001$; gain-down: $F(4,40)=11.2$, $P<0.001$).

Learned changes in gain are normally greater at lower frequencies than higher frequencies. This is illustrated in Figure 6-6A, when learning was in the correct direction for the gain-up protocol (as it was for PBS and baclofen), the amount learned was greater at 0.5 Hz than it was at 8 Hz ($P<0.05$, paired t-tests). However, when the direction of learning was reversed by blocking the $GABA_B$ receptor and/or mGluR1 during the gain-up protocol, learning was no longer frequency-dependent (Fig 6-6A). In the presence of YM 298198, CGP 52432 or a combination of the drugs, after gain-up learning 0.5 Hz was no longer greater than 8 Hz ($P>0.05$, paired t-tests). These results were consistent across the individual subjects (Table 6-1).

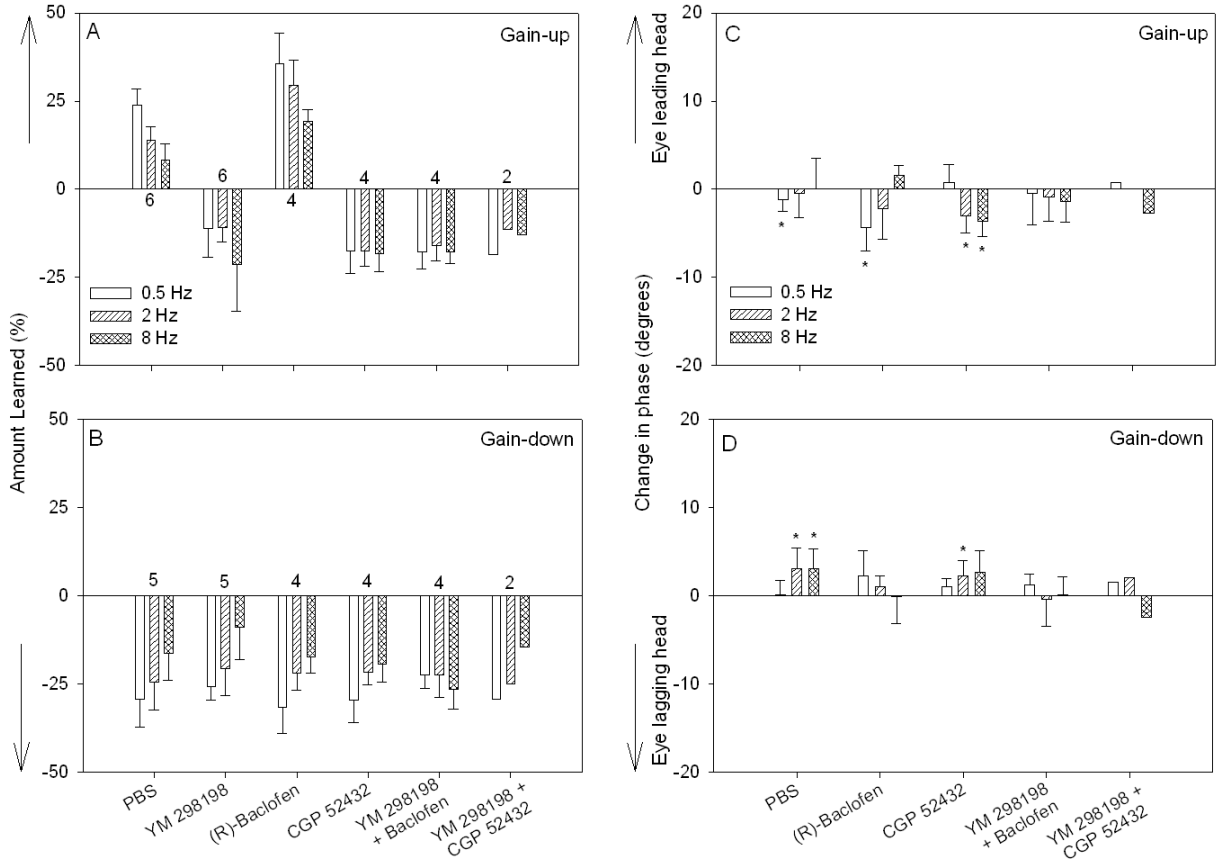

Figure 6-6. Summary of learned changes in VOR gain and phase with $GABA_B$ drugs. A: The gain change in the gain-up protocol as a percentage of the pre-injection gain. The groups of bars represent the total sample of measurements for each substance. Each test substance is indicated on the abscissa. The numbers on the plots are the sample sizes for each condition. Different fills indicate the different test frequencies. B: The gain change in the gain-down protocol. C: The phase change, in degrees, in the gain-up protocol. Lag and lead polarities are indicated along the ordinate. Data are from the same experiments as in A. D: The phase change in the gain-down protocol. Data are from the same experiments as in B. Asterisks (in C and D) indicate a significant change in phase during the learning period. $P<0.05$

We found that learning was always in the correct direction during the gain-down learning protocol. In most cases, after gain-down learning, the amount of learning decreased as the test frequency increased (Fig 6-6B). Neither CGP 52432 nor baclofen alone had any effect on the frequency-dependence. The amount of learning at 0.5 Hz was significantly greater than 8 Hz for the baclofen group ($p<0.005$, paired t-test). In the CGP 52432 group, the amount learned at 0.5 Hz was not significantly greater than the amount learned at 8 Hz (see Table 6-2), however, it was significantly greater than the amount learned at 2 Hz ($P<0.05$, paired t-test).

Even though learning was in the correct direction, when baclofen was injected with YM 298198, the frequency-dependent trend was abolished after gain-down learning (Fig 6-6B). There was no significant difference in the amount learned between any frequencies in this group (see Table 6-2). This observation was the same across both subjects tested in this drug group (Table 6-1).

6.3.7 $GABA_B$ receptors had little effect on VOR phase

Learning was also associated with small changes in the phase angle, or the temporal relationship between the head and eye velocities. These results are summarized in Figure 6-6 (C and D). We performed post-hoc paired t-tests to compare the pre- and post-learning values in phase. Significant values are shown in Figure 6-6 (C and D) as asterisks. In general, the eye velocity tended to lag behind the head (phase lag) following gain-up learning (Fig 6-6C), but shifted to a phase lead following gain-down learning (Fig 6-6D). The effects of YM 298198 on the VOR phase were excluded from this analysis as they have been reported earlier (Chapter 5). A repeated measures ANOVA of the phase revealed a significant effect of time during both learning protocols (gain-up: $F(2,22)=20.5$, $P<0.001$; gain-down: $F(2,20)=7.53$, $P<0.005$). There was a significant interaction between time and drug group with regard to phase in both gain-up ($F(4,22)=3.62$, $P<0.03$) and gain-down learning ($F(4,20)=3.61$, $P<0.03$). There was no effect of drug group or frequency on the phase angle during gain-up or gain-down learning, indicating that the $GABA_B$ receptor drugs did not cause any significant changes in the VOR phase.

6.3.8 Modeling learning using spine populations

We have recently shown using immunohistochemistry in both mice (Broussard et al., 2011) and cats (see Fig 1-6) that mGluR1 and $GABA_B$ receptors are not co-localized. These results suggest that the majority of the mGluR1 and $GABA_B$ receptors are not located on the same dendritic spines. It is thought that the dendritic spines on the Purkinje cells are capable of LTP or LTD depending on the calcium concentration within each spine (Coesmans et al., 2004). We suggest that at a single Purkinje cell some spines may undergo LTD, while other spines may experience

LTP, or no change at all. It is thought that the combined effect of all spines determines the direction of plasticity of the Purkinje cell. In turn, the population of Purkinje cells are thought to determine the overall direction of learning (see: Broussard et al., 2011).

To test the validity of these ideas, we simulated our present results, using Microsoft Excel, with a simple basic calculation based on a population of spines. In our model, an individual spine could be in one of three possible states: stable (s), potentiated (p), or depressed (d). We assumed a small population of 100 spines ($n_s + n_p + n_d = 100$), and each spine was assigned a weight (w) of 0.01. The equal weights assumed in this model are only a first approximation and are unlikely to be accurate. The predicted change in the VOR gain during learning was calculated as follows:

$$C_P = w^* [(n_s * L_s) + (n_p * L_p) + (n_d * L_d)]$$

Where C_P is the predicted change in gain, n is the number of spines in each state, and L is a number representing the direction of learning for each group of spines. L was assigned one of three values depending on the effect each state (s, p, or d) was thought to have on learning: $L_s = 0$, $L_p = -1$, and $L_d = 1$. Note that the negative value assigned to the potentiating state reflects that LTP is thought to be responsible for gain-down learning (Boyden and Raymond, 2003), while the positive value for the depressed state reflects that LTD is thought to be responsible for gain-up learning (Hansel et al., 2006).

We assumed that mGluR1 and $GABA_B$ receptors are not located on the same spine, meaning no co-localization. For the different drug conditions, the state of each spine was assumed to be determined by either mGluR1 or the $GABA_B$ receptor, not both. In other words, the mGluR1 drug (YM 298198) could affect only 50% of the spines, while the $GABA_B$ receptor drugs (CGP 52432 and baclofen) would affect the remaining 50%. We further assumed that the effects of the antagonists would shift some spines from a depressing to a potentiating state, and from a potentiating state to a stable state. This shift is thought to reflect the calcium levels required for plasticity (Coesmans et al., 2004). As the calcium concentration in a spine decreases, LTD would shift to LTP, and LTP would shift to a stable state. An agonist would have the opposite effect,

shifting spines from stable to potentiating to depressing. Outcomes were calculated independently for each test frequency (0.5, 2 and 8 Hz). We have suggested that 0.5 Hz frequency has a larger representation in the flocculus than 8 Hz (Chapter 5). In our model, we assumed that the number of "active" spines (in LTP or LTD) would be greatest at 0.5 Hz, and the least at 8 Hz. With these limitations, we adjusted the number of spines in each state by hand to mimic the change in gain (difference between post- and pre-learning gain values) under the different drug conditions.

Figure 6-7 shows that we were able to match the simulated changes in VOR gain (white bars) to the actual experimental data (black bars) in all conditions. The required numbers of spines in each state for the different drug conditions and learning protocols are given in Table 6-3. Note that the numbers given in Table 6-3 represent only one of many possible solutions, but effectively demonstrate that a working model was possible with the assumptions and limitations outlined above.

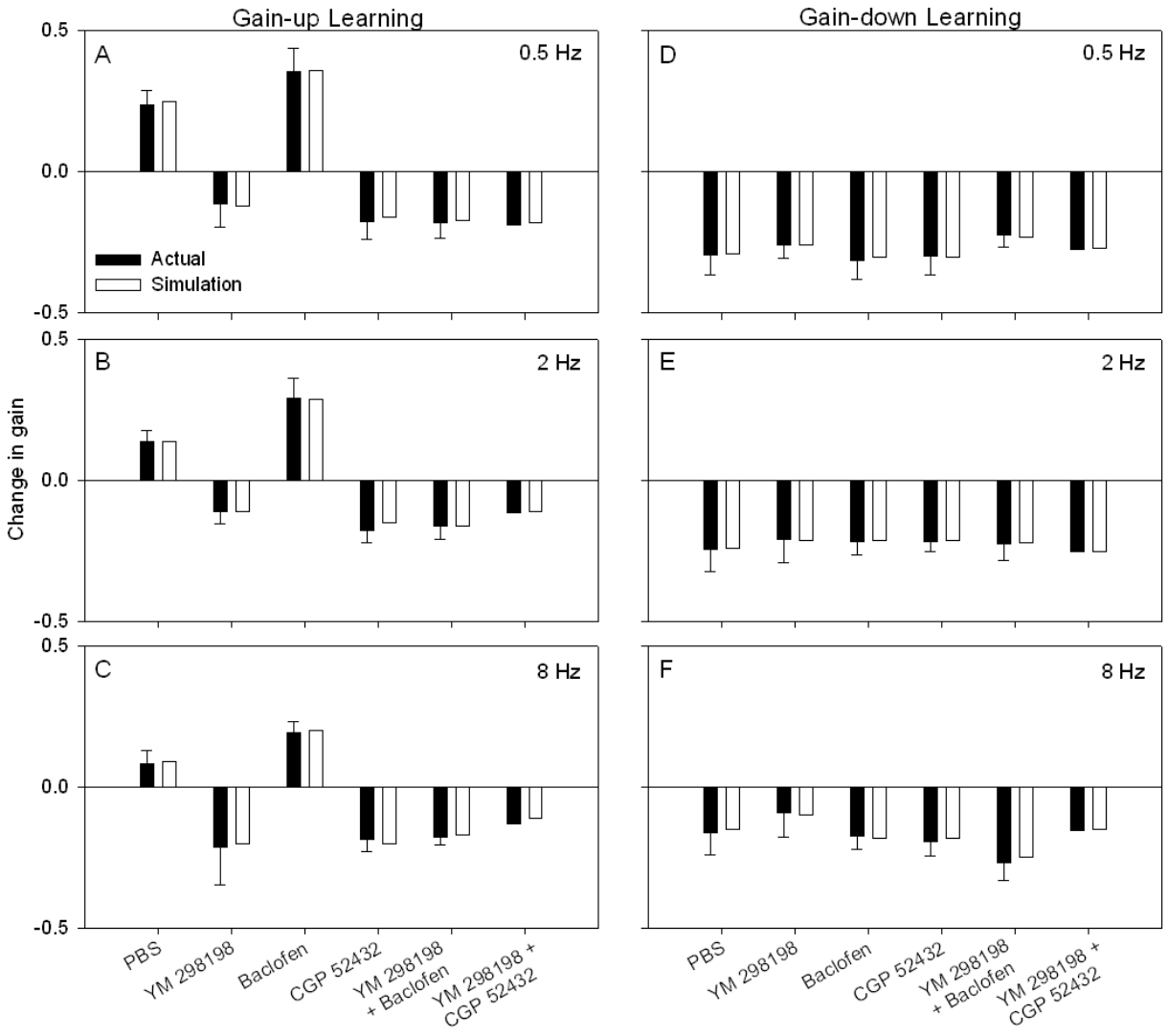

Figure 6-7. The change in gain can be accurately modelled. The change in gain was modelled by varying the amount of spines in different states of learning. A-C: The change in gain during gain-up learning, and the different test frequencies. Black bars: The actual change in gain measured as the difference between the pre- and post-learning time points. Error bars indicate S.D. White bars: The result of the simulation obtained by the consensus of spines model (see text for equation). Different test substances are indicated along the abscissa. D-F: The change in gain during gain-down learning, at the different test frequencies.

In Figure 6-7 the inversion of learning by the antagonists during the gain-up protocol was mimicked by shifting the number of spines toward the stable state (i.e. fewer spines in the depressed state, and more in the potentiated state) (see Table 6-3; compare CGP 52432, YM 298198 and both to PBS). Accordingly, our equation yielded a negative change in gain (Fig 6-7). Conversely, to simulate the effects of the agonist, baclofen, during gain-up learning, we shifted

the number of spines toward the depressed state (Table 6-3; compare PBS vs. baclofen). This resulted in a positive change in the VOR gain (Fig 6-7).

	0.5 Hz			2 Hz			8 Hz		
Drug	n_s	n_p	n_d	n_s	n_p	n_d	n_s	n_p	n_d
Gain-up Learning									
PBS	55	10	35	60	13	27	65	13	22
YM 298198	60	26	14	65	23	12	70	25	5
Baclofen	50	7	43	55	8	37	60	10	30
CGP 52432	60	28	12	65	25	10	70	25	5
YM 298198 + Baclofen	55	31	14	60	28	12	65	26	9
YM 298198 + CGP 52432	65	24	6	75	18	7	85	13	2
Gain-down Learning									
PBS	55	37	8	60	32	8	65	25	10
YM 298198	60	33	7	65	28	7	70	20	10
Baclofen	50	40	10	55	33	12	60	29	11
CGP 52432	60	35	5	65	28	7	70	24	6
YM 298198 + Baclofen	55	34	11	60	31	9	65	30	5
YM 298198 + CGP 52432	65	31	4	75	25	0	85	15	0

Table 6-3. Numbers used to generate model of learning using populations of spines. Our simple quantitative model assumed a population of independent spines. Each spine could be in one of three states: stable (s), potentiated (p), or depressed (d). Spines were assumed to have either mGluR1 or $GABA_B$ receptors, but not both. The table shows the number of spines in each state to make the predictions in Fig. 6-7, using the equation outlined in the text.

In the model, when YM 298198 was given in combination with a $GABA_B$ drug, the overall change represented a simple additive interaction among the spines containing mGluR1 and $GABA_B$ receptors. In other words, even when drugs for both receptors were present, we could still accurately mimic the change in gain without assuming that the drugs would act on the same spines. For example, when baclofen and YM 298198 were given together, spines could be shifted toward the depressing state due to the baclofen, but this effect was overwhelmed by the opposing shift toward the potentiating state due to the YM 298198.

To simulate gain-down learning, we made qualitatively similar shifts among drug groups. By using separate populations of spines for each testing frequency (0.5, 2 and 8 Hz) we could

accurately imitate the reversed frequency dependence caused by inverted learning (Fig 6-7 A-C). This model, although simple, demonstrated that it is possible, given certain assumptions, for mGluR1 and $GABA_B$ receptors to determine the direction of learning, via the addition of inputs from separate populations of spines.

6.4 Discussion

$GABA_B$ receptors have been shown to be involved in PF-LTD (see: Tabata and Kano, 2010). In cerebellar slices, application of a $GABA_B$ receptor agonist enhances LTD, while an antagonist impairs LTD (Kamikubo et al., 2007). Here, we show that when $GABA_B$ receptors were blocked in the cerebellar flocculus, gain-up learning was inverted. These results are consistent with LTD being the mechanism of gain-up learning, and show a role for $GABA_B$ receptors in VOR motor learning.

6.4.1 Role of inhibitory synapses in motor learning

Our finding of a role for the $GABA_B$ receptor in cerebellar motor learning raises the issue of whether local inhibitory neurons participate in learning. During periods of increased activity in inhibitory interneurons, GABA has been shown to spill over from stellate and basket cell terminals and activate the $GABA_B$ receptors in their vicinity (Dittman and Regehr, 1997). The excitatory inputs to stellate and basket cells are capable of synaptic plasticity (see: Jörntell et al., 2010), as are the inhibitory synapses onto the Purkinje cells (Kano et al., 1992; Mittmann and Häusser, 2007).Therefore both of these sites of plasticity could potentially contribute to motor learning in the cerebellum. mGluR1 and $GABA_B$ receptors are primarily located on the Purkinje cells (Hampson et al., 1994; Fritschy et al., 1999; Ige et al., 2000; Kulik et al., 2002), and are less abundant on the inhibitory interneurons (Fritschy et al., 1999; Than and Szabo, 2002), suggesting that our drugs acted primarily on Purkinje cells.

$GABA_B$ receptors are located on spines across from excitatory terminals (i.e. parallel fibres), and are not located on the inhibitory synapses on Purkinje cells (Fritschy et al., 1999; Ige et al., 2000; Kulik et al., 2002). In general, these receptors are thought to act at these excitatory synapses, and not at the inhibitory synapses (see: Tabata and Kano, 2010). At the inhibitory synapses, inhibition of the $GABA_A$ receptors in mice impaired VOR memory consolidation and the reversal of phase learning, and had no effect on the initial learned changes in gain (Wulff et al., 2009). We propose that the inhibitory synapses in the cerebellum may regulate post-learning changes, while the effects of the $GABA_B$ receptors on the mGluR1 signalling pathway is most likely due to plasticity at the parallel fibre-Purkinje cell synapses.

6.4.2 Learned gain increases require $GABA_B$ receptors

We found that a $GABA_B$ receptor antagonist inverted learning during the gain-up protocol, resulting in a learned gain decrease instead of an increase. This is very similar to our results involving a mGluR1 antagonist (see Chapter 5). We have suggested that this inversion of learning is consistent with the post-synaptic calcium concentration determining the direction of plasticity. It is thought that a relatively low increase in calcium is required for post-synaptic LTP, whereas a higher concentration of calcium is required for LTD (Coesmans et al., 2004; Jörntell and Hansel, 2006). A substitution of LTP for LTD could change gain increases to gain decreases. In our study, blocking the $GABA_B$ receptor could have limited the intracellular release of calcium, preventing PF-LTD, and causing PF-LTP instead.

6.4.3 $GABA_B$ receptors interacts with mGluR1

One important question is how would the $GABA_B$ receptor block the intracellular release of calcium? $GABA_B$ receptors are known to interact with mGluR1. It has been found that baclofen can potentiate the increase in intracellular calcium caused by the activation of mGluR1 (Hirono et al., 2001; Kamikubo et al., 2007; Rives et al., 2009). It is thought that the G protein coupled to $GABA_B$ receptors (Gi/o) enhances the action of phospholipase C (PLC) (Rives et al., 2009). PLC is part of the mGluR1 signalling pathway and is activated by the Gq protein, which is coupled to mGluR1. Activation of PLC leads to the release of intracellular calcium, activation of protein

kinase C, and the expression of LTD by the internalization of AMPA receptors (see: Ito, 2001). This supports the idea of mGluR1 and $GABA_B$ receptors contributing to a common signalling pathway involving PLC.

However, it has been shown, in transfected cells, that baclofen does not generate a calcium signal when given alone (Rives et al., 2009). This suggests that mGluR1 and $GABA_B$ receptors work synergistically. Indeed in transfected cells, it is assumed that the potentiating effect of baclofen depends on the interaction between mGluR1 and $GABA_B$ receptors, which leads to increase in calcium via PLC (Rives et al., 2009). However, transfected cells do not have dendritic spines, and this interaction has not been demonstrated in Purkinje cells. Moreover, the PLC pathway does not affect branchlet-wide calcium signalling, but is thought to be confined to the individual spines on the Purkinje cell dendrite (Wang et al., 2000). This suggests that if mGluR1 and $GABA_B$ receptors do work synergistically to regulate LTD, they must be located on the same dendritic spines.

6.4.4 mGluR1 and $GABA_B$ receptors are not located on the same spines

Both mGluR1 and $GABA_B$ receptors are present on Purkinje cell spines (Luján et al., 1997; Fritschy et al., 1999; Kulik et al., 2002; Rives et al., 2009), and have been assumed to be present on the same spines (Kamikubo et al., 2007). However, we have recently shown in both the mouse cerebellum (Broussard et al., 2011) and the flocculus of the cat (see Fig 1-6), that mGluR1 and $GABA_B$ receptors do not co-localize, and are rarely present, in any great amount, on the same dendritic spines.

Here, we also looked at possible synergistic effects by combing the mGluR1 antagonist with either the $GABA_B$ receptor antagonist or agonist. It was thought that if mGluR1 and $GABA_B$ receptors were acting on the same spines, the effects of these drugs would have been expected to combine or “add together”. For example, it was reasoned that if the effects of mGluR1 and $GABA_B$ converge within an individual spine, then blocking mGluR1 should prevent any effects

of $GABA_B$ activation during gain-up learning. However, during gain-up learning, we found that the inverted learning was slightly increased by baclofen, even in the presence of YM 298198. This suggests that the $GABA_B$ receptor could still affect learning, even when the mGluR1 receptor was blocked. This could be due to mGluR1 and $GABA_B$ receptors acting on different populations of spines.

Similarly, it was thought that if mGluR1 and $GABA_B$ receptors were working synergistically within the same spines to control calcium levels, and LTD, it would be possible to prevent learning entirely. We have shown that both gain-up learning was inverted in the presence of either a mGluR1 antagonist (Chapter 5) or a $GABA_B$ antagonist (present results). If during gain-up learning, the two antagonists work on the same spines, the combined decrease in the level of calcium should be below the threshold for even gain-down learning, preventing any learning from occurring. Instead, we observed a gain decrease that was comparable to either antagonist on its own. This could suggest that the two antagonists were actually affecting different populations of spines.

6.4.5 Learning is determined by a summation of two populations of spines

Based on the co-localization data in our recently submitted manuscript, we have suggested that mGluR1 and $GABA_B$ receptors are located on different spines, and determine the plasticity (LTD or LTP) within their own spines (Broussard et al., 2011). In this scenario, the combined effect of all spines then determines the direction of plasticity of the Purkinje cell and the population of Purkinje cells determines the overall direction of learning (see: Broussard et al., 2011). Using a simple model, we were able to mimic our experimental data, while assuming mGluR1 and $GABA_B$ receptors were located on separate populations of spines. While our simple model did not take into account the biophysical properties of neurons, it does show that our data are consistent with an additive interaction among different populations of spines, given certain conditions.

6.4.6 Conclusions

Our results indicate that the activity of the $GABA_B$ receptor is required for gain-up learning, but not gain-down learning in the VOR. Although mGluR1 and $GABA_B$ receptors are not located at the same spines, they do interact. Co-activation of both receptors is required for learned gain increases. We suggest that mGluR1 and $GABA_B$ receptors are located on separate Purkinje cell spine populations, each controlling the direction of plasticity within their individual spines. The direction of learning in the VOR is determined by a consensus of all Purkinje cell dendritic spines within the cerebellar flocculus.

Chapter 7

7 General Discussion and Future Directions

In this thesis we describe results which support the idea that memory for the VOR gain is initially encoded within the cerebellar flocculus. We present data that suggest that learned gain increases or decreases can be disrupted or rapidly consolidated, and we propose that these disruption and rapid consolidation processes do not represent new learning. Furthermore, our results suggest that learned gain increases depend on the activation of mGluR1 and $GABA_B$ receptors.

7.1 Rapid consolidation and disruption are not frequency selective

We showed in Chapter 3 that the memory of learned gain increases and decreases can be disrupted if the cat is rotated in the dark immediately after learning had stopped. Conversely, the gain of the VOR can also consolidate rapidly, if vision is restricted and the cat is stationary for an hour immediately following learning. We conclude that motor memory in the VOR is capable of rapid consolidation that occurs within 1 hour after learning has stopped. The rapid consolidation in the VOR is similar to what was found in motor learning of the NMR (Cooke et al., 2004). Given the uniform nature of the cerebellum, these findings suggest that other forms of cerebellar dependent learning could also be capable of rapid consolidation.

Learning in the VOR shows frequency dependence. Learning is greatest when measured at the frequency at which training took place (Chapter 4; Robinson, 1976; Lisberger et al., 1983; Raymond and Lisberger, 1996; De Zeeuw et al., 1998; Kimpo et al., 2005). We found that disruption and rapid consolidation did not show the same pattern of frequency dependence as the initial learning did. We therefore suggest that disruption and rapid consolidation are not forms of new learning occurring at a different set of synapses. Instead, we suggest that disruption and rapid consolidation is a local mechanism that affects the synapses that were originally modified during learning. However, since we did not record from these or any other synapses, we cannot

rule out the involvement of non-synaptic plasticity or learning in another area such as the brainstem, which may not be frequency dependent. Further research in this area could benefit from electrophysiological recordings. By simultaneously recording from synapses in the cerebellum and brainstem during learning, consolidation and disruption, it could be possible to determine which brain area is preferentially involved in these processes.

One way to explain the selectivity of learning is the idea of frequency channels (Lisberger et al., 1983) or microzones within the cerebellar cortex (Dean et al., 2010). If frequency channels have a physical basis in the distribution of signals in the cerebellar cortex, the Purkinje cells at the centre of a channel would have some synapses that are preferentially tuned to a particular frequency (Broussard et al., 2011). The amount of learning or change at the frequency tuned synapses might be greatest when training occurs at that particular frequency. The greater change at the tuned synapses would explain why learning is greatest at the frequency at which training occurred (Lisberger et al., 1983). However, our research does not confirm the existence of such channels; we merely offer this as one possible explanation. Further research is required to substantiate the idea of frequency selective channels in the cerebellum.

Unlike learning, we found that the amount of normalized disruption was slightly greater as the training and testing frequencies diverged, while less disruption occurred at the disruption frequency. We have suggested that the synapses within the channel corresponding to the training frequency might be preferentially stabilized (Broussard et al., 2011). Mechanisms of selectively identifying synapses have been described before with the idea of synaptic tagging (Frey and Morris, 1997; Redondo and Morris, 2011). Synapses within the frequency channel could be "tagged" to promote preferential protein synthesis enabling selective stabilization. Synapses outside the frequency channel might be quicker to reverse their learned changes without this synaptic tag. However, synaptic tagging has not yet been shown to exist in the cerebellar cortex, and has yet to be linked to cerebellar motor learning. Further research in this area, could look for possible "synaptic tags" after cerebellar motor learning.

Here we found that rapid consolidation did not show that same frequency selectivity that is consistent with new learning. We suggest that rapid consolidation of the VOR takes place within the cerebellar cortex. It is believed that while learning initially takes place in the flocculus, in the long-term the memory is transferred to include the vestibular nuclei (Kassardjian et al., 2005; Shutoh et al., 2006; Anzai et al., 2010). However, it is not clear how this transfer in memory is related to consolidation. It has been proposed that the transfer of memory to the brainstem may begin soon after the memory has been encoded (Menzies et al., 2010), if so this would overlap with the onset of consolidation. Further research is required to clarify the relationship between consolidation and memory transfer. A more focused study to clarify the time course of memory transfer after consolidation would be beneficial. On such experiment might involve using local injections of the glutamate antagonist such as CNQX in the flocculus or vestibular nucleus at various intervals such as 30, 60 or 90 minutes to determine where the memory is located.

To summarize, we conclude that the VOR is capable of rapid consolidation, which can occur within 1 hour after learning has stopped. We can further conclude that rapid consolidation and disruption do not show the same frequency selectivity that is seen during learning. We therefore suggest that rapid consolidation does not involve new learning, which would be required for memory transfer. This suggests that rapid consolidation is a local mechanism, taking place within the cerebellar cortex.

7.2 mGluR1 and $GABA_B$ receptors are required for gain-up learning

Fortunately, more is known of the initial encoding of memory during the learning stage than the mechanisms of consolidation. In our research, we found that learned gain increases, but not decreases, require both mGluR1 and $GABA_B$ receptors. This is consistent with the idea of gain-up learning being the result of LTD (Hansel et al., 2006), while gain-down learning is dependent on another mechanism, perhaps LTP (Boyden and Raymond, 2003). mGluR1 is required for LTD, and its signalling cascade causes the activation of PKC as well as the release of calcium from the internal stores via the activation of PLC (see: Kano et al., 2008). $GABA_B$ receptors are

known to enhance mGluR1 signalling, and most likely act through the activity of PLC (see: Tabata and Kano, 2010). Although mGluR1 and $GABA_B$ receptors are not located on the same dendritic spines on Purkinje cells (Broussard et al., 2011; Fig 1-6), we found that they do interact, and the co-activation of mGluR1 and $GABA_B$ receptors is required for gain-up learning. This finding suggests that mGluR1 and $GABA_B$ receptors are required for other forms of cerebellar motor learning as well. Further research into the requirements of mGluR1 and $GABA_B$ receptors in the OKR, OKR or saccadic motor learning systems could be interesting.

We propose a model in which mGluR1 and $GABA_B$ receptors each contribute to gain-up learning, but are located on different dendritic spines. This is further illustrated in Figure 7-1. Both receptors contribute to the calcium signalling within their respective spines, and thus LTP or LTD, via their interaction with PLC. We further propose that the calcium within these two populations of spines would interact at the dendritic branches, leading to protein synthesis (Fig 7-1). Thus, in our model, each spine is independently capable of LTD or LTP based on its calcium level, but it is the overall consensus of the spines that determines the direction of plasticity in a Purkinje cell. The direction of learning is determined by the overall population of Purkinje cells within the cerebellar flocculus.

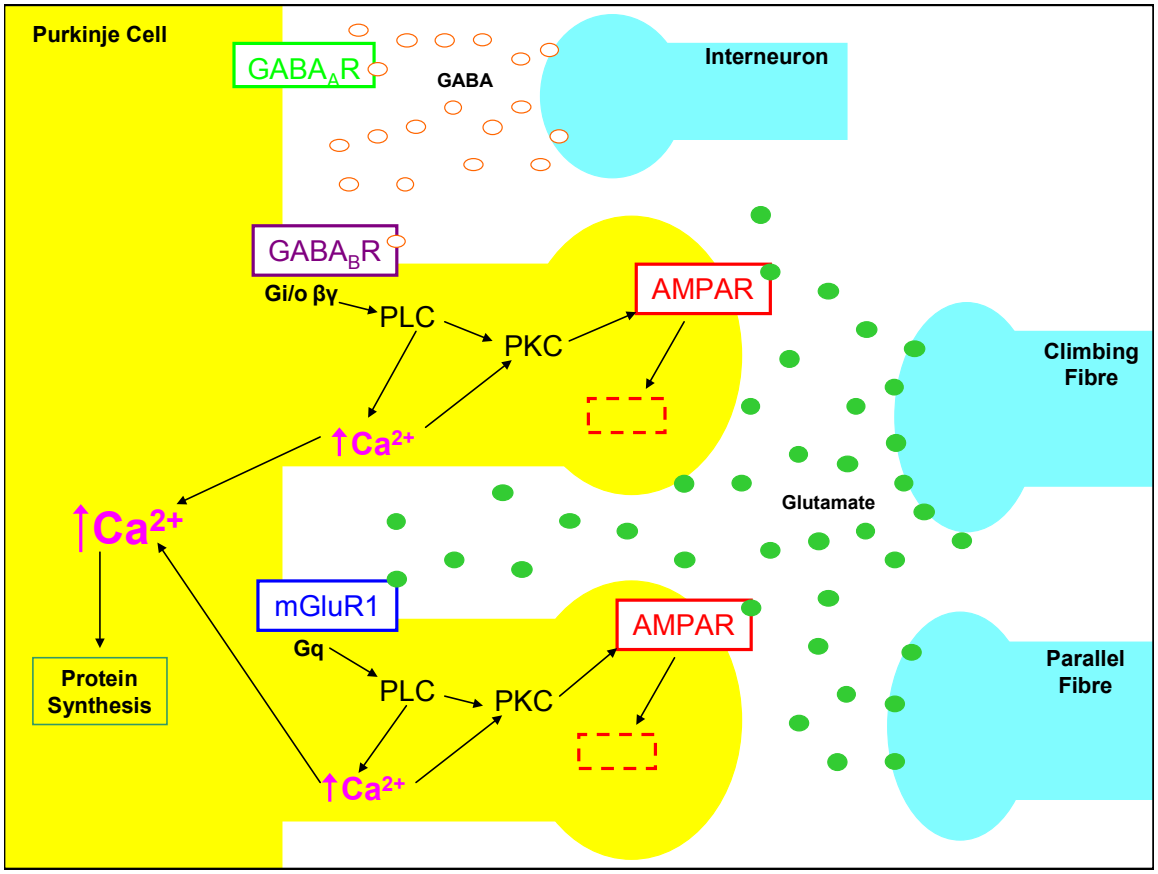

Figure 7-1. mGluR1 and GABA$_B$ receptors contribute to LTD from different spines. Model showing how mGluR1 and GABA$_B$ receptors located on different Purkinje cell spines could contribute to LTD and the concentration of calcium within their spines. During LTD, the combined activation of the parallel fibres and climbing fibres allows glutamate to bind to mGluR1 (as well as AMPA receptors). Spillover from inhibitory interneurons allows GABA to bind to the GABA$_B$ receptors. G proteins (Gq or the βγ subunit from Gi/o) activate phospholipase C (PLC). PLC releases calcium (Ca^{2+}) from the internal stores raising the levels of calcium within the individual spines. PLC can also activate PKC which leads to the internalization of AMPA receptors from cellular membrane during LTD. The increase of calcium within the dendrite of the Purkinje cells can lead to protein synthesis and the long-term effects of LTD. Note that for simplicity not all pathways and second messengers are shown.

In our study, blocking the mGluR1 or GABA$_B$ receptors (or both) during the gain-up protocol inverted learning, meaning the gain of the VOR decreased instead of increasing. This is consistent with the idea of these receptors contributing to the post-synaptic calcium level in the Purkinje cell spines, ensuring that the higher threshold of calcium is met for LTD. By blocking mGluR1 or GABA$_B$ receptors during gain-up learning, we may have limited the amount of calcium, preventing LTD, and causing LTP at many spines instead. Similarly, by enhancing mGluR1 or GABA$_B$ receptors, we may have increased the calcium levels, ensuring that more spines reached the threshold needed for LTD. Therefore both mGluR1 and GABA$_B$ receptors

contribute to gain-up learning in the VOR via their influence on calcium levels at their respective spines.

However, the idea of decreasing calcium levels causing the inversion in gain-up learning is based on the assumption that an mGluR1 or $GABA_B$ receptor antagonist can inhibit or prevent calcium transients within their post-synaptic spines. The antagonists, YM 298198 and CGP 52432, used in our studies have not yet been shown to influence calcium levels. With our methods, we are limited to measuring eye movements only, and thus we cannot be certain what effects are happening at individual synapses. Further studies are needed to verify what effect YM 298198 and CGP 52432 are having on the internal calcium levels in Purkinje cells. As our study was focused on the behavioural aspect, this research would benefit from in vitro studies, focusing on the effects of these drugs in slice experiments.

7.3 Summary

In summary, we provide evidence that the VOR is capable of rapid consolidation, and suggest that this process may be a local mechanism, stabilizing the previously changed synapses within the cerebellar cortex. We also provide support for the idea that PF-LTD is involved in learned gain increases, and that gain-up learning depends on the co-activation of mGluR1 and $GABA_B$ receptors.

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Appendix 1
Controls for Butorphanol and PBS Injections

Butorphanol was necessary in cat V

Before any penetration with a needle or electrode through the dura for an injection or stimulation, we always applied topical lidocaine on the dura for approximately 20 minutes. After the lidocaine, most cats were unaware or not bothered by penetration through the dura. Cat V however, was more sensitive to the passage of the needle than other cats. In this cat, to ensure his comfort and safety, we also administered the opioid analgesic butorphanol, 0.2 mg/kg (s.c.), 30 minutes before the application of lidocaine in each trial.

Methods

To evaluate the effects of butorphanol, which was always necessary in cat V, we compared learning under three separate conditions. In the first condition, "no injection" group, we measured the amount of learning, after gain increases or decreases, where no injection was given (gain-up: n=11; gain-down: n=24). In the second condition, "vehicle injection" we measured the amount of learning in experiments where the vehicle PBS was injected, but no butorphanol was given (gain-up: n=4; gain-down: n=3). Finally, in the "butorphanol/injection" group, we measured the amount of learning in experiments where PBS was injected and butorphanol was also administered (gain-up: n=2; gain-down: n=3). The following cats were used for each condition: R, S, T, V, A, B, C and E for no injection, S, B, C and E for PBS without butorphanol, and V and C for PBS with butorphanol.

PBS and opioid injections did not affect learning

Any effect of PBS on motor learning due to the pressure caused by an injection would confound the results of any study involving injections (Chapters 5 and 6). We therefore compared, across previous experiments, instances of motor learning after an injection of PBS alone (vehicle group)

with all instances of motor learning using the same protocol but with no injection (no injection group). As a control for the opioid analgesic (required in cat V), we also compared the effects of butorphanol on learning. In Figure A1-1, panels A and C show the breakdown for gain-up and gain-down learning. Neither the vehicle injection alone nor the combination of PBS and butorphanol had any effect on the amount of learning. We found no difference when we compared the total sample of all PBS injections with the no injection condition for both gain-up and gain-down learning conditions ($P>0.05$, unpaired t-tests). The scatter plots (Fig. A1-1 B and D) show all measurements in the "no injection" and "injection with butorphanol" conditions.

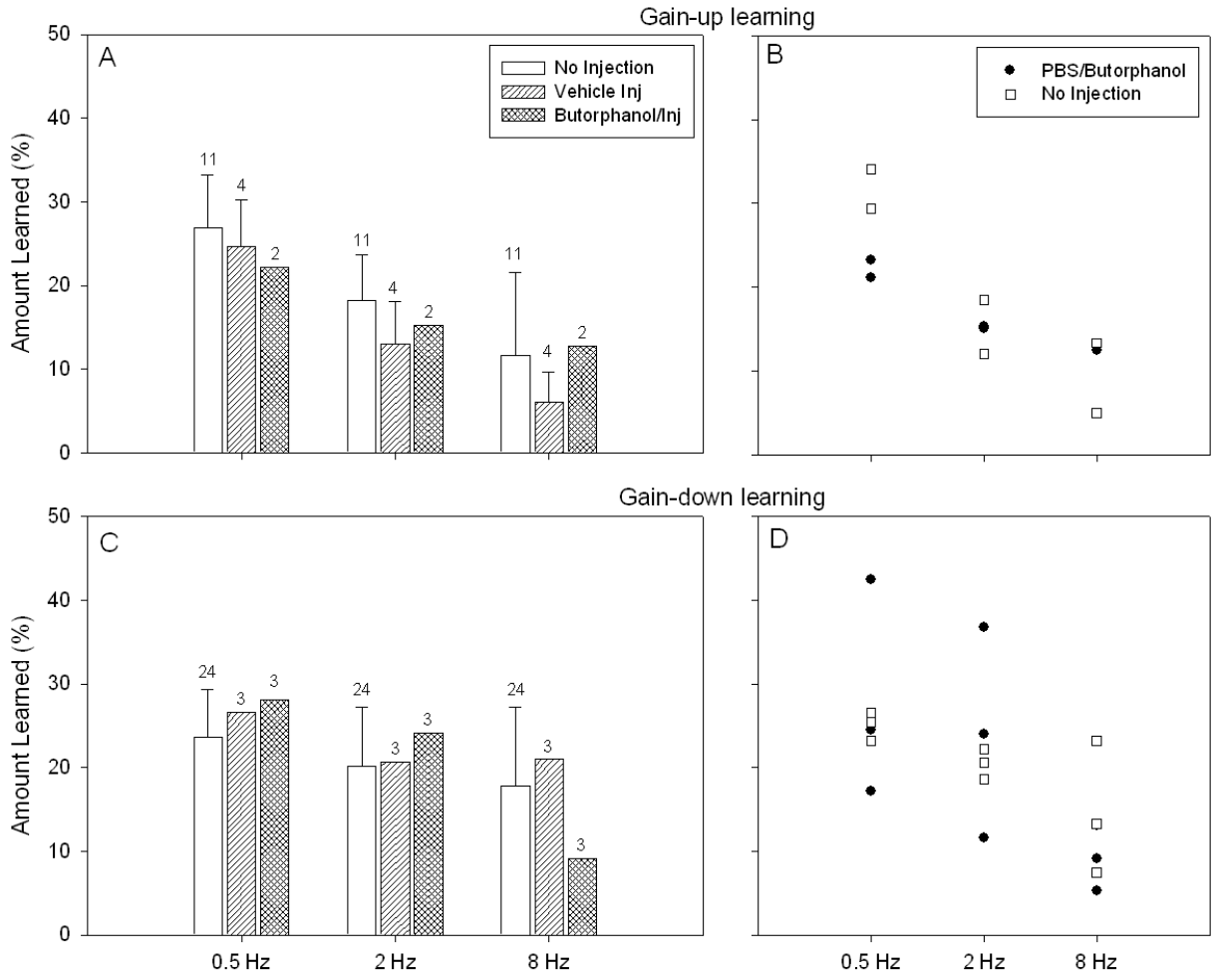

Figure A1-1. Controls for PBS and butorphanol injections. Figure A1-1. Neither bilateral floccular injections of PBS alone, nor systemic butorphanol, affected learning. A: The amount learned in the gain-up protocol was similar across conditions for all test frequencies. White bars: Trials in which cats received neither PBS injections nor butorphanol (data from cats A, B, C, E, R, S, T, and V). Single-hatched bars: Trials in which cats received PBS injections, but no butorphanol (data from S, B, C and E). Double-hatched bars: Trials in which cats received butorphanol followed by PBS injection into the flocculus (cats V and C). Sample sizes are shown above each bar. B:

The amount learned during the gain-up protocol, in individual trials in which butorphanol was administered before a PBS injection (black circles) and trials in which no butorphanol and no injection was given (open squares). C: The amount learned in the gain-down protocols was also similar across conditions, at all test frequencies. D: Individual trials for the gain-down protocol.

Conclusions

Here, we show that an injection of an opioid analgesic before learning, or the pressure effects of a PBS injection did not affect the ability of cat to increase or decrease the gains of their VOR. We conclude that the butorphanol given to cat V before each trial did not affect the results obtained. Furthermore, we conclude that a PBS vehicle injection had no affect on subsequent learning periods.

Appendix 2
Effects of Repeated learning

Gain-up and gain-down learning reverse with unequal efficacy

The expression of LTD and LTP at the PF-PC synapse is not equal. Whereas PF-LTD is primarily expressed post-synaptically (Sakurai, 1987), PF-LTP has been shown to be expressed both pre- and post-synaptically (Salin et al., 1996; Lev-Ram et al., 2002). This asymmetry at the level of the synapse leads to the prediction that learned gain increases and decreases themselves may show an asymmetry.

Although in the long-term, Miles and colleagues have shown that in monkeys learning is capable of reversing learned gain increases and decreases repeatedly without showing any residual learning or asymmetry in the gain (Miles and Eighmy, 1980). In the short-term, Boyden and Raymond have shown in mice that learned gain increases cannot fully reverse previously learned gain decreases (Boyden and Raymond, 2003). In the study by Boyden and Raymond, mice underwent multiple training sessions of gain-up or gain-down learning. It was found that two gain-down learning sessions fully reversed three prior gain-up learning sessions, and resulted in an overall gain decrease. However, two gain-up learning sessions could not fully reverse three sessions of prior gain-down learning, and even resulted in a overall gain decrease (Boyden and Raymond, 2003). This suggests that gain-up and gain-down learning reverse each other with unequal efficacy.

These results clearly show that gain-up and gain-down learning in the VOR rely on different plasticity mechanisms. The mechanism for learned gain decreases seems to be more potent than that of learned gain increases, at least in the short-term. We asked whether repeated learning in cats would result in a gain asymmetry, or an accumulation of gain-down learning. Some of these results were previously published (Titley et al., 2010).

Methods

Since gain-up learning was found to be affected by prior gain-down learning within a trial, we looked to see if gain decreases accumulated over many trials. In a post-hoc analysis we tested to see if the baseline VOR gain at the start of each experiment decreased over repeated trials. We analyzed data from 8 different cats that underwent repeated gain-up and gain-down learning experiments. We sub-divided the data into two groups, subjects who did not receive any intra-floccular injections (cats R, T, and A), and subjects who received injections prior to learning trials (cats S, V, B, C, and E).

The baseline gain measured at 2 Hz was plotted from trials before gain-up, gain-down or from control trials without telescopes. To allow comparisons we only plotted the learning trials that were induced with a sum-of-sines rotational stimulus, and thus did not include gain increases that were induced at a single rotational frequency (see Chapter 4). The median inter-trial interval in both groups was 7 days.

Repeated learning trials had no effect on gain

In both groups we plotted the normalized baseline VOR gain value measured at 2 Hz over the trial number for all subjects. In a separate analysis we plotted percent gain change after gain-up learning trials. Because both mGluR1 and $GABA_B$ receptor drugs affect gain-up learning (see Chapters 5 and 6), we only included trials after a PBS (vehicle) injection. To each plot we fitted a line of best fit through all the data from all subjects.

The results are illustrated in Fig A2-1. We found no difference in the normalized baseline VOR gain over time in cats that did not receive drugs (Fig A2-1 A), and cats that did receive previous drug injections (Fig A2-1 B). In both groups, we saw no correlation between the baseline VOR

gain and time (trial number). We also looked to see if repeated trials affected the amount of gain-up learning (Fig A2-1 C and D). In both groups, we did not see a consistent effect over trials.

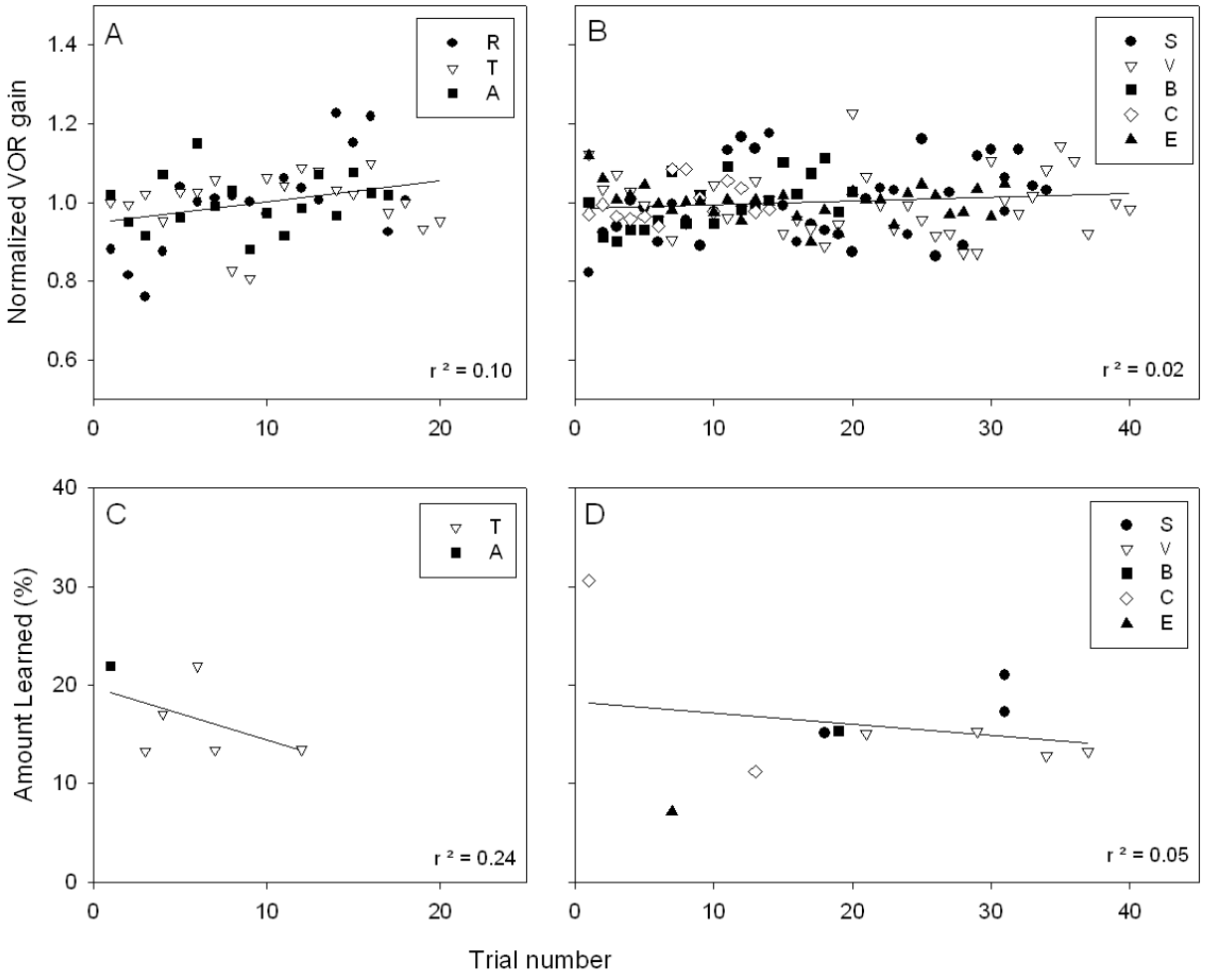

Figure A2-1. Repeated learning trials had no long-term effects. Repeated learning trials did not affect the baseline VOR gain at the beginning of each trial or the amount of gain-up learning. A: The baseline gain did not change in cats R, T and A, during studies that did not involve drug injections (data from Chapters 3 and 4). The baseline VOR gain was normalized to the average value for each cat, and is plotted as a function of a number trials experienced by each cat. B: The baseline gain did not change in subjects S, V, B, C and E, that did receive an injection of an mGluR1 or GABAB drug (data from Chapters 5 and 6). C and D: The amount of learning after gain-up trials. In D, only trials in which PBS was injected are shown, trials in which a drug was injected were excluded.

Discussion

Recent evidence has suggested that in the short term gain increases and decreases reverse each other with unequal efficacy (Boyden and Raymond, 2003). We later confirmed these results with preliminary data from naive cats. Four cats that had no prior experience with telescopic lenses, showed that 2 hours of gain-down learning would not only reverse 1 hour of prior gain-up learning, but resulted in a learned gain decrease. On the other hand, 2 hours of gain-up learning failed to reverse 1 hour of gain-down learning (Broussard et al., 2011). This suggests that in the short-term (within a trial) learned gain increases and decreases are asymmetric, and gain-down is more potent than gain-up learning.

However, in the long term (repeated trials) we did not see any asymmetry in either the baseline gains over time or the amount learned after gain-up learning trials. These results are consistent with previous studies in monkeys (Miles and Eighmy, 1980), and suggest that over the long-term the VOR is able to compensate for the unequal weights of gain increases and decreases.

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Appendix 3
Raw Traces from each cat

For each study presented in this thesis, we provided a representative example of the data from an individual cat. Here, we present examples traces from each cat in all studies.

Rapid consolidation of gain changes in the VOR

In Chapter 3, we presented data showing rapid consolidation of learned gain changes in the VOR. Figure 3-1 showed examples of learned gain increases and decreases from cat S. Here we present example of learned changes from each cat in that study (see: Fig A3-1). All cats were able to successfully change the gain of their VOR after 1 hour of either gain-down or gain-up learning.

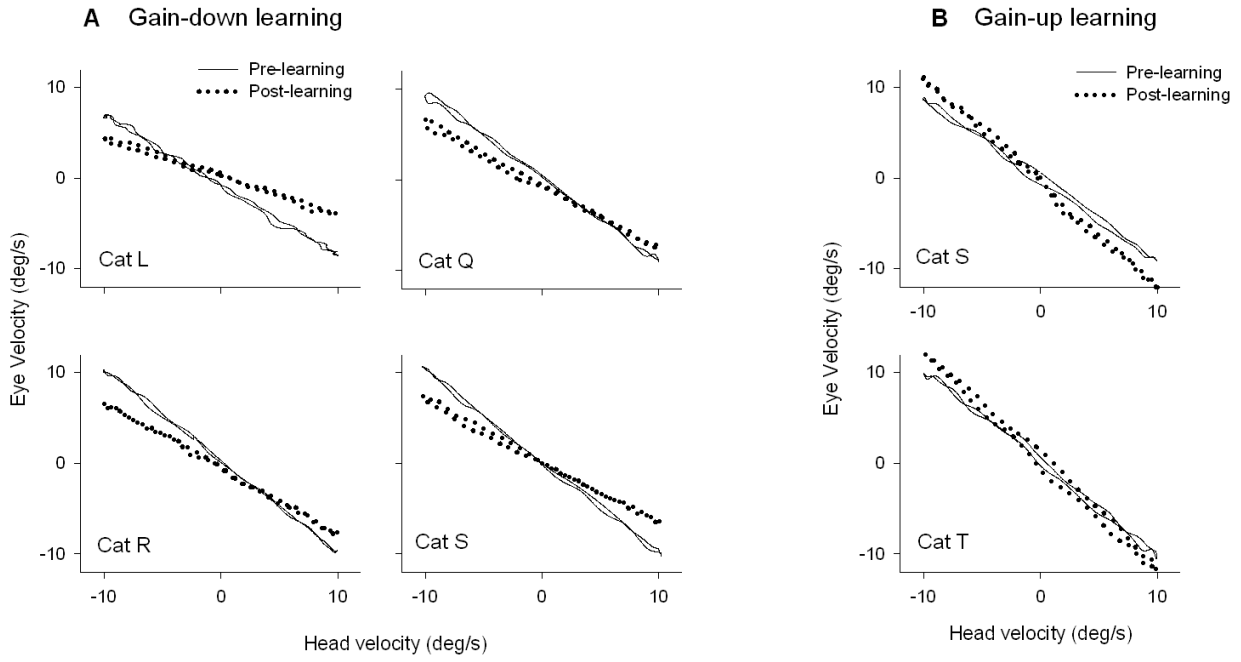

Figure A3-1. Examples of learned gain changes from each cat in Chapter 3. Eye velocity plotted against head velocity during the VOR before (solid line) and after learning (dotted line) at 2 Hz. Traces are averages of 10 cycles or more. A: Gain-down learning (cats L, Q, R and S). B: Gain-up learning (cats S and T).

Disruption and consolidation generalize across frequencies

In Chapter 4, we showed evidence that disruption and rapid consolidation did not show frequency selectivity. In Figure 4-1, we showed example traces from cat A of learning and disruption at each training frequency used (0.5, 2 and 8 Hz). Here we show traces from all cats in this study (Fig A3-2).

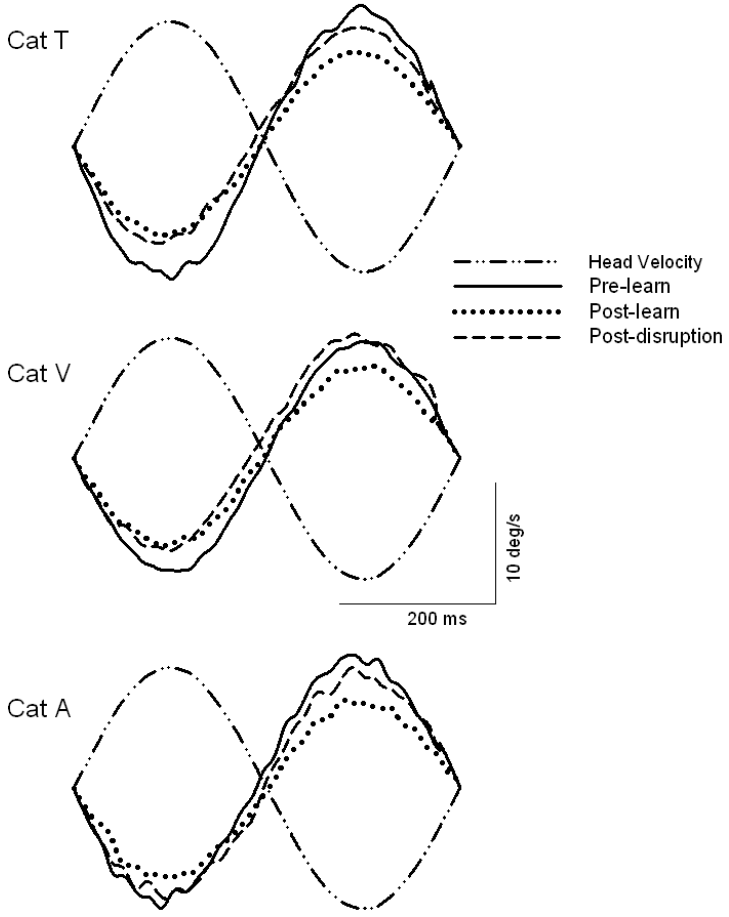

Figure A3-2. Examples of learning and disruption from each cat in Chapter 4. Sample traces of head (dashed-dotted line) and eye velocity (solid line: pre-learning; dotted line: post-learning; dashed line: post-disruption) plotted against time at 2 Hz. Examples are from trials with no neutral period, and show that each cat in this study (cats T, V and A) was able to disrupt learned changes in gain.

Motor learning in the VOR requires cerebellar mGluR1 receptors

In Chapter 5, we described how gain-up but not gain down learning in the VOR depends on mGluR1 receptors. We showed examples from cat V depicting how the mGluR1 antagonist, YM 298198, inverted gain-up learning, resulting in a gain decrease (Fig 5-3). We also showed examples from cat B showing how the mGluR1 agonist, DHPG, augmented gain-up learning (Fig 5-4). Here we show examples showing the effects of these drugs on each cat (Fig A3-3).

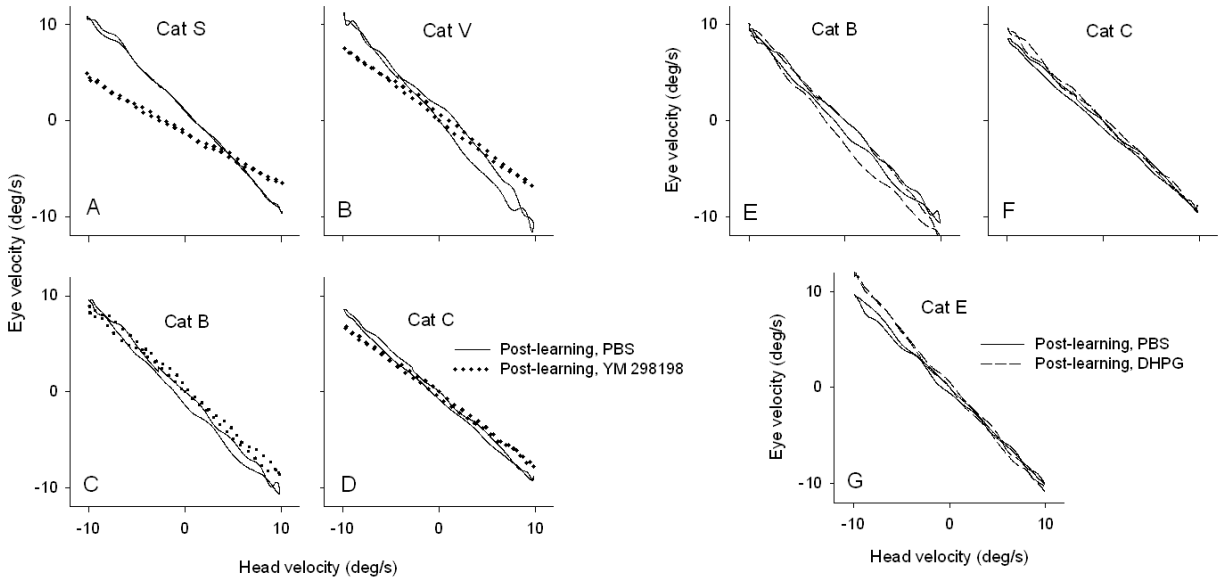

Figure A3-3. The effects of mGluR1 drugs on each cat in Chapter 5. Eye velocity as a function of head velocity at 2 Hz after gain-up learning. All traces are averages of 10 or more cycles. A-D: In the presence of the mGluR1 antagonist all cats (cats S, V, B and C) showed a gain decrease. Solid line: PBS. Dotted line: YM 298198. E-F: In the presence of the mGluR1 agonist gain-up learning was enhanced in all cats (cats B, C, and E). Solid line: PBS. Dashed line: DHPG.

$GABA_B$ receptors are required for VOR motor learning

In Chapter 6, we showed that gain-up learning also required $GABA_B$ receptors. We showed examples from cat E showing the effects of the $GABA_B$ receptor antagonist (CGP 52432) and agonist (baclofen) on gain-up learning (Fig 6-2). Here we show the effects of these $GABA_B$ receptor drugs on all cats in this study (Fig A3-4).

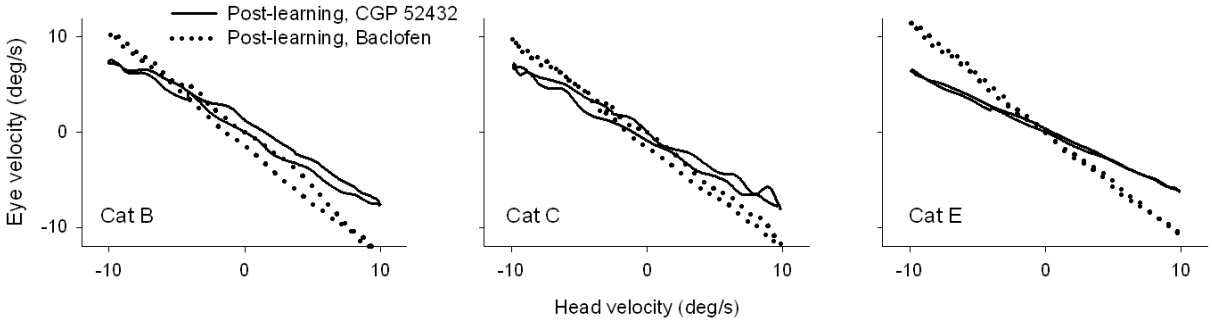

Figure A3-4. The effects of $GABA_B$ receptor drugs on each cat in Chapter 6. During the gain-up learning protocol, the $GABA_B$ receptor antagonist, CGP 52432, inverted gain-up learning, while the agonist, baclofen, augmented gain-up learning. Eye velocity as a function of head velocity after gain-up learning at 2 Hz in all cats that received these drugs (cats B, C and E). Solid line: CGP 52432. Dotted line: Baclofen. Traces are averages of 10 or more cycles.