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Synaptic Plasticity in Cerebellar-dependent Learning: the Role of Endocannabinoids

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Plasticidade Sináptica em Aprendizagem dependente do Cerebelo: o Papel dos Endocanabinóides

Dissertação apresentada à Faculdade de Medicina da Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Investigação Biomédica-Neurobiologia, realizada sob orientação científica da Dr. Megan R. Carey, Investigadora Principal no Champalimaud Neuroscience Programme da Fundação Champalimaud e do Prof. Dr. Miguel Castelo-Branco, Investigador Principal/Professor Assistente na Faculdade de Medicina da Universidade de Coimbra

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Dissertation presented to the Faculty of Medicine of the University of Coimbra to comply with the requirements necessary for the degree of Master in Biomedical Research-Neurobiology, performed under the scientific orientation of Dr. Megan R. Carey, Principal Investigator in the Champalimaud Neuroscience Programme at the Champalimaud Foundation and Prof. Dr. Miguel Castelo-Branco, Principal Investigator/Assistant Professor in the Faculty of Medicine at the University of Coimbra.

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Palavras-chave

Circuitos Neuronais e Comportamento; Cerebelo; *Delay eyelid conditioning*; Plasticidade Sináptica; Endocanabinóides; Receptores Canabinóides Tipo 1 (CB1Rs).

Resumo

O cerebelo é essencial para a coordenação e aprendizagem motora, no entanto ainda não se compreende totalmente como estas funções são reguladas. Os endocanabinóides são reguladores importantes da atividade sináptica em todo o cérebro. Estes são libertados de neurónios pós-sinápticos e atuam retrogradamente em receptores canabinóides pré-sinápticos tipo 1 (CB1Rs) para suprimir a neurotransmissão. Recentemente, foi observado que estes mensageiros são necessários para a modulação de várias formas de plasticidade a curto- e a longo-prazo no cerebelo. De acordo com isto, ratinhos com deleção global de CB1Rs foram vistos como tendo deficiente aprendizagem motora dependente do cerebelo, sugerindo que os endocanabinóides são importantes para a aprendizagem e para a memória sensorial-motora. Contudo, nestas experiências os CB1Rs foram eliminados de todas os tipos de células. Neste projeto tivemos como objetivo perceber em que tipos celulares e através de que mecanismos os endocanabinóides atuam para regular a aprendizagem motora. Para tal, ferramentas genéticas de ratinho foram combinadas com um comportamento animal dependente do cerebelo e foi avaliada a aprendizagem motora de diferentes linhas de ratinho com deleções em diferentes tipos celulares específicos. Assim, para além de ratinhos com deleção global de CB1Rs, três linhas de ratinhos com deleções condicionais, sem expressão de CB1Rs 1) nas *granule cells*, cujos axónios formam as *parallel fibers* e possivelmente nas *mossy fibers*, 2) nas *granule cells* ou 3) nos *inhibitory interneurons* foram testadas em *delay eyelid conditioning*.

Surpreendentemente, nas nossas condições experimentais, os resultados obtidos indicam que os ratinhos com deleção global de CB1Rs são muito menos debilitados em *delay eyelid conditioning* do que observado anteriormente. De facto, a nível geral a aprendizagem parece normal. De acordo com isto, as diferentes linhas de ratinhos sem CB1Rs em tipos celulares específicos, testadas pela primeira vez em *delay eyelid conditioning*, também não apresentam defeitos na aprendizagem a nível geral. Mesmo aspetos mais subtis da aprendizagem, como a amplitude e o tempo das respostas, não parecem ser afetados nem pela deleção global nem pela deleção condicional de CB1Rs.

Através da análise teste-a-teste, foi possível observar que a experiência em testes prévios afeta a aprendizagem em testes seguintes, independentemente do genótipo. Ou seja, ratinhos com deleção global de CB1Rs não foram afetados de forma diferente dos controlos pela experiência em testes prévios. No entanto, eles aparentaram ser mais afetados pelo intervalo de tempo entre testes (escala de tempo de segundos). O mesmo efeito foi observado em ratinhos sem expressão de CB1Rs nas *granule cells* e nas *mossy fibers*.

Os nossos resultados sugerem que os endocanabinóides presentes no cerebelo podem estar a atuar no sentido de suster a memória entre testes. Análises preliminares também revelaram que o sistema endocanabinóide pode ser importante para a consolidação da memória motora (escala de tempo de horas/dias), porque tanto os ratinhos com deleção global como os ratinhos com deleção de CB1Rs nas *mossy fibers/granule cells* aparentam lembrar menos após um longo período de tempo sem treino. Curiosamente, a atividade locomotora também parece estar relacionada com a aprendizagem em *delay eyelid conditioning*.

No geral, os nossos resultados sugerem que a modulação mediada por CB1Rs nas sinápses entre as *parallel fiber-Purkinje cell* ou *mossy fiber-deep cerebellar nuclei* pode estar envolvida em fenómenos a curto- e a longo-prazo em *delay eyelid conditioning*, especificamente na memória a curto-prazo e na consolidação a longo-prazo da memória motora.

Keywords Neural Circuits and Behavior; Cerebellum; Delay eyelid conditioning; Synaptic Plasticity; Endocannabinoids; Type 1 Cannabinoid Receptors (CB1Rs).

Abstract

The cerebellum is crucial for motor control, timing and learning, but exactly how these functions are regulated is still not fully understood. Endocannabinoids are powerful regulators of synaptic function throughout the brain. They are released from postsynaptic neurons and act retrogradely onto pre-synaptic type 1-cannabinoid receptors (CB1Rs) to suppress neurotransmission. Recently, these messengers were observed to be required in the modulation of various forms of short- and long-term plasticity within the cerebellum. In line with this, global CB1Rs knockout mice were shown to have impaired cerebellum-dependent motor learning, suggesting the importance of endocannabinoids function for sensorimotor learning and memory. However, in these experiments CB1Rs were eliminated from all cell types.
In this project we aimed to understand in which cell types and through what mechanisms endocannabinoids act to regulate motor learning. For that, mouse genetic tools were combined with a cerebellum-dependent behavioral assay and motor learning of different cell-specific CB1Rs knockouts was evaluated. Thus, apart from the global CB1R KO mice, three different conditional KO lines with mice lacking CB1Rs at 1) granule cells and possibly mossy fibers, 2) granule cells or 3) inhibitory interneurons were tested in delay eyelid conditioning.
Surprisingly, under our experimental conditions, the results obtained indicate that global CB1R KO mice are far less impaired in delay eyelid conditioning than what described previously. In fact, gross features of learning appear normal. Accordingly, the different cell-specific CB1R KO mice tested for the first time in delay eyelid conditioning also do not present major impairments. Even more subtle aspects of the response, like amplitude and timing, do not seem to be affected in both global and conditional CB1R KO mice.
By performing trial-to-trial analysis, it was possible to observe that previous trial experience affects learning on the following trial, regardless of genotype. Global CB1R KO mice were not differentially affected by previous trial experience. Nonetheless, they appeared to be more affected by the interval between trials (timescale of seconds). The same effect was seen in mice lacking CB1Rs at the granule cells and mossy fibers. Our results suggest that endocannabinoids within the cerebellum might be acting to sustain memory between trials. Preliminary analysis also revealed that the endocannabinoid system might be important for the consolidation of motor memory (timescale of hours/days), as both global and mossy fiber/granule cell-specific CB1R KO mice appear to remember less after a long period of time without training. Interestingly, running activity also appears to be related with learning in delay eyelid conditioning.
Overall, our results suggest that CB1R-mediated modulation at the parallel fiber-Purkinje cell or mossy fiber-deep cerebellar nuclei might be involved in both short- and long-term phenomena in delay eyelid conditioning, specifically in working memory and consolidation of motor memory.

Contents

List of Figures

Chapter 1

Introduction

1.1 Neural Circuits and Behavior

Many of the issues that have been successfully explored concern neurons as the basic structural and functional unit of the nervous system. These studies of the cellular and molecular components of neurons have revealed much about their individual functions and provided a basis for understanding how they are organized into circuits and circuits into systems that process information underlying sensation, perception, action and learning. Understanding how these neural systems are organized and how they function to generate behavior is currently a fundamental goal in Neuroscience.

Neurons do not function alone; they are organized into circuits that process specific information and provide the foundation of sensation, perception and behavior [1].

The mammalian brain has billions of neurons that can be classified into thousands of different types. These neurons are connected within circuits by trillions of synaptic contacts. Interestingly, the behavior complexity depends less on the variety of neurons than on their organization into anatomical circuits with precise functions. One key organizational principle of the brain, therefore, is that neurons with similar properties can produce different actions because of the way they are interconnected. Thus, studying single neurons might not be sufficient to understand how the brain works. The patterns of their connections and the strengths and properties of their functional interactions determine how neural circuits process information [2; 3].

In trying to understand in detail how neurons act to generate complex behaviors there is the need to carefully analyze the circuitry in which neurons participate. Thus, key questions in neuroscience include [2]:

- What are the computations processed by neural circuits and how does this activity contributes to behavior?
- How are neural circuits modified during learning and memory?
- What are the changes in neural circuits that giving rise to neurological and psychiatric disorders?

In order to explore these questions a combination of molecular and cellular biology together with neural anatomy, physiology and behavioral biology approaches can be used [1].

Recent progress has been made in the development of tools that allow genetic dissection of neural circuits. Genetic targeting is critical to dissect the role of specific cell types in neural circuits, including mapping connectivity, measuring activity, activating and inactivating specific neurons and understanding the contribution of particular neuronal components [3].

Since behavior is the observable output of the brain, animal behavior experiments have been regarded as crucial to understand brain function. Monitoring the changes that occur in a known circuit when an animal is participating in a task provides the opportunity to reveal what actually happens in the brain during behavior. To facilitate the identification of brain networks devoted to specific complex functions, more sophisticated and sensitive analyses of behavioral tasks have been developed [1; 4].

A powerful way to access how neural circuits process information is by combining genetic tools that allow manipulation of gene expression in identified cell-types together with animal behavior paradigms. This methodology ultimately enables the establishment of causality between the activity in specific groups of neurons, the function of neural circuits and contribution to specific features of animal behavior.

1.2 Neural Circuits underlying Movement

Even though the organization of neural circuits varies greatly according to the function, there are some characteristic features. One major feature is the direction of information flow in any circuit, which is crucial to understand its purpose. Generally, neurons carrying information towards the brain or the spinal cord are called afferent neurons. Those carrying information away from the brain or the spinal cord are named efferent neurons. There are also neurons that are only involved in more local aspects of a circuit, that are named interneurons or local circuit neurons. These three functional classes - afferent, efferent and interneurons - are the basic constituents of all neural circuits [1].

A simple reflex circuit, the knee-jerk response (also known as the myotatic reflex), exhibits several characteristics regarding the functional organization of neural circuits. First, the stimulation of peripheral sensors (receptors associated with muscle stretch in this case) triggers action potentials which travel along the afferent axons of the sensory neurons. This information then stimulates motor neurons in the spinal cord via synaptic contacts. The action potentials triggered in motor neurons then travels in efferent axons, giving rise to muscle contraction; a behavioral response. In detail, the excitatory synaptic connections between the sensory afferents and the extensor efferent motor neurons cause the extensor muscles to contract; at the same time, the interneurons activated by the afferents are inhibitory and their activation suppresses electrical activity in flexor efferent motor neurons causing flexor muscles to become less active. This results is a complementary activation and inactivation of the agonist and antagonist muscles con-

trolling the position of the leg [1].

Circuits that compute similar types of information constitute neural systems that are associated with broad behavioral purposes. Generally, these systems are divided into sensory, motor and cognitive systems. Movements, whether voluntary or involuntary, are produced by spatial and temporal patterns of muscular activity regulated by the motor system [1].

Motor regions of the cortex and brainstem contain upper motor neurons that initiate movement by controlling the activity of interneurons and lower motor neurons in the brainstem and spinal cord. Nonetheless, two additional regions of the brain are crucial in motor control: the basal ganglia and the cerebellum, both influencing movement by regulating the activity of upper motor neurons. Thus, the neural circuits responsible for controlling movement can be divided into four different but highly interactive subsystems: the lower motor neurons, the upper motor neurons, the basal ganglia and the cerebellum [1].

The first is the local circuitry located in the gray matter of the spinal cord and its similar circuitry in the brainstem. The relevant cells include the lower motor neurons (that project to out of the brainstem and spinal cord in order to innervate the skeletal muscles of the head and body, respectively) and the interneurons (that are the represent the major synaptic input to the lower motor neurons). Information regarding the control of movements, whether reflexive or voluntary, is ultimately conveyed to the muscles by the activity of the lower motor neurons; thus these neurons are responsible for the final common path for movement. The interneurons receive sensory inputs as well as projections from higher centers. Thus, the circuits formed by interneurons provide much of the control between different muscle groups that is essential for coordinated movement. For example, even after the spinal cord is disconnected from the brain in an experimental animal, appropriate stimulation of local spinal circuits elicits highly coordinated limb movements that despite being involuntary still resemble walking [1].

The second motor subsystem is constituted by the upper motor neurons whose cell bodies are located in the brainstem or cerebral cortex and whose axons descend to contact with the interneurons or, more rarely, with the lower motor neurons directly. The upper motor neuron pathways located in the cortex are essentially responsible for the initiation of voluntary movements and for complex spatiotemporal sequences of skilled movements. In particular, projections descending from the cortical areas in the frontal lobe, including the motor cortex, are involved in planning, initiation and direction of sequences of voluntary movements. Upper motor neurons originating in the brainstem are responsible for regulating muscle tone and for orienting the eyes, head and body according to vestibular, somatic, auditory and visual sensory information. Hence, the contributions of upper motor neurons are essential for movements related to basic navigation and the control of posture [1].

There are also complex circuits with output pathways that do not directly access either the interneurons or the lower motor neurons; instead, they control movement by controlling the activity of the upper motor neurons. The third subsystem consists of a

group of structures collectively referred to as the basal ganglia. The basal ganglia suppress unwanted movements and prepare (or 'prime') upper motor neuron circuits for the initiation of movements [1].

The fourth and larger of these subsystems, the cerebellum, acts via its efferent pathways to the upper motor neurons as a servomechanism, detecting the difference (or 'motor error') between the intentional movement and the movement actually performed. This 'error-sensing machine' then uses this information to mediate both real-time and long-term reductions in these motor errors [1].

1.3 Cerebellar Structure

The cerebellum has a well-defined structure constituted by a series of highly regular repeating units, each of which containing the same basic microcircuit. Different regions of the cerebellum receive projections from different parts of the brain and spinal cord and project to different motor systems. Nonetheless, the similarity of the architecture and physiology in all regions of the cerebellum might imply that different cerebellar regions perform similar computational operations on different inputs [5].

The cerebellum is located at the bottom of the brain, with the cerebral cortex above it. Such as the cerebral cortex, the cerebellum is divided into two hemispheres but it also contains a narrow midline zone - the vermis (Figure 1.1A). Superficially, the cerebellum consists of a cortex with three layers folded in thin, parallel strips called folia, which are transverse to the long axis of the body. Thus, the surface aspect of the cerebellum is due to the fact that it is made up of a very tightly folded layer of gray matter, the cerebellar cortex. Underneath the gray matter of the cortex lies white matter, formed by myelinated nerve fibers projecting to and from the cortex. Within the white matter there are three pairs of deep cerebellar nuclei, composed of gray matter. The cerebellum can be subdivided on the basis of phylogeny, anatomy and function [5].

1.3.1 Organization of the Cerebellum

Based on the cerebellar surface appearance, two deep transverse fissures divide the cerebellum into three lobes: anterior, posterior and flocculonodular. The primary fissure on the dorsal surface separates the anterior and posterior lobes, which together form the body of the cerebellum (Figure 1.1A). The posterolateral fissure on the ventral surface separates the body of the cerebellum from the smaller flocculonodular lobe (Figure 1.1B). Each lobe extends through the entire cerebellum, from the midline to the most lateral part [5; 6].

The cerebellar hemispheres can be divided into three longitudinal zones: medial, intermediate and lateral. These zones differ in the type of information they receive and thus on their inputs. The medial zone is dominated by information from vestibular, somatosensory, visual and auditory regions. The intermediate zone receives proprioceptive and somatosensory information from the spinal cord, and information from the motor cortex via the pontocerebellar fibers. The lateral zone receives information via the pontine nuclei from the motor cortex, the premotor cortex and most of the cerebral cortex [5].

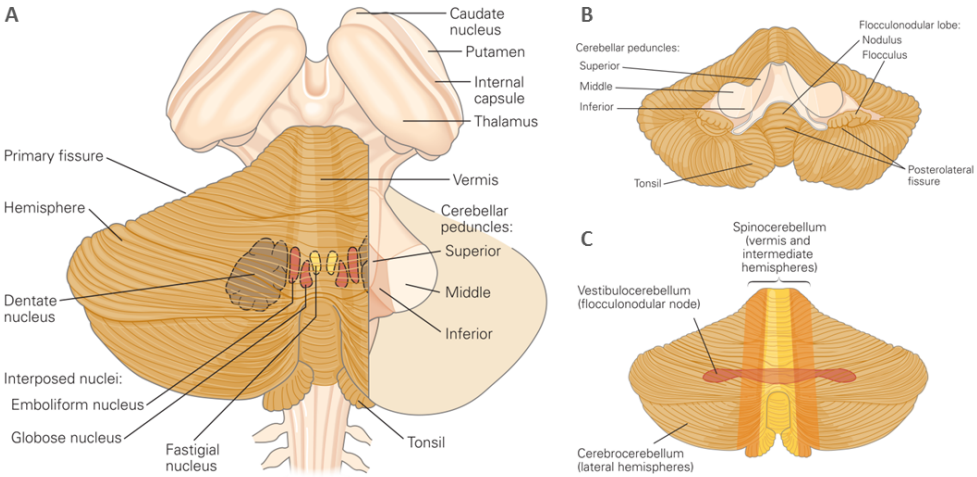

Figure 1.1: **Overall organization of the cerebellum.** A) Dorsal view of the left hemisphere, showing the location of the deep cerebellar nuclei. Part of the right hemisphere has been removed to reveal the underlying cerebellar peduncles. B) The cerebellum is shown detached from the brainstem, revealing the cerebellar peduncles on the anterior aspect of the inferior surface. C) Functional cerebellar regions. Adapted from [6]

The cerebellum can be subdivided into three main parts based on differences in their sources of input and their distinctive roles in different kinds of movements; vestibulocerebellum, spinocerebellum and cerebrocerebellum (Figure 1.1C) and (Figure 1.2). The vestibulocerebellum consists of the flocculonodular lobe and is the most primitive part. As its name suggests, the vestibulocerebellum receives vestibular and visual inputs and projects to the vestibular nuclei in the brainstem. It is primarily concerned with the regulation of movements underlying posture and equilibrium but it also participates in other vestibular reflexes and eye movements [1; 6].

Other main part of the cerebellum is the spinocerebellum, which comprises the vermis and intermediate parts of the hemispheres. It is named so because it is the only part that receives somatosensory and proprioceptive inputs directly from the spinal cord. The central part, called the vermis, receives visual, auditory and vestibular input as well as somatosensory input from the head and proximal parts of the body. It is primarily concerned with movements of proximal muscles and also regulates eye movements in response to vestibular inputs. The lateral part of the spinocerebellum also receives somatosensory input from the limbs and is primarily concerned with movements of distal muscles, such as relatively gross movements of the limbs in walking [1; 6].

The last of the major subdivisions is the cerebrocerebellum, which occupies the lateral cerebellar hemisphere and receives input from many areas of the cerebral cortex. It is the phylogenetically most recent and by far the largest subdivision in humans. Almost all of the inputs and outputs related to this region involve connections with the cerebral cortex. The cerebrocerebellum is concerned with the regulation of highly skilled movements, especially the planning and execution of complex spatial and temporal sequences

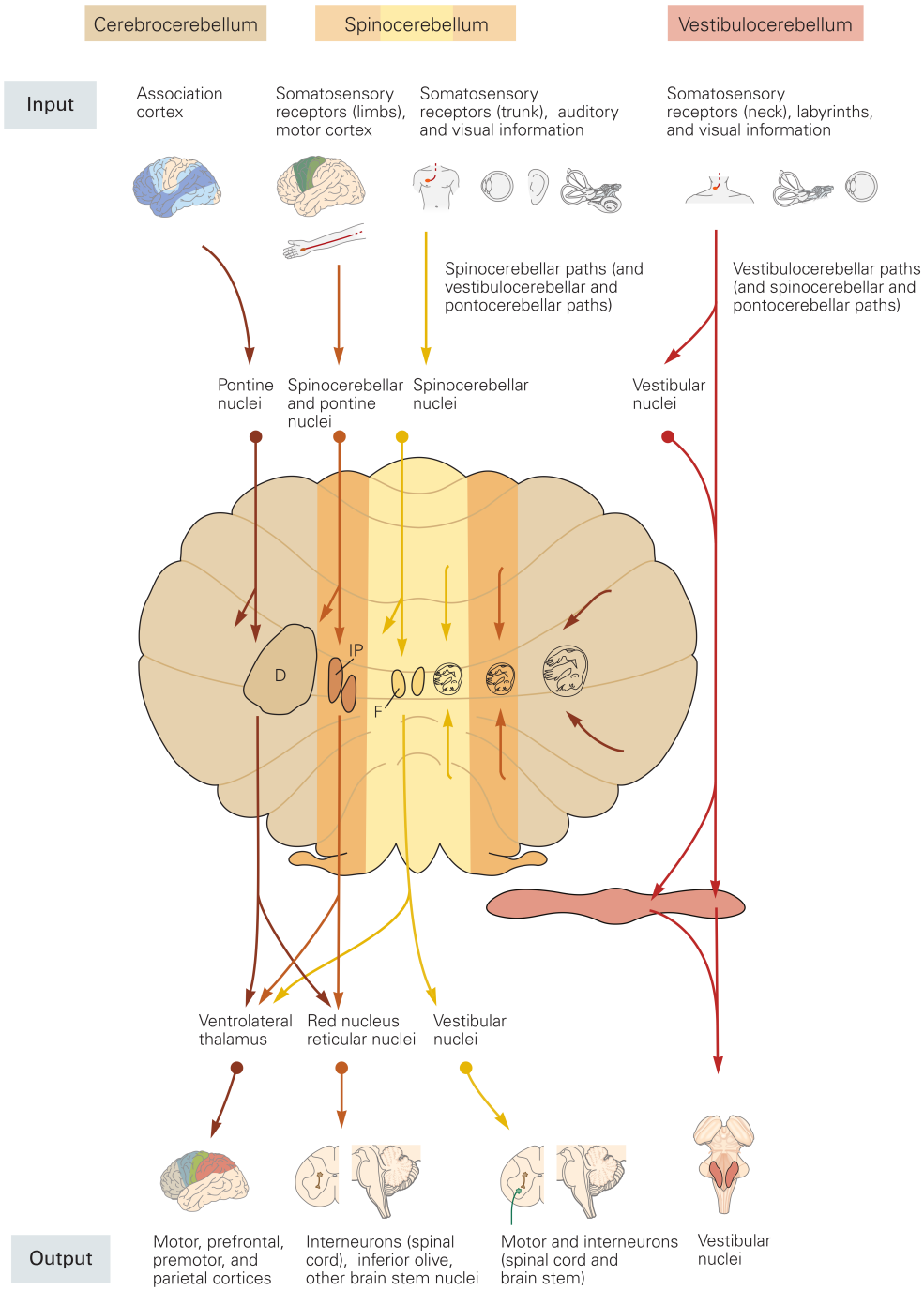

Figure 1.2: **Functional areas of the cerebellum.** The cerebellum is shown unfolded with arrows elucidating the inputs and outputs of the different functional areas. Major cerebellar subdivisions: cerebrocerebellum, spinocerebellum and vestibulocerebellum. Deep cerebellar nuclei: dentate nucleus (D), interposed nucleus (IP) and fastigial nucleus (F). [6]

of movement. It may also have a role in working memory [1; 6].

The connections between the cerebellum and other parts of the nervous system occur through three large pathways called cerebellar peduncles; superior, middle and inferior (Figure 1.1A). Most of the output axons from the cerebellum come from the deep cerebellar nuclei and project through the superior cerebellar peduncle. Nonetheless, the output from the flocculonodular lobe projects to the vestibular nuclei in the brainstem [1; 6].

The superior cerebellar peduncle is almost entirely an efferent (output) pathway. The nuclei of the neurons that give rise to this pathway are in the deep cerebellar nuclei and their axons project to upper motor control neurons in the red nucleus, the deep layers of the superior colliculus and, after a relay in the dorsal thalamus, the primary motor and premotor areas of the cortex [1].

The middle cerebellar peduncle is an afferent (input) pathway to the cerebellum; most of the cell bodies that give rise to this pathway are in the base of the pons where they form the pontine nuclei. The pontine nuclei receive input from a high variety of sources, including almost all areas of the cerebral cortex and the superior colliculus. The axons of the pontine nuclei cross the midline and enter the cerebellum via the middle cerebellar peduncle. Each of the two middle cerebellar peduncles contains over 20 million axons, making this one of the largest pathways in the brain. Most of these pontine axons carry information from the cortex to the cerebellum [1].

Lastly, the inferior cerebellar peduncle is the smallest but the most complex of the cerebellar peduncles, containing several afferent and efferent pathways. Efferent pathways in this peduncle project to the vestibular nuclei and the reticular formation. The afferent pathways include axons from the vestibular nuclei, the spinal cord and different regions of the brainstem [1].

1.3.2 Inputs to the Cerebellum

The cerebral cortex is considered the largest source of inputs to the cerebellum, and most of these inputs project to the cerebrocerebellum. The majority of these pathways originate in the primary motor and premotor cortices of the frontal lobe, the primary and secondary somatic sensory cortices of the anterior parietal lobe and the secondary visual regions of the posterior parietal lobe. The visual input to the cerebellum is mainly originated in association areas responsible for the processing moving visual stimuli. Indeed, coordination of ongoing movement guided by visual input is one of the major tasks carried out by the cerebrocerebellum. Most of these cortical relay in the pontine nuclei before entering the cerebellum [1].

The cerebellum also receives input from sensory areas. Vestibular axons from the cranial nerve and axons from the vestibular nuclei in the medulla project to the vestibulocerebellum. In addition, interneurons in the spinal cord send their axons to the spinocerebellum. The vestibular and spinal inputs transmit the cerebellum information from the labyrinth in the ear, from muscle spindles and from other mechanoreceptors that are responsible to monitor the position and motion aspects of the body. The somatic sen-

sory input is topographically mapped in the spinocerebellum such that representations of the body surface exist within the cerebellum. Currently it is thought that each small area of the body surface is represented multiple times by spatially separated clusters of cells rather than by a specific site [1]. However, this scheme differs from others in which each major subdivision controls all parts of the body for a specific range of behaviors [5]. The vestibular and spinal inputs are ipsilateral concerning their side of entry in the brainstem, running in the inferior cerebellar peduncle. This arrangement allows the right cerebellum to regulate the right half of the body and the left cerebellum the left half, in contrast to most areas of the brain [1].

Finally, the entire cerebellum receives modulatory inputs from the inferior olive and the brainstem. These nuclei participate in the motor learning and memory functions of the cerebellar circuitry [1].

1.3.3 Outputs of the Cerebellum

Except for a direct projection from the vestibulocerebellum to the vestibular nuclei, the cerebellar cortex projects to the deep cerebellar nuclei. Apart from the vestibular nuclei, which are outside of the cerebellum, there are four major deep cerebellar nuclei: the dentate nucleus, two interposed nuclei and the fastigial nucleus (Figure 1.1A). Each receives input from a different region of the cerebellar cortex. And although the limits are not clear, in general, the cerebrocerebellum projects primarily to the dentate nucleus, the spinocerebellum to the interposed nuclei and the vestibulocerebellum to the vestibular and fastigial nuclei [1].

The vestibular nuclei and the deep cerebellar nuclei project to upper motor neurons situated in the cortex (via a relay in thalamus) and in the brainstem. The thalamic nuclei that receive projections from the deep cerebellar nuclei project directly to primary motor and premotor association cortices. Thus, the cerebellum has access to the upper motor neurons that organize the sequence of muscular contractions underlying complex voluntary movements. Pathways originated in the deep cerebellar nuclei also project to upper motor neurons in the red nucleus, the superior colliculus, the vestibular nuclei and the reticular formation [1]. Since each cerebellar hemisphere is concerned with the ipsilateral side of the body, each deep cerebellar nucleus controls movement of ipsilateral parts of the body. Nonetheless, different deep nuclei differentially control movement. The dentate controls voluntary movements of the extremities of the body, such as reaching and grasping for objects. The interposed nuclei control stretch, contact, placing and other reflexes. The vestibular and the fastigial nucleus control eye and head movements, equilibrium, upright stance and gait [5].

1.3.4 Circuits within the Cerebellum

The cerebellar cortex contains three distinct layers that are constituted by distinct types of neurons performing different operations (Figure 1.3). The granule layer is the deepest and is the input layer to the cerebellum. It contains a very high number of granule cells, estimated to be 100 billion, which appear like small, densely packed and darkly stained nuclei. These cells are thought to be the most abundant class of neurons in the brain. Interneurons, such as Golgi cells, cells of Lugaro, unipolar brush cells and chandelier cells

are also present in this layer. The mossy fibers, one of the major inputs to the cerebellum, project to this layer. Mossy fibers excite the granule cells and Golgi interneurons, and in turn Golgi cells inhibit granule cells; these three cells interact in a structure called cerebellar glomeruli [6].

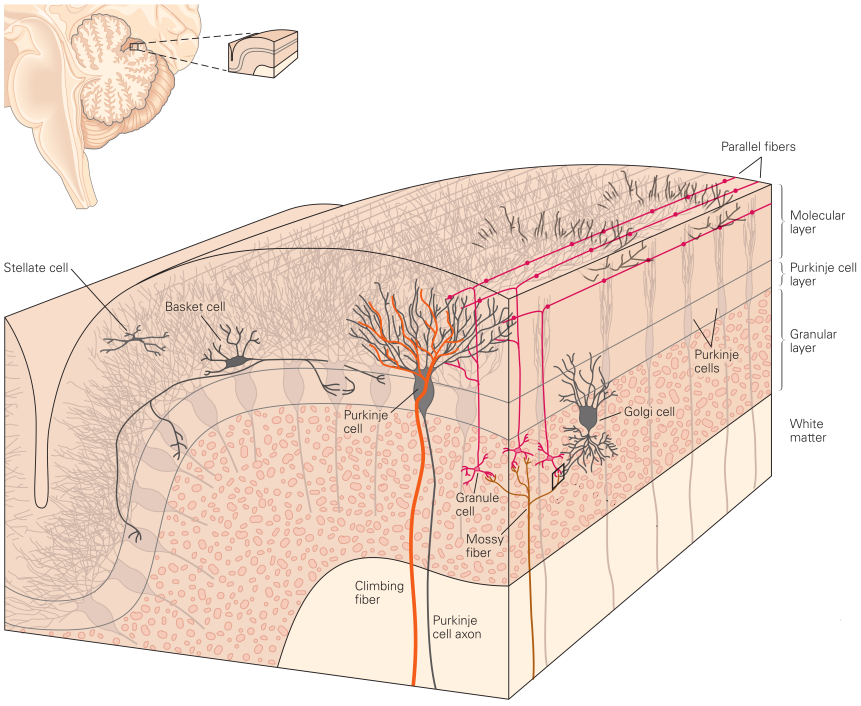

Figure 1.3: **Neurons and circuits within the cerebellum.** Vertical section of a single cerebellar folium illustrating the general organization of the cerebellar cortex. Adapted from [6]

In the middle is the Purkinje cell layer, the output layer of the cerebellar cortex. This layer consists mainly on Purkinje cell bodies. Purkinje cells dendrites extend upward into the molecular layer where they receive inputs from the other major input to the cerebellum, the climbing fibers. Interneurons also synapse onto the Purkinje cells. Purkinje cell axons are responsible for the output of the cerebellar cortex and project to the deep nuclei that located in the white matter that is underneath or exceptionally to the vestibular nuclei in the brainstem [6].

The most superficial is the molecular layer, which is an important processing layer. It contains the cell bodies and dendrites of two types of inhibitory interneurons, the stellate and the basket cells, as well as the extensive dendrites of the Purkinje cells. It also contains the axons of the granule cells, which are called parallel fibers due to the fact that they run parallel to the long axis of the folia. Parallel fibers branch to form a T-shape in order to transmit information via excitatory synapses onto the dendritic spines of the Purkinje cells. Parallel fibers run in the medial-lateral direction, and so they are oriented perpendicularly to the dendritic arbors of the Purkinje cells. Due to

its parallel fibers, each granule cell has the potential to make a few synapses with a large number of Purkinje cells. However, each granule cell also synapses onto Purkinje cells as its axon ascends into the molecular layer, forming very dense connections [6].

The ultimate destination of the two major inputs to the cerebellum, mossy fibers and climbing fibers, is the Purkinje cells. Both mossy fibers and climbing fibers form excitatory synapses with cerebellar neurons, however, they terminate in different layers of the cerebellar cortex, induce different patterns of firing in the Purkinje cells and are also thought to mediate different functions [1; 6].

Mossy fibers originate from cell bodies in the spinal cord and brainstem and are responsible for carrying sensory information from both the periphery and the cerebral cortex. Neurons in the pontine nuclei (a part of the pons, located in the brainstem) and other sources receive a projection from the cerebral cortex and then transmit that information to the contralateral cerebellar cortex. The axons from the pontine nuclei and other sources, the mossy fibers, do not project directly onto the Purkinje cells; instead they excite granule cells in the granular cell layer of the cerebellar cortex. Each granule cell receives input from just a few mossy fibers, but the anatomic features of the granule cells allows for a wide distribution from each mossy fiber to a large amount of Purkinje cells. In this way each Purkinje cell receives a huge amount of granule cell inputs; on the order of tens of thousands [1; 6].

The Purkinje cells also receive a direct modulatory input from the climbing fibers, which originate in the inferior olive and carry sensory information from both the periphery and the cerebral cortex. Each climbing fiber surrounds the cell body and proximal dendrites of one Purkinje cell, making numerous synaptic contacts. Each climbing fiber contacts 1 to 10 Purkinje cells, but each Purkinje cell receives input from only one climbing fiber. The terminals of the climbing fibers are arranged in such a way that the axons from clusters of related neurons in the inferior olive terminate in strips that extend across several folia. In turn, Purkinje cells within one strip project to a common group of deep nuclear neurons. They are the only output of the cerebellar cortex. Since Purkinje cells are GABAergic, the output of the cerebellar cortex is therefore inhibitory. However, the neurons in the deep cerebellar nuclei receive excitatory input from the mossy and climbing fibers. The Purkinje cell inhibition of the deep cerebellar nuclei neurons is thought to modulate the level of this excitation [1; 6].

The high specificity of the climbing fiber connections contrasts with the large convergence and divergence of the mossy fibers/parallel fibers, which suggests a specialization of the climbing fiber system for the precise control of the Purkinje cells activity [6].

Mossy and climbing fibers have very different effects on Purkinje cells electrical activity (Figure 1.4). Climbing fibers influence Purkinje cells activity in an unusual and very strong way. Each action potential in a climbing fiber results in prolonged depolarization of the Purkinje cell producing a complex spike: an initial large-amplitude action potential followed by a high-frequency burst of smaller-amplitude action potentials. Climbing fibers spontaneously generate complex spikes at low rates; no more than one to three spikes per second. When stimulated by specific sensory events, these cells fire single

action potentials. Therefore, the climbing fiber system seems specialized to detect specific events. Although climbing fibers do not fire very frequently, synchronous firing of multiple climbing fibers is thought to signal important events. This synchrony is in part due to the fact that neurons in the inferior olive often are connected to each another through electric synapses [6].

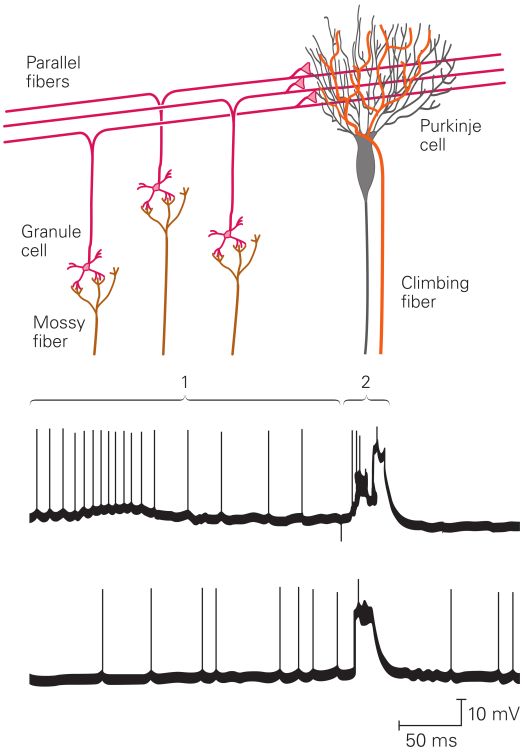

Figure 1.4: **Simple and complex spikes recorded from a cerebellar Purkinje cell.** Simple spikes are produced by mossy fiber input (1), whereas complex spikes are triggered by climbing fiber inputs (2). [6]

On the other hand, parallel fibers produce brief, small excitatory potentials which generate very small postsynaptic potentials in Purkinje cells - simple spikes. Thus, inputs from a large amount of parallel fibers are needed to have a significant effect on the frequency of simple spikes. Like climbing fibers, parallel fibers also have a spontaneous firing; however, it is much higher, up to 100 spikes per second. Nonetheless, when stimulated during active movement, Purkinje cells fire at rates of several hundred of spikes per second. This happens presumably due to the large amount of somatosensory, vestibular and other sensory signals that arrive to the granule cells via the mossy fibers. Thus, the mossy fiber system is responsible for carrying information about the amplitude and duration of behaviors generated by peripheral stimuli, by controlling the firing rate of simple spikes in Purkinje cells [6].

Generally, parallel fibers are thought to be responsible for transmitting sensory context information and climbing fibers to transmit the message of motor error to the Purk-

inje cells, being therefore responsible for the sensory feedback [1].

An important characteristic of the cerebellar circuit is that excitatory and inhibitory inputs converge both in the cerebellar cortex and in the deep cerebellar nuclei (Figure 1.5). The deep nuclei receive not only inhibitory inputs from Purkinje cells but also excitatory inputs from mossy and climbing fibers. Mossy fibers influence the neurons in the deep nuclei in two ways: by direct excitatory synapses or indirectly via the granule cell/parallel fiber circuit excitation and consequent Purkinje cell inhibition. Thus, the inhibitory output of the Purkinje cells modulates the excitatory signals transmitted from the mossy fibers to the deep nuclei. Most climbing fibers also project to the deep nuclei, in addition to the projection onto Purkinje cells [6].

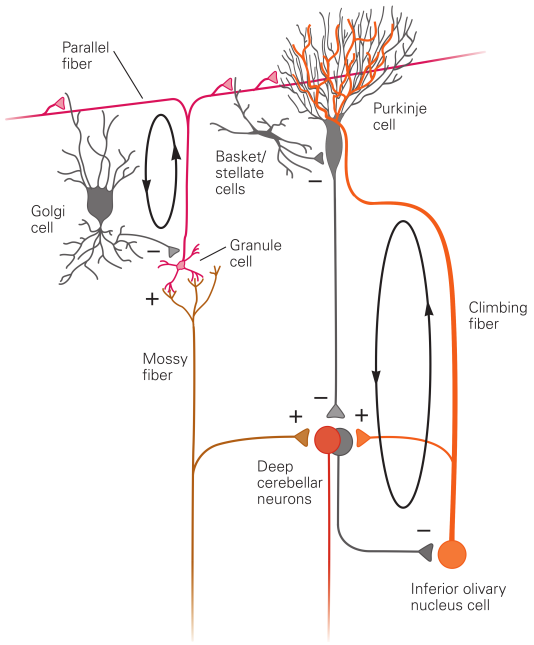

Figure 1.5: **Synaptic organization of the cerebellar circuit.** Excitatory and inhibitory information converge both at the cerebellar cortex and the deep nuclei. Recurrent loops involve Golgi cells within the cerebellar cortex and the inferior olive outside the cerebellum. [6]

As mentioned before, in the cerebellar cortex, both excitatory and inhibitory inputs converge on Purkinje cells. Parallel fibers directly excite Purkinje cells but also indirectly inhibit them by modulating the activity of interneurons. Inputs from interneurons modulate the inhibitory activity of Purkinje cells at both the dendrites and the cell body. The short axons of stellate cells contact the nearby dendrites of Purkinje cells, whereas the long axons of basket cells run perpendicular to the parallel fibers and form synapses on the Purkinje cell bodies. The stellate cells have a local inhibitory effect; a stellate cell and a Purkinje cell are both excited by the same parallel fibers. On the other hand, the basket cells are excited by several parallel fibers and represent the most powerful of these local inputs [6].

This basic circuit is repeated over and over throughout the cerebellum acting as a fundamental functional module. Modulation of signal flow through these modules provides the basis for both real-time regulation of movement and long-term changes in regulation that underlie motor learning [1].

Summarizing, the Purkinje cells receive two types of excitatory input from outside of the cerebellum, one directly from the climbing fibers (sensory feedback) and other indirectly from mossy fibers (sensory context) via the parallel fibers of the granule cells. The Golgi, stellate and basket cells are inhibitory interneurons that control the level of excitation of the Purkinje cells. The Purkinje cells modulate the activity of the deep cerebellar nuclei, which in turn are driven by the direct excitatory input they receive from the collaterals of the mossy and climbing fibers. The modulation of cerebellar output also occurs at the level of the Purkinje cells. These modulatory signals may be responsible for the motor learning aspect of the cerebellar function [1].

1.3.5 Recurrent Loops

The cerebellum has many check points within its circuitry. Generally, many parts of the cerebellum form recurrent loops with the cerebral cortex. The lateral cerebellum receives input from the cerebral cortex through relays in the pontine nuclei. In turn, the cerebral cortex receives input from the lateral cerebellum through relays in the thalamus. This recurrent circuit is organized as a series of parallel closed loops, such that one part of the cerebellum connects reciprocally with one part of the cerebral cortex [6].

Other cerebellar recurrent loop involves the inferior olivary nuclei, which are the source of all climbing fibers (Figure 1.5). The deep cerebellar nuclei are formed by GABAergic inhibitory neurons that project to the inferior olive. When the inhibitory inputs from the deep nuclei increase, the frequency of firing in the inferior olive decreases, reducing the amount of excitatory climbing fiber input to the deep cerebellar nuclei and Purkinje cells. In this way, each part of the cerebellum is able to regulate its own climbing fiber inputs. Curiously, the GABAergic fibers from the deep nuclei can regulate the electrical conductivity between neurons in the inferior olive. By selectively disconnecting neurons in the inferior olive through inhibition, one specific array of Purkinje neurons can be synchronously activated [6].

The last loop occurs within the cerebellar cortex and involves the Golgi interneurons (Figure 1.5). Each Golgi cell receives excitatory input from parallel fibers and in turn, its GABAergic terminals provide inhibitory input to the granule cells. Thus, Golgi cell firing suppresses mossy fiber excitation of the granule cells and consequently regulates the firing of the parallel fibers. This loop might be responsible for controlling the duration of activity in the granule cells. Other idea is that it can also control the amplitude of the excitatory response of the granule cells to their mossy fiber inputs; so that the responses of granule cells can only occur when a certain number of mossy fiber inputs are reached or only when the frequency of firing reaches a certain threshold. So, Golgi cells might make sure that only a small number of granule cells are active in each moment, creating a sparse code input to the cerebellar cortex [6].

1.4 Cerebellar Function

Ideas about cerebellar function have been driven largely by the effects of dysfunction or lesion of the cerebellum together with anatomical and physiological properties of cerebellar inputs, outputs and circuitry. From such evidence it has long been clear that the cerebellum is a crucial component of the motor system [5].

1.4.1 Cerebellar Disorders

Patients with cerebellar damages, regardless of the causes or location, exhibit persistent errors in movement. These movement errors are on the same side of the body as the damage to the cerebellum, reflecting the cerebellar special structure in which sensory and motor information is represented ipsilateral instead of contralaterally. Furthermore, the fact that somatic, visual and other inputs are represented topographically within the cerebellum makes the movement deficits to be quite specific. Hence, the topographical organization of the cerebellum allows cerebellar damage to disrupt the coordination of movements performed by some muscle groups but not others [1].

1.4.2 Lesions of Cerebellar Subdivisions

Cerebellar deficits can vary depending on which cerebellar structures have been damaged, and whether the lesion is bilateral or unilateral. Fulton and Dow have described behavioral abnormalities resulting from damage of the three functional divisions of the cerebellum [5].

Damage to the vestibulocerebellum impairs the ability to stand upright and maintain the direction of the eyes; as the eyes exhibit difficulty in fixate and drift from the target and then jump back with a corrective saccade, a phenomenon called nystagmus. Disruption of the pathways to the vestibular nuclei may also result in loss of muscle tone and consequent muscle atrophy. In contrast, patients with damage to the spinocerebellum have difficulty controlling movements related to walking. These patients have a wide-based gait with shuffled movements, which represents the inappropriate operation of ensembles of muscles that normally rely on sensory feedback to produce smooth and coordinated actions. The patients also have difficulty performing rapid alternating movements such as the finger-to-nose test, a sign referred to as dysdiadochokinesia. Over and under reaching may also occur - dysmetria. During these over and undershooting movements, there are tremors (also called action or intention tremors) due to the disruption of the mechanisms responsible for detecting and correcting errors associated with movements. Finally, lesions of the cerebrocerebellum produce impairments in highly skilled sequences of learned movements, such as speech or playing a musical instrument [1; 5].

1.4.3 Lesions of Cerebellar Nuclei

Observations of damage to cerebellar subdivisions were confirmed and extended with lesions of the deep cerebellar nuclei in cats and monkeys by Sprague, Chambers, Thach, Dow, among others [5].

Ablations of the fastigius dramatically impair movements that require control of equilibrium, such as unsupported sitting, stance and gait. Longitudinal splitting of the cerebellum along the midline also produces long lasting disturbances of equilibrium. These data suggest that the fastigius is likely involved in movements like gait and stance, which is consistent with disturbed vestibular function. Lesions of the interpositus primarily cause tremor. Temporary inactivation of both the interpositus and the dentate elicits tremor that is dependent on proprioceptive feedback but is also influenced by vision. In monkeys, interpositus inactivation minimally perturbs gait but causes an action tremor of high amplitude as the animals reach food. These studies support the idea that the interpositus is more responsible for the balance of agonist and antagonist muscle activity of a limb as it moves, which is in agreement with the fact that it is the major output of the spinocerebellum. The interpositus may use the afferent input information received from the periphery to generate predictive signals that decrease alternating stretch reflexes capable of causing limb oscillation. Impairments of the dentate nucleus produce slight delays in reaction time, poor terminal control of actions and impaired multi-jointed movements. Lesions of the dentate nucleus can also produce a slight delay in the reaction time of movements associated with visual and auditory inputs. In single-jointed movements, dentate ablation causes subjects to overshoot very slightly or moderately, whereas in multi-jointed movements it results in highly impaired reaching patterns. Thus, dentate lesions profoundly impair movements requiring coordination of multiple joints but affects single-jointed movements only slightly, which is consistent with the knowledge about the cerebrocerebellum [5].

Summarizing, cerebellar diseases and lesions disrupt the modulation and coordination of movement. Thus, the hallmark of patients with cerebellar damage, regardless of the site of lesion, is difficulty in producing smooth, well-coordinated movements. Instead, movements tend to be jerky and imprecise, a condition referred to as cerebellar ataxia. Disruption of the error-correcting cerebellar mechanisms - motor learning - is thought to account for many of these difficulties in performing movements [1].

Research on the anatomy, physiology and function have been crucial to understand that the cerebellum and its associated systems enable an internal comparison of an intended movement with the actual movement leading to a reduction in the difference, or 'motor error'. The corrections of motor error produced by the cerebellum occur both in real-time and over longer periods, as motor learning. It remains to be fully established what are the learning rules that determine these corrections and which specific neurons and/or synapses are altered.

1.4.4 Motor Learning

One hypothesis regarding cerebellar function considered the cerebellum as a primary site for motor learning. The general hypothesis of cerebellum-dependent motor learning has been supported by lesion, stimulation and electrophysiological recordings in several motor learning tasks. Nonetheless, how the cerebellum functions to contribute to both movement and motor learning is yet to be established. To determine the contribution of the cerebellum to motor error reduction, there is the need to establish cause-effect relationships between learning of motor responses and specific changes in neuronal responses

[7].

The fact that the cerebellum has a very standardized anatomy and physiology suggests that the cerebellum performs the same general computation for many different motor tasks [7]. Thus, progress regarding the dissection of cerebellar function has been made by studies using different cerebellum-dependent motor learning tasks, such as adaptation of the vestibulo-ocular reflex adaptation and classical eyelid conditioning.

1.4.5 Vestibulo-Ocular Reflex Adaptation

The adaptation of the vestibulo-ocular reflex (VOR) has been greatly used to study the neural mechanisms that underlie cerebellum-dependent learning. Anatomic, physiological, behavioral and computational studies about the VOR have contributed to the current knowledge about the neural circuits underlying this behavior and the synaptic sites that mediate this sensorimotor learning [8].

The VOR is essential to keep the eyes on a visual target during head movements. These reflex responses prevent visual images from sliding on the surface of the retina as head position varies. This means that when a visual image on the retina shifts its position as a result of head movement, the eyes must move at the same velocity in the opposite direction in order to stabilize the stimulus. The action of the vestibulo-ocular movements can be appreciated by fixating an object and moving the head from side to side; the eyes automatically compensate for the head movement by moving the same distance in the opposite direction, thus keeping the object nearly on the same place of the retina [1]. Turning the head in one direction causes the eyes to rotate in the opposite direction in nearly equal amplitude, even in complete darkness. Therefore, the gain of the VOR, which is defined as eye speed divided by head speed in darkness, is usually close to 1 [8].

In VOR studies, the adaptability to changes in the nature of incoming sensory information is assessed by making subjects use either magnifying or minifying glasses. By modifying the optical stimulus, a VOR with a gain of 1 is no longer appropriated to stabilize retinal images. Because the glasses alter the size of the visual image on the retina, the compensatory eye movements are either too large or too small. On the one hand, if subjects wear magnifying glasses there is image motion in the opposite direction from head motion and it requires increases in the gain of the VOR. On the other hand, minifying glasses cause image motion in the same direction as head motion and require decreases in VOR gain [1; 8].

Over time, subjects learn to adjust the distance that the eyes must move in response to head movements in order to compensate for the artificially altered size of the visual field. Learning in the VOR usually takes several days and is retained even after the glasses are removed. Information that reflects this change in the sensory context of the VOR must therefore be relearned and remembered to eliminate error that was artificially introduced during the experiment. An altered VOR returns to normal over approximately the same time course as for the learning. Repeated learning with magnifying and minifying glasses does not appear to be long-term stored [1; 8].

Each head turn is associated with the motion of images in the retina. Thus, at the circuit level, the VOR adaptation consists of two stimuli: the vestibular stimulus (head turn) and the visual stimulus (image motion). The vestibular stimulus is transmitted via mossy fibers and the visual stimulus is transmitted via both mossy fibers and climbing fibers. Both stimuli converge at the level of the cerebellar cortex and cerebellar nuclei. For adaptation of the VOR, the floccular complex is a relevant region of the cerebellar cortex and the nucleus involve is the parallel to the deep cerebellar nucleus for the VOR; the vestibular nucleus [7].

1.5 Eyelid Conditioning

Classical eyelid conditioning has been used to demonstrate that the cerebellum contributes to the correction of movements through experience and to investigate mechanisms underlying this behavior. In particular, studies on eyelid conditioning have been important in understanding the differential role of the cerebellar cortex and the cerebellar nuclei in sensorimotor learning and in characterizing the role of the cerebellum in the timing of learned responses [7; 9].

Eyelid conditioning is a form of classical or Pavlovian conditioning. Pavlovian conditioning is a simplest form of associative learning by which animals learn relations among external events so that their future behaviors are better adapted to their environments [10]. Through several studies using different techniques, including lesion, recordings, stimulation, reversible inactivation, imaging and computer simulation it was show that the cerebellum is essential for classical eyelid conditioning motor learning [9].

Like for the VOR adaptation, there are several advantages in using eyelid conditioning to understand cerebellar-dependent learning mechanisms: 1) it can be precisely induced with stimuli that can be easily controlled, 2) there is a relatively direct mapping of stimuli into the cerebellar circuit 3) the output behavior is modified so that learning can be measured [9].

1.5.1 Paradigm

The eyelid conditioning involves paired presentations of a neutral conditioned stimulus (CS; such as a light) and a reinforcing unconditioned stimulus (US; such as an air puff directed to the cornea of the eye). Light and puff stimuli are separated from each other by several hundred milliseconds - Inter Stimulus Interval (ISI) - and the light + puff paired trials are in turn separated by several seconds - Inter Trial Interval (ITI). A naive animal only closes its eye in response to the air puff and this unlearned behavior is referred to as the unconditioned response (UR). However, over the course of training, the animal gradually develops an eyelid response to the light, before the air puff is presented - conditioned response (CR) [11]. The CR mimics the UR, precedes the air puff onset time and is accurately timed, so that its peak coincides with the onset of the air puff. Another hallmark feature is that the CR amplitude is scaled according to the intensity of the stimuli. Learning can be induced by a wide range of contextual stimuli that include not only visual but also auditory and somatosensory. In the standard eyelid conditioning

paradigm, the CS is often a light, a tone or mild electrical skin stimulation. The US is usually a mild air puff directed to the cornea or a weak periocular electrical stimulus [12]. For simplicity purposes, from now on the conditioned stimulus (CS) will be referred to as light and the unconditioned stimulus (US) as a puff; even though the presented work was performed using different CS and US stimuli.

1.5.2 Delay and Trace

There are two variants of eyelid conditioning paradigms: delay and trace. In the delay paradigm, the light precedes the puff several hundred milliseconds and the stimuli temporally overlap and co-terminate. In the trace paradigm the light is followed by a period of time without stimulus before the puff is presented [13].

This subtle difference makes trace eyelid conditioning to be generally considered as relatively more complex than delay eyelid conditioning. It is considered that the animal must use some sort of internal memory or timing mechanism to successfully associate both light and puff stimuli [14]. Thus, whereas delay eyelid conditioning involves learning that is dependent on the cerebellum but can occur without the forebrain, trace eyelid conditioning requires the hippocampus, caudate nucleus, primary sensory cortex and medial prefrontal cortex in addition to the cerebellum [13].

Recent studies suggest that cerebellar learning of trace eyelid conditioning involves mossy fiber input driven by the medial prefrontal cortex. Trace eyelid conditioning requires a region of the medial prefrontal cortex that projects to the cerebellum via the lateral pontine nuclei. Moreover, during trace eyelid conditioning the medial prefrontal cortex seems to provide activity related to the conditioned stimulus that persists during the stimuli interval, suggesting that the forebrain provides a delay conditioning-like input to the cerebellum. Nonetheless, mossy fibers involved in trace conditioning are different from the ones involved in delay eyelid conditioning [13; 15].

Even though trace eyelid conditioning involves different brain areas, the cerebellum and its associated circuitry seem to constitute the entire essential circuit for delay eyelid conditioning [14].

1.5.3 Circuit

Cerebellar inputs and outputs related to eyelid conditioning learning have a relatively well-mapped circuit (Figure 1.6). Sensory context information related to the light stimulus is conveyed to the cerebellum via mossy fibers input [16; 17]. Sensory feedback information related to the stimulus of air puff directed to the eye, however, is conveyed via climbing fibers [17; 18; 19].

Both inputs converge at the level of the cerebellar cortex and cerebellar nuclei. Output from the cerebellum via one of the cerebellar nuclei drives the expression of conditioned responses [20]. Therefore, the behavioral properties of eyelid conditioning reflect the input/output processing of the cerebellum. The ability of paired light + puff presentations to promote conditioned eyelid responses underlies two basic features of cerebellar processing: 1) paired activation of mossy fiber and climbing fiber inputs eventually leads to

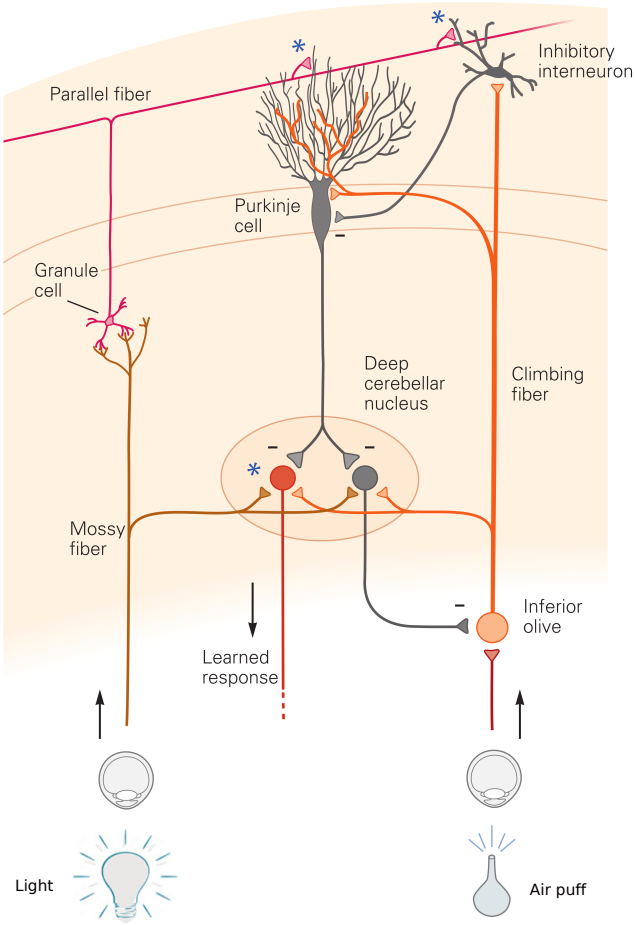

Figure 1.6: **Cerebellar circuit underlying classic eyelid conditioning.** Learning is thought to occur in the cerebellar cortex and in the deep cerebellar nuclei. Sites of learning are denoted by asterisks. [6]

increased cerebellar output in response to the same mossy fiber input alone and 2) the particular set of climbing fiber inputs activated by the puff determines which cerebellar nuclei cells and consequently muscles will become activated after learning. During eyelid conditioning, activation of climbing fiber input by the puff will result in increased activity in the deep cerebellar nucleus, which contains the subset of cells responsible for closing the eyelid [9].

Depending on the CS sensory modality (light, tone or electrical skin stimulation) the relevant regions involved in eyelid conditioning can vary. The light CS pathway seems to consist of unilateral parallel input from the lateral geniculate nucleus and optic tract to the medial pontine nuclei (contralateral to the conditioned eye) and its mossy fiber projection to the cerebellar cortex and nuclei [21]. On the other hand, despite the US sensory stimulus used (air puff or periocular electrical stimulus) the relevant cerebellar regions involved do not seem to vary. Thus, the air puff US pathway seems to consist of input from the trigeminal and spinal areas that receive sensory afferents from both the cornea/face to the inferior olive and its climbing fiber projection to the cerebellar cortex and nuclei [22]. For eyelid conditioning using these stimuli, the anterior lobe is a relevant region of the cerebellar cortex and the deep cerebellar nucleus involved is the anterior interpositus nucleus [22].

1.5.4 Temporal Properties

The fact that delay eyelid conditioning experiments allow to explore temporal properties of the motor responses has been useful in understanding important features about cerebellar processing. Initial studies have shown that for delay eyelid conditioning to be successful, the light must precede the puff onset (ISI) by at least 100ms and learning worsens as the interval increases beyond 500ms [23; 24]. Moreover, the ISI also affects an important feature of delay eyelid conditioning learning, the timing of the CR. Most experimental work supports the idea that, despite of the ISI used, the timing of the CR is achieved by adjusting the speed of the response so that the maximum eyelid closure occurs at the time when the puff is expected [25; 26; 27]. Thus, precise timing is achieved by modulating the velocity of the CR but not its onset. In addition, maximum eyelid speed seems to occur in the first 100ms after movement onset and does not seem to scale with the ISI, suggesting that the adaptive timing does not seem to be accomplished by modulating the movement speed in a uniform way throughout the duration of the response [27].

Moreover, the CR that is created when a particular mossy fiber input (in response to a light) reliably predicts the need to move a particular muscle as signaled by the climbing fiber input (in response to the puff) does not simply depend on learning a new cerebellar output. Because there is the need of protecting the eye by closing the eyelid right before the puff is delivered, the timing of the generated movement is also of extreme importance [9]. Thus, this temporally specific learning appears to be an intrinsic property of cerebellar processing.

Despite early controversy about the role of the cerebellar cortex in motor learning [20] several studies combining cerebellar cortex lesions with delay eyelid conditioning experiments managed to establish its involvement [28; 29]. Subsequently, cerebellar

cortex lesions were shown to disrupt the timing of previous learned responses, even though the animals still exhibited learned responses [30]. Post-lesion responses displayed a short and relatively fixed latency to the onset of the light; that was independent of their pre-lesion timing. This timing effect suggested a role of the cerebellar cortex in temporally specific learning but also provided basis to understand its role in the acquisition and extinction of the CRs [31; 32].

1.5.5 Acquisition and Extinction

Eyelid conditioning experiments involve an acquisition phase that gradually leads to the establishment of the CRs and is induced by successive presentation of paired light + puff trials. Extinction consists in a gradual decline of previously established CRs due to the repeatedly presentation of unpaired trials with light but not puff stimulus [12].

Acquisition is accompanied by a consolidation phenomenon in which the information is long-term stored and can be recalled. Reversible inactivation of cerebellar function during either acquisition or consolidation allows the identification of the anatomical and temporal location of related components [12]. Independent inactivation of cerebellar cortex [33], cerebellar nuclei [34] or inferior olive [35] prevents acquisition. These findings suggest that the three components are essential for normal acquisition, but do not localize where is the essential plasticity taking place. The role of the cerebellar cortex and cerebellar nuclei in memory formation has been dissociated by looking at consolidation of learning. Reversible inactivation of the cerebellar cortex with the GABA(A) receptor antagonist muscimol immediately after training prevents memory consolidation, whereas a similar procedure in the cerebellar nuclei does not, suggesting the importance of the cerebellar cortex in consolidation of learning [36]. These consolidation mechanisms have a delayed time course, beginning about one hour after training and ending around one hour after that [37]. This slow time course is similar to parallel fiber-Purkinje cell synaptic plasticity with normal activation of climbing fiber inputs [38].

Extinction phenomena can be prevented by blocking the inhibition in the inferior olive nuclei, suggesting that information related with the puff is normally signaled through the olive and climbing fiber system [39]. Despite the fact that convergence of information through mossy and climbing fiber systems into Purkinje cells is especially consistent with a role of the cerebellar cortex in motor learning, lateral projections of mossy and climbing fibers to the deep cerebellar nuclei also raise the possibility of an associative mechanism at the level of the cerebellar nuclei [12; 40].

Generally, and in parallel with work in the VOR, delay eyelid conditioning studies suggest that cerebellar learning dependents on plasticity in both the cerebellar cortex and cerebellar nuclei and that these sites contribute to different aspects of the learning. Lesion, stimulation and modeling studies have been used to dissociate their functions, suggesting a central role of the cerebellar cortex in temporally specific motor learning [9; 12].

1.6 Cerebellar Synaptic Plasticity Mechanisms

The hypothesis that cerebellar function is crucial for motor learning was based on the convergence of different input pathways at the level of Purkinje cells. As mentioned before, mossy fiber and climbing fiber pathways convey two very different types of sensory signals to the cerebellum. Mossy fibers convey contextual sensory signals required for the generation and coordination of movement. Climbing fibers convey sensory feedback, acting as a teaching signal [6].

The original cerebellar learning theory, the Marr-Albus-Ito theory, postulated that if parallel fiber-Purkinje cell synapse activation (which originates simple spikes in the Purkinje cell) and climbing fiber-Purkinje cell activation (which originates complex spikes in the Purkinje cell) coincided reliably, the parallel fiber-Purkinje cell synapse would be modified (Figure 1.7) [41; 42; 43]. This theory was originally based on models of Marr [41]. Later, Albus [42] elaborated it, suggesting that due to its anatomical features the cerebellar cortex is optimally designed for converging information and modifying Purkinje cell output. Recordings obtained by Ito confirmed this concept by showing that combined activation of mossy and climbing fiber inputs resulted in long-term depression (LTD) at the parallel fiber-Purkinje cell [43]. Changes in parallel fiber-Purkinje cell synaptic strength would cause changes in behavior through alterations in Purkinje cell output to downstream motor areas. Because climbing fibers appear to act as feedback cells in order to signal error, climbing fiber-dependent LTD would improve motor performance by weakening parallel fiber-Purkinje cell synapses that were consistently associated with errors [44]. In other words, sensory inputs conveyed by climbing fibers would report errors in a prior movement, and serve as instructions for LTD at the synapses from parallel fibers to Purkinje cells. LTD would then cause changes in Purkinje cell's simple-spike responses and thus improve the following movement.

During the past two decades several new types of cerebellar plasticity have been identified in vitro and numerous advances have been made in understanding their underlying molecular mechanisms [44; 45]. Apart from LTD at the parallel fiber-Purkinje cell synapse, also presynaptic and postsynaptic parallel fiber long-term potentiation (LTP) [46; 47; 48], climbing fiber LTD [49], inhibitory interneuron-Purkinje cell synapse LTP [38; 50; 51; 52] and long-term synaptic and non-synaptic changes of cerebellar and vestibular nuclei neurons (Figure 1.8) [53; 54; 55; 56]. Cooperatively or independently these synaptic plasticity processes might contribute to cerebellum-dependent sensorimotor learning.

1.6.1 Parallel Fiber-Purkinje Cell Synapse

Long-term synaptic plasticity at the parallel fiber-Purkinje cell synapse includes both pre and postsynaptic expression of LTD and LTP [44].

In parallel fiber LTD, a persistent attenuation of the parallel fiber-Purkinje cell synapse is produced when parallel fiber and climbing fiber inputs to the Purkinje cell are stimulated simultaneously at low frequency [57]. The requirements for parallel fiber LTD induction are typically associative; parallel fiber synaptic strength is not attenu-

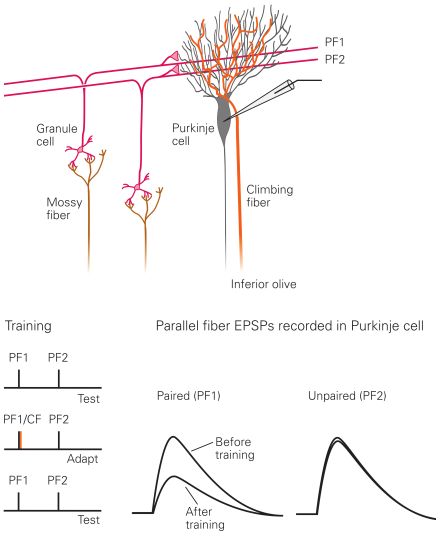

Figure 1.7: **Original theory of cerebellar motor learning.** Long-term depression (LTD) at the parallel fiber-Purkinje cell synapse induced by in vitro stimulation of both parallel (PF) and climbing fibers (CF). Co-activation of one set of parallel fibers (PF1) and climbing fibers produces a long-term reduction in the responses of those parallel fibers. The responses of a second set of parallel fibers (PF2) are not depressed because they are not simultaneously stimulated with the climbing fibers. Excitatory postsynaptic potential (EPSP). [6]

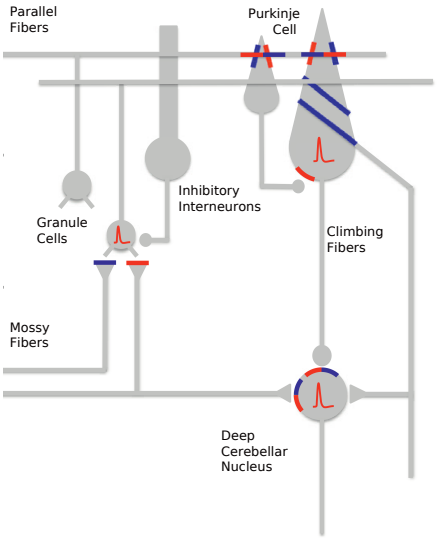

Figure 1.8: **Synaptic plasticity mechanisms in the cerebellum.** Long-term plasticity mechanisms described for the cerebellar circuit. Blue and red bars represent LTD and LTP, respectively. The bars are placed either pre or postsynaptically according to the most likely locus of expression. Changes in intrinsic excitability are represented by action potentials. [43]

ated neither by climbing fiber stimulation alone nor by parallel fiber stimulation alone nor by stimulation of both non-simultaneously [58]. Parallel fiber LTD expressed postsynaptically has three main requirements for induction: 1) climbing fiber-dependent influx of calcium through voltage-gated calcium channels, which occurs during the complex spike, 2) parallel fiber-dependent release of glutamate, which acts upon both mGluR1 metabotropic receptors and AMPA ionotropic receptors and 3) protein kinase C (PKC) activation [45]. Postsynaptic expressed LTD consists in the reduction in the number of functional AMPA receptors, via clathrin-mediated endocytosis [59; 60].

Presynaptic expressed parallel fiber LTP is mediated by the activation of calcium-sensitive adenylyl cyclase, leading to an increase in the concentration of cAMP and the consequent activation of protein kinase A (PKA). In fact, this type of plasticity can be induced by bath application of an adenylyl cyclase activator or membrane-permeable cAMP analogs [46]. Moreover, cerebellar LTP induced by granule cell stimulation is attenuated in the absence of calcium-sensitive adenylyl cyclase [61]. When using PKA inhibitors (either applied in the bath or presynaptically) induction of LTP is blocked [47]. Postsynaptic parallel fiber LTP is associated with a decrease in the rate of failures and the extent of neural facilitation. In addition, LTP can be detected in granule cell-glial cell interactions when postsynaptically recording evoked AMPA/kainite receptor-mediated currents [62] or glutamate transport currents [63]. All together, these observations supported, until relatively recently, the existence of a unique presynaptic site of expression. Nonetheless, there is now evidence showing that parallel-fiber LTP can also be expressed postsynaptically, is enhanced by chelating postsynaptic calcium and depends on nitric oxide but not cAMP or cGMP [48].

Several work have been suggesting that at the parallel fiber-Purkinje cell synapse the induction of postsynaptic LTD or LTD was different from the observed at other glutamatergic synapses, with LTD requiring a higher calcium threshold than LTP [64]. Moreover, it was observed that the climbing fiber-Purkinje cell synapse induction is able to change the probability of LTD or LTP induction by controlling the levels of postsynaptic calcium; with activity in the climbing fiber promoting postsynaptic LTD and impairing LTP [64; 65; 66]. However, postsynaptic calcium levels alone might not be sufficient to account for the bidirectional induction of long-term plasticity and more complex mechanisms might be involved as well [67]. Actually, it has been shown that, in addition to postsynaptic long-term plasticity, parallel fiber-Purkinje cell synapses are also modulated by both short and long-term presynaptic plasticity [46; 68]. Moreover, it was seen that climbing fiber-Purkinje cell synapse activity can also control presynaptic parallel fiber-Purkinje cell plasticity [68].

1.6.2 Climbing Fiber-Purkinje Cell Synapse

In vitro brief stimulation of climbing fibers induces parallel fiber-Purkinje cell LTD. Because climbing fiber-Purkinje cell LTD was not associated with a change in neural facilitation, it is thought this type of plasticity might be expressed postsynaptically. The molecular machinery required for LTD at the parallel fiber-Purkinje cell synapse (mGluR1, GluR2-containing AMPA receptors and PKC) is also present at the climbing fiber-Purkinje cell synapse. And similar to parallel fiber-Purkinje cell LTD, climbing

fiber-Purkinje cell LTD is blocked by loading the Purkinje cell with a calcium chelator or by bath application of mGluR antagonist or PKC inhibitor [49].

Despite the fact that only near 20% attenuation of excitatory postsynaptic currents (EPSCs) amplitude is produced, climbing fiber LTD can still influence the integrative response of the Purkinje cell [45]. Complex spikes are associated with a large dendritic calcium transient [69], which is likely attenuated as consequence of climbing fiber LTD originating a reduction in the second complex spike. This climbing fiber-evoked calcium transient has several important effects. It can briefly modulate dendritic excitability through effects on ion channels, such as calcium-dependent potassium channels, or on electrogenic proteins, such as the sodium-calcium exchanger [45]. Moreover, it is critical for inducing parallel fiber-Purkinje cell LTD [69], short-term potentiation of mGluR-mediated responses at the parallel fiber-Purkinje cell synapses [70] and both LTP [50] and transient depression [71] of GABAergic interneurons-Purkinje cell synapses.

1.6.3 Inhibitory Interneuron-Purkinje Cell Synapse

Repetitive climbing fiber activation may induce LTP of both spontaneous and evoked GABA(A) receptor-mediated inhibitory postsynaptic currents (IPSCs) [50]. This inhibitory LTP requires postsynaptic calcium transient, because it can be blocked by loading Purkinje cells with a calcium chelator and can be induced with application of depolarizing voltage instead of climbing fiber stimulation. The calcium transient that triggers inhibitory LTP requires calcium mobilization from internal stores [51]. Inhibitors of either calcium/calmodulin-dependent kinase II (CaMKII) [72] or PKA [73] have been shown to block interneuron-Purkinje cell synapse LTP.

The inhibitory interneurons in the cerebellar cortex fire spontaneously and regularly in the absence of synaptic input. However, parallel fiber inputs can affect the activity of the interneurons [74] and in turn, interneurons can regulate both timing and frequency of Purkinje cell spiking. Indeed, single action potentials evoked in inhibitory interneurons can generate delays in Purkinje cell activity. Furthermore, tonic inhibitory input can drastically modulate the spike firing pattern of the Purkinje cells [75]. Different plasticity mechanisms of interneuron inputs and outputs also influence the relationship between parallel fiber activity and Purkinje cell output [44]. Given the properties of the molecular layer inhibitory interneurons, they have been seen as important regulators of the cerebellar output; their high spatiotemporal precision indicates their role in the optimization of cerebellar learning processes [76].

1.6.4 Mossy Fiber-Granule Cell Synapse

Mossy fibers excite granule cells and Golgi interneurons, and in turn Golgi interneurons inhibit granule cells; these three cell types interact in a complex named glomerulus. Mossy fiber-granule cell LTP requires postsynaptic depolarization, NMDA receptor activation and consequent calcium influx. Thus, it is sensitive to the level of inhibitory input [77], that in this case is provided by Golgi cells [78]. Mossy fiber LTP can also be blocked by an mGluR antagonist or a PKC inhibitor [79]. During mossy fiber LTP expression, amplitudes of both the AMPA and the NMDA receptor-mediated synaptic currents are increased. Apart from LTP, mossy fiber-granule cell synapse also presents

LTD plasticity [45].

Granule cells exhibit low spontaneous firing rates and require summation of multiple, almost coincident mossy fiber EPSCs to integrate sensory information. Therefore, even during very high frequency mossy fiber stimulation there are several mechanisms at the mossy fiber-granule cell synapse responsible to maintain synaptic efficacy, such as: 1) spillover of glutamate, 2) rapid reloading of release-ready vesicles, 3) AMPA receptor desensitization and 4) suppression of GABA release [44].

AMPA receptor EPSCs at the mossy fiber-granule cell synapse were shown to be mediated by both direct release of glutamate and rapid diffusion of glutamate from neighboring synapses - glutamate spillover. Glutamate spillover is thought to improve transmission efficacy by both increasing the amplitude and duration of the EPSCs and reducing fluctuation [80]. Moreover, glutamate release seems to be maintained at each release site by rapid reloading of release-ready vesicles from an unusually large releasable pool of vesicles [81]. In addition, AMPA receptors exhibit resistance to desensitization and this property seems to be crucial for sustaining high-frequency transmission at a synapse in which glutamate accumulates - glutamate pooling [82]. There is also evidence that mossy fiber-mediated spillover of glutamate inhibits GABA release from Golgi interneurons terminals by activating presynaptic mGluRs. This multisynaptic mechanism at the glomerulus level is thought to improve the efficacy of excitatory transmission by reducing the level of inhibition [83].

The principles underlying the processing of sensory-evoked synaptic inputs are still not fully understood, however, several studies have been suggesting that individual granule cells receive inputs from mossy fibers activated by the same sensory stimulus [44]. Regardless of the input type, the temporal patterns of granule cell spike activity, both spontaneous and evoked, seem to primarily follow the activity in the presynaptic mossy fibers. Nonetheless, granule cells seem to filter non synchronized mossy fiber input, acting as signal-to-noise discriminators [84]. It was even suggested a coding-specific innervation, so that mossy fiber input codes are preserved in the synaptic responses of granule cells [85]. On the other hand, it has also been proposed that granule cells integrate sensory and motor signals conveyed by distinct mossy fiber classes - multimodal integration - and that granule cells selective output enhances the capacity of Purkinje cells to acquire selectivity through associative plasticity [86]. This last proposal is in agreement with the original idea of Marr, that highly selective or sparse coding in granule cells would optimize the number of patterns that could be stored by parallel fiber-Purkinje cell plasticity [44].

The temporal pattern of granule cell activity generated by mossy fiber sensory input, and the role of Golgi interneurons in generating this pattern, is still not very well understood [44]. At the Golgi cell-granule cell synapse both fast synaptic currents and sustained inhibition can occur. Consequently, fast and sustained inhibition differentially influences granule cell spike timing during persistent mossy fiber input [87]. One possibility is that mossy fiber inputs to granule cells are transformed into well-timed spike bursts by intrinsic granule cell processing and that delayed Golgi cell inhibition sets a limit to the duration of such bursts [88], but prolonged granule cell activity can also occur [84].

The mossy fiber-granule cell synapse presents bidirectional long-term synaptic plasticity, with larger postsynaptic calcium increases being responsible for LTP and small ones being responsible for LTD [89]. The role of mossy fiber-granule cell LTP/LTD is not completely understood, but these long-term plasticity mechanisms seem to be regulated by glutamate receptors that control the frequency and duration of mossy fiber discharges onto granule cells [90]. Regulation of synaptic strength at this synapse together with changes in the strength of Golgi cell-granule cell synapses might have a critical influence in the selection of mossy fiber patterns that are transmitted to the Purkinje cell [91].

1.6.5 Cerebellar Nuclei-Related Synapses

Deep cerebellar nuclei neurons fire action potentials spontaneously unless strongly inhibited by the Purkinje cells. Moreover, they receive excitatory inputs from both mossy and climbing fibers. Several work has suggested the importance of deep cerebellar nuclei plasticity in cerebellar learning [44]. Actually, it was even hypothesized by Miles and Lisberger [92] that learning-related plasticity could take place not at the parallel fiber-Purkinje cell synapse, as suggested by the original theory of learning, but at the excitatory synapses onto the Purkinje cell target neurons in the deep cerebellar nuclei.

Recently, it was observed that not only inhibitory plasticity of Purkinje cells inputs to the deep cerebellar nuclei but also excitatory plasticity of mossy fibers to the deep cerebellar nuclei neurons can modulate their activity. High-frequency burst stimulation of mossy fibers, either alone or paired with postsynaptic depolarization, gives rise to LTD at the mossy fiber-deep cerebellar nucleus synapse [93]. On the other hand, high-frequency stimulation of mossy fibers together with postsynaptic hyperpolarization gives rise to LTP at the mossy fiber-deep cerebellar nucleus synapse, suggesting that Purkinje cell inhibition guides the strengthening of excitatory synapses in the cerebellar nuclei [94]. Therefore, unlike associative plasticity in the cerebellar cortex that depends on the simultaneous activation of excitatory inputs (parallel fiber and climbing fiber inputs), plasticity at the cerebellar nuclei seems to depend on the simultaneous activation of excitatory and inhibitory inputs (mossy fiber and Purkinje cell inputs). Plasticity at the climbing fiber-Purkinje cell synapse is still poor understood [44].

Spontaneously active deep cerebellar nucleus neurons can be inhibited by a strong Purkinje cells input. After Purkinje cell-mediated hyperpolarization they exhibit rebound depolarization [95]. This rebound phenomenon might be an important mechanism for the cerebellar nuclei neurons to differentially process the mossy and climbing fiber inputs [96].

1.6.6 Synaptic Plasticity and Motor Learning

Diverse mice models with impaired synaptic plasticity at different sites of the cerebellar circuit have been created and tested in both the VOR adaptation and delay eyelid conditioning paradigms. Most of these studies are in agreement with the original cerebellar learning theory. They support the role of LTD at the parallel fiber-Purkinje cell synapse, nonetheless, they also revealed that other sites of plasticity may also contribute to delay

eyelid conditioning sensorimotor learning [12].

Studies with mutant animals show that parallel fiber-Purkinje cell LTD is important for delay eyelid conditioning acquisition, corroborating the original cerebellar learning theory. Metabotropic glutamate receptor (mGluR) type 1 knockout (KO) mice have impaired parallel fiber-Purkinje cell LTD and impaired delay (in which light and puff temporally overlap) and trace (in which there is an interval between light and puff) eyelid conditioning. Nonetheless, Purkinje cell specific rescue of mGluR1 mice restored delay eyelid conditioning, which is cerebellum-dependent, but not trace eyelid conditioning, which also requires the hippocampus [97]. GluRdelta2 subunit (predominantly expressed in Purkinje cell spines) KO mice are also deficient in parallel fiber-Purkinje cell LTD and in delay eyelid conditioning [98]. L7-PKCi mutant mice, in which various forms of PKC are effectively inhibited, also exhibit impairments in delay eyelid conditioning; there was not generation of a response accurately timed to the puff onset [26].

As mentioned above, one of the phases of eyelid conditioning task consists of extinction; the amplitude of the responses gradually decrease due to the presentation of light-only trials. Such as parallel fiber-Purkinje cell LTD is important in the acquisition of the responses, parallel fiber-Purkinje cell LTP might be important in the extinction phase of learning. However, spontaneous recovery and fast relearning after the extinction phase indicate that it might consist of a process where new learning inhibits the original rather than a process of unlearning [99]. One possible explanation is the involvement of plasticity mechanisms, not only at the cortical but also the nuclear level [100].

One experimental approach to differentiate the role of both cerebellar cortex- and nuclei-dependent plasticity in the expression of learning (after the animal have learned) also consisted in the use of GABA(A) antagonists in order to deprive the cerebellar nuclei of inhibitory cortical control. In several instances, this revealed new, inappropriately short latency eyelid responses driven by the light. This effect is thought to depend on plasticity in the mossy fiber collateral projections to the cerebellar nuclei [101] or on additional cerebellar postsynaptic excitability changes [102]. [102]. Due to the fact that these short-latency responses are well related with the light properties, they are more likely to arise from synapse-specific plasticity and indeed there is evidence of changes in nuclear excitatory synapses after delay eyelid conditioning [103]. Nonetheless, even though such changes are important in the generation of the responses, the use of the AMPA receptor antagonist in the cerebellar nuclei has no impact on the frequency or kinematics of the responses [104]. There are some evidence that short latency responses are not cerebellar-dependent [105; 106]. An interesting hypothesis is that the delay eyelid conditioning learning process is composed of two stages: 1) an early stage, characterized by short-latency responses that are at least partly controlled by extracerebellar structures such as the amygdala and 2) a later stage, which is represented by well-timed conditioned responses that are mainly controlled by the pontocerebellar and olivocerebellar systems [107].

Even though most of the work related with synaptic plasticity in the cerebellum supports the idea that LTD at the parallel fiber-Purkinje cell synapse is crucial for cerebellar motor learning, there is evidence suggesting the opposite. Recently, De Zeeuw and

co-workers [108] challenged the original theory of cerebellar learning by testing mutant mice, in which blockade of parallel fiber-Purkinje cell LTD expression is done by targeting the late events of the LTD signaling cascade. Surprisingly, LTD mutant mice were not impaired in any form of cerebellar learning tested (such as delay eyelid conditioning and VOR adaptation). Their data shows that LTD is not essential for motor learning and suggests that the other forms of synaptic plasticity present in the cerebellar cortex might contribute to learning through compensatory mechanisms. Moreover, there is also evidence that impairments related with parallel fiber-Purkinje cell LTD might have been due to other processes in Purkinje cells and not to specifically to LTD [109; 110; 111; 112]. In addition, it was also observed that blocking of parallel fiber-Purkinje cell LTP seems to produce impairments similar to blocking of LTD and not contrary [44; 113]. Despite being different forms of long-term plasticity both LTD and LTP were shown to be important for learning, a possible explanation is that bidirectional synaptic plasticity at parallel fiber-Purkinje cell synapse might be important for the reversibility observed in motor learning [52].

Overall, studies on delay eyelid conditioning together with studies on VOR adaptation, have provided detailed information about the potential functions of several types and sites of neuronal plasticity that underlie cerebellum-dependent sensorimotor learning. Hence, there are two likely sites of synaptic plasticity important for cerebellar learning: the cerebellar cortex and the cerebellar nuclei. These sites seem to contribute to different aspects of the learning and it has been suggested that the temporal specificities of motor learning are processed within the cerebellar cortex [9; 12].

Apart from long-term changes, short-term synaptic plasticity mechanisms have been also implicated in other forms of motor learning. By looking at trial-to-trial effects, Lisberger and co-workers have shown that the presence of a Purkinje cell-complex spike (induced by the climbing fiber, signaling error) in one learning trial induces a large and properly timed depression of the Purkinje cell-simple spike (induced by the parallel fiber) on the following trial [114]. In a follow up study, it was shown that this single-trial plasticity and learning occur quickly and are forgotten within 4-10s. Due to the properties of the single-trial learning, it is suggested that motor learning might result from many processes that work together over multiple timescales. Single-trial changes are the result of one of those processes that might occur in the early phases of motor learning and might be necessary for gradual long-term modifications to take place [115].

1.7 Endocannabinoids

The original hypothesis of cerebellar learning, which supported correlation between LTD induction and motor learning, has been confirmed by several studies. However, parallel fiber-Purkinje cell LTD, by itself, is unlike to account for all the properties of cerebellar motor learning [45]. Recent work even suggests that parallel fiber-Purkinje cell LTD might not be essential for learning at all [108]. Meanwhile, new types of cerebellar plasticity involved in learning are still being identified [12; 44; 45]. In particular, there is evidence suggesting that, apart from long-term, short-term plasticity might be also important for cerebellar-dependent motor learning [114; 115; 116]. The endocannabinoid

system is an attractive neural candidate to mediate both short- and long-term cerebellar associative plasticity that occurs during learning.

Endocannabinoids (eCBs) are powerful regulators of synaptic function throughout the brain. They generally act as retrograde messengers and suppress neurotransmitter release in a transient or long-lasting way, at both excitatory and inhibitory synapses (Figure 1.9) [117; 118].

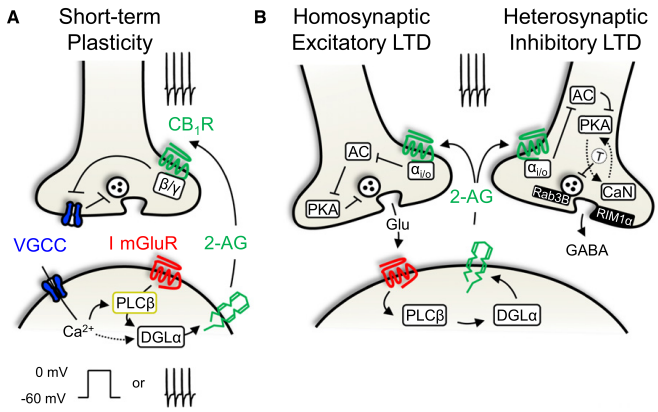

Figure 1.9: **Molecular mechanisms underlying endocannabinoid-mediated short- and long-term synaptic plasticity.** A) Endocannabinoid-mediated short-term depression. B) Endocannabinoid-mediated long-term depression (LTD) of excitation and inhibition. Dashed lines indicate putative pathways. [118]

1.7.1 Signaling

The eCB signaling system is constituted by: 1) at least two G protein-coupled receptors, known as the cannabinoid type 1 and type 2 receptors (CB1R and CB2R, respectively), 2) endogenous ligands (eCBs), of which anandamide (AEA) and 2-arachidonoyl-glycerol (2-AG) are the best characterized and 3) synthetic enzymes, degradative enzymes and transporters that regulate the levels of eCBs and their action on the receptors [118].

CB1Rs are widely expressed throughout the brain and are mainly localized at presynaptic boutons. eCBs are lipophilic messengers primarily produced in the postsynaptic neuron. Thus, the principal mechanism by which eCBs regulate synaptic function is though retrograding signaling. Postsynaptic activity leads to the production of eCBs that move backwards across the synapse, bind presynaptic CB1Rs and suppress neurotransmitter release [117; 119]. This synaptic mechanism is thought to be of greater physiological importance, allowing postsynaptic neurons to modulate their synaptic inputs [120]. However, there is also evidence suggesting that the eCB signaling can occur in a non-retrograde manner [118].

eCBs mediate forms of short-term synaptic plasticity known as depolarization-induced suppression of inhibition (DSI) [121] and depolarization-induced suppression of excitation (DSE) [122]. eCBs also mediate presynaptic forms of long-term depression at both

excitatory [123] and inhibitory synapses [124].

CB1Rs and CB2Rs belong to the class of G protein-coupled receptors and mediate almost all effects of both exogenous and endogenous cannabinoids. CB1Rs are one of the most widely expressed G protein-coupled receptors in the brain [118]. CB1R activation inhibits neurotransmitter release at the synapses through two main mechanisms. Short-term plasticity, in which CB1Rs are activated for a few seconds, requires direct G protein-dependent inhibition of presynaptic calcium influx through voltage-gated calcium channels (VGCCs) [122; 125]. Long-term plasticity, however, generally requires inhibition of adenylyl cyclase and downregulation of cAMP/PKA pathway [126]. Moreover, CB1Rs only need to be act during the induction but not the expression of eCB-LTD. Induction also requires combined presynaptic firing with CB1R activation, providing a mechanism for input specificity; only active synapses detecting eCBs express long-term plasticity [127].

Regarding the eCB molecules, despite several have been identified, only AEA and 2-AG emerged as important regulators of synaptic function. 2-AG seems to be the principal eCB required for activity-dependent retrograde signaling. However, there is evidence for functional crosstalk between 2-AG and AEA signaling [128] and differential recruitment of both molecules from the same postsynaptic neuron, depending on the type of presynaptic activity [129].

Synthesis and degradation are crucial for controlling the spatiotemporal expression of eCB signaling. Postsynaptic neuronal depolarization elevates intracellular calcium via VGCCs and elicits 2-AG production, most likely through activation of calcium-sensitive enzymes. In addition, activation of postsynaptic mGluRs by glutamate release can induce 2-AG production via the activation of the enzyme phospholipase C beta (PLCβ) [130]. Probably, calcium entry via VGCCs together with the downstream signaling from mGluRs activation is able to mobilize 2-AG. PLCβ appears to be a coincidence detector for postsynaptic activity and G protein-coupled receptor signaling. Nonetheless, activation of mGluRs is sufficient to mobilize eCBs and trigger short- and long-term forms of plasticity [118]. Upon activation, PLCβ hydrolyzes phosphatidylinositol to generate diacylglycerol (DAG), which is then converted to 2-AG by DAG-lipase alpha (DGLα). DGLα is localized postsynaptically [131] and seems to be required for calcium-dependent 2-AG production contributing to both short- and long-term synaptic plasticity [132; 133]. OAfter synthesized, 2-AG moves backwards across the synapse and activates CB1Rs. Monoacylglycerol is the principal degradative enzyme of 2-AG [134]. This enzyme is found presynaptically and controls the duration and magnitude of 2-AG-mediated synaptic plasticity [130]. In contrast to 2-AG signaling, AEA synthesis and degradation seems more complex. Postsynaptic depolarization and intracellular calcium influx seem to support AEA production [135]. AEA is in part synthesized by NAPE-PLD phospholipase expressed postsynaptically [136] but there also seem to be alternative synthetic pathways [118]. AEA transport across membranes might be facilitated by a lipophilic carrier protein [137], which presumably supports AEA delivery to intracellular compartments where the principal enzyme responsible for AEA degradation, the fatty acid amide hydrolase, is located [138].

1.7.2 Endocannabinoids and Cerebellum-Dependent Plasticity

eCBs-dependent synaptic plasticity seems to be important in the cerebellar cortex. Purkinje cell depolarization and consequent postsynaptic calcium increase lead to eCBs release, thus triggering depolarization-induced suppression of inhibition (DSI) or excitation (DSE). When Purkinje cell mGluRs are activated by glutamate and eCBs are released, synaptically evoked suppression of excitation (SSE) takes place [117; 118].

Depolarization of Purkinje cells triggers eCBs release and transiently reduce the strength of inhibitory interneuron-Purkinje cell synapses for tens of seconds [119]. In addition to DSI at the interneuron-Purkinje cell synapse, DSE seems to occur at both parallel fiber- and climbing fiber-Purkinje cell synapses [122; 139]. eCB retrograde inhibition at the synapses onto the Purkinje cell is prevented by postsynaptic application of a calcium chelator, suggesting that postsynaptic calcium increase triggers the release of eCBs that are involved as retrograde messengers in cerebellar DSI and DSE [122]. Also, Purkinje cell-dependent eCB signaling requires very large calcium signals, which suggests that release of eCBs by the Purkinje cell might occur only in localized regions of the dendritic arbor that experience large elevations of calcium [140]. These short-term synaptic mechanisms are expressed presynaptically and were shown to be absent in global CB1R KO mice, suggesting that they specifically involve CB1R activation [141]. In fact, the cerebellar cortex contains the highest level of CB1R in the brain and CB1Rs were shown to be present in all Purkinje cell-related synapses [142; 143].

In addition to short-term plasticity, Regehr and co-workers [144] showed that CB1Rs are also required for cerebellar long-term forms of plasticity, such as parallel fiber-Purkinje cell LTD. This suggests that Purkinje cells release eCBs that activate CB1Rs, not only to transiently inhibit neurotransmission release but also to induce a postsynaptic form of LTD. Interestingly, activity at the climbing fiber-Purkinje cell synapse seems to regulate this inhibition; compared to parallel fiber stimulation alone, co-activation of parallel fiber and climbing fiber greatly enhances eCB-mediated inhibition at parallel fiber-Purkinje cell synapses [116]. Moreover, LTD and eCBs release was more prominent when parallel fiber activation preceded by 50-150ms climbing fiber activation, suggesting that the properties of eCB release might contribute to the timing dependence of some forms of motor learning [145]. This is in agreement with the associative motor learning occurring in the cerebellum, since parallel fiber-Purkinje cell synapse LTD required for learning was observed to occur when climbing fiber activity follows parallel fiber activity by 100ms [146]. Interestingly, mice that lack CB1Rs specifically at the granule cells (whose axons form parallel fibers) were shown to be deficient in DSE, SSE, and LTD at the parallel fiber-Purkinje cell synapse [147]. It is known that, on the timescale of hours to days, neurons respond to changes in their activity by regulating the strength of their synapses. eCBs could underlie homeostatic regulation of synapses that occur in shorter timescales [148]. Physiologically, the involvement of eCBs at both short- and long-term forms of plasticity at the parallel fiber-Purkinje cell synapse might be important to fine-tune motor behavior on both short and long timescales.

At the behavioral level, mice that globally lack CB1R and thus have impaired eCB signaling have been reported to display various symptoms, including hypoactivity in the

open field, increased immobility in the ring catalepsy test, hypoalgesia in hot the plate and formalin tests [149], impaired extinction of aversive memory [150] and spatial memory [151] and reduction of some aspects of anxiety [152].

Importantly, and given its involvement in cerebellar plasticity, global CB1R KO mice were shown to have impaired acquisition of learned responses in a cerebellum-dependent motor learning task, the delay (which is cerebellum-dependent) but not in trace eyelid conditioning (which also involves other brain areas, like the hippocampus) [153]. In addition, when administering several doses of CB1R antagonist and agonist to rats the impairments on acquisition of the CRs were seen to be dose-dependent [154]. Accordingly, when tested in delay eyelid conditioning, human cannabis users exhibit impairments in both acquisition and timing of CRs; cannabis use appears was associated with both downregulation and desensitization of CB1Rs in the mammalian brain [155]. All together, these results indicate that CB1R-mediated eCB signaling is essential for cerebellum-dependent motor learning.

Chapter 2

Materials and Methods

2.1 Animals

Animals were kept on a 12:12 hour light/dark cycle, set for 8am-8pm darkness so that all experiments were performed during the dark period while mice were more active. Before surgery, mice were housed in groups of ∼5-6, after surgery they were single-housed; with food and water ad *libitum* in both situations. Mice were between 10 and 14 weeks before surgery and had not been used in any prior experiments. All procedures were submitted to the Portuguese National Authority for Animal Health (*Direcção-Geral de Alimentação e Veterinária*; DGAV) and to the Commission for Experimentation and Animal Welfare of the University of Coimbra (*Orgão para o Bem Estar Animal*; ORBEA).

2.1.1 Global and Conditional KO

C57BL/6 mice (*Mus musculus*) of different strains were used: global CB1R KO (n=11) and littermate controls (n=12); alpha6Cre;CB1flox (n=9) and littermate controls (n=6); gabra6Cre;CB1flox (n=8) and littermate controls (n=9); parvalbuminCre;CB1flox (n=12) and littermate controls (n=9). All strains had already been developed previously in the lab. Global CB1R KO mice are transgenic mice that lack CB1Rs everywhere in the brain. Global CB1R KO (CB1R -/-) mice and their controls (CB1R +/+) were obtained by intercrossing heterozygous breeding pairs (CB1R +/-).

Alpha6Cre;CB1flox, gabra6Cre;CB1flox and parvalbuminCre;CB1flox are conditional KO lines. Cre;CB1flox mice were generated using the Cre/loxP recombination system. In this system, transgenic mice in which Cre recombinase expression is restricted to individual cell-types through the use of cell specific promoters, are crossed with mice carrying floxed allels of the gene of interest, in this case the Cnr1 gene that encodes CB1Rs. The result is that part of the progeny will lack CB1R where Cre recombinase is expressed, providing a cell-type specific KO (Figure 2.1A). CB1R are expressed in several cell types within the cerebellum [143]. Taking advantage of the well-defined cerebellar circuitry, the fact that the cerebellum is well served with cell type specific promoters and the Cre/loxP recombination system, it is possible to abolish the expression of CB1R in specific cerebellar cell-types [147; 156; 157; 158; 159]. Thus, alpha6 and gabra6 KO mice lack CB1R at the granule cells and respective parallel fibers. But the gabra6 Cre line also targets brainstem nuclei that give rise to mossy fibers, ablating

possible expression of CB1Rs at the mossy fibers; expression of CB1Rs at the mossy fibers has not been confirmed so far. Parvalbumin KO lack CB1R at the parvalbumin expressing interneurons, which are not only cerebellar (Figure 2.1B). In the cerebellum, both inhibitory interneurons and Purkinje cells express parvalbumin; however, Purkinje cells do not express CB1Rs.

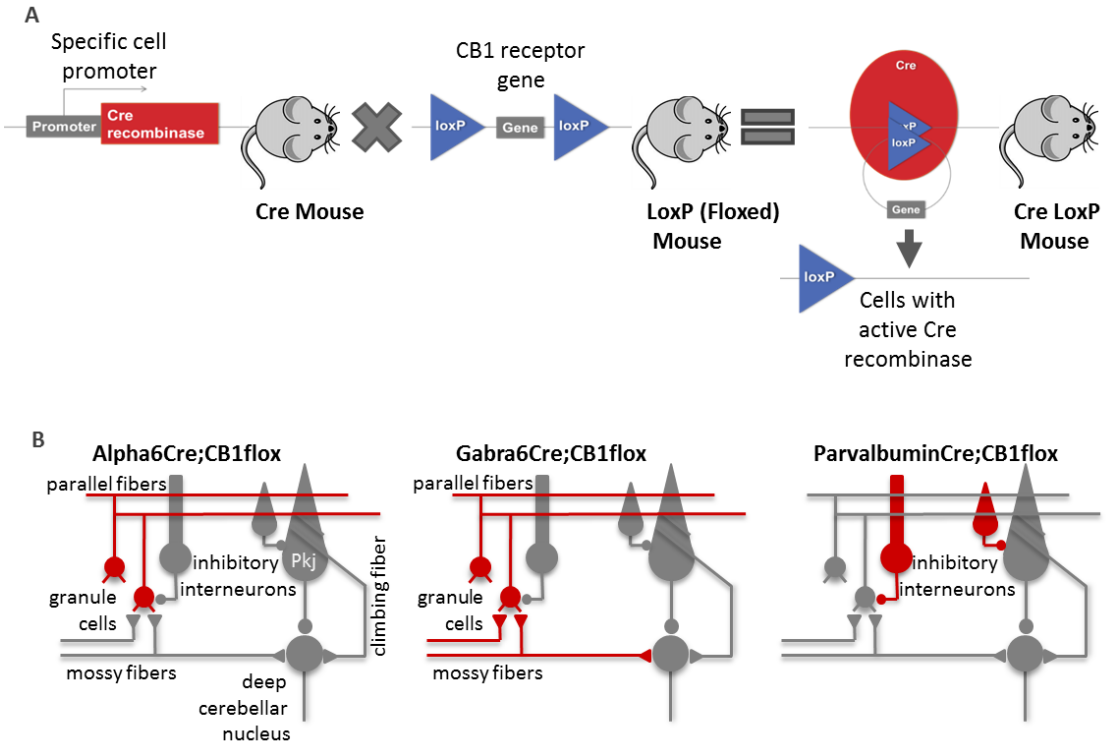

Figure 2.1: **Cre/loxP recombination system allows the generation of conditional KO mice.** A) In this genetic approach, mice carrying loxP sequences that flank the gene coding for CB1Rs are crossed with mice carrying a gene coding for the Cre recombinase enzyme in a different chromossome. As a result some of the progeny will have both CB1 alleles flanked by loxP sequences and also express Cre recombinase. Because Cre recombinase recognizes and removes the flanked CB1 gene, in cells expressing this enzyme there is no CB1R expression. B) Conditional KO mice: alpha6Cre;CB1flox lack CB1Rs at the granule cells, whose axons form the parallel fibers gabra6Cre;CB1flox lack CB1Rs at both the mossy fibers and granule cells, parvalbuminCre;CB1flox lack CB1Rs at the parvalbumin expressing inhibitory interneurons.

2.1.2 Head Fix Implant Surgery

For the preparation to be head-fixed, mice were anesthetized with isofluorane (Vetflurane - Virbac) and placed in a stereotaxic apparatus (VetEquip) providing continuous flow of air and isofluorane. In the beginning of the surgeries, both eyes were abundantly covered with a gel to prevent drying (Vidisic Gel - Bausch&Lomb). A ∼1cm diameter circle of skin was cut off from the top of the head to expose the skull. To increase the adhesion surface, a dental drill was used to gently scrape the skull and a thin layer of dental

cement (Super Bond - C&B) was distributed over. Then, a custom-cut rectangular metal head piece was transversally attached to the skull using an extra layer of dental cement. Approximately 20min before the end of the surgery, an analgesic was injected intraperitoneally (Dolorex - Intervet). After surgery procedure mice were single-housed and allowed ∼1 day of recovery from surgery.

2.2 Conditions

2.2.1 Setup

The experimental setup was based on a previous work [27], where head-fixed mice placed on top of a treadmill were allowed to walk/run on top of it. The treadmill used was a Fast-Trac Activity Wheel (InnoWheel) placed on top of a mouse igloo (InnoDome), both from Bio-Serv. This freely rotating treadmill was positioned below a custom-built head-fixing device in which the mouse head piece could be mounted. For the motorized treadmill experiments it was used a DC motor with an encoder (Maxon) to accurately control the speed of the treadmill. The entire experimental setup was placed inside a soundproof box kept in the dark and mice were monitored using a surveillance camera PlayStation Eye (Figure 2.2). For a better habituation to the experimental setup, at the moment of single-housing, the same type of treadmills was placed inside each mouse home cage.

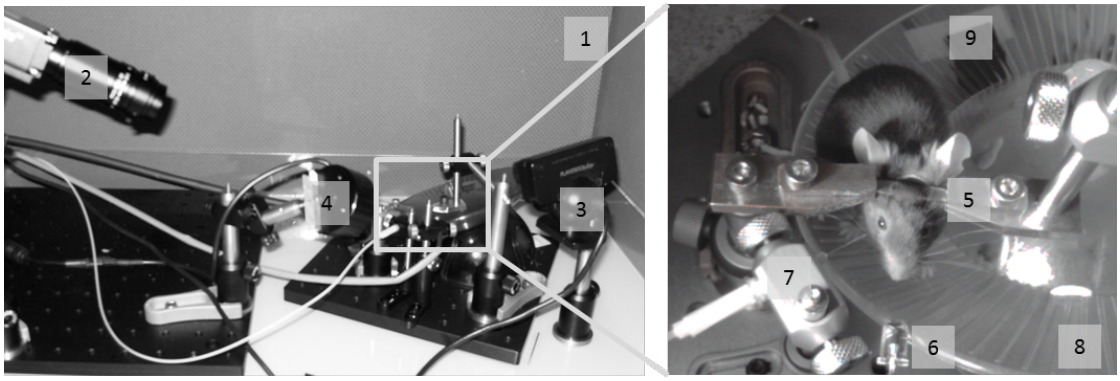

Figure 2.2: **Experimental setup with head fix apparatus.** 1) soundproof box, 2) high-speed video camera, 3) surveillance video camera, 4) infrared light, 5) head-fixed mouse, 6) LED, 7) air puff needle, 8) treadmill and 9) infrared sensor.

2.2.2 Stimuli

The experimental stimuli used were chosen to reliable elicit delay eyelid conditioning in mice [107]. The unconditioned stimulus (US) was an air puff (50psi, 50ms duration) controlled by a Picospritzer (Parker) and delivered via a 27 gauge needle positioned ∼0.5cm from the cornea of the right eye of the mouse. Because inherent delays in the electronics and the time it takes for the air to travel from the pressure injector to the top of the needle, the puff hits the cornea some milliseconds after the stimulus is triggered; nonetheless, this time of delay was taking into account when calculating the time at which

the puff should arrive. The conditioned stimulus (CS) was a white LED, positioned 2-3cm directly in front of the mouse. Because the experimental box was kept dark, the white light was salient and easily detectable by both eyes.

2.2.3 Data Acquisition

For data acquisition it was performed high-speed video recording of eyelid movements, using a Genie HM640 (DALSA) monochromatic camera to monitor a 172 x 160 pixel region (so that the eye was completely enclosed) at 900 fps. Videography was performed so that each trial has a correspondent video. Lighting was provided by an infrared illuminator. A custom-written code in LabVIEW together with a board responsible for digital to analog signal conversion (both from National Instruments) was used to trigger and control all the hardware in a synchronized way. Videography not only allowed to overpass difficulties in measuring the eyelid response seen in previous works [107] but also increased temporal resolution, allowing to measure more subtle aspects of behavior.

2.3 Design

2.3.1 Protocol

Behavioral experiments consisted of four sequential phases: ∼6 sessions of habituation, 20 sessions of acquisition (S1-S20), 2 test sessions (T1, T2) and 4 extinction sessions (E1-E4) (Figure 2.3). Each session consisted of 110 trials, separated by a randomized inter trial interval (ITI) of 5-20s, and within each trial stimuli were separated by a fixed interval (ISI) of 300ms. Generally, each mouse performed two sessions a day, one in the morning and one in the afternoon; each session lasted for around 30min. For the motorized treadmill experiments, the ITI used was 5-10s.

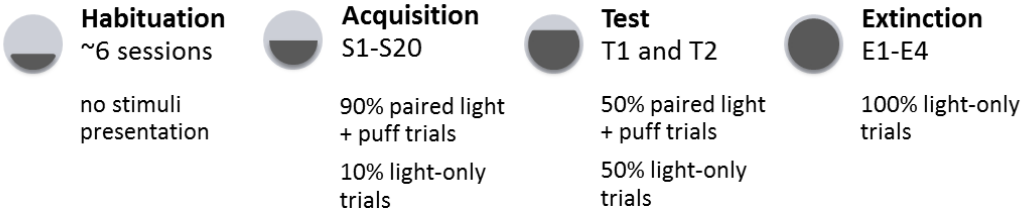

Figure 2.3: **Delay eyelid conditioning protocol.** Different phases of the conditioning experiments and respective percentage of paired and unpaired trials.

2.3.2 Habituation

Before the starting of the behavioral experiments, mice were handled for ∼20min. For each of the ∼6 habituation session, mice were placed on top of the treadmill with the head-fixed for increased duration from 10-30min and no stimuli were presented. To facilitate head fixation and diminish stress induction, mice were head fixed under light isofluorane

anesthesia. Prior to each session of the learning protocol, there was a habituation period of ∼15min where mice were head fixed but no stimuli were presented; to make sure that mice were fully recovered from the light anesthesia.

2.3.3 Acquisition, Test and Extinction

Acquisition sessions consisted in the presentation of 90% paired light + puff trials and 10% light-only trials in a randomized order, so that each block of 10 trials would have 9 paired and 1 light-only trial. By analyzing light-only trials it is possible to observe the conditioned response (CR) in a more clear way, since in these trials the CR is not masked by the unconditioned response (UR). Test sessions consisted in the presentation of 50% paired and 50% light-only trials in a randomized order, so that each block of 10 trials would have half of each type of trials. Because these sessions have a higher number of light-only trials when compared to the acquisition sessions, they allow a more detailed and robust analysis of the full CRs. In extinction sessions 100% of the trials presented were light-only.

2.3.4 Constrains

Some constrains were imposed for a trial to start: 1) at least the attributed ITI time had to elapse, 2) the eyelid had to be open between a predetermined threshold and 3) eyelid had to be open for at least 1s. Threshold parameters were adjusted for each session by the experimenter. The direction of the air puff was adjusted before each session for each mouse so that the unconditioned stimulus (air puff; US) elicited a normal eye blink; puff reaction was assessed by presenting only the puff for ∼1-3 times.

2.4 Analysis

All data analysis was performed using both Microsoft Excel (Microsoft Office) and Matlab (MathWorks) software.

2.4.1 Image Processing

Recorded videos were analyzed using a custom-written code in Matlab, previously developed in the lab. For each video recording, the distance between the eyelids was calculated in each frame by thresholding the gray scale image and summing the pixels that constitute the minor axis of the low-intensity eye-region of the resulting image (Figure 2.4).

2.4.2 CR Definition

Eyelid closure was normalized for each session of each mouse so that the size (in pixels) of a full eye blink was 1 and eyelid position in the 100ms preceding each trial was subtracted from that trial, resulting in all trials beginning from a position of 0. For paired light + puff trials, an eyelid movement was considered a CR if it occurred between 100ms after the time of light onset and the time of puff onset and exceeds 10% of full closure within that trial (>0.1 normalized pixel values). For light-only trials, a CR was considered when occurring between 100ms after the time of light onset and 100ms after the time of puff onset and exceeding 10% of maximum closure within that trial.

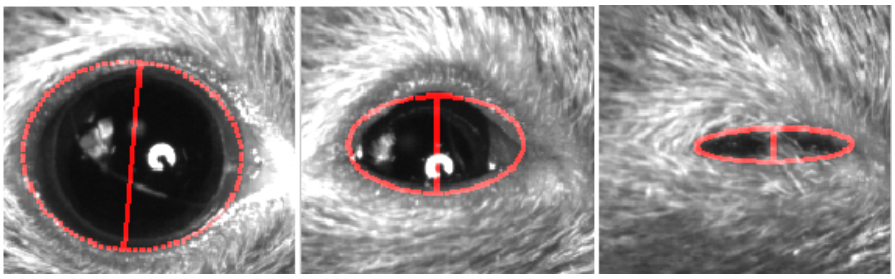

Figure 2.4: **Eyelid movements were recorded for each trial using videography.** To analyze the eye blinks kinematics, the distance between two eyelids was measured in pixels for each trial.

2.4.3 CR Features

Example average traces of the normalized eyelid response as a function of time were calculated for each session as the mean value of all trials per time point. In Figure 2.5A it is possible to see example average traces of sessions performed by one animal. In the beginning, the animal only closes the eye in response to the air puff - unconditioned response (UR). However, after the presentation of several hundred paired light + puff trials, the animal learns to close the eye in response to the light only, before the onset of the puff - conditioned response (CR). In Figure 2.5B it is possible to observe example response curves of a paired light + puff trial and a light-only trial. The ISI used was 300ms and the ITI was randomized from 5 to 20s (Figure 2.5C).

Averaged CR amplitude was calculated for each session as the mean value of the peak CR eyelid closures occurring every trial. The averaged timing of the CR was calculated for each session as the mean value of the time correspondent to the peak CR.

2.4.4 Statistics

Statistical analysis was performed using Matlab software, to determine the significance of differences found between genotypes or conditions. One-way repeated measures ANOVA was performed when comparing all sessions of both genotypes. Post hoc comparisons were made using either the two-sample t-Test (for independent variables; such as comparing the same session of two genotypes) or the paired t -Test (when comparing two dependent variables, such as comparing two different conditions of the same genotype). Differences were considered statistically significant when the calculated p-value was <0.05.

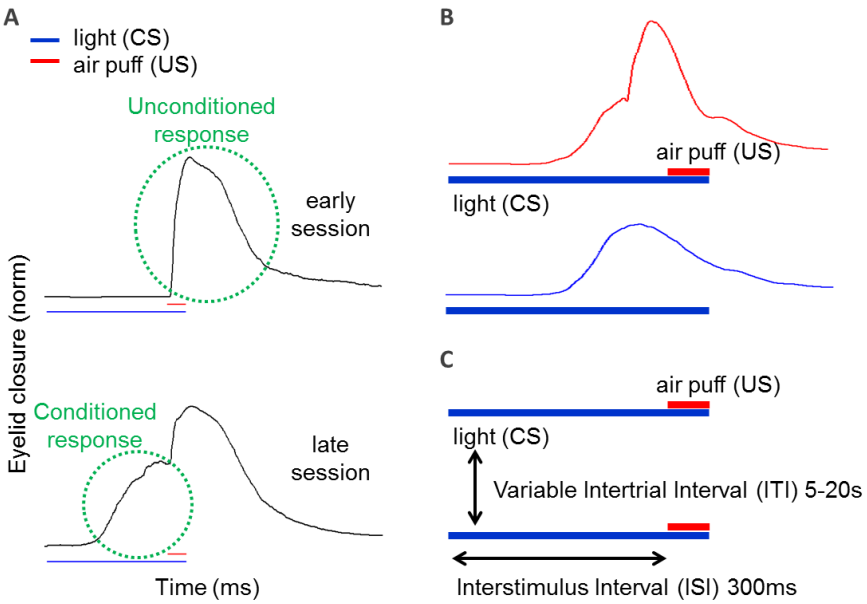

Figure 2.5: **Features of delay eyelid conditioning.** A) Example sessions of one control animal; in early sessions the mouse only responds to the air puff (unconditioned stimulus; US) whereas in late sessions it exhibits a learned blink in response to the light alone (conditioned stimulus; CS). B) Response curves to paired light + puff trials (in red) are different from the ones of light-only trials (in blue). C) Within each trial stimuli are separated by an interval on the order of milliseconds (ISI), trials are then separated by an interval (ITI) on the order of seconds.

Chapter 3

Results

We assessed the role of endocannabinoids in cerebellum dependent-learning by testing control, global and cell-specific CB1R KO mice in the delay eyelid conditioning paradigm. Global CB1R KO mice lack CB1Rs everywhere in the brain. Thus, to understand where in the cerebellar circuit endocannabinoids are playing a role, three different conditional KO mice were tested: 1) alpha6Cre;CB1flox, mice that lack CB1Rs at the granule cells and respective parallel fibers, 2) gabra6Cre;CB1flox, mice lacking CB1Rs at granule cells/parallel fibers and possibly at the mossy fibers and 3) parvalbuminCre;CB1flox, which lacks CB1Rs at the inhibitory interneurons.

In these experiments we measured the ability of mice to learn closing their eyes in response to a light (conditioned stimulus; CS) after consistent pairing it with an air puff eliciting an eye blink (unconditioned stimulus; US); the learned response to the light before the puff is presented is called a conditioned response (CR). To establish correlations between endocannabinoid-mediated plasticity and delay eyelid conditioning, several aspects of the behavior were evaluated.

3.1 CB1R KO mice do not show major impairments in delay eyelid conditioning

First, we measured the percent of successful trials (trials in which the animal exhibited a CR). The percent CR of each session for each mouse was then averaged by session for each genotype.

CB1Rs were observed to influence delay eyelid conditioning [153; 154; 155], possibly by an effect of CB1R-mediated endocannabinoid plasticity in the cerebellum [147; 148]. When comparing the averaged percentage of successful trials of wild-type and CB1R KO mice there is a tendency for CB1R KO to have lower %CR, especially in the first sessions of learning (Figure 3.1A, S3 and S4). Nonetheless, no significant differences were found (Figure 3.1A, wild-type in black and global CB1R KO mice in grey; acquisition $F(1,19) = 0.17$, $p = 0.688$; test $F(1,1) = 0.04$, $p = 0.835$; extinction $F(1,3) = 0.86$, $p = 0.363$). These results indicate that despite exhibiting a slower rate of acquisition in an early phase, CB1 KO mice eventually reach levels of learning similar to the wild-type mice. Accordingly, when compared to littermate controls, alpha6, gabra6 and parval-

bumin conditional CB1R KO mice exhibit similar %CR (Figure 3.1B, alpha6 littermate controls in dark green and alpha6 CB1R KO mice in light green, acquisition $F(1,19) = 0.02$, $p = 0.892$; test $F(1,1) = 0.42$, $p = 0.528$; extinction $F(1,3) = 0.03$, $p = 0.871$; Figure 3.1C, gabra6 littermate controls in dark blue and gabra6 CB1R KO mice in light blue, acquisition $F(1,19) = 0.87$, $p = 0.367$; test $F(1,1) = 2.22$, $p = 0.157$; extinction $F(1,3) = 2.34$, $p = 0.147$; Figure 3.1D, parvalbumin littermate controls in purple and parvalbumin CB1R KO mice in pink, acquisition $F(1,19) = 0.94$, $p = 0.345$; test $F(1,1) = 1.91$, $p = 0.183$; extinction $F(1,3) = 0.6$, $p = 0.450$).

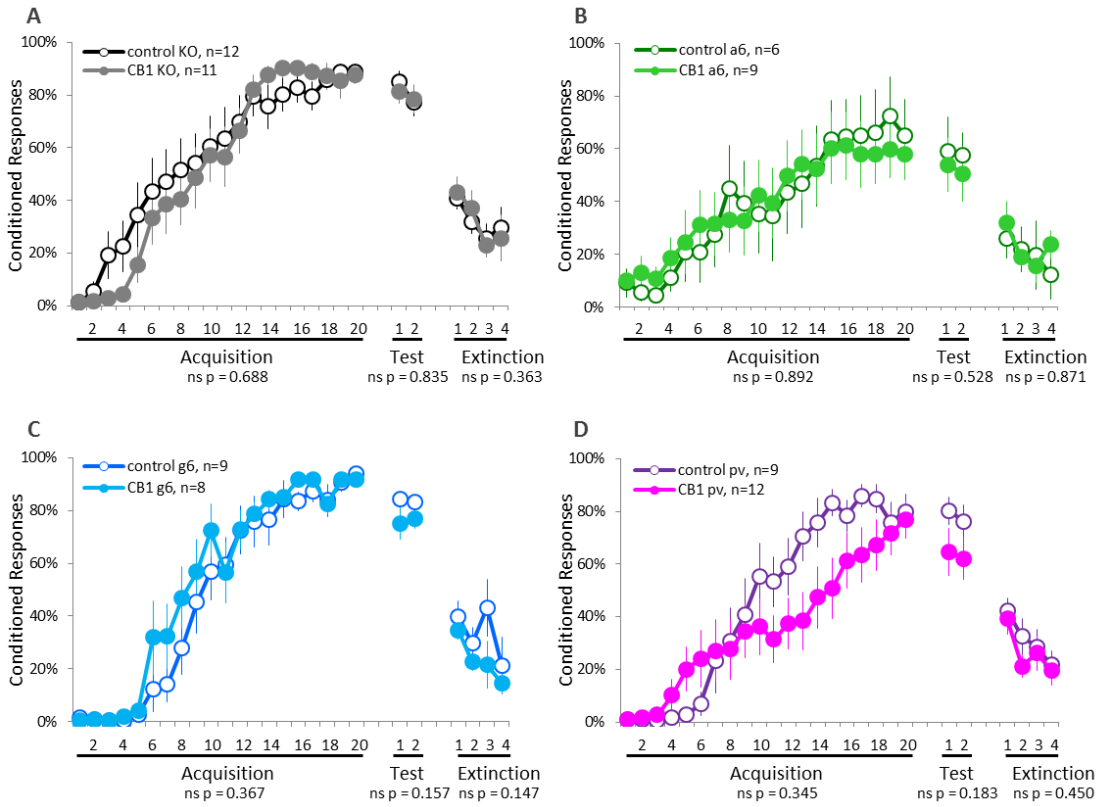

Figure 3.1: **Global and selective CB1R KO mice show normal learning in delay eyelid conditioning, as they exhibit similar learning rates comparing to littermate controls.** A-D) Averaged percentage of conditioned responses across sessions for global, alpha6 (a6), gabra6 (g6) and parvalbumin (pv) strains, respectively. Error bars represent standard error of the mean (SEM). One-way repeated measures ANOVA was performed to compare both controls and KO for each strain, in each phase of the learning protocol.

When looking at the average of the peak amplitude of the CR, global CB1R KO mice tend to have lower amplitude of responses and this effect seems to accentuate in the test phase (Figure 3.2A, T1 and T2). But overall, the learning curves of both global and selective CB1R KO seem to be similar to littermate controls (Figure 3.2A, wild-type vs global CB1R KO mice, acquisition $F(1,19) = 0.89$, $p = 0.356$; test $F(1,1) = 1.34$, $p = 0.260$; extinction $F(1,3) = 2.57$, $p = 0.124$; Figure 3.2B, alpha6 littermate controls

vs alpha6 CB1R KO mice, acquisition F(1,19) = 0, p = 0.984; test F(1,1) = 0, p = 0.969; extinction F(1,3) = 0.34, p = 0.570; Figure 3.2C, gabra6 littermate controls vs gabra6 CB1R KO mice, acquisition F(1,19) = 0.24, p = 0.630; test F(1,1) = 0.01, p = 0.909; extinction F(1,3) = 6.27, p = 0.02; Figure 3.2D, parvalbumin littermate controls and parvalbumin CB1R KO mice, acquisition F(1,19) = 0.89, p = 0.357; test F(1,1) = 1.86, p = 0.188; extinction F(1,3) = 0.36, p = 0.557). These results suggest that, when looking at gross features of learning, neither global nor conditional CB1R KO mice have impaired learning in delay eyelid conditioning.

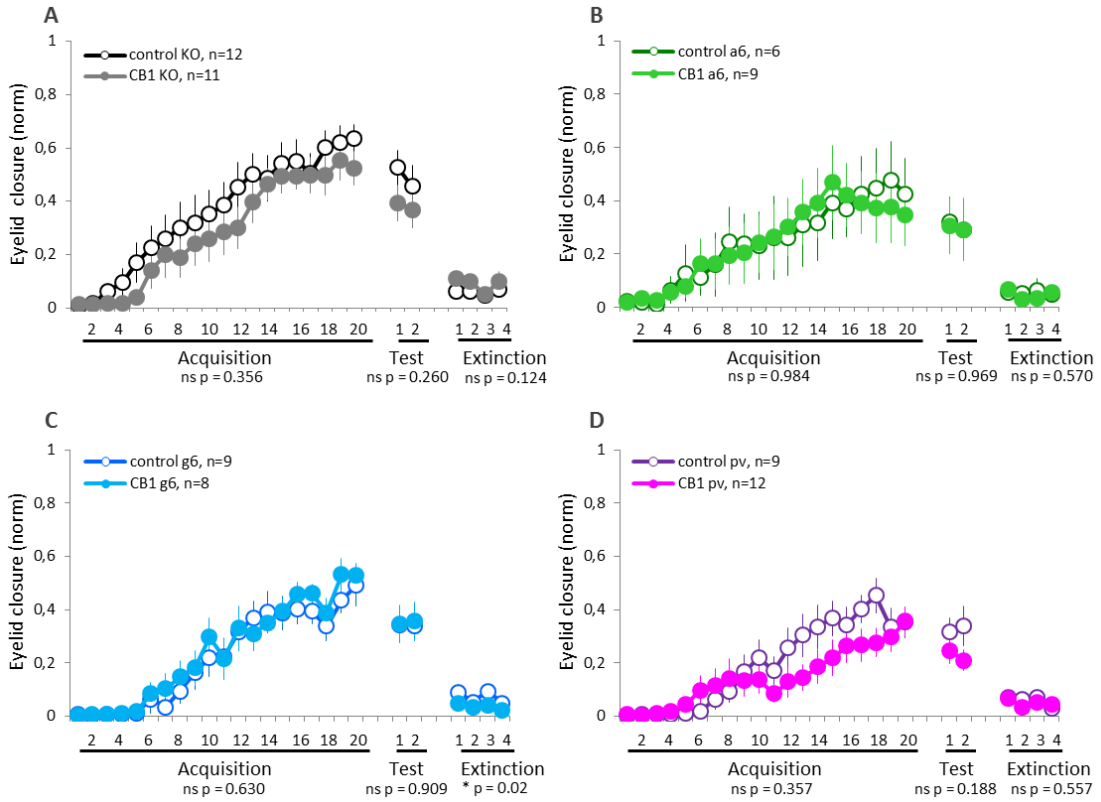

Figure 3.2: **Global and selective CB1R KO mice show normal amplitude of the learned responses, when compared to littermate controls.** A-D) Averaged peak amplitude across sessions for global , alpha6 (a6), gabra6(g6) and parvalbumin (pv) strains, respectively. Error bars represent standard error of the mean (SEM). One-way repeated measures ANOVA was performed to compare both controls and KO for each strain, in each phase of the learning protocol.

3.2 CB1R KO mice seem to time and adapt their learned eyelid responses

During delay eyelid conditioning, animals learn the adaptive timing of CR expression [27], so that peak CR amplitude occurs around the time of puff onset. The cerebellar cortex has been shown to be involved in the temporal aspects of the eyelid response

[30]. Interestingly, endocannabinoid release was observed during LTD at the parallel fiber-Purkinje cell synapse and endocannabinoids suggested to contribute to the timing dependence of some forms of motor learning [145]. Endocannabinoid-mediated suppression of neurotransmission at this synapse was seen to occur through the activation of CB1Rs [147]. Thus, in the present study we assessed the ability of CB1R KO mice to time their learned responses appropriately to the temporal interval between the light and the puff onset. Because the response to the puff (unconditioned response) masks the kinematics of the response to the light (conditioned response) only trials where just a light was presented were analyzed. To diminish variability associated with different levels of learning and have a representative number of trials, only trials of the first test session exhibiting a CR were taking into account.

Regarding the timing of the response, global CB1R KO mice exhibit similar average latency to peak CR amplitude (Figure 3.3A, wild-type vs global CB1R KO mice, p = 0.702). When comparing the conditional CB1R KO mice with their littermate controls there is also no significant difference in the timing of the response, suggesting that both global and conditional CB1R KO mice are able to generate well-timed CRs (Figure 3.3B, C and D; alpha6 littermate controls vs alpha6 CB1R KO mice, p = 0.108; gabra6 littermate controls vs gabra6 CB1R KO mice, p = 0.357; parvalbumin littermate controls vs parvalbumin CB1R KO mice, p = 0.360).

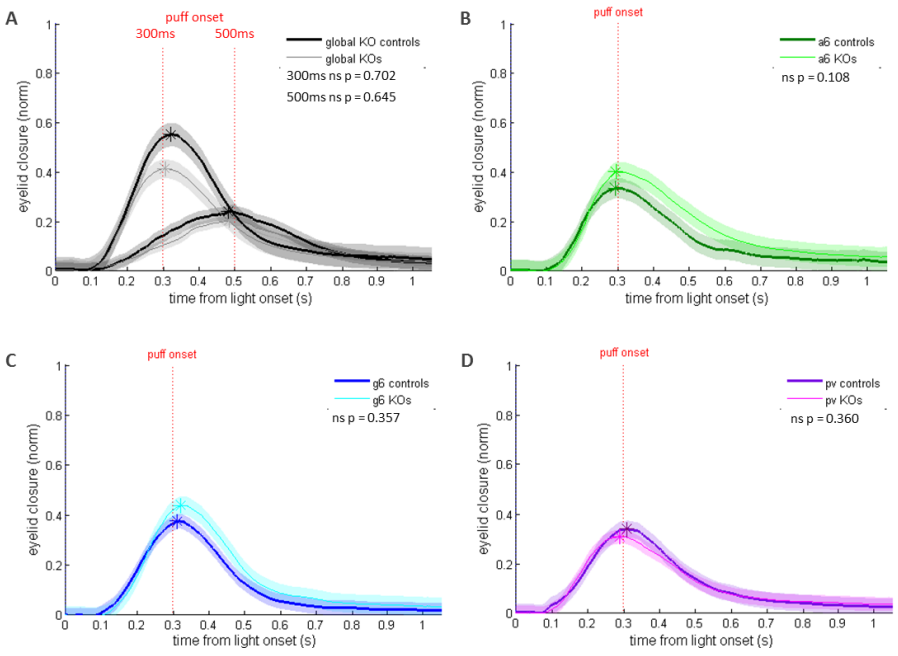

Figure 3.3: **CB1R KO mice can time their learned responses, since no relevant differences were found in the timing of the CR peak.** A-D) Averaged response curves of the light-only trials exhibiting a CR in test 1 sessions. For global 300ms and 500ms group, alpha6 (a6), gabra6 (g6) and parvalbumin (pv) strains, respectively. Shadows represent standard error of the mean (SEM). Two-sample t-Test was used to compare control and KO mice.

To further understand if global CB1R KO mice could adjust the time of their learned responses, we manipulated the interval between stimuli (ISI). In this case, instead of 300ms, it we used an ISI of 500ms in order to increase the spatiotemporal resolution and thus observe in more detail possible changes in the kinematics of the CR. Firstly, it was observed that both KO and their littermate controls were able to time their responses appropriately. Under these conditions, also no differences of timing between global CB1R KO mice and controls were found (wild-type vs global CB1R KO 500ms ISI mice, p = 0.645). In both groups, mutant mice tend to have lower CR amplitude when comparing to their respective controls and the difference of amplitudes is larger in the 300ms ISI group. It is also worth noting that the amplitude of the responses in the 500ms ISI group is largely lower when comparing to mice that did 300ms ISI, regardless of being mutant mice or control (Figure 3.3A).

3.3 CB1R KO mice show normal trial-to-trial learning dependent on previous experience

Trial-to-trial analysis in other cerebellar-dependent behavior revealed motor learning to be dependent not only on long- but also short-term changes in synaptic strength [114; 115]. Interestingly, endocannabinoid signaling has been associated with short-term cerebellar synaptic plasticity [116; 122; 147] through the activation of CB1R [141]. Thus, we examined trial-to-trial effects in CB1R KO mice during learning in delay eyelid conditioning. To do so, two groups of trials were compared: trials preceded by trials with a puff (paired light + puff trials; 1-x) and trials preceded by trials without puff presentation (light-only trials; 0-x). In order to diminish variability associated with different levels of learning within and across mice and to have a representative number of 0-x trials, the sessions analyzed were T1 and T2. To analyze modulation of learning there is the need to eliminate confounds related with poor learning, thus, only trials with a CR were analyzed.

By comparing the average curves of both 1-x and 0-x trials, it was possible to observe that the presence or absence of a puff on the previous trial affects the CR on the next trial (Figure 3.4A and B). Analyzing the curve of the difference between the 1-x and 0-x average curves over time it is possible to observe that all curves are positive, meaning that on average trials preceded by trials with a puff have higher CR amplitude than trials preceded by trials where no puff was presented, regardless of the genotype (Figure 3.4C-F).

When quantifying these short-term effects, overall, both global and selective CB1R KO mice exhibit higher percentage of trials with CR and higher CR amplitude in 1-X trials than in 0-x (Figure 3.4G, wild-type: %CR p = 0.008 and amplitude p = 0.002, global CB1R KO: %CR p = 0.042 and amplitude p = 0.0004; Figure 3.4H, alpha6 littermate controls: %CR p = 0.085 and amplitude p = 0.323, alpha6 CB1R KO: %CR p = 0.0002 and amplitude p = 0.008; Figure 3.4I, gabra6 littermate controls: %CR p = 0.126 and amplitude p = 0.002, gabra6 CB1R KO: %CR p = 0.061 and amplitude p = 0.001; Figure 3.4J, parvalbumin littermate controls: %CR p = 0.004 and amplitude p = 0.003, parvalbumin CB1R KO: %CR p = 0.006 and amplitude p = 0.0001). Results regarding modulation of amplitude CR by previous trial experience for individual animals

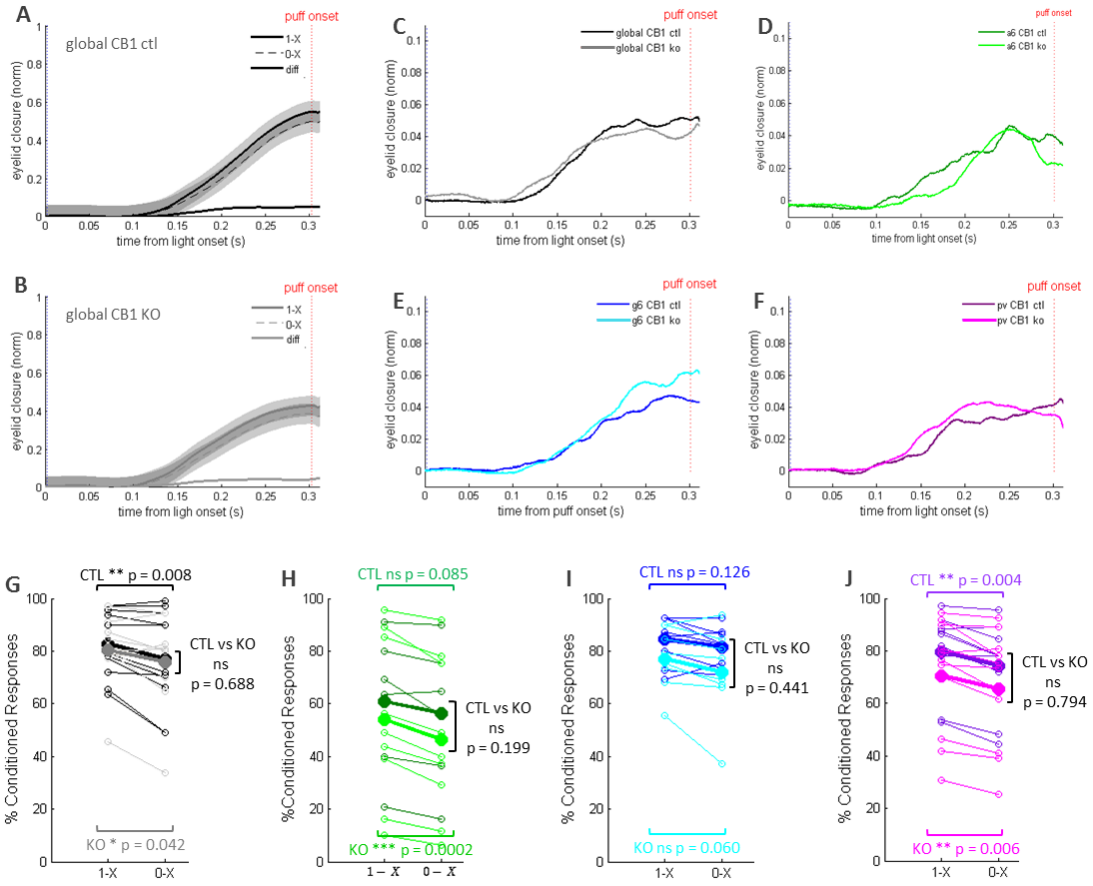

Figure 3.4: **Previous trial experience affects the CR on the next trial, regardless of genotype; trials preceded by a trial with a puff exhibit more and stronger CRs than trials preceded by trials where only light was presented.** A and B) Averaged response curve of trials from test 1 and test 2 sessions preceded by a trial with (1-x; solid line) or without a puff (0-x, dashed line) for both controls and global CB1R KO, respectively. Difference between 1-x and 0-x average curves over time is also shown (diff). Shadows represent standard error of the mean (SEM). C-F) Difference between 1-x and 0-x trials over time for global, alpha6 (a6), gabra6 (g6) and parvalbumin (pv) strains, respectively. G-J) Averaged CR percentages of each condition (1-x and 0-x) for each genotype (thicker lines) are plotted on top of performances from individual animals (thinner lines). Each plot shows data for both genotypes of each strain. Paired t-Test was used to compare 1-x with 0-x conditions for each genotype. Two-sample t-Test was used to compare 1-x and 0-x difference between controls and KOs.

are shown as supplementary data (SuppFigure 1A-D).

To understand if these differences were more prominent in control or CB1R KO mice, the differences between 1-x and 0-x trials were compared. However, no differences were found between genotypes (wild-type vs global CB1R KO: %CR p = 0.688 and amplitude p = 0.981; littermate controls vs alpha6 CB1R KO: %CR p = 0.199 and amplitude p = 0.911; littermate controls vs gabra6 CB1R KO: %CR p = 0.441 and amplitude p = 0.414; littermate controls vs parvalbumin CB1R KO: %CR p = 0.794 and amplitude p = 0.386). These results indicate that CB1R mutants and their littermate controls respond similarly to alterations in the previous trial.

3.4 Global and gabra6 CB1R KO mice appear to be more affected by the duration of the interval between trials

Apart from effects related with the presence and absence of puff, short-term plasticity can also be investigated by analyzing the changes in learned responses that different intervals between trials produce. The intertrial interval (ITI) used for delay eyelid conditioning was randomized between 5 and 20s. To understand how elapsed time between trials affects the learned response, we divided trials from test sessions in 3 groups, according to the preceded ITI: 1) short, which includes trials preceded by an interval of less than 10s, 2) medium, which includes trials preceded by an interval of 10-16s and 3) long, which includes trials preceded by an interval of more than 17s. As mentioned before, the presence or absence of a puff on a previous trial affects the learned response on the following trial, thus, to eliminate the confound of this variable, the ITI effects were analyzed by looking only at 1-x trials so that the trials analyzed were all preceded by a trial where a puff was presented.

On average, shorter ITIs are associated with a slightly larger CR amplitude and longer ITIs with a slightly decrease in CR amplitude (Figure 3.5A and B, average curve of 1-x trials in solid line and 0-x trials in dashed line for both control and global CB1R KO mice, respectively). By analyzing the curve of the difference between the short and long ITI average curves over time it is possible to observe that all curves are slightly positive, meaning that regardless of genotype, if the time preceding the current trial is longer the amplitude of the learned response is slightly lower (Figure 3.5C-F). Interestingly, global CB1R KO mice seem to be more affected by the duration of the interval between trials than their littermate controls (Figure 3.5C). Gabra6 CB1R KO mice also seem to be more affected than littermate controls, especially right before the time that the puff would have arrived (Figure 3.5E). Unexpectedly, parvalbumin littermate controls seem to be more affected than the parvalbumin CB1R KO mice (Figure 3.5F).

When quantifying these interval effects, both global and gabra6 CB1R KO mice presented slightly lower percent and amplitude of the learned responses after a long interval, despite no statistical significant differences were found (Figure 3.5G, wild-type: %CR p = 0.365 and amplitude p = 0.988, global CB1R KO: %CR p = 0.116 and amplitude p = 1.115, wild-type vs global CB1R KO: %CR p = 0.332 and amplitude p = 0.321; Figure 3.5H, alpha6 littermate controls: %CR p = 0.871 and amplitude p =

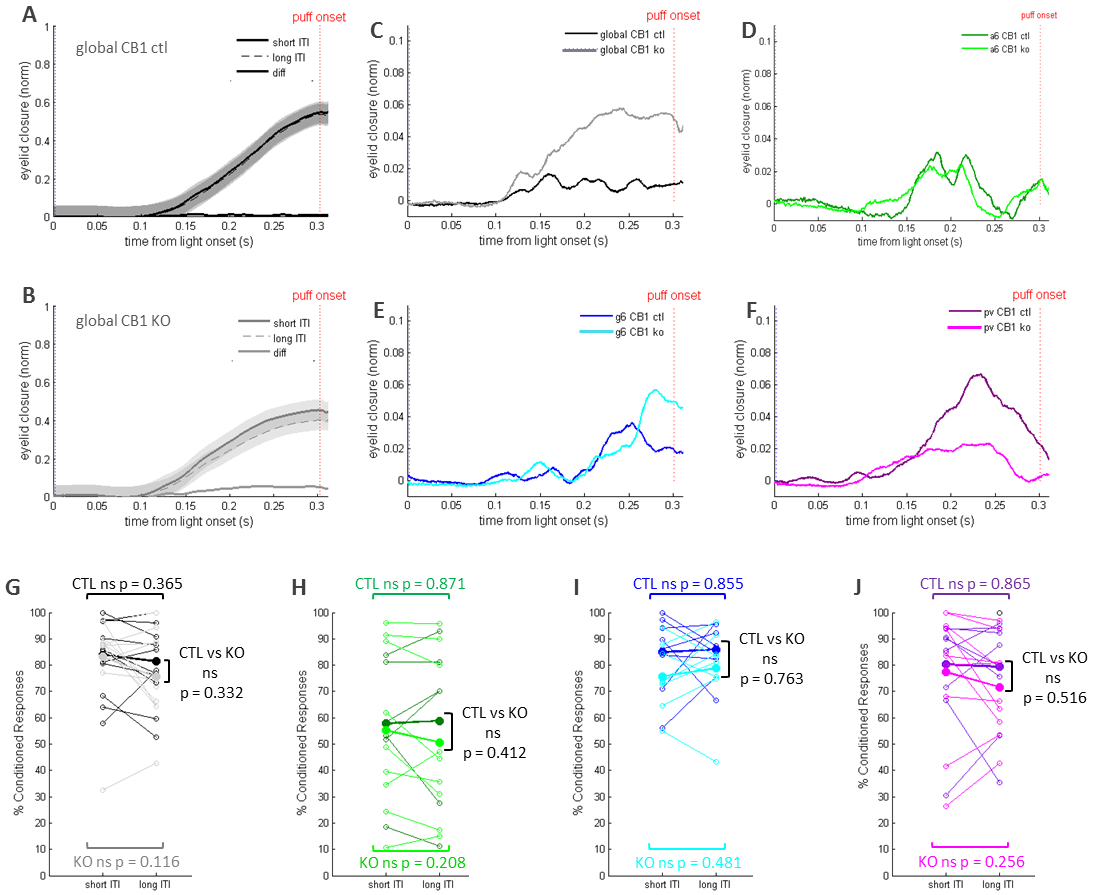

Figure 3.5: **Global and gabra6 CB1R KO mice appear to be more affected by the interval between trials; global KO mice exhibit less and decreased responses after a longer interval than after a shorter interval.** A and B) Averaged response curve of trials from test1 and test2 sessions preceded by a short (short ITI; solid line) or long interval (long ITI; dashed line) for both controls and global CB1R KO, respectively. Difference between short and long average curves over time is also shown (diff). Shadows represent standard error of the mean (SEM). C-F) Difference between trials preceded by a short and a long interval over time for global, alpha6 (a6), gabra6 (g6) and parvalbumin (pv) strains, respectively. G-J) Averaged CR percentages of each condition (short and long) for each genotype (thicker lines) are plotted on top of performances from individual animals (thinner lines). Each plot shows data for both genotypes of each strain. Paired t-Test was used to compare short with long interval conditions for each genotype. Two-sample t-Test was used to compare the difference of short and long intervals between controls and KOs.

0.723, alpha6 CB1R KO: %CR p = 0.208 and amplitude p = 0.587, littermate controls vs alpha6 CB1R KO: %CR p = 0.412 and amplitude p = 0.908; Figure 3.5I, gabra6 littermate controls: %CR p = 0.855 and amplitude p = 0.279, gabra6 CB1R KO: %CR p = 0.481 and amplitude p = 0.133, littermate controls vs gabra6 CB1R KO: %CR p = 0.763 and amplitude p = 0.056; Figure 3.5J, parvalbumin littermate controls: %CR p = 0.865 and amplitude p = 0.198, parvalbumin CB1R KO: %CR p = 0.256 and amplitude p = 0.967, littermate controls vs parvalbumin CB1R KO: %CR p = 0.516 and amplitude p = 0.358). Results regarding modulation of amplitude CR by the interval between trials for individual animals are shown as supplementary data (SuppFigure 2A-D).

3.5 Global and gabra6 CB1R KO mice appear to have impaired motor memory consolidation

For motor learning to occur, learned responses must be long-term stored. Thus, acquisition of eyelid learned responses is associated with their consolidation in a form of motor memory [36]. To investigate the consolidation phenomenon we quantified how much mice recall from the previous session. Thus, changes in both %CR and amplitude CR were examined by comparing the average of the last third of one session with the first third of the following one. Pairs of sessions exhibiting less than 10% of CR in the first session of comparison were excluded. Because of the protocol used, it was possible to analyze learning on different timescales: 1) morning to afternoon of the same day, which are separated by ∼3-6 hours, 2) afternoon of one day to the morning of the next one, which are separated by ∼17-20 hours and 3) afternoon to morning of two days after, which are separated by ∼65-68 hours.

Overall and regardless of genotype, mice show increased percentage of CR when comparing learning on short timescales (morning to afternoon of the same day and afternoon to morning of the next day) (Figure 3.6A-D). This increase seems to be more accentuated in littermate controls than in CB1R KO mice. When looking at longer timescales, whereas control mice do not exhibit relevant changes in the percent CR from Friday afternoon to Monday morning, global and gabra6 CB1R KO mice appear to remember less (Figure 3.6A and C). The same effect can be seen in the parvalbumin CB1R KO mice, but in a much smaller extent (Figure 3.6D). No significant differences were found for longer timescales, which can be related with the low number of animals in that condition. When comparing the differences of the last and first sessions between genotypes of the same strain also no significant differences were found (statistics for %CR are presented for 1st, 2nd and 3rd condition, respectively: Figure 3.6A, wild-type p = 0.015/0.00003/0.405, global CB1R KO p = 0.064/0.058/0.186, wild-type vs global CB1R KO p = 0.476/0.029/0.114; Figure 3.6B, alpha6 littermate controls p = 0.013/0.0005/0.388, alpha6 CB1R KO p = 0.346/0.065/0.986, littermate controls vs alpha6 CB1R KO p = 0.146/0.258/0.665; Figure 3.6C, gabra6 littermate controls p = 0.005/0.0001/0.836, gabra6 CB1R KO p = 0.08/0.770/0.098, littermate controls vs gabra6 CB1R KO p = 0.429/0.064/0.083; Figure 3.6D, parvalbumin littermate controls p = 0.008/0.01/0.215, parvalbumin CB1R KO p = 0.001/0.0001/0.418, littermate controls vs parvalbumin CB1R KO p = 0.507/0.678/0.812).

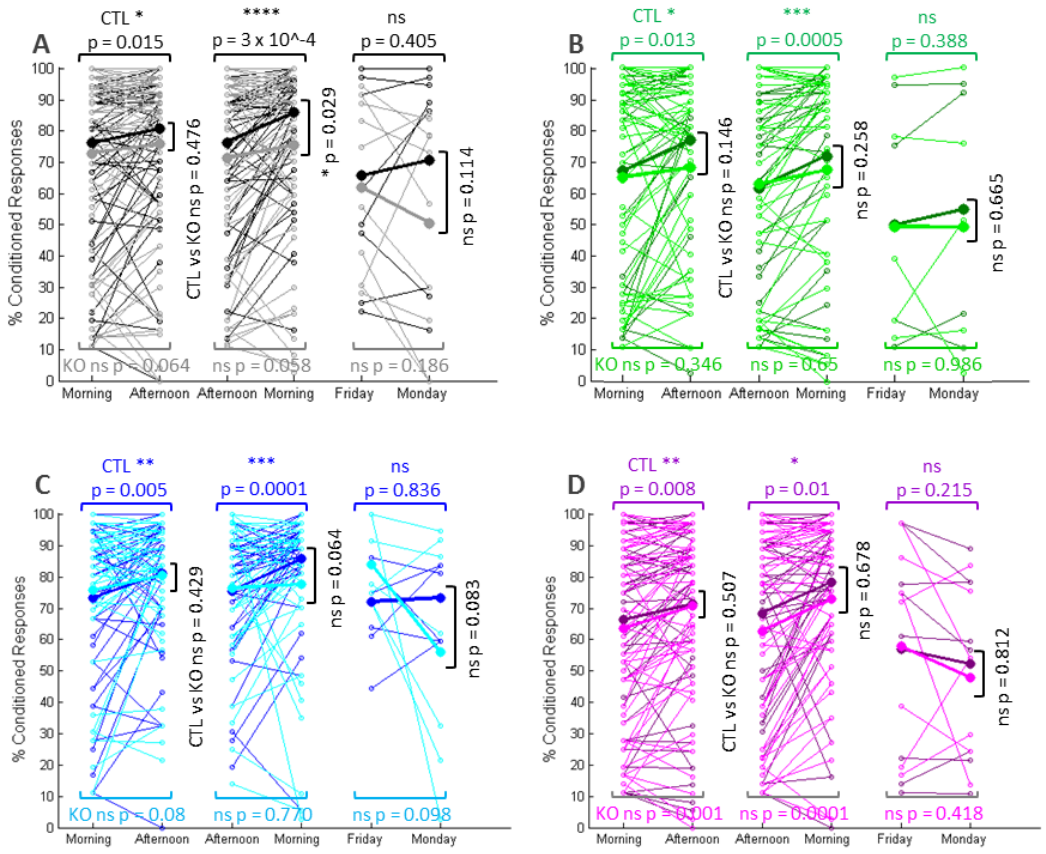

Figure 3.6: **Global and gabra6-specific CB1R KO mice appear to remember less after a long period of time without training.** Learning was analyzed on different timescales by comparing performance of pairs of sessions from: 1) morning to afternoon, 2) afternoon to morning of the next day and 3) Friday afternoon to Monday morning. For each pair of sessions, it was compared the percentage of trials with CR of the last third of one session with the first third of the following session. Pairs of sessions with less than 10% of conditioned responses by the first session of comparison were excluded. A-D) Averaged CR percentages of each condition for each genotype (thicker lines) are plotted on top of performances from individual animals (thinner lines). Each plot shows data for both genotypes of one strain: global, alpha6 (a6), gabra6 (g6) and parvalbumin (pv), respectively. Paired t-Test was used to compare conditions for each genotype. Two-sample t-Test was used to compare the difference of the last and the first session between controls and KOs.

3.6 Mice that run more appear to learn faster

Global CB1R KO mice were shown to be hypoactive [149]. The experimental setup used for delay eyelid conditioning allows the quantification of how much mice voluntarily walk or run on the treadmill during the sessions. When quantifying the distance mice ran on the treadmill on average during the acquisition sessions, both global and alpha6 CB1R KO mice were seen to be hypoactive, as they ran around half of what littermate control mice did (Figure 3.7). However, gabra6 and parvalbumin CB1R KO mice presented similar locomotor activity to their respective littermate controls.

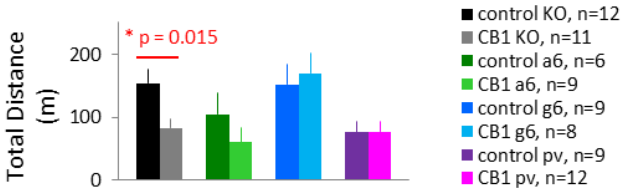

Figure 3.7: **Global and alpha6-specific CB1R KO mice appear to be hypoactive, as they ran around half of the distance their littermate controls did.** Mice were allowed to walk or run voluntarily on a treadmill during learning. Averaged total distance ran during all acquisition sessions for each genotype is presented. Error bars represent standard error of the mean (SEM).

To understand whether differences in activity could account for learning, for the running analysis we sorted mice by how much they ran on average during the acquisition sessions. Mice that ran less than approximately one fourth of what control mice ran by session on average (∼38m) were considered non-runners, the others were considered runners. Regardless of genotype, mice that run more seem to learn better than non-runners. Overall, mice considered as runners exhibit increases in the percentage of the learned response much earlier than non-runners, exhibiting a faster learning (Figure 3.8A-D). Curiously, and even though on average both global and alpha6 CB1R KO mice were considered hypoactive (Figure 3.7), mice from these genotypes that run more appear to learn better. In fact, control and KO mice considered runners have similar rates of learning and appear to learn better than control and KO mice considered non-runners (Figure 3.8A and B). For the gabra6 and parvalbumin strains the difference between runners and non-runners does not seem so evident (Figure 3.8C and D), this could be due to the fact that the activity profiles of control and KO mice for both strains are not as different as for the global and alpha6 strains (Figure 3.7). The relation between CR amplitude and running activity was also analyzed and results are in agreement with the described for the CR amplitude (SuppFigure 3A-D).

Due to the relation between running activity and learning and to understand whether mice than run the same learn similarly, we carried out an experiment where instead of running voluntarily mice had to run in a motorized treadmill. When making a group of control mice run the same per session at a constant speed of 1.6m/s (which corresponds to ∼80% of the distance performed on average by control mice freely walking; SuppFigure 4A), mice learned faster and reached high levels of learning much earlier (∼S5; Supp-

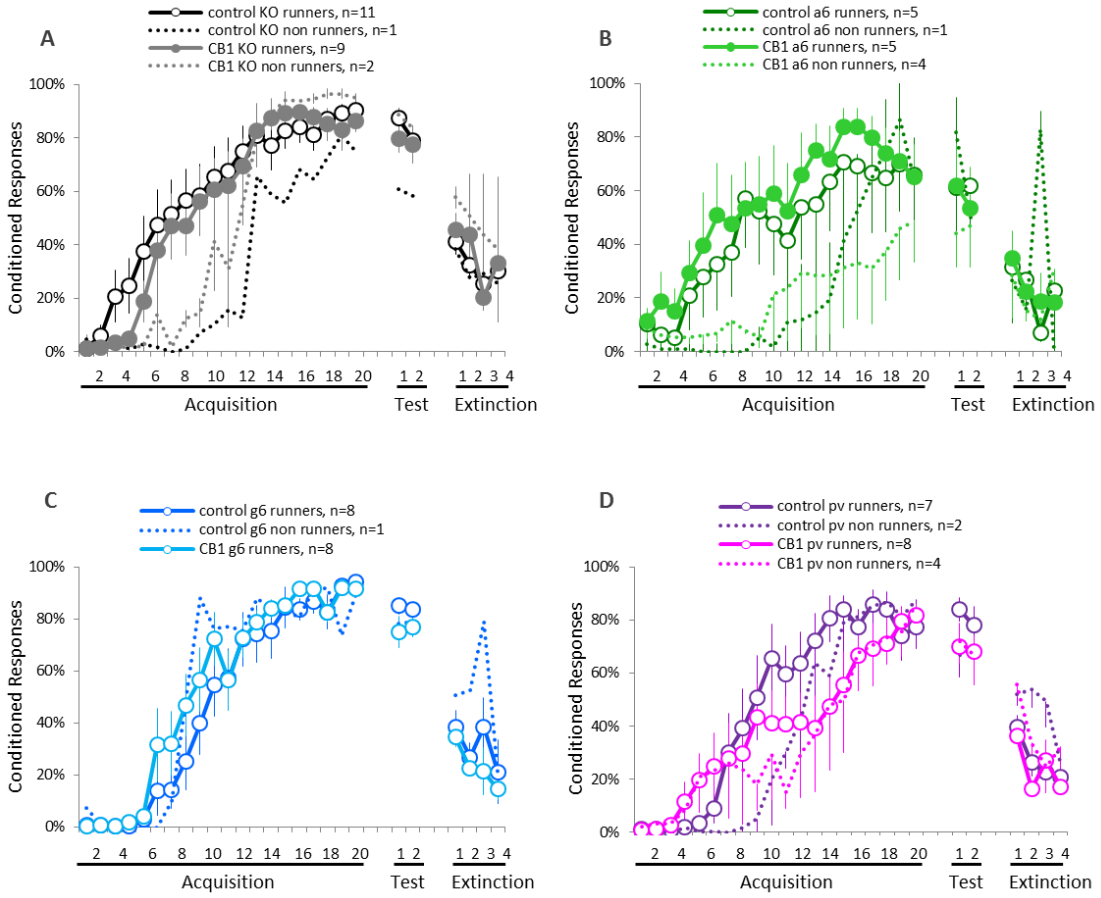

Figure 3.8: **Mice that run more appear to learn faster, regardless of genotype; as they exhibit increases in percent CR earlier than non-runners.** Mice were allowed to walk or run voluntarily on a treadmill during learning. For the running analysis of each genotype, mice were sorted by how much they run during training; mice running less than around one fourth of what control mice did per session on average (38m) were considered non-runners. A-D) Averaged percentage of conditioned responses of both runners and non runners across sessions for global, alpha6 (a6), gabra6 (g6) and parvalbumin (pv) strains, respectively. Error bars represent standard error of the mean (SEM).

Figure 4B) than control mice considered runners and running voluntarily (∼S11; Figure 2). Moreover, mice running on the motorized treadmill exhibited a more standardized learning, with much less variability.

Because this is a preliminary analysis of running effects on learning that was performed based only on the present data set, the number of animals in most of the conditions is relatively low, thus no statistical tests were applied. By doing more experiments using the motorized treadmill, a more accurate analysis of possible effects of running in delay eyelid conditioning can be performed.

Due to the observed relevance of running activity in learning, we re-analyzed some features of behavior including only mice considered runners. Interestingly, neither the ITI effect seen in global CB1R KO mice nor the consolidation effect observed in both global and gabra6 CB1R KO mice suffered relevant alterations (data not shown).

Chapter 4

Discussion

The cerebellar circuit is crucial for motor control, timing and learning [44]. Over the past two decades, several neural substrates have been indicated as mediators of synaptic plasticity that underlies motor learning [12; 44]. Endocannabinoids are key modulators of synaptic function that generally activate presynaptic cannabinoid receptors type 1 (CB1Rs) through retrograde signaling suppressing neurotransmitter release [117]. CB1Rs are expressed throughout the cerebellum [143] and were suggested to influence delay eyelid conditioning [153; 154; 155]. This could be due to an effect of CB1R plasticity in the cerebellum [147; 148] or to extracerebellar modulation.

Our main objective with this project was to understand how the endocannabinoid signaling contributes to cerebellar plasticity and learning. Specifically, we aimed to determine in which cell-types of the cerebellar circuit and through what mechanisms, endocannabinoids act to regulate motor learning. To do so, a genetic approach to specifically eliminate eCBs receptors (CB1Rs) from specific cell-types of the cerebellar circuit was combined with a simple form of learning. The conditional KO lines used for the present experiments were the alpha6 (which lacks CB1Rs at the granule cells), gabra6 (that also lacks CB1Rs at granule cells and possibly at the mossy fibers) and parvalbumin (that lacks CB1Rs at the inhibitory interneurons). By testing these different cell-type specific KO mice in delay eyelid conditioning and measuring different features of behavior, it was possible to begin revealing the role of endocannabinoid-mediated plasticity in sensorimotor learning.

The parameter usually used to evaluate performance in delay eyelid conditioning consists on the percent of successful trials in a session, which corresponds to the number of trials that had a response to the light (conditioned stimulus; CS), before the puff (unconditioned stimulus; US) was presented - conditioned response (CR). Nonetheless, because of the high-speed video capture system we used to record the eyelid movements, it was possible to accurately look at other aspects of the movement kinematics of the learned responses in individual trials, such as amplitude and timing of the CR.

Surprisingly, the results obtained are not in agreement with previous work suggesting a role for endocannabinoid signaling in motor learning [44; 148; 154; 155]. The impairments we found when testing global CB1R KO mice in delay eyelid conditioning were less severe than what was previously described [153].In fact, when looking at gross fea-

tures of learning like CR percent, almost no impairments were observed. For the CB1R conditional KO mice tested for the first time in delay eyelid conditioning, also no major impairments were found. Even some more subtle aspects like CR amplitude and timing do not seem to be affected. When looking at trial to trial effects, there is modulation of the current trial by previous trial experience, which appears to be independent of CB1R activation. Nonetheless, we did observe two possible differences in learning due to CB1Rs. First, both global and gabra6 CB1R KO mice appear to be negatively affected by a longer interval elapsed between trials. Second, when analyzing motor memory consolidation, global and gabra6 CB1R KO mice appear to remember less after a long period of time. The discrepancy between our results and previous ones will be addressed below. Running activity also appears to be related with learning in delay eyelid conditioning and might explain some of the difference between the present results and previous work.

4.1 eCB signaling might not be essential for cerebellar delay eyelid conditioning

Mice lacking CB1Rs everywhere in the brain were shown to have impaired acquisition in delay eyelid conditioning [153]. In the present work, however, even though both percent and amplitude of the learned responses tended to be lower, global CB1R KO mice acquired learned responses normally (Figure 3.1A and Figure 3.2A).

One possible explanation for this discrepancy might be related with the type of learning induced in the previous work [153]. The experimental protocol of Kishimoto and Kano appears to share some features with cued fear conditioning. Cued fear conditioning is a behavior that has been described to be amygdala-dependent. During typical cued fear conditioning, a tone (CS) is paired with an aversive foot shock (US) and after a few tone + shock paired trials the tone starts to elicit a wide range of defensive responses [107]. Kishimoto and Kano used a tone as a CS and a periocular electrical stimulation as a US. In fact, CR acquisition seen by Kishimoto and Kano is faster than the normal cerebellar-dependent type of learning, as their control mice achieve 30% CR by the first session of acquisition. Thus, it can be questioned whether the type of learning observed in their work was only mediated by the cerebellum, or also depended on extracerebellar structures such as the amygdala. Actually, some of the work done regarding delay eyelid conditioning in mice is now being questioned due to this issue [107].

In the present study we used a light as a CS to avoid the traditional learned response induced by a tone stimulus, which includes a non-associative auditory startle reflex that happens right after the tone onset and that may interfere with the expression of the eye movement related with the actual CR; the startle reflex is also controlled by the amygdala [26; 107]. For the US we used a mild air puff stimulus due to the suggestion that the strong periorbital stimulation usually used in eyelid conditioning leads to fear-based learning and can induce an amygdala-dependent short-latency response that can interfere with the expression of the CR [27; 107]. Because small differences in stimuli used for conditioning can seriously affect performance of mice in delay eyelid conditioning it is important to take them into account when comparing the present results with previous ones.

Regarding the fast conditioning seen by Kishimoto and Kano [77], it does not seem to be in agreement with what has been described as normal cerebellar-dependent learning. Delay eyelid conditioning typically develops along several sessions of learning, so that paired presentation of stimuli during training gradually leads to the development of learned responses and their strengthening [107]. Amygdala-dependent learning has been seen to enhance effectiveness of eyelid conditioning, as lesions to this area affect the speed of acquisition of learned responses [160]. Along these lines, it was hypothesized that learning in delay eyelid conditioning using severe stimuli might consist of two phases: 1) an initial amygdala-dependent phase that occurs rapidly and is characterized by the expression of short-latency responses (occurring right after the tone onset) and 2) a later cerebellar-dependent phase where there is the expression of well-timed conditioned responses (occurring right before the shock) that slowly evolve as the training proceeds [107].

Having all this in consideration, one can argue that maybe the impaired performance of global CB1R KO mice in delay eyelid conditioning seen by Kishimoto and Kano might be to some extent related to the deletion of CB1Rs from the amygdala. Moreover, other studies suggesting the involvement of endocannabinoid plasticity in delay eyelid conditioning also appear to involve extracerebellar learning. In a previous study in which rats were subjected to delay eyelid conditioning, a pharmacological approach was used to show a CB1R dose-dependence effect on learning [154]. Another study, testing humans in this paradigm to assess how consumption of cannabis and consequent CB1R downregulation affects cerebellum-dependent learning also supports a role for the endocannabinoid system in this behavior [155]. For the study performed in rats a tone (CS) and a shock (US) were used as stimuli to elicit the eyelid learned response. The stimuli used for the study in humans consisted of a tone (CS) and an air puff (US). Curiously, in both studies the baseline of the percentage of CRs by the first session was around $\sim$25%, thus suggesting amygdala-dependent learning. In fact, eCBs were seen to be involved in plasticity mechanisms related to learning dependent on the amygdala [150]. This leads to the question of whether eCB signaling is important for cerebellum-dependent learning at all.

Under our experimental conditions, acquisition of learned responses appears to be cerebellum-dependent so that both the percent of trials presenting a learned response and the strength of that learned response developed gradually in control mice (Figure 3.1A and Figure 3.2A). When testing global CB1R KO mice, despite the slightly lower level of learning, mice acquired levels of learning comparable to control mice. In line with this, no gross impairments were found when testing the different conditional KO mice in delay eyelid conditioning (Figure 3.1B-D and Figure 3.2B-D), despite the fact that gabra6 KO mice were shown to have deficient parallel fiber-Purkinje cell LTD [147]. Thus, and by looking at gross features of learning, the present results suggest that eCB signaling might not be essential for cerebellum-dependent delay eyelid conditioning.

Apart from the type of learning induced, other factors can account for the differences between the presented and previous work [153]; in particular the length of the acquisition protocol. Kishimoto and Kano protocol consisted of 7 learning sessions whereas the experimental design of the current study was based on 20 acquisition sessions. Thus, and

because cerebellum-dependent learning is thought to be gradual, the hypothesis cannot be excluded that if the same protocol would have been applied to global CB1R KO mice run in Kishimoto and Kano's study, mice with the deletion would have reached levels of learning similar to those observed in control mice.

4.2 eCBs might not be required for the timing of learned eyelid responses

The cerebellar cortex has been shown to be responsible for the appropriate timing of the learned eyelid response [30]. CB1R-dependent endocannabinoid plasticity was seen mediate LTD at the parallel fiber-Purkinje cell synapse [144; 147], suggesting that this form of plasticity not only transiently inhibits neurotransmission but it also induces long-term changes. Moreover, endocannabinoid suppression at this synapse was also seen to be related with the timing of climbing fiber activation; with higher endocannabinoid release occurring when both parallel fiber and climbing fiber were co-activated [116]. This is in agreement with the associative motor learning thought to occur during cerebellar learning, and suggests that endocannabinoids might contribute to the timing dependence of motor learning [145].

Thus, despite the fact that gross features of behavior do not seem to be affected by the deletion of CB1Rs throughout the brain, more subtle aspects as the timing of the learned response could be. However, in the present work, we found no significant differences in the timing of the learned responses between CB1R KO and control mice (Figure 3.3A-D). When testing global CB1R KO mice in delay eyelid conditioning using a longer interval between stimuli (ISI) to increase the spatiotemporal resolution, KOs adapted the timing of their responses as well as littermate controls (Figure 3.3A). Even though the timing was appropriate, there was an overall decrease in the amplitude of the learned responses when using a longer ISI. This is in line with a previous work where the effect of different ISIs in the kinematics of the learned response was analyzed [27]. Our results suggest that CB1Rs at the parallel fibers are not necessary for the proper timing of the learned eyelid responses, suggesting that other forms of plasticity might compensate or be modulating the temporal features of the CRs.

4.3 Previous trial experience affects the eyelid learned response on the following trial, independently of eCB signaling

It has been suggested that motor learning might result from different processes occurring at different timescales. Apart from the initially proposed long-term changes, short-term synaptic plasticity mechanisms have also been implicated in cerebellar-dependent learning [114; 115].

Relatively recently, Medina and Lisberger [114] have been able to establish causality between the mechanisms proposed in the original cerebellar-learning theory by analyzing trial-to-trial changes in smooth eye pursuit learning in monkeys. They showed that

when a climbing fiber fires onto a Purkinje cell to signal motor error in one trial, there is induction of a large and well-timed depression of the activity between the parallel fiber and the Purkinje cell on the next trial, allowing movement to be corrected. Single-trial learning in delay eyelid conditioning has never been explored.

By analyzing trial-to-trial changes in delay eyelid conditioning we were able to observe that the presence or absence of an air puff on the previous trial affects the learned response on the following one. Trials that are preceded by a trial with a puff appear to have larger amplitude of the learned responses than trials preceded by a trial where the puff stimulus was not presented (Figure 3.4A). These results seem to be in agreement with what has been observed before in smooth eye pursuit experiments [114; 115]. In eyelid conditioning, climbing fibers are activated by the air puff stimulus and carry the motor error signal to the Purkinje cells in order to accurately depress the activity generated by parallel fibers onto the Purkinje cells when a light stimulus occurs on the following trial, gradually generating or amplifying the eyelid learned response [6]. Thus, if in the previous trial an air puff is presented, the learned response is expected to be larger in the following trial. On the other hand, when there was not an air puff stimulus on the previous trial, the learned response is expected to be reduced in the following trial.

Interestingly, endocannabinoid signaling has been shown to underlie several short-term synaptic plasticity mechanisms in the cerebellum. Endocannabinoid retrograde inhibition was seen to occur at the parallel fiber-Purkinje cell synapse in response to elevated calcium (eCB-mediated DSE) [122; 147] and climbing fiber-Purkinje cell synapse activity was shown to regulate this inhibition [116]. These results suggest that endocannabinoids might regulate the pause in activity that occurs at the parallel fiber-Purkinje cell synapse after climbing fiber input to Purkinje cell signals error, during motor learning. This short-term mechanism was seen to be absent in global CB1R KO mice [141].

Surprisingly, when tested in delay eyelid conditioning, both CB1R mutants and littermate controls responded similarly to alterations in the previous trial (Figure 3.4C-F and SuppFigure 1A-D). Overall, our results indicate that there is trial-to-trial learning in delay eyelid conditioning but intriguingly it does not seem to be mediated by endocannabinoid plasticity. Curiously, not only the CR amplitude, but also the percent of successful trials seems to be decreased in trials following trials where there was no puff presentation, for all genotypes (Figure 3.4G-J). This suggests that not only the amplitude, but also the actual presence of a learned response can be modulated by previous trial experience and this seems to be independent of the endocannabinoid signaling.

4.4 eCB signaling might be important for short-term memory related to the interval duration between trials

Although endocannabinoid-mediated plasticity does not seem to contribute to trial-to-trial learning depending on previous trial experience, it could contribute to short-term plasticity mechanisms necessary for sustaining memory between two trials. Moreover, the effect of different intervals between trials on the learned eye blink response has not been assessed in mice.

The results obtained in our study indicate that trials following a light + puff paired trial by a short interval exhibit slightly larger CR amplitude than trials preceded by a longer interval (Figure 3.5A), for all genotypes (Figure 3.5C-F; SuppFigure 2A-D). Interestingly, global deletion of CB1Rs appears to affect in a greater extent the responses associated with different intervals between trials (Figure 3.5C). This indicates that global CB1R KO mice do not hold on to the memory of the previous trial for as long as control mice. Gabra6 CB1R KO mice also appear to be slightly more affected, especially right before the puff onset (Figure 3.5E). These results suggest that there is a slight modulation of the current trial response that is dependent on the time elapsed since the previous trial and that the endocannabinoid signaling might modulate this phenomenon.

It could be that endocannabinoid-mediated plasticity is necessary for the maintenance of memory during consecutive trials. Indeed, global CB1Rs KO mice seem to be negatively affected by a longer interval between trials (Figure 3.5C). It should also be noted that the interval between trials used by Kishimoto and Kano (20-40s) [153] was much longer than the one used our study (5-20s), suggesting that part of the discrepancy between the two studies could arise from the loss of endocannabinoid-dependent motor memory. Because this effect was mainly observed upon global deletion of CB1Rs (Figure 3.5C and SuppFigure 2A), it remains to be accurately seen where endocannabinoids might be acting to control and assure working memory between consecutive trials. Nonetheless, because gabra6 but not alpha6 CB1R KO mice were more affected by a long intertrial interval (Figure 3.5E and SuppFigure 2C), working memory could be influenced by endocannabinoid-mediated plasticity at the mossy fiber-deep cerebellar nuclei neuron synapse.

4.5 CB1RS might be important for motor memory consolidation

Storage of motor memories is thought to be important for motor learning to occur. In fact, there is evidence for a critical period after training in delay eyelid conditioning, where memory consolidation mediated by the cerebellar cortex takes place [36; 37; 161]. These results seem to support a 'systems consolidation' view, where consolidation might involve shifts in memory location; meaning that motor memory is stored at different loci early and late in the learning process [162]. Thus, it was proposed that initially motor memory would be located at the cerebellar cortex, and during long-term consolidation motor memories would be converted to a more distributed representation that includes not only the cerebellar cortex but also the deep cerebellar nuclei [7].

Even though the performed experiments were not projected to study consolidation effects, we were able to analyze some of the features regarding motor memory consolidation in delay eyelid conditioning with the data set available. The results obtained indicate that on shorter timescales (for example, sessions within the same day), control mice appear to improve their performance from one session to the next one (Figure 3.6A-D), which is consistent with the normal consolidation described to take place during learning. When comparing performance of sessions separated by longer periods (sessions separated

by two days), control mice also appear to remember well, as no relevant differences in the percentage of CR trials appear to occur (Figure 3.6A-D). However, both global and gabra6 mice appear to have impaired memory consolidation, exhibiting less percent CR after a long period without training than their littermate controls (Figure 3.6A, B and C). This seems to be consistent with the idea that motor memory consolidation might involve a shift from the cerebellar cortex to the cerebellar nuclei [7; 39]. Because only gabra6 but not alpha6 conditional KO mice were impaired, perhaps endocannabinoid signaling at the mossy fiber-deep cerebellar nuclei neuron synapse could be important for regulating long-term memory at the deep cerebellar nuclei. Curiously, endocannabinoid signaling has also been involved with memory consolidation in other brain structures, such as hippocampus [163].

Interestingly, this impairment of motor memory after a long period of time seen in global, gabra6 and to less extent, parvalbumin-specific CB1R KO mice (Figure 3.6), could be a partial explanation for their biphasic learning curves (Figure 3.1 and Figure 3.2); for the majority of mice a long time period between sessions occurred from S10 to S11 and this interval appears to be related with a decrease in both percent and amplitude of the conditioned responses. Nonetheless, there is still the need to increase the number of mice of the parvalbumin strain to better understand whether the pattern observed in their learning curve is only due to the fact that they presented many fluctuations in learning or to an actual effect in motor memory consolidation.

4.6 Running activity might be important for motor learning

Locomotor activity has been mentioned to be important for delay eyelid conditioning. Mice have frequent periods of quiet wakefulness that might be partially responsible for the low levels of conditioning observed in previous studies. During these periods, even a well-conditioned mouse does not exhibit CRs. Thus, the occurrence of quiet wakefulness periods can strongly negatively influence the outcome of the learning process [107]. Nonetheless, these periods appear to be reduced when they are engaged in running on a treadmill, which is one of their favorite activities [27].

Even though the performed experiments were not designed to investigate in detail the potential running effects in delay eyelid conditioning, it was possible to quantify and analyze how running affects learning using the present data set. The experimental setup used in the present experimental conditions allowed mice to run voluntarily on a treadmill while being conditioned. For the running analysis, we quantified this activity and sorted mice by their activity profile during acquisition. The results obtained show that mice that run more, and probably are more active throughout each session, seem to learn better than non-runners (Figure 3.8A and SuppFigure 3A); supporting the hypothesis that activity during delay eyelid conditioning increases the efficacy of learning. By quantifying activity in the treadmill during learning it was possible to observe that mice lacking CB1Rs either globally or at the granule cells appear to have decreased locomotor activity when compared to littermate controls (Figure 3.7). Our finding is in agreement with what was described before for the global CB1R KO mice, as they were shown to exhibit decreased levels of locomotor activity, even though not revealing any apparent

health problems or physiological abnormalities [149]. It could be that the impairments seen previously by Kishimoto and Kano [153] were to some extent due to hypoactivity affecting learning. Because CB1R KO mice are on average less active, they would have more frequent quiet wakefulness moments and this decrease in alertness state would negatively affect learning. Actually, in Kishimoto and Kano experiments mice were conditioned in a small chamber, in contrast with the experimental setup used in this study where mice were allowed to walk/run on a treadmill, thus being more engaged throughout the sessions. In fact, when sorting CB1R KO mice by how much they ran, it was possible to see that runners of both control and global CB1R KO genotypes appeared to learn very similarly (Figure 3.8A and B, and SuppFigure 3A and B). Overall, our results suggest a relation between running activity and learning in delay eyelid conditioning, with a possible indirect effect of the endocannabinoid signaling.

In line with this hypothesis, when a group of control mice was tested on a motorized version of the treadmill (where mice ran constantly and same throughout the sessions), they all learned fast and at similar rates (SuppFigure 4A and B), suggesting that much of the variability seen in delay eyelid conditioning might be related with variations in locomotor activity. Whether this is a binary modulation (awake vs asleep) and thus a proxy for alertness or an actual modulation of learning by speed is still to be determined. Curiously, there is some evidence that responses to visual stimuli are increased if an animal is running when compared to stationary [164] and for running speed to modulate responses in the visual cortex [**?**]. Thus, it seems reasonable to hypothesize that running activity might have a role in the cerebellum and thus in motor learning.

When re-analyzing previously observed effects of possible CB1R-mediated plasticity taking into account the running activity, the results remained similar (data not shown). Thus, the long intertrial interval effect on global CB1R KO mice or the consolidation impairment after longer periods on both global and gabra6 CB1R KOs, might be directly related with the endocannabinoid signaling and not to indirect running effects.

Chapter 5

Conclusions and Future Perspectives

The possibility to relate sensory and motor signals with identifiable cells within a well-defined circuit makes the cerebellum an attractive system to study how synaptic plasticity relates to learning. Delay eyelid conditioning is a form of associative motor learning that requires an intact cerebellum. In this paradigm, mice learn to close their eyes in response to a neutral conditioned stimulus (CS; such as a light) after it has been paired with an unconditioned stimulus reliably eliciting a blink (US; such as an air puff). The circuit underlying this behavior is relatively well-established, with both sensory input and motor output information being associated with different cerebellar cell types.

Because CB1Rs were observed to influence delay eyelid conditioning possibly by an effect of CB1R-mediated plasticity in the cerebellum, the goal of this project was to determine the role of CB1R-mediated plasticity in cerebellum-dependent learning. More specifically we aimed to understand in which cells and through what mechanisms are these receptors acting to regulate motor learning. This was done by assessing how CB1R deletion from different cerebellar cell types can affect different aspects of learning in delay eyelid conditioning.

Surprisingly, our results indicate a much milder contribution of the endocannabinoid system to delay eyelid conditioning than what was previously seen. Actually, when assessing the learning of global CB1R KO mice, no major impairments were found at all. Moreover, when testing cerebellar cell-specific CB1R KO mouse lines for the first time in delay eyelid conditioning, KO mice presented similar percentage and amplitude of the learned responses when compared to littermate controls. One possible explanation for this discrepancy might be related with the type of learning induced in previous works, which appears to be not only cerebellar- but also extracerebellar-dependent. In fact, the present results suggest that the impairments seen previously in delay eyelid conditioning might be indirectly related to deletion of CB1Rs from the amygdala and not to a direct modulation in the cerebellum.

When looking at more subtle aspects of the behavior, such as the CR timing, also no significant differences were found, as in general mice were able to appropriately time their conditioned responses. Thus, despite the fact that endocannabinoids are involved in suppressing neurotransmitter release during parallel fiber-Purkinje cell LTD, suggesting a role of CB1Rs in controlling the timing of the learned response, the endocannabinoid sig-

naling at this synapse does not appear to regulate the timing aspect of learning. Nonetheless it could be the case that endocannabinoids contribute to parallel fiber-Purkinje cell LTD indirectly via the modulation of activity at the climbing fiber-Purkinje cell synapse. To test this hypothesis mice lacking CB1Rs at the climbing fibers could be tested in delay eyelid conditioning.

By analyzing trial-to-trial changes in behavior, it was possible to observe that mice are affected by previous trial experience during delay eyelid conditioning. When a puff is presented on the previous trial, the learned response is increased on the following trial. But despite evidence suggesting a role for endocannabinoids in mediating this short-term form of plasticity, no relevant differences were found between global or conditional CB1R KO and their littermate controls. However, when we looked at how different intervals between trials can affect the learned response, both global and gabra6 CB1R KO mice appeared to have lower amplitude responses after a longer time period between trials than after a shorter interval when comparing to their littermate controls. This modulation appears to be endocannabinoid dependent, with CB1R-mediated plasticity probably being responsible for sustaining motor memory between trials. This effect might also help explaining the discrepancy with previous results, since the intervals of time between trials used in previous studies were longer than in the ones we used.

Apart from the intertrial interval short-term related plasticity, long-term consolidation also appears to be affected by the deletion of CB1Rs. Both global and gabra6 mice appear to remember less after a longer period without training than littermate controls, suggesting that endocannabinoid signaling might be involved in the consolidation of motor memory. Testing both global and gabra6 CB1R KO mice in delay eyelid conditioning with a protocol accounting for more variable timescales would help clarifying the possible consolidation impairment upon CB1R deletion. Curiously, the endocannabinoid signaling was also associated with memory consolidation in other brain areas, such as the hippocampus.

Overall, our results suggest that CB1R-mediated modulation at the parallel fiber-Purkinje cell or mossy fiber-deep cerebellar nuclei neuron synapse might be involved in both short- and long-term phenomena in delay eyelid conditioning. This plasticity would assure working memory between trials in a short timescale and be involved in the recall process of motor memory after a long time period. Because only global and gabra6 CB1R KO mice but not alpha6 were affected in both cases, this modulation might occur at the mossy fiber-deep cerebellar nuclei neuron synapse. This would be consistent with the idea that plasticity related to motor learning occurs in two phases and temporally depends on different cerebellar structures. Early in learning mainly the cerebellar cortex is involved and at later phases there is a shift of motor memory so that not only the cerebellar cortex but also the deep cerebellar nuclei are involved; this phenomenon is hypothesized to allow the cerebellar cortex to efficiently regulate the formation of new motor memories. The fact that the effects observed occurred after mice had already shown reasonable levels of learning and appear associated with plasticity at the cerebellar nuclei also seems to be in agreement with this idea. Together with the fact that endocannabinoid signaling has been shown to mediate both short- and long-term cerebellar plasticity, it seems reasonable to hypothesize that endocannabinoid modulation might play a role in both working

memory and recall of motor memory after learning has been established.

Nonetheless, more experiments are needed to specifically evaluate this possibility. Performing electrophysiology in slices to measure activity at the mossy fiber-deep cerebellar nuclei synapses of both global and gabra6 CB1R KO mice would help to understand whether deletion of possible existent CB1Rs is affecting synaptic modulation and thus influencing learning. It would also be interesting to understand whether activity at this synapse suffers changes during learning; by being, for example, silent at early phases and exhibiting activity related to delay eyelid conditioning after a certain threshold of learning has been acquired.

Curiously, under our experimental conditions, running activity also appears to modulate learning in delay eyelid conditioning, with mice that run more learning faster than mice considered non-runners. Because we observed this effect for all genotypes, running activity does not seem to be endocannabinoid-dependent. Nonetheless, global CB1Rs are hypoactive and this, together with the fact that running activity and learning can be related, suggests that some of the impairments seen in other studies could be indirectly related to running activity and not to direct endocannabinoid modulation. One possible way to assess this could be by making both control and mutant mice having the same running activity profile. It would be interesting to test if by homogenizing the amount of running across sessions by using a motorized treadmill, learning would also be more uniform across all mice. Moreover, it would be worth to see if different velocities of running are associated with different strengths of the eyelid learned response, so that there is a modulation by speed. It would also be interesting to investigate whether locomotion modulates activity of different cerebellar cell-types. This could be done by using electrophysiology to understand if running is associated, for example, with increased spontaneous activity or weakening of suppressive signals, which is thought to diminish the signal-to-noise ratio and thus contribute to neural coding.

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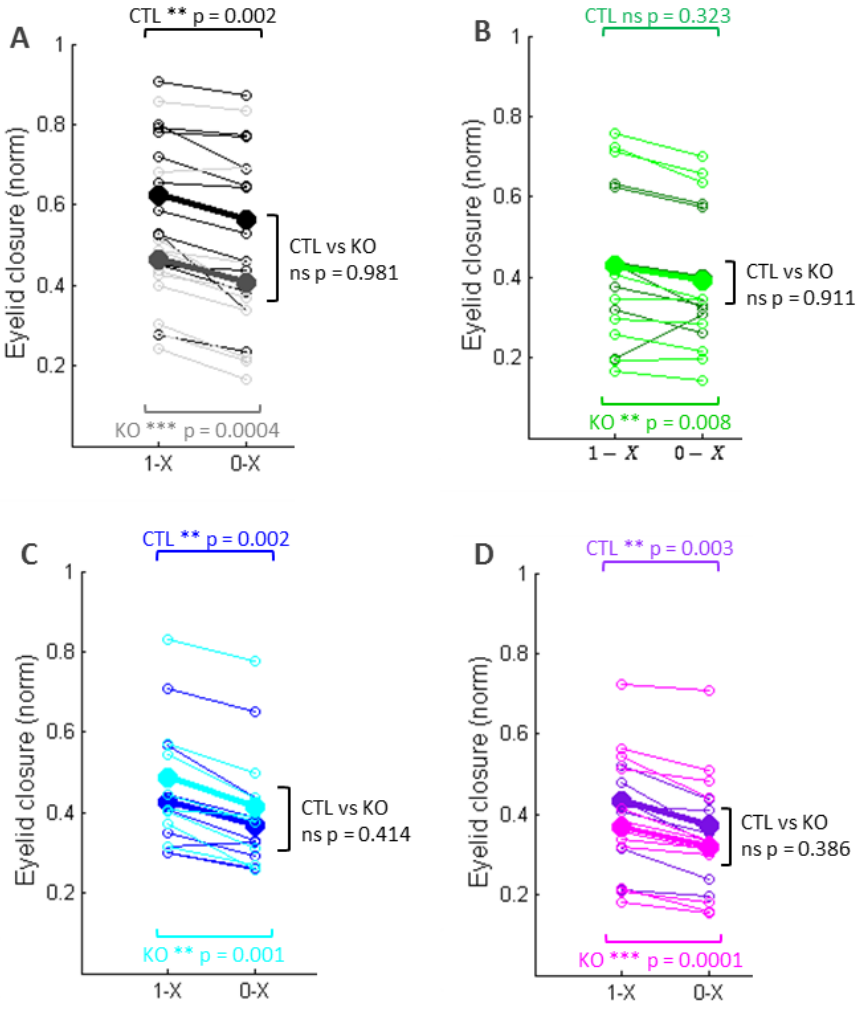
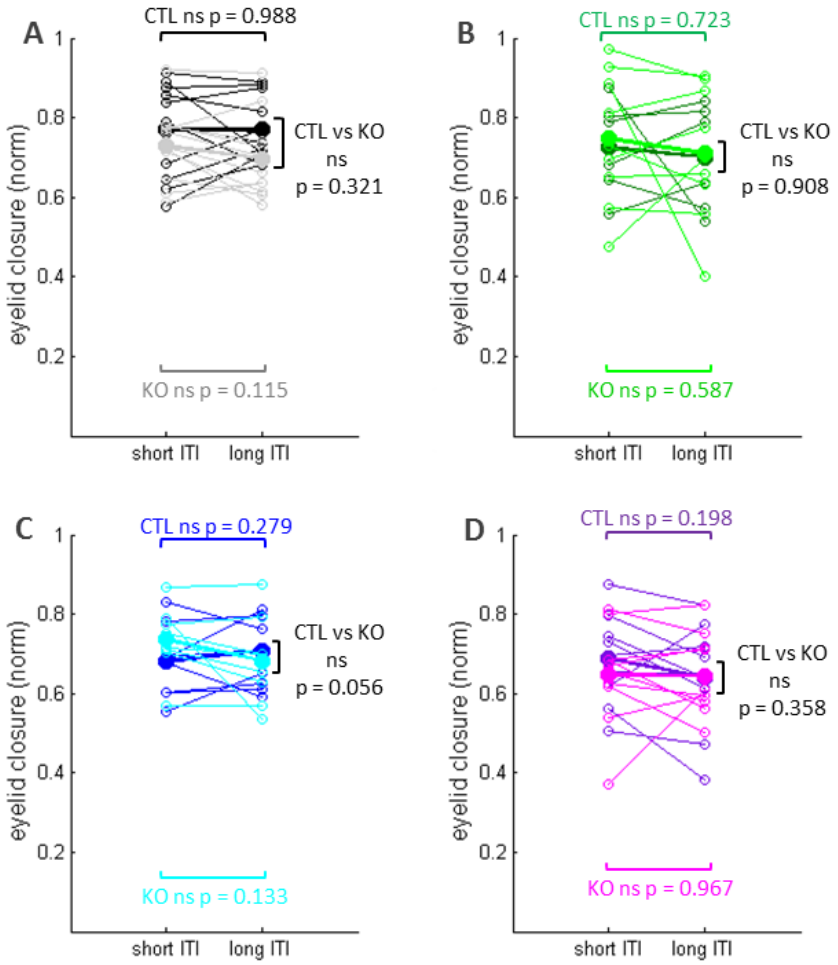
Annexes

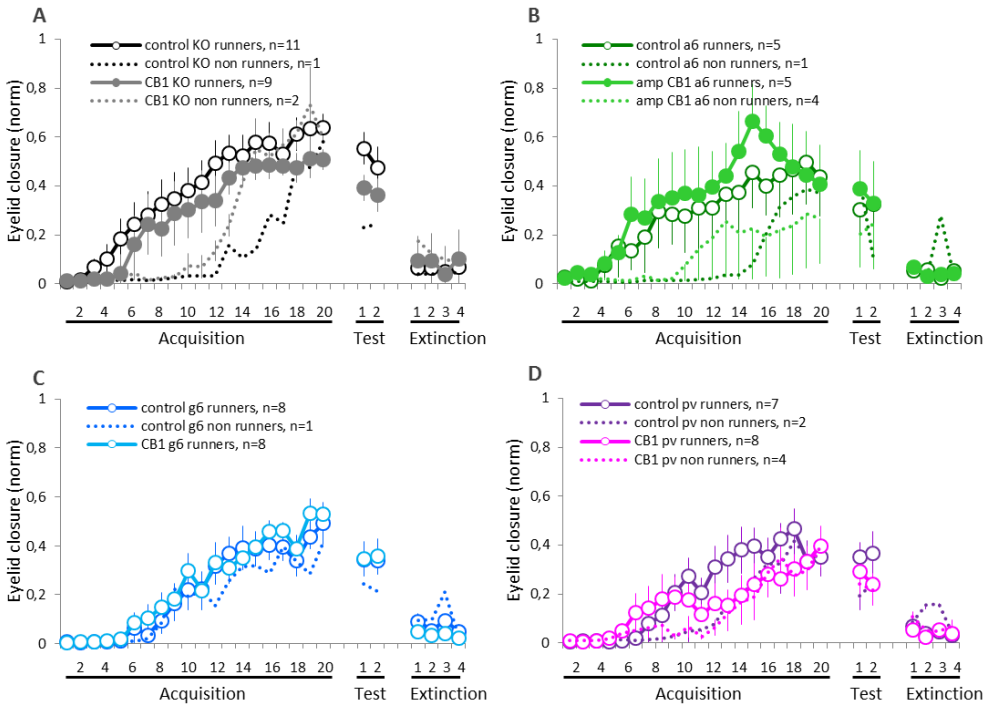
Supplementary Figures



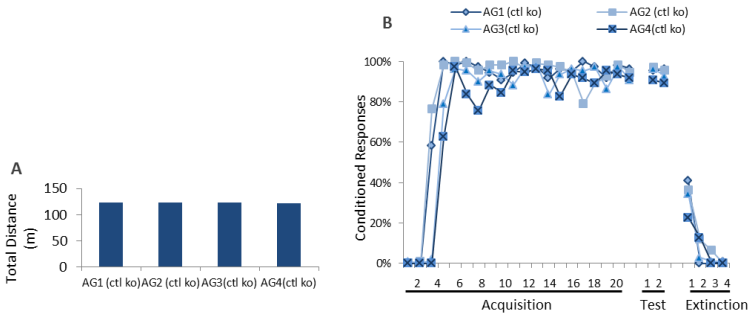
SuppFigure 1: **Previous trial experience affects the amplitude of the CRs on the next trial, regardless of genotype.** A-D) Averaged amplitude of CR of each condition (1-x and 0-x) for each genotype (thicker lines) is plotted on top of performances from individual animals (thinner lines). Each plot shows data for both genotypes of one strain: global, alpha6 (a6), gabra6 (g6) and parvalbumin (pv), respectively. Paired t-Test was used to compare 1-x with 0-x conditions for each genotype. Two-sample t-Test was used to compare 1-x and 0-x difference between controls and KOs.



SuppFigure 2: **Global and gabra6 CB1R KO mice appear to be more affected by a long inter trial interval; they exhibit decreased responses after a longer interval than after a shorter interval.** A-D) Averaged amplitude of CR of each condition (trials preceded by a short or a long interval) for each genotype (thicker lines) is plotted on top of performances from individual animals (thinner lines). Each plot shows data for both genotypes of one strain: global, alpha6 (a6), gabra6 (g6) and parvalbumin (pv), respectively. Paired t-Test was used to compare short with long conditions for each genotype. Two-sample t-Test was used to compare short and long difference between controls and KOs.



SuppFigure 3: **Mice that run more appear to learn faster, regardless of genotype.** Mice were allowed to walk or run voluntarily on a treadmill during learning. For each genotype, mice were divided by how much they run during training; mice running less than around one fourth of what control mice did per session on average (38m) were considered non-runners. A-D) Averaged CR amplitude of both runners and non runners across sessions for global, alpha6 (a6), gabra6 (g6) and parvalbumin (pv) strains, respectively. Error bars represent standard error of the mean (SEM).



SuppFigure 4: **Mice that run the same seem to learn similarly.** Mice were imposed to run at a constant speed of 1.6m/s (which corresponds to ∼80% of the distance performed on average by control mice freely walking) on a motorized treadmill during learning. A) Averaged total distance ran during all acquisition sessions for each mouse. B) Averaged CR amplitude of each wild-type mouse. Error bars represent standard error of the mean (SEM).