Structural Correlates of Motor Learning

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February 2021

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A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

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Abstract

The cerebellum is known to support motor learning – however, the synaptic substrates of learning are a subject of controversy. A previously well-established model of motor learning suggested that long-term depression at parallel fiber-to-Purkinje cell synapses supports motor learning; however how this model works has recently been brought into question. In order to determine what form of plasticity is induced at synapses in the cerebellum during learning, we established and adapted a form of cerebellum-dependent forelimb-reach learning in mice, followed by assessing structural plasticity in the relevant region of the cerebellum. Specifically, we used a sparse-labeling technique to assess the density of dendritic spines onto Purkinje cells, which are the sites of parallel fiber-to-Purkinje cell synapses. Our results demonstrate an inverse correlation between the amount of learning and Purkinje cell spine density, at the level of individual mice. Thus, we provide evidence that depression-like changes do indeed occur at parallel fiber-to-Purkinje cells synapses during motor learning. Moreover, the degree of such plasticity correlates with the amount of learning.

Le résumé

Le cervelet est connu pour soutenir l'apprentissage moteur - cependant, les substrats synaptiques de l'apprentissage sont un sujet de controverse. Un modèle d'apprentissage moteur précédemment bien établi suggérait que la dépression à long terme au niveau des synapses entre les fibres parallèles et les cellules de Purkinje soutient l'apprentissage moteur; cependant la façon dont ce modèle fonctionne a été récemment remis en question. Afin de déterminer quelle forme de plasticité est induite au niveau des synapses du cervelet pendant l'apprentissage, nous avons établi et adapté une forme d'apprentissage dépendante du cervelet au niveau des membres antérieurs chez la souris, puis nous avons évalué la plasticité structurelle dans la région concernée du cervelet. Plus précisément, nous avons utilisé une technique de marquage clairsemée pour évaluer la densité des épines dendritiques des cellules de Purkinje, qui sont les sites de synapse entre les fibres parallèles et les cellules de Purkinje. Nos résultats démontrent une corrélation inverse entre l'apprentissage et la densité des épines des cellules de Purkinje, ce pour chaque souris. Ainsi, nous apportons la preuve que des changements de type « dépression » se produisent effectivement au niveau des synapses entre les fibres parallèles et les cellules Purkinje pendant l'apprentissage moteur. De plus, le degré d'une telle plasticité est en corrélation avec l''apprentissage.

Acknowledgements

I would first especially like to acknowledge my supervisor Aparna Suvrathan for her guidance and help in getting through every phase of this project, especially through figuring out things to test and optimize when the project wasn't running so well, as well as helping me through writing everything. She was patient through every phase of this project, and I can't imagine working through this project with anyone else as my supervisor. I would also like to thank my lab mates Riya Thomas and Camilla Ciapponi for their help on optimizing behaviour, as well as suggestions in Golgi and antibody staining. My project would not be possible without their help. I would like to acknowledge the Sjöström Lab for their help in teaching me and looking after me during the initial days of my project as well as giving me training, and allowing me use of their equipment, and Aurore Thomazeau for helping me with translating the abstract to French. I would also like to acknowledge the David, van Meyel, and Chen labs for the use of their lab space, equipment, as well as training. I would like to acknowledge the Murai lab for their help in assisting me in staining and processing Golgi and fluorescent sections, and especially for the use of the Vibratome, and showing me how to section and process some slides.

Contribution of Authors

This thesis was written by Richard Zhang, the author, under guidance from Aparna Suvrathan. The author also performed all experiments and analyses described in the thesis, with the exception of the establishment of the behaviour task, which was optimized with the assistance and collaboration of Riya Thomas, and the acquisition of the CAMKIICre:TdTomato mouse, which was generously provided by the Murai lab, with tissue processing and imaging performed by the author. French translation was provided by Aurore Thomazeau.

Introduction

The cerebellum allows us to perform precisely coordinated and well-timed movements and is critical for motor learning. The well-mapped cytoarchitecture of the cerebellum, and its well-established role in motor learning makes it an ideal system in which to investigate the synaptic substrates of learning. However, several open questions remain. A key question relates to whether plasticity at parallel fiber-to-Purkinje cell synapses supports motor learning and, if so, whether synapses are strengthened or weakened. This thesis aims to answer this question by investigating structural plasticity induced by motor learning.

We hypothesize that long-term depression-like changes occur at parallel fiber-to Purkinje cell synapses during learning. The background and previous findings that form the basis for this hypothesis are described below.

Background

1. CYTOARCHITECTURE OF THE CEREBELLAR CORTEX

The primary inputs of the cerebellar cortex are the mossy fibers, which carry relevant information into the cortex for processing, and originate in brain areas such as the motor cortex, premotor cortex, and prefrontal cortex, as well as from sensory systems[1]. These mossy fibers then synapse onto granule cells (GCs), whose axons form parallel fibers (PFs). The PFs, in turn, synapse onto Purkinje Cells (PCs), the sole output of the cerebellar cortex. A secondary synaptic input into the cerebellar cortex exists in the form of the climbing fiber, originating from the cells of the inferior olive of the medulla oblongata. Each Purkinje cell is also innervated by a single climbing fiber. Both parallel fiber and climbing fiber synapses are excitatory, glutamatergic connections, while the PC output is inhibitory. In addition to these excitatory inputs, there exist inhibitory interneurons within the cerebellar cortex [2,3](Fig 1)

Information from these excitatory and inhibitory synaptic inputs are integrated in the Purkinje cell, which then projects out of the cerebellar cortex, to the deep cerebellar nuclei (DCN). Purkinje cells form inhibitory, gamma-aminobutyric acid (GABA)-ergic synapse with the DCNs. The DCN, in turn, projects to ascending and descending pathways in the brainstem.[4]

Each of the major cell types and synaptic inputs in the cerebellar cortex is described briefly below.

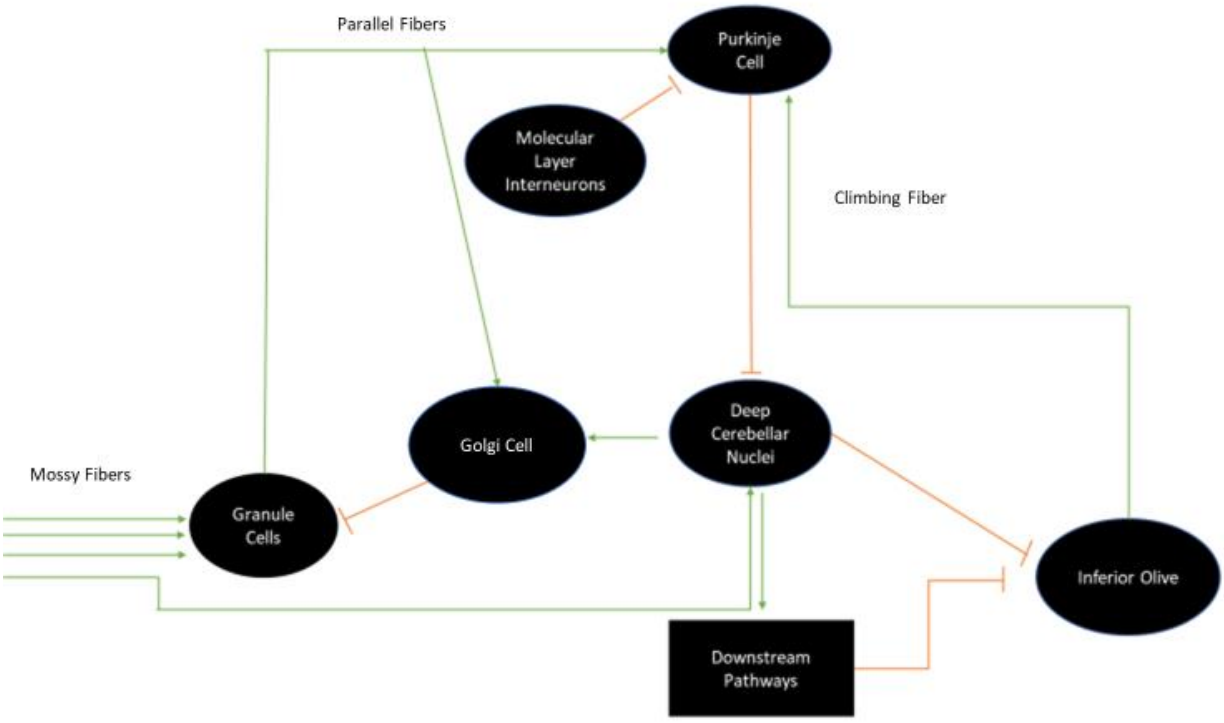

Fig 1. A schematic showing major connections and major components of the cerebellar cortical circuit. Excitatory connections are shown in green, while inhibitory connections are shown in orange.

1A. Purkinje Cells

The Purkinje cells are the single output from the cerebellar cortex. These inhibitory cells possess an extensive dendritic arbor, concomitant with their role in integrating a large number of signals. These signals include the connections they receive from the Molecular Layer Interneurons (MLIs), Granule Cells (GCs)s via the PFs, and cells of the inferior olive via the climbing fibers[2]. The PCs possess intrinsic pacemaker activity, which can be modified by the synaptic inputs to the PC[5]. As the PC is the junction of many input sources, and is the sole output, it has long been speculated that the PC is the primary location of plasticity in the cerebellum during cerebellum-dependent learning[6]. Notably, PC activity and characteristics appear to be heterogeneous across different regions of the cerebellum, which follows the expression of the marker Aldolase-C, or Zebrin.

Zebrin-negative PCs fire at a much higher frequency than Zebrin-positive PCs. In addition, PCs in different Zebrin zones have differences in the regularity of simple spikes as well as in the length of the post-complex spike pause[7]. (Simple spikes are Purkinje cell action potentials, while complex spikes are the characteristic spike waveform recorded at the PC cell body when there is input from the climbing fiber.) Thus, PCs are heterogeneous in their cellular properties.

1B. Mossy Fibers

The mossy fibers form one of the primary inputs into the cerebellar cortex. These fibers carry contextual information (i.e. proprioceptive, sensory), as well as motor command copies into the cerebellar cortex[8]. Inside the cerebellar cortex, the MFs form several connections with neighboring cells. Firstly, the MFs contact the GCs, through MF-GC rosettes. This connection is of note, since a single MF contacts multiple GCs via these rosettes, thus creating a divergent, expanded, and higher dimensional signal. This has been speculated to assist with pattern separation, redundancy, and temporal coding, allowing for features such as movement timing for instance[9]. Additionally, the MFs also provide input to the inhibitory Golgi cells[10]. Finally, MFs also directly contact the deep cerebellar nuclei, bypassing the cerebellar cortical circuitry entirely[11]. In conclusion, the MFs carry information into both the cerebellar cortex as well as the DCN and form the primary input into the cerebellum (Fig 1).

1C. Golgi Cells

Golgi cells are a type of inhibitory interneuron found in the granule cell layer, which receive inputs from both MFs and PFs[12].These cells then output inhibitory signals to GCs. It was initially believed that Golgi cells maintain an inhibitory baseline, which allows for the focusing of MF signals, as

only the strongest MF signals would be able to overcome baseline inhibition[10]. Additionally, it was also thought to allow for a consistent number of MFs to be active at any one time[13]. More recent observations speculate that the Golgi cells could help amplify contrasts between activated and inactivated groups of MFs, creating a form of center-surround organization[12].

1D. Granule Cells

GCs receive input from the MFs and are the most numerous neurons in the entire brain (approximately 70 billion)[14,15] . Each GC possesses ~4 dendrites, each contacting one mossy fiber[16] which, when combined with their vast numbers, is ideal for the purposes of pattern separation, as discussed previously. The axons of these cells form PFs, and provide input to the PC. It was found in the rat that each PC receives inputs from roughly 170,000 PF synapses[17]. Parallel fibers also contact the molecular layer interneurons, comprised of Stellate and Basket cells, which provide inhibitory input to PCs. The connectivity and extremely large number of GCs suggests that incoming signals are split into many components, creating a very complex layer of computation, allowing for the expansion of inputs, where every granule cell is able to code for a very specific and unique set of conditions[9].

1E. Molecular Layer Interneurons

MLIs, as their name suggests, are found in the molecular layer of the cerebellar cortex. These cells form inhibitory inputs into the PC. These interneurons receive input from parallel fibers and provide feedforward inhibition to the Purkinje cell. There are two types of MLIs, Stellate and Basket cells, the former of which contacts the distal dendrites of the PC, while the latter synapses closer to the soma of the PC. Due to their location close to the axon initial segment of PCs, Basket

cells can strongly affect PC firing rate[18]. Stellate cells on the other hand play an important role in the integration of synaptic inputs in the dendrites, and play a role in modulating PC firing patterns[18]. Notably, the MLI-PC synapse is known to show plasticity, which has implications for the investigation into mechanisms of cerebellar memory. MLIs are also able to receive inhibitory inputs from at least two other MLIs, allowing for further regulation of inhibitory activity. MLIs also are able to receive inputs from multiple CFs through glutamate spillover[19]. Finally, optogenetic stimulation of MLIs, resulting in the inhibition of PCs, is sufficient to generate movement such as eyelid closure[20]. Thus, MLIs occupy a very important role in the cerebellum, by being able to directly inhibit the PC and thereby affect and modify the output of the entire cerebellar cortex.

1F. Climbing Fibers

The CFs serve as a secondary input into the PC, and originate from neurons in the inferior olive, which receive sensory input. CFs possess a unique relationship with PCs where every PC receives only one CF input. Structurally, CFs wrap themselves around the dendrites of the PC, creating multiple synapses onto each PC[21]. CFs are thought to encode error signals during cerebellum-dependent motor learning. To elaborate, motor errors cause firing of inferior olive neurons, which results in a complex spike at the Purkinje cell body. When complex spikes are paired with parallel fiber inputs, plasticity of parallel fiber synapses is induced in the form of long-term depression. Thus, climbing fiber signals carry error information that can correct Purkinje cell output in order to correct motor behaviors. It should be noted that climbing fiber inputs influence MLIs and project to the DCN, in addition to their direct connection to Purkinje cells[19].

2. FUNCTIONS OF THE CEREBELLUM

The cerebellum has long been associated with motor function, most notably in facilitating balance, coordination and motor learning; i.e., movements and actions are, with practice, improved in their accuracy, smoothness, and precision. Historically, it was observed that lesions and damage to the cerebellum resulted in loss/impairment of motor coordination and balance, in both animal studies, as well as in clinical observations[22,23]. In cerebellar ataxia, which arises from the degeneration of cerebellar neurons[24], it was observed that patients lack motor coordination and balance and will consequently display a defective gait while walking[25,26]. Motor deficiencies have also been observed in other complex motor tasks, specifically those requiring coordination and precise timing. For example, while throwing a ball, it was observed that patients with cerebellar dysfunction had issues in timing the release of the ball from their hand[27]. Interestingly, damage to different areas of the cerebellum results in different symptoms present in ataxia (i.e. damage to the cerebellar hemispheres result in issues with fine motor control such as tremor and lack of coordination, while damage to the vermis region results in issues such as the aforementioned defective gait), showing the compartmentalization of functions in the cerebellum[24]. More recently, there has also been a wealth of evidence showing cerebellar activity in certain cognitive tasks, such as language. Patients with cerebellar degeneration were found to have deficiencies in verbal fluency[28], as well as in memory, planning, organization, and spatial tasks. In particular, fMRI activity was found in specific lobules of the cerebellum during these tasks[29]. Indeed, patients with injury or dysfunction in cerebellar areas responsible for cognitive tasks also show cognitive deficiencies, including symptoms such as impaired working memory,

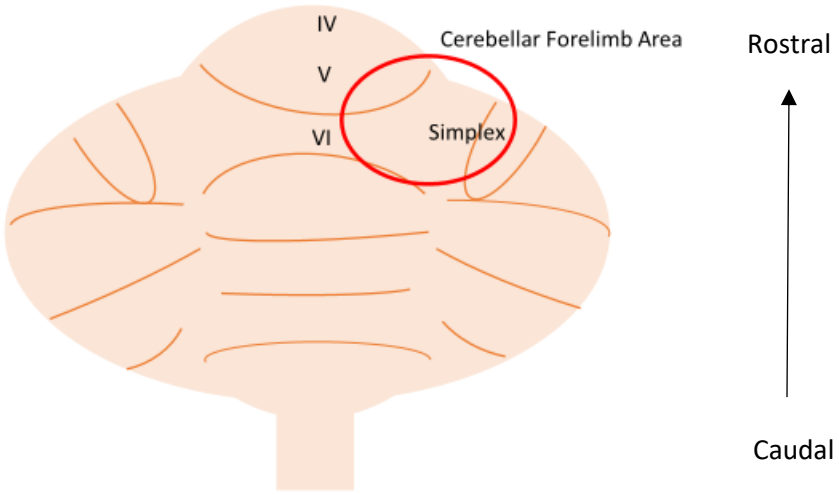

Fig 2. This cartoon indicates the forelimb area of the cerebellum consisting of the lobule simplex, IV, and V, thought to be involved in forelimb movement and coordination.

This is now collectively termed Cerebellar Cognitive Affective Syndrome (CCAS), or Schmahmann's syndrome[29].

While our understanding of many of the functions of the cerebellum come from patient case studies, animal models provide a unique advantage for studying the cellular mechanisms of learning. In cerebellum-dependent learning studies in animal models, perhaps the most studied tasks are Vestibulo-Ocular Reflex (VOR) learning and Optokinetic Reflex (OKR) adaptation. These forms of learning are known to be dependent on a region of the cerebellum known as the flocculus. Other well-studied cerebellum-dependent forms of learning include eyeblink conditioning which is thought to engage the lobule simplex[30], forelimb reaching which is also thought to engage neurons in the lobule simplex (Fig 2)[31], and rotarod learning, which is thought to also broadly engage the cerebellum[32].

3. PLASTICITY IN THE CEREBELLUM – The Marr-Albus-Ito Model

The Marr-Albus-Ito model is regarded as the classic model of cerebellar memory, summarizing the findings and theories of Marr, Albus, and Ito. Briefly, the model describes the cerebellar cortex as a supervised learning machine, where errors in motor output are fed back into the PF-PC synapse, via the climbing fibers, and result in the depression of PF synapses, thereby correcting erroneous motor output[6].

The model had its origins in the ideas of David Marr, who first hypothesized that PF-PC synapses are facilitated through stimulation of both the PFs and the CFs[33]. Briefly, Marr suggested that there are 2 phases to cerebellar learning, a "rehearsal" phase, where the cellular circuitry is trained, followed by a "trained" phase. Marr noted that every PC contains two inputs: a PF input as well as a CF input, and a single output leading to downstream motor effectors. During the "rehearsal" or training phase, the inferior olive reacts to a cerebral movement command and signals to the PC via the CF, which in turn signals the motor command downstream. The PC then receives two signals, an input from the CF, as well as the context in which this input occurred, via the MFs. In essence, CFs signal for elemental movements, whose contexts are provided by MFs. PCs would then, through repeated pairings of CF and MF, learn the context in which the CFs fire, thus training the circuit, and sensitizing the PC through LTP. Eventually, the PCs would be able to recognize all contexts and situations signaled by the cerebrum, and independently propagate motor signals downstream, resulting in forms of elemental movement. Thus, the PCs are then trained to recognize the situations that they are supposed to fire, and as a result are able to sequentially guide elemental movements[6,33].

This view would be later modified by Albus in 1971. Albus instead likened the cerebellar cortical circuit to that of a perceptron, a theoretical learning machine/algorithm. Here, MF signals

undergo pattern separation as a result of the GCs, which then synapse onto the PCs. Albus also suggested that the CFs send error signals to correct erroneous PC activity, and that stimulation of both CFs and PCs conjunctively results in depression, not potentiation as had been suggested by Marr[6,13]. Unfortunately, due to technological limitations, evidence of parallel fiber depression would not be observed until 1982.

In 1982, Masao Ito and colleagues investigated the PF-PC synapse, by recording from PCs in decerebrate rabbit flocculi[34]. In accordance with the then-theoretical Marr-Albus model, the group stimulated vestibular afferents (which lead to MFs) conjunctively with the inferior olive of the animals, and were able to observe depression in the PC firing, including a quick phase lasting 10 minutes as well as a much slower phase of depression lasting 4 hours. The group also observed that glutamate sensitivity after conjunctive stimulation of the inferior olive and vestibular afferents is reduced in the PC, thus providing the first experimental observations of the model first envisioned by Marr. The observations suggested a form of post-synaptic plasticity in the PC, by extension establishing the PF-PC synapse as a location of memory. Taken together, the Marr-Albus-Ito model suggests that cerebellar motor learning is driven by climbing fiber error signals inducing long-term depression at parallel fiber synapses. Subsequent experiments have provided a wealth of evidence in favor of this model, and it is now currently the dominant model to explain the synaptic substrates of motor learning.

4. EVIDENCE IN SUPPORT OF THE MARR-ALBUS MODEL:

After the observations of Ito and colleagues in 1982, experiments were performed to identify the cellular mechanisms underlying the Marr-Albus-Ito model, and the role of parallel fiber LTD in

motor learning[34]. It was found that disruption of CF inputs by interfering with the inferior olive results in impaired locomotion and motor behavior, consistent with predictions from the model, and observed in numerous animal models[35-37]. In addition, experiments using pharmacological, transgenic, and optogenetic means to disrupt LTD demonstrated a perturbation of motor learning. Some specific examples are listed below. Pharmacological blockade of nitrous oxide signaling, which is involved in the signaling cascade for LTD[38], resulted in deficits in Vestibular Ocular Reflex (VOR) learning[3]. Genetic knockout or perturbation of kinases involved in the signaling pathway for LTD, such as PKC[37], and alphaCAMKII[39], also showed the same result: inhibition of LTD and consequent deficits in VOR learning. In addition, knockout of mGluR1, a key receptor at the PF-PC synapses, results in both deficiency in LTD and in behavioural deficiencies consistent with cerebellar dysfunction, not unlike those found in ataxia, including tremor and, disrupted gait. Furthermore, eyeblink conditioning was impaired in these animals, also suggesting that LTD plays a central role in motor learning. More recently, optogenetic techniques have been used to transiently inhibit LTD in vivo during learning, by inhibiting AMPA internalization. Here, too, disruptions in LTD were paralleled by deficits in VOR and OKR learning[40].

In summary, the importance of LTD in cerebellum-dependent learning, as predicted by the Marr-Albus-Ito model, has been well established as numerous studies have shown impairments in motor learning resulting from the impairment of LTD at in the PF-PC synapse.

5. CONTROVERSIES REGARDING THE MARR-ALBUS MODEL.

5A. Contrasting effect of disruption of LTD on learning

However, the importance of PF-PC LTD in motor learning has also been called into dispute. In two studies, both genetic and pharmacological perturbation of LTD failed to impact cerebellum-dependent motor learning, in direct contrast to previous results[41,42]. Schonewille et al. created mutant mouse lines in which signaling pathways necessary for AMPA receptor internalization were perturbed. AMPA receptor internalization is the final step in the process of LTD and is downstream of the activity of kinases such as PKC. The aim of these experiments was to minimize the side effects of broad perturbation of cellular signaling, which is likely to impact other, non-LTD-related, functions of the cell[41]. The mutant mice were then tested in several motor learning tasks such as VOR learning, eyeblink conditioning, and locomotion on an Erasmus ladder. No significant impairment in motor learning was observed, despite impairing AMPAR internalization, and hence LTD. These results suggest that LTD at the PF-PC does not play a critical role in cerebellum-dependent motor learning. Notably, a follow-up study succeeded in inducing LTD in the same mice by utilizing a much stronger LTD induction protocol[43], suggesting that the knockout of LTD was incomplete, and raising the possibility that LTD may still be important for motor learning.

Another approach to perturbing LTD was by using T-588, which inhibits PKC, a key protein in in the pathway of LTD induction[42]. Two studies[41,42] found that, although LTD induction was impaired, cerebellum- dependent motor learning was not. These studies therefore also suggest that LTD is not essential for cerebellum-dependent motor learning in mice. This, however, is in contrast with a previous study which reported pharmacological LTD blockage in mice using the COX-2 inhibitor nimesulide, showing impairment in OKR adaption[44]. Furthermore, blockage of LTD using T-588 as well as nimesulide in marmosets have shown impairment in VOR adaptation, suggesting that

blockage of LTD using these drugs is sufficient to impair motor learning[45]. Taken together, these results show that while T-588 impairs VOR learning in marmosets, this does not hold true for mice, and that other forms of pharmacological blockade of LTD are effective in perturbing motor learning. It has been thus been argued that the divergence in results could stem from the use of different pharmacological blockers, as well as how various species react to those pharmacological blockers[6].

In any case, these experiments present the possibility that LTD is not a central component of motor learning in the cerebellum, and thus the Marr-Albus-Ito model which places a very central view on LTD of the PF-PC synapse, is either inaccurate or incomplete.

5B. Additional sites of plasticity

The original model proposed by Marr in 1969 assumed that the PF-PC synapse is the only synapse undergoing plasticity. While Marr noted that this exclusivity was not necessary for the purposes of the model[33], despite his initial assumptions, the overall Marr-Albus-Ito model does not consider the contributions of other sites of plasticity in the cerebellum. However, there is a body of evidence supporting both the existence of other forms and sites of plasticity, and their relevance for motor learning. These are briefly summarized below:

5B.1 Plasticity at other sites in the cerebellar cortex

Recently, a more distributed view of plasticity has arisen following evidence that plasticity in the cerebellar cortex is not limited to the PF-PC synapse[46]. Aside from the PF-PC synapse, plasticity has been found in several other synapses within the cortex, including the Purkinje cell-molecular

layer interneuron synapse, the mossy fiber-granule cell synapse, and the parallel fiber-Golgi cell synapse[46].

The MLI-PC synapse is known to undergo rebound potentiation, where a large influx of calcium in the PC, arising from a complex spike, results in an upregulation of $GABA_A$ channels, increasing sensitivity to GABA, and thereby causing potentiation of MLI-PC synapses[47]. Furthermore, in transgenic mice where rebound potentiation is inhibited, VOR adaption is impaired, suggesting rebound potentiation at the MLI-PC synapse is relevant for motor learning[47].

PF-MLI synapses have also been observed to display forms of plasticity including LTD[46]. Repeated high frequency PF stimulation is known to induce LTD in the MF-MLI synapse through an increase in postsynaptic calcium concentration, along with the activation of the metabotropic receptor mGluR1, which will activate the production of endocannabinoids and the induction of LTD through the activation of the CB1 cannabinoid receptor on the PF, thereby reducing glutamate release[48]. This is followed by a replacement of calcium-permeable AMPA channels with calcium-impermeable AMPA channels, thus creating a limit on calcium entry, effectively creating a method of regulating plasticity[49]. However, disruptions in molecular pathways involved in LTD have not resulted in impairments in learning, suggesting that LTD in the PF-MLI synapse is not essential for motor learning[41,46].

MF-GC synapses are also thought to undergo both potentiation and depression. It is believed that this is regulated by calcium entry guided by the intensity of MF inputs, where rapid signaling from MFs induce calcium uptake by GCs, resulting in LTP, while low signaling from MFs and low calcium uptake conversely results in LTD[50]. Transgenic mice, where potentiation of the GC is inhibited,

show deficits in VOR adaption, suggesting that the MF-GC synapse plays an important role in facilitating motor learning[51].

PF-Golgi cell synapses also have been shown to exhibit LTD following rapid stimulation of PFs[52]. It has also been observed that plasticity in the Golgi cell may be influenced by CF signaling, where conjunctive stimulation of Golgi cell afferents and CFs results in potentiation of Golgi cell firing[53]. It has been reported that mice with impaired PF transmission, as well as animals treated with blockers to impair PF terminals, also suffer from impairments in cerebellum-dependent learning, including VOR adaption and eyeblink conditioning, although it can be argued that this may be the effect of a broader PF impairment as opposed to a specific impairment of the PF-Golgi synapse.

5B.2. Plasticity in the deep cerebellar nucleus

Plasticity in the cerebellum is not exclusively associated with the cerebellar cortex, as there exists evidence of plasticity occurring at sites such as the outside the cerebellar cortex, such as in the Deep Cerebellar Nuclei (DCN).

The DCN, like the PC, is in a very suitable location for integrating information, due to its connections from the mossy fibers, inferior olive, and Purkinje cells[54]. Like the PC, there is a wealth of information suggesting that not only is there plasticity in the DCN, this plasticity is induced during motor learning, and is essential for motor learning. For instance, eyeblink conditioning performed in rats was found to result in an increased number of synapses in the DCN, compared to both untrained animals and animals that underwent unpaired training[55]. Additionally, the lesioning of areas of the DCN, but not the cerebellar cortex, was able to abolish eyeblink conditioning in trained animals[56]. It was later found that following lesion of the

cerebellar cortex, timing deficiencies in eyeblink responses were observed, even though the eyeblink responses themselves were intact, suggesting that the cerebellar cortex is responsible for controlling timing of learned motor actions[57], Thus, evidence indicates that plasticity exists in both the cerebellar cortex as wells as the DCN. Furthermore, pharmacological inactivation of afferents to and efferents from the DCN have also shown that learning is only induced if these pathways into the DCN are intact and not disrupted, suggesting that plasticity in the DCN plays a role in motor learning[58].

Recent studies into the role of the DCN in cerebellar memory have supported the idea that the DCN plays a role in long term memory storage. Initial learning, driven by the CF, is thought to occur in the cerebellar cortex. This plasticity then eventually drives plasticity in the DCN for longer-term storage[59] Although there is currently no direct evidence of this occurring, it has been observed that lesions in the cerebellar cortex after learning, such as eyeblink conditioning, is established do not fully block learned responses[57]. Moreover, lesioning of the cerebellar cortex blocks acquisition of a learned response[45]. In contrast, lesions of the DCN results in the loss of previously learned responses[60]. Additionally, it was also found that pharmacological blockage of the cerebellar cortex was able to block one-day old OKR adaptions, however one-week old OKR responses were not affected. Furthermore, it was found that mice trained one-week previously displayed enhanced responses in the DCN in response to vestibular stimuli, relative to untrained animals, suggesting that plasticity has occurred[61].

The additional sites of plasticity create a need for further investigation, in order to determine the functional role of these forms of plasticity, and how they relate to the overarching Marr-Albus-

Ito model. Consequently, the Marr-Albus-Ito model is currently incomplete, and more research is needed to incorporate plasticity found in other regions of the cerebellum.

5B.3. LTP at PF-to-PC synapses.

Currently, the Marr-Albus-Ito model focuses on the role of LTD in cerebellar motor learning, however, there has also been evidence for the involvement of LTP at the PF-PC synapse. It is well known that stimulation of the PF without CF input results in LTP of the PF-PC synapse[6]. Notably perturbation of LTP at the PF-PC synapse also appears to result in deficiencies in VOR adaption as well as in eyeblink conditioning, as is seen with perturbation of LTD[62,63]. It has been speculated that LTP may play an opposing role to LTD, where LTP allows the synapse to reset and relearn after prior induction of LTD[64]. However, it has also been suggested that LTP may be the primary substrate for motor learning, based on evidence that blockade of LTP is able to impair motor learning. It is also possible that LTP could work in parallel with LTD in different areas in the cerebellum[65]. In summary, there is a growing body of evidence that LTP, like LTD, plays an important role in cerebellar motor learning. Therefore, further investigation is required to determine the purpose of LTP, in relation the LTD and how it affects the Marr-Albus-Ito model.

Considering all the evidence showing the involvement of LTP as well as alternative sites of memory in the cerebellum, such as the MLIs, or the DCN, a controversy has naturally erupted on the significance of LTD in cerebellar motor memory. While classically it was believed that LTD is the primary substrate of memory in the cerebellum, several alternative views have also appeared challenging the classic view. These include views suggesting that LTP may play a prominent role

in addition to LTD, or that the cerebellum may possess multiple sites of memory, and that studies of memory in the cerebellum should not only be limited to the PF-PC synapse.

While many studies have pointed to the fact that LTD at the PF-PC synapse is essential for motor learning, it is still unclear how plasticity in the cerebellum serves to support motor learning. Nonetheless, evidence that LTD at the PF-PC synapse supports motor learning is the strongest. We propose to test if this is true in the context of goal-directed movement learning. We will use a cerebellum-dependent paw reach task to drive motor learning in the cerebellum. We will then investigate whether we see a structural signature of synaptic plasticity, a change in spine density onto Purkinje cells, in order to determine whether synaptic strengthening or weakening occurs during motor learning. This approach has the advantage of not causing any molecular perturbations, which could have broad impact and recruit compensatory mechanisms of plasticity. In addition, our results will identify the net effect of any potential combination of plasticity mechanisms, acting concurrently or independently during learning.

Hypothesis and Rationale

1. OVERARCHING HYPOTHESIS:

Based on past theories and models of cerebellum-dependent motor learning, our overarching hypothesis is that depression of PF-PC synapses supports motor learning. After training the animals in a cerebellum-dependent motor learning task, we anticipate a reduction in the strength and number of synapses and therefore a reduction in spine density as a result of training.

2. RATIONALE/EXPERIMENTAL APPROACH:

Previous electrophysiological studies on LTP and LTD, while insightful, are limited in that the experiments do not actually attempt to observe the effects of LTD in an awake, behaving animal, instead relying on an underlying supposition that an LTD blockage, as tested in brain slice preparations, is equivalent to blocking in vivo forms of synaptic weakening[6].

An alternative approach to investigate motor learning in the cerebellum in awake animals is to directly observe the cellular and synaptic signatures of behavioural learning. One way to do this is by assaying structural plasticity, notably spine density counts, which we are considering as a proxy for LTD[66-68]. Spines are dendritic protrusions that are the site of PF-to-PC synapses. Activity-driven synaptic plasticity is known to affect cytoskeletal machinery and drive changes in spine morphology and density[69], supporting the link between synaptic plasticity and change in spine number. Moreover, both mechanisms for LTD and spinogenesis regulation share the same pathway[66]. Furthermore, several studies on structural changes as a result of learning have shown differences between PC spine density in animals which have undergone motor learning versus

those who have not. One study[67], found a decrease in spine density in the PC dendrites as a result of optokinetic reflex training, consistent with electrophysiological observations after similar training protocols[6,40]. In contrast, another study found an increase in the dendritic spine density of Purkinje cells in rats subjected to obstacle course training versus those who were not, suggesting that LTP had occurred[70], echoing previous ambiguities on the relative roles of LTP and LTD in cerebellum-dependent motor learning.

This thesis addresses the question of whether motor learning in a voluntary goal-directed task recruits plasticity at parallel fiber-to-Purkinje cell synapses in the cerebellum. Specifically, we aim to identify whether learning-induced structural plasticity supports the hypothesis of depression or that of potentiation at these synapses. We utilized a form of motor learning involving a paw reaching task, in order to observe learning-induced spine changes in PC dendrites. Based on our overarching hypothesis that synaptic depression of the PF-PC synapses supports motor learning, we anticipate a reduction in spine density in animals which were trained in our paw reaching task, versus animals which were untrained. Alternatively, if we do not observe a depression in the spine density, this would suggest that either depression exists but spine density is not an appropriate proxy for depression, or that depression of PF-PC synapses does not support motor learning, at least for this task.

METHODS

1.TEST CHAMBER:

The test chamber consisted of a Perspex box with a base of 9 cm x 15 cm, and a height of 20 cm. A 9 cm slit of width 5 mm is present in one wall. Footage was recorded using a Logitech C920® Webcam secured directly over the slit using a retort stand and clamp, thereby providing a full view of the area in front of the slit (Fig 3.).

2. ANIMALS:

All animal procedures were conducted using protocols approved by the Facility Animal Care Committee of the Montreal General Hospital and the RIMUHC. C57Bl/6 mice aged 2 to 4 months were used to perform paw reach tasks. Animals were given access to *ad libitum* food until 3 to 7 days before the start of the experiment. Animals were kept on an inverted 9:00 to 9:00 day/night cycle. All experiments were conducted during the dark phase of the cycle. Both male and female mice were included in the experiment.

3. ANIMAL STARVATION:

Cohorts of C57Bl/6 mice were gradually reduced to 90% of their natural body weight, defined as the baseline weight prior to food restriction, over 3 to 7 days. This body weight was consistently maintained over the entire course of the experiment. This was achieved by removing access to *ad libitum* food 3 to 7 days prior to the start of the experiments, for the entirety of the experiment, and giving fixed amounts of food per day. Food amounts given were calculated to be roughly 3g for a 25g mouse initially. Over the course of experiments food amounts were continually adjusted

based on animal weight trends, and animal weight was monitored each day to ensure that the weight remains around 90% and does not drop beneath 85%.

4. ANIMAL HABITUATION AND SHAPING:

Animals were habituated by placing them in the test chamber with roughly 10 Bioserv® 0.254 cm diameter DPP (Dustless Precision Pellets) chocolate test pellets for 20 minutes over 2 days. Animals were then shaped by being placed in the test chamber with DPP pellets placed in an elevated Perspex platform directly outside of the slit at a height of 1 cm, and a distance adjusted to be just out of reach of the animal's tongue. Habituation continued until the animal made 5-7 reaches within a time limit of 20 minutes. Animals unable to make 5-7 reaches within 3 trials were dropped from the training. During the experiment, pellets are placed on the platform in offset divots (Fig 3B) such that the pellets are only accessible with the opposite limb, restricting the task to one limb only. To this end, animals would be designated as right or left-handed based on the animal's initial preferred limb during shaping. If the animal failed to make the requisite 5-7 reaches within 20 minutes in 3 days, the animal would be dropped from the experiment or used as a control.

5. ANIMAL TRAINING:

During Training, the Perspex food platform is raised such that the DPP pellets now sit at a height of 1.5 cm from the ground and a distance of 1.5 cm from the slit. The pellets are placed into one of 2 divots drilled into the platform spaced 1 cm apart, depending on the left or right handedness of the animal. The animals were then placed individually in the test chamber for 20 minutes or 50 reach attempts whichever came first, over the course of 8 days. 1280 x 720-pixel video was

recorded during testing for future analysis at a frame rate of 30 fps using the Logitech C920 Webcam. All behaviour was performed during the dark phase, in the afternoon (2:00pm – 6:00pm).

Control animals were placed in the box for a period of 15-20 minutes with roughly 15 DPP pellets placed into the box.

6. BEHAVIOURAL DATA ACQUISITION AND ANALYSIS:

Animals behaviours were graded based on several criteria, including success, pellets eaten, as well as failures and errors made. Data for success, failure, and pellets eaten were gathered during behavior, by an observer sitting outside the behavior space directly observing the animal, while errors were assessed using video recordings.

Success was defined as a single motion from the animal, retrieving the pellet from outside the test chamber, and eating it, using the designated limb, without dropping the DPP pellet. Anything aside from such a motion was classified as failure. Any reaches made with the non-designated limb were not counted.

Analysis of errors was performed using video recorded from behavior sessions. Three types of errors including “Miss”, “Grasp”, and “Drop” errors were counted.

A “Miss” error was defined as a reach attempt where the animal’s paw fails to contact the DPP pellet at all, and thus misses completely.

A “Grasp” error was defined as a reach attempt where the animal is able to make contact with the pellet but fails to secure the pellet within its paw, and either fails to grasp the pellet or knocks the pellet.

A ”Drop” error was defined as a reach attempt where the animal is able to make contact with the pellet, secure the pellet within it’s paw, but fails to successfully retrieve the pellet in a single motion resulting in the animal dropping the pellet, on its way to its mouth.

Success rates were calculated by dividing the number of successes by the number of total reach attempts. Additionally, all failures were calculated similarly by dividing number of failures by reach attempts.

7. GOLGI COX SOLUTION PREPARATION:

Golgi-cox staining performed as described previously[71]. Stock solutions of 5% potassium dichromate (Fisher), 5% potassium chromate (Fisher), and 5% mercuric chloride (Ricca Chemical Company) were created. The Golgi-Cox staining solution was composed of 50 ml of 5% potassium dichromate, 50 ml of 5% ml mercuric chloride, and 40 ml of 5% potassium dichromate mixed with 110 ml of H_2O. The solution was then set aside in the dark for 48 hours to precipitate at room temperature. The supernatant was then poured into small glass bottles for staining, with care taken to not disturb the precipitate.

8. GOLGI COX STAINING

Animals were deeply anesthetized using isoflurane, and decapitated. Animal brains were removed and cut into 2 sagittal hemispheres and rinsed with PBS to remove any blood. The

hemispheres were then put into small bottles containing Golgi-Cox solution, ensuring that the hemispheres were submerged. The hemispheres then were put in the dark for 24 hours, before Golgi-Cox solution was replaced with new Golgi-Cox solution, and the brains were set aside for another 7-10 days, before being submerged in 7% sucrose dissolved in a 0.1 M phosphate buffer (10.1 g Sodium phosphate dibasic (Sigma), 1.7 g Sodium phosphate monobasic (Sigma), 500 ml dH2O) for at least 1 week.

9. GELATIN SLIDES

Gelatin coated slides were created by first rinsing Superfrost® (Fisher) slides with double-distilled water (ddH_2O) and letting them dry for 2 hours in a fume hood. A 10% gelatin (Sigma) solution of gelatin and distilled water was then created and heated to 70^oC. Washed and dried Superfrost® slides were then immersed into the gelatin solution for 10 minutes. Slides were then removed from the solution and placed onto paper towels and allowed to dry overnight at room temperature in a dustless area.

10. GOLGI SECTIONING

Sectioning was performed on a Vibratome 1000®. Stained hemispheres were removed from the bottles containing 7% phosphate buffered sucrose, and the cerebellum isolated. The cerebellum was then secured on a chuck with a cyanoacrylate adhesive (Loctite®). The chuck was then secured in the cutting chamber and submerged with phosphate buffered saline as a cutting solution. Sections were cut to a thickness of 150 µm and were transferred to a gelatin (Sigma) coated Superfrost® slide, using a dropper, and dried by removing excess PBS with a dropper and pressing gently with a paper towel.

11. GOLGI PROCESSING

Slides were processed using a slide dipping rack and immersion in several solutions to both develop and clear the sections. First, slides were immersed in ddH_2O for 10 minutes. Slides were then submerged into 5% sodium carbonate (ACP chemicals Inc.) for 30 minutes in the dark. Slides were then washed by submerging again in ddH_2O for 10 minutes, before being submerged for 40 minutes in 70% ethanol, followed by 95% and 100% ethanol for 10 minutes each to dehydrate the sections. Finally, the slides were cleared in xylene (Fisher) to improve contrast for 2 minutes before the addition of Dibutyl phthalate Polystyrene Xylene/DPX (Sigma) mountant and a cover slip. Slides were left to cure in the fume hood for 3-4 days before analysis to ensure that the DPX was fully dry.

12. ANALYSIS OF SPINES

Spines were analyzed using a Zeiss Axiovert® 10, with a 40X lens. Spines were traced in ImageJ. Cells with intact primary and secondary dendrites ~150 micrometers from the cell body, based in the forelimb area (lobules 4,5, simplex) of the cerebellum, straddling the vermis and the hemispheres (Fig 2), ipsilateral to the animal's preferred paw were selected for analysis. Dendrites were selected and traced based on being the first dendrite off the primary dendrite, for roughly 100 µm. All observable dendritic spines within this 100 µm section of dendrite was counted (Fig 12A).

Spines were quantified by density (Spine count/ Length of Dendrite) using ImageJ.

13. DATA AND STATISTICS

All Graphs were created using GraphPad Prism® software. Behavioural graphs were statistically analyzed either using a one way repeated measures ANOVA with a Bonferroni post-hoc test when analyzing trends occurring over the entire course of the experiment, or a paired 2-tailed Student's t-Test when analyzing specific groups such as behavior on Day 1 vs Day 8 or between different cohorts of animals. A p value of <0.05 was considered statistically significant.

During spine analysis, the experimenter was kept blind throughout spine quantification. All statistical tests were performed using GraphPad Prism®.

All data was shown as mean +/- SEM.

14. PERFUSION

Animals were deeply anesthetized with Isoflurane. A series of cuts from the abdomen through the ribcage were carefully made to expose the thoracic cavity. A needle connected to tubing leading through a perfusion pump was secured to the left ventricle of the animal using hemostats. The right atrium was cut, and PBS pumped through the heart for roughly 10 minutes. PBS was then substituted for a 4% Paraformaldehyde (PFA) solution in PBS and pumped throughout the animal for 15 minutes to fix the animal. Finally, the fixed brain was then extracted and placed in 4% PFA-PBS for 24 hours before use.

Results

1. PAW-REACH TASK IN RODENTS

In order to induce cerebellum-dependent motor learning, we utilized a forelimb reaching task. In this task, a mouse is placed in a behavior box containing a narrow slit in one wall. The animal then has to learn to reach through the slit with a forepaw, in order to retrieve a reward (i.e. a food pellet). The animal is allowed to make paw reaches over 8 successive days, during which the success rate of the paw reach increases as the animal learns the task and is able to make an increased number of accurate reaches.

This task is well-established and has been extensively utilized previously, in the context of neural activity in several parts of the nervous system, including the motor cortex[72] the spinal cord[73], and the cerebellum[31,74]. In particular, it has been observed that 1) stimulation of Purkinje cells induces forelimb movements[74], 2) stimulation of the DCN is able to alter the trajectory of mouse forelimb reaches, as well as affect the ability of a mouse to make a successful reach[31]. More generally, the activity of cerebellar cortical neurons in the forelimb area of the cerebellar cortex is associated with limb movements[75]. This area includes the lobule simplex[74] as well as areas of lobules IV and V of the vermis[76,77] (Fig 2).

The task we have chosen offers unique advantages compared to some commonly used cerebellar tasks, such as learning on the rotarod or the Erasmus ladder. Those tasks involve balance and whole-body coordination, which engages the cerebellum more broadly. In contrast, the forelimb task allows us to focus our investigation on the relatively small and localized forelimb area of the cerebellum. The task is also limited to a single limb, allowing us to use the other hemisphere of

the cerebellum as an internal control. In addition, the forelimb area is highly accessible to in vivo measurement or perturbation of neural activity, permitting future experiments that expand on the current research.

2. DEVELOPMENT OF THE TASK

The first component of creating the task is the design of the behavior box. Our initial box design was based on an existing design and protocol[78]. The box featured a base of 8.5 cm x 15 cm and a height of 25 cm, 3 walls of 1 cm thickness, and a front wall of 0.5 cm thickness. The design featured slits on both the top and bottom sides on the front wall the box, with the bottom

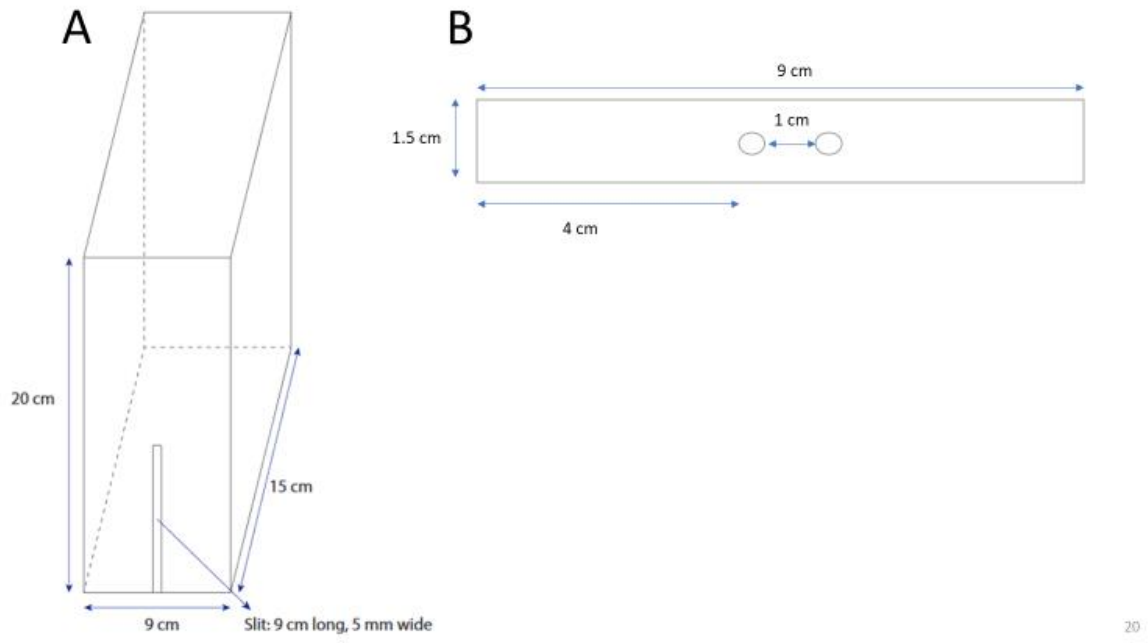

Fig. 3 A) Dimensions of the final box design. B) Dimensions of the reward platform

having a 9 cm vertical slit, while the top had two 9 cm long vertical slits, with the idea that the box could be inverted to use either the single slit or double slits. This design however, had a few shortcomings, namely that the mice kept on trying to climb out of the box by using the slits as

supports. We addressed this issue by removing the double slits from the design, as they were not used in the final behavior protocol. Secondly, the animals also kept chewing on the slits, becoming too distracted to complete the task. We thus modified the box to be substantially thicker to address this issue. The new box design features a wall thickness of 1 cm on all 4 walls. The box retained similar dimensions of a 9 cm x 15 cm base, and a 20 cm height, with a single slit of 9 cm x 0.5 cm. This design was able to resolve all the issues associated with the previous box design (Fig 3A).

A smaller Perspex platform of 3 cm x 9 cm was used to hold the reward. This platform contained 2 divots to stabilize the food pellet, as well as restrict handedness, such that a successful paw reach is only possible with one paw. These divots are spaced 2 cm apart from each other. The reward is placed in the divot at a height of 1.5 cm and a distance of 1.5 cm away from the box (Fig 3B).

Next, we had to adjust the reward for the behavior task. The reward is vital to the success rate of the task as it affects both the difficulty (how easy the reward is to grab) as well as the motivation for the animals to complete the task. The initial reward we decided to use was a millet seed, based on a previous protocol[78]. Unfortunately, while millet seeds were regular in size and shape, animals suffered from poor motivation and were unwilling to perform the training task. Next, we experimented with pieces of dried fruit. While the animals were readily willing to perform the task for such a reward, pieces of dried fruit were irregular in size and shape, creating too much variation in the success rates of the animals. Furthermore, these pieces of dried fruit had to be manually cut into the correct shape and size, which was both time consuming and unfeasible. Finally, we decided on pre-manufactured chocolate Dustless Precision Pellets (DPP)

from Bioserv®[31]. These pellets were regular in size and shape and were sufficiently appealing for the animals to perform the task.

Aside from rewards, we also adjusted other parameters such as shaping parameters, as well as platform distance, height and divot spacing, each of which is described in more detail below. Changing these parameters modifies the difficulty of the task. While easier tasks result in higher success rates, it is more difficult to induce learning because animals start with a high baseline success rate. In contrast, if the task is too difficult, animals lose motivation and do not learn. For example, we originally had the reward platform height level with the ground, with the reward at a distance of 1 cm, however, this height and distance was too easy for the animals. We then changed the height and distance to at 2 cm from the base of the box, and 1 cm away from the wall[31]. However, this height was too difficult for some animals. Therefore, finally, the reward was finally placed at a height of 1.5 cm and a distance of 1.5 cm, which we found was more optimal.

The final task involved a cohort of both male and female mice, aged 2-4 months, housed on an inverted 9 am to 9 pm day-night cycle. Habituation, shaping, and training were always performed during the dark phase of the day-night cycle, which is when mice are awake, although the training itself was conducted in a well-lit room. Care was taken to ensure that the training was conducted at roughly the same time every day. The animals were first gradually brought down to 90% of their baseline bodyweight 3-7 days prior to the start of the experiment, by reducing their food supply. Habituation began on the first day, where the animals were placed in the test chamber for 20 minutes, with roughly 10 pellets of food reward placed in the box along with the animal. This is repeated for two days, to allow the animal to become familiar with the experimenter, the room, lighting, the box, and the reward. After habituation, the animals are then shaped to allow

the animals to become familiar with the concept that they need to make a forelimb movement, without sufficient trials to actually become trained. This is accomplished by lowering the food platform to 1 cm, and gradually moving the pellet away from the slit until the pellet is just out of reach of the animal's tongue. The animal is allowed to make 5-7 paw reaches. Shaping continues for a maximum of 3 days, if by the end of the 3 days, the animal is unable to make paw reaches, the animal is dropped from the training cohort. During shaping, it is also important to note the preferred paw of the animal as during training the pellets will be placed in divots slightly offset from the center of the slit, thereby limiting access to one limb only (Fig 3B).

The final step is the training where the food reward is brought back up to the training height of 1.5 cm and distance of 1.5 cm. The animal is allowed to make up to 50 reach attempts or make attempts for 20 minutes, whichever comes first, as per the original protocol[78]. Then, the total success rate is determined (number of successful reaches/numbers of total reaches). The training continued for 8 consecutive days. Control animals, on the other hand, were placed in the behavior box for 15-20 minutes, with roughly 10-20 food pellets placed in the box, such that a paw reach is unnecessary to retrieve them.

We observed robust improvement in the paw reach success rate of the animal from Day 1 to Day 8. Animals were able to improve their success rate from roughly 20% on Day 1 to 40% by Day 8 (Fig 4A, B). Accordingly, the number of pellets eaten by the animals increased significantly during the training period to reflect this increased success rate (Fig 4C, D). Both the increase in success rate, as well as the number of pellets eaten improved significantly as measured by a repeated

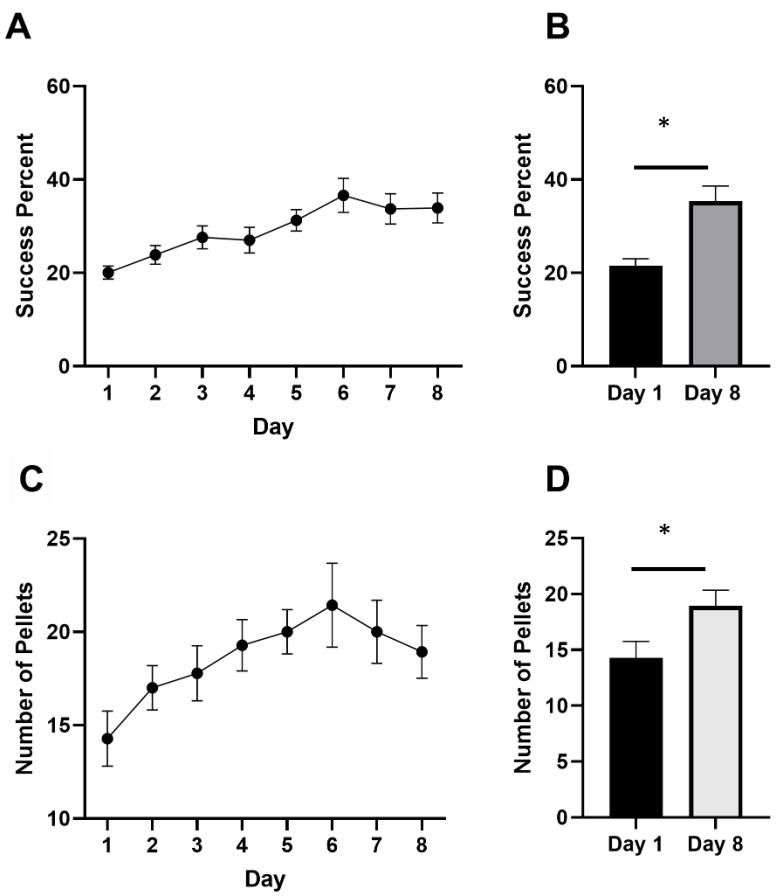

Fig 4. Learning in the forelimb-reach task. A, B) There is a significant improvement in success rate over the time course of training, as measured with A) a repeated measures ANOVA (** = p<0.01, N=24), or B) a Student's t-test comparing success rate on Day 1 vs. Day 8 (* = p<0.05,N=24). C, D) There is an increase in the number of pellets eaten over the time course of training, as measured with C) a repeated measures ANOVA(*= p<0.05,N=14), or D) a Student's t-test comparing pellets eaten on Day 1 vs. Day 8 (* = p<0.05, N=14). Error bars indicate SEM.

measures ANOVA (Fig 4A, C), over the course of the experiment. Furthermore, we observed a significant difference between the success rates of Day 1 and Day 8 (Student's t-test, $p < 0.05$) (Fig 4B), as well as in the number of pellets eaten between Day 1 and Day 8 (Student's t-test, $p < 0.05$). Notably, we observed a steady increase in success rate over a period of 6 days, followed by a plateau period of 2 days, consistent with the success rates observed by other groups using similar forelimb reaching tasks[78,79].

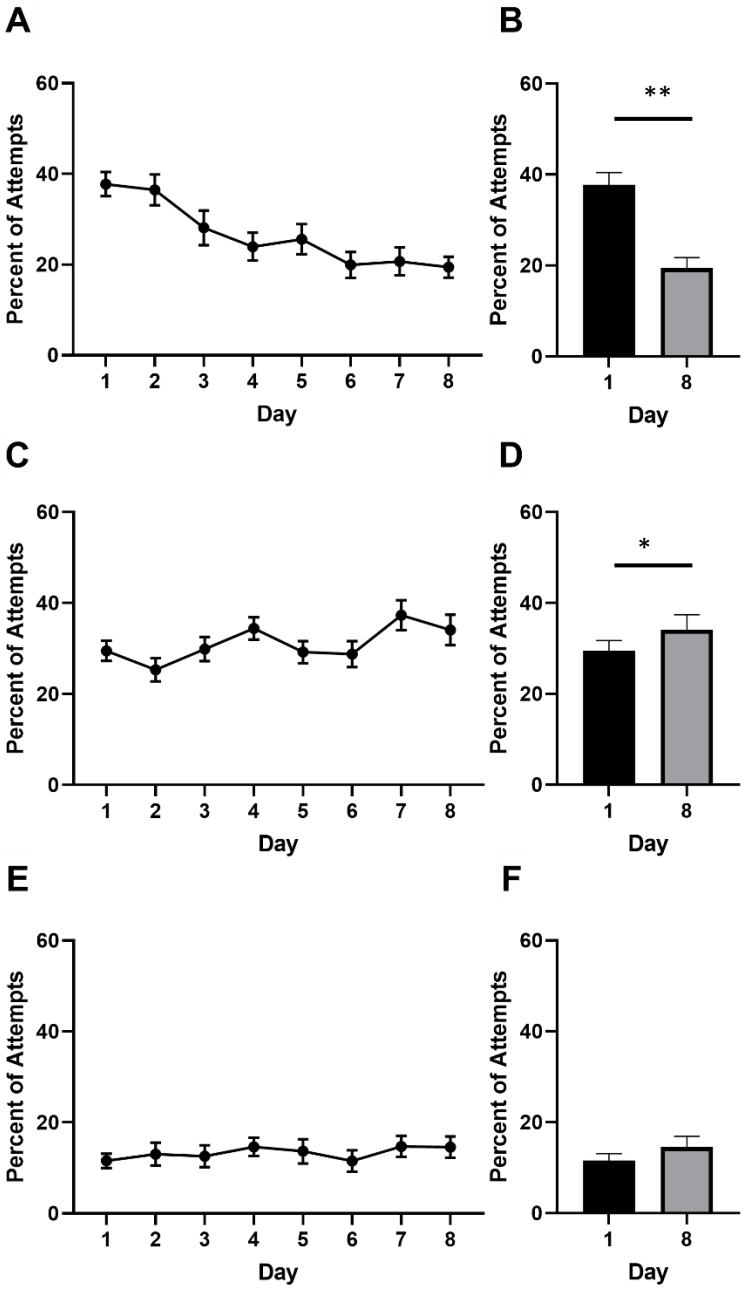

Fig 5. Types of motor errors during training. A, B) Miss-type errors, measured as a percentage of total reach attempts, reduce significantly over the course of training, as measured with A) RM ANOVA, ** = $p<0.01$, N=14 or B) Student's t-test ** = $p<0.01$, N=14. C, D) Grasp-type errors increase, as a percentage of total reach attempts, over the time course of training as measured with C) RM ANOVA, * = $p<0.05$, N=14 or E) Student's t-test * = $p<0.05$,N=14. D, F) However, Drop-type errors do not change significantly. Error bars represent standard error of the mean.

3. ANALYSIS OF PAW REACH ERRORS

An alternative method to characterize improvement in the tasks is to analyze the types of errors made during the paw reach task, as well as the number of these types of errors.

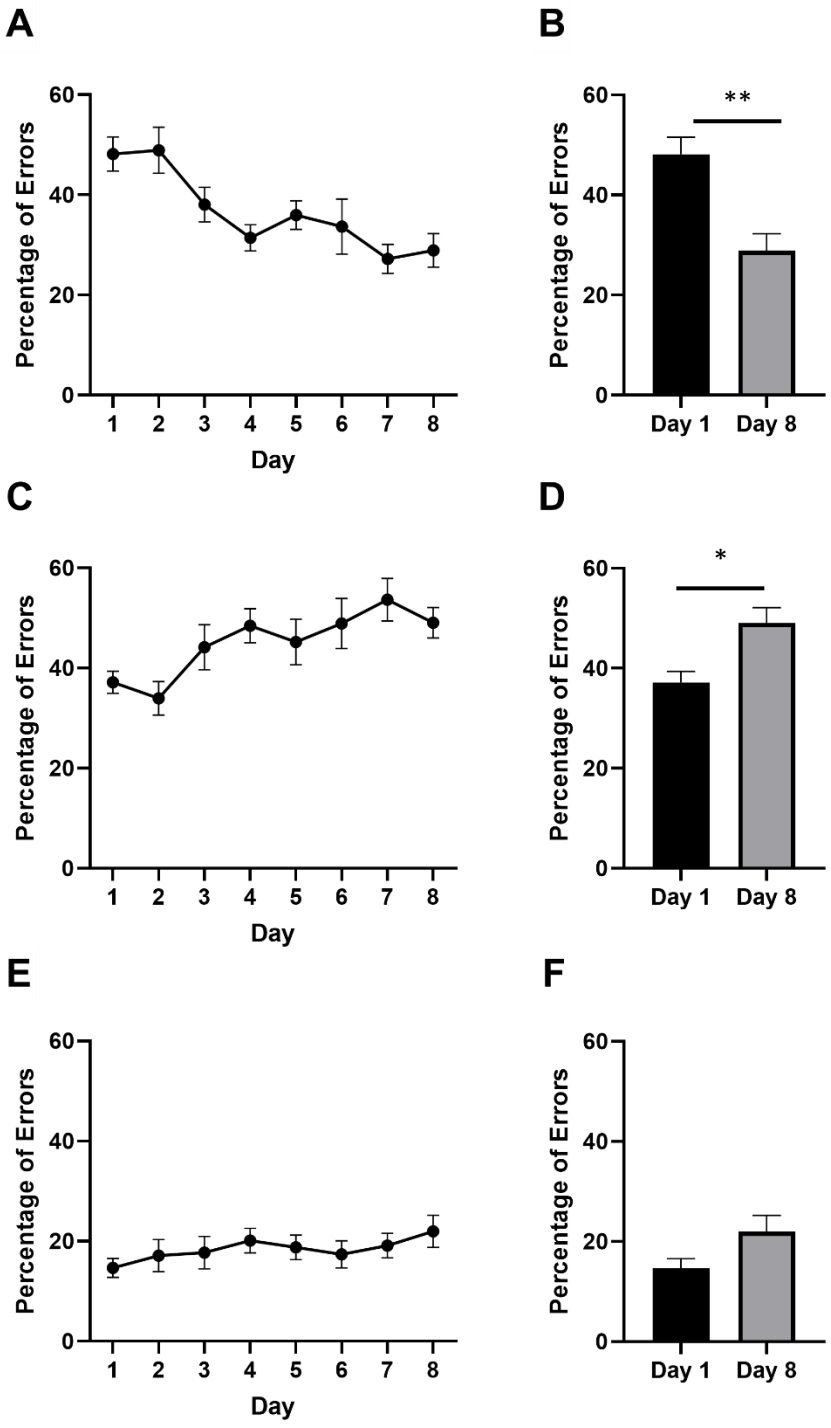

Fig 6. Types of motor errors during training. A, B) Miss-type errors, measured as a percentage of total errors, reduce significantly over the course of training, as measured with A) RM ANOVA, ** = P<0.01,N=14 or B) Student's t-test, ** = p<0.01,N=14. C, D) Grasp-type errors increase, as a percentage of total errors, over the time course of training as measured with C) RM ANOVA, * = p<0.05, N=14 or E) Student's t-test * = <0.05, N=14. D, F) However, Drop-type errors do not change significantly. Error bars represent standard error of the mean.

We adopted an approach previously used on a very similar task[80] which analyzed different phases within a paw reach. A paw reach can be deconstructed into 3 main phases: a precontact phase, a contact phase, and a retrieval phase; errors are characterized by whichever phase they occur

in. These result in a miss-type error where the animal fails to contact the pellet (i.e. during the precontact phase), a grasp-type error where the animal is able to contact the pellet but is unable to grasp the pellet (i.e. during the contact phase), and a drop-type error where the animal drops the pellet while attempting to move the pellet into its mouth (i.e. the retrieval phase).

During the course of the learning period, we noted a significant decrease in the percent of miss-type errors over total errors, showing that the main area of improvement during the course of the learning task (Fig 5A, D) is the ability to coordinate and make contact with the pellet. Notably, while we observed a significant decrease in the proportion of misses, we observed a significant increase in the proportion of grasp-type errors (Fig 5B, E), while drop-type errors did not change significantly (Fig 5C, F). To clarify the relationship of the drop in the proportion of miss-type errors and the increase in grasp-type errors to overall reach attempts, we also plotted the number of miss, grasp, and drop-type errors, as a percentage of total reaches. We observed a significant decrease in the percentage of miss-type, as seen in the previous data (Fig 6A, D), likewise the percentage of the grasp-type errors increased significantly (Fig 6B, E), however drop-type errors did not change significantly (Fig 6C, F), suggesting that only the accuracy of the reach, but not the grasp or retrieval improved during the course of the training. As the improvement in the miss-type errors, like the overall improvement, appears to plateau around day 6, this suggests that the bulk of improvement in the paw reach task derives from the animal's ability to make contact as opposed to grasping or retrieval. The significant increase in the amount of grasp-type errors likely is the result of the increase in accuracy of the paw reaches due to the reduction in miss-type errors. It is currently unknown whether it is possible for the animals to improve their success rate at all with regards to grasping or retrieval since the plateau at Day 6 implies that the animal

has reached their maximum success rate, although extending the training protocol beyond 8 days could help clarify this.

4. LONG-TERM LEARNING

It has been suggested that motor memories move from the cerebellar cortex to the deep cerebellar nuclei during long-term learning[61]. However, this progression is not well understood. Since this project is aimed at identifying synaptic signatures of motor learning, we were interested in understanding how long this form of learning lasts. This would provide the potential for future experiments on testing whether synaptic changes in the cerebellar cortex are short-lived and, if so, what the exact time-course of such plasticity is. This experiment lays the framework to eventually determine whether the cortical memory trace (if any) is removed completely over time, is retained by the PC, or is transferred to another location such as the DCN. To assess the persistence of learning, we trained a cohort of 6 mice using the standard 8-day training protocol and returned 1 week after the 8-day training period to assess the success rate after the 1-week gap in training. We found that learning did not decrease significantly from day 8, suggesting that long-term learning has occurred, over the time course of at least 1 week (Fig 7). This result will allow us to perform future experiments on how synaptic plasticity in the cortex evolves over the time course of memory storage.

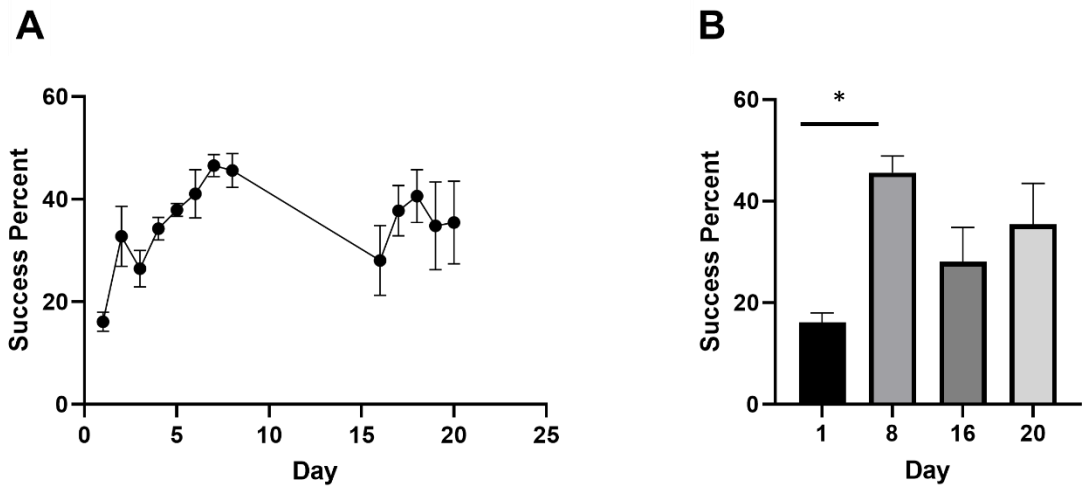

Fig 7. Learning is long-lasting. A) Time course of learning, measured as success percentage, during training, and 1 week after training. B) Success percentage compared between days 1,8, 16, and 20 (RM ANOVA, N=6, *=p<0.05).

5. VARIABLES THAT INFLUENCE LEARNING

In order to account for potential factors affecting the success rate of the animals, we looked at relationships between learning and the sex, handedness, and age of the animals tested (Fig 8).

We looked at 19 animals across 4 cohorts comprising a range of ages from 1.5 to 3.5 months. Animals were binned into age ranges of roughly 2 weeks. We found that there was no significant difference in learning between mice binned into different age bins (Fig 8A), as defined by success rate on day 8. While every age cohort showed a slightly different profile of learning over the course of training, we believe that this is more likely due to inter-cohort variation, as well as small cohort size. Overall, animals of different age had similar initial and final success rates (Fig 9).

Additionally, we looked at whether sex had any effect on success rate in these 19 animals. Interestingly, while we observed improvements in both male and female animals, we only observed significant improvement in female animals (Fig 8B). However, it should be noted that

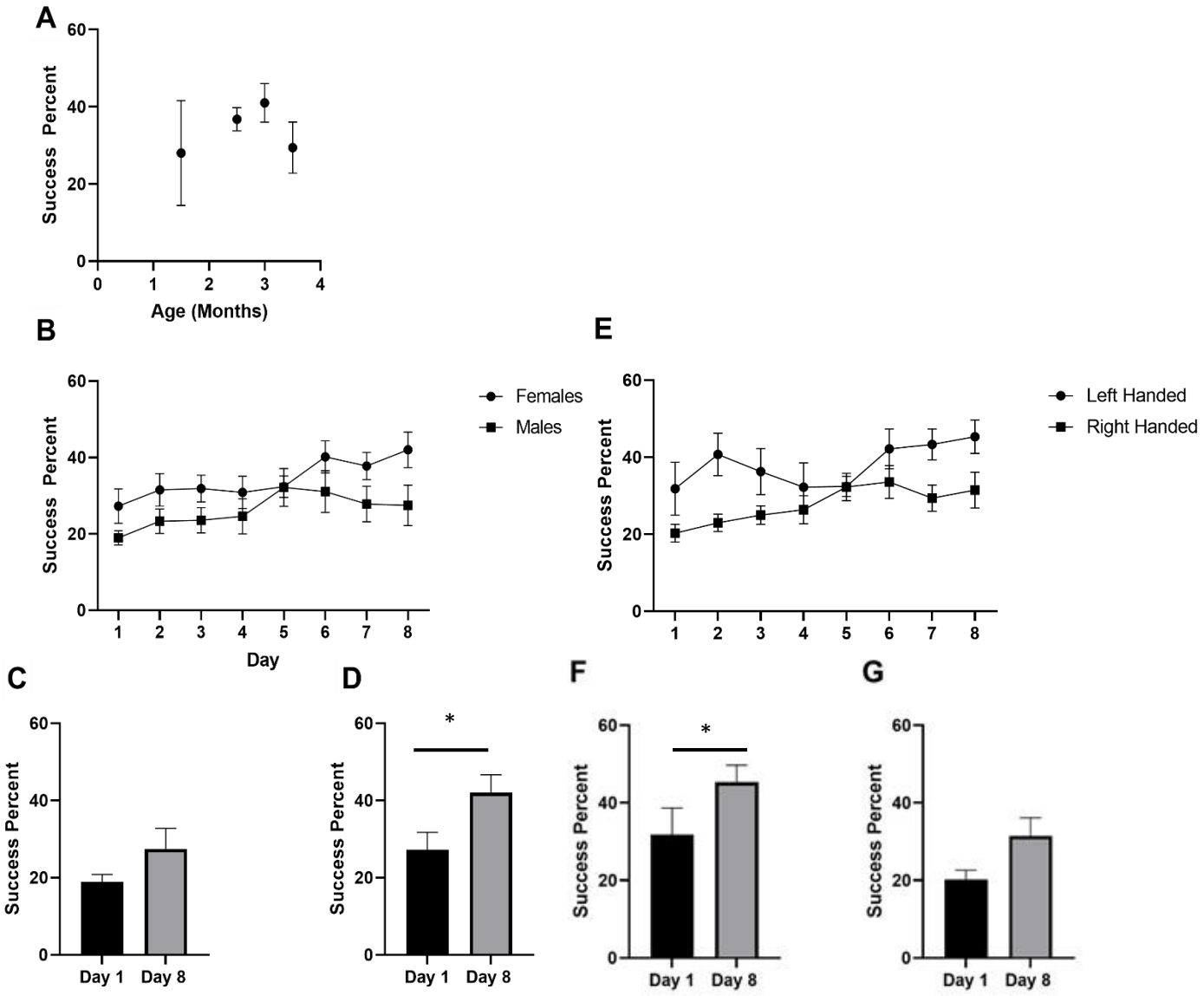

Fig 8. Age, sex, and handedness as determinants of learning A) There is no difference between the final success percentage in animals of different age-bins (ANOVA, N = 19, p>0.05). B) Only female mice showed a significant difference in learning during the time course of the experiment (RM ANOVA, N= 10, *=p<0.05) , or D) in the increase in success percentage from Day 1 to Day 8 (Student's t-test, * = p< 0.05). E)Likewise, only right handed mice show an significant difference in learning during the time course of the experiment (RM ANOVA, N=14, *=P<0.05), or F) in the increase in success percentage from Day 1 to Day 8 (t-test, *=p<0.05).

the number of females is slightly greater than the number of males, which could contribute to the lack of statistical significance observed in the amount of learning in male animals. Finally, we looked at the effect of handedness on the success rate of the animals. Animals were segregated based on their preferred limb, and success rates were again compared. As was the case with sex,

while both right and left-handed animals improved, we only found a significant difference in the right-handed animals (Fig 8C). We believe the differences, however, may be an artifact of much lower number of left-handed animals.

6. LEARNING-INDUCED STRUCTURAL PLASTICITY IN PURKINJE CELL DENDRITIC SPINES

In order to quantify the changes in structural plasticity that occur during cerebellum-dependent motor learning, we determined the spine density in dendrites of the PC. Since previous studies on distal PC dendrites have found no differences in spine density as a result of LTD induction[81] as well as the fact that preserving and staining distal dendrites are much more difficult, we decided to focus on proximal dendrites. We counted all visible proximal dendritic spines on a section of the first branch off of the primary dendrite extending for approximately 100µms.

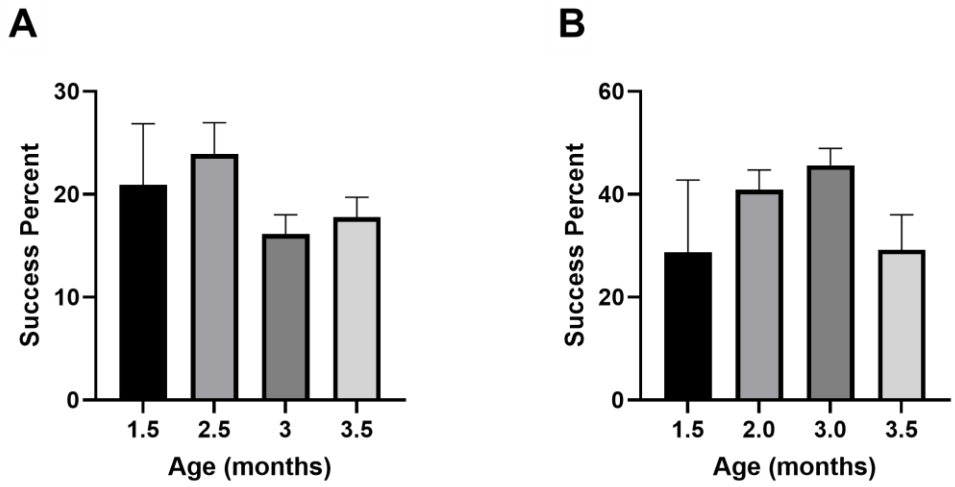

Fig 9. A) Comparison of the initial success percentage between differentially aged cohorts showed no significant difference (ANOVA, p>0.05). B) Additionally, a comparison of the final success percentage between the same cohorts showed no significant difference (ANOVA, p>0.05). Together, these comparisons suggest that there is no significant difference in the learning between these differentially aged cohorts.

All analysis was performed exclusively on PCs inhabiting areas associated with forelimb movement, namely lobule simplex, lobule IV and lobule V[74,76,77](Fig 2). Spine density was quantified as the number of spines per unit length of dendrite.

6a. Golgi-Cox Staining:

In order to observe and quantify dendritic features, a method of sparse labeling is required. We decided on Golgi-Cox staining as it is a well-established technique, and is capable of visualizing PC spines[71]. Our initial staining procedures were based upon an established protocol for staining PCs[71], (**Protocol 1,** *described below*). Despite following the established protocol, we observed a large amount of cracking in our sections, extensive damage to the PCs, and sections falling off the microscope slide (Fig 10 A, B). We believed that this was due in part to the drying step used in the protocol, PCs dying before fixation, as well as damage during section processing. To address these issues, we attempted several strategies to reduce damage to the section during staining. We first attempted perfusing brains with a 4% paraformaldehyde in PBS solution, in order to fix PCs before immersion into Golgi-Cox solution, however, this resulted in large precipitates forming in the cells rendering dendritic observations impossible (Fig 10 C, D) (**Protocol 2**). Additionally, cracks still formed in some areas of the cerebellum, especially after the drying step and during processing suggesting that these steps are contributing to damage. Thus, we attempted to use more mild solutions during slide processing, namely substituting ammonium hydroxide for sodium carbonate as seen in another Golgi-Cox procedure[82], carefully transferring sections using a water dropper instead of a brush to minimize damage to the sections, and drying by blotting with paper towel instead of air-drying sections (**Protocol 3)**. This improved section quality immensely, allowing dendritic observations to be possible, however there were still issues

with sections not adhering to slides, which would limit the amount of data we could gather from each animal (Fig 10 E, F). Therefore, we began using gelatin coated slides to improve adhesion of sections, which finally resolved this issue **(Protocol 4)**. The results showed excellent staining, clearly showing dendritic spines (Fig 10 G, H). Furthermore, sections were more easily retained during all processing steps allowing a greater number of sections per animal. The different protocols are listed below:

Protocol 1 (Fig 10 A, B):

1. The animal was anesthetized, then euthanized. The brain was extracted, and the cerebellum was isolated. The cerebellum was then divided into 2 hemispheres via a sagittal cut.
2. The hemispheres were rinsed with PBS and placed in a Golgi-Cox solution of 5% Potassium chromate, 5% potassium dichromate, and 5% Mercuric chloride in the dark. Golgi-cox solution was emptied and replaced after the first day. The brains were kept in solution for 7-10 days.
3. Brains were sectioned on a vibratome, mounted on slides and developed using an 28% ammonium hydroxide solution, before clearing with xylene for 2 minutes and mounting on slides in DPX

Protocol 2 (Fig 10 C, D):

The animal was anesthetized, then euthanized. The animal was perfused using a 4% Paraformaldehyde solution in PBS to fix the brain prior to extraction. All following steps remained the same as in Protocol 1.

Protocol 3 (Fig 10 E, F):

1. The animal was anesthetized, then euthanized. The brain was extracted, and the cerebellum was isolated.
2. The cerebellum was rinsed and placed intact in a Golgi-Cox solution of 5% Potassium chromate, 5% Potassium dichromate, and 5% Mercuric chloride, in the dark. Golgi-cox solution was emptied and replaced after the first day. The brains were kept in solution for 14 days.
3. Brains were sectioned on a vibratome and mounted on slides and developed using 5% sodium carbonate, before clearing with xylene and mounting in DPX.

Protocol 4 (Fig 10 G, H):

Identical to Protocol 3, except for the use of gelatinized slides.

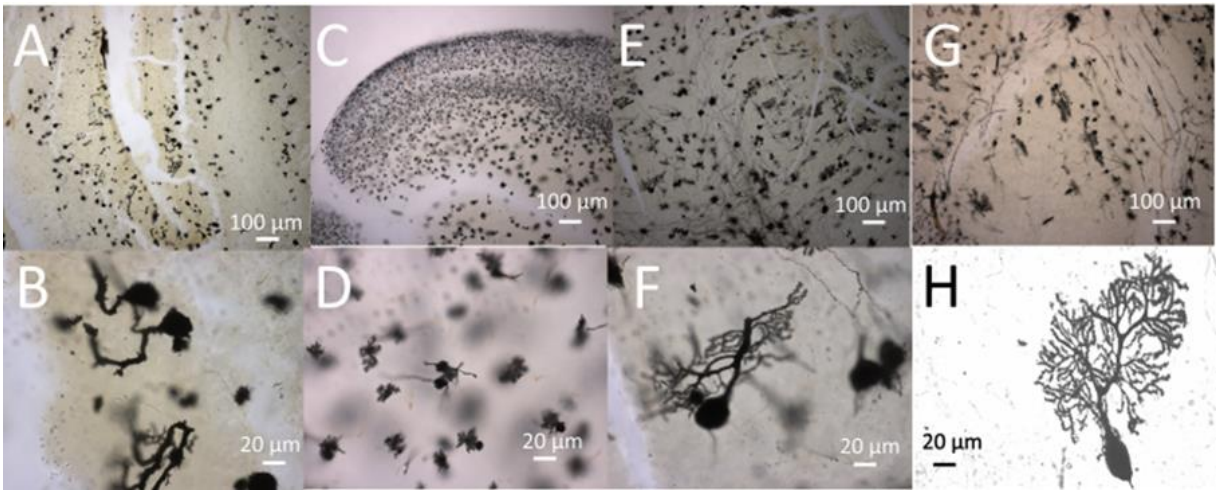

Fig 10. Images of staining derived from various staining protocols. A, B staining from Protocol 1, showing cracking and fracturing, as well as damaged PCs (B). C, D shows images of Protocol 2, and shows precipitate, obscuring dendritic analysis. E, F Shows staining following Protocol 3. G, H images following Protocol 4.

6b. Alternate Sparse Labelling Technique:

We also attempted an alternate technique for sparse labelling. We were able to obtain access to a CamKIICre:TdTom transgenic animals, which show sparse labeling of PCs (Fig 11). Paraformaldehyde-fixed brains from the CamKIICre:TdTom mice were sectioned, and we were able to observe robust sparse TdTomato fluorescent labeling of Purkinje cells. Labelling was observed across multiple lobules, including simplex and lobules IV and V, the forelimb areas (Fig 2). This genetic approach to sparse labeling Purkinje cells provided promising results, and offers a complementary method for future experiments.

6c. Spine density

We compared the number of dendritic spines between trained and untrained animals. We observed no difference in Purkinje cell spine density between trained and untrained animals (Student's t-test, n=17, p= 0.244) (Fig 12). However, we noted a large variability in learning

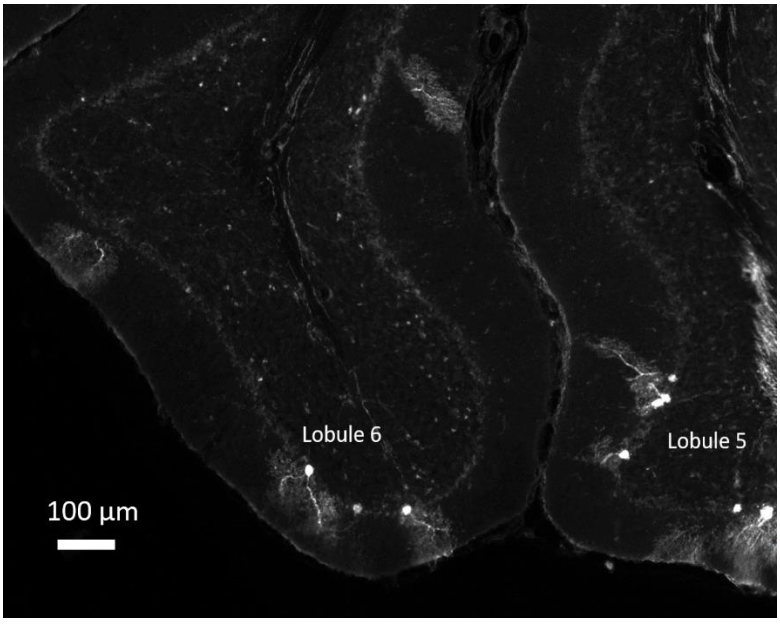

Fig 11. Sparse labelling of Purkinje cells in CamKIICre:TdTom transgenic animals, with the TdTomato in white. Notably sparse labelling is visible in Lobule V, within the forelimb-reach area.

between animals (Fig 12C). Therefore, we looked at correlations between spine density and learning (defined as the improvement in success rate from Day 1 of training to Day 8 of training) on an animal-by-animal basis. Strikingly, we found a significant inverse correlation ($r = -0.75$, $p < 0.05$) between the amount of learning and the spine density (Fig 12D). This implies that increased learning is associated with a reduction in spine density, and an increase in synaptic pruning. Since

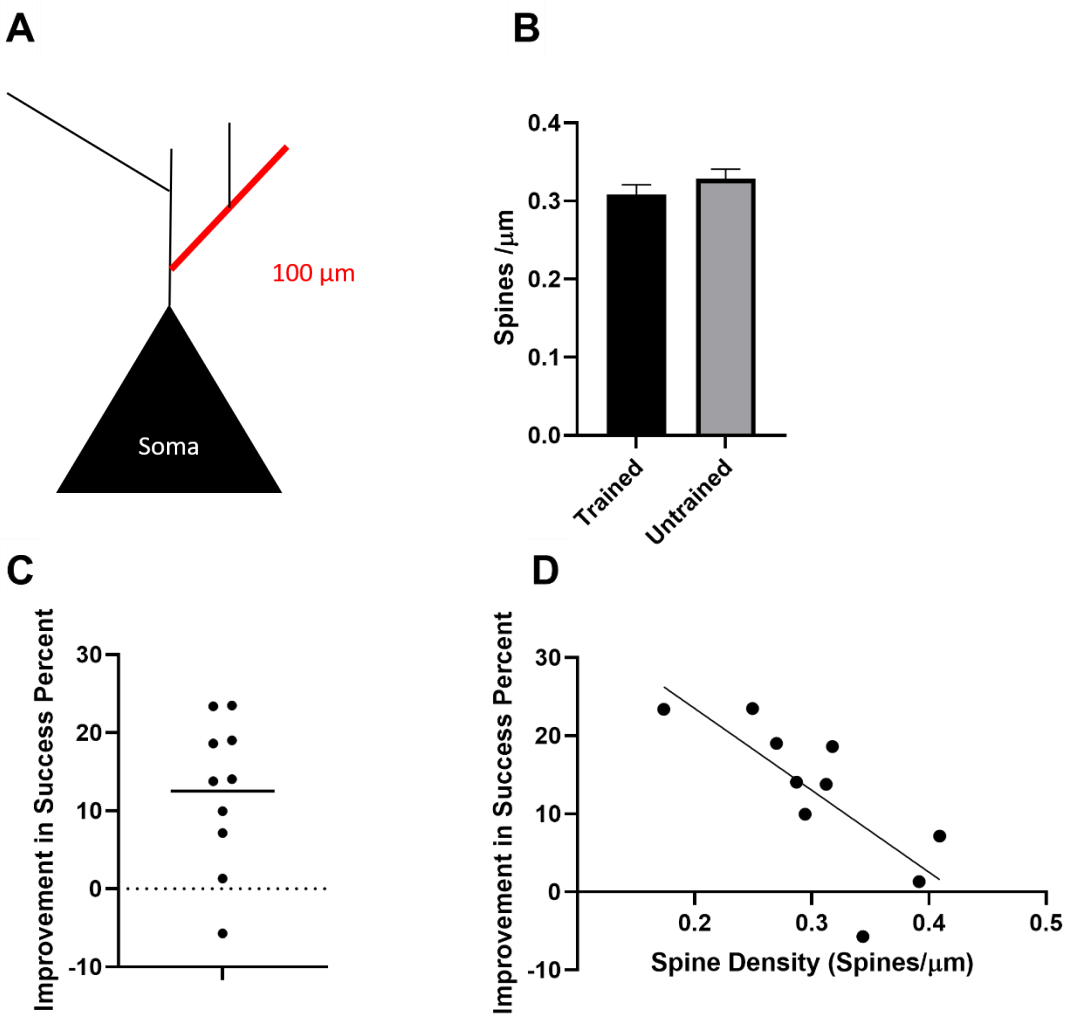

Fig 12. A) Dendritic branch selected for spine quantification is the first branch off the primary dendrite, and is marked in red B) There is no significant difference in spine density between trained and untrained animals (Student's t-test, N= 17, $p>0.05$) C) Difference in amount of learning, defined as the improvement in success percentage varies across animals D) There is a correlation between the amount of learning, measured as the improvement in success percentage, and the spine density. (N=9, R= -0.75, *=$p<0.05$)

reduction in synaptic strength via LTD is consolidated as an eventual reduction in spine number[66], this result suggests that an LTD-like change has occurred in animals that have learned. Moreover, the amount of learning is associated with a reduction in the number of synapses, suggesting that motor learning is associated with weakening of parallel fiber synapses onto Purkinje cells (since PC spines mark sites of parallel fiber synapses). Therefore, our observations suggest that, in the case of our paw reach task, an LTD-like form of plasticity is the main substrate of memory, in accordance with what is predicted by the Marr-Albus-Ito model[6].

The animals used for dendritic spine analysis consisted of both sexes, ranging from 1.5 to 3.5 months old. Animals were also both left and right-handed. We found that there was no significant difference in the spine density between animals of different sexes (Fig 13A), or handedness (Fig 13B).

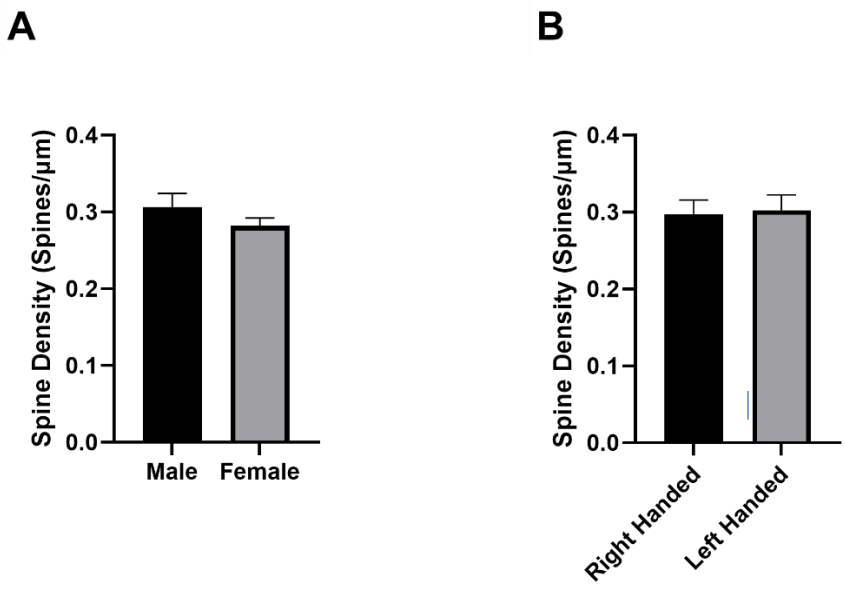

Fig 13. There are no differences in spine density in animals of A) different sexes or B) different handedness. (Student's t-test, N=17, p>0.05)

Discussion

1. PAW REACH TASK

Here we established a paw reach-based cerebellum-dependent learning task. We have observed that animals show a significant improvement in the success of the paw reach over the training period. Furthermore, training parameters such as starting or end success rate were consistent with previous protocols[78]. Notably in both our protocol, as well as in previous protocols success rate does not appear to approach 100%, instead showing a plateau at roughly 40%. This suggests that roughly 40% is the maximum success rate possible, with no further improvement capable of being supported. Interestingly in our data, we see a decrease in the number of miss type errors, however we do not see a drop in the number of drop or grasp errors. This could suggest that the system may be incapable of adapting to the point where success is able to approach 100% since the system is only capable of modulating miss type errors as opposed to grasp and retrieval errors. While the cerebellum in humans and mice is known to control elements essential in grasping and retrieval such as grip force and motor coordination[83,84] the fact that there does not seem to be an improvement observed in the task could suggest that this level of control is beyond the ability of the circuit. This would then imply that the bulk of the changes in synaptic density in the cerebellar cortex, is associated with an improvement in vision-directed hand-eye coordination in contacting the food pellet, as opposed to other elements of the paw reach task such as grasping, which may involve more tactile systems. To clarify this, a longer study where more longer-term trends in errors and success rates may help clarify if very fine skills such as grasping improve at all.

2. SPINE COUNT

We observed a significant inverse negative relationship between spine density and improvement at our paw reach task. The results suggest that a lower spine density is associated with greater improvement at the paw reaching task. Previous work has found a shared pathway between AMPA internalization, a characteristic of LTD, and spinogenesis, suggesting that LTD may be able to be observed through changes in synaptic density. Thus, our observation of an inverse relationship between spine density and improvement is consistent with the idea that LTD is the primary substrate of learning, as suggested by the Marr-Albus-Ito model.

However, the results do not conclusively demonstrate this idea. Notably, of the roughly 170,000 synapses in the PC, most are silent and do not actively contribute to changes in PC firing associated with motor learning[85]. Thus, the decrease in synaptic density could be associated with a reduction in silent synapses, as opposed to active synapses, thus obscuring the relationship between spine density and LTD. Furthermore, while silent synapses could be pruned, active synapses could concurrently be potentiated. Such a situation would imply that LTP is the primary substrate of memory, even though a reduction in spine density was observed. To resolve this, a functional analysis measuring changes in mEPSCs and LTD saturation in PCs in both trained and untrained animals is necessary to determine whether LTD has occurred.

Given that this type of movement is strongly associated with the forelimb area of the cerebellum (Fig 2)[31,74], we would anticipate that much of the learning would be limited to the forelimb area of the cerebellum, since this task involves mostly the improvement of coordination of the forearm. Consequently, we would expect to find spine changes largely limited to the forelimb

area to reflect this. While the task may involve other aspects aside from forelimb coordination, such as balancing, the success of the task is mostly dependent on the improvements to the reach and coordination of the forearm, and we would expect the forelimb area of the correct limb to show the most synaptic change. In the future, this could be verified by checking and quantifying other areas of the cerebellum, which should show slight, or negligible changes compared to the forelimb area of the trained limb.

Additionally, since animals were restricted in only using 1 limb during the paw reach task, a similar phenomenon should also be observed with regards to handedness. The trained forelimb and it's corresponding forelimb area, should show indicators of learning such as a reduced spine count, while the opposite forelimb should show negligible change regardless of improvement in the task. Much like with observing the specificity of the task by checking changes in spine density in areas outside of the "trained" forelimb area, we should also be able to compare the forelimb areas of both hemispheres, to determine if there is any learning in the opposite forelimb area.

LONG TERM MEMORY STORAGE

Finally, our results do not address the issue of overall long-term memory location. Previous research on memory in the DCN has suggested that learned information stored in the PCs may be later transferred to the DCN for long term storage[61]. Indeed, it has been observed that changes in the membrane properties (i.e. intrinsic plasticity) of deep cerebellar nuclear neurons have been observed following with eyeblink conditioning in rats[86]. Moreover, another group found that there was a recovery of PC dendritic spine density to baseline levels 2 weeks after OKR training, associated with a recovery of baseline HOKR gain[67]. This finding suggests that PC spine

densities are closely associated with long-term learning, in addition to the intrinsic plasticity in the cerebellar nuclei. Currently, we observe an decrease in spine density associated with an increased improvement at the motor learning task, however whether such a relationship holds true for longer periods of training, or training protocols with large gaps between training sessions as done in (Fig 7)**,** is unclear. Studying spine densities with cohorts trained with longer protocols could help clarify whether PC dendritic spines play a role in long term memory storage directly, or whether these spines are gradually removed, as motor memories are transferred to the DCN.

CONCLUSION

We have demonstrated an adapted cerebellum-dependent forelimb reaching task and its improvements in mice. We have shown that animals improve significantly during training, and thus our task is suitable to investigate the cerebellar substrates of motor learning. Additionally, we have investigated the role of LTD in cerebellum-dependent motor learning, using spine density as a proxy for depression in the synapses. We found an association between overall success rate at the forelimb reaching task, and a lower spine density in the forelimb reaching area of the cerebellum, suggesting that LTD is the primary substrate of cerebellar motor learning, consistent with the classical Marr-Albus-Ito model. Despite this, our results are not yet conclusive, as it is still unclear whether the observations of a decrease in spine density truly represent LTD. Further work is required to determine whether there was any functional change to the PC itself.

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