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Mapping the neural circuitry underlying spatial and temporal locomotor adaptation

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Abstract

Locomotor adaptation is a basic form of learning required for stable mobility in an unpredictable environment. Locomotor adaptation has been widely studied in humans using a split-belt treadmill that controls the speed of each side of the body independently. A previous study has shown that mice adapt on a split-belt treadmill in a way that is remarkably similar to humans. Similar to human locomotor adaptation, mice learn to adapt their locomotor patterns to achieve a more symmetric gait by adjusting spatial and temporal aspects of interlimb coordination. Split-belt locomotor adaptation is a cerebellar-dependent form of learning that requires the interposed deep cerebellar nucleus. Both spatial and temporal components of locomotor adaptation are impaired by chemogenetic manipulations of the interposed nucleus. However, while unilateral manipulations of the interposed impair spatial adaptation regardless of the belt-speed condition, temporal adaptation is only affected with manipulations ipsilateral to the fast treadmill belt. This differential lateralization suggests that spatial and temporal adaptation might be processed independently in the cerebellum and/or downstream motor areas. Here, we investigate specific output pathways from the interposed nucleus and examine their contributions to spatial and temporal locomotor learning. First, we show that the interposed nucleus sends direct excitatory projections to the thalamus, red nucleus and reticular nuclei. Next, we targeted inhibitory DREADDs to some of these downstream nuclei to examine their role in spatial and temporal adaptation. We found distinct regions specifically involved in spatial vs temporal learning, demonstrating that for locomotor learning space and time are processed by differential neural circuits.

Key words: Spatial adaptation, Temporal adaptation, Interposed Nucleus, Red nucleus, Gigantocellular reticular nucleus

Resumo

A adaptação locomotora é uma forma de aprendizagem motora necessária para uma mobilidade estável e equilibrada em ambientes dinâmicos e em constante mudança. Em humanos, este tipo de aprendizagem tem sido estudada usando uma passadeira com duas cintas que permite controlar a velocidade de cada lado do corpo independentemente. Os ratinhos adaptam nesta passadeira de uma forma muito semelhante aos humanos e, tal como estes, aprendem a adaptar a sua locomoção de modo a caminharem de uma forma mais simétrica. Esta simetria é alcançada através de ajustes em parâmetros espaciais e temporais relativos à coordenação entre membros. A adaptação locomotora é dependente do cerebelo e requer o *interposed deep cerebellar nucleus*. Os componentes espaciais e temporais da adaptação locomotora são afetados por manipulações chemogenéticas deste núcleo. No entanto, enquanto manipulações unilaterais do *interposed* afetam a adaptação espacial independentemente da velocidade das cintas da passadeira, a adaptação temporal só é afetada quando as manipulações são ipsilaterais ao lado da passadeira com a velocidade mais rápida. Esta diferente lateralização sugere que a adaptação espacial e temporal pode ser processada independentemente no cerebelo ou em áreas motoras que recebam projeções do mesmo. No presente trabalho, identificamos as regiões que recebem projeções do *interposed nucleus* e examinamos a sua contribuição para a adaptação espacial e temporal. Em primeiro lugar, demonstramos que o *interposed nucleus* envia projeções excitatórias para o *thalamus*, *red nucleus* e *reticular nucleus*. Seguidamente, usamos DREADDs inibitórios para manipular alguns destes núcleos motores e analisar o seu envolvimento na adaptação locomotora. Diferentes regiões envolvidas especificamente em cada tipo de adaptação (espacial e temporal) foram identificadas, demostrando que estas formas de aprendizagem são processadas por circuitos neuronais distintos.

Palavras chave: Adaptação espacial, Adaptação temporal, *Interposed Nucleus*, *Red nucleus, Gigantocellular reticular nucleus*

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Chapter I

Introduction

1.1. Locomotion

The ability to move actively from one place to another is an essential behavior performed by all animals. Despite seemingly effortless, locomotion is a complex motor behavior that requires the interplay between multiple supraspinal centers and spinal mechanisms to plan, initiate, execute and coordinate movement [1–3].

Planning and initiation of locomotion involves the coordinated activation of several brain regions including motor cortex, basal ganglia, midbrain and hindbrain, but basic rhythmic gait patterns can be generated by the spinal cord alone [1–10]. The spinal cord is the final output of motor systems and, together with brainstem circuits, is responsible for the execution of movement [2,11]. Furthermore, efficient locomotion requires whole-body coordination. To accomplish this, further layers of modulation to keep movements calibrated come from the cerebellum [2,3,12].

1.1.2. Cerebellar contributions to locomotion

The cerebellum is essential for the control of movements, especially those required for balance and coordination [13]. This structure is involved in the correction and modulation of motor patterns by adapting and fine-tuning movements in order to make them more accurate, through a trial and error process [14]. Thus, cerebellar damage is associated with reduced postural control and balance, as well as profound impairments on interlimb coordination, timing of muscle activation and a decreased ability to learn from movement errors, symptoms referred to as ataxia [13,15,16].

1.1.2.1. Cerebellar functional anatomy for the control of locomotion

The cerebellum receives inputs from different parts of the brain and spinal cord and it is directly or indirectly connected with several motor structures, such as motor cortex, brainstem and spinal cord [17,18]. Based on its afferent and efferent connectivity, the cerebellum can be divided into distinct functional zones (medial, intermediate and lateral), each having different roles in locomotion [19,20]. These functional zones are defined by the sole output centers of the cerebellum, the deep cerebellar nuclei (DCN), which is

composed by three distinct nuclei with different output connectivity: the medial nucleus, the interposed nucleus and the lateral nucleus (Fig. 1) [13].

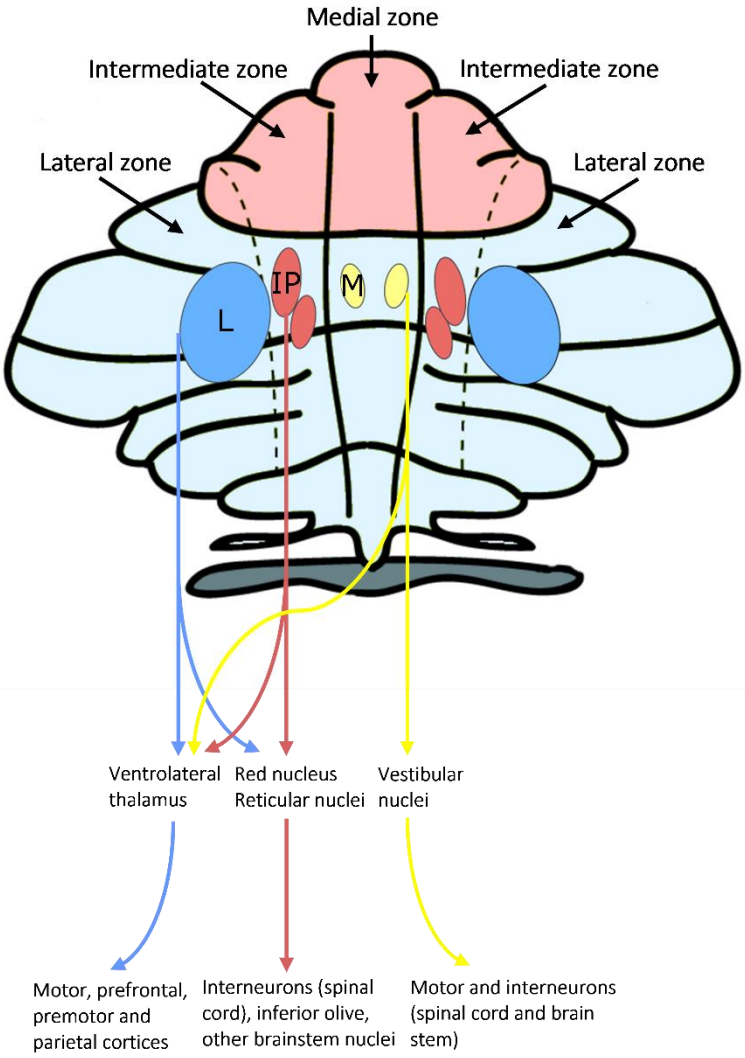

Figure 1 - Medial to lateral organization of the cerebellum. The cerebellum can be divided into three distinct zones: medial, intermediate and lateral. Each zone contains a distinct deep cerebellar nuclei (medial, interposed and lateral nucleus) that send output projections to different regions of the brain. (**L**, lateral nucleus; **IP**, interposed nucleus; **M**, medial nucleus). Adapted from Kandel *et al.*, 2000 [23].

1.1.2.1. A) Medial zone

The most medial zone of the cerebellum, the vermis, receives vestibular, visual and auditory inputs, as well as somatic sensory inputs from the head and proximal parts of the body via ascending spinal pathways [21–23]. More recently, it has also been reported that the vermis receives dense inputs from the motor areas of the cerebral cortex [24]. The medial zone projects primarily to the vestibular and reticular nuclei, via the medial

nucleus, which has as outputs the cerebral cortex and brainstem, thereby modulating the descending motor systems responsible for the control of the proximal muscles of the body and limbs [25–27]. Thus, the medial zone integrates both spinal and vestibular information, thereby influencing important motor pathways for walking.

The role of the medial zone of the cerebellum in balance and locomotion has been extensively reported in animals. Lesions or inactivation of the medial nucleus in cats and monkeys have been associated with alterations in upright postural tone, disturbances in maintaining sitting and standing balance and walking difficulties characterized by balance deficits that include frequent falls to the side of the lesion [28–33]. Specifically, cats with lesions in this nucleus show adduction combined with ipsilateral limb flexion and contralateral limb extension [28,29,33]. These animals also display irregular interlimb timing and shorter stride length during locomotion on a motorized treadmill [34]. Furthermore, a recent study performed in rats showed that unilateral lesion of the medial nucleus impairs motor coordination as demonstrated by poor motor performances in beam-walking, grid run-away and rota-rod [35]. Consistently with the results obtained from animal studies, human patients with medial nucleus lesions show impairments in gait balance-related parameters [36]. Thus, this data suggests that the medial region of the cerebellum plays a key role in the control of posture, dynamic balance and locomotion.

1.1.2.1. B) Intermediate zone

The intermediate zone of the cerebellar hemispheres receives inputs from ascending spinal pathways, reticular nuclei and cerebral cortical areas [37–41]. The output of this zone is the interposed nucleus which projects to the red nucleus, reticular nuclei and to cerebral cortex via the thalamus [13,26,27]. The intermediate cerebellar hemispheres can therefore integrate both spinal and cortical information and then convey it to motor cortical areas, thus influencing walking.

The influence of the intermediate zone in locomotion is not as well understood. Damage of this region is associated with deficits in walking which are not as pronounced as the ones caused by lesions in the medial zone [28–30]. Studies in cats with lesions in the interposed nucleus have shown that these animals have no difficulties walking on a flat surface [29,34]. Furthermore, cats and monkeys with lesions in the intermediate zone of the cerebellum have little or no impairment in upright posture and balance during standing

and walking [28–30]. Nonetheless, this region is involved in a more discrete control of the ipsilateral limbs since that some abnormalities such as loss of postural placing and hopping reflexes, hypermetric movements during swing phase and abnormal timing of coordinated movements are observed in these limbs [28,29,42,43]. The pattern of movement associated with lesions in this region is characterized by higher activation of the ipsilateral flexor muscle in late stance and during the entire swing phase, mainly due to an increase in swing phase and a decrease in stance phase in the ipsilateral limb and a decrease in swing phase and an increase in stance phase in the contralateral limb [42,44]. Consistently with this data, humans with lesions in the interposed nucleus show deficits in interlimb coordination [36].

Recordings of simple spikes from Purkinje cells terminating in the interposed nucleus during locomotion show that each cell discharge rhythmically in time with the stepping movements of the limbs. The greatest activity of the Purkinje cell population occurs in the transition from stance to swing phases in the ipsilateral forelimb, whereas the least activity occurs in the mid-stance [45]. Armstrong and Edgley (1988) have shown that the activity of the interposed nucleus is only slightly altered upon change of the locomotor paradigm from slow to fast speed or to an uphill incline [45]. On the other hand, Schwartz and colleagues (1987) have shown that the cells of the interposed nucleus are modulated during locomotion on a treadmill and that their discharge is strongly associated with the muscular activity of the ipsilateral forelimb. Furthermore, when the locomotor cycle is perturbed, this modulation ceases [46].

The role of the intermediate zone in locomotion seems to be largely distinct from the medial zone, in the way that lesions in the interposed do not strongly affect walking over ground but do induce impairments in more complex tasks such as walking on a treadmill or horizontal bars [29,34]. However, more precise and accurate analysis should be performed to characterize with detail movement abnormalities. Moreover, the interposed nucleus does not have a direct influence in upright posture and balance but its lesions cause hypermetria during swing phase and abnormal timing of forelimb stance and swing phases [28,29,34,42]. Therefore, the interposed nucleus seems more likely to be involved in the control of limb movements by regulating the timing of coordination between limbs, as well as the activity of the ipsilateral flexor and extensor muscles, thus controlling limb trajectories during locomotion.

1.1.2.1. C) Lateral zone

The lateral zone of the cerebellar hemispheres receives inputs exclusively from the cerebral cortex and its outputs are conveyed through the lateral nucleus to the red nucleus and to cortical regions such as the motor, pre-motor and pre-frontal cortices, via the thalamus [25,37,47–51]. The lateral zone can therefore be important for the control of voluntary movements and walking by tightly interacting with the motor cortex.

Animals with lesions in the lateral nucleus can walk rather effectively on a flat surface and do not present any detectable change in postural tone and supporting reflex. However, these animals displayed some misplacements of the ipsilateral limbs during more complex locomotor tasks, such as beam walking [29,30]. Patients with lesions in the lateral nucleus show impairments in leg placement and goal-directed limb movements [36].

Recordings of lateral nucleus neurons have shown that these cells are poorly modulated during normal treadmill locomotion and are not coupled to the forelimb step-cycle. Nonetheless, studies performed in cats show step-related rhythmic modulation of these cells during visually guided stepping [52]. Likewise, cats that are exposed to a rung that moves up as they approach display an increase in the firing rate of lateral nucleus cells, thereby showing modulation upon the unpredictable movements of the rung [53]. Thus, the lateral nucleus seems to play a key role in limb placement during precise motor tasks and in making adjustments to the normal locomotor pattern under novel walking conditions in which strong visual guidance is required.

In sum, the medial zone of the cerebellum is strongly involved in dynamic balance control, upright stance and locomotion. On the other hand, the intermediate zone does not play such significant role in posture and balance but seems involved in interlimb coordination by regulating the timing of movement between limbs. The lateral zone also does not play a crucial role in balance, but it is particularly important under novel walking conditions that require the control of complex, visually guided limb movements.

1.1.2.2. Cerebellar motor outputs for the control of locomotion

The cerebellum has been shown to be specifically involved in coordinated locomotion [13]. Before conveying motor commands to spinal motor neurons, this structure

can influence several downstream motor pathways, including the corticospinal, rubrospinal and reticulospinal tracts [23].

The corticospinal tract has been extensively implied in locomotor control [10,23,54]. This pathway originates primarily in the motor cortex and parietal areas of the cortex and terminates in the intermediate region of the spinal cord [23,55,56]. The motor cortex has been shown to be essential for gait modifications that require strong visuomotor coordination like walking on a horizontal ladder or stepping objects placed on a treadmill [57,58]. Several studies using a variety of locomotor tasks have demonstrated that when the gait has to be modified motor cortical neurons projecting to the spinal cord change their discharge activity [57–61]. Moreover, motor cortex inactivation during gait modifications induces alterations in limb trajectories and placement of the paw [58,62,63]. In addition to this, it has been demonstrated that the posterior parietal cortex is essential for planning locomotion by estimating the position of an animal relatively to obstacles in its path [10,64]. Further evidence for this comes from recordings on the posterior parietal cortex: while some cells increased their discharge as the animal approaches the object, others sustained their activity when the animal goes over it [23,64]. Thus, the corticospinal tract seems mostly involved in the planning and execution of gait modifications when visual guidance is required [10].

The rubrospinal tract has also been shown to be involved in locomotor control. This pathway arises from the magnocellular red nucleus and projects predominantly to the cervical spinal cord [65–67]. Lesion studies suggest that the red nucleus is not required for movement initiation but that it is important during ongoing locomotion [67,68]. Bilateral lesions of the red nucleus do not prevent walking but do induce impairments on gait coordination [67,69,70]. Furthermore, animals with lesions of the rubrospinal tract demonstrate impaired braking with the forelimb ipsilateral to the rubrospinal tract injury, as well as reduced weight support by the ipsilateral hindlimb [71]. Recordings from red nucleus neurons show that the maximal activity of these cells occurs during the swing phase of the step cycle, thereby influencing the activity of flexor muscles [72,73]. Nevertheless, more than half of the neurons showed phasic activity during both swing and stance phases [73]. These neural recordings, together with evidence from microstimulation studies, suggest that the red nucleus might also influence the activity of extensor muscles at the end of the swing and during the stance phases [68,70,73]. Similarly to the corticospinal tract, the rubrospinal tract has been shown to be important for voluntary

gait modifications. Recordings from the red nucleus demonstrate that these neurons increase their discharge activity when the contralateral forelimb is the first to step over an obstacle [73]. Taken together, this data suggests that the rubrospinal tract is involved in ongoing locomotion and that it might contribute to its modulation when adaptive gait changes to the environment are required [67,68,73].

The reticulospinal tract is thought to be very important for locomotion, but the exact function of this pathway and its associated nuclei has not yet been well established. The difficulties in understanding the role of the reticulospinal tract on locomotion come mainly from the non-discrete boundaries between these nuclei combined with the multiple cell types within each nucleus, leading to the report of a variety of diverge functions including initiation and stopping of locomotion, as well as speed modulation [5,74–76]. The reticulospinal tract has been shown to project bilaterally to cervical and lumbar segments of the spinal cord [77,78]. Thus, it is not surprising that stimulation of the reticular formation results in multi-joint and multi-limb movements, with responses in either fore- or hindlimbs ranging from ipsilateral flexion to contralateral extension [62]. Moreover, reticulospinal neurons integrate information from multiple upstream centers to send commands to spinal motor neurons. It is also thought that there might be processing of motor commands within the reticular formation, though this has not yet been addressed in detail [74].

Thus, the cerebellum can impact multiple downstream pathways important for the control of locomotion. But how does the cerebellum interact with these output pathways to generate coordinated movement? This question will require further investigation.

1.2. Cerebellum and motor learning

The idea that the cerebellum is involved in adaptation and learning of movements has emerged in the early 1970s with the work of Marr, Albus and Ito [79–81]. Their general hypothesis has been supported by much research, although the exact role of the cerebellum in motor learning is not yet fully resolved [30,82,83]. To understand the neural mechanisms underlying this form of learning, two forms of cerebellar-dependent motor learning have been extensively studied, namely the classical eyelid conditioning and the adaptation of the vestibulo-ocular reflex (VOR). These studies have revealed much about

the cerebellum's role in motor learning, but it is still not clear whether more complex motor tasks share the same mechanisms.

1.2.1. Cerebellar circuitry

The cerebellum is essential for motor learning and, therefore, its anatomy and physiology are crucial to the ideas about the sites of plasticity and the underlying learning mechanisms. Thus, the cerebellar circuitry will be first briefly described.

The cerebellum is composed by a series of neuronal units displaced in highly regular arrays, each sharing the same basic microcircuitry (Fig. 2). The similarity between the architecture and physiology in all regions of the cerebellum suggests that the cerebellum performs similar computational operations for many different motor, and perhaps non-motor, tasks [17,18].

The cerebellum receives information from two major excitatory inputs: mossy fibers and climbing fibers, which produce different patterns of firing in cerebellar cortical Purkinje cells, conveying distinct types of information [17,18]. Mossy fibers carry sensory information from the spinal cord, brainstem and cerebral cortex and they form excitatory synapses on the dendrites of granule cells [84,85]. The sensory signals that converge on granule cells through mossy fibers are essential for the generation and coordination of movements [86]. The axons of granule cells constitute the parallel fibers that pass through and make synapses with several Purkinje cells, thereby controlling the firing rate of simple spikes in Purkinje cells. Moreover, granule cells also contact with Golgi and basket/stellate cells, which form inhibitory synapses onto granule

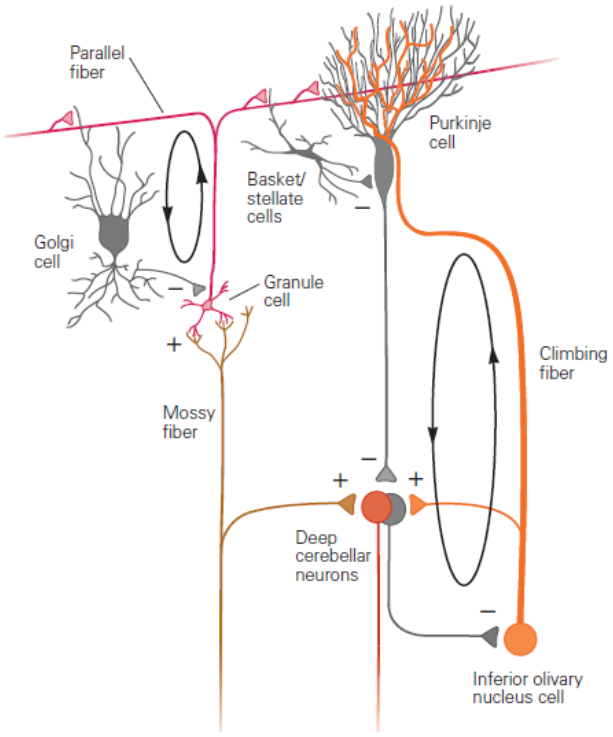

Figure 2 – Organization of the cerebellar microcircuitry. Excitatory and inhibitory inputs can be compared both at the cerebellar cortex and deep cerebellar nuclei. Recurrent loops occur at the cerebellar cortex and outside the cerebellum, in the inferior olive [18].

cells or Purkinje cells, respectively [17,18]. Climbing fibers arise from the inferior olive and directly synapse onto Purkinje cells on the cerebellar cortex [87]. Each climbing fiber contacts 1 to 10 Purkinje cells, however, in contrast to the parallel fibers, each Purkinje cell receives inputs from only a single climbing fiber [86]. Climbing fibers enwrap the dendritic tree of the Purkinje cells, making numerous synaptic contacts [17]. A single action potential from a single climbing fiber results in a prolonged depolarization that yields a complex spike [18,88]. Climbing fiber activity is assumed to convey sensory feedback information, particularly error signals [88].

An important characteristic of the cerebellar circuitry consists in the fact that the excitatory and inhibitory inputs are compared both in the cerebellar cortex and deep cerebellar nuclei [18]. Purkinje cells exert inhibitory effects on the deep cerebellar nuclei, the sole output of the cerebellar circuitry. In contrast, mossy and climbing fibers send excitatory projections to the deep nuclei [17]. Thus, the inhibitory input from the latter modulates the excitatory inputs from the former. Another important hallmark of the cerebellar circuitry is the presence of several recurrent loops at different levels [17,18].

1.2.2. Eyelid conditioning and vestibulo-ocular reflex

Extensive analysis of simple forms of motor learning such as classical eyelid conditioning and adaptation of the vestibulo-ocular reflex (VOR) has provided several evidences for the cerebellum's involvement in motor learning [30,82,83].

In the classical conditioning of the eyelid response, an air puff to the cornea serves as an unconditional stimulus (US) that elicits an eyeblink response. If a neutral conditioned stimulus (CS) such as a tone repeatedly precedes the US, the tone will gradually elicit a blink in advance to the air puff. If this paradigm is repeated many times, the CS alone is sufficient to induce a blink thus indicating that there was a reliable, learned eyelid response [89,90].

Another form of cerebellar-dependent motor learning is VOR adaptation [91]. The VOR stabilizes images on the retina by generating compensatory eye movements in the opposite direction to the head movement [92]. Turning the head can always induce eye movements, even without training. However, when the head turn is successively paired with image motion there is a change in the size of the vestibular response in order to improve image stabilization on the retina [92–95]. This gradual adaptation can be considered

a learning response since that, overtime, the head turn alone begins to elicit an altered eye movement response that is similar to the one triggered by the presence of both head turn and image motion. Thus, the tone and the head turn can be considered the stimuli that induces the learned response and the image motion and air puff the instructive stimuli [92,96].

It is clear that the two learning paradigms are very similar at the behavior level. The only difference is that in the eyelid conditioning the CS needs to be paired with an air puff to elicit a response, whilst in the VOR the CS triggers a response before training, though its amplitude changes throughout practice, when associated with image motion.

1.2.2.1. Similarities between eyelid conditioning and VOR neural circuits

Eyelid conditioning and VOR responses are processed by different motor pathways, though both in extracerebellar structures. The red nucleus and other brainstem nuclei are involved in eyelid conditioning, whereas the vestibular nuclei and other brainstem nuclei are involved in VOR adaptation [91,97–101] . Likewise, the sensory inputs associated with conditioning are different (auditory inputs for eyelid conditioning and vestibular inputs for VOR), but both project in parallel to the cerebellar cortex and deep cerebellar nuclei. In the cerebellar cortex, the anterior lobe is relevant for eyelid conditioning and the floccular complex for VOR, while in the deep cerebellar nuclei the anterior interposed nucleus is important for eyelid conditioning and the floccular target neurons in the vestibular nucleus (correspondent to the deep cerebellar nuclei) for VOR [102–107].

In each paradigm, the two stimuli that need to be paired in order to cause a conditioned response converge both in the cerebellar cortex and deep cerebellar nuclei. In both eyelid conditioning and VOR, sensory information about the CS (tone or head turn) is conveyed to the cerebellum through mossy fibers and somatosensory information about the US (air puff or image motion) is carried to the cerebellum by climbing fibers [108–112]. Thus, the functionally homologous sensory stimuli for the two behaviors use similar pathways to convey them to the cerebellum.

1.2.2.2. Memory in the cerebellar cortex and deep cerebellar nuclei

The role of the cerebellum in motor learning in both eyelid conditioning and VOR has been demonstrated by lesion experiments. For instance, lesions in the anterior interposed nucleus block eyelid responses to the tone (CS) thereby preventing learning, but do not affect responses to the air puff (US) alone [113,114]. On the other hand, for the VOR, lesions in the vestibular nuclei cause deficits in overall behavior [113]. However, other mechanisms rather than learning could have been affected and, therefore, only the FTNs on the vestibular nuclei should have been ablated in this experiment.

The role of the cerebellum in eyelid conditioning has been further confirmed by two studies that demonstrated that learning occurs downstream mossy fiber cerebellar inputs and upstream the red nucleus. In the first study, the replacement of the CS and US by stimulation of mossy fibers or climbing fibers, respectively, induced a conditioned response, indicating that learning occurs downstream the input of the cerebellar circuitry (mossy fibers) [110,112]. In the second study, the red nucleus was reversibly inactivated and the CS and US were delivered at the same time, during several sessions. During training, the expression of conditioned responses was abolished. However, when the activity of the red nucleus was reestablished, there was expression of conditioned responses, showing that learning occurs upstream the red nucleus, apparently in the cerebellum [114,115].

Although these studies suggest that the cerebellum is involved in memory storage in motor learning, they do not demonstrate the role of the cerebellar cortex and the deep cerebellar nuclei. In eyelid conditioning, reversible inactivation of the interposed nucleus allows the acquisition of a conditioned response but blocks its expression, thereby indicating that the cerebellar cortex is probably a site of plasticity [116]. Still, in both eyelid conditioning and VOR adaptation, lesions of relevant parts of the cerebellar cortex prevent further learning but abolish only some of the memory acquired during training, suggesting that in cerebellar-dependent learning there is storage of memory both within and outside the cerebellar cortex, probably in the relevant deep cerebellar nuclei [102,105,106,117–119].

Eyelid conditioning and VOR adaptation are quite different motor problems, however, it is clear that they share remarkable similarities both at behavioral and circuit level. Even though the two systems receive different sensory inputs and send outputs to distinct parts of the brain, the pathways that convey them to the cerebellum are the same

and the underlying neural mechanisms of these forms of learning seem to be similar. Thus, these two behaviors may represent general principles underlying cerebellar-dependent motor learning that could perhaps be applied in more complex tasks.

1.2.3. Locomotor adaptation

The cerebellum is involved in simple motor learning paradigms such as the previously mentioned classical eyelid conditioning and VOR adaptation. The learning mechanisms underlying these behaviors appear to be quite similar, but could this be applied to more complex motor tasks such as locomotor adaptation?

Locomotor adaptation is essential for our everyday life. We must constantly adapt locomotor patterns to meet changing environmental demands. Successful locomotion in these unpredictable environments requires relatively fast adjustments (reactive feedback adaptation), as well as more gradual adjustments that require time and practice under exposure to novel conditions (predictive feedforward adaptation) [120]. In humans, locomotor adaptation has been extensively studied using a split-belt treadmill that controls the speed of opposite sides of the body independently [120–122]. In this experimental paradigm, first subjects are tested walking with belts tied (baseline), followed by belts split (adaptation) and again belts tied (post-adaptation). In this locomotor task, healthy individuals adapt interlimb coordination over the course of the split-belt trials to achieve a more symmetric pattern of gait. In the post-adaptation phase, healthy individuals show negative aftereffects, indicating that they have stored a new pattern of movement [120,122]. On the other hand, individuals with cerebellar damage make reactive changes of individual limbs to match the speed of the belts, but do not adapt interlimb coordination and do not show negative aftereffects [120]. Thus, the cerebellum seems to be specifically involved in controlling interlimb coordination. In agreement with these data, mutant mice with post-natal Purkinje cell degeneration (*pcd* mice) show impairments in interlimb and whole-body coordination during free walking, but do not demonstrate abnormalities in forward motion of individual limbs if changes in body size and walking speed are considered [123].

Split-belt locomotor adaptation can also be studied in mice, using high-speed videography and 3D whole-body tracking [124] (Fig. 3A and 3B). Mice placed on a split-belt treadmill learn to adapt their locomotor patterns to achieve a more symmetric and

stable gait. Similar to human locomotor adaptation, intralimb parameters scale to match the speed of the belts (Fig. 3C and 3D), while interlimb and whole-body coordination adapt over several minutes of split-belt walking to enable a more symmetric gait (Figure 3E and 3F). *Pcd* mice, however, are able to respond appropriately to changes in belt speed but do not adapt interlimb parameters over the course of the split-belt trials and do not show negative aftereffects in the post-adaptation phase [124]. Thus, split-belt adaptation in mice, as in humans, seems to be a cerebellar-dependent form of learning.

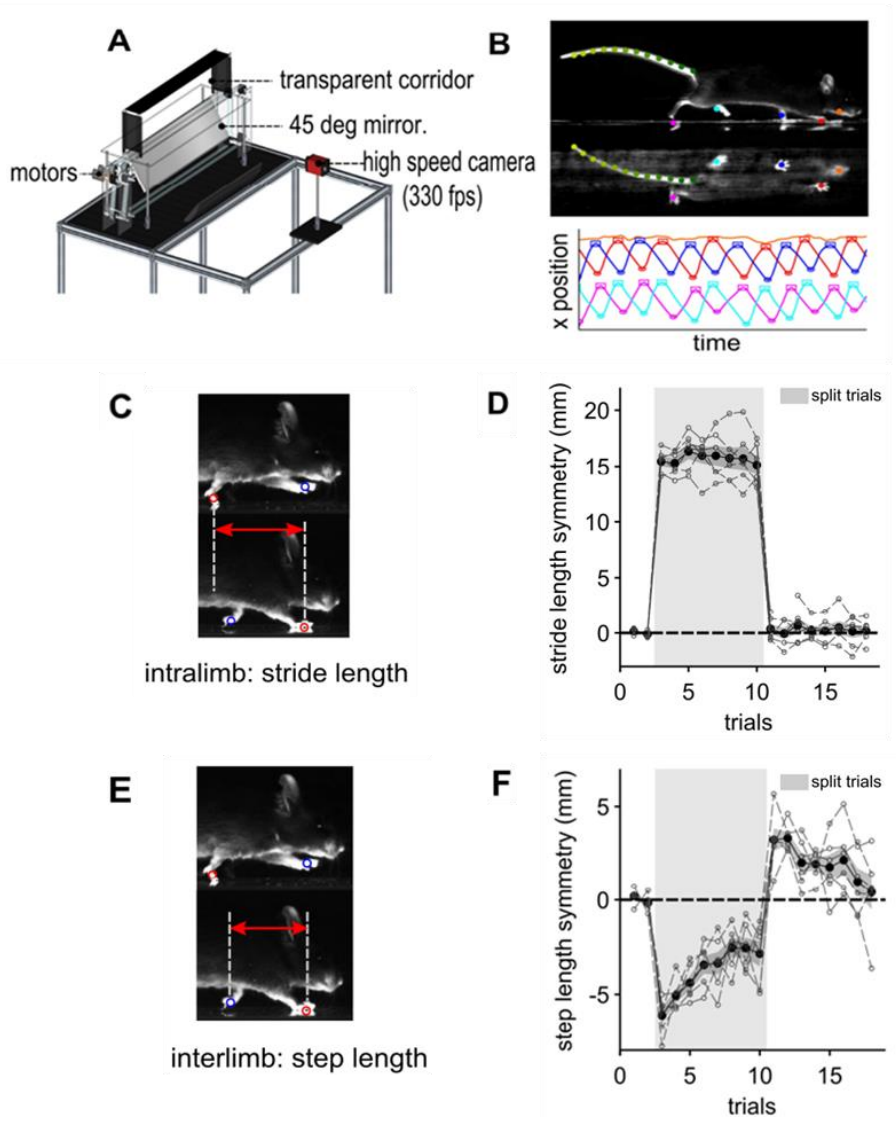

Figure 3 – Mice on a split-belt treadmill learn to adapt interlimb coordination. A) Split-belt treadmill setup schematic. Mice are placed on a transparent corridor where they freely walk on two belts driven by motors that control the speed of the belts independently. A 45-degree mirror below the corridor allows a high-speed camera (330 fps) to capture both side and bottom views of the mice. **B)** 3D tracking of mouse body features (paws, nose and tail) (top). Tracking example for all paws and nose represented as their position in x overtime (bottom). **C)** Intralimb parameters (stride length) consist in the distance that a single limb takes from stance to stance during a stride cycle. **D)** Average stride length symmetry for front limbs shows that mice do not adapt intralimb parameters. Dashed lines represent individual animals' adaptation.

E) Interlimb parameters are computed as step length which consists in the relative distance between two homologous limbs at stance onset. **F)** Average step length symmetry for front limbs shows that mice adapt interlimb coordination. Individual animals' adaptation are represented as dashed lines. From Darmohray *et al.*, 2018 [124].

Human and mouse locomotor adaptation share remarkable similarities. However, there are evident differences in their gait (quadrupedal vs bipedal) which demand different behavioral analysis. For instance, analysis of front limbs vs hind limbs movements in mice during locomotor adaptation shows that front limbs adapt faster and with a higher magnitude than hind limbs [124].

Locomotor adaptation on a split-belt treadmill requires specifically adjustments in interlimb coordination on both species. Interlimb coordination is usually measured as step length. However, step length adaptation has been shown to have both spatial and temporal components in mice (Fig. 4A and 4B) and humans [120,124,125]. Spatial adaptation is achieved by adjusting the excursion of the limbs during a stride relative to the body center (Fig. 4C), while temporal adaptation is accomplished by adjusting the relative timing between limbs (Fig. 4D).

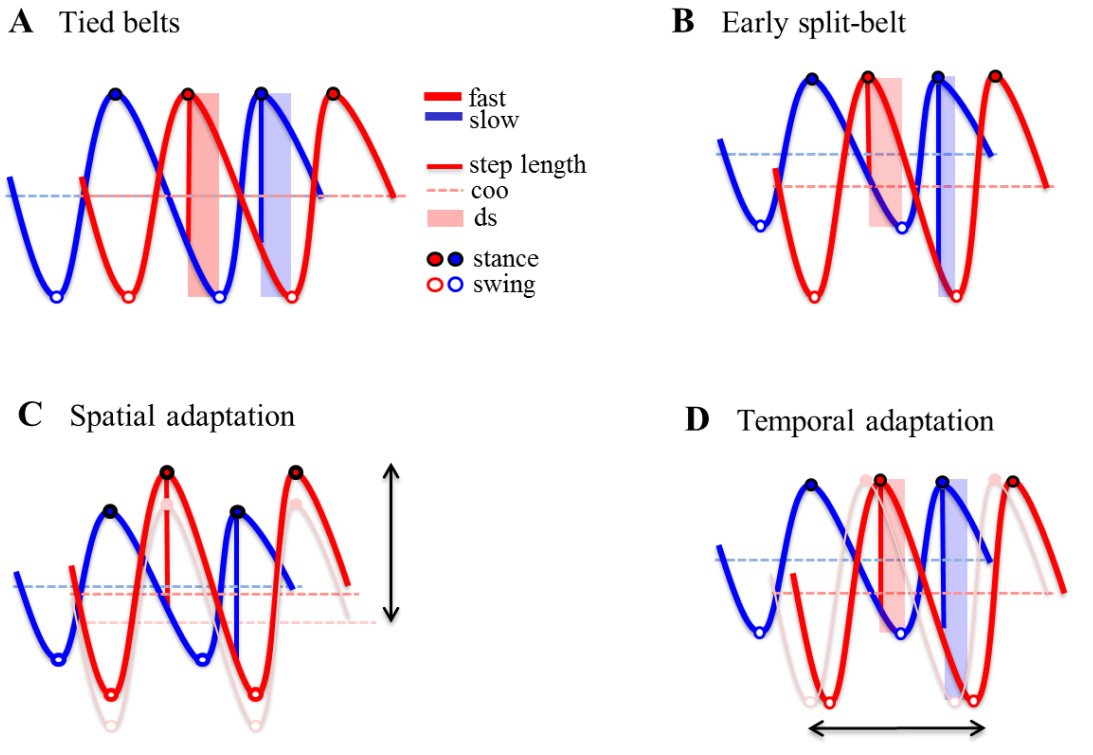

Figure 4 - Interlimb parameter step length can be broken down into spatial and temporal adaptation. A) Schematic representing stride cycles during tied belt walking for fast (red) and slow (blue) limb. Step length (vertical solid lines) symmetry is achieved by symmetry on both spatial (center of oscillation (coo), dashed horizontal lines) and temporal (shaded vertical patches) parameters. **B)** Early split-belt period is characterized by asymmetries on step length which result from changes on spatial and temporal components. **C)** Spatial adaptation is accomplished by changing the center of oscillation (coo) of the limbs overtime. Light red lines represent fast limb stride cycles during early split-belt adaptation. **D)** Temporal adaptation occurs by adjusting the relative timing between the limbs.

The mechanisms underlying locomotor adaptation seem therefore to be conserved across species, opening up the possibility of using mice as a model to understand how this behavior is processed at the circuit and synaptic level. This form of learning has been shown to involve the cerebellum and, more recently, a specific cerebellar region required for this behavior has been identified: the interposed nucleus and overlying paravermal cortex [124]. Furthermore, this experimental paradigm can also help to better understand the role of the cerebellum in coordinated locomotion. The cerebellum is thought to control the ipsilateral side of the body but interlimb coordination requires comparison of movements between different limbs. So how does the cerebellum integrate sensory information from multiple limbs and then translate it into coordinated movements? A previous study has started to address this by analyzing how unilateral manipulations of the Purkinje cells terminating in the interposed affect spatial and temporal adaptation. Interestingly, a differential laterality of contributions to spatial and temporal learning was observed: while double support symmetry was only affected when the fast belt was run ipsilateral to the injection site, spatial adaptation was impaired regardless of the side that the fast belt was set (ipsilateral or contralateral) [124]. These results suggest that on one side the cerebellum is involved in the bilateral control of spatial parameters of locomotor adaptation and on the other that space and time might be processed independently in the interposed and/or its associated circuitry. Therefore, it is necessary to continue to dissect the circuitry behind the interposed to understand whether spatial and temporal adaptation are processed by distinct neural populations.

1.3. Objectives

Despite the tremendous growth in research on cerebellar functions in recent years, the neural mechanisms underlying cerebellar-dependent motor learning are still largely unknown, especially the ones regarding locomotor adaptation. The role of the cerebellum in locomotor adaptation has been widely assessed in patients with cerebellar damage, however, these studies do not provide information about which specific regions of the cerebellum are mediating adaptation [120]. Mice provide several advantages to study coordinated locomotion. Aside from the high availability of genetic tools that allow to manipulate their neural circuits, they are also very small making it possible to analyze movements across different parts of the body with high spatial and temporal resolution

using high-speed videography and 3D whole-body tracking [123]. These recent technologies can be used to study locomotor adaptation on a split-belt treadmill [124].

Split-belt locomotor adaptation has been shown to be a cerebellar-dependent form of learning that involves spatiotemporal changes in interlimb coordination [120,124,125]. A specific cell type and region of the cerebellum, the Purkinje cells in the paravermal cortex overlying the interposed nucleus, have been shown to be necessary for this form of learning. This study has started to narrow down the potential pathways involved in split-belt adaptation. Moreover, manipulations of this region suggest that spatial and temporal components of locomotor adaptation might be processed distinctly in the interposed and/or its associated circuitry [124].

Thus, our main goal is to map the interposed downstream circuitry required for split-belt adaptation in order to find out whether spatial and temporal adaptation are processed by differential neural circuits.

To achieve this, our aims are:

- Extend the results that demonstrate that perturbing the activity of the Purkinje cells terminating in the interposed impairs locomotor adaptation by using an alternative viral strategy to manipulate directly the interposed nucleus.
- Trace the projections of the interposed to its downstream motor targets.
- Manipulate the interposed downstream structures to assess whether specific output nuclei contribute differently to spatial vs temporal components of locomotor adaptation.

Chapter II

Methods

2.1. Animals

Animal procedures were carried out in accordance with the Champalimaud Centre for the Unknown Ethics Committee guidelines and approved by the Portuguese Direcção Geral de Veterinária. Animals were kept in a temperature-controlled room with a reverse light cycle (12h light/12h dark) so that all the experiments were performed during the dark period in which mice are more active. Male and female mice were separately housed in groups of 2-5 mice with *ad libitum* access to water and food. All experiments were carried out in C57BL/6 mice with approximately 10-14 weeks of age. Vglut2-cre mice obtained from the Jackson Lab (#016963, Slc17a6$^{tm2(cre)Lowl}$/J) were used in the interposed manipulation experiments.

2.2. Surgical procedures

In all surgeries, mice were placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) and anesthetized with isoflurane (3% for induction, 1.5% for maintenance). For virus injections, the skin was cut to expose the skull and craniotomies were drilled over the region of interest. A nanoject (Nanoject II, Drummond) with a pulled beveled glass pipette (30-40 μm of diameter) was used for local administration of the virus with multiple short pulses. After injection, the craniotomy was filled with a silicone-based elastomer (Kwik-cast, World Precision Instruments). Antibactericide and lidocaine were applied on the exposed skin and the skull was covered with dental cement (Super Bond – C&B). Buprenorphine (0.1 mg/kg) was intraperitoneally injected for postoperative analgesia.

2.3. DREADDs manipulations

For interposed manipulation experiments, mice were injected with 200-240 nL of doubled floxed Gi-coupled hM4D DREADDs (AAV8-hSyn-DIO-hM4D(Gi)-mCherry, addgene plasmid #44362) into the interposed nucleus of vglut2-cre animals for expression of inhibitory DREADDs in the glutamatergic neurons of the interposed. The following coordinates were used for targeting the interposed: -6.24 mm anterior-posterior from bregma, 1.6 mm lateral and -2.3 mm ventral.

For manipulations of the magnocellular red nucleus and gigantocellular reticular nucleus, wild-type mice were injected with 200-250 nL of Gi-coupled hM4D DREADDs (AAV8-hSyn-hM4D(Gi)-mCherry, addgene plasmid #50475). The coordinates used for targeting the magnocellular red nucleus were -3.5 mm anterior-posterior from bregma, -0.6 mm lateral and -3.6 ventral, while for the gigantocellular reticular nucleus were -6.4 mm anterior-posterior from bregma, -0.5 mm lateral and -4.6 mm ventral.

Split-belt adaptation experiments were performed two weeks post-injection to allow time for virus expression. To activate DREADDs receptors, clozapine N-oxide (CNO) was administrated intraperitoneally (10 mg/kg) and mice were run 30-40 minutes post-injection.

All animals were perfused after the behavioral experiments and histology was performed to confirm virus expression and injection site location.

2.4. Anatomical tracing

Vglut2-cre mice were injected into interposed nucleus with 200 nL of AAV8-hSyn-DIO-mCherry (addgene plasmid #50459) for anterograde expression of mCherry in the glutamatergic neurons of the interposed. Mice were perfused after 2 weeks to allow time for virus expression and their brains were histologically analyzed to identify the distinct excitatory outputs of the interposed nucleus.

2.5. Histology

Mice were given intraperitoneal injections of ketamine/xylazine (10 ml/kg) and posteriorly perfused with 0.01 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA). Their brains were dissected, placed overnight in PFA 4% and then cryopreserved for at least 24h in 30% sucrose in 0.01 M PBS and 10% sodium azide. The brains were embedded in optimum cutting temperature (OCT) compound (Tissue-Tek, 4583) and 50 μm-thick coronal sections were cut in a cryostat (CM3050S, Leica). Immunohistochemistry was then performed. Images were acquired in a Z1 AxioScan Microscope (Zeiss) with a 20x magnification objective.

2.6. Immunohistochemistry

Immunohistochemistry was performed on interposed nucleus and gigantocellular reticular nucleus slices. Floating sections of the regions of interest were washed three times with 0.01 M PBS and then incubated in 0.01 M PBS with 0.4% Triton-X (T9284-100ML, Sigma-Aldrich®) for 60 min to increase tissue penetration. The slices were then incubated overnight at room temperature with rabbit anti-mCherry primary antibody (ab167453, Abcam) diluted 1:1000 in 0.01 M PBS with 0.4% Triton-X. Afterwards, the slices where washed three times with 0.01 M PBS and incubated with the secondary antibody, Alexa anti-Rabbit 488 (A-11008, ThermoFisher Scientific), diluted at 1:1000 in 0.01M PBS with 0.4% Triton-X for two hours at room temperature. The slices were then washed twice with 0.01 M PBS. Interposed nucleus sections were counterstained with DAPI (4,6-Diamidine-2′-phenylindole dihydrochloride) solution diluted 1:1000 in 0.01 M PBS for 20 min and washed again twice with 0.01 M PBS. Sections were then mounted and coverslipped with mowiol mounting medium.

2.7. Experimental setup

All behavioral experiments were performed using a split-belt treadmill previously developed in the lab [124]. The setup was composed by two transparent melinex belts driven by two DC motors with high-resolution encoders and an Escon 50/5 motor controller (Maxon). Mice were placed in a transparent corridor (4 x 30 cm) that restrained their walking to the place of the belts. A 45-degree angled mirror below the corridor allowed a single high-speed camera (Pike F-032 B/C, Allied Vision Technologies) to capture both side and bottom views of the mice. This camera allowed to capture images at 330 fps. The setup was illuminated by a set of LEDs that emitted cool white light and that was positioned to increase contrast and decrease reflection. Image acquisition was controlled using a software previously written in Labview. Body features (paw, nose and tail) were tracked in 3D using a previously developed tracking algorithm [124].

2.8. Experimental protocols

Animals were habituated to the behavioral setup for 3 daily sessions before starting the experiment. During each session, mice were subjected to 10 trials of short duration (approximately 20 seconds) with increasingly different speeds (0.1 – 0.275 m/s) until they were able to walk without turns, maintain a regular position and keep up with the speed of the belts required for the subsequent experiments.

Split-belt locomotor adaptation experiments were composed by three distinct phases: baseline tied (3 trials), split (10 trials) and post-adaptation tied (10 trials) in which each trial had the duration of 1 minute. For the split adaptation phase the fast belt was set at 0.375 m/s and the slow belt at 0.175 m/s, while for the baseline and post-adaptation phases both belts were set at an intermediate speed between slow and fast belts (0.275 m/s). 40 minutes before the experiment, intraperitoneal injections of either saline (control) or CNO were given. Every split-belt adaptation protocol was followed by a washout on the next day that consisted on 10 trials at tied speed (0.275 m/s).

2.9. Gait analysis and parameters definition

For the computation of all gait parameters, strides were defined as the period from stance to stance. Symmetry was computed by subtracting trial average values of each individual limb on the fast side per individual limb on the slow side.

Step length was defined as the relative distance between the two homologous limbs at stance onset. Spatial parameters were measured by computing the center of oscillation (coo) which consists in the midpoint between swing and stance phases relative to the body center. Temporal parameters were assessed by computing the % of double support which is defined as the percentage of the stride cycle duration in which two homologous limbs are in stance at the same time.

2.10. Statistics

Statistical analyses were performed using MATLAB. For split-belt adaptation experiments, learning was quantified by measuring the difference between early post-adaptation period (first aftereffect trial) relative to the mean baseline period. To compare

learning between control and manipulation groups, paired *t*-test were applied. Differences were considered significant at $p < 0.05$ and are reported as * for $p < 0.05$, ** for $p < 0.01$; and *** for $p < .001$.

Chapter III

Results

3.1. Split-belt locomotor adaptation requires interposed nucleus

Locomotor adaptation on a split-belt treadmill has been shown to be specific to measures of interlimb coordination which involve spatiotemporal gait changes [120,124]. A previous study narrowed down the neural circuitry underlying this form of learning by targeting the Purkinje cells terminating in each of the three deep cerebellar nuclei (DCN) (Fig. 5A). Perturbation of the interposed-associated circuitry has shown that this nucleus, but not the medial or lateral, is necessary for split-belt adaptation. Moreover, this study suggests that spatial and temporal adaptation might be processed differentially by the interposed and/or its associated circuitry since unilateral manipulations of this region show bilateral and unilateral contributions for spatial and temporal learning, respectively [124]. Thus, to understand whether spatial and temporal adaptation are independently processed it is necessary to continue to dissect the circuitry behind the interposed nucleus.

We started by expanding the previous results by targeting directly the interposed nucleus (Fig. 5B). We injected an adeno-associated virus into the right interposed nucleus of vglut2-cre mice for cre-dependent expression of inhibitory DREADDs on glutamatergic neurons (Fig. 6A). Virus expression in the interposed nucleus was broad and spread throughout the entire anteroposterior axis (Fig. 6B). Mediolateral spreading within the interposed was variable: in some animals, spillover to the medial and lateral nucleus was observed, while in others was restricted to a small region of the interposed. The injections were performed unilaterally so that split-belt adaptation protocol could be run with the fast belt ipsilateral or contralateral to the injection site, thereby providing us the ability of identifying potential differences in lateralization.

Split-belt adaptation experiments were performed two weeks after virus injection. Mice locomotor adaptation was assessed after intraperitoneal injection of either saline (control) or clozapine-N-oxide (CNO). The same mice were then compared across conditions to assess whether inhibition of interposed glutamatergic neurons impaired locomotor learning. The adaptation curves presented here correspond always to front limbs symmetry because it was previously shown that split-belt adaptation occurs mainly through adjustments in front limb movements [124]. Separate groups of mice were used when the belts were run ipsi- and contra-fast.

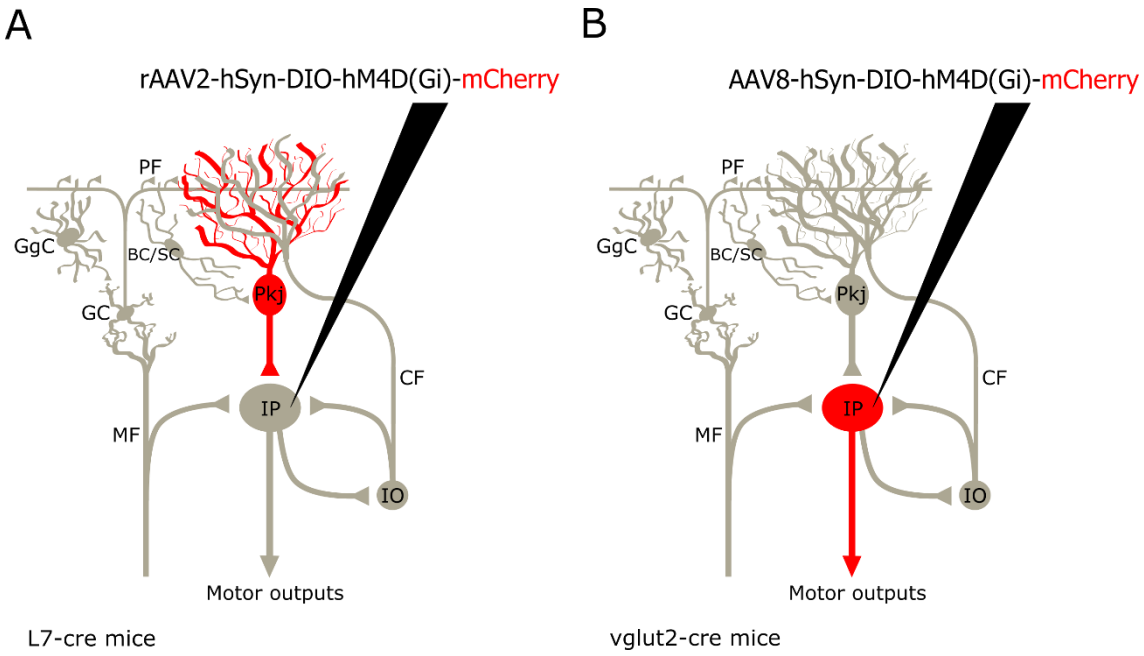

Figure 5 – Schematic of cerebellar circuitry representing experimental design for Purkinje cells vs interposed nucleus manipulations. A) Schematic representing viral strategy used in a previous study performed by Darmohray *et al.*, 2018 [124]. A retrograde virus (rAAV2-hSyn-DIO-hM4D(Gi)-mCherry) was injected into the interposed nucleus of L7-cre mice for cre-dependent expression of inhibitory DREADDs on Purkinje cells. **B)** Schematic representing viral strategy used in the present work. Vglut2-cre mice were injected in the interposed nucleus with a virus (AAV8-hSyn-DIO-hM4D(Gi)-mCherry) to express inhibitory DREADDs in the interposed glutamatergic neurons.

We examined how direct manipulation of the interposed nucleus affected spatial and temporal adaptation on ipsi- and contra-fast conditions. Control animals showed normal adaptation on both conditions, with symmetric spatiotemporal parameters during baseline tied, followed by an asymmetry during split trials which improved overtime and then a prominent aftereffect when the belts returned to tied, demonstrating that there was learning. However, inhibition of interposed glutamatergic neurons impaired spatial adaptation (Fig. 6C and 6D), as measured by the center of oscillation, both on ipsi- (CNO v saline aftereffect: $t_{(8)} = 2.4$, $p = 0.04$) and contra-fast (CNO v saline aftereffect: $t_{(7)} = 7.06$, $p = 2.01\ e^{-04}$) conditions (Fig. 6E), while double support symmetry (Fig. 6F and 6G) was only affected on the ipsi-fast (CNO v saline ipsi-fast aftereffect: $t_{(8)} = -2.25$, $p = 0.05$; CNO v saline aftereffect contra-fast: $t_{(7)} = -0.06$, $p = 0.95$) (Fig. 6H). These results are consistent with what was observed when the activity of the Purkinje cells terminating in the interposed is perturbed [124]. Moreover, the effects observed are dependent on DREADDs expression, as mice injected with an adeno-associated virus expressing only mCherry adapted similarly with saline and CNO (CNO v saline spatial aftereffect: $t_{(4)} = 1.7$ $p = 0.16$; CNO v saline temporal aftereffect: $t_{(4)} = 1.68$ $p = 0.17$) (Annex 1).

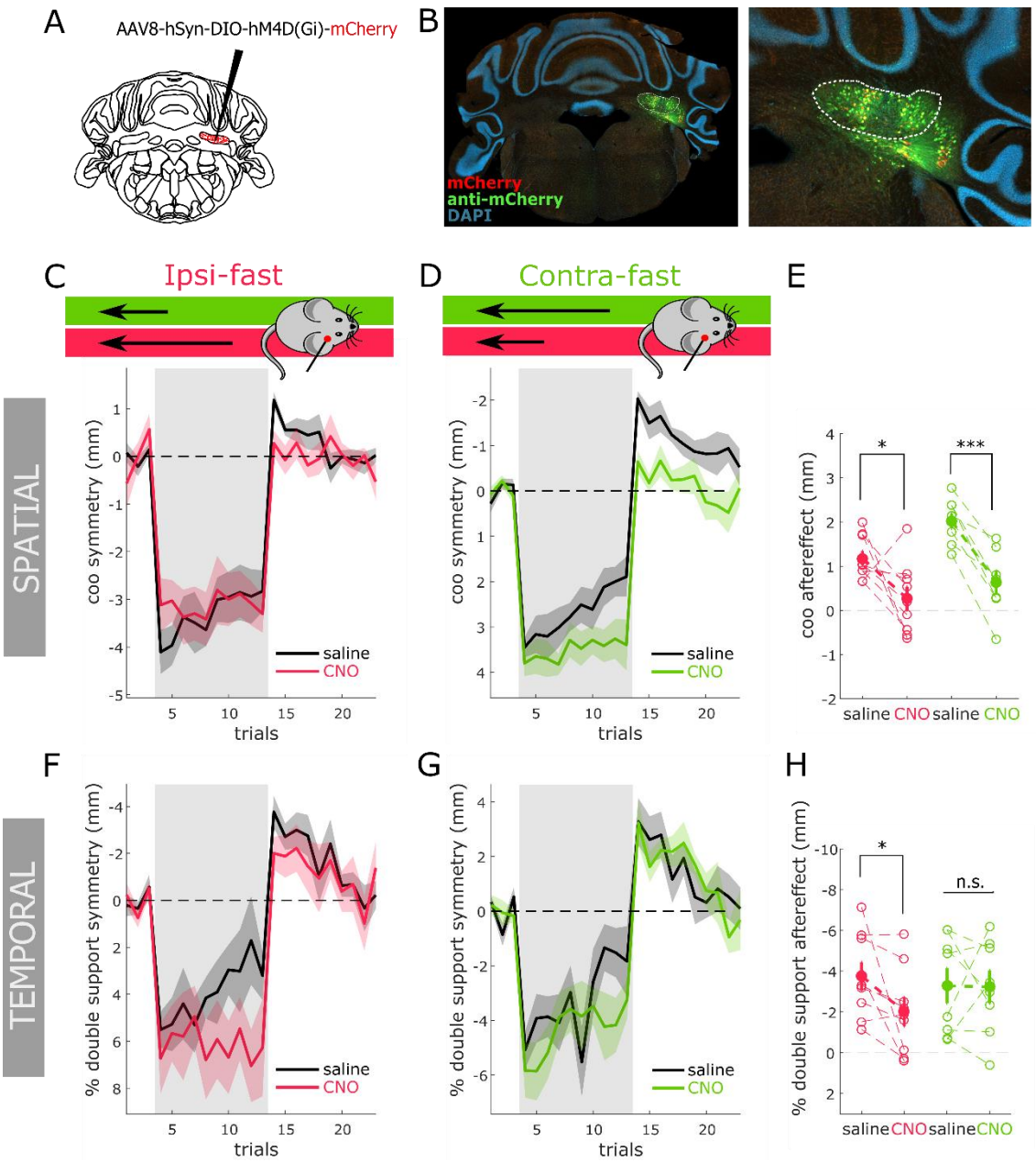

Figure 6 - Interposed nucleus has different laterality of contributions to spatial and temporal adaptation. A) Schematic of injection site location. AAV8-hSyn-DIO-hM4D(Gi)-mCherry was injected into the right interposed nucleus of vglut2-cre mice. **B)** Left: Example of coronal section showing expression of inhibitory DREADDs in the interposed nucleus. Right: Magnification of the interposed nucleus. Virus expression is seen throughout the entire interposed with some spillover to the lateral nucleus. DREADDs expression can be observed by either mCherry (red) or anti-mCherry (green) labelling. DAPI staining (blue) was used to visualize the cerebellar structure. **C)** Top: Schematic representing ipsi-fast condition. The fast belt was set ipsilateral to the injection site. Bottom: Average spatial adaptation (± s.e.m.) curves for interposed-injected mice run on the ipsi-fast condition after saline (black) or CNO (pink) administration (n=9). Grey patches represent split-belt trials. **D)** Top: Schematic representing contra-fast condition. The fast belt was set contralateral to the injection site. Bottom: Average spatial adaptation (± s.e.m.) curves for animals injected in the interposed and run on the contra-fast condition after administration of saline (black) or CNO (green) (n=8). **E)** Average center of oscillation aftereffect size (first post-adaptation trial) for saline and CNO-injected animals on the ipsi-fast (pink) and contra-fast (green) conditions. Dashed thin lines represent individual animals' aftereffect size. **F)** Average temporal adaptation (± s.e.m.) curves for interposed-injected animals run on the ipsi-fast condition after saline (black) or CNO (pink) administration (n=9). **G)** Average temporal adaptation (± s.e.m.) curves for mice injected in the interposed and run on the contra-fast condition after saline (black) or CNO (green) injections (n=8). **H)**

Average % double support aftereffect size (first trial post-adaptation) for mice administered with saline and CNO and run on the ipsi-fast (pink) and contra-fast (green) conditions. Individual animals' aftereffect size is shown in dashed thin lines.

3.2. Interposed nucleus projections to pre-motor and motor areas

The interposed nucleus and overlying paravermal cortex has been shown to be required for locomotor adaptation. The demonstration that the interposed, but not the other deep nuclei, is necessary for locomotor learning decreases the downstream candidate sites for split-belt adaptation [124]. Moreover, the differences observed in the laterality of contributions of the interposed to spatial and temporal learning suggest that these strategies might be dissociable in the deep nuclei and/or its associated circuitry.

Thus, to map the cerebellar downstream circuitry involved in split-belt adaptation, we started by anatomically identifying the interposed pre-motor and motor projecting areas. We injected an adeno-associated virus into the interposed nucleus of five vglut2-cre mice for anterograde expression of mCherry in the interposed glutamatergic neurons (Fig. 7A and 7B).

We observed axonal labelling in all animals in the following brain regions:

- Forebrain: basolateral amygdaloid nucleus (BLA); ventrolateral thalamic nucleus (VL), central medial thalamic nucleus (CM), paracentral thalamic nucleus (PC) (Fig. 7C), ventromedial thalamic nucleus (VM) (Fig. 7D);
- Midbrain: superior colliculus (SC), mesencephalic reticular formation (mRt), magnocellular red nucleus (RMC) (Fig. 7E);
- Hindbrain: reticulotegmental nucleus of the pons (RtTg) (Fig. 7F), pontine reticular nucleus caudal part (PnC), parvocellular reticular nucleus alpha part (PCRtA), facial nerve (7n) (Fig. 7D), intermediate reticular nucleus (IRt), gigantocellular reticular nucleus (Gi), gigantocellular reticular nucleus alpha part (GiA), lateral paragigantocellular nucleus (LPGi) (Fig. 7H), medullary reticular nucleus ventral part (MdV), medullary reticular nucleus dorsal part (MdD) (Fig. 7I).

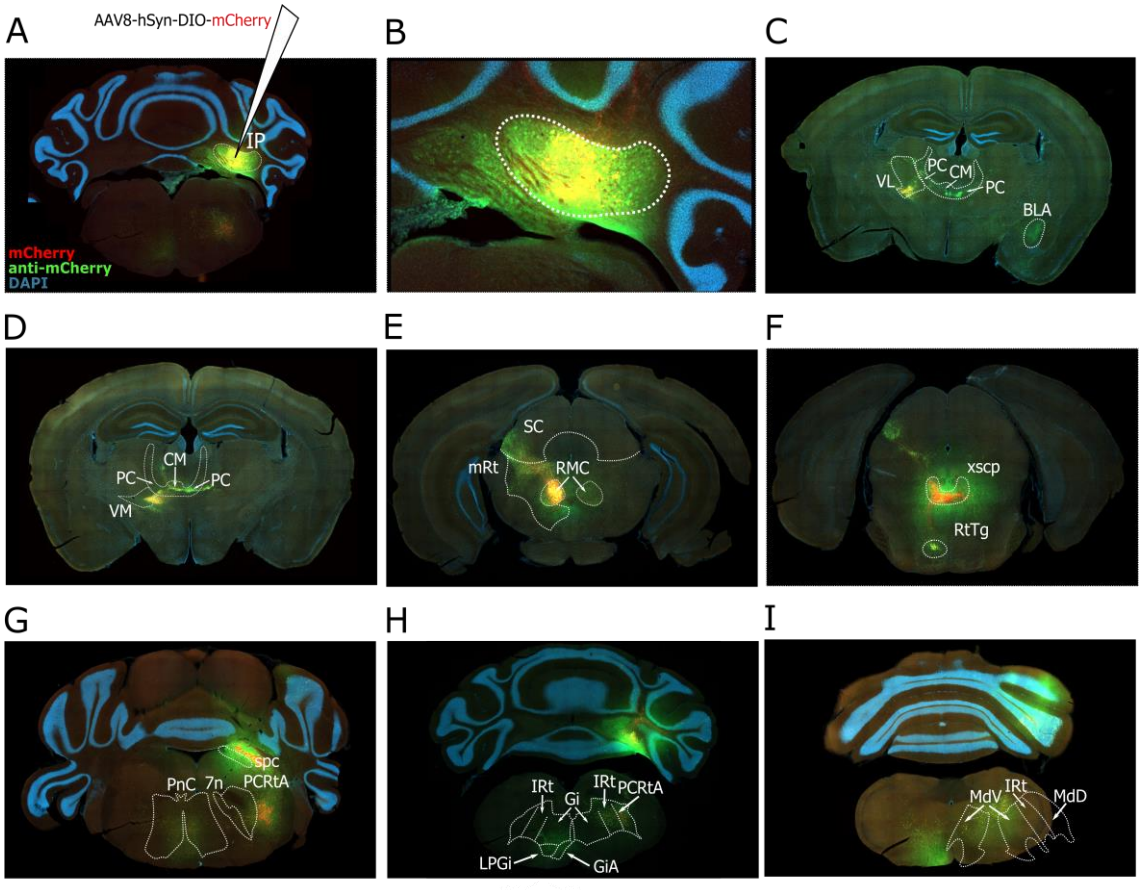

Figure 7 – Interposed-downstream motor structures. A) Example of a cerebellar coronal section with mCherry (red), anti-mCherry (green) and DAPI (blue) staining showing virus spread on injection site location. AAV8-hSyn-DIO-mCherry was injected into the interposed nucleus of vglut2-cre mice for anterograde expression of mCherry in glutamatergic neurons. **B)** Interposed nucleus magnification. **C-E)** Interposed-downstream targets: BLA, VL **(C)**; PC, CM **(C, D)**; VM **(D)**; mRt, SC, RMC **(E)**; xscp, RtTg **(F)**; spc, PnC, 7n **(G)**; PCRtA **(G, H)**; Gi, LPGi, GiA **(H)**; IRt **(H, I)**; MdV, MdD **(I)**. Abreviations used in all figures: IP: interposed nucleus; BLA: basolateral amygdaloid nucleus; VL: ventrolateral thalamic nucleus; CM: central medial thalamic nucleus; PC: paracentral thalamic nucleus; VM: ventromedial thalamic nucleus; mRt: mesencephalic reticular formation; SC: superior colliculus; RMC: magnocellular red nucleus; xscp: decussation of the cerebellar peduncle; RtTg: reticulotegmental nucleus of the pons; spc: superior cerebellar peduncle; PnC: pontine reticular nucleus caudal part; 7n: facial nerve; PCRtA: parvocellular reticular nucleus alpha part; Gi: gigantocellular reticular nucleus; LPGi: lateral paragigantocellular nucleus; GiA: gigantocellular reticular nucleus alpha part; IRt: intermediate reticular nucleus; MdV: medullary reticular nucleus ventral part; MdD: medullary reticular nucleus dorsal part.

3.3. Red nucleus manipulations specifically impair temporal learning

The interposed nucleus projects to many motor-related brain structures. The magnocellular red nucleus is one of the major targets of the interposed, receiving massive contralateral glutamatergic inputs from the previous. Thus, to start identifying the cerebellar outputs involved in spatial and temporal locomotor learning, we started by targeting this structure.

We injected an adeno-associated virus into the left magnocellular red nucleus of wild-type mice for pan-neuronal expression of inhibitory DREADDs (Fig. 8A). Virus expression was observed in the entire nucleus, though in some mice expression was sparse (Fig. 8B). The interposed nucleus projects to the contralateral red nucleus, which then sends contralateral projections to the spinal cord [66]. Thus, we performed virus injections on the contralateral red nucleus relatively to the interposed and then run split-belt adaptation experiments with the fast belt contralateral to the injection site (Fig. 8C).

We then analyzed how inhibition of magnocellular red nucleus neurons affected spatial (Fig. 8D) and temporal (Fig. 8F) components of locomotor adaptation. While spatial adaptation upon administration of CNO was not different from the control (CNO v saline aftereffect: $t_{(5)} = -0.29$, $p = 0.79$) (Fig. 8E), double support symmetry was impaired (CNO v saline aftereffect: $t_{(5)} = 3.58$, $p = 0.02$) (Fig. 8G). The magnocellular red nucleus seems therefore to be specifically involved in temporal adaptation.

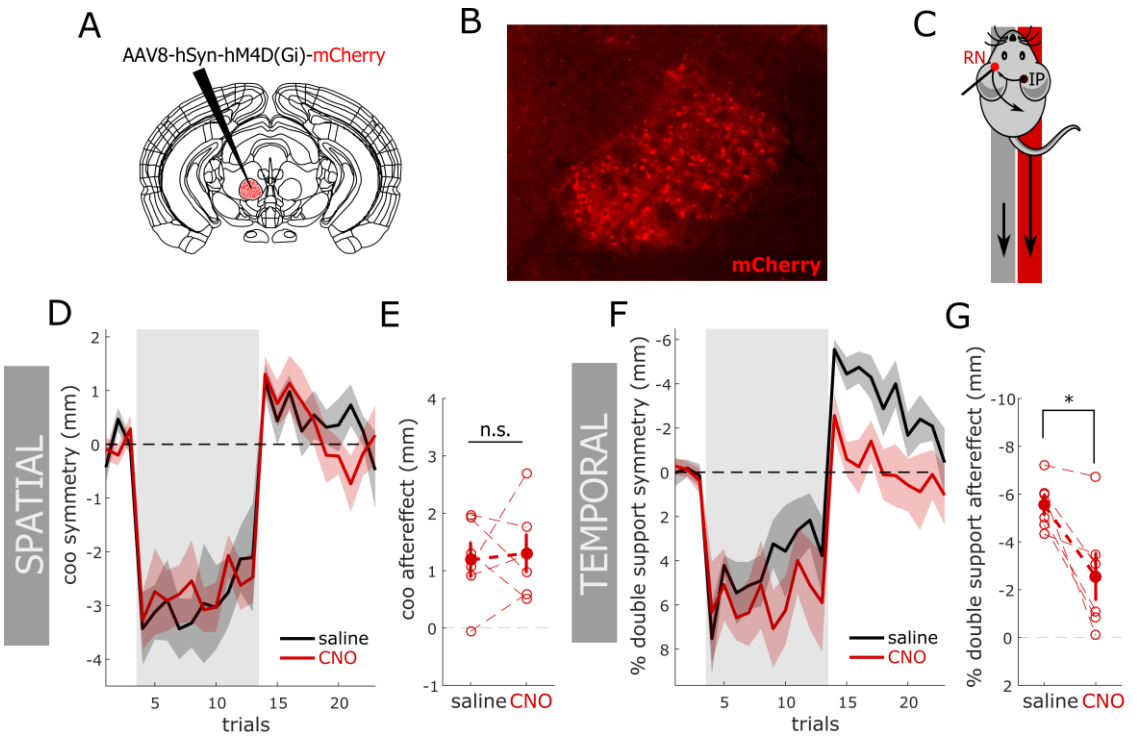

Figure 8 – Temporal adaptation specifically requires the red nucleus. A) Schematic representing virus injection. AAV8-hSyn-hM4D(Gi)-mCherry was injected into the left red nucleus of wild-type mice. **B)** Magnification of coronal section showing the red nucleus. DREADDs expression can be observed through mCherry labelling on the cell bodies of the magnocellular red nucleus. **C)** Schematic representing injection site location (RN) and split-belt adaptation protocol. The interposed nucleus (IP) projects to the contralateral red nucleus (RN) which then projects contralaterally to the spinal cord. Fast belt (red) was run contralateral to the injection site (RN). **D)** Average spatial adaptation (± s.e.m.) curves for red nucleus-injected animals run with saline (black) or CNO (red) (n=6). Grey patches represent split-belt trials. **E)** Average center of oscillation aftereffect size (first post-adaptation trial) for mice injected with saline and CNO. Dashed thin lines show individual animals' aftereffect size. **F)** Average temporal adaptation (± s.e.m.) curves for mice injected in the red nucleus and administered with saline (black) and CNO (red) (n=6). **G)** Average % double support aftereffect size (first trial post-adaptation) for saline and CNO-injected animals. Individual animals' aftereffect size is represented in dashed thin lines.

3.4. Gigantocellular reticular nucleus is necessary for spatial adaptation

The identification of a pre-motor region involved only in temporal adaptation indicates that space and time are dissociable at the circuit level. Consequently, we wanted to find out the interposed downstream circuitry responsible for spatial adaptation. Our previous results suggest that spatial adaptation is influenced bilaterally and, therefore, we sought to target a region that could potentially impact movement on both sides of the body. The reticulospinal tract has been described as projecting bilaterally to the cervical and lumbar spinal cord, so we decide to target inhibitory DREADDs to one of these nuclei, the gigantocellular reticular nucleus [74,78].

For this, we injected a pan-neuronal adeno-associated virus into the left gigantocellular reticular nucleus of wild-type mice (Fig. 9A). The anteroposterior and mediolateral spread of the virus was broad, with some spillover to the dorsal paragigantocellular nucleus (DPGi), intermediate reticular nucleus (IRt) and gigantocellular reticular nucleus alpha part (GiA) (Fig. 9B). The gigantocellular reticular nucleus receives contralateral inputs from the interposed, but sends both ipsi- and contralateral projections to the spinal cord [66]. Since cerebellar control is thought to be predominantly ipsilateral, we started by running split-belt experiments with the fast belt contralateral to the injection site (Fig. 9C).

The impact of gigantocellular reticular nucleus manipulations on spatial (Fig. 9D) and temporal adaptation (Fig. 9F) was then examined. Interestingly, CNO-injected animals showed impairments on spatial learning (CNO v saline aftereffect: $t_{(3)} = 5.24$, $p = 0.01$) (Fig. 9E), while temporal remained intact (CNO v saline aftereffect: $t_{(3)} = -1.29$, $p = 0.28$) (Fig. 9G).

These results demonstrate that spatial and temporal adaptation are mediated through distinct output pathways, identifying specific regions required for each form of locomotor learning.

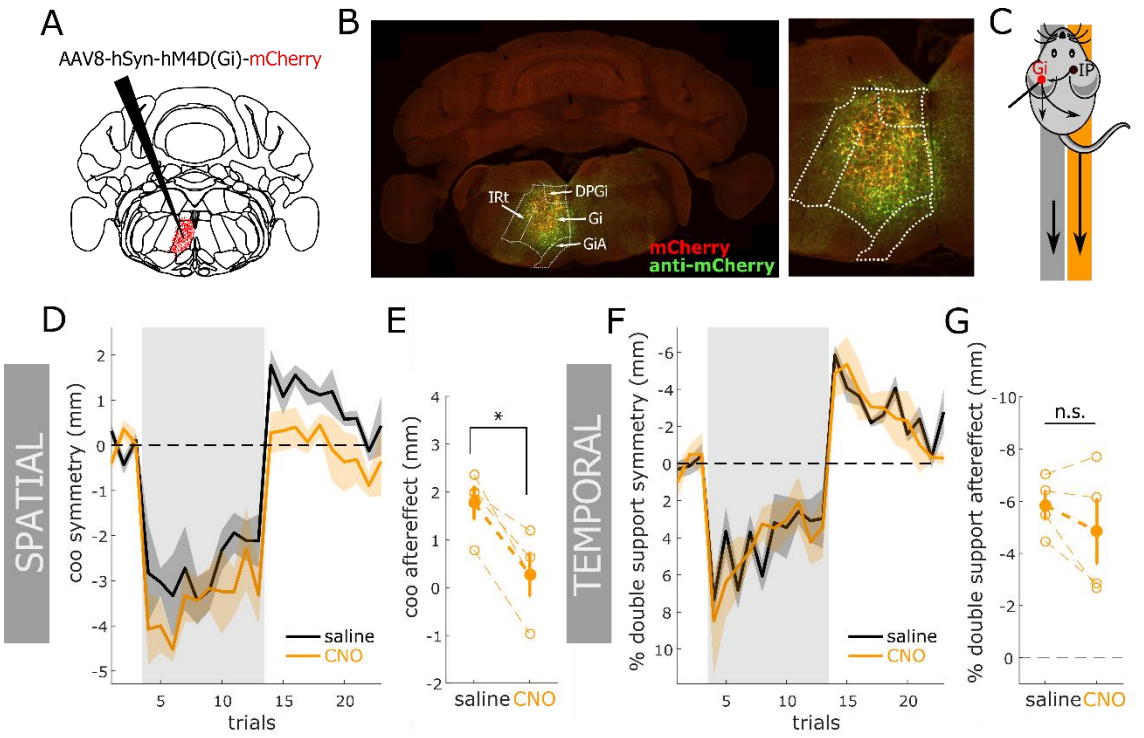

Figure 9 - Gigantocellular reticular nucleus is specifically required for spatial adaptation. A) Schematic representation of virus injection. AAV8-hSyn-hMD4(Gi)-mCherry was injected into the gigantocellular reticular nucleus of wild-type mice. **B)** Left: example of coronal section with mCherry (red) and anti-mCherry (green) showing virus spread on the gigantocellular reticular nucleus. Spillover to the dorsal paragigantocellular nucleus (DPGi), intermediate reticular nucleus (IRt) and gigantocellular reticular nucleus alpha part (GiA) can be observed. Right: Gigantocellular reticular nucleus magnification. **C)** Schematic of injection site location and split-belt adaptation protocol. The interposed nucleus (IP) projects to the contralateral gigantocellular reticular nucleus (Gi) which in turn sends bilateral projections to the spinal cord. Split-belt experiments were performed with the fast belt (orange) contralateral to the injection site. **D)** Average spatial adaptation (± s.e.m.) curves for mice injected in the gigantocellular reticular nucleus and run following administration of saline (black) and CNO (orange) (n=4). Grey patches represent split-belt trials. **E)** Average center of oscillation aftereffect size (first trial post-adaptation) for saline and CNO-injected mice. Individual mice' aftereffect size is represented in thin dashed lines. **F)** Average temporal adaptation (± s.e.m.) curves for gigantocellular reticular nucleus-injected animals after saline (black) and CNO (orange) injection (n=4). **G)** Average % double support aftereffect size (first post-adaptation trial) for animals injected with saline and CNO. Dashed thin lines show individual animals' aftereffect size.

Chapter IV

Discussion

Locomotor adaptation is essential to ensure safe and successful mobility in an environment that is constantly changing. Locomotor adaptation has been extensively studied in humans using a split-belt treadmill that imposes different speeds on opposite sides of the body [120,122]. In this paradigm, individuals need to adapt interlimb coordination overtime to achieve a more symmetric and stable gait. Like in humans, mouse split-belt adaptation is cerebellar-dependent and involves changes in interlimb coordination that are accomplished through adjustments in spatial and temporal kinematic parameters [124,125]. Recently, a sub-region of the cerebellum, the paravermal cortex overlying the interposed nucleus, has been shown to be required for locomotor learning [124].

Here, we show that unilateral manipulations of interposed glutamatergic neurons impair spatial and temporal adaptation with different degrees of lateralization, providing further evidence for what was observed when the activity of the Purkinje cells terminating in this nucleus is perturbed [124]. These results suggest that the neural mechanisms underlying space and time might be different. Moreover, we identify the interposed-downstream motor targets and found distinct regions specifically involved in either spatial or temporal learning. Spatial adaptation was impaired when gigantocellular reticular nucleus neurons were inhibited, while temporal was only affected by red nucleus manipulations. Thus, we demonstrate for the first time that space and time are independently processed by cerebellar downstream pathways.

4.1. Separability of spatial and temporal learning

Locomotor adaptation on a split-belt treadmill specifically involves spatiotemporal changes in interlimb coordination. Many lines of evidence suggest that spatial and temporal locomotor adaptation are independent processes that work in parallel to achieve coordinated locomotion and that might be dissociable at the circuit level [120,122,124,125].

In humans, spatial and temporal adaptation have been shown to have different development onsets and adaptation rates, with temporal parameters adapting faster than spatial [122,125,126]. Furthermore, subjects can consciously prevent spatial adaptation without affecting temporal, demonstrating that space and time can be separately adapted [125]. Similarly, mouse split-belt adaptation has spatial and temporal components that adapt at different rates. Moreover, analysis of individual limb contributions to mouse locomotor

adaptation showed that spatial adaptation was comprised of changes across all limbs whereas temporal seems to involve only the fast front limb [124].

To investigate whether the neural mechanisms underlying spatial and temporal adaptation are independent, a previous study performed unilateral manipulations of the Purkinje cells overlying the interposed nucleus and run split-belt protocols with the fast belt ipsilateral or contralateral to the injection site so that potential differences in lateralization could be identified. The authors observed that while temporal learning was only affected on the ipsi-fast condition, spatial adaptation was impaired regardless of the belt-speed condition [124]. Likewise, when we manipulated the interposed nucleus directly by targeting its glutamatergic projection neurons, the same differences in the lateralization of spatial and temporal parameters were observed. Interestingly, individual limb contributions to space and time are in agreement with the differential lateralization observed with unilateral manipulations of the interposed nucleus: while spatial adaptation involves changes across multiple limbs and is affected by the interposed bilaterally, temporal adaptation seems to require only the fast front limb and is impaired only when interposed manipulations are ipsilateral to the fast treadmill belt.

Taken together, these results further indicate that the neural mechanisms underlying spatial and temporal adaptation might be distinct.

4.2. Red nucleus and temporal learning

The demonstration that the interposed nucleus, but not the medial or lateral nucleus, is necessary for split-belt adaptation narrows down the required downstream circuitry for locomotor adaptation [124]. Our anatomical tracing revealed interposed glutamatergic projections to several regions known to influence locomotion like motor cortex, midbrain and brainstem, with the most prominent projections observed in the magnocellular red nucleus.

The red nucleus has been extensively described as required for skilled forelimb movements, with unilateral lesions affecting reaching on the contralateral limb by causing dysmetria and impairments in grouped digit extension and distal muscles movements [65,127–129]. Moreover, this structure has also been shown to play an important role in locomotion. Lesion studies suggest that the red nucleus is not necessary for movement

initiation but that it plays a role on ongoing locomotion, with bilateral lesions impairing gait coordination. Thus, it has been hypothesized that the red nucleus might be important during conditions that require correction and adaptation of ongoing movements [67,68].

We manipulated red nucleus neurons activity and showed that this nucleus is required for temporal adaptation alone, thereby showing that space and time are separable at the circuit level. We have previously demonstrated that interposed nucleus manipulations affect temporal adaptation only on the ipsi-fast condition. Interestingly, the red nucleus controls the same body side as the interposed, since it receives contralateral inputs from the previous and then sends contralateral projections to the spinal cord [66,130]. Furthermore, the magnocellular red nucleus targets predominantly cervical spinal segments, thus exerting control mainly in forelimb motor neurons [65,131]. Thus, both interposed and red nucleus might be involved in temporal learning by controlling the timing of the fast forelimb. Nonetheless, it would be important to perform split-belt experiments with the fast treadmill belt ipsilateral to the injection site to confirm that the red nucleus only affects temporal adaptation on the contra-fast condition. Our anatomical tracing of interposed-downstream targets revealed a few interposed excitatory projections to the ipsilateral magnocellular red nucleus. It is the first time that ipsilateral projections from the interposed to the red nucleus are reported and it would be important to determine whether they also play a role in split-belt learning. However, this scenario would be unlikely since we show that the red nucleus is specifically involved in temporal adaptation. So, its influence on locomotor adaptation should be equal to what is observed on temporal learning after interposed nucleus manipulations.

The neural mechanisms by which the red nucleus influences temporal adaptation remain to be further elucidated. The magnocellular red nucleus does not only receive inputs from the interposed, but it also sends collaterals back to the deep nuclei, creating a feedback loop between the two structures. The red nucleus contacts multiple cell types within the interposed, thereby being in position to exert influence on interposed neuronal activity by modulating the integration of Purkinje cells inputs or by acting on the nucleo-olivary pathway. Moreover, red nucleus projections to the interposed are collaterals of rubrospinal axons that project to the cervical spinal cord, suggesting that this pathway copies pre-motor information necessary for the control of forelimb movements [130]. This raises the question of whether the red nucleus serves only as a relay between motor commands and the motor neurons responsible for its execution or if it instructs learning

via its projections to the cerebellum. In eyelid conditioning, the red nucleus has been shown to be involved in expression, but not acquisition, of the learned response [114,115]. But would this function be conserved across distinct forms of cerebellar-dependent learning like locomotor adaptation?

4.3. Gigantocellular reticular nucleus and spatial learning

After identifying a specific role for the red nucleus in temporal adaptation, we sought to find out whether there was a specific region required solely for spatial adaptation. Spatial adaptation has been previously shown to involve changes across all limbs [124]. Because of this, we sought to manipulate an interposed target that could potentially impact the spinal circuits involved in multi-limb control. The reticulospinal tract projects bilaterally to both cervical and lumbar spinal segments, thus being implied in coordination of movements that involve multi-joint and multi-limb motor commands [74]. This tract is comprised of multiple nuclei that usually exhibit biases for either fore- or hindlimb-innervating spinal motor neurons. Gigantocellular reticular nucleus neurons have been reported as projecting bilaterally to both fore- and hindlimb motor neurons with similar ratios of distribution [78]. Thus, motor commands from the gigantocellular reticular nucleus can potentially elicit changes across all four limbs, like what is observed on spatial adaptation [124]. Consequently, we perturbed the activity of the gigantocellular reticular nucleus and assessed its importance to locomotor adaptation. We found that gigantocellular reticular neurons were specifically required for spatial, but not temporal adaptation.

There are few studies addressing the role of the gigantocellular reticular nucleus on locomotion with diverge functioning being reported, including bilateral forelimb stepping elicited by electric stimulation and halting of locomotion induced by optogenetic stimulation of V2a glutamatergic spinal projecting neurons [76,132]. Thus, how gigantocellular reticular nucleus neurons impact locomotion remains largely unknown. Our results demonstrate a specific role for this nucleus in locomotor learning by showing its involvement in spatial adaptation. Furthermore, it would also be interesting to examine whether gigantocellular reticular nucleus neurons project to the interposed nucleus. If so,

the gigantocellular reticular nucleus, similarly to the red nucleus, could be in position to provide key information to instruct learning within the cerebellum.

The demonstration that unilateral manipulations of the interposed nucleus affect spatial adaptation independently of whether the fast belt is set ipsi- or contralateral to the injection site and that the interposed motor output involved in this form of learning has the potential to influence movement across all limbs suggests that the cerebellum might be involved in bilateral control of movement. This challenges the view of cerebellar control as ipsilateral, though there are some studies that have implied the cerebellum in bilateral control [133,134]. In fact, interlimb coordination involves comparison of movement between multiple limbs, so how would the cerebellum integrate information between different limbs and then translate it into coordinated locomotion without exerting some bilateral influence?

With this work, we have identified specific motor nuclei located in distinct spinal descending pathways required for either spatial or temporal learning: while the magnocellular red nucleus, which gives rise to the rubrospinal tract, is necessary for temporal adaptation, the gigantocellular reticular nucleus, which belongs to the reticulospinal tract, is involved in spatial adaptation. These results show that for locomotor learning space and time are processed by distinct neural circuits.

Chapter V

Conclusion and Future Perspectives

The present study was the first to demonstrate that spatial and temporal locomotor adaptation are independently processed by cerebellar output pathways. We show that the interposed nucleus contributes to spatial and temporal learning differently and identified pre-motor regions involved in either space (gigantocellular reticular nucleus) or time (red nucleus).

We show that unilateral manipulations of the interposed impact spatial adaptation regardless of the side that the fast treadmill belt is set, whereas temporal adaptation is only affected when the fast belt is run ipsilateral to the injection site. Accordingly, spatial learning involves the gigantocellular reticular nucleus which can potentially exert bilateral control on limb movements, while temporal learning requires the red nucleus, which seems to influence only one side of the body. In the previous experiments targeting the pre-motor projection nuclei of the interposed, split-belt adaptation protocols were performed only in one belt-speed condition. Thus, to confirm that gigantocellular reticular nucleus manipulations affect spatial learning regardless of the belt-speed condition and the red nucleus affects temporal only on the contra-fast, we plan to perform the same unilateral manipulations but set the belts at opposite speeds. Moreover, we are going to manipulate more interposed-downstream targets and assess whether they are involved in locomotor adaptation.

The demonstration that spatial and temporal adaptation require the interposed nucleus but are distinctly processed in its outputs raises the question of how this information is processed within the cerebellum. To start addressing this, we intent to perform a retrograde tracing from the red nucleus and gigantocellular reticular nucleus to examine whether they are distinct neural populations within the interposed. If that is the case, this would indicate that the deep nuclei processes space and time independently, but if the neural population that gives rise to red nucleus and gigantocellular reticular nucleus is the same, how does the cerebellum integrate and processes both information to convey it to distinct outputs so that different motor commands can be executed?

In case the neural populations in the interposed that give rise to red nucleus and gigantocellular reticular nucleus-projecting neurons are discrete, we intend to specifically manipulate the interposed-projecting neurons to these downstream targets to confirm that these projections are indeed responsible for either space or time.

Furthermore, if these neural populations are independent, we aim to use a combination of viral approaches to trace the inputs to red nucleus or gigantocellular

reticular nucleus-projecting interposed neurons. This experiment would allow us to map the cerebellar circuitry specifically involved in spatial and temporal adaptation, namely the Purkinje cells, mossy fibers and climbing fibers inputs. Interestingly, this would indicate whether the information required for spatial and temporal adaptation is processed independently by the cerebellar cortex or if it only diverges in the interposed nucleus and whether the information conveyed by mossy and climbing fibers is carried by distinct neural populations.

References

1. Grigoriĭ Nikolaevich Orlovskiĭ, T. G. Deliagina, S. G. *Neuronal Control of Locomotion: From Mollusc to Man*. (Oxford University Press, 1999).
2. Arber, S. & Costa, R. M. Connecting neuronal circuits for movement. *Science (80-.).* **360,** 1403–1404 (2018).
3. Kiehn, O. Decoding the organization of spinal circuits that control locomotion. *Nat. Rev. Neurosci.* **17,** 224–238 (2016).
4. Dubuc, R. *et al.* Initiation of locomotion in lampreys. *Brain Res. Rev.* **57,** 172–182 (2008).
5. Caggiano, V. *et al.* Midbrain circuits that set locomotor speed and gait selection. *Nature* **553,** 455–460 (2018).
6. Garcia-Rill, E., Hyde, J., Kezunovic, N., Urbano, F. J. & Petersen, E. The physiology of the pedunculopontine nucleus: implications for deep brain stimulation. *J. Neural Transm.* **122,** 225–235 (2014).
7. Grillner, S. & Robertson, B. The basal ganglia downstream control of brainstem motor centres-an evolutionarily conserved strategy. *Curr. Opin. Neurobiol.* **33,** 47–52 (2015).
8. Garcia-Rill, E. The basal ganglia and the locomotor regions. *Brain Res. Rev.* **11,** 47–63 (1986).
9. Takakusaki, K. Neurophysiology of gait: From the spinal cord to the frontal lobe. *Mov. Disord.* **28,** 1483–1491 (2013).
10. Drew, T. & Marigold, D. S. Taking the next step: Cortical contributions to the control of locomotion. *Curr. Opin. Neurobiol.* **33,** 25–33 (2015).
11. MacKay-Lyons, M. Central pattern generation of locomotion: a review of the evidence. *Phys. Ther.* **82,** 69–83 (2002).
12. Mauk, M. D., Medina, J. F., Nores, W. L. & Ohyama, T. Cerebellar function: Coordination, learning or timing? *Curr. Biol.* **10,** 522–525 (2000).
13. Morton, S. M. . & Bastian, A. J. Cerebellar Control of Balance and Locomotion. *Neurosci.* **10,** 247–259 (2004).
14. Bastian, A. J. Understanding sensorimotor adaptation and learning for rehabilitation. *Curr. Opin. Neurol.* **21,** 628–633 (2008).
15. Marsden, J. & Harris, C. Cerebellar ataxia: pathophysiology and rehabilitation. *Clin. Rehabil.* **25,** 195–216 (2011).
16. Bastian, a J., Martin, T. a, Keating, J. G. & Thach, W. T. Cerebellar ataxia: abnormal control of interaction torques across multiple joints. *J. Neurophysiol.* **76,** 492–509 (1996).
17. Roostaei, T., Nazeri, A., Sahraian, M. A. & Minagar, A. The human cerebellum: A review of physiologic neuroanatomy. *Neurol. Clin.* **32,** 859–869 (2014).
18. Lisberger, Stephen G.; Thach, T. W. The Cerebellum. in *Principles of Neural Science* 37–41 (2012). doi:10.1080/14734220510007914
19. Jansen, J. & Brodal, A. Experimental studies on the intrinsic fibers of the cerebellum. II. The

cortico-nuclear projection. *J. Comp. Neurol.* **73,** 267–321 (1940).

20. Voogd, J. & Glickstein, M. The anatomy of the cerebellum. *Trends Cogn. Sci.* **2,** 307–313 (1998).
21. Precht, W., Volkind, R. & Blanks, R. H. I. Functional organization of the vestibular input to the anterior and posterior cerebellar vermis of cat. *Exp. Brain Res.* **27,** 143–160 (1977).
22. Snider, Ray S.; Stowell, A. Receiving areas of the tactile, auditory and visual systems in the cerebellum. 331–357 (1942).
23. Kandel, E. R., Schwartz, J. H. & Jessell, T. M. *Principles of Neural Science*. (2000).
24. Coffman, K. A., Dum, R. P. & Strick, P. L. Cerebellar vermis is a target of projections from the motor areas in the cerebral cortex. *Proc. Natl. Acad. Sci.* **108,** 16068–16073 (2011).
25. Barlow, J. S. *The Cerebellum and Adaptive Control*. (2002). doi:10.1017/CBO9780511529771
26. Asanuma, C., Thach, W. T. & Jones, E. G. Distribution of cerebellar terminations and their relation to other afferent terminations in the ventral lateral thalamic region of the monkey. *Brain Res. Rev.* **5,** 237–265 (1983).
27. Asanuma, C., Thach, W. T. & Jones, E. G. Anatomical evidence for segregated focal groupings of efferent cells and their terminal ramifications in the cerebellothalamic pathway of the monkey. *Brain Res. Rev.* **5,** 267–297 (1983).
28. Chambers, William W.; Sprague, J. Functional localization in the cerebellum. I. Organization in longitudinal cortico-nuclear zones and their contribution to the control of posture, both extrapyramidal and pyramidal. 105–129 (1955).
29. Chambers, William W.; Sprague, J. M. Functional localization in the cerebellum. II Somatic Organization in Cortex and Nuclei. *Cortex* **47,** 59–80 (1955).
30. Thach, W. The Cerebellum And The Adaptive Coordination Of Movement. *Annu. Rev. Neurosci.* **15,** 403–442 (1992).
31. Milak, M. S. *et al.* Effects of inactivating individual cerebellar nuclei on the performance and retention of an operantly conditioned forelimb movement. *J. Neurophysiol.* **78,** 939–59 (1997).
32. Martin, J. H., Cooper, S. E., Hacking, A. & Ghez, C. Differential effects of deep cerebellar nuclei inactivation on reaching and adaptive control. *J. Neurophysiol.* **83,** 1886–1899 (2000).
33. Sprague, J. M. & Chambers, W. W. Regulation of posture in intact and decerebrate cat: I. Cerebellum, reticular formation, vestibular nuclei. *J Neurophysiol* **16,** 451–463 (1953).
34. Yu, J. & Eidelberg, E. Recovery of locomotor function in cats after localized cerebellar lesions. *Brain Res.* **273,** 121–131 (1983).
35. Wankhar, W. & Rathinasamy, S. Unilateral lesion of Fastigal Nucleus in Wistar Albino Rats and its effect on motor coordination - a preliminary study. *J. Behav. Heal.* **4,** 122 (2015).
36. Ilg, W., Giese, M. A., Gizewski, E. R., Schoch, B. & Timmann, D. The influence of focal cerebellar lesions on the control and adaptation of gait. *Brain* **131,** 2913–2927 (2008).
37. Brodal, P. & Bjaalie, J. G. Salient anatomic features of the cortico-ponto-cerebellar pathway. *Prog. Brain Res.* **114,** 227–249 (1997).
38. Clendenin, M., Ekerot, C. F., Oscarsson, O. & Rosén, I. Functional organization of two

spinocerebellar paths relayed through the lateral reticular nucleus in the cat. *Brain Res.* **69,** 140–143 (1974).

39. Clendenin, M., Ekerot, C. F., Oscarsson, O. & Rosén, I. The lateral reticular nucleus in the cat I. Mossy fibre distribution in cerebellar cortex. *Exp. Brain Res.* **21,** 473–486 (1974).
40. Matsushita, M. & Okauo, N. D. N. Spinocerebellar Projections to Lobules I and II of the Anterior Lobe in the Cat , as Studied by Retrograde Transport of Horseradish Peroxidase. *J. Comp. Neurol.* **208,** 411–424 (1981).
41. Grant, G. Spinal course and somatotopically localized termination of the spinocerebellar tracts: an experimental study in the cat. *Acta Physiol Scand Suppl.* **56,** 1–61 (1962).
42. Udo, M., Matsukawa, K., Kamei, H. & Oda, Y. Cerebellar control of locomotion: effects of cooling cerebellar intermediate cortex in high decerebrate and awake walking cats. *J. Neurophysiol.* **44,** (1980).
43. Perciavalle, V. *et al.* Consensus paper: Current views on the role of cerebellar interpositus nucleus in movement control and emotion. *Cerebellum* **12,** 738–757 (2013).
44. Udo, M., Matsukawa, K. & Kamei, H. Effects of partial cooling of cerebellar cortex at lobules V and IV of the intermediate part in the decerebrate walking cats under monitoring vertical floor reaction forces. *Brain Res.* **160,** 559–564 (1979).
45. Armstrong, D. M. & Edgley, S. A. Discharges of interpositus and Purkinje cells of the cat cerebellum during locomotion under different conditions. *J. Physiol.* **400,** 425–445 (1988).
46. Schwartz, A. B., Ebner, T. J. & Bloedel, J. R. Responses of interposed and dentate neurons to perturbations of the locomotor cycle. *Exp. Brain Res.* **67,** 323–338 (1987).
47. Brodal, P. & Bjaalie, J. G. Organization of the pontine nuclei. *Neurosci. Res.* **13,** 83–118 (1992).
48. Middleton, F. A. & Strick, P. L. Cerebellar output channels. *Int. Rev. Neurobiol.* **41,** 61–82 (1997).
49. Asanuma, C., Thach, W. T. & Jones, E. G. Brainstem and spinal projections of the deep cerebellar nuclei in the monkey, with observations on the brainstem projections of the dorsal column nuclei. *Brain Res. Rev.* **5,** 299–322 (1983).
50. Dum, R. P. An Unfolded Map of the Cerebellar Dentate Nucleus and its Projections to the Cerebral Cortex. *J. Neurophysiol.* **89,** 634–639 (2002).
51. Orioli, P. J. & Strick, P. L. Cerebellar connections with the motor cortex and the arcuate premotor area: An analysis employing retrograde transneuronal transport of WGA-HRP. *J. Comp. Neurol.* **288,** 612–626 (1989).
52. Marple-Horvat, D. E. & Criado, J. M. Rhythmic neuronal activity in the lateral cerebellum of the cat during visually guided stepping. *J. Physiol.* **518,** 595–603 (1999).
53. Marple-Horvat, D. E., Criado, J. M. & Armstrong, D. M. Neuronal activity in the lateral cerebellum of the cat related to visual stimuli at rest, visually guided step modification, and saccadic eye movements. *J. Physiol.* **506,** 489–514 (1998).
54. Barthélemy, D., Grey, M. J., Nielsen, J. B. & Bouyer, L. Involvement of the corticospinal tract in the control of human gait. *Prog. Brain Res.* **192,** 181–197 (2011).

55. Martin, G. F., Megirian, D. & Conner, J. B. The origin, course and termination of the corticospinal tracts of the Tasmanian potoroo (Potorous apicalis). *J. Anat.* **111,** 263–281 (1972).

56. Nudo, R. J., Nudo, R. J., Masterton, R. B. & Masterton, R. B. Descending Pathways to the Spinal Cord, III: Sites of Origin of the Corticospinal Tract. *Names* **597,** 584–597 (1990).

57. Drew, T., Andujar, J. E., Lajoie, K. & Yakovenko, S. Cortical mechanisms involved in visuomotor coordination during precision walking. *Brain Res. Rev.* **57,** 199–211 (2008).

58. Drew, T., Jiang, W., Kably, B. & Lavoie, S. Role of the motor cortex in the control of visually triggered gait modifications. *Can. J. Physiol. Pharmacol.* **74,** 426–442 (1996).

59. Widajewicz, W., Kably, B. & Drew, T. Motor cortical activity during voluntary gait modifications in the cat. II. Cells related to the hindlimbs. *J. Neurophysiol.* **72,** 2070–2089 (1994).

60. Beloozerova, I. N. & Sirota, M. G. The role of the motor cortex in the control of vigour of locomotor movements in the cat. *J. Physiol.* **461,** 27–46 (1993).

61. Amos, A., Armstrong, D. M. & Marple-Horvat, D. E. Changes in the discharge patterns of motor cortical neurones associated with volitional changes in stepping in the cat. *Neurosci. Lett.* **109,** 107–112 (1990).

62. Friel, K. M., Drew, T. & Martin, J. H. Differential Activity-Dependent Development of Corticospinal Control of Movement and Final Limb Position During Visually Guided Locomotion. *J. Neurophysiol.* **97,** 3396–3406 (2007).

63. Drew, T., Jiang, W. & Widajewicz, W. Contributions of the motor cortex to the control of the hindlimbs during locomotion in the cat. *Brain Res. Rev.* **40,** 178–191 (2002).

64. Lajoie, K., Andujar, J. E., Pearson, K. & Drew, T. Neurons in Area 5 of the Posterior Parietal Cortex in the Cat Contribute to Interlimb Coordination During Visually Guided Locomotion: A Role in Working Memory. *J. Neurophysiol.* **103,** 2234–2254 (2010).

65. Miller, L. E. & Gibson, A. The Red Nucleus. *New Encycl. Neurosci.* **6,** 55–62 (2009).

66. Liang, H., Paxinos, G. & Watson, C. Projections from the brain to the spinal cord in the mouse. *Brain Struct. Funct.* **215,** 159–186 (2011).

67. Armstrong, D. M. Supraspinal contributions to the initiation and control of locomotion in the cat. *Prog. Neurobiol.* **26,** 273–361 (1986).

68. Muir, G. D. & Whishaw, I. Q. Red nucleus lesions impair overground locomotion in rats: A kinetic analysis. *Eur. J. Neurosci.* **12,** 1113–1122 (2000).

69. Evans, Byron H.; Ingram, W. R. The effects of combined red nucleus and pyramidal lesions in cats. *J. Comp. Neurol.* **70,** 461–476 (1939).

70. Ingram, W. R. & Ranson, S. W. Effects of lesions in the red nuclei in cats. *Arch Neurol Psychiat* **28,** 483–512 (1932).

71. Webb, A. A. & Muir, G. D. Unilateral dorsal column and rubrospinal tract injuries affect overground locomotion in the unrestrained rat. *Eur. J. Neurosci.* **18,** 412–422 (2003).

72. Orlovsky, G. N. Activity of rubrospinal neurons during locomotion. *Brain Res.* **46,** 99–112 (1972).

73. Lavoie, S. & Drew, T. Discharge characteristics of neurons in the red nucleus during voluntary gait

modifications: A comparison with the motor cortex. *J. Neurophysiol.* **88,** 1791–1814 (2002).

74. Brownstone, R. M. & Chopek, J. W. Reticulospinal Systems for Tuning Motor Commands. *Front. Neural Circuits* **12,** 1–10 (2018).

75. Capelli, P., Pivetta, C., Esposito, M. S. & Arber, S. Locomotor speed control circuits in the caudal brainstem. *Nature* **551,** 373–377 (2017).

76. Bouvier, J. *et al.* Descending Command Neurons in the Brainstem that Halt Locomotion. *Cell* **163,** 1191–1203 (2015).

77. Peterson, B. W., Maunz, R. A., Pitts, N. G. & Mackel, R. G. Patterns of projection and branching of reticulospinal neurons. *Exp. Brain Res.* **23,** 333–351 (1975).

78. Esposito, M. S., Capelli, P. & Arber, S. Brainstem nucleus MdV mediates skilled forelimb motor tasks. *Nature* **508,** 351–356 (2014).

79. Ito, M. Neural design of the cerebellar motor control system. *Brain Res.* **40,** 81–84 (1972).

80. Marr, D. A Theory of Cerebellar Cortex. *J. Physiol* **202,** 437–470 (1969).

81. Albus, J. S. A theory of cerebellar function. *Math. Biosci.* **10,** 25–61 (1971).

82. du Lac, S., Raymondl, J. L., Sejnowski, T. J. & Lisberger, S. G. Learning and memory in the vestibulo-ocular reflex. *Annu. Rev. Neurosci.* **409,** (1995).

83. Thompson, R. F. & Krupa, D. J. Organization of memory traces in the mammalian brain. *Annu. Rev. Neurosci.* **17,** 519–49 (1994).

84. Maekawa, K. & Kimura, M. Mossy fiber projections to the cerebellar flocculus from the extraocular muscle afferents. *Brain Res.* **191,** 313–325 (1980).

85. Stone, L. S. & Lisberger, S. G. Visual responses of Purkinje cells in the cerebellar flocculus during smooth-pursuit eye movements in monkeys. II. Complex spikes. *J. Neurophysiol.* **63,** 1262–1275 (1990).

86. Carey, M. R. Synaptic mechanisms of sensorimotor learning in the cerebellum. *Curr. Opin. Neurobiol.* **21,** 609–615 (2011).

87. Thach, W. T. Discharge of Purkinje and cerebellar nuclear neurons during rapidly alternating arm movements in the monkey. *J. Neurophysiol.* **31,** (1968).

88. Schmolesky, M. T., Weber, J. T., De Zeeuw, C. I. & Hansel, C. The making of a complex spike: Ionic composition and plasticity. *Ann. N. Y. Acad. Sci.* **978,** 359–390 (2002).

89. Jirenhed, D. A. & Hesslow, G. Are Purkinje Cell Pauses Drivers of Classically Conditioned Blink Responses? *Cerebellum* **15,** 526–534 (2016).

90. Freeman, J. H. Cerebellar learning mechanisms. *Brain Res.* **1621,** 260–269 (2015).

91. Robinson, D. A. Adaptive gain control of vestibuloocular reflex by the cerebellum. *J. Neurophysiol.* **39,** (1976).

92. Cohen, M. R., Meissner, G. W., Schafer, R. J. & Raymond, J. L. Reversal of motor learning in the vestibulo-ocular reflex in the absence of visual input. *Learn. Mem.* **11,** 559–565 (2004).

93. Ito, Masao; Shiida, Takashi; Yagi, Nobuya; Yamamoto, M. The Cerebellar Modification of Rabbit's Horizontal Vestibulo-Ocular Reflex Induced by Sustained Head Rotation Combined with

Visual Stimulation. *Proc. Japan Acad.* **50,** 85–89 (1974).

94. Miles, F. A. & Fuller, J. H. Adaptive plasticity in the vestibulo-ocular responses of the rhesus monkey. *Brain Res.* **80,** 512–516 (1974).

95. Gonshor, A. & Jones, G. M. Extreme vestibulo-ocular adaptation induced by prolonged optical reversal of vision. *J. Physiol.* **256,** 381–414 (1976).

96. Raymond, J., Lisberger, S. & Mauk, M. The Cerebellum: A Neuronal Learning Machine? *Sci. Ser.* **272,** 1126–1131 (1996).

97. McCormick, D. A. & Thompson, R. F. Cerebellum: Essential Involvement in the Classically Conditioned Eyelid Response. *Science (80-.).* **223,** 296–299 (1983).

98. Welsh, J. & Harvey, J. Cerebellar lesions and the nictitating membrane reflex: performance deficits of the conditioned and unconditioned response. *J. Neurosci.* **9,** 299–311 (1989).

99. Yeo, C., Hardiman, M. & Glickstein, M. Classical conditioning of the nictitating membrane response of the rabbit: I. Lesions of the cerebellar nuclei. *Exp. brain Res.* **60,** 87–98 (1985).

100. Daum, I., Schugens, M. M., Ackermann, H., Lutzenberger, W. & et al. Classical conditioning after cerebellar lesions in humans. *Behav. Neurosci.* **107,** 748–756 (1993).

101. Lisberger, S. G., Miles, F. A. & Zee, D. S. Signals used to compute errors in monkey vestibuloocular reflex: possible role of flocculus. *J. Neurophysiol.* **52,** (1984).

102. Garcia, K. S., Steele, P. M. & Mauk, M. D. Cerebellar Cortex Lesions Prevent Acquisition of Conditioned Eyelid Responses. *J. Neurosci.* **19,** 10940–10947 (1999).

103. Stephen G . Lisberger and Terri A . Pavelko. Brain Stem Neurons in Modified Pathways for Motor Learning in the Primate Vestibulo-Ocular Reflex. *Science (80-.).* **242,** 771–773 (1988).

104. Nagao, S. Effects of vestibulocerebellar lesions upon dynamic characteristics and adaptation of vestibulo-ocular and optokinetic responses in pigmented rabbits. *Exp. Brain Res.* **53,** 36–46 (1983).

105. Perrett, S. P. & Mauk, M. D. Extinction of Conditioned Eyelid Responses Requires the Anterior Lobe of Cerebellar Cortex. *Struct. Large-Scale Synchronized Firing Primate Retin.* **15,** 2074–2080 (1995).

106. Perrett, S. P., Ruiz, B. P. & Mauk, M. D. Cerebellar cortex lesions disrupt learning-dependent timing of conditioned eyelid responses. *J. Neurosci.* **13,** 1708–1718 (1993).

107. McCormick, D. A. & Thompson, R. F. Neuronal responses of the rabbit cerebellum during acquisition and performance of a classically conditioned nictitating membrane-eyelid response. *J. Neurosci.* **4,** 2811–2822 (1984).

108. Alley, K., Baker, R. & Simpson, J. I. Afferents to the vestibulo-cerebellum and the origin of the visual climbing fibers in the rabbit. *Brain Res.* **98,** 582–589 (1975).

109. Sears, L. L. & Steinmetz, J. E. Dorsal accessory inferior olive activity diminishes during acquisition of the rabbit classically conditioned eyelid response. *Brain Res.* **545,** 114–122 (1991).

110. Steinmetz, J. E., Lavond, D. G. & Thompson, R. F. Classical conditioning in rabbits using pontine nucleus stimulation as a conditioned stimulus and inferior olive stimulation as an unconditioned stimulus. *Synapse* **3,** 225–233 (1989).

111. Berthier, N. E. & Moore, J. W. Cerebellar Purkinje cell activity related to the classically conditioned nictitating membrane response. *Exp. Brain Res.* **63,** 341–350 (1986).

112. Mauk, M. D., Steinmetz, J. E. & Thompson, R. F. Classical conditioning using stimulation of the inferior olive as the unconditioned stimulus. *Proc. Natl. Acad. Sci. U. S. A.* **83,** 5349–5353 (1986).

113. Welsh, B. Y. J. P. & Harveyt, J. A. Pavlovian Conditioning in the Rabbit During Inactivation of the Interpositus Nucleus. *J. Ph* 459–480 (1991).

114. Krupa, D. J., Thompson, J. K. & Thompson, R. F. Localization of a memory trace in the mammalian brain. *Science (80-.).* **260,** 989 LP-991 (1993).

115. Chapman, P. F., Steinmetz, J. E., Sears, L. L. & Thompson, R. F. Effects of lidocaine injection in the interpositus nucleus and red nucleus on conditioned behavioral and neuronal responses. *Brain Res.* **537,** 149–156 (1990).

116. Uemura, T. & Cohen, B. Effects of vestibular nuclei lesions on vestibulo-ocular reflexes and posture in monkeys. *Acta Otolaryngol.* **315,** 56–71 (1973).

117. Luebke, A. E. & Robinson, D. A. Gain changes of the cat's vestibulo-ocular reflex after flocculus deactivation. *Exp. Brain Res.* **98,** 379–390 (1994).

118. Pastor, A. M., de la Cruz, R. R. & Baker, R. Cerebellar role in adaptation of the goldfish vestibuloocular reflex. *J. Neurophysiol.* **72,** 1383 LP-1394 (1994).

119. Partsalis, A. M., Zhang, Y. & Highstein, S. M. Dorsal Y group in the squirrel monkey. II. Contribution of the cerebellar flocculus to neuronal responses in normal and adapted animals. *J. Neurophysiol.* **73,** 632 LP-650 (1995).

120. Morton, S. M. & Bastian, A. J. Cerebellar Contributions to Locomotor Adaptations during Splitbelt Treadmill Walking. *J. Neurosci.* **26,** 9107–9116 (2006).

121. Prokop, T., Berger, W., Zijlstra, W. & Dietz, V. Adaptational and learning processes during human split-belt locomotion: interaction between central mechanisms and afferent input. *Exp. Brain Res.* **106,** 449–456 (1995).

122. Reisman, D. S. Interlimb Coordination During Locomotion: What Can be Adapted and Stored? *J. Neurophysiol.* **94,** 2403–2415 (2005).

123. Machado, A. S., Darmohray, D. M., Fayad, J., Marques, H. G. & Carey, M. R. A quantitative framework for whole-body coordination reveals specific deficits in freely walking ataxic mice. *Elife* **4,** 1–22 (2015).

124. Darmohray, D., Jacobs, J., Marques, H. G. & Carey, M. R. Spatial and temporal locomotor learning in mouse cerebellum. *bioRxiv* 389965 (2018). doi:10.1101/389965

125. Malone, L. A., Bastian, A. J. & Torres-Oviedo, G. How does the motor system correct for errors in time and space during locomotor adaptation? *J. Neurophysiol.* **108,** 672–683 (2012).

126. Vasudevan, E. V. L., Torres-Oviedo, G., Morton, S. M., Yang, J. F. & Bastian, A. J. Younger Is Not Always Better: Development of Locomotor Adaptation from Childhood to Adulthood. *J. Neurosci.* **31,** 3055–3065 (2011).

127. Whishaw, I. Q. & Gorny, B. Does the red nucleus provide the tonic support against which fractionated movements occur? A study on forepaw movements used in skilled reaching by the rat.

Behav. Brain Res. **74,** 79–90 (1996).

128. Sybirska, E. & Gorska, T. Effects of red nucleus lesions on forelimb movements in the cat. *Acta. Neurobiol. Exp.* **40,** 821–841 (1980).

129. Van Kan, P. L. E. & McCurdy, M. L. Contribution of primate magnocellular red nucleus to timing of hand preshaping during reaching to grasp. *J. Neurophysiol.* **87,** 1473–1487 (2002).

130. Beitzel, C. S., Houck, B. D., Lewis, S. M. & Person, A. L. Rubrocerebellar feedback loop isolates the interposed nucleus as an independent processor of corollary discharge information in mice. *J. Neurosci.* **37,** 1093–17 (2017).

131. Ekerot, C. F., Garwicz, M. & Jörntell, H. The control of forelimb movements by intermediate cerebellum. *Prog. Brain Res.* **114,** 423–9 (1997).

132. Ross, G. S. & Sinnamon, H. M. Forelimb and hindlimb stepping by the anesthetized rat elicited by electrical stimulation of the pons and medulla. *Physiol. Behav.* **33,** 201–208 (1984).

133. Edgley, S. A.; Lidierth, M. Step-related discharges of Purkinje cells in the paravermal cortex of the cerebellar anterior lobe in the cat. *J. Physiol.* **401,** 339–415 (1988).

134. Soteropoulos, D. S. & Baker, S. N. Bilateral representation in the deep cerebellar nuclei. *J. Physiol.* **586,** 1117–1136 (2008).

Annexes

Annex 1 – DREADDs control

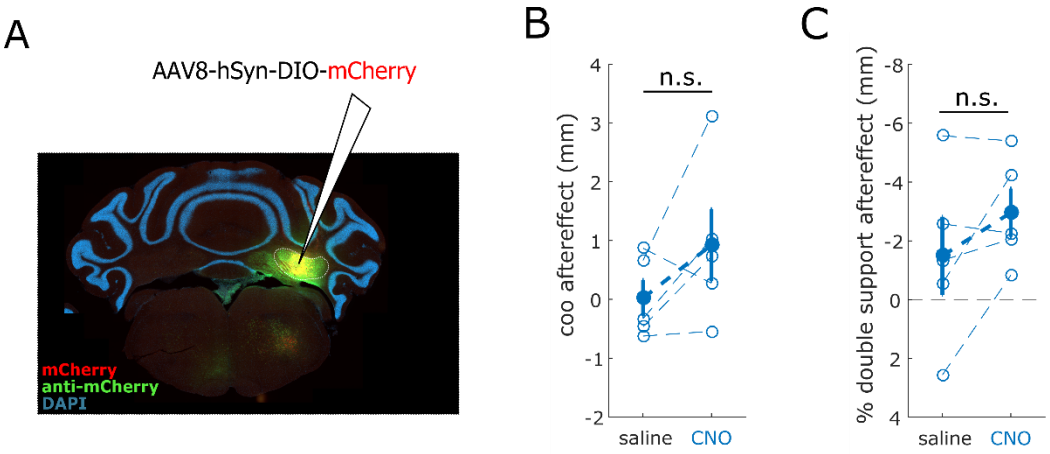

Figure 10 - Mice injected with an adeno-associated virus without DREADDs expression adapt similarly to controls on the ipsi-fast condition. A) Example of a coronal section with mCherry (red), anti-mCherry (green) and DAPI staining demonstrating virus spread on the interposed nucleus. Vglut2-cre mice were injected into the interposed with AAV8-hSyn-DIO-mCherry. **B)** Average center of oscillation aftereffect size (first trial post-adaptation) for mice injected with saline and CNO. **C)** Average % double support aftereffect size (post-adaptation first trial) for saline and CNO-injected animals. Individual mice' aftereffect size is shown in thin dashed lines.

Annex 2 – List of abbreviations

7n – facial nerve

BLA – basolateral amygdaloid nucleus

CM – central medial thalamic nucleus

CS – conditional stimulus

Gi – gigantocellular reticular nucleus

GiA – gigantocellular reticular nucleus alpha part

IP – interpose nucleus

IRt – intermediate reticular nucleus

LPGi – lateral paragigantocellular nucleus

MdD – medullary reticular nucleus dorsal part

MdV – medullary reticular nucleus ventral part

mRt – mesencephalic reticular formation

PC – paracentral thalamic nucleus

PCRtA – parvocellular reticular nucleus alpha part

PnC – pontine reticular nucleus caudal part

RMC – magnocellular red nucleus

RN – red nucleus

RtTg – reticulotegmental nucleus of the pons

SC – superior colliculus

scp – superior cerebellar peduncle

US – unconditional stimulus

VL – ventrolateral thalamic nucleus

VM – ventromedial thalamic nucleus

VOR – vestibulo-ocular reflex

VSCT – ventral spinocerebellar tract

xscp – decussation of the cerebellar peduncle