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For correspondence:
lena@biologie.ens.fr
dpopa@biologie.ens.fr

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Multiple Functions of Cerebello-Thalamic Neurons in Learning and Offline Consolidation of a Motor Skill in mice

Andres P Varani, Caroline Mailhes-Hamon, Romain W Sala, Marie Sarraudy, Sarah Fouda, Jimena L Frontera, Clément Léna*, Daniela Popa*

Institut de biologie de l'Ecole normale supérieure (IBENS), Ecole normale supérieure, CNRS, INSERM, PSL Research University, Paris, France

eLife Assessment

Varani et al present **important** findings regarding the role of distinct cerebellothalamic connections in motor learning and performance. The evidence supporting the main claims is **convincing**, with multiple replications, validation of their techniques, and appropriate controls. The work will be of broad interest to neuroscientists interested in central mechanisms of motor learning and control, as well as thalamic physiology.

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Abstract

Motor skill learning is a complex and gradual process that involves the cortex and basal ganglia, both crucial for the acquisition and long-term retention of skills. The cerebellum, which rapidly learns to adjust the movement, connects to the motor cortex and the striatum primarily via the ventral and intralaminar thalamus respectively. Here, we evaluated the contribution of cerebellar neurons projecting to these thalamic nuclei in a skilled locomotion task in mice. Using a targeted chemogenetic inhibition that preserves the motor abilities, we found that cerebellar nuclei neurons projecting to the intralaminar thalamus contribute to learning and expression, while cerebellar nuclei neurons projecting to the ventral thalamus contribute to offline consolidation. Asymptotic performance, however, required each type of neurons. Thus, our results show that cerebellar neurons belonging to two parallel cerebello-thalamic pathways play distinct, but complementary, roles functioning on different timescales and both necessary for motor skill learning.

Introduction

Learning to execute and automatize certain actions is essential for survival and, indeed, animals have the ability to learn complex patterns of movement with great accuracy to improve the outcomes of their actions (Krakauer, Hadjiosif et al. 2019). Two categories of learning are often engaged in motor skill acquisition (Seidler 2010, Krakauer, Hadjiosif et al. 2019): 1) sequence learning, which is needed when series of distinct actions are required, and 2) adaptation which corresponds to learning a variation of a previous competence, and typically takes place when motor actions yield unexpected sensory outcomes. The neurobiological substrate of motor skills involves neurons distributed in the cortex, basal ganglia and cerebellum, each structure using different learning algorithms (Doya 1999). On one hand, the cerebellum is a site where supervised learning takes place (Raymond and Medina 2018). The cerebellum is thought to form associations between actions and predicted sensory outcome at short-time scale (typically under one second), which are seen as internal models (Ito 2008), and are essential for the precise

timing and coordination required for motor skill execution. The cerebellum has been shown to be central for the adaptation of skills such as oculomotor movements (Nguyen-Vu, Kimpo et al. 2013, Yang and Lisberger 2014, Herzfeld, Kojima et al. 2018), reaching (Hewitt, Popa et al. 2015), locomotion (Morton and Bastian 2006, Darmohray, Jacobs et al. 2019), as well as conditioned reflexes (Clopath, Badura et al. 2014, Longley and Yeo 2014). On the other hand, reinforced learning takes place in the basal ganglia and is required to learn complex actions involving sequences of movements, for which the involvement of the cerebellum is much less understood (Seidler, Purushotham et al. 2002, Bernard and Seidler 2013, Krakauer, Hadjiosif et al. 2019, Baetens, Firouzi et al. 2020).

In general, motor skills are progressively acquired (e.g. Karni, Meyer et al. 1998) and their execution may ultimately recruit sets of brain structures distinct from those involved in the initial training (e.g. Brashers-Krug, Shadmehr et al. 1996, Muellbacher, Ziemann et al. 2002, Korman, Raz et al. 2003). Strikingly, motor skill consolidation does not occur simply "online", during repeated task execution, but also "offline" during the resting periods (Brashers-Krug, Shadmehr et al. 1996, Muellbacher, Ziemann et al. 2002, Cohen, Pascual-Leone et al. 2005, Doyon, Korman et al. 2009). Indeed, a single resting period may be sufficient to change the brain regions recruited in task execution (Shadmehr and Holcomb 1997). Moreover, motor memories may also persist in the form of "savings", which facilitate relearning of the task at a later time (Huang, Haith et al. 2011, Mauk, Li et al. 2014). Overall, motor skill learning is a dynamical process distributed in time and across brain regions.

Comprehending the role of cerebellum in motor skill learning, beyond its role in motor coordination, requires considering its integration in brain-scale circuits including the cortex and basal ganglia (Caligiore, Pezzulo et al. 2017). In the mammalian brain, both cerebellum and basal ganglia receive the majority of their inputs from the cerebral cortex, via the pontine nuclei for the cerebellum. The cerebellum and basal ganglia send projections back to the cortex via anatomically and functionally segregated channels, which are relayed by predominantly non-overlapping thalamic regions (Bostan, Dum et al. 2013, Proville, Spolidoro et al. 2014, Hintzen, Pelzer et al. 2018). Furthermore, anatomical and functional reciprocal di-synaptic connections have been described between the basal ganglia and the cerebellum (Bostan and Strick 2010, Carta, Chen et al. 2019). Cerebellar projections to the striatum and to the motor cortex are relayed primarily through distinct thalamic regions, respectively the intralaminar thalamus and ventral thalamus (Steriade 1995, Chen, Fremont et al. 2014), suggesting distinct contributions of these cerebello-diencephalic projections.

In the present study, we hypothesized that the cerebellum may contribute to some phases of learning in a complex motor task via its projections to thalamic nuclei embedded in thalamo-cortical and thalamo-striatal networks. We studied the accelerating rotarod task, which learning depends on the cortex and basal ganglia (Costa, Cohen et al. 2004, Cao, Ye et al. 2015, Kida, Tsuda et al. 2016). We then focused on the cerebellar nuclei (CN) and their projections to the centrolateral (intralaminar) thalamus and ventral anterior lateral complex (motor thalamus), which are known to relay their activity to the striatum and the motor cortex (Chen, Fremont et al. 2014, Proville, Spolidoro et al. 2014, Gornati, Schäfer et al. 2018), and which inhibition does not impair the ability to walk on a rotating rod. We examined the contribution of CN and CN neurons belonging to distinct cerebello-thalamic pathways to learning using chemogenetic disruptions either during or after the learning sessions and revealed a differential contribution of the distinct CN-thalamic neurons to the online learning and the offline consolidation.

Results

In the present study, we used the paradigm of the accelerating rotarod, in which the animals walk on an accelerating rotating horizontal rod. Over multiple repetitions of the tasks, the rodents develop locomotion skills to avoid falling from the rod. This paradigm allows to study the neurobiological basis of motor skill learning (e.g. Costa, Cohen et al. 2004, Yang, Pan et al. 2009, Rothwell, Fuccillo et al. 2014), especially at multiple time scales. When repeated over

multiple days, distinct phases of learning, with different rate of performance improvement and organization of locomotion strategies, can be distinguished (Buitrago, Schulz et al. 2004) and selectively disrupted (Hirata, Takahashi et al. 2016, Sathyamurthy, Barik et al. 2020).

Partial inhibition of the CN neurons using hM4D(Gi)-DREADD does not impair basic motor abilities

To evaluate the contribution of the cerebellar outputs during and after the accelerating rotarod, we employed a chemogenetic approach (Roth 2016) using the inhibitory DREADD (hM4D-Gi) activated by the synthetic drug Clozapine-N-Oxide (CNO).

In order to validate this approach, mice were injected with AAV5-hSyn-hM4D(Gi)-mCherry (DREADD mice) or AAV5-hSyn-EGFP (Sham mice) and implanted with microelectrode arrays in the CN (Fig. 1a-c). Post-hoc histology confirmed the position of the electrodes (Fig. 1d) and showed that the expression of hM4D(Gi)-mCherry was confined to the CN and expressed in neurons, with a large proportion of the cells expressing hM4D(Gi)-DREADD in the CN (sup Fig. 1). A week after surgery, the neuronal activity was recorded in the CN in an open-field arena before and after accelerating rotarod sessions. CNO injection in mice which received DREADD-expressing virus yielded a ~35% decrease in firing rate, which was not observed following saline (SAL) injection or following saline or CNO injection in Sham mice (Fig. 1e-g; Sham mice which received either SAL or CNO are respectively referred to as Sham+SAL and Sham+CNO while DREADD mice which received SAL or CNO are referred to as DREADD+SAL and DREADD+CNO).

We then examined whether the reduction of CN firing impacted the motor function. We first assessed spontaneous motor activity, strength and motor coordination. No significant differences were observed between the experimental groups in footprint patterns (Fig. Sup2a), grid test, horizontal bar and vertical pole (Fig. Sup2b) indicating that motor coordination and strength were not affected by the reduction of CN firing induced by the administration of CNO 1mg/kg. The average velocity of open-field locomotion was not altered by CNO or SAL injection in DREADD or Sham mice (Fig. Sup2c) consistent with intact basic locomotion skills and motivation. As locomotion on a rotating rod may require specific sensorimotor abilities, a crucial control measure is to confirm that the animals can move normally on a fixed-speed rotarod when not subjected to acceleration challenges. We observed that mice with CN inhibition (DREADD+CNO group) exhibited similar performance to the ones of the control groups (DREADD+Saline, Sham+CNO, Sham+Saline, Fig 1h). Thus, these results indicate that the reduction of the CN firing induced by the CNO had a limited impact on the motor abilities of the mice and it is thus appropriate to examine the cerebellar contribution to motor learning rather than to motor function.

Partial CN inhibition during or after the task has a different impact on motor learning

To test the effect of a CN activity reduction on motor learning, we first examined the impact of CN inhibition by injecting CNO (1 mg/kg) each day before the first trial of an accelerating rotarod session (Fig. 1i) over seven days. Most of the performance improvement took place in the first four days, referred to as “Early Phas”, whereas stable performances were observed in the last three days, here referred to as “Late phas”.

Inhibition of CN activity during the task (Fig 1i) did not affect significantly the learning of DREADD-expressing mice on the first day of the Early Phase, but reduced their performance on the following days compared to the control groups. During the Early Phase, overnight loss of performance was observed in the DREADD+CNO mice between the last trial of one day and the first trial of the following day (Supplementary Table 4), indicating an impairment in motor learning consolidation. As the effects of systemic CNO administration last longer than the duration of the trials, the results above do not allow to distinguish the effect of cerebellar inhibition during the trials, or after training (offline consolidation). We therefore injected another set of mice with CNO after the training sessions (Fig. 1j). CN inhibition after the task in the Early Phase indeed reduced the performance on the first trials of the next day, consistent with a disruption of offline

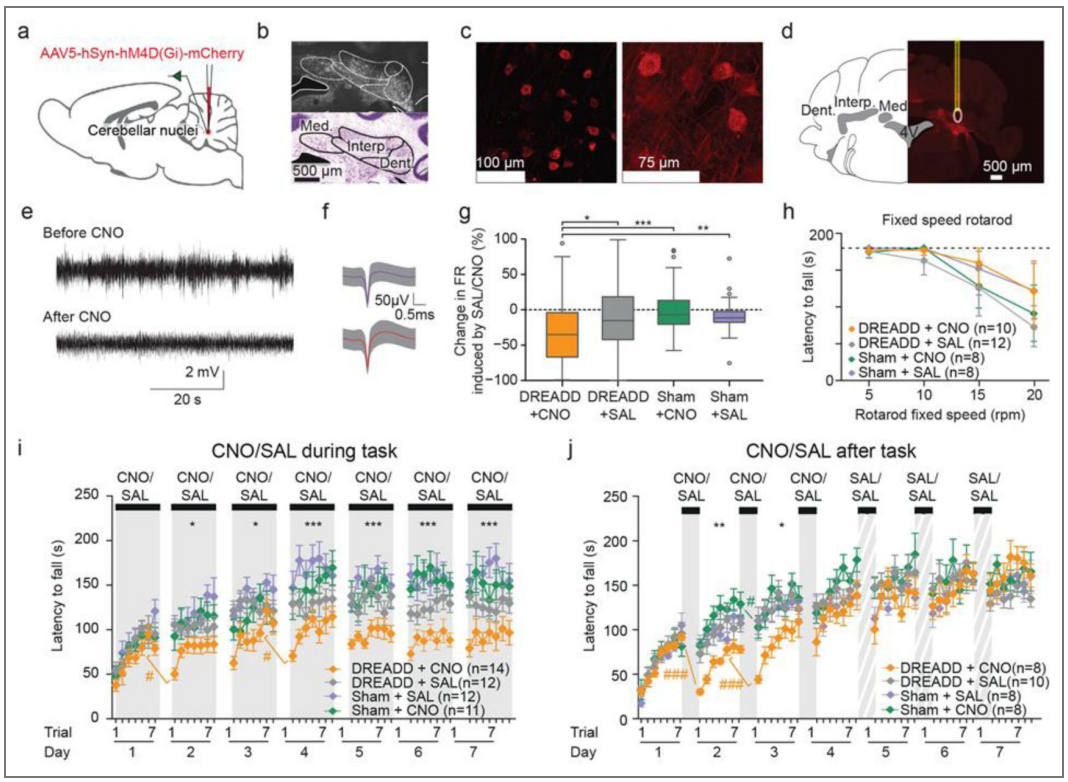


Figure 1. Cerebellar nuclei partial inhibition during and after a motor task impairs learning.

a) Scheme of the implantation and injection. **b**) Coronal section of the cerebellum showing hM4Di-DREADD expression in the three CN. **c**) Representative confocal image showing hM4Di-DREADD expression on neuronal membranes. **d**) Electrode placement close to cells expressing hM4Di-DREADD (red: lesion site, yellow: electrode track). **e)** Examples of high-pass filtered traces of CN recordings before and after CNO injection. **f**) Examples of spike shapes obtained from spike sorting in CN (line+shading indicates mean +/- SD). **g)** Boxplots displaying the percentage of modulation of CN neurons average firing rate induced by CNO or SAL injections during an open field session. CN firing rate was reduced after 1 mg/kg of CNO injection in DREADD injected mice compared to other groups (Wilcoxon test* p<0.05, ***p<0.001. Boxes represent quartiles and whiskers correspond to range; points are singled as outliers if they deviate more than 1.5 x interquartile range from the nearest quartile). **h)** Latency to fall during fixed speed rotarod (5, 10, 15, 20r.p.m.) for all experimental groups. One way repeated-measure ANOVA on averaged values for all the speed steps in each experimental group followed by Tukey Posthoc pairwise comparison (p>0.05 in all cases). **i**) Impact of daily of injections of CNO before trial 1 on accelerating rotarod performance. Summary of the performance for each trial/day (repeated measure ANOVA Group effect *p<0.05, ***p<0.001; # p<0.05, ###p<0.001 Tukey pairwise test last trial of each day vs first trial of next day). **j**) Impact of daily of injections of CNO after the task (30 min after trial 7). All treatment is switched to saline in the Late phase. Same presentation as in i). Data represents mean ± S.E.M. *n* indicates the number of mice. (See Supplementary Tables 1-5 for detailed statistics)

consolidation. The performance of DREADD+CNO mice on the last day of the Early Phase was not different from the control groups, indicating that the lack of offline consolidation was overcome by the training the next day. To test whether the skill consolidated under CNO remained stable thereafter, we then shifted the treatment, removing CNO during the Late phase, and we found that mice did not exhibit any further difference between groups. Overall, these results indicate that the offline CN activity participates in the consolidation of the accelerating rotarod learning.

Accelerating rotarod learning is a cumulative process over multiple trials and multiple days. To disentangle differences in learning from differences in consolidation, we examined the change of performance of single animals within and across days. In order to minimize the effect of inter-trial variability on the estimation of performance and learning, we simplified the data by performing a linear regression on the performance of each day where the slope indicates the daily learning rate (Fig 2a). The endpoints at trial 1 and 7 of this regression are used as estimates of the initial and final skill level on each day (Fig. 2b) allowing us thus to follow the daily learning, overnight changes and offline consolidated learning (Fig. 2b). In the control groups, we found an inverse correlation between the initial performance of each day and the amount of daily learning: animals with strong initial performance on a given day showed weaker improvements than animals with poor initial performances (Fig 2c, top). This result indicates that the comparison of the daily learning between groups of animals requires to take into account the daily initial performance of each animal. In the control mice, daily learning was almost completely preserved overnight consistent with an effective consolidation across days (Fig 2c, bottom). Strikingly, DREADD-expressing mice which received CNO during the task exhibited both a lower learning performance (lower daily increase in performance for similar daily initial performance) (Fig 2d, top) and lower consolidation (lower consolidated learning for similar daily learning) (Fig 2d, bottom) than control mice. Therefore, the lower performance of these DREADD-expressing mice receiving CNO during the task was not due solely to a disrupted consolidation. In contrast, DREADD-expressing mice, which received CNO after the task, showed normal learning performance (i.e. similar daily increase in performance for similar daily initial performance) (Fig. 2e, top) but lower consolidation (Fig 2e, bottom).

Selective inhibition of cerebellar neurons projecting to distinct thalamic regions differentially impacts motor learning

Since the CN project to a wide array of targets (Teune, van der Burg et al. 2000), we specifically examined whether cerebellar neurons projecting to ventral anterior/lateral (VAL) and central lateral (CL) thalamus differentially contribute to the rotarod learning and execution. For this purpose, an AAV5-hSyn-DIO-hM4D(Gi)-mCherry virus expressing an inhibitory DREADD conditioned to the presence of Cre-recombinase was injected into the CN, while a retrograde CAV-2 virus expressing the Cre recombinase was injected either in the CL or in the VAL. In both cases, we found an expression of hM4D(Gi)-mCherry throughout the CN mostly in the Interposed and Dentate for the CL injections (Supplementary Figure 3). Since there was no effect of CNO in Sham mice (Fig. 1), we only compared DREADD-injected animals receiving either CNO or SAL.

We examined the impact of the inhibition of cerebello-thalamic neurons on spontaneous locomotion, motor coordination and strength. No significant differences were observed between the experimental groups for footprint patterns (Fig. Sup4a), grid test (Fig. Sup4b), horizontal bar (Fig. Sup4c) and vertical pole (Fig. Sup4d) indicating that coordination and strength are not affected by the inhibition of cerebellar-thalamic pathways induced by the administration of CNO 1mg/kg. We also determined locomotor activity in open-field experiments (Fig. Sup4e) which revealed that velocity was generally not affected by CNO injection in DREADD or Sham mice (Fig. Sup4e), although CNO-injected CN→VAL and CN→CL groups, respectively exhibited slightly higher velocity in day 1 and lower velocity in day 4 in the first open field session compared to control group. No significant differences were observed between Saline- vs. CNO-treated CN-CL mice in the fixed speed rotarod test (Fig. Sup4f). However, we found a decrease in the latency to

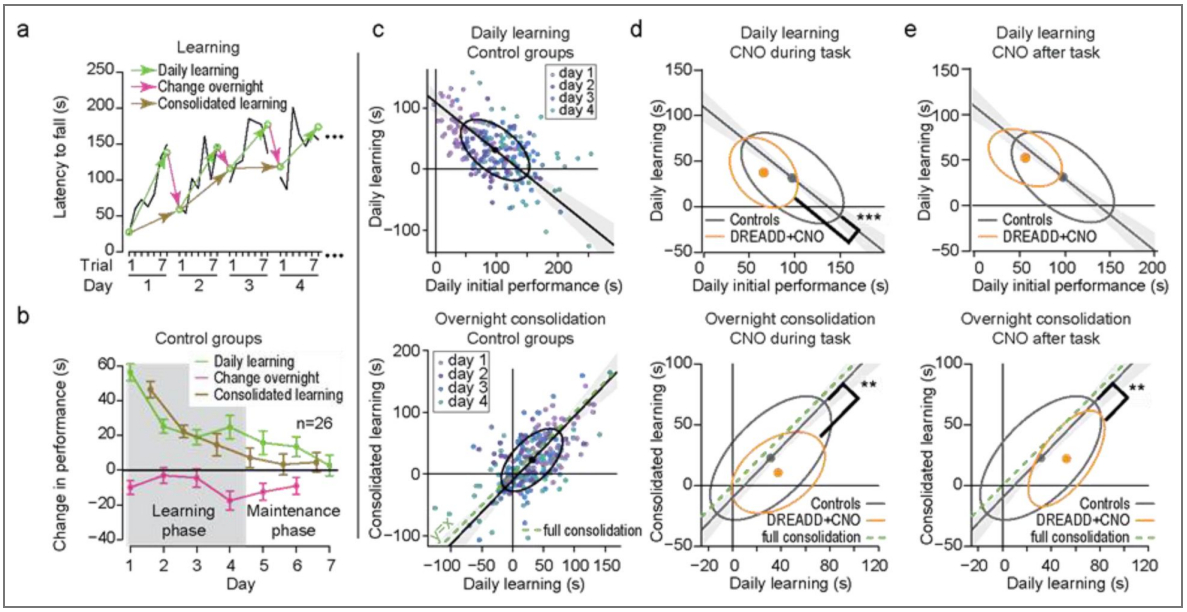


Figure 2. changes in learning and consolidation of motor memory induced by cerebellar nuclei inhibition.

a) Example of evolution of latencies to fall for a mouse during the accelerating rotarod protocol. Linear regressions estimated values of trial 1 and 7 during each day are shown using green hollow dots, within-day learning (green arrows), overnight change (red arrows) and day+night learning (brown arrows). **b)** Evolution of estimated daily learning, overnight change and consolidated learning shows learning mostly in days 1-4 (Early Phase). **c)** within-day learning vs daily initial performances (top) and within-day learning vs consolidated learning (bottom) in the Early Phase. Scatterplot of performance from all control mice; the ellipse contains 50% of a bivariate normal distribution fitted to the values and the dot indicates the center of the distribution. Deming linear regression outcomes are represented with 95% confidence interval in shaded color. **d)** same as panel c) with superimposition of Controls (black) and mice expressing DREADD in DCN and CNO during the task (orange); only ellipses and regression line are included for clarity of the graph (**p<0.01 ***p<0.001 Wilcoxon test for difference between groups of residuals, i.e. signed distance of performances to Deming regression line of Control mice). **e)** same as panel d) for CNO administered after the task, (See Supplementary Table 8 for detailed statistics)

fall for 15 and 20 r.p.m. in the CN→VAL+CNO group suggesting that the ability to locomote on a rotarod was slightly decreased though the animals were still able to remain more than one minute on the rotarod at 20 r.p.m. (Fig. Sup4f).

We then examined how the accelerating rotarod learning was impaired by the inhibition of CN neurons involved in cerebello-thalamic pathways during (Fig. 3b, e) and after (Fig. 3c, f) the task. Inhibition of the CN→CL neurons during the task (Fig. 3b) produced a progressive deviation from the performance of the control group during the Early Phase, yielding to a substantial reduction of performance in the Late phase. In contrast, when the inhibition took place after the task during days 1, 2 and 3 (Fig. 3c), the performances remained similar to the control group. This suggests that the CN→CL neurons mostly contribute to the learning during training.

In contrast, the specific inhibition of the CN→VAL neurons yielded another pattern of performance evolution. First, when inhibition took place during the task (Fig. 3e), there was a marked drop in performance from the last trial of one day to the first trial of the following day, as observed for the full DCN inhibition (Fig 1i), and the performances saturated at lower level than control mice during the Late phase. Second, when inhibition took place after the task on days 1, 2 and 3 (Fig. 3f), a similar overnight drop in performance was found during the Early Phase. Interestingly, this drop was maintained when the treatment after the task was shifted from CNO to Saline after 4 days, suggesting the cerebellar contribution to the consolidation of the task is critical early in the learning process and cannot be easily reinstated later.

Then, we examined to which extent learning and consolidation were affected during the Early Phase by CN→CL versus CN→VAL neurons inhibition (Fig. 3g-k). Control mice also showed a pattern of decreased learning for higher initial performance and full overnight maintenance of the improvement of performance (Fig. 3g). Inhibition of the CN→CL neurons reduced the learning compared to controls (i.e. smaller daily learning for similar daily initial performance) when performed during (Fig. 3h) but not after (Fig. 3i) the task and preserved learning consolidation (i.e. gain of performance is preserved overnight) in all cases (Fig. 3hi). In contrast, inhibition of the CN→VAL neurons during or after the task preserved the learning (i.e. same daily learning for similar initial performance) but selectively disrupted learning consolidation in all cases (Fig. 3jk). This indicates that CN→CL and CN→VAL neurons play different roles during the Early Phase, the former primarily during the task, and the latter after the task.

The differential impact of chemogenetic inhibition of CN neurons retrogradely infected from the VAL and the CN suggest that different CN populations are targeted. To verify this, we performed combined retrograde injections of fluorescent rAAV centered on the VAL and CL (n=2 mice, Fig. Suppl 5). Retrograde injections yielded labelling in the three main divisions of the CN (Dentate, interposed and fastigial). Retrogradely labeled cell soma from CL, VAL or both were observed in each CN division and were distributed along anteroposterior axis (Fig. Suppl. 5c). Counts of labeled cells in each CN at 7 anteroposterior levels from each injection was highly correlated between the mice (r=0.92, 51 measures/mice) and counts were thus pooled. Overall, very few double-labeled neurons were found (3.4%, 30 double-labeled, 305 green, 549 red cells), consistent with a limited overlap of the CN populations retrogradely infected by the two types of injections.

Inhibition of each type of cerebello-thalamic neurons impairs execution once maximal performance has been achieved

Mice that learned the task while either CN→CL or CN→VAL neurons were inhibited showed lower performance compared to controls after 7 days of training (“Day 7” in Figure 3b,e and Fig 4a,c). To examine whether this result is due to the deficits in learning and consolidation described above or whether at this late stage, CN→CL or CN→VAL neurons participate in the task execution, we administered CNO to mice that had learned the accelerating rotarod during 7 days receiving Saline (Fig. 4, black symbols). These CNO injections were thus performed at the end of the Late phase, when the performances of the mice were stable across days. In mice expressing inhibitory DREADD in CN→CL (Fig. 4a) or CN→VAL neurons (Fig 4c), the injection of CNO before the task induced a significant reduction in performance both at the start and end of the

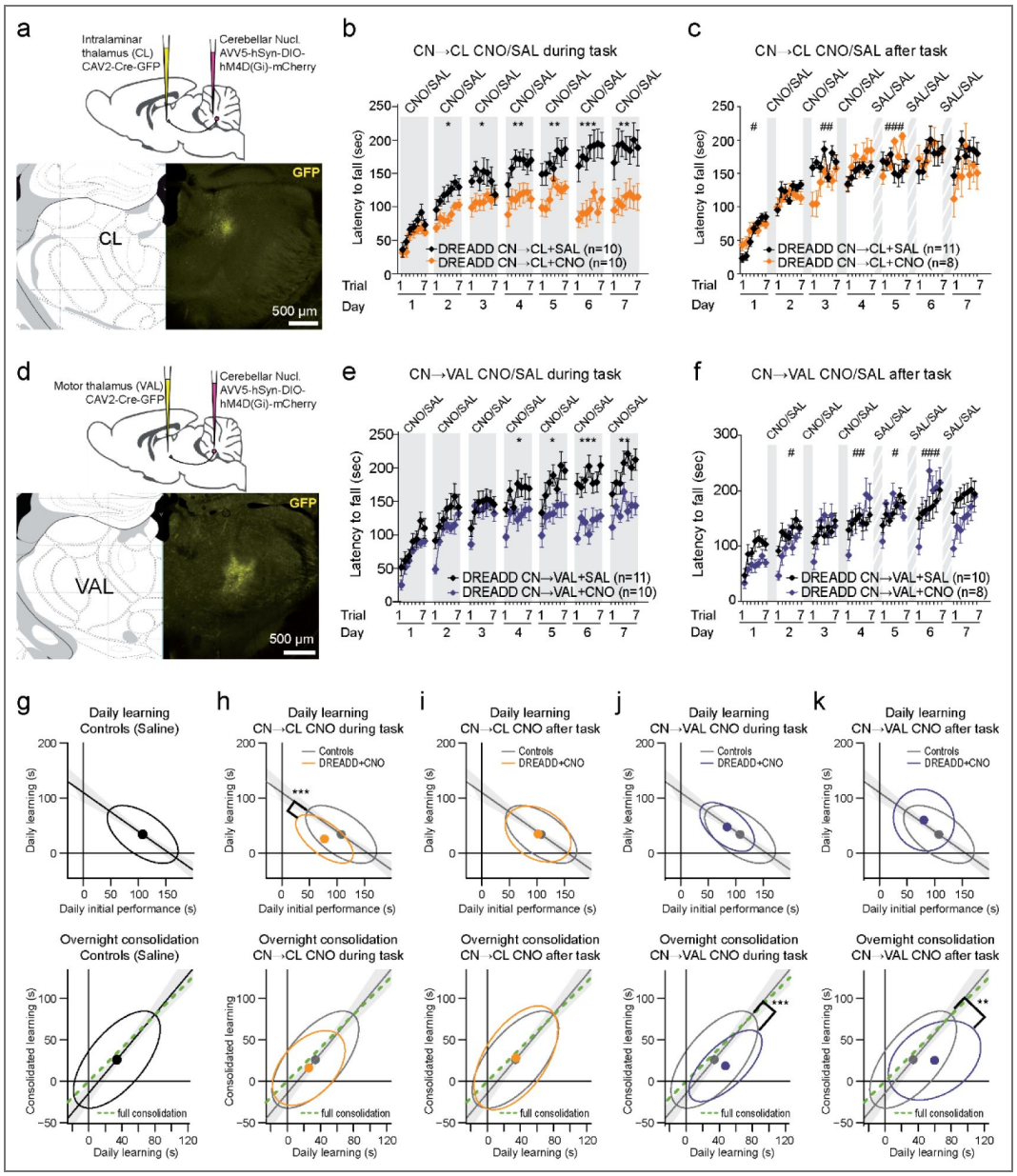


Figure 3. Inhibition of cerebellar nuclei (CN) neurons projecting to the centrolateral thalamus (CL) and to the ventral anterior lateral (VAL) thalamus during and after the training sessions differentially impairs motor learning.

a) scheme of the combined viral injections targeting the CN->CL neurons using a retrograde virus expressing the Cre in the thalamus and a virus inducing Cre-dependent expression of inhibitory DREADD in the CN. *Top*: schematic of the viral injections. *Bottom*: GFP fluorescence revealing the site of injection of the CAV viruses. **b**) comparison of the effect of daily injections before trial 1 of CNO (orange) or Saline (black) in mice described in panel a) (Data represents mean ± S.E.M, *n* indicates the number of mice; *p<0.05, **p<0.01, ***p<0.001; repeated measure ANOVA Group effect). **c**) Same as b) for daily CNO injections 30 minutes after trial 7 during the Early Phase. **d**) scheme of combined viral injections targeting the CN->VAL neurons. **e,f**) same as (b,c) for CNO-injected (blue) and control (black) mice obtained as described in panel d. **g)** same as figure 2c for the Saline-treated groups of panels b-f. **h-k)** same as figure 2d-e for the different groups receiving injections CNO during or after the task compared to Saline-treated mice. Same color coding as panels b,c and e,f. (**p<0.01 ***p<0.001 Wilcoxon test for difference between groups of residuals i.e. signed distance of performances to Deming regression line of Control mice). CN: cerebellar nuclei, VAL: ventral anterior lateral thalamus, CL: central lateral thalamus. (See Supplementary Tables 9,10 for detailed statistics)

daily training sessions (Fig 4b,d). Interestingly, daily learning and deficit in consolidation still took place under inhibition of CN→VAL neurons while neither of these effects were present following inhibition of CN→CL neurons, consistent with the primary role of the later for task learning and of the former for offline consolidation. These results moreover indicate that CN→CL and CN→VAL neurons contribute to task execution and/or memory retrieval in the late stage of learning in normal conditions.

Since CN→CL or CN→VAL neurons participate to the performance in the Late phase, we expected that the removal of the inhibition after 7 days of training would result in an improvement of performance. Indeed, in mice expressing inhibitory DREADD in CN→CL neurons, there was a significant performance improvement (but no further learning) when the treatment was shifted from CNO to Saline administration (Fig 4a,b) consistent with the involvement of CN→CL neurons in task execution. Strikingly, such improvement was not observed when the inhibition of CN→VAL neurons was lifted (Fig 4c,d), indicating that contribution of CN→VAL neurons in early phases of task learning are needed for a proper encoding of the task and that cannot be easily recovered if it was impaired at the early phases.

Discussion

In this study, we found that a transient and mild chemogenetic inhibition which reduces the cerebellar nuclei activity preserves the motor coordination but disrupts motor learning in a complex motor task, the accelerating rotarod task, known to depend on the motor cortex and basal ganglia. Moreover, we distinguish two contributions of the cerebellum to learning; one is carried by CN neurons projecting toward the intralaminar thalamus, and is needed for learning and recall/execution. The other is carried by CN neurons projecting toward the motor thalamus and is required to perform an offline consolidation of a latent memory trace into a consolidated, readily available, motor skill (Fig 5). Finally, our results show that, beyond its role in learning and consolidation and independently from a role in basic motor coordination, the cerebellum becomes more strongly engaged via its projections toward the cerebral cortex and basal ganglia when the performance in the task progresses.

A role for the cerebellum in the multi-nodal network of motor skill learning

By showing an involvement of the cerebellum in the accelerating rotarod task, our results complement the previous results which demonstrated that the basal ganglia and motor cortex are recruited and required to complete the task (Costa, Cohen et al. 2004, Cao, Ye et al. 2015, Kida, Tsuda et al. 2016). Our chemogenetic experiments also indicate that the involvement of the cerebellum changes along the multiple phases of motor learning, ranging from a minimal contribution in the initial phase to a stronger contribution in the later phases. These observations parallel the converging evidence indicating that the areas of the basal ganglia involved in the accelerating rotarod evolve along the phases of learning (Yin, Mulcare et al. 2009, Durieux, Schiffmann et al. 2012, Cao, Ye et al. 2015). Therefore, the three main central nodes of motor function (cortex, basal ganglia and cerebellum) are differentially recruited along the multiple phases of the accelerating rotarod task.

The impact of various cerebellar disruptions on accelerating rotarod performance have previously been examined in too many studies to be listed exhaustively here, but the contribution of cerebellum to learning is generally difficult to interpret. The reported effects range from ataxia and disruption of the ability to run on a rod (Sausbier, Hu et al. 2004), to normal learning (Galliano, Potters et al. 2013), defects in learning (Groszer, Keays et al. 2008, Galliano, Potters et al. 2013), defects in consolidation (Sano, Kohyama-Koganeya et al. 2018) and even increase in learning (Iscru, Serinagaoglu et al. 2009). However, the cerebellum is critical for inter-limb coordination (Machado, Darmohray et al. 2015, Sathyamurthy, Barik et al. 2020), and most studies lack proper motor controls to test the ability to walk on a rotating rod: poor performance may thus simply result from problems of running on the rod rather than problems of learning. Moreover many studies involve genetic mutations which leave room from variable compensations

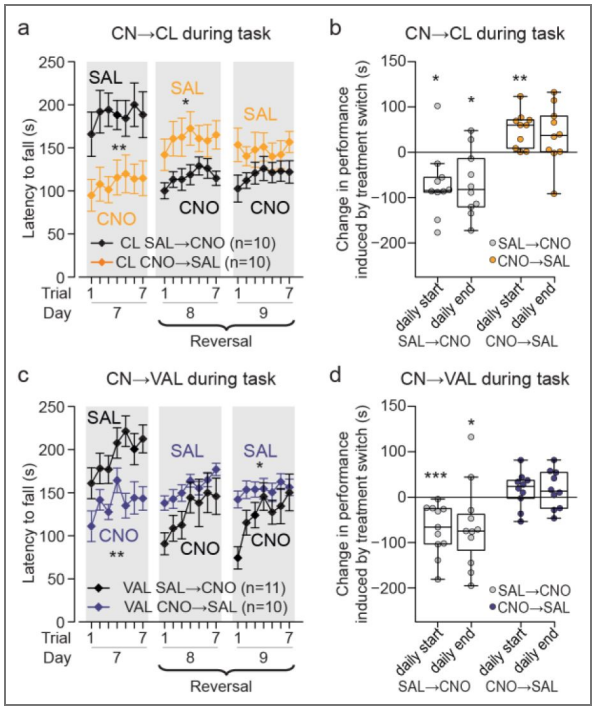


Figure 4. Reversal of the inhibition of the cerebello-thalamic neurons at the end of the Late phase yields lasting impairments.

a) Performance of mice with DREADD expression in CL-projecting CN neurons, which learned the task under respectively CNO and Saline treatment (Fig. 3a) during 7 days and then receive respectively Saline and CNO treatment during a Reversal phase (*: p<0.05 repeated-measure ANOVA group effect; Data represented as mean ± S.E.M, *n* indicates the number of mice). **b)** change induced by treatment switch on skill levels for the first trial (daily start) and last trial (daily end); values are estimated as in Fig. 2a, and start and end values for day 8 and 9 are averaged for each animal; *p<0.05, **p<0.01, ***p<0.001 paired Wilcoxon test. Boxes represent quartiles and whiskers correspond to range; points are singled as outliers if they deviate more than 1.5 x interquartile range from the nearest quartile. **c,d**) same as panels a and b for mice with DREADD expression in VAL-projecting CN neurons (Fig. 3d). CN: cerebellar nuclei, VAL: ventral anterior lateral thalamus, CL: central lateral thalamus. (See Supplementary Table 15 for detailed statistics)

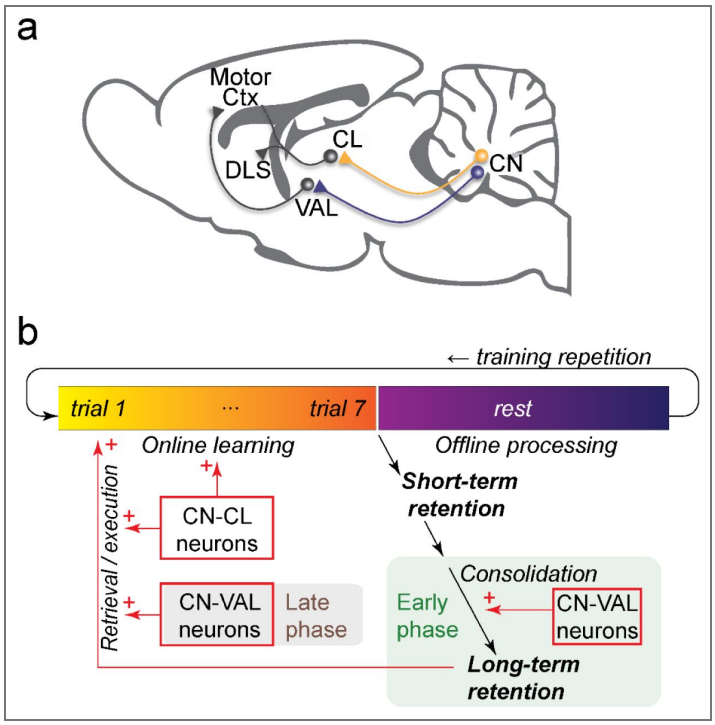


Figure 5. **Summary of the behavioral findings.**

a) Schematic representation of mouse brain CN: Cerebellar Nuclei, CL: centrolateral thalamus, VAL: ventral and anterior thalamus, DLS: dorsolateral striatum, Motor Ctx: motor cortex. **b**) Summary of the controls exerted by CN-CL and CN-VAL neurons on the skill learning. The CN-CL neurons contribute to online-learning and retrieval/execution of the learned skill. The CN-VAL neurons contribute to consolidate offline the recent learning into a form of consolidated, readily-available, memory; a defect in consolidation in the early phases (first days) cannot be rescued in the late phase. CN-VAL neurons also participate to the retrieval/execution in the late phases of learning.

along development and adult life, as exemplified by the diversity of the motor phenotypes of mice with different timing of degeneration of Purkinje cells (Porras-Garcia, Ruiz et al. 2013). Finally, the multiphasic nature of rotarod learning is often overlooked.

Studies of cerebellar synaptic plasticity support the involvement of cerebellum in rotarod learning. Indeed, the targeted suppression of parallel-fiber to Purkinje cell synaptic long-term depression in the cerebellar cortex disrupts rotarod learning after an initial phase, without altering any other motor ability tested (Galliano, Potters et al. 2013). Consistently, Thyrotropin-releasing hormone (TRH) knock-out mice do not express long-term depression at parallel fiber-Purkinje cell synapses and exhibit impaired performance in the late phase of rotarod learning, while the administration of TRH in the knock-out mice both restores long-term depression and accelerating rotarod learning (Watanave, Matsuzaki et al. 2018). More generally, studies in mutant mice suggest that cerebellar plasticity is required for adapting skilled locomotion (Vinueza Veloz, Zhou et al. 2015). This suggests that cerebellar plasticity is involved in accelerating rotarod learning and thus contributes to learning processes and not simply to the execution of the task. Yet it remains unclear whether this cerebellar learning is needed to improve descending control of the motor system, or whether it is needed by the motor centers in the forebrain.

A specific impact on learning of CL-projecting CN neurons

In our study, we found that the chemogenetic inhibition of CN-CL neurons during the task reduces the learning performances of the mice. This effect unlikely results from basic motor deficits: we found that the chemogenetic modulation did not significantly alter 1) limb motor coordination in footprint analysis, 2) strength in the grid test, 3) speed in spontaneous locomotion in the open-field test, 4) locomotion speed and balance required to complete the horizontal bar test and 5) body-limb coordination and balance required in the vertical pole test. Since all these motor parameters may be affected by cerebellar lesions, this suggests that CN-CL neurons are not necessary to maintain those functions, which might thus be relayed by other cerebellar nuclei neurons; indeed focal lesions in the intermediate cerebellum (thus projecting to the Interposed nuclei) has been reported to induce ataxia without altering rotarod learning (Stroobants, Gantois et al. 2013). Alternatively, the effect of the partial inhibition induced by CNO in our study (typically ~35% reduction in firing rate) might be compensated at other levels in the motor system to ensure normal performances in these tasks; indeed, the selective ablation of CN-CL neurons has been reported to yield locomotor deficits in the initial performances on the accelerating rotarod (Sakayori, Kato et al. 2019), which contrasts with the lack of significant deficit in the early phase following CN-CL neurons inhibition in our study. A possible explanation for this discrepancy could be that our intervention, being milder than a full ablation, selectively disrupted the advanced patterns of locomotion only needed at the higher speeds of the rotarod and thus did not impact on the slow rotarod locomotion typically performed in the initial learning phase of the task. However, the highest speeds reached on the rotarod correspond to the average locomotion speed in the open-field, which is unaffected by the chemogenetic inhibition. Moreover, in our conditions, the inhibition of the CN-CL neurons did not produce significant deficits in the fixed-speed rotarod; CNO-treated animals ran in average for about two minutes at 20 r.p.m. while they fell in average after the same amount of time on the accelerating rotarod, corresponding to a rotarod speed below 20 r.p.m. at the time of the fall. This rules-out a contribution of weakness, or fatigue, to the latency to fall in the accelerating rotarod, the CNO-treated animals being able to run on the fixed-speed rotarod more than twice the distance, at a higher speed, than the distance they run on the accelerating rotarod before falling. Overall, this indicates that the partial inhibition of the CN-CL neurons does not disrupt the elementary motor abilities needed in the task.

We observed that the daily gain in latency depended on the initial latency to fall of the day, both for control mice and for CN-CL inhibited mice. Therefore, we can compare learning intensity across conditions by examining how much learning takes place within a single day for a given initial performance. We found that inhibition of the CN-CL neurons yielded a lower increase in latency to fall for a given initial performance than in control mice, indicating a weaker learning. Moreover, the administration of CNO in animals that learned under Saline treatment after

reaching maximal performance induced a sudden drop in performance, revealing a deficit in the execution of the learned task. Interestingly, animals that learned under CNO and were switched to Saline treatment after 7 days of treatment from the cerebello-thalamic inhibition showed little learning, suggesting that the cerebello-thalamic contribution is essential in the initial phase, possibly because of the encoding of the skill in different brain circuits once fully acquired (Yin, Mulcare et al. 2009). Overall, our results show that the CN-CL neurons contribute to both learning and execution of motor skills.

The inhibition of CN-VAL neurons during the task also yielded lower levels of performance in the Late stage, suggesting that these neurons contribute also to learning and/or execution of motor skills. Indeed, the mild defect in fixed speed rotarod could indicate the presence of a locomotor deficit, only visible at high speed. Although this locomotor deficit may not be critical in the first days (where the mice do not reach rotarod speeds of 20rpm – i.e. 130s) and where a learning deficit is already observed, the reduced maximal performance of mice following CN-VAL inhibition may thus in part result from motor deficit. Interestingly, both Dentate and Interposed nuclei contain some neurons with collaterals in both VAL and CL thalamic structures (Aumann and Horne 1996, Sakayori, Kato et al. 2019), suggesting that the effect on learning could be mediated by a combined action on the learning via the CL thalamus and via the VAL thalamus. However, consistent with (Sakayori, Kato et al. 2019), we found that the manipulations of cerebellar neurons retrogradely targeted either from the CL or from the VAL produced different effects in the task. Indeed, our tracing experiments showed that retrograde infection from the CL and VAL labelled different populations. This indicates that either the distinct functional roles of VAL-projecting or CL-projecting neurons reported in our study is carried by a subset of pathway-specific neurons without collaterals, or that our retrograde infections in VAL and CL preferentially targeted different cerebello-thalamic populations even if these populations had axon terminals, probably of different densities, in both thalamic regions. Future studies mapping the different subpopulations of CN neurons using anterograde markers and single axon reconstruction shall help clarify this point.

Beyond the thalamus, the CL and VAL may project to overlapping targets (Hunnicutt, Long et al. 2014), but the density of the projections may differ substantially. Since the CL is a narrow structure, accurate anterograde tracing is primarily available for the rat, and it shows only limited projections to the primary motor cortex, and the primary target seems to be the striatum and the cingulate and retrosplenial cortices (Fig 13, (Van der Werf, Witter et al. 2002)), even if some retrograde labelling from the motor cortex in the CL has indeed been observed (Cicirata, Angaut et al. 1986). Comparison of single axon reconstruction from the CL and VAL shows weaker branching patterns in the cortex for the CL (Kuramoto, Furuta et al. 2009) vs (Deschenes, Bourassa et al. 1996). Given the scarce existing evidence for retrosplenial and cingulate cortices involvement in accelerating rotarod learning, an effect of the CL in learning through the dorsal striatum (Costa, Cohen et al. 2004) seems more likely; however, the CL could also modulate learning through more minor cortical targets (secondary and primary motor cortices). Finally, the CL and VAL belong to different class of thalamic profile (Phillips, Schulmann et al. 2019) consistent with their distinct connectivity, and distinct functional role as found in our results.

Contribution of VAL-projecting CN neurons to offline consolidation

While in control mice, the final performance at the end of a session could be reproduced at the beginning of the next session, this preservation of performance across night was heavily altered when CN-VAL neurons were inhibited after the task, suggesting an impairment of offline consolidation. However, in this group of mice, the daily gain of performance increased across days, instead of decreasing, and compensated the overnight loss, this faster relearning might reveal the presence of “savings”. Therefore, if the inhibition of CN-VAL neurons alters the offline consolidation, a latent trace of the learning might remain, unaltered by CN-VAL inhibition, and allow for a faster relearning on the next day.

The effect of CNO peaks in less than an hour and lasts for several hours afterwards (Alexander, Rogan et al. 2009); therefore the disruption of offline consolidation reported above is produced by a disruption of the cerebellar activity in the few hours that follow the learning session. This falls in line with a number of evidence indicating that cerebellar-dependent learning is consolidated by the passage of time, even in the awake state (Shadmehr and Holcomb 1997, Muellbacher, Ziemann et al. 2002, Cohen, Pascual-Leone et al. 2005, Doyon, Korman et al. 2009, Nagai, de Vivo et al. 2017), although very few studies in Human have examined the impact of offline cerebellar stimulations on motor learning (Samaei, Ehsani et al. 2017). However, the recent observation of coordinated sleep spindles in the cortex and cerebellar nuclei (Xu, De Carvalho et al. 2022) provides a potential mechanism to a cerebellar involvement in consolidation since spindles are a cortical rhythm also associated with consolidation of motor learning (Barakat, Carrier et al. 2013, Lemke, Ramanathan et al. 2021).

In the case of rotarod, it has been noted that sleep is not required for the overnight preservation of performance (Nagai, de Vivo et al. 2017); however sleep may still be required for the change of cortical (Cao, Ye et al. 2015, Li, Ma et al. 2017) or striatal neuronal substrate of the accelerating rotarod skill (Yin, Mulcare et al. 2009). Therefore, while the offline activity of cerebellar nuclei could be more specifically associated in converting savings into readily available skills distributed over a wide circuit including the cortex and basal ganglia, multiple processes of memory consolidation would co-exist and operate at different timescales.

The existence of multiple timescales for consolidation has already been described in Human physiology where the movements could be consolidated without sleep while consolidation of goals (Cohen, Pascual-Leone et al. 2005) or sequences (Doyon, Korman et al. 2009) would require sleep. It is indeed difficult, as for most real-life skills, to classify the accelerating rotarod as a pure locomotor adaptive learning, or a pure locomotor sequence learning: on one hand, the shape of the rod and its rotation induce a change in the correspondence between steps and subsequent body posture and thus require some locomotor “adaptation”. On the other hand, the acceleration of the rod introduces sequential aspects: 1) speed increases occur in a fixed sequence, and 2) asymptotic performances require to use successively multiple types of gaits as the trial progresses (Buitrago, Schulz et al. 2004). Following offline inhibition of CN-VAL neurons, which aspect of the accelerating rotarod skill would be maintained and which would be lost? Faster relearning has been proposed to reflect an improved performance at selecting successful strategies (Morehead, Qasim et al. 2015, Ruitenberg, Koppelmans et al. 2018). An attractive possibility could be that novel sensory-motor correspondences encountered on the rotarod would remain learned, possibly leaving a memory trace within the cerebellum. Nevertheless, these elementary ‘strategies’ would not be properly temporally ordered into a sequence over the duration of a trial (~2 minutes) if the transfer to the cortex is disrupted. The learning session the next day would benefit from the existence of these fragments of skill (savings) in the cerebellum, but learning would still be required to order them properly. A similar idea has indeed been proposed for the contribution of cerebellum to sequence learning (Spencer and Ivry 2009). Alternatively, recent studies revealed cerebellar mechanisms which could serve sequence learning (Ohmae and Medina 2015, Khilkevich, Zambrano et al. 2018) and the offline inhibition of CN-VAL neurons could disrupt the consolidation of these sequences via the feedback collaterals of CN-VAL neurons to the cerebellar cortex (Houck and Person 2015). However, our study does not allow us to conclude on the nature of savings remaining after the offline inhibition of CN-VAL neurons.

Finally, our results on cerebellar consolidation of a task learning dependent on forebrain regions extend previous findings showing that simple oculomotor learning or adaptive reflex which are learned in the cerebellar cortex, undergo complete or partial consolidation via a transfer to cerebellar nuclei or brainstem structures (Bao, Chen et al. 2002, Kassardjian, Tan et al. 2005, Shutoh, Ohki et al. 2006). Our findings are also consistent with a dependance on post-learning neuronal activity (Okamoto, Shirao et al. 2011). Moreover, these paradigms suggest that such consolidation processes may indeed support savings (Medina, Garcia et al. 2001). While combined changes in the metabolic activity have been observed in the cerebellum and forebrain motor circuits along learning (e.g. Shadmehr and Holcomb 1997, Grafton, Hazeltine et al.

2002, Della-Maggiore and McIntosh 2005, Mawase, Bar-Haim et al. 2017), such studies provide little information on cerebellar output since the metabolic activity mostly reflect input activity (Howarth, Gleeson et al. 2012). Our study thus complements such studies by providing support for an increasing role in cerebellar output neurons during the task along learning and consolidation.

In conclusion, our results provide clear evidence for the existence of online contributions of neurons belonging to distinct cerebello-thalamic pathways to the acquisition and execution of motor skills encoded in a cerebello-striato-cortical network. They also show a contribution to the offline consolidation by a distinct cerebello-thalamic population. Thus, our work highlights the importance of studying the contribution to learning of single nodes in the brain motor network from an integrated perspective (Caligiore, Pezzulo et al. 2017, Krakauer, Hadjiosif et al. 2019) and supports a functional heterogeneity of cerebellar contributions to brain function (Diedrichsen, King et al. 2019).

Materials and methods

Animals

Adult male C57BL/6J mice (Charles River, France, IMSR Cat# JAX:000664, *RRID:IMSR_JAX:000664*), 8 weeks of age and 24 ± 0.4 g of weight at the beginning of the experiment were used in the study. Mice were fed with a chow diet and housed in a 22 °C animal facility with a 12-hr light/dark cycle (light phase 7am–7pm). The animals had free access to food and water. All animal procedures were performed in accordance with the recommendations contained in the European Community Directives (authorization number APAFIS#1334-2015070818367911 v3 and APAFIS #29793-202102121752192).

1. Behavioral experiments

1.1. Accelerating rotarod task

The rotarod apparatus (mouse rotarod, Ugo Basile) consisted of a plastic roller with small grooves running along its turning axis (Bearzatto, Servais et al. 2005). One week after injections, mice were trained with seven trials per day during seven consecutive days. This training protocol was chosen since performance progression takes several days and reaches a plateau over a few days. During each trial, animals were placed on the rod rotating at a constant speed (4 r.p.m.), then the rod started to accelerate continuously from 4 to 40 r.p.m. over 300 s. The latency to fall off the rotarod was recorded. Animals that stayed on the rod for 300 s were removed from the rotarod and recorded as 300 s. Mice that clung to the rod for two complete revolutions were removed from the rod and time was recorded. Between each trial, mice were placed in their home cage for a 5-minutes interval.

1.2. Open-field activity

Mice were placed in a circular arena made of plexiglas with 38 cm diameter and 15 cm height (Noldus, Netherlands) and video recorded from above. Each mouse was placed in the open-field for a period of 10 minutes before and after the accelerating rotarod task with the experimenter out of its view. The position of center of gravity of mice was tracked using an algorithm programmed in Python 3.5 and the OpenCV 4 library. Each frame obtained from the open-field videos were analyzed according to the following process: open-field area was selected and extracted in order to be transformed into a grayscale image. Then, a binary threshold was applied on this grayscale image to differentiate the mouse from the white background. To reduce the noise induced by the recording cable or by particles potentially present in the Open-field, a bilateral filter and a Gaussian blur were sequentially applied, since those components have a higher spatial frequency compared to the mouse. Finally, the OpenCV implementation of Canny algorithm was applied to detect the contours of the mouse, the position of the mouse was computed as mouse's center of mass. The trajectory of the center of mass were interpolated in x and y using scipy's Univariate Spline function (with smoothing factor s=0.2 x length of the data), allowing the

extraction of a smoothed trajectory of the mouse. The distance traveled by the mouse between two consecutive frames was calculated as the variation of position of the mouse multiplied by a scale factor, to allow the conversion from pixel unit to centimeters. The total distance traveled was obtained by summing the previously calculated distances over the course of the entire open-field session. The speed was computed as the variation of position of center points on two consecutive frames divided by the time between these frames (the inverse of the number of frames per seconds). This speed was then averaged by creating sliding windows of 1 second. After each session, fecal boli were removed and the floor was wiped clean with a damp cloth and dried after the passing of each mouse.

1.3. Horizontal bar

Motor coordination and balance were estimated with the balance beam test which consists of a linear horizontal bar extended between two supports (length: 90 cm, diameter: 1.5 cm, height: 40 cm from a padded surface). The mouse is placed in one of the sides of the bar and released when all four paws gripped it. The mouse must cross the bar from one side to other and latencies before falling are measured in a single trial session with a 3-minutes cut-off period.

1.4. Vertical pole

Motor coordination was estimated with the vertical pole test. The vertical pole (51 cm in length and 1.5 cm in diameter) was wrapped with white masking tape to provide a firm grip. Mice were placed heads up near the top of the pole and released when all four paws gripped the pole. The bottom section of the pole was fixated to its home-cage with the bedding present but without littermates. When placed on the pole, animals naturally tilt downward and climb down the length of the pole to reach their home cage. The time taken before going down to the home-cage with all four paws was recorded. A 20 seconds habituation was performed before placing the mice at the top of the pole. The test was given in a single trial session with a 3-minutes cut-off period.

1.5. Footprint patterns

Motor coordination was also evaluated by analyzing gait patterns using the approach used in (Simon, Seznec et al. 2004).. Mouse footprints were used to estimate foot opening angle and hindbase width, which reflects the extent of muscle loosening. The mice crossed an illuminated alley, 70 cm in length, 8 cm in width, and 16 cm in height, before entering a dark box at the end. Their hindpaws were coated with nontoxic water-soluble ink and the alley floor was covered with sheets of white paper. To obtain clearly visible footprints, at least 3 trials were conducted. The footprints were then scanned and examined with the Dvrtk software (Jean-Luc Vonesch, IGBMC). The stride length was measured with hindbase width formed by the distance between the right and left hindpaws. Linearity, defined as the average change in angle between consecutive right-right steps is calculated by drawing a line perpendicular to direction of travel, starting at first right footprint. After determining the angle between this perpendicular line and each subsequent right footprint, differences in angle were estimated between each consecutive step pair, and the average of absolute values of all angles was calculated. A high linearity score is indicative of nonlinear movement. Sigma, describing the regularity of step length, is defined as the standard variation of all right-right and left-left step distance. Gait width, the average lateral distance between opposite left and right steps, is determined by measuring the perpendicular distance of a given step to a line connecting its opposite preceding and succeeding steps. Alternation coefficient, describing the uniformity of step alternation, is calculated by the mean of the absolute value of 0.5 minus the ratio of right-left distance to right-right step distance for every left-right step pair.

1.6. Grid test

The grid test is performed to measure the strength of the animal. It consists in placing the animal on a grid which tilts from a horizontal position of 0° to 180°. The animal is registered by the side and the time until it falls is measured. The time limit for this experiment is 30 seconds. In the cases where the mice climbed up to the top of grid, a maximum latency of 30 seconds was applied.

1.7. Fixed speed rotarod

Motor coordination, postural stability and fatigue were estimated with the rotarod (mouse rotarod, Ugo Basile). Facing away from the experimenter's view, the mice placed on top of the plastic roller were tested at constant speeds (5, 10, 15 and and 20 r.p.m). Latencies to fall were measured for up to 3 minutes in a single trial.

2. Cerebellar outputs inactivation

We used evolved G-protein-coupled muscarinic receptors (hM4Di) that are selectively activated by the pharmacologically inert drug Clozapine-N-Oxide (CNO) (Alexander, Rogan et al. 2009). In our study, non-cre and cre dependent version of the hM4Di receptor packaged into an AAV were used in order to facilitate the stereotaxic-based delivery and regionally restricted the expression of hM4Di. As demonstrated previously (Anaclet, Ferrari et al. 2014, Anaclet, Pedersen et al. 2015, Venner, Anaclet et al. 2016, Pedersen, Ferrari et al. 2017, Anaclet, De Luca et al. 2018), hM4Di receptor and ligand are biologically inert in the absence of ligand. Moreover, at the administered dose of 1 mg/kg, CNO injection induces a maximum effect during the 1–3 h post-injection period (Anaclet, Ferrari et al. 2014, Anaclet, De Luca et al. 2018) which enables us to confirm that during the whole duration of our protocols the CNO was still effective. CNO administration in sham-operated animals, and saline injection in sham-operated and DREADD-expressing animals were also tested to distinguish the effect of specific inhibition of the targeted neuronal population from a nonspecific effect of CNO or its metabolite clozapine (Gomez, Bonaventura et al. 2017) or from the expression of DREADD without CNO.

In order to globally inactivate the cerebellar outputs, stereotaxic surgeries were used to inject DREADD viral constructs bilaterally into the Dentate, Interposed and Fastigial nucleus. Mice were anesthetized with isoflurane for induction (3% in closed chamber during 4-5 minutes) and placed in the Kopf stereotaxic apparatus (model 942; PHYMEP, Paris, France) with mouse adapter (926-B, Kopf), and isoflurane vaporizer. Anesthesia was subsequently maintained at 1–2% isoflurane. A longitudinal skin incision was performed before removing the connective tissue on the skull and expose the bregma and lambda sutures of the skull. The coordinates for the Dentate nucleus injections were: 6.2 mm posterior to bregma, +/-2.3 mm lateral to the midline and -2.4 mm from dura while the Interposed injections were placed anteroposterior (AP) -6.0 mm, mediolateral (ML) = +/-1.5 mm in respect to bregma and dorsoventral (DV) -2.1 mm depth from dura. Finally, the Fastigial injections were placed -6.0 AP, +/-0.75 ML in respect to bregma and -2.1 depth from dura. Small holes were drilled into the skull and DREADD (AAV5-hSyn-hM4D(Gi)-mCherry, University of North Carolina Viral Core, 7.4×10^{12} vg per ml, 0.2 µl) or control (AAV5-hSyn-EGFP, UPenn Vector Core, the same concentration and amount) virus were delivered bilaterally via quartz micropipettes (QF 100-50-7.5,Sutter Instrument, Novato, USA) connected to an infusion pump (Legato 130 single syringe, 788130-KDS, KD Scientific, PHYMEP, Paris, France) at a speed of 100 nl/minutes. The micropipette was left in place for an additional 5 minutes to allow viral dispersion and prevent backflow of the viral solution into the injection syringe. The scalp wound was closed with surgical sutures, and the mouse was kept in a warm environment until resuming normal activity. All animals were given analgesic and fluids before and after the surgery.

3. Chronic in vivo extracellular recordings in non-DREADD or DREADD mice

In a set of mice sham EGFP-injected or DREADD-injected mice, (Dentate, Fastigial and Interposed) bundles of electrodes were implanted into the cerebellar nuclei. Both non-DREADD or DREADD injections (AAV5-hSyn-hM4D(Gi)-mCherry or AAV5-hSyn-EGFP) and electrodes implantation were performed the same day. This experiment was performed in order to evaluate and validate that hM4D(Gi) receptors decrease the activity within the three cerebellar nuclei.

Recordings were performed in awake behaving control mice during the open-field sessions. Recordings and analysis were performed using an acquisition system with 32 channels (sampling rate 25kHz; Tucker Davis Technology System 3) as described in (de Solages, Szapiro et al. 2008,

Popa, Spolidoro et al. 2013). Bundles of electrodes consisting in nichrome wire (0.005 inches diameter, Kanthal RO-800) folded and twisted into six to eight channels were implanted (electrode tip located at Fastigial: -6.0 AP, +/-0.75 ML, -2.1 depth from dura; Interposed: -6.0 AP, +/-1.5 ML, -2.1 depth from dura; Dentate: -6.2 AP, +/-2.3 ML, -2.4 depth from dura). To protect these bundles and ensure a good electrode placement, they were then held through a metal tube (8-10mm length, 0.16-0.18mm inner diameter, Coopers Needle Works Limited, UK) attached to an electrode interface board (EIB-16 or EIB-32; Neuralynx) by Loctite universal glue. Microwires of each bundle were connected to the EIB with gold pins (Neuralynx). The entire EIB and its connections were secured in place by dental cement for protection purpose. Electrodes were cut to the desired length before implantation (extending 0.5mm below tube tip). The 1kHz impedance of each electrode was measured and lowered by gold-plating to 200–500 kΩ. Mice were anesthetized with isoflurane and placed in the stereotaxic apparatus, then the skull was drilled and dura were removed above cerebellar nuclei recording site (see section 2 for a detailed description of the surgical procedure). Electrodes bundles were lowered into the brain, the ground was placed above the cerebellar cortex and the assembly was secured with dental cement. One week after the surgery to allow for virus expression, we started to record cellular activity in the cerebellar nuclei in freely moving mice placed in the open-field. Mice were habituated to the recording cable for 2–3 d before starting the recording. Recordings in the open-field were performed before and after CNO or saline (SAL) injection. The mice were recorded for a 10 minutes baseline period followed by intraperitoneal injections of CNO 1mg/kg or SAL which were performed in a random sequence using a crossover design. After CNO or SAL injection, the mice were recorded during 30 minutes before and 15 minutes after the accelerating rotarod task protocol. Signal was acquired by headstage and amplifier from TDT (RZ2, RV2, Tucker-Davis Technologies, USA) and analyzed with Matlab and Python 3.5. The mice were not recorded until CNO washout but successive days yielded similar starting firing rates (Fig. Suppl. 6a)

The spike sorting was performed with MountainSort version 4 (Chung, Magland et al. 2017) (*https://github.com/flatironinstitute/mountainsort*). Single units were isolated based on quantitative quality metrics computed after MountainSort 4 spike sorting (Phyton 3.8). Units were required to have a signal-to-noise ratio (SNR) greater than 5, inter-spike interval (ISI) violations less than 1%, an amplitude cutoff below 0.1, a presence ratio above 0.9, a firing rate greater than 0.1 Hz, and at least 50 detected spikes. In addition, units were assessed for temporal stability across the recording using autocorrelograms and presence over the recording, ensuring there were no prolonged periods of total inactivity. Units meeting these criteria were deemed well-separated and reliable for further analysis.

The average firing rates were computed from the recordings during the openfield sessions. At the end of experiments, the placement of the electrodes was verified. Firing rate modulation was computed as the difference of firing rate after and before CNO injection divided by the average of these two firing rates; it is therefore bounded between -100% (total suppression) and +100% (discharge only post-treatment).

4. Cerebellar-thalamic outputs inactivation

In order to inhibit specifically cerebellar outputs to the centrolateral (CL) and/or ventral anterior lateral (VAL) thalamus, we applied a pathway-specific approach (Boender et al., 2014). The technique comprises the combined use of a CRE-recombinase expressing canine adenovirus-2 (CAV-2) injected in the thalamus and an adeno-associated virus (AAV-hSyn-DIO-hM4D(Gi)-mCherry) that contains the floxed inverted sequence of the DREADD hM4D(Gi)-mCherry injected in the cerebellar nuclei. It entails the infusion of these two viral vectors into two sites that are connected through direct neuronal projections. AAV-hSyn-DIO-hM4D(Gi)-mCherry is infused in the site where the cell bodies are located, while CAV-2 is infused in the area that is innervated by the corresponding axons. After infection of axonal terminals, CAV-2 is transported towards the cell bodies and expresses CRE-recombinase (Kremer et al., 2000; Hnasko et al., 2006). AAV-hSyn-DIO-hM4D(Gi)-mCherry contains the floxed inverted sequence of hM4D(Gi)-mCherry, which is reoriented in the presence of CRE, prompting the expression of hM4D(Gi)-mCherry. This ensures that hM4D(Gi)-mCherry is not expressed in all AAV-hSyn-DIO-hM4D(Gi)-mCherry infected neurons,

but exclusively in those that are also infected with CAV-2. Using the same procedures described above, 0.4 µl of the retrograde canine adeno-associated cre virus (CAV-2-cre, titter ≥ 2.5×10^8) (Plateforme de Vectorologie de Montpellier, Montpellier, France) was bilaterally injected in the CL (from bregma: AP -1.70 mm, ML ±0.75 mm, DV −3.0 mm) and VAL (from bregma: AP -1.4 mm, ML ±1.0 mm, DV −3.5 mm). In addition, 0.2 µl of AAV-hSyn-DIO-hM4D(Gi)-mCherry (UNC Vector Core, Chapel Hill, NC, USA) was bilaterally injected one week later into the cerebellar nuclei, focusing on the Dentate (from bregma: AP −6.2 mm, ML ±2.3 mm, DV −2.4 mm) and Interposed (from bregma: AP −6.0 mm, ML ±1.5 mm, DV −2.1 mm) nucleus. All the stereotactic coordinates were determined based on The Mouse Brain Atlas (Paxinos and Franklin 2004).

5. Behavioral experiments design

Behavioral tests were performed one week following stereotaxic surgery to allow for virus expression. Balance beam, vertical pole, footprint patterns, grid test and fixed speed rotarod experiments were performed 30 minutes after CNO (1 mg/kg, ip) or SAL injections. Two different strategies were used for the accelerating rotarod motor learning task experiments: 1) CNO (1 mg/kg, ip) or SAL was injected every day 30 minutes before the 1st trial of the accelerating rotarod task. Four days later to ensure a proper CNO washout, mice were retested by receiving 7 trials for two consecutive daily sessions. Drug-free mice received CNO (1mg/kg) or SAL 30 minutes before the first trial in both days. The treatments were inverted meaning that those animals that received CNO during the preceding 7 days in this case were injected with SAL and the other way around. 2) CNO (1 mg/kg, ip) was injected 30 minutes after last trial at the day 1, 2 and 3; subsequently mice received SAL 30 minutes after last trial at the day 4, 5 and 6 of the accelerating rotarod task.

The DREADD ligand Clozapine-N-Oxide (CNO, TOCRIS, Bristol, UK) was dissolved in SAL (0.9% sodium chloride) and injected intraperitoneally at 1mg/kg.

5. Histology

Mice were anesthetized with ketamine/xylazine (100 and 10 mg/kg, i.p., respectively) and rapidly perfused with ice-cold 4% paraformaldehyde in phosphate buffered SAL (PBS). The brains were carefully removed, postfixed in 4% paraformaldehyde for 24 h at 4 °C, cryoprotected in 20% sucrose in PBS. The whole brain was cut into 40-µm-thick coronal sections on a cryostat (Thermo Scientific HM 560; Waltham, MA, USA). The sections were mounted on glass slides sealed with Mowiol mounting medium (Mowiol® 4-88; Sigma-Aldrich, France). Verification of virus injection site and DREADDs expression was assessed using a wide-field epifluorescence microscope (BX-43, Olympus, Waltham, MA, USA) using a mouse stereotaxic atlas (Paxinos and Franklin 2004). We only kept mice showing a well targeted viral expression centered on the targeted nucleus. Representative images of virus expression were acquired a Zeiss 800 Laser Scanning Confocal Microscope (×20 objective, NA 0.8) (Carl Zeiss, Jena, Germany). Images were cropped and annotated using Zeiss Zen 2 Blue Edition software.

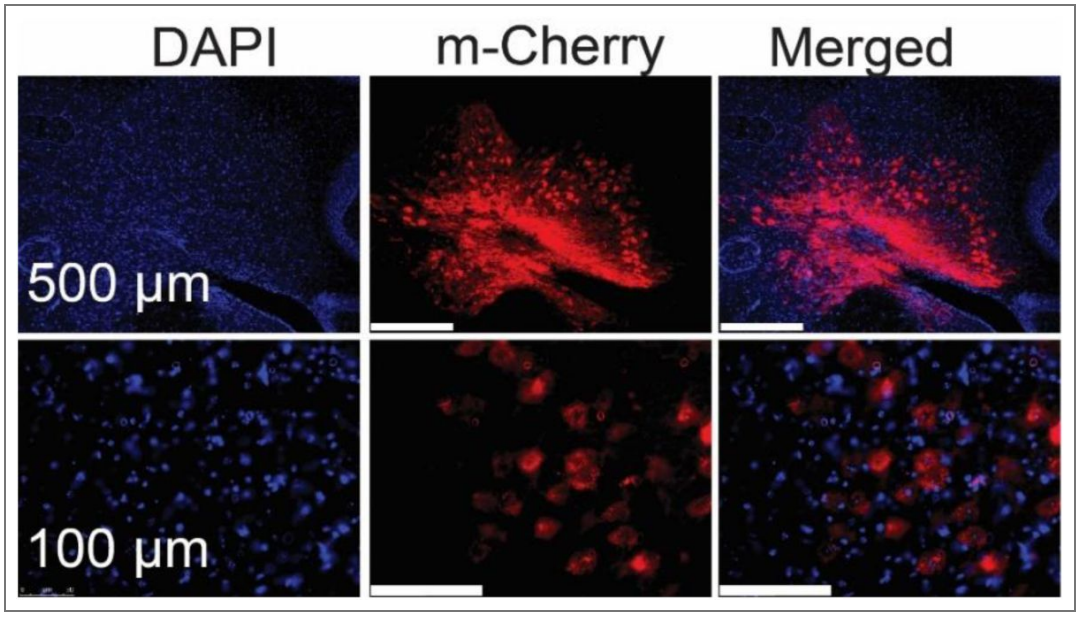
Quantification and statistical analysis

Latency to fall (mean ± S.E.M) in rotarod for chemogenetic experiments were analyzed using one-way ANOVA repeated measure followed by two types for Posthoc tests: paired t-test for repeated-measure comparison and independent t-test for cross group comparisons. Locomotor activity (velocity) in open-field (mean ± S.E.M) was analyzed using two-way ANOVA repeated measure (treatment×moment) followed by t-test Posthoc (comparisons between treatments for each open-field session). Fixed speed rotarod (mean ± S.E.M) was analyzed using two-way ANOVA repeated measure (treatment×speed) followed by t-test Posthoc (comparisons between treatments for each speed steps). Footprint patterns parameters, horizontal bar, vertical pole and grid test were analyzed using one-way ANOVA. Data are represented as boxplots (median quartiles and interquartile range plus outliers).

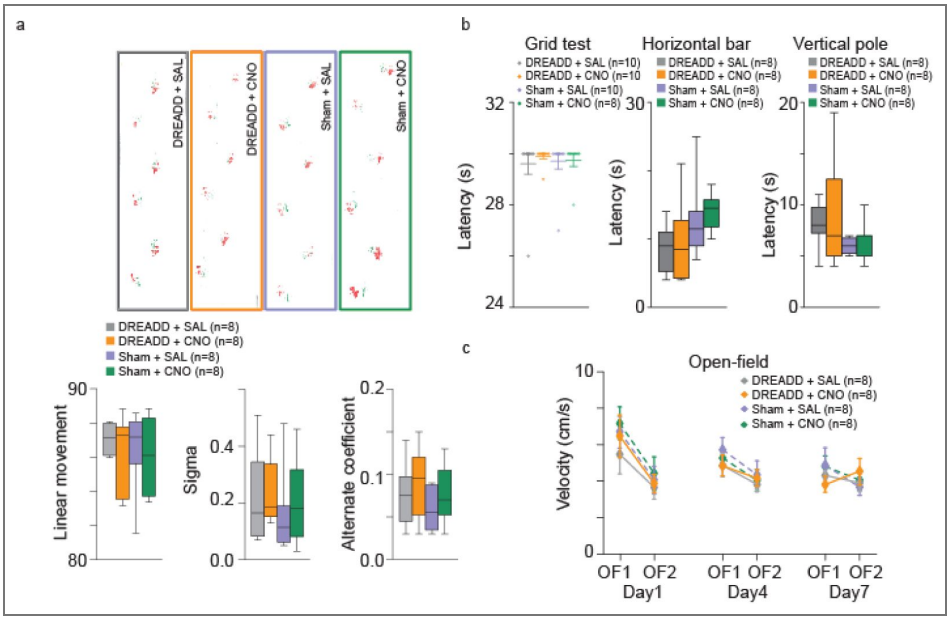
Latency to fall exhibit variations between successive trials, so that single trial performance are poor estimators of the skill level. To get a more robust estimate of the initial and final skill level and thus of learning of each day, we performed a linear regression on the latency to fall for each

day and each animal; the within-day learning and overnight loss was estimated from the start- and end-points (corresponding to trial 1 and 7) of each regression segment (Figure 2a). To estimate the interdependence of initial performance of the day, within day learning and inter-day learning, we used Deming linear regression, assuming equal variance of the random noise of the measured quantity on the x- and y-axis. This assumption is made because the measures used in the Deming regressions are all derived from latency to fall, which is modeled as a combination of skill level and random noise. Deming confidence intervals were obtained by bootstrap. These values were computed with the R package mcr (version 1.3.3.1). To test if treatments altered the relationship between *initial performance* vs *learning* or *daily learning* vs *overnight learning*, we compared the distribution of signed distance ('residuals') to the control Deming regression line between groups. Indeed, while in control groups these quantities show clear linear relationships, this is far less the case in treatment groups (possibly due to the variability of the effect of the treatment -efficacy of viral injections- and/or to the disruption of the neurobiological processes underlying these relationships) and thus precludes direct comparisons of the regressions between groups. Therefore, to test if there is a change in these relationships following treatments, we examined to which extent data points from treatment groups in bivariate comparisons (*initial perf * daily learning*, *daily learning x consolidated learning*) are distributed around the control group regression line differently from the data points of control groups. We therefore use a Wilcoxon comparison of the distributions of residuals of the treatment groups vs residuals of control groups around control regression line to test if the relationship examined is disrupted by the treatment for this group: e.g. if the median of residuals of the treatment group is significantly lower than the median of the control group in the *initial performance * daily learning* comparison, it indicates that learning is slower taking into account its dependance in initial performance. Similarly, if the residuals of the treatment group are lower than those of the control group in the *daily learning * consolidated learning* comparison, it indicates that consolidation is weaker.

Supplementary figures

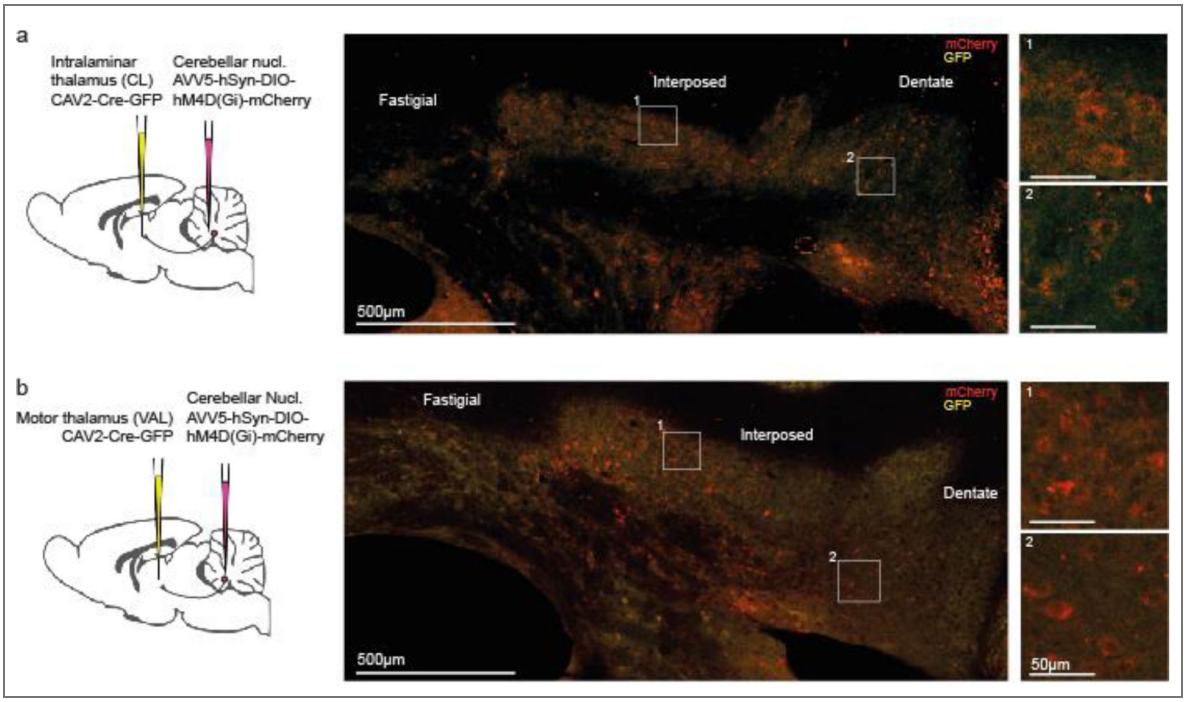


Supplementary Figure 1. **DAPI positive neurons expressing hM4Di-DREADD-mCherry in cerebellar nuclei.**



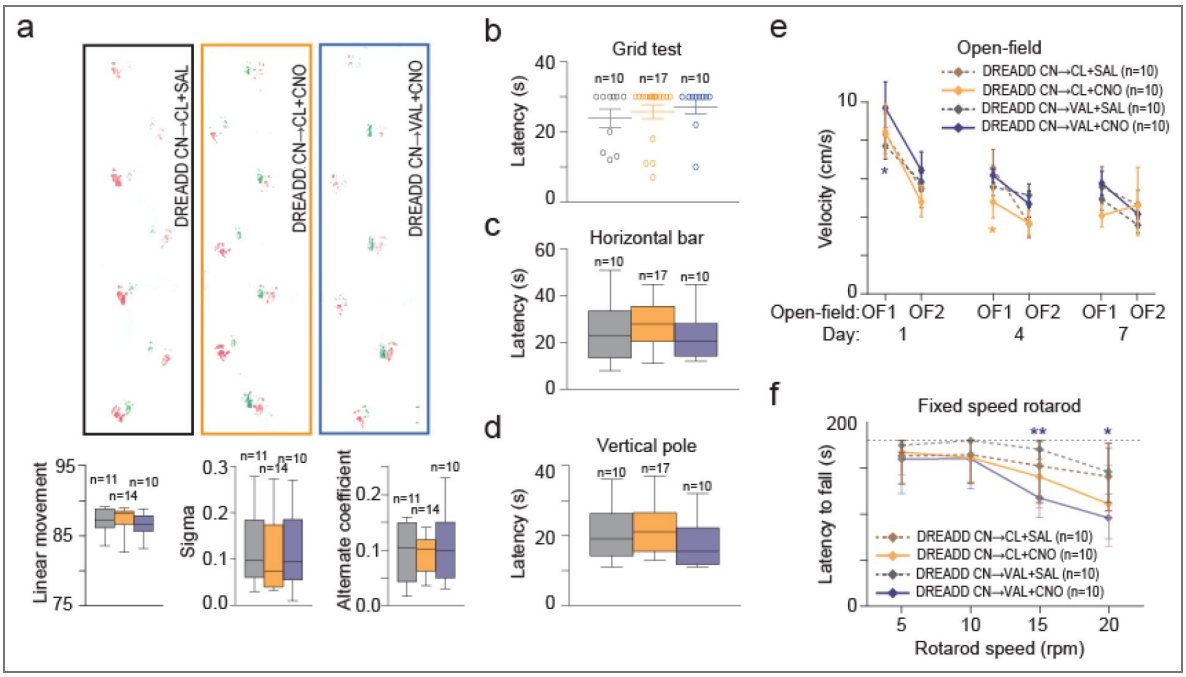
Supplementary Figure 2. Cerebellar nuclei inhibition did not affect execution and fatigue, locomotion, motor coordination, balance and strength.

a) Footprint patterns were quantitatively assessed for 3 parameters as shown on representative footprint patterns for all experimental groups. Three parameters are represented graphically: linear movement (bottom left), sigma (bottom middle) and alternation coefficient (bottom right). **b**) tests of strength and coordination. Grid test: latency reflecting the time before falling from the grid. 30 seconds of cut-off of was established as the maximum latency (dotted line on figure). Horizontal bar: latency to cross the horizontal bar (balance beam test) for all experimental groups. Vertical pole: latency to reach home cage in vertical pole test for all experimental groups. **c**) Locomotor activity (Velocity) in DREADD and non-DREADD (Sham) injected mice after CNO or SAL injection during open-field sessions before (OF1) and after (OF2) rotarod for experimental days 1, 4 and 7. *n* indicates the number of mice. Boxes represent quartiles and whiskers correspond to range; points are singled as outliers if they deviate more than 1.5 x interquartile range from the nearest quartile. No posthoc significant difference was found for the data presented in this figure (see Supplementary Tables 6-7 for detailed statistics).



Supplementary Figure 3. Expression of hM4D(Gi)-mCherry in the cerebellar nuclei following CL and VAL injections.

a) left: schematics of the experiment for CN-CL groups, right: distribution of labeled neurons. b) same as a for CN-VAL groups.

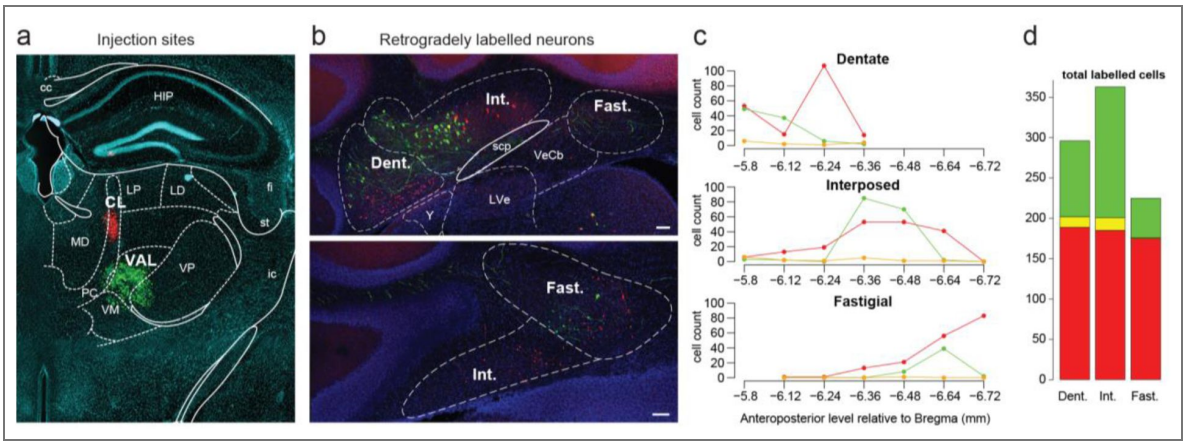


Supplementary Figure 4. Inhibition of CN-CL or CN-VAL by 1mg/kg CNO does not affect execution and fatigue, locomotion, motor coordination, balance and strength.

a) Footprint patterns were quantitatively assessed for 3 parameters as shown on representative footprint patterns (top) for all experimental groups. Three parameters are represented graphically: linear movement (bottom left), sigma (bottom middle and alternation coefficient (bottom right). b) Latency reflecting the time before falling from the grid. 30 seconds of cut-off of was established as the maximum latency (dotted line on figure). c) Latency to cross the horizontal bar (balance beam test) for all experimental groups. d) Latency to reach home cage in vertical pole test for all experimental groups. e) Locomotor activity (Velocity) in DREADD injected mice after CNO or SAL injection during open-fields sessions before (OF1) and after (OF2) rotarod for day 1, 4 and 7 (**p<0.01 paired t-test OF1 vs OF2). f) Latency to fall during fixed speed rotarod (5, 10, 15, 20 r.p.m.) for all experimental groups. One-way repeated measure ANOVA was performed on averaged values for each speed steps in each experimental group followed by a Tukey Posthoc pairwise comparison (*p<0.05, **p<0.01 for CN->VAL group CNO vs SAL). CN, cerebellar nuclei, CL, centrolateral thalamus; VAL, ventral anterior lateral thalamus. *n* indicates the number of mice. Boxes represent quartiles and whiskers correspond to range; points are singled as outliers if they deviate more than 1.5 x interquartile range from the nearest quartile. (See Supplementary Tables 11-13 for detailed statistics)

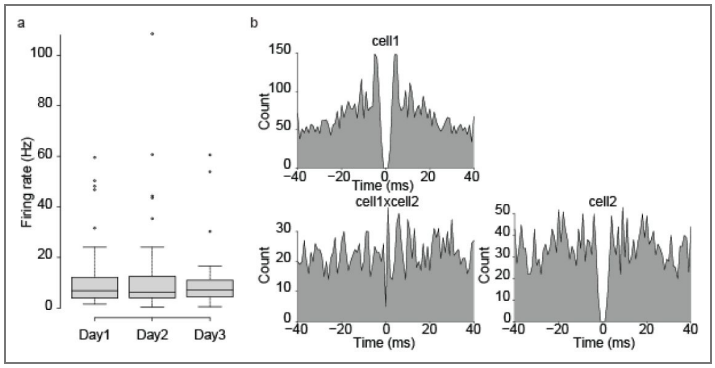
Supplementary Figure 5. Retrograde infections from the CL and VAL differentially label cerebellar nuclei populations.

a. injections sites targeted at the VAL (AAVrg-CAG-GFP) and CL (AAVrg-CAG-mCherry). b. example of retrogradely infected neurons at two different levels of the cerebellar nuclei. c. summary of neuronal counts at 7 different anteroposterior levels (cumulated from 2 mice, where counts at each level and each cerebellar nuclei exhibit high correlation r=0.91). Red infection from the CL, green infection from VAL, orange: infection from both structures. d. summary of total cell counts per nucleus.



Supplementary Figure 6. Extracellular recordings in the CN.

a. similarity of initial openfield firing rates for cerebellar units isolated on successive days. b. example of auto- and cross-correlogram from stimultaneously recorded units.



Data availability

The data will be made available on a Dryad repository upon acceptance of the manuscript.

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Additional information

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Author contributions

Conceptualization, A.P.V., D.P. and C.L.; Methodology, A.P.V. and D.P.; Software, R.W.S. and C.L.; Formal Analysis, A.P.V., R.W.S., S.F. and C.L.; Investigation, A.P.V., R.W.S., C.M.H, J.L.F. and M.S.; Data Curation, A.P.V., R.W.S., S.F. and C.L.; Writing –Original Draft, A.P.V., C.L. and D.P.; Writing –Review & Editing, D.P. and C.L.; Visualization, A.P.V., R.W.S., J.L.F and C.L.; Supervision, D.P. and C.L.; Funding Acquisition, D.P. and C.L.

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Author ORCID iDs

Andres P Varani: https://orcid.org/0000-0003-4227-5785
Caroline Mailhes-Hamon: https://orcid.org/0000-0002-7009-7798
Romain W Sala: https://orcid.org/0000-0002-6768-043X
Marie Sarraudy: https://orcid.org/0009-0004-4546-1203
Sarah Fouda: https://orcid.org/0009-0007-1844-5373
Jimena L Frontera: https://orcid.org/0000-0002-3167-8768
Clément Léna: https://orcid.org/0000-0002-1431-7717
Daniela Popa: https://orcid.org/0000-0002-8389-1122

Additional files

Supplementary tables and figures

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Peer reviews

Reviewer #2 (Public review):

Summary:

This study examines the contribution of cerebello-thalamic pathways to motor skill learning and consolidation in an accelerating rotarod task. The authors use chemogenetic silencing to manipulate activity of cerebellar nuclei neurons projecting to two thalamic subregions that target motor cortex and striatum. By silencing these pathways during different phases of task acquisition (during task vs after task), the authors report valuable finding of the involvement of these cerebellar pathways in learning and consolidation.

Strengths:

The experiments are well-executed. The authors perform multiple controls and careful analysis to solidly rule out any gross motor deficits caused by their cerebellar nuclei manipulation. The finding that cerebellar projections to the thalamus are required for learning and execution of the accelerating rotarod task adds to a growing body of literature on the interactions between the cerebellum, motor cortex, and basal ganglia during motor

learning. The finding that silencing the cerebellar nuclei after task impairs consolidation of the learned skill is interesting.

Revision comment:

The revised manuscript is improved in clarity and methodological detail. An important addition is the retrograde labeling data showing a degree of anatomical segregation between CN->CL and CN->VAL pathways that strengthens their reported different functional roles. I still think that potential effects on motor execution when cerebellar nuclei are silenced during task performance may complicate interpretations specifically related to learning. However, the evidence supporting a role of the cerebellar nuclei in off-line consolidation is convincing.

Overall, the study outlines a multifaceted role of the cerebellum in motor learning, consolidation, and execution. The demonstration that cerebellar projections to distinct forebrain structures contribute to these processes is significant.

https://doi.org/10.7554/eLife.102813.2.sa2

Reviewer #3 (Public review):

Summary:

Varani et al present important findings regarding the role of distinct cerebellothalamic connections in motor learning and performance. Their key findings are that: 1) cerebellothalamic connections are important for learning motor skills, 2) cerebellar efferents specifically to the central lateral (CL) thalamus are important for short-term learning, 3) cerebellar efferents specifically to the ventral anterior lateral (VAL) complex are important for offline consolidation of learned skills, and 4) that once a skill is acquired, cerebellothalamic connections become important for online task performance. The authors went to great lengths to separate effects on motor performance from learning, for the most part successfully. While one could argue about some of the specifics, there is little doubt that the CN-CL and CN-VAL pathways play distinct roles in motor learning and performance. An important next step will be to dissect the downstream mechanisms by which these cerebellothalamic pathways mediate motor learning and adaptation.

Strengths:

(1) The dissociation between on-line learning through CN-CL and offline consolidation through CN-VAL is convincing.

(2) The ability to tease learning apart from performance using their titrated chemogenetic approach is impressive. In particular, their use of multiple motor assays to demonstrate preserved motor function and balance is an important control.

(3) The evidence supporting the main claims is convincing, with multiple replications of the findings and appropriate controls.

(4) The retrograde tracing experiments (Supplementary Figure 5) demonstrate convincingly that the CN-VAL and CN-CL projections are almost entirely segregated,

Weaknesses:

(1) Despite the care the authors took to demonstrate that their chemogenetic approach does not impair online performance, there is (as they acknowledge in the Discussion) impaired rotarod performance at fixed higher speeds in Supplementary Figure 4f for CN-VAL projections, suggesting that there could be subtle changes in motor performance below the level of detection of their assays. There is also a trend in the same direction that did not pass

significance for CN-CL at higher speeds, suggesting that part of the effects could be related to subtle deficits in performance.

https://doi.org/10.7554/eLife.102813.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***eLife Assessment***
>
> *This study provides evidence that cerebellar projections to the thalamus are required for learning and execution of motor skills in the accelerating rotarod task. This important study adds to a growing body of literature on the interactions between the cerebellum, motor cortex, and basal ganglia during motor learning. The data presentation is generally sound, especially the main observations, with some limitations in describing the statistical methods and a lack of support for two separate cerebello-thalamic pathways, which is incomplete in supporting the overall claim.*

We completed the MS by adding a double retrograde labelling study showing that the two pathways have limited overlap and by addressing the other concerns.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *This is an interesting manuscript tackling the issue of whether subcircuits of the cerebellum are differentially involved in processes of motor performance, learning, or learning consolidation. The authors focus on cerebellar outputs to the ventrolateral thalamus (VL) and to the centrolateral thalamus (CL), since these thalamic nuclei project to the motor cortex and striatum respectively, and thus might be expected to participate in diverse components of motor control and learning. In mice challenged with an accelerating rotarod, the investigators reduce cerebellar output either broadly, or in projection-specific populations, with CNO targeting DREADD-expressing neurons. They first establish that there are not major control deficits with the treatment regime, finding no differences in basic locomotor behavior, grid test, and fixed-speed rotarod. This is interpreted to allow them to differentiate control from learning, and their inter-relationships. These manipulations are coupled with chronic electrophysiological recordings targeted to the cerebellar nuclei (CN) to control for the efficacy of the CNO manipulation. I found the manuscript intriguing, offering much food for thought, and am confident that it will influence further work on motor learning consolidation. The issue of motor consolidation supported by the cerebellum is timely and interesting, and the claims are novel. There are some limitations to the data presentation and claims, highlighted below, which, if amended, would improve the manuscript.*

We thank the reviewer for the positive comments and insightful critics.

> *(1) Statistical analyses: There is too little information provided about how the Deming regressions, mean points, slopes, and intercepts were compared across conditions. This is important since in the heart of the study when the effects of inactivating CL- vs VL-projecting neurons are being compared to control performance, these statistical methods become paramount. Details of these comparisons and their assumptions should be added to the Methods section. As it stands I barely see information about these tests, and only in the figure legends. I would also like the authors to describe whether there is a criterion for significance in a given correlation to be then compared to another. If I have a weak correlation for a regression model that is non-significant, I would not want to*

> *'compare' that regression to another one since it is already a weak model. The authors should comment on the inclusion criteria for using statistics on regression models.*

We thank the reviewer for pointing out this weakness of description. The description of the Methods has thus been expanded and better justified in the "Quantification and statistical analysis" section.

We agree with the reviewer that comparison between Deming regressions would be fragile due to the weakness of these regression in treatment groups (while they are quite robust for control groups) and they are not included in the MS, although Deming regression coefficients with their confidence intervals are now provided for all groups in the statistical tables. As now more clearly explained in the Methods, the comparisons between groups are based on the distribution of residuals around regressions of the control regression lines. If we understand correctly the reviewer's request, the control groups are all included.

> *(2) The introduction makes the claim that the cerebellar feedback to the forebrain and cortex are functionally segregated. I interpreted this to mean that the cerebellar output neurons are known to project to either VL or CL exclusively (i.e. they do not collateralize). I was unaware of this knowledge and could find no support for the claim in the references provided (Proville 2014; Hintzer 2018; Bosan 2013). Either I am confused as to the authors' meaning or the claim is inaccurate. This point is broader however than some confusion about citation.*

The references are not cited in the context of collaterals from the DCN but for the output channels of the basal ganglia and cerebellum: "They [basal ganglia and cerebellum] send projections back to the cortex via anatomically and functionally segregated channels, which are relayed by predominantly non-overlapping thalamic regions (Bostan, Dum et al. 2013, Proville, Spolidoro et al. 2014, Hintzen, Pelzer et al. 2018)." Indeed, the thalamic compartments targeted by the basal ganglia and cerebellum are distinct, and in the Proville 2014, we showed some functional segregation of the cerebello-cortical projections (whisker vs orofacial ascending projections). Hintzen et al. have indeed performed an extensive review indicating the limited overlap between cerebellar- and basal ganglia-recipient territories. The sentence has been corrected to clarify what the "They" referred to.

> *The study assumes that the CN-CL population and CN-VL population are distinct cells, but to my knowledge, this has not been established. It is difficult to make sense of the data if they are entirely the same populations, unless projection topography differs, but in any event, it is critical to clarify this point: are these different cell types from the nuclei? how has that been rigorously established?; is there overlap? No overlap? Etc. Results should be interpreted in light of the level of this knowledge of the anatomy in the mouse or rat.*

There is indeed a paragraph devoted to the discussion of this point (last part of the section "A specific impact on learning of CL-projecting CN neurons."). Briefly, we actually know from the literature that there is a degree of collateralization (CN neurons projecting to both VAL and CL, see refs cited above), but as the reviewer says, it does not seem logically possible that the exact same population would have different effects, which are very distinct during the first learning days. The only possible explanation is the CN-CL and CN-VAL infections recruit somewhat different populations of neurons. We have now added more experiments to support our finding using retrograde infections using two rAAV viruses expressing red and green fluorescent reporter. These experiments confirm the limited overlap of the two populations of interest obtained by retrograde infection. We feel thus confident that while some CN neurons may project to both structures, retrograde infection strategies thus appear to differentially infect CN populations.

> *(3) It is commendable that the authors perform electrophysiology to validate DREADD/CNO. So many investigators don't bother and I really appreciate these data.*

> *Would the authors please show the 'wash' in Figure 1a, so that we can see the recovery of the spiking hash after CNO is cleared from the system? This would provide confidence that the signal is not disappearing for reasons of electrode instability or tissue damage/ other.*

The recordings were not extended to the wash period, but examination of the firing rate before CNO on successive days did not evidence major changes in the population firing rate (this is now shown in a new supplementary figure 6).

> *(4) I don't think that the "Learning" and "Maintenance" terminology is very helpful and in fact may sow confusion. I would recommend that the authors use a day range " Days 1-3 vs 4-7" or similar, to refer to these epochs. The terminology chosen begs for careful validation, definitions, etc, and seems like it is unlikely uniform across all animals, thus it seems more appropriate to just report it straight, defining the epochs by day. Such original terminology could still be used in the Discussion, with appropriate caveats.*

Since reference to these time windows is repeatedly used in the text we have shifted to “Early” and “Late” phase terminology.

> *(5) Minor, but, on the top of page 14 in the Results, the text states, "Suggesting the presence of a 'critical period' in the consolidation of the task." I think this is a non-standard use of 'critical period' and should be removed. If kept, the authors must define what they mean specifically and provide sufficient additional analyses to support the idea. As it stands, the point will sow confusion.*

This has been corrected to: “suggesting the cerebellar contribution to the consolidation of the task is critical early in the learning process and cannot be easily reinstated later”

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *This study examines the contribution of cerebello-thalamic pathways to motor skill learning and consolidation in an accelerating rotarod task. The authors use chemogenetic silencing to manipulate the activity of cerebellar nuclei neurons projecting to two thalamic subregions that target the motor cortex and striatum. By silencing these pathways during different phases of task acquisition (during the task vs after the task), the authors report valuable findings of the involvement of these cerebellar pathways in learning and consolidation.*
>
> *Strengths:*
>
> *The experiments are well-executed. The authors perform multiple controls and careful analysis to solidly rule out any gross motor deficits caused by their cerebellar nuclei manipulation. The finding that cerebellar projections to the thalamus are required for learning and execution of the accelerating rotarod task adds to a growing body of literature on the interactions between the cerebellum, motor cortex, and basal ganglia during motor learning. The finding that silencing the cerebellar nuclei after a task impairs the consolidation of the learned skill is interesting.*

We thank the reviewer for the positive comments and insightful critics below.

> *Weaknesses:*
>
> *While the controls for a lack of gross motor deficit are solid, the data seem to show some motor execution deficit when cerebellar nuclei are silenced during task performance. This deficit could potentially impact learning when cerebellar nuclei are silenced during task acquisition.*

One of our key controls are the tests of the treatment on fixed speed rotarod, which provides the closest conditions to the ones found in the accelerating rotarod (the main difference between the protocols being the slow steady acceleration of rod rotation in the accelerating version). Indeed, small but measurable deficits are found at the highest speed in the fixed speed rotarod in the CN-VAL group, while there was no measurable effect on the CN-CL group, which actually shows lower performances from the second day of learning; we believe this supports our claim that the CN-CL inhibition impacted more the learning process than the motor coordination. In contrast, the CN-VAL group only showed significantly lower performance on day 4 consistent with intact learning abilities. Yet, under CNO, CN-VAL mice could stay for more than a minute and half at 20rpm, while in average they fell from the accelerating rotarod as soon as the rotarod reached the speed of ~19rpm (130s). Overall, we focused our argument on the first days of learning where the differences between the groups are more pronounced. We clarified the discussion (section "A specific impact on learning of CL-projecting CN neurons.")

> *Separately, I find the support for two separate cerebello-thalamic pathways incomplete. The data presented do not clearly show the two pathways are anatomically parallel. The difference in behavioral deficits caused by manipulating these pathways also appears subtle.*

There is indeed a paragraph devoted to the discussion of this point (last part of the section "A specific impact on learning of CL-projecting CN neurons."). Briefly, we actually know from the literature that there is a degree of collateralization (CN neurons projecting to both VAL and CL, see refs cited above), but it does not seem logically possible that the exact same population would have different effects, which are very distinct during the first learning days. The only possible explanation is the CN-CL and CN-VAL infections recruit somewhat different populations of neurons. We have now added more experiments to support our finding using retrograde infections using two rAAV viruses expressing red and green fluorescent reporter. These experiments confirm the limited overlap of the two populations of interest obtained by retrograde infection. We feel thus confident that while some CN neurons may project to both structures, retrograde infection strategies thus appear to differentially infect CN populations.

While we agree that after 3-4 days of learning the difference between the groups becomes elusive, we respectfully disagree with the reviewer that in the early stages these differences are negligible.

> ***Reviewer #3 (Public review):***
>
> *Summary:*
>
> *Varani et al present important findings regarding the role of distinct cerebellothalamic connections in motor learning and performance. Their key findings are that:*
>
> *(1) Cerebellothalamic connections are important for learning motor skills*
>
> *(2) Cerebellar efferents specifically to the central lateral (CL) thalamus are important for shortterm learning*
>
> *(3) Cerebellar efferents specifically to the ventral anterior lateral (VAL) complex are important for offline consolidation of learned skills, and*
>
> *(4) That once a skill is acquired, cerebellothalamic connections become important for online task performance.*
>
> *The authors went to great lengths to separate effects on motor performance from learning, for the most part successfully. While one could argue about some of the*

> *specifics, there is little doubt that the CN-CL and CN-VAL pathways play distinct roles in motor learning and performance. An important next step will be to dissect the downstream mechanisms by which these cerebellothalamic pathways mediate motor learning and adaptation.*
>
> *Strengths:*
>
> *(1) The dissociation between online learning through CN-CL and offline consolidation through CN-VAL is convincing.*
>
> *(2) The ability to tease learning apart from performance using their titrated chemogenetic approach is impressive. In particular, their use of multiple motor assays to demonstrate preserved motor function and balance is an important control.*
>
> *(3) The evidence supporting the main claims is convincing, with multiple replications of the findings and appropriate controls.*

We thank the reviewer for the positive comments and insightful critics below.

> *Weaknesses:*
>
> *(1) Despite the care the authors took to demonstrate that their chemogenetic approach does not impair online performance, there is a trend towards impaired rotarod performance at higher speeds in Supplementary Figure 4f, suggesting that there could be subtle changes in motor performance below the level of detection of their assays.*

This is now better acknowledged in the discussion in the section "A specific impact on learning of CL-projecting CN neurons." However, we want to underline that the strongest deficit in learning is found in animals with CN->CL inhibition which latency to fall saturates at about 100s on the rotarod; this indicates that mice fall as soon as the accelerating rotarod speed reaches about 16rpm. In fixed speed rotarod, the inhibition of CN->CL neurons shows not even a trend of difference at 15rpm with control mice, and the animals run 2 minutes without falling at this speed. This makes us confident that the CN->CL pathway interfers more with the learning than with the actual locomotor function on the rotarod.

> *(2) There is likely some overlap between CN neurons projecting to VAL and CL, somewhat limiting the specificity of their conclusions.*

This issue is treated in the discussion. (see also replies to reviewers 1 and 2 above). We added experiments with simultaneous retro-AAV infections in CL and VAL and the data are presented in Supplementary Figure 5. We found that retrograde infection targeted different populations of CN neurons; although collaterals in both CL and VAL may be present for (some of) these two populations of neurons, they are likely strongly biased toward one or the other thalamic regions, explaining the differential retrograde labelling in the CN. We hope these experiments will answer the reviewer' s concern.

> ***Recommendations for the authors:***
>
> ***Reviewer #2 (Recommendations for the authors):***
>
> *(1) Multiple studies have reported on the effect of cerebellar nuclei (CN) manipulation on locomotion. Here the authors perform several controls and careful analysis to rule out gross motor deficits caused by DREADD-mediated CN silencing. As the authors point out in the discussion, part of the difference from prior studies could be the mild degree of inhibition here. However, it is possible that the CN inhibition here induces a subtle motor deficit and the accelerating rotarod task is challenging and more readily reveals this motor deficit, rather than a deficit in motor learning per se. Two pieces of data seem to suggest this:*

> *(a) under CN inhibition during the task (Figure 1i), mice could never achieve the level of performance as mice under CN inhibition after the task, even after several days of training, which suggests the CN inhibition is interfering with task performance;*
>
> *(b) in highly trained mice (after learning), applying the CN inhibition impaired performance to a similar extend as mice in Figure 1i (Figure 4).*
>
> *Can the authors rule out the possibility that CN inhibition during the task is impairing motor execution rather than motor learning?*

We do not rule out a contribution of impaired motor coordination at the highest speed (last paragraph of the section "A specific impact on learning of CL-projecting CN neurons."). Indeed, most of our argument in favor of deficit in learning is primarily in the first days (Early phase), particularly for the CN->CL CNO group (Fig 3h). A crucial control in our work is the use of fixed speed rotarod, where no deficit is observed. The difference between the fixed and accelerating rotarod is rather minimal since the acceleration of the rotarod is rather small (0.12rpm/s for speed up to >20 rpm).

Interpreting the effect of treatment reversal is challenging. If the only effect of CNO was a motor deficit, the animals who learned under CNO should rapidly regain higher performance under saline, which is not observed. When switching from CNO to Saline after 7 days of training, it is difficult to disentangle which part is due to a crude motor deficit (which would not show in fixed speed rotarod), and which part is due to an unability to resume motor learning after the task has been (mis-)consolidated.

> *(2) The separation of the cerebellar pathways to the intralaminar thalamus (IL) and ventral thalamus (VAL) is not clear to me. It is not clear the CN neurons projecting to these nuclei are distinct. In addition, although IL projects to the striatum and VAL does not, both IL and VAL project to motor cortex. It is unclear to what extent these pathways can be separated. The argument for distinct pathways (as laid out in the discussion) is the distinct behavior deficits when manipulating these two pathways, but this difference seems subtle (point 3).*

We now clarify that CN populations are different help to retrograde labelling experiments (new Suppl Fig 5). A discussion on the differences in IL and VAL projections is now discussed in the last paragraph of the section "A specific impact on learning of CL-projecting CN neurons." Briefly, we argue that the despite some overlap of their targets, the profiles of the CL and VAL differ substantially.

> *(3) The pattern of behavioral deficits induced by CN->CL and CN->VAL neurons appear similar in Figure 3b-c and e-f. I have difficulty seeing how these data lead to the differences in the regression fits in panels 3g-k, which seem to show distinct patterns of performance change within and across sessions. One notable difference in Figure 3b-c and e-f seems to be that CN->VAL CNO treated mice exhibit lower performance on the very first trial for most days. Somehow, this pattern is present even after the CNO treatment is switched to saline (Figure 3f). I wonder if this data point is driving the difference. One control analysis the authors could do is to exclude the 1st trial and test if the effects are preserved.*

Since the learning is cumulative and involves varying degree of consolidation it is indeed difficult to substantiate the difference from the average performance: a performance on day 3 may be limited by slow learning and perfect consolidation or good learning and imperfect consolidation. That is why we designed an analysis which takes into account the observed relationships between initial performance, within session gain of performance and acrosssession carry-over of this gain of performance (Fig 2). This analysis focuses on the first days of learning, before the performance plateau is reached in the CNO groups. While a clear

deficit in consolidation is observed with full CN inhibition, this is not the case for the CN→CL CNO groups, despite their weaker performance after 3 days, similar to that seen with full CN inhibition. In contrast, normal learning is observed in the CN→VAL CNO group during these three days. The consolidation deficit in the CN→VAL CNO group is more subtle than in the CN CNO group and is indeed largely driven by the first data point. This is consistent with the idea that CN→VAL inhibition only partially impairs consolidation (compared to full CN inhibition), leaving some "savings" that allow rapid reacquisition.

> *(4) The quantification of locomotion in Figure S2 needs more information. What is linear movement? What is sigma? What is the alternation coefficient? These are not defined in the legends or the Methods as far as I can tell. Related to point 1 above, the authors should provide some analysis of the stride length and hindlimb to forelimb distance as measures of locomotion execution.*

These measures were taken from Simon J Neurosci 2004 24(8):1987-1995 which is now cited and their description is now provided in the Methods.

> *Minor:*
>
> *(5) To help readers follow the logic of experimental design, please explain why CNO was switched to saline after day 4 in Figures 1j, 3c, and f. Specifically, is the saline manipulation meant to test something as opposed to applying CNO throughout the entire course of the behavioral test?*

Since we had no difference between the groups at the end of the Early phase, we decided to test whether the skill consolidated under CNO remained available when the CNO was removed (and it indeed was). This is now more clearly stated in the Results.

> *(6) I have difficulty understanding what is plotted in Figure 4b and d. The legend says the change in performance is calculated the same way as in Figure 2a, so the changes are presumably the regression slopes. But how are the regression slopes calculated for daily start (1st trial) and daily end (last trial)?*

Skill level at the beginning and end of each trial correspond to the values of the regression line for abscissae values of trial 1 and trial 7 (green points). This has been added to the figure legend.

> *(7) Do CN-CL and CN-VAL neurons also project to other brain regions besides the thalamus? Might these pathways also contribute to learning and consolidation of the accelerating rotarod task? Please discuss.*

This is now discussed in more detail in the last paragraph of the section "A specific impact on learning of CL-projecting CN neurons."

> ***Reviewer #3 (Recommendations for the authors):***
>
> *(1) Please check the anatomic evidence for the strict dichotomy between intralaminar (specifically central lateral nucleus) nuclei projecting to the striatum and the ventral-anteriorlateral (VAL) complex projecting to the cortex. For example, while the Chen et al paper shows that there are cerebellar-intralaminar-striatal projections, it does not exclude intralaminar cortex projections, which have at least been demonstrated in rats. Similarly, VAL has projections to striatum (see, e.g., Smith et al, "The thalamostriatal system in normal and diseased states", Frontiers in Systems Neuroscience, 2014). It may be that some of these projections are stronger, but I don't think it's true that these pathways are as well-separated as the authors suggest. I also don't think this changes the fundamental conclusions but is important for potential mechanisms by which differential learning could occur and necessitate modification of Figure 5.*

We have toned down the interpretation of CL and VAL relaying specifically to different brain structures and mostly put forward the duality of the pathways. The connections with the cortex are now discussed at the end of the section “A specific impact on learning of CL-projecting CN neurons.”

> *(2) Please provide more details on the spike sorting. By what metrics were single units declared to be well-separated? How many units were identified under each condition? What was the distribution of firing rates with and without CNO treatment? Are the units shown in panel 1f from before and after CNO as in panel E or are just 2 examples of isolated units? The units by themselves are not very helpful to the reader. Showing sample auto and/or crosscorrelograms for units recorded on the same electrode would be more helpful to show how well-isolated the units are.*

Single units were considered well-isolated based on quantitative quality metrics computed after MountainSort 4 spike sorting (Phyton 3.8). Units were required to have a signal-to-noise ratio (SNR) greater than 5, inter-spike interval (ISI) violations less than 1%, an amplitude cutoff below 0.1, a presence ratio above 0.9, a firing rate greater than 0.1 Hz, and at least 50 detected spikes. In addition, units were assessed for temporal stability across the recording using autocorrelograms and presence over the recording, ensuring there were no prolonged periods of total inactivity. Units meeting these criteria were deemed well-separated and reliable for further analysis. This has been added to the Methods.

Cell numbers are provided with the statistics in the supplementary table for fig panel 1g. Panels are from the same unit before and after CNO. Example of auto- crosscorr- are provided in the new Supplementary Figure 6.

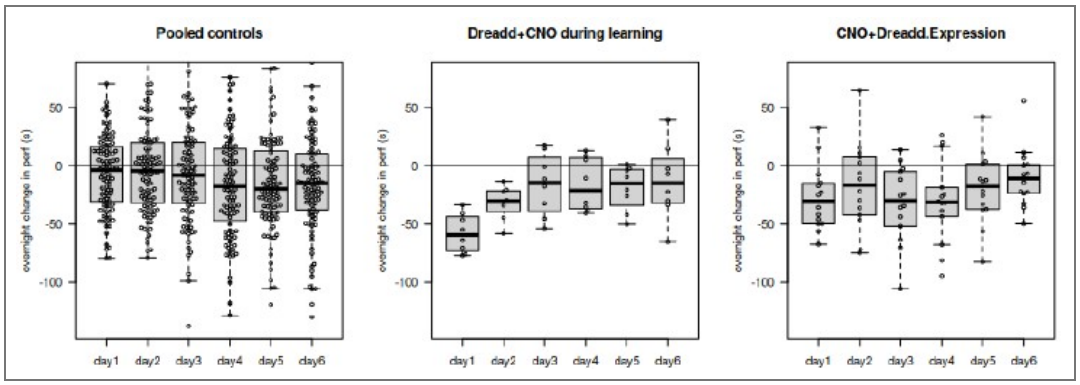
> *(3) Panel 2g - "firing rate modulation" is unclear. I think the authors are showing the mean firing rate with DREADD+CNO treatment divided by the mean firing rate in the pre-CNO condition for the same group (I couldn't find that in the Methods, my apologies if I missed it)? However, firing rate modulation to me means variability in firing rate within a recording. Perhaps "relative firing rate" or "% pre-CNO firing rate" would be clearer?*

The definition has been added to the Method and the axis has been changed to ‘Change in FR induced by SAL/CNO’

> *(4) Figure 3f - why does consolidation appear to be impaired after the transition from CNO to saline between sessions, when in panel 1j suppressing the CN does not have a similar effect once CNO is switched to saline? Could this be driven by a small number of mice? Since a central conclusion of the paper is that CN-VAL connections are uniquely important for posttraining consolidation, this discrepancy is important to explain - if the results post-saline are spurious, how do we know that the results post-CNO aren't also spurious? Panels similar to Figure 4b and d showing all the data from the last/first trial of each session I think would be convincing.*

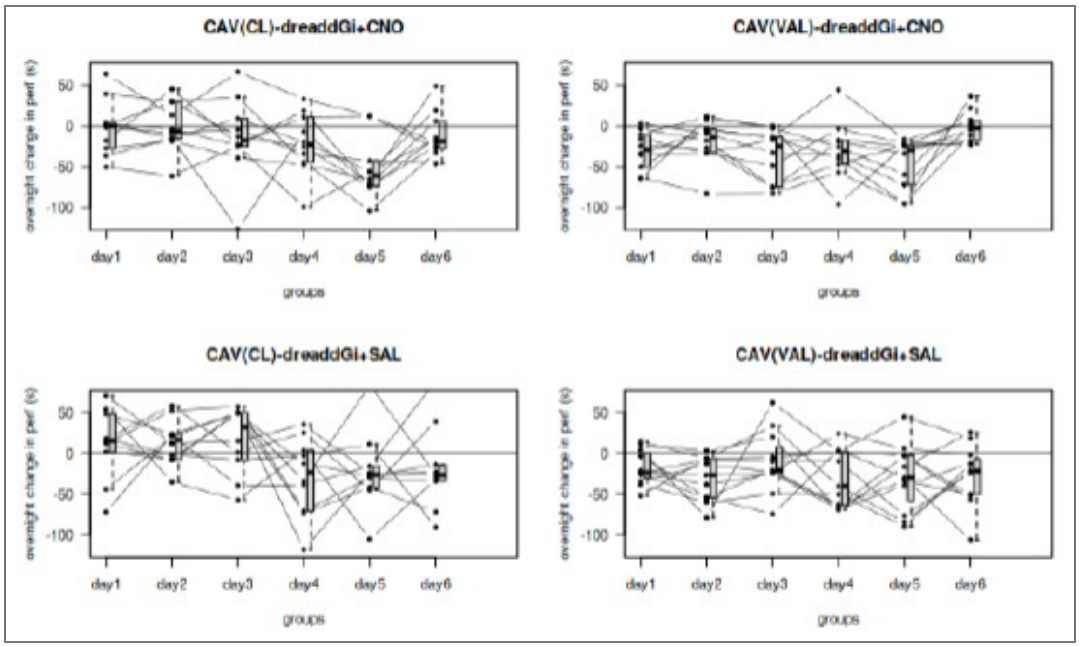
Our results overall indicate that the overnight consolidation of the improvement in performance seem only effective in the early phase (as pointed out on the summary figure 5). We do not believe then that the saline results are spurious.

It can be seen indeed in the control groups of the figure 1; to make this more visible, we plot in Author response image 1 the difference between trial 7 and trial 1 the next day. An overnight drop in performance becomes visible in the late phase.



Author response image 1.

The decrement on the first trial in the first 3 days is visible for the majority of the mice. The plot asked by the reviewer is represented in the Author response image 2.



Author response image 2.

> *Minor points:*
>
> *(5) In panel 1a, the solid yellow line obscures a lot of the image and I don't think adds anything.*

We assume this was referring to a line on fig1d, which has been removed.

> *(6) Panel 2a - color selection could present problems for those with red-green color blindness.*

This has been fixed.

> *(7) Supplementary Figure 3 - what are the arrows and arrowheads indicating?*

These have been removed.

> *(8) In the Discussion: "Studies of cerebellar synaptic plasticity provide clearly support the involvement of cerebellum in rotarod learning..." Delete the word "provide"*

This has been fixed

> *(9) "This indicates that either the distinct functional roles of VAL-projecting or CLprojecting." The second "of" should be "or", I think.*

This has been fixed.

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