Increase of vesicular glutamate transporter 2 co-expression in the deep cerebellar nuclei related to skilled reach learning

Haian Mao a,1, Tomás Mediavilla b,1, Héctor Estévez-Silva b,c, Daniel Marcellino b, Fahad Sultan b,

a Department of Rehabilitation Medicine, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou 510080, China
b Dept. of Integrative Medical Biology, Umeå University, Johann Bures väg 12, 901 87 Umeå, Sweden

c Instituto de Tecnologías Biomédicas, Departamento de Ciencias Médicas Básicas, Universidad de La Laguna, 38071 Tenerife, Spain

ARTICLE INFO
Keywords:
Cerebellum
Motor learning
Synapse
3D reconstruction

ABSTRACT
Motor learning induces plasticity in multiple brain regions involving the cerebellum as a crucial player. Synaptic plasticity in the excitatory collaterals to the cerebellar output, the deep cerebellar nuclei (DCN), have recently been shown to be an important part of motor learning. These synapses are composed of climbing fiber (CF) and mossy fiber synapses, with the former conveying unconditioned and the latter conditioned responses in classical conditioning paradigms. The CF synapse on to the cerebellar cortex and the DCN express vesicular transporter 2 (vGluT2), whereas mossy fibers express vGluT1 and /or vGluT2 in their terminals. However, the underlying regulatory mechanism of vGluT expression in the DCN remains unknown. Here we confirm the increase of vGluT2 in a specific part of the DCN during the acquisition of a skilled reaching task in mice. Furthermore, our findings show that this is due to an increase in co-expression of vGluT2 in vGluT1 presynapses instead of the formation of new vGluT2 synapses. Our data indicate that remodeling of synapses – in contrast to synaptogenesis - also plays an important role in motor learning and may explain the presence of both vGluT1's and vGluT2 in some mossy fiber synapses.

1. Introduction

The cerebellum has long been known to play an important role in motor coordination and skill acquisition (Glickstein, Strata et al., 2009, Glickstein, Sultan et al., 2011). Multiple recent studies have pointed out that the cerebellum uses internal models to provide sensory predictions of motor outcome. Cerebellar learning is thought to be driven by error signals that indicate differences between the intended movement and the one that was actually executed (Wolpert et al., 1998; Smith and Shadmehr, 2005). The process often involves a transition from “controlled” to “automatic” processing in which movements that initially require problem-solving and attention become increasingly efficient, stereotyped, resistant to online feedback, and importantly, require much less attention (Shiffrin and Schneider, 1984). Central to these proposals of the cerebellum as an internal model-updating device is its dual input system. The cerebellum has two different excitatory afferents that have been at the base of the earliest learning theories: the mossy fibers (MF) and the climbing fibers (CF). These theories of motor learning (Marr, 1969; Ito, 1970; Albus, 1971) have suggested that the MF mediates the conditioned stimulus, while the CF the unconditioned stimulus.

The excitatory synapses of the cerebellum are divided into two subsets according to different vesicular transporters (vGluT1 and vGluT2) that are specifically utilized within the presynaptic bouton to load glutamate into the vesicles (Bellochio et al., 1998). In the brain the two transporters are often expressed in a complementary pattern (Fremau et al., 2001; Varoqui et al., 2002): vGluT1 mRNA is expressed in the neocortex, cerebellar cortex and hippocampus whereas vGluT2 is expressed in most subcortical brain regions (such as hypothalamus, thalamus, midbrain and amygdala). A simple mapping of the vGluT1 and 2 onto the cerebellar fiber system would attribute vGluT1 to the MF and vGluT2 to the CF. Indeed, the CF within the cerebellar cortex and nuclei use vGluT2, while the MF-parallel fiber system in the molecular layer of the cerebellar cortex uses vGluT1 (Hisano et al., 2002). This fits well with the general observation that vGluT2 is often found in reliable, hard-wired “driver” connections, while vGluT1 is observed in flexible
“modulator” connections (Guillery and Sherman 2002; Nakakubo et al., 2020). Nevertheless, a number of studies have shown that the MF terminals (within both the cerebellar cortex and nuclei) co-express vGluT2 (Hisano et al., 2002). Furthermore, studies looking at plasticity within eye-blink conditioning (Lee et al., 2015) and within forelimb conditioning (Boele et al., 2013) have shown that the vGluT2 presynapses increase in numbers following sensorimotor learning. Therefore, there appears to be a certain contradiction in that the supposedly hard-wired increase in numbers following sensorimotor learning. So far, it is not clear what functional consequence this has, but our study provides an important observation how vGluT2 are involved in synaptic plasticity.

2. Results

2.1. Skilled-reaching task and animal behavior

All trained animals showed an increase in their grasping skill as determined by their successful retrieval of pellets from the groove. Successful reaches during the 12 days of motor skill training increased significantly with time ($\chi^2 = 19.71, p < 0.05$) in trained mice. At training day 12, trained animals successfully retrieved 68% ($\pm 8$ SEM) pellets with a mean accuracy of 33% ($\pm 13$ SEM). All animals in the training group presented learning rates with slopes significantly different from zero ($p < 0.05$) at an individual level (Fig. 1).

2.2. Qualitative description of vGluT 1 and 2 staining in the cerebellum

Immunostaining was checked for specificity by comparing vGluT1 and 2 channels in the cerebellar cortex and DCN (deep cerebellar nuclei). The excitatory presynapses in the molecular layer display exclusive staining patterns (Hisano et al., 2002), with the parallel fibers staining for vGluT1 and the climbing fibers for vGluT2 only. This pattern was confirmed in our sections where we observed no cross reactivity between the two channels in the cerebellar cortex (Fig. 2). A more complex picture emerges when we look at the DCN. Here we find presynapses that show colabelling, however we also find exclusive labelling for either vGluT1 or 2 (Fig. 2).

2.3. Quantification of vGluT 1 and 2 bouton volume and density

We quantified a total of 471 3D probes from two controls and three trained animals (213 from controls and 258 from trained animals). We used a random systematic sampling scheme to ensure that our probes location selection (slide selection and within slide location selection) was unbiased. Such a single 3D probe was obtained from 30 optical sections (z-stacks) and covered a volume of $71.4 \times 71.4 \times 9.6$ μm. The 3D sections were deconvoluted and the boutons’ surfaces were reconstructed after smoothing and thresholding. The results for surface volumes and counts are summarized in Fig. 3.

The largest general variability was observed in the volumes of vGluT2 boutons. In contrast, vGluT1 volume variability was smaller. The bouton density of the untrained hemisphere was comparable to the trained side with the exception of the vGluT2 bouton density within the PIN (posterior interposed nucleus, see Supplementary Fig. 1 and 2). We tested whether training had any effect on the PIN vGluT2 bouton density by using a linear mixed effect model comparing trained vs. untrained hemisphere and found a significant effect of training ($\chi^2 = 5.73, df = 1, p = 0.0167$). A similar comparison of the vGluT2 bouton density of the left and right side of the controls PIN revealed no significant difference ($\chi^2 = 0.11, df = 1, p = 0.75$).

2.4. vGluT 1 and 2 colabelling in PIN

In a next step we wanted to know whether the increase in vGluT2 boutons in the PIN was due to increased colabelling with vGluT1 or to an increase in expression and/or new synapse formation of vGluT2 only. A pixel-wise colabelling analysis was performed by calculating the Pearson’s correlation coefficient between the thresholded vGluT1 and 2 channels (sees examples in Fig. 4A and D). A comparison of the trained vs. untrained Pearson’s correlation coefficient for all probes from the PIN showed larger correlations on the trained side (Fig. 4H). A linear mixed effect model analysis confirmed a highly significant effect of training on the coefficient ($\chi^2 = 17, df = 1, p = 0.001$), indicating a larger probability of pixel-wise vGluT1 and 2 colabelling due to training. We also tested whether the thresholding was affected by difference in background staining. However, we found no difference (linear mixed model; effect of training: $\chi^2 = 0.6127, df = 1, p > 0.43$; effect of vGluT1/2 $\chi^2 = 2.6979, df = 1, p > 0.1$) between the trained and untrained vGluT1 and 2 thresholds (Supplementary Fig. 3).

We next quantified the percentage of colabelling in vGluT1 and vGluT2 synapses (Fig. 4I) based on object surface analysis. Applying a linear mixed effect model showed that training had a significant effect on the percentage of colabelling in vGluT1 boutons ($\chi^2 = 10.02, df = 1, p < 0.001$), whereas no such significant effect was observed in vGluT2 boutons $\chi^2 = 1.87, df = 1, p = 0.17$.

![Fig. 1. Behavioral results of mice performing skilled reaching task. Data shown are for three mice included in this study. A: Average successful reaches increased over time ($\chi^2 = 19.71, p < 0.05$). B: Average successful reaches increased from 8% ($\pm 2$) at training day 1 to 68% ($\pm 8$) at training day 12. Accuracy at training day 12 was 33 ($\pm 13$). The learning rates for the three animals trained in this study were significantly different from zero ($p < 0.05$). Each color represents an individual animal. Data represented as mean $\pm$ SEM.](image-url)
3. Discussion

We studied learning mechanisms in the cerebellum by looking at the effect of learning a skilled task on the quantitative morphology of the excitatory DCN synapses of mice. To our knowledge, this is the first unbiased systematic quantification of excitatory synapses in the DCN following learning a skilled task. With our approach, we can confirm that learning such a task can lead to an increase in vGluT2 boutons (Boele et al., 2013). Our statistical analysis, however, was limited to the PIN and smaller morphometric changes in the other DCN may have gone unnoticed. Nevertheless, the important finding of this study is that vGluT2 density increases in the PIN are due to an increase in co-expression of vGluT2 in vGluT1 boutons. In the following, we will first compare our findings to previous results on the excitatory synapses in the DCN, before we return to discussing the implications of our findings on learning models of the cerebellum.

3.1. Comparison to previous studies

A comparison of our quantification in mice shows that they have higher densities of vGluT boutons than was estimated by us previously in rats. Mice vGluT1 and 2 bouton densities in the DCN amount to $10^6$ mm$^{-3}$ and $18*10^6$ mm$^{-3}$, respectively, compared to vGluT1 and 2 densities in the rat of $5.6*10^6$ mm$^{-3}$ and $4.3*10^6$ mm$^{-3}$. One reason for this difference could be the higher neuron density in the mice DCN: $4.73 \times 10^4$ vs. $2.53 \times 10^4$ (Sultan et al., 2002; Hamodeh et al., 2017). However, this cannot fully explain this difference. An alternative confounding explanation is that our surface reconstruction algorithm may have split the boutons more frequently in the mouse, leading to higher densities. This would then also lead to smaller bouton volumes. However, a comparison of the volumes shows the opposite. vGluT1 and 2 boutons had smaller volumes in the rat (vGluT1: 2.58 mm$^3$ vs. 0.69 mm$^3$ and vGluT2: 1.04 mm$^3$ vs 0.5 mm$^3$ for the mice and rats, respectively).

3.2. Role of vGluT 1 and 2 in plasticity: Confirmation and reinterpretation

Learning and plasticity is a well described phenomena in the cerebellum (Gao et al., 2012, Freeman 2015). LTD (long term depression) was first described to occur at the parallel fibre-Purkinje cell synapse

---

Fig. 2. Staining specificity of vGluT1 and vGluT2 in the mouse cerebellar cortex and DCN. The cerebellar cortex displayed an exclusive pattern of vGluT1 (large number of parallel fibers) or vGluT2 (climbing fiber presynapse with some pointed out with blue arrows), indicating immunostaining specificity. In contrast the DCN (D-I) displayed vGluT1 (blue arrows) or vGluT2 (white arrows) only as well as double staining’s (yellow surfaces in G). Arrows are added to D-I to facilitate comparisons. D-F: show merged deconvoluted confocal images, vGluT1 only channels and vGluT2 (F) channel. G-I shows the co-labelling surfaces (G), vGluT1 surfaces (H) and vGluT2 surfaces (I), respectively. Scale bar in C is 5um and also applies to A and B. The scale bar in l is 2 μm and also applies to D-H.
following CF and parallel fibre activation (Ito 1970). Other forms of plasticity have also been described within DCN neurons, which include LTP (long term potentiation) and increased intrinsic excitability (Aizenman and Linden 2000). A link between synaptic plasticity (LTD) and vGluT2 expression was shown in the study by He and colleagues (He et al., 2012). They demonstrated a role for vGluT2 in the early development of vGluT1-encoded transmission and plasticity in the hippocampus. Genetic deletion of vGlut2 during early development prevented the induction of LTD and produced impaired learning and memory (He et al., 2012). This could point to the importance of vGluT2 in LTD formation. Nevertheless, future studies are required to elucidate this connection and to establish the importance of LTD in DCN neurons.
Fig. 4. Comparison of vGlut1, 2 and colabelled synapses in untrained (A-C) and trained (D-G) PIN. The columns from left to right indicate the merged deconvoluted confocal microscopy images (A, D), the reconstructed surfaces (B, F) for vGlut1 (green) and vGlut2 (red) and the surfaces (C, G) showing co-labelling (yellow). The inserts A’ and D’ shows the 2D intensity histograms (both axis 0 to 255 intensity levels) of the vGlut1 and vGlut2 channels. Pixel-wise intensity colabelling in A’ and D’ was calculated with the Pearson’s coefficient (PC). In this example the number of vGlut1, vGlut2 and colabelled surfaces for the untrained is 126, 202, 49 and for the trained side 126, 303 and 97, respectively. Scale bar in C = 3 μm, also applies for A, B, D, F, and G. H) Quantifying pixel-wise Pearson’s correlation coefficient for all probes from the PIN showed higher coefficients in the trained side of the mice (p < 0.001, linear mixed effect model in R). I) Percentage of colabelling in vGlut1 and vGlut2 in trained and untrained mouse PIN. Percentage of colabelled vGlutI boutons was calculated as a percentage of colabelled bouton density normalized by either the density of vGlut1 or vGlut2 boutons. The colabelling in vGlut1 boutons from the trained PIN are significantly higher compared to the untrained PIN (p < 0.001, linear mixed effect model in R).
The cerebellum is one of the few brain regions that contains both kinds of vGlutTs in their climbing and mossy fibres. In addition, both kinds of vGlutTs are expressed in a subset of mossy fibres. Previous studies have shown that during the early stages of development, vGluT2 are switched to vGluT1 synapses. This follows the switch of vGluT2 mRNA expression to vGluT1 mRNA expression in the neurons’ somata (Miyazaki et al., 2003). Our results in the adult brain could imply that the mossy fibres retain a status comparable to that of the early stages of development, allowing for prolonged modification from vGluT2 to vGluT1. A different scenario emerges if we look at studies of plasticity in the DCN. Boele and colleagues showed an increase in basilar pontine nuclei vGluT2 mossy fibre presynapses in the DCN following Pavlovian eyelblink conditioning (Boele et al., 2013). Our study now shows that this is due to the co-expression of these transporters in the same presynapse.

Recent studies (Nakakubo et al., 2020) on the Calyx of Held synapse, which expresses both transporters showed that vGluT1 together with vGluT2 is required to allow synaptic transmission to follow high frequency (>100 Hz) firing. In a seminal study on the quantitative structure of the synaptic vesicle (Takamori et al., 2006) it was estimated that one synaptic vesicle contains 10–14 transporter channels on average and that these are sufficient to load a vesicle in a reasonable time for most synapses of about 20 secs (Takamori, 2016). However the Nakakubo study shows that this may become challenging in high frequency firing synapses as found within the auditory and cerebellar system for instance (Sultan et al., 2012). Our observation of synaptic plasticity showing an increased coexpression of vGluT1 and 2 in the DCN may point to an interesting mechanism of changes that allow higher frequency firing that may be important in learning a skilled reaching task.

4. Experimental procedure

4.1. Animal training

All animal experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Umeå Regional Ethics Committee for Animal Research (ethical permit: A35-2015). Male 9-week-old C57BL/6J mice were purchased from Jackson Laboratory. All animals used in this project were part of an ongoing study using non-invasive MRI to detect structural brain plasticity and the trained animals were anesthetized every other day for a total of six times with isoflurane for Animal Research (ethical permit: A35-2016). Male 9-week-old mice were placed on a limited diet and were weighed and monitored each day. These body weights were then used to calculate the lower boundary of tolerated weight loss (85–90% of their previous body weight). Animals were food restricted throughout the 15-day experimental paradigm and were provided the daily food ration, immediately after completing the daily training session. In all animals, the daily food ration secured their weights to remain above the set weight loss boundary. Three days prior to training, mice were placed in the training cage with food pellets and forelimb dominance for grasping was identified. Once the forelimb preference was determined for each individual animal, the mice were repeatedly trained to grasp and retrieve food pellets through a narrow slit positioned contralateral to the preferred forelimb for each individual animal. During the subsequent 12 days, each animal in the trained group was given 15-minute training sessions consisting of 30 discrete trials (one pellet per trial). Successful reaches was calculated as the percent of trials for which food pellets were successfully retrieved from the groove without exhibiting any abnormal behavior (i.e., without dropping the pellet or without the use of the tongue to aid in retrieving the pellet from the groove), normalized by the number of trials completed by each individual animal during a training session. Accuracy was calculated as the number of successful reaches divided by the number of attempts to reach the pellet for each training session (Sampaio-Baptista et al., 2013). The learning rate for each individual animal was calculated as the slope of a fitted model to the learning curve based on successful reaches.

4.2. Tissue preparation

The mice were transcardially perfused with Tyrode’s solution and then freshly prepared 4% room temperature paraformaldehyde in 0.1 M phosphate buffer (PB) at pH 7.4 after a deep anesthesia induced by sodium pentobarbital (i.p. 100 mg/kg). The brain was immediately dissected out of the skull and then cryoprotected in ascending sucrose (10% – 20% – 30%) in 0.1 M PB. The cerebellum was removed from the brainstem and a small cut was made on the right hemisphere before mounting the whole cerebellum on a microtome. Coronal sections of 40 μm were serially acquired and stored into 4 series in 0.1 M PB. Immunofluorescence staining was carried out on free-floating sections which belonged to the same section series of each mouse. In the case of trained animals this assured that trained and untrained sides were treated equally. Prior to primary antibody incubation, sections were washed three times in 0.1 M PB for 5 min each and then blocked in 0.1% horse serum (PAA Laboratories, Coelbe, Germany) and 0.3% Triton X-100 at room temperature for 1 h.

4.3. Immunostaining

A double staining with vGluT1 and vGluT2 antibody was performed on the cerebellar sections from 2 control mice and 3 trained mice. The sections were first incubated with goat anti vGluT1 (sc-13532, Santa Cruz Biotechnology, Texas) at 1:1000 at 4 ◦C for 16 h, followed by 3x washes in 0.1 M PB and then incubated with donkey anti goat Alexa Fluor 488 at 1:500 for 2 h at room temperature. The sections were then washed again before incubation with guinea pig anti vGluT2 (1:1000, lot number: 135404, synaptic systems, North Saanich British Columbia), at 4 ◦C for 16 h. The sections were then washed again and incubated with the goat anti guinea-pig Alexa Fluor 633 (1:500, A21105, Invitrogen, California) for 2 h at room temperature. Sections were washed and mounted with Mowiol 4-88 (Merck, Darmstadt, Germany) in glycerol on glass slides. The slides were stored at 4 ◦C.

4.4. Data acquisition

3D images were acquired on a laser scanning confocal microscope (LSM 510, Carl Zeiss, Jena, Germany). Overview images of the vGluT1 channel for every section were taken under 488 nm excitation and emission band pass filtering 505–550 nm at low magnification (10 × objective). Probe positions were determined by first marking an identifiable origin (x, y coordinates set to 0, 0) and then scanning positions in the DCN region were defined at a regular spacing of 200 μm. The location of the origin point was chosen from an easily identifiable structure (i.e., vessels) within a core region of the DCN. However, this location differs from slice to slice and is not related to the structures to be analyzed, thereby providing random systematic sampling. A z-stack was
acquired using a 63 × (NA 1.4, oil immersion) objective, applying a 2 × zoom at each predetermined position. The pinhole was set to one airy unit and subsequently optimized for every detection channel to achieve an equal optical slice thickness for all channels. XY voxel size was set to 0.14 μm and image matrix size was set to 512 × 512 pixels. The z-stack step size was 0.32 μm and we took an average of 30 optical sections. In total, 20 cerebellar sections from five mice were stained (7 slices from the 2 control mice and 13 slices from the trained mice) and a total of 471 3D probes were sampled. The vGluT2 channel was recorded under excitation of 633 nm and emission wavelength at 650–750 nm. The probes were named with the hemisphere information (left vs. right).

The shrinkage of section thickness was calculated by taking four random positions within DCN region on every first stained section of each mouse. The upper and lower sections border was determined by the staining signal for vGluT1 channel at a magnification of 20x (NA 0.8, dry). 20 probes were taken from 5 sections. The mean thickness of the slices after mounting was 28.17 μm, while the original sectioning thickness was 40 μm. Classification of the mouse DCN was based on the Nissl staining of the Allen atlas.

4.5. Data preprocessing and analysis

All z-stacks were acquired at 8-bit in Zeiss lsm format and then deconvoluted using the iterative (n = 10) “blind deconvolution” of AutoQuant X3 (Media Cybernetics, Bethesda, MD) with the maximum likelihood estimation and constrained iteration. Data were saved in AutoQuant to 8-bit lsm format after deconvolution.

4.6. Surface generation for density analysis

The deconvoluted 3D stacks were further processed in Imaris (Bitplane AG, Switzerland). The surfaces of the vGluT1 and vGluT2 boutons were constructed based on the fluorescent intensity of vGluT1 and vGluT2 channel. The surface bounding of vGluT1 and 2 boutons was smoothed at 0.15 μm and thresholding levels was set to be determined automatically based on the absolute intensity and using the Ridler and Calvard (R-C) algorithm (Ridler and Calvard 1978). The option of splitting touching objects was activated by setting the seed point diameter at 0.5 μm during the vGluT1 surface construction. At the last step surfaces were filtered by excluding those with <10 voxels. The surface quantifications were manually exported and saved in Excel files.

4.7. Calculating the co-labelling of vGluT1 and vGluT2 boutons

The quantification of colabelled vGluT1 and vGluT2 boutons in the PIN from the three trained mice was performed by either using a pixel-wise intensity colabelling and calculating the Pearson’s coefficient or through an object based colabelling analysis. The object based colabelling was performed by obtaining the intersection of the surface boundaries of the 2 vGluT channels (vGluT1 ∩ 2). However, we could only perform channel and not surface subtractions in Imaris. Therefore, we subtracted vGluT1-only channel from vGluT1. The vGluT1-only channel was obtained for each probe by subtracting the vGluT2 surfaces from the original vGluT1 channel. The density of labelled boutons was divided by the vGluT1 or vGluT2 bouton density to obtain the percentages, which were then compared between the trained and untrained hemisphere.

4.8. Statistical analysis

Improvement in successful reaches was evaluated using a non-parametric Friedman test of differences among repeated measures. Behavioral data was analyzed using Prism 9.2.0 (GraphPad Software, San Diego, California USA). The general effect of training on the volume and densities of vGluT boutons was tested with a linear mixed effect model to take into account multiple measurement within individual subjects (Aarts et al., 2014). The linear mixed effect model was performed with lme4 package in R using maximum likelihood estimations. Shapiro-tests (package lme4 in R) were used to test for normal distribution and data were transformed (transformTukey) in case of deviations from normal distribution. Model response was the vGluT2 bouton density in PIN with the training condition as a fixed effect, while the subject ID was treated as a random effect. We also constructed a null model with only the random effect. We then compared the two models using a likelihood ratio test to ascertain whether these two models differed significantly from each other. The significance level α was set to 0.05.

5. Role of authors

HM planned the project, conducted the staining, acquired and analyzed the data and wrote the manuscript. TM planned and conducted the behavioral experiments, analyzed the behavioral data and wrote the manuscript. HES planned and conducted the behavioral experiments and analyzed the behavioral data. DM planned the behavioral experiments and analyzed the behavioral data. FS planned the project, analyzed the data and prepared the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

H.M. was funded by the Chinese Scholarship Council and by the GuangDong Basic and Applied Basic Research Foundation (grant number 2021A1515110254). Grants were received from Umeå University Medical Faculty (FS and DM).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.brainres.2022.147842.

References

Aarts, E., Verhage, M., Veenvliet, J.V., Dolan, C.V., van der Sluis, S., 2014. A solution to dependency: using multilevel analysis to accommodate nested data. Nat. Neurosci. 17 (4), 491–496.
Altenman, C.D., Linden, D.J., 2000. Rapid, synaptically driven increases in the intrinsic excitability of cerebellar deep neurons. Nat. Neurosci. 3 (2), 109–111.
Albus, J., 1971. A theory of cerebellar function. Math Bioscience 10, 25–61.
Belloccchio, E.E., Hu, H., Pohorille, A., Chan, J., Pickel, V.M., Edwards, R.H., 1998. The localization of the brain-specific inorganic phosphate transporter suggests a specific presynaptic role in glutamatergic transmission. J. Neurosci. 18 (21), 8648-8659.
Boele, H.-J., Koekkoek, S.K.E., De Zeeuw, C.I., Ruigrok, T.J.H., 2013. Axonal sprouting and formation of terminals in the adult cerebellum during associative motor learning. J. Neurosci. 33 (45), 17897–17907.
Freeman, J.H., 2015. Cerebellar learning mechanisms. Brain Res. 1621, 260–269.
Freeman, R.T., Troyer, M.D., Paehner, I., Nygaard, G.O., Tran, C.H., Reimer, R.J., Belloccchio, E.E., Fortin, D., Storm-Mathisen, J., Edwards, R.H., 2001. The expression of vesicular glutamate transporters defines two classes of excitatory terminals in the adult cerebellum during associative motor learning. J. Neurosci. 31 (2), 247–260.
Gao, Z., van Beugen, B.J., De Zeeuw, C.I., 2012. Distributed synergistic plasticity and cerebellar learning. Nat. Rev. Neurosci. 13 (9), 619–635.
Glickstein, M., Strata, P., Voogd, J., 2009. Cerebellum: History. Neuroscience 162, 549-559.
Glickstein, M., Sultan, F., Voogd, J., 2011. Functional localization in the cerebellum. Cortex 47 (1), 59-80.
Guillery, R.W., Sherman, S.M., 2002. Thalamic relay functions and their role in corticocortical communication: generalizations from the visual system. Neuron 33 (2), 163–175.
Hammelé, S., Bodurk, A., Mao, H., Sultan, F., 2017. Uncovering specific changes in network wiring underlying the primate cerebrotype. Brain Struct. Funct. 222 (7), 3255–3266.
He, H., Mahnke, A.H., Doyle, S., Fan, N., Wang, C.-C., Hall, B.J., Tang, Y.-P., Inglis, F.M., Chen, C., Erickson, J.D., 2012. Neurodevelopmental role for VGLUT2 in pyramidal
neuron plasticity, dendritic refinement, and in spatial learning. J. Neurosci. 32 (45), 15886–15901.

Hisano, S., Sawada, K., Kawano, M., Kanemoto, M., Xiong, G., Mogi, K., Sakata-Haga, H., Takeda, J., Fukui, Y., Nogami, H., 2002. Expression of inorganic phosphate/vesicular glutamate transporters (BNPI/VGLUT1 and DNPI/VGLUT2) in the cerebellum and precerebellar nuclei of the rat. Brain Res. Mol. Brain Res. 107 (1), 23–31.

Ito, M., 1970. “Neurophysiological aspects of the cerebellar motor control system.” Int. J. Neurol. 7 (2), 162–176.

Lee, K., Mathews, P., Reeves, A.B., Choe, K., Jami, S., Serrano, R., Otis, T., 2015. Circuit mechanisms underlying motor memory formation in the cerebellum. Neuron 86 (2), 529–540.

Marr, D., 1969. A Theory of Cerebellar Cortex. Journal of Physiology-London 202, 437–470.

Mediavilla, T., Özalay, H. Estevez-Silva, B. Frias, G. Oradl, F. Sultan, C. Brozzoli, B. Garzon, M. Lovden and D. Marcellino “Experience-dependent contraction of grey matter in rodent sensorimotor cortex during learning is associated with adaptive myelination.” Manuscript submitted for publication.

Miyazaki, T., Fukaya, M., Shimizu, H., Watanabe, M., 2003. Subtype switching of vesicular glutamate transporters at parallel fibre-Purkinje cell synapses in developing mouse cerebellum. Eur. J. Neurosci. 17 (12), 2563–2572.

Molina-Luna, K., Pekanovic, A., Rohrich, S., Hertler, B., Schubring-Giese, M., Rioult-Pedotti, M.S., Luft, A.R., 2009. Dopamine in motor cortex is necessary for skill learning and synaptic plasticity. PLoS ONE 4 (9), e7082.

Nakakubo, Y., Abe, S., Yoshida, T., Takami, C., Isa, M., Wojcik, S.M., Brose, N., Takamori, S., Hori, T., 2020. Vesicular Glutamate Transporter Expression Ensures High-Fidelity Synaptic Transmission at the Calyx of Held Synapses. Cell Rep 32 (7), 108040.

Ridler, T.W., Calvard, S., 1978. Picture Thresholding Using an Iterative Selection Method. Ieee Trans. Systems Man Cybern. 8 (8), 630–632.

Sash, B.J., Maclean, A.J., Kaniek, M., Zurek, A.A., Martin, L.J., Roder, J.C., Orser, B.A., 2010. Short-term memory impairment after isoflurane in mice is prevented by the alpha5 gamma-aminobutric acid type A receptor inverse agonist L-655,708. Anesthesiology 113 (5), 1061–1071.

Sampao-Baptista, C., Khripitchev, A.A., Fordyce, S., Schlapple, T., Scholz, J., Jhabdi, S., DeLuca, G.C., Miller, K.L., Taylor, A., Thomas, N., Kleim, J., Sibson, N.R., Banker, D., Johansen-Berg, H., 2013. Motor Skill Learning Induces Changes in White Matter Microstructure and Myelination. J. Neurosci. 33 (50), 19499–19503.

Shiffrin, R., Schneider, W., 1984. Automatic and controlled processing revisited. Psychol. Rev. 91, 269–276.

Smith, M.A., Shadmehr, R., 2005. Intact ability to learn internal models of arm dynamics in Huntington’s disease but not cerebellar degeneration. J. Neurophysiol. 93 (5), 2809–2821.

Sultan, F., Augath, M., Hamodreh, S., Murayama, Y., Oeltmann, A., Rauch, A., Thier, P., 2012. Unravelling cerebellar pathways with high temporal precision targeting motor and extensive sensory and parietal networks. Nat. Commun. 3, 924.

Takamori, S., König, T., Mock, M., Thier, P., 2002. Quantitative organization of neurotransmitters in the deep cerebellar nuclei of the Lurcher mutant. J. Comp. Neurol. 452 (4), 311–323.

Wolpert, D.M., Miall, R.C., Kawato, M., 1998. Internal models in the cerebellum. Trends Cognitive Sci. 2 (9), 338–347.