NMDARs expressed in Granule Cells are involved in cerebellar plasticity and motor learning

Martijn Schonewille[a], Allison E. Girasole[b,c], Philippe Rostaing[d], Caroline Mailhes-Hamon[e], Alexandra B. Nelson[b], Antoine Triller[d], Mariano Casado[e], Chris I. De Zeeuw[a,f*] & Guy Bouvier[e,g*]

a. Department of Neuroscience, Erasmus MC, Rotterdam, The Netherlands.

b. Neuroscience Graduate Program, UCSF, San Francisco, CA 94158, USA; Department of Neurology, UCSF, San Francisco, CA 94158, USA; Kavli Institute for Fundamental Neuroscience, UCSF, San Francisco, CA 94158, USA; Weill Institute for Neurosciences, UCSF, San Francisco, CA 94158, USA.

c. Current address: Department of Neurobiology, Harvard Medical School, Boston, MA, USA; Howard Hughes Medical Institute, Harvard Medical School, Boston, MA, USA.

d. Cell Biology of the Synapse. Ecole Normale Supérieure, IBENS, Paris, F-75005, France. CNRS, UMR 8197, Paris, F-75005, France. INSERM, U 1024, Paris, F-75005, France. PSL, Paris, France.

e. Ecole Normale Supérieure, IBENS, Paris, F-75005, France. CNRS, UMR 8197, Paris, F-75005, France. INSERM, U 1024, Paris, F-75005, France. PSL, Paris, France.

f. Netherlands Institute for Neuroscience, Royal Dutch Academy of Arts &Sciences, Amsterdam, The Netherlands.

g. Current address: Department of Physiology, University of California, San Francisco, San Francisco, CA, USA; Howard Hughes Medical Institute, University of California, San Francisco, San Francisco, CA, USA.

* Corresponding authors: bouvier.ga@gmail.com (G.B) & c.dezeeuw@erasmusmc.nl (C.I.D.Z)

Abstract

Long-term synaptic plasticity is believed to be the cellular substrate of learning and memory. Synaptic plasticity rules are defined by the specific complement of receptors at the synapse and the associated downstream signaling mechanisms. In young rodents, at the cerebellar synapse between granule cells and Purkinje cells, bidirectional plasticity is shaped by the balance between transcellular nitric oxide driven by presynaptic NMDA receptor (NMDAR) activation and postsynaptic calcium dynamics. However, the role and the location of NMDAR activation in these pathways is still debated in mature animals. Here, we show in adult rodents that NMDARs are present and functional in presynaptic terminals where their activation triggers nitric oxide (NO) signaling. In addition, we find that selective genetic deletion of presynaptic, but not postsynaptic, NMDARs prevents synaptic plasticity at parallel fiber-Purkinje cell (PF-PC) synapses. Consistent with this finding, the selective deletion of granule cells NMDARs affects adaptation of the vestibulo-ocular reflex. Thus, NMDARs presynaptic to PCs are required for bidirectional synaptic plasticity and cerebellar motor learning.

Significance Statement

Learning depends on synaptic plasticity. The signaling mechanisms that control induction of this plasticity determines the learning rules at the specific synapse involved. Moreover, the relationship between the activity patterns of synaptic inputs and the type, direction, and level of plasticity induced may evolve during development. Here, we establish a key link between NMDA receptor activation presynaptic to cerebellar Purkinje cells, downstream signaling, and the ability of adult animals to learn a cerebellar motor task.

Introduction

The ability of an organism to adjust its behavior to environmental demands depends on its capacity to learn and execute coordinated movements. The cerebellum plays a central role in this process by optimizing motor programs through trial-and-error learning (1). Within the cerebellum, the synaptic output from granule cells (GCs) to Purkinje cells (PCs) shapes computational operations during basal motor function and serves as a substrate for motor learning (2). Several forms of motor learning depend on changes in the strength of the PF to PC synapse (3, 4).

In the mammalian forebrain, synaptic plasticity typically relies on postsynaptic NMDAR activation, which alters AMPAR turnover at the postsynaptic site (5). However, this may not extend to the cerebellar synapse between GCs and PCs, since no functional postsynaptic NMDARs have been identified in young or adult rodents (6, 7). Pharmacological approaches, however, have shown that plasticity induction still depends on NMDAR activation at the PF-PC synapse in young rodents (8-11). Alternative mechanisms for NMDAR-dependent synaptic modulation may involve presynaptic NMDARs activation (12-14; for review: 15,16). Besides depending on NMDARs, PF-PC synaptic plasticity also requires nitric-oxide signaling (19, 20). As nitric-oxide synthase (NOS) is expressed in GCs, but not in PCs (21), the activation of presynaptic NMDARs might allow Ca^{2+} influx that activates NO synthesis, which in turn may act upon the PCs. In the mature cerebellum, the existence of presynaptic NMDARs on PFs and the role of NO in PF-PC plasticity remains a matter of debate. Previously, we have proposed that the activation of putatively presynaptic NMDARs in young rodents is necessary for inducing PF-PC synaptic plasticity without affecting transmitter release or presynaptic calcium dynamics (8, 16, 9, 17). More recently, it has been shown that a subset of PFs express presynaptic NMDARs containing GluN2A subunits and that these receptors are functional (10, 11). Thus, in contrast to their role at other synapses, presynaptic NMDARs at part of the PF-PC synapses might act via the production of NO to induce postsynaptic synaptic plasticity, without altering neurotransmitter release (8, 19-22).

In the cerebral cortex, the expression of presynaptic NMDARs is developmentally regulated (23, 24). However, little is known about the presence and function of presynaptic NMDARs in adult tissue. In the adult cerebellum, PCs only show NMDARs postsynaptic to their climbing fibers (CFs) (17). It has been proposed that the activation of these receptors could have heterosynaptic effects during PF-PC long-term depression (LTD). This mechanism would explain why LTD in adults depends on NMDARs. According to this model, presynaptic NMDARs would be a transient feature of developing tissue and not necessary for induction of synaptic plasticity and motor learning in adult animals (17). Combining electron microscopy, 2-photon calcium imaging, synaptic plasticity experiments, and behavioral measurements, we show that presynaptic NMDARs are not

developmentally regulated and are required for cerebellar motor learning.

Here, we show that presynaptic NMDARs are present and functional in PFs of mature rodents. By specifically deleting the NMDAR subunit GluN1 either in the post- (PC) or the presynaptic cells (GCs), we demonstrate that NMDAR activation in GCs plays a key role in bidirectional synaptic plasticity and in vestibulo-ocular reflex (VOR) adaptation, an important paradigm for testing cerebellar motor learning (25-27). In contrast, NMDARs in PCs are neither involved in PF-PC synaptic plasticity nor required for cerebellar motor learning.

Results

NMDARs are presynaptically expressed and are activated by high-frequency stimulation of parallel fibers. First, we established the presence of presynaptic NMDARs in PFs of adult rodents by performing both pre-embedding and post-embedding electron microscopy immunohistochemistry, using antibodies directed against either GluN1 or GluN2 subunits. Pre-embedding immunoperoxidase staining revealed numerous GluN2 (Fig. 1*A*) and GluN1 (Fig. 1*B*) reactive profiles in PF varicosities facing small postsynaptic elements. The latter had cytological characteristics of PC dendritic spines (28). Pre-embedding immunogold labelling of GluN2 (Fig. 1*C*) and GluN1 (Fig. 1*D*) and distance measurements (Fig. 1*E*) indicated that particles are predominantly observed at the edge of the presynaptic active zones (82.5% are out of the active zone with 50% of the total at less than 125 nm from its edge). The same result was observed with post-embedding techniques (Fig. 1*F*). Next, we tested whether PF varicosities showed activity-dependent NMDAR-associated calcium transients. To follow calcium dynamics in PFs, we injected adeno-associated viruses carrying floxed GCaMP6f and td-Tomato in the cerebellar vermis of α6-Cre mice; α6 is a promoter specific of cerebellar GCs (29). Three to four weeks post viral injection, td-Tomato fluorescence was observed to the GC somatodendritic compartment in the granule cell layer and their PFs axons in the molecular layer (Fig. 1*G*). We stimulated PFs in cerebellar horizontal slices using an extracellular electrode and measured calcium transients 50 to 250 µm from the stimulated site. This distance ensured the absence of presynaptic calcium dynamics perturbations due to stimulation-induced local electroporation (11). We stimulated PFs in bursts every 15 sec (25 pulses at 200 Hz). PF stimulation produced calcium transients of variable amplitude between varicosities. After a stable baseline was achieved, we blocked NMDARs by applying a mix of D-APV (150 µM) and buffered Zn^{2+} (300 nM). In the presence of these drugs, the mean calcium transient amplitude was reduced (Fig. 1*H-J*, 95.3 ± 1.1% of baseline, p = 7.5e−5, n = 107 detected objects). Upon drug washout, calcium transient amplitudes returned to baseline values (98.2 ± 0.8% of baseline, p = 4e−3 vs NMDAR blockers; p = 0.02 vs

control). The ratio between APV and baseline values presented a skewed distribution (Fig. 1*J*), consistent with the heterogeneous expression of NMDARs at these varicosities reported in young animals (11). Thus, in mature PF boutons, presynaptic NMDARs are located at the periphery of the active zone and may serve a functional role.

Presynaptic, but not post-synaptic, NMDARs are required to induce PF-PC plasticity. In young rodents, we have previously shown that PF-PC synaptic plasticity required high frequency burst activation of PFs to recruit presynaptic NMDARs (10, 11). Moreover, we have previously confirmed that high frequency bursts also induce long-term potentiation (LTP) in adult mice at the age of 2-5 months (30). Therefore, using bursts of 5 stimulations at 200 Hz every second (300 repetitions) of PFs, we first induced a postsynaptically-expressed potentiation of 227±19% compared to baseline (n = 12, p = 4.4e−05; PPR = 98.8 ± 2.8% of baseline, p = 0.23; Fig. S1*A-D*). In contrast to 200 Hz, PF bursting activity at 16.7 Hz did not result in a robust potentiation of the EPSC (125±5% of baseline, n = 6; p = 3e−04 vs. 200 Hz control condition, p = 0.09 vs. baseline, Fig. S1B-*D*) as shown in young rodents for both LTD and LTP (10, 11; respectively). The LTP induction at 200 Hz required NMDAR activation, as potentiation was blocked in the presence of D-APV (111 ± 7% of baseline; n = 7, P = 4e−04 vs. control, P = 0.12 vs. baseline) (Fig. S1B-D). To determine the location of the NMDARs involved in LTP induction, we generated mice lacking NMDARs specifically in GCs by crossing α6-Cre with GluN1-floxed animals (GC-GluN1 ko, Fig. 2*A,* see Methods). Mice homozygous for the deleted GluN1 allele were viable and their cerebellum appeared to develop normally. Burst stimulation at 200 Hz revealed that PF-PC LTP was impaired in these animals (109 ± 7 %, n = 10 vs 200 ± 40%, n = 8 in slices from control animals GluN1 flox/flox without Cre, GC-GluN1 wild-type (wt), p = 3e-5; Fig. 2*B*,*D-F*). Likewise, PF-PC LTD was also abolished in these animals (110 ± 3%, n = 6 versus 70 ± 0.5 %, n = 7 in slices from control animals GC-GluN1 wt, p = 0.0009; Fig. 2C-*F*). These results show that NMDARs expressed by granule cells in adult animals are necessary for both LTP and LTD induction at PF-PC synapses. To determine whether NMDARs expressed by Purkinje cells also contribute to synaptic plasticity in mature mice, we produced mice lacking NMDARs specifically in PCs by crossing L7-Cre with GluN1 floxed mice (PC-GluN1 ko, Fig. 2*A*). L7 is a promoter that is specific for cerebellar PCs (30, see Methods). PF-PC LTP was indistinguishable from controls (206 ± 17 %, n = 9 versus 209 ± 26%, n = 10 in slices from control animals PC-GluN1 wt, p = 0.56; Fig. 2B,*G-I*). Likewise, PF-PC LTD was unaffected in slices from these animals (70 ± 4 %, n = 6 versus 76 ± 2 %, n = 7 in slices from control animals GluN1 flox/flox without Cre, PC-GluN1 wt, p = 0.36; Fig. 2*C*,*G-I*). Thus, the NMDARs necessary for LTP and LTD induction at mature PF-PC synapses are located presynaptically in the GCs and not in the postsynaptic PCs.

In contradiction to our results, Piochon and colleagues (17) observed no effect of NMDAR

blocking on LTP induction. We therefore tried to identify the experimental factors responsible for this difference. In the direct vicinity of the stimulation electrode (up to a few tens of microns), presynaptic (and presumably post-synaptic) calcium dynamics are severely disturbed, potentially due to local electroporation (11). Since the stimulation electrode is usually in the immediate vicinity of the recorded synapse in sagittal sections, the requirements for presynaptic NMDAR activation may be bypassed under these conditions. To check for a potential bias created by slice orientation, we tested plasticity induction in sagittal slices. Indeed, using the same conditions as Piochon and colleagues (17) (7 PF stimulations at 100 Hz, 300 repetitions at 1 Hz) and our LTP induction protocol used in horizontal slices (5 PF stimulations at 200 Hz, 300 repetitions at 1 Hz), we successfully induced LTP (142 ± 11% of baseline; n = 9, p = 0.0002; Fig. S1*E*-*G*; and 149 ± 10% of baseline; n = 7, P = 6e-4; Fig. S1*E*,*G*; respectively). In contrast with horizontal slice orientation experiments, this form of LTP was not abolished by APV (50 µM) application (148 ± 16% of baseline, n = 6, p = 0.003, p = 0.95 vs. sagittal control condition, Fig. S1*E*-*G*; and 139 ± 14% of baseline, n = 6, p = 0.008, p = 0.43 vs. sagittal control condition, Fig. S1*E*,*G*; respectively). Therefore, we propose that the absence of an effect of NMDAR blockade reported by Piochon and colleagues (17) might be due to direct perturbation of the presynaptic terminal triggering spurious Ca^{2+} entry.

To potentially connect the requirement for high frequency stimulation of PFs to induce PF-PC plasticity and the kinetics properties of NMDARs, we investigated the involvement of presynaptic NMDARs containing the GluN2A subunits. It has been demonstrated that presynaptic NMDARs at PF-PC synapses in young animals show pharmacological properties characteristic of GluN2A-containing receptors (10, 11, 31), which may largely determine receptor kinetics (23, 24, 33). To determine if NMDARs containing GluN2A subunits are involved in LTP induction in adult mice, we investigated LTP induction in GluN2A-KO mice (34). In these mutants lacking GluN2A subunits, LTP could not be induced with our standard protocol (5 PF stimulations at 200 Hz, 300 repetitions (106 ± 15% of baseline, n = 7, P = 0.21, Fig. S1*B*,*D*), in contrast to their wild-type littermates (177 ± 18% of baseline, n = 6). This result shows that GluN2A-containing NMDARs are required for plasticity induction at PF-PC synapses in adults. These data are consistent with earlier demonstrations in younger animals (11), highlighting persistent expression patterns of NMDAR subunits at PF-PC synapses during postnatal development and beyond. Thus, PF-PC bidirectional synaptic plasticity in the mature cerebellum is driven by NMDARs containing GluN2A subunits.

Nitric oxide dependent LTP requires activation of GCs expressing NMDARs. Both LTP and LTD induction depend on nitric oxide (NO) signaling in young rodents in the cerebellum (19, 20, 35). However, this finding has yet to be shown in adult animals. To test the involvement of NO signaling in adult LTP, we applied our standard LTP induction protocols in the presence of L-NAME (100 µM),

a specific antagonist of nitric oxide synthase. Under these conditions LTP was abolished (101 ± 5% of baseline, n = 7, p = 0.47; Fig. S2*A*-*C*). NO synthesis during PF bursting activity may result from activation of presynaptic NMDARs (8, 10, 11) and/or from activation of receptors located in interneurons (18, 36). To understand the link between NMDAR activation and NO synthesis and determine the cell types involved, we used an electrochemical probe to measure NO production during high-frequency burst stimulation in the molecular layer (see Methods). We stimulated PFs with a protocol mimicking the previous configuration used to image NMDAR-dependent calcium signals (Fig. 1). This protocol resulted in a significant enhancement of the current recorded at the electrode (Fig. S2*D*,*E*; 112.8 ± 32.8pA , p = 8e-5, n = 20, GC-GluN1 wt). The signal was specific for NO production, as it was absent in the presence of L-NAME (Fig. S2*D*,*E*; -33.5 ± 34.1pA, p = 0.18, n = 12). Moreover, NO production was due to NMDAR activation, because it was also abolished in the presence of APV (Fig. S2*D*,*E*; -68.1 ± 27.1pA, p = 1.3e-4 APV vs control, n = 15). Finally, the signal was absent in slices from mice lacking GluN1 in GCs (Fig. S2*D*,*E*; -10.4 ± 27.2pA, n =14, wt vs. ko, p = 0.003). Therefore, we conclude that high-frequency activity of PFs results in activation of their NMDARs and that subsequent NO production participates in the induction of plasticity within the molecular layer.

Cerebellum-dependent basal motor behavior is virtually unaffected after ablation of NMDARs from GCs or PCs. To test the specific contributions of NMDARs in GCs and PCs in basal reflexive eye movements, we subjected animals lacking NMDARs specifically in either GCs or PCs to a cerebellum-dependent behavioral assay involving compensatory eye movements. Vestibular and full-field visual inputs drive compensatory eye movements, known as the vestibulo-ocular reflex (VOR) and optokinetic reflex (OKR), respectively (37, 38). While the VOR compensate head movements with eye movement, the OKR causes eye movement in response to a visual flow, while the head is stationary. These reflexes work in conjunction and together generate the visually-enhanced VOR (or VVOR) to maintain a stable image on the retina when an animal is moving through their environment. To preserve optimal stabilization throughout life the VOR is subject to adaptation based on visual feedback, a process that depends on the cerebellar cortex (3). To evaluate the contribution of pre- and postsynaptic GluN1 receptors to motor performance, we tested baseline OKR, VOR, and VVOR in wt, PC-GluN1 ko mice, and GC-GluN1 ko mice (Fig. S3*A*). In PC-GluN1 ko mice, eye movements evoked by sinusoidal rotation of the visual field (OKR) or table (VOR) did not differ in gain (reflecting amplitude) or phase (timing) from those of controls (Fig. S3*B*, OKR and VOR: all p > 0.5). In the VVOR, combining both inputs, the gain was also not affected (p = 0.55), but there was a small difference in phase (p = 0.010, Δphase = 1.1 ± 0.1° across tested frequency range). Likewise, ablating GluN1 from GCs mildly affected compensatory eye movements (Fig. S3*C*). VOR phase was lower

in GC-GluN1 ko mice ($p = 0.043$, Δphase = 6.2 ± 2.2°), whereas all other parameters did not differ from those of controls (all $p > 0.3$), despite a trend in the gain of the VVOR to be lower in GC-GluN1 ko mice ($p = 0.056$). Taken together, ablating GluN1 from Purkinje or granule cells has minimal effects on baseline performance of the VOR and OKR, suggesting that NMDARs and NMDAR-dependent plasticity are only marginally important for basal cerebellum-related motor behavior.

VOR adaptation deficits are more pronounced after ablating NMDARs from GCs. Genetic interventions, particularly those targeted at components of plasticity processes, typically affect motor learning more than motor performance (27, 39, 40). To investigate the role of GCs and PCs NMDARs in cerebellar motor learning, we first tested the contribution of GluN1 to short-term learning by subjecting mice to a VOR learning paradigm which induces a decrease in VOR gain. Five sessions of sinusoidal rotation of the mice and the visual input in the same direction at the same amplitude (in-phase) for 10 min resulted in a progressive decrease of VOR gain in control mice (recorded in dark, $p < 0.001$, before vs. after 50 min; Fig. 3*A,B*). This type of short-term cerebellar learning was not affected in PC- or GC-GluN1 ko mice (both $p > 0.14$ vs respective controls; Fig. 3*A,B*). Next, we tested mice on a longer-term learning paradigm by continued training the following day (after mice remain in the dark for 23 hours) with the same parameters. However, the amplitude of visual stimulus was increased. This paradigm was designed to reverse the direction of the original VOR (i.e. VOR phase adapt towards 180°). Over three days of reversal training, VOR phase increased from values ≤ 30° to an average maximum of 144 ± 15° in control and 132 ± 13° in PC-GluN1 ko mice (Fig. 3*A*). This phase increase was comparable between groups (day 2, 3, and 4, all $p > 0.3$). While the VOR gain increased again after the phase had reversed, this gain increase was significantly less pronounced in PC-GluN1 ko mice (day 4, $p = 0.011$), indicating a subtle impairment in VOR learning (Fig. 3*A*). In contrast, the effects in GC-GluN1 ko mice were more severe. These mice did not achieve the same phase-shift as control mice, reaching an average maximum of 58 ± 10° (GC-GluN1 ko) compared to 115 ± 16° (wt; Fig. 3*B*). This difference was significant throughout the training period ($p < 0.015$ for days 2, 3, and 4). In addition to its role in VOR adaptation, the cerebellum is also important for adaptation of the OKR. Prolonged exposure to sinusoidally rotating visual input, combined with or without a conflicting vestibular input, will result in an adaptive increase of OKR gain (39). OKR gains after the reversal training were significantly higher in all mice, but this increase was attenuated in GC-GluN1 ko mice (Fig. 3*C*, $p = 0.045$), whereas there was no impairment in PC-GluN1 ko mice ($p=0.27$). The presence of an evident phenotype in both cerebellum-dependent learning paradigms, VOR phase-reversal and OKR gain-increase, confirms the functional relevance of NMDARs in GCs in cerebellum-dependent learning.

Overnight consolidation, the stabilization of acute adaptive changes to long-term

modifications, is crucial for persistent motor adaptation (41, 42). To uncover whether the GC-GluN1 phenotype is linked to the ability to adapt during training or the ability to store and maintain the adaptation overnight, we tested the consolidation of the adaptive changes (43). As this measure is relatively sensitive to noise, we compared the most stable, consistent change: i.e. the difference between the VOR gain-decrease during the first day and that on the second day. Consolidation, the percentage of change persisting overnight, was impaired in GC-GluN1 ko mice (Fig. 3*D*, 39 ± 8% vs. 71 ± 8% in controls, p = 0.011, unpaired Student's t-test), implying that at least part of the deficit can be explained by impaired consolidation. Not surprisingly, consolidation was not impaired in PC-GluN1 ko mice (Fig. 3*D*, 41 ± 6% vs. 52 ± 7% in controls, p = 0.38, unpaired Student's t-test). This selective impact on consolidation underscores the long-term impact of the GluN1-dependent mechanisms hypothesized above.

Discussion

Here we present immunohistochemical and functional evidence for the presence of presynaptic NMDARs at PF-PC synapses in the cerebellum of adult mice. These receptors are required to induce both postsynaptic forms of synaptic plasticity, LTP and LTD. Using cell-specific deletion of NMDARs either in GCs or PCs, we demonstrated that only GC NMDARs are robustly involved in PF-PC synaptic plasticity and VOR adaptation. Counter to previous theories, our results support the idea that these receptors are persistently expressed in the adult cerebellum, and the roles of PC NMDARs in PF-PC synaptic plasticity and cerebellar learning should be reconsidered.

Location of presynaptic NMDARs. Presynaptic receptors are often found within the active zone where they modulate synaptic transmission and plasticity. This tendency is seen in various types of presynaptic receptors throughout the brain (45), including GABA receptors (44), NMDARs (13) and kainate receptors (15), all of which are found on PF terminals (46). However, presynaptic NMDARs at the cerebellar PF-PC synapse control plasticity in a distinct manner: these receptors participate in the induction of plasticity by promoting NO synthesis without changes in transmitter release (8, 10, 11). How the activation of a calcium-permeable presynaptic receptor results in calcium-dependent signaling (NO synthesis) without interfering with transmitter release has been a puzzle, leading to doubt regarding the existence of such receptors. Here we propose an explanation for this paradox. Immunogold staining shows that most of the presynaptic NMDARs are not located within the active zone, but at its periphery, thus capable of being active without affecting calcium dynamics at release sites. For comparison, this location is beyond the 30 nm coupling distance between calcium influx

through voltage-dependent calcium channels and the release sensor (47). We also show that PF-PC synaptic plasticity is NO-dependent, and that NMDAR activation leads to NO production, suggesting a tight coupling between NMDARs and NOS. This coupling may minimize crosstalk between the Ca^{2+} entry involved in transmitter release and the Ca^{2+}signaling implicated in plasticity induction through the NMDAR-NOS system.

Pivotal role of presynaptic NMDARs in synaptic plasticity. We have previously described that presynaptic NMDARs are required to induce LTP (11) and LTD (10) at PF-PC synapses in cerebellar slices from immature rodents. Here, we show that both LTP and LTD induction are NMDAR-dependent in adult animals. These results differ with previous reports (17,18). Using cell-specific deletions of the GluN1 subunit either in GCs (α6-Cre mice) or PCs (L7-Cre mice), we demonstrate that only the NMDARs expressed in GCs, and not those in PCs, are involved in both LTP and LTD at the PF-PC synapse. We propose two explanations for this discrepancy. First, in the parasagittal slices used by Piochon and colleagues (17), electroporation of the presynaptic elements by the extracellular stimulation may bypass the requirements for NMDAR activation (11; Fig. S2). Second, climbing fibers (CFs) *in vivo* fire in bursts at high frequency (reaching 400 Hz: 48, 49), well above frequency of stimulation by Piochon and colleagues. We induced LTD using a high frequency burst of the CF 100 ms after the PF burst, as described previously *in vitro* (11, 29, 49) and *in vivo* (49, 51). This bursting parameter may be crucial, since calcium spike propagation in the PC dendritic arbor depends on membrane excitability (52). Therefore, a gentle hyperpolarization of the dendrite due to CF-NMDARs blockade could decrease the probability of propagation and consequently impair LTD induction (11). This phenomenon is of potential physiological interest, since it may attribute a subtle role in dendrite excitability to the NMDARs present at the CF-PC synapse.

Motor performance and motor learning. The robust cellular effects on synaptic plasticity raise the question of the functional impact of ablating NMDARs. Cerebellum-dependent learning depends on the synergy of several forms of plasticity distributed across the molecular layer (reviewed in ref. 3). Here we demonstrate 1) that in line with the absence of an effect on plasticity, ablating NMDARs from PCs virtually does not affect VOR adaptation, and 2) that the combined deletion of LTD and LTP impairs, but does not completely block, cerebellar learning. The absence of the complete block of cerebellar learning could also be due to the potential involvement of molecular layer interneurons in oculomotor learning (19). Whereas the role of NMDARs in PCs is limited, NMDARs in GCs are necessary for plasticity induction. The connection between mossy fibers (MF) and GC dendrites is glutamatergic and may also undergo NMDAR-dependent LTP (53). Here we show that deleting GluN1 from GCs blocks bidirectional plasticity at the PF-PC synapse. Deleting GluN2A from GCs

was also found to block MF-GC LTP in the dendritic compartment (39). Interestingly, GluN2A/C double knockouts were found to have motor deficits in the rotarod test (54) and GluN2A knockout mice have an attenuated, but not blocked, VOR phase-reversal learning (39). Deleting GluN1 in GCs may also disrupt MF-GC LTP. Hence, the deficits in cerebellar learning observed in the α6Cre-GluN1 mice could in principle also be related to the putative effect on MF-GC LTP. Conversely, given the dramatic effects on both LTD and LTP at the PF-PC synapse after GluN1 deletion, one could also conclude that GluN2A/C deletion would result in a comparable effect on these forms of plasticity, and thereby alter cerebellar motor learning. This potential alternative explanation can only be valid if GluN2A is also required for presynaptic plasticity, which is indeed what we observed here (Fig. S1*D*). The lack of an apparent phenotype on basal cerebellar motor performance function, even when dealing with demanding functional tests, indicates that NMDARs in GCs as well as synaptic plasticity at PF-PC synapses are involved specifically in the mechanisms necessary for cerebellum-dependent learning and memory. Indeed, the diverse presynaptic machinery of PF terminals may endow its target, the PC, with ample means to fine-regulate its output in the long-term (46, 55).

Material and Methods

Electron immunohistochemistry. Pre-embedding immunoperoxidase or immunogold and post-embedding immunogold detections were performed with specific anti-GluN1 mouse monoclonal antibody and anti-GluN2A rabbit polyclonal antibody. See details in SI Material and Methods.

Electrophysiology in slices. Whole-cell patch-clamp recoding in cerebellar slices from C57Bl6 mice (8- to 20 weeks-old) were performed the same as in ref. 11. Details are provided in SI Material and Methods.

NO detection. NO efflux from cerebellar slices was monitored using a NO-selective amperometric microprobe with a 7 µm tip (WPI), similar to previous work (52). A constant voltage of 0.9V was applied using an Axon Multiclamp 700B amplifier. The probes were routinely cleaned in 0.1M H_2SO_4 to remove debris. The microprobe was positioned at least 30 µm away from the stimulating electrode to avoid spurious Ca-NO signaling (11). Before use, probes were equilibrated for 30 min in the incubation medium.

Viral injection. 2-3 month-old C57BL/6 Bac6-cre mice, in which recombinase is expressed exclusively in the cerebellar granule cells, were used. A mix of flexed GCaMP6f and td-Tomato adeno-associated viruses were stereotaxically injected into the cerebellar cortex. See details in SI Material and Methods.

Calcium imaging. All experiments were performed using a custom-built random-access two-photon

laser-scanning microscope. Details are provided in SI Material and Methods.

Compensatory eye movement adaptation. At least 5 days after preparatory surgery, adult male mutant and control mice were head-fixed in the middle of a turntable with surrounding screen. Compensatory eye movements were evoked with rotational visual and/or vestibular input, and cerebellar motor learning was tested by mismatching these two inputs. Video-recorded eye movements were calibrated and gain and phase, representing the amplitude and timing of movement respectively, were determined. Details are provided in SI Material and Methods.

Acknowledgements. This work was supported by the program "Investissements d'Avenir" from the French Government, implemented by Agence Nationale de la Recherche, references: ANR-10-LABX-54 MEMOLIFE, ANR-11-IDEX-0001-02 PSL* Research University. MS was funded by the Funding Dutch Scientific Organization – Life sciences (NWO ALW Veni) and ERC-Stg. CIDZ was supported by FP7-C7 European Commission, ZonMw, Netherlands Organization for Scientific Research (ENW-Klein), European Research Council (Advanced Grant, and Proof of Concept Grant), Medical NeuroDelta Programme, Topsector Life Sciences & Health (Innovative Neurotechnology for Society or INTENSE), and the Albinism Vriendenfonds Netherlands Institute for Neuroscience. GB was funded by Région Ile de France, FRM, and Labex MEMOLIFE. AEG was supported by the NSF GRFP and the UCSF Discovery Fellows Program. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. We thank A Ayon, R de Avila Freire and L Post for technical assistance and B Barbour, RS Larsen, PL Reeson, and Chenyu Wang for comments on the manuscript.

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Figure Legends:

Figure 1: NMDARs are present and functional in PF varicosities.

(A-F) Electron immunohistochemistry reveals the presence of GluN1 and GluN2 on presynaptic sites of PF-PC synapses. NMDAR labelling of profiles presynaptic to dendritic spines with antibodies recognizing GluN2 (A, C, E) or GluN1 (B, D) subunits. GluN2 (A) or GluN1 (B) subunit immunoperoxidase deposit is detected in the presynaptic element of asymmetric synapses. The presynaptic particles associated with GluN2 (C) or GluN1 (D) antigenic determinants (arrows) are detected at the edge (arrowheads) of the active zone following pre-embedding immunogold labelling. With post-embedding immunogold labeling GluN2 **(E)** associated particles are also found at the edge of presynaptic actives zones showing that the access to antigenic determinant was not limited by the cytoskeleton of the presynaptic differentiation. **(F)** Gold particle quantification of the distance to the edge of the active zone for GluN2: values along the x-axis represent the distance between the edge of the synaptic complex and the nearest immunogold labeling (mean = 100.1 nm (red), median = 50 nm, SEM = 24.3, n = 41), negative values represent particles within the active zone. Note: 82.5% of the gold particles are outside the active zone (right to the dark vertical line). **(G-J)** Calcium imaging of GCaMP6f-expressing PF varicosities. **(G)** Acousto-optic deflectors based 2-photon snapshot projection (1 plane, 10 images) of a granule cell expressing td-Tomato. PFs are directly stimulated in the molecular layer (ml) and images are recorded at least at 50 µm from the stimulation point (gcl: granule cell layer, pcl: Purkinje cell layer, ml: molecular layer). Enlarged: varicosity calcium image example before (top), during (middle), and after (bottom) blocking NMDARs (top to bottom, respectively). **(H)** Calcium transient example of a another labeled varicosity (30 stimulations at 200Hz). Baseline, APV and Zn^{2+} application, wash-out, and ([baseline]-[NMDAR-blockade]) subtraction (purple dashed). Note the NMDAR-blockade effect on the calcium transient. **(I)** Time course of the normalized ΔF/F in control conditions (n = 107 varicosities, 31 slices from 14 mice). 150 µM D-APV and 300 nM Zn^{2+} were bath applied from minute 8 to 28. **(J)** Normalized ΔF/F data distribution (smoothed histogram; kernel=20). Box plot of normalized data comparing the signal in control conditions, during NMDAR block, and after washout (blue). Mean (red dots) and median (red lines) are shown.

Figure 2: Synaptic plasticity in animals lacking NMDARs in specific neuronal populations

(A) Schematic representation of the strategy used to knock out the GluN1 gene either in cerebellar granule cells (GC-GluN1, wt:blue/ko:green) or in Purkinje cells (PC-GluN1, wt:red; ko:black) **(B, C)** Representative recordings before (grey) and after (dashed line) LTP **(B)** and LTD **(C)** induction. The colored dots beside the traces are the same as in (D- I). **(D,E)** Time course of the normalized EPSC

charge (top) and PPR (bottom), in GC-GluN1wt (blue) and GC-GluN1ko (green) for LTP (D) and for LTD (E). **(F)** Normalized EPSC charge (top) and PPR (bottom) after plasticity induction (t = 30-35 min) for all individual experiments in GC-GluN1wt (LTP, n = 8; LTD, n= 7) and GC-GluN1ko (LTP, n = 10; LTD, n= 6). **(G, H)** Time course of the normalized EPSC charge in PC-GluN1 wt (red) and GC-GluN1 ko (black) for LTP (G) and for LTD (H). **(I)** Normalized EPSC charge (top) and PPR (bottom), after plasticity induction (t = 30-35 min) for all individual experiments in PC-GluN1wt (LTP, n = 10; LTD, n= 7) and PC-GluN1ko (LTP, n = 9; LTD, n= 6). PPR was not changed after synaptic plasticity induction (GC-GluN1: D-F, bottom; PC-GluN1: G-I, bottom). Boxes represent median (black), the upper and lower quartile of the distribution. ns $p > 0.05$; ** $p < 0.001$.

Figure 3: Cerebellum-dependent VOR phase reversal is affected in mice lacking NMDARs in GCs

(A) Top panels: Schematic representation of the VOR phase reversal experiment. Mice were subjected to a mismatched combination of vestibular and visual input. VOR phase reversal is induced by five 10 min training sessions during which the visual stimulus is rotated in-phase with the vestibular input (amplitude, 5°) at increasing amplitudes (day 1, 5°; day 2, 7.5°; day 3-4, 10°). Bottom panels: VOR phase reversal training starts with a decrease of VOR gain (day 1), followed by the increase of the VOR phase (aimed at a phase value of 180°, day 2-4) and finally the increase in the VOR gain (day 4). PC-GluN1 ko mice show VOR phase reversal with similar phase values as controls (both n = 12), but are impaired in the VOR gain increase after reversal (day 4, p = 0.011). **(B)** Mice lacking NMDARs from GCs have a more pronounced deficit in that the phase increases more slowly (day 2, 3, 4: p = 0.001, 0.013, 0.006, respectively). **(C)** VOR phase reversal training also results in an enhanced gain of the OKR. This cerebellum-dependent adaptive change was also impaired in mice lacking NMDARs (n = 11 mice) in GCs (vs. 7 controls, p = 0.045), but in PC-GluN1 ko mice (both n = 11, p = 0.27). **(D)** VOR gain consolidation, the percentage of learned response present on day 2 (b) relative to the adaptive change on day 1 (a) (see inset), was not affected in PC-GluN1 mice (p = 0.38, Students *t*-test). In contrast, impaired long-term adaptation could be linked to a deficit in consolidation in GC-GluN1 ko mice, compared to controls (p = 0.011). (D) Error bars denote SEM (please note that error bars can fall within symbols), * $p < 0.05$, ** $p < 0.001$.

Fig. 1. Schonewille et al.

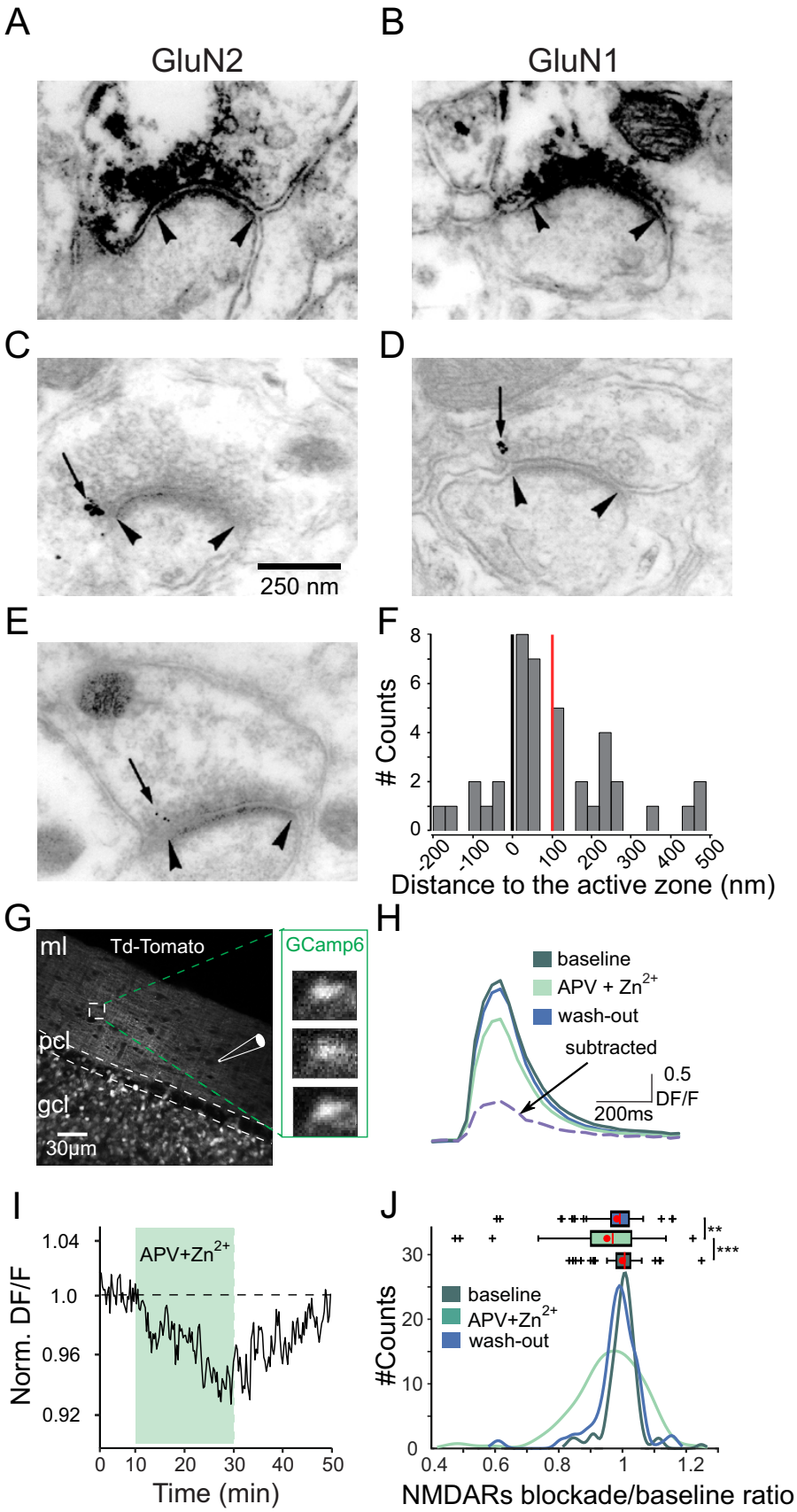

Fig. 2. Schonewille et al.

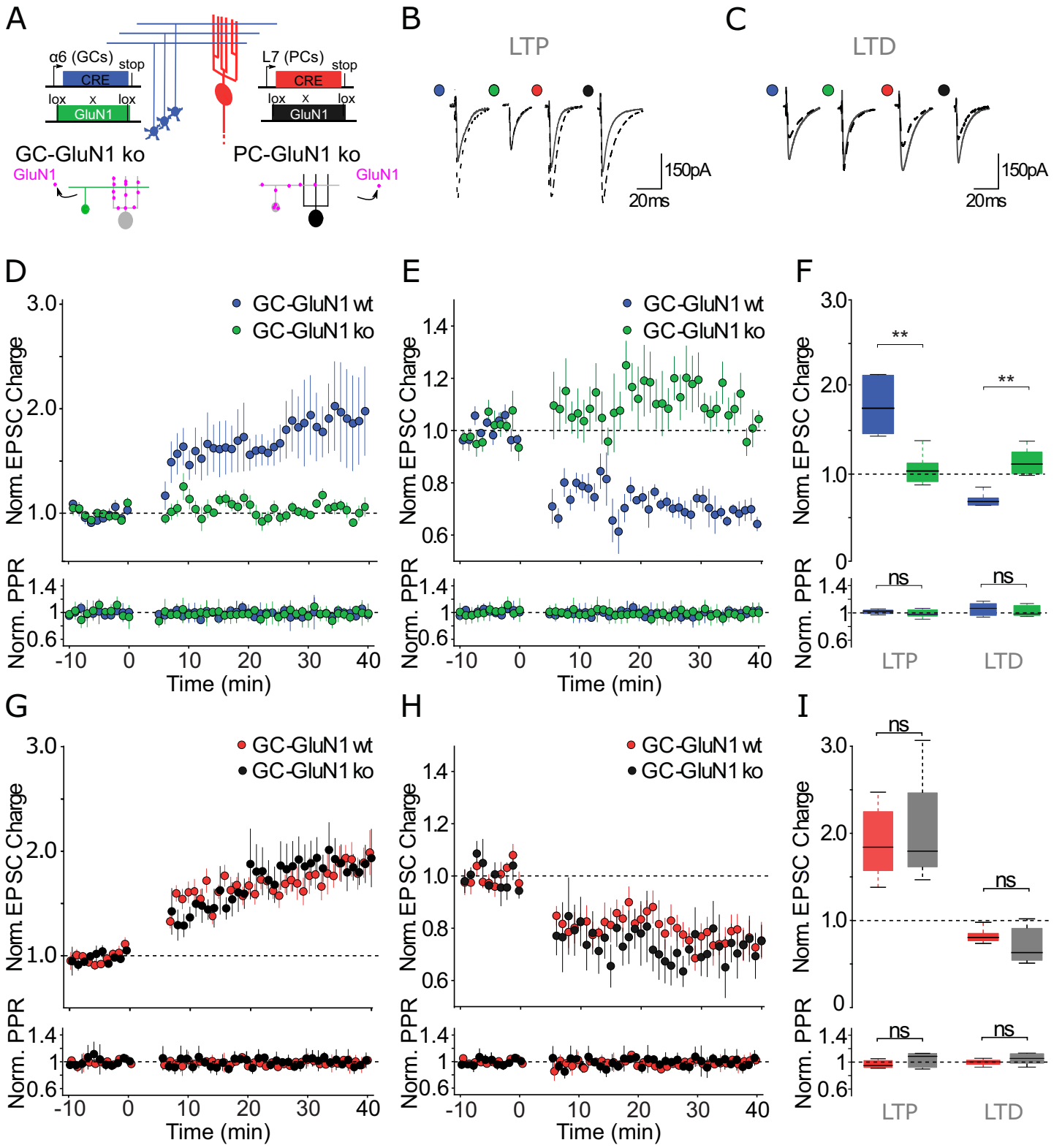
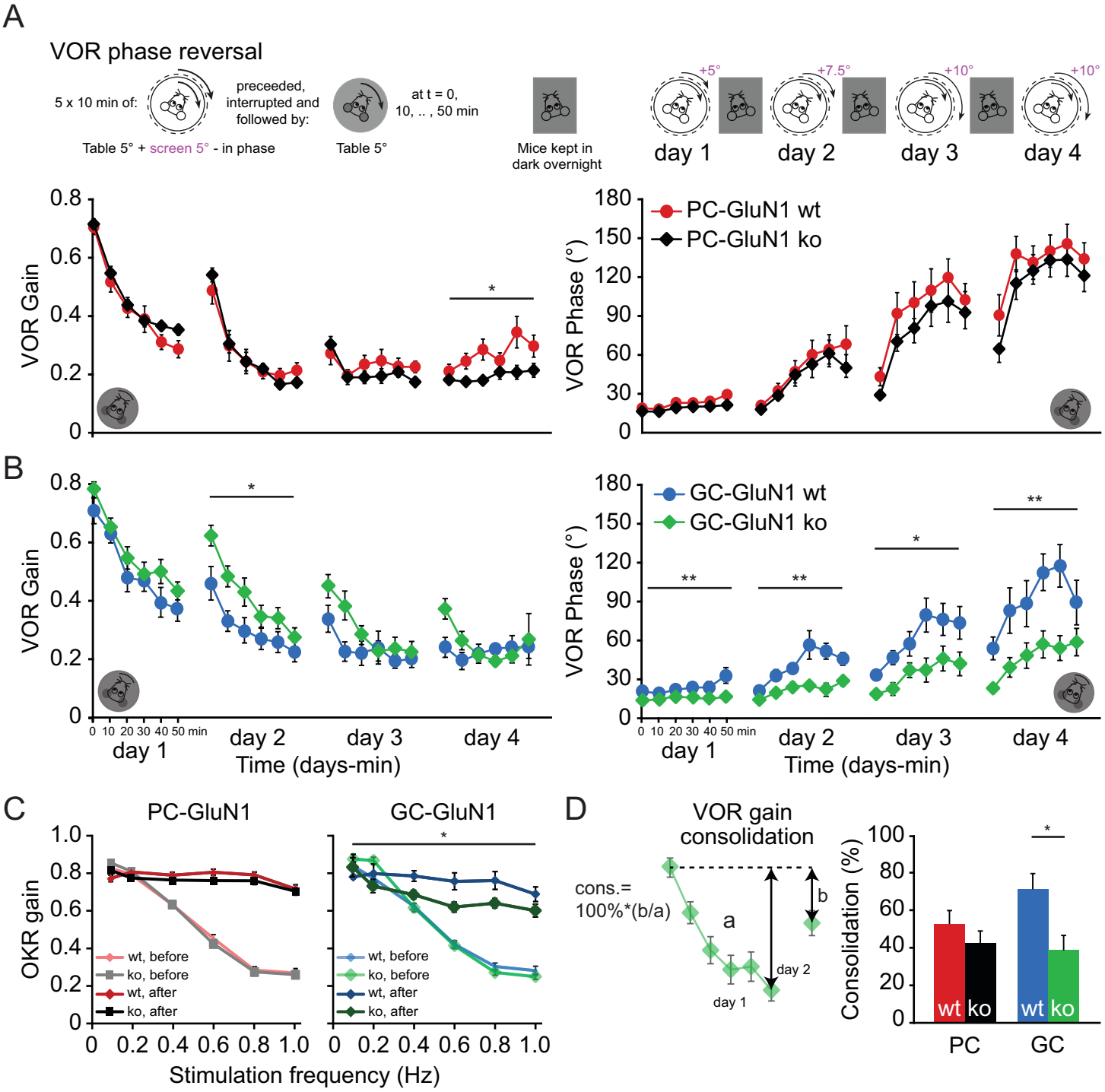
Fig. 3. Schonewille et al.