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# NR2A SUBUNIT OF THE N-methyl D-aspartate RECEPTORS ARE REQUIRED FOR POTENTIATION AT THE MOSSY FIBER TO GRANULE CELL SYNAPSE AND VESTIBULO-CEREBELLAR MOTOR LEARNING

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**Abstract**—Traditionally studies aimed at elucidating the molecular mechanisms underlying cerebellar motor learning have been focused on plasticity at the parallel fiber to Purkinje cell synapse. In recent years, however, the concept is emerging that formation and storage of memories are both distributed over multiple types of synapses at different sites. Here, we examined the potential role of potentiation at the mossy fiber to granule cell synapse, which occurs upstream to plasticity in Purkinje cells. We show that null-mutants of NMDA-NR2A receptors (NMDA-NR2A<sup>-/-</sup> mice) have impaired induction of postsynaptic long-term potentiation (LTP) at the mossy fiber terminals and a reduced ability to raise the granule cell synaptic excitation, while the basic excitatory output of the mossy fibers is unaffected. In addition, we demonstrate that these NR2A<sup>-/-</sup> mutants as well as mutants in which the C terminal in the NR2A subunit is selectively truncated (NR2A<sup>ΔC/ΔC</sup> mice) have deficits in phase reversal adaptation of their vestibulo-ocular reflex (VOR), while their basic eye movement performance is similar to that of wild type littermates. These results indicate that NMDA-NR2A mediated potentiation at the mossy fiber to granule cell synapse is not required for basic motor performance, and they raise the possibility that it may contribute to some forms of vestibulo-cerebellar memory formation. © 2011 Published by Elsevier Ltd on behalf of IBRO.

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**Abbreviations:** DNA, deoxyribonucleic acid; EPSP, excitatory postsynaptic potential; GABA<sub>A</sub>, A subunit of the gamma-aminobutyric acid receptor; LTP, long-term potentiation; NMDA, N-methyl D-aspartate receptor; NMDA-NR2A, NR2 subunit of the NMDA receptor; OKR, optokinetic reflex; PCR, polymerase chain reaction; SEM, standard errors of the mean; VOR, vestibulo-ocular reflex; VVOR, visually-enhanced vestibulo-ocular reflex.

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**Key words:** motor learning, NMDA receptor, LTP, plasticity, behavior.

A variety of disturbances, such as developmental malformations, disease, or fatigue, can lead to aberrations in motor performance, and often these factors induce secondary processes in the brain, which allow us to adapt and limit the performance errors to a certain level. Such secondary compensatory processes usually employ the same learning mechanisms as those used during our daily acquisition of new motor skills, and they are generally mediated by various forms of neuronal plasticity, which are often located at multiple sites. For example, many studies on cerebellar motor learning indicate that formation and storage of procedural memory is situated in at least two sites including the Purkinje cells of the cerebellar cortex and their target neurons in the cerebellar and vestibular nuclei (for reviews see Lisberger, 1998; De Zeeuw and Yeo, 2005). More recent studies have raised the possibility that the granular layer of the cerebellum also contributes to procedural memory formation, because the mossy fiber to granule cell input has been demonstrated to show NMDA-mediated long-term potentiation (LTP) *in vitro* (D'Angelo et al., 1999; Armano et al., 2000; Maffei et al., 2002, 2003; Rossi et al., 2002; Sola et al., 2004; Mapelli and D'Angelo, 2007). However, the possible contribution of this latter form of plasticity to motor learning has yet to be confirmed in intact awake behaving animals, which show otherwise a normal motor coordination.

NMDA receptors are heteromeric ligand-gated ion channels assembled from two families of subunits. Until now two types of NR1 (a–b), four types of NR2 (A–D) and two types of NR3 (A–B) subunits have been described (Llansola et al., 2005). Most NMDA receptors contain two NR1 and two NR2 subunits (Premkumar and Auerbach, 1997). In the developing cerebellum NMDA receptors can occur in multiple types of cells and they play a crucial role in their differentiation (Rabacchi et al., 1992; Komuro and Rakic, 1993). During this process NR1a and NR2B are gradually replaced by NR1b and by NR2A or NR2C, respectively (Cathala et al., 2000; Llansola et al., 2005). In the mature cerebellar cortex NMDA receptors are most abundantly expressed in granule cells, in which they are formed by NR2A and NR2C subunits (Watanabe et al., 1994; Piochon et al., 2007; Renzi et al., 2007). The C-terminal of these subunits can interact with proteins in the postsynaptic density, which retains the NMDA receptor at the synapse and mediates interactions between signal

transduction molecules downstream (Llansola et al., 2005). The NMDA receptors in granule cells control high-frequency repetitive neurotransmission by enhancing and protracting membrane depolarization during excitatory postsynaptic potential (EPSP) trains and they allow the induction of LTP through postsynaptic calcium entry (D'Angelo et al., 1999; Armano et al., 2000; Maffei et al., 2002, 2003; Rossi et al., 2002; Sola et al., 2004; Mapelli and D'Angelo, 2007). Thus, in the adult cerebellum NMDA receptors play a central role in both basic synaptic transmission and plasticity at the mossy fiber to granule cell synapse (D'Angelo et al., 1990, 1993, 1994, 1995, 1997).

To find out whether a disruption of NR2A leads to altered synaptic plasticity at the mossy fiber-granular cell synapse, and, if so, whether such a deficit can be associated with an impairment in motor performance and/or motor learning, we tested granule cell responses and compensatory eye movements in null-mutant mice that lack the NMDA-NR2A receptor (NR2A<sup>-/-</sup>) (Sakimura et al., 1995; Kadotani et al., 1996; Kishimoto et al., 1997; Zhao and Constantine-Paton, 2007). Plasticity in the granular layer was investigated following high-frequency stimulation of mossy fibers (HFS; 100 Hz for 1 s), while cerebellar motor performance and motor learning were investigated by studying the optokinetic reflex (OKR), vestibulo-ocular reflex (VOR), visually-enhanced vestibulo-ocular reflex (VVOR) as well as adaptation following visuo-vestibular training (Stahl et al., 2000; Andreescu et al., 2005).

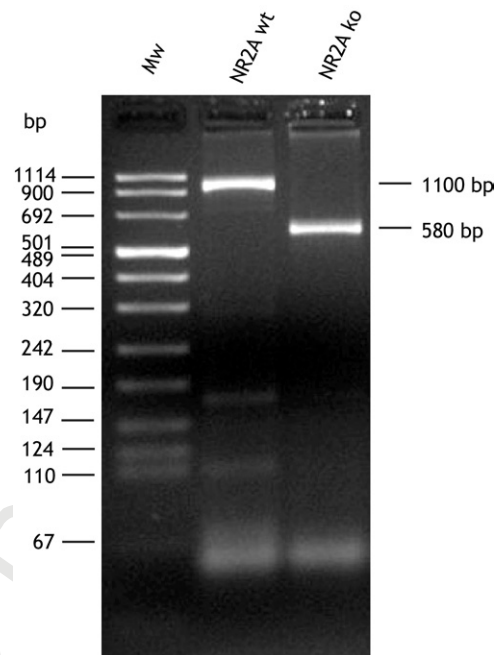
## EXPERIMENTAL PROCEDURES

### Subjects

In this study, we used seven NMDA-NR2A<sup>-/-</sup> mutant mice and 10 of their wild type littermates for electrophysiological recordings and eight NMDA-NR2A<sup>-/-</sup> mutant mice and nine of their wild type littermates for behavioural studies (Sakimura et al., 1992). In addition, we employed seven NMDA-NR2A<sup>ΔC/ΔC</sup> mutant mice and 10 of their wild type littermates for comparison (Sprengel et al., 1998; Rossi et al., 2002). Mice were housed on a 12 h light/dark cycle with food and water available *ad libitum*. All experiments were performed without any knowledge of the genotype and all animal procedures described were in accordance with the rules of the local ethical committee.

### Genotyping

Mice were genotyped by PCR analysis. Briefly, DNA was extracted from tail biopsies (Purelink Genomic DNA Kits-Invitrogen Srl), frozen at -80 °C, and PCR was performed on 2 μg DNA using specific primers for NR2A<sup>-/-</sup> mice (Primer PGK Prom2: 5'-CAGACTGCCT-TGGGAAAAGCG-3'; Primer 2AIN10N\*do: 5'-GGGAATTCGCGGC-CGCAAGAGCAAGAAGACTCC-3'; Primer 2AIN11x\*up: 5'-GGAG-GTACCTCGAGCTCTTCTACAG-3'). Same technique was used for NR2A<sup>ΔC/ΔC</sup> mice (Primer rsp26: 5'-AGAAGTAATGTACCTGAGG-3'; Primer rsp25: 5'-ATCTGCCAGACTGCTCCAG-3'). An initial denaturation of 3 min at 96 °C was followed by 20 s at 96 °C, 30 s at 55 °C and 75 s at 72 °C for 35 cycles. A final extension of 10 min at 72 °C was performed with the Taq Polymerase Eurobiotaq. The molecular weight of the PCR products was compared to the DNA molecular weight marker VIII (Roche Molecular Biochemicals, Italy). The bands acquired with the Image Master VDS (Amersham Bioscience Europe) were at the expected size of 1100 bp for NR2A<sup>+/+</sup> and 580 bp for NR2A<sup>-/-</sup> (Fig. 1).



**Fig. 1.** PCR analysis of NR2A receptor in the NR2A<sup>-/-</sup> and wild type mice. Gel electrophoresis of PCR products taken from wild type mice (wt) and NR2A<sup>-/-</sup> mice (ko). The 1100 and 580 bp bands are indicated at the right of the panel, and correspond to the wild type and knockout PCR products, respectively. Mw, molecular weight.

### Electrophysiological recordings

Whole-cell patch-clamp recording was performed as previously reported (D'Angelo et al., 1995, 1999; Armano et al., 2000). Longitudinal slices (220 μm thick) of the cerebellar flocculus and nodulus were prepared from mice 18–22 days old using a Vibratome (Dosaka, Kyoto, Japan) and cold Krebs solution containing 120 mM NaCl, 2 mM KCl, 2 mM CaCl<sub>2</sub>, 1.9 mM MgSO<sub>4</sub>, 1.18 mM NaH<sub>2</sub>PO<sub>4</sub>, 26 mM NaHCO<sub>3</sub>, and 10 mM glucose (pH 7.4, equilibrated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>). After slicing, the slices were incubated for at least 1 h at room temperature (20–23 °C) in oxygenated Krebs solution. For P 40–50 slices, Krebs solution for cutting and recovery was modified as reported by Goldfarb and Prestori to improve tissue viability (Goldfarb et al., 2007; Prestori et al., 2008). Whole-cell patch-clamp recordings were performed using a recording chamber mounted on the stage of an upright microscope (Zeiss, Oberkochen, Germany). The Krebs solution used for slice perfusion (1–1.5 ml/min) was supplemented with 10 μM bicuculline (Sigma) and 500 nM strychnine (Sigma) to block GABA<sub>A</sub> and glycine receptors. Patch pipettes were pulled from borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany), and, when filled with the intracellular solution, had a resistance of 7–9 MΩ before seal formation. The recording electrodes were filled with a solution containing 126 mM K-gluconate, 4 mM NaCl, 5 mM HEPES, 15 mM glucose, 1 mM MgSO<sub>4</sub>, 0.1 mM BAPTA-free, 0.5 mM BAPTA-Ca<sup>2+</sup>, 3 mM Mg<sup>2+</sup>-ATP, and 0.1 mM Na<sup>+</sup>-GTP (pH 7.2 adjusted with KOH). This solution maintained resting free [Ca<sup>2+</sup>] at 100 nM.

Recordings were made with an Axopatch 200B amplifier (Molecular Devices, Union City, CA, USA) at 32 °C (D'Angelo et al., 1995, 1997, 2001; D'Angelo and Rossi, 1998). All recordings were made at a cutoff frequency of 10 kHz and subsequently digitized at 20 kHz using Clampex 9 in combination with Digidata1200B analog-to-digital converter (Molecular Devices). Just after obtaining the cell-attached configuration, electrode capacitive transients were carefully cancelled to allow for electronic compensation of

pipette charging during subsequent current clamp recordings (D'Angelo et al., 1995; D'Angelo and Rossi, 1998).

The mossy fiber bundle was stimulated with a coaxial tungsten electrode (Clark Instruments, Pangbourne, UK) via a stimulus isolator using 200  $\mu$ s pulses at a basal frequency of 0.33 Hz. According to previous measurements (Sola et al., 2004), we stimulated between one and two mossy fibers per granule cell. A step current protocol was used to monitor intrinsic excitability. The resting membrane was set at  $-80$  mV, and 800 ms or 2 s steps of current with  $-8$  to 48 pA in 2 or 4 pA increments were injected (Armano et al., 2000). After evoking EPSPs at basal frequency for 10 min (control period) synaptic plasticity was induced from a membrane potential of  $-50$  mV by delivering a stimulus train at 100 Hz for 1 s (HFS). After tetanization, the recording was returned to the basal mossy fiber stimulation frequency. The stability of recordings can be influenced by modification of series resistance and neurotransmitter release. To ensure that series resistance remained stable during recordings, passive cellular parameters were monitored throughout the experiments.

### Behavioral testing

All mice used were males and they were 12–20 weeks old. Three days before behavioral testing, a prefabricated piece equipped with two nuts was cemented to the skull to allow fixation of the mouse's head in a restrainer device. The surgical procedures were performed under general anesthesia using a mixture of isoflurane (Isofloran 1–1.5%; Rhodia Organique Fine Ltd) and oxygen. During the experiment the mouse was placed in a restrainer, with the head fixed above the center of the turntable. A cylindrical screen (diameter 63 cm) with a random-dotted pattern (each element 2°) surrounded the turntable (diameter 60 cm), and both the screen and turntable were driven independently by AC servomotors (Harmonic Drive AC, the Netherlands). The table and drum position signal were measured by potentiometers, filtered (cut-off frequency 20 Hz), digitized (CED Limited, UK) and stored on a computer. A CCD camera was fixed to the turntable to monitor the mouse's eye using an eye-tracking device of ISCAN (Iscan Inc.). Both video calibrations and subsequent eye movement computations were performed as described previously (Andreescu et al., 2007). Angular OKR, VOR and VVOR were evoked by rotating the surrounding screen, the turntable in dark and the turntable in light, respectively (rotations of 0.2–1 Hz at 5° rendering a velocity of 6.3–31.4 deg/s). Before measuring the VOR pilocarpine (4%; Laboratoires Chauvin, France) was used in order to limit the pupil dilatation in darkness. Gain and phase of the eye movements were calculated according to standard procedures (Stahl, 2002). VOR adaptation was evoked by using 5 $\times$ 10 min of visuo-vestibular mismatch training during three consecutive days. On the first day, VOR gain decrease was induced by subjecting the animals to 5 $\times$ 10 min of sinusoidal vestibular and visual stimuli that were rotating exactly in phase (table and drum  $\pm 5^\circ$ ; 0.6 Hz); on the second day VOR phase reversal was induced by subjecting the animals to 5 $\times$ 10 min sinusoidal vestibular and visual stimuli that were rotating in phase, while the amplitude of the visual stimuli was increased to 7.5° (table 0.6 Hz,  $\pm 5^\circ$ ; drum 0.6 Hz,  $\pm 7.5^\circ$ ); and on the third day, VOR phase reversal was completed by subjecting the animals also to 5 $\times$ 10 min sinusoidal vestibular and visual stimuli that were rotating in phase, but now the amplitude of the visual stimuli was increased to 10° (table 0.6 Hz  $\pm 5^\circ$ ; drum 0.6 Hz  $\pm 10^\circ$ ). VOR was measured every day before the training and after each 10 min of training ( $\pm 5^\circ$ ; 0.6 Hz). Mice were kept in complete darkness in between the daily measurements.

### Statistical tests

Data are presented as mean  $\pm$  SEM. *n* refers to the number of mice. For statistical comparisons we used the two-way ANOVA with repeated measures and Student's *t*-test (SPSS 11.0 Inc.).

## RESULTS

### Absence of long-term synaptic plasticity in granule cells of the vestibulo-cerebellum in NR2A<sup>-/-</sup> mice

Transmission at the mossy fiber to granule cell synapse was investigated by whole-cell patch clamping from granule cells in cerebellar slices obtained from NR2A<sup>-/-</sup> and wild type mice. EPSPs were elicited before and after tetanization with high-frequency stimulation train (HFS). EPSPs elicited by mossy fiber stimulation could either remain sub-threshold or be combined with spikes forming EPSP-spike complexes (Figs. 2 and 3). Recordings were obtained from juvenile adult mice (P18–P22) as well as at a later stage (P45–P50) when cerebellar maturation is complete (e.g. see Goldfarb et al., 2007; Prestori et al., 2008).

At all ages, in the cerebellar slices obtained from wild type mice, tetanization increased the EPSP amplitude, the proportion of EPSP-spike complexes, and the number of spikes per EPSP (Figs. 2A–C and 3A–C). The amplitude change of the EPSPs that did not generate spikes after tetanization was 29.6 $\pm$ 4.5% in juvenile mice (*n*=4; *P*<0.05, paired Student's *t*-test) and 48.4 $\pm$ 13.7% (*n*=4; *P*<0.03, paired Student's *t*-test) in adult mice. In the wild types, the probability of EPSP-dependent firing increased remarkably both in juvenile mice (from 13.7 $\pm$ 6% before to 77.1 $\pm$ 16% after tetanization; *n*=6; *P*<0.01, paired Student's *t*-test) and in mature mice (from 3.0 $\pm$ 1.6% before to 43.8 $\pm$ 17.1% after tetanization; *n*=7; *P*<0.05, paired Student's *t*-test). Thus, mossy fiber-granule cell synapses of the vestibulo-cerebellum can make LTP similarly to those of other parts of the cerebellar vermis (Armano et al., 2000) and hemispheres (Roggeri et al., 2008).

The picture was different in the cerebellar slices obtained from NR2A<sup>-/-</sup> mutants. In juvenile mice, after tetanization, the EPSP amplitude change was 6.3 $\pm$ 8.8% (*n*=3; *P*>0.5, paired Student's *t*-test; Fig. 2C) and the probability of EPSP-dependent firing did not show any remarkable change (from 14.7 $\pm$ 10.1% before to 29.6 $\pm$ 27% after induction; *n*=5; *P*>0.8, paired Student's *t*-test; Fig. 2B). The difference between wild types and NR2A<sup>-/-</sup> was statistically significant after tetanization (*P*<0.005, Student's *t*-test). In mature mice, after tetanization, the EPSP amplitude change was  $-11.6\pm 11.4\%$  (*n*=4; *P*>0.45, paired Student's *t*-test; Fig. 3C), while the probability of EPSP-dependent firing increased significantly (from 2.25 $\pm$ 0.6% before to 26.2 $\pm$ 20.1% after induction; *n*=4; *P*<0.05, paired Student's *t*-test; Fig. 3B). The difference after tetanization between wild types and NR2A<sup>-/-</sup> was not statistically significant (*P*>0.6, Student's *t*-test). Therefore, probably some changes occurred in intrinsic excitability (Armano et al., 2000). It should be noted that, whereas the ability to generate a spike was recovered, the granule cell output pattern remained deficient,

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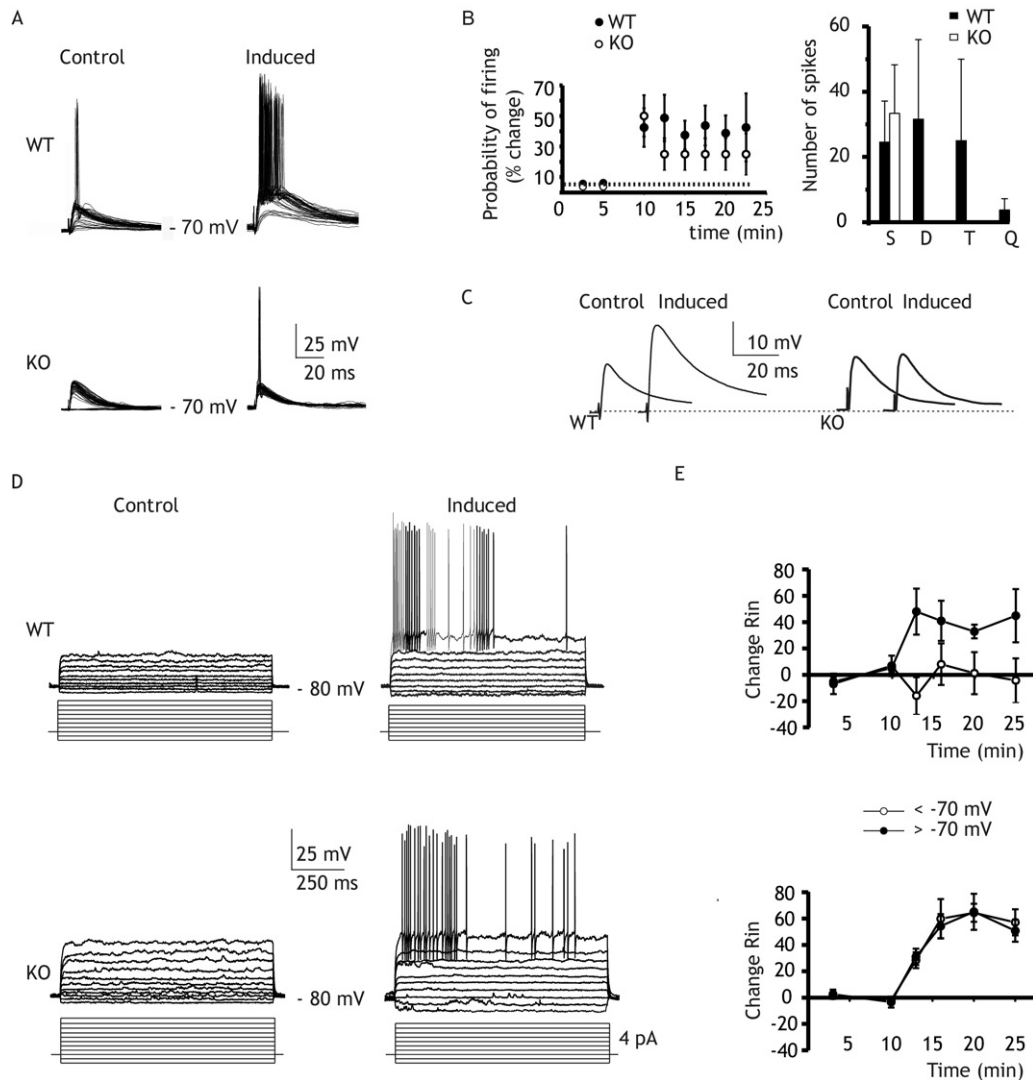
**Fig. 2.** Synaptic and non synaptic plasticity at the mossy fiber–granule cell relay: P18–P22 mice. Responses recorded from granule cells are shown before and after mossy fiber tetanization (100 Hz for 1 s) in wild type (wt) and NR2A<sup>-/-</sup> mice (ko). (A) EPSPs and EPSP-spike complexes were elicited from -70 mV (several traces are superimposed). Note the marked increase in EPSP-spike complexes in the wild types but not in NR2A<sup>-/-</sup> mutants. (B) The plots on the left show the change in the probability of firing in the wild types and NR2A<sup>-/-</sup> mice during induction experiments (the induction trains were delivered at time 0 and the action potential threshold in the two cells is indicated). The wild type EPSPs show a persistent potentiation of EPSP-spike complexes after tetanization, whereas no noticeable increase is observed in the NR2A<sup>-/-</sup>. The histogram on the right shows the number of spikes (singlet, doublet, triplet, quadruplet) generated in each EPSP-spike complex after tetanization. Note that wild type mice can generate more than one spike, while this does not usually occur in the NR2A<sup>-/-</sup> mice. (C) An increase in EPSP peak amplitude is observed in the wild type cells, whereas no amplitude change is observed in NR2A<sup>-/-</sup> cells. Only EPSPs remaining subthreshold have been used for the average. (D) Voltage responses elicited from -80 mV in wild types (wt) and NR2A<sup>-/-</sup> mice (ko) to 2 pA steps current injection. The wild type cell shows an increase of firing following induction, whereas the NR2A<sup>-/-</sup> cell shows no remarkable increase. (E) Granule cell input resistance ( $R_{in}$ ) measured in the low potential range (<-70 mV) and in the high potential range (>-70 mV). Note that  $R_{in}$  increase in the high potential range in wt but not in ko mice, while it remains stable in both cases in the low potential range.

since the neurons were usually unable to generate more than one spike (Figs. 2B and 3B).

The possibility that changes in intrinsic excitability might differentially regulate EPSP-spike coupling in NR2A<sup>-/-</sup> mutants during development was assessed by measuring the granule cell input resistance. Membrane potential changes were caused by current steps in the 10 mV potential range either below -70 mV ( $R_{in-low}$ ) or above -70 mV ( $R_{in-high}$ ). In the wild types, after tetanization, the change of  $R_{in-high}$  was  $58.1 \pm 14.9\%$  in juvenile mice ( $n=4$ ;  $P<0.05$ , paired Student's *t*-test) and  $48.0 \pm 16.4\%$  in adult mice ( $n=4$ ;  $P<0.05$ , paired Student's *t*-test; Figs. 2E and

3E). It should be noted that  $R_{in-low}$  remained unchanged in all ages, providing an internal control for recording stability. In NR2A<sup>-/-</sup> mutants, after tetanization, both  $R_{in-high}$  and  $R_{in-low}$  increased remarkably only in adult mice ( $50.9 \pm 6.4\%$  and  $52.4 \pm 7.7\%$ , respectively;  $n=4$ ,  $P<0.05$ ), whereas in juvenile mice  $R_{in-high}$  and  $R_{in-low}$  did not show any increase ( $0.7 \pm 8.0\%$  and  $-2.0 \pm 3.9\%$ , respectively;  $n=4$ ,  $P>0.8$ ; Figs. 2E and 3E). Therefore, the partial recovery observed in EPSP-spike coupling following tetanization can be explained by the increased input resistance.

In conclusion, mossy fiber-granule cell long-term synaptic plasticity is impaired in the vestibulo-cerebel-



**Fig. 3.** Synaptic and non synaptic plasticity at the mossy fiber–granule cell relay: P45–P50 mice. Responses recorded from granule cells are shown before and after mossy fiber tetanization (100 Hz for 1 s) in wild type (wt) and NR2A<sup>-/-</sup> mice (ko). (A) EPSPs and EPSP-spike complexes were elicited from -70 mV (several traces are superimposed). Note the much stronger increase in EPSP-spike complexes in the wild types than in NR2A<sup>-/-</sup> mutants. (B) The plots on the left show the change in the probability of firing in the wild types and NR2A<sup>-/-</sup> mice during induction experiments (the induction trains were delivered at time 0 and the action potential threshold in the two cells is indicated). The wild type EPSPs show a stronger persistent potentiation of EPSP-spike complexes after tetanization than the NR2A<sup>-/-</sup>. The histogram on the right shows the number of spikes (singlet, doublet, triplet, quadruplet) generated in each EPSP-spike complex after tetanization. Note that wild type mice can generate more than one spike, while this does not usually occur in the NR2A<sup>-/-</sup> mice. (C) An increase in EPSP peak amplitude is observed in the wild type cells, whereas no amplitude change is observed in NR2A<sup>-/-</sup> cells. Only EPSPs remaining subthreshold have been used for the average. (D) Voltage responses elicited from -80 mV in wt and ko mice to 2 pA steps current injection. Both the wild type and NR2A<sup>-/-</sup> cell show an increase of firing following induction. (E) Granule cell input resistance (R<sub>in</sub>) measured in the low potential range (<-70 mV) and in the high potential range (>-70 mV). Note that R<sub>in</sub> increases in the high potential range in wt but not in ko mice, while it increases in both cases in the low potential range.

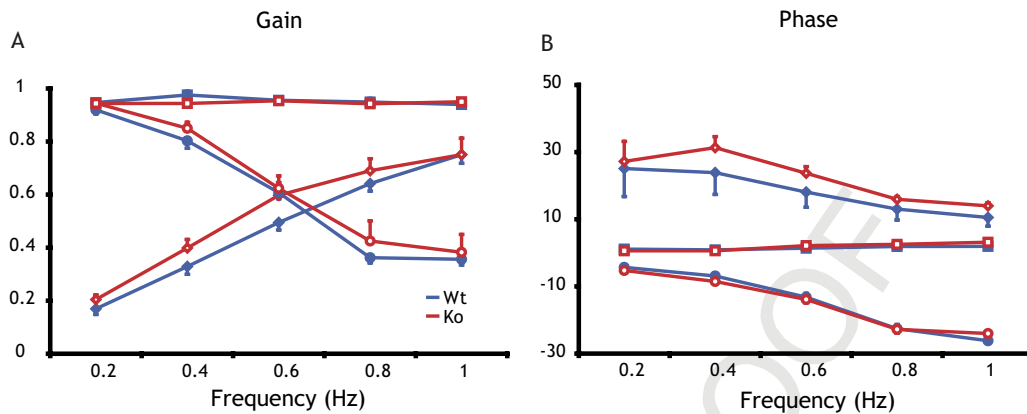
lum of both juvenile and mature NR2A<sup>-/-</sup> mutants, although a partial recovery can be observed in the mature mutants.

#### Eye movement performance is not impaired in NR2A<sup>-/-</sup> mice

NR2A<sup>-/-</sup> mice were subjected to vestibular stimulation to investigate the amplitude (gain) and timing (phase) of their angular VOR, while a whole field visual stimulus was used to investigate the gain and phase of their OKR and VVOR.

VOR gain values of NR2A<sup>-/-</sup> mice ( $n=8$ ) ranged from  $0.20 \pm 0.02$  to  $0.75 \pm 0.06$  over the tested frequency band (0.2–1 Hz), while those of wild type littermates ( $n=9$ ) ranged from  $0.17 \pm 0.02$  to  $0.75 \pm 0.03$ ; these differences were not significant ( $P > 0.2$ ; two-way ANOVA; Fig. 4A). Following this turntable stimulation at 0.2–1 Hz phase leads of NR2A<sup>-/-</sup> mice ranged from  $27.2 \pm 6.0$  to  $14.0 \pm 0.9$  degrees, while those of their wild type littermates varied from  $25.1 \pm 8.4$  to  $10.5 \pm 2.6$  degrees; these were no significant difference ( $P > 0.4$ ; two-way ANOVA; Fig. 4B). Simi-

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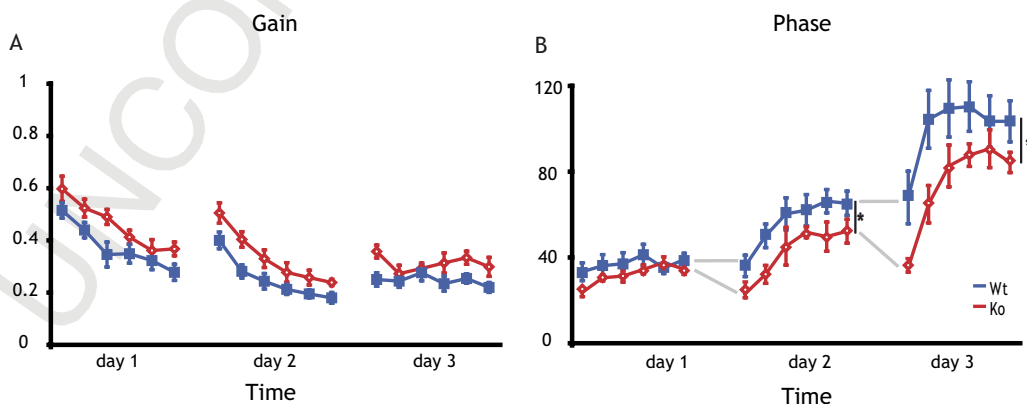
**Fig. 4.** NMDA-NR2A<sup>-/-</sup> mutant mice show no motor performance deficit. (A) Presentations of gain values (i.e. eye velocity/stimulus velocity) of the VOR (diamonds), OKR (circles) and VVOR (squares) at stimulus frequencies ranging from 0.2 to 1 Hz in NMDA-NR2A<sup>-/-</sup> mice (ko) and wild type mice (wt). (B) Plots of phase values (i.e. phase eye velocity–phase stimulus velocity in degrees) of eye movements in NMDA-NR2A<sup>-/-</sup> mice and their wild type littermates. Empty symbols represent mutant mice, while filled symbols represent wild type mice. Data are mean ± SEM. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

larly, the OKR and VVOR of the NR2A<sup>-/-</sup> mice also showed no significant deficits in gain or phase over the entire frequency range (0.2–1 Hz;  $P > 0.3$  for all gain and phase comparisons; two-way ANOVA; Fig. 4). Thus, we conclude that NR2A<sup>-/-</sup> mutant mice show no abnormalities in motor performance when the vestibular and visual systems are investigated separately or when they operate together, as under natural conditions.

#### Deficits in motor learning in NR2A<sup>-/-</sup> mutant mice

To assess motor learning capabilities in NR2A<sup>-/-</sup> mice, we subjected them to a paradigm that was meant to reduce the gain of the VOR (day 1) and to subsequently reverse the VOR phase (days 2 and 3). VOR gain adaptation was studied on day 1 by presenting perfectly in phase drum and table rotations each with an amplitude of 5° at 0.6 Hz. VOR phase reversal was studied on days 2 and 3 by increasing

the amplitude of the in-phase drum rotations to 7.5° and 10°, respectively, while the table rotation parameters were maintained (0.6 Hz; 5°). When the adaptation was tested on day 1 no significant differences in gain reduction were observed among NR2A<sup>-/-</sup> ( $n = 8$ ) and control mice ( $n = 9$ ) ( $P > 0.3$ , two-way ANOVA; Fig. 5A). Moreover, when the measurements were resumed after mice spent 24 h in darkness, the gain in NR2A<sup>-/-</sup> mice was also similar to that of their wild type littermates indicating that they also do not show any difference in gain consolidation ( $P > 0.3$ , Student's *t*-test). Thus, NR2A<sup>-/-</sup> mice show a normal capacity for gain-decrease motor learning as well as for gain consolidation. However, when the phase leads were measured on the second and third day of training, NR2A<sup>-/-</sup> mice showed a significantly smaller phase change than the wild types (in both cases  $P < 0.05$ ; two-way ANOVA; Table 1, Fig. 5B). Possibly, these differences



**Fig. 5.** NMDA-NR2A<sup>-/-</sup> mutant mice show a deficit in phase reversal learning. On day 1 short-term adaptation was studied using in phase, drum and table stimulation (both at 5°; 0.6 Hz). On days 2 and 3 gain adaptation, phase reversal and consolidation were studied by rotating the drum 7.5° at 0.6 Hz (day 2) and 10° at 0.6 Hz (days 3) in phase with the table (5°; 0.6 Hz). (A) We observed no difference in gain reduction among NMDA-NR2A<sup>-/-</sup> mice (ko) and control mice (wt) during these 3 d of training. (B) NMDA-NR2A<sup>-/-</sup> mice were able to reverse their phase values (i.e. to move their eyes during the VOR in the same direction as the table instead of in the opposite direction), but not as prominently as wild types. NMDA-NR2A<sup>-/-</sup> mice were not able to consolidate the phase changes from day 1 to day 2 and from day 2 to day 3. \*  $P < 0.05$ . Data are mean ± SEM. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

**Table 1.** Statistics during and following learning paradigm

	Mutant	Two-way ANOVA with repeated measures	P value day 1	P value day 2	P value day 3
Gain	NMDA-NR2A <sup>-/-</sup> mice	Test of within subjects effects	0.38	0.77	0.26
		Test of between subjects effects	0.97	0.32	0.84
	NMDA-NR2A <sup>ΔC/ΔC</sup> mice	Test of within subjects effects	0.33	0.41	0.22
		Test of between subjects effects	0.24	0.79	0.87
Phase	NMDA-NR2A <sup>-/-</sup> mice	Test of within subjects effects	0.27	0.95	0.38
		Test of between subjects effects	0.21	0.04	0.05
	NMDA-NR2A <sup>ΔC/ΔC</sup> mice	Test of within subjects effects	0.60	0.06	0.49
		Test of between subjects effects	0.12	0.02	0.03

All *P* values of two-way ANOVA tests of between and within subject comparisons are listed. All mutant mice were tested against their wild type littermates.

were partly due to a difference in phase consolidation, because the level of phase changes carried forward from the previous day was significantly smaller in NR2A<sup>-/-</sup> mice ( $P < 0.05$  for both days, Student's *t*-test; Fig. 5B). Thus, NR2A<sup>-/-</sup> mice were able to reverse the phase of their VOR, but not as prominently as wild types, and they did, unlike wild types, not show any significant sign of phase consolidation overnight.

#### NR2A<sup>ΔC/ΔC</sup> mice show same phenotype as NR2A<sup>-/-</sup> mice

Since the C-terminal of NR2A subunits can interact with proteins in the postsynaptic density, which retains the NMDA receptor at the synapse and mediates interactions between signal transduction molecules downstream (Llanos et al., 2005), we wanted to find out whether truncation of this domain was sufficient to induce the learning deficits described above. Moreover, complete deletion of the NR2A subunits as in the NR2A<sup>-/-</sup> mice might have induced various secondary compensations, which themselves might have contributed to the learning deficits in phase reversal. We therefore subjected mice with just a truncated C-terminal in the NR2A subunit (NR2A<sup>ΔC/ΔC</sup> mice) to the same paradigms as the NR2A<sup>-/-</sup> mice (Kadotani et al., 1996; Sprengel et al., 1998). The NR2A<sup>ΔC/ΔC</sup> mice showed the same phenotype as the NR2A<sup>-/-</sup> mice in that they showed no deficits in motor performance or in motor learning during the 1-day gain decrease training, while they did show significant deficits in the subsequent 2-day phase reversal learning (Fig. 6). All significance levels of the comparisons of the gain and phase VOR, OKR and VVOR motor performance values between NR2A<sup>ΔC/ΔC</sup> mice ( $n = 7$ ) and controls ( $n = 10$ ) were higher than 0.1 over the entire frequency range from 0.2 Hz to 1.0 Hz (two-way ANOVA), while the significance level of that for the VOR gain decrease learning paradigm was higher than 0.2 (two-way ANOVA; Table 1). In contrast, the NR2A<sup>ΔC/ΔC</sup> mice showed, just like the NR2A<sup>-/-</sup> mice described above, significantly smaller phase changes than the wild types on the 2 days of phase reversal training (phase difference on day 2,  $P < 0.02$ ; and on day 3,  $P < 0.03$ , two-way ANOVA; Table 1, Fig. 6D). Interestingly, the differences in levels of consolidation overnight between the NR2A<sup>ΔC/ΔC</sup> mice and controls showed the same

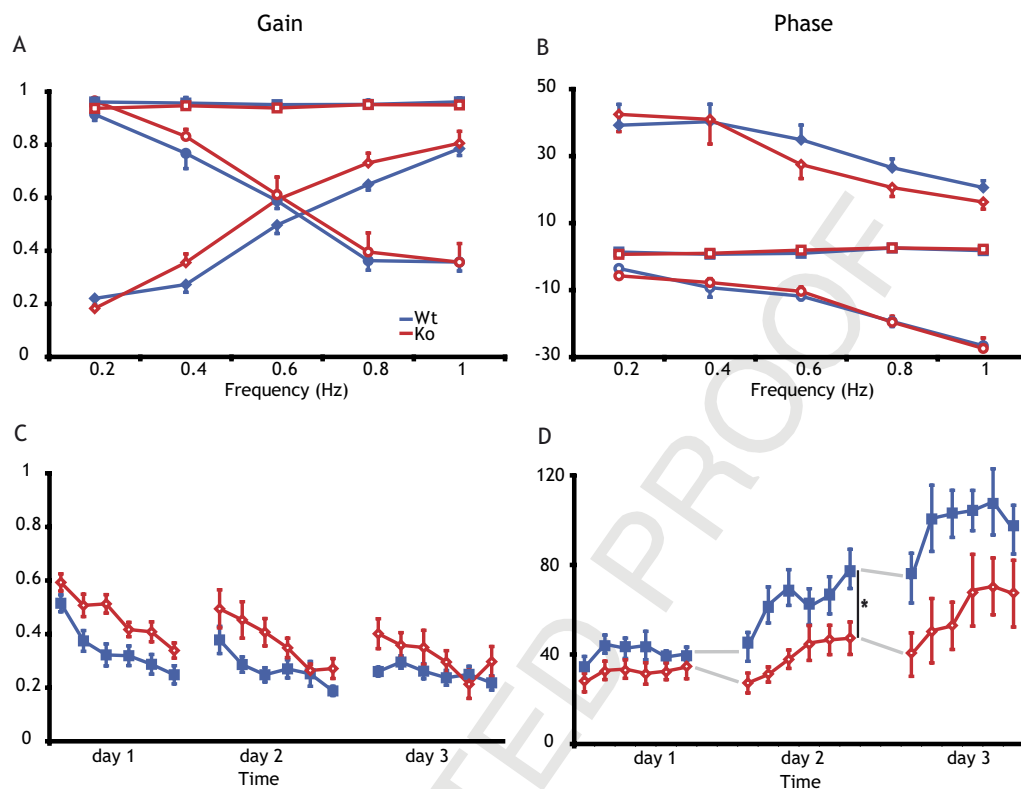
trend as described above for the NR2A<sup>-/-</sup> mutants, but these differences were not significant (Fig. 6D;  $P > 0.1$ , Student's *t*-test). These data obtained in the NR2A<sup>ΔC/ΔC</sup> mice suggest that the C-terminal of the NR2A subunit is necessary for a normal level of phase reversal learning.

## DISCUSSION

Here we investigated the functional role of the NR2A subunit in glutamatergic transmission between mossy fibers and granule cells as well as the effects of NR2A deficiency on motor behavior. We show that NR2A is necessary for the induction of LTP at the mossy fiber to granule cell synapse, that basic eye movement performance and gain-decrease learning do not require NR2A activation, and that NR2A activation is necessary for optimal phase reversal learning. The fact that NR2A is more prominently distributed in the granular layer than in all other parts of the olivocerebellar system (Watanabe et al., 1992, 1994; Monyer et al., 1994) raises the possibility that the affected phase reversal learning is a consequence of inefficient LTP induction at the mossy fiber to granule cell synapse. However, since the NR2A subunit is also expressed in Purkinje cells, molecular interneurons, and Golgi cells as well as in other neurons of the vestibulo-ocular pathway, this potential causal relation awaits confirmation in cell-specific mutants.

The mossy fiber-granule cell synapse in the vestibulo-cerebellum shows the ability to potentiate and this form of LTP is manifested both as an increase in synaptic transmission and in intrinsic excitability (D'Angelo et al., 1999; Armano et al., 2000). The enhanced EPSP-spike coupling observed in wild type slices was probably determined by a combination of increased EPSPs and increased ability of generating spikes (for review see Hansel et al., 2001). Conversely, LTP was severely impaired in the NR2A<sup>-/-</sup> mice, resembling the cell physiological phenotype observed in mice with an NR2A-NR2C C-terminal deletion (Rossi et al., 2002). Therefore, different mutations in the NMDA receptor cause a similar impairment in LTP induction at the mossy fiber to granule cell synapse suggesting that the common mechanism is a reduction in the efficiency of calcium entry during high-frequency repetitive synaptic transmission (Gall et al., 2005).

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**Fig. 6.** NMDA-NR2A<sup>ΔC/ΔC</sup> mutant mice also show a normal motor performance, while their ability for phase reversal learning is also affected. (A) Gain values of the vestibular-ocular reflex (VOR; diamonds), of the optokinetic reflex (OKR; circles) and of the visually increased VOR (VVOR; squares) at stimulus frequencies ranging from 0.2 to 1 Hz in NMDA-NR2A<sup>ΔC/ΔC</sup> mice and wild type mice are presented. (B) Phase values from NMDA-NR2A<sup>ΔC/ΔC</sup> mice are plotted. Empty symbols represent mutant mice (ko) while filled symbols represent their wild type littermates (wt). (C) A 3 d paradigm did not induce differences in gain reduction among NMDA-NR2A<sup>ΔC/ΔC</sup> mice and control mice. (D) NMDA-NR2A<sup>ΔC/ΔC</sup> mice were able to reverse their phase values but not as prominently as wild types. \*  $P < 0.05$ . Data are mean  $\pm$  SEM. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

Interestingly, in the juvenile adult NR2A<sup>-/-</sup> mutant (P20) there is no LTP and there are no long-term changes in intrinsic excitability (or even less than in wt), whereas in the mature adult (P50) there is still no LTP, but there is an evident change in intrinsic excitability. It is possible that the granule cells are partly compensating during postnatal development by reducing their inward rectifier current following tetanization (Rossi et al., 2006). However, phase reversal learning of the VOR never becomes optimal. Thus, if the observed cell physiological and behavioral phenotypes in the NR2A<sup>-/-</sup> mutants are causally related, it is most likely the specific change in the individual synaptic weights that matters. This change probably results in an inability of the granule cell to fire multiple spikes (doublets or triplets). Facilitated by compensatory non-synaptic plasticity many granule cells may be able to generate single spikes, but due to the lack of LTP induction the patterns of their output lacking high-frequency patterns remain abnormal.

Motor performance is not affected in our mouse models, despite the absence or modification of NR2A in the cerebellar cortex (Monaghan and Cotman, 1985). Apparently, NR2As modulate mainly the effectiveness of plasticity at the mossy fiber-granule cells synapse and control only mildly the ability of the granule cells to convey infor-

mation from the mossy fibers to the cerebellar cortex (see also Rossi et al., 2002). Motor performance is generally the consequence of a long period of training and it is constantly recalibrated by learning mechanisms in order to meet the demands of a continuously changing environment. The multi-day, visuo-vestibular reversal training paradigm is a challenging task and revealed that the vestibulo-ocular system of our mutant mice has a limited capability in motor learning. These data are in line with other investigations focused on classical conditioning processes. Mice lacking NR2A or both NR2A and NR2C have impaired eyeblink conditioning (Kishimoto et al., 1997), while systemic blocking of NMDA receptors by pharmaceutical intervention has also been shown to impair eyeblink conditioning (Thompson and Disterhoft, 1997; Takatsuki et al., 2001). Interestingly, when both the NR2A and NR2C subunits are affected, such as in the NR2A/C<sup>ΔC/ΔC</sup> mouse (Kadotani et al., 1996; Imamura et al., 2000) or NR2A/C<sup>-/-</sup> mouse (Sprengel et al., 1998), the motor deficits are not restricted to motor learning deficits; in these mutants the motor performance is also impaired, which makes it difficult to interpret the cause of the learning disability. Fortunately, the present specific findings in the NR2A<sup>-/-</sup> mouse and NR2A<sup>ΔC/ΔC</sup> mutants allow us to exclude this potential caveat and they suggest that the cer-



ebellar learning deficits are not secondary to performance deficits.

In both the NR2A<sup>-/-</sup> mouse and NR2A<sup>ΔC/ΔC</sup> mutants the inefficient induction of LTP at the cerebellar mossy fiber–granule cell synapse is based on NMDA receptor alteration. In the NR2A<sup>-/-</sup> mice, the NMDA current is reduced and has altered kinetics (Sprengel et al., 1998; Rossi et al., 2002). In the NR2A<sup>ΔC/ΔC</sup> mutants there is a deficit in channel opening probability and in receptor coupling to intracellular transduction pathways (Rossi et al., 2002). Thus, in the end, both mutations are likely to alter critical steps in NMDA receptor-dependent LTP induction lying between the NMDA receptor and the biochemical mechanisms triggering LTP. By showing that both mutants do not only share a similar deficit in LTP induction at the mossy fiber to granule cell synapse, but also a similar behavioural phenotype, we promote the possibility that plasticity in the granular layer also contributes to some aspects of cerebellar motor learning. Thus, the forms of cellular plasticity that may underlay cerebellar motor learning may not be restricted to plasticity at the parallel fiber to Purkinje cell synapse, and/or to LTD and LTP at the cerebellar and vestibular nuclei downstream (Schonewille et al., 2010; Ito, 1972; Miles and Lisberger, 1981; De Zeeuw et al., 1998). How this contribution may come about mechanistically remains to be shown. LTP in the granule cells could play an important role in determining the efficacy of synaptic summation and the output spike frequency in the mossy fiber to granule cell synapse during repetitive mossy fibers activation (D'Angelo et al., 1995). LTP in the granule cells could thus contribute to the memory formation by controlling the diverse, temporal activity patterns of granule cells and by influencing the efficacy of information transmission to inhibitory neurons and Purkinje cells (D'Angelo and De Zeeuw, 2009). Future experiments in granule cell-specific transgenic mice are needed to further clarify an actual causal relationship between NMDA receptors, LTP and VOR control.

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