Behavioral/Systems/Cognitive

# Estradiol Improves Cerebellar Memory Formation by Activating Estrogen Receptor $\beta$

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**Learning motor skills is critical for motor abilities such as driving a car or playing piano. The speed at which we learn those skills is subject to many factors. Yet, it is not known to what extent gonadal hormones can affect the achievement of accurate movements in time and space. Here we demonstrate via different lines of evidence that estradiol promotes plasticity in the cerebellar cortex underlying motor learning. First, we show that estradiol enhances induction of long-term potentiation at the parallel fiber to Purkinje cell synapse, whereas it does not affect long-term depression; second, we show that estradiol activation of estrogen receptor $\beta$ receptors in Purkinje cells significantly improves gain-decrease adaptation of the vestibulo-ocular reflex, whereas it does not affect general eye movement performance; and third, we show that estradiol increases the density of parallel fiber to Purkinje cell synapses, whereas it does not affect the density of climbing fiber synapses. We conclude that estradiol can improve motor skills by potentiating cerebellar plasticity and synapse formation. These processes may be advantageous during periods of high estradiol levels of the estrous cycle or pregnancy.**

*Key words:* **cerebellum; estrogen; motor learning; neuronal plasticity; estrogen receptor $\beta$; mutant mice**

## Introduction

Learning and memory are crucial for acquiring and storing new information, and these processes are potentially affected by hormones. The gonadal hormone estradiol (E2) has beneficial effects on the formation of hippocampus-dependent memory (Farr et al., 1995; O'Neal et al., 1996; Gibbs et al., 1998; Shors et al., 1998; Leuner et al., 2004; Rhodes and Frye, 2004). During the estrous cycle, pyramidal cells in the hippocampus are subject to major changes including morphological changes (Woolley and McEwen, 1993; Adams et al., 2001) and modifications in synaptic efficacy (Warren et al., 1995; Cordoba Montoya and Carrer, 1997; Good et al., 1999; Vouimba et al., 2000; Mukai et al., 2007). Yet, it is not known whether E2 also affects cerebellar memory formation, despite the prominent presence of estrogen receptors (ERs) in the cerebellum (Shughrue et al., 1997; Price and Handa, 2000). If activated ERs are involved in cerebellar memory formation, one expects that at least some forms of motor learning and cellular plasticity are affected concomitantly by E2. Moreover, if E2 action is mainly cortical and the relationship between the cellular and behavioral effects is causal in a direct manner, one expects that the behavioral effects on the learning are rather specific with relatively mild or no effects on motor performance (Welsh and Harvey, 1991; Jiménez-Díaz et al., 2004). Here we tested the impact of E2 on adaptation of the vestibulo-ocular reflex (VOR) after visuovestibular training (Robinson, 1976; Ito, 1991; De Zeeuw et al., 1998) (Fig. 1). The main type of plasticity that may underlie this form of cerebellar motor learning is historically thought to be long-term depression (LTD) at the parallel fiber to Purkinje cell synapse (Marr, 1969; Albus, 1971; Ito 1991). However, recent behavioral studies on VOR adaptation suggest that different plasticity mechanisms may be involved in increasing and decreasing VOR gains (Boyden et al., 2003; De Zeeuw and Yeo, 2005). Induction of LTD at the parallel fiber to Purkinje cell synapse may be responsible for increasing the gain (Hansel et al., 2006), whereas other mechanisms such as long-term potentiation (LTP) at the same synapse may contribute to decreasing the gain (Hansel et al., 2001; Lev-Ram et al., 2002; Boyden et al., 2003; Coesmans et al., 2004). Considering the impact of E2 on hippocampal memory formation (Leuner et al., 2004; Rhodes and Frye, 2004; Mukai et al., 2007), one might expect E2 to affect LTP rather than LTD in the cerebellum. Thus, the present experiments were designed to study the effects of E2 on LTP at the parallel fiber to Purkinje cell synapse, on gain-decrease training of the VOR, and on the morphology of synaptic inputs to the Purkinje cells. In addition, we investigated the distribution of E2 receptors in the mouse flocculus, which is known to be the main cerebellar lobule involved in the control of VOR adaptation (Ito, 1991; Lisberger et al., 1994; Blazquez et al., 2007). Together, these studies should shed

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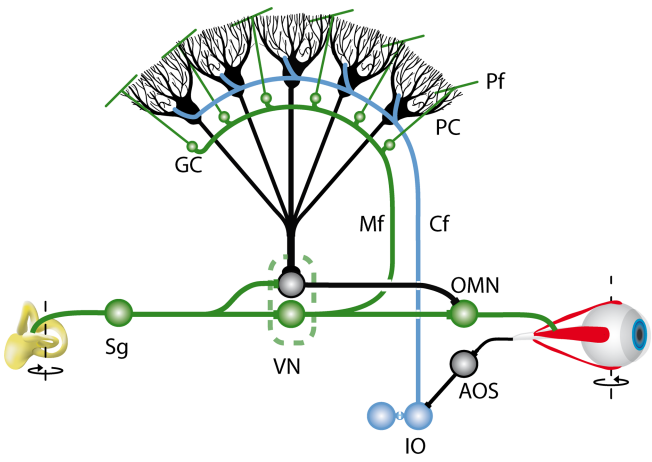


**Figure 1.** Scheme showing VOR circuitry. The VOR is an eye movement reflex that stabilizes retinal images during head movements (Collewijn and Grootendorst, 1979; Iwashita et al., 2001; Van Alphen and De Zeeuw, 2002; Boyden et al., 2004; Faulstich et al., 2004; Stahl, 2004). Primary afferents from the vestibular system [Scarpa's ganglion (Sg)] converge on second-order vestibular nuclei neurons (VN) that innervate the oculomotor nucleus (OMN) to control eye movements. Information about head movements reaches the cerebellar cortex via the mossy fibers (Mf) innervating the granule cells (GC) that generate parallel fibers (Pf). Information about retinal slip, processed by the accessory optic system (AOS) and inferior olive (IO), reaches the cerebellar cortex via the climbing fibers (Cf). This information is processed in the Purkinje cells (PC), which form the sole output of the cerebellar cortex. We tested the effect of E2 on motor performance and motor learning by investigating the VOR in the dark, before and after visuovestibular training. This reflex can be altered by the cerebellar side loop that can modulate the activity of the vestibular nuclei.

light on the potential facilitating role of E2 in motor memory formation.

## Materials and Methods

*Subjects.* A total of 70 female C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME), 17 male C57BL/6 mice, and 8 Purkinje cell-specific ERβ knock-out female mice (L7-ERβ) were housed on a 12 h light/dark cycle with food and water available *ad libitum* and subjected to the various tests described below. In one group of C57BL/6 females (Eovx mice), E2 levels were fixed at a high level by ovariectomy (OVX) and subsequent daily subcutaneous injections of 5 μg of estradiol benzoate dissolved in 0.1 ml sesame oil, whereas in another group of C57BL/6 females (Covx mice), E2 levels were kept at a constant low level by OVX and subsequent daily subcutaneous injections of only 0.1 ml of sesame oil. In addition, we investigated groups of intact females and males for control. The Purkinje cell-specific ERβ mutants were generated by crossing flox-ERβ mutant mice (Dupont et al., 2000) with L7-cre mice (Barski et al., 2000). All experiments described below were performed blindly, and all animal procedures described below were in accordance with the rules of the ethical committee of the Erasmus Medical Center, Rotterdam.

*Hormonal status.* To check the hormonal status in female mice, daily vaginal smears were taken. To confirm this hormonal status, blood and uterus were collected on the day of the last experiment. In intact mice also, ovaries were collected. Levels of E2 were estimated using an ultrasensitive double-antibody radioimmunoassay (Diagnostic Systems Laboratories, Webster, TX). Eovx mice had uterus weights and serum E2 levels that were significantly higher than in Covx mice (both $p < 0.001$, $t$ tests) (Table 1). Intact females in proestrus day had a bigger uterus, less follicles of class IV (diameter, 310–370 μm), more follicles of class V (diameter, >370 μm), and a higher E2 level than females in diestrus day (all $p < 0.001$, two-tailed paired $t$ tests) (Table 1).

*Electrophysiology.* Sagittal slices of the cerebellar vermis (200 μm) were prepared from 6- to 8-week-old C57BL/6 females (OVX females that received daily subcutaneous injection for 14 d; Eovx, $n = 10$; Covx, $n = 10$) and males ($n = 10$). Slices were kept in artificial CSF containing (in mM) 124 NaCl, 5 KCl, 1.25 $Na_2HPO_4$, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, and 10 D-glucose bubbled with 95% $O_2$ and 5% $CO_2$ and supplemented with 20 μg of bicuculline methiodine to block $GABA_A$ receptors (Coesmans et al., 2004). Whole-cell patch-clamp recordings were performed at room temperature. The recording electrodes were filled with a solution containing (in mM) 9 KCl, 10 KOH, 120 K gluconate, 3.48 $MgCl_2$, 10 HEPES, 4 NaCl, 4 $Na_2ATP$, 0.4 $Na_3GTP$, and 17.5 sucrose, pH 7.3. The holding potential of Purkinje cells in voltage-clamp mode ranged between −65 and −75 mV. Paired-pulse facilitation (PPF) was investigated by stimulating the parallel fibers with two 3 μA pulses with an interval of 50 ms (pulse width, 700 μs; pair-pulse frequency, 0.05 Hz) and by recording the responses in voltage-clamp mode. For tetanization, cells were switched to the current-clamp mode and parallel fibers were stimulated either alone (LTP) or in combination with climbing fibers (LTD). By measuring the PPF ratio, the presynaptic or postsynaptic nature of LTP and LTD can be estimated. The facilitated response to the second pulse is attributable to a very short-term enhancement in synaptic efficacy that is caused by residual presynaptic $Ca^{2+}$, facilitating more transmitter release. A presynaptic form of long-term plasticity is coupled to alteration in the PPF ratio because it affects the transmitter release mechanism, whereas the postsynaptic form of long-term plasticity does not change the PPF ratio because the transmitter release mechanism is not affected by this plasticity change (Zucker, 1989; Lev-Ram et al., 2002; Coesmans et al., 2004). Recordings were excluded from the study if the access or series resistance varied >15% during the experiment.

*Behavioral tests.* Three days before the behavioral tests, the mice received a prefabricated piece equipped with two nuts cemented to the skull so as to be able to fixate their head in a restraining device (Andreescu et al., 2005). The surgical procedures were performed under general anesthesia using a mixture of isofluoran (Isofloran, 1–1.5%; Rhodia Organique Fine, Paris, France) and oxygen. In OVX mice, angular optokinetic reflex (OKR), VOR, and visually enhanced VOR (VVOR) were measured (±5°, 0.2–1 Hz). Before VOR recordings, 4% pilocarpine (Laboratories Chauvin, Montpellier, France) was used to limit the pupil dilatation in darkness for video detection. VOR gain-decreases (VOR learning) were induced by presenting in phase sinusoidal vestibular and visual stimuli (± 5°, 0.6 Hz) for 50 min. Before and after training, VOR was measured (±5°, 0.6 Hz). OKR gain-increases (OKR learning) were induced by presenting 180° out-of-phase sinusoidal vestibular and visual stimuli (±5°, 0.6 Hz) for 50 min. OKR was measured before and after training (±5°, 0.6 Hz). The following experimental protocol was used for Eovx and Covx mice: day 0–OVX; day 17–18–OKR, VOR, and VVOR; day 19–VOR adaptation; day 20–VOR; day 26–OKR adaptation; and day 27–OKR measurements. Mice were treated daily with E2 or solvent between days 0 and 28. Between the measurements done on days 19–20 and 26–27, mice were kept in complete darkness. Intact C57BL/6 female mice were presented with VOR gain-decrease paradigm for 30 min on one specific day of the estrous cycle (i.e., diestrus or proestrus), and VOR was measured not only at the beginning and the end of the training but also every 10 min. Intact L7-ERβ female mice were presented with the VOR gain-decrease paradigm in proestrus day. Intact male mice were presented with the VOR gain-decrease paradigm for 1 d. Video eye movement recording and data analysis were performed as described previously (Stahl et al., 2000; Andreescu et al., 2005).

*Morphology.* Cerebellar slices (40 μm) from 16-week-old females were processed for light microscopy (Price and Handa, 2000). In short, slices were rinsed four times for 10 min in Tris-buffered saline (TBS; pH 7.6, 0.05 M), followed by a preincubation of 1 h in ice-cold TBS containing 10% normal horse serum and 0.5% Triton. Subsequently, sections were incubated with a primary antibody for 48 h at 4°C [ERα (1:200) and ERβ–N-term (1:1000); Affinity BioReagents, Golden, CO], rinsed four times for 10 min in TBS, incubated with a secondary antibody (1:200) for 90 min at room temperature, and rinsed again four times for 10 min in TBS. Finally, tissue was incubated for 90 min at room temperature in ABC Elite (PK 6100; Vector Laboratories, Burlingame, CA), rinsed three times for 10 min in TBS, and rinsed three times for 10 min in 0.05 M Tris HCl, followed by a DAB staining.

The flocculus (from OVX females that received a daily subcutaneous injection for 14 d; Eovx, $n = 8$; Covx, $n = 8$) was processed for electronic microscopy (CM 100; Philips, Eindhoven, The Netherlands) and labeled

with an antibody against calbindin (De Zeeuw et al., 1989; Koekkoek et al., 2005). The "conventional" estimation procedure was used to determine the synaptic density at the parallel fiber and climbing fiber to Purkinje cell, respectively (Woolley and McEwen, 1992). From each brain (80 electron micrographs), the amount of synapses ($N_s$) was counted, and the total postsynaptic density area ($PSD_A$) and the PSD perimeter ($PSD_P$) were measured (Soft Imaging System Analysis 3.0; Olympus Imaging America, Center Valley, PA). In addition, the exact area sampled (corrected A) was computed by subtracting all areas covered by large synapse-free structures from the total area sampled. Estimated synapse density ($D_s$) was calculated according to the following: $D_s = (N_s/\text{corrected A}) \times PSD_A$.

*Statistical tests.* Data are presented as mean ± SEM. For statistical comparisons, we used the two-way ANOVA with repeated measures followed by a *post hoc* analysis, if required, and the two-sample Student's *t* tests (SPSS 11.0; SPSS, Chicago, IL).

## Results

### E2 improves LTP but not LTD in Purkinje cells

To find out whether E2 exerts a potentiating effect on plasticity at the parallel fiber to Purkinje cell synapse, we investigated the level of induction of LTP in both Eovx and Covx female mice as well as in males (Fig. 2*A*). LTP was induced by stimulating the parallel fiber input at 1 Hz for 5 min in current-clamp mode (Lev-Ram et al., 2002; Coesmans et al., 2004). After tetanization, EPSC amplitude was significantly increased to 145.8 ± 2.7% in Eovx mice ($n = 10$; $t = 37–42$ min after; $p < 0.001$, two-tailed paired *t* test), to 117.7 ± 1.5% in Covx mice ($n = 10$; $t = 37–42$ min after; $p < 0.001$, two-tailed paired *t* test), and to 121.6 ± 0.1% in males ($n = 9$; $t = 37–42$ min after; $p < 0.001$, two-tailed paired *t* test). The differences in LTP induction among Eovx mice and Covx mice as well as among Eovx mice and males were significant ($p < 0.01$ and $p < 0.02$, respectively; two-way ANOVA) and probably of a postsynaptic nature, because the PPF ratio did not change after tetanization (Eovx mice: 98.8 ± 0.5, $p = 0.57$; Covx mice: 97.8 ± 0.4, $p = 0.99$; males: 98.6 ± 0.3, $p = 0.70$; two-tailed paired *t* test) (Fig. 2*B*,*D*). Moreover, because the input resistance, rise time kinetics, decay time, and amplitude of the EPSCs were not significantly different among Eovx mice and Covx mice ($p = 0.55$, $p = 0.75$, $p = 0.72$, and $p = 0.76$ respectively; two-tailed paired *t* test) (Table 2), it appears unlikely that the differences in LTP induction are biased because of potential differential effects of E2 on basal synaptic transmission at the parallel fiber to Purkinje cell synapse in the three groups of animals.

To investigate whether the impact of E2 on plasticity at the parallel fiber to Purkinje cell synapse is specific for induction of LTP, we also investigated its effect on induction of LTD. We therefore subjected slices of both Eovx and Covx female mice as well as of normal males to paired stimulations of parallel fibers and climbing fibers at 1 Hz for 5 min in current-clamp mode (Fig. 2*C*). After tetanization, the amplitude of the EPSC was significantly decreased to 85.8 ± 0.6% in Eovx mice ($t = 37–42$ min after; $p < 0.001$, two-tailed paired *t* test; $n = 7$) and to 82.2 ± 0.7% in Covx mice ($t = 37–42$ min after; $p < 0.001$, two-tailed paired *t* test; $n = 6$). These values were not significantly different ($p = 0.49$, two-way ANOVA). Interestingly, the level of LTD induction in Purkinje cells of male mice was also similar to those of Covx and Eovx female mice ($p = 0.18$ and $p = 0.40$, respectively, two-way ANOVA, *post hoc* LSD). We conclude that E2 significantly increases induction of LTP, but not of LTD, at the parallel fiber to Purkinje cell synapse via a postsynaptic mechanism.

### E2 improves VOR motor learning but not motor performance

When basic eye movement performance was measured in female mice that were ovariectomized and received daily subcutaneous

**Table 1. Vaginal smear feature, uterus size, number of follicles, and E2 level in C57BL/6 female mice**

| Mice | Vaginal smear | Uterus (mg) | Follicles | | E2 (pmol/L) |
|---|---|---|---|---|---|
| | | | Class IV | Class V | |
| Eovx ($n = 9$) | Irregularly shaped cornified cells | 132 ± 7 | | | 465 ± 97 |
| Covx ($n = 9$) | Leucocyte; few parabasal cells | 18 ± 2 | | | <3 |
| Proestrus ($n = 7$) | Irregularly shaped cornified cells | 109 ± 5 | 1 ± 1 | 9 ± 1 | 58 ± 2 |
| Diestrus ($n = 8$) | Leucocyte; few parabasal cells | 76 ± 5 | 7 ± 1 | 2 ± 1 | 49 ± 1 |

All values are mean ± SEM.

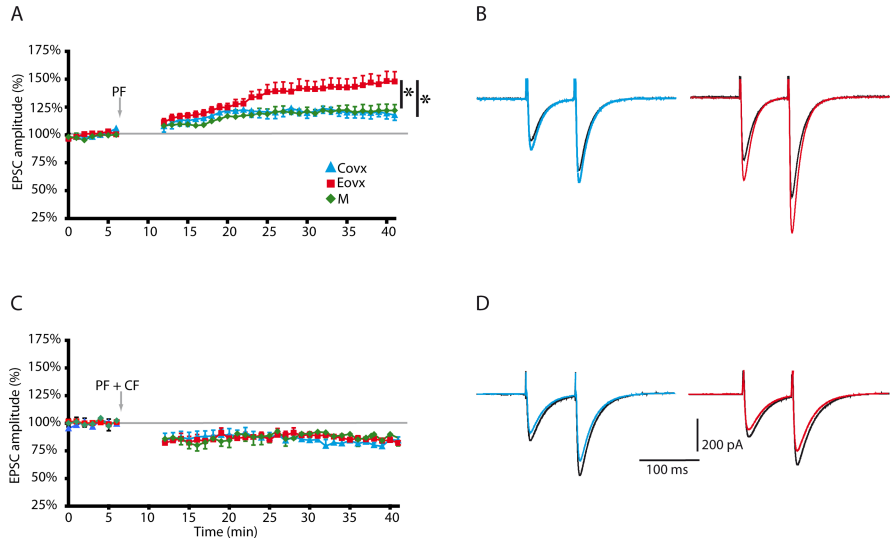


**Figure 2.** E2 enhances LTP but not LTD. ***A***, ***B***, Induction of parallel fiber-LTP by parallel fiber (PF) stimulation at 1 Hz for 5 min results in a more robust response in the slices from Eovx mice (square) compared with those from Covx (triangle) and male (M; diamond) mice. ***C***, ***D***, Induction of parallel fiber-LTD by parallel fiber (PF) and climbing fiber (CF) stimulation at 1 Hz for 5 min generated the same response in slices from Eovx (square), Covx (triangle), and male (diamond) mice. Traces show superimposed PF-EPSCs from Purkinje cells from a Covx (left) and an Eovx (right) mouse recorded before conjunctive stimulation (black) and 25 min after conjunctive stimulation (blue and red, respectively); each trace represents an average of 30 traces. *$p < 0.05$. All values are mean ± SEM.

**Table 2. Basal synaptic parameters from Purkinje cells from Covx, Eovx, and male mice**

| Mice | R input (MΩ) | EPSC amplitude (pA) | 10–90% rise time (ms) | Decay time constant (ms) | PPF ratio (%) |
|---|---|---|---|---|---|
| Covx ($n = 5$) | 94.7 ± 8.2 | −349.0 ± 23.0 | 3.1 ± 0.2 | 19.7 ± 3.1 | 97.8 ± 0.4 |
| Eovx ($n = 5$) | 105.4 ± 14.8 | −374.4 ± 5.8 | 3.0 ± 0.2 | 21.0 ± 1.4 | 98.8 ± 0.5 |
| Male ($n = 5$) | 92.6 ± 4.9 | −350.2 ± 6.0 | 3.0 ± 0.1 | 21.6 ± 1.1 | 98.6 ± 0.3 |

All values are mean ± SEM. R input, Input resistance.

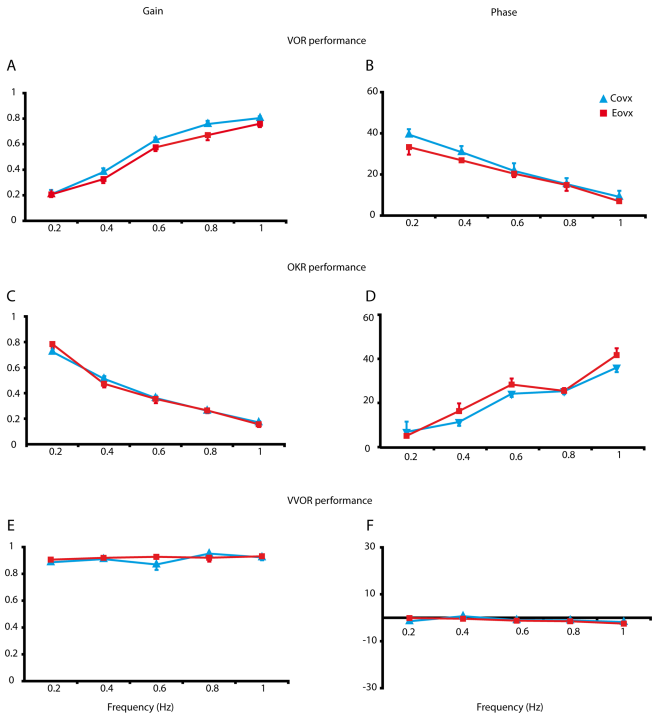


**Figure 3.** E2 does not affect motor performance. Robust compensatory eye movements are generated by head rotations in the dark (VOR) (***A***, ***B***), by environment rotations in the light (OKR) (***C***, ***D***), and by head rotations in light (VVOR) (***E***, ***F***) with frequencies ranging from 0.2 to 1 Hz at an amplitude of 5°. The amplitude (gain) was computed as the ratio of eye velocity to stimulus velocity; timing (phase) was expressed as the difference (in degrees) between the eye velocity and stimulus velocity. In all conditions, no differences were observed in gain or phase between mice with low (Covx; triangle) and high (Eovx; square) levels of E2 (all $p > 0.05$, 2-way ANOVA). Error bars indicate SEM.

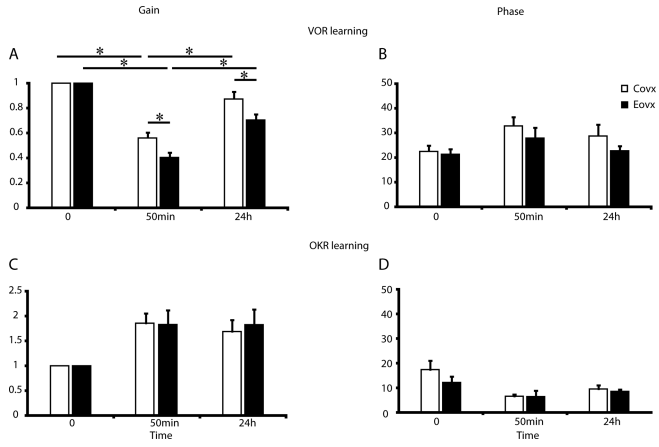


**Figure 4.** E2 enhances gain-decrease VOR learning. ***A***, ***B***, Normalized VOR gain and phase before training, after 50 min of training, and 24 h later in Covx mice (open bar) and Eovx mice (filled bar). Note that Eovx mice learned significantly better than Covx mice. Twenty-four hours in darkness reduced the effect of the training on gain, but the difference between groups persisted. No differences were observed in phase (*$p < 0.05$). ***C***, ***D***, No differences were noticed in gain-increase OKR learning between Eovx mice and Covx mice either after 50 min of training or 24 h later, either in gain or phase. Error bars indicate SEM.

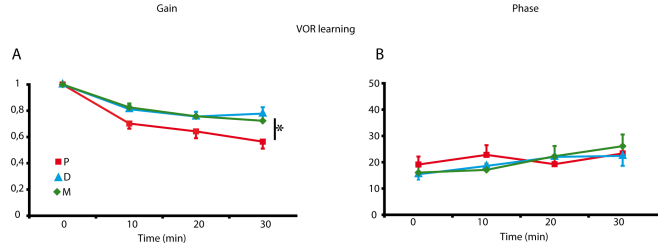


**Figure 5.** Motor learning is enhanced by endogenous high levels of the E2. ***A***, ***B***, Thirty minutes of training induced a higher VOR gain-decrease in female mice with natural high levels of E2 [proestrus (P); squares] than in female mice with natural low levels of E2 [diestrus (D); triangles] or than in male mice (M; diamonds). No differences were observed in phase. *$p <$ 0.05. All values are mean ± SEM.

injections with either estradiol benzoate dissolved in sesame oil (Eovx) or sesame oil alone (Covx), VOR amplitude (i.e., gain) and timing (i.e., phase) of the VOR in mice with a high level of E2 (Eovx, $n = 9$) were similar to those in mice with a low level of E2 (Covx, $n = 9$; $p = 0.14$ and $p = 0.31$, respectively, two-way ANOVA) (Fig. 3*A*,*B*). In addition, eye movement responses of Eovx mice to visual stimulation (OKR) (Fig. 3*C*,*D*) and to head rotation in light (VVOR) (Fig. 3*E*,*F*) were also similar to those of Covx mice (OKR gain, $p = 0.98$; OKR phase, $p = 0.12$; VVOR gain, $p = 0.91$; VVOR phase, $p = 0.30$; two-way ANOVA) and showed the classical relationship to stimulus frequency (Fig. 3). Consequently, we conclude that E2 does not influence the general eye-motor performance.

In contrast, when both groups of mice were subjected to a visuovestibular training paradigm in which head rotation was paired for 50 min with in-phase rotation of the visual environment (±5°, 0.6 Hz), significant differences were observed (Fig. 4*A*,*B*). The training paradigm induced a robust decrease in VOR gain of 60% in Eovx mice (from an initial value of 0.56 ± 0.03 to a final value of 0.23 ± 0.02; $n = 9$), whereas in the Covx mice, a significantly smaller learning effect was observed ($p < 0.001$, two-tailed paired *t* test; 44% from 0.57 ± 0.03 to 0.32 ± 0.03; $n = 7$). In both groups, the change in VOR gain induced by the training was reduced after spending 24 h in darkness (Fig. 4*A*) (Eovx, $p < 0.001$; Covx, $p < 0.001$; two-tailed paired *t* test), but the difference between Eovx and Covx mice was preserved (Fig. 4*A*) ($p < 0.02$, two-tailed paired *t* test). In contrast, no significant differences were observed in phase values (Fig. 4*B*) (after training, $p = 0.40$; at 24 h, $p = 0.22$; two-tailed paired *t* test). This discrepancy may be explained by the fact that the timing of the response (VOR phase) might be regulated by different forms of plasticity or at different places that are not subject to estrogen action (Lisberger et al., 1983; Faulstich et al., 2004; De Zeeuw and Yeo, 2005).

As a control, OKR adaptation was tested by pairing head rotation with out of phase rotation of the environment. All mice responded to the training stimulus by adaptively increasing OKR gain and decreasing OKR phase, but no significant differences were observed in gain or phase between OVX mice with and without E2 replacement (after training for gain, $p = 0.94$; after training for phase, $p = 0.95$; at 24 h for gain $p = 0.70$; at 24 h for phase, $p = 0.60$; two-tailed paired *t* tests) (Fig. 4*C*,*D*).

Thus, artificially generated high levels of E2 specifically improved gain-decrease motor learning and memory maintenance, whereas the overall motor performance was unaffected. The basic motor performance remained normal because the visuovestibular system was not challenged in this test situation.

## Endogenously induced high levels of E2 are sufficient to alter motor learning

Artificially induced changes in levels of motor learning do not necessarily imply functional differences that are relevant under

physiological circumstances. To test to what extent variations in the endogenous levels of E2 affect motor learning, VOR adaptation was measured in "intact" (non-OVX) female mice at different days of their normal estrous cycle. In female mice in diestrus (low E2 level of 49 ± 1 pmol/L; $n = 8$), 30 min of training resulted in a change in VOR gain of 22%, whereas in mice in proestrus (high E2 level of 58 ± 2 pmol/L; $p < 0.001$, two-tailed paired $t$ test; $n = 7$), the same training resulted in a significantly greater change of 44% ($p < 0.005$, two-way ANOVA) (Fig. 5*A*). The same paradigm applied to male mice (E2 level of 48 ± 14 pmol/L; $n = 7$) induced a change in the VOR gain of 28%, which was comparable to the change observed in female mice in diestrus ($p = 0.47$, two-way ANOVA, *post hoc* LSD) (Fig. 5*A*) but significantly smaller than that in females in proestrus ($p < 0.03$, two-way ANOVA, *post hoc* LSD). No significant changes were found for phase values (all $p > 0.5$, two-way ANOVA, *post hoc* LSD) (Fig. 5*B*). Thus, female mice with endogenous high levels of E2 show significantly better levels of motor learning than females with endogenous low levels of E2 or male mice.

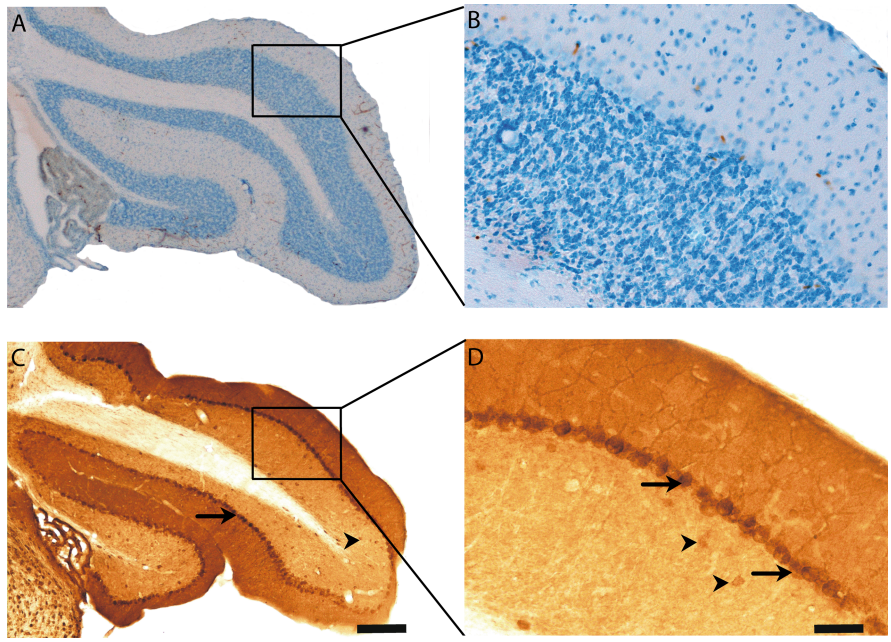


**Figure 6.** E2 receptor expression in the vestibulocerebellum. ***A***, ***B***, ERα immunoreactivity in sagittal cerebellar slices counterstained with thionin reveals no ERα in any of the cerebellar neurons. ***C***, ***D***, ERβ immunoreactivity in sagittal cerebellar slices shows a high expression of the ERβ. ERβ is present in Purkinje cells (arrows) and Golgi cells (arrowheads) in the flocculus and paraflocculus. Scale bars: ***A***, ***C***, 400 μm; ***B***, ***D***, 100 μm.

### ERβ but not ERα are present in the adult vestibulocerebellum

In the developing cerebellum, both ERα and ERβ are localized in Purkinje cells, granular cells, and molecular layer interneurons (Jakab et al., 2001; Perez et al., 2003). ERβ has also been shown to occur in the adult cerebellum (Price and Handa, 2000), but it is still not clear to what extent it is located in the vestibulocerebellum (i.e., the area that controls adaptation of the VOR) (Robinson, 1976). We therefore investigated the localization of both α ERs (ERα) and β ERs (ERβ) in the flocculus and paraflocculus of the vestibulocerebellum in the adult mouse. Although ERα expression could not be detected in the flocculus or paraflocculus (Fig. 6*A*,*B*), ERβ occurred in the cell soma and dendrites of Purkinje cells in both flocculus and paraflocculus as well as in their terminals in the vestibular nuclei (Fig. 6*C*,*D*). In addition, labeling for ERβ could be observed in Golgi cells in the granular layer of the flocculus and paraflocculus (Fig. 6*C*,*D*). Therefore, E2 may exert its effects on VOR adaptation either by directly activating the Purkinje cells in the flocculus and/or via activation of the Golgi cells.

### Activation of ERβ in Purkinje cells contributes to enhanced adaptation

To further pinpoint the location of the ERs responsible for the differences in motor learning, we created and tested mouse mutants in which ERβ was specifically removed in Purkinje cells (L7-ERβ−/−). When female L7-ERβ−/− mice were presented in the proestrus day with the training paradigm, the training still induced a change in VOR gain of 29% in L7-ERβ−/− mice ($n = 4$) (Fig. 7*A*). However, in the female L7-ERβ wild-type littermates, we observed a significantly larger effect on the adaptation (51%; $n = 4$; $p < 0.05$, two-way ANOVA) (Fig. 7*A*), indicating that the activation of the ERβ in the Purkinje cells plays an important role in this form of motor learning. No significant

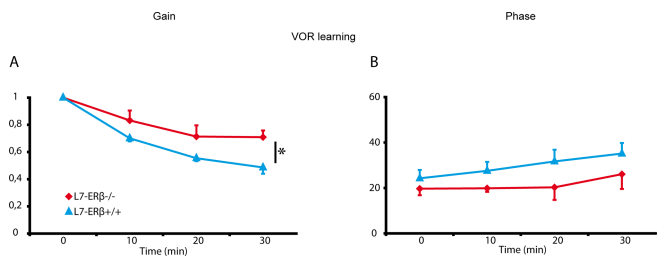


**Figure 7.** Motor learning is enhanced by the presence of the ERβ in the Purkinje cells. ***A***, ***B***, Thirty minutes of training induced a higher VOR gain-decrease in L7-ERβ+/+ littermates (triangles) than in L7-ERβ−/− (diamonds). No significant differences were observed in phase. *$p < 0.05$. All values are mean ± SEM.

changes were found in the phase ($p = 0.17$, two-way ANOVA) (Fig. 7*B*). Thus, by subjecting mice in which the ERβ receptor is specifically ablated in Purkinje cells (i.e., the L7-ERβ−/− mutants) to the visuovestibular training paradigms, we show here that activation of ERβ in Purkinje cells is sufficient to enhance gain-decrease adaptation of the VOR in the Eovx mice and in the intact mice in proestrus described above.

### E2 alters parallel fiber to Purkinje cell synapses at structural level

The effects of E2 on synaptic efficacy described above may, in principle, be correlated to effects on the morphology of the synapses involved. We therefore investigated Purkinje cells of Eovx mice and Covx mice at the ultrastructural level. Cerebellar sagittal sections were stained against calbindin, processed for electron microscopy, and quantitatively analyzed at the level of the parallel fiber input (Fig. 8*A*,*B*). We found that high levels of E2 significantly increased the number of synapses (Eovx mice, 0.55 ± 0.03/$\mu m^2$; Covx mice, 0.47 ± 0.02/$\mu m^2$; $p = 0.051$, two-tailed paired $t$ test). The postsynaptic density area ($PSD_A$) and the postsynaptic

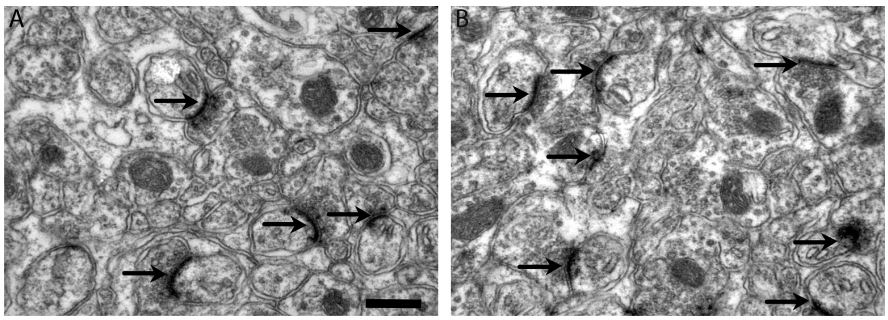


**Figure 8.** E2 increases synaptic density in the vestibulocerebellum. Electron micrographs of the molecular layer of the flocullus that are labeled with antibody against calbindin showing ultrastructural characteristics of Purkinje cell synapses in OVX mice that received oil (***A***) or E2 (***B***). Arrows mark synapses between Purkinje cell and parallel fibers. Scale bar, 0.5 μm.

density perimeter ($PSD_P$) were slightly, but not significantly, increased in Eovx mice ($n = 8$) compared with those in Covx mice ($n = 8$). The $PSD_A$ area and $PSD_P$ in Eovx mice were 7788 ± 515 $nm^2$ and 662 ± 14 nm, respectively, whereas those in Covx mice were 7177 ± 287 $nm^2$ and 634 ± 15 nm, respectively ( $p = 0.32$ and $p = 0.18$, respectively, two-tailed paired *t* test). However, when the synaptic density was considered (for formula, see Materials and Methods), it became apparent that the total postsynaptic area was significantly increased by 30% in Eovx mice (Eovx mice, 376 ± 35; Covx mice, 289 ± 20; $p < 0.05$, two-tailed paired *t* test). In contrast, the total postsynaptic surface area available per vibratome section at the climbing fiber to Purkinje cell synapse did not show any significant change ( $p = 0.61$, two-tailed paired *t* test). We conclude that E2 specifically enhances the availability of the synaptic complexes at the parallel fiber to Purkinje cell input.

## Discussion

Our data show via different lines of evidence that the gonadal hormone E2 promotes cerebellar plasticity in a specific manner. We show that E2 enhances induction of LTP at the parallel fiber to Purkinje cell synapse, whereas it does not affect LTD; that E2 activation of ERβ receptors of Purkinje cells significantly improves gain-decrease adaptation of the VOR, whereas it does not affect gain-increase adaptation of the OKR or general eye movement performance; and that E2 increases the total size of the postsynaptic complex of the parallel fiber to Purkinje cell synapse, whereas it does not affect that of the climbing fiber synapse.

These data support one another, and they are in line with several other investigations. First, Boyden and colleagues (Boyden and Raymond, 2003; see also Miles and Eighmy, 1980; Van Alphen and De Zeeuw, 2002; Faulstich et al., 2004) pointed out that gain-decrease paradigms show different dynamics compared with those of gain-increase paradigms and that it is therefore unlikely that they both depend on LTD. Second, LTP induction is supposed to increase the insertion of AMPA receptors and thereby enlarge their postsynaptic complex, whereas LTD induction at these synapses could only decrease their size because of the endocytosis of these receptors and their associated protein complexes (Strata and Rossi, 1998; Hansel et al., 2001; Song and Huganir, 2002; Coesmans et al., 2004). Moreover, because climbing fiber activity can only promote LTD at the parallel fiber synapse (Coesmans et al., 2004), one does not expect a change at the climbing fiber synapse if the promoting effect of E2 is specific for LTP induction. Thus, although LTD and LTP do certainly not provide the only push–pull mechanisms in the olivocerebellar system (De Zeeuw et al., 2004), the present study does provide for the first time experimental evidence for the potential functional role of cerebellar LTP, and it puts together currently available cell physiological and structural mechanisms into a new integrative hypothesis that is compatible with several existing lines of research.

Our finding that female gonadal hormones can improve motor learning may have several teleological implications. First, the improved ability for particular forms of cerebellar motor learning may be related to specific needs during the short period of proestrus in which females need to find, attract, and have intercourse with appropriate males. In this sense, the observed effect can be considered as a sexually dimorphic trait, because females with naturally occurring high levels of E2 outperformed males, who performed as well as females with naturally occurring low levels of E2. This characteristic is in line with other sexually dimorphic traits such as verbal abilities and fine motor skills in which females are also superior (Epting and Overman, 1998). Together, these procedural skills stand in contrast to some other traits in which males may be superior, such as visuospatial abilities (Epting and Overman, 1998). Second, changes in sexual hormones occur not only during the estrous cycle but also during pregnancy. Therefore, changes in Purkinje cell activities caused by changes in levels of E2 may also be related to pregnancy. During pregnancy, the increment of the abdominal size changes the center of gravity, which leads to an altered postural balance forcing the vestibulocerebellum to adapt to the new situation so as to make smaller movements such as error-free walking. Nonstumbling pregnant females have reproductive advantages. Obviously, during pregnancy, other types of behavior such as "nest making" also require hormonal changes, but it remains to be seen to what extent E2 alone can explain such complicated behaviors. In this respect, it is important to note that we did not only observe differences in VOR adaptation during the natural estrous cycle, in which the levels of other hormones such as progesterone also vary substantially, but that we also observed the same effects when we artificially altered the level of E2 alone.

The impact of E2 on cerebellar motor learning and plasticity does not stand on itself. E2 can promote dendritic outgrowth, spinogenesis, and synaptogenesis of Purkinje cells during neonatal life (Sakamoto et al., 2003; Shikimi et al., 2004), and it can enhance their responses to natural and pharmacological stimulation during adulthood (Smith et al., 1987; Smith, 1989). E2 also improves other forms of memory formation such as spatial reference memory and trace conditioning controlled by the hippocampus and forms of visual memory and strategy solving tasks controlled by the cerebral cortex (Frick et al., 2002; Luine et al., 2003; Leuner et al., 2004; Rhodes and Frye, 2004). In the hippocampus, E2 has also been shown to facilitate the formation of new synapses (Woolley and McEwen, 1993) and to exert a positive impact on induction of LTP (Cordoba Montoya and Carrer, 1997; Good et al., 1999; Vouimba et al., 2000). Thus, although short-term potentiating mechanisms such as those mediated by levels of calcium concentration and activation of kinases and phosphatases can vary substantially between hippocampal and cerebellar learning (Cordoba Montoya and Carrer, 1997; Good et al., 1999; Vouimba et al., 2000), sex hormone-related mechanisms appear to be able to overrule these differences and exert general effects in different brain regions.

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