Unconventional Peripheral, Central and Hormonal Factors in Cerebellar Plasticity

Corina Emilia Andreescu

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Unconventional Peripheral, Central and Hormonal Factors in Cerebellar Plasticity

Onconventionele perifere, centrale en hormonale factoren in cerebellaire plasticiteit

Proefschrift

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For my dear parents

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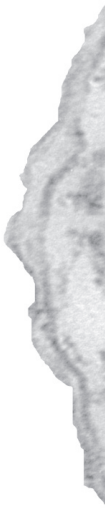

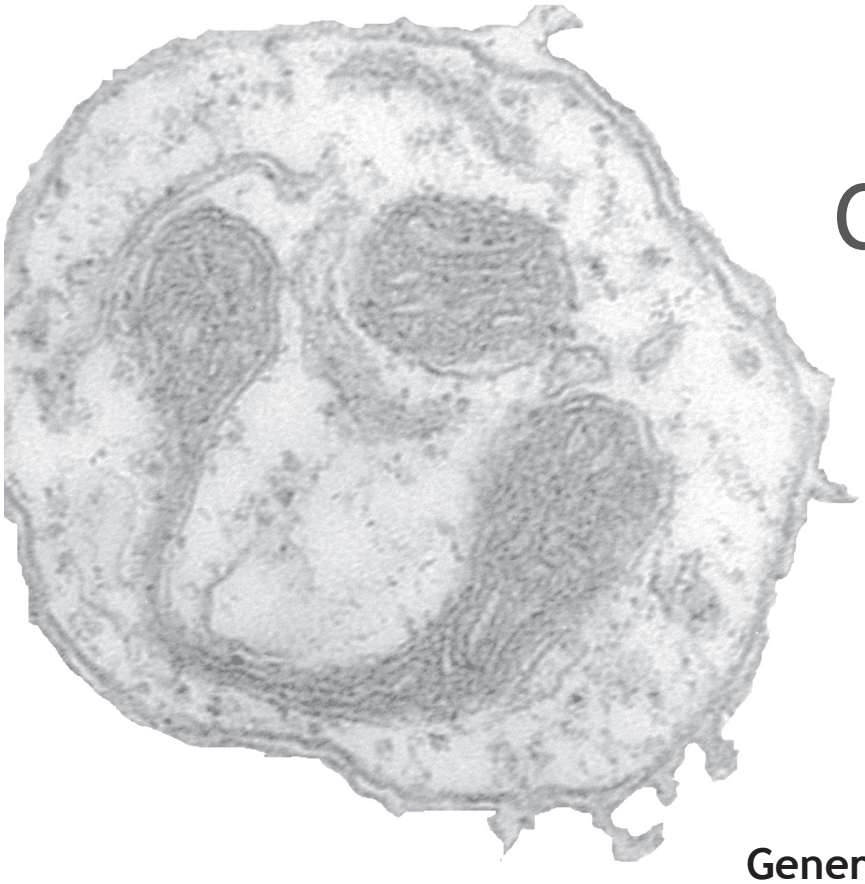

Chapter 1

General Introduction

General introduction

How do we learn new things and how do we acquire new motor skills?
Learning is defined as the mechanism by which the central nervous system adapts to environmental pressures and constraints by generating appropriate behaviors.
Motor skills are typically acquired gradually without any noticeable conscious memory of what information has been gained. The olivocerebellar system has the capacity to use its molecular machinery to convert information into a form that can be stored as short-term or long-term motor memory. This circuitry that facilitates motor skills is subjected to many factors.
In this thesis, we describe the effects of alterations in the vestibular system, central factors and hormone levels on the motor performance and the motor learning.

1.1 Compensatory ocular reflexes

1.1.1 Vestibulo-ocular reflex and optokinetic reflex

The vestibulo-ocular reflex (VOR) is a compensatory ocular movement in response to stimulation of the vestibular organ. The vestibular organ contains specialised areas with receptor cells that translate head movement into neuronal signals. Detection of head movements generates eye movements in the opposite direction, in order to stabilize a visual target on the retina. The vestibulo-ocular reflex occurs for rotational movements, linear movements, or a combination of both.

The connection between the vestibular organ and the extraocular muscles of the eye ball was shown by Lorente de Nó (Lorente de Nó, 1933) to be a three-neuron reflex arc, in which signals are transmitted from the vestibular organ to the ipsilateral vestibular nucleus, from there to the contralateral oculomotor, trochlear or abducens nucleus, and finally to the appropriate eye muscle (Figure 1). Attached to this three-neurons VOR arc is an important side loop that comprises projection to and from a portion of the cerebellum called the flocullus and ventral paraflocullus (Langer et al. 1985b; Langer et al. 1985a).

The optic system acts in concert with the vestibulo-ocular system to maintain stability of images on the retina over a broad range of head movements. The VOR compensates for “fast” head movements using signals from the vestibular system, while “slow” movements of images that cover a large portion of the visual field generate compensatory eye movements (i.e. optokinetic reflex: OKR) in the same direction as the visual stimulus (Collewijn, 1969; van Alphen et al. 2001). Visual signals necessary for OKR are transmitted from the accessory optic system to extraocular motor neurons via the vestibular nuclear complex.

Stable images on the retina are maintained under various movement conditions by using both reflexes: vestibulo-ocular reflex and optokinetic reflex. Both reflex systems can be easily and accurately investigated by measuring the eye movements. In laboratory, eye movements can be induced either vestibularly by rotating the animal in the dark (VOR; Figure 2), or visually by rotating a well-lighted random dotted surrounding around the animal (OKR; Figure 2). By rotating the animal in the light, vestibular and optokinetic information are both available and eye movement responses are most accurately performed under these circumstances.

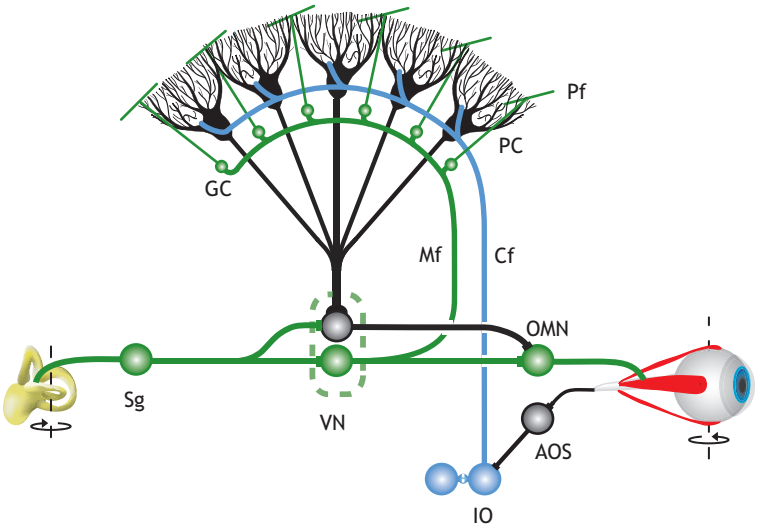

Figure 1. VOR circuitry. *Primary afferents from the vestibular system (Scarpa's ganglion; Sg), converge upon second order vestibular nuclei neurons (VN) that innervate the oculomotor nucleus (OMN) to control eye muscles. Information about head movements and retinal slip reaches the cerebellar cortex via the mossy fibres (Mf) and via the climbing fibers (Cf). This information is processed in the Purkinje cells (PC), which form the sole output of the cerebellar cortex. GC – granule cells; Pf – parallel fibers; AOS – accessory optic system and IO – inferior olive.*

Figure 2. Video eye movement recording. *The eye movements are recorded during various stimulations by using an eye-tracking device. Optokinetic reflex (OKR) is induced by presenting in light sinusoidal visual stimuli (left). Vestibulo-ocular reflex (VOR) is induced by presenting in dark sinusoidal vestibular stimuli (right).*

Stable images on the retina are maintained under various movement conditions by using both reflexes: vestibulo-ocular reflex and optokinetic reflex. Both reflex systems can be easily and accurately investigated by measuring the eye movements. In laboratory, eye movements can be induced either vestibularly by rotating the animal in the dark (VOR; Figure 2), or visually by rotating a well-lighted random dotted surrounding around the animal (OKR; Figure 2). By rotating the animal in the light, vestibular and optokinetic information are both available and eye movement responses are most accurately performed under these circumstances.

1.1.2 VOR and OKR adaptation

Throughout life VOR and OKR remain accurate as long as they are recalibrated by teaching or by error signals provided by the visual system (Sharpe et al. 1981; Estanol and Lopez-Rios, 1984). Adaptation of the VOR can be induced by providing subjects with magnifying, miniaturizing, or reversing spectacles during normal behavior or by pairing head turns with image motion (Gonshor and Jones, 1973; Ito et al. 1974b; Miles and Fuller, 1974; Gauthier and Robinson 1975). If for instance, we suddenly and constantly align the image motion with the head motion, the system is confronted with an overestimated VOR amplitude, and consequently VOR amplitude will be decreased adaptively (Figure 3).

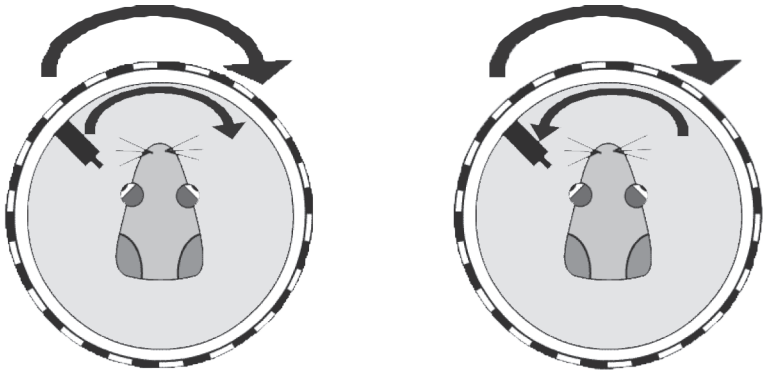

Figure 3. VOR adaptation. *VOR amplitude (gain) decrease is induced by presenting in phase sinusoidal vestibular and visual stimuli (left). VOR amplitude (gain) increase is induced by presenting 180º out-of phase sinusoidal vestibular and visual stimuli (right).*

Visual-vestibular training paradigms meant to change the VOR are also effective in changing OKR gain. Both VOR adaptation aimed at increasing VOR gain as well as adaptation aimed at decreasing VOR gain result in an increase of the OKR gain (Collewijn and Grootendorst, 1979; Nagao, 1983). Furthermore, OKR gain can be increased by prolong visual stimulation in the presence of sufficient retinal slip (Iwashita et al. 2001).

The process of learning leads to dynamic changes in amplitude and timing of VOR and OKR (Collewijn and Grootendorst, 1979; Nagao, 1983; Iwashita et al. 2001; van Alphen and De Zeeuw, 2002; Boyden et al. 2004; Faulstich et al. 2004; Stahl, 2004). Several hypotheses regarding the site(s) where motor learning occurs and is stored have been suggested. Marr and Albus proposed that the cerebellar flocculus is the exclusive site of learning (Marr, 1969; Albus, 1971; Ito, 1982). We now know that the cerebellum is necessary for motor learning to proceed, because complete bilateral ablation of the flocculus and ventral paraflocculus or of the whole cerebellum abolishes learning in the VOR and has little or no effect on normal VOR performance (Robinson, 1976; Flandrin et al. 1983; Nagao, 1983; Lisberger et al. 1984). Furthermore, newly learned responses are precluded after cerebellar removal (Zee et al. 1981; Rambold et al. 2002). Miles and Lisberger suggested that the brainstem is exclusively responsible for motor learning (Miles and Lisberger, 1981). Nowadays, the most accepted hypothesis is the multisite hypothesis, which suggests motor learning roles for both the flocculus and the brainstem (Lisberger and Sejnowski, 1992; Partsalis et al. 1995a, b; Hirata and Highstein, 2001).

Even though there are still controversies regarding how and where motor learning occurs, there are a few principles that are generally agreed upon. Motor learning requires an intact cerebellum (Ito et al. 1974a; Robinson, 1976; Lisberger et al. 1984) and adaptation of eye movements requires vision in combination with a vestibular or a visual signal (Collewijn and Grootendorst, 1979; Lisberger et al. 1984; Shelhamer et al. 1994). Finally it is important to keep in mind that although motor learning is induced in the laboratory using optical methods, it is generally assumed that the system is plastic in order to deal with changes due to injuries, diseases, or aging in various components of the vestibular system (Maioli and Precht, 1985; Broussard et al. 1999).

1.2 Peripheral factors

The peripheral component of the vestibular system, the labyrinth, is buried deep within the skull and it is composed of the three semicircular canals and the two otolith organs, the utricle and the saccule. Specialised areas in the membranous labyrinth, which are filled with endolymph, contain receptor cells that translate movement into neuronal signals. Each semicircular canal and otolith organ is spatially aligned in order to be most sensitive to movements in specific planes of the three-dimensional space (Figure 4).
Signals related to rotational movements of the head are derived from the three semicircular canals (the anterior canal, the posterior canal and the horizontal canal) via specialised cells, named hair cells. The hair cells are located within the crista ampullaris and their cilia are embedded into a gelatinous mass called the cupula. Angular acceleration of the head will lead to movement of the endolymph relative to the canal and will cause a deviation of the cupular membrane. Deviation of the cupular membrane will cause a displacement of the hair bundles and will excite or inhibit hair cells activity depending on the direction of the movement.

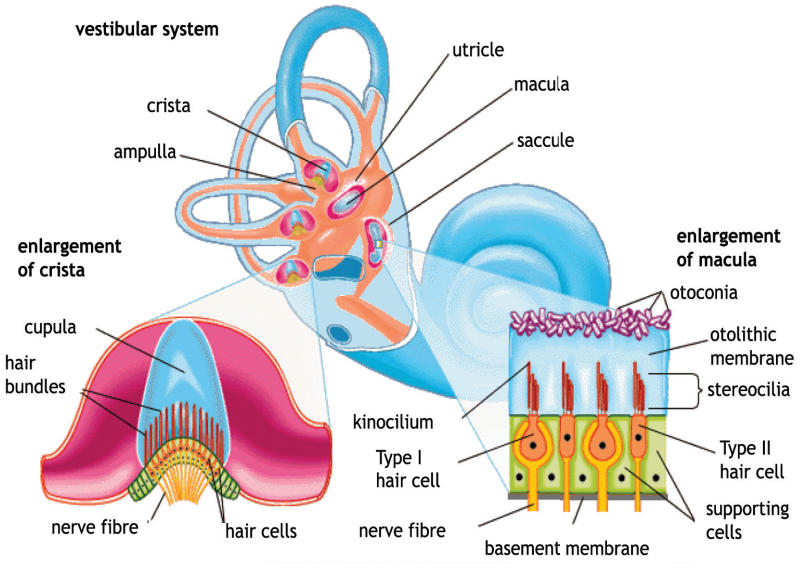

Figure 4. The inner ear. *The cross section of the ampulla of the posterior semicircular canals shows the hair cells, hair bundles and the position of the cupula (left). The cross section of the utricular macula shows hair bundles projecting into the otolithic membrane which is covered by otoconia (right).*

Additionally, displacements and linear accelerations of the head, such as those induced by tilting or translational movements, are detected by the utricle and the saccule. Both organs contain a sensory epithelium, the macula, which consist of hair cells and associated supporting cells. Overlying the hair cells and their hair bundles is a gelatinous layer, and above this is a fibrous structure, the otolithic membrane, in which calcium-carbonate crystals called otoconia are embedded. The crystals give the otolith organs their name (otolith is Greek for "ear stone"). Linear acceleration due to translational movement or reorientation of the head with respect to the gravity leads to displacement of the otolithic membrane and will generate a receptor potential in the hair cells.

The information provided by these peripheral endorgans reaches the vestibular nuclear complex of the brainstem and the vestibular part of the cerebellum (Dino et al. 2001; Maklad and Fritzsch, 2003). Furthermore, information from the linear and angular acceleration sensors of the ear are compared in the brain to eliminate the inherent ambiguity of the otolith system to distinguish gravity from non-gravity-related linear acceleration (Angelaki et al. 1999).

In order to investigate how linear acceleration and gravitational inputs of the inner ear contribute to the generation of adequate eye movements we used a mutant mouse that is lacking the otoconia (tilted mouse; tlt). The spontaneous tilted (tlt) mutation was identified as a mutation of a transmembrane protein, named otopetrin 1 (Otop1) that induces a deficit of otoconia (Hurle et al. 2003). By eliminating the otoconia and hence all forms of linear acceleration one can investigate the contribution of otolith organs to eye movement and whether compensatory processes are induced by this deficit.

1.3 Central factors

It is generally accepted that motor learning results from changes in the underlying circuits and in the neuronal signals they convey. Yet, it continues to be a matter of debate how specific disruptions in the underling circuits affect motor learning and whether, and if so how, these deficits induce compensations.

The attractiveness of cerebellar learning theory led to decades of experiments focused on determining whether or not long-term depression (LTD) could be the neuronal mechanism of cerebellar motor learning (Ito, 1986; Lev-Ram et al. 1997; Daniel et al.

1998; De Zeeuw et al. 1998; Lisberger, 1998; Hansel et al. 2006). The "LTD theory" suggested that error signals in motor performance are conveyed by the climbing fibers that will initiate an error correction by promoting depression at synapses between parallel fibers and Purkinje cells (Box 1) when climbing fibers and parallel fibers are simultaneously active.

Targeted genetic disruption of the LTD process in Purkinje cells has little effect on baseline oculomotor behaviour, but impairs short-term adaptation of the VOR that is induced by visual–vestibular mismatch training (De Zeeuw et al. 1998). Interestingly, long-term adaptation of the VOR does not appear to rely on cerebellar LTD; both increases and decreases in VOR gain could be induced after multiple days of visual-vestibular mismatch training in LTD-deficient mice, although gain increases were impaired when compared with those in wild type mice (van Alphen and De Zeeuw, 2002). The signal transduction pathway that underlies this parallel fibers – LTD involves the activation of PKC (protein kinase C), mGluR1 (metabotropic glutamate receptor 1), cGKI (cGMP – dependent protein kinase type I), CAMKII (calcium/calmodulin - dependent protein kinase II) that causes subsequentely AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor internalization (Linden and Connor, 1991; Aiba et al. 1994; De Zeeuw et al. 1998; Feil et al. 2003; Hansel et al. 2006).

The current state of knowledge endorses the original idea that LTD is only one of the mechanism underling cerebelar learning (Ito, 1972; Miles and Lisberger, 1981; Thompson and Krupa, 1994). In recent years, it has become clear that one synapse and one molecular mechanism are not enough to explain motor learning (Hansel et al. 2001; Carey and Lisberger, 2002; Boyden and Raymond, 2003; Coesmans et al. 2004; De Zeeuw and Yeo, 2005). The recent discovery of postsynaptic long-term potentiation (LTP) at the parallel fiber – Purkinje cell synapse (Lev-Ram et al. 2002; Coesmans et al., 2004) provided a new candidate for synaptic plasticity contributing to learning and memory (Boyden and Raymond, 2003; De Zeeuw and Yeo, 2005). This form of plasticity involves lower levels of intracellular calcium than the one necessary for LTD induction and involves the activation of phosphatases (Coesmans et al. 2004; Belmeguenai and Hansel, 2005). These two molecular mechanisms, LTD and LTP, might cooperate to increase and decrease the VOR gain, respectively (Boyden and Raymond 2003; Carey and Lisberger 2002; De Zeeuw and Yeo 2005).

Box 1

"Corpuscles surrounding the yellow substance [the junction between gray and white matter] in large numbers, are seen everywhere in rows in the laminae of the cerebellum. Each of these corpuscles faces the inside [of the organ], with the blunt, roundish endings towards the yellow substance, and it displays distinctly in its body the central nucleus; the tail-like ending faces the outside, and, by means of two processes, mostly disappears into the gray matter which extends close to the outer surface which is surrounded by the pia mater." (Purkinje over … Purkinje cells, Prague, 1837).

Jan Evangelista Purkinje (native Czech name Purkyně) was born on 17 December 1787 in Bohemia (now in the Czech Republic). At the age of ten, Purkinje was admitted to a monastery where he became a choirboy and an outstanding student. Just before he was to become a priest, however, Purkinje decided to study philosophy at the University of Prague. While at the University, he became interested in medicine and he began his medical education in 1814. Purkinje's doctoral dissertation, published in 1819, was entitled "Contributions to the Knowledge of Vision in its Subjective Aspect". In 1823 he was appointed professor at Breslau, Prussia (now Wrocław in Poland) – perhaps through the influence of Germany's greatest poet and writer, Johann Wolfgang von Goethe, who had befriended him. At Breslau, Purkinje founded the world's first official Institute of Physiology in 1839. In 1850, he returned to Prague University. He devoted the rest of his life to making science more accessible to his countrymen. He is buried in the Czech National Cemetery in Vyšehrad, Prague.

Purkinje made important contributions to cell biology, physiology and early neuroscience – in fact, every scientist knows his name, as it is linked forever to our body. He described: 1) Purkinje effect (1819) – the visual phenomenon in which, as light intensity decreases, red objects fade faster than blue objects; 2) Purkinje images (1832) – a threefold reflection seen in the eye of another person, caused by an object reflecting from the corneal surface and both sides of the lens; 3) Purkinje tree – the shadows of the retinal vessels seen as dark lines on a reddish field when light enters the eye; 4) **Purkinje cells in the cerebellum** (1837) – large neurons with piriform cell bodies with many branching dendrites; and 5) Purkinje fibers in the heart (1839) – the fibrous tissue that conducts electrical impulses from the atrioventricular node to all parts of the ventricles of the heart. Purkinje believed that experiments performed on oneself were more valuable than those performed on cadavers or animals. He performed a total of 35 self-experiments, deliberately overdosing himself with various drugs (i.e. belladonna, digitalis, opium), and graphically recording their visual and other effects.

Purkinje was one of the best known scientists of his time. Such was his fame that when people from outside Europe wrote letters to him, all that they needed to put as the address was "Purkyně, Europe".

"….and should you fail to understand, let Purkinje give you a hand"! (Goethe)

- **Purkinje, J.** Congress of Physicians and Scientists in Prague, 1837
- **Mazzarello P**. Purkinje: more than just a name, *Trends in Neurosciences* 25 (8): 432, 2002
- **Nicholas J. Wade, Josef Brožek, Jiří Hoskovec**. Purkinje's Vision: The Dawning of Neuroscience. *Lawrence Erlbaum Associates*, Mahwah, 2001
- **Tan S Y and Lin K H.** Johannes Evangelista Purkinje (1787-1869): 19th century's foremost phenomenologist. *Singapore Med J* 46(5): 208-9, 2005

LTP and LTD at the parallel fiber – Purkinje cell synapse are not the only forms of plasticity present in the cerebellum that are involved in different aspect of learning (Hansel et al. 2001). Recent studies have also demonstrated that a NMDA-mediated long-term potentiation at the mossy fiber to granule cell synapse can be induced (D'Angelo et al. 1999; Armano et al., 2000; Maffei et al. 2002; Rossi et al. 2002; Maffei et al. 2003; Sola et al. 2004; Mapelli and D'Angelo, 2007). By using null-mutant mice that lack the NMDA receptors one can investigate the contribution of NMDA-mediated long-term potentiation to compensatory eye movement (i.e. VOR, OKR) and to motor learning.

Maintenance of the learned behaviour requires the memory trace to be stored. Furthermore, motor memory, like other types of memory, undergoes a process of consolidation. Consolidation usually differentiates short-term from long-term memory and may involve a change in the locus of the memory storage (Shadmehr and Holcomb, 1997). According to the "transfer hypothesis", short-term changes may take place in the cerebellar cortex. Subsequently the cerebellar cortex generates an appropriate error signal to drive the formation of the long-term memory in the brain stem (Figure 5) (Galiana, 1986; Peterson and Houk, 1991; Raymond et al. 1996; Medina et al. 2001; Shutoh et al. 2006).

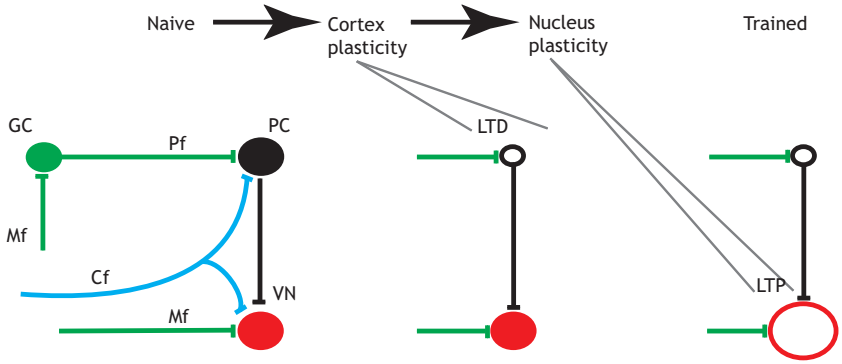

Figure 5. Hypothesized sequence of events during VOR adaptation. *In the naive animal (left column) vestibular information are transmitted to both Purkinje cells (PC) and vestibular nuclei (VN). The Purkinje cells are inhibitory cells that synapse on vestibular nuclei. Paired out-of-phase vestibular and visual stimulation leads to LTD at the pf- PC synapses (cortex plasticity, middle column). For this reason, Purkinje cell activity decreases and this new condition could serve as a signal to induce LTP at mf – VN synapses. Adapted from (Medina et al., 2000). Cf - climbing fibres; GC - granular cells; LTD - long-term depression; LTP - long-term potentiation; Mf - mossy fibres; PC - Purkinje cells; Pf - parallel fibres; VN - vestibular nuclei.*

1.4 Hormonal factors

1.4.1 Estrous cycle

Estrogens are steroid hormones that are present in the circulation of females and males. It is now well known that estrogens influence the development, growth, differentiation, maturation and function of various tissues throughout the body, including the peripheral and central nervous systems (McEwen and Alves, 1999; Gibbs and Gabor, 2003; Sherwin, 2003).

In premenopausal women, 95% of circulating estradiol is derived from the ovary. At that time, peripheral tissues, such as adipose and breast tissues, the osteoblasts and the chondrocytes of the bone, the vascular endothelium and aortic smooth muscle cells, and numerous sites in the brain play a minor role in estrogen synthesis. The human menstrual cycle is a 28 ± 7 days event which begins at the onset of menses, and includes an estrogen-dominant follicular phase of variable duration which culminates in a mid-cycle peak of 17ß-estradiol (E2) and ovulation, followed by a 12- to 14-day progesterone- dominant luteal phase (Figure 6). After menopause ("meno" is the Greek for month and "pausis" is the Greek for pause, cessation), when gonadal estrogen synthesis ceases, the peripheral tissues become the most important sites of estrogen production (Nelson and Bulun, 2001). In males estradiol is mainly derived from peripheral conversion of circulating testosterone and only a small amount is directly produced by the testes or originates from peripheral conversion of estrone and androstenedione (de Ronde et al. 2003).

Like primates, mice have reproductive cycles, but rather than the 28 days menstrual cycle, young adult female mice experience a 4 - 5 days estrous cycle (estrus is the Latin for "frenzy"). In middle aged mice (between 11 and 14 months of age), the estrous cycles begin to become irregular and eventually cease with further aging (between 17 and 18 months of age) (Nelson et al. 1982).

1.4.2 Estrogen receptors in the cerebellum

Estrogens are known to exert their actions through members of the nuclear hormone receptor superfamily, estrogen receptor-α (ERα) (Greene et al. 1986), and the more recently identified estrogen receptor-ß (ERß) (Ogawa et al. 1998). ERα and ERß are the products of two different genes that are located on separate chromosomes (Enmark et al. 1997). ERs share common structural features but differ in their affinities and

specificities for ligands, and tissue distribution (Klinge, 2000; Hewitt and Korach, 2002). Both ERs are widely distributed throughout the body, including the brain.

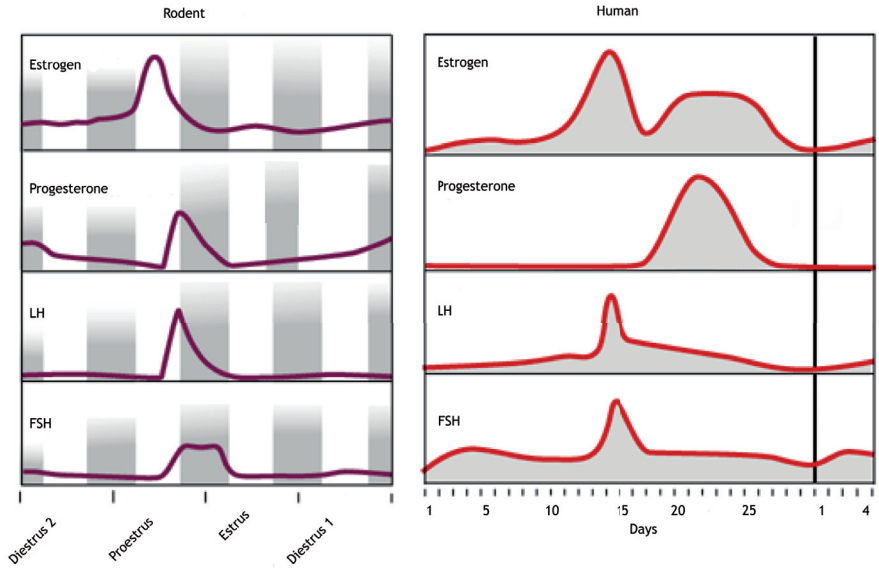

Figure 6. Comparison of the estrous cycle of the rodent and menstrual cycle of the human. *The 4-day estrous cycle of the rat (gray bars indicate night, 6 p.m. to 6 a.m.), showing fluctuations in estrogen, progesterone, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) is presented (left). In the mouse, the estrous cycle is similar, but varies in length (4-7 days). The human 28-day menstrual cycle is presented (right). Adapted from (Knobil and Neill, 1994).*

In the cerebellum both ERs are expressed but their expression level changes during development suggesting that the two ERs play distinct roles in cerebellar development. ERα is localized in Purkinje cells in the developing cerebellum (Ikeda and Nagai, 2006) and its expression profile appears to correlate with Purkinje cell differentiation (Altman, 1972b, a). During development, ERα is firstly expressed in Golgi neurons, secondly in Purkinje cells and thirdly in basket cells (Shughrue et al. 1992; Price and Handa, 2000; Ikeda and Nagai, 2006). During neonatal life, estradiol promotes Purkinje dendritic growth, spinogenesis and synaptogenesis (Sakamoto et al. 2003). In adulthood only ERß is expressed in the cerebellum, being present in Purkinje and in Golgi cells (Price and Handa, 2000). During adult life, estradiol improves Purkinje cell responses to excitatory neurotransmitters and enhances Purkinje cell discharges related to limb movements

(Smith et al. 1987; Smith, 1989). These results support the idea that ERß is functional in the adult brain, participating in the regulation of non-reproductive brain function.

1.4.3 Estradiol mechanisms of action

The complex physiological responses to steroids are generally presumed to involve transcriptional regulation of many genes, mediated via the so-called classic, or genomic, steroid pathway. This mechanism is summarized in the following steps: 1) free steroid enters the target cell and binds with high affinity to a receptor; 2) the steroid-receptor complex activates, which enables the receptor to bind to selective sites on the DNA; 3) the activated steroid-receptor complex interacts with specific DNA sequences referred to as steroid response elements, usually located in the promoter region of the steroid-responsive genes; and 4) the activated steroid-receptor complex initiates transcription of these genes (Hewitt and Korach, 2002).

Evidence of non-genomic effects exerted by steroids are observed in a wide variety of cell types (Revelli et al. 1998; Falkenstein et al. 2000; Levin, 2002) and have the following characteristics: 1) these effects are too rapid (from seconds to few minutes) to be compatible with the involvement of changes in mRNA and protein synthesis; 2) these effects can be elicited even by steroids coupled with high-molecular weight substances that cannot cross the plasma membrane and do not enter the cell (e.g. BSA-conjugates); 3) these effects are not blocked by inhibitors of mRNA or protein synthesis; and 4) these effects can be observed even in highly specialized cells that do not accomplish mRNA and protein synthesis (e.g. spermatozoa) or by cells lacking nuclear steroid receptors (Revelli et al., 1998; Moore and Evans, 1999). Such non-genomic effects are postulated to be initiated at the plasma membrane and to be mediated by a membrane-bound receptor (Falkenstein et al. 2000; Losel et al. 2003). The controversies in this field are not about whether or not E2 has non-genomic effects, but rather whether or not all of these effects are of physiological relevance and whether or not they are mediated by the nuclear estrogen receptors (ERs). Since the plasma membrane is not a barrier for E2 entry into cells, it is not necessary for estrogen receptors (whatever their nature) to be membrane-bound in order for them to be activated by E2 and trigger changes in ion channels or kinases at the cell surface. The variety of estrogen effects has extensively expanded to include rapid actions on excitability of neurons, activation of cAMP (cyclic adenosine monophosphate) and MAP (mitogen activated protein) kinase pathways, effects on calcium channels and calcium ion entry, and protection of neurons against

damage by excitotoxins and free radicals (McEwen, 2002; Segars and Driggers, 2002). Whether the classical genomic mechanism or a non-genomic mechanism is responsible for estradiol effects on the cerebellum needs still to be investigated.

1.4.4 Estradiol effects on central nervous system

Much research has been focused on the estradiol effects on hippocampus and cerebral cortex. This body of work revealed that E2 improves forms of memory formation such as spatial reference memory, trace conditioning, visual memory and strategy solving tasks (Frick et al. 2002; Luine et al. 2003; Daniel and Lee, 2004; Leuner et al. 2004; Rhodes and Frye, 2004). Anyhow, the literature concerning estrogen effect on Morris water maze task shows that E2 effect on learning are not always straight forward, but depend on the age of the subjects (Foster et al. 2003), and method and time of administration (Markham et al. 2002). Several studies showed that E2 did improve memory (O'Neal et al. 1996; Packard, 1998; Yamada et al., 1999; Sandstrom and Williams, 2001; Frick et al. 2002). Other studies showed that E2 had no effect on the memory at all (Singh et al. 1994; Berry et al. 1997) or even impaired memory (Frye, 1995; Galea et al. 1995; Chesler and Juraska, 2000). These different results might be due to the fact that a critical time window for estradiol administration might exist in order to induce a positive effect on the brain (Morrison et al. 2006; Siegfried, 2007).

In the hippocampus estradiol regulates the density and number of excitatory synaptic inputs and increases hippocampal excitability as well as the potential for synaptic plasticity (Woolley, 1998; Liu et al. 2008).

Studies of estrogen effects in non-primate species such as rodents have broadened our understanding of the interaction between hormones and the nervous system (Figure 7). The female mouse is an interesting model. It has several advantages: mice have a short gestation period, are big enough for blood sampling, and can be altered genetically.

Knocking out a gene (a targeted gene is completely deleted from the genome) may cause functional and behavioural changes that could indicate the contribution of that gene in a normal mouse physiology. For example, ERα deficient mice do not display normal female sexual behaviour and they do not show a learning deficit (Rissman et al. 1999). ERßs are essential for migration of neurons to their correct location in the developing cortex and the lack of this receptor induces morphological abnormalities in the cortex (Wang et al. 2001). These mutant mice also show increased anxiety and impaired spatial learning (Krezel et al. 2001; Rissman et al. 2002).

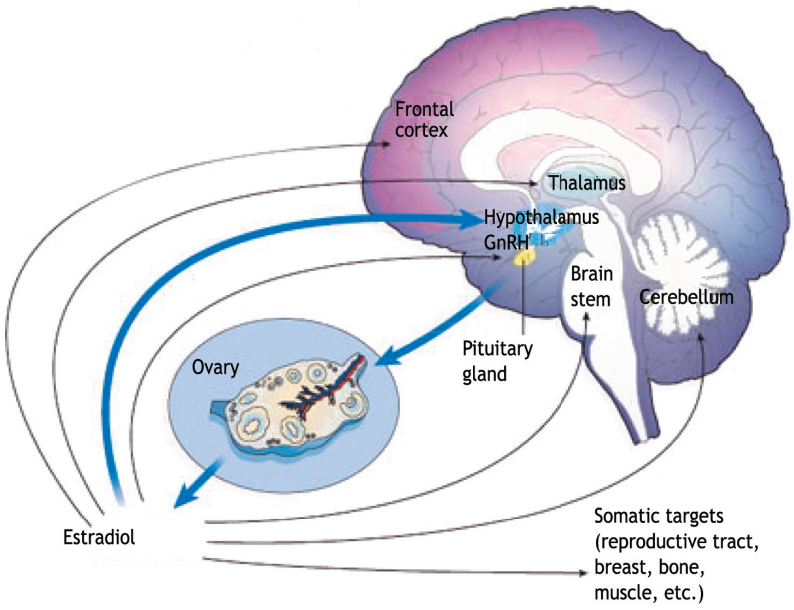

Figure 7. Interactions between the brain and the reproductive endocrine system. *The hypothalamic-pituitary-gonadal axis of females is shown, with the three levels of regulation of reproductive function. GnRH neurons in the hypothalamus release the decapeptide into the portal capillary vasculature, leading to the pituitary gland. Pituitary gonadotropes release the gonadotropins LH and FSH into the general circulation. LH and FSH act on their receptors on the ovary to regulate sex steroid hormone production and release, folliculogenesis and ovulation. Sex steroids in turn are released into the circulation and exert effects on the body. They also exert feedback actions on the hypothalamus and pituitary gland. In addition, steroids exert regulatory actions on receptors in nonreproductive brain regions, including (but not limited to) prefrontal cortex and hippocampus, thalamus, brainstem and cerebellum. Adapted from (Morrison et al. 2006).*

The disadvantage of these mouse models is that the genes are knocked out globally and therefore ERs are not expressed in any of the cells. It is clear that the behaviour of a null mutant mouse is not only influenced by the deleted gene but also by a number of secondary or compensatory developmental or physiological changes. Fortunately, by using genetic techniques it is nowadays possible to disrupt a gene in selective cell types and sometimes the process of gene deletion can be controlled in time. Generation of these cell specific mutant mice is necessary to further pinpoint the exact role of estradiol in various central nervous system dependent processes.

By generating mutant mice that lack the ERßs from the Purkinje cells one can investigate the contribution of estradiol action on these cells to compensatory eye movement (i.e. VOR, OKR) and to motor learning.

1.5 Scope of this thesis

This thesis investigates the role of peripheral, central and hormonal factors in compensatory eye movements and cerebellar plasticity.

In order to answer the question if and how these factors can alter the compensatory eye movements and how the cerebellum contributes to these changes we investigated the eye movements of a laboratory mouse. By using an integrated and multidisciplinary approach of genetic techniques, cellular physiology, and behavioral experiments in various mouse models, we were able to tackle some of these questions.

According to the multisensory integration theory, peripheral inputs like vestibular, optokinetic and proprioceptive inputs act in concert to maintain a stable retinal image of the visual world. Activation of the vestibular system via the otolith organs is accomplished through a shearing action between the stereociliary bundles of the hair cells and the overlying otoconial membrane. An efficient way to investigate the contribution of the otolith organs to the eye movement is to completely remove their stimulus. This is possible by investigating mice in space (i.e. under microgravity conditions) or by studying the space mouse model: the tilted mouse. The tilted mouse does not have functional otolith organs because it lacks otoconia due to a mutation in otopetrin 1. We investigated in these mice how linear acceleration and gravitational inputs contribute to compensatory eye movements and whether and, if so, what kind of compensation this deficit induces (Chapter II).

Several studies have demonstrated that multiple plasticity processes take place at the level of Purkinje cells and with the AMPA receptors as the key element. Yet, it remains elusive to what extent NMDA receptors contribute to cerebellar plasticity and whether a specific deficit of neuronal signals via NMDA receptors is important for VOR adaptation (Chapter III).

So far many extra- and intracellular factors have been shown to be involved in the induction and maintenance of cerebellar plasticity underling motor learning. Yet it is not known to what extent and, if so, how physiological variation of estradiol affects motor learning in females (Chapter IV).

In conclusion, in this thesis, we describe the effects of alterations in the vestibular system, central factors and hormone levels on the motor performance and motor learning.

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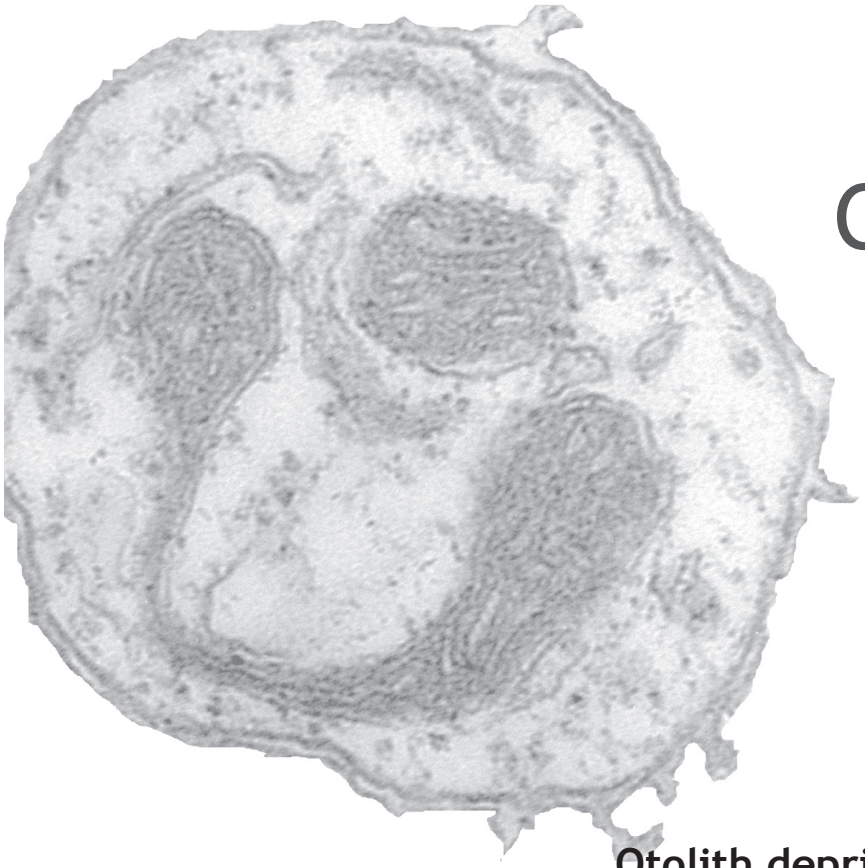

Chapter 2

Otolith deprivation induces optokinetic compensation

Corina E. Andreescu, Martijn M. De Ruiter, Chris I. De Zeeuw and Marcel T.G. De Jeu

Department of Neuroscience, Erasmus MC, Rotterdam, The Netherlands

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Abstract

According to the multisensory integration theory vestibular, optokinetic and proprioceptive inputs act in concert to maintain a stable retinal image of the visual world. Yet, it remains elusive to what extent the otolith organs contribute to this process and whether a specific loss of otolith input is compensated for. Here we investigated the compensatory eye movements in *tilted* mice, which lack otoconia due to a mutation in otopetrin 1. *Tilted* mice showed very small displacements of the eyes in the orbit during static roll paradigms, suggesting the absence of functional otolith organs. Independent of head position with respect to gravity, the gain and phase lead of angular vestibulo-ocular reflex of *tilted* mice were decreased and increased, respectively (frequencies 0.2 to 1 Hz and peak accelerations 8 to 197 deg/sec^2, respectively). Furthermore, lack of otolith input increases the dependency of the vestibular system on stimulus frequency. In contrast, the gain of optokinetic reflex in *tilted* mice was significantly higher in the low frequency range than in control mice, regardless of the position of the mice in space or the plane of the eye movements. Thus our data support the presence of the multisensory integration system and revealed a compensatory enhanced optokinetic reflex in *tilted* mice, indicating an adaptive synergism in the processing of otolith and visually driven signals.

Introduction

To maintain a stable retinal image one needs access to information provided by sensory systems controlling the vestibulo-ocular reflex (VOR), optokinetic reflex (OKR) and cervico-ocular reflex. Moreover, the control systems of these individual reflexes need to be sufficiently integrated in the central nervous system in order to weigh the impact and need of the different components under a wide variety of physical circumstances (Raphan et al. 1979; Merfeld and Zupan 2002; Mergner et al. 2003; Angelaki et al. 2004). Otolith organs provide a specific contribution to this multisensory system. Electrical stimulation of otolith organs (Fluur and Mellstrom 1971) or otolith nerves (Suzuki et al. 1969) demonstrated that otoliths control eye movements. The otolith signals are conveyed via primary otolith afferents (Fernandez and Goldberg 1976a, b) to the vestibular nuclei (Bush et al. 1993). These signals are able to generate an angular maculo-ocular reflex, which operates as a low-pass filtered response (rabbits: Barmack 1981; Van der Steen and Collewijn 1984; rats: Brettler et al. 2000; mice: Harrod and Baker 2003; cats: Rude and Baker 1988; Tomko et al. 1988; monkeys: Paige and Tomko 1991; Angelaki and Hess 1996). In addition, information provided by the otolith organs influences the orientation of the eyes with regard to gravity and linear acceleration (Baarsma and Collewijn 1975; Cohen et al. 2001). This information must be combined with input from semicircular canals to obtain proper compensatory eye movements (Angelaki et al. 2004). Moreover, converged otolith-canal neural activity significantly changes its modulation in different behavioral contexts such as active and passive head movements (McCrea and Luan 2003). In some of these situations the otolith information may be used to transform primary semicircular canal signals into space-reference angular motion (Angelaki and Hess 1995). Thus, several lines of evidence suggest that the otolith organs control eye movements via vestibular and oculomotor nuclei that are also used by the semicircular canal system.

According to the multisensory integration theory, one expects not only that deficits in the otoliths can cause a variety of problems in the canal-driven system, but also that compensation must take place. Yet, at present it is not clear whether dysfunctional otoliths can be compensated for, and if so, how and under which circumstances such compensations can occur? Eye movement recordings of primates under microgravity in space have not been conclusive due to small sample sizes, limited experimental time and the fact that the otoliths can still sense accelerations in this situation (Dizio

and Lackner 1992; Clement et al. 1993; Correia 1998; Moore et al. 2003). Moreover investigations on this topic have been hampered by the inability to mechanically lesion the otolith organs or nerves without affecting the input from the semicircular canals or without the loss of afferent fibers that will induce a reactive synaptogenesis (Goto et al. 2002).

To investigate potential vestibular compensatory processes, unilateral stimulation experiments on patients with unilateral vestibular nerve dissections (Clarke and Engelhorn 1998) and gravity-aligned/misaligned rotation experiments on patients with vestibular neuritis were performed (Schmid-Priscoveanu et al. 2004). However, these pathological circumstances were not specific enough to elucidate the compensatory mechanism induced by dysfunctional otoliths.

In the present study, we investigated potential mechanisms for compensation using *tilted* mice, which lack otoconia due to a spontaneous recessive mutation in otopetrin 1 gene (Otop 1) located on chromosome 5 (Hurle et al. 2003). Although their vestibular ganglion does develop relatively slowly (Smith et al. 2003), the projections from the otolith organs to the vestibular nuclei seems to be at least grossly normal in these mutant mice (Crapon de Caprona et al. 2004). Furthermore, *tilted* mice do not show any permanent abnormal phenotype in organ systems other than the otoliths (Ornitz et al. 1998). The linear vestibular evoked potential (linear VsEPs) is absent in *tilted* mice (Jones et al. 2004), as a consequence of the absence of otoconia. Apart from confirming their deficiency in gravito-inertial information by determining their eye position following static roll paradigms, we investigated their angular vestibulo-ocular reflex in the dark (aVOR) and the light (angular visually enhanced VOR or aVVOR) as well as their optokinetic reflex (OKR) over a wide range of stimulus parameters. The lack of otoconia decreases the gains and increases the phase errors not only during "otolith-mediated" aVOR but also during "canal-mediated" aVOR and increases the vestibular system dependency on frequency, especially at stimulus frequencies smaller than 1 Hz. We demonstrate that a frequency dependent enhancement of the optokinetic system can be used as a compensatory mechanism for a lack of functional otoliths. Our data is in line with the idea of an adaptive integration system where canal, otolith and visual signals share centrally processing.

Materials and Methods

In this study, we used eighteen homozygous *tilted* mice (Otop 1^{tlt}) and twenty-two heterozygous control littermates (age: 12 – 20 weeks; The Jackson Laboratory). The recessive *tlt* mutation arose spontaneously in 1983 on the STOCK p^{6H}/p^{d} and was backcrossed onto the C57BL/6J background. The mutant mice were not deaf or blind (Ornitz et al. 1998). They were housed on a 12 hour light/dark cycle with food and water available *ad libitum*. All animal procedures described were in accordance with the guidelines of the ethical committee of Erasmus MC, Rotterdam.

Phenotype assessment

The homozygous *tilted* mice were easily identified by their inability to swim when they were dropped from at least 20 cm height into a deep tank of water. *Tilted* mice cannot find the surface of the water and need rescuing to prevent drowning. Heterozygous control littermates mice can find the surface of the water and swim easily (Ornitz et al. 1998).

Surgical procedures

An acrylic pedestal was formed on the animal's skull under general anesthesia of a mixture of isofluoran (Isofloran 1 – 1.5%; Rhodia Organique Fine Ltd), nitrous oxide and oxygen. The pedestal construction was made as follows: a midline incision was made to expose the dorsal cranial surface and four stainless steel screws (1x1.5mm) were implanted in the calvarium and then embedded in dental acrylic. A pre-fabricated piece equipped with two nuts was attached to the pedestal in order to fixate the mouse in the restrainer device.

Video eye movement recording apparatus

After a recovery period (3 days), each mouse was handled daily for 2 days. During the experiment it was placed in an acrylic tube, with their head secured. The tube was inserted into the setup via a carrier that allowed orientation of the mouse (from mouse upright to mouse with its nose up or down; ± 90 degree). The carrier on which the mouse was fixed also permitted translation of the mouse in the left-right direction and near-far direction from the camera. The purpose of these translations was to position the mouse's eyeball on the rotation axis of the video camera, which ran through the center of the table (Stahl et al. 2000).

A cylindrical screen (diameter 63 cm) with a random-dotted pattern (each element 2°) surrounded the turntable (diameter 60 cm). Both the surrounding screen and the turntable were driven independently by an AC servo-motor (Harmonic Drive AG, The Netherlands). The table and drum position signal were measured by potentiometers, filtered (cut-off frequency 20 Hz), digitized (CED Limited, Cambridge, UK) and stored on a computer.

Three infrared emitters (maximum output 600 mW, dispersion angle 7°, peak wavelength 880 nm) illuminated the eye during the recording. The camera and two infrared emitters were fixed to the turntable. The third infrared emitter was connected to the camera and aligned horizontally with the camera's optical axis. This third emitter produced the tracked corneal reflection (CR).

The eye movements were recorded using the eye-tracking device of Chronos Vision. The images of the eye were captured using an infrared sensitive CMOS camera (frame rate 50 Hz) and were relayed to a personal computer equipped with acquisition software from Chronos Vision (IRIS).

Behavioral testing

A head-fixed coordinate frame was defined as follows: the yaw (z) axis was the ventro-dorsal axis, the roll (x) axis was naso-occipital and the pitch (y) axis was interaural. Four different approaches were used to test the eye movement performance. First, the eye movement counterroll performances were measured during different static horizontal roll stimuli. Mice in upright stance (naso-occipital axis along an earth-horizontal plane) were positioned at different roll angles between ± 20°. The mice were rotated very slowly (5°/s) around their naso-occipital axis from one to another position. All tilt positions of the mouse were held at least for 20 sec or until the eye position was stable. Second, the optokinetic eye movements (OKR), the angular vestibulo-ocular eye movements (aVOR) and the angular visually enhanced vestibulo-ocular eye movements (aVVOR) were measured during different paradigms. The amplitude was kept at 5° while the frequency of the sinusoidal stimulus ranged from 0.2 to 1 Hz (generating a peak velocity between 6 deg/sec and 31 deg/sec, and a peak acceleration between 8 deg/sec^2 and 197 deg/sec^2) during the following paradigms:

1. Dynamic horizontal yaw (Yh): mice upright (naso-occipital axis along the earth-horizontal plane), rotation around ventro-dorsal axis,
2. Dynamic vertical roll (Rv): mice nose up (naso-occipital axis along the earth-vertical plane), rotation around naso-occipital axis,

3. Dynamic horizontal roll (Rh): mice upright (naso-occipital axis along the earth-horizontal plane), rotation around naso-occipital axis.

Third, aVOR was tested at constant peak velocities (8 deg/sec and 30 deg/sec) while the frequency varied between 0.1 and 1.6 Hz. Fourth, aVOR was tested at constant peak acceleration (18 deg/sec^2) while the frequency varied between 0.1 and 1.6 Hz. The constant peak velocity and acceleration were tested using the dynamic horizontal yaw paradigm (Yh).

Each paradigm was presented to the mice at least for three days, but not all the paradigms were delivered on the same day. Each animal was recorded no more than once a day. Before aVOR recordings, pilocarpine 4% (Laboratories Chauvin, France) was used to limit the pupil dilatation in darkness.

Data analysis

A calibration was made before any of the recordings were started. The camera was rotated several times by ± 10° around the earth-vertical axis passing through the center of the table. The positions of the pupil (P) and corneal reflection (CR) recorded at the extreme positions of the camera rotation were used to calculate Rp, the radius of rotation of the pupil (Stahl et al. 2000).

The gain and the phase of the eye movements were calculated by using a custom-made Matlab programme (Mathworks Inc., Natick, USA). The eye position (E) was calculated using the CR and P positions from the recorded file and the Rp value was computed from the calibration (Stahl et al. 2000).

$$E = \arcsin[(CR-P)/Rp]$$

To obtain slow-phase eye velocity the eye-position data (E) was differentiated. The quick phases were identified using a velocity-threshold filter. The trace parts containing the quick phase (0.02 sec before the saccades and 0.08 sec after the saccades) were removed from the eye velocity data. To obtain stimulus velocity, the stimulus (table or drum) trace was also differentiated. Both eye and stimulus velocity signals were filtered by a Butterworth low pass filter with a cut off frequency of 40 Hz, before they were fitted by a sine wave function using a least-squares method. Gain was computed as the ratio of eye velocity to stimulus velocity whereas phase was expressed as the

difference (in degrees) between the eye velocity and stimulus velocity traces. In the static roll paradigm, gain was computed as a ratio of eye position (degrees) to head position (degrees), whereas the sensitivity was computed as a ratio of eye position (degrees) to the sine of the head roll angle, which is equal with the linear acceleration along the interaural axis (head y axis) in unit of g = 981 cm/s^2 (Maruta et al. 2001).

Statistics

To compute the session average, gain and phase values were combined per trial. Session averages from at least three days were used to calculate the final gain and phase value per mouse. Data were presented as mean ± SD. For statistical comparisons we used the two-way ANOVA for repeated measures and the standard *t*-test. Statistical analysis was performed using the commercial software package SPSS 11.0 (SPSS Inc.).

Results

a. Otolithic function during static roll

Tilted mice lack otoconia in both otolith organs (Ornitz et al. 1998). To test the function of the otolith organs, mice were subjected to static horizontal roll. Mice were rotated very slowly (5°/s) towards the end-point roll angle where they were held at least for 20 sec or until the eye position was stable. Head roll angles around an earth horizontal axis varied between ± 20° generating a projection of the gravity vector along the interaural axis ranging from -0.34 g to +0.34 g. Sensitivity and gain of the eye counterroll in *tilted* mice were 3 ± 3°/g and 0.06 ± 0.005 (n = 7), respectively (Figure 1A-B). Both values were significantly lower than those in control mice (n = 7; sensitivity 26 ± 4°/g, $p < 0.001$, *t*-test; gain 0.45 ± 0.07, $p < 0.001$, *t*-test; Figure 1A-B). In control mice, but not in *tilted* mice, there was a linear relationship ($r^2 = 0.79$) between eye position and linear acceleration along the interaural axis. *Tilted* mice did not show any relationship between the eye position and the head roll angle ($r^2 = 0.002$). Together, these data indicate that the static contribution of the otoliths to compensatory eye movements is negligible in otoconia-deficient mice.

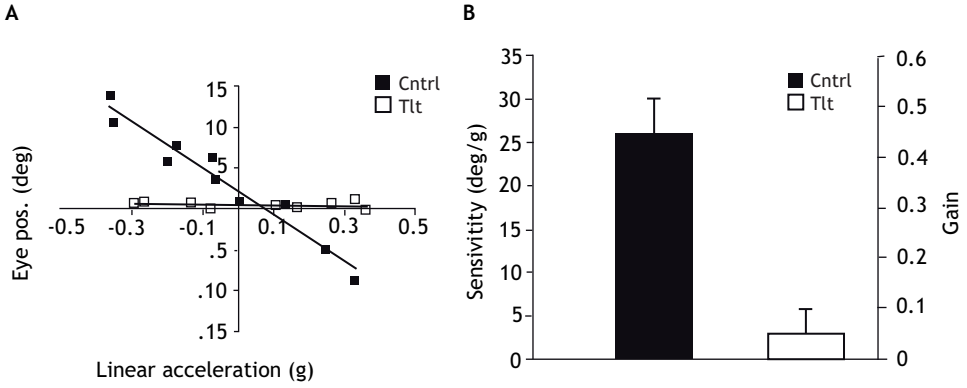

Figure 1. Static contribution of otolith organs to compensatory eye movement in normal and tilted mice. *In A), one example is given for how the sensitivity was calculated: the eye position was plotted against the sine of the roll angle (the linear acceleration along the interaural axis) for a control mouse (filled squares) and a tilted mouse (open squares). B) Eye positions recorded during different static roll angles showed smaller sensitivity and gain values in tilted mice. Data present the mean + SD.*

b. Contribution of otoliths to the VOR

If the static contribution of otoliths to the eye position is affected in the mutants, one expects that the dynamic contribution of otoliths to the VOR is also affected. We therefore subjected control and *tilted* mice to horizontal roll (Rh), which activates conjunctively the vertical semicircular canals and otolith organs (Figure 2A). The vertical roll (Rv; Figure 2B) and horizontal yaw (Yh; Figure 2C) paradigms were used to dynamically stimulate the vertical semicircular canals or the horizontal semicircular canals, respectively. An eye movement recording from each stimulus paradigm is shown in Figure 2.

In control mice (n = 8) the gains of the horizontal roll (Rh) aVOR varied from 0.38 ± 0.11 at 0.2 Hz to 0.59 ± 0.1 at 1.0 Hz, while their phase leads were relatively fixed around 10 degrees at all frequencies (Figure 3A). *Tilted* mice (n = 7) had significantly lower gains (varying from 0.14 ± 0.08 at 0.2 Hz to 0.44 ± 0.12 at 1 Hz; $p < 0.005$, ANOVA) and significantly higher phase leads (varying from 93.00 ± 19.8° at 0.2 Hz to 33.6 ± 4.9° at 1 Hz; $p < 0.001$, ANOVA) at all frequencies. Both the gain and phase differences between mutants and control mice decreased as the frequency increased. The finding that the eye movement performance during horizontal roll is severely affected in *tilted* mice does not necessarily mean that the eye movement performance during vertical roll and

horizontal yaw stimuli are also impaired, because these are more selectively driven by the vertical and horizontal semicircular canals, respectively. On the other hand, these types of reflexes may also be impaired in *tilted* mice, if otolith information is needed for the central integration process preceding these compensatory eye movements. We therefore investigated the aVOR during vertical roll and horizontal yaw. Although the differences between mutants (n = 9) and control mice (n = 7) were less prominent than during horizontal roll, vertical roll paradigm showed lower gains and higher phase leads in *tilted* mice (Figure 3B; for gain and phase values $p = 0.08$ and $p < 0.01$ (ANOVA), respectively).

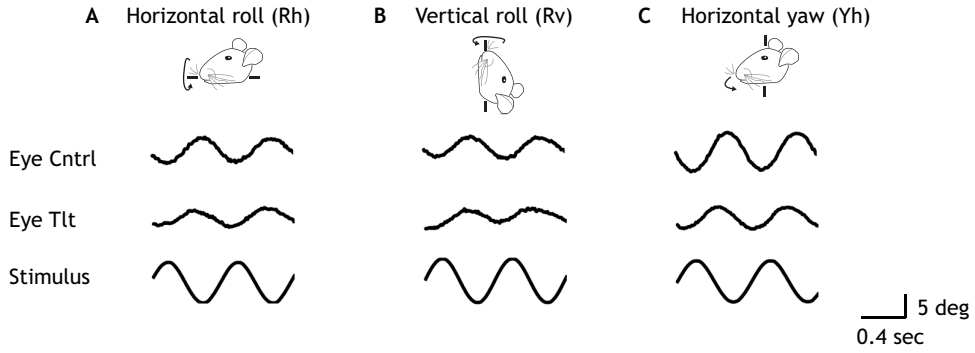

Figure 2. Example of compensatory eye movements during sinusoidal rotation in dark at 0.6 Hz and 5 degree. *Vertical eye movements measured during horizontal roll aVOR (Rh; A) and vertical roll aVOR (Rv; B), and horizontal eye movements during yaw aVOR (Yh; C) are shown. Inset: 5 degree amplitude (vertical) and 0.4 sec (horizontal).*

The otolith input contribution to the aVOR can be determined by subtracting the vertical roll from horizontal roll eye responses in control mice or by subtracting the horizontal roll responses in *tilted* mice from the horizontal roll responses in control mice. Figure 3C shows that these two subtractions do not lead to the same outcome in terms of gain or phase. It appears unlikely that this difference is due to some interaction between the horizontal and vertical canals, because subtraction of the eye movement performance during vertical roll in *tilted* mice from that during horizontal roll in *tilted* mice renders an outcome of approximately zero (Figure 3C). Thus, it is likely that a static-otolith driven component combined with a dynamic-otolith-canals driven component lead to higher gains and lower phases in control mice during horizontal roll aVOR as compare to *tilted* mice during vertical roll aVOR and horizontal roll aVOR.

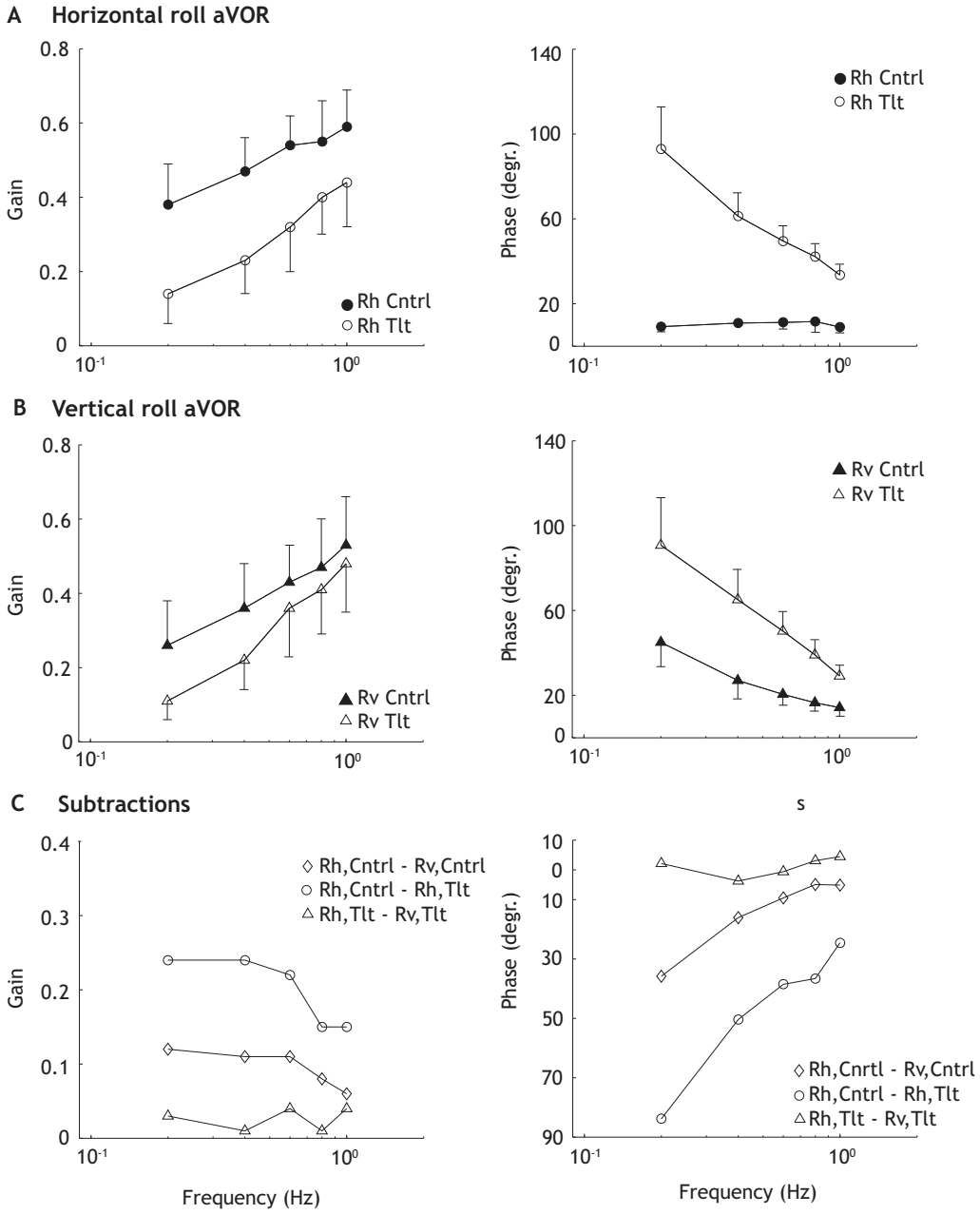

Figure 3. Tilted mice show deficits during roll aVOR. *A) During horizontal roll at which both the semicircular canals and otoliths are stimulated both gain and phase values of tilted mice (open circles) are severely affected. B) During vertical roll at which only the vertical canals are directly stimulated both gain and phase values of tilted mice (open triangles) are moderately affected. C)*

Subtractions of the vertical roll from horizontal roll eye responses in control mice (diamonds) and tilted mice (triangles), as well as subtractions of the horizontal roll eye responses in tilted mice from horizontal roll eye responses in control mice (circles) are shown. Dynamic stimulation of otolith organs increases the gain values and decreases the phase leads of the aVOR in a frequency dependent manner (diamonds) less than the combined dynamic-static stimulation of the otolith organs (circles). In tilted mice there is no difference between canals only and canals-otolith mediated aVOR (triangles). Rh and Rv indicate horizontal roll and vertical roll, respectively. Data present the mean + or - SD.

As the aVORs over the studied frequency range depend on peak acceleration (van Alphen et al. 2001), we tested the horizontal yaw aVOR not only during constant amplitude (5 degrees) but also during constant peak velocity and constant peak acceleration paradigms. In control mice (n = 8) the gains of the horizontal yaw (Yh) aVOR at constant amplitude stimulation varied from 0.43 ± 0.11 at 0.2 Hz to 0.77 ± 0.11 at 1 Hz, whereas their phase leads decreased from 27.9 ± 10.2° at 0.2 Hz to 6.1 ± 2.6° at 1 Hz (Figure 4A). *Tilted* mice (n = 7) had significantly lower gains (varying from 0.15 ± 0.04 at 0.2 Hz to 0.44 ± 0.07 at 1 Hz; $p < 0.005$, ANOVA) and significantly higher phase leads (varying from 103.6 ± 23.2° at 0.2 Hz to 23.2 ± 4.7° at 1 Hz; $p < 0.001$, ANOVA). When the performance of the horizontal yaw (Yh) aVOR was tested during constant peak velocity (8 deg/sec and 30 deg/sec; Figure 4B) and constant peak acceleration (18 deg/sec^2; Figure 4C), *tilted* mice (n = 5) showed again significantly lower gains and significantly higher phase leads than control mice (for all gain and phase values $p < 0.001$ (ANOVA)). The aVOR gains and phases in control mice were dependent not only on frequency but also on acceleration of the stimulus (Figure 4C), increasing and decreasing, respectively, as the acceleration increased. If in *tilted* mice the aVOR gains and phases had been also dependent on amplitude and/or acceleration of the stimulus, then separate curves should have emerged in figure 4B. In *tilted* mice aVOR gains do not depend on amplitude and/or acceleration at low frequencies (0.1 and 0.2 Hz), but depend only on the frequency of the stimulus, whereas aVOR phases depend only on stimulus frequency over the studied frequency range.

These data indicate that inputs from the otolith organs improve the eye movement performance during aVOR especially at the lower frequencies. In the absence of the otolith input, at low frequencies, the vestibular system of the mouse converts from a system dependent on frequency, peak velocity and peak acceleration of the stimulus to a system primarily dependent on the frequency of the stimulus.

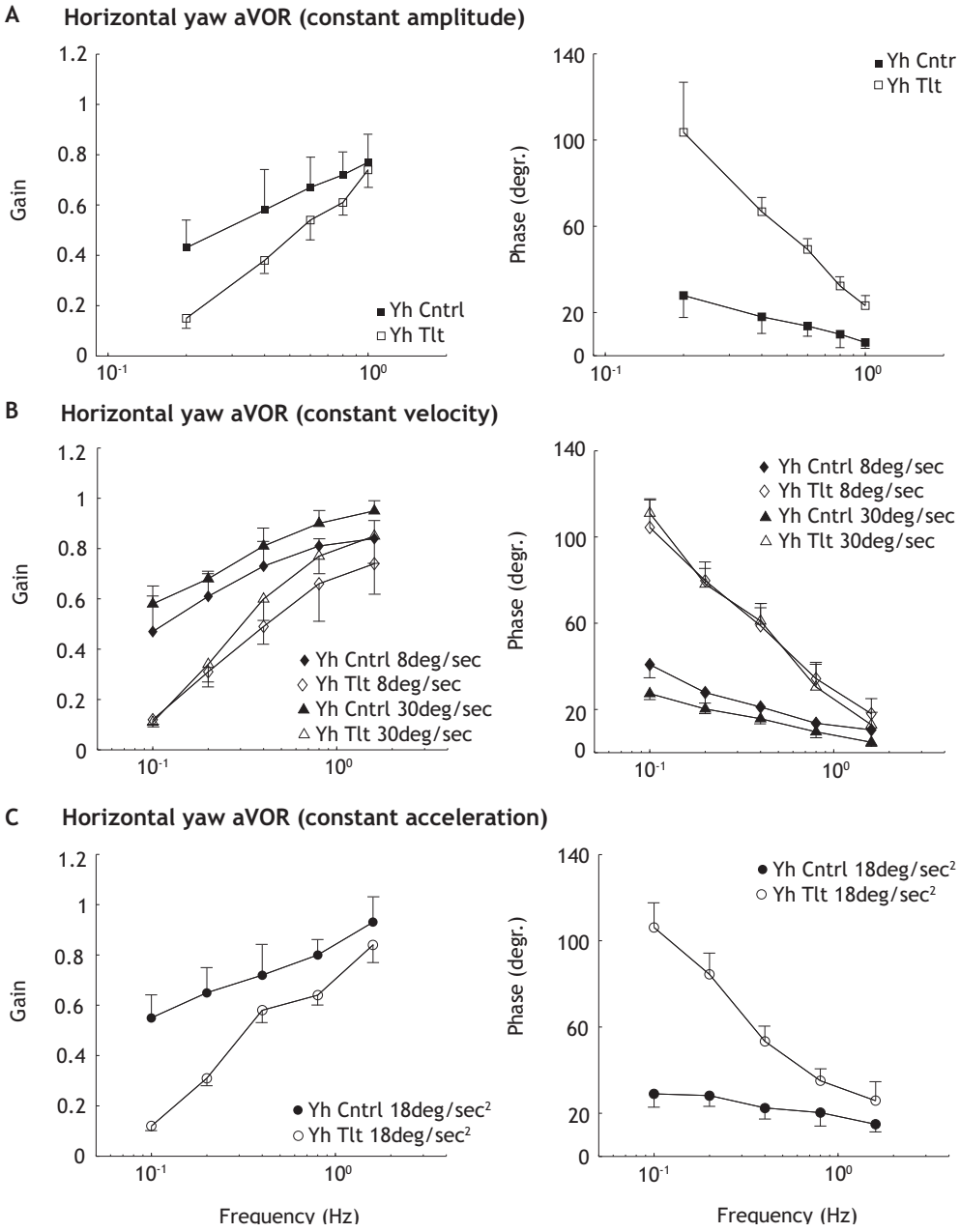

Figure 4. Tilted mice show deficits during yaw aVOR. *During horizontal yaw in which only the horizontal canal is directly stimulated both gain and phase values of tilted mice are moderately affected in all tested conditions: A) constant amplitude (5 degree), B) constant velocities (8 deg/sec and 30 deg/sec) and C) constant acceleration (18 deg/sec/sec). Data present the mean + or - SD.*

c. OKR compensation

The data described above showed that the otolith organs can improve both via static and dynamic mechanisms the eye movement performance during aVOR around different axes in space. This contribution is most prominent at lower frequencies. These findings raise the question whether the deficits that occur in otoconia-deficient mice during aVOR can be compensated by a secondary enhanced OKR, which can particularly dominate oculomotor performance at the lower frequency range (Collewijn and Grootendorst 1979). We therefore tested the OKR under the same set of body orientations and frequencies that were used for the experiments described above. For the vertical eye movement OKR, i.e. that of the horizontal roll and vertical roll, the gain values of the OKR of *tilted* mice (n = 11) were significantly higher than those of control mice (n = 10) at the two lowest frequencies of 0.2 Hz and 0.4 Hz which correspond to velocities 6 deg/sec and 8 deg/sec but not at the higher frequencies (Figure 5A-B). In control mice (n = 8) the gains of the horizontal yaw OKR varied from 0.69 ± 0.08 at 0.2 Hz to 0.15 ± 0.04 at 1 Hz whereas *tilted* mice (n = 7) had significantly higher gains (varying from 0.80 ± 0.05 at 0.2 Hz to 0.26 ± 0.08 at 1 Hz; data not shown). The significance levels varied from $p < 0.05$ in vertical roll position to $p < 0.001$ in yaw position (ANOVA). In contrast, no significant differences were observed in the phase values of the OKR among the mutants and controls (p-levels varied from 0.54 in horizontal roll to 0.98 in vertical roll; ANOVA). The subtraction of the OKR gain values of control mice in horizontal roll position from those of *tilted* mice in the same position did not differ from the same subtraction in vertical roll position (Figure 5D). Thus, the position of the mouse does not influence the gains of the vertical eye movement OKR. Although the optokinetic compensation was significant and robust in all body positions in *tilted* mice, it was not sufficient to obtain a normal gain of the VVOR (Table I). For example, the VVOR gains during yaw movements were significantly higher in control mice (n = 9; 0.88 ± 0.06) than in *tilted* mice (n = 8; 0.80 ± 0.06); ($p < 0.01$, ANOVA).

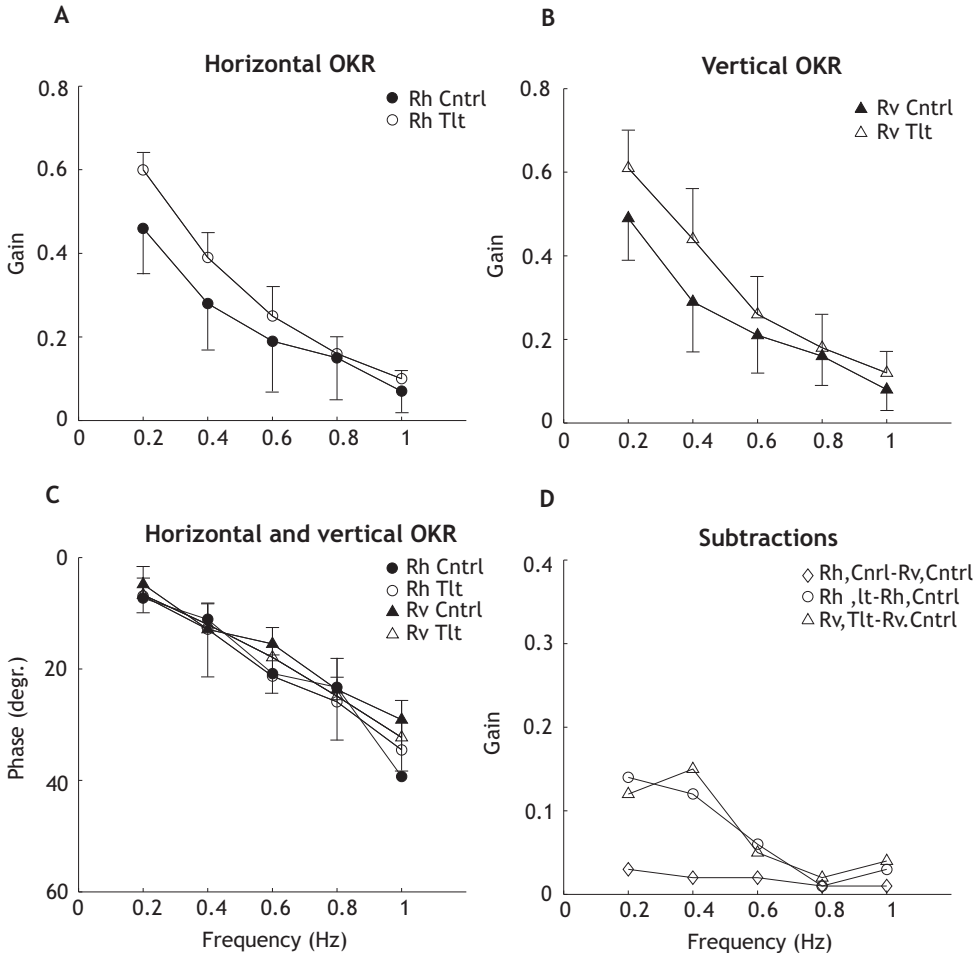

Figure 5. Low-frequency OKR compensation due to otolith dysfunction. *A-C) Tilted mice show higher gains during horizontal roll OKR (A) and vertical roll OKR (B) at the lower frequencies, while their phase values are normal at all frequencies (C). D) The position of the mouse does not influence the compensatory OKR gains (circles and triangles). Rh and Rv indicate horizontal roll and vertical roll, respectively. Data present the mean + or - SD.*

***Table I** A) Angular visually enhanced vestibulo-ocular reflexes induced by three different vestibular stimuli in wild type and tilted mice (gains).*

Paradigm	Mice	0.2 Hz	0.4 Hz	0.6 Hz	0.8 Hz	1 Hz
Rh	**Wt** (n = 8)	0.78 ± 0.11	0.82 ± 0.11	0.82 ± 0.10	0.83 ± 0.13	0.82 ± 0.13
	Tlt (n = 10)	0.59 ± 0.06	0.57 ± 0.04	0.58 ± 0.05	0.63 ± 0.07	0.67 ± 0.06
Rv	**Wt** (n = 12)	0.70 ± 0.13	0.68 ± 0.14	0.65 ± 0.14	0.73 ± 0.15	0.72 ± 0.13
	Tlt (n = 7)	0.65 ± 0.08	0.62 ± 0.08	0.63 ± 0.08	0.67 ± 0.11	0.71 ± 0.09
Yh	**Wt** (n = 9)	0.85 ± 0.06	0.90 ± 0.05	0.89 ± 0.05	0.88 ± 0.06	0.88 ± 0.08
	Tlt (n = 8)	0.84 ± 0.05	0.76 ± 0.09	0.78 ± 0.04	0.81 ± 0.06	0.84 ± 0.05

Table I B) Angular visually enhanced vestibulo-ocular reflexes induced by three different vestibular stimuli in wild type and tilted mice (phases).

Paradigm	Mice	0.2 Hz	0.4 Hz	0.6 Hz	0.8 Hz	1 Hz
Rh	**Wt** (n = 8)	0.4 ± 2.8	-0.8 ± 2.4	-0.7 ± 2.9	-1.4 ± 3.0	-0.5 ± 1.5
	Tlt (n = 10)	-1.7 ± 4.4	4.2 ± 4.5	8.6 ± 5.4	5.2 ± 4.0	7.3 ± 5.0
Rv	**Wt** (n = 12)	3.9 ± 2.4	1.5 ± 2.3	0.8 ± 2.6	2.2 ± 2.2	2.7 ± 5.3
	Tlt (n = 7)	-0.7 ± 4.2	1.0 ± 4.9	3.8 ± 7.2	7.1 ± 4.7	4.8 ± 5.8
Yh	**Wt** (n = 9)	0.4 ± 1.8	0.3 ± 1.0	-0.1 ± 2.6	0.1 ± 1.3	0.7 ± 1.5
	Tlt (n = 8)	-1.1 ± 1.9	0.5 ± 3.7	3.7 ± 2.3	1.6 ± 5.1	3.5 ± 4.1

Table I. Data are presented as mean ± SD. Gain values (eye velocity / stimulus velocity) and phase values (eye velocity − stimulus velocity in degrees) of the angular visually enhanced vestibulo-ocular reflex (aVVOR) at stimulus frequencies ranging from 0.2 to 1 Hz during horizontal roll (Rh), vertical roll (Rv) and horizontal yaw (Yh).

Discussion

Our data show 1) that absence of otoconia leads to dysfunctional otolith organs impairing both the static contribution to the vestibulo-ocular counterroll and the static and dynamic contribution to the angular vestibulo-ocular reflex; 2) that the deficits occur most prominently at the lower frequencies of the aVOR; 3) that the absence of functional otolith organs results in greater frequency dependence of the aVOR and 4) that in light these deficits are to a large extent compensated by an enhanced optokinetic response. In conjunction, they provide supportive evidence for an adaptive multisensory integration system for stabilizing the retinal image.

VOR deficits

The lack of otoconia in *tilted* mice resulted in dysfunctional otolith organs that were virtually unable to evoke correct eye movement responses following static or dynamic displacement of the head. With regard to the static stimuli, we found that the gain and sensitivity of their eye-counterrolls were approximately 10% of those in control mice littermates. The residual counterroll eye movements in *tilted* mice might be driven by inputs from extracervical somato-sensory receptors (Krejcova 1971; Yates et al. 2000) or by inputs from giant otoconia that are sometimes present in *tilted* mice (Ornitz et al. 1998). The sensitivity in control mice (26°/g) was in between that of rabbits (17°/g) (Maruta et al. 2001) and fish (30°/g) (Benjamins 1918; Cohen et al. 2001). With regard to the dynamic stimuli in *tilted* mice, we found the most prominent aberrations during horizontal roll, indicating that this paradigm evokes a relatively high activity in the otolith organs. Interestingly, comparison between control mice and *tilted* mice also revealed deficits in eye movement performance following horizontal yaw and vertical roll stimulation even though these paradigms are thought to evoke relatively little dynamic activity in the otolith organs (see also Harrod and Baker 2003). Moreover, we showed that subtraction of the eye movement performance during horizontal roll in the mutants from that during horizontal roll in control mice is not equal to the difference between eye movement performance during vertical roll in control mice and that during horizontal roll in control mice (Figure 3C). Altogether, these results suggest the presence of an otolith component during both horizontal yaw and vertical roll stimulations and show that by placing control mice in the vertical position, the contribution of the otolith organs to aVOR is not completely removed. The most obvious explanation of these unexpected otolith contributions in this situation is that the otolith organs are statically stimulated and that this static otolith signal is partially able to correct the "vertical semicircular canals aVOR" in these control mice (Figure 3B). The presence of a static otolith signal during rotation of mice in the plane of the horizontal canals will also explain the eye movement aberration found in *tilted* mice during horizontal yaw stimulations. An alternative explanation is that otolith organs were not precisely placed in the centre of rotation during stimulation. Consequently, a dynamic stimulation of the otolith organs was induced that would be large enough to contribute substantially to the aVOR. This possiblity is very unlikely, because the tangential and centripetal acceleration are under these circumstances too low (tangential acceleration = 0.002g; centripetal acceleration = 0.0002g) to elicit a response (Clarke and Engelhorn 1998).

The tilt angle of the rotation axis with respect to gravity elicits the otolith responses, therefore the facts that the macular surface of the otolith organs is curved, not planar (Flock 1964) and that otolith organs do not lie in the plane of the semicircular canals (Curthoys et al. 1999) are also no plausible explanations for our results. Nevertheless, it remains unclear whether the mechanism suggested, is entirely responsible for this large otolith-dependent aVOR component. The possibility that otolithic deprivation in *tilted* mice altered the neuronal activity of utricular afferents still needs to be elucidated. In order to unravel this mechanism, electrophysiological measurements of either utricular afferents or vestibular nuclei neurons are necessary.

The similarities in eye movement responses evoked by horizontal linear acceleration and off-vertical axis rotation in rat led to the conclusion that utricular driven eye movement in rodents complements the semicircular canal activations in order to achieve gaze stability during horizontal roll stimulation (Hess and Dieringer 1990, 1991). The horizontal roll aVOR can be explained by the fact that signals derived from otolith organs and semicircular canals converge at the level of vestibular neurons, which send their eye movement commands to the oculomotor nuclei (Sato et al. 2000; Zhang et al. 2001; Dickman and Angelaki 2002; Zhang et al. 2002). The missing static otolith correction in the *tilted* mice and the convergence of the otolith and canal driven signals might also explain our finding that the aberrations in aVOR of *tilted* mice were frequency-dependent not only during horizontal roll, but also during vertical roll and horizontal yaw. This is the first study that shows that in the absence of otolith input, the dependency of the vestibular system on the frequency of the stimulus is increased. Taken together our aVOR data support the multisensory integration theory in that the brain must combine information from semicircular canals and otolith organs in order to make proper compensatory eye movements (Harrod and Baker 2003; Angelaki et al. 2004).

OKR compensation

We found that OKR gain values of *tilted* mice were significantly increased. Several findings support the argument that this increase reflects a mechanism that will compensate for deficits in the aVOR. First, the increases in OKR gain occurred in every position at which deficits in the aVOR were detected, i.e. that of the horizontal roll, horizontal yaw and vertical roll. Second, the increases in OKR gain occurred predominantly at the lower frequencies, which corresponds to the frequency range at which the aVOR gain

values were most prominently affected. Furthermore, the VVOR gain values were not increased in *tilted* mice, suggesting that the OKR increase was not a primary effect but a secondary effect in an attempt to correct the VVOR gain values that were partly reduced.

The mechanism that underlies OKR compensation in *tilted* mice probably resembles that underlying OKR and VOR adaptation following visuo-vestibular or visual training paradigms (Collewijn and Grootendorst 1979; Nagao 1983; Iwashita et al. 2001). During these adaptations OKR or VOR gain values change in response to enhanced retinal slip. While a change in VOR gain depends on the direction of retinal slip in relation to the direction of the eye movement, the OKR gain always increases when there is enhanced retinal slip, independent from the direction of the slip (Collewijn and Grootendorst 1979; De Zeeuw et al. 1998). In *tilted* mice the aVOR gains are reduced due to dysfunctional otoliths, which in turn increase the retinal slip triggering a compensatory change in the OKR. Similarly, the low-frequency aVOR can be enhanced as a mechanism to compensate for a decrease in OKR gain; this reversed process occurs in lurcher mice, which suffer from reduced OKR gain values due to a lack of floccular Purkinje cells (Van Alphen et al. 2002). Even so, it should be noted that a total blockage of the VOR such as occurs in shaker mutants, Usher Syndrome Type 1B patients or subjects after bilateral labyrinthectomy does not necessarily result in increased OKR gains (Cohen et al. 1973; Barmack et al. 1980; Sun et al. 2001). In these cases the vestibular deficits fall too much in the high frequency range and/or the increased retinal slip levels fall outside the optimal range that can drive optokinetic signals mediating adaptation in the flocculus of the cerebellum (Simpson et al. 1996). Thus, the optokinetic system may be particularly suited to compensate for the lack of otolith-driven information necessary for a proper aVOR as both systems have similar low pass filter characteristics, while it may not be well designed to compensate for deficits in the vestibular-canal system, which dominates the higher frequencies. The cerebellar cortex might be a suitable site for this OKR compensatory mechanism.

In conclusion, by analysing mutants with specific deficits in their otoliths we provide evidence that the otolithic input shows central cross-talk with the input of the semicircular canals and that the otolith organs provide indispensable information for the angular vestibulo-ocular reflex. The lack of otolith input increases the dependency of the vestibular system on the stimulus frequency. The optokinetic reflex can compensate for the lack of gravito-inertial perception in the low frequency range. All

these phenomena support the presence of an adaptive multisensory integration system that combines information from otolith organs, semicircular canals, and retina in order to make proper compensatory eye movements.

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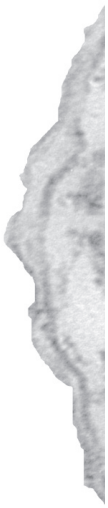

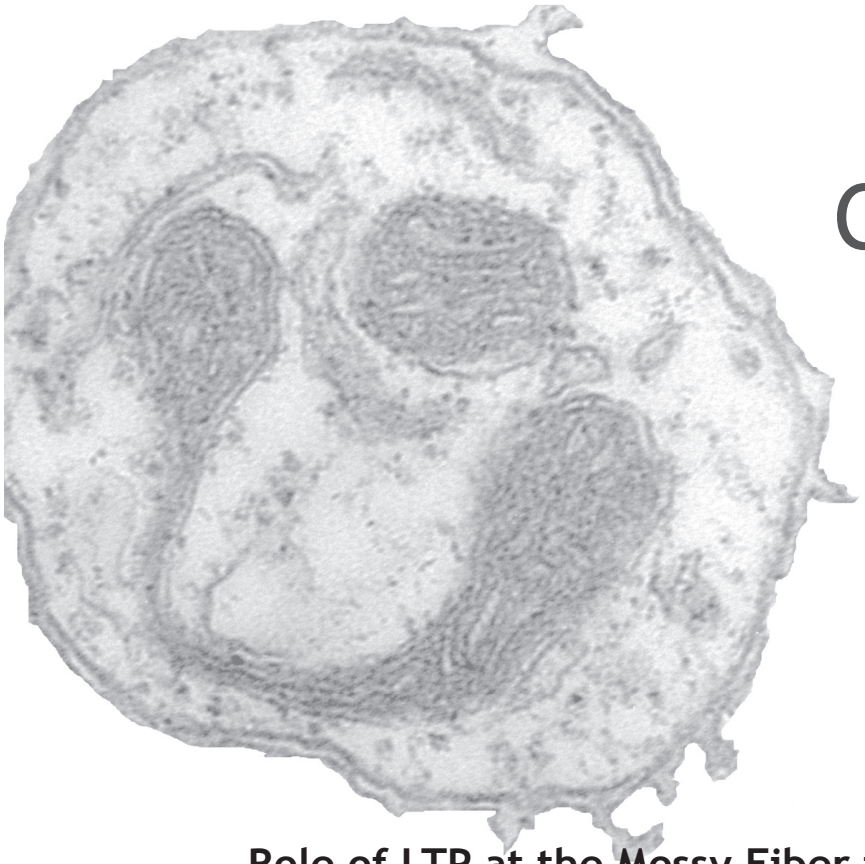

Chapter

3

Role of LTP at the Mossy Fiber to Granule Cell Synapse in Cerebellar Motor Learning

Corina E. Andreescu[1], Anna D'Errico[2], Marcel T.G. De Jeu[1], Paola Rossi[2], Laura Botta[2], Georg Kohl[3], Paola Perin[2], Egidio D'Angelo[2], Chris I. De Zeeuw[1,4]

[1]Department of Neuroscience, Erasmus MC, Rotterdam, The Netherlands
[2]Dept. of Cellular and Molecular Physiological and Pharmacological Sciences, University of Pavia and CNISM, Pavia, Italy
[3]Department of Molecular Neurobiology, Max Plank Institute for Medical Research, Heidelberg, Germany
[4]Netherlands Institute for Neuroscience, Royal Academy of Arts & Sciences (KNAW), Amsterdam, The Netherlands

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$^{-/-}$) have impaired induction of postsynaptic long-term potentiation (LTP) at their mossy fiber terminals and a reduced ability to increase the intrinsic excitability of their granule cells, while the basic excitatory output of their mossy fibers is unaffected. In addition, we demonstrate that these mutants have deficits in the adaptation of their vestibulo-ocular reflex (VOR), while their basic eye movement performance is similar to that of wild type littermates. These results demonstrate that NMDA-NR2A mediated potentiation at the mossy fiber to granule cell synapse can contribute to cerebellar memory formation.

Introduction

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; Rossi et al. 2002; Maffei et al. 2003; 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 that 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 (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). In effect, the NMDA receptors in granule cells control high-frequency repetitive neurotransmission by enhancing and protracting membrane depolarization during 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; Rossi et al. 2002; Maffei et al. 2003; 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; D'Angelo et al. 1993, 1994; D'Angelo et al. 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 NMDA-NR2A receptor ($NR2A^{-/-}$) (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 (100 Hz for 1 sec), 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) and a visuo-vestibular training (Stahl et al. 2000; Andreescu et al. 2005).

Materials and Methods

Subjects

In this study, we used 6 NMDA-$NR2A^{-/-}$ mutant mice and 5 of their wild type littermates for electrophysiological recordings and 8 NMDA-$NR2A^{-/-}$ mutant mice and 9 of their wild type littermates for behavioural studies (Sprengel et al. 1998). In addition, we employed here 7 NMDA-$NR2A^{\Delta C/\Delta C}$ mutant mice and 10 of their wild type littermates (Sprengel et al. 1998). All mice used were males. Mice were housed on a 12 hours 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 2µl DNA using specific primers for $NR2A^{-/-}$ mice (Primer PGK Prom2: 5'-CAGACTGCCTTGGGAAAAGCG

- 3'; Primer 2AIN10N*do: 5' – GGGAATTCGCGGCCGCAAGAGCAAGAAGACTCC – 3'; Primer 2AIN11x*up: 5' - GGAGGTACCTCGAGCTCTTCTACAG – 3'). Same technique was used for NR2A$^{\Delta C/\Delta C}$ mice (Primer rsp26: 5'-AGAAGCTAATGTACCTGAGG-3'; Primer rsp25: 5'-ATCTGCCAGACACTGCTCCAG-3'). An initial denaturation of 3 min at 96ºC was followed by 20 sec at 96ºC, 30 sec at 55ºC and 75 sec at 72ºC for 35 cycles. A final extension of 10 min at 72ºC 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$^{+/+}$ and 580 bp for NR2A$^{-/-}$ (Figure 1).

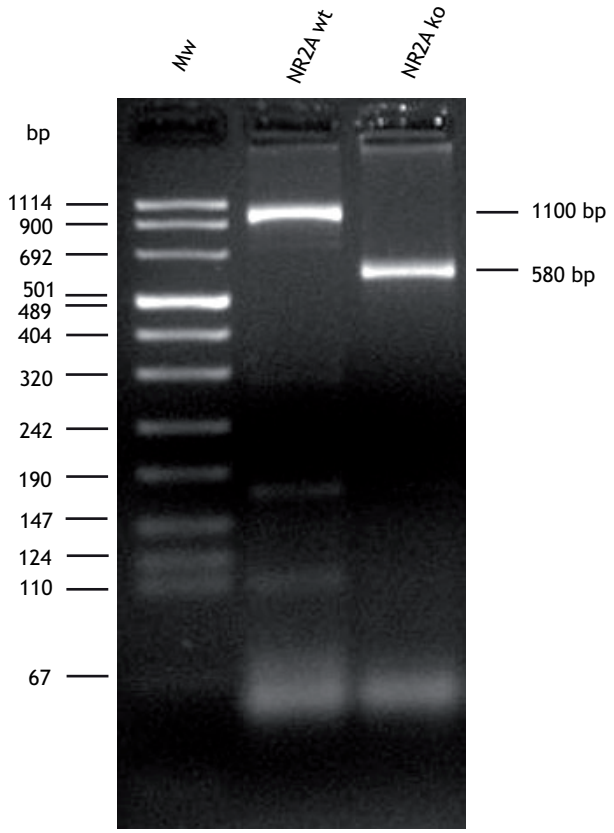

Figure 1. PCR analysis of NR2A receptor in the NR2A$^{-/-}$ and wild type mice. *Gel electrophoresis of PCR products taken from wild type mice (wt) and NR2A$^{-/-}$ mice (ko). The 1100 and 580 bp bands are indicated at the right of the panel, and correspond to the wild type and knockout PCR products,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

Electrophysiological recordings

Whole-cell patch-clamp recording were performed as previously reported (D'Angelo et al. 1995; D'Angelo et al. 1999; Armano et al. 2000). Longitudinal slices (220 µm thick) of the cerebellar flocculus and nodulus were prepared from mice 18 to 23 days old using a Vibratome (Dosaka, Kyoto, Japan) and cold krebs solution containing 120 mM NaCl, 2 mM KCl, 2 mM CaCl2, 1.9 mM MgSO4, 1.18 mM NaH2PO4, 26 mM NaHCO3, and 10 mM glucose (pH 7.4, equilibrated with 95% O2 and 5% CO2). After slicing, the slices were incubated for at least 1h at room temperature (20 – 23ºC) in oxygenated krebs solution. Whole-cell patch-clamp recording 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 stricnine (Sigma) to block $GABA_A$ 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 $MgSO_4$, 0.1 mM BAPTA-free, 0.5 mM BAPTA-Ca^{2+}, 3 mM Mg^{2+} -ATP, and 0.1 mM Na^+ -GTP (pH 7.2 adjusted with KOH). This solution maintained resting free [Ca^{2+}] at 100 nM.

Recordings were made with an Axopatch 200B amplifier (Molecular Devices, Union City, CA) at 32ºC (D'Angelo et al. 1995, 1997; D'Angelo and Rossi, 1998; D'Angelo et al. 2001). All recordings were made at a cutoff frequency of 10 kHz and subsequently digitized at 20 kHz using Clampex 8 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 µs pulses at a basal frequency of 0.33 Hz. According to previous measurements (Sola et al. 2004), we stimulated between 1 and 2 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 steps of current with 2 to 28 pA in 2 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 sec. 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 12 to 20 weeks old. Three days before the behavioral testing, a prefabricated piece equipped with two nuts was cemented to the skull in order to fixate the mouse's head in a restrainer device. The surgical procedures were performed under general anaesthesia using a mixture of isofluoran (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 (Stahl et al. 2000).

Angular optokinetic reflex (OKR), angular vestibulo-ocular reflex (VOR) and visually enhanced angular vestibulo-ocular reflex (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 5º rendering a velocity of 6.3 – 31,4 deg/sec). Before measuring the VOR pilocarpine 4% (Laboratories 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 et al. 2002).

VOR learning was evoked by using 5 x 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 x 10 min of sinusoidal vestibular and visual stimuli that were rotating exactly in phase (table and drum ± 5°; 0.6 Hz); on the second day VOR phase reverse was induced by subjecting the animals to 5 x 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, ± 5°; drum 0.6 Hz, ± 7.5°); and on the third day, VOR phase reverse was completed by subjecting the animals also to 5 x 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, ± 5°; drum 0.6 Hz, ± 10°). VOR was measured every day before the training and after each 10 minutes of training (± 5°, 0.6 Hz). Mice were kept in complete darkness between the measurements done on day 1 and day 2, and between the measurements done on day 2 and day 3.

Statistical Tests

Data are presented as mean ± SEM. For statistical comparisons we used the 2-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$^{-/-}$ 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$^{-/-}$ and wild type mice. EPSPs were elicited before and after tetanization with a high-frequency stimulation train (100 Hz for 1 min). EPSPs elicited by mossy fiber stimulation could either remain sub-threshold or be combined with spikes forming EPSP-spike complexes. 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. No comparable changes could be observed after tetanization in the NR2A$^{-/-}$ granule cells (Figure 2A; $p > 0.2$ in all cases; Student's *t*-test). The frequency of occurrence of EPSP–spike complexes is considered an index of plasticity (Armano et al. 2000). In the wild types the probability of EPSP-dependent firing increased remarkably (from 13.7 ± 6% before to 77.1 ± 16% after tetanization; $n = 6$; $p < 0.006$, paired Student's *t*-test), whereas no comparable change was observed in the NR2A$^{-/-}$ mutants (from 14.7 ± 10.1% before to 29.6 ± 27% after induction; $n = 5$; $p = 0.85$, paired Student's *t*-test). The difference between wild types and NR2A$^{-/-}$ was statistically significant after tetanization ($p < 0.005$, Student's *t*-test). The amplitude of the EPSPs that did not generate spikes after tetanization was 29.6 ± 4.5% in wild types ($n = 4$; $p < 0.08$, paired Student's *t*-test) and 14.8 ± 11.1% in the NR2A$^{-/-}$ mutants ($n = 3$; $p = 0.31$, paired Student's *t*-test; Figure 2). Here, the difference between EPSP amplitude of wild types and NR2A$^{-/-}$ mutants did not reach statistical significance ($p = 0.2$, Student's *t*-test).

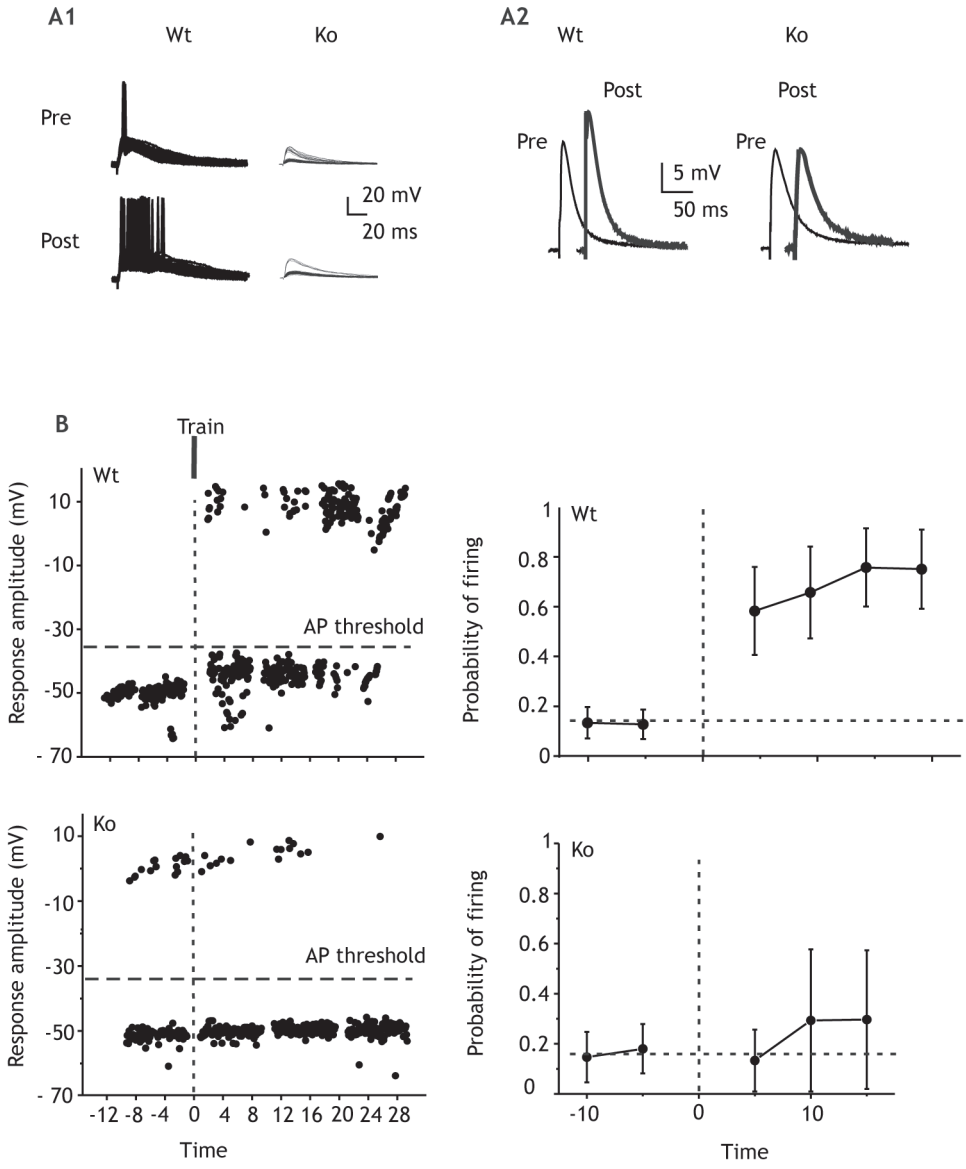

Figure 2. EPSP changes before and after tetanization (100 Hz for 1 min). *Synaptic responses were elicited from -70 mV in wild types (wt) and NR2A$^{-/-}$ mice (ko) before and after stimulation with a high-frequency train. (**A1**) EPSPs and EPSP-spike complexes before and after high-frequency stimulation are shown (several traces are overimposed). Note the marked increase in EPSP-spike complexes in the wild types but not in NR2A$^{-/-}$. (**A2**) An increase in EPSP peak amplitude is present*

*in the wild type cells, whereas no amplitude change is observed in NR2A$^{-/-}$ cells. Only EPSPs remaining subthreshold have been used for the average. (**B**) The plots on the left show the time course of EPSP amplitude in the wild types and NR2A$^{-/-}$ mice during induction experiments. The wild type EPSPs show a marked amplitude increase leading to formation of EPSP-spike complexes after tetanization, whereas no noticeable increase in EPSP or EPSP-spike complexes is observed in the NR2A$^{-/-}$. The plots on the right show the average change in the probability of action potential firing (i.e. formation of EPSP-spike complexes) in wild types and NR2A$^{-/-}$ mice after induction. The induction trains were delivered at time 0 and action potential threshold in the two cells is indicated.*

Usually changes in LTP induction at the mossy fiber to granule cell synapse are associated with an increase in intrinsic excitability (Armano et al. 2000). This possibility was assessed by measuring the average firing frequency in response to step current injection (over the whole response range from 10 pA to 20 pA) and by calculating the difference before and after induction (Figure 3). In wild types firing frequency increased remarkably (89.2 ± 7.5%; n = 4; p < 0.008, paired Student' *t*-tests), whereas no significant increase was observed in the NR2A$^{-/-}$ mutants (4.8 ± 15.6%; n = 3; p = 0.71, paired Student' *t*-tests). We therefore conclude that NR2A is necessary for LTP induction at the mossy fiber to granule cell synapse.

Eye movement performance is not impaired in NR2A$^{-/-}$ mice

NR2A$^{-/-}$ 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$^{-/-}$ mice (n = 8) ranged from 0.20 ± 0.02 to 0.75 ± 0.06 over the tested frequency band (0.2 – 1 Hz), while those of wild type littermates (n = 9) ranged from 0.17 ± 0.02 to 0.75 ± 0.03; these differences were not significant (p > 0.2; 2-way ANOVA; Figure 4A). Following this turntable stimulation at 0.2 Hz to 1 Hz phase leads of NR2A$^{-/-}$ mice ranged from 27.2 ± 6.0 to 14.0 ± 0.9 degrees, while those of their wild type littermates varied from 25.1 ± 8.4 to 10.5 ± 2.6 degrees; these data were also not significant (p > 0.4; 2-way ANOVA; Figure 4B). Similarly, the OKR and VVOR of the NR2A$^{-/-}$ 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; 2-way ANOVA; Figure 4). Thus, we conclude that NR2A$^{-/-}$ 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.

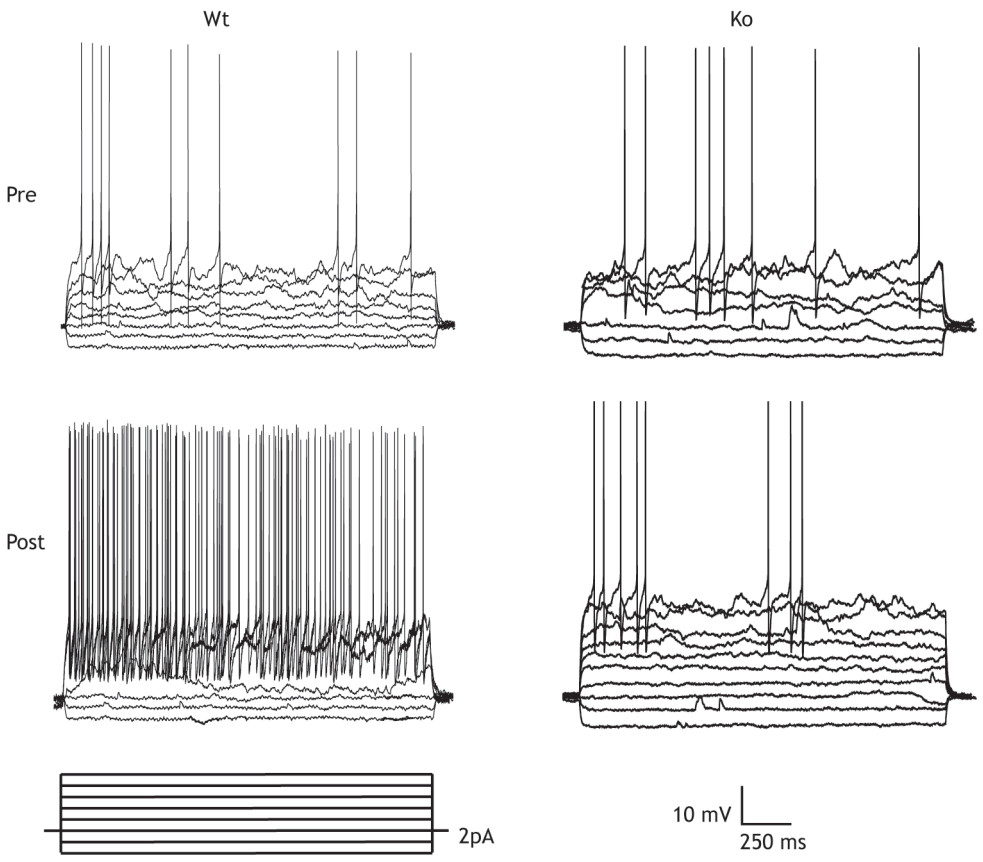

Figure 3. Intrinsic excitability changes following tetanization*. Voltage responses were elicited from -80 mV in wild types (wt) and NR2A$^{-/-}$ mice (ko) before and after stimulation with a high-frequency train. The figure shows responses to 2 pA steps current injection. The wild type cell shows an increase of firing consistent with LTP induction, whereas the NR2A$^{-/-}$ cell shows no remarkable increase.*

Deficits in motor learning in NR2A$^{-/-}$ mutant mice

To find out whether the impairment in LTP induction at the mossy fiber to granule cell synapse might lead to deficits in motor learning, we subjected the NR2A$^{-/-}$ mice 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$^{-/-}$ (n = 8) and control mice (n = 9) ($p > 0.3$, 2-way ANOVA; Fig. 5A). Moreover, when the measurements were resumed after mice spent 24 hours in darkness, the gain in NR2A$^{-/-}$ mice was also similar to that of their wild type littermates indicating that they also don't show any difference in gain consolidation ($p > 0.3$, Student's t-test). Thus, NR2A$^{-/-}$ 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$^{-/-}$ mice showed a significantly smaller phase change than the wild types (in both cases $p < 0.05$; 2-way ANOVA; Table 1; Fig. 5B). Possibly, this difference was 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$^{-/-}$ mice ($p < 0.05$ for both days, Student's t-test; Fig. 5B). Thus, NR2A$^{-/-}$ 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.

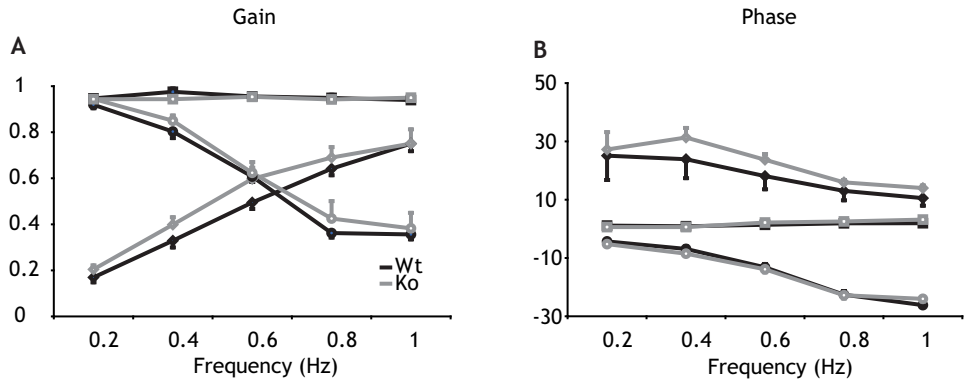

Figure 4. NMDA-NR2 mutant mice show no motor performance deficit. *(**A**) Gain values (= eye velocity/stimulus velocity) of the vestibular-ocular reflex (VOR; circles), of the optokinetic reflex (OKR; diamonds) and of the visually increased VOR (VVOR; squares) at stimulus frequencies ranging from 0.2 to 1 Hz in NMDA-NR2$^{-/-}$ mice (ko) and wild type mice (wt) are presented. (**B**) Phase values (= eye velocity − stimulus velocity in degrees) from NMDA-NR2$^{-/-}$ mice and their wild type littermates are plotted. Empty symbols represent mutant mice while filled symbols represent wild type mice. Data are mean ± SEM.*

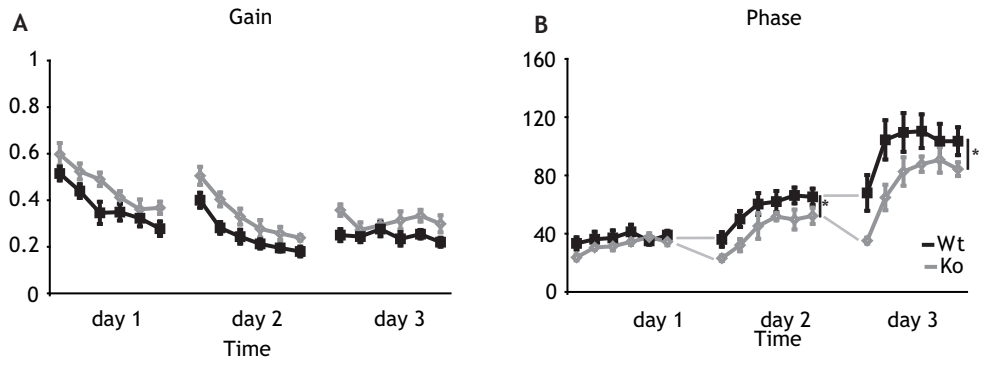

Figure 5. NMDA-NR2$^{-/-}$ mutant mice show motor learning deficit during a three days training paradigm. *On day 1 short-term adaptation was studied using in phase, drum and table stimulation (both at 5°; 0.6 Hz). On days 2 and 3 long-term adaptation, phase reversal and consolidation were studied by 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**) No difference in gain reduction among NMDA-NR2$^{-/-}$ mice (ko) and control mice (wt) during these three days of training were observed. (**B**) NMDA-NR2$^{-/-}$ mice were able to reverse their phase values, that is to move their eyes during the vestibulo-ocular reflex in the same direction as the table instead of in the opposite direction, but not as prominently as wild types. NMDA-NR2$^{-/-}$ 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.*

NR2A$^{\Delta C/\Delta C}$ mice show same phenotype as NR2A$^{-/-}$ mice

Since the C-terminal of NR2A subunits is essential for triggering signal transduction molecules downstream and thereby presumably for synaptic plasticity at the mossy fiber to granule cell synapse (Llansola 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$^{-/-}$ mice might have induced various secondary compensations, which themselves might have caused the learning deficits in phase reversal. We therefore subjected mice with just a truncated C terminal in the NR2A subunit (NR2A$^{\Delta C/\Delta C}$ mice) to the same paradigms as the NR2A$^{-/-}$ mice (Kadotani et al. 1996; Sprengel et al. 1998). The NR2A$^{\Delta C/\Delta C}$ mice showed the same phenotype as the NR2A$^{-/-}$ mice in that they showed no deficits in motor performance or in motor learning during the 1-day gain decrease training, while they showed significant deficits in the subsequent two-day phase reversal learning (Figure 6). All significance levels of the comparisons of the gain and phase VOR, OKR and VVOR motor performance

values between NR2A$^{\Delta C/\Delta C}$ 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 (2-way ANOVA), while the significance level of that for the VOR gain decrease learning paradigm was higher than 0.2 (2-way ANOVA; Table 1). In contrast, the NR2A$^{\Delta C/\Delta C}$ mice showed, just like the NR2A$^{-/-}$ mice described above, significantly smaller phase changes than the wild types on the two days of phase reversal training (phase difference on day 2, $p < 0.02$; and on day 3 $p < 0.03$, 2-way ANOVA; Table 1; Figure 6D). Interestingly, the differences in levels of consolidation overnight between the NR2A$^{\Delta C/\Delta C}$ mice and controls showed the same trend as described above for the NR2A$^{-/-}$ mutants, but these differences were not significant (Figure 6D; $p > 0.1$; Student's *t*-test). These data obtained in the NR2A$^{\Delta C/\Delta C}$ mice suggest that the C-terminal of the NR2A subunit is necessary for a normal level of phase reversal learning.

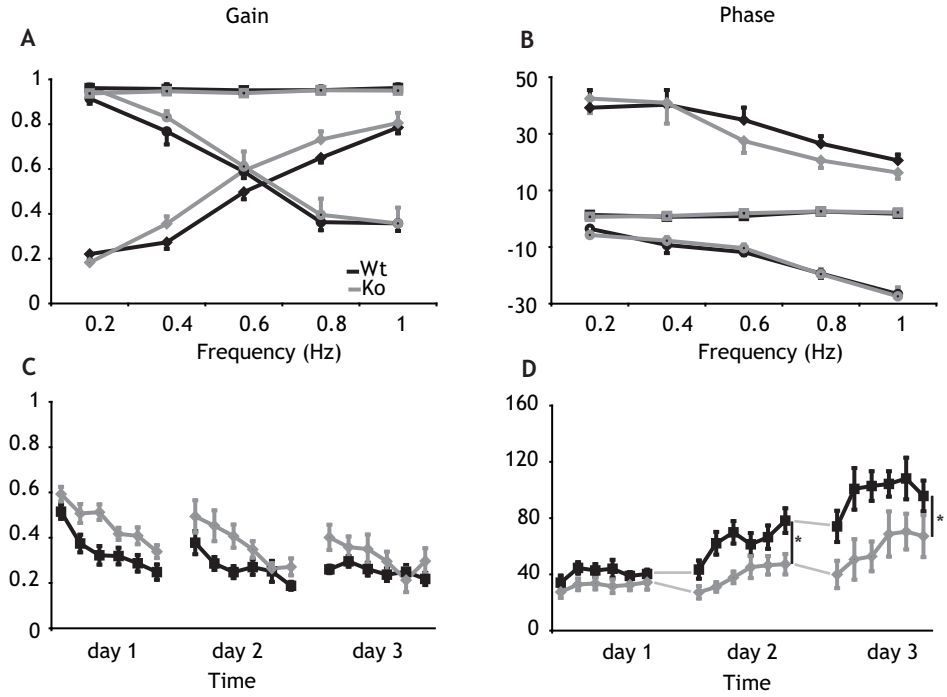

Figure 6. NMDA-NR2$^{\Delta C/\Delta C}$ mutant mice show normal motor behaviour and motor learning deficit during a three days training paradigm. *(A) Gain values of the vestibular-ocular reflex (VOR; circles), of the optokinetic reflex (OKR; diamonds) and of the visually increased VOR*

*(VVOR; squares) at stimulus frequencies ranging from 0.2 to 1 Hz in NMDA-NR2$^{\Delta C/\Delta C}$ mice and wild type mice are presented. (**B**) Phase values from NMDA- NR2$^{\Delta C/\Delta C}$ mice and wild type mice are plotted. Empty symbols represent mutant mice (ko) while filled symbols represent their wild type littermates (wt). (**C**) A three days paradigm did not induce differences in gain reduction among NMDA-NR2 $^{\Delta C/\Delta C}$ mice and control mice. (**D**) NMDA-NR2 $^{\Delta C/\Delta C}$ mice were able to reverse their phase values but not as prominently as wild types. * p < 0.05. Data are mean ± SEM.*

Table 1. Statistical analysis of the motor learning performances.

		2-way ANOVA with repeated measures	*p* value Day 1	*p* value Day 2	*p* value Day 3
Gain	NMDA-NR2$^{-/-}$	Test of within subjects effects	0.38	0.77	0.26
		Test of between subjects effects	0.97	0.32	0.84
	NMDA-NR2$^{\Delta C/\Delta C}$	Test of within subjects effects	0.33	0.41	0.22
		Test of between subjects effects	0.24	0.79	0.87
Phase	NMDA-NR2$^{-/-}$	Test of within subjects effects	0.27	0.95	0.38
		Test of between subjects effects	0.21	*0.04*	*0.05*
	NMDA-NR2$^{\Delta C/\Delta C}$	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 from two-way ANOVA tests of between and within subjects effects are listed. All mutant mice were tested against their wild type littermates.

Discussion

Here we investigated the functional role of the NR2A subunit in glutamatergic transmission between mossy fibers and granular 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 granular cell synapse, that NR2A activation does not affect general eye movement performance and gain-decrease learning, and that NR2A activation is essential for phase reversal learning. The fact that NR2A is much more prominently distributed in the granular layer than in all the other parts of the olivocerebellar system (Monaghan and Cotman, 1985; Capocchi et al. 1992; Grassi et al. 1996; Chen et al. 2006; Piochon et al. 2007), suggests that these three main findings may be directly correlated.

The mossy fiber-granule cell synapse in the vestibulo-cerebellum shows the ability to potentiate and this form of long-term potentiation 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 is severely impaired in the NR2A$^{-/-}$ mice, resembling the result previously reported in mice with 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).

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 information 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 training paradigm is much more challenging 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$^{\Delta C/\Delta C}$ mouse (Kadotani et al. 1996; Imamura et al. 2000) or NR2A/C$^{-/-}$ 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$^{-/-}$ mouse and NR2A$^{\Delta C/\Delta C}$ mutants allow us to exclude this potential caveat and they suggest that the cerebellar learning deficits cannot be secondary to performance

deficits, but that they are probably mainly due to a impaired induction of LTP at the mossy fiber to granule cell synapse.

Thereby we provide one of strongest pieces of evidence, which suggests that the forms of cellular plasticity that may underlay cerebellar motor learning are not restricted to long term depression (LTD) and/or LTP at the parallel fibers to Purkinje cell synapse, and/or to LTD and LTP at the cerebellar and vestibular nuclei downstream (Ito, 1972; Miles and Lisberger, 1981; De Zeeuw et al. 1998). In fact, we show here for the first time that plasticity in the granular layer upstream may well contribute to cerebellar motor learning. How this contribution may come about mechanistically remains to be shown. LTP in the granular 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 (see also D'Angelo et al. 1995). LTP in the granule cells could thus contribute to the memory formation by controlling the 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, submitted).

Acknowledgments

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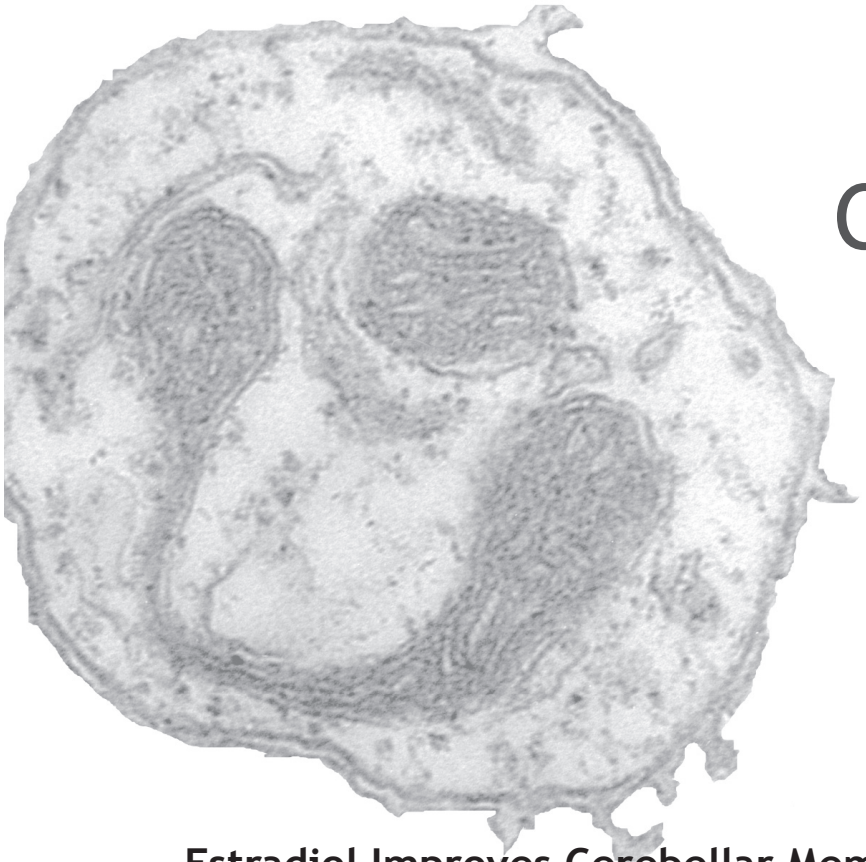

Chapter 4

Estradiol Improves Cerebellar Memory Formation by Activating ERß

Corina E. Andreescu[1], Bogdan A. Milojkovic[1], Elize D. Haasdijk[1], Piet Kramer[2], Frank H. De Jong[2], Andrée Krust[3], Chris I. De Zeeuw[1,4]* and Marcel T.G. De Jeu[1]

[1]Department of Neuroscience, Erasmus MC, Rotterdam, The Netherlands
[2]Department of Internal Medicine, Erasmus MC, Rotterdam, The Netherlands
[3]Institut de Genetique et de Biologie Moleculaire et Cellulaire, CNRS/INSERM/ULP, College de France, Strasbourg, France
[4]Netherlands Institute for Neuroscience, Royal Netherlands Academy of Sciences, Amsterdam, The Netherlands

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Abstract

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 (LTP) at the parallel fiber to Purkinje cell synapse, while it does not affect long term depression (LTD); second, we show that estradiol activation of ERß-receptors in Purkinje cells significantly improves gain-decrease adaptation of the vestibulo-ocular reflex (VOR), while it does not affect general eye movement performance; and third, we show that estradiol increases the density of parallel fiber to Purkinje cell synapses, while 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.

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 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 (ER) 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 relation between the cellular and behavioral effects is causal in a direct fashion, 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) following visuovestibular training (Robinson 1976; Ito 1991; De Zeeuw et al. 1998) (Figure 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 (Albus 1971; Marr, 1969; 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), while other mechanisms such as long-term potentiation (LTP) at the same synapse may contribute to decreasing the gain (Boyden et al. 2003; Hansel et al. 2001; Lev-Ram et al. 2002; 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 estradiol 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 light on the potential facilitating role of estradiol in motor memory formation.

Materials and Methods

Subjects

A total of seventy female C57/BL6 mice (Jackson Lab), seventeen male C57/BL6 mice and eight Purkinje cell specific ERß knock-out female mice (L7-ERß) were housed on a 12 hours light/dark cycle with food and water available ad libitum and subjected to the various tests described below. In one group of C57/BL6 females (Eovx mice) estradiol levels were fixed at a high level by ovariectomy (OVX) and subsequent daily subcutaneous injections of 5 µg estradiol benzoate dissolved in 0.1 ml sesame oil, while in another group of C57/BL6 females (Covx mice) estradiol levels were kept at a constant low level by ovariectomy and subsequent daily subcutaneous injections of only 0.1 ml sesame oil. In addition, we investigated groups of intact females and males for control. The Purkinje cell specific ERß mutants were generated by crossing flox-ERß 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 MC, 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 estradiol were estimated using an ultra-sensitive double-antibody radio-immunoassay (Diagnostic Systems Laboratories, TX, USA). Eovx mice had uterus weights and serum estradiol levels that were significantly higher than 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 estradiol level than females in diestrus day (all $p < 0.001$, *t*-tests; Table 1).

Electrophysiology

Sagittal slices of the cerebellar vermis (200 µm) were prepared from 6 - 8 weeks old C57/BL6 females (OVX females that received daily subcutaneous injection for 14 days; Eovx, n = 10; Covx, n = 10) and males (M, n = 10). Slices were kept in ACSF containing 124 mM NaCl, 5 mM KCl, 1.25 mM Na_2HPO_4, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 26 mM$NaHCO_3$, and 10 mM D-glucose bubbled with 95 % O_2 and 5 % CO_2 and supplemented with 20 µg 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 9 mM KCl, 10 mM KOH, 120 mM K gluconate, 3.48 mM $MgCl_2$, 10 mM HEPES, 4 mM NaCl, 4 mM Na_2ATP, 0.4 mM Na_3GTP, 17.5 mM sucrose (pH 7.3). Holding potential of Purkinje cells in voltage-clamp mode ranged between -65 and -75 mV. Pair-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 and 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 pair-pulse facilitation ratio, the pre- or postsynaptic nature of LTP and LTD can be estimated. The facilitated response to the second pulse is due 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 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 more than 15% during the experiment.

Table 1. Vaginal smears feature, uterus size, number of follicles, and estradiol level in C57/BL6 female mice.

	Vaginal smear	Uterus (mg)	Follicles class IV	Follicles class V	Estradiol pmol/L)
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.

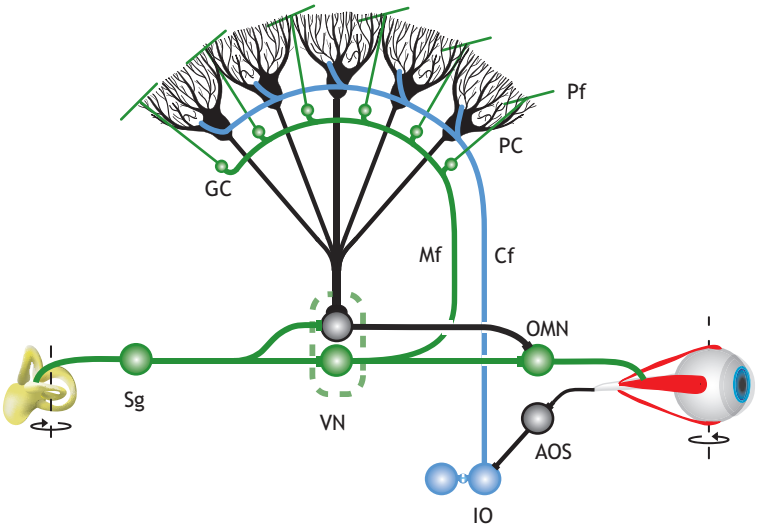

Figure 1. Scheme showing VOR circuitry. *The VOR is an eye movement reflex that stabilizes retinal images during head movements (Collewijn and Grootendorst 1979; Iwashita et al. 2001; Van Alphen and De Zeeuw 2002; Boyden et al. 2004; Faulstich et al. 2004; Stahl 2004). Primary afferents from the vestibular system (Scarpa's ganglion; Sg), converge upon 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 fibres (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 vestibulo-ocular reflex (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.*

Behavioural tests

Three days before the behavioural 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 Ltd) and oxygen. In OVX mice angular optokinetic reflex (OKR), VOR,

and visually enhanced vestibulo-ocular reflex (VVOR) were measured (± 5°; 0.2 – 1 Hz). Before VOR recordings pilocarpine 4% (Laboratories Chauvin, France) was used in order 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 minutes. 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 minutes. 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. Mice were treated daily with estradiol or solvent between day 0 and day 28. Between the measurements done on day 19 – 20 and 26 – 27 mice were kept in complete darkness. Intact C57/BL6 female mice were presented with VOR gain decrease paradigm for 30 minutes 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 minutes. Intact L7-ERß female mice were presented with VOR gain decrease paradigm in proestrus day. Intact male mice were presented with VOR gain decrease paradigm for one day. Video eye movement recording and data analysis were done as previously described (Stahl et al. 2000; Andreescu et al. 2005).

Morphology

Cerebellar slices (40 µm) from 16 weeks old females were processed for light microscopy (see Price and Handa 2000). In short, slices were rinsed for 4 x 10 min in TBS (Tris Buffered Saline; pH 7.6; 0.05M) followed by a pre-incubation of 1 hour in ice-cold TBS containing 10% normal horse serum and 0.5% Triton. Subsequently sections were incubated with primary antibody for 48 hours at 4°C (ERα (1:200) and ERß-N-term (1:1000); Affinity BioReagents, Golden), rinsed for 4 x 10 min in TBS, incubated with secondary antibody (1:200) for 90 minute at room temperature and rinsed again for 4 x 10 min in TBS. Finally, tissue was incubated for 90 minute at room temperature in ABC-elite (PK 6100 Vector labs), rinsed for 3x 10 min in TBS, and for 3 x 10 min in 0.05M Tris HCl , followed by a DAB staining.

The flocculus (from OVX females that received daily sc injection for 14 days; Eovx n = 8; Covx n = 8) was processed for electronic microscopy (Philips CM 100) and labelled with 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 fibre and climbing fibre 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). 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:

$$Ds = (N_s/\text{corrected A})*PSD_A$$

Statistical Tests

Data are presented as mean ± SEM. For statistical comparisons we used the 2-way ANOVA with repeated measures followed by a post hoc analysis if required and the two samples Student' *t*-tests (SPSS 11.0 Inc.).

Results

Estradiol 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 (Figure 2A). 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, $p < 0.001$, 2-tailed paired *t*-test), to 117.7 ± 1.5% in Covx mice (n = 10, t = 37 − 42 min after, $p < 0.001$, 2-tailed paired *t*-test), and to 121.6 ± 0.1% in males (n = 9, t = 37 − 42 min after, $p < 0.001$, 2-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; 2-way ANOVA), and probably of a postsynaptic nature, because the paired-pulse facilitation ratio did not change after tetanization (for Eovx mice 98.8 ± 0.5, $p = 0.57$; for Covx mice 97.8 ± 0.4, $p = 0.99$; for males 98.6 ± 0.3, $p = 0.70$; 2-tailed paired *t*-test) (Figures. 2B and 2D).

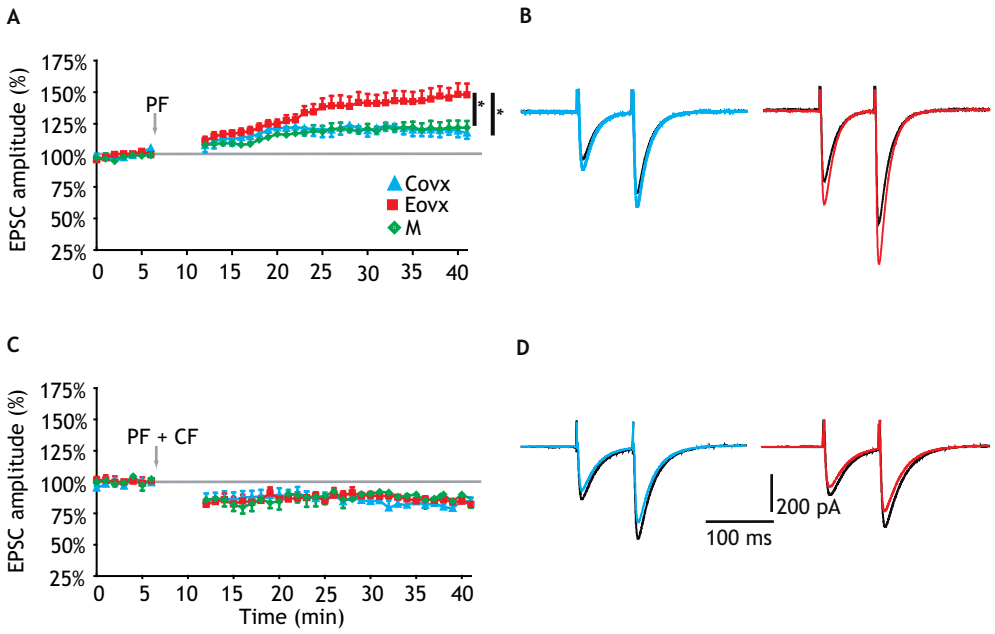

Figure 2. Estradiol enhances LTP but not LTD. *(**A** and **B**), Induction of parallel fiber – LTP by parallel fibers (PF) stimulation at 1 Hz for 5 min result in a more robust response in the slices from Eovx (square) mice as compared with those from Covx (triangle), and male mice (diamond). (**C** and **D**), Induction of parallel fiber – LTD by parallel fibers (PF) and climbing fibers (CF) stimulation at 1 Hz for 5 min generated the same response in slices from Eovx (square), Covx (triangle), and male mice (diamond). Traces show superimposed PF-EPSCs from Purkinje cell 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 representing an average of 30 traces. * $p < 0.05$. All values are mean ± SEM.*

Moreover, since the input resistance, rise time kinetics, decay time and amplitude of the EPSC's were not significantly different among Eovx mice, Covx mice and males ($p = 0.55$, $p = 0.75$, $p = 0.72$, $p = 0.76$ respectively; 2-tailed paired *t*-test; Table 2), it appears unlikely that the differences in LTP induction are biased due to potential differential effects of estradiol 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 mins in current-clamp mode (Figure 2C). After tetanization, the amplitude of the excitatory postsynaptic current (EPSC) was significantly decreased to 85.8 ± 0.6% in Eovx mice (t = 37 − 42 min after, $p < 0.001$, 2-tailed paired *t*-test; n = 7) and to 82.2 ± 0.7% in Covx mice (t = 37 – 42 min after, $p < 0.001$, 2-tailed paired *t*-test; n = 6). These values were not significantly different (p = 0.49, 2-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, 2-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.

Table 2. Basal Synaptic Parameters from Purkinje Cells from Covx, Eovx and Male 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
M (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.

Estradiol 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 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; Figures 3A-B; p = 0.14 and p = 0.31, respectively; 2-way ANOVA). In addition, eye movement responses of Eovx mice to visual stimulation (optokinetic reflex; OKR; Figures 3C-D) and to head rotation in light (visually enhanced vestibulo-ocular reflex; VVOR; Figures 3E-F) were also similar to those of Covx mice (for OKR gain p = 0.98, for OKR phase p = 0.12, for VVOR gain p = 0.91, for VVOR phase p = 0.30; 2-way ANOVA) and showed the classical relation to stimulus frequency (Figure 3). Consequently, we conclude that E2 does not influence the general eye-motor performance.

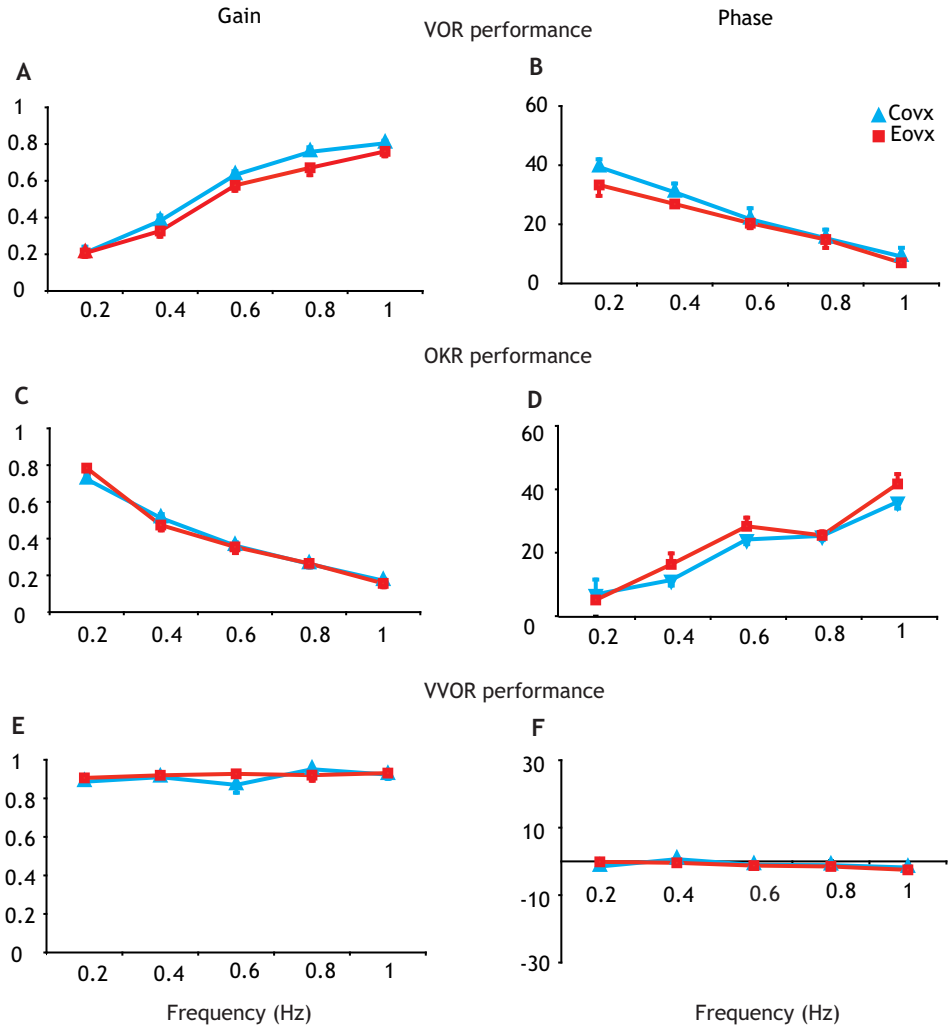

Figure 3. Estradiol does not affect motor performance. *Robust compensatory eye movements are generated by (**A** and **B**) head rotations in dark (vestibulo-ocular reflex; VOR), by (**C** and **D**) environment rotations in light (optokinetic reflex; OKR) and by (**E** and **F**) head rotations in light (visually-enhanced vestibulo-ocular reflex; VVOR) and with frequencies ranging from 0.2 to 1 Hz at 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) level of estradiol (all p>0.05, 2-way ANOVA). Error bars indicate SEM.*

In contrast, when both groups of mice were subjected to a visuovestibular training paradigm in which head rotation was paired for fifty minutes with in phase rotation of the visual environment (5°, 0.6 Hz) significant differences were observed (Figures 4A-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 ($p < 0.001$, 2-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 hours in darkness (Figures 4A; $p < 0.001$ Eovx, $p < 0.001$ Covx; 2-tailed paired *t*-test), but the difference between Eovx and Covx mice was preserved (Figure 4A; $p < 0.02$, 2-tailed paired *t*-test). In contrast, no significant differences were observed in phase values (Figure 4B, $p = 0.40$ after training and $p = 0.22$ at 24h, 2-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 $p = 0.94$ and for phase $p = 0.95$; at 24h for gain $p = 0.70$ and for phase $p = 0.60$, 2-tailed paired *t*-tests; Figures 4C-D).

Thus, artificially generated high levels of E2 specifically improved gain-decrease motor learning and memory maintenance, while 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) thirty minutes of training resulted in a change in VOR gain of 22%, whereas in mice in proestrus (high E2 level of 58 ± 2 pmol/L; $p < 0.001$, 2-tailed paired *t*-test; n = 7), the

same training resulted in a significantly greater change of 44% ($p < 0.005$, 2 way ANOVA; Figure 5A). 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 ($p = 0.47$, 2 way ANOVA, post hoc LSD; Figure 5A), but significantly smaller than that in females in proestrus ($p < 0.03$, 2 way ANOVA, post hoc LSD). No significant changes were found for phase values (all $p > 0.5$, 2-way ANOVA, post hoc LSD; Figure 5B). 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.

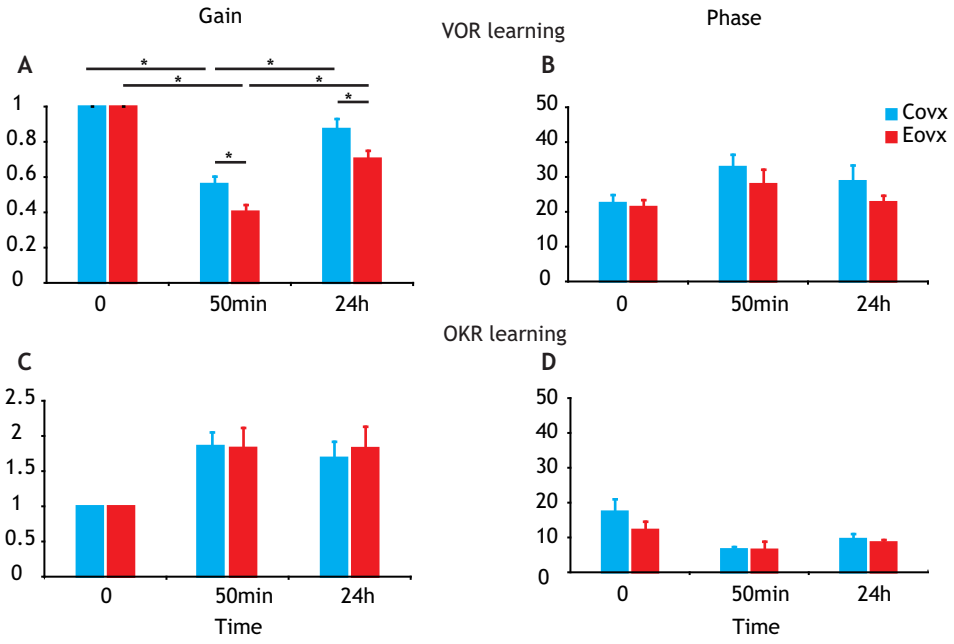

Figure 4. Estradiol enhances gain-decrease VOR learning. *(**A** and **B**), Normalized VOR gain and phase before training, after 50 minutes of training, and 24 hours later in Covx mice (blue) and Eovx mice (red). Note that Eovx mice learned significantly better than Covx mice. Twenty-four hours in darkness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

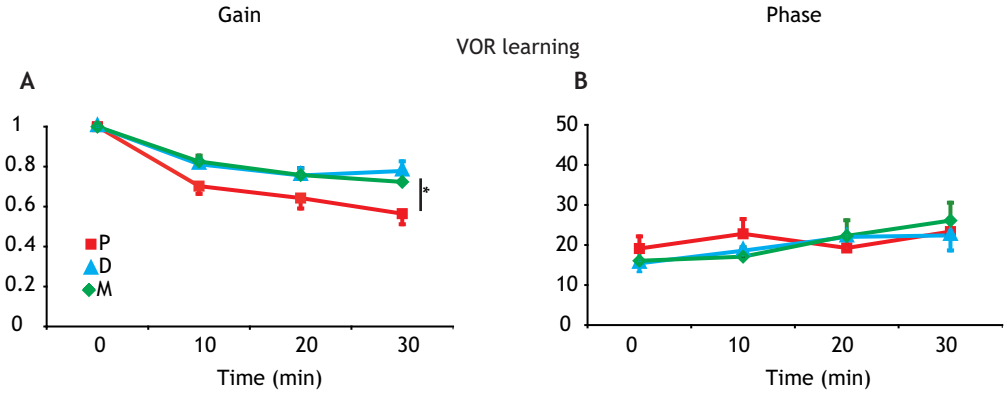

Figure 5. Motor learning is enhanced by endogenous high levels of the estradiol. *(**A** and **B**), Thirty minutes of training induced a higher VOR gain decrease in female mice with natural high levels of E2 (proestrus (P), squares) than in female mice with natural low levels of E2 (diestrus (**D**),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

ER-ß but not ER-α are present in the adult vestibulocerebellum

In the developing cerebellum both ERα and ERß are localized in Purkinje cells, granular cells and molecular layer interneurons (Jakab et al. 2001; Perez et al. 2003). ERß 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 estrogen receptors (ERα) and ß estrogen receptors (ERß) in the flocculus and paraflocculus of the vestibulocerebellum in the adult mouse. While ERα expression could not be detected in the flocculus or paraflocculus (Figures 6A-B), ERß 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 (Figures 6C-D). In addition, labeling for ERß could be observed in Golgi cells in the granular layer of the flocculus and paraflocculus (Figures 6C-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 their Golgi cells.

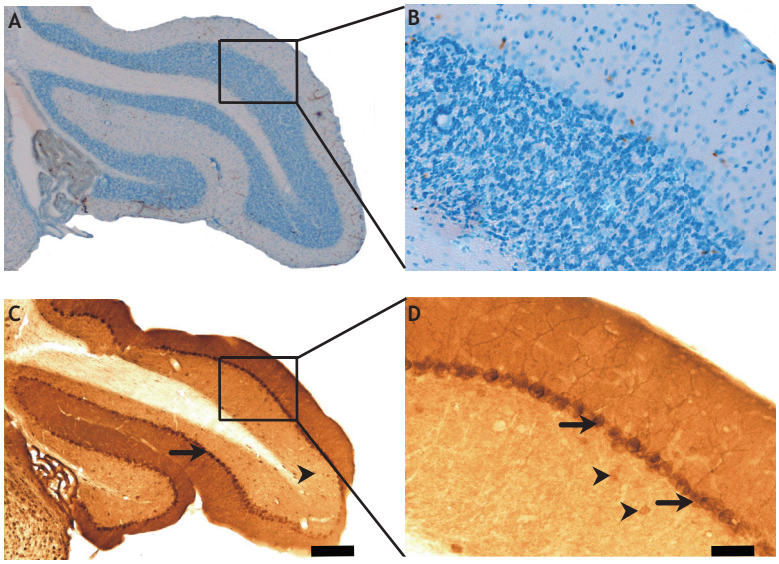

Figure 6. Estradiol receptors expression in the vestibulocerebellum. *(**A** and **B**), ERa immunoreactivity in sagital cerebellar slices counterstained with thionin reveals no ERa in any of the cerebellar neurons. (**C** and **D**), ERß immunoreactivity in sagital cerebellar slices shows a high expression of the ERß. ERß is present in Purkinje cells (arrows) and Golgi cells (head arrows) in the flocculus and paraflocculus. Scale bars: 25 µm (**A** and **C**) and 100 µm (**B** and **D**).*

Activation of ER-ß in Purkinje Cells contributes to enhanced adaptation

To further pinpoint the location of the estrogen receptors responsible for the differences in motor learning, we created and tested mouse mutants in which ERß was specifically removed in Purkinje cells (L7-ERß$^{-/-}$). When female L7-ERß$^{-/-}$ mice were presented in the proestrus day with the training paradigm, the training still induced a change in VOR gain of 29% in L7-ERß$^{-/-}$ mice (n = 4; Figure 7A). However, in the female L7-ERß wild-type littermates we observed a significantly larger effect on the adaptation (51%; n = 4; $p < 0.05$, 2 way ANOVA; Figure 7A) indicating that the activation of the ERß in the Purkinje cells plays an important role in this form of motor learning. No significant changes were found in the phase ($p = 0.17$, 2 way ANOVA, Figure 7B). Thus, by subjecting mice in which the ERß-receptor is specifically ablated in Purkinje cells (i.e. the L7-ERß$^{-/-}$ mutants) to the visuovestibular training paradigms, we show here that activation of ERß 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.

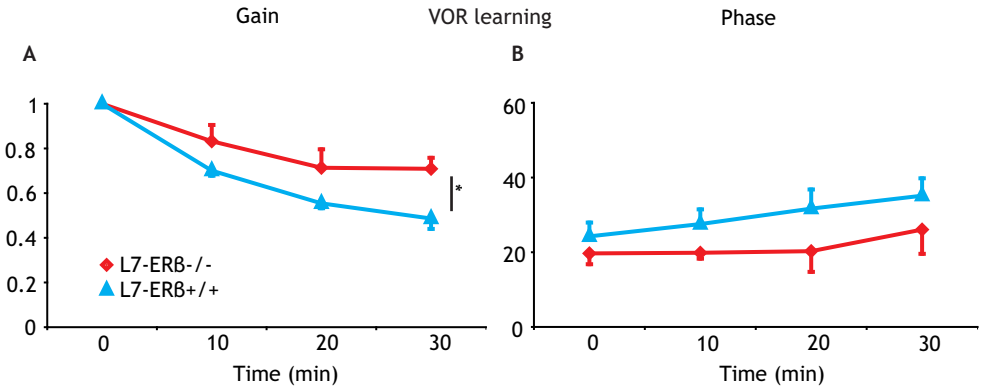

Figure 7. Motor learning is enhanced by the presence of the ERβ in the Purkinje cells. *(**A** and **B**), Thirty minutes of training induced a higher VOR gain decrease in L7- ERβ+/+ littermates (triangles) than in L7- ERβ$^{-/-}$ (diamonds). No significant differences were observed in phase. * $p <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

Estradiol 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 (Figure 8A-B). We found that high levels of estradiol significantly increased the number of synapses (0.55 ± 0.03 / μm^2 in Eovx mice and 0.47 ± 0.02 / μm^2 in Covx mice; p = 0.051, 2-tailed paired *t*-test). The postsynaptic density area (PSD_A) and the postsynaptic density perimeter (PSD_P) were slightly, but not significantly, increased in Eovx (n = 8) as compared to those in Covx (n = 8) mice. 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 (p = 0.32 and p = 0.18, respectively, 2-tailed paired *t*-test). However, when the synaptic density was considered (for formula see methods), it became apparent that the total postsynaptic area was significantly increased by 30% in Eovx mice (376 ± 35 in Eovx mice and 289 ± 20 in Covx mice; $p < 0.05$, 2-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, 2-tailed paired *t*-test). We conclude that E2 specifically enhances the availability of the synaptic complexes at the parallel fiber to Purkinje cell input.

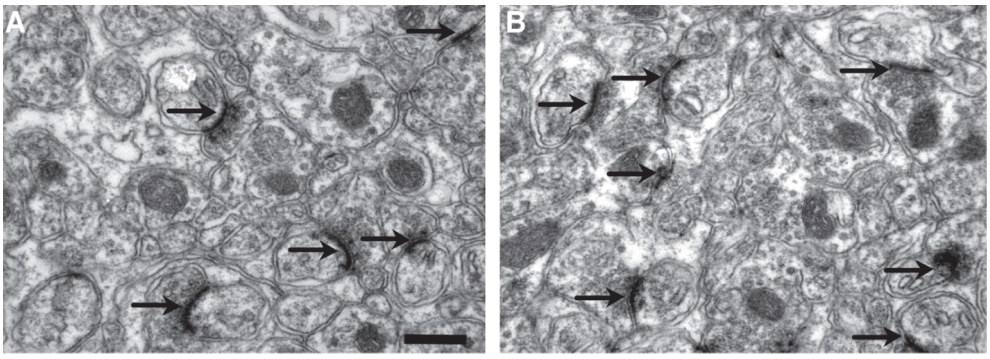

Figure 8. Estradiol increases synaptic density in the vestibulocerebellum. *(**A** and **B**), Electron micrographs of the molecular layer of the flocullus that are labelled with antibody against calbindin showing ultrastructural characteristics of Purkinje cells synapses in OVX mice that received oil (*A*) or estradiol (*B*). Arrows mark synapses between Purkinje cell and parallel fibers. Scale bar: 50 µm.*

Discussion

Our data show via different lines of evidence that the gonadal hormone estradiol promotes cerebellar plasticity in a specific fashion. We show that estradiol enhances induction of LTP at the parallel fiber to Purkinje cell synapse, while it does not affect LTD; that estradiol activation of ERß-receptors of Purkinje cells significantly improves gain-decrease adaptation of the VOR, while it does not affect gain-increase adaptation of the OKR or general eye movement performance; and that estradiol increases the total size of the postsynaptic complex of the parallel fiber to Purkinje cell synapse, while 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 pointed out that gain-decrease paradigms show different dynamics compared to those of gain-increase paradigms, and that it is therefore unlikely that they both depend on LTD (Boyden and Raymond 2003; see also Miles and Eighmy, 1980; Van Alphen and De Zeeuw, 2002; Faulstich et al. 2004). Second, LTP induction is supposed to increase the insertion of α-amino-3-hydroxi-5-methyl-4-isoxazolepropionic acid receptors (AMPA receptors) and thereby enlarge their postsynaptic complex, while 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, since 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 estradiol is specific for LTP induction. Thus, even though LTD and LTP do certainly not provide the only push-pull mechanisms in the olivo-cerebellar 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 estrous cycle but also during pregnancy. Therefore, changes in Purkinje cell activities due to changes in levels of estradiol 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 like error free walking. Non-stumbling 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 estradiol 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 estradiol alone.

The impact of estradiol on cerebellar motor learning and plasticity it 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, even though 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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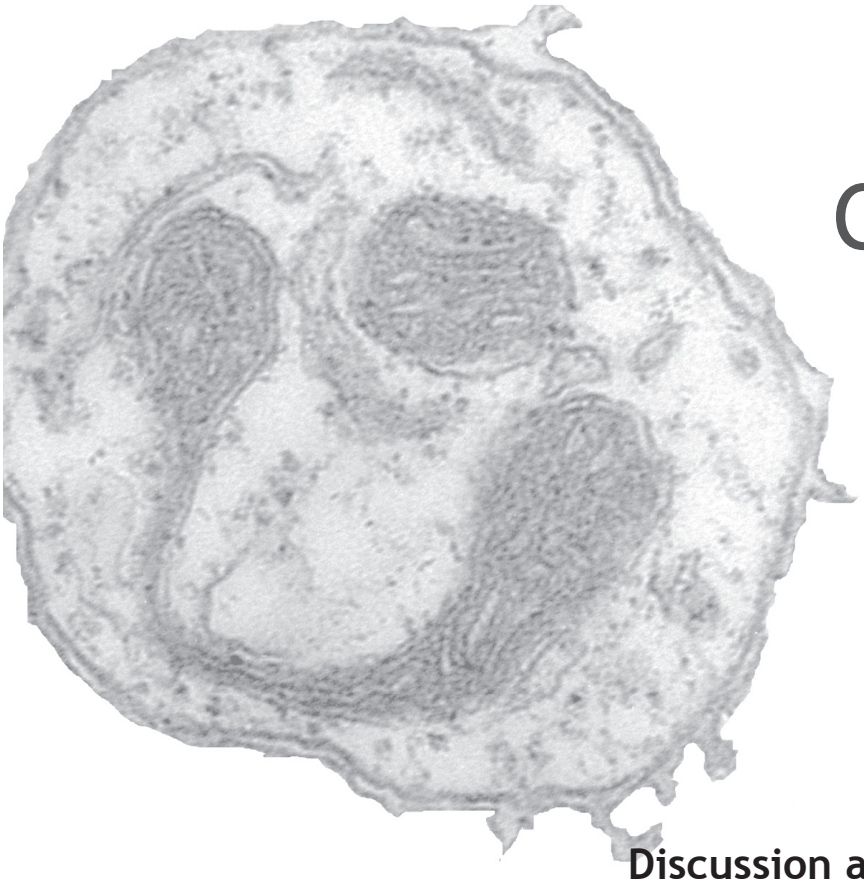

Chapter 5

Discussion and conclusions

In this thesis, we describe the effects of alterations in the vestibular system, central factors and hormone levels on cerebellar plasticity and on the motor performance and motor learning.

One way to investigate the role of cerebellar plasticity in motor performance and motor learning is to measure eye movements (Raymond et al., 1996; Ito, 2002; Blazquez et al., 2004; De Zeeuw and Yeo, 2005). The term "eye movements" actually refers to a set of behaviors that are generally divided into two classes: gaze-shifting and gaze-stabilizing movements (Leigh and Zee, 1999).

Gaze-shifting movements are present mainly in animals whose retina have specialized regions equivalent to a fovea. Such ''foveate'' animals must orient each eye to align the fovea with the optical projection of the visual target. Gaze-shifting movements include saccades, smooth pursuit and vergence.

Mammals lacking a fovea or area centralis, the ''afoveate'' mammals, show robust gaze-stabilizing movements, also named compensatory eye movements. Compensatory eye movements (vestibulo-ocular reflex – VOR and optokinetic reflex – OKR) prevent the image of the surrounding to slip across the retina; this retinal slip is detrimental to "clear" vision. Therefore, compensatory eye movements are constantly adapted to best fit the condition at that instance. Inputs from the vestibular labyrinth and retina drive compensatory eye movements that are open to rigorous quantitative analyses. Thus, the oculomotor system serves as an excellent model to evaluate the effects of alterations in the vestibular system, central factors and hormone levels at the behavior level.

Video-oculography is a powerful technique for recording eye movements in different species and it is nowadays also used in mice (see Box 1). This technique is successfully used to investigate ocular motor performance (OKR; VOR; visually enhanced VOR - VVOR) as well as motor learning (VOR adaptation; OKR adaptation). Mice are extensively used as a model for eye movement measurements. The major advantage of mice models is that mutant strains are available. These mutants provide tools for exploring the connections between genes, cell biology, and the development and function of neuronal circuits. Furthermore, these mutants can mimic human diseases. The major disadvantages of measuring eye movements in mice are that they do not accept restrainment easily and are quickly stressed.

Box 1

Many methodological variations of compensatory eye movements and VOR and/or OKR adaptation have been studied and are being used by research groups for many different purposes. Nevertheless, researchers have become increasingly aware that several requirements are needed for high-quality video eye movement recordings in mice.

- The system used for eye movement recordings includes a video pupil tracking system, a servo-operated turntable, a servo-operated optokinetic cylinder (drum) and a computer equipped with data acquisition software.
- Infrared emitters are needed to illuminate the eye during the recording. However, the video-recording system must track only one corneal reflection in order to compensate for any relative movement between the camera and the eye.
- Several characteristics of the experimental animals need to be controlled for or taken into account when planning eye movement experiments or analyzing results. Mice should have pigmented irises ("black eyes" i.e. C57Bl6/J background) since pupil tracking is impossible in mice with no pigmented irises ("red eyes"). Factors like body weight (i.e. the fit of the mouse in the apparatus used to restrain the animal), age (Faulstich et al., 2004; Stahl, 2004b), gender (Andreescu et al., 2007) as well as strain (Koekkoek et al., 1997; Katoh et al., 1998; Stahl, 2004a) of the animal subject can affect eye motor responses.
- The head of the mouse must be fixed with respect to the camera; therefore, each animal must be surgically implanted with a head fixation pedestal, a procedure that requires approximately thirty minutes and a mice surgery facility.
- Pilocarpine (or other miotic drug) needs to be used to perform video-oculography in darkness. In the absence of such drug the pupil of the mouse becomes enormous in darkness, making detection of the pupil impossible. Furthermore, the effect of the pilocarpine lasts for 75 - 90 minutes making very long experiments difficult.
- A good calibration (i.e. free of eye movements) is needed for consistent data. The eye video-recording system stores video images from the calibration and recordings on a disc and extracts eye positions from these files offline. Although this procedure is disc space and time consuming, it is advantageous by making possible offline data analysis and, if necessary, data reanalysis.
- Finally, acquiring and analyzing the data requires two to three hours per animal. Thus eye movement recordings will likely remain a specific procedure applied to selected mice.

A number of disadvantages remain in using video-oculography. This technique is subject to artifacts like blinks and loss of pupil detection due to interference from illuminating light and/or important changes in the pupil diameter. Occasionally illumination and software tracking parameters must be changed. Therefore video-tracking the eye continuously requires close attention. Extremely nervous or anxious animals have to be handled for several days before starting the experiments and gently restrained to avoid breaking of the head fixation pedestal during the experiment. Sometimes, during the experiment, extremely nervous or anxious animals close their eyes, making video pupil tracking impossible. Even so, an increasing number of studies that incorporate eye movements' data from mice will be performed in the future.

Peripheral impact

A critical step in self-motion perception and spatial awareness is the integration of motion cues from multiple sensory organs that individually do not provide an accurate representation of the physical world. One of the sensory ambiguities arises in identifying the actual motion associated with linear accelerations sensed by the otolith organs in the inner ear (Fernandez and Goldberg, 1976; Angelaki and Dickman, 2000). These internal linear accelerometers respond identically during translational motion (for example, walking forward) and gravitational accelerations experienced as we reorient the head relative to gravity (that is, head tilt). Activation of the sensory receptors of the otolith organs is accomplished via a shearing action between the stereociliary bundles of the hair cells and the overlying otoconial membrane.

Using a mutant model mouse (tilted, *tlt*) we identified, here, the contribution of otolith organs to compensatory eye movements. Light microscopy images taken from the inner ear of tilted mice reveals the absence of the otoconia in both otolith organs, which indicated that otolith organs might be dysfunctional and therefore the otolith-driven reflexes affected. The absence of otoconia leads to impaired vestibulo-ocular reflex and therefore, we can conclude that otolith-driven impulses complement semicircular canals activation to achieve gaze stability during various displacements of the head. The deficits in VOR can be explained by the fact that signals derived from the otolith organs and the semicircular canals converge at the level of vestibular neurons, which send their eye movement commands to the oculomotor nuclei (Sato et al. 2000; Zhang et al. 2001; Dickman and Angelaki, 2002; Zhang et al. 2002b). The ability to combine information from multiple sensory organs is needed in order to have stable retinal images.

Tilted mice present an enhanced optokinetic response that indicates that the oculomotor system is able to compensate for the deficits. The optokinetic system may be particularly suited to compensate for the lack of otolith-driven information necessary for a proper VOR as both systems have similar low-pass filter characteristics. In tilted mice the decreased vestibular and increased optokinetic performance induced partially reduced responses when combining vestibular and visual information was possible. The optokinetic system appears to be able to make up, to some extent, for inherited and perhaps even acquired deficiencies in the ability of the vestibular system to stabilize images on retina during head movement and to generate a reliable perception of one's motion in space.

Optokinetic compensation induced by the otoliths deficit does not necessarily imply similar compensation processes when aberrant configurations of the semicircular canals and/or vestibular dysfunction are present. Optokinetic compensation may not be well designed to compensate for deficits in the vestibular-canal system, which acts more like a high-pass filter. However, acute unilateral semicircular canal dysfunction triggers gain increase in the OKR (Paige, 1985; Fetter and Zee, 1988; Faulstich et al. 2006) and this increase depends on the integrity of the olivo-cerebellar circuit (Faulstich et al. 2006). Furthermore, visual experience after unilateral labyrinthectomy is essential for the recovery of VOR (Fetter et al. 1988). Future research of multisensory integration system will mainly focus on unrevealing if chronic vestibular-canal system deficit induces compensation and if so, of what nature.

The mechanism that underlies OKR compensation in tilted mice probably resembles that underlying OKR and VOR adaptation following visual-vestibular or visual training paradigms (Collewijn and Grootendorst, 1979; Nagao, 1983; Iwashita et al. 2001). In both compensation mechanisms and adaptation processes, the adaptive changes in the amplitude and timing of the eye movement serve to restore gaze stability during head motion.

Central impact

VOR adaptation can be detected by measuring the gain of the VOR in darkness after the subject has been wearing magnifying, miniaturizing, or reversing spectacles (natural behavior) or has been exposed to a particular combination of visual stimuli and head movement (controlled behavior). If, for instance, image motion is consistently in the same direction as head motion then initially the amplitude of the VOR will be too large and consequently the VOR amplitude will be adaptively reduced. Humans, monkeys, rabbits and rodent are all able to adapt their VOR (Gonshor and Jones, 1973; Collewijn and Grootendorst, 1979; Miles and Eighmy, 1980; Lisberger, 1984; Tan et al. 1992; Shelhamer et al. 1994; Kramer et al. 1995; De Zeeuw et al. 1998). VOR amplitude can be either increased or decreased and the persistence of amplitude changes depends on the nature and duration of the adapting stimuli (Miles and Eighmy, 1980; Boyden and Raymond, 2003; Kuki et al. 2004).

Experimental control over stimuli and the good understanding of the underling circuit have been essential in distinguishing the relative contributions of the cerebellum and vestibular nuclei in VOR adaptation. Previous work has shown that during VOR

adaptation the head acceleration and eye motion information are relayed to the cerebellum via mossy fiber afferents, and the image motions on the retina are relayed via climbing fibers (Raymond et al., 1996). Moreover, output from the cerebellum via vestibular nuclei drives the expression of VOR adaptation that adjusts the amplitude of the VOR and reduces the image motion. Thus, the behavioral properties of VOR adaptation reflect the input/output transformations of its underling circuit.

One of the most fundamental challenges in neuroscience is to link behavioral properties of learning with cellular properties of plasticity. Historically, long-term depression (LTD) of the parallel fiber to Purkinje cell synapse (pf-PC) constitute a candidate cellular mechanism for explaining different forms of cerebellum dependent motor learning including VOR adaptation (Ito, 1986; Thompson et al. 1997). However, in recent years many synapses in the cerebellum have been shown to be modifiable. Plasticity in the cerebellar cortex is not limited to LTD of the parallel fiber to Purkinje cell synapse (Hansel et al. 2001). Evidence to date suggest that LTD and long-term potentiation (LTP) at pf-PC synapses are mutually reversing; a property that is required, otherwise both LTP and LTD soon saturate and no further learning is possible (Coesmans et al. 2004).

Genetically altered mice, in which key molecules were targeted, have broadened our understanding about LTD or LTP participation in the VOR adaptation. By using L7-promotor, Pukinje cell specific mutant mice have been generated. For example, Pukinje cell specific metabotropic glutamate receptor 1 (mGluR1) deletion impaired LTD and caused motor coordination deficit (Ichise et al. 2000). Furthermore, transgenic mice selectively expressing an inhibitor of protein kinase C in Purkinje cells (L7-PKCI) mice present impaired LTD and VOR adaptation (De Zeeuw et al. 1998).

Here we investigate null – mutant mice that lack the NR2A subunit of the N-methyl D-aspartate receptor (NMDA-NR2A$^{-/-}$) and that have an impaired LTP at their mossy fiber terminals and a reduced ability to increase the intrinsic excitability of their granule cells, while the basic excitatory output of their mossy fibers is unaffected. In addition, we demonstrate that these mutants have deficits in the adaptation of their vestibulo-ocular reflex (VOR), while their basic eye movement performance is similar to that of wild type littermates. These results demonstrate that NMDA-NR2A mediated potentiation at the mossy fiber to granule cell synapse can contribute to cerebellar memory formation. We can conclude that several sites of cerebellar plasticity exist and are likely to be involved in different aspects of VOR adaptation.

Accumulating evidence from adaptation of the VOR indicates that plasticity in the cerebellar cortex is not sufficient to account for all learning aspects that take place during cerebellum dependent motor learning (du Lac, 1996; Raymond et al. 1996).

Debates over the role of the cerebellar cortex and cerebellar and vestibular nuclei in motor learning have been focused on the identification of the region relevant to VOR learning and to VOR memory. Although, the cerebellum is most likely the critical site for motor learning, its precise role in motor memory is still a controversial topic (Ito, 1984; du Lac et al. 1995). Regarding this issue, several hypotheses have been launched. One hypothesis suggests that motor learning and memory are located in the cerebellar cortex (Ito, 1986), while another one suggests that motor memory is formed in the vestibular or cerebellar nuclei by means of signals mediated by the cerebellar cortex (Lisberger, 1998). Recently, it has been shown that multiple plasticity mechanisms and/or multiple neuronal sites may be involved in cerebellum dependent motor learning (Blazquez et al. 2004; Boyden et al. 2004; De Zeeuw and Yeo, 2005) and that learning takes place first in the cerebellar cortex and is then transferred to the vestibular nuclei (Shutoh et al. 2006).

Hormonal impact

Hormones can also modulate various motor skills, including motor learning and VOR adaptation. Here, we show that endogenous high levels of estradiol (E2) significantly improve motor learning when compared with endogenous low levels of E2. Furthermore, females at the day of proestrus prevail over males when exposed to the same training paradigm.

In the olivo-cerebellar circuit that is responsible for VOR adaptation, estrogen receptor ß (ERß) has been shown to be expressed in the cerebellum (Price and Handa, 2000), in the vestibular nuclei and in the inferior olive (Shughrue et al. 1997; Zhang et al. 2002a). Therefore, to further pinpoint the role of Purkinje cell estrogen receptors in motor learning, Purkinje cells – specific ERß mutant mice (L7-ERß$^{-/-}$) were presented with the same learning paradigm. This approach allowed us to analyze, for the first time, the specific role(s) of E2 and ERß in cerebellar function in vivo. Furthermore, the tissue-specific knockout strategy (used to generate the L7-ERß$^{-/-}$ mice) circumvented potential limitations of the conventional gene targeting technique, such as the lack of regional specificity, the presence of multiple defects and hence the presence of compensatory mechanisms. The lack of ERß in Purkinje cells decreases the ability

of gain-decrease VOR adaptation in female mice in proestrus day, indicating that activation of ERß in Purkinje cells is sufficient to enhance this specific type of learning. Interestingly preliminary data show that when female L7-ERß$^{-/-}$ mice are presented at the day of proestrus with the gain-increase training paradigm, the training induces a change in VOR gain of 20% in L7-ERß$^{-/-}$ mice that is similar with the wild-type female littermates change in the VOR gain. This discrepancy may be explained by the fact that the gain increase and gain decrease processes might be regulated by different forms of plasticity (Faulstich et al. 2004; De Zeeuw and Yeo, 2005). It has been proposed that parallel fiber – Purkinje cell LTP might underlie adaptive changes in VOR gain decrease and that parallel fiber – Purkinje cell LTD might underlie adaptive changes in VOR gain increase (Boyden and Raymond, 2003; De Zeeuw and Yeo, 2005). Interestingly, however, E2 enhances induction of LTP at the parallel fiber to Purkinje cell synapse, while it does not affect LTD at the same synapses. This finding supports the hypotheses that gain increase and decrease depend on different molecular mechanisms.

The impact of E2 on cerebellar motor learning does not stand on itself. E2 also improves other forms of memory formation such as spatial reference memory, trace conditioning, visual memory and strategy solving tasks (Frick et al., 2002; Luine et al. 2003; Daniel and Lee, 2004; Leuner et al. 2004; Rhodes and Frye, 2004).

Furthermore, E2 mediates not only functional synaptic plasticity but also structural synaptic changes. In the cerebellum E2 regulates the synaptic density by increasing the total size of the postsynaptic complex of the parallel fiber to Purkinje cell synapse, while it does not affect that of the climbing fiber synapse. E2 mediates fluctuation of the synaptic density during the estrous cycle also in the hippocampus (Woolley and McEwen, 1992). It should not necessarily be assumed that more versus fewer synapses will lead to better versus worse circuitry function. It might be possible that during a period of high spine synapse number such as in proestrus, rapid synapse turnover actually impairs various performances. Results from studies that attempted to relate behavior typically associated with the hippocampus to stages of the estrous cycle or estradiol treatment have been equivocal (Woolley, 1998). However, in the cerebellum estradiol-induced morphological and electrophysiological synaptic plasticity are well correlated with the behavioral output.

It might be that the behavioral significance of hormonal-induced cerebellar changes is not restricted to the estrous cycle itself. Pregnancy and motherhood may

be life stages in which the behavioral significance of hormone-induced changes in the cerebellum is more relevant than during the estrous cycle.

The fact that estradiol has the potential to alter neuronal morphology and function in only a few hours (Woolley and McEwen, 1992) leads to the speculation that, if similar changes occur in women, various aspects could also change during the menstrual cycle. Further experiments should investigate whether in women motor performance and motor learning systematically fluctuate with phases of the menstrual cycle. Anyhow it might be possible that humans are capable of overruling hormonally modulated changes by using systems or mechanisms that are not available to or not sufficiently developed in other animals.

In summary, we can conclude that various factors influence cerebellar plasticity and cerebellar motor learning and that measuring compensatory eye movements is a powerful technique to investigate these factors. Compensatory eye movements rely on a multisensory integration system that induces execution of accurate eye movements in time and space. This multisensory integration system has also the capacity to compensate for certain deficits.

Adaptation of compensatory eye movements is mediated by the olivo – cerebellar circuit that utilizes multiple mechanisms to recalibrate its output. The processes that involve acquisition and storage of the altered behavioural responses do not solely rely on LTD and LTP at parallel fiber to Purkinje cell synapse but also on LTP at mossy fiber to granule cell synapse.

Estradiol induces morphological changes and electrophysiological synaptic plasticity changes in the cerebellum that are well correlated with the cerebellum-dependent behavioral output.

In Greek mythology, Pandora (which means all-gifted) was the first woman on earth. Zeus ordered her creation as a punishment for Prometheus' having stolen fire and then giving it to humans. She brought with her down to earth a box which she was supposed not to open under any circumstances. However, her curiosity was stronger and she opened the box releasing its content. The very last thing to fly out of the box was hope. Hope remained ever since a powerful driving force for scientific research. Therefore, we still hope that one day the cerebellum contribution to VOR learning and memory will be completely answered.

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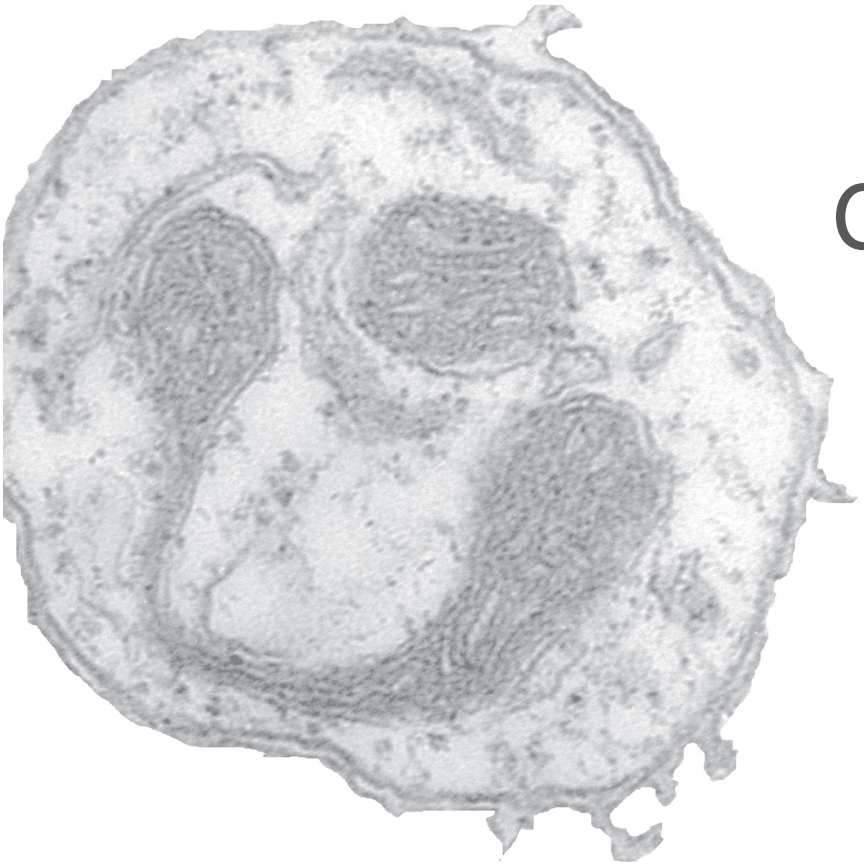

Chapter 6

Summary

Samenvatting

Summary

Motor learning is the mechanism by which the central nervous system adapts motor skills to comply with environmental pressures and constraints. Motor skills are typically acquired gradually without any noticeable conscious memory of what information has been gained.

The most prominent and most frequently studied form of motor learning and memory is the vestibulo-ocular reflex (VOR) adaptation. The VOR is a compensatory eye movement in response to stimulation of the vestibular organ. The vestibular organ contains specialised areas with receptors cells that translate head movement into neuronal signals: the semicircular canals and the otolith organs. Detection of head movements generates eye movements in the opposite direction, in order to stabilize a visual target on the retina. The mechanisms underlying adaptation of the VOR have been investigated with a variety of experimental paradigms in a number of different species.

In order to answer the question if and how various factors can alter compensatory eye movements and how the cerebellum contributes to these changes, we investigated the eye movements of a laboratory mouse. By using an integrated and multidisciplinary approach of genetic techniques, cellular physiology, and behavioral experiments in various mouse models, we were able to tackle some of these questions.

According to the multisensory integration theory, peripheral inputs like vestibular, optokinetic and proprioceptive inputs act in concert to maintain a stable retinal image of the visual world. It remains elusive to what extent the vestibular otolith organs contribute to this process and whether a specific loss of otolith organs input is compensated for. An efficient way to investigate the contribution of the otolith organs to the eye movements is to remove their stimulus. This is possible by investigating mice in space (i.e. under microgravity conditions) or by studying the “space mouse model”: the tilted mouse. The tilted mouse does not have functional otolith organs because it lacks otoconia due to a mutation in otopetrin 1 (**Chapter II**). Independent of head position with respect to gravity, the amplitude and timing of the VOR of tilted mice are significantly impaired compared to their wild type littermates. Furthermore, lack of otolith input increases the dependency of the vestibular system on stimulus frequency.

In contrast, the amplitude of optokinetic reflex in tilted mice is significantly higher in the low frequency range than in control mice. Thus our data support the presence of the multisensory integration system and revealed a compensatory enhanced optokinetic reflex in tilted mice, indicating an adaptive synergism in the processing of otolith-driven and visually-driven signals.

Several studies have demonstrated that multiple plasticity processes take place at the level of Purkinje cells and with AMPA receptors as the key element. However it is still unclear to what extent NMDA receptors contribute to cerebellar plasticity and whether a specific deficit of neuronal signals via NMDA receptors is important for VOR adaptation (**Chapter III**). We investigated the induction of long-term potentiation and adaptation of the vestibulo-ocular reflex in a mouse model (NMDA NR2A$^{-/-}$) in which the NR2 subunit was deleted. In NMDA NR2A$^{-/-}$ mice long-term potentiation (LTP) at mossy fiber – granular cell synapse was severely impaired. Furthermore, although these mice showed a normal motor performance, their capability for motor learning was affected. Mutant mice did show an adaptive capacity of the vestibulo-ocular reflex during a multi-day visuo-vestibular training paradigm, but were unable to consolidate their adapted phase values across training sessions. These data show that VOR gains and phases can be adapted in mice that lack LTP at mossy fiber – granular cell synapse, but at the same time that the newly obtained phase responses can not be consolidated due to the absence of NR2A.

Hormones can also affect the way various motor skills are learned, including VOR adaptation. Yet, it is not known how and to what extent gonadal hormones influence the achievement of accurate movements in time and space. Here (**chapter IV**), 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 LTP at the parallel fiber to Purkinje cell synapse, while it does not affect long term depression (LTD); second, we show that estradiol activation of ERß-receptors in Purkinje cells significantly improves gain-decrease adaptation of the vestibulo-ocular reflex, while it does not affect general eye movement performance; and third, we show that estradiol increases the density of parallel fiber to Purkinje cell synapses, while it does not affect the density of climbing fiber synapses. We conclude that estradiol can improve motor learning by potentiating cerebellar plasticity and synapse formation.

These processes may be advantageous during periods of high estradiol levels of the estrous cycle or pregnancy.

In summary, we can conclude that various factors influence cerebellar plasticity and cerebellar motor learning and that measuring compensatory eye movements is a powerful technique to investigate these factors. Compensatory eye movements rely on a multisensory integration system that induces execution of accurate eye movements in time and space. This multisensory integration system has also the capacity to compensate for certain deficits.

Adaptation of compensatory eye movements is mediated by the olivo - cerebellar circuit that utilizes multiple mechanisms to recalibrate its output. The processes that involve acquisition and storage of the altered behavioural responses do not solely rely on LTD and LTP at parallel fiber to Purkinje cell synapse but also on LTP at mossy fiber to granule cell synapse.

Estradiol induces morphological changes and electrophysiological synaptic plasticity changes in the cerebellum that are well correlated with the cerebellum-dependent behavioral output.

Samenvatting

Motorisch leren is het proces waarbij het centrale zenuwstelsel de motorische vaardigheden verbeterd om te kunnen voldoen aan de eisen die door het organisme of de omgeving gesteld worden. Motorische vaardigheden worden over het algemeen geleidelijk verkregen zonder een bewuste gewaarwording over datgene wat verkregen is. Het olivocerebellaire systeem heeft de capaciteit om de nieuw verkregen motorische vaardigheden tijdelijk op te slaan. Dit systeem en zijn moleculaire machinerie (die deze verkregen motorische vaardigheden opslaat) is onderhevig aan perifere, centrale en hormonale factoren.

Een zeer veel gebruikte methode om motorisch leren en het motorische geheugen te onderzoeken is het aanpassen van het vestibulo-oculair reflex (VOR). Het VOR is een compenserende oogbeweging, die geïnduceerd wordt door activatie van het evenwichtsorgaan. Het evenwichtsorgaan bevat een aantal gespecialiseerde structuren; de half cirkelvormige kanalen en de otolith organen. In deze structuren zitten haarcellen die de bewegingen van het hoofd omzetten in neuronale signalen. Het waarnemen van beweging van het hoofd door het evenwichtsorgaan veroorzaakt reflexmatige oogbewegingen in de tegenovergestelde richting, waardoor de visuele waarneming stabiel op de retina geprojecteerd wordt. Het mechanisme waarmee de VOR zich kan aanpassen is een geliefd model om motorisch leren te bestuderen. Dit model is dan ook al vele malen bestudeerd met een groot aantal verschillende methoden en in vele verschillen diersoorten.

Om de vraag te beantwoorden welke factoren (en hoe) deze compenserende oogbeweging kunnen veranderen en op welke wijze het cerebellum hieraan bijdraagt, zijn we oogbewegingen gaan meten in het proefdier “de muis”. Door gebruik te maken van de muis kunnen we deze factoren mbv een multidisciplinaire benadering (genetische technieken, cellulaire en systeem fysiologische technieken en gedragstudies) onderzoeken.

Volgens de multisensorische integratie theorie, werken perifere informatie stromen (zoals informatie van de evenwichtsorganen, de optokinetische informatie van de ogen en de proprioceptische informatie van de spieren) samen om beelden te stabiliseren op de retina. Tot nu toe was het onbekend wat de bijdrage was van de otolith organen aan dit proces en of het wegvallen van otolith informatie gecompenseerd wordt door andere systemen. Dit kun je onderzoeken door de wijze waarop deze organen gestimuleerd

worden weg te nemen. Een van de mogelijkheden is om muizen te onderzoeken onder gewichtloze condities, maar een goedkoper alternatief is om het “space mouse model” te onderzoeken: de tilted muis. De tilted muis heeft geen functionele otolith organen omdat deze muis geen otoconia (oorstenen) heeft ontwikkeld in deze organen door een mutatie in het otopetrin 1 gen (**Hoofdstuk II**). Onafhankelijk van de positie van de kop in relatie tot de zwaartekracht vertoont deze mutant muis een sterk verslechterde VOR ten opzichte van zijn niet gemuteerde familieleden. Het gebrek aan otolith informatie veroorzaakte ook een sterke afhankelijkheid van het VOR systeem voor de stimulatiefrequentie. Hiertegenover staat dat de amplitude van de OKR groter is, in het lage stimulusfrequentie bereik, bij de mutant muizen dan in zijn niet gemuteerde familieleden. Dit resultaat was onafhankelijk van de positie van de muis (ten opzichte van de gravitatie) en van de richting van de oogbeweging (i.e. vertikaal en horizontaal). Onze resultaten bevestigen de aanwezigheid van een multisensorische integratie systeem en laat de aanwezigheid van een compenserend mechanisme zien (OKR) in de mutant muizen, waarmee het (“multisensorische”) aanpassingsvermogen tussen de otolith en visuele signalen wordt aangegeven.

De algemene gedachtegang is, dat motorisch leren of de compensatie van de verschillende motorische tekortkomingen, komt door veranderingen in het olivocerebellaire circuit en de signalen die ze overbrengen. Verschillende studies hebben laten zien dat plasticiteitprocessen plaatsvinden op het nivo van de Purkinje cellen en hierbij neemt de AMPA receptor een centrale plaats in. Het is nog steeds onduidelijk in hoeverre NMDA receptoren een bijdrage kunnen leveren aan cerebellaire plasticiteitprocessen en in hoeverre deze NMDA receptoren belangrijk zijn voor motorisch leergedrag. In **hoofdstuk III** hebben we een cerebellaire plasticiteitproces (i.e. mossy fiber – granule cel synaps long term potentiatie: mf-gc LTP) en de adaptatie van de VOR onderzocht in muizen met en zonder de NMDA receptor subunit NR2A. In de mutant muizen zonder de NMDA NR2A was het vermogen om de synaptische verbindingen tussen de mosvezels en granule cellen te versterken enorm afgenomen. Deze mutant muizen hebben normale oogbewegingreflexen (OKR, VOR en VVOR), echter het aanpassen van de VOR vertoonde afwijkingen. De niet gemuteerde muizen zijn instaat om een fase verandering in de VOR te consolideren over een aantal die verspreid zijn over een aantal dagen. Onze resultaten laten zien dat in muizen die de NMDA receptor subunit NR2A missen, normaal de fase en de amplitude van de VOR kunnen aanpassen tijdens de leerperiode. Echter,

ze zijn niet instaat zijn om de nieuw aangeleerde VOR fase in het motorische geheugen vast te leggen. De NMDA receptoren zijn dus belangrijk voor een specifiek onderdeel van het motorische leren: het lange termijn motorische geheugen.

Hormonen beïnvloeden eveneens het motorische leergedrag, inclusief het aanpassen van de VOR. Het is echter nog niet bekend hoe en in welke mate geslachtshormonen het genereren van accurate beweging beïnvloed. In dit proefschrift (**hoofdstuk IV**), laten we met behulp van verschillende experimenten zien dat estradiol een bepaalde vorm van plasticiteit in de cerebellaire schors vergemakkelijkt; het proces dat vermoedelijk tengrondslag ligt aan een specifieke vorm van motorisch leren. Teneerste laten de experimenten zien dat estradiol het versterken van de synaptische verbinding tussen parallel vezel en Purkinje cel vergemakkelijkt, maar niet het verzwakkingproces van de synaptische verbinding beinvloedt. Ten tweede laten de experimenten zien dat oestradiol activatie van de estrogen receptors in de Purkinje cellen niet de normale oogbewegingreflexen (OKR, VOR en VVOR) beïnvloedt, maar wel het aanpassingsvermogen van de VOR verbetert. Ten slotte zien we ook dat door de aanwezigheid van oestradiol er meer parallel vezel-Purkinje cel synapsen zijn ontstaan, maar niet meer climbing fiber-Purkinje cel synapsen. Uit deze serie experimenten concludeerden wij dat oestradiol het motorische leerproces kan versnellen door het versterken van de synaptische verbinding te vergemakkelijken en meer synaptische verbinding aan te maken. Hierdoor is het motorische leerproces efficiënter gedurende de periodes met hoge oestradiol gehaltes in de menstruatie cycli of gedurende de hoge oestradiol gehalte tijdens de zwangerschap.

In het kort kunnen we concluderen dat er een groot aantal factoren zijn die het proces van cerebellaire plasticiteit en dientengevolge het proces van motorisch leren kunnen beïnvloeden. Het meten van compenserende oogbewegingreflexen is een kwantitatief sterke methode gebleken om de invloed van de verschillende factoren op het motorische leergedrag te onderzoeken. De compenserende oogbewegingen vertrouwt op een multisensorisch integratie systeem voor de accurate uitvoering van de oogbeweging in tijd en plaats. Dit multisensorisch integratie systeem heeft ook de capaciteit om te compenseren voor bepaalde tekortkomingen. Het is duidelijk dat zowel het cerebellum als de vestibulaire kernen bijdragen aan de juiste afstelling van de oogbewegingen. De processen, die betrokken zijn bij de geheugenopslag en uitvoering van opnieuw

afgestelde oogbeweging zijn niet alleen afhankelijk van synaptische processen tussen de parallel vezels en de Purkinje cellen, maar ook van de synaptische processen tussen de mosvezels en granule cellen. Dus het aanpassingsvermogen van de compenserende oogbewegingen wordt gemedieerd door het olivocerebellaire circuit die door meerdere mechanismen/factoren beïnvloed kan worden om de oogbeweging accuraat af te stellen.

List of abbreviations

ACSF	artificial cerebrospinal fluid
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	adenosine triphosphate,
AOS	accessory optic system
aVOR	angular vestibulo-ocular reflex
aVVOR	angular visually enhanced vestibulo-ocular reflex
BAPTA	1,2-bis-(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid)
BSA	bovine serum albumin
CAMKII	calcium/calmodulin-dependent protein kinase II
cAMP	cyclic adenosine monophosphate
cGK I	cGMP-dependent protein kinase type I
cGMP	cyclic guanosine monophosphate
CF	climbing fiber
CR	corneal reflection
DNA	deoxyribonucleic acid
E	the position of the eye
E2	17ß-estradiol
EPSP	excitatory postsynaptic potential
ERα	estrogen receptor-α
ERß	estrogen receptor-ß
ERs	estrogen receptors
FSH	follicle-stimulating hormone
GnRH	gonadotropin-releasing hormone
GABA	γ-aminobutyric acid receptor
GC	granular cells
GTP	guanosine triphosphate,
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
IO	inferior olive
KO	knockout mice
L7-PKCI	mutant mice selectively expressing an inhibitor of protein kinase C in Purkinje cells
L7-ERß$^{-/-}$	mutant mice selectively missing estrogen receptor beta in Purkinje cells

LH	luteinizing hormone
LTD	long-term depression
LTP	long-term potentiation
MAP	mitogen activated protein
mGluR1	metabotropic glutamate receptor 1
MF	mossy fibers
MF – VN	mossy fiber - vestibular nuclei synapse
mRNA	messenger ribonucleic acid
NMDA	N-methyl D-aspartate receptor
NMDA-NR2A$^{-/-}$	null - mutant mice with NR2A expression lost thoughout the brain
NMDA-NR2AΔC/ΔC	NMDA mutanat mice with C-terminal deletions of NR2A
NR 1, 2, 3	subunits of the NMDA receptor
OKR	optokinetic reflex
OMN	oculomotor nucleus
Otop1	otopetrin 1
P	the position of the pupil
PC	Purkinje cells
PKC	protein kinase C
PF	parallel fibers
PF- PC	parallel fibers - Purkinje cells synapse
PPF	paired-pulse facilitation
Rh	horizontal roll
Rp	the radius of rotation of the pupil
Rv	vertical roll
Sg	Scarpa's ganglion
Tlt	tilted mice
VOR	vestibulo-ocular reflex
VVOR	visually enhanced vestibulo-ocular reflex
VN	vestibular nuclei
Yh	horizontal yaw
Wt	wild type mice

Curriculum Vitae et Studiorum

CORINA EMILIA ANDREESCU

Corina Andreescu, was born in Brasov, Romania. She attended both primary and secondary school in Brasov. After graduating from high school, she started her medical studies at the University of Medicine and Pharmacy "Iuliu Haţieganu" in Cluj-Napoca. After six years of university studies she obtained her Medical Doctor degree in 1999. Immediately after, she was offered the position of teaching assistant at the department of Physiology of the University of Medicine and Pharmacy "Iuliu Haţieganu" Cluj-Napoca. In this capacity Corina participated in many scientific and educational projects while also being involved in teaching physiology to medical students. During this period a research project of the department of Physiology drew her interest towards neuroscience. In 2001 she started her residency in endocrinology in Cluj-Napoca. In January 2003 she received a PhD position at the Neuroscience department of the Erasmus Medical Center in Rotterdam that resulted in this thesis. Corina Andreescu will continue practicing medicine at the department of internal medicine at the Rainier de Graaf hospital in Delft.

List of publications

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Andreescu CE, D'Errico A, De Jeu MTG, Rossi P, Botta L, Kohl G, Perin P, D'Angelo E, De Zeeuw CI Role of LTP at the Mossy Fiber to Granule Cell Synapse in Cerebellar Motor Learning (*to be submitted*)

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CE Andreescu, CI De Zeeuw, MTG De Jeu (2004) Keeping balance in space. T*hird Dutch Endo-Neuro-Physo Meeting*, Doorwerth, The Netherlands

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