Plasticity in eye movement control

Plasticiteit in de controle van oogbewegingen

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Chapter 1

General Introduction

Introduction

Learning is the process by which new information is acquired and implemented by the central nervous system in order to adapt to changes of the environment. Memory is the process by which this new information is encoded, stored, and later retrieved.

Humans have at least two qualitatively different systems of information storage, which are generally referred to as declarative (explicit) memory and nondeclarative (implicit) memory. Declarative memory is the storage (and retrieval) of material that is available to consciousness and can therefore be expressed by language, images, sound and so on. Nondeclarative memory, on the other hand, is not available to consciousness, at least not in any detail. Nondeclarative memory includes motor skills, cognitive skills, simple classical conditioning, priming effects, and other information that is acquired and retrieved unconsciously. Nondeclarative memory builds up slowly, through repetition over many trials, and is expressed primarily in performance, not in words. It includes perceptual and motor skills and the learning of certain types of procedures and rules (Kandel, 2000). In this thesis, we investigated one specific process of nondeclarative memory formation, namely motor learning. The cerebellum is thought to contain the site for nondeclarative information storage of "successful" motor learning tasks (i.e. amplitude and timing of movements).

Lesions of the cerebellum can cause general dysfunctions like ataxia and hypotonia and, in addition, rather specific defects can be attributed to lesions of particular parts of the cerebellum. For example, lesions of the nodulus and flocculus of the vestibulocerebellum predominantly evoke disturbances in balance and eye movement control, respectively (Ito et al., 1982). Cerebellar cortical lesions can also prevent and abolish motor learning (Ito et al., 1982; McElligott et al., 1998; Robinson, 1976; Thach et al., 1992; Thompson, 1990; Yeo et al., 1984). In general, the studies on cerebellar function show that the cerebellar cortex plays an important role in the recalibration and adaptive adjustment of movements, as well as in learning new motor skills and motor related associations (Houk et al., 1996; Ohyama et al., 2003; Raymond et al., 1996; Thach, 1996).

To get a better understanding in how the cerebellum processes and stores information, we use specific perturbations that affect the information processing of the cerebellum. With the use of genetic tools or by application of antibodies, signal transduction pathways were affected that were considered important for cerebellar function. To study normal and altered cerebellar processing, we used the oculomotor system. The relative simplicity of the oculomotor system and the fact that the behavioural output and sensory input can easily be measured makes this a highly accessible system for quantitative analysis. Furthermore, the extremely regular anatomical organization throughout the cerebellar cortex (Eccles et al., 1967) suggests that the computation performed by the cerebellum are similar for all its target systems (Albus, 1971; Marr, 1968). Understanding cerebellar contribution to the control of eye movements may therefore lead to a general comprehension of the cerebellar role in other forms of motor behavior.

Vestibulo-ocular and optokinetic reflexes

Compensatory eye movements are generated in order to minimize movement of the image

on the retina during self motion. Such retinal image motion would degrade visual acuity. Information from the vestibular and optokinetic sensory systems are used to compensate the oculomotor plant, which encompasses the eye ball, the extra ocular muscles and non-muscular supporting tissues. The vestibulo-ocular reflex (VOR) is a compensatory eye movement in response to a head movement. Head accelerations are detected by the semicircular canals (angular accelerations) and otolith organs (linear accelerations). This vestibular information is centrally processed and transformed into an appropriate command signal that will drive the oculomotor plant in opposite direction of the head movement. The optokinetic reflex (OKR) is an eye movement in response to movement of images across the retina (retinal slip). This optokinetic eye movement reduces the amount of retinal slip, which defines the OKR as a closed negative feedback system that will keep the retinal image motion as small as possible. The optokinetic system acts in concert with the vestibulo-ocular system to maintain stability of images on the retina over a broad range of head movements. In order to maintain accurate compensatory eye movements throughout life, recalibration and adaptive adjustment of the VOR and OKR are necessary. Vision provides that calibrating mechanism. The VOR and OKR circuitry possesses a measure of plasticity that allows its internal parameters to be tuned by vision to appropriately scale eye movements output to head movement input. Such long term modifications can be experimentally achieved by accompanying head rotations with an altered visual feedback (Harrison et al., 1986; Ito, 1979).

Anatomy of the floccular cerebellum

The flocculus of the cerebellum is involved in the control of the gain and phase dynamics of the OKR, the VOR, adaptation of the VOR, and in the case of primates in the control of smooth pursuit eye movements (Ito, 1984). To fulfil these functions, the flocculus receives many different afferents conveying different signals related to eye movements, and, in turn, it projects to various nuclei in the complex of vestibular and cerebellar nuclei to influence ultimately the activity of oculomotor neurons (Figure 1). These afferents include the climbing fibers, mossy fibers, and mono-aminergic fibers, while the efferent pathway of the flocculus is formed by the Purkinje cell projections.

The climbing fiber input to the flocculus is, similar to that of other parts of the cerebellum, strongly topographically organized. The climbing fiber input to Purkinje cells in the rabbit flocculus can be divided into five zones: four visual zones and one nonvisual zone (De Zeeuw et al., 1994b; Tan et al., 1995). The visual Purkinje cells receive their climbing fiber input from the dorsal cap and/or ventrolateral outgrowth of the inferior olive. The climbing fiber activities (i.e. complex spikes) of these Purkinje cells modulate optimally to optokinetic stimulus (De Zeeuw et al., 1994b). Optokinetic slip signals are conveyed from the retina through the nuclei of the accessory optic system and inferior olive (Simpson et al., 1996).

The mossy fiber input to the flocculus, which is mostly bilateral, is much more diverse than the climbing fiber input and is derived from many different sources. The sources of the mossy fiber projections to the flocculus include the medial vestibular nucleus, descending vestibular nucleus, and superior vestibular nucleus: [rabbits (Alley et al., 1975; Yamamoto, 1979b), cats (Kotchabhakdi and Walberg, 1978; Sato et al., 1983), rats (Blanks et al., 1983)], group y: [rhesus macaque (Langer et al., 1985b)], nucleus prepositus hypoglossi: [cats (Kotchabhakdi et al., 1978),

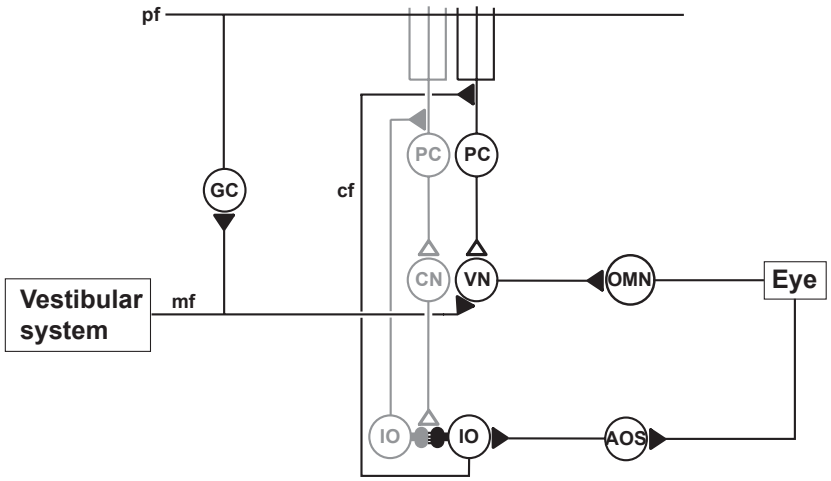

Figure 1: A simplified schematic representation of the oculomotor pathway involved in the vestibulo-ocular reflex (VOR). The major pathway that mediates the VOR is the three-neuron arc. This open loop is formed by the vestibular ganglion cells that receive input from the vestibular system, the second-order vestibular nuclei (VN) neurons that are innervated by the primary afferents from these ganglion neurons, and the oculomotor (OMN) neurons that innervate the oculomotor muscles. The floccular side loop is able to mediate the VOR by innervating the deep cerebellar nuclei (CN) neurons and VN neurons. Vestibular information is relayed to the floccular Purkinje cells (PC) via mossy fibers (mf), Granule cells (GC) and parallel fibers (pf). Retinal slip, detected by the eye, is processed by the accessory optic system (AOS) and inferior olive (IO). Neurons of the IO innervate the floccular PC's through climbing fibers (cf). Note that the PC's involved in horizontal compensatory eye movements project to the medial VN and the nucleus prepositus hypoglossi and that the PC's involved in vertical compensatory eye movement project to superior VN and ventral dentate nuclei/ dorsal y group. Closed loop indicated in grey; open triangle: inhibitory synapse; closed triangle: excitatory synapse.

rabbits (Barmack et al., 1992a; Barmack et al., 1992b; Yamamoto, 1979a)], nucleus abducens: [cats (Kotchabhakdi and Walberg, 1977), rabbits (Yamamoto, 1979a)], nucleus reticularis tegmenti pontis: [cats (Hoddevik, 1977), rabbits (Yamamoto, 1979a)], and, via the output of the brush cells, the flocculus itself (Mugnaini et al., 1997). Similar to the climbing fibers, most of the mossy fibers in the flocculus use glutamate as their neurotransmitter (Voogd et al., 1996).

The mono-aminergic inputs to the flocculus are mainly formed by serotonergic and noradrenergic fibers; a dopaminergic input to the flocculus is virtually absent (Ikai et al., 1992; Panagopoulos et al., 1991). The serotonergic input to the flocculus, which is relatively weak, is mainly derived from the nucleus pontis oralis and the nucleus reticularis (para)giga ntocellularis(Bishop et al., 1985). The fibers consist mainly of long axons containing many boutons de passage terminating in all three cortical layers, i.e. the granular cell layer, Purkinje cell layer, and molecular layer (Bishop et al., 1985). The serotonin receptors (type 1b) are primarily located in the molecular layer (Pazos and Palacios, 1985). The noradrenergic projection to the flocculus originates from the locus coeruleus (van Neerven et al., 1990). These fibers also terminate in both the granular cell layer and molecular layer of the flocculus (Kimoto et al., 1981). The noradrenergic receptors (mainly β type) are predominantly concentrated in irregular patches in the Purkinje cell layer (Sutin and Minneman, 1985).

The Purkinje cell axons form the one and only output of the flocculus. They project to target cells in the cerebellar and vestibular nuclei that themselves do not project

to the flocculus. The projections of Purkinje cells from the flocculus to the complex of cerebellar and vestibular nuclei in the rabbit are, similar to the climbing fiber projections, strongly topographically organized [rabbit (De Zeeuw et al., 1994b); monkey (Langer et al., 1985a); cat (Sato et al., 1988); rat (Umetani, 1992)]. Furthermore, Purkinje cell axons from the flocculus can branch and innervate different nuclei (De Zeeuw et al., 1994b). Individual Purkinje cell of the flocculus can participate in two pathways that are involved in compensatory eye movements: a closed (via inhibitory neurons) and an open (via predominantly excitatory neurons) anatomical pathway (De Zeeuw et al., 1994b). The ventral dentate nucleus, dorsal group y and nucleus prepositus hypoglossi, which project to the dorsal cap and ventrolateral outgrowth, are part of a circuit linking the inferior olive, the cerebellar cortex, and the cerebellar or vestibular nuclei (De Zeeuw et al., 1994a; De Zeeuw et al., 1993; Voogd and Bigaré, 1980). This circuit is referred to as the closed olivofloccular pathway (Figure 1: grey loop), because a specific olivary subnucleus provides the climbing fibers to a particular zone of Purkinje cells that innervates a specific cerebellar nucleus that, in turn, projects to the corresponding olivary subnucleus (De Zeeuw et al., 1997; Voogd and Bigaré, 1980). In contrast, the medial vestibular nucleus and superior vestibular nucleus do not project to the dorsal cap and ventrolateral outgrowth, and, thus, this arrangement is referred to as the open olivofloccular pathway. Interestingly, the floccular receiving neurons in the open pathway (medial vestibular nucleus and superior vestibular nucleus) receive a direct input from the semicircular canals and are second-order neurons in the three-neuron arc that underlie the vestibulo-ocular reflex (Buttner and Buttner-Ennever, 1988), whereas the flocculus receiving neurons in the closed pathway (ventral dentate nucleus, dorsal y and nucleus prepositus hypoglossi) do not receive a direct input from the primary afferents of the semicircular canals. It is noteworthy that an axon of an individual Purkinje cell can branch and innervate two different nuclei, one branch can be part of the open pathway while the other is part of the closed pathway, and thereby the Purkinje cell can simultaneously influence loops that are intimately connected with either the primary vestibular afferents or the inferior olive, establishing a site for cross-coupling.

Floccular processing and plasticity

Potential cell physiological and molecular mechanisms underlying VOR adaptation

The open floccular pathway is anatomically placed as a side loop to direct vestibular pathways that mediate the VOR. Based on this anatomical architecture (Figure 1), the cerebellar flocculus is a good candidate to provide a site for the adaptive mechanism that ensures precision of visual stability during movements (Ito, 1982). Changes in VOR can be induced in altered visuovestibular environments (Gonshor and Jones, 1973) and this adaptation process can be eliminated by lesioning the flocculus (Ito, 1982; Lisberger et al., 1984; Nagao, 1983; Robinson, 1976; Zee et al., 1981). Experiments of McElligott et al. (McElligott et al., 1998) and Nagao and Kitazawa (Nagao and Kitazawa, 2003), in which the VOR was adapted by visual-vestibular mismatch training and this adaptation once established was abolished following inactivation of the flocculus strongly suggest that the modification of the VOR are, at least initially, stored in the flocculus. Whether the memory trace for the adapted VOR always remains in the flocculus is still unclear.

The models of cerebellar function and plasticity that were proposed by Marr (Marr, 1968) and Albus (Albus, 1971) provide the basis for understanding how the flocculus could

contribute to plasticity in the VOR (Fujita, 1982). In general, these models propose that the modulated discharge of the mossy fiber/parallel fiber system represents the conditions and context in which a movement is learned and performed, and the inferior olive/climbing fiber system transmits performance error signals. When such an error signal is conveyed by the climbing fiber, the strength of the parallel fiber/purkinje cell synapses that are active at the same time, is reduced (i.e. parallel fiber-Purkinje cell long-term depression (PF-LTD)) (Albus, 1971). Single unit recordings of floccular Purkinje cells provided indirect evidence for PF-LTD and showed that spiking activity of Purkinje neurons could indeed be depressed by pairing activation of vestibular mossy fibers to the flocculus with stimulation of climbing fibers (Ito, 1982). Confirmation of cellular plasticity in the flocculus led to the hypothesis that climbing fibers, which carry retinal error signals (Maekawa and Kimura, 1974; Maekawa and Takeda, 1976), could cause a depression for the appropriate parallel fiber inputs to keep the VOR calibrated and hence reduce retinal error (Ito, 1982). Intracellular recordings in cerebellar slices provided the additional evidence that parallel fiber to Purkinje cell transmission can be depressed (Ito and Kano, 1982). However, this does not prove that PF-LTD is the only cellular mechanism to store information obtained during cerebellar motor learning. There is also evidence that information storage at the deep cerebellar nuclei (DCN) and VN play an important role in several types of motor learning (Bear and Linden, 2000; Mauk, 1997; Raymond et al., 1996).

Molecular mechanisms of long-term depression/potentiation

Three initial signals are required for the induction of PF-LTD: activation of AMPA and of mGluR1 receptors by glutamate release from PF, and depolarization of the Purkinje cell by the climbing fiber (Figure 2)(Ito, 2001). AMPA receptor activation causes a local depolarization of PC spines, leading to influx of Ca^{2+} through voltage-gated Ca^{2+} channels (VGCC), which in turn enhances IP_3-mediated Ca^{2+} release from intracellular stores (Okubo et al., 2001). Activation of mGluR1 results in G-protein coupled activation of phospholipase C and the subsequent production of IP_3 and DAG. IP_3 binds to intracellular IP_3 receptors, resulting in a release of Ca^{2+} from intracellular stores (Finch and Augustine, 1998; Takechi et al., 1998). The climbing fiber activity causes a widespread AMPA-mediated depolarization in the dendritic tree. This depolarization in turn initiates dendritic calcium entry via VGCC (Watanabe et al., 1998). Altogether, paired activation of climbing and parallel fibers generates a supralinear Ca^{2+} signal (Wang et al., 2000). Both this supralinear increased Ca^{2+} signal and DAG are required for activation of PKC. Activated PKC phosphorylates AMPA-receptors containing GluR2/3 (Chung et al., 2003). These phosphorylated AMPA-receptors are internalized, resulting in a reduction of parallel-fiber/climbing fiber synaptic transmission (Wang and Linden, 2000).

Another pathway involved in PF-LTD is mediated by nitric oxide (NO) (Lev-Ram et al., 1995). Genetic ablation of neuronal NO synthase impairs cerebellar LTD (Lev-Ram et al., 1997) and adaptation of compensatory eye movements (Katoh et al., 2000). The molecular and cellular mechanisms of cerebellar NO signalling are not completely understood. Indirect evidence from experiments with cerebellar slices suggests that NO induces LTD via activation of soluble guanylyl cyclase and subsequent cGMP synthesis in PCs (Boxall and Garthwaite, 1996; Daniel et al., 1993; Hartell, 1996; Hartell, 2001; Lev-Ram et al., 1997). The identification of the signalling components downstream of cGMP is complicated by the existence of multiple receptors for cGMP (Beavo and Brunton, 2002).

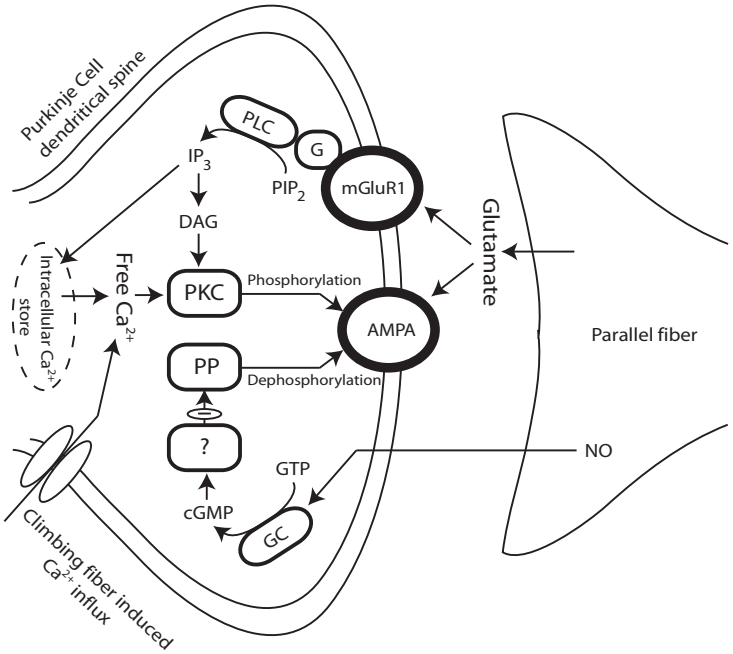

Figure 2: A simplified schematic representation of the biochemical processes underlying cerebellar long-term depression. Phosphorylation of the AMPA receptors results in an internalization of these AMPA receptors, which subsequently leads to a reduction of synaptic strength. MGluR1, metabotropic Glutamate Receptor type 1; G, G-protein; PLC, Phospholipase C; IP_3, Inositol 1,4,5-triphosphate; PIP_2, Phosphatidylinositol 4,5-biphosphate; DAG, 1,2,-diacylglycerol; PKC, Protein Kinase C; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; NO, Nitric oxide; GC, Guanylyl cyclase; GTP, guanosine triphosphate; cGMP, cyclic guanosine monophosphate; PP, protein phosphatases; VGCC, Voltage-gated Calcium channel.

The parallel fiber-Purkinje cell synapses cannot only be depressed, but can also show long term potentiation (PF-LTP). Two different forms of PF-LTP can be induced; a presynaptic PF-LTP and a postsynaptic PF-LTP. The presynaptic PF-LTP is not an appropriate mechanism for reversing postsynaptic PF-LTD, it might only mask the postsynaptic PF-LTD. The postsynaptic form of PF-LTP does provide a good cellular basis for the reversal mechanism of PF-LTD (Coesmans et al., 2004; Lev-Ram et al., 2003). In general, LTP and LTD reversibility is essential for maximizing information storage (Willshaw and Dayan, 1990). Physiologically, the up- and down-modulation of the vestibulo-ocular reflex can also be best explained by LTD and LTP reversing each other at the same synapses (Boyden and Raymond, 2003).

Scope of this thesis

The general aim of our studies was to investigate the mechanisms underlying cerebellar motor coordination and motor learning. In this thesis, we used the oculomotor system of genetically altered or pharmacologically treated mice to study these mechanisms.

The anatomical and/or physiological zones of the flocculus have been mapped

for many species, but little is know about the organization of the flocculus in the mouse. Therefore, we first studied the zonal organization of the flocculus in mice (Chapter 2). With the use of electrophysiological and immunohistochemical techniques, a "physiologically zonal map" of mouse flocculus has been constructed. In Chapter 3, we investigated our first mutant mouse: the Lurcher mouse. This Lurcher mouse completely lacks cerebellar Purkinje cells, which allowed us to investigate the importance of the flocculus pathway in controlling and modifying eye movements. In Chapter 4, we looked further into the Purkinje cell mechanisms involved in the modification of eye movements which are known to impair the induction of LTD. By using a PC specific cGKI knockout mouse, we investigated the role of this protein (potential target of cGMP in NO pathway) in LTD and cerebellar learning. In Chapter 5, we investigated potential mechanisms underlying motor coordination deficits in Paraneoplastic cerebellar ataxia (PCA) patients with mGluR1 auto-antibodies. By using the mouse model, we were able to investigate in what way these auto-antibodies affect Purkinje cell functioning and motor learning. In Chapter 6, we investigated another plastic eye movement paradigm, i.e. vestibular compensation following unilateral labyrinthectomy that does not depend on LTD. This thesis is concluded by a general discussion (Chapter 7).

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Chapter 2

Zonal Organization of the Mouse Flocculus: Physiology, Input and Output

Zonal Organization of the Mouse Flocculus: Physiology, Input and Output

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Abstract

The zones of the flocculus have been mapped in many species with a noticeable exception, the mouse. Here, the functional map of the mouse was constructed via extracellular recordings followed by tracer injections of biotinylated-dextran-amine and immunohistochemistry for heat-shock protein- 25 (HSP-25). Zones were identified based on the Purkinje cell complex spike modulation occurring in response to optokinetic stimulation. In zones 1 and 3 Purkinje cells responded best to rotation about a horizontal axis that is perpendicular to the ipsilateral anterior semicircular canal, whereas in zones 2 and 4 they responded best to rotation about the vertical axis. The tracing experiments showed that Purkinje cells of zone 1 projected to the parvicellular part of lateral cerebellar nucleus and superior vestibular nucleus, while Purkinje cells of zone 3 projected to group Y and the superior vestibular nucleus. Purkinje cells of zones 2 and 4 projected to the magnocellular and parvicellular parts of the medial vestibular nucleus, while some also innervated the lateral vestibular nucleus or nucleus prepositus hypoglossi. The climbing fiber inputs to Purkinje cells in zones 1 and 3 were derived from neurons in the ventrolateral outgrowth of the contralateral inferior olive, whereas those in zones 2 and 4 were derived from the contralateral caudal dorsal cap. Purkinje cells in zones 1 and 2, but not in zones 3 and 4, were positively labeled for HSP25. The present study illustrates that Purkinje cells in the murine flocculus are organized in discrete zones with specific functions, specific input – output relations, and a specific histochemical signature.

Abbreviations

AchE	acetylcholinesterase
BDA	biotinylated dextran amine
CDC	caudal dorsal cap
DC	dorsal cap
DM	dorsomedial group
Fl	flocculus
FRN's	flocculus receiving neurons
HA	horizontal axis
HSP-25	heat-shock protein-25
icp	inferior cerebellar peduncle
IntA	anterior interposed nucleus of the cerebellum
IntP	posterior interposed nucleus of the cerebellum
IO	inferior olive
LatPC	parvicellular part of lateral cerebellar nucleus
LVN	lateral vestibular nucleus
Med	medial cerebellar nucleus
MVe	medial vestibular nucleus
MVem	magnocellular part of medial vestibular nucleus
MVep	parvicellular part of medial vestibular nucleus
OKR	optokinetic reflex
PFl	paraflocculus
PrH	nucleus prepositus hypoglossi
RDC	rostral dorsal cap
scp	superior cerebellar peduncle
SuVe	superior vestibular nucleus
VA	vertical axis
VLO	ventrolateral outgrowth
VOR	vestibulo-ocular reflex
WGA-HRP	wheat germ agglutinated horseradish peroxidase

Introduction

The flocculus of the cerebellum plays an important role in the control of compensatory eye movements (for review see De Zeeuw et al., 2004). The anatomical and/or physiological zones of the flocculus have been mapped for various species, including monkey (Voogd et al., 1987), cat (Groenewegen and Voogd, 1977; Gerrits and Voogd, 1982), rabbit (De Zeeuw et al., 1994; Van der Steen et al., 1994; Tan et al., 1995a), and rat (Ruigrok et al., 1992; Sugihara et al., 2004). The organization of the flocculus in mammals generally follows that of the cerebellar cortex in that sagittal zones of Purkinje cells project to specific parts of the cerebellar and vestibular nuclei (Yamamoto et al., 1978; Voogd and Bigaré, 1980; Sato et al., 1982a, b; Tan et al., 1995a; Balaban et al., 2000; Sugihara et al., 2004) and that they receive their climbing fibers from a specific group of neurons in the contralateral inferior olive (IO) (Groenewegen and Voogd, 1977; Groenewegen et al., 1979; Ruigrok et al., 1992; Tan et al., 1995c; Sugihara et al., 2004). In most mammals the zones can be morphologically discriminated by using acetylcholinesterase staining as a biochemical marker and/or by tracing the climbing fiber inputs anterogradely from the olive (Voogd and Bigaré, 1980; Tan et al., 1995c). To date, little is known about the floccular organization in the mouse; so far no biochemical marker has been correlated to their zones and the topography of their climbing fiber projections has not been elucidated.

Climbing fibers potentials evoke complex spikes in Purkinje cells of the cerebellar cortex (Eccles et al., 1966; Thach, 1967). In the flocculus of both rabbits and mice, the complex spike activity of Purkinje cells is modulated optimally in response to rotational optokinetic stimulation about either the vertical axis (VA) or the horizontal axis (HA) that is approximately perpendicular to the ipsilateral anterior semicircular canal (Graf et al., 1988; Goossens et al., 2004; Hoebeek et al., 2005). In zones 1 and 3 of the rabbit flocculus, complex spike activity is optimally modulated in response to optokinetic stimulation about the HA oriented at 45º contralateral azimuth/135º ipsilateral azimuth, while complex spike activities of Purkinje cells in zones 2 and 4 are optimally modulated in response to optokinetic stimulation about the vertical axis (De Zeeuw et al., 1994). Whether the distribution of Purkinje cells with different preferred axes of optokinetic modulation in mice follows the same zonal pattern as in rabbits is unknown.

Purkinje cells in the flocculus are known to project to various parts of the cerebellar and vestibular nuclei, as demonstrated in primate (Haines, 1977; Langer et al., 1985), cat (Voogd, 1964; McCrea et al., 1979; Sato et al., 1982a), rabbit (Alley, 1977; Yamamoto and Shimoyama, 1977; Balaban, 1987), and rat (Balaban et al., 2000). These studies used degeneration and/or tracing of large groups of axons, mostly of multiple zones. Until recently studies on the efferent projection of individual zones of the flocculus were mostly done with retrograde axonal labeling from injections in the vestibular nuclei (Yamamoto et al., 1978; Sato et al., 1982b; Tan et al., 1995a) and therefore did not provide detailed information on the precise termination of the Purkinje cell axons. The only study using anterograde axonal transport from discrete injections of individual Purkinje cells is the study by De Zeeuw et al. (1994). However, the Purkinje cell projections from this study could not be directly correlated to the specific olivary origins of the climbing fiber inputs to the flocculus due to the use of biocytin, which is exclusively an anterograde tracer.

To overcome this limitation as well as to find out whether and how the flocculus

of the mouse is organized in zones, we placed small injections of biotinylated-dextran-amine (BDA), which acts as both an anterograde and a retrograde tracer, into areas that were identified electrophysiologically by recording climbing fiber responses of Purkinje cells during optokinetic stimulation. The injections labeled small areas of Purkinje cells as well as their climbing fiber inputs, and thus allowed us to trace both their afferent and efferent projections of identified floccular zones in the same experiment. In addition, we investigated to what extent immunoreactivity for heat-shock protein-25 (HSP-25), which has recently been demonstrated to reveal distinct bands in the vestibulocerebellum of mice (Armstrong et al., 2000), could be correlated to the physiological identity and projection pattern of these zones. Thus in conjunction, the current study provides a full description of the anatomical and physiological organization of the flocculus of the mouse, which nowadays with the advent of transgenics, is the first mammal of choice.

Material and Methods

Surgery

Eighteen adult C57BL/6 mice were prepared for neurophysiological experiments under anesthesia consisting of a 1:2 mixture of O_2, N_2O, and 1.5% isoflurane. An acrylic head fixation pedestal was fixed to the skull by M1 screws, and a recording chamber was made following a craniotomy (diameter ~3 mm) of the left occipital bone (Goossens et al., 2001). The animals were allowed to recover for 5 days before the start of the recording and injection sessions.

Recordings and injections

The animals were restrained in a custom made plastic tube, which was placed in the center of a random dotted drum that could be rotated around a variety of vertical or horizontal axes in space (Stahl et al., 2000; van Alphen et al., 2001). Extracellular Purkinje cell activity was recorded using filament containing borosilicate glass pipettes (OD 2.0 mm, ID 1.16 mm, tip diameter 2.0 μm) filled with 2M NaCl. For amplification, data acquisition, and analysis, we used Cyberamp (CED, Cambridge, UK), Spike 2 software (CED) and custom-written Matlab routines (Mathworks, USA), respectively. A floccular zone was identified by its complex spike responses to optokinetic stimulation (De Zeeuw et al., 1994); for tuning curves in mice see (Hoebeek et al., 2005), and subsequently the recording pipette was exchanged for a pipette (tip diameter 2 μm) that was filled with 10% BDA. After the floccular zone was re-identified based on the complex spike modulation recorded with the BDA pipette, an iontophoretic injection was made using a constantly monitored anodal current of 1-4 μA, pulsed 7 seconds on, 7 seconds off, for a period of 10 minutes. Following the injection, the brain was covered with gramicidin-containing ointment and sealed with bone wax (Hoebeek et al., 2005).

Tissue processing

After 5-7 days the mice were deeply anesthetized with pentobarbital (200 mg/kg) and perfused transcardially with 4% paraformaldehyde. The brains were postfixed for 1 hour in 4% paraformaldehyde, embedded in gelatin (11%) and sectioned transversally at 40 mm with a freezing microtome. Sections were serially collected in four glass vials in which subsequent rinsing and incubation procedures were performed. Vials 1 and 3 were incubated for both BDA

and HSP25 staining, whereas vials 2 and 4 only served for BDA histochemistry. All sections were rinsed for 30 min in Tris buffered saline (TBS, pH 7.6), incubated for 90 min in a solution of TBS / 0,05% Triton-X100 / 10% normal horse serum to block non-specific protein-binding sites, incubated overnight at 4°C in avidin-biotin-peroxidase complex (Vector Laboratories, Inc. Burlingame, Ca), rinsed again, and finally incubated in diaminobenzidine (75 mg/100 ml). In vials 1 and 3, cobalt ions were added to the incubation bath in order to obtain a black reaction product. The reaction was stopped after 15-20 minutes by rinsing in TBS. Vials 1 and 3 were subsequently incubated with the rabbit-anti-mouse HSP25 antibody (SPA-801, StressGen, Victoria, BC) diluted in TBS containing 0.5% Triton-X100 and 2% normal serum. After 48 hours incubation in the dark at 4°C, the sections were rinsed in TBS (3 times) and then incubated 90 min with the secondary antibody biotinylated goat-anti-rabbit antibody (1:200, Vector Laboratories, Burlingame, CA). After this incubation, the sections were rinsed in TBS for 60 minutes, and incubated again 90 min with the Avidin-Biotin-Complex (Vector Laboratories, Inc., Burlingame, CA) in TBS with 0,5% Triton-X100. This step was followed by 3 x 5 min rinsing in TBS and 3 x 5 min in 0.1 M Tris buffer (pH 7.6). This time, the complex was visualized by diaminobenzidine (DAB, 75mg/100 ml) only, thus resulting in a brown precipitate at places where HSP25 was present. Sections of vials 2 and 4 in which only BDA was visualized as a brown precipitate, served as additional sections for analysis and plotting. The sections were mounted, air-dried, counterstained with thionin, dehydrated through graded alcohol series, cleared in xylene, and cover-slipped with Permount (Fisher Scientific).

Histological analysis

The histological material was analyzed with a Leica DMR light microscope equipped with a DC 300 digital camera. Graphical plots of anterograde and retrograde labeling in the cerebellar and vestibular nuclei were made with an Olympus BH-2 microscope equipped with a Lucivid miniature monitor (Microbrightfield, Colchester, VT) and a motorized X, Y and Z stage drive, and Neurolucida software (Microbrightfield). Similar plots were made of retrograde labeling within the inferior olivary complex from which a standardized diagram was prepared of the caudal inferior olivary nucleus (Ruigrok et al., 1992). Olivary labeling was subsequently entered within this standardized diagram. To map the flocculus, digital prints were prepared of all consecutive sections from the caudal-most to the rostral-most part of the flocculus. In all sections four reference points were indicated which served to construct a map of the unfolded flocculus (Ruigrok et al., 1992; Balaban et al., 2000) (see Figure 2). The unfolded flocculus map was transformed by custom-written MATLAB routines.

Results

Purkinje cell recordings and injections

Most of the complex spike activities of the Purkinje cells in the flocculus of the mouse responded optimally to optokinetic stimulation along either the VA or the HA perpendicular to the ipsilateral anterior semicircular canal (Figure 1). In all these cases the simple spikes modulated out of phase with respect to the complex spike activities (Figure 1C, D). The 18 small BDA injections that were made were distributed throughout the entire caudal-rostral and medio-lateral extent of the areas in the flocculus that responded to optokinetic stimulation

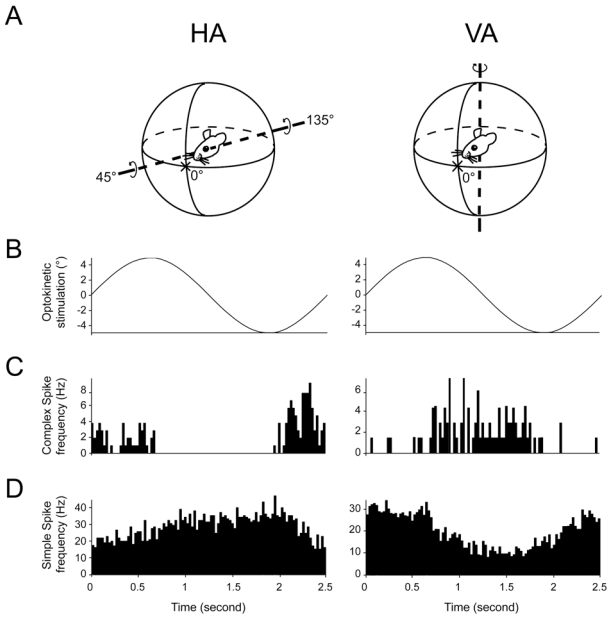


Figure 1.
Complex spike and simple spike activities of Purkinje cells in the flocculus are modulated by HA or VA visual field rotation. **A**, This panel shows the spatial orientation of the optokinetic stimulation used to determine the preferred axis for each Purkinje cell. The visual field was rotated around the ipsilateral 135° posterior - contralateral 45° anterior axis in the horizontal plane (HA; left column) or the vertical axis (VA; right column). Arrows in A indicate the direction of rotation that results in increased complex spike activity in the left flocculus. **B**, Sinusoidal optokinetic stimulation was presented at 0.4 Hz. **C**, Examples of peristimulus time histograms of complex spike activity of two Purkinje cells showing an optimal response to HA (left) or VA (right) optokinetic stimulation. **D**, As in **C** but for simple spikes; note reciprocal modulation in that increased simple spike activity is accompanied by decreased complex spike activity and vice versa. The histograms in **C** and **D** show the average firing frequency over 40 cycles per bin (bin width is 25 msec).

(Figure 2). The cells that modulated optimally to the HA were clustered either in a zone located caudally in the flocculus (referred to as zone 1) or in a zone located in its rostral half (referred to as zone 3) (see Figure 2D). In contrast, the VA cells were all located in between or rostrally to these zones (referred to as zone 2 and zone 4). Caudal to zone 1, no cells were found that responded to optokinetic stimulation; in this area, which probably corresponds to the C2-zone (see De Zeeuw et al., 1994; De Zeeuw and Koekkoek, 1997), no injections were made. Among the eighteen BDA injections that were made in the floccular areas that showed responses to optokinetic stimulation, four were made into zone 1, eight in zone 2, five in zone 3, and one in zone 4 (Table 1). Two of these injections, i.e. nr 17770-8 in zone 1 and nr 15733-1 in zone 3, extended somewhat into the ventral paraflocculus. A large part of the Purkinje cells that were located in the three most caudal zones, i.e. zone C2, zone 1 and zone 2, were positively labeled for HSP-25 (Figure 2B, C). In contrast, none of the Purkinje cells in zones 3 and 4 showed any immunoreactivity for HSP-25. In conjunction, these data indicate that the vast majority of Purkinje cells in the flocculus of the mouse respond to optokinetic

stimulation about axes that run through the horizontal semicircular canal or ipsilateral anterior semicircular canal, that these cells are organized in parasagittal zones, and that they can be partly identified by immuno-histochemical staining.

Projections from the inferior olive to the flocculus

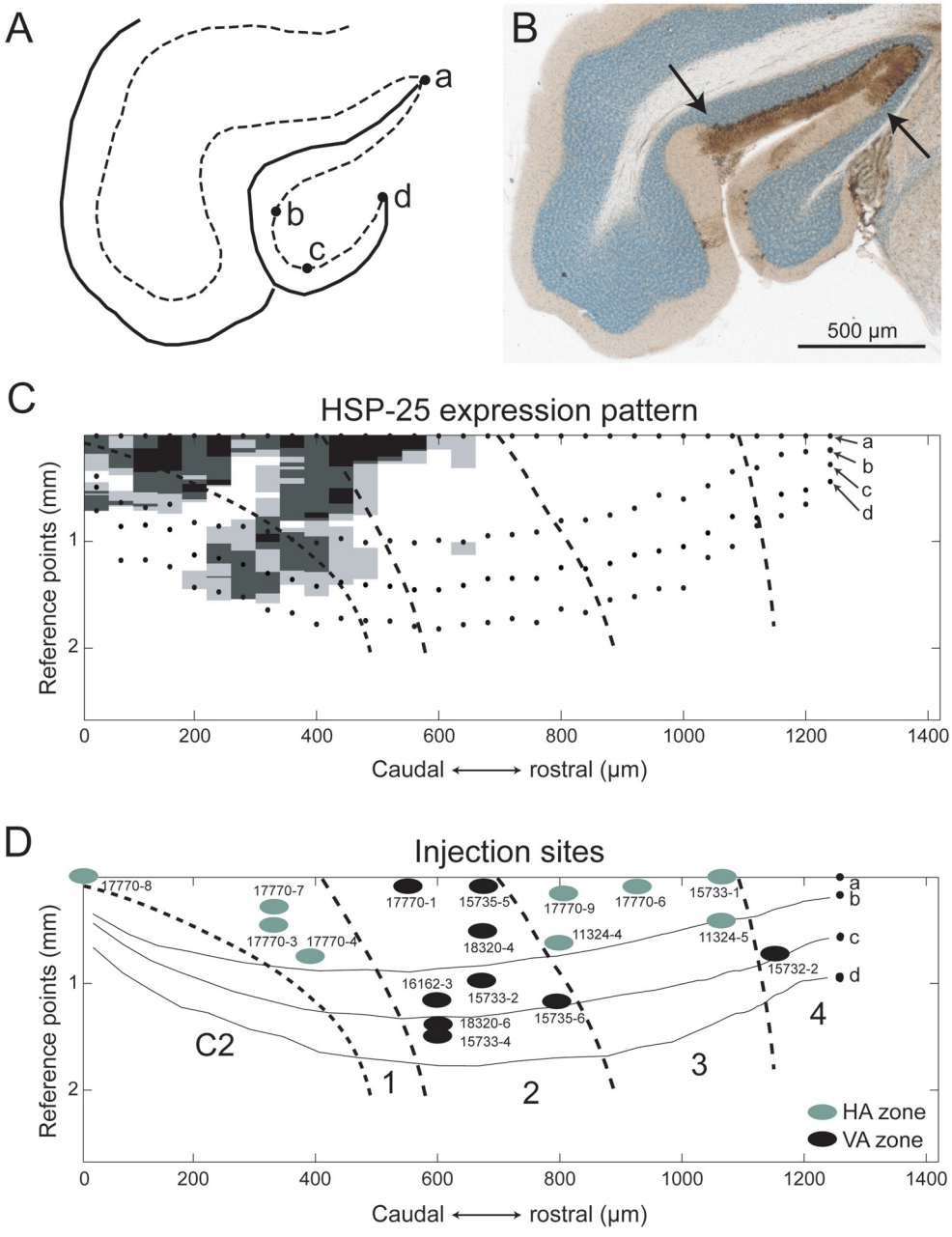
The data described above indicate that the complex spike activities of Purkinje cells in zones 1 and 3 modulate optimally around an axis that is perpendicular to the preferred axis of Purkinje cells in zones 2 and 4. These results suggest that the climbing fibers that evoke these different activities originate from different clusters of neurons in the inferior olive. We therefore investigated whether the BDA injections in zones 1 and 3 resulted in different sets of retrogradely labeled neurons than those in zones 2 and 4. This prediction did hold (Table 1). The injections of BDA in zones 1 and 3 resulted in labeled neurons in the contralateral ventrolateral outgrowth (VLO), whereas those in zones 2 and 4 resulted in retrogradely labeled neurons in the contralateral caudal dorsal cap (CDC) (Figure 3). In all cases the injections were relatively small and the number of retrogradely labeled neurons never exceeded 15. In 1 case, mouse 15735-5, the cluster of retrogradely labeled neurons in the caudal dorsal cap extended into a more rostral area approaching the rostral dorsal cap and VLO. Note that the injection site in this case was located near the border between zones 2 and 3 (Figure 2D). In 3 cases we did not observe any retrogradely labeled neuron in the inferior olive (Table 1). Thus, collectively from these results we conclude that the sources of the climbing fiber projections to the different floccular zones in the mouse are compatible with the preferred complex spike modulations of their Purkinje cells. The climbing fiber projections to both HA zones (1 and 3) are derived from the same olivary subnucleus, the VLO, while those to the VA zones (2 and 4) are both derived from a different subnucleus, the caudal DC.

Purkinje cell projections

The specific climbing fiber inputs as well as the preferred complex spike and simple spike modulations of the Purkinje cell zones described above would theoretically be most effective were it maintained in the nuclei receiving Purkinje cell inputs as well. We therefore investigated whether the BDA injections in the flocculus that provided the zone-specific retrograde labeling in the different olivary subnuclei, also showed anatomically discrete anterograde labeling of Purkinje cell axons and terminals.

The injections into the HA zones 1 and 3 demonstrated similar, yet not identical, projection patterns. Labeled fibers originating from zone 1 followed the floccular peduncle from which they turned caudally towards the parvicellular aspect of the lateral cerebellar nucleus (LatPC) where a dense axonal terminal plexus was observed in a confined narrow region (Figures 3C and 4A). Other fibers followed an arching route through the LatPC passing just dorsal to the inferior cerebellar peduncle and Y-nucleus. At that point the fibers turned rostromedially and reached the SuVe where a few fibers and terminals were found. The fibers of zone 3 followed the same route as the fibers of zone 1, but they provided more terminal boutons in the SuVe and showed terminals in group Y instead of the LatPC (Figure 4).

The injections into VA zones 2 and 4 demonstrated projection patterns that clearly diverged from those of zones 1 and 3 (Table 1). Purkinje cell axons derived from zone 2 coursed ventrally to the LatPC and passed group Y without providing terminals to these regions. They descended ventromedially to the lateral vestibular nucleus (LVN), nucleus prepositus


A
a
b
c
d
B
500 µm
C
HSP-25 expression pattern
Reference points (mm)
Caudal ⟷ rostral (µm)
D
Injection sites
17770-8
17770-7
17770-3
17770-4
17770-1
15735-5
17770-9
17770-6
15733-1
11324-4
11324-5
18320-4
16162-3
15733-2
18320-6
15733-4
15735-6
15732-2
C2
1
2
3
4
HA zone
VA zone

Mouse	Physiology	Zone	Ipsilateral projection	Contralateral inferior olive
17770-3	HA	1	LatPC, SuVe	No labeling
17770-4	HA	1	LatPC, SuVe	VLO
17770-7	HA	1	LatPC, SuVe	VLO
17770-8	HA	1	No projection	VLO
18320-4	VA	2	No projection	CDC
15735-6	VA	2	MVep, some in LVN	No labeling
15733-4	VA	2	MVep, MVem, some in LVN	CDC
18320-6	VA	2	MVep, MVem	CDC
15733-2	VA	2	No projection	No labeling
17770-1	VA	2	MVem, MVep, some in PrH	CDC
16162-3	VA	2	MVep	CDC
15735-5	VA	2	SuVe and MVem, MVep	CDC
11324-4	HA	3	SuVe and MVep, group Y	VLO
15733-1	HA	3	Group Y	VLO
11324-5	HA	3	SuVe, group Y	VLO
17770-6	HA	3	SuVe, group Y	VLO
17770-9	HA	3	SuVe, group Y	VLO
15732-2	VA	4	MVem, MVep, some in LVN	CDC

▲Table 1. List of mice. Complex spike responses to HA or VA optokinetic stimulations, Injection, projection sites of BDA-labeled floccular Purkinje cells, and retrogradely labeled neurons in the contralateral IO.

◀Figure 2.

The flocculus of the mouse can be divided into five functional zones. **A**, Schematic representation of the flocculus showing the four reference points (a, b, c and d) used in each floccular section (40 μm) for analysis. **B**, This panel illustrates an example of HSP-25 staining in the (para)flocculus (borders of the HSP-25-immunopositive area are indicated by arrows). **C**, Schematic representation showing the HSP-25 expression pattern (from gray to black in increasing intensity) in the flocculus of the mouse with reference points a-d unfolded along the y-axis and consecutive sections presented in the rostrocaudal direction along the x-axis. Dashed lines indicate the borders of the functional zones identified by electrophysiological recordings. **D**, This panel shows the BDA injection sites in HA (gray) and VA (black) zones superimposed on the scheme presented in C. The numbers refer to the animals involved. Note that HSP-25 labeling is restricted to zones 1, 2, and C2.

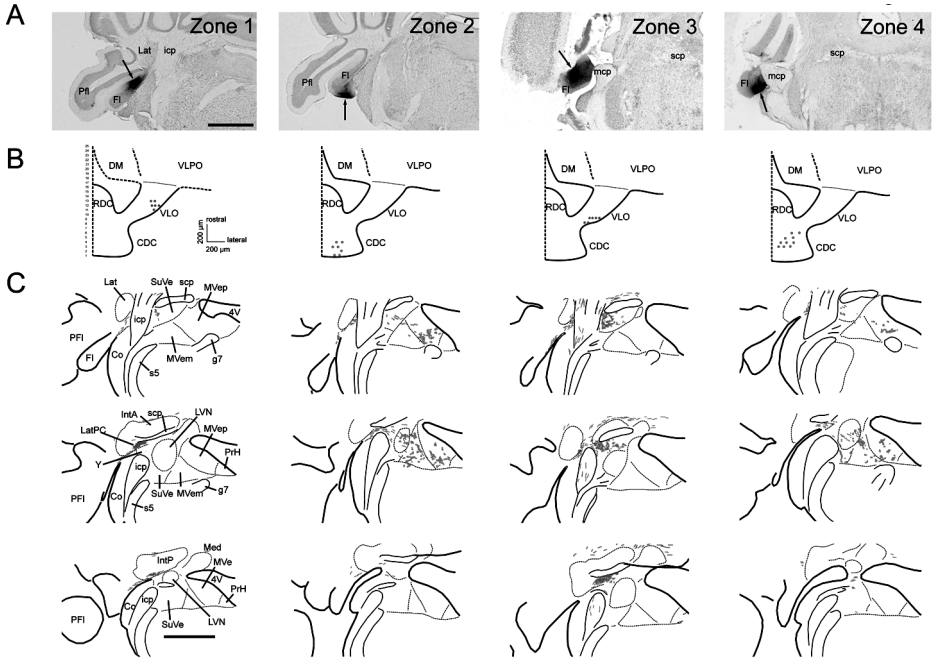


Figure 3.
Floccular zones receive specific climbing fiber inputs and project to discrete regions in the midbrain. **A**, Micrographs showing examples of BDA injection sites in zones 1, 2, 3 and 4 in the mouse flocculus. **B**, Reconstructions of corresponding retrograde labeling in the contralateral inferior olive illustrate that the injections in zones 1 and 3 labeled olivary neurons (dots) in the ventrolateral outgrowth (VLO), while the injections in zone 2 and 4 labeled olivary neurons in the caudal dorsal cap (CDC). **C**, Reconstructions of corresponding projection patterns shows that the injection in zone 1 (HA) resulted in Purkinje cells projecting to LatPC and SuVe, that the injection in zone 3 (HA) resulted in Purkinje cells projecting to group Y and SuVe, and that the injections in zones 2 and 4 (VA) resulted in Purkinje cells projecting to MVem and MVep, and in these two cases to lesser extend also in the LVN.

hypoglossi (PrH) and/or the magnocellular and parvicellular parts of the medial vestibular nucleus (MVem and MVep). Axon terminals were observed in all these regions, but the terminal arborizations were much more extensive in the MVem and MVep than in the LVN or PrH. Within the MVem many labeled terminals were distributed around large somata and proximal dendrites (Figure 4G). In the case in which the injection site was close to the border with zone 3 (nr 4-15735-5), some of the fibers also projected to the SuVe. The labeled Purkinje cell axons derived from zone 4 demonstrated a similar pattern of terminal arborizations as those of zone 2; most labeling was observed in the MVem and MVep, some in the LVN, and no axonal varicosities were seen within the confines of the LatPC or group Y.

These data allow us to conclude that the Purkinje cell projections from HA zones (1 and 3) diverge from those of VA zones (2 and 4) and that the specifics of these projection patterns match remarkably well with the characteristics of the climbing fiber inputs described above.

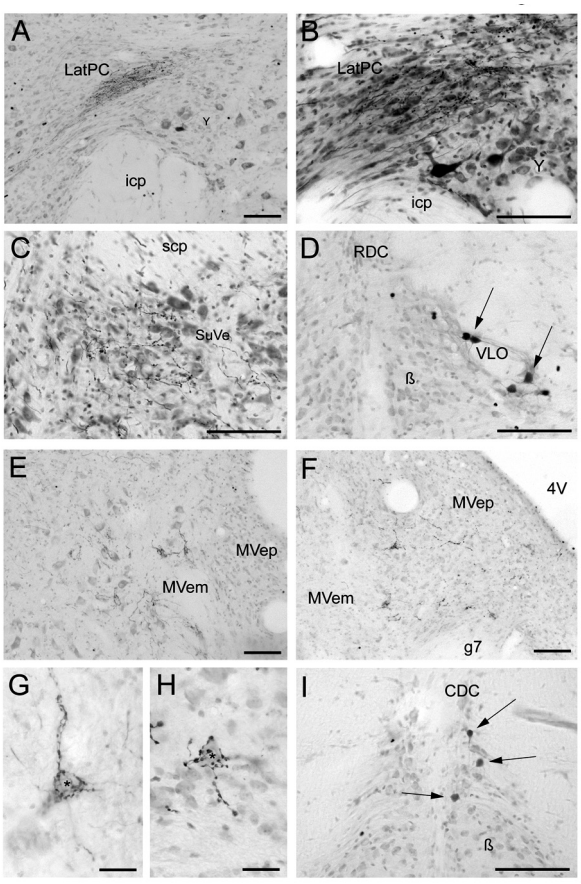


Figure 4.
Micrographs showing labeling characteristics after BDA injections into identified zones of the mouse flocculus. **A**, Anterograde labeling of Purkinje cell axons and terminals within the LatPC following injection in zone 1 (case 17770-7). **B**, Purkinje cell terminal arborizations are labeled within LatPC and group Y after BDA injection in zone 3 (case 11324-5). **C**, Same case also shows efferent labeling within the lateral part of the SuVe. **D**, Retrograde labeled neurons (arrows) are present in the VLO after BDA injection in zone 3 of the contralateral flocculus (case 17770-6). **E**, Varicose fibers and terminals in the MVem are labeled after injection into zone 2 of the flocculus (case 15733-4). **F**, Same case also shows labeled fibers and terminals in the MVep. **G** and **H,** These panels show examples of vestibular nucleus neurons (asterisks) that are surrounded by labeled terminal fibers after BDA injections into zone 2 (case 15733-4) and zone 4 (case 15732-2), respectively. ***I***, Retrograde labeling of olivary neurons (arrows) in the CDC after BDA injection into zone 4 of the flocculus (case 15732-2). Scale bars represent 25 µm in **G** and **H** and 100 µm in all other panels.

Discussion

In the present study, for the first time, we demonstrated that subpopulations of Purkinje cells reside in anatomically and physiologically discrete zones in the mouse flocculus. In addition, we have shown that the individual zones of Purkinje cells receive climbing fiber inputs from different regions of the inferior olive and project to different sets of nuclei and subnuclei, thereby retaining structural and functional separation at all three levels of the cerebellar modules.

Zones in the mouse flocculus

The mouse flocculus was found to have four parasagittal zones that responded to optokinetic stimulation about particular axes in space (zones 1 to 4) and one zone that was non-responsive to optokinetic stimulation (C2 zone). Zones 1 and 3 contained Purkinje cells responding optimally to visual field rotation around the 45º contralateral azimuth - 135º ipsilateral azimuth HA, while Purkinje cells located in zones 2 and 4 showed a maximal response to rotation around the VA. These data agree with the divisions that have been found in the

rabbit flocculus following both zone-specific recordings and zone-specific stimulations. De Zeeuw and colleagues (De Zeeuw et al., 1994) showed a near-identical organization of zones in the rabbit flocculus following recordings of complex spike and simple spike activities, while Van der Steen and colleagues (Van der Steen et al., 1994) were able to evoke binocular eye movements about the same HA and VA axes in space by electrical microstimulation of the corresponding zones. In these studies the sites of the recordings or stimulations were marked by a tracer and/or electrical lesion and subsequently correlated to anatomical zones, the borders of which were visualized with the use of acetylcholine-esterase (AchE) (Tan et al., 1995b). In mice, however, neither AchE nor any other biochemcial marker tested to date clearly labels the borders between the zones in the vestibulocerebellum (De Zeeuw et al., 2004). However, Armstrong and colleagues have recently made note of HSP-25 patterning in the vestibulocerrebelum (Armstrong et al., 2000). Therefore, we tested the usefulness of Hsp-25 as a morphological marker for zonal borders and found that this protein is expressed by Purkinje cells in HA zone 1, VA zone 2, and the non-HA / non-VA zone C2, but not by those in zones 3 and 4. Thus, although we have not been able to find a specific morphological marker for either the VA zones or HA zones, we did find a marker to segregate the two most rostral zones from the three caudal zones in mice.

The most rostral floccular VA zone (zone 4), which we have found both in mice and rabbits, has not been described for the cat at the physiological level. Sato and Kawasaki (1984) found three zones in the flocculus of the cat following electrical stimulation; stimulation of a caudal zone produced downward eye movements, while stimulation of a middle and rostral zone produced ipsilateral horizontal and upward eye movements, respectively. Thus, the organization of these zones suggests that the caudal zone of the cat flocculus corresponds to zone 1 of the mouse flocculus, the middle zone to zone 2 and the rostral zone to zone 3. It appears likely though that the cat flocculus also has a rostral VA zone, because it can be found at the anatomical level following anterograde tracing of its climbing fibers (Gerrits and Voogd, 1982). Possibly, the rostral VA zone was missed in the physiological study in the cat, because it is too narrow to be reliably detected by electrical stimulation. In support of this point, it should be noted that in rabbits too zone 4 could not be detected by electrical stimulation (Van der Steen et al., 1994), while it was evident following single unit recordings of modulating Purkinje cells (De Zeeuw et al., 1994).

Similarly, Gerrits and Voogd (1982) found the existence of the C2 zone in the cat flocculus based on its 3D-structure, its relation with adjacent cerebellar structures, and its connections. Presumably the cat C2 zone is like that in mice and rabbits in that it is not directly involved in the optokinetic reflex. The function of the C2 zone could be related to head movements, as stimulation of this zone in the flocculus evokes short-latency head movements (De Zeeuw and Koekkoek, 1997).

Climbing fiber input to the mouse flocculus

The observation that complex spike activities in the mouse flocculus can be divided into discrete zones based on their responses to optokinetic stimulation around particular axes in space agreed well with the finding that these zones receive their climbing fiber inputs from different olivary subnuclei. We showed that Purkinje cells in HA zones 1 and 3 receive their climbing fibers from the VLO, while those in VA zones 2 and 4 receive their climbing fibers from the caudal DC. These data largely agree with the findings by Tan and colleagues (1995c)

in rabbit, except that zones 1 and 3 in that species also receive inputs from the rostral DC. The zonal organization of the olivocerebellar projection to the flocculus in the mouse also shows a pattern similar to that in the rat (Ruigrok et al., 1992; Sugihara et al., 2004); zones FD and FD' in the rat correspond to zones 1 and 3 and are innervated by the VLO, while zones FE and FE' are innervated by the dorsal cap and correspond to zone 2 and zone 4, respectively. Thus, one can conclude that the VLO and caudal DC in animals in the grandorder glires are generally involved in vertical and horizontal optokinetic eye movements, respectively, and that the olivary projections to the flocculus in the mouse resemble those that have been described for rats and rabbits.

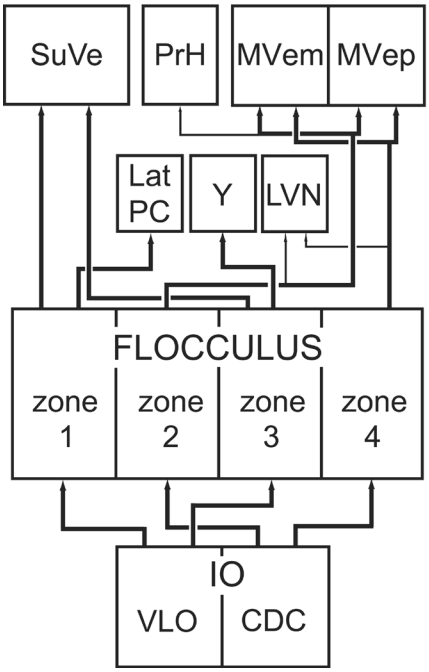


Figure 5.
Summary of afferent and efferent pathways of Purkinje cells in the mouse flocculus. Line width indicates the relative strength of the projection.

Purkinje cell projections of the mouse flocculus

The organization of the output of the flocculus in mice was as zone-specific as that of its climbing fiber input and its Purkinje cell activities. Our findings reveal that Purkinje cells of zone 1 project to the LatPC and/or SuVe, that Purkinje cells of zone 3 project to group Y and/or SuVe, and that those of zone 2 and 4 project to the MVem and MVep, and occasionally to the LVN and PrH. These results largely agree with degeneration and tracing studies in rabbits (Yamamoto et al., 1978; Yamamoto, 1979; De Zeeuw et al., 1994; Tan et al., 1995a), cats (Voogd, 1964; Sato et al., 1982ab; Carleton and Carpenter, 1983; Dietrichs, 1983; Langer et al., 1985), monkeys (Balaban et al., 1981; Carleton and Carpenter, 1983; Langer et al., 1985) and rats (Bernard, 1987; Umetani, 1992; Balaban et al., 2000). The present study provides the first direct evidence that Purkinje cells in VA zone 2 project directly to neurons in the PrH. A projection from the flocculus to the PrH has been suggested before in the rabbit (Yamamoto,

1978), but De Zeeuw and colleagues (1994) were unable to confirm this, let alone to determine from which zone it was derived. In addition, we observed a projection from zone 3, but not zone 1, to group Y in mice, while in rabbits both zones have been found to project to group Y (De Zeeuw et al., 1994). Thus, there may be subtle differences between mice and other mammals, but the main topographical organization appears very similar.

Finally, the current study provides the first direct evidence for mammals that the specific output connections of the floccular zones perfectly match their specific climbing fiber inputs. We have been able to directly show this relation by combining anterograde and retrograde transport of a single tracer, BDA. Fortunately, the speed and efficiency of the anterograde and retrograde transport of BDA were sufficiently comparable to allow us to identify both labeled neurons in the inferior olive and sites of Purkinje cell terminations in the cerebellar and vestibular nuclei in single sections following single injections. Whenever the source of the climbing fibers was not discrete, for example when an injection site was close to the border between two zones, the Purkinje cell projections were similarly more diffuse. Therefore, the current data allow us to conclude that the olivocerebellar modules as originally described by Voogd and colleagues (Voogd and Bigaré, 1980) hold valid and reach an extremely high level of precise topography.

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Chapter 3

Motor Performance and Motor Learning in Lurcher Mice

Motor Performance and Motor Learning in Lurcher Mice

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ABSTRACT: In adult Lurcher mice virtually all cerebellar Purkinje cells have degenerated as a direct consequence of mutant gene action, providing a natural model for studying the effect of cerebellar cortical lesions on the generation of compensatory eye movements. Lurcher mice possess both optokinetic (OKR) and vestibular (VOR) compensatory reflexes. However, clear differences were observed in control of the OKR consisting of a large reduction in gain and a moderate increase in phase lag. Minor differences were also observed in the VOR in that gain and phase lead of the reflex were both increased in Lurcher animals. Subjecting Lurcher animals to eight days of visuovestibular training tested the assumption that increased VOR gain reflected an adaptive mechanism within remaining brainstem oculomotor pathways to compensate for the reduced OKR. Contrary to control animals, Lurcher animals were unable to modify either VOR or OKR in the course of training and therefore confirmed that an intact cerebellum is indispensable for the implementation of adaptive modifications to the oculomotor system.

KEYWORDS: Lurcher mouse; OKR; VOR; visuovestibular adaptation

INTRODUCTION

Oculomotor reflexes like the optokinetic reflex (OKR) and the vestibulo-ocular reflex (VOR) reduce slip of visual images across the retina by generating eye movements that are compensatory to head motion or movement of the visual surround. Both reflexes are mediated by a neural network that is largely located in the brain stem[1–3] and supported by a parallel pathway through the cerebellum. The cerebellar side loop has been deemed particularly necessary for maintaining accuracy of compensatory ocular reflexes,[4,5] a process referred to as motor learning.

With the discovery of Purkinje cell–specific gene promotors, it has become possible to switch individual genes on or off at will, allowing us to study cerebellar information processing and motor learning in a controlled way at the level of individual proteins.[6,7] As a reference against which to compare such specific molecular modifications, it seems prudent to quantify in mice the worst intervention possible, that is, complete ablation of the cerebellar cortex. Rather than attempting

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extensive surgical procedures, we chose to use Lurcher mice, a strain of mutant mice that completely lacks cerebellar Purkinje cells, making their deficit equivalent to a complete cerebellar cortical lesion. The underlying pathology of Purkinje cell death has been traced to a mutation of δ2-glutamate receptor (GluRδ2) resulting in a gain of this receptor's function that triggers apoptotic cell death of Purkinje cells in these animals.[8–10]

The Lurcher strain provides us with a model for cerebellar cortical lesions, which is highly reproducible between individual animals and allows us to judge how ocular reflexes are controlled on a subcortical level. Previous studies have attempted to describe the oculomotor performance of Lurcher mice, but they failed to be quantitatively accurate due to the use of a large scleral search coil that partially impeded eye movements.[11,12] We therefore expand upon these previous studies by using a less invasive "mini"-search coil technique to investigate Lurcher oculomotor behavior over a wider range of stimulus parameters and by assessing the extent to which motor plasticity still occurs in these Purkinje cell– deficient animals.

METHODS

The eye movement performance of 15 Lurcher mutant mice and 13 normal control littermates was tested. The procedures for implanting a head fixation pedestal and a mini-search coil were identical to those previously described for this laboratory.[13] Animals were allowed to recover for at least three days prior to the first recording session. The local ethical committee of the Erasmus University Rotterdam approved animal care and experimental protocols.

The optokinetic reflex (OKR) and vestibulo-ocular reflex (VOR) and visually enhanced VOR (VVOR) were measured. Two different approaches were used to test the performance of the optokinetic system. First, the frequency response of the OKR was tested by sinusoidally rotating a vertically striped (width of bars 4°) optokinetic drum around the animal at frequencies of 0.1, 0.2, 0.4, 0.8, and 1.6 Hz while peak stimulus velocity was kept constant at 8°/s. Second, the effect of peak velocity on gain of the OKR was evaluated by varying peak stimulus velocity between 2, 4, 8, 16, and 32°/s at 0.4 Hz stimulus frequency. The VOR was elicited by sinusoidal, whole-body rotation in the dark. Sinusoidal stimulation was performed at 0.1, 0.2, 0.4, 0.8, and 1.6 Hz and 10° stimulus amplitude. The VVOR was tested using the VOR stimulus parameters while the animal viewed the illuminated, earth-fixed drum.

Gain of the eye movement (eye velocity/head velocity) and phase of eye movement with respect to stimulus movement were calculated by fitting a sine wave to the average response using least-square optimization. When eye movement lagged stimulus movement, phase was expressed with a negative sign. Phase relations of VOR were shifted by 180°, making the phase angle zero for perfectly compensatory responses. All values reported are mean and standard error, weighting each mouse equally. All signal analysis was done with MATLAB (Mathworks Inc., Natick, MA).

A VOR reversal paradigm was used to test VOR adaptation. In this paradigm the animal had to learn to reverse the natural direction of the VOR. Reversal training lasted for eight days and began by rotating the optokinetic drum in phase, that is, 0° phase difference, with table rotation at 5° amplitude. In the following five days, am-

plitude of the optokinetic drum was increased by 1°/day until it was 10° on day 6. At this point the optokinetic drum was rotating in phase with table rotation but at twice the amplitude. Amplitude of the optokinetic drum remained 10° from day 6 on. Every day the VOR was checked before training to monitor progression of VOR change.

Differences between Lurcher and control animals in gain or phase of OKR, VOR, and VVOR were tested for statistical significance using a two-way, repeated measures ANOVA. The effect of visuovestibular training on OKR and VOR was tested for each mouse separately using a one-way, repeated measures ANOVA. Statistical analysis was performed, using a commercial software package called SAS-6.12 (SAS Institute Inc., Cary, NC).

RESULTS

Motor Performance

Lurcher mice were easily distinguished from control littermates by their ataxic behavior, which was visible as a wobbly, lurching gate. Oculomotor control of Lurcher mice was considerably less accurate than that of normal control animals, as reflected by a large decrease in OKR gain across all tested peak velocities (FIG. 1A,

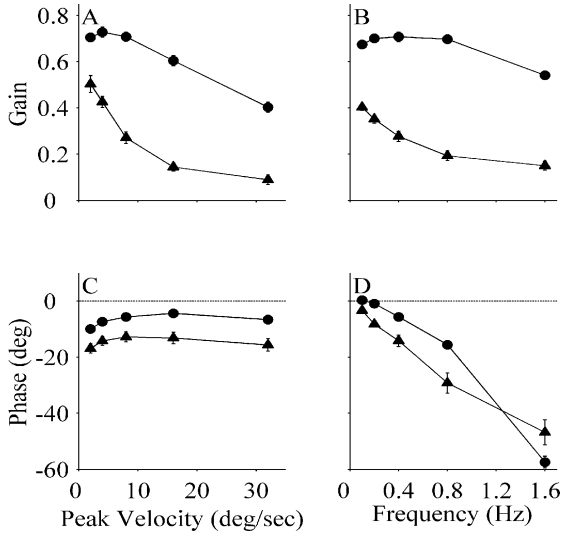

FIGURE 1. The OKR of Lurcher (*triangles*) and control (*circles*) animals is presented in BODE plots relating gain and phase of the response to peak stimulus velocity (**A** and **C**) and stimulus frequency (**B** and **D**). Lurcher OKR gain was found to be smaller than that of control animals across all stimulus conditions. In addition to a reduced OKR gain, Lurcher animals also showed increased phase lag of eye velocity with respect to stimulus velocity. Phase lag at 1.6 Hz was not significantly different from that of control animals ($p = 0.14$). The error bars indicate 1 SEM.

p <0.001). While OKR gain of normal animals remained fairly constant at 0.7 for stimulus velocities smaller than 8°/s and decreased for higher peak velocities, that of Lurcher animals was already considerably smaller at 2°/s and declined immediately when peak stimulus velocity was increased. The increased dependence of Lurcher OKR on peak velocity was reflected in the two-way ANOVA by a significant interaction (p <0.001) between the factors: animal type and peak stimulus velocity. Performance of the OKR was also tested against stimulus frequency while peak stimulus velocity was kept constant at 8°/s. OKR gain in Lurcher animals again showed a significant depression over the entire range of tested frequencies when compared to control animals (FIG. 1B, p <0.001). Again, interaction between animal type and stimulus frequency proved significant (p <0.001) in the analysis, reflecting the fact that OKR gain remained relatively constant up to 0.8 Hz in control animals, but showed no such stability in Lurcher animals. The reduced OKR gain of Lurcher animals was accompanied by an increase in phase lag of the eye movement with respect to stimulus movement. Eye movements of Lurcher mice generally lagged stimulus movement more than those of control animals (FIG. 1D, $p = 0.03$). Phase lag was significantly different at all tested frequencies (p <0.01, two-tailed t test with Bonferroni correction) except 1.6 Hz. The stronger dependence of OKR gain on frequency and the increased phase lag indicate that the absence of cerebellar cortical output in Lurcher animals has changed the low-pass filter characteristics of the OKR in the closed-loop situation by an apparent increase of the overall time constant.

The VOR of Lurcher animals was similar to that of control animals in that VOR gain increased with frequency, measuring 0.07 ± 0.008 and 0.59 ± 0.02 at 0.1 Hz and 1.6 Hz, respectively (FIG. 2A). The relation of gain with frequency was complemented by a reduction in phase lead for increasing stimulus frequencies. At 0.1 Hz phase lead measured 110 ± 6.5° and decreased to 12 ± 1.2° at 1.6 Hz (FIG. 2C). In normal animals, VOR gain increased from 0.02 ± 0.009 to 0.55 ± 0.02, and VOR phase decreased from 75 ± 9.5° to 7.8 ± 0.7° at 0.1 Hz and 1.6 Hz, respectively. Both VOR gain and phase curves were significantly different between Lurcher and control animals (p <0.01). A post hoc t test with Bonferroni correction for multiple comparisons pinpointed the differences between Lurcher and control animals at 0.1, 0.2, and 0.4 Hz. At these three frequencies, VOR gain of Lurcher mice was significantly larger (p <0.05) than that of control animals, while phase lead of the VOR in Lurcher mice was significantly larger than that of control animals (p <0.05) at 0.1 and 0.2 Hz. Because both phase lead and gain of the Lurcher VOR increased, the difference between control and Lurcher animals cannot be attributed to a different overall VOR time constant between Lurcher and control animals. In fact, a decrease in the dominant time constant of the VOR could be expected in Lurcher mutants, but such a decrease would result in a concomitant decrease in VOR gain at low frequencies. From the present results we witness the exact opposite: VOR gain is relatively increased in Lurcher animals at low-stimulus frequencies.

One of the functions assigned to the flocculus is that it provides the site of direct interaction between OKR and VOR,[4] in such a way that the OKR would supplement VOR to provide a stable, combined response. In control animals, the VVOR indeed proved stable across the range of tested frequencies. Gain was 0.74 ± 0.02 at 0.1 Hz and 0.67 ± 0.03 at 1.6 Hz, while phase measured 2.5 ± 0.4 and 6.1 ± 2.1 at the same frequencies (FIG. 2B and D). The VVOR response of Lurcher mice was more variable, gain first declined from 0.51 ± 0.03 at 0.1 Hz to 0.44 ± 0.02 at 0.2 Hz and then

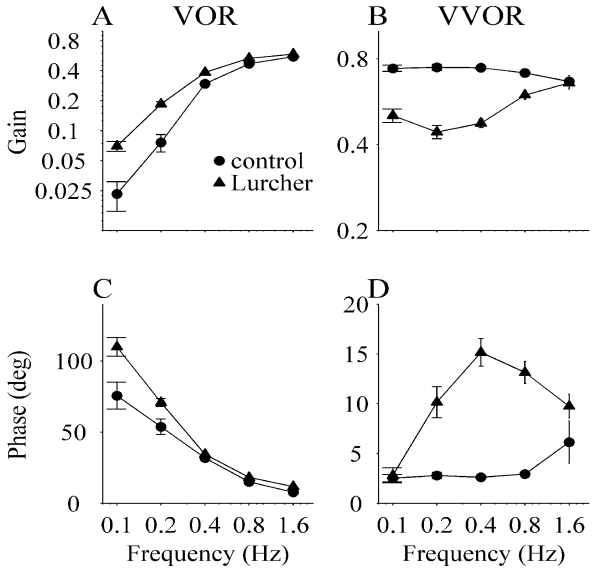

FIGURE 2. BODE plots for Lurcher (*triangles*) and control (*circles*) animals are shown of the VOR (**A** and **C**) and VVOR (**B** and **D**). VOR gain increased with increasing stimulus frequency for both Lurcher and control animals. The interaction of VOR and OKR provided a stable VVOR in control animals across the tested frequency range. VVOR gain and phase were more variable in Lurcher animals, reflecting the inability of their severely impaired OKR system to complement VOR.

increased again to 0.66 ± 0.03 at 1.6 Hz. VVOR phase followed a similar change in Lurcher mice, measuring 3 ± 0.8° and 10 ± 1.2° at 0.1 and 1.6 Hz, respectively, and reaching a peak of 15 ± 1.4° at 0.4 Hz. The variable response of Lurcher mice can be reasonably well explained from a linear addition of VOR and OKR. Because VVOR is a combination of both vestibular and optokinetic inputs, the response at high-stimulus frequency is largely driven by the VOR, while that at low-stimulus frequency will be relatively more dependent on the OKR. The OKR of Lurcher animals did not extend far enough into the higher-frequency range to complement VOR and achieve a stable VVOR across all tested frequencies. The validity of explaining differences in VVOR between Lurcher and controls from a defect in Lurcher OKR may be illustrated by a linear reconstruction of the VVOR at 0.4 Hz using separately measured OKR and VOR responses.

By vectorially subtracting VOR of Lurcher mice from the VVOR stimulus, the residual vector that drives the optokinetic system can be estimated. At 0.4 Hz this estimate comes to a residual optokinetic stimulus of 18°/s at 162° phase angle with ipsilateral head velocity. FIGURE 1A can now be used as a reference table that maps the response of the OKR at 0.4 Hz for different peak stimulus velocities. OKR gain and phase at 18°/s can be read from the curve at 0.15 and –14°, with respect to stimulus movement. Converting these values to the VVOR coordinate system yields an eye velocity of 2.7°/s at a phase lag of –32° with ipsilateral head velocity. Vectorially

adding the estimated eye velocity vector for optokinetic component with vestibular component yields a VVOR gain of 0.44 and a phase lead of 21° with ipsilateral head velocity. These values are close enough to the real values (0.48 and 15°, respectively) to assume that deficits in Lurcher VVOR are largely caused by a normal integration of an impaired OKR and VOR.

Motor Learning

Visuovestibular adaptation was tested in 4 Lurcher mice and 5 control animals. Both Lurcher and control animals displayed a VVOR in response to the training stimulus that was different from their normal VOR at the same vestibular stimulus. The difference between VVOR and VOR indicates that visual information was used to guide the eye movements during training. Control animals responded to the training stimulus by slowly decreasing VOR gain at 0.6 Hz from 0.30 ± 0.03 on the first to 0.13 ± 0.02 on the sixth day and then slightly increasing VOR gain again to 0.16 ± 0.03 on day 8 (FIG. 3A). Changes in VOR gain were accompanied by a slow rise in phase of the eye movement with respect to turntable movement, measuring 30 ± 5°, 111 ± 26° and 115 ± 31° on the same days (FIG. 3B). No changes were observed in Lurcher mice. VOR gain measured 0.37 ± 0.1 before and 0.42 ± 0.03 after train-

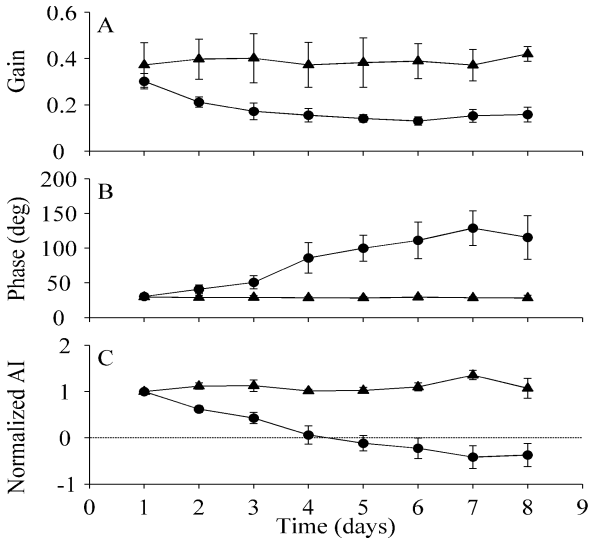

FIGURE 3. Change in properties of the VOR at 0.6 Hz in response to visuovestibular training is plotted against time. (**A**) Absolute VOR gain for Lurcher (*triangles*) and control animals (*circles*) is presented against time. VOR in control animals is reduced with training while that of Lurcher animals does not change. Error bars indicate SEM. (**B**) Phase of the eye movement with respect to table movement is plotted in the same way as gain. Control animals show a pronounced advance in phase lead with training reflecting a change in direction of the VOR. (**C**) Normalized adaptation index is shown to outline two distinct features. First, VOR reverses direction as phase lead is larger than 90° and the adaptation index changes sign. Second, normalizing the data of each animal to their initial performance clearly shows that the Lurcher animals do not adapt their VOR.

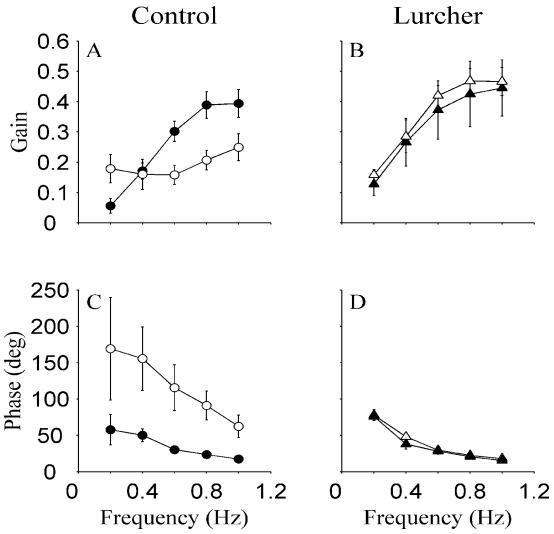

FIGURE 4. Change in properties of the VOR in control (**A** and **C**) and Lurcher (**B** and **D**) animals is presented in the form of BODE plots. *Closed symbols* represent data before and *open symbols* after eight days of visuovestibular training. Error bars indicate SEM.

ing, while phase of the eye movement with respect to table movement was 30 ± 2° before and 28 ± 4° after training. In control animals, phase lead changed so much that direction of the VOR actually reversed from out of phase with table rotation to in phase. In order to accentuate the actual reversal of direction we calculated an adaptation index (AI) according to the equation: $AI = G^{*}\cos(\theta)$. In this equation, G equals VOR gain, and θ is the phase angle of the eye movement with respect to table movement. As phase lead of the eye movement with respect to table movement increased to values larger than 90°, AI became negative, indicating that VOR had reversed direction. FIGURE 3E shows AI, normalized to its initial value, for all animals at the training frequency (0.6 Hz). As expected, control animals differed significantly from Lurcher animals ($p = 0.002$, two-way ANOVA with repeated measures), in that control animals showed a steady decrease of AI, while Lurcher animals showed no change in AI throughout the entire eight days of training. In addition to the training frequency, VOR was also tested for four other frequencies centered about the training frequency (FIG. 4). Although training was performed exclusively at 0.6 Hz, changes in VOR of control animals were not limited to the training frequency but encompassed the entire tested spectrum. AI changed significantly at all frequencies in control animals ($p < 0.001$ at all frequencies), while in Lurcher animals it did not change at any of the tested frequencies ($p > 0.05$ at all frequencies).

After visuovestibular training, behavioral changes in control animals were not limited to the VOR but also included the OKR. Every day the OKR was tested at the same amplitude (5°) and frequencies as the VOR with the addition of 0.1 Hz. The experimental protocol to monitor progression of learning in the OKR therefore differed from that described earlier in that peak stimulus velocity increased as frequen-

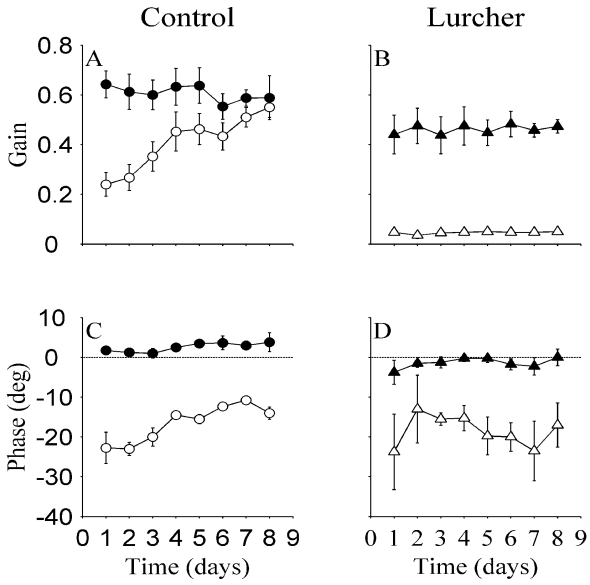

FIGURE 5. The progression of OKR changes in time is presented for control (**A** and **C**) and Lurcher (**B** and **D**) animals. Only data taken at 0.1 Hz (*closed symbols*) and 1 Hz (*open symbols*) are shown because those data represent the smallest and largest effect of training on OKR, respectively.

cy was increased. During visuovestibular training, OKR gain progressively increased and phase lag decreased in control animals (FIG. 5A and C). At 1 Hz (31°/s peak stimulus velocity), OKR gain increased from 0.24 ± 0.05 to 0.55 ± 0.04 ($p = 0.007$), and phase lag decreased from –24 ± 9° to –14 ± 2° ($p = 0.006$). In Lurcher animals, neither OKR gain nor phase changed during training (FIG. 5B and D). Adaptive changes of the OKR in control animals were limited to higher-stimulus frequencies (FIG. 6A and C). Changes in gain proved significant at 0.6, 0.8, and 1 Hz ($p = 0.02$, $p < 0.001$, and $p < 0.001$), while changes in phase were significant at all frequencies except 0.1 Hz ($p = 0.03$ at 0.2 Hz and $p < 0.001$ for all other frequencies). In Lurcher mice, the OKR remained unchanged at all tested frequencies (FIG. 6B and D). An intact cerebellar cortex is therefore required both for adaptation of the VOR as well as for adaptation of the OKR.

DISCUSSION

The oculomotor performance of Lurcher mice is largely dominated by an impaired OKR. Closed-loop OKR gain is severely reduced, and phase lag of the eye movement with respect to stimulus movement is increased compared to control animals. Lurcher OKR, like that of control animals, is clearly nonlinear, rendering accuracy of the OKR not only dependent on stimulus frequency but also on stimulus

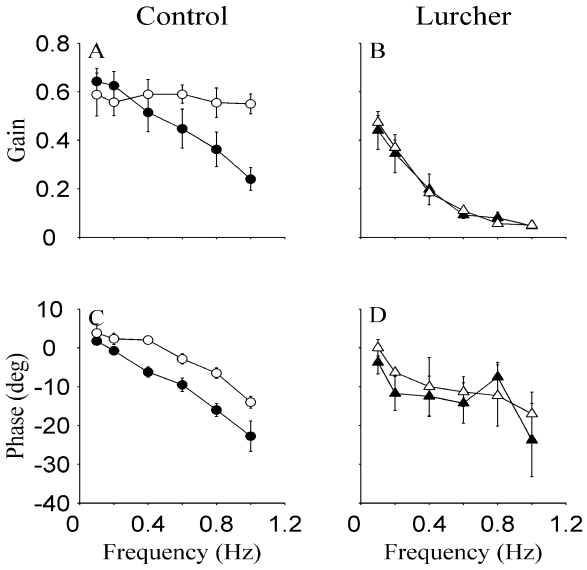

FIGURE 6. Changes in OKR due to visuovestibular training are represented in the form of BODE plots for control (**A** and **C**) and Lurcher (**B** and **D**) animals. *Closed symbols* represent data before and *open symbols* after eight days of training. BODE plots of the OKR before training differ from those shown in FIGURE 1 because a constant stimulus amplitude was used to assess the OKR during training. Because of the constancy of stimulus amplitude, peak stimulus velocity increased as frequency was increased. Stimulus frequencies of 0.4, 0.8, and 1.2 correspond to peak stimulus velocities of 12.6, 25.1, and 37.7°/s, respectively.

velocity. This nonlinear relation of the OKR appeared more pronounced in Lurcher animals as reflected by the stronger dependence of OKR gain on peak stimulus velocity. Control animals showed a stable OKR gain for sinusoidal stimuli with a peak velocity smaller than 9°/s, while Lurcher mice did not exhibit any such plateau response. In relation to frequency, the OKR (peak velocity 8°/s) both in Lurcher and control animals revealed typical low-pass characteristics of declining gain and increased phase lag with increasing stimulus frequency. However, in addition to a severely reduced OKR gain, phase lag was also larger in Lurcher mice than in control animals. The Lurcher OKR represents the function of remaining extracerebellar pathways, which are known to exist at the level of the brain stem.[14–16]

Changes in the OKR of Lurcher mice may be explained by considering the OKR, in its reduced form, to be a low-pass system within a negative feedback loop. The internal filter element can be expressed as the Laplacian transfer function $g_{int}/(s\tau+1)$ in which g_{int} represents the internal gain, s denotes complex frequency, and τ represents the time constant. Placing such an element inside a negative feedback loop results in a systems performance $E'/D' = (g_{int}/g_{int}+1)/(s(\tau/1+g_{int})+1)$. A reduction in the internal gain of the system would therefore result in a reduction of the closed-loop OKR gain and an increase in its time constant. The result of such a deficit was present in Lurcher animals as reduced OKR gain and increased phase lag with respect to control animals. In addition to explaining the increased phase lag, this inter-

pretation of a reduced internal gain could also explain the more pronounced dependence of Lurcher OKR on stimulus velocity, because a reduction of the internal gain would emphasize the effect of a saturation element in the system. Saturation is known to occur in the optokinetic system at the level of the accessory optic system involving the detection of retinal slip.[17,18] The nature of the deficits observed in Lurcher mice hint at the possibility that cerebellar output is necessary in mice to partially compensate for the nonlinear nature of the OKR by boosting internal gain of the system.

Assigning a role to the cerebellum in gain control is definitely not new.[4,19] The cerebellum has been assigned a short-term role in oculomotor control concerned with directly fine-tuning VOR based on optokinetic information,[4] and also a long-term role in which vestibular and optokinetic interaction would shape dynamics of the underlying VOR.[4,5,20] In this respect it was striking that in Lurcher mice no severe impairments emerged in either VOR alone or in the ability to combine VOR and OKR. VVOR gain was reduced in Lurcher mice, but this impairment is attributable to the obvious OKR deficit. Lack of a functional cerebellum did not appear to prevent normal integration of optokinetic and vestibular signals into a VVOR.

VOR of Lurcher mice was similar to that of control animals and only differed significantly at 0.1, 0.2, and 0.4 Hz. Contrary to expectation,[14,21,22] at these low frequencies Lurcher mice performed better, that is, they had a higher VOR gain than normal animals. An increase in phase lead of the eye movement with respect to stimulus movement was also observed. Increased phase lead of the VOR has been described before in Lurcher[23] and PCD[24] mice and can be attributed to a reduced function of the velocity to position neural integrator,[25] which depends on cerebellar output.[26] However, a reduction in performance of the neural integrator would invariably lead to a reduction in VOR gain at low-stimulus frequencies. Rather than a reduction, we observed an increased VOR gain in Lurcher animals, which is comparable to that reported in previous studies of Purkinje cell–deficient mutants.[23,24] It is unclear whether increased VOR gain in cerebellar mutant mice is a primary effect of the cerebellar lesion or a secondary compensatory mechanism that allows the animals to achieve a reasonable performance of the VVOR despite an impaired OKR. Lesion studies provide no unequivocal answer to this question because lesions of the flocculus have been reported to cause both a decrease[14,21,22,27] or an increase[5,28] of VOR gain, and some studies report no effect at all.[2,22] The same ambivalent results have been reported for lesions of the nodulus, ascribing both an increasing[24,27] and decreasing[29] influence on angular VOR gain to the lesion. Rather than adding to this list of lesion studies, we asked whether Lurcher animals might have retained some adaptive capacity in the remaining, extracerebellar circuitry that would allow them to adapt VOR gain.

The extent to which Lurcher and control animals were able to adapt their VOR was investigated by subjecting both groups to a vision-reversal paradigm. In this paradigm, control animals responded by an initial drop of VOR gain followed by an increase in phase lead. Changes in VOR were paralleled by an increased OKR gain and reduced phase lag of the OKR. Lurcher animals proved incapable of adapting their VOR or OKR, demonstrating once more the necessity of an intact cerebellum to express these forms of plasticity.[5,20,22,27] Though cerebellar involvement in adaptation of the VOR and OKR is evident, long-term depression of the parallel fiber to Purkinje cell synapse[4] does not appear to be an absolute prerequisite to express the adaptive

behavior because L7-PKCi transgenic animals, in which LTD was selectively disrupted, did show attenuated adaptation when subjected to the same adaptation protocol.[30] The present data differ from previous adaptation studies[27,31–33] in that control mice did not show frequency-specific adaptation of either VOR or OKR. The different findings are possibly caused by differences in experimental protocols. Particularly the fact that we extended training to eight consecutive days may have prevented us from seeing any frequency-specific changes.

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Chapter 4

Impairment of LTD and Cerebellar Learning by Purkinje Cell–Specific Ablation of cGMP-Dependent Protein Kinase I

Impairment of LTD and cerebellar learning by Purkinje cell–specific ablation of cGMP-dependent protein kinase I

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The molecular basis for cerebellar plasticity and motor learning remains controversial. Cerebellar Purkinje cells (PCs) contain a high concentration of cGMP-dependent protein kinase type I (cGKI). To investigate the function of cGKI in long-term depression (LTD) and cerebellar learning, we have generated conditional knockout mice lacking cGKI selectively in PCs. These cGKI mutants had a normal cerebellar morphology and intact synaptic calcium signaling, but strongly reduced LTD. Interestingly, no defects in general behavior and motor performance could be detected in the LTD-deficient mice, but the mutants exhibited an impaired adaptation of the vestibulo-ocular reflex (VOR). These results indicate that cGKI in PCs is dispensable for general motor coordination, but that it is required for cerebellar LTD and specific forms of motor learning, namely the adaptation of the VOR.

Introduction

The cerebellum offers a unique opportunity to identify and study the components necessary for neuronal plasticity and learning (Raymond et al., 1996; Mauk et al., 1998; Carey and Lisberger, 2002). The presumed mechanisms of learning and memory formation are changes of the efficacy of synaptic transmission. Cerebellar long-term depression (LTD), the activity-dependent attenuation of synaptic transmission at the parallel fiber–Purkinje cell (PC) synapse, has been extensively studied. However, the interaction between multiple signaling pathways involved in cerebellar LTD remains largely unresolved. LTD is readily evoked when climbing and parallel fibers are conjunctively activated and has been implicated in particular forms of motor learning, such as adaptation of the vestibulo-ocular reflex (VOR) (Nagao and Ito, 1991; De Zeeuw et al., 1998). It has been shown that the two messengers Ca^{2+} and NO are sufficient to induce LTD (Lev-Ram et al., 1995). Furthermore, genetic ablation of neuronal NO synthase impaired cerebellar LTD (Lev-Ram et al., 1997b) and adaptation of compensatory eye movements (Katoh et al., 2000).

The molecular and cellular mechanisms of cerebellar NO signaling are not completely understood. Indirect evidence from experiments with cerebellar slices suggested that NO induces LTD via activation of soluble guanylyl cyclase and subsequent cGMP synthesis in PCs (Daniel et al., 1993; Boxall and Garthwaite, 1996; Hartell, 1996; Lev-Ram et al., 1997a; Hartell et al., 2001). The identification of the signaling components downstream of cGMP is complicated by the existence of multiple receptors for cGMP (Beavo and Brunton, 2002) and by the lack of highly specific activators and inhibitors for a given cGMP receptor protein (Smolenski et al., 1998; Schwede et al., 2000). Cerebellar PCs express high levels of cGMP-dependent protein kinase type I (cGKI) (Hofmann and Sold, 1972; Lohmann et al., 1981),

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Key words: cGMP kinase; Cre recombinase; intracellular signaling; synaptic plasticity; learning

Abbreviations used in this paper: cGKI, cGMP-dependent protein kinase type I; DSCT, delayed synaptic Ca^{2+} transient; EPSC, excitatory postsynaptic current; ESCT, early synaptic Ca^{2+} transient; LTD, long-term depression; OKR, optokinetic reflex; PC, Purkinje cell; VOR, vestibulo-ocular reflex; VVOR, VOR in the light.

whereas cGK type II was not detected in the cerebellum (unpublished data). Interestingly, agents that inhibit cGKI in vitro, particularly the widely used "cGK inhibitor" KT5823, have been shown to impair LTD in cerebellar slices, indicating a role for cGKI in LTD induction (Hartell, 1994; Lev-Ram et al., 1997a). However, it was recently observed that KT5823 may not inhibit cGKI in certain intact cells (Burkhardt et al., 2000), including cerebellar PCs (Rybalkin, S.D., and J.A. Beavo, personal communication). These findings suggest that the effects of cGKI inhibitors should be interpreted with caution, particularly if inhibition of kinase activity was not demonstrated, for example, by monitoring the phosphorylation of a known cGKI substrate protein (Burkhardt et al., 2000; Shimizu-Albergine et al., 2003). It has been noted that it might be difficult to study LTD with pharmacological tools, as they can exaggerate the importance of certain pathways in LTD induction that might be less important, or not even used, in physiological conditions (Daniel et al., 1998). Furthermore, the specific relevance of cGKI in PCs to cerebellar motor learning has not been investigated yet.

As a first step toward an understanding of the in vivo function of cerebellar cGKI signaling, we have used a genetic, rather than a pharmacological, approach, namely PC-specific disruption of the cGKI gene in mice by using Cre/loxP-assisted conditional somatic mutagenesis (Metzger and Feil, 1999). PC-specific cGKI knockout mice perform normal in several tasks testing general motor performance, but exhibit strongly reduced cerebellar LTD and impaired adaptation of the VOR. Thus, cGKI-dependent signaling in PCs contributes to synaptic plasticity and particular forms of motor learning.

Results

PC-specific ablation of cGKI

The tissue-specific knockout strategy was necessary because conventional null mutants with a global cGKI deficiency show multiple defects and have a short life expectancy of ~4 wk (Pfeifer et al., 1998). Furthermore, the interpretation of phenotypes of conventional knockout mice is often complicated by the absence of the gene product of interest in all cells of the animal throughout ontogeny. PC-specific cGKI knockout mice (cGKI^{pko} mice) were generated by using the Cre/loxP recombination system. In mice carrying a conditional cGKI allele (L2 allele) with two loxP sites flanking the critical exon 10 of the cGKI gene, Cre-mediated recombination of the loxP sites results in excision of exon 10 and, thus, in a cGKI null allele (L− allele) (Wegener et al., 2002). Conversion of the cGKI L2 allele into the L− allele will take place only in cells expressing active Cre recombinase.

To ablate cGKI specifically in PCs, we generated mice carrying the cGKI L2 allele as well as the L7-Cre transgene (Barski et al., 2000), which expresses the Cre recombinase in almost all cerebellar PCs. The expression of cGKI was first analyzed by Western blot analysis of extracts from various tissues. As compared with control mice, cGKI^{pko} mice showed a strong reduction of cGKI protein in the cerebellum, but normal cGKI levels in other brain regions

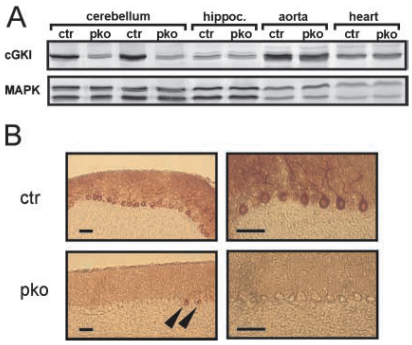

Figure 1. **Conditional ablation of cGKI in cerebellar PCs.** (A) Western blot analysis of cGKI expression (top) in various tissues of control mice (ctr) and cGKI^{pko} mice (pko). Equal loading of protein extracts from tissues of control and cGKI^{pko} mice was confirmed by staining the blot with an antibody against p44/42 MAPK (bottom). (B) Immunohistochemical detection of cGKI on sagital cerebellar sections of control mice (ctr, top) and cGKI^{pko} mice (pko, bottom). Arrowheads indicate cGKI-positive PCs in cGKI^{pko} mice. Bars, 50 μm.

and peripheral tissues, such as hippocampus, aorta, and heart (Fig. 1 A). Immunohistochemical detection of cGKI at the cellular level indicated that the protein was highly expressed in almost all PCs of control animals, whereas <5% of the PCs in cGKI^{pko} animals expressed cGKI (Fig. 1 B). These results correlate well with the recombination pattern of the L7-Cre mouse line as revealed by expression of β-galactosidase in "Cre indicator" mice (Barski et al., 2000) and of a loxP-flanked calbindin target gene (Barski et al., 2003), i.e., strong Cre activity in cerebellar PCs and weak to undetectable Cre activity in other brain regions or peripheral tissues. The finding that the cGKI protein was not completely absent in extracts from the cerebellar region of cGKI^{pko} mice (Fig. 1 A) can be attributed to its residual expression in few PCs (Fig. 1 B), and to the presence of cGKI in cerebral vessels (Lohmann et al., 1981). Taken together, these data demonstrated that our knockout strategy resulted in efficient and selective ablation of cGKI in cerebellar PCs.

Cerebellar structure is normal in cGKI^{pko} mice

Based on gross morphology, brains of cGKI^{pko} animals could not be distinguished from those of their control littermates. Basic histological analysis showed that the cerebellum of cGKI^{pko} mice was of normal size and external appearance with a regular foliation and that the cerebellar cortex had a normal layering (Fig. 2 A, a'–d'). As in control animals, PCs of cGKI mutants could be specifically labeled for calbindin D28k, were regularly arranged, and had a characteristic morphology (Fig. 2 A, e' and f'). Detailed analyses by immunofluorescence and electron microscopy revealed a normal fine structure of the cerebellum of cGKI^{pko} mice, particularly a typical appearance of PC dendrites, dendritic spines, and synapses (Fig. 2 B). Thus, the absence of cGKI in PCs of cGKI^{pko} mice had apparently no effect on cerebellar structure.

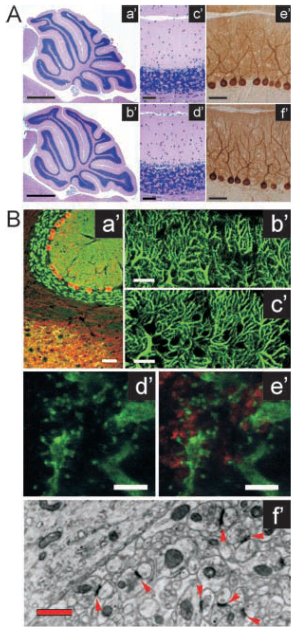

Figure 2. **Cerebellar anatomy is normal in cGKI^{pko} mice.** (A) Basic histology of the cerebellum. Sagittally oriented sections of control mice (a′, c′, and e′) and cGKI^{pko} mice (b′, d′, and f′) were stained with hematoxylin and eosin (a′–d′) or with an antiserum to calbindin D28K (e′ and f′). Bars: (a′ and b′) 1 mm; (c′–f′) 50 μm. (B) Fine structure of the cerebellum. (a′) Double labeling of a section from a cGKI^{pko} mouse for calbindin D28k (red) and synaptophysin (green). Note the regular arrangement of PC bodies (red), their main dendrites, and their axons reaching the deep cerebellar nuclei located in the bottom part of this micrograph. The antiserum to synaptophysin (green) labels the molecular layer and reveals the presence of characteristic glomeruli within the granule cell layer. Yellow signals within the deep nuclei represent PC synapses onto deep nuclear neurons. (b′ and c′) Dendrites of PCs from control mice (b′) and cGKI^{pko} mice (c′) labeled with an antiserum to calbindin D28k. Note the irregular outline of the dendritic branchlets, reflecting the presence of numerous dendritic spines in both controls and mutants. (d′ and e′) High power view of dendritic spines on a tertiary dendritic branchlet of a PC (green) from a cGKI^{pko} mouse. Synaptophysin-positive presynaptic elements of parallel fibers (red in e′) are contact spines. (f′) Electron micrograph of the molecular layer of the cerebellar cortex of a cGKI^{pko} mouse. A major PC dendritic branch can be seen in the upper left corner. Synapses on PC dendritic spines show typical dense membrane thickening (arrowheads). Bars: (a′) 50 μm; (b′ and c′) 20 μm; (d′ and e′) 5 μm; (f′) 1 μm.

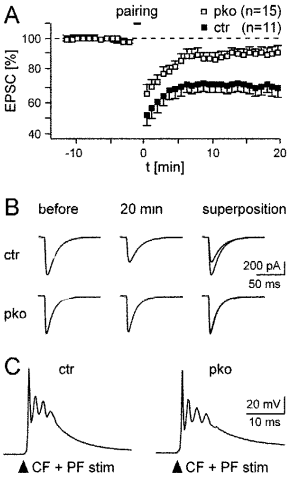

Figure 3. **Cerebellar LTD is impaired in acute cerebellar slices of cGKI^{pko} mice.** (A) LTD at parallel fiber–PC synapses of control mice (filled squares, $n = 11$ experiments) and cGKI^{pko} mice (open squares, $n = 15$ experiments). The black bar indicates the time of pairing. (B) Representative EPSCs recorded 5 min before and 20 min after LTD induction in a control mouse (ctr) and in a cGKI^{pko} mouse (pko). Traces are averages of 10 consecutive EPSCs. (C) Averages of all 120 combined climbing/parallel fiber responses recorded during the pairing procedure in two representative experiments obtained in PCs of a control mouse (ctr) and a cGKI^{pko} mouse (pko). The arrowheads indicate the time of concomitant parallel fiber and climbing fiber stimulation.

Impaired LTD but normal synaptic calcium signaling in cGKI^{pko} mice

Synaptic transmission and LTD was investigated at the parallel fiber–PC synapse in acute cerebellar slices using whole-cell patch-clamp recordings. When stimulating afferent parallel fibers with similar intensities (12.1 ± 2.7 V in control [$n = 11$] and 13.0 ± 2.0 V in cGKI^{pko} [$n = 15$] mice), the amplitudes of the excitatory postsynaptic currents (EPSCs) evoked in PCs were 509 ± 47 pA in control experiments ($n = 11$) and 460 ± 57 pA in experiments with cGKI^{pko} mice ($n = 15$). These values did not differ significantly between both experimental groups, indicating that basal synaptic transmission was not changed. Instead, the simultaneous activation of parallel and climbing fibers induced robust LTD in PCs of control mice, but not in PCs of cGKI^{pko} mice (Fig. 3). After LTD induction, EPSCs were decreased by 31.0 ± 5.9% in control mice, whereas only a marginal reduction of 9.8 ± 4.5% was detected in cGKI^{pko} mice (Fig. 3, A and B). Thus, cerebellar LTD was strongly depressed in the absence of cGKI. It is important to note that, despite the marked difference in LTD, the electrical responses recorded during LTD induction were highly similar in both genotypes (Fig. 3 C). These responses, produced by the coincident stimulation of parallel and climbing fibers, were dominated by the well-known climbing fiber–mediated complex spike (Eccles et al., 1966). We expected that a subpopulation of PCs in cGKI^{pko} mice would exhibit normal LTD, because cGKI was still expressed in few PCs of the mutant mice (Fig. 1 B). Consistent with this assumption, we found that 2 out of 15 cells of cGKI^{pko} mice exhibited LTD. When these two cells were excluded from the analysis, the mean EPSC amplitude of the remaining cells in cGKI^{pko} mice was 97.1 ± 6.8%. This analysis suggested that deletion of cGKI completely suppressed LTD induction. However, for showing unbiased data, these two experiments were included in the results of Fig. 3 A.

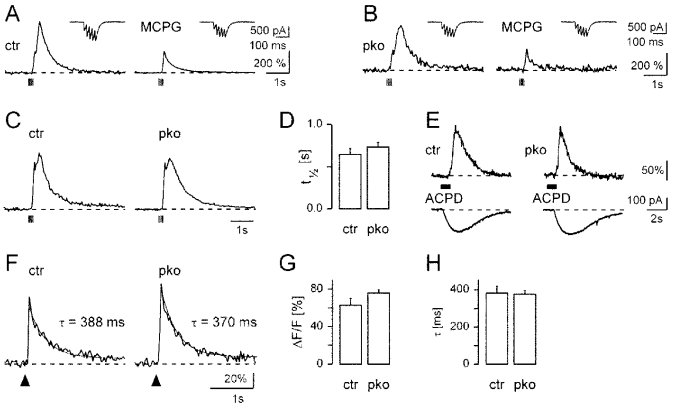

Figure 4. **Synaptic calcium signaling is normal in PC dendrites of cGKI^{pko} mice.** Complex synaptic Ca^{2+} signals were recorded from active dendritic microdomains in response to repetitive synaptic stimulation of parallel fibers (five pulses, 50 Hz, delivered at the time indicated by vertical bars) of control mice (ctr) and cGKI^{pko} mice (pko). (A and B) Representative recordings under standard conditions (left) and in the presence of 2 mM MCPG ([R,S]-α-methyl-4-carboxyphenylglycine) (right), which blocks the delayed mGluR1-mediated Ca^{2+} signal component. The insets show the corresponding EPSCs. (C) Average of complex synaptic Ca^{2+} signals obtained from all synaptic inputs tested (19 inputs in control mice, 28 inputs in cGKI^{pko} mice). Ca^{2+} signals are normalized to the peak of the early, MCPG-insensitive component. (D) Summary plot of the average duration of synaptic Ca^{2+} signals (time at which the Ca^{2+} level reached half amplitude of the early component, $t_{1/2}$) in control (n = 19 synaptic inputs) and mutant mice (n = 28 synaptic inputs). (E) Ca^{2+} signals (top) and corresponding inward currents (bottom) in response to local application of the mGluR agonist t-ACPD (bar). Signals were evoked by pressure ejection of t-ACPD (200 μM, 1 s) from an application pipette placed ~15 μm above the dendrites of a voltage-clamped PC. (F) Ca^{2+} transients evoked by climbing fiber activation (marked by arrowheads) in a representative control (ctr) and a cGKI^{pko} (pko) mouse. The respective decay time constants (τ) are indicated. (G) Summary plot of the average amplitude of climbing fiber–evoked Ca^{2+} transients. (H) Summary plot of the average decay time constant of climbing fiber–evoked Ca^{2+} transient. Bar graphs in G and H contain data from 14 responses obtained in three cells for control and 25 responses obtained in six cells from cGKI^{pko} mice. Neither in G nor in H was a significant difference between both experimental groups observed.

Induction of LTD at the parallel fiber–PC synapse essentially requires an increase in postsynaptic calcium (Konnerth et al., 1992), especially through the release of Ca^{2+} from IP_3-sensitive stores in PC dendrites (Miyata et al., 2000; Wang et al., 2000). Because the IP_3 receptor has been assumed to be one of the possible cGKI substrates (Haug et al., 1999), we tested whether synaptic calcium signaling in PCs was altered in cGKI^{pko} mice compared with control mice (Fig. 4). Complex Ca^{2+} signals in whole-cell patch-clamped PCs were evoked by repetitive stimulation of parallel fibers. The resulting Ca^{2+} transients consisted of two distinct components, an early response with a fast rising phase (the early synaptic Ca^{2+} transient [ESCT]) and a delayed second component (the delayed synaptic Ca^{2+} transient [DSCT]). The ESCT reflects Ca^{2+} influx through voltage-gated Ca^{2+} channels after AMPA receptor activation, whereas the DSCT is due to mGluR-mediated Ca^{2+} release from intracellular stores (Finch and Augustine, 1998; Takechi et al., 1998). Compared with control mice, the ESCT/DSCT ratio was not significantly altered in cGKI^{pko} mice (Fig. 4, A and B). The duration of the synaptically evoked Ca^{2+} signal ($t_{1/2}$) was also not significantly different between genotypes (Fig. 4, C and D), being 651 ± 65 ms (n = 19) and 738 ± 50 ms (n = 28) in control and cGKI mutant mice, respectively. Moreover, local dendritic application of the mGluR agonist t-ACPD (1-amino-cyclopentane-trans-1,3-dicarboxylic acid) produced similar Ca^{2+} signals in both genotypes (Fig. 4 E). These data indicated that parallel fiber–evoked synaptic calcium signaling was intact in cGKI-deficient PCs.

Finally, we analyzed Ca^{2+} transients after single climbing fiber activation in PC dendrites. The Ca^{2+} transients measured did not differ significantly between control and cGKI^{pko} mice (Fig. 4, F–H). The average amplitude of the Ca^{2+} transients was 64 ± 7% in control mice (n = 14) and 77 ± 4% in cGKI^{pko} mice (n = 25) (Fig. 4 G). The calcium transients decayed monoexponentially with an average time constant of 384 ± 36 ms (n = 14) and 379 ± 15 ms (n = 25) in control and cGKI^{pko} mice, respectively (Fig. 4 H). Thus, deficiency in cGKI does not alter synaptic calcium signaling in PCs. It is also useful to note that in all these experiments, we used a molecular layer stimulation protocol, which allows one to test for aberrant multiple climbing fiber innervation (Hashimoto et al., 2001). In cGKI^{pko} mice, all PCs tested (6/6) were innervated by a single climbing fiber.

Adaptation of the VOR but not general motor performance is impaired in cGKI^{pko} mice

Visual inspection of cGKI^{pko} mice did not reveal any gross abnormalities or overt behavioral phenotypes. No differences in weight, growth, life expectancy, and activity in the open field test were observed between control mice and cGKI mutants (unpublished data). Despite their defect in cerebellar LTD, cGKI^{pko} mice showed normal motor coordination as analyzed by the footprint, runway, and rotarod test (Fig. 5), suggesting that cerebellar cGKI is dispensable for general motor control.

Several groups have previously pointed out that cerebellar LTD might not be involved in general motor performance but in learning of particular motor tasks, such as adaptation of the VOR (De Zeeuw et al., 1998). Basic properties of the optokinetic reflex (OKR) and of the VOR in the dark and in the light (VVOR) were assessed to find out whether naive cGKI^{pko} mice showed an oculomotor performance identical to that of control mice. No significant differences were found in dynamics of the OKR, VOR, and VVOR between cGKI mutants and control animals (Fig. 6). In both cases

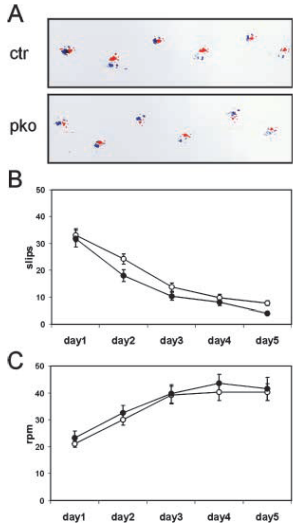

Figure 5. **General motor coordination is not affected in cGKI^{pko} mice.** (A) Footprint pattern of a control mouse (ctr, top) and a cGKI^{pko} mouse (pko, bottom). Prints of the forepaws and hindpaws are red and blue, respectively. For each group, 20 footprints of two mice (10 consecutive footprints per mouse) were analyzed. The hindbase width of control mice (2.4 ± 0.1 cm) and cGKI^{pko} mice (2.4 ± 0.1 cm) was identical. (B) Runway test performance of control mice (filled circles, $n = 15$) and cGKI^{pko} mice (open circles, $n = 15$). Mice traversed a narrow horizontal beam with obstacles, and the number of slips was recorded. (C) Rotarod performance of control mice (filled circles, $n = 14$) and cGKI^{pko} mice (open circles, $n = 12$). The duration of retention on an accelerating rotarod was measured by means of the rotational speed (rpm) at which a mouse fell off the rod.

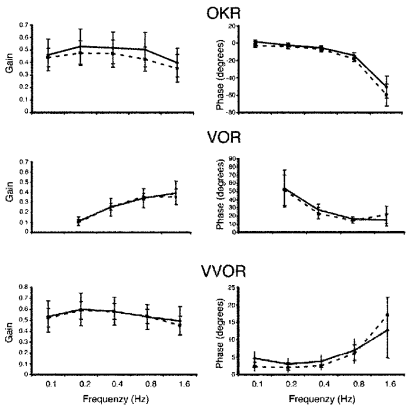

Figure 6. **General eye movement performance is normal in cGKI^{pko} mice.** Baseline oculomotor performance of control mice (solid lines, $n = 13$) and cGKI^{pko} mice (broken lines, $n = 10$) was measured during OKR (top), VOR (middle), and VVOR (bottom). The gain (left) and phase (right) values were determined at different stimulus frequencies. Depending on the frequency, OKR gain varied from 0.46 ± 0.07 to 0.35 ± 0.11 in mutants and from 0.52 ± 0.13 to 0.4 ± 0.11 in control animals, while phase lag increased from −2.0 ± 0.2° at 0.1 Hz to −60 ± 13° at 1.6 Hz in mutants and from 1 ± 0.3° at 0.1 Hz to −51 ± 11° at 1.6 Hz in control animals. The VOR of both types of mice showed the familiar characteristics of a high-pass system in that gain increased and phase lead decreased as stimulus frequency was increased. Gain rose from 0.09 ± 0.03 to 0.35 ± 0.08 in cGKI^{pko} mice and from 0.11 ± 0.04 to 0.39 ± 0.12 in control animals, when stimulus frequency was increased from 0.2 to 1.6 Hz. Phase lead decreased from 52 ± 19° at 0.2 Hz to 21 ± 11° at 1.6 Hz in cGKI mutants and from 54 ± 23° to 15 ± 7° in control mice. Finally, the VVOR gain and phase values of both the mutants and the controls were dominated by vision at the lower frequencies and by the vestibular input at the higher frequencies.

the OKR and VOR showed the familiar characteristics of a low-pass and high-pass system, respectively, while their VVOR gain and phase values were dominated by vision at the lower frequencies and by vestibular input at the higher frequencies. Thus, one can conclude that baseline responses of compensatory eye movements did not differ between cGKI mutants and control mice before training.

In response to "in phase" training during five consecutive days, both the cGKI^{pko} mice and control animals responded with changes in VOR dynamics, but at a different pace and level (Fig. 7). After one training session, the controls, but not the mutant mice, showed a significant reduction in their normalized gain value at 0.6 Hz ($P < 0.002$). In contrast, at 0.2, 0.4, 0.8, and 1.0 Hz, no significant differences were observed after 1 d of training, neither in the control nor in the mutant mice (unpublished data). Yet, after a longer training period of 5 d, both the controls and the mutants showed significantly reduced normalized gain values at multiple frequencies. For example, after training sessions four and five, both the controls and cGKI mutants showed significant reductions at 0.4, 0.6, or 0.8 Hz (Fig. 7 and not depicted). The reductions in control mice were significantly larger than those in cGKI mutants (e.g., $P < 0.03$ and $P < 0.02$ at 0.6 Hz after sessions four and five, respectively). Thus, the LTD-deficient cGKI^{pko} mice showed no frequency-specific training behavior after one training session, and after a prolonged training period, they showed significantly less VOR adaptation than control animals.

Discussion

Here we used conditional gene targeting to ablate cGKI, a potential mediator of cerebellar NO/cGMP signaling, selectively in cerebellar PCs of mice in order to study the role of downstream components of the cerebellar NO/cGMP cascade in LTD and motor learning. This approach allowed us to analyze, for the first time, the specific role(s) of PC cGKI in cerebellar function in vivo. Furthermore, the tissue-specific knockout strategy circumvented potential limitations of the conventional gene targeting technique, such as the lack of regional specificity, the presence of multiple defects, and early postnatal lethality. PC-specific cGKI knockout mice (cGKI^{pko} mice) were characterized by the absence of cGKI protein in almost all PCs and normal cGKI expression in other brain regions and peripheral tissues. Thus, the cGKI^{pko} mice used in this study represent a model of PC-specific cGKI deficiency. Importantly, the lack of cGKI in PCs did not alter cerebellar structure and synaptically evoked den-

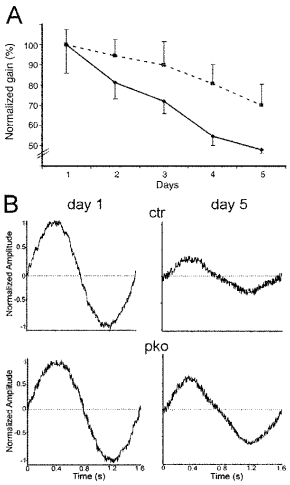

Figure 7. **Adaptation of the VOR is impaired in cGKI^{pko} mice.** (A) Changes in VOR dynamics in response to visuo-vestibular training over 5 d were measured in control mice (solid lines, $n = 13$) and cGKI^{pko} mice (broken lines, $n = 10$). Normalized gain values at 0.6 Hz are shown. (B) Representative raw traces of eye movement amplitudes in control (top) and cGKI^{pko} (bottom) mice before (day 1, left) and after (day 5, right) training.

dritic Ca^{2+} signals in PCs. These results indicate that the cGKI mutants had no basal physiological abnormalities that could confound the analysis of LTD and behavior.

The main finding of the present study is that cGKI^{pko} mice showed nearly complete absence of cerebellar LTD, as measured by whole-cell patch clamping in acute slices, and impaired adaptation of the VOR, while their general eye movement performance was normal. This phenotype demonstrates a specific role for PC cGKI signaling in cerebellar LTD and motor learning. Despite impaired LTD, cGKI^{pko} mice showed no overt behavioral phenotype and performed normal in several tests of general motor coordination, i.e., the footprint, runway, and rotarod test, suggesting that cGKI in PCs is dispensable for general motor coordination. A highly similar phenotype was observed in transgenic mice expressing a PKC inhibitor peptide selectively in PCs (De Zeeuw et al., 1998; Goossens et al., 2001; Van Alphen and De Zeeuw, 2002). Together, these previous and our present results strongly support the concept that cerebellar LTD is involved in specific forms of motor learning, such as adaptation of the VOR, but not in general motor performance. Impaired motor coordination in various knockout mouse models correlates with aberrant multiple innervation of PCs by climbing fibers, which, even though LTD is retained, is expected to impair the function of the cerebellar neuronal circuit (Ito, 2001). Furthermore, these mouse mutants lacked the gene of interest in all cells of the body, questioning the specific relationship of motor discoordination to the cerebellum. In contrast, cGKI^{pko} mice lacked cGKI selectively in cerebellar PCs and showed normal climbing fiber innervation. Thus, the phenotype of the cGKI^{pko} mouse model may be more informative with respect to the specific role of cerebellar LTD in motor learning compared with other mouse mutants in which multiple climbing fiber innervation and noncerebellar defects might also contribute to motor phenotypes.

How could activation of cGKI in PCs contribute to LTD and cerebellum-dependent learning? LTD induction requires an appropriate balance between protein kinases and phosphatases (Ito, 2002) and can be facilitated by inhibition of protein phosphatase 1/2A (Ajima and Ito, 1995). Indeed, cGKI may phosphorylate G-substrate, a well-characterized cGKI target in PCs (Schlichter et al., 1978; Aswad and Greengard, 1981), which would in turn inhibit protein phosphatase 1/2A (Endo et al., 1999; Hall et al., 1999). Inhibition of protein dephosphorylation would increase the levels of phosphoproteins generated by the action of various protein kinases, including PKC and cGKI itself. It is assumed that phosphorylation of the AMPA receptor complex, presumably by PKC, allows the removal of AMPA receptor subunits from the synaptic membrane via clathrin-mediated endocytosis (Wang and Linden, 2000; Chung et al., 2003). Thus, we propose the following molecular model for cerebellar LTD and motor learning: NO/cGMP-dependent activation of cGKI results in phosphorylation of G-substrate, inhibition of protein phosphatases, extended endocytosis of phosphorylated AMPA receptor subunits, LTD, and motor learning. Future studies, for example, the analysis of the effects of phosphatase inhibitors on LTD in cGKI^{pko} mice, should help to validate this model.

In conclusion, this study demonstrates that cGKI-dependent signaling in PCs contributes to cerebellar LTD and a particular form of motor learning, adaptation of the VOR. To the best of our knowledge, this is the first cell-specific demonstration that cGKI is involved in cerebellar synaptic plasticity and learning in vivo in a way that cannot be compensated for by PKC. Based on these and previous results, we propose that cGKI in PCs is indispensable for cerebellar learning.

Materials and methods

Experimental animals

The generation of mice carrying a conditional loxP-flanked (floxed) cGKI allele (L2) or a recombined cGKI null allele (L−) and the detection of the cGKI wild-type (+), L2, and L− alleles by PCR have been described previously (Wegener et al., 2002). To achieve the Cre-mediated conversion of the floxed L2 allele into the excised L− allele in cerebellar PCs, L7-Cre transgenic mice were used (Barski et al., 2000). L7-Cre mice express the Cre recombinase under the control of the L7/pcp-2 gene promoter, which is active in cerebellar PCs (Oberdick et al., 1993). The L7-Cre transgene was detected by PCR analysis of tail DNA with *cre*-specific primers (Feil et al., 1996). Mice with modified cGKI alleles were crossed with L7-Cre mice to generate PC-specific cGKI knockout mice (cGKI^{pko} mice; genotype: cGKI$^{L-/L2}$; L7-Cre$^{tg/0}$) and control mice (genotype: cGKI$^{+/L2}$; L7-Cre$^{tg/0}$). For experiments, litter-matched adult (3–6-mo-old) control mice and cGKI^{pko} mice on a mixed 129Sv/C57BL6 genetic background were used, with the investigator being unaware of the genotype of the animals. Experiments had been approved by the committee on animal care and welfare of the local government.

Western blot analysis of cGKI expression

cGKI was detected using a rabbit polyclonal antibody to cGKI (Pfeifer et al., 1998). Equal loading of gels for immunoblots was confirmed by staining with p44/42 MAP kinase antibodies (New England Biolabs, Inc.).

Morphological and immunohistochemical analysis

Animals were deeply anesthetized and perfused through the ascending aorta with either 10% phosphate-buffered formalin (for detection of cGKI),

Bouin's fixative, or, for ultrastructural analysis, a buffered mixture of 2% freshly depolymerized paraformaldehyde and 2% glutaraldehyde. Brains were dissected and postfixed in the same fixatives overnight. For light microscopic studies, brains were embedded in paraffin, cut at 10 μm, and routinely stained with hematoxylin and eosin. Immunohistochemistry was performed as previously described (Mertz et al., 2000) using antisera to cGKI (Pfeifer et al., 1998), calbindin D28k (mouse, clone CL-300, 1:400; Sigm-Aldrich), or synaptophysin (rabbit, G95, 1:2,000; a gift of R. Jahn, Max-Planck Institut für Biophysikalische Chemie, Göttingen, Germany). For detection of primary antibodies, we used either the avidin-biotin method with diaminobenzidine as a chromogen (Vector Laboratories) or species-specific secondary antibodies tagged with Cy-3, Cy-2, or Alexa®488. For ultrastructural analysis, specimens were postfixed in 1% osmium tetroxide in PBS. Specimens were rinsed in water, dehydrated, and embedded in Durcopan resin. Semithin (1 μm) sections were stained with toluidine blue. Ultrathin sections were cut with a diamond knife and contrasted with uranyl acetate and lead citrate.

Electrophysiology and calcium imaging

Slices (300 μm) were prepared from mice that were decapitated after anesthesia with CO_2. Whole-cell recordings were obtained from PCs in slices perfused with artificial cerebro-spinal fluid composed of (in mM) 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 20 glucose, and 0.01 bicuculline (Sigma-Aldrich), bubbled with 95% O_2 and 5% CO_2. Pipettes (2–4 MΩ resistance) were pulled from borosilicate glass and coated with silicon. The pipette solution contained (in mM) 148 potassium gluconate, 10 Hepes, 10 NaCl, 0.5 $MgCl_2$, 4 Mg-ATP, and 0.4 Na_3-GTP, pH 7.3. Oregon green BAPTA-1 (Molecular Probes) was added to the pipette solution (100 μM for LTD experiments and 200 μM for calcium imaging). Synaptic stimulation was performed by using pipettes filled with 1 mM NaCl (1 MΩ resistance) placed in the molecular layer. The threshold for climbing fiber activation was identified in the voltage clamp mode by gradually increasing the voltage pulse through a stimulation pipette placed over the PC's dendritic tree. In contrast to the parallel fiber responses, the large-amplitude climbing fiber EPSC is characterized by an all-or-none behavior. The stimulus pulse amplitude (150 μs duration) was 3–33 V for parallel fiber stimulation and 20–90 V for climbing fiber stimulation. Parallel fibers were stimulated at 0.2 Hz, and EPSCs were recorded in the voltage clamp mode until a stable baseline amplitude was obtained for at least 10 min. LTD was induced according to published procedures (Barski et al., 2003). In brief, the stimulus intensity was raised to a value at least 20% over climbing fiber threshold (identified beforehand), and 120 stimuli were repeated at 1 Hz in the current clamp mode. The successful stimulation of the climbing fiber was verified by the recording of complex spikes accompanying each stimulation. After pairing and returning to the voltage clamp mode, the stimulus intensity was set to the initial value and the recording of parallel fiber EPSCs at 0.2 Hz was resumed for 20 min. Passive membrane properties of PCs were monitored by applying 5 mV hyperpolarizing pulses. The series resistance was kept constant throughout the measurement at around 10–20 MΩ. The holding current during LTD measurements ranged from −100 to −500 pA and the input resistance from 29 to 111 MΩ without differences between both experimental groups. For calcium imaging, a confocal laser-scanning microscope (Odyssey; Noran Instruments) attached to an upright microscope was used. Fluorescence images were acquired at 30 Hz and analyzed off-line with custom-made software. Ca^{2+} transients were recorded in regions of interest in active dendritic regions. The Ca^{2+}-dependent fluorescence signals were expressed as increases in fluorescence divided by the prestimulus fluorescence values (ΔF/F) and further analyzed using Igor Pro software (Wavemetrics).

Motor coordination tests

Footprint patterns of mice were analyzed using a narrow tunnel (10 cm wide, 35 cm long, 10 cm high) with white paper on the bottom. Before traversing the tunnel, the hind- and forepaws of the animals were dipped in nontoxic blue and red ink, respectively. The runway test was performed as follows. The animal was placed at one end of a horizontally fixed beam (1 cm wide, 100 cm long, separated into 11 segments by low obstacles, height from a table 40 cm), facing the opposite end, and allowed to move on the beam to reach an escape platform on its home cage. One investigator counted the number of slips of the forepaw and hindpaw on the right side of the beam, and another investigator counted the slips on the left side. Each day, each mouse underwent one trail (five consecutive runs) on five consecutive days. The ability to maintain balance on a rotating cylinder was assayed by using a computerized rotarod (Technical & Scientific Equipment GmbH) in its accelerating mode. Each mouse was tested once per day on five consecutive days. During each test session, animals were placed on the stationary rod for ~30 s, and the rod was started and accelerated continuously from 5 to 58 rpm over 270 s. The rotational speed at which a mouse fell off the rotating cylinder was recorded automatically. Mice that did not fall off during the 270-s trail period were given a score of 58 rpm.

Eye movement recordings

Mice were anesthetized with a mixture of halothane, nitrous oxide, and oxygen. The procedures for implanting a head fixation pedestal and a "mini" search coil were identical to those previously described (van Alphen et al., 2001). Baseline measurements were taken for their OKR, VOR, and VVOR. The OKR and VVOR in response to sinusoidal movement of the drum or table in the light were tested at five different frequencies (0.1, 0.2, 0.4, 0.8, and 1.6 Hz) and two different amplitudes (58 and 108; 0-peak). VOR in response to sinusoidal whole body rotation in the dark was tested at the same set of frequencies and amplitudes except that the stimulus frequency 0.1 Hz was omitted, because at this frequency, the vestibular signals driving the VOR are insufficient to obtain a powerful and reliable response. Subsequently, the animals were subjected to visuo-vestibular training for 5 d, which lasted 1 h per day. Animals were trained to reverse its direction using the "in phase" training protocol, which is the most effective training paradigm (van Alphen et al., 2001). Training began on the first day by rotating the optokinetic drum in phase, i.e., 0° phase difference, with table rotation at 5° amplitude. In the following 4 d, amplitude of the optokinetic drum was increased at 1° per day until it was 9° on day 5. At this point, the optokinetic drum was rotating in phase with the table but at twice the amplitude. For both turntable and drum movement, we chose a stimulus training frequency of 0.6 Hz, which is an optimal compromise to ensure both a reliable vestibular input to the VOR and a visual input with a peak velocity well within the physiological range of the mouse optokinetic system (van Alphen et al., 2001). Gain of the eye movement and phase of eye movement with respect to stimulus movement were calculated by fitting a sine wave to the average response using least-square optimization. When eye movement lagged stimulus movement, phase was expressed with a negative sign. Phase relations of VOR were shifted by 180°, making the phase angle zero for perfectly compensatory responses.

Statistics

Data shown are mean ± SEM, and statistical analysis was performed using ANOVA for repeated measures or the *t* test for two independent means. Significance was accepted if $P < 0.05$.

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Chapter 5

Mechanisms Underlying Cerebellar Motor Deficits Due to mGluR1-Autoantibodies

Mechanisms Underlying Cerebellar Motor Deficits Due to mGluR1-Autoantibodies

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Patients with Hodgkin's disease can develop paraneoplastic cerebellar ataxia because of the generation of autoantibodies against mGluR1 (mGluR1-Abs). Yet, the pathophysiological mechanisms underlying their motor coordination deficits remain to be elucidated. Here, we show that application of IgG purified from the patients' serum to cerebellar slices of mice acutely reduces the basal activity of Purkinje cells, whereas application to the flocculus of mice in vivo evokes acute disturbances in the performance of their compensatory eye movements. In addition, the mGluR1-Abs block induction of long-term depression in cultured mouse Purkinje cells, whereas the cerebellar motor learning behavior of the patients is affected in that they show impaired adaptation of their saccadic eye movements. Finally, postmortem analysis of the cerebellum of a paraneoplastic cerebellar ataxia patient showed that the number of Purkinje cells was significantly reduced by approximately two thirds compared with three controls. We conclude that autoantibodies against mGluR1 can cause cerebellar motor coordination deficits caused by a combination of rapid effects on both acute and plastic responses of Purkinje cells and chronic degenerative effects.

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Paraneoplastic cerebellar ataxia (PCA) occurs secondary to certain forms of cancer such as small cell lung cancer, ovarian cancer, breast cancer, or Hodgkin's disease.[1,2] The syndrome results from an immune response against components of cerebellar neurons that usually are homologous with antigens present in the tumor. Recently, we showed that PCA after Hodgkin's disease can be caused by autoantibodies against the metabotropic glutamate receptor type I (mGluR1-Abs).[3] In the cerebellum, this receptor is abundantly present in dendrites of Purkinje cells, at the perisynaptic site of both their parallel fiber and climbing fiber input.[4–7] However, the mechanisms by which mGluR1-Abs evoke cerebellar motor coordination deficits remain to be demonstrated.

Several potential pathophysiological mechanisms can be considered based on recent mGluR1 agonist/antagonist studies. First, an in vitro block of mGluR1 has been shown to exert acute effects on the excitability[8–10] and firing rate of Purkinje cells[9] (see Neale and colleagues[10]). Second, an in vitro mGluR1 block has been shown to interfere with long-term depression (LTD) at the parallel fiber–Purkinje cell synapse,[11–13] a form of synaptic plasticity commonly thought to play a role in cerebellar motor learning behavior. A third possible pathophysiological mechanism for which indications recently have emerged[14] could be a more chronic degenerative effect of an mGluR1 block on Purkinje cells, affecting dendritic arbor morphology or even killing the cells.

These potential pathophysiological mechanisms, which all may contribute to motor coordination deficits in PCA patients, probably are not mutually exclusive and may in fact interact during the various stages of the disease. To determine whether all three mechanisms indeed can play a role in the cerebellar coordination deficits of PCA patients with Hodgkin's disease, we investigated the motor behavior of these patients in more detail, and we studied the impact of their mGluR1-Abs on the activity of Purkinje cells of mice

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in vitro and on their motor behavior in vivo. In addition, we analyzed postmortem the brain of PCA Patient A who was subjected to mGluR1-Abs for at least 5 years.

Subjects and Methods

IgG Purification

IgG was purified from the first plasma exchange of Patients A and B and from normal control plasma using a protein A–Sepharose column (Bio-Rad, Richmond, CA). The purified IgG was dialyzed against phosphate-buffered saline and then concentrated using a centrifugal filter (Centricon; Millipore, Bedford, MA).

Basal Activity Recordings from Purkinje Neurons in Mouse Cerebellar Slices

The whole-cell patch-clamp recording in a cerebellar slice was conducted as previously described.[9,15] Transverse slices cut from the cerebellum of an ICR mouse were perfused continuously with external solution saturated with O2 and CO2. A cell body of a Purkinje neuron was perforated patch clamped with a patch electrode. The holding potential was −70mV, and the current was low pass filtered at 1Hz. Anti–mGluR1 IgG from Patient B was added to the slice bath.

Chronic mGluR1-Ab Infusion in Floccular Region and Eye Movement Recordings in Mice

Animal procedures described in this section were performed under an animal care protocol that was approved by the local ethical committee of Erasmus MC.

Sixteen C57Bl6 wild-type mice were prepared for eye movement recordings as previously described.[16] Optokinetic reflex (OKR) responses were tested using sinusoidal stimuli at peak velocities of 2, 4, 8, 16 and 32 degrees per second at a frequency of 0.4Hz. Vestibuloocular reflex responses in dark (VOR) and light (VVOR) were evaluated during sinusoidal whole-body rotation at 0.1, 0.2, 0.4, 0.8, and 1.6Hz and stimulus amplitudes of 5 degrees, in complete darkness or light. After 6 days of baseline recordings, a second operation was performed. Animals were anesthetized with a mixture of O2, N2O and 1,5 to 2% halothane. An incision was made behind the ear, the dura over the paraflocculus was opened, and a small polyvinylchloride catheter was connected to the hole. The catheter was led under the skin and connected to a microosmotic pump (Alza, Mountainview, CA), which had been filled earlier according to the Alza instructions, and then was implanted under the skin on the back. Mice were allowed 1 day recovery before the eye movement recordings continued. The microosmotic pumps used in these experiments deliver their contents at a rate of 0.5μl/hr for 7 days and were filled with purified and concentrated IgG (5–10mg/ml) from either Patient B or a healthy control. In the first series of experiments, the eye movements were recorded for another 7 days. Seven mice received mGluR1-Abs and five mice received control IgG. In the second series of experiments (n = 4, all receiving mGluR1-Abs) pump and catheter were removed 2 days after onset of the effects, and eye movements were recorded for an additional 5 days. After the eye movement recordings were finished, the mice were perfused transcardially with 4% paraformaldehyde in 0.1M sodium phosphate buffer (pH 7.2). Brains were removed, postfixed, and gelatin embedded, and coronal sections (40μm) were cut on a freezing microtome. Free-floating slices were incubated with biotinylated anti–human IgG (Vector, Burlingame, CA) and then with avidin-biotine complex (Vector), reacted with diaminobenzidine tetrahydrochloride, and counterstained with thionin.

Long-term Depression Induction Experiments in Embryonic Mouse Cerebellar Cultures

Mouse embryonic cerebellar cultures were prepared as previously described[11] and maintained in vitro for 9 to 16 days before their use in patch-clamp experiments. Patch electrodes were attached to Purkinje cell somata and contained CsCl (135mM), HEPES (10mM), EGTA (0.5mM), and Na2-ATP (4mM). Iontophoresis electrodes were filled with 10mM glutamate in 10mM HEPES. Glutamate test pulses were delivered using negative current pulses (600–900nA, 30–110-millisecond duration) applied at a frequency of 0.05Hz. After acquisition of baseline responses, six conjunctive stimuli were applied at 0.05Hz, each consisting of a glutamate test pulse combined with a 3-second depolarization step to 0mV, timed so the depolarization onset preceded the glutamate pulse by 500 milliseconds. Cells were bathed in a solution that flowed at a rate of 0.5ml/min and contained NaCl (140mM), KCl (5mM), CaCl2 (2mM), MgCl2 (0.8mM), HEPES (10mM), glucose (10mM), tetrodotoxin (0.005mM), and picrotoxin (0.1mM) and was adjusted to pH 7.35 with NaOH. Membrane currents were recorded with an Axopatch 200A amplifier (Axon Instruments, Burlingame, CA) in resistive voltage-clamp mode, low pass filtered at 5kHz, and digitized at 10 to 20kHz using an ITC-16 interface (Instrutech, Great Neck, NY). Bis-fura-2 ratio imaging of intracellular free Ca^{2+} was accomplished by measuring the background corrected fluorescence ratio at 340 and 380nm excitation using a cooled charge-coupled device camera system.[17] Exposure times were 100 to 400 milliseconds per wavelength image. Human IgG was either bath applied (extracellular application) or added to the internal saline in the patch electrode (intracellular application).

Saccade Adaptation in Humans

We performed a standard saccade adaptation paradigm.[18] During the experiment, changes were imposed on the visuomotor system by altering the visual input during saccades.

Subjects

Patient A (female, 49 years old) still harbored mGluR1-Abs and was suffering from severe cerebellar symptoms. Patient B (female, 23 years old) expressed mGluR1-Abs earlier but no longer harbored mGluR1-Abs and had no remaining symptoms at the time of the experiment. Clinical details on Patients A and B can be found elsewhere.[3] Patients C (female, 64 years old) and D (male, 56 years old) suffered from PCA with different well-characterized autoantibodies: anti–Yo[19] and anti–VGCC[20] autoantibodies, respectively. Four male

healthy control persons, ranging in age from 24 to 34 years, also participated in the experiments. The study was performed with the approval of the ethics committee at our institution, and informed consent was obtained from the patients.

Experimental Setup

Subjects were seated in a darkened room, and their head was fixated using a biteboard. Visual targets were red dots on a monitor, which was positioned 50cm from the subjects' head. Two-dimensional eye position was recorded with noninvasive infrared corneal reflection techniques. In Patient A, eye movements were measured using an Eyelink system (SensoMotoric Instruments, Berlin, Germany).[21] In Patient B, eye movements were measured using a Ober2-system (Permobil Meditech, Timra, Sweden). Controls were tested in both systems. The sampling rate of the Ober2-system was 500Hz per channel, whereas the sampling rate of the Eyelink was 250Hz. In the calibrated eye position signal, the onset and offset of saccadic eye movements were detected by the computer on the basis of both velocity and mean acceleration criteria.

Experimental Paradigm

In each trial, a red fixation spot appeared on the monitor at an eccentricity of 10 degrees to the left of the straight-ahead position. After a random period of 1 to 2 seconds, the target was displaced over 20 degrees to the right. Subjects had to redirect their gaze as quickly as possible to the new target. However, triggered by the saccade to this new target, the red dot was again displaced over 6 degrees to the left, thus making the first saccade appear too large. The timing of the saccade-triggered target displacement was approximately 30 milliseconds after saccade onset, which is the expected moment of the saccade peak velocity. The simultaneous occurrence of target jump and saccade peak velocity is optimal for good adaptation.[18]

Anatomical and Immunohistochemical Analysis

Brains were obtained from Patient A and three controls. Patient A (female, 49 years old) died of a cardiac infarct, while Hodgkin's disease was still in remission. Control 1 (female, 49 years old) died of cerebral contusion by metastasized carcinoma of ovarian origin. Control 2 (male, 68 years old) died of respiratory insufficiency, and Control 3 (male, 85 years old) died of cardiac decompensation. All controls were without known cerebellar disease. Consent was obtained from the next of kin for autopsy. Brain samples were taken to obtain frozen material. The rest of the brain was fixed by suspension in 10% formalin in phosphate-buffered saline for approximately 6 weeks. Blocks of tissue were taken from the culmen of the lobus anterior, nodulus, and flocculus and embedded in paraffin.

For examination of Purkinje cell dendritic morphology, 20μm sections were cut from tissue of all four subjects, incubated with rabbit anti–calbindin D28k antibodies (1:6,000; Swant, Bellinzona, Switzerland), processed using the avidin-biotin peroxidase diaminobenzidine tetrahydrochloride method (Vector), and counterstained with hematoxylin and eosin (Sigma, St. Louis, MO).

For Purkinje cell counts, sections were cut perpendicularly to the cerebellar folia at 7μm thickness. Every 10th section was selected and counterstained with hematoxylin and osin (Sigma). To obtain a reliable estimate of Purkinje cell density, we determined the number of Purkinje cells, divided by the length of the Purkinje cell layer, per section. From Patient A and the two male controls, the flocculus, nodulus, and lobus anterior of the vermis were analyzed; from the female control, only the nodulus and lobus anterior were analyzed.

We examined the possibility of immune-mediated degeneration of Purkinje cells with a range of immunological markers. We cut 5μm sections from frozen nonfixed cerebellar tissue of Patient B and incubated these with rabbit anti–CD3 (1:200; DAKO, Copenhagen, Denmark), mouse anti-CD4 (1:3,000; TNO-PG, Leiden, Germany), mouse anti-CD8 (1:800; TNO-PG), mouse anti-CD20 (1:25; Coulter, Hialeah, FL), mouse anti–human IgM (1:1,000; Nordic Tilburg, the Netherlands), rabbit anti–human IgG (1:400; TNO-PG), mouse anti–CD68 (1:800; DAKO), mouse anti–CD83 (1:150; Immunotech, Marscille, France), rabbit anti–nitric oxide synthase (1:4,000; Calbiochem, San Diego, CA), and acid phosphatase. Secondary antibodies used were biotine-conjugated donkey anti–rabbit (Amersham, Buckinghamshire, UK) for CD3 and nitric oxide synthase, rabbit anti–mouse (DAKO) for CD8, CD20, and CD68, and peroxidase-conjugated rabbit anti–mouse for CD4, swine anti–rabbit (DAKO) for IgG. Slices were processed with avidin-peroxidase (Sigma) if applicable and counterstained with hematoxylin and eosin (Sigma).

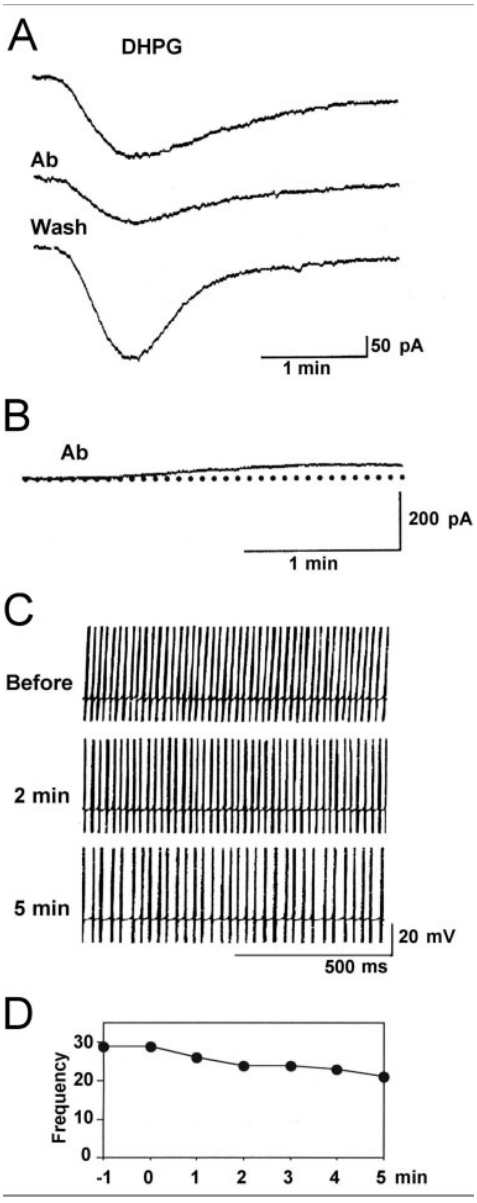
Results

Acute Effects on Currents Evoked by Exogenous mGluR1 Agonist

We tested the effects of IgG purified from patients' sera in a cerebellar slice preparation of mice. In voltage-clamp modem bath application of (*RS*)-3,5-dihydroxyphenylglycine (DHPG; Tocris Bristol, UK), a selective agonist for Group I mGluRs, induced an inward current consisting of a large transient phase and a smaller sustained phase (Fig 1A, top trace). Because Purkinje neurons express mGluR1 but not mGluR5, we considered that these currents were induced through activation of mGluR1. When the mGluR1-Abs were added, both the transient and sustained phases of the DHPG-induced inward current were suppressed significantly. After a 20 to 40-minute wash, the DHPG-induced transient inward current and the steady current increased again (see Fig 1A, bottom trace). Application of mGluR1-Abs without any other ligands reduced the holding inward current (see Fig 1B).

Acute Effects on Basal Activity of Purkinje Cells

This change in excitability induced by mGluR1-Abs suggests that these human antibodies can directly affect



the spontaneous firing frequency of Purkinje neurons. We investigated this issue in current-clamp recordings. Bath application of mGluR1-Abs to spontaneously firing Purkinje neurons slightly hyperpolarized them (three of five neurons) and significantly reduced their action potential firing frequency over the course of 5 minutes (see Fig 1C and D). Together these data show that acutely blocking mGluR1 on Purkinje cells by human mGluR1-Abs reduces Purkinje neuronal excitability and firing rate in unstimulated conditions.

Acute Impact on Performance of Compensatory Eye Movements

The observed effects of mGluR1-Abs on excitability and firing rate of Purkinje cells raise the possibility of an acute impact on motor performance. To test this possibility in a quantifiable manner, we recorded the compensatory eye movements of 16 C57/Bl6 mice after infusion of the human antibodies into their flocculus. After baseline recordings were done, we implanted an osmotic minipump, which delivered either patients' mGluR1-Abs (n = 11) or control IgG (n = 5) to the flocculus. All mice that received mGluR1-Abs showed a strong effect on their eye movement behavior after implant of the minipump. OKR gain values (eye velocity/stimulus velocity) decreased significantly at all frequencies, whereas VOR gain values did not change in any of them (Fig 2, middle panel). VVOR gain values were more prominently affected at the lower frequencies than at the higher frequencies (see Fig 2, bottom panel). Infusion of control IgG did not exert any effect on the gain nor on the phase of both OKR and VOR (see Fig 2, right column). Finally, in all cases tested (n = 7 for mGluR1-Abs, n = 5 for control IgG), immunohistochemical analysis showed the integrity of the floccular region including the injection site as well as the presence of human IgG in the flocculus. Removal of the pump resulted in increase of both the OKR and VVOR gain values in all cases tested (n = 4, all receiving mGluR1-Abs; data not shown). These re-

Fig 1. Acute effects of mGluR1-Abs on Purkinje neuronal activity. (A) DHPG-induced inward current before, during (Ab), and after (Wash) application mGluR1-Abs (Patient B). Two hundred micrograms IgG/ml suppressed the transient inward current induced by 50μM DHPG to 72.3 ± 11.7% and the steady current to 62.7 ± 56.7% (paired Student's t *test,* $p < 0.001$*; middle trace). After a 20 to 40-minute wash, the transient current increased again to 107 ± 25.6% and the steady current increased to 94.6 ± 68.7%. (B) mGluR1-Abs without other ligands reduce the holding inward current by 5 ± 8pA (n = 5). (C, D) mGluR1-Abs reduce Purkinje cell action potential frequency from 31.8 ± 10.4Hz to 24.4 ± 11.1Hz (paired Student's* t *test,* $p < 0.02$*). Application of mGluR1-Abs started at 0 minutes.*

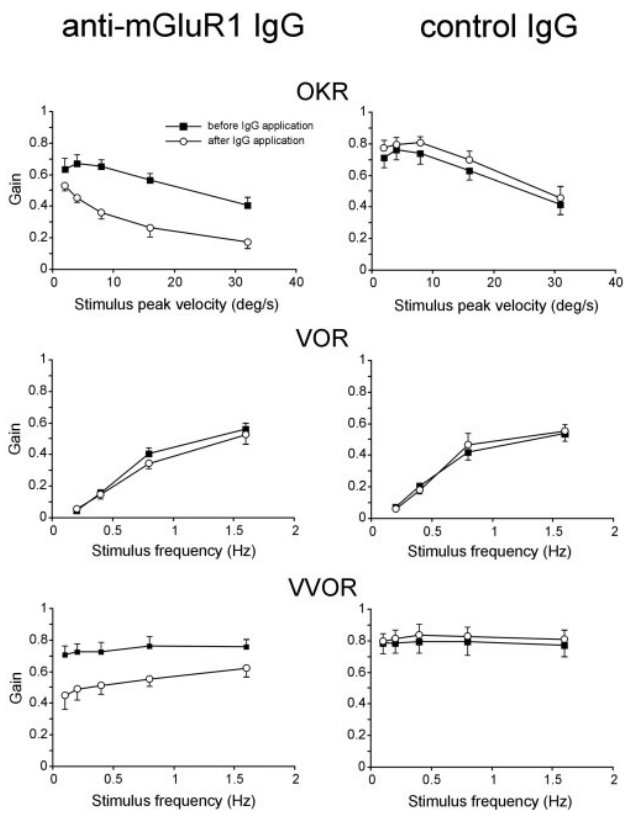


Fig 2. Effects on mice compensatory eye movements after mGluR1-Ab (Patient B) infusion into the flocculus. Optokinetic reflex (OKR) gain values (eye velocity/stimulus velocity) shown were plotted against stimulus peak velocity at a frequency of 0.4Hz. Vestibuloocular reflex (VOR) and visually enhanced vestibuloocular reflex (VVOR) gain values shown were plotted against frequency for 5-degree stimulus amplitude. Data shown were taken on the day before and on the second day after implantation of the microosmotic pump. Infusion of mGluR1-Abs reduces OKR values significantly at all frequencies (paired Student's t *test;* $p < 0.005$ *for all tested frequencies, except at 0.1Hz;* $p < 0.05$*; top panel), but not VOR gain values (middle panel). VVOR gain values show a larger mGluR1-Ab effect at the lower peak velocities (from 0.71* $\pm$ *0.16 to 0.46* $\pm$ *0.26 for a 0.1Hz, 5-degree stimulus;* $p < 0.02$*) than at the higher peak velocities (from 0.76* $\pm$ *0.14 to 0.62* $\pm$ *0.16 for a 1.6Hz, 5-degree stimulus;* $p < 0.05$*; bottom panel). Infusion of human control IgG shows no effects on compensatory eye movements.*

sults indicate that application of mGluR1-Abs to the flocculus reduces selectively, acutely, and reversibly the amplitude of the visual component of compensatory eye movements.

Patients' mGluR1-Abs Block Induction of Long-term Depression

Apart from an acute role in the excitability of Purkinje cells, mGluR1 also may be involved in the induction of LTD at the parallel fiber–Purkinje cell synapse.[22] To determine whether this process is also impaired, we applied mGluR1-Abs to cultured embryonic Purkinje cells during LTD induction.[11] After acquisition of baseline responses to glutamate test pulses, glutamate/depolarization conjunctive stimulation was applied to cultured mouse Purkinje cells to induce LTD. Glutamate test pulses then were resumed (Fig 3A). Previous experiments show a decrease in the amplitude of the Excitatory Postsynaptic Current (EPSC) to approximately 60% of the baseline.[11,17] However, when this experiment was performed in the presence of patients' mGluR1-Abs in the extracellular medium, LTD was strongly attenuated. In contrast, when mGluR1-Abs were added intracellularly via the patch pipette, or when IgG obtained from a healthy control person was added extracellularly, the decrease in EPSC amplitude was similar to that previously seen when no IgG was added.[11,17] These results indicate that patients' mGluR1-Abs are directed against the extracellular part of the receptor and that they can block LTD. To determine whether this blockage may be partly caused by an effect on calcium currents, we measured the effect of mGluR1-Abs on Ca^{2+} influx and/or mobilization. Figure 3B illustrates that neither basal Ca^{2+} nor Ca^{2+} influx via voltage-gated calcium channels was significantly altered by adding mGluR1-Abs from either patient or IgG from a healthy control person. In contrast, mGluR1-mediated Ca^{2+} mobilization was significantly reduced after adding the patients' antibodies. This result is consistent with the reduction in mGluR1-

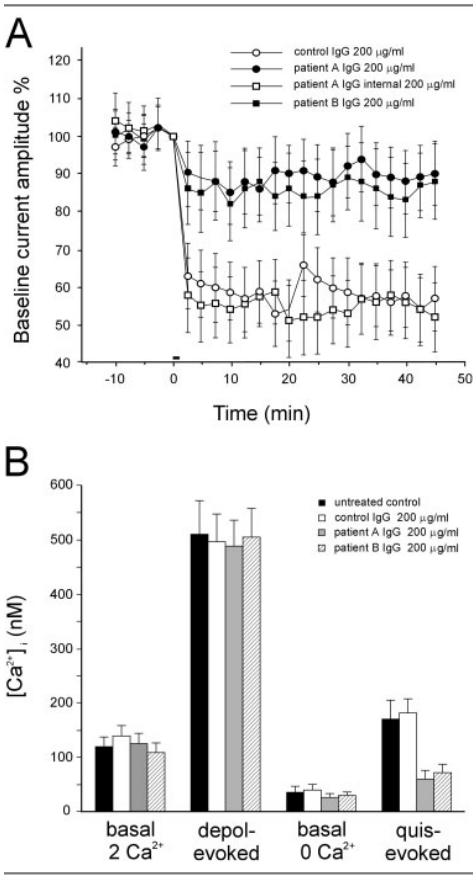


Fig 3. mGluR1-Abs block induction of long-term depression (LTD) and mGluR1-mediated Ca^{2+} mobilization. (A) After glutamate/depolarization conjunctive stimulation, the amplitudes of EPSCs induced by glutamate test pulses generally decrease to approximately 60% of their baseline values. However, this LTD cannot be normally induced after extracellular application of mGluR1-Abs (Patient A: 90 ± 8.4% of baseline, n = 6; Patient B: 88 ± 10.0%, n = 7; mean ± SEM). LTD induction remains intact upon extracellular application of human control IgG (57 ± 8.6%, n = 6), or intracellular application of mGluR1-Abs (52 ± 9.2%, n = 6). (B) Bis-fura-2 microfluorimetry was used to assess depolarization-evoked Ca^{2+} influx in the dendrites of Purkinje cells bathed in normal Ca^{2+} external saline (2mM) as an index of voltage-gated Ca^{2+} channel function, whereas quisqualate-evoked Ca^{2+} mobilization in Ca^{2+}-free external saline was used as an index for mGluR1 function. Depolarization-evoked Ca^{2+} influx via voltage-gated calcium channels is unaffected by mGluR1-Abs or control IgG. In contrast, quisqualate-evoked mGluR1-mediated Ca^{2+} mobilization is reduced by application of the patients' mGluR1-Ab, whereas control IgG application shows no effect.

mediated inward current shown in Figure 1A, and the reduction of phosphatidyl-inositol turnover measured in mGluR1-expressing CHO cells.[3] These results indicate that patients' mGluR1-Abs can block mGluR1-evoked processes, such as diacylglycerol production and IP3-mediated Ca^{2+} mobilization, which both normally contribute to LTD induction.

Adaptation of Saccadic Eye Movements Is Impaired in Paraneoplastic Cerebellar Ataxia Patients

Because LTD may be a mechanism underlying certain forms of cerebellar motor learning,[23,24] the data described above suggest that motor learning capabilities of PCA patients with mGluR1-Abs could be impaired. Yet, potential deficits in particular forms of cerebellar motor learning in these patients may be difficult to ascribe specifically to a lack of LTD, because deficits in motor performance itself also will directly impair cerebellar motor learning.[25] We therefore investigated a cerebellar learning paradigm, which was only blurred by performance deficits during relatively small periods of the training process: adaptation of saccadic eye movements. In this paradigm (Fig 4D), which is mediated by the oculomotor vermis,[26–28] fast adaptation of saccade amplitude can be obtained by consistently changing the visual target during visually guided saccades.[18,29]

Four patients were tested. Patient A had severe PCA and a positive titer for mGluR1-Abs at the time of testing; Patient B had been successfully treated for PCA due to mGluR1-Ab.[3] Patients C and D suffered from severe PCA associated with anti–Yo and anti–VGCC autoantibodies, respectively.[19,20] PCA associated with the latter two autoantibodies is characterized by massive Purkinje cell loss,[2,20] and these patients served as positive controls. The general dynamic properties of the saccadic eye movements of both Patients A and B were within the normal range[30] (see Fig 4A). Still, whereas Patient B showed normal adaptation of her saccadic eye movements after amplitude reduction training (data not shown), Patient A did not show adaptation (see Fig 4E). Thus, the lack of adaptation in Patient A may be caused by impaired LTD induction due to the activity of the mGluR1-Ab. However, because these autoantibodies may also contribute to degeneration, this association does not need to be directly causal. We therefore compared the saccadic eye movements of Patient A with Patients C and D who were likely to suffer from severe Purkinje cell loss. Patients C and D also did not show any adaptation of the amplitude of their saccadic eye movements after the training process, but in contrast with Patient A they showed typical cerebellar motor performance deficits such as saccadic dysmetria and postsaccadic drift (see Fig 4B). This difference suggests that possible degeneration in

Patient A must be insufficient to result in performance deficits as observed in Patients C and D, and that the lack of adaptation in Patient A probably is not predominantly caused by massive Purkinje cell loss.

Even though Patient A did not have the gross performance deficits of Patients C and D, she still had a slight opsoclonus and she frequently made a small gaze step toward the right from the initial fixation spot before the presentation of the target (see Fig 4A). Thus, in some trials the stimulus was presented at different retinal positions, affecting the amplitude of the required saccade. Because saccade adaptation is amplitude specific,[18] this might interfere with the saccade adaptation process. To determine whether this failure could contribute to her lack of adaptation, we devised a second, slightly modified, adaptation paradigm for four healthy control subjects. In this paradigm, we presented the control persons with a fixation spot that was at the exact same position where Patient A fixated at the onset of the target. Thus, the sequence of presented motor errors was identical in the controls as in Patient A. All four control persons subjected to this paradigm showed a considerable and gradual decrease of saccade amplitude (see Fig 4F), indicating that the lack of adaptation in Patient A was not caused by the presence of scatter in initial fixation position. Together, these data show that adaptation of the amplitude of saccadic eye movements is impaired in PCA patients with mGluR1-Ab, and that this deficit cannot be explained by a deficit in motor performance.

mGluR1-Abs Can Cause Purkinje Cell Loss

As discussed above, we demonstrated that the motor coordination deficits in PCA patients with mGluR1-Abs can be caused by acute disturbances in the excitability and plasticity of Purkinje cells. Yet, although magnetic resonance imaging scans of these patients did not show robust atrophy or degeneration (data not shown), it remains possible that degeneration occurred and contributed to their motor deficits. We therefore performed postmortem analysis on the cerebella of PCA Patient A, described above, and of three control persons. Control 1 was of the same age and gender as Patient A and died from cancer without cerebellar disease. In general, the size of Patient A's cerebellum including its granular layer, molecular layer, and white matter appeared normal. The density of Purkinje cells, however, was affected in all parts of the cerebellar hemispheres and vermis (Fig 5A, top panels). Purkinje cell morphology also was affected. As visualized with calbindin immunostaining, it was evident that the dendritic trees of the remaining Purkinje cells were severely amputated (see Fig 5A). In the quantitative analysis, special emphasis was put on the areas that are involved in the control of eye movements, that is, the flocculus and nodulus of the vestibulocerebellum, which control compensatory eye movements, and the lobus anterior, which includes the oculomotor vermis controlling saccadic eye movements. Figure 5B shows that the densities of Purkinje cells in these areas are approximately a third of those in three age-matched controls. In general, PCA is characterized by extensive inflammatory infiltrates consisting of cytotoxic T lymphocytes,[2,20,31] which progressively disappear over time when the immune response fades away, leaving the patient severely disabled with almost complete loss of Purkinje cells. To examine immune-mediated degeneration of Purkinje cells in Patient A, we performed immunohistochemical analysis of selected areas of the cerebellum. We did not observe any signs of an ongoing inflammatory reaction, and no cytotoxic $CD8^+$ T lymphocytes were detected. In areas with Purkinje cell loss, reactive Bergmann gliosis was noted. Although these findings do not support a role for a specific cellular immune reaction in the degeneration of the Purkinje cells, it cannot be excluded that such a process had occurred previously.[2] However, in contrast with other reported PCA autopsy cases, the loss of Purkinje cells in Patient A was much less prominent and may indicate, together with the absence of inflammatory infiltrates, that other mechanisms than a cytotoxic cellular immune response caused Purkinje cell degeneration.

Discussion

In this study, we investigated potential mechanisms underlying motor coordination deficits in PCA patients with mGluR1-Abs after Hodgkin's disease. The major findings indicate that mGluR1-Abs can affect Purkinje cells at three different levels including their excitability, plasticity, and survival. We suggest that these consequences at the cellular level, in turn, will result in deficits of both cerebellar motor performance and motor learning.

The acute effects of patients' mGluR1-Abs on Purkinje cells in the cerebellar slice preparation were a reduction of their excitation by mGluR1 agonists as well as their spontaneous firing rate, which is largely in line with the effects that have been shown at the in vitro level for antagonists such as MCPG,[8] CPCCOEt[9] (cf Neale and colleagues[10]), and LY367385.[32] The major difference was the relatively long time constant of the effect of the mGluR1-Abs as compared with the antagonists,[9] which might be caused by slower diffusion of the human IgG into the slice preparation.

The short-term effects of the mGluR1-Abs at the Purkinje cell level most likely explain the acute performance deficits of the compensatory eye movements that we observed after application of the mGluR1-Abs with the infusion pump into the flocculus of mice. The

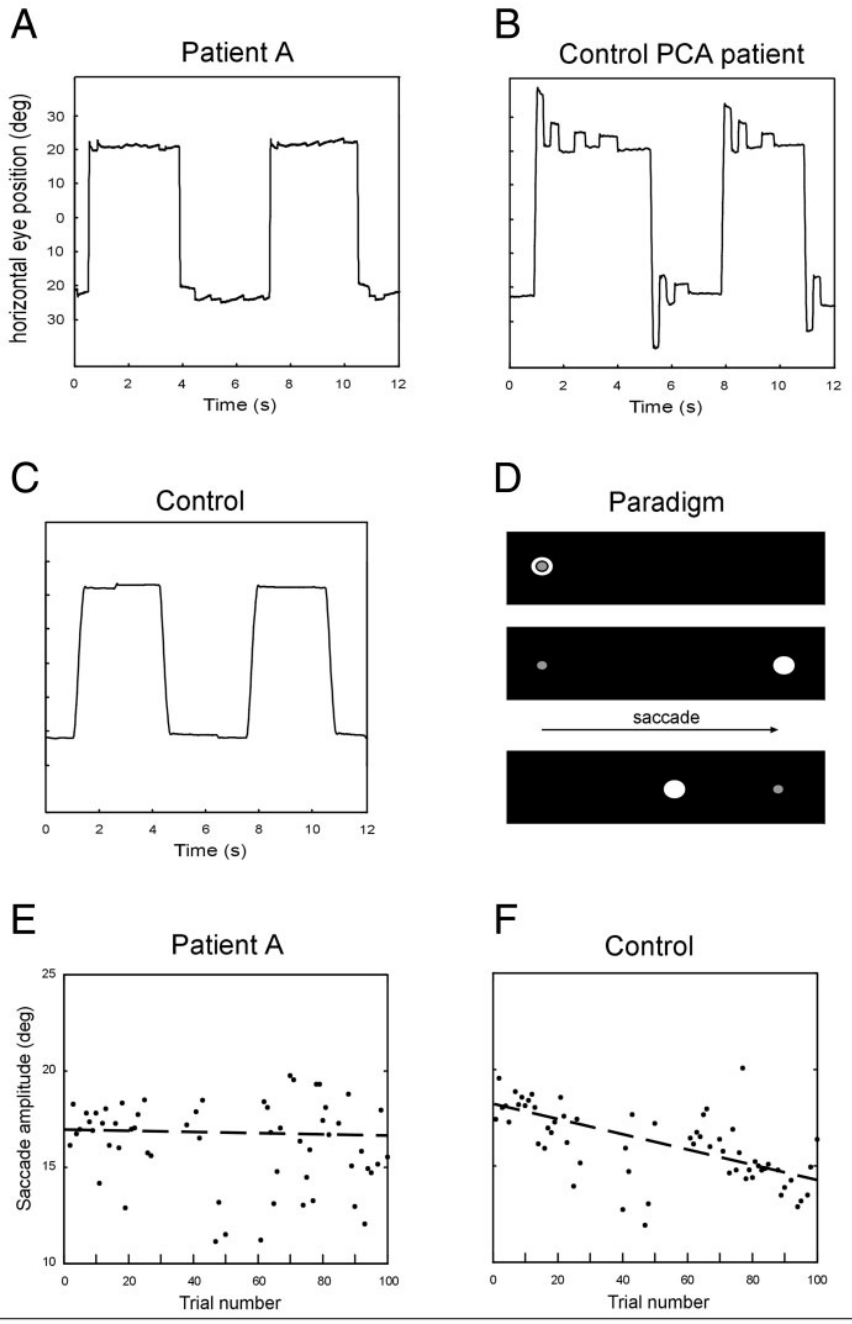

A
Patient A
horizontal eye position (deg)
30
20
10
0
10
20
30
0
2
4
6
8
10
12
Time (s)
B
Control PCA patient
0
2
4
6
8
10
12
Time (s)
C
Control
0
2
4
6
8
10
12
Time (s)
D
Paradigm
saccade
E
Patient A
Saccade amplitude (deg)
25
20
15
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Figure 4

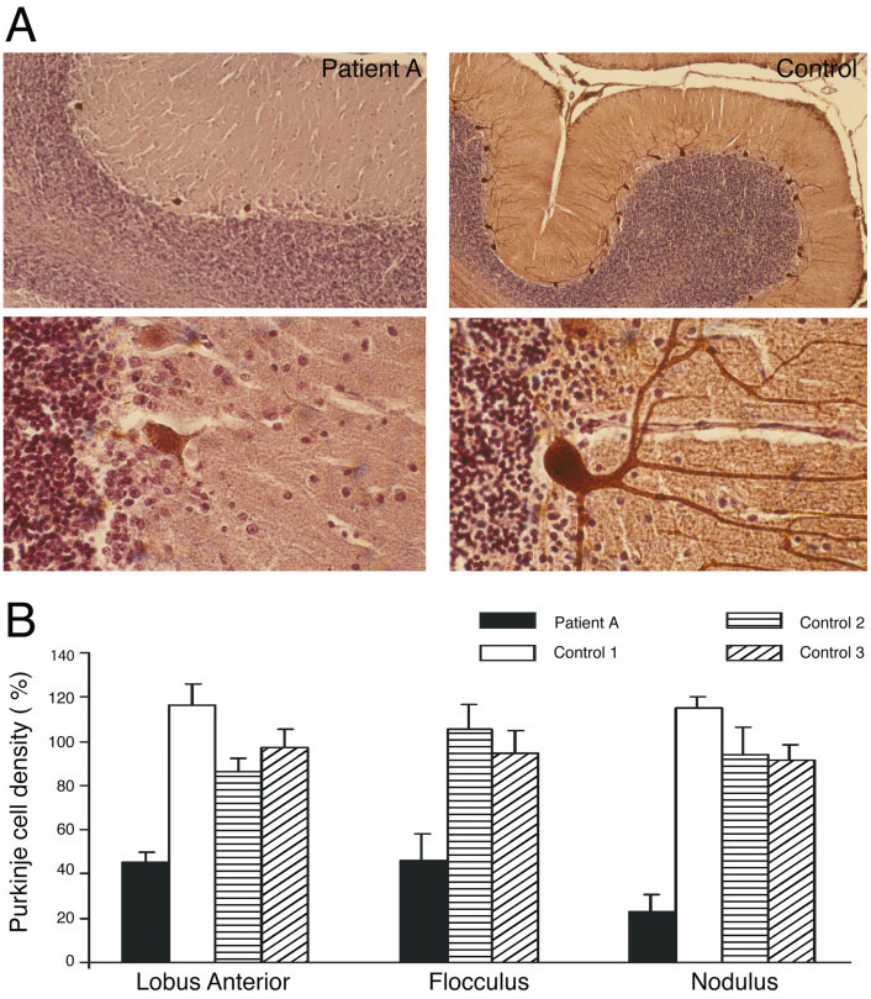

Fig 5. Effects of chronic exposition to mGluR1-Abs on Purkinje cell number and dendritic morphology. (A) Purkinje cell density in the cerebellum of Patient A is significantly lower than in control cerebella (top panels). Dendritic morphology of the remaining Purkinje cells is severely affected in the cerebellum of Patient A. (B) Purkinje cell loss in the cerebellum of Patient A. The Purkinje cell density in the lobus anterior was 45.1 ± 4.5%, in the flocculus 46.3 ± 11.9% (no floccular tissue from Control 1 was available), and in the nodulus 23.1 ± 7.9% of the average density in the controls (two-sample unequal variance Student's t *test,* $p < 0.0001$ *for all analyzed regions).*

Fig 4. Saccadic properties and saccade adaptation. Saccadic eye movements of Patient A (A) compared with those of control PCA patient C (B) and a healthy control person (C). (D) Saccade adaptation paradigm. First the target (white) is fixated by the test subject (gaze position shown in gray). The target jumps 20 degrees toward the right. When the test subject makes a saccade toward the target, the target simultaneously jumps back 6 degrees to the left. (E) Patient A shows no saccade adaptation when presented with the adaptation paradigm. Note the large scatter in saccade amplitude. (F) Healthy control persons show a gradual decrease in saccade amplitude when tested in the adjusted control adaptation paradigm.

decreased spike activity that we see after mGluR1-Ab application to a cerebellar slice suggests that mGluR1-Ab injection into the flocculus reduced floccular spike activity and thereby the gain of the OKR and VVOR. This is in line with the fact that an artificially increased floccular activity leads to an increased OKR gain.[33]

Other possible explanations such as a blockage of LTD or a degeneration of Purkinje cells probably do not contribute to this acute process. For example, a blockage of LTD induction at the parallel fiber–Purkinje cell synapse impairs adaptation of the VOR but does not necessarily result in performance deficits or ataxia.[24] Similarly, we showed in the autopsy study of PCA patients that chronic exposure to mGluR1-Abs can lead to a severe loss of Purkinje cells, but histological analysis of the floccular region of mice that were treated with mGluR1-Ab showed that this form of degeneration does not occur within a couple of days. Moreover, the infusion experiments in mice also demonstrated that the gain reducing effects were reversible indicating that no damage with permanent functional consequences was done. Thus, direct exposure to the patients' mGluR1-Abs can lead to acute changes in the excitability and firing rate of Purkinje cells, and this effect in itself is sufficient to evoke acute deficits in cerebellar motor performance.

The learning deficits on the other hand are most likely caused by a blockage of LTD. The experiments on cultured Purkinje cells indicated that the patients' mGluR1-Abs can block mGluR1-mediated Ca^{2+} mobilization as well as LTD induction, whereas the behavioral investigations of the patients showed that fast adaptation of the amplitude of their saccadic eye movements is impaired. The fact that the general performance of their saccades was relatively normal suggests that for this particular cerebellar paradigm the lack of motor learning was not caused by such severe performance deficits as are seen in the PCA-positive control patients with anti–Yo and anti–VGCC autoantibodies. Even so, for other cerebellar motor learning paradigms such as adaptation of the VOR, we were unable to determine whether impairment of LTD induction was the major cause underlying the learning deficit, because in both the patients and the mice treated with the infusion pumps the general motor performance of their compensatory eye movements was insufficiently normal.[25] In this respect, it is noteworthy that the normal saccadic performance of Patient B was relatively spared. Why the general performances of different cerebellar motor behaviors were affected differently eludes us.

The essential role of mGluR1 for LTD induction at the parallel fiber–Purkinje cells synapse has been demonstrated in various studies both in cultured Purkinje cells and in the slice preparation.[11–13,34,35] The role of LTD induction at the parallel fiber–Purkinje cell synapse in cerebellar motor learning originally was suggested by Marr[36] and Albus,[37] and the first experimental evidence for the relation between LTD induction and cerebellar motor learning was provided by Ito and colleagues.[23,38] Both the role of mGluR1 in LTD induction and the role of LTD induction in cerebellar motor learning recently have been supported by experiments in various mouse mutants. For example, a global knockout of mGluR1 shows a blockage of LTD induction and impaired eye blink conditioning.[39,40] However, the investigations of these animals also showed the general caveats of global knockouts such as developmental deficits and a lack of cell specificity. The mGluR1 knockout showed a persistent multiple climbing fiber innervation of their Purkinje cells,[41–43] and because the expression of mGluR1 is not restricted to Purkinje cells or the cerebellar cortex,[4–6] they showed a complex of several behavioral deficits which makes it difficult to specifically attribute the impaired eye blink conditioning to a lack of LTD induction at the parallel fiber–Purkinje cell synapse. The problem of cell specificity, however, has been largely solved by creating and testing a cell-specific rescue of the mGluR1 knockout, in which LTD induction and the major cerebellar motor deficits were rescued.[44] The present study adds to the body of evidence that LTD is probably necessary for cerebellar motor learning, because with the use of the mGluR1-Abs it was possible to circumvent the developmental problems of the mutants and still observe the same relationship between LTD and motor learning.

Although we have in part successfully dissected here the various direct molecular, cellular, and behavioral effects of the mGluR1-Ab, note that in the patients all these direct effects act in concert with their chronic degenerative impact on both the morphology and number of Purkinje cells. Although this latter effect is not as severe as in many forms of PCA caused by other immune reactions,[2,45–47] it probably still was sufficiently prominent to contribute substantially to part of the symptoms. PCA associated with antineuronal antibodies generally is caused by a specific cytotoxic immune reaction involving $CD8^+$ lymphocytes.[2,20,31] The absence of inflammatory infiltrates combined with the relatively high number of spared Purkinje cells suggests that mGluR1-Abs may cause Purkinje cell degeneration by other mechanisms.

In addition, we have demonstrated that mGluR1-Abs can cause neurological symptoms in vivo upon transfer into experimental animals,[3] whereas other paraneoplastic autoantibodies do not cause neurological damage upon passive transfer into animals.[46,47] With the exception of anti-VGCC, mGluR1-Ab is the only PCA associated autoantibody directed against a cell surface epitope. The accessibility of the target antigen

to mGluR1-Abs can explain the ability of mGluR1-Abs to directly interfere with Purkinje cell function. Interestingly, degeneration of Purkinje cells has recently been demonstrated in vitro and in vivo by application of the Group 1 mGluR antagonist CPCCOEt.[14] How this degeneration exactly comes about is still a matter of debate. Further studies of chronic application of mGluR1-Abs in vitro and in vivo may help elucidate the role of chronic mGluR1 blockade and antibody-mediated immune mechanisms involved in the chronic Purkinje cell degeneration in PCA caused by mGluR1-Abs.

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Chapter 6

Role of LTD in Vestibular Compensation

Role of LTD in vestibular compensation

M. Faulstich, C. Luo, A.M. van Alphen, S. du Lac and C.I. De Zeeuw

Abstract

Recovery from a unilateral labyrinthectomy is a clear example of plasticity in the central nervous system. To investigate the contribution of cerebellar LTD in this form of plasticity we made unilateral lesions of the labyrinth in Lurcher mice, l7-PKCi transgenic mice and control mice. Lurcher is a mutant mouse strain that is characterized by absence of cerebellar Purkinje cells in adult life and hence has no functional cerebellum. L7-PKCi transgenic animals lack a form of cellular plasticity called cerebellar long-term depression, which is a candidate cellular learning mechanism thought to underlie different forms of motor learning. Vestibular compensation following unilateral labyrinthectomy reflects a long-term adaptive process and comparison of Lurcher and control mice confirmed that this process depends on the cerebellum. However, absence of differences in VOR recovery between L7-PKCi transgenic animals and control animals shows that, although dependent on cerebellar function, recovery of the VOR does not rely on LTD of the parallel fiber to Purkinje cell synapse.

Introduction

De-afferentation of one labyrinth results in a characteristic syndrome of oculomotor and postural disorders. These disorders can be divided into two categories: dynamic and static symptoms (Smith and Curthoys, 1989; Dieringer, 1995). Static symptoms include a deviation of the eyes towards the lesioned side and spontaneous nystagmus. Dynamic symptoms encompass alterations in reflexes that derive their input from the vestibular organ, like the vestibulo ocular reflex (VOR). The VOR typically exhibits a severely reduced gain and increased phase lead following unilateral labyrinthectomy (Baarsma and Collewijn, 1975; Maioli et al., 1983; Fetter and Zee, 1988; Vibert et al., 1993). Vestibular compensation is a clear example of plasticity in the central nervous system.

Resolution of dynamic symptoms is analogous to a well-known form of motor learning, i.e. VOR adaptation. In VOR adaptation the relation between head movement and image slip is altered, for instance by placing an optical device like reversing prisms in front of the eyes, which results in a recalibration of the underlying VOR (Jones and Davies, 1976; Jones, 1977). After unilateral labyrinthectomy a recalibration of the VOR is also required because this intervention essentially halves the input to this reflex. Over time VOR gain recovers to almost normal values in monkey and man for low stimulus velocities, while at higher stimulus velocities recovery is less complete and remains asymmetric (Allum et al., 1988; Fetter and Zee, 1988; Curthoys and Halmagyi, 1995). Recalibration of the VOR following unilateral labyrinthectomy does not occur in the absence of vision (Courjon et al., 1977) and requires intact cerebellar function (Courjon et al., 1982), which lends further support to the analogy between this phenomenon and VOR adaptation.

A candidate cellular mechanism underlying VOR adaptation is long-term

depression (LTD) of the parallel fiber to Purkinje cell synapse (Ito, 1982). L7-PKCi transgenic mice, in which cerebellar LTD was selectively blocked, were unable to increase VOR gain when subjected to a visuo-vestibular adaptation paradigm for one hour (De Zeeuw et al., 1998). In the present study we investigate whether vestibular compensation in the mouse is indeed dependent on the cerebellum and whether it involves the same cellular mechanism as short term VOR adaptation. Unilateral labyrinthectomies were therefore performed in three types of mice, including L7-PKCi transgenic mice, Lurcher mice and control mice. Lurcher mutant mice effectively model a complete lesion of the cerebellar cortex since at three months of age all cerebellar Purkinje neurons have degenerated in this mutant strain.

Methods

Surgical procedures

Mice were anaesthetized with a mixture of halothane, nitrous oxide and oxygen. An acrylic head fixation pedestal was formed and fixed to the skull with small screws (M1, 1.5 mm) implanted on the frontal, parietal and interparietal bone plates. A small incision was made in the conjunctiva on the temporal side of the eyeball. A pocket was bluntly dissected anterior to the insertion of the lateral rectus muscle. A copper wire coil (1 mm outside diameter, 60 turns, 1.0 mg) was placed in the pocket. The coil was fixed to the sclera with 2 sutures (10/0 nylon, Ethicon). The sutures were approximately aligned in the equatorial plane of the eye. The conjunctiva was closed over the coil with an additional suture. The leads of the coil were carefully tunneled underneath the conjunctiva and the skin to a miniature coaxial MMCX connector that was attached to the top of the acrylic head pedestal. All animals were allowed to recover for at least 3 days before the first eye movement recordings were made.

Following recovery, 2-3 days of baseline eye movement measurements were made, after which a labyrinthectomy was performed under halothane/nitrous oxide anesthesia. A retro-auricular incision was made to expose the temporal bone and the horizontal semicircular canal. The horizontal canal was drilled open over an extent of at least 1 mm with a .5 mm burr. The endolymphatic fluid was drained passively with absorbent points (Henry Schein Inc. NY, USA). After the flow of endolymph ceased, air was flushed through the canal via a blunted 30-gauge needle connected to a 3 cc syringe. The airflow was aimed anteriorly into the canal, in the direction of the horizontal canal crista. After draining and flushing, a dental paper tip was inserted into the rostral opening of the canal and fixed in place with bone wax.

Damage to the vestibular apparatus was verified post-mortem in a subset of animals. Following transcardial perfusion, the temporal bones were embedded in resin, sectioned at 10 μm, and stained with a mixture of toluidine blue and methylene blue (1:1; final concentration 1%). Microscopic evaluation revealed a complete loss of tectorial structure over the cristae of the semicircular canals and the utricle and saccule on the side of the lesion. As shown in Fig. 1A, all stereocilia were missing, and hair cells showed signs of degeneration. Corresponding sections of the temporal bone from the intact side showed normal hair cells and intact stereocilia and tectorial structure (Fig. 1B).

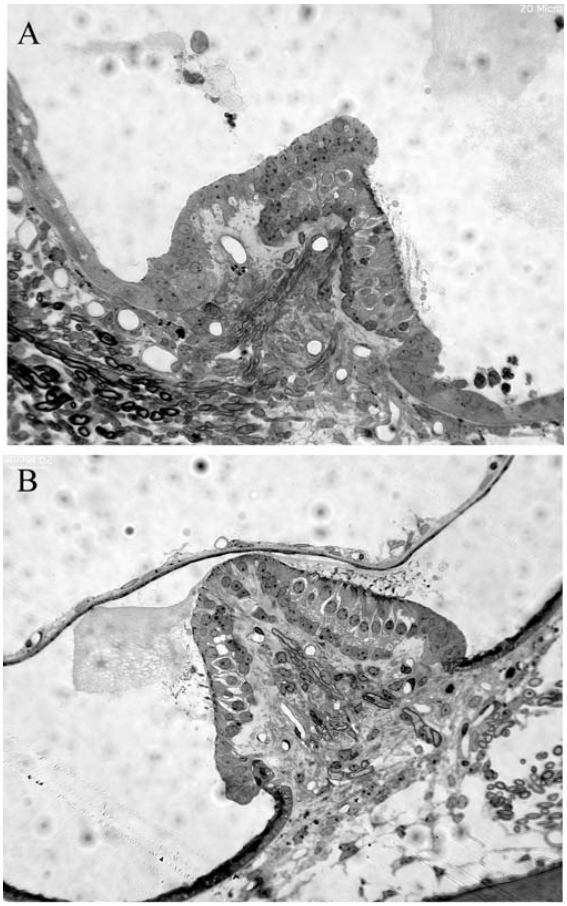

Figure 1: Histology of the crista of the horizontal canal in a canal-lesioned mouse. Sections show corresponding locations from the lesioned (A) and intact (B) side. Note in A the lack of tectorial structure, the absence of stereocilia and the degeneration of hair cells with the resulting disarray of cellular structure. Hair cells in B show the normal regular arrangement and stereocilia. Part of the tectorial structure can be seen attached to the cilia. The thin structure spanning the canal lumen just above the crista in B is the lining of the canal, which detached during histological preparation.

Behavioral testing

During eye movement measurements, mice were immobilized in a custom-made restrainer incorporating an aluminum plate to which the head fixation pedestal was bolted. The restraint assembly was mounted within magnetic field coils (CNC Engineering, Seattle, WA) atop a vertical-axis turntable (Biomedical Engineering Co., Thornwood, NY). The midpoint of the interaural axis was positioned in the center of rotation of the turntable. The field coils were attached to the turntable and moved with the animal. Optokinetic stimuli were delivered with a striped drum (bar width: 4 deg). The drum had a diameter of 26 centimeters and surrounded the animal. The vestibulo-ocular reflex (VOR) was evaluated during sinusoidal, whole body rotation in the dark. Eye position and stimulus recordings were filtered on-line at 20 Hz using a 4th order Bessel filter (Axon Instruments, Foster City, CA) and digitized at 250 Hz with 32-bit precision (CED, Cambridge, UK). Before each recording session the eye coil was calibrated by rotating the field coils around the animal. During rotation of the field coils

of 20 degrees and the resulting voltage was taken as a reference for the following recording session.

The optokinetic reflex (OKR) and vestibulo-ocular reflex (VOR) were measured in three different strains of mice: L7-PKCi (De Zeeuw et al., 1998), wildtype C57bl/6, and Lurcher. The OKR was tested with sinusoidal rotation of the striped drum at 5 different frequencies: 0.2, 0.4, 0.6, 0.8 and 1 Hz. The amplitude of the drum was constant at 5 deg (0-peak), resulting in peak velocities that ranged from 3 to 31 deg/sec. The VOR was tested by rotating the animal sinusoidally in the dark over the same frequency range as the OKR and with amplitudes of 5 and at 10 deg (0-peak). The visually enhanced VOR (VVOR) was tested in the light by whole body rotation with respect to an earth fixed drum. The order in which stimuli were delivered was randomized. Each recording session lasted approximately 30 minutes.

Data analysis

Eye, head and drum position were differentiated off-line, using a 3-point software algorithm. Fast phases were identified algorithmically whenever eye velocity exceeded 2.5 times the peak stimulus velocity. Identified fast phases were excised from the eye velocity trace with a margin of 20 ms before to 80 ms after detection. Gain and phase of eye movement with respect to stimulus movement were calculated by fitting a sine wave to the average using least-square optimization. When eye movement lagged stimulus movement, phase was expressed with a negative sign. Phase relations of VOR and VVOR were shifted by 180 deg, making the phase angle 0 for perfectly compensatory responses.

Nystagmus was analyzed by counting the number of fast phases (eye velocity > 150 deg/s) that occurred in 3 minutes when the animal was stationary in the dark.

Results

Effects of labyrinthectomy on wildtype mice

Following labyrinthectomy, mice displayed the postural symptoms of unilateral vestibular dysfunction that have been observed in other species, including head tilt, rolling, and circling. Ocular nystagmus with quick phases directed toward the side contralateral to the lesion was prominent immediately after labyrinthectomy and declined to control levels over a time course that varied from hours to days across mice. Fig 2A plots the rate of quick phases as a function of time with respect to labyrinthectomy averaged across 6 wildtype mice. In one mouse, a spontaneous nystagmus redeveloped 5-7 days following labyrinthectomy, as has been observed in monkeys (Fetter and Zee, 1988).

Labyrinthectomy impaired the ability of mice to stabilize gaze during head movements. Within a period of days, however, adaptive changes in the VOR restored gaze stabilization. Fig. 3 shows gain (A) and phase (B) of VVOR in wildtype mice as a

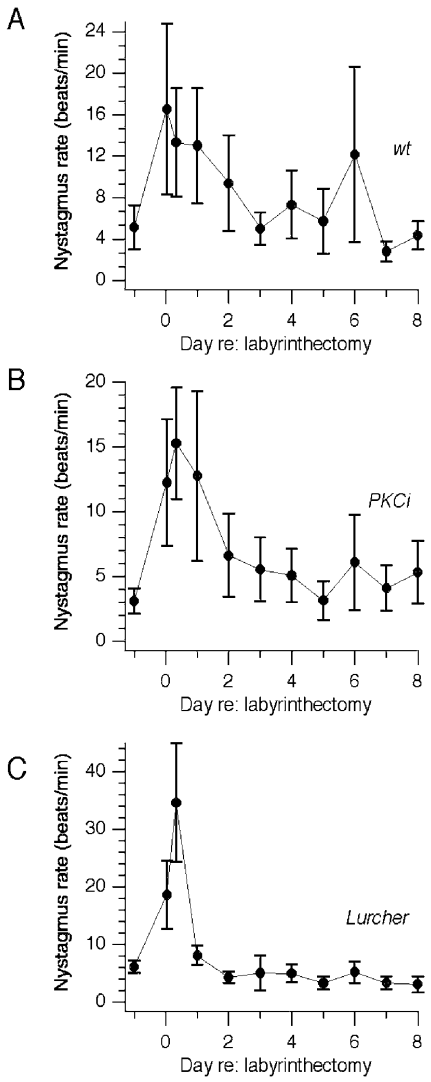

Figure 2: Timecourse of spontaneous nystagmus following labyrinthectomy. Each panel plots the mean rate of rapid (> 150 deg/s) eye movements recorded during 3 minutes in darkness in the absence of vestibular stimulation, as a function of day with respect to labyrinthectomy. A. Wildtype mice (n=5). B. L7-PKCi mice (n=6). C. Lurcher mice (n=6). Error bars are standard error of the mean.

function of time with respect to labyrinthectomy. Data are the mean ± SEM of 6 animals in response to head rotation at 0.6 Hz, ± 5 deg. In control conditions, the average VVOR gain in the two days preceding the lesion was 0.77. The gain measured 8 hours after the lesion dropped to 0.46, and then increased over the subsequent three days. By the third day following the lesion, gain had increased to 0.65 and showed little change for the duration of the experiment (8 days). VVOR phase was almost perfectly compensatory in control conditions and was affected relatively little by labyrinthectomy (Fig. 3B)

To assess the relative contributions of vestibular and visual pathways to the adaptive changes in gain stabilization observed in VVOR, we analyzed recovery of VOR and OKR following labyrinthectomy. Fig. 3C shows VOR gain and Fig. 3D shows VOR

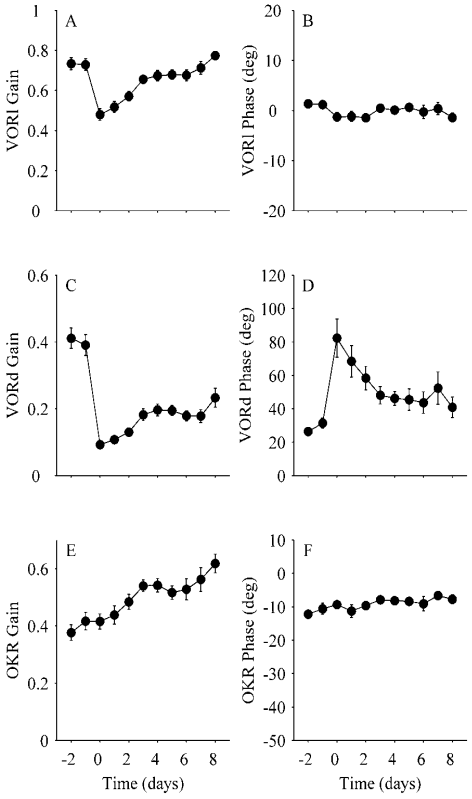

Figure 3: Effects of labyrinthectomy on evoked eye movements in wildtype mice. A and B plot gain and phase, respectively, of VOR in the light (VVOR) as a function of day with respect to labyrinthectomy. C and D plot gain and phase of VOR in the dark. E and F plot gain and phase of the OKR. Stimuli in all conditions were 0.6 Hz, ± 5 deg. Plots show averages and standard errors of data from 6 mice. Phase in B and D is shifted by 180 deg such that a phase response of 0 deg represents perfectly compensatory VOR. Positive phase indicates a phase lead with respect to compensatory.

phase at 0.6 Hz, ± 5 deg for our population of 6 wildtype mice. In control conditions VOR gain averaged 0.41, with a phase lead relative to compensatory of 33 deg. Following labyrinthectomy, VOR gain dropped to 0.09. Gain increased to 0.18 over the following 3 days, after which it remained relatively constant (ranging from 43 to 55% of control values). Labyrinthectomy resulted in a large and variable phase lead in VOR that averaged 82 deg. Within 3 days, VOR phase lead dropped to 52 deg and was relatively stable from the third to the eighth day following the lesion.

Fig. 3E shows OKR gain prior to and following labyrinthectomy. The OKR gain in control conditions was 0.4 when measured at 0.6 Hz, ± 5 deg, and eye movement phase lagged that of the visual stimulus by 13 deg. Labyrinthectomy had no immediate effect on OKR gain. During the 8 subsequent days, OKR gain increased to values that were higher than in control conditions. OKR phase remained relatively constant throughout the testing period (Fig 3F).

Adaptive increases in VOR gain following labyrinthectomy were evident across all but the lowest frequency that we tested. Fig. 4A plots VOR gain prior to, 8 hours following, and 6-8 days following labyrinthectomy as a function of stimulus frequency. In control mice, VOR gain depended on frequency, increasing to a plateau level from 0.2

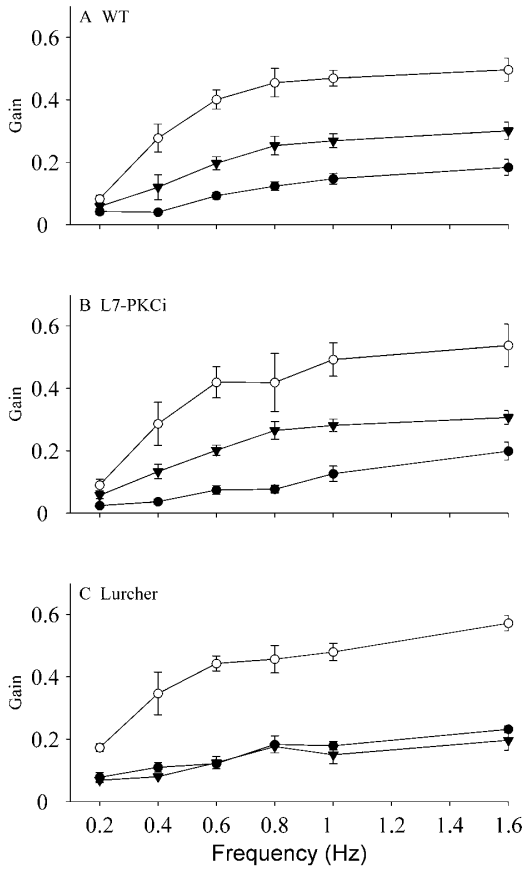

Figure 4: Frequency-dependence of adaptive increases in VOR gain. The gain of VOR in the dark is plotted as a function of rotation frequency in control conditions (open circles), 8 hours following labyrinthectomy (filled circles), and 6-8 days following labyrinthectomy (filled triangles). Plots indicate the mean and standard error of 6 mice; stimulus amplitude was ±5 deg. Panel A shows data from wildtype mice; B shows L7-PKCi mice, and C. shows Lurcher mice.

Hz to 0.8 Hz, as has been reported previously. Following labyrinthectomy, gain dropped to 48% of control levels when tested at 0.2 Hz and to between 16 and 32% at higher stimulus frequencies. No significant change in gain was observed after 6-8 days in response to head rotations at 0.2 Hz. The largest increases in gain were observed in response to head rotations at 1.6 Hz, the highest frequency that we tested. Following labyrinthectomy, eye movements directed away from the side of the lesion were slightly greater than those oppositely directed; however, these differences were not statistically significant (paired t-test, $p > .05$ at all frequencies, n=6 animals).

L7-PKCi mice show normal adaptive gain increases following labyrinthectomy

To determine whether cerebellar long term depression (LTD) is required for the adaptive increases in VOR gain that are induced by unilateral damage to the vestibular periphery, we analyzed eye movements in transgenic mice in which an inactivating peptide of

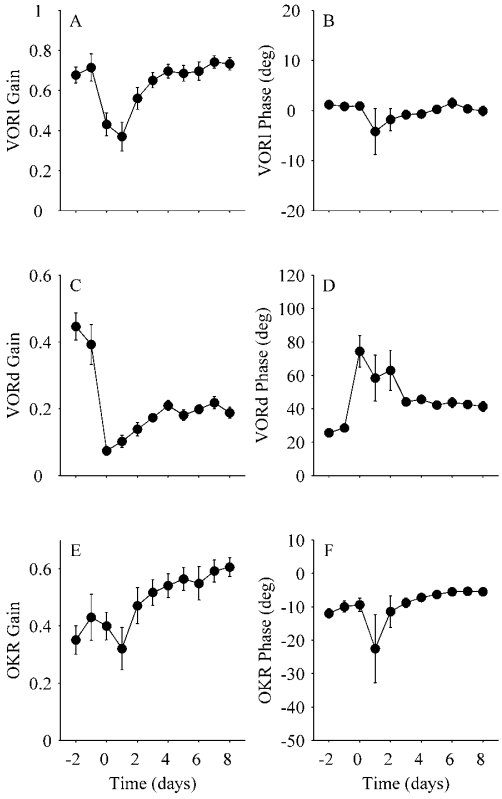

Figure 5: Effects of labyrinthectomy on evoked eye movements in L7-PKCi mice. A and B plot gain and phase, respectively, of VOR in the light (VVOR) as a function of day with respect to labyrinthectomy. C and D plot gain and phase of VOR in the dark. E and F plot gain and phase of the OKR. Stimuli in all conditions were 0.6 Hz, ± 5 deg. Plots show averages and standard errors of data from 6 mice. Phase in B and D is shifted by 180 deg such that 0 phase represents perfectly compensatory VOR. Positive phase indicates a phase lead with respect to compensatory.

protein kinase C was expressed under control of the Purkinje cell promotor L7. Previous studies have shown that L7-PKCi mice do not exhibit cerebellar LTD, either in culture (De Zeeuw et al., 1998) or in brain slices (Goossens et al., 2001). As described previously (De Zeeuw et al., 1998), we found that under control conditions, eye movements are normal in L7-PKCi mice; at 0.6 Hz ± 5 deg, VVOR gain averaged 0.70, VOR gain averaged 0.42, and OKR gain averaged 0.39. Although L7-PKCi mice are unable produce rapid adaptive increases in VOR gain following an hour of visual-mismatch training (De Zeeuw et al., 1998), they exhibited gain increases following labyrinthectomy that were indistinguishable from those in wildtype mice. Fig. 5A shows VVOR prior to and during the 8 days following unilateral labyrinthectomy in 6 L7-PKCi mice. Labyrinthectomy induced an initial drop in VVOR gain to 0.43, which was followed by ncreases in gain over the subsequent 3-4 days. Gain reached plateau values of 0.69-0.74 between days 4 and 8. As in wildtype animals, after labyrinthectomy, the phase of VVOR remained nearly perfectly compensatory (Fig. 5B). L7-PKCi mice showed adaptive increases in VOR, as seen in Fig 5C. The gain of VOR dropped to 0.16 within 8 h following labyrinthectomy and recovered to a plateau value of 0.28 within 4

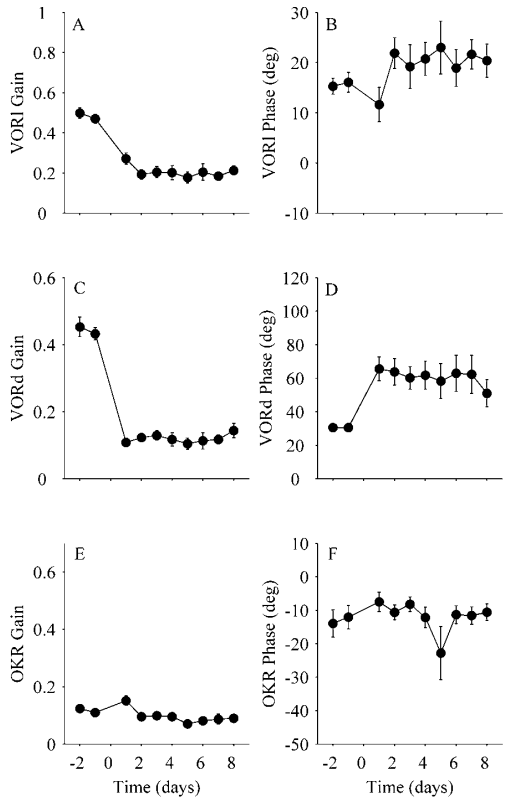

Figure 6: Effects of labyrinthectomy on evoked eye movements in Lurcher mice. A and B plot gain and phase, respectively, of VOR in the light (VVOR) as a function of day with respect to labyrinthectomy. C and D plot gain and phase of VOR in the dark. E and F plot gain and phase of the OKR. Stimuli in all conditions were 0.6 Hz, ± 5 deg. Plots show averages and standard errors of data from 6 mice. Phase in B and D is shifted by 180 deg such that 0 phase represents perfectly compensatory VOR. Positive phase indicates a phase lead with respect to compensatory.

days. As shown in Fig. 5B Labyrinthectomy resulted in large and variable phase lead over the first 2 post-lesion days. By the third post-lesion day VOR phase a value of 44 deg, which remained stably and significantly elevated over control conditions during the subsequent 5 days.

These adaptive increases in VOR gain were not significantly different from those in wildtype mice on any of the days tested ($p > .20$; unpaired t-tests). As in wildtype mice, OKR gain in L7-PKCi mice increased following labyrinthectomy (Fig. 5E), and OKR phase was unaffected by labyrinthectomy (Fig. 5F). The patterns of gain changes across frequency were similar in L7-PCKi and wildtype mice (Fig. 4B).

Impaired VOR plasticity in Lurcher mice

The results described above indicate that PKC expression in Purkinje cells is not required for adaptive increases in VOR gain following unilateral loss of the vestibular periphery. To investigate whether labyrinthectomy-induced gain increases depend on intact cerebellar function, we measured eye movements in the Lurcher strain of mice, in which

Purkinje cells degenerate by the sixth postnatal week (Caddy and Biscoe, 1979; Wetts and Herrup, 1982).

Labyrinthectomy produced a pronounced nystagmus in Lurcher mice that returned to control values within 2 days (Fig. 2C). In contrast with wildtype and L7-PKCi mice, Lurcher mice did not show long-lasting adaptive increases in gaze stabilization following unilateral vestibular dysfunction. One day after labyrinthectomy, VVOR gain in Lurcher mice dropped to approximately one-half of the control value and remained depressed for the subsequent 7 days tested (Fig 6A). Labyrinthectomy had relatively little effect on VVOR phase (Fig. 6B).

VOR gains did not show adaptive changes following labyrinthectomy (Fig. 6C). VOR gain dropped to 23% of control conditions within one day and remained at depressed levels of between 21% and 32% of control over the subsequent 7 days. VOR gains in Lurchers were significantly lower than those in both wildtype and L7-PKCi mice from the third through the eighth day following labyrinthectomy ($p < .05$; unpaired t-test). No recovery of VOR gain was observed at any frequency tested (Fig 4C). VOR phase lead increased from 30 to 69 following labyrinthectomy and remained elevated for the subsequent testing period (Fig. 6D). Neither OKR gain nor phase, were significantly affected by labyrinthectomy in Lurcher mice (Fig 6E and F).

Discussion

This study analyzed a form of long-term plasticity of the VOR in mice that is induced by damage to the peripheral vestibular endorgan. Following unilateral labyrinthectomy, VOR gains in wildtype mice dropped and then increased over the course of about a week. To determine whether the mechanisms of these adaptive gain increases are similar to those underlying short-term plasticity of the VOR, we analyzed the consequences of labyrinthectomy in L7-PKCi transgenic mice that express a peptide inhibitor of protein kinase C in cerebellar Purkinje cells. Although these mice are impaired in both short-term plasticity of the VOR and cerebellar long-term depression (De Zeeuw et al., 1998; Goossens et al., 2001), they exhibited VOR gain increases following unilateral labyrinthectomy that were indistinguishable from those in wildtype mice. These adaptive gain increases depend on the integrity of the cerebellum, since they were absent in Lurcher mice, which lack functional Purkinje cells. These findings indicate that cerebellar-dependent long and short-term gain increases in the VOR are mediated by distinct mechanisms.

Although the method that we used to damage the vestibular periphery in mice differs from those described in studies of other animals (Maioli and Precht, 1985; Fetter and Zee, 1988; Vibert et al., 1993; Gilchrist et al., 1998; Balaban et al., 1999), a number of lines of evidence indicate that vestibular hair cells suffered permanent damage and thus could not signal information related to head motion. Histological analyses of the inner ears in lesioned animals revealed complete loss of tectorial structure, damage to hair cells, and loss of stereocilia, as would be expected from the combination of endolymph loss and physical shearing due to perfusion of the canal with air. Some of the increases in VOR gain following physical plugging of the semicircular canal have been attributed to peripheral rather than central processes (Hess et al., 2000). The fact that

Lurcher mice did not show increases in VOR gain indicates that the plasticity we measured in this study resulted from central rather than peripheral adaptive mechanisms.

The consequences of a peripheral vestibular lesion for the gain of the VOR were qualitatively similar between mice and other species (Maioli and Precht, 1985; Fetter and Zee, 1988; Vibert et al., 1993) but showed some quantitative differences. Following the lesion, VOR gains dropped to about 20% of control values in mice, whereas they dropped to about 50% in monkeys and cats (Maioli and Precht, 1985; Fetter and Zee, 1988; Lasker et al., 2000) and to about 30-50 % of control in guinea pigs (Vibert et al., 1993). A velocity-dependent asymmetry in the VOR following peripheral damage has also been reported (Fetter and Zee, 1988; Vibert et al., 1993; Broussard et al., 1999; Lasker et al., 2000), but was not apparent with the low velocity stimuli used in the present study. VOR gain increased rapidly in mice following a peripheral lesion, recovering to 50-60% of control values within less than a week. Although most published studies do not measure VOR gain during the first week following vestibular damage, rapid increases in VOR gain following unilateral loss of vestibular inputs have been documented in monkeys and cats (Maioli et al., 1983; Fetter and Zee, 1988; Broussard et al., 1999). A slower time course of vestibular compensation appears to exist in guinea pigs (Vibert et al., 1993). Unfortunately, limitations on the durability of implanted eye coils used in this study precluded extended measurements of VOR gain following labyrinthectomy. Studies that measured eye movements with a video tracker, which are not hampered by such restrictions, indicate that following vestibular damage VOR gains in mice recover to about 70% of control values with a time constant of 5-6 days and show no further increase over many months (Faulstich and du Lac, personal communication).

Increases in VOR gain following labyrinthectomy depended on the integrity of the cerebellum, as evidenced by the lack of gain increase in Lurcher mice. Lurcher animals, named after their obviously ataxic lurching gate, have a mutation in the gene that encodes the delta-2-glutamate receptor (Zuo et al., 1997). The mutation results in postnatal Purkinje cell death and consequent degeneration of cerebellar granule cells and inferior olive neurons (Caddy and Biscoe, 1979). Degeneration of vestibular nucleus neurons or other neurons in the circuitry for the VOR due to the Lurcher mutation have not been reported. However, the fact that the VOR of intact Lurcher mice is very similar to that of wildtype mice indicates that vestibular compensation was impaired because of the defective cerebellar circuitry and not because of possible aberrations in extra-cerebellar vestibular pathways. A role for the cerebellum in vestibular compensation has been reported (Courjon et al., 1982), as has a role for inferior olive neurons in postural recovery (Llinas et al., 1975).

Persistent image motion during head movement evokes cerebellar-dependent changes in the gain of the VOR that occur over a time course of hours to days (reviewed in: du Lac et al., 1995; Raymond et al., 1996). The time course and magnitude of these adaptive gain changes appears to depend, in part, on the duration of the discrepant visual and vestibular stimuli (Raymond and Lisberger, 1996). Small changes in VOR gain that occur over 1-2 hours of forced visual/vestibular mismatch training require protein kinase C expression in cerebellar Purkinje cells, as they are abolished in L7-PKCi mice that express a PKC inhibitor (De Zeeuw et al., 1998). The finding that L7-PKCi mice show normal VOR plasticity following labyrinthectomy but show impaired plasticity during visual-vestibular mismatch training may indicate different cellular mechanisms for these

two forms of cerebellar-dependent plasticity. Given that cerebellar LTD is impaired in L7-PKCi mice (De Zeeuw et al., 1998; Goossens et al., 2001), our data suggest that LTD is not a mechanism subserving vestibular compensation following peripheral vestibular dysfunction, and that this form of plasticity can proceed independently of LTD.

Changes in PKC expression in cerebellar Purkinje cells have been observed following labyrinthectomy (Goto et al., 1997; Barmack et al., 2001). Pharmacological blockade of PKC causes an increase in spontaneous nystagmus following labyrinthectomy in guinea pigs (Sansom et al., 2000) and delays the normally rapid recovery from nystagmus in rats (Balaban et al., 1999). In contrast, we observed neither a difference in nystagmus nor in gain recovery between wildtype and L7-PKCi mice. Increases in PKC expression may be a mechanism for the earliest phases of recovery from unilateral vestibular damage. Our data indicate, however, that PKC activation in Purkinje cells is not a critical step in VOR plasticity following peripheral lesions.

In summary, our data indicate that multiple mechanisms of cerebellar-dependent adaptive gain control exist in the circuitry for the VOR. Short-term plasticity induced by visual-vestibular mismatch training requires PKC activation in Purkinje cells and may utilize LTD as a mechanism of learning. Long-term plasticity following peripheral vestibular dysfunction proceeds independently of LTD and Purkinje cell PKC activation but requires the integrity of the olivo-cerebellar circuitry. Multiple sites of cellular plasticity in cerebellar dependent motor learning have been postulated (Raymond et al., 1996). It will be intriguing to determine how different components of the circuit are responsible for plasticity that occurs over different time scales and in response to different stimulus conditions.

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Chapter 7

Discussion and Conclusions

The VOR is a compensatory eye movement that stabilizes retinal images during head movements. The VOR needs to be adapted for example when subjects are starting to wear corrective spectacles or during development when their oculomotor plant reaches its normal proportions (Faulstich et al., 2004; McMullen et al., 2004). As explained in the introduction (Chapter 1) in the lab VOR adaptation can be readily induced by providing visual stimuli that are in conflict with the vestibular stimulus (De Zeeuw et al., 1998; Faulstich et al., 2004) and such visuo-vestibular training paradigms can modify both the gain and phase of the VOR (De Zeeuw et al., 2004). These parameters can increase or decrease after short-term or long-term training periods, the changes induced can persist for short or long periods, and the adaptations can depend on the history of the eye movement behaviour before the training period (Boyden et al., 2004). For my thesis I investigated the role of the cerebellum in forms of motor learning such as adaptation of the VOR. Since many mouse mutants with specific molecular deficits in the cerebellar network have been created over the past decade, I focussed on studying cerebellar function in mouse mutants, in particular in those in which postsynaptic LTD at the parallel fiber to Purkinje cell synapse is affected. To prepare the groundwork for these studies we first describe the zonal organization of the Purkinje cell activities as well as the input and output of these cells in the mouse flocculus (Chapter 2). It is shown for the first time that subpopulations of Purkinje cells reside in anatomically and physiologically discrete zones in the mouse flocculus. In addition, we show that the individual zones of Purkinje cells receive climbing fiber inputs from different regions of the inferior olive and project to different sets of nuclei and subnuclei, thereby retaining structural and functional separation at all three levels of the cerebellar modules. Subsequently, we show the overall function of the vestibulo-cerebellar flocculus in oculomotor control by investigating the eye movements of lurcher mice, which are known to lack all their Purkinje cells after about four weeks of age and to show abnormal adaptation of their vestibulo-ocular reflex (Chapter 3). The oculomotor performance of Lurcher mice is largely dominated by an impaired OKR. Closed-loop OKR gain is severely reduced, and phase lag of the eye movement with respect to stimulus movement is increased compared to control animals. Lurcher OKR, like that of control animals, is clearly nonlinear, rendering accuracy of the OKR not only dependent on stimulus frequency but also on stimulus velocity. In addition to a severely reduced OKR gain, phase lag was also larger in Lurcher mice than in control animals. In conjunction, these deficits explain why Lurcher mice show abnormal adaptation of their compensatory eye movements. While the Lurcher reveals the ultimate boundaries of what the flocculus can do in eye movement control, other studies were aimed at unraveling the role of LTD at the parallel fiber to Purkinje cell synapse in cerebellar motor learning. To date, two major molecular pathways have been described for the induction of LTD; these include the NO-PKG pathway and the mGluR1-PKC pathway. For this thesis we investigated the roles of both pathways in eye movement control. In Chapter 4 we used conditional gene targeting to ablate cGMP-dependent protein kinase type I (cGKI), a potential mediator of cerebellar NO/cGMP signalling, selectively in cerebellar Purkinje cells of mice in order to study the role of downstream components of the cerebellar NO/cGMP cascade in LTD and motor learning. This approach allowed us to analyze, for the first time, the specific role(s) of Purkinje cell cGKI in cerebellar function in vivo. Furthermore, the tissue-specific knockout strategy circumvented potential limitations of the conventional gene targeting technique, such as the lack of regional specificity, the presence of multiple defects, and early postnatal lethality. The lack of cGKI in Purkinje cells did not alter cerebellar structure and synaptically evoked dendritic Ca^{2+} signals

in PCs. These results indicate that the cGKI mutants had no basal physiological abnormalities that could confound the analysis of LTD and behavior. cGKI^{pko} mice showed nearly complete absence of cerebellar LTD, as measured by whole-cell patch clamping in acute slices, and impaired adaptation of the VOR, while their general eye movement performance was normal. Thus, because both the basic electrophysiological properties of the Purkinje cells and the basic motor performances were intact, this study provides a strong case in favor of the hypothesis that LTD underlies cerebellar motor learning. To show that this relation does not only hold for the NO-PKG but also for the mGluR1-PKC pathway and that the relation between LTD and cerebellar motor learning does not only hold for mice but also for humans we investigated for Chapter 5 the impact of mGluR1-auto-antibodies in patients with PCA that generated these antibodies as a secondary process to Hodgkin's disease. We showed that mGluR1-Abs can affect Purkinje cells at three different levels including their survival, excitability and plasticity (LTD). We suggest that these consequences at the cellular level, in turn, will result in deficits of both cerebellar motor performance and motor learning. Thus, these findings in PCA patients also support the hypothesis that LTD underlies at least in part cerebellar motor learning and they show that deficits in this mechanism can lead to neurological aberrations in humans. The findings described above raise the question as to whether PF-LTD has a general and functional role in all forms of cerebellar motor learning. We therefore investigated for Chapter 6 the role of LTD in vestibular compensation following unilateral labyrinthectomy in both lurcher mice and LTD-deficient PKCi mutants (De Zeeuw et al., 1998). These data showed that Purkinje cells in the cerebellar cortex are indeed necessary for this type of cerebellar motor learning, while the presence or absence of LTD has no impact. Thus taken together, we can conclude that LTD generally has an important role in cerebellar motor learning, but there are forms of cerebellar motor learning that do not depend on it. Possibly, other forms of plasticity in the cerebellar cortex also play a role (for review see Hansel et al., 2001); these may include presynaptic and postsynaptic PF-LTP (Salin et al., 1996; Linden and Ahn, 1999; Lev-Ram et al., 2002), climbing fiber LTD (CF-LTD)(Hansel and Linden, 2000), LTP of the inhibitory interneuron to Purkinje cell synapse (Kano et al., 1992; Khodakhah et al., 1997; Jorntell and Ekerot, 2002 and 2003), and long-term, synaptic and non-synaptic changes in neurons in the cerebellar and vestibular nuclei (Aizenman and Linden, 1998 and 2000; Nelson et al., 2003 and 2005).

Below we will discuss our findings on the possible role of postsynaptic LTD in the context of the current literature and we will review and discuss the possible role of postsynaptic LTP at the parallel fiber to Purkinje cell synapse as well as the possible role of plasticity at the vestibular nuclei neurons in VOR adaptation. Moreover, we will propose experiments which may result in further insight in the possible roles of these forms of plasticity.

Postsynaptic parallel fiber LTD

So far all mouse mutants in which PF-LTD is impaired, have shown abnormal adaptation of their compensatory eye movements (Shutoh et al., 2002; Katoh et al., 2000; Katoh et al., 2005; Feil et al., 2003; De Zeeuw et al., 1998; see also Coesmans et al., 2003). These also include mutants in which insertion of the GluRdelta2 receptors (Katoh et al., 2005), signalling in the NO-PKG pathway (Feil et al., 2003; current thesis), or phosphorylation in the mGluR1-PKC

pathway (De Zeeuw et al., 1998) is affected in a Purkinje cell specific manner. Yet, in all these cases in which there are no problems of region- or cell specificity, there are still caveats in showing an unequivocal causal relation between PF-LTD induction and VOR adaptation. For example, the knockout of GluRdelta2 also shows deficits in motor performance raising the possibility that its deficits in VOR adaptation result from impairments in their optokinetic and vestibular baseline responses rather than a blockage of LTD (Katoh et al., 2005). In contrast, our Purkinje cell specific knockout of cGKI does not show signs of basal electrophysiological or behavioural abnormalities, but here developmental aberrations and compensation by related proteins may have occurred as the protein knocked out is expressed from early on (Feil et al., 2003). The potential problem of compensation by iso-enzymes was at least partly solved in the transgenic L7-PKCi mutant in which various isoforms of Protein Kinase C are effectively inhibited by overexpression of an inhibitory peptide, PKC19-31 (De Zeeuw et al., 1998; Gao et al., 2003). However, in this mutant the transition from multiple- to monoclimbing fiber innervation is delayed by several months (De Zeeuw et al., 1998; Goossens et al. 2001), and CF-LTD, which is another form of plasticity in Purkinje cells that depends on PKC (Hansel and Linden, 2000), may also be affected. One could argue that L7-PKCi mutants older than 6 months show neither a persistent multiple climbing fiber innervation nor any sign of abnormal discharge dynamics in their Purkinje cell activities (Goossens et al. 2001 and 2004), while the correlation between their impaired PF-LTD induction and VOR adaptation is maintained (De Zeeuw et al., 2004), but the wide range of potential effects that PKC may exert makes it hard to exclude that any developmental factor contributed significantly to the deficits in VOR adaptation observed in the L7-PKCi mutant. Thus, one can conclude that PF-LTD certainly remains one of the major potential mechanisms underlying VOR adaptation, but new more specific mutants downstream of the pathways involved will have to determine whether it is in fact the core of the engram or not.

Postsynaptic parallel fiber LTP

Raymond and colleagues observed that increases in VOR gain can be more readily reversed by visuovestibular training than decreases in VOR gain and therefore proposed that PF-LTP and PF-LTD may underlie adaptive changes in VOR gain decrease and increase, respectively (Boyden and Raymond, 2003; Boyden et al., 2004). Asymmetry in the gain and phase dynamics during gain-up and gain-down training indeed appears to be a general phenomenon in wild type mice (van Alphen and De Zeeuw, 2002; Faulstich et al., 2004), but all Purkinje cell specific, LTD-deficient mice mentioned above show deficits in both VOR increase and VOR decrease paradigms (De Zeeuw et al., 1998; van Alphen and De Zeeuw, 2002; Feil et al., 2003; Katoh et al., 2005). Interestingly, however, while PF-LTD contributes in the VOR increase paradigm most prominently to direct gain changes, in the VOR decrease paradigm it exerts its main effect by modifying the phase value (van Alphen and De Zeeuw, 2002; Feil et al., 2003; current thesis). Thus, because postsynaptic PF-LTD can be directly reversed by postsynaptic PF-LTP at the cellular level by a common calcium mediated mechanism (Lev-Ram et al., 2002 and 2003; Coesmans et al., 2004), the main roles of this form of LTP at the behavioural level may be to reduce the gain in a VOR decrease paradigm and to reduce the phase lead in a VOR increase paradigm.

Plasticity in vestibular nuclei

The LTD-deficient cGKI knockouts and L7-PKCi transgenics mentioned above show both severely impaired VOR adaptation, yet their gain and phase dynamics are normal during baseline measurements (current thesis; Feil et al., 2003; De Zeeuw et al., 1998). How is it possible that these animals achieve a normal eye movement performance, while their motor learning is affected? Possibly, forms of plasticity other than those at the parallel fiber to Purkinje cell synapse come into play when prolonged periods of training are available (Blazquez et al., 2004). Indeed, if one trains LTD-deficient L7-PKCI mutants for periods longer than a few days, it does adapt its VOR (van Alphen and De Zeeuw, 2002). This form of chronic learning shows less frequency-specificity than acute learning and without LTD it occurs at a slower rate than normal, but ultimately it reaches a normal saturation level. Thus postsynaptic LTD and LTP may be essential for rapid motor learning within a few hours, but other forms of plasticity may contribute when longer time periods are available. One of the interesting candidates for this mechanism is firing rate potentiation in the vestibular nuclei neurons that are innervated by Purkinje cells from the flocculus (Nelson et al., 2003). Du Lac and colleagues have demonstrated that transient changes in inhibitory input to these cells can lead to long-lasting increases in intrinsic excitability, which are relatively difficult to reverse, making this form of plasticity well suited for chronic motor learning.

Plans for future experiments

The studies on VOR adaptation presented in this thesis and reviewed above have provided detailed information about the potential functions of the various types and sites of cellular plasticity in the olivocerebellar system. Yet, in all cases the evidence that is presented is correlational and cannot be regarded as causal or conclusive. The main advances that may be at hand in the near future include new mutants, which are based on new insights in the molecular mechanisms underlying the various types of cerebellar plasticity. For example, Linden and colleagues have demonstrated that the PKC-isoform that is responsible for induction of PF-LTD is PKCα (Leitges et al., 2004), and that the phosphorylation site in the AMPA receptor subunit that is essential for this induction is GluR2 Ser880 (Chung et al., 2003). Thus these two molecular substrates form ideal downstream targets to interfere with LTD induction and hopefully to do so without developmental and acute side-effects. Similarly, recent studies which show that inhibition of myosin/moesin phosphatase promotes LTD (Eto et al., 2002; Launey et al., 2004), raise the interesting possibility that such protein phosphatases are involved in mediating postsynaptic PF-LTP. If correct, precisely blocking this pathway may allow us to create for the first time a mutant in which this form of LTP is specifically affected in Purkinje cells and to find out what the functional roles of this form of plasticity are for cerebellar motor learning. Finally, du Lac and colleagues showed that firing rate potentiation in vestibular nuclei neurons can be controlled by Calcium/calmodulin-dependent protein kinase II (CaMKII) (Nelson et al., 2005). Since various CaMKII mutants are available (e.g. Elgersma et al., 2002), and since the electrophysiological properties of cerebellar nuclei neurons closely resemble those of vestibular nuclei neurons (Sekirnjak et al., 2003; Aizenman and Linden, 2000), mouse behaviourists should also be able to elucidate the role of plasticity in the target neurons of

cerebellar Purkinje cells for VOR adaptation. Thus, we can conclude that the morphological, molecular and behavioural, preparing groundwork has been done in the past few years to create and/or test now a new generation of mutants that may well unravel an essential part of the holy grail of memory formation. In this respect the cerebellar field may distinguish itself from other areas, because it allows a unique combination of cell-specific molecular approaches with controllable stimulations, recordable electrophysiological responses, and tractable behaviours. It is time and tide for research in cerebellar memory formation.

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Summary/Samenvatting

Summary

The cerebellum plays an important role in the recalibration and adaptive adjustment of movements, as well as learning new motor skills and motor related associations. In this thesis, we investigated the mechanisms underlying cerebellar motor learning. To obtain a better understanding, in how the cerebellum processes and stores information, we used specific perturbations that affected the information processing of the cerebellum. Signal transduction pathways were affected that were considered important for cerebellar motor learning by using genetic tools (transgenic mice) and the application of antibodies. Alterations in cerebellar motor learning were studied by monitoring the oculomotor system of these transgenic and treated mice.

The anatomical and physiological organization of the cerebellar flocculus of the mouse is still unknown. Therefore, we first described the zonal organization of the Purkinje cell activities as well as the input and output of these cells in the mouse flocculus (Chapter 2). In the mouse, the flocculus could be divided into five anatomical and physiological zones, based on the Purkinje cell complex spike modulation occurring in response to optokinetic stimulation. Floccular Purkinje cells are organized in a set of two alternating zones, zones 1, 3 and zones 2, 4, responded best to rotation about a horizontal axis and about the vertical axis respectively, which is similar as in rabbit and rat. Each zone of Purkinje cells receive climbing fiber inputs from different regions of the inferior olive and project to different sets of nuclei and subnuclei.

In Chapter 3, we showed the functional importance of Purkinje cells in the oculomotor system by investigating the eye movements of Lurcher mice, which are known to lack all their Purkinje cells after four weeks of age. The oculomotor performance of Lurcher mice revealed an impaired optokinetic reflex and an incapability of adapting their vestibulo-ocular and optokinetic reflex, demonstrating the importance of Purkinje cells for both controlling and modifying eye movements.

In Chapter 4 and 5, we looked further into one of the mechanisms that could be involved in this modification process; the long-term depression (LTD) at the parallel fiber to Purkinje cell synapse. To date, two major molecular pathways have been described for the induction of LTD; these include the NO-PKG pathway and the mGluR1-PKC pathway. In chapter 4, we revealed the function of the NO-PKG pathway in controlling and modifying eye movements by using conditional knockout mice that lack cGKI selectively in Purkinje cells. The lack of cGKI in Purkinje cells did not alter the cerebellar morphology and synaptic dendritic calcium signaling in Purkinje cells. These cGKI mutants did reveal a nearly complete absence of cerebellar LTD and an impaired adaptation of the VOR, while their eye movement performance was normal. This result showed that cGKI participates in the NO-PKG pathway and it confirms the involvement of this pathway in cerebellar motor learning.

In Chapter 5, we investigated the consequences of blocking the mGluR1-PKC pathway on cerebellar processes by using human auto-antibodies against the metabotropic glutamate receptor type 1 (mGluR1-Ab) from patients with paraneoplastic cerebellar ataxia (PCA). We showed that the human mGluR1-Ab reduced Purkinje cell survival, neuronal excitability and plasticity (i.e. LTD). We suggest that these consequences at the cellular level will result in the observed deficits of both cerebellar motor performance and motor learning.

The findings described above raise the question as to whether LTD has a general and

functional role in all forms of cerebellar motor learning. We therefore investigated in Chapter 6 the role of LTD in vestibular compensation following unilateral labyrinthectomy in both Lurcher mice and LTD-deficient PKCi mutants. The result of this study showed that Purkinje cells in the cerebellar cortex are indeed necessary for this type of cerebellar motor learning, while the presence or absence of LTD has no impact.

In conclusion cerebellar cortex is necessary for motor performance and the motor learning. Purkinje cell electrophysiological properties contribute to eye movement performance and cerebellar LTD facilitates cerebellar motor learning (Chapter 4 and 5), but not all forms of motor learning; e.g. vestibular compensation following unilateral labyrinthectomy (Chapter 6).

Samenvatting

Het cerebellum speelt een belangrijke rol in het kalibreren en aanpassen van motorische taken, alsmede het aanleren van nieuwe motorische vaardigheden en het aanleren van motorisch gerelateerde verbanden. In dit proefschrift onderzoeken wij het mechanisme dat ten grondslag ligt aan het aanleren en aanpassen van motorische taken. Om meer inzicht te krijgen in hoe het cerebellum informatie verwerkt en opslaat, gebruiken we specifieke verstoringen die het informatie verwerkingsproces van het cerebellum beïnvloeden. Door gebruik te maken van genetische gemodificeerde muizen en de toediening van antilichamen aan normale muizen, hebben we processen beïnvloed die belangrijk worden geacht voor motorisch leren. Veranderingen in motorisch leergedrag van deze muizen werd vervolgens bepaald met behulp van het oogbewegingsysteem.

De anatomisch en fysiologische organisatie van de cerebellaire flocculus van de muis is nog steeds onbekend. In hoofdstuk 2 beschrijven we daarom als eerste de zonale organisatie van de Purkinje cel activiteiten alsmede de anatomische in- en output van deze cellen. De flocculus van de muis kan verdeeld worden over vijf anatomische en fysiologische zones. Deze zijn gebaseerd op de Purkinje cel complex spike modulatie die optreed gedurende optokinetische stimulatie. De flocculus van de muis is opgebouwd uit vier Purkinje cel responderende zones, die alternerend gevoelig zijn voor verschillende optokinetische stimulaties. Zones 1 en 3 responderen het best op een rond de horizontale as roterende optokinetisch stimulatie, terwijl zones 2 en 4 responderen het best op een rond de verticale as roterende optokinetische stimulatie. Dit resultaat komt overeen met de zonale organisatie van het konijn en de rat. Iedere zone van Purkinje cellen ontvangt klimvezel input uit aparte delen van de inferior olive en projecteert vervolgens naar aparte delen van kernen en subkernen.

In hoofdstuk 3 laten we het functionele belang zien van Purkinje cellen binnen het oogbewegingsysteem. Dit hebben we gedaan door oogbewegingen te onderzoeken van Lurcher muizen, die na een leeftijd van 4 weken geen Purkinje cellen meer bezitten. De oogbewegingmetingen aan de Lurcher muizen lieten een duidelijke vermindering zien in de optokinetische reflex en een sterk verminderde aanpassingsvermogen van de vestibulo-ocular en optokinetische reflex. Dit resultaat geeft het belang aan van de Purkinje cellen voor de uitvoering en het aanpassingsvermogen van oogbewegingen.

In hoofdstuk 4 en 5 verdiepen we ons in het proces dat ten grondslag kan liggen aan dit aanpassingsvermogen van oogbewegingen, namelijk een vorm van plasticiteit van de parallel vezel – Purkinje cel synaps genaamd “long-term depression” (LTD). Heden ten dage zijn er twee moleculaire processen bekend waarmee LTD geïnduceerd kan worden, namelijk via de NO-PKG en via de mGluR1-PKC signaal transductie route. In hoofdstuk 4 laten we de relevantie van de NO-PKG signaal transductie route voor de uitvoering en het aanpassingsvermogen van oogbewegingen zien door gebruik te maken van conditionele knock-out muizen die selectief cGKI mist in zijn Purkinje cellen. Het gebrek aan cGKI in de Purkinje cellen had geen effect op de morfologie van het cerebellum en liet geen wijzigingen zien in de dendritische calcium signalen van de Purkinje cellen. In deze cGKI mutant muizen was nagenoeg geen cerebellaire LTD meer aanwezig en deze muizen vertoonde eveneens een sterk verminderde aanpassingsvermogen van de vestibulo-ocular reflex, terwijl de uitvoering van de oogbewegingreflexen normaal verliepen. Dit resultaat geeft de bijdrage van cGKI in de NO-PKG signaal transductie route aan en bevestigt eveneens de betrokkenheid van de NO-

PKG signaal transductie route in het cerebellair motorisch leerproces.

In hoofdstuk 5 onderzoeken we de consequenties van het blokkeren van de mGluR1-PKC signaal transductie route op de verschillende cerebellaire processen. De mGluR1-PKC signaal transductie route werd geblokkeerd door gebruik te maken van menselijke autoantilichamen tegen de metabotrope glutamaat receptor type 1 (mGluR1-Ab) verkregen uit patiënten met paraneoplastische cerebellaire ataxia (PCA). De mGluR1-Ab verminderde de overlevingskans, de exciteerbaarheid en de plasticiteit (LTD) van de Purkinje cellen. Eveneens zien we een afname in de uitvoering en het aanpassingsvermogen van oogbewegingen. Het is naar ons inziens aannemelijk dat de effecten op cellulair niveau verantwoordelijk zijn voor deficiënties op gedragsniveau.

Uit de bovengenoemde resultaten rijst automatisch de vraag of LTD een algemene en functionele rol speelt in alle vormen van cerebellair motorisch leren. Om deze vraag te beantwoorden hebben we in hoofdstuk 6 de rol van LTD in vestibulaire compensatie na unilaterale labyrinthectomie in zowel Lurcher als LTD-deficiënte PKCi muizen onderzocht. De resultaten van deze studie geven aan dat Purkinje cellen nodig zijn voor dit type cerebellair motorisch leren, maar dat de aanwezigheid of afwezigheid van LTD geen enkele invloed op dit proces uitoefent.

Samengevat kunnen we concluderen dat het cerebellum nodig is voor een correcte uitvoering en het aanpassingsvermogen van motorische taken. Purkinje cel eigenschappen dragen bij tot het correct uitvoeren van motorische taken en cerebellair LTD draagt bij tot het correct aanpassen van de motorische taken, echter het draagt niet bij tot iedere vorm van adaptatie; bijvoorbeeld vestibulaire compensatie door unilaterale labyrinthectomie.

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