Electrophysiological Responses of Floccular Purkinje Cells during Compensatory Eye Movements in Mutant Mice

Electrofysiologische afleidingen van flocculaire Purkinje cellen tijdens compensatoire oogbewegingen in muizen mutanten

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General Introduction

The role of the cerebellum in motor control has been a subject of study for many decades. The cerebellum has always attracted numerous scientists due to its well-described and relatively simple cyto-architecture, the unique set of electrophysiological properties of its main neuron, the Purkinje cell, as well as the possibility to readily quantify the various kinds of motor behaviour that it controls. With the advent of the genomics era, the role of the cerebellum as a general model system for understanding neural networks has even gained more attention, as the cerebellum is a system with several cell-specific promotors, which allow cell-specific genetic manipulation. And thus, many cerebellar physiologists start experiments in mice, which not only permits the generation of transgenics, but also homologous recombination using ES-cells.

In our efforts to unravel cerebellar function in motor control we are trying to combine the bests of all worlds described above. The present thesis describes the combination of molecular manipulation in mouse mutants with electrophysiological recordings of Purkinje cells in a cerebellar area that controls compensatory eye movements. To date, such a combinational approach has not been successfully applied, and by doing this we hope to be able to further elucidate the molecular and electrophysiological mechanisms that may underlie cerebellar motor coordination. In this general introduction we will first lay out the details of the morphological characteristics and electrophysiological properties of Purkinje cells, which form both the core integrative element of the cerebellar cortex and its sole output (**Chapter 1.1**). Subsequently, we will explain the advantages of investigating compensatory eye movements (**Chapter 1.2**) as well as the relevant background information about recording eye movements in mouse mutants in general (**Chapter 1.3**), and finally we will present the general scope of the thesis as a whole (**Chapter 1.4**).

1.1 Cerebellar Purkinje cells

The cerebellum encompasses the highly organized cerebellar cortex and the cerebellar nuclei. The Purkinje cells, which were first described by the Bohemian physiologist Jan Evangelista Purkynje (1787-1869), form the sole output of the cerebellar cortex. These large neurons directly innervate both the inhibitory and excitatory neurons in the cerebellar and vestibular nuclei so as to ultimately alter motor behaviour. Since this thesis addresses the significance of the electrophysiological responses of Purkinje cells in mutants in which calcium mediated mechanisms are affected, we will address here the general morphology and connectivity of the Purkinje cell (**Chapter 1.1.1**), its general electrophysiological properties (**Chapter 1.1.2**), its homeostasis of calcium (**Chapter 1.1.3**), and its forms of synaptic plasticity (**Chapter 1.1.4**).

1.1.1 Purkinje cell morphology and connectivity

Purkinje cells have a distinctive morphology characterized by a flat but extensively branched dendritic tree, a large soma, and a long, collateral-forming axon. The dendritic tree, which protrudes into the molecular layer, is 250 μm wide, 250 μm high and 6 μm thick. All dendrites of the Purkinje cells are studded with numerous small spines. Their cell bodies are organized in a single layer that covers the whole cerebellar cortex (Marr et al., 1969); they are beet-shaped and lie 50 μm apart in the longitudinal and 60 μm apart in the transverse direction. Apart from prominent inhibitory inputs from local cortical interneurons, Purkinje cells receive two major excitatory inputs: the mossy fiber - parallel fiber pathway and the climbing fibers. The mossy fibers innervate the granule cells, which in turn give rise to the parallel fiber input that innervates the dendritic spines of Purkinje cells. Mossy fibers arise from various precerebellar nuclei and give rise to 100-250 rosettes per fiber, and each rosette contacts approximately 20 granule cell dendrites. In addition, the axons that give rise to the mossy fibers also provide collaterals to the cerebellar and vestibular nuclei neurons. Granule cell axons ascend through the granule cell layer and protrude into the molecular layer where they contact the thin distal Purkinje cell dendrites numerous times (Bower and Woolston, 1983; Jaeger and Bower, 1994; Gundappa-Sulur et al., 1999). The parallel fibers are actually the distal T-parts that arise following the bifurcation of the ascending granule cell axons (Figure 1). Parallel fibers pass through the molecular layer perpendicularly to the planes of the Purkinje cell dendritic trees. Each Purkinje cell dendritic tree is contacted approximately 150.000 times by parallel fibers, but, in contrast to ascending granule cell axons, each parallel fiber makes one or two synaptic contacts per dendritic tree. A single parallel fiber is 0.6 μm thick and can stretch over millimeters. Each fiber is thought to contact approximately 300 Purkinje cells.

The other major excitatory input to Purkinje cells arises from the inferior olive: the climbing fiber. Climbing fibers are olivary axons that climb into the proximal part of the dendritic tree Purkinje cells and innervate the Purkinje cells in a 1:10 ratio. Although during early postnatal development each Purkinje cell receives 2 to 4 climbing fibers, adult Purkinje cells receive input from just a single climbing fiber. In addition, climbing fibers pass through the granule cell layer where they give off collaterals to Golgi cells. The olivocortical projections are characterized by a strict sagittal patterning such that neurons from a particular region of the olive project to one or two specific sagittal zone(s) (as reviewed by Voogd et al., 1996). However, due to collateralization, olivary axons can reach two different zones in related parts of the cerebellum. For example, a single axon can innervate Purkinje cells in both the nodulus and flocculus of the vestibulocerebellum (Ruigrok, 2003).

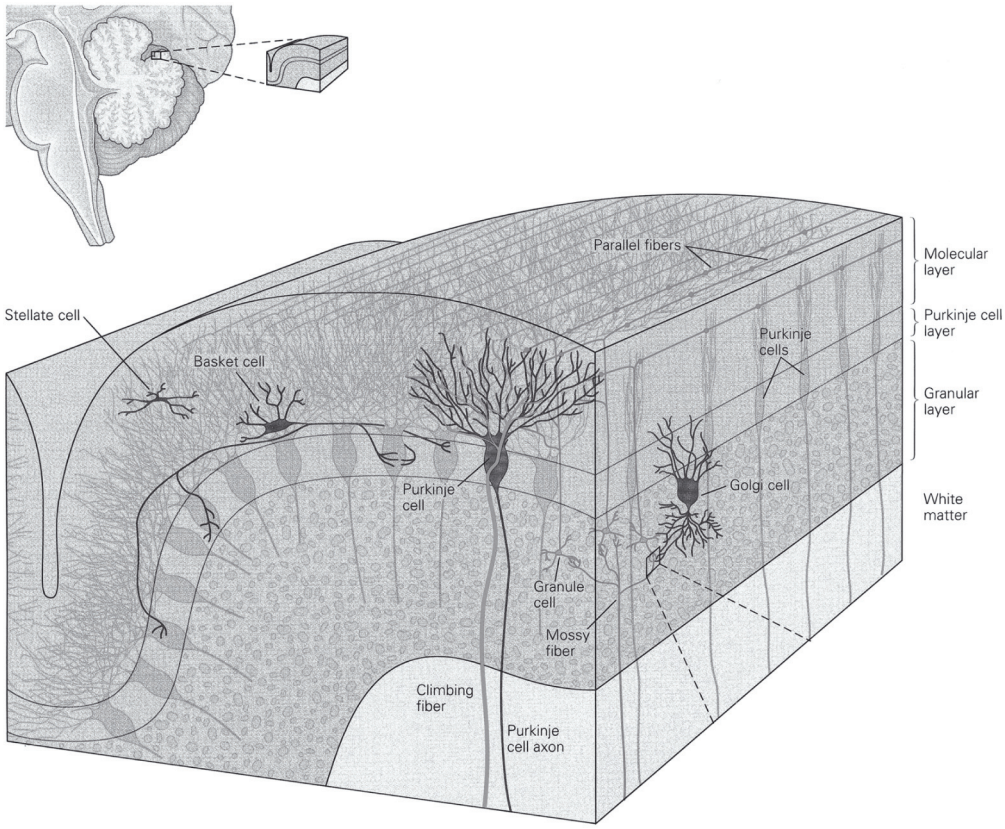

Figure 1: Schematic representation of the morphology of the cerebellar cortex. Purkinje cells form the output of the cerebellum and are aligned along the parallel fibers. Granule cells innervate Purkinje cells through their ascending axons and parallel fibers and are inhibited by golgi cells. Golgi cells are large neurons, which form large radial shaped dendritic and axonal projections, and are innervated by parallel fibers and climbing fibers. Basket cell axons form a basket-like shaped terminal surrounding the Purkinje cell soma, which forms an effective inhibitory input to Purkinje cells. In addition, stellate cells form inhibitory synapses in the dendritic tree of Purkinje cells. See text for details.

The axon terminals of Purkinje cells, which are all GABAergic (Ito, 1984), make inhibitory connections with various sorts of neurons in the cerebellar and vestibular nuclei. Individual Purkinje cells from different sagittal zones project to different sets of regions of the cerebellar and vestibular nuclei (De Zeeuw et al., 1994). It has been estimated that a single Purkinje cell innervates 30-50 different cerebellar nucleus cells and that one cerebellar nucleus cell receives input from about 20-30 different Purkinje cells (Chan-Palay, 1977). Besides the GABA-ergic input from Purkinje cells, neurons in the various cerebellar nuclei receive excitatory input from mossy fibers and climbing fibers collaterals.

1.1.2 Electrophysiological properties of Purkinje cells

Mainly due to the well organized cyto-architecture of the cerebellar cortex, it is relatively easy to record extracellularly from single units of Purkinje cells. Purkinje cells generate two characteristic types of spikes: simple spikes and complex spikes. While the simple spikes resemble

action potentials, complex spikes are formed by an initial spike followed by multiple waveforms. Since a substantial part of my thesis deals with the recording and analysis of these Purkinje cell activities, I will lay out below the details of the generation of both the simple spikes and complex spikes.

Simple spikes

Simple spikes are action potentials driven by synaptic input. Without excitatory and inhibitory synaptic input simple spikes are non-existent (Llinas and Sugimori, 1980b; Hausser and Clark, 1997). These spontaneous action potentials can also occur in acutely dissociated cell bodies of Purkinje cells indicating the ability of Purkinje cells to act as a pacemaker (Raman and Bean, 1997, 1999). In such a preparation action potentials are fired in a highly regular fashion with frequencies ranging from 17-150 Hz (Raman and Bean, 1999).

The firing of simple spikes as well as spontaneous action potentials is mainly mediated by sodium, calcium and potassium currents (Llinas and Sugimori, 1980b; Raman and Bean, 1997). Sodium currents initiate the actual simple spike or spontaneous action potential. This sodium current is constituted by sodium ions that enter the Purkinje cell through tetrodotoxin-sensitive sodium channels. The increase in positively charged ions on the inside of the Purkinje cell depolarizes the cell membrane. In addition to fast inward sodium currents, calcium currents further depolarize the Purkinje cell membrane. These calcium currents are mediated predominantly by the high-voltage activated P-type voltage-gated calcium channels (Llinas et al., 1989). Calcium influx activates calcium-dependent potassium efflux, which in turn counteracts the depolarization of the Purkinje cell membrane. Large conductance calcium-dependent potassium channels are found in both the Purkinje cell soma and dendrites. Because of their fast activation kinetics and voltage-dependence these large conductance channels are likely to contribute to rapid return of the Purkinje cell membrane potential to its resting values (Jacquin and Gruol, 1999). In addition to calcium-dependent potassium channels, so-called Kv3 potassium channels are localized in the Purkinje cell soma and dendritic tree. Since these channels require strong depolarization in order to be activated, they are also thought to be suited to induce repolarization following action potentials (Martina et al., 2003). Following this potassium-mediated hyperpolarization, several mechanisms restore the membrane potential to its resting values. The so-called I_h-current is mediated by cyclic nucleotide-gated channels. These channels open upon hyperpolarized membrane potentials and gate sodium, potassium (Robinson and Siegelbaum, 2003), and calcium ions (Yu et al., 2004). The I_h-current reverses positive to the resting membrane potential and depolarizes the cell. In addition, low-voltage activated T-type calcium currents and “resurgent” sodium currents may play a role in depolarization of the membrane potential.

The presence of synaptic input to the Purkinje cell dendritic tree and soma distinguishes action potentials from simple spikes. As explained above, the excitatory input to the cerebellar Purkinje cell is mediated by parallel fibers and climbing fibers. Following parallel fiber activation, glutamate is released from parallel fiber synapses. Glutamate binds to ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors and metabotropic glutamate type 1 receptors (mGluR1). Activation of ionotropic AMPA receptors leads to fast excitatory postsynaptic potentials (EPSPs), which are mediated by sodium influx through the channel formed by this receptor. Activation of mGluR1 leads to G-protein-coupled activation of phospholipase C, which induces di-acyl-glycerol (DAG) production. This latter compound promotes the conversion of phosphatydil-inositol-phosphate to IP_3, which is bound by intracellular IP_3-receptors and triggers calcium release from internal calcium stores (Finch and Augustine, 1998; Takechi et al., 1998). Thus, massive activation of mGluR1 leads to a delayed EPSP, which is mediated by a slow rise in intracellular calcium concentrations. Subsequently, this increase in the intracellular calcium

concentration may cause activation of voltage-gated calcium channels, which can be visualized up to the spiny level with calcium imaging (Eilers et al., 1995). In addition to these local calcium currents, granule cell activity initiates sodium currents in Purkinje cell dendrites that spread passively towards the Purkinje cell soma. From here then, ultimately, voltage-gated sodium channels in the axon hillock will be activated and initiate the simple spike (Callaway and Ross, 1997; Kuruma et al., 2003).

Simple spikes will probably be evoked by parallel fiber stimulation. Yet, the efficiency of the parallel fiber - Purkinje cell synapses is low. Stimulation of a single granule cell will elicit in only 10% of the cases an excitatory postsynaptic current (EPSC) in the Purkinje cell dendrites (Isope et al., 2004). However, since normally 30 to 150 parallel fibers may be simultaneously active, simple spikes may still be readily evoked by a regular activation of parallel fibers (Roth and Hausser, 2001; Isope and Barbour, 2002; Isope et al., 2004). Moreover, due to synchronous activation of the postsynaptic Purkinje cell, boutons of the ascending granule cell axons may be more effective than those of the parallel fibers (Isope and Barbour, 2002). Thus, it is not surprising that simple spike frequencies during specific motor behaviour may exceed well beyond 200 Hz.

In addition to excitatory innervation by granule cells, Purkinje cells receive inhibitory input from basket and stellate cells. Basket cells are found in the most proximal part of the molecular layer. Basket cells have a basket-like axonal terminal that embraces the lower part of Purkinje cell bodies. Stellate cells are found in the distal parts of the molecular layer where they innervate the dendrites of the Purkinje cells. Both basket and stellate cells are excited by granule cells via parallel fibers. Activation of the basket and stellate cells will activate both postsynaptic ionotropic $GABA_A$- and G-protein-coupled $GABA_B$-receptors. Activation of $GABA_A$-receptors leads to a fast hyperpolarization of the cell by eliciting a fast inhibitory postsynaptic potential (IPSP) that has a reversal potential of ~ -70 mV indicative for an increase in conductance of chloride. Activation of $GABA_B$-receptors is slower than activation of $GABA_A$-receptors. By G-protein coupling $GABA_B$-receptors mediate a decrease in calcium channel activity and an increase in potassium permeability, which results in an efflux of potassium and hyperpolarization of the Purkinje cell (Catterall, 2000; Mintz and Bean, 1993). Antagonizing the GABA-ergic innervation of Purkinje cells will both increase their firing rate and their regularity of firing (Hausser and Clark, 1997; Miyashita and Nagao, 1984). Thus, the simple spike firing pattern is not only controlled by the parallel fiber input but also by the inhibitory interneurons.

Complex spikes

Excitatory synaptic input to the Purkinje cells originates not only from parallel fibers, but also from climbing fibers. Although each Purkinje cell is innervated by a single climbing fiber, the synaptic connection is extremely powerful, because each climbing fiber makes numerous synaptic contacts; it has been estimated that over 1500 synaptic contacts operate simultaneously at a high release probability (Dittman and Regehr, 1998). This means that when an olivary neuron fires a single action potential, a Purkinje cell receives innervation by 1500 synaptic contacts simultaneously. Activation of this large number of climbing fiber – Purkinje cell synaptic contacts triggers a postsynaptic response that overrides all other synaptic input. Purkinje cells that are connected by the same olivary neuron are activated synchronously. Groups of Purkinje cells may also become synchronized because of electrotonic coupling between inferior olivary cells (Llinas et al., 1974). Electrotonic coupling of inferior olive neurons is mediated by dendrodendritic gap junctions (Sugihara et al., 1993; Lang, 2003). Due to specific conductances in the olivary membranes (Llinas and Yarom, 1981), the average spontaneous firing frequency of inferior olive neurons and thereby of complex spike activities is ~ 1 Hz, but can reach up to 10 Hz for very short

periods (Goossens et al., 2001; Simpson et al., 1996).

The generation of a complex spike comprises the following events. When a neuron in the inferior olive fires an action potential, glutamate is released from climbing fiber terminals. Postsynaptic metabotropic glutamate and AMPA-receptors bind glutamate and are activated. Due to the anatomical spreading of climbing fiber synaptic contacts and more importantly, the simultaneous activation of all these synaptic contacts, a strong depolarization is produced. This strong widespread dendritic depolarization elicits dendritic calcium spikes (Llinas and Sugimori, 1980a), which are mediated by P/Q- and T-type voltage-gated calcium channels (Pouille et al., 2000; Schmolesky et al., 2002). The depolarization that follows climbing fiber activation spreads to the axon hillock where a spike is initiated by opening of voltage-gated sodium currents (Stuart and Hausser, 1994). This fast sodium spike seems to be similar to the spontaneous action potential and simple spike (Ito, 1984). The climbing fiber activated somatic sodium spike causes sodium to diffuse into the primary dendrite (Stuart and Hausser, 1994). No AMPA-receptor independent dendritic sodium currents have been found to be elicited by direct climbing fiber activity or direct depolarizations of Purkinje cell soma or dendrite (Lasser-Ross and Ross, 1992; Callaway and Ross, 1997), which is in line with the passive spreading of the sodium transient following parallel fiber activity (Kuruma et al., 2003). Possibly the absence of active propagation of sodium currents is due to the low density and poor spreading of sodium channels in the Purkinje cell dendritic tree. However, a modelling study suggests that not the distribution of sodium channels, but the morphology of the dendritic Purkinje cell determines the spreading of sodium transients (Vetter et al., 2001). This study suggests that irrespective of the density of cation channels, the back- or forward propagation of action potentials is strongly, negatively correlated with the dendritic membrane area at distance x from the soma. In addition to the initial spike, climbing fiber activity elicits two rather slow Purkinje cell responses. The first slow component of the complex spike consists of a so-called plateau phase on top of which spikelets appear (Schmolesky et al., 2002). Both the plateau phase and the spikelets are probably mediated by dendritic P/Q- and T-type voltage-gated calcium channels (Schmolesky et al., 2002) and deinactivation of somatic sodium currents (Llinas and Sugimori, 1980a; Raman and Bean, 1997). The second slow component of the complex spike consists of a repolarization and a slow after-hyperpolarization of the Purkinje cell membrane potential. As described for simple spikes, repolarization of the membrane potential is likely to be mediated by large-conductance calcium-dependent potassium channels and resurgent sodium currents (Jacquin and Gruol, 1999; Raman and Bean, 1997). The slow after-hyperpolarization is likely to be mediated by the small-conductance potassium channels and non-inactivating sodium currents (Schmolesky et al., 2002). When recorded *in vivo*, the complex spike is followed by a pause in the simple spike activity, which is termed “climbing fiber pause” (Simpson et al., 1996). The mechanisms behind the climbing fiber pause are still under debate (as reviewed by Schmolesky et al., 2002).

Glutamate released by climbing fibers not only affects Purkinje cells by depolarizing them, but also affects the inhibitory synaptic input to Purkinje cells. Kano et al. (1992) showed that following climbing fiber activation and the subsequent depolarization of the Purkinje cell, the inhibitory synaptic input is subject to a long-lasting potentiation known as rebound potentiation, which is mediated by the sensitization of $GABA_A$-receptors. Rebound potentiation can be counteracted by the simultaneous release of AMPA from climbing fibers and GABA from inhibitory interneurons (Kawaguchi and Hirano, 2000). However, it has been shown that glutamate released by climbing fibers not only binds to AMPA-receptors on the Purkinje cell membrane, but also to AMPA-receptors located on nearby inhibitory interneuron axonal terminals (Satake et al., 2000). Activation of these presynaptic AMPA-receptors decreases the amount of GABA released (Satake et al., 2000; Satake et al., 2004). This glutamate-mediated decrease in presynaptic release of

GABA causes a dysinhibitory effect on Purkinje cells. AMPA-receptor mediated heterosynaptic interactions of glutamate are not found following parallel fiber stimulation, indicating that this effect is dependent on the amount of glutamate that is released or the spatial arrangement of the climbing fiber and parallel fiber synapses and AMPA-receptors (Satake et al., 2000). Together these results indicate that climbing fiber activity not only depolarizes the Purkinje cell, but also facilitates the following inhibitory rebound potentiation by temporarily decreasing the inhibitory synaptic input, which is in line with the fact that rebound potentiation displays a slower time course than the climbing fiber induced disinhibition (Kano et al., 1992; Kawaguchi and Hirano, 2000; Satake et al., 2000).

1.1.3 Calcium homeostasis of Purkinje cells

Calcium homeostasis is important for the functioning of Purkinje cells. The internal calcium concentration affects several important mechanisms, such as gene transcription, regulation of synaptic plasticity and initiation of programmed cell death by excitotoxicity. How can a single second-messenger encode all of these functions? The answer must lie in the temporal and spatial differences in the intracellular calcium concentration. Both Purkinje cell spines and dendrites are subject to massive influx of calcium ions following synaptic inputs and action potential back-propagation. Fierro and Llano (1996) showed that the calcium buffering capacity of proximal Purkinje cell dendrites is approximately an order of magnitude larger than that of apical or distal dendrites of hippocampal CA1 pyramidal neurons (Helmchen et al., 1996; Sabatini et al., 2002). The buffering capacity for calcium is the highest in the Purkinje cell compared to the buffering capacity for other cations (Fierro and Llano, 1996; Maeda et al., 1999). Cerebellar Purkinje cells contain various sorts of calcium binding proteins like calbindin D-28k, parvalbumin, calmodulin, S100, and various calcium sensor proteins like neurocalcin (Celio, 1990; Bastianelli, 2003).

Calbindin D-28k and parvalbumin are especially prevalent in cerebellar Purkinje cells (de Talamoni et al., 1993). Both calcium buffers are found throughout the whole Purkinje cell and have a high affinity for calcium and to a lesser extent for magnesium. Calbindin D-28k is a member of the EF-hand superfamily of proteins. The morphology of these proteins permits specific reversible binding of calcium (Kawasaki et al., 1998). Calbindin D-28k incorporates four binding sites for calcium, two of which have a high affinity for calcium and binding kinetics comparable to those of parvalbumin. Two other calcium binding sites have medium calcium affinity and binding kinetics about an order of magnitude faster than parvalbumin (Schmidt et al., 2003). The affinity and binding kinetics of the binding sites of parvalbumin are thus partially overlapping with those of two binding sites of calbindin D-28k. When calcium enters the Purkinje cell dendrite, the decay of the intracellular calcium concentration can be best described a double exponential equation. In contrast, dendritic calcium concentrations of other cell types like neocortical and hippocampal CA1 pyramidal neurons return to resting values with a decay that is well described by a single exponential function (Helmchen et al., 1996; Lee et al., 2000). This difference between calcium concentration decay kinetics is most likely due to the partial overlap between calbindin D-28k and parvalbumin binding kinetics (Schmidt et al., 2003). The amplitude of dendritic calcium transients has been shown to be affected only by calbindin D-28k, and most likely by the two binding sites that are of medium affinity and fast kinetics. The somewhat slower calbindin D-28k and parvalbumin binding sites accelerate the calcium decay significantly, but do not affect the amplitude of the peak calcium current (Schmidt et al., 2003). When the intracellular calcium concentration is lowered, calcium is released again by calbindin D-28k and parvalbumin. Gradually this slows down the decay of the intracellular calcium concentration.

Free calcium is removed from the cytosol by various sorts of calcium clearance mechanisms. Plasma membrane calcium pumps and the sodium-calcium exchangers extrude free calcium from the cytosol, while smooth endoplasmic reticulum pumps sequestrate free calcium. Quantitative measurements of the amounts of calcium removed from the cytosol by various calcium clearance mechanisms are hindered by the large variability of spine morphology, but various studies show their limiting effects on spreading and magnitude of calcium transients (Fierro et al., 1998; Sabatini et al., 2002; Schmidt et al., 2003). Besides active calcium clearance mechanisms, diffusion of calcium contributes passively to the decay of local increases in intracellular calcium concentration. It remains to be elucidated to what extent diffusion of calcium, either bound by calcium binding proteins or unbound, from spines to dendrites exists in dendritic regions of the Purkinje cell (e.g., Sabatini et al., 2002; Schmidt et al., 2003a). In addition to calcium extrusion mechanisms and calcium binding proteins, the complex morphology of the dendritic tree is postulated to influence back- and forward propagation of calcium transients (Vetter et al., 2001). In summary it seems that the intracellular calcium concentration is controlled by a wide variety of intracellular and molecular mechanisms and that it evokes its effects through an equally diverse range of processes. In conclusion, the diversity of mechanisms that regulate the intracellular calcium concentration indicates the importance of its temporal and spatial specificity for the regulation of the second messenger funcions of calcium.

1.1.4 Long term changes in strength of synaptic input to Purkinje cells

Influx of calcium ions into dendritic spines is of particular importance to synaptic plasticity. Long-lasting changes in synaptical strength have been assumed to be important for learning. Marr (1969), Albus (1971), and Ito (1972) proposed that plastic changes in the strength of the parallel fiber – Purkinje cell synapses might underlie cerebellar learning. In addition, Marr (1969) proposed that the climbing fiber acts as a teacher signal for the coincident parallel fiber input. Ito and Kano (1982) were the first to describe long term depression (LTD) of the parallel fiber – Purkinje cell synapse (PF-LTD), induced by conjunctive stimulation of the parallel fiber and the climbing fiber. Various mechanisms underlying PF-LTD have been demonstrated (Figure 2) (see Ito, 2001 for review). Climbing fiber activity causes glutamate binding on AMPA-receptors, causing widespread depolarization in the dendritic tree. This depolarization in turn initiates dendritic calcium entry via P/Q-type and T-type voltage-gated calcium channels (Watanabe et al., 1998; Pouille et al., 2000; Schmolesky et al., 2002). Parallel fiber activity also initiates AMPA-receptor and mGluR1 activity. Activation of mGluR1 evetually leads to IP_3-mediated calcium release from internal calcium stores (Finch and Augustine, 1998; Takechi et al., 1998). Besides increasing intracellular calcium concentration, parallel fiber activity activates DAG. Both DAG and increased calcium concentrations are required for activation of protein kinase C (PKC). Inhibition of PKC blocks PF-LTD (De Zeeuw et al., 1998). Activated PKC phosphorylates AMPA-receptors containing GluR2/3-subunits at a serine residue on the intracellular C-terminal (Chung et al., 2003). These phosphorylated AMPA-receptors are internalized by clathrin-mediated endocytosis (Wang and Linden, 2000). In this way PKC-mediated LTD reflects a reduction in the number of AMPA-receptors in the Purkinje cell membrane.

Intracellular calcium concentrations determine whether or not synaptic strength is altered. The exact timing of the parallel fiber activity-induced and climbing fiber activity-induced increases in the intracellular calcium concentration is critical for the initiation and spreading of synaptic plasticity. Wang et al. (2000) showed that when parallel fiber activity precedes climbing fiber activity not more than 200 ms the resulting calcium transient is larger than the linear sum of

the two separate calcium transients (Miyakawa et al., 1992; Wang et al., 2000). This supralinear calcium signal can thus be seen as a coincidence detector for paired activity of parallel fibers and climbing fibers. In addition to supralinear calcium signals, LTD is equally dependent on the timing of parallel and climbing fiber activation. These data indicate that supralinear calcium currents are coincidence detectors for LTD. When a small number of parallel fibers is stimulated, supralinearity of the resulting calcium transient is confined to single spines and dependent on mGluR1-activation (Wang et al., 2000). Calcium release from IP_3-mediated internal calcium stores is not only essential for supralinear calcium currents in single spines, but also for LTD induction in dendrites (Miyata et al., 2000). When larger numbers of parallel fibers are activated, coincidence

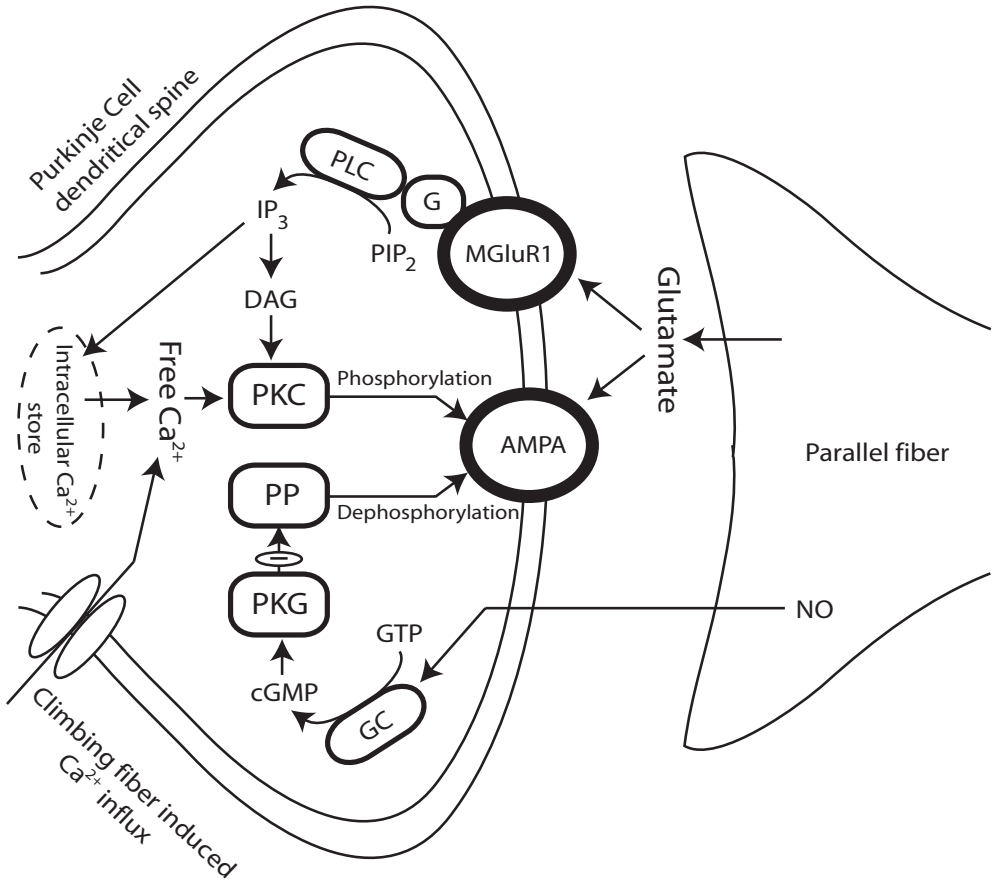

Figure 2: Simplified view of mechanisms underlying the induction of cerebellar long term depression. A central role for free calcium is depicted. Calcium enters the cell following climbing fiber activation through voltage-gated calcium channels. In addition, calcium is released from ionotsitol-tri-phosphate (IP_3)- or ryanodine-mediated internal calcium stores following parallel fiber activation. The subsequent increase in internal calcium concentration plus the presence of dia-acyl-glycerol (DAG) are needed to activate protein-kinase C (PKC), which eventually leads to the internalization of AMPA-receptors. Depicted also is the NO-PKG pathway. This pathway is activated by the presynaptic release of nitric oxide (NO) and results in the activation of protein-kinase G (PKG). Activation of PKG inhibits protein phosphatases (PP) and thus inhibits the dephosphorylation of AMPA-receptors. Both pathways result in the internalization of AMPA-receptors. PLC, phospholipase C; MGluR1, metabotropic glutamate receptor I; G, G-protein; GC, guanylate cyclase.

detection does not depend on mGluR1-activation, but relies on voltage-gated calcium channels (Wang et al., 2000). Supralinear calcium currents are likely to spread beyond the region of the activated synapses and thus are likely to spread PF-LTD to inactive neighboring synapses (Hartell, 1996; Wang et al., 2000a). These neighboring synapses need not be parallel fiber – Purkinje cell synapses; thus LTD may also serve as a mechanism for heterosynaptic effects of LTD.

Another possible pathway involved in parallel fiber LTD is mediated by nitric oxide (NO) and protein kinase G (PKG). NO is released by parallel fibers and spreads beyond the synapse of origin. This spread is large and fast; it can encompass 4000 synapses within 10 ms, and thus can serve as a mediator of heterosynaptic LTD (Reynolds and Hartell, 2001). NO acts on guanylyl cyclase, which converts guanosine-tri-phosphate into cyclic guanosine-mono-phosphate (cGMP). cGMP activates protein kinase G (PKG). PKG blocks dephosphorylation of AMPA-receptors by inhibiting protein phosphatases. Thus, PKG activation will shift the phosphorylation / dephosphorylation balance toward phosphorylation and results in less AMPA-receptors in the membrane of Purkinje cells dendritic spines and hence a depressed amplitude of EPSPs generated by parallel fiber activation.

Recently, it has been shown that the parallel fiber – Purkinje cell synapse can also be potentiated (PF-LTP). PF-LTP can be both presynaptically and postsynaptically mediated (Salin et al., 1996; Storm et al., 1998; Lev-Ram et al., 2002; Lev-Ram et al., 2003). Presynaptic LTP is unlikely to be an appropriate mechanism to reverse postsynaptically located parallel fiber LTD, while postsynaptic LTP may do this quite well without unnecessary inefficiencies (Coesmans et al., 2004). These data show a mechanism of bidirectional plasticity of the parallel fiber – Purkinje cell synapse which may underlie adaptation of motor performance.

Climbing fiber – Purkinje cell synapses have been found to be subject to plasticity as well (Hansel et al., 2001; Weber et al., 2003). Climbing fiber – Purkinje cell LTD (CF-LTD) is induced by electrical stimulation of the climbing fiber at 5 Hz for 30 sec and is mediated postsynaptically (Hansel and Linden, 2000). A more physiological stimulus paradigm that consists of 5 Hz train stimulation for 5 s with 10 s intertrain intervals also elicits CF-LTD (Schmolesky et al., 2002). CF-LTD results in reduction of climbing fiber EPSC amplitudes by ~ 20% and a significant decrease in calcium transients (Hansel et al., 2001; Weber et al., 2003). Coesmans et al. (2004) used CF-LTD to show that calcium currents affect the polarity of synaptic plasticity at the parallel fiber – Purkinje cell synapse. These results show that the internal calcium concentration may act like a switch via CF-LTD; PF-LTD induction occurs at higher calcium concentrations and PF-LTP occurs at lower concentrations.

1.2 Optokinetic and vestibulocular 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. During compensatory eye movements vestibular and/or optokinetic sensory information is transformed into appropriate command signals that drive the oculomotor plant, which encompasses the eyeball, the extra ocular muscles and non-muscular supporting tissues. The performance of the optokinetic reflex (OKR), vestibulo-ocular reflex (VOR), and visually-enhanced VOR (VVOR) is usually quantified by gain and phase values (Figure 3). Gain values correspond to the ratio of the amplitude of the eye velocity to the amplitude of the stimulus velocity. Phase values represent the time difference between the eye and the stimulus, expressed in degrees.

1.2.1 Vestibulocular reflex

The VOR generates eye movements in response to head movements. Angular head acceleration is detected by the semicircular canals, which are situated in the petrous temporal bone. Angular head acceleration leads to movement of the endolymph relative to the canal wall and this in turn causes a deviation of the gelatinous cupular membrane (cupula), which traverses the membranous canal. Deviation of the cupular membrane causes a deviation in the stereo- and kinocilia, which are embedded in the cupula. Depending on the direction of movement, this deviation excites or inhibits hair cells. Excitation and inhibition here corresponds, respectively, to the increase and decrease in neurotransmitter release from the basilar side of the hair cells. Movement of the stereocilia away from the kinocilium decreases neurotransmitter release, while movement towards the kinocilium increases neurotransmitter release. Due to inertia and viscosity of the endolymphatic fluid and the elastic properties of the cupula, the semicircular canals work as angular accelerometers with low-pass frequency tuning (Steinhausen, 1933). Over a broad range of stimulus frequencies (especially those encompassing the range of frequencies generated by the species during normal head movements) deflection of the cupula is proportional to angular head velocity instead of angular head acceleration (Jones and Milsum, 1971; Wilson and Melvill Jones, 1979). The oculomotor plant requires a mixture of velocity and position signals; for a proper functioning of the VOR a head position signal is required in addition to the canal-derived head velocity signal. The position signal is derived from the head velocity signal by a process of mathematical integration, conducted by various brainstem nuclei. The nucleus prepositus hypoglossi and the central medial vestibular nucleus form the neuronal integrator for movement about the vertical axis (Cannon and Robinson, 1987; Cheron and Godaux, 1987; Mettens et al., 1994), and the interstitial nucleus of Cajal, superior vestibular nucleus and dorsal group y may integrate signals related to rotations about the horizontal axes (Fukushima, 1987; Fukushima et al., 1992). These neural velocity-to-position integrators are found to be imperfect (leaky). Thus when the eye is displaced by rotating the animal in a lighted surrounding to one side, the eyes will drift back to the resting position if the lights are extinguished. The drift is quasi-exponential and thus can be quantified by its amplitude and time constant. This time constant is indicative of the leakiness of the integrator, shorter values indicating greater "leakiness". For humans, cats, and monkeys, the neural integrator time constant is approximately 20 s (Becker and Klein, 1973; Robinson, 1974; Cannon and Robinson, 1987). For mice it is 2.1 s, indicating the comparative weakness of the neural integrator in mice (Van Alphen et al., 2001).

The VOR is also characterized by a time constant. The time constant of the VOR is measured by providing a step in velocity in the dark and fitting a single exponential equation to the evoked eye movements. In mice the VOR time constant is 690 ms (Van Alphen et al., 2001). The VOR time constant in mice is surprisingly small considering that Skavenski and Robinson (1973) showed that in the absence of velocity storage, the VOR time constant should be approximately equal to the cupular time constant, which is proportional to the time constant of the primary vestibular afferent fibers. In both rats and guinea pigs, the primary vestibular afferent time constants measure on average 3.2 s (Curthoys, 1982). Primary afferent time constants in gerbils are found to range between 2 and 3 s (Schneider and Anderson, 1976). Considering the close relation in species and size between mice, rats, guinea pigs, and gerbils, one might assume that their cupular time constants are comparable too. There are two possible reasons for why the VOR time constant is so much shorter than this assumed length of the cupular time constant. First, the search coil might have artificially reduced the time constant, as suggested by the large VOR phase leads that developed in mice after coils were implanted (Stahl et al., 2000). Second, velocity storage might act in a negative way as has been found in the pigeons (Anastasio and Correia, 1994). In pigeons

the velocity storage is not added to the cupular time constant but subtracted, which results in a shorter VOR time constant. The first explanation seems most probable, since the velocity storage is so weak in mice it seems unlikely that the substraction of the velocity storage time constant can account for large difference between the assumed cupular time constant and the measured VOR time constant (Anastasio and Correia, 1994; Van Alphen et al., 2001). One might argue that in mice, in contrast to the monkey, the VOR time constant is not the approximate sum of the cupular and velocity storage time constants in mice (Skavenski and Robinson, 1973). In addition, one might argue that despite the close relation between rats and mice, the cupular time constant is much shorter in mice than in rats.

Ultimately, a short VOR time constant could confer advantages, as it would correspond to a higher optimal frequency-range of the VOR. Mice are likely to benefit from a short VOR time constant because they are fast-moving animals, and thus the visual motions they experience are likely to have frequency components higher than for the rat, cat, monkey, or human. In addition, mice in the wild are found in the field where they do not stick out above the surrounding vegeta-

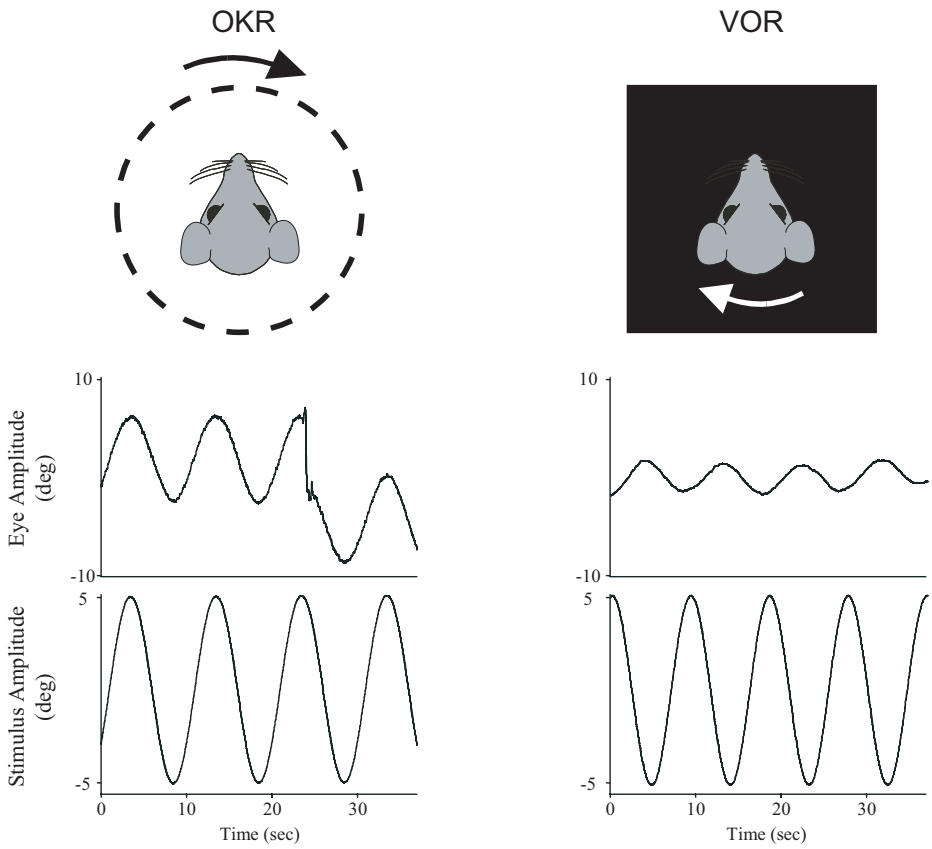

Figure 3: Schematic representation of the optokinetic reflex (OKR) and vestibulocular reflex (VOR). The OKR (left panel) is elicited by rotating a black-and-white surrounding around the animal. The VOR (right panel) is elicited by rotating the mouse in the dark. Middle graphs show the eye position in response to sinusoidal optokinetic (left column) and vestibular (right column) stimulation of 0.1 Hz. Note that the OKR is better than the VOR at this particular stimulus frequency. Gain values are calculated by dividing the amplitude of the eye velocity response by the stimulus velocity. Phase values are calculated as the difference between the phases of the sinusoidal fits of the eye velocity and stimulus velocity traces.

tion. This results in that all objects of interest will be nearby and thus rapid vestibularly-evoked eye movements need to be accurate in terms of survival.

1.2.2 Optokinetic reflex

Retinal slip, which is defined as movement of images across the retina, drives the OKR. The purpose of the OKR is to keep this image motion as small as possible. Optokinetically-driven eye movements reduce the amount of retinal slip, which indicates that the OKR is a negative feedback system. The distribution of velocity- and direction-sensitive retinal ganglion cells is species-specific. Higher animals have a fovea, an area in the centre of the retina with the highest spatial resolution (where photoreceptors are in a 1:1 ratio with ganglion cells). Due to this fovea higher animals like primates have comparable or higher OKR gain values over a wider range of frequencies than afoveate animals. Rabbits have a visual streak, which indicates an increased density of retinal ganglion and cone photoreceptor cells. Other afoveate animals, like guinea pigs and mice, have a more uniform distribution of the photoreceptor cells over their retina (Szel et al., 1992; Juliusson et al., 1994; Rohlich et al., 1994; Szel et al., 2000). Interestingly however, most horizontal cells, which connect cones, rods and retinal bipolar cells are found in the inferior retina, indicating that this part of the retina is equipped best for lateral integration of images in mice (Peichl and Gonzalez-Soriano, 1994).

Because mice lack a fovea, there is no need for eye movements intended to accurately position a specific part of the retina with respect to small visual targets, i.e., movements such as smooth pursuit and saccades. Thus, afoveate animals show poor eye movements in response to a moving object on a stationary background. Compensatory eye movements on the other hand, are easily elicited in afoveate animals. OKR gain values of both foveate and afoveate animals are close to one at low stimulus frequencies and decrease with increasing stimulus frequencies; hence the OKR system resembles a low-pass system (Collewijn, 1969a, b; Nagao, 1983; Van Alphen et al., 2001). In both foveate and afoveate animals the OKR acts secondarily to the VOR, a high-pass system that produces good compensatory eye movements at high peak acceleration and high frequencies (Collewijn, 1969b; Hess et al., 1985; Van Alphen et al., 2001). The low-pass OKR system is well suited to compensate for the residual retinal slip resulting from any minor inaccuracies of the high-pass VOR system, provided that the velocity and frequency content of the retinal slip are low. The combination of VOR and OKR (VVOR) results in accurate eye movements over a broad range of stimulus frequencies. Thus, eye movements are accurate when animals are rotated in the light.

The OKR is dependent on stimulus velocity. In the rabbit, the velocity dependence is most likely caused by the limited slip velocity sensitivity of retinal ganglion cells (Oyster et al., 1972) and a similar explanation likely applies to the mouse. Velocity dependence of the optokinetic system is shown by providing a velocity step to an optokinetic stimulus; the response is biphasic. The initial response to the velocity step of the optokinetic stimulus is a jump in eye velocity. The height of the initial jump in eye velocity reflects the retinal slip velocity at which the visual system saturates. The visual system is mediated by the optokinetic pathway, which traverses the flocculus (Collewijn, 1969b; Hess et al., 1985; Van Alphen et al., 2001). Lesioning of the flocculus significantly decreases the initial jump in eye velocity following a velocity step in the optokinetic stimulus (Zee et al., 1981; Waespe et al., 1983). Following the fast initial jump in eye velocity a slow rise in the eye velocity appears, which is mediated by the velocity storage mechanism (Cohen et al., 1977; Cohen et al., 1981; Maioli and Precht, 1984; Hess et al., 1985). For mice, in which the velocity storage mechanism is poorly developed (Van Alphen et al., 2001;

Stahl, 2004), the OKR is mediated mainly by the optokinetic pathway (Figure 4). In mice, optokinetic stimulus velocities > 8º/s elicit sub-maximal responses (Van Alphen et al., 2001; Stahl, 2004).

The OKR is influenced not only by the velocity sensitivity of the retinal receptors, but also by the dynamics of the compensatory eye movement circuit and the oculomotor plant. Thus the leakiness of the neural integrator, already mentioned in connection to the VOR, impacts on the OKR too (Van Alphen et al., 2001; Stahl, 2004). This weak neural integrator function is indicated by the following: In the mouse the optokinetic transport delay is 70 ms (Van Alphen et al., 2001). This transport delay is dictated by the latency between the detection of movement by a retinal photoreceptor cell and the generation of an electrical signal in these cells. A transport delay of 70 ms predicts a larger OKR phase lag at stimulus frequencies under 1.0 Hz than was found (Van Alphen et al., 2001). A weak neural integrator would add a smaller OKR phase lag than would be generated by an ideal integrator. This subnormal lag can be seen as a relative phase lead, which would reduce the resulting OKR phase lag. At frequencies above 1.0 Hz, the observed phase lag was larger than that expected on the basis of the 70 ms delay (Van Alphen et al., 2001). The fact that the observed phase values differ from those expected on the basis of the transport delay, indicates that the transport delay alone can not account for the OKR phase dynamics. Only a combination with the neural integrator (of which the lead-producing effects fall off at higher frequencies) and the transport delay could account for the OKR phase dynamics.

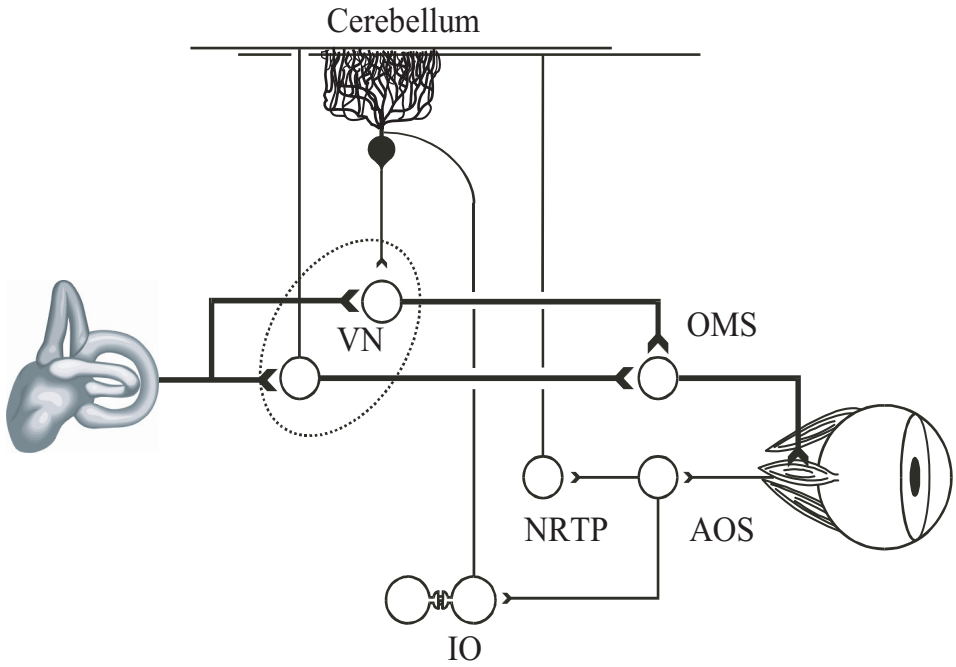

Figure 4: Schematic representation of the oculomotor pathways that include the floccular side loop, which lies parallel to the vestibular nucleus (VN). The floccular side loop is the substrate of the optokinetic pathway. Both the flocculus and the VN are positioned at key positions where the vestibular and optokinetic pathways cross. Primary vestibular efferents transmit information about head movement to the VN. Neurons from the VN innervate the cerebellum via mossy fibers and parallel fibers. Retinal slip, detected by the retina, is processed by the accessory optic system (AOS) and inferior olive (IO). Neurons of the IO innervate the cerebellar Purkinje cells through climbing fibers. Besides the IO, neurons of the AOS innervate the nucleus reticularis tegmentum pontis (NRTP), which form another mossy fiber – parallel fiber input for floccular Purkinje cells.

1.3 Measuring eye movements in mice

1.3.1. Technical aspects

Compensatory eye movements can be quantified in mice using various methods. The early attempts of measuring compensatory eye movements in mice used either video tracking recordings or electroculography (Grusser-Cornehls and Bohm, 1988; Mitchiner et al., 1976), but the eye movements were calibrated by the unsupported assumption that at lower stimulus velocities the OKR gain values reached unity (Stahl et al., 2000). Koekkoek et al. (1997) used magnetic search coils with a diameter of 3 mm and reported low gain values and large phase lags. The magnetic search coil method has been updated by using 1 mm diameter coils, which reduces the mechanical restrictions on the movement of the eye. The research presented in the current thesis was performed using this magnetic mini-search coil as well as optimized video tracking techniques (Stahl et al., 2000; Stahl, 2004). The scleral search coils with a diameter of 1 mm that we use in our laboratory weigh 1 mg and are made of 60 turns of copper wire. The search coils are implanted under the conjunctiva on the temporal side of the eyeball. Leads of the coil are routed around the eyeball under the conjunctiva and exit the orbit superiorly. Coils are fixed to the sclera with sutures and the conjunctiva is closed over the coil with another suture. Although magnetic search coils are small in size and some researchers are capable of surgically placing coils with minimal damage to the retina, movement of the eye is still affected by their presence. In contrast to scleral search coil techniques, video tracking techniques are non-invasive and thus in principle unlikely to influence the movement of the eye. Video tracking techniques use commercially available cameras, software and infrared illumination. Infrared light is invisible to mice. The emitters are positioned on both sides of the camera. In addition, one infrared emitter is fixed to and positioned above the camera, providing a reference reflection. Camera and software detect the corneal reference reflection of the reference emitter and the pupil. Because the software needs the whole image of the pupil to calculate its position relative to the corneal reflection, it is important that the pupil diameter does not become too large. Recordings of eye movements in the dark require the use of parasympathetic agonists, which limit pupil dilation. Although we cannot exclude the possibility that such drugs affect eye movement performance, this effect seems to be limited as relatively high gain values can be obtained (Stahl et al., 2000).

1.3.2 Background and transgenics of mice

Generally the gain and phase values of the eye movements measured in mice strongly depend on the recording technique used as well as on the type of background or mutant investigated. Various mouse strains are used in eye movement studies. The most commonly used strain is the C57BL/6. Mice of this strain have been shown to produce clear eye movements in response to optokinetic stimuli (Katoh et al., 1998; Van Alphen, 2002). However, because stem cells derived from inbred mouse strains, such as C57BL/6 and BALB/c, are technically challenging to use for homologous recombination (Gerlai, 1996, 2001), artificially engineered mouse mutants often have a 129/Sv background. The use of transgenic mice generated from 129/Sv stem cells can specifically influence the results of oculomotor studies, since these mice carry the pink eye dilution allele, which results in albino appearance and abnormal visual acuity (Crawley et al., 1997). Similar to humans, rabbits, and rats that are affected by ocular albinism (Precht and Cazin, 1979; Winterson and Collewijn, 1981; Lannou et al., 1982; Collewijn et al., 1985), albino mice have several retinal

deficits (Williams et al., 1998; Raven and Reese, 2002). Therefore it is not surprising that 129/Sv mice show decreased OKR and VOR amplitudes compared to C57BL/6 mice (Katoh et al., 1998). Katoh et al. (1998) proposed that besides retinal aberrations, deficits in the accessory optic system contribute to the abnormal OKR, and deficits in the vestibular system cause altered VOR function. In addition to changes in eye movement performance, 129/Sv mice have also been shown to perform more poorly on a variety of behavioural and cognitive tests (Balogh et al., 1999). These behavioural deficits differ in nature between the various sub-lines of 129/Sv mice (Lathe, 1996). Thus, there are numerous disadvantages of using 129/Sv mice for behavioural studies. Nevertheless their stem cells are most commonly used for the generation of transgenic mice by homologous recombination, because these cells are easily bred *in vitro*.

The undesirable behavioural qualities of 129/Sv-derived mice are avoided by backcrossing the progeny to C57BL/6 mice to replace 129/Sv for C57BL/6 genes. Although the genetic background of mice of a single strain may on average be similar in all offspring of all generations, the heritage of individual genes is quite different from animal to animal due to cross-over during meiosis. Cross-over during meiosis does not affect the heritage of individual genes when littermates are used for mating, because the genome of the litters is identical. But when mice from different litters are used to create offspring, cross-over during meiosis causes the wild type offspring to be genetically different from their littermates in more than the single targeted gene. The polymorphic background probably has consequences on the expression of effects of mutating the targeted gene. For instance, when the targeted gene activates a second gene, the effects of the target mutation would depend on whether the second gene was inherited from the 129/Sv stem cell or the C57BL/6 breeding strain. This effect of secondary gene activation will lead to a larger statistical variation within a genotypical group, making the effect of the genetic alteration harder to detect. Hence, data will need to be retrieved from a larger number of animals. Fortunately, the polymorphic background does not lead to systematic differences between control and experimental animals since the distribution of alleles is random. Reducing the genetic variation by increasing the number of backcrosses seems more desirable than measuring more animals. However, it has been calculated that mice are 99% genetically identical only after 12 backcrosses. This number of crosses requires approximately 2 years to achieve (Festing, 1992), so it may actually require less work to measure a larger number of heterogeneous animals, provided that the measurements are not time consuming.

Another complication is the issue of genetic linkage. Genetic linkage refers to the fact that even in heavily backcrossed offspring, genes that immediately flank targeted genes are most likely to originate from the embryonic stem cells. When 129/Sv stem cell-based mice are used, special attention should be paid to genetic linkage, since numerous 129/Sv genes are significantly different from the genomic DNA of other mouse strains (Lathe, 1996). A commonly used technique to check whether the phenotype of a null-mutant mouse is caused by deletion of the targeted gene as opposed to mutations in the genes flanking the targeted gene is to create a “rescue” mouse, in which a copy of the targeted gene is reinserted into the genome. Creating rescue mice is done by taking a fertilized egg from the mother and injecting a cDNA-probe containing the targeted gene into the pronucleus. In this way the targeted gene is re-inserted into the genome and located randomly. When resulting phenotypes are normal, one concludes that the abnormal phenotype of the original mutant mouse is not the result of the mutated 129/Sv genes flanking the targeted gene. Another method of excluding the possibility that flanking genes account for an abnormal phenotype is to insert genetic marker genes next to the targeted gene by the “knock-in” technique (Gerlai, 1996). Inserting a marker next to the targeted gene enables one to screen for 129/Sv flanking genes in wild type and mutant alleles. When genetic markers are found on alleles of both wild type and mutant offspring, this shows that on all these alleles the flanking genes are

similar and thus the phenotypical difference is to be ascribed to the absence of the targeted gene and not to the presence of the flanking genes on the mutant allele. In order to be sure that the insertion of the DNA marker does not influence the expression of the targeted gene, the mouse in which the marker is inserted should be extensively tested, which takes a considerable amount of time.

Interpretation of results from transgenic or null-mutant mice is confounded by the lack of spatial restriction of the genetically induced effect. This lack of spatial specificity can be prevented when cell-specific constructs are used. For instance, in the brain Purkinje cell protein-2 (L7) is expressed exclusively in cerebellar Purkinje cells (Oberdick et al., 1990). L7 is a gene that consists of 4 exons and codes for a protein that contains a G protein-regulatory motif. Expression of L7 starts during the first two postnatal weeks (as reviewed by Barski et al., 2002). Inserting a target gene into the fourth exon of a modified form of the L7-vector, L7ΔAUG, results in the target gene being expressed selectively in cerebellar Purkinje cells (De Zeeuw et al., 1998). A method of creating knock-outs that are cell-specific involves the L7-gene and the so-called Cre / LoxP system. Two transgenic mice are needed for this system - a mouse in which the targeted gene is surrounded by loxP sites (floxed transgene), and another mouse in which the L7 promotor is coupled to Cre recombinase expression (L7Cre mice). When L7Cre and floxed mice are crossed, the floxed transgene is excised from the genome in any cells that express L7.

The use of cell-specific promotors like L7 largely solves the problem of spatial specificity of the mutation, but does not diminish the effect of developmental compensation. Genetic alteration may cause additional developmental changes. Any observed behavioural deficit or lack thereof may be caused by these secondary changes, rather than the primary genetic alterations. An example of a method that can achieve high temporal specificity is the tetracycline-controlled transactivator system. This system allows gene transcription to be fully inducible and is based on the tetracycline resistance gene of E.coli (Gossen et al., 1995). Expression of the transgene coupled to the tetracycline operator is inhibited or activated by administration of tetracycline or doxycycline, respectively (Gossen and Bujard, 1992; Gossen et al., 1995). In order to provide mouse models in which the mice are “equipped” with both the inducible and cell-specific gene transcription systems, a considerable amount of work has to be done. At least 3 mouse lines should be created: a floxed transgene mouse line, a cell-specific Cre recombinase mouse line, and a tetracycline transgenic mouse (as reviewed by Hedou and Mansuy, 2003). Another powerful approach that uses only two mouse lines couples oestrogen and progesterone receptor genes to Cre recombinase expression (Indra et al., 1999; Wunderlich et al., 2001). For instance, Purkinje cell-specific mice are generated by using transgenic mice in which L7Cre is coupled to the gene coding for progesterone receptor. When progesterone ligands that pass the blood - brain barrier are administered, progesterone receptor transcription in these mice is initiated and Purkinje cell-specific Cre recombinase expression will be triggered. When these L7Cre / progesterone mice are crossed with a floxed trangene mouse line, offspring will be generated in which excision of the targeted gene is fully inducible and Purkinje cell-specific. In addition to achieving temporal specificity, this method also greatly increases the statistical power; analysis can be performed in a paired fashion, i.e. each mouse is its own control. The ability to use paired analysis reduces the number of mice needed for an experiment or the number of backcrosses needed.

1.4 Scope of this thesis

The current thesis describes electrophysiological characteristics of cerebellar Purkinje cells in mouse mutants during compensatory eye movements. The central question is whether alterations

of calcium homeostasis will result in altered Purkinje cell activity and / or altered motor behaviour, and whether these parameters can be correlated to each other. To this end we investigated three types of mutant mice: 1) a spontaneous mutant carrying a mutation in the voltage-gated calcium P/Q type channel; 2) a Purkinje cell-specific knock-out of the calcium buffer, calbindin D-28k; and 3) a Purkinje cell specific transgenic mutant in which a calcium-dependent kinase of motor learning is affected, PKC. Together, these studies should provide a broad view of the various potential functions that calcium may play in cerebellar Purkinje cells. We have focused in particular on the role of Purkinje cells in the flocculus during compensatory eye movements, because such movements form a readily tractable behaviour mediated by well known neuro-anatomical pathways. In addition, we can readily identify the Purkinje cell activity in the floccular zones that is responsible for the control of these movements about particular axes in space (eg. De Zeeuw et al., 1994).

We have organized the thesis in the following order. In this chapter (**Chapter 1**) we give a general introduction on cerebellar Purkinje cells, compensatory eye movements, and the use of mutant mice. In **Chapter 2** we review the general function of the flocculus, its afferent and efferent pathways, and the activity patterns of its Purkinje cells in response to optokinetic and vestibular stimuli in various species. Recordings of floccular Purkinje cells during compensatory eye movements in monkeys, rabbits, and rats have revealed the contribution of the eye velocity, eye position, and retinal slip to their firing rates. Similar relations are likely to be found in mice. In **Chapter 3** we describe how a mutation in the P/Q-type voltage gated calcium channel as occurs in *tottering* mutants (*tg*) affects Purkinje cell activity and how this altered Purkinje cell activity in turn may explain the predominant cause of ataxia in *tg* mice. Since the intracellular calcium concentration is not only regulated by the influx of calcium ions but also by the calcium buffering capacity, we investigated in **Chapter 4** whether Purkinje cell specific deletion of calbindin D-28k alters the amplitude of calcium transients evoked by synaptic input and whether a decrease in calcium buffering capacity affects simple spike and complex spike firing patterns in a way that may ultimately affect motor behaviour. Apart from a direct regulation of Purkinje cell output, calcium functions as the single most important regulator of long term changes in synaptic efficacy (Coesmans et al., 2004). We therefore investigated in **Chapter 5** whether a change in the activity of a calcium-dependent regulator (PKC) of synaptic efficacy (LTD) results in a change in Purkinje cell output during motor behaviour. Finally, in **Chapter 6** we discuss how altered calcium entry, calcium buffering, and a lack of LTD induction may and may not change the electrophysiological responses of cerebellar Purkinje cells and thereby their effect on oculomotor behaviour. In conjunction, the findings and hypotheses described in these chapters provide several new ideas that may open up various new lines of cerebellar research that can be addressed in future Ph.D.-projects.

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Chapter 2

Gain and phase control of compensatory eye movements by the flocculus of the vestibulo-cerebellum

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2.1 Introduction

The cerebellum influences the amplitude and timing of movements. Neurons in the cerebellar nuclei are thought to coordinate the management of commands, corrections, and feedback related to limb and trunk movements through coactivation of paths descending to alpha-motoneurons (for generation of muscle power), as well as to gamma-motoneurons (for adjustment of muscular position sensors and velocity sensors) (Brooks and Thach, 1981). In this way, the cerebellum probably exerts two supportive actions in order to govern posture and motor control, namely phasic triggering of programs and tonic support of reflexes (Bloedel and Courville, 1981). Lesions of the cerebellum can cause general dysfunctions like ataxia and hypotonia and, in addition, rather specific defects attributable to lesions of particular parts of the cerebellum. For example, lesions of the spinocerebellum can produce abnormality in the sequence of muscle contractions during rapid movements, and dysmetria plus tremor during slow movements. Lesions of the cerebrocerebellum are more characterized by delays in movement initiation and in coordination of distal limb movements (Kennedy et al., 1982; Keele and Ivry, 1990). Lesions of the nodulus and flocculus of the vestibulocerebellum evoke predominantly disturbances in balance and eye movement control, respectively (Ito, 1984).

This chapter focuses on the role of the flocculus in the control of the amplitude and the timing of compensatory eye movements. Separate sections are devoted to gain (i.e. amplitude of eye velocity / amplitude of stimulus velocity) and phase (i.e. time difference between eye and stimulus x frequency x 360) of these movements. Special emphasis will be placed on cellular and molecular factors that directly influence these parameters. Apart from supplementary reports of experiments in rats, cats, and monkeys, the bulk of the material reviewed here has been obtained from studies in rabbits and mice.

2.2 Floccular pathways involved in the control of compensatory eye movements

The flocculus of the cerebellum is involved in the control of the gain and phase dynamics of the optokinetic reflex (OKR), the vestibulo-ocular reflex (VOR), adaptation of the VOR, and in the case of primates, smooth pursuit eye movements (for review, see Ito 1984). To fulfill these functions the flocculus receives afferents conveying many different signals related to eye movements. In turn, the flocculus projects to various vestibular and cerebellar nuclei that ultimately influence the activity of oculomotor neurons (see Figure 4 in chapter 1). The afferents include the climbing fibers, mossy fibers, and mono-aminergic fibers, while the efferent pathways of the flocculus are formed by the Purkinje cell projections.

2.2.1 Floccular input

The climbing fiber input to the flocculus is, as in other parts of the cerebellum, strongly topographically organized (for review see Voogd et al, 1996). The climbing fiber input to Purkinje cells in the rabbit flocculus can be divided into five zones - four visual zones and one non-visual zone (De Zeeuw et al., 1994b; Tan et al., 1995). The visual Purkinje cell zones (zones 1-4) receive their climbing fiber input from the dorsal cap and/or ventrolateral outgrowth of the inferior olive, while the Purkinje cells of the non-visual zone (zone C2) receive their climbing fibers from the rostral tip of the rostral medial accessory olive. The C2 zone is presumably involved in the control of neck movements, because electrical stimulation of this zone elicits short latency neck move-

ments (De Zeeuw and Koekkoek, 1997). The climbing fiber activities (i.e. complex spikes) of the visual Purkinje cells modulate optimally to an optokinetic stimulus rotating about the vertical axis (zones 2 and 4) or about the horizontal axis perpendicular to the plane of the ipsilateral anterior semicircular canal (zones 1 and 3) (see Figure 1) (De Zeeuw et al. 1994a). These different slip signals are conveyed from the retina through different sets of nuclei of the accessory optic system and inferior olive (for review, see Simpson et al., 1996). The optokinetic input to the horizonal axis zones are subsequently mediated by the contralateral medial terminal nucleus, the ipsilateral visual tegmental relay zone of the accessory optic system, and via either the rostral dorsal cap, or the ventrolateral outgrowth of the inferior olive. The visual signals to the vertical axis zones are subsequently mediated by the ipsilateral dorsal terminal nucleus, the pretectal nucleus of the optic tract, and via the caudal dorsal cap (Simpson et al., 1988; Soodak and Simpson, 1988). To a large extent the complex spike modulation of Purkinje cells in the different zones is already encoded in the descending projections from the accessory optic system and the neurons in the different olivary subnuclei. Apart from the visual signals, evidence has been gathered that the climbing fiber input to the visual zones of the flocculus may also relay non-visual, vestibular-like signals (De Zeeuw et al., 1995a; Simpson et al., 2002). In the rabbit, the majority of the climbing fibers in these zones modulate when the rabbit is oscillated in the dark at frequencies and amplitudes higher than 0.8 Hz and 5 degrees, respectively. This complex spike modulation is approximately in-phase with the concomitant simple spike modulation, and the activity increase consistently occurs when the head rotates contralaterally. This non-reciprocal relation between the complex spike and simple spike modulation during vestibular stimulation stands in marked contrast to the reciprocal relation found when the rabbit is afforded vision. Possibly, the complex spike signals that arise with vestibular stimulation in the dark reflect the inhibitory input to the caudal dorsal cap from the nucleus prepositus hypoglossi, which has been shown to code for both eye position and velocity (De Zeeuw et al., 1993). The third possible signal carried by climbing fibers to the flocculus may be more directly related to the actual movement (Frens et al., 2001). Similar to the vestibular-like signals, it remains to be determined how and where this motor-related signal is generated. It becomes apparent when presenting transparently moving optokinetic stimuli. Applying this stimulation paradigm to alert rabbits has demonstrated that identical retinal slip patterns can result in different complex spike modulation depending on eye movement behaviour.

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. Sources of the mossy fiber projections to the flocculus include the medial vestibular nucleus, descending vestibular nucleus, superior vestibular nucleus (rabbits, Alley et al., 1975; Yamamoto, 1979; Barmack et al., 1992b; 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; rabbits, Yamamoto, 1979; Barmack et al., 1992a; Barmack et al., 1992b), nucleus abducens (cats, Kotchabhakdi and Walberg, 1977; rabbits, Yamamoto 1979), nucleus reticularis tegmenti pontis (cats, Hoddevik, 1978; rabbits, Yamamoto 1979), and, via the output of the brush cells, the flocculus itself (Mugnaini et al., 1997). In addition to mossy fibers, recurrent Purkinje cell collaterals are known to project to neighboring cells (Bishop, 1988). Based upon electrophysiological recordings that showed short latencies consistent with a monosynaptic connection, it was originally assumed that primary vestibular afferents from the vestibular ganglion also directly innervate the flocculus (Shinoda and Yoshida, 1975; Ito 1984). However, using sensitive autoradiography, Gerrits et al. (1989) demonstrated that primary vestibular afferents are virtually absent in the flocculus. In retrospect, the short latencies may have been due to axosomatic gap junctions at the terminals of vestibular afferents innervating second-order vestibular neurons (De Zeeuw and Berrebi, 1995). Instead, most, if not all, vestibular mossy fiber input to the flocculus is

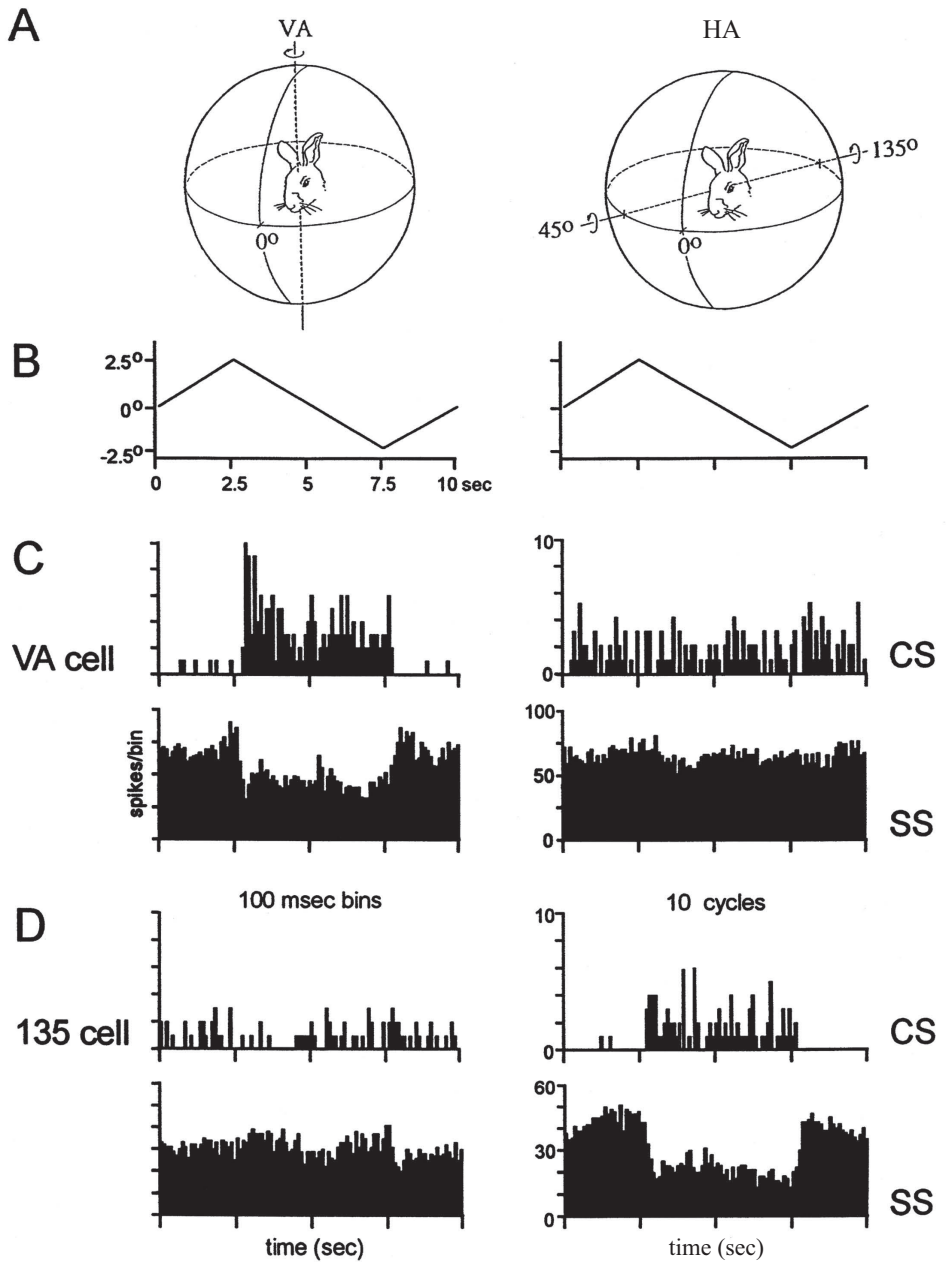

Figure 1: Peristimulus time histograms of the CS and SS responses of a representative vertical axis Purkinje cell (VA cell; C) and horizontal axis Purkinje cell (135 cell; D) in the rabbit flocculus to optokinetic stimulation (see A and B) about the vertical axis (left column) and horizontal axis perpendicular to the ipsilateral anterior canal (right column). The horizontal axis Purkinje cell is referred to as a 135 cell, because the horizontal axis is oriented at 135˚ ipsilateral azimuth. Note the reciprocity of the CS and SS modulations at the preferred axis of stimulation (adapted from De Zeeuw and Simpson; see De Zeeuw et al. 1995).

probably derived from second-order neurons in the vestibular nuclei. The information transmitted from the nucleus reticularis tegmenti pontis to the flocculus is probably mainly visual, while information from the nucleus prepositus hypoglossi include vestibular, visual, and eye movement related signals, and information from the abducens nucleus may relay eye movement signals only (Maekawa and Kimura, 1980; Miyashita et al., 1980).

The preferred axes of modulation for floccular simple spike responses are mostly the same as those of complex spike responses (Figure 1). This concordance is found despite the patchy distribution of the mossy fiber projection and despite the fact that the mossy fiber projection is bilateral and that the parallel fibers cross the climbing fiber zones orthogonally (Brand et al., 1976; Mugnaini, 1983). The concordance of preferred axis, despite the extent of the parallel fiber ramification, may be explained in part by the theory that the ascending axons of granule cells (not their parallel fibers) serve as the most powerful route by which granule cells influence Purkinje cells (Llinas, 1982; Bower, 1997). In addition, the spatial alignment may be supported by a subpopulation of the Golgi cells that exert their inhibitory actions preferentially in neighbouring (micro)zones (De Zeeuw et al., 1995b). In particular, the glycinergic Lugaro cells traverse perpendicularly through the sagittal zones, and these cells are densely innervated by zone-crossing recurrent Purkinje cell axon collaterals (Laine and Axelrad, 2002; De Zeeuw, unpublished observations).

Most of the mossy fibers in the flocculus use glutamate as a neurotransmitter (for review, see Voogd et al. 1996). Phosphate-activated glutaminase and/or conjugates of glutamate are found in many neurons of the medial vestibular nucleus, superior vestibular nucleus, group y, nucleus prepositus hypoglossi, and nucleus reticularis tegmenti pontis. However, there is considerable evidence for more heterogeneity in mossy fibers of the flocculus, because many have been shown to contain neuroactive peptides or to be cholinergic. For example, in the flocculus of the opossum, cholecystokinin occurs in mossy fibers, while enkephalin and corticotropin-releasing factor are colocalised in both climbing fibers and mossy fibers (Cummings and King, 1990; Madtes and King, 1994). In rabbits mossy fibers that use corticotrophin-releasing factor for neurotransmission originate from the nucleus prepositus hypoglossi, vestibular nuclei, reticular nucleus, nucleus reticularis gigantocellularis, and raphe nuclei (Errico and Barmack, 1993; Overbeck and King, 1999). The cholinergic mossy fiber input to the flocculus originates predominantly in the caudal medial vestibular nucleus and the nucleus prepositus hypoglossi (Ojima et al., 1989; Barmack et al., 1992a; Barmack et al., 1992b; Jaarsma et al., 1995), and innervates both granule cells and unipolar brush cells (Jaarsma et al., 1996). The distribution of acetylcholine receptors in the cortex of the flocculus appears more prominent and widespread than that of the cholinergic fibers, but there is some variability across species (Jaarsma et al. 1997). For example, in most species examined (i.e. mouse, rat, cat, rabbit and monkey) muscarinic m2-receptors are localised on the dendrites of Golgi cells and a subset of mossy fibers in the flocculus, but in the rabbit they are also prominent on a subset of parallel fibers and on most of the Purkinje cells in zone 1 of the flocculus, one of the two horizontal axis zones (Jaarsma et al., 1995; Jaarsma et al., 1997). Vertical axis zone Purkinje cells do not show immunoreactivity for muscarinic receptors (Jaarsma et al., 1995). Nicotinic acetylcholine receptors are generally sparse in the cerebellar cortex, occurring at low levels in granule cells (Jaarsma et al. 1997). However, α7-nicotine receptor subunits have been identified in the flocculonodular lobe and their distribution suggests a specific association with unipolar brush cells (Hunt and Schmidt, 1978).

Mono-aminergic inputs to the flocculus are mainly formed by serotonergic and noradrenergic fibers; dopaminergic input to the flocculus is virtually absent (Panagopoulos et al., 1991; Ikai et al., 1992). Serotonergic input to the flocculus, though relatively weak, is mainly derived from the nucleus pontis oralis and the nucleus reticularis (para)gigantocellularis (Bishop et al.,

1985). The fiber bundles consist mainly of long axons that terminate in all three cortical layers of the cerebellum (Bishop et al. 1985). Ligand binding autoradiography demonstrates a low density of serotonin receptors in the cerebellar cortex with the highest density in the molecular layer consisting primarily of $5HT_{1b}$-receptors (Pazos and Palacios, 1985). Noradrenergic projections to the flocculus originate from the locus coeruleus (for review, see Van Neerven et al., 1990). These fibers terminate in both the granule cell layer and molecular layer of the flocculus (Kimoto et al., 1981). Autoradiographic studies have shown that the noradrenergic receptors are mainly of the β-type, and that they are predominantly concentrated in irregular patches in the Purkinje cell layer (Sutin and Minneman, 1985).

2.2.2 Floccular output

Despite early reports on an efferent floccular pathway formed by the axons of unipolar brush cells (Mugnaini and Floris, 1994), Purkinje cell axons form the sole output of the flocculus (Mugnaini et al., 1997). They project to target cells in the cerebellar and vestibular nuclei that themselves do not project to the flocculus. Projections of Purkinje cells from the visual floccular zones to the complex of cerebellar and vestibular nuclei in the rabbit are, similar to climbing fiber projections, topographically organized. Collectively, Purkinje cells of horizontal axis zone 1 project to the ipsilateral ventral dentate nucleus, dorsal group y, and superior vestibular nucleus; Purkinje cells of vertical axis zones 2 and 4 project to the ipsilateral magnocellular and parvocellular medial vestibular nucleus; and Purkinje cells of horizontal axis zone 3 project to the ipsilateral dorsal and ventral group y, and the superior vestibular nucleus (for review on rabbits, see De Zeeuw et al., 1994b; monkey, Langer et al., 1985a; cat, Sato et al., 1988; rat, Umetani, 1992) (Figure 2). Individual Purkinje cell axons originating in the flocculus can branch and innervate different vestibular and cerebellar nuclei (De Zeeuw et al., 1994a). Branching axons from zone 1 either innervate both the ventral dentate nucleus and superior vestibular nucleus or both dorsal group y and the superior vestibular nucleus. Branching axons from zones 2 and 4 innervate both the magnocellular and parvocellular medial vestibular nucleus (and the nucleus prepositus hypoglossi, if folium p is included). Branching axons from zone 3 innervate both dorsal group y and the superior vestibular nucleus, or both ventral group y and the superior vestibular nucleus. The terminal varicosities of an individual floccular Purkinje cell axon can contact both smaller inhibitory and larger excitatory neurons within their target nuclei (De Zeeuw and Berrebi 1995). For example, individual Purkinje cell axons of the horizontal axis zones give off terminals in both the dorsolateral and central parts of the superior vestibular nucleus, regions containing, respectively, the excitatory and inhibitory second-order vestibular neurons innervated by primary afferents from the ipsilateral anterior semicircular canal (Highstein and Reisine, 1979; Sato and Kawasaki, 1990; De Zeeuw et al., 1994b).

2.3 Open and closed floccular pathways

Individual Purkinje cells of the flocculus mediate two pathways that are involved in compensatory eye movements: an open (via predominantly excitatory neurons) and a closed (via inhibitory neurons) anatomical pathway (Figure 2) (De Zeeuw et al., 1994a). All nuclei that receive input from the visual floccular zones (i.e. the medial vestibular nucleus, the superior vestibular nucleus, the ventral dentate nucleus, dorsal group y, and the nucleus prepositus hypoglossi) project to the oculomotor complex (Highstein et al., 1971; Graybiel and Hartwieg, 1974; Yamamoto et al.,

1986; Buttner and Buttner-Ennever, 1988; Evinger, 1988). The ventral dentate nucleus, dorsal group y, and nucleus prepositus hypoglossi, which all 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 (Voogd and Bigaré, 1980; De Zeeuw et al., 1993; De Zeeuw et al., 1994a). This circuit is referred to as the closed olivofloccular pathway (Figure 2A and B), 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 (Voogd and Bigaré, 1980; De Zeeuw et al., 1997). In contrast, the medial vestibular nucleus and superior vestibular nucleus do not project to the dorsal cap or ventrolateral outgrowth, and, thus, this arrangement is referred to as the open olivofloccular pathway (Figure 2C and D). 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 underlies the VOR (for review see Buttner and Buttner-Ennever, 1988), whereas the flocculus receiving neurons in the closed pathway (ventral dentate nucleus, dorsal y group, and nucleus prepositus hypoglossi) do not receive a direct input from the primary afferents of the semicircular canals. Because 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 circuits that are intimately connected with either the primary vestibular afferents or the inferior olive.

The open and closed pathways each contribute to the control of both horizontal (Figure 2A and C) and vertical (Figure 2B and D) compensatory eye movements. With regard to the open pathway, the medial vestibular nucleus neurons (when group y is included) are excited by primary afferents from all ipsilateral semicircular canals (Büttner and Büttner-Ennever 1988), but the flocculus inhibits only those neurons that are monosynaptically innervated by the primary afferents from the anterior and horizontal canals (Ito et al., 1973; Sato et al., 1988). The finding that the neurons receiving innervation from the horizontal canal primary afferents are inhibited by the flocculus is consistent with the fact that floccular zones 2 and 4, which are involved in the horizontal compensatory eye movements, project to the medial vestibular nucleus. In cats, flocculus receiving neurons in the medial vestibular nucleus can be either excitatory or inhibitory (Kawaguchi, 1985; Sato et al., 1988). The excitatory flocculus receiving neurons in the medial vestibular nucleus project to the ipsilateral medial rectus motoneurons in the ipsilateral oculomotor nucleus. The inhibitory flocculus receiving neurons in the medial vestibular nucleus project to the ipsilateral abducens nucleus and thus inhibit the excitatory action of the abducens nucleus on the ipsilateral lateral rectus muscle and the contralateral medial rectus muscle (through the excitatory innervation on the contralateral oculomotor nucleus) (rabbit, Highstein, 1973; cat, Highstein and Reisine 1979; Reisine and Highstein, 1979; Reisine et al., 1981; Uchino et al., 1982; Uchino and Suzuki, 1983; Sato et al., 1988; Sato and Kawasaki, 1990; monkey, McCrea et al., 1987). There has been some debate though on the innervation of the contralateral oculomotor nucleus by the inhibitory vestibular nucleus neurons, which in turn are innervated by floccular Purkinje cells; in contrast to experiments in the cat, this connection has not been found in the rabbit (Ito et al., 1977; Sato et al., 1988). These results seem in line with the fact that ipsilateral flocculus stimulation elicits only ipsilateral eye movement in rabbits but movements of both eyes in cats (Sato et al., 1984; Dufosse et al., 1977). Flocculus receiving neurons in the superior vestibular nucleus receive vestibular primary afferents from the anterior canal (Ito et al., 1973; Ito, 1982). These findings are consistent with the fact that floccular zones 1 and 3, which are involved in the vertical compensatory eye movements (i.e. eye movements dominated by the vertical recti and oblique muscles), project to the superior vestibular nucleus (see also Van der Steen et al., 1994). The excitatory second-order flocculus receiving neurons in the superior vestibular nucleus

innervate the contralateral oculomotor nucleus, which leads to excitatory input to the ipsilateral superior ocular rectus muscles and the contralateral inferior ocular oblique muscles. The inhibitory flocculus-receiving neurons innervate the ipsilateral trochlear and oculomotor nuclei, which leads to suppression of the contralateral superior oblique muscles and ipsilateral inferior rectus muscle (Highstein, 1973; Ito et al., 1976; Yamamoto et al., 1978; Highstein and Reisine, 1979; Sato and Kawasaki, 1990).

Turning to the closed floccular pathway, the ventral dentate nucleus and dorsal group y provide a GABAergic input to the rostral dorsal cap and ventrolateral outgrowth of the inferior olive (De Zeeuw et al., 1994a), which in turn give rise to climbing fibers innervating Purkinje

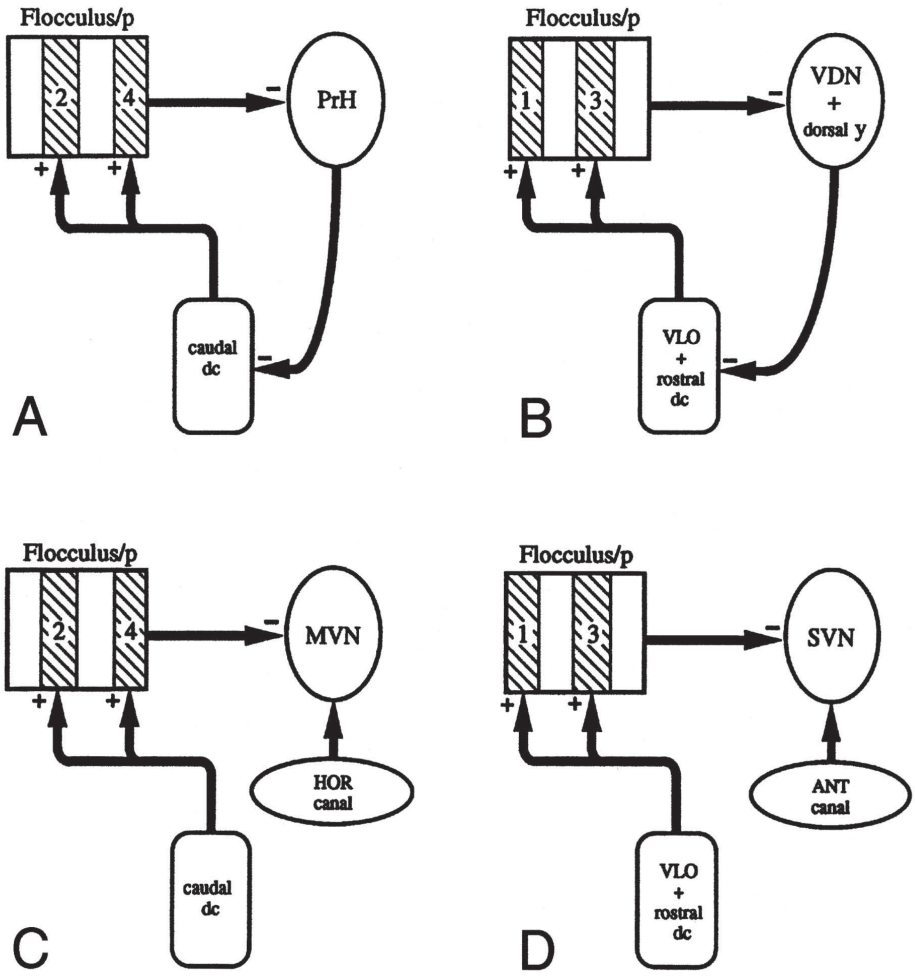

Figure 2: Summary of closed (A and B) and open (C and D) loops between the flocculus including folium p (p), the cerebellar and vestibular nuclei, and corresponding olivary subnuclei involved in horizontal (A and C) and vertical (B and D) compensatory eye movements (HCEM and VCEM) (from De Zeeuw et al. 1994a). It should be noted that individual Purkinje cell axons can collateralize into two branches one of which is involved in the open loop and another one that is involved in the closed loop system. PrH, dc, VLO, VDN, dorsal y, MVN, SVN, HOR canal, and ANT canal indicate nucleus prepositus hypoglossi, dorsal cap, ventrolateral outgrowth, ventral dentate nucleus, dorsal group y, medial vestibular nucleus, superior vestibular nucleus, horizontal canal, and anterior canal, respectively.

cells in zones 1 and 3. These Purkinje cells innervate the ventral dentate nucleus and dorsal group y and hence close the loop. The nucleus prepositus hypoglossi provides the major GABAergic input to the caudal dorsal cap (De Zeeuw et al. 1993), which projects to zones 2 and 4 of the flocculus and the adjacent folium p (Ruigrok et al., 1992; Tan et al., 1995). Folium p in turn innervates the nucleus prepositus hypoglossi (Yamamoto 1979). Therefore, the ventral dentate nucleus and dorsal y are part of the closed olivofloccular pathway mainly involved in the vertical compensatory eye movements, and the nucleus prepositus hypoglossi is part of an analogous pathway mainly involved in horizontal compensatory eye movements (see also Blanks and Bok, 1977; Chubb and Fuchs, 1982; McCrea and Baker, 1985; Delgado-Garcia et al., 1989). Taken together, we can conclude that in both the open and closed floccular pathways, the nuclei involved in horizontal and vertical eye movements are segregated from each other.

2.4 Gain control

2.4.1 Effects of floccular lesions on gain of compensatory eye movements

The possible roles of the flocculus in gain control have been extensively studied in multiple species including rabbits, primates, and cat. Ito and colleagues initially proposed that the flocculus augments OKR gain and mediates enhancement of VOR gain by vision (Ito et al., 1974; Ito et al., 1982; Ito, 1984). In line with this suggestion were results of numerous lesion studies; ablation of the flocculus significantly decreases OKR gain, but VOR gain to a lesser extent (Takemori, 1975; Robinson, 1976; Zee et al., 1981; Ito, 1982; Nagao, 1983; Waespe et al., 1983; Lisberger et al., 1984; Nagao, 1989a). Unilateral lesion of the flocculus in rabbits did not change VOR gain values (Barmack and Pettorossi, 1985). VOR gain values are increased in pcd and lurcher mice, both of which mutants suffer from degeneration of cerebellar Purkinje cells (Killian and Baker, 2002; Van Alphen et al., 2002). Floccular lesions not only decrease compensatory eye movements, but also induce a spontaneous conjugate nystagmus characterized by contralaterally directed slow phases when the animal is placed dark (Nagao, 1983; for unilateral lesions see Barmack and Pettorossi, 1985). Bilateral injections of GABAergic agonist muscimol or baclofen in the flocculus cause a functional ablation and induce the same behavioural gain changes (Van Neerven et al., 1989). This demonstrates that when the flocculus is chemically or mechanically lesioned, gain values of compensatory eye movements are severely affected.

2.4.2 Effects of floccular afferent ablation on gain of compensatory eye movements

A unilateral lesion of the inferior olive evokes in the rabbit: an immediate, spontaneous conjugate drift of the eyes to the side contralateral to the lesion; and a reduction of the OKR gain of the contralateral eye during optokinetic stimulation to the side ipsilateral to the lesion (Barmack and Simpson, 1980). Unilateral, electric lesions of the inferior olive did not result in a decrease in VOR gain, but in a velocity bias to the contralateral side at low stimulus frequencies (Barmack and Simpson, 1980). In contrast, Demer and Robinson (1982) reversibly lesioned the unilateral olive in cats by infusion of lidocaine, which temporarily increased the VOR gain. They proposed a model in which the flocculus or vestibulocerebellum is seen as a multiplication site for the climbing fiber and mossy fiber inputs. When either of the excitatory inputs to the flocculus is zero (e.g., following an inferior olive lesion), the floccular output is zero, following which the gain value will rise towards the internal gain value, which they hypothesized to be ~2. This model

would thus account for an increase in the VOR gain following cerebellar afferent lesions. In order to test the validity of this model regarding the mossy fiber input, either numerous precerebellar nuclei have to be lesioned, or neurotransmission from mossy fibers to granule cells have to be blocked. To date no study was able to lesion all mossy fiber input to the cerebellar cortex, which makes it hard to test the validity of the model proposed by Demer and Robinson (1982). Various studies only provide weak indications on the models validity. Unilateral lesion of the nucleus reticularis tegementis pontis reduce the OKR gain values in rabbits and rats, and did not decrease the VOR gain values, except when lesioned rats were rotated with continuous velocity in the dark (Miyashita et al., 1980; Hess et al., 1989). In addition, infusion of substances into the cerebellum that attenuate or even block neurotransmission from mossy fibers to granule cells was performed by Tan and colleagues. The inhibition of one sort of neurotransmitter does not lesion all mossy fiber input to the cerebellar cortex, since various substances act as neurotransmitter for this input. In order to investigate the function of the cholinergic input to the flocculus of the rabbit various substances were injected intrafloccularly (for a review see Van der Steen and Tan, 1997). Their studies on the effect of cholinergic agonist and antagonists suggest that acetylcholine may play a modulatory role in the rabbit eye movement circuitry. Injection of carbachol, a non-specific choline agonist that binds both muscarinic and nicotinic receptors strongly enhanced the OKR and moderately enhanced the VOR (0.46 and 0.14 gain increase, respectively). In addition, application of specific muscarinic agonist or antagonist also resulted in a significant increase or decrease of OKR gain values, respectively. Specific nicotinic agents had no effect (Tan, 1992). In conclusion, these results suggest a role of the muscarinic cholinergic system in signal processing in the flocculus. In contrast to cholinergic agents, serotonergic and noradrenergic agonists and antagonists do not have a strong effect on the compensatory eye movement performance of rabbits (van Neerven et al., 1990; Tan and Collewijn, 1991).

2.4.3 Relations between gain of compensatory eye movements and Purkinje cell activity in the flocculus

The relations between the complex spike and simple spike activities of Purkinje cells in the flocculus and the control of short term dynamics and long term adjustments of compensatory eye movements have been subject of intense research over the past decades. Moreover, these relations still evokes intense debates in the field of vestibular research (Lisberger, 1998). The possible causes and consequences of climbing fiber activity are briefly discussed above and extensively reviewed by Simpson et al. (1996). Relations between the simple spike activity and the compensatory eye movements are at least as controversial. As mentioned above, simple spike firing patterns of floccular Purkinje cells probably reflect a variety of feed-forward signals that converge onto these neurons, such as vestibular, retinal slip, eye position, eye velocity, vergence, and accommodation (Miles et al., 1980; Ito, 1984; Shidara et al., 1993; De Zeeuw et al., 1995b). In addition, other proprioceptive signals are feed back to the flocculus (Ito, 1984). Although it is difficult, if not impossible, to measure the exact contribution of each of these individual signal components to a particular eye movement response, one can still describe a general trend in the relation between gain and simple spike response. In the rabbit, the modulation amplitude (amplitude of simple spikes modulation / mean discharge rate x 100%) generally increases as the OKR and VOR gain increases (Miles et al., 1980; Ito, 1984; Nagao, 1989a, b; Shidara et al., 1993; De Zeeuw et al., 1995a). In one study, 180° out-of-phase sinusoidal visual and vestibular stimulation for one hour (0.1 Hz, 5° drum and 5° turntable) increased the VOR gain by 0.16 on average, while the modulation amplitude increased by 4.7% (Nagao 1989a,b). Similarly, the modulation

has been shown to be higher during VVOR than during VOR stimulation, paralleling the higher gain values found during VVOR stimulation (De Zeeuw et al., 1995a). Although it is difficult to determine if the change in simple spike activity is causal to or the consequence of changed eye movements, Van der Steen and colleagues found an indication that the former is most likely to be true. They injected acetylcholine iontophoretically into the flocculus during continuous sinusoidal optokinetic stimulation and found that the modulation amplitude, nut not the baseline firing frequency increased, paralleling the increase in OKR gain values (Van der Steen and Tan, 1997). These results are in line with what was found by Tan et al. (1992) who indicated that the injection of another cholinergic agent (carbachol) resulted in increased simple spike modulation amplitude. Moreover, Van der Steen et al. (1994) showed that electrical stimulation of the white matter containing Purkinje cell axons from the vertical and horizontal axes zones in the flocculus result in temporal and temporal/downward eye movement. Together these experiments show that stronger simple spike modulation might cause a gain increase of compensatory eye movements. For example, an increase in simple spike activity in the VA zone of the Purkinje cells during contralateral head movements is expected to exert an inhibitory effect on both the excitatory and inhibitory flocculus-receiving neurons in the medial vestibular nucleus, disinhibiting the ipsilateral medial rectus, and de-exciting the ipsilateral lateral rectus motoneurons, respectively (see chapter 2.2).

The notion that an increase in simple spike modulation correlates with an increase in compensatory eye movement gain values raises the question of how induction of LTD of the parallel fiber – Purkinje cell synapses could possibly lead to a gain increase (Ito, 1982, 1998). The flocculus hypothesis predicts that LTD occurs specifically in in-phase cells (Purkinje cells responding to ipsilateral head movements) during a gain-enhancing visuovestibular training paradigm, and that, thereby, the out-of-phase cells (Purkinje cells responding to contralateral head movements and thereby enhancing VOR gain) come to determine flocculus output (Ito, 1982, 1998). However, recent recordings in the rabbit and the mouse showed that there are hardly any in-phase cells to be found in the untrained state, making it unlikely that an increase in gain could be mediated by a decrease in the in-phase activity (De Zeeuw et al., 1995a; Hoebeek and De Zeeuw, unpublished observation). Moreover, recordings of Purkinje cell activity during VOR adaptation in monkeys showed that changes in the vestibular sensitivity of vertical axis Purkinje cells (that is, horizontal gaze velocity cells; see also below) are in the opposite direction from that predicted by the classical LTD-hypothesis of Ito (1998) (Lisberger et al., 1994; Raymond and Lisberger, 1998). The same conclusion can be drawn from experiments done by Hirata et al. (1998), which showed that changes in the transfer function between flocculus and eye do not correlate consistently with a change in VOR gain. Since it has been shown that parallel fiber – Purkinje cell LTD plays a role in long-term adaptive changes of compensatory eye movements (De Zeeuw et al., 1998; Van Alphen and De Zeeuw, 2002), a different mechanism than that proposed by Ito (1982; 1998) must mediate these adaptive changes.

2.5 Phase control

2.5.1 Effects of floccular lesions on the phase dynamics of compensatory eye movements

The role of the flocculus in control of the phase of compensatory eye movements is not as extensively documented as that of the gain. In general, eye movements in flocculectomized animals lag those of normal animals during OKR and to a lesser degree during VOR (Ito et al., 1982; Nagao, 1983). It must be said that OKR and VOR gain changes that can be induced by application of cho-

linergic antagonists and agonists are more prominent than the associated phase shifts (Frens and van der Steen, unpublished observations). In pigmented rabbits, bilateral flocculectomy produces a significant phase lag during OKR at a broad range of frequencies and during VOR in the dark at the higher frequencies (Nagao, 1983, 1989b). In the cat, flocculectomy decreases the phase lead by 7° during VOR at 0.05 Hz, which is non-significant (Robinson, 1976). Contradictory results have also been published; Keller and Precht (1979) found an increase in phase lead following flocculectomy. During VVOR at lower frequencies (0.01-0.5Hz), flocculectomy in pigmented rabbits has been reported to induce a phase lag varying from 8° to 15° (Kimura et al., 1991), but also no change in VVOR phase values (Keller and Precht, 1979). In addition, a bilateral lesion of the flocculus by kainic acid can abolish the ability to increase VOR phase lead by exposure to optokinetic stimulation in phase with vestibular stimulation but at twice the amplitude (Nagao, 1983). In monkeys, lesions of the ventral paraflocculus and the flocculus do not produce consistent effects on phase values (Zee et al., 1980; Zee et al., 1981). This difference may be due to the fact that a large portion of the primate's floccular Purkinje cells encode gaze velocity (Miles et al., 1980), which contrasts with the rabbit, whose flocculus is dominated by Purkinje cells encoding eye position, eye velocity, and retinal slip (Leonard, 1986). In mice, flocculectomy induces considerable phase lag during OKR (Koekkoek et al., 1997). The optokinetic responses of the lurcher mice generally also lagged the stimulus movement more than those of control mice, while their phase lead during VOR was significantly increased at the lower frequencies compared to control animals (Stahl, 2002; Van Alphen et al., 2002). The phase values of lurcher and wild type mice during VVOR can be roughly predicted by the addition of their OKR and VOR performance (that is, phase is dominated by OKR at lower frequencies and by VOR at the higher frequencies) (Stahl, 2002; Van Alphen et al., 2002). Similar effects on phase values of the OKR and VOR can be observed in weaver mice, which lack cerebellar granule cells (Grusser-Cornehls and Bohm, 1988; Koekkoek and De Zeeuw, unpublished observation). A phase lag in the OKR can also be induced by a lesion of the visual mossy fiber pathway to the flocculus; Hess et al. (1989) showed that unilateral lesions of the nucleus reticularis tegmentum pontis in rats not only decrease of OKR gain but also produce an OKR phase lag. This result implies that visual inputs to the flocculus normally advance the phase of command signals underlying the OKR. In general, the effects of a mechanical or genetic flocculectomy on the phase of eye movements in rabbits, cats, and mice suggest that the flocculus advances the phase of the net preoculomotor signal for the OKR and stabilizes that of the VOR. The data obtained from mouse mutants suggest that the phase regulation role of the flocculus is implemented in the circuitry of the oculomotor system, rather than by molecular factors that do not have a prominent secondary effect on the wiring.

2.5.2 Phase relations of activity patterns of floccular Purkinje cells and flocculus-receiving neurons in the vestibular nuclei

If, as described above, eye movements in flocculectomized animals lag those of normal animals, one would expect that in normal animals the phase of the neurons in vestibular nuclei that project to the oculomotor nucleus and receive input from the flocculus lead the neurons that also project to the oculomotor nucleus but do not receive floccular input. Stahl and Simpson (1995) proved this to be true by demonstrating that in the awake pigmented rabbit, flocculus receiving neurons in the medial vestibular nucleus have a phase lead with respect to eye movement that is significantly greater than that of those neurons that do not receive floccular input. This phase difference is present for vertical axis OKR, VOR and VVOR at a wide range of frequencies (0.05 – 0.8 Hz). This finding indicates that the phase lead of floccular receiving neurons could be due to a phase

lead of the floccular Purkinje cells, which was proven to be the case by (De Zeeuw et al., 1995a); vertical axis Purkinje cells lead the flocculus-receiving neurons in the medial vestibular nucleus at all frequencies tested (0.05 – 0.8 Hz) during VOR and VVOR. Similar phase relations may be present in mice (Grusser-Cornehls, 1995; Grusser-Cornehls et al., 1995). Although no direct comparisons were made between flocculus-receiving and non-flocculus-receiving neurons, and no eye movement data was collected, Grusser-Cornehls and colleagues were able to show that the phase of simple spike activity of floccular Purkinje cells leads that of vestibular nuclei neurons. Moreover, they showed that the phase values of the floccular Purkinje cells and vestibular nuclei neurons in weaver mutant mice vary more widely in wild type littermates, indicating that a proper organization of the granule cell layer is necessary to establish stable phase characteristics of the neuronal signals (Grusser-Cornehls, 1995). Whether the same Purkinje cell – vestibular nucleus neuron phase relations hold true for the monkeys is unclear. The primate Purkinje cells comparable to those of the rabbit and the mice are probably the "eye movement only" group, which make up approximately a quarter of the recorded population in the monkey (Miles et al., 1980). This cell type has been investigated less than the the gaze-velocity Purkinje cells, and it is unknown whether these "eye movement only" Purkinje cells lead their medial vestibular targets (Miles et al., 1980; Lisberger et al., 1994).

Stahl and Simpson (1995) showed that the phase-leading property of the floccular signal indicates a role of the flocculus in compensating for excessive intergration by the brain stem neural circuitry. In addition, these authors proposed that if the brain stem integrator network has limitations in the precision of the phase lag that it produces, a separate structure may be required for fine-tuning. The flocculus could perform this role by taking the over-integrated premotor signal from the brain stem, emphasizing the component in-phase with eye velocity relative to the component in-phase with eye position, and then injecting it into the vestibular nuclei (Stahl and Simpson, 1995). As such, during sinusoidal stimulation, the flocculus creates a signal that leads the original input signal, e.g. putting emphasis on the velocity in stead of the position means differentiation and thus creation of phase lead. This scenario predicts the exsistence of a cell group producing an over-integrated signal whose phase lies between the motorneurons and eye position. The weighted sum of the flocculus receiving neurons, the non-flocculus receiving neurons, and the phase-lagged premotor cell group would be in phase with the abducens neurons. The proposed lagging cell group could be located in the prepositus hypoglossi, which projects to the flocculus, the oculomotor nucleus and the abducencs nucleus (for a review see McCrea et al., 1979; Barmack et al., 1992a; Barmack et al., 1992b) and which contains some cells with a phase close to eye position (cat, Lopez-Barneo et al., 1982; Escudero et al., 1992; monkey, McFarland and Fuchs, 1992).

The idea that integration can become excessive has a long precedent. Skavenski and Robinson (1973) proposed that at high stimulus frequencies the integrator would produce an excessive phase lag due to the presence of additional lag engendered by the orbital mechanics of the oculomotor plant. They hypothesized the need for a bypass around the neural integrator, via the medial longitudinal fasciculus (Skavenski and Robinson, 1973). Another compensatory mechanism is provided by the phase leading effect of the flocculus on the flocculus receiving neurons in the medial vestibular nucleus, which tune down the net phase lag in the output of the vestibular nuclei (Stahl and Simpson, 1995; De Zeeuw et al., 1995a).

The phase lag of the compensatory eye movements in flocculectomized animals is particularly prominent during OKR, even more so than during VOR, and consequently, one would expect that the flocculus-receiving neurons would have a stronger visual signal than the non-flocculus receiving neurons in the medial vestibular nucleus (Stahl and Simpson, 1995). The difference between the phase during the VOR and the VVOR is greater for the flocculus receiving

neurons than for the non-flocculus receiving neurons. This difference proved to be attributable to the contribution of the vertical axis Purkinje cells, since they showed an even larger difference between the VOR and VVOR phase lead values (De Zeeuw et al., 1995a). Thus, the visual signals of the floccular Purkinje cells not only evoke a prominent effect in gain control but also in phase control of compensatory eye movements.

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Chapter 3

Increased noise level of Purkinje cell activities minimizes impact of their modulation during sensorimotor control

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3.1 Abstract

While firing rate is well established as a relevant parameter for encoding information exchanged between neurons, the significance of other parameters is more conjectural. Here, we show that regularity of neuronal spike activities can have an important effect on sensorimotor processing by investigating cerebellar function in *tottering* mutants, which suffer from a mutation in P/Q-type voltage gated calcium channels. While the modulation amplitude of the simple spike firing rate of their floccular Purkinje cells during optokinetic stimulation is indistinguishable from that of wild types, the regularity of their firing is markedly disrupted. This irregularity is so robust that the normal influence of the cerebellar cortex on eye movements cannot be detected; the gain and phase values of *tottering's* compensatory eye movements are indistinguishable from those of wild types in which the flocculus is ablated. In addition, the abnormal gain observed in *tottering* can be largely mimicked in wild types by applying blockers of P/Q-type channels to the flocculus. Moreover, normal eye movements can be evoked in *tottering* when the flocculus is electrically stimulated with regular spike trains mimicking the firing pattern of normal simple spikes. To our knowledge, the present study provides the first demonstration of the importance of regularity of firing in Purkinje cells, and one of the most dramatic demonstrations to date of the relevance of this parameter in neuronal information processing.

3.2 Introduction

Modulation of firing rate is typically the only parameter of neural activity that is considered when assessing the influence of that activity on sensorimotor behaviour. Modulation - behaviour relationships have been demonstrated in many experimental contexts, including lower and higher, sensory and motor systems (Frazor et al., 2004; Li et al., 1999). Theoreticians have pointed out the impact of noise levels (i.e. regularity of firing) in neuronal network models (Mar et al., 1999; Steinmetz et al., 2001; Tiesinga et al., 2002), but it remains to be demonstrated at the experimental level whether a change in noise without a change in spike modulation can alter sensorimotor behaviour. We looked for such a dissociation between neuronal firing rate modulation and neuronal noise in the context of calcium channelopathies, because various mutations in voltage-gated calcium channels result in both cell physiological and behavioural aberrations (Cao et al., 2004; Llinas et al., 1989; Mintz et al., 1992; Ophoff et al., 1996; Qian and Noebels, 2000; Stahl, 2002). We focused on the *tottering* mutant (*tg*), which suffers from a point-mutation in CACNA1A, the gene encoding the $\alpha 1_a$-subunit of the P- and Q-type voltage-gated calcium channel (Bourinet et al., 1999; Fletcher et al., 1996). The mutation affects the extracellular membrane domain of the pore forming loop. The cerebellar Purkinje cells of these mutants show a complex combination of cellular abnormalities including a reduction in Ca^{2+}-channel current density (Wakamori et al., 1998), a reduction in the amplitude of the parallel fibre – Purkinje cell EPSC (Matsushita et al., 2002), and an increased susceptibility to inhibitory modulation by GABAergic interneurons (Zhou et al., 2003). The general importance of these deficits for motor behaviour is suggested by the fact that mutations in the $\alpha 1_a$-subunit of the P- and Q-type channels are associated with motor coordination problems in both mice (Campbell et al., 1999; Fletcher et al., 1996; Green and Sidman, 1962; Stahl, 2004) and humans (Ducros et al., 1999; Ophoff et al., 1996; Zuchenko et al, 1997). We therefore set out experiments in *tg* mutants and controls to systematically investigate the relationship between motor performance and the modulation and regularity of Purkinje cell simple and complex spike firing rates. The flocculus of the vestibulocerebellum, which controls compensatory eye movements, was used as a model system to determine these correlations quan-

titatively. In this system relationships between sensory input, motor output, and intermediate Purkinje cell activities can be rigorously defined (De Zeeuw et al., 1995; Goossens et al., 2004; Stahl and Simpson, 1995ab; Simpson et al., 1996).

3.3 Experimental Procedures

3.3.1 Animal preparation

Data were collected from 54 *tg* mice and 54 wild type littermates (C57BL/6J background; Jackson laboratory, Bar Harbor, ME, USA), which were prepared for chronic experiments (Goossens et al., 2001; van Alphen et al., 2001). All preparations were done with approval of the European Communities Council Directive (86/609/EEC).

3.3.2 Optokinetic and vestibular Stimulation

The OKR was assessed by rotating a planetarium sinusoidally about both vertical and horizontal axes at various frequencies (0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 Hz) and a fixed peak velocity (8˚/sec) (Stahl et al., 2000; van Alphen et al., 2001). The (V)VOR was assessed by rotating the animal sinusoidally around the vertical axis at various frequencies (0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 Hz) and fixed amplitude (10˚) in the dark or light. Stimuli were controlled and monitored by a 1401plus unit (CED, Cambridge, UK).

3.3.3 Eye movement recordings

The position of the left eye was measured using a video method operating at 240 samples/sec (ETL-200, ISCAN, Burlington, MA, USA) (Stahl, 2004; Stahl et al., 2000; van Alphen et al., 2001). For baseline compensatory eye movements (see Figure 1) the eye position signal was resampled at 500 Hz (1401plus unit, CED). Data were stored for off-line analysis using Spike 2 and Matlab (Mathworks Inc. Natick, MA, USA).

3.3.4 Single cell recordings

Extracellular Purkinje cell activities were recorded from either the flocculus or non-floccular regions in the hemisphere of the left cerebellar cortex (Goossens et al., 2004). The cells were recorded either during spontaneous activity in the light or during optokinetic stimulation (see above). Signals were amplified, filtered, digitized and stored for off-line analysis. Purkinje cells were identified by their brief pause in simple spike activity following each complex spike. Once a floccular Purkinje cell was isolated, the preferred axis of rotation was determined by rotating the planetarium around the vertical axis or a horizontal axis at 135˚ azimuth, ipsilateral to the side of recording. In a limited number of cells, a wider range of axes was tested to allow for the construction of a spatial tuning curve.

3.3.5 Floccular lesions and histology

After locating the left flocculus by recording its typical complex spike response to optokinetic stimulation, we lesioned the flocculus by suction (n = 6 *tg* and 3 wild type animals). Eye movement recordings were done for two days pre-lesion and averaged and subsequently compared to data collected on day three post-lesion. To check the extent of the lesions, the animals were anaesthetised and perfused, and subsequently Nissl and silver stainings were prepared from the cerebellum and brainstem (Jaarsma et al., 1992; Nadler and Evenson, 1983).

3.3.6 Electrical stimulation

Custom-made urethane-insulated tungsten electrodes were used to electrically stimulate the left floccular peduncle. Regular trains of pulses (200 ms, 400 ms, 600 ms, 800 ms, 1000 ms and 1500 ms trains; 80 μs pulse duration; 100 Hz pulse frequency with various stimulation intensities) were used to evoke eye movements in the ipsilateral eye. Each paradigm was applied to at least 4 wild types and 4 *tg* mutants. Averages of more than 10 stimulus responses per paradigm were used to analyze the latencies and waveforms of the evoked eye movements. Latencies were calculated using averages of the short stimulus paradigms using linear regression analysis (Van der Steen et al., 1994). To quantify eye movement waveforms, we averaged responses to 600 ms, 1000 ms and 1500 ms pulse trains, normalized them, and fitted them iteratively by a single exponential function of the form $y = 1 - e^{(-t/\tau)}$. The resulting time constants were tested for significance.

3.3.7 Injections of ω-Agatoxin IVA

The border of the left flocculus was identified using the electrophysiogical recordings described above. Subsequently, the recording electrode was replaced by a borosilicate glass electrode filled with 100 nM ω-Agatoxin IVA (diluted in 0.9% saline; Alomone Labs, Jerusalem, Israel) (Knight et al., 2003). Approximately 10 μl of the ω-Agatoxin IVA solution was injected by pressure at multiple sites evenly distributed over the entire flocculus. Compensatory eye movements were measured before the localization of the flocculus and three days after the injection of the blocker in 6 wild type mice. For control, we injected the vehicle (saline) in 6 wild types and the ω-Agatoxin IVA solution in 6 *tg* mice. Juxtacellular recordings of Purkinje cell activities were made with the use of multiple barrel electrodes following iontophoretic injections of ω-Agatoxin IVA solution as described by Shields et al. (2004).

3.3.8 Data analysis

Off-line analysis of eye movements and neuronal firing rates was performed in MATLAB (Mathworks) (Goossens et al., 2004). Gain and phase values were determined by fitting sine functions to the slow-phase eye velocity traces. Simple spikes and complex spikes were discriminated using custom-made routines based on cluster analysis (Goossens et al., 2004). Simple spike PSTH's (100 bins per cycle) were compiled at each stimulus frequency and fit by a sine function. Neuronal amplitude of modulation was calculated by dividing the amplitude of the fitted sine wave by its offset. The phase of the simple spike activity relative to the eye velocity (θ) was calculated from the difference of the phase of the sinusoidal fits to firing rate and eye velocity (De Zeeuw et

al., 1995; Stahl and Simpson, 1995b). CV values of spontaneous spike activities were calculated by dividing the standard deviations of the interspike interval lengths by their means. Autocorrelograms of spiking data were constructed using custom made routines in Matlab. A fixed bin size of 50 ms and a 7th order low pass filter were used to calculate the correlogram coefficient of the spiking data (ratio of the amplitude of the first off-centre peak in the filtered data and the amplitude of the centre peak of the raw data). Autocorrelograms of interspike intervals were created by correlating the duration of each interval with the duration of the neighbours. While the CV values and autocorrelations were used to determine the regularity of the interspike interval, the trial-to-trial variability was determined by averaging of the standard deviation of the mean difference between the number of spikes in the on-phase and that in the off-phase over the cycles. With respect to the relation between firing rate and saccadic eye movements, epochs containing quick phases were analysed separately. Saccades were detected with a velocity threshold of 25°/s (for details see Van der Steen & Bruno, 1995). More than 40 saccades per animal were used to calculate the main sequence parameters. Data are presented as mean ± SEM.

3.4 Results

3.4.1 Mutation in P/Q-type calcium channel leads to abnormal sensorimotor behaviour

The *tg* mutants under investigation showed the general ataxic behaviour during locomotion as

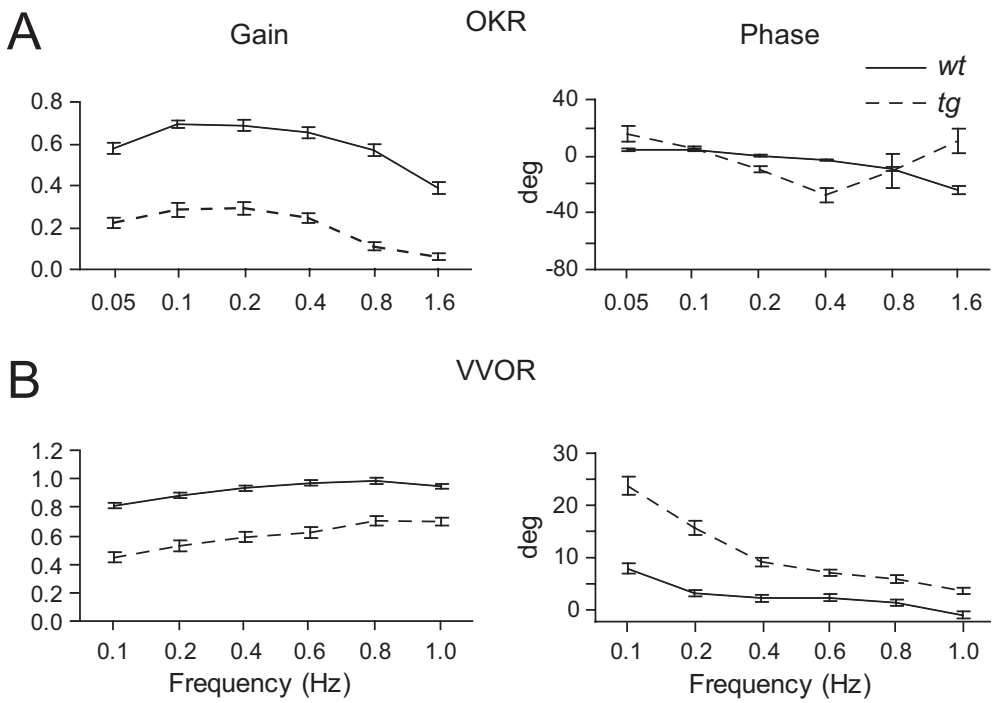

Figure 1. Compensatory eye movements in the presence of vision are impaired in *tottering* (*tg*) mice. Gain values (left panels) of *tg* mutants are decreased during the optokinetic reflex (OKR) (A) and vestibulo-ocular reflex in the light (VVOR) (B). Phase curves (right column) of the *tg* mutants only show significant changes during VVOR. Solid lines and dashed lines indicate data of wild types (*wt*; n = 13) and mutants (*tg*; n = 9), respectively.

described previously (eg. Campbell et al., 1999). To quantify motor dysfunction, we determined gain and phase of eye movements during sinusoidal optokinetic and vestibular stimulation at frequencies varying from 0.05 Hz to 1.6 Hz (Figure 1). We studied 9 *tg* mutants and 13 wild type littermates. During the optokinetic reflex (OKR) the gain values of *tg* mice ranged from 0.06 to 0.29 over the tested frequency band, while those in wild types ranged from 0.39 to 0.69; these values were significantly different ($p < 0.001$; repeated measures ANOVA) (Figure 1A). During vestibular stimulation in the light (VVOR) the gain values ranged from 0.44 to 0.70 in *tg* mice and from 0.81 to 0.99 in wild types; these gain vs. frequency relationships also differed significantly ($p < 0.001$; repeated measures ANOVA) (Figure 1B). During the vestibulo-ocular reflex in the dark (VOR) the gain values of *tg* mutants were hardly different from those in wild type littermates. The phase vs. frequency curves of *tg* mutants differed significantly from those in wild types during VVOR ($p < 0.001$; repeated measures ANOVA), but not during OKR ($p = 0.5$; repeated measures ANOVA). These data indicate that compensatory eye movements driven in part or in totality by vision (i.e. VVOR and OKR) are markedly abnormal in *tg* mutants.

3.4.2 Purkinje cells with abnormal P/Q-type channels fire irregularly but modulate normally

To find out whether the motor deficits in *tg* mice can be caused by abnormal firing of their Purkinje cells, we recorded Purkinje cell simple spike and complex spike activity at rest (i.e. spontaneous activity) and during optokinetic stimulation. Extracellular recordings of Purkinje cells in alert *tg* mutants ($n = 86$ from 22 animals) and wild type mice ($n = 79$ from 14 animals) during spontaneous activity in the light did not reveal gross anomalies in amplitude, shape or duration of their simple spikes or complex spikes. However, the firing pattern of the simple spikes was much more irregular in *tg* mutants than in wild types (Figure 2A). This difference held equally true for all cerebellar regions from which we recorded, i.e. the flocculus as well as extra-floccular regions including crus I and II, paramedian lobule, and paraflocculus. The coefficient of variance (CV; for details see experimental procedures) of the spontaneous simple spike activities in the flocculus of *tg* mice (2.57 ± 0.25) did not differ ($p = 0.4$; *t*-test) from that in the extra-floccular regions (2.18 ± 0.19) indicating that the abnormal firing pattern was a general phenomenon. After pooling Purkinje cells from all regions, the average CV of spontaneous activity in *tg* was almost four times higher than that of wild types (2.25 ± 0.16 vs. 0.63 ± 0.07, $p < 0.001$; *t*-test). The increase in irregularity in *tg* mice was also reflected in a shorter climbing fibre pause (defined by the period between the start of the complex spike and the start of the first simple spike after this event). In *tg* and wild types the pause was 13.6 ± 0.55 ms and 16.4 ± 0.07 ms, respectively ($p < 0.05$; *t*-test). The reduction in pause length cannot be explained by a higher firing frequency of the simple spikes in *tg*, because the average firing frequency was in fact slightly lower than in wild type (52 ± 3 spk/s and 65 ± 4 spk/s, respectively).

In order for the irregularity to account for abnormalities of motor behaviour, the irregularity must be present during movements as well as at rest. We therefore investigated the activities of floccular Purkinje cells in *tg* mutants ($n = 25$ from 10 animals) and wild types ($n = 14$ from 5 animals) that made compensatory eye movements in response to sinusoidal optokinetic stimulation (Figure 2B). During this behaviour as at rest, the simple spikes activities in the *tg* proved to be more irregular, both during the excitation (on-phase) and suppression (off-phase) halves of each stimulus cycle. A raster diagram generated from a typical *tg* and wild type neuron is shown in Figure 2C, and demonstrates qualitatively the greater irregularity of the *tg* neuron. To quantify the irregularity, we calculated the autocorrelation and correlation coefficients of both the binned simple spike counts using a bin size of 50 ms (Figures 2D and E) and of the individual interspike

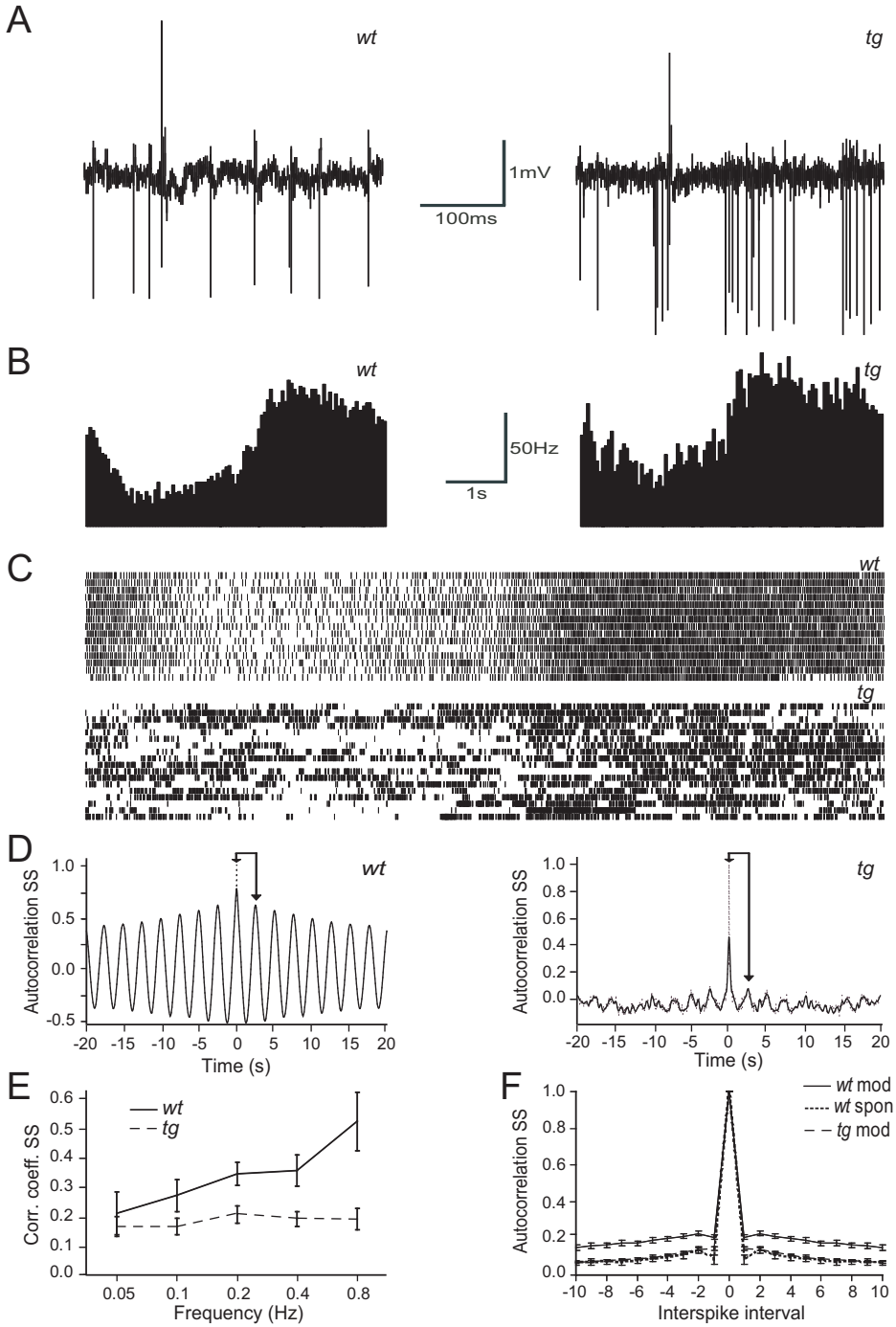

Figure 2. Simple spikes (SS) of Purkinje cells in *tg* mutants modulate normally during optokinetic stimulation, but fire irregularly. (A) Extracellular recordings of spontaneous Purkinje cell activities of a *wt* (left panel) and a *tg* mouse (right panel). Note the increased irregularity of the simple spikes in *tg*. Complex spikes are positive and simple spikes are negative. (B) Per-Stimulus Time Histograms (PSTH) show that the modulation of the simple spike activities of a floccular Purkinje cell during optokinetic stimulation in *tg* is indistinguishable from that in *wt* (for population data see Figure 3A).

(C) Raster plots of the same spikes that are presented in the PSTH's in B demonstrate the qualitatively obvious nature of the greater irregularity of *tg* Purkinje cells. Each small stripe represents a single spike and each row of stripes contains spike data of a complete stimulus cycle. (D) Autocorrelations during modulation show that the predictability of simple spikes in *wt* (left panel) is much better than that in *tg* (right panel; stimulus at 0.4 Hz and 8°/s; bin size 50 ms). Unfiltered traces are indicated in dashed lines and low pass filtered traces are indicated in solid lines. Arrows indicate values used for calculation of correlation coefficients (see methods). (E) Correlation coefficients between the number of spikes in the center 50 ms bin and the number of spikes in the neighbouring bins during optokinetic stimulation in *tg* mice are lower than those of wild types and they are not influenced by stimulus frequency, in contrast to those of wild types. (F) Autocorrelation of simple spike interspike intervals in *wt* during optokinetic modulation (solid line; *wt* mod) is significantly higher than that in *wt* during spontaneous activity (dotted line; *wt* spon) as well as than that in *tg* during modulation (dashed line; *tg* mod).

intervals (Figure 2F). In both calculations, the correlation coefficients were much lower in *tg* than in controls ($p < 0.001$ in both analyses; ANOVA). Moreover, while the correlation coefficient in wild types increased with increasing stimulus frequency, that of the *tg* mutants did not (Figure 2E). In addition, the average autocorrelation of the interspike intervals in wild types during modulation was significantly higher than that during spontaneous activity ($p < 0.001$; ANOVA), while that of the *tg* mutants was not ($p = 0.4$; ANOVA). Apart from the greater irregularity of the interspike intervals, there was a greater variability in the modulation depth from cycle to cycle (trial-to-trial variability). We calculated the standard deviation of the difference between the number of spikes during the on- and off-phase of OKR at all frequencies (i.e. 0.05 Hz, 0.1 Hz, 0.2 Hz, 0.4 Hz and 0.8 Hz all with a peak velocity of 8 deg/s), and compared these standard deviations for *tg* and wild type. The standard deviations were significantly increased in *tg* mutants ($p < 0.001$; non-orthogonal ANOVA), while the mean difference in spikes between the on-phase and off-phase did not differ among *tg* and wild type littermates ($p = 0.7$; non-orthogonal ANOVA). Although modulation depth was more variable in *tg* than in controls, its average value was comparable; sine waves fitted to the simple spike responses to the optokinetic stimulation showed that the average modulation amplitudes did not differ ($p = 0.7$; non-orthogonal ANOVA) (Figure 3A). Together these data demonstrate that Purkinje cells in *tg* mice fire much more irregularly during modulation than those in wild types and that the predictive power of a single interspike interval of a modulating Purkinje cell is larger in wild types than that in *tg* mice.

A factor that could potentially confound the comparison of simple spike regularity in *tg* and wild types would be the presence of modulation related to fast phases, either if *tg* and wild type generated different numbers of fast phases, or if the modulation associated with each fast phase differed. We eliminated both possibilities by compiling saccade - triggered averages of the simple spike data (Figure 3B). These triggered averages showed no significant peaks in firing frequency before or after saccades, neither in wild types ($n = 51$ from 12 animals) nor in *tg* mutants ($n = 55$ from 13 animals) ($p = 0.9$ and $p = 0.2$, respectively; Kolmogorov-Smirnov). Since there was no saccade-associated modulation, differences in the occurrence of saccades or activity associated with each saccade could not have influenced the assessments of regularity during OKR. Taken together, we conclude from our floccular Purkinje cell recordings during optokinetic responses that a change in regularity of simple spike activities but not in modulation amplitude or saccade related activity may explain the abnormal eye movement behaviour of *tg* mutants.

3.4.3 Output of modulating Purkinje cells in vestibulocerebellum of tg mutants is not functional

Even though OKR and VVOR gain values as well as the regularity of simple spike responses during these movements are affected in tg mutants, the modulating Purkinje cells in their floccu-

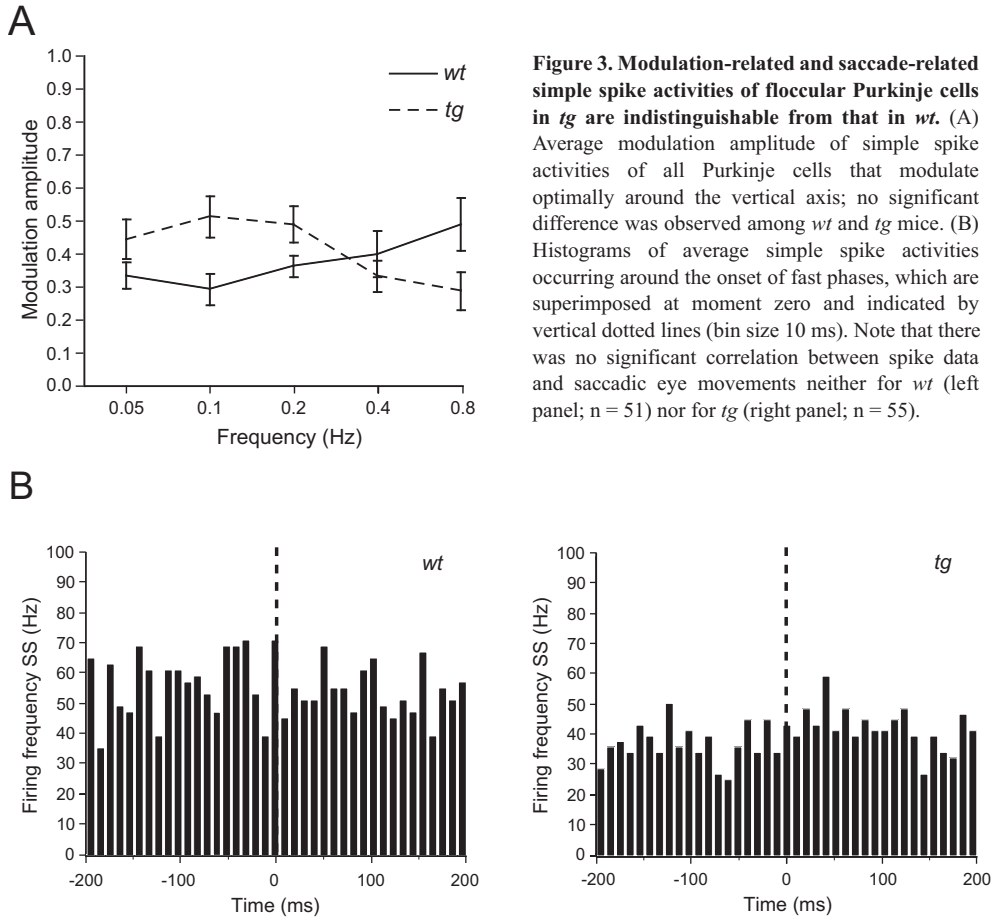

Figure 3. Modulation-related and saccade-related simple spike activities of floccular Purkinje cells in *tg* are indistinguishable from that in *wt*. (A) Average modulation amplitude of simple spike activities of all Purkinje cells that modulate optimally around the vertical axis; no significant difference was observed among *wt* and *tg* mice. (B) Histograms of average simple spike activities occurring around the onset of fast phases, which are superimposed at moment zero and indicated by vertical dotted lines (bin size 10 ms). Note that there was no significant correlation between spike data and saccadic eye movements neither for *wt* (left panel; n = 51) nor for *tg* (right panel; n = 55).

lus may still contribute, although less effectively than in wild types, to increase the gain of their compensatory eye movements (De Zeeuw et al., 2004). To determine the extent to which signal processing in the flocculus of the tg contributes to compensatory eye movements under vision, we ablated the flocculus of *tg* (n = 6) and wild type (n = 3) mice and evaluated the differences between the pre- and post-lesion gain values (Figures 4A and B). In *tg* mutants we did not observe any significant decrease in OKR or VVOR gain values after the lesions ($p = 0.1$ and $p = 0.4$, respectively; ANOVA). In contrast, the gains did decrease significantly in wild types during OKR and VVOR ($p < 0.03$ and $p < 0.05$, respectively; ANOVA) (see also Koekkoek et al., 1997). For both OKR and VVOR, we found that the absolute gain values of the wild types after the lesions did not differ from those of the *tg* mutants before the lesions ($p = 0.3$ and $p = 0.2$, respectively; ANOVA). Both wild type and *tg* VOR gain values did not change after the lesion ($p = 0.1$ and $p = 0.9$, respectively; ANOVA; data not shown). The completeness of the lesions was verified by applying a silver staining to reveal the degenerating fibres in the floccular peduncle (Figures 4C and D) and by reconstructing the core of the lesions in each animal (Figures 4E and F). These data indicate that the output of the flocculus in *tg* mutants does not contribute significantly to the gain of the optokinetic reflex or visually enhanced vestibulo-ocular reflex.

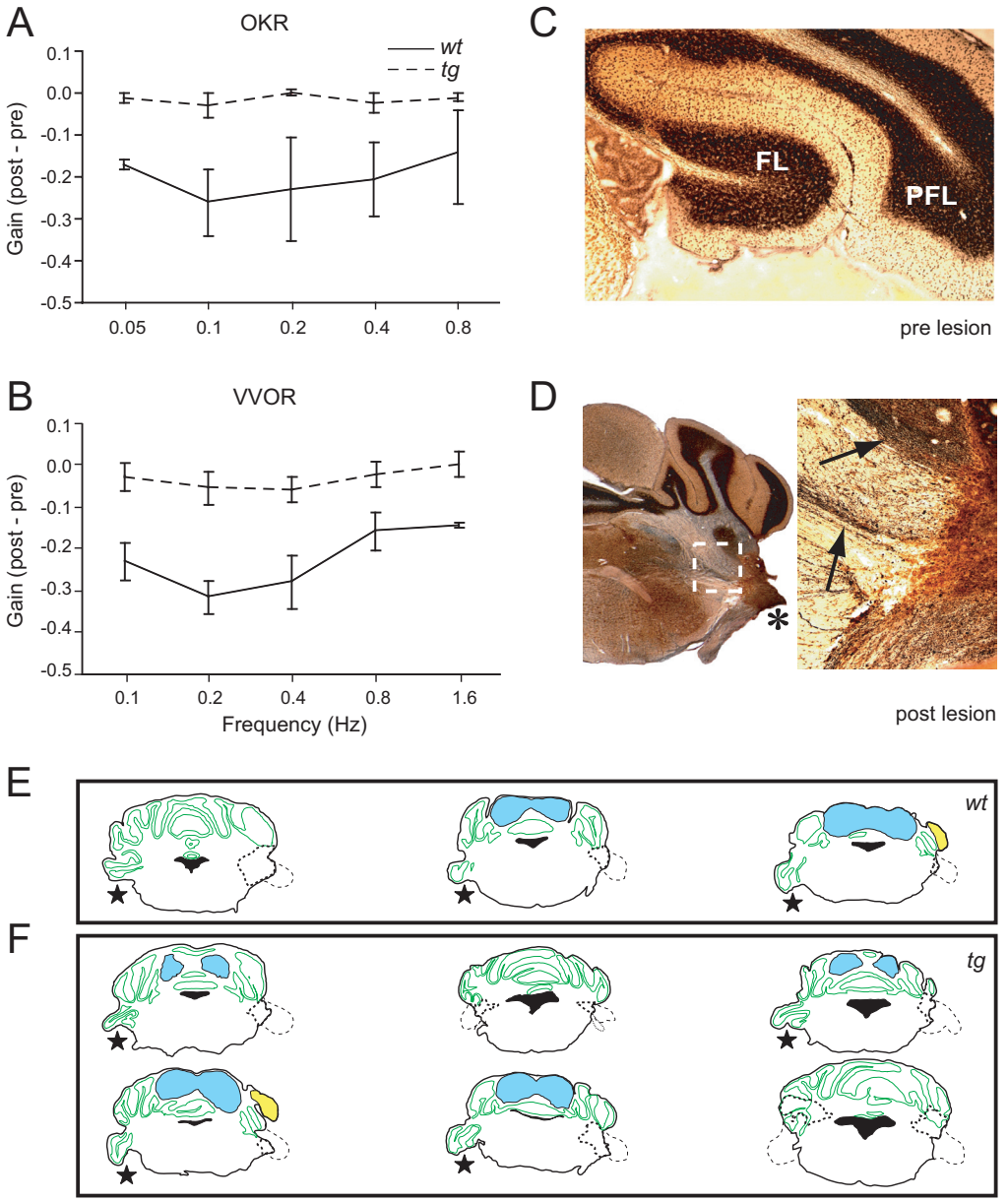

Figure 4. Flocculus of tg mutant does not contribute to compensatory eye movements. (A and B) Gain values of *tg* mutants during OKR (A) and VVOR (B) are not decreased by ablation of their flocculus, whereas those in wild types are. (C and D) A silver-stained section of an intact flocculus and paraflocculus of a *tg* mouse in which no lesion was placed (C) and a silver stained section of degenerated fibers in the floccular peduncle of a *tg* mouse in which the flocculus was ablated (D). Asterisk in D indicates the location of the lesion. Inset in low magnification panel on the left corresponds to high magnification panel on the right. Arrows indicate degenerated fiber bundles originating from paraflocculus (upper arrow) and floccular lobes (lower arrow). (E and F) Reconstructions of core of lesions of floccular complex in 3 *wt* (E) and 6 *tg* mice (F). Dashed lines indicate core of the lesions; dotted lines indicate area with abundant degenerated fibers as visualized by silver staining; dark and light grey indicate colliculi and cerebral cortex, respectively; and stars indicate intact floccular complex on the contralateral side. Black areas indicate the 4th ventricle. Note that two *tg* mice received a bilateral ablation of their flocculus. Drawing of lesion at the middle bottom panel in F corresponds to panels C and D.

3.4.4 Connections downstream of cerebellar cortex are functionally intact

The recording and lesion experiments described above suggest that the floccular output is functionally absent in *tg* due to irregular simple spike activities. However, an alternative or additional explanation could be that the floccular output is blocked downstream, at the synapses between Purkinje cells and their targets, or possibly at more distal locations, such as the neuromuscular junctions with the eye muscles (Plomp et al., 2000). To find out whether such a downstream blockade contributes to the behavioural deficits, we investigated whether saccadic eye movements are impaired in *tg* mutants. Saccades require the highest levels of muscular force and would be expected to be slowed if there was a functionally meaningful defect of neuromuscular transmission (Zee and Leigh, 1983). Saccades manifest an orderly relationship between their duration re amplitude and peak velocity re amplitude (i.e. main sequence), and in mice this relationship is linear over a broad range of amplitudes (Stahl, 2004). Figure 5A shows typical examples of the main sequences for fast phases of optokinetic nystagmus of a *tg* and a wild type mouse. The average slopes of duration re amplitude (1.2 ± 0.2 ms/deg) as well as the offsets of the linear fits (15.3 ± 4.5 ms) in *tg* mice (n = 6) did not differ from those in wild types (n = 5) (0.9 ± 0.2 ms/deg and 19.6 ± 3.3 ms, respectively) (p = 0.2 and p = 0.3; *t*-test). Moreover, the slope (39.6 ± 1.8 rad/s/deg) and offset (7.4 ± 1.4 rad/s) of the peak velocity to amplitude fits in *tg* did not differ either (p = 0.2 and p = 0.9, respectively; *t*-test) from those in wild types (36.7 ± 0.8 rad/s/deg and 7.3 ± 1.6 rad/s, respectively). These data suggest that the presence of abnormal P/Q calcium channels at synapses within the vestibular and ocular motor nuclei, as well as at neuromuscular junctions, does not limit the development of muscular force, at least on the short time scales associated with saccades (Robinson, 1970).

We also tested the integrity of post-floccular pathways by electrical stimulation of the flocculus. The short latencies of the eye movements that could be evoked by electrical stimulation of the floccular vertical axis zone with a 100 Hz pulse train of short duration (200 ms) supported the observation described above. The average latency in *tg* mice (12.1 ± 1.2 ms; n = 7) did not differ (p = 0.4; *t*-test) from that in wild types (10.5 ± 0.7 ms; n = 7) (Figure 5B). Moreover, the stimulus thresholds that were necessary to evoke these short-latency, temporally directed eye movements were the same in both types of animals (varying from 10 μA to 18 μA in both groups). Since CACNA1A mutations have been associated with a more rapid run-down of neurotransmitter release (Plomp et al., 2000), the possibility remained that *tg* mutants suffer from a functionally significant neuromuscular blockade that only becomes apparent during periods of activation longer than those associated with saccades or 200 ms pulse trains. To investigate this possibility, we also tested pulse trains up to 1500 ms (Figure 5C). Independent of the duration of the stimulation, the dynamics of the movements elicited in the ipsilateral eye in *tg* mutants (n = 4) were comparable to those in wild types (n = 4), and in both *tg* mutants and wild types these movements never stopped before the stimulus stopped. Moreover, the average time constants of the curves fitted to the responses in *tg* mutants did not differ significantly from those in wild types, neither for the long stimulations nor for the shorter stimulation protocols (600 ms, p = 0.5; 1000 ms, p = 0.9; 1500 ms, p = 0.4; *t*-tests). These results argue against the presence of a defect of signal transmission downstream of the flocculus, and thereby they suggest that the reduced visual compensatory eye movement performance in tg should not be attributed to such a barrier.

3.4.5 Olivary connections upstream of cerebellar cortex are functionally intact

Since P/Q-type calcium channels are also moderately expressed in the inferior olive neurons

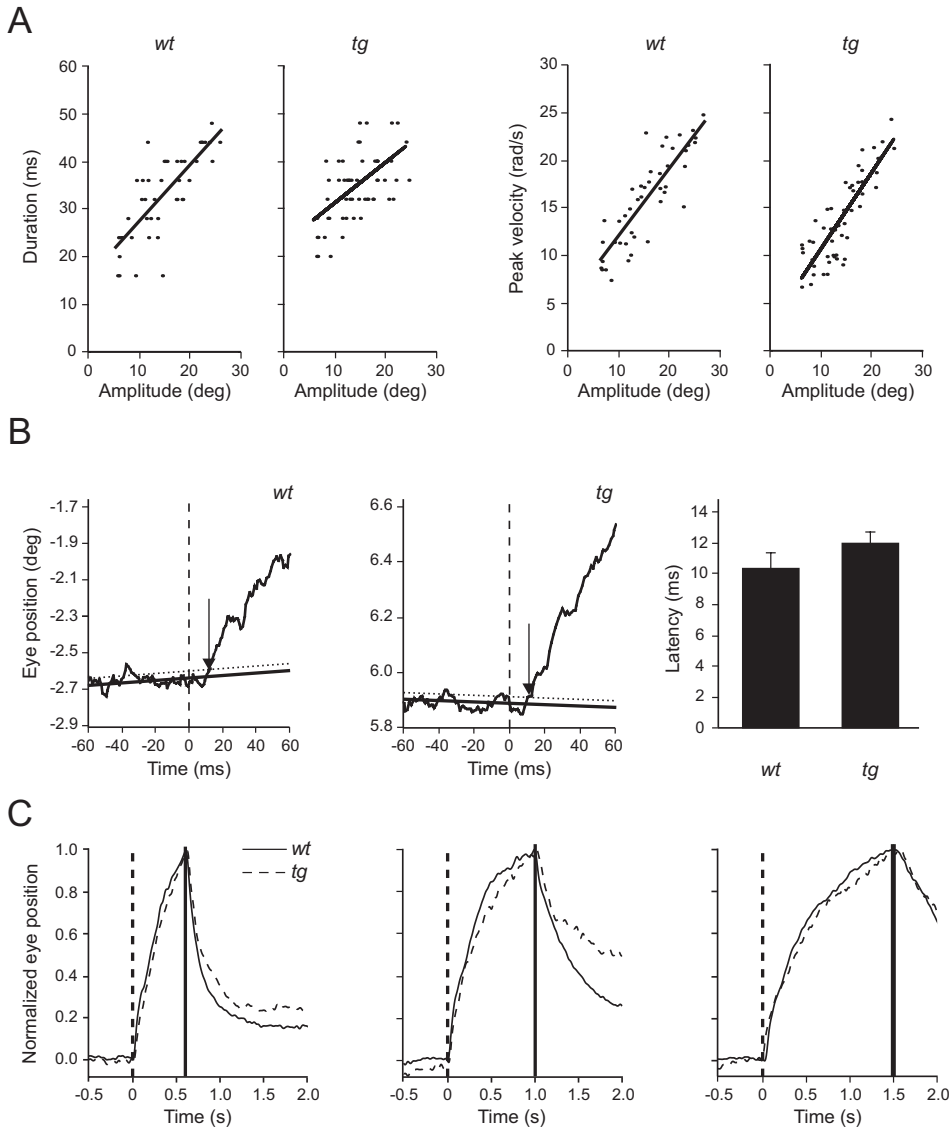

Figure 5. Oculomotor connections downstream of the flocculus appear functionally intact. (A) Typical examples of the dynamics of saccadic eye movements in *tg* and *wt*. Data show representative of linear fits to the duration re amplitude (left panels) and peak velocity re amplitude (right panels). The average main sequence parameters of *tg* are not different from those in wild type littermates (see text for numbers). (B) Eye movements evoked by electrical stimulation in the flocculus show normal latencies in *tg* mutants. The left and middle panel show eye position traces before and after the onset (dashed line) of electrical stimulation in *wt* and *tg*, respectively. Horizontal oriented black straight lines indicate result of linear regression of the 200 ms period prior to the onset of the stimulus with (dotted line) and without +1 SD (bold line). Arrows indicate crossings of eye position traces and SD lines, which are used to determine response latency. Right panel indicates that the average latency of the eye movements following electrical stimulation of the flocculus in 7 *wt* mice is indistinguishable from that in 7 *tg* mice. (C) Left, middle and right panel show normalized eye positions following 600 ms, 1000 ms and 1500 ms stimulus trains, respectively (for all paradigms n = 4 wild types and n = 4 *tg* mutants). Dashed and solid vertical lines indicate onset and stop of the electrical stimulus, respectively. The finding that the eyes in *tg* mutants did not drift back during the application of the stimulus suggests that the neurotransmitter stores in its oculomotor synapses and neuromuscular junctions are not depleted during the stimulus.

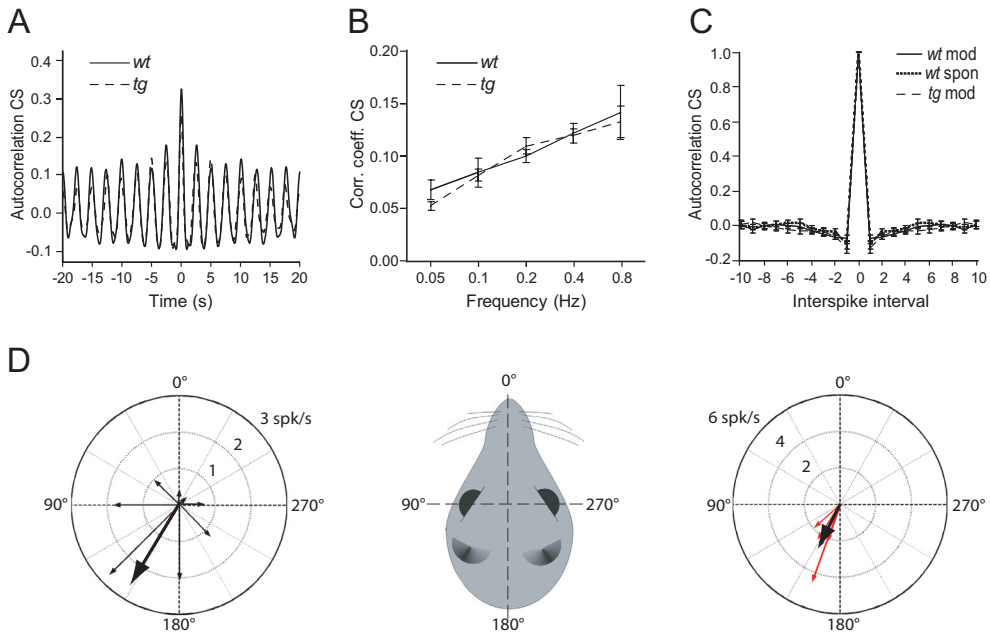

Figure 6. Olivary connections upstream of flocculus of tg mutants are functionally intact. (A) Autocorrelations show that there is no difference in the predictability of complex spike activities (CS) during optokinetic modulation between *wt* and *tg* data (stimulus frequency 0.4 Hz; bin size 50 ms). Solid and dashed lines indicate low-pass filtered autocorrelation for *wt* and *tg*, respectively. (B) Correlation coefficients of *wt* and *tg* CS activities during modulation are equally dependent on stimulus frequency. (C) Autocorrelations of CS interspike intervals in *wt* during modulation did not differ from that in *wt* during spontaneous activity nor from that in *tg* mice during modulation. Note that all CS data presented in panels A, B and C show opposite results to the simple spike data presented in Figures 2D, E and F. (D) Tuning curves of climbing fibre responses of floccular Purkinje cells are normal in *tg* mutants. Left panel shows an example of the depth of modulation of the climbing fibre responses of a single cell for 8 different horizontal axes in space. Lengths of arrows indicate depth of modulation and the bold arrow indicates vector for optimal modulation (close to 135˚ azimuth). Right panel shows these vectors for five different cells (bold arrow indicates average vector).

(Fletcher et al., 1996; Hillman et al., 1991; Stea et al., 1994; Westenbroek et al., 1995), the increased irregularity in simple spike activities may also be imposed by changes upstream of the flocculus, that is, in climbing fiber activities generated in the olive (De Zeeuw et al., 1998). This speculation is plausible since sudden increases or decreases of climbing fibre activities decrease and increase the simple spike frequency, respectively (Montarolo et al., 1982). We therefore evaluated the normality of complex spike activities (Figure 6). During spontaneous activity the mean firing frequency and CV of complex spikes in *tg* mice (n = 86 from 22 animals) equalled 0.98 ± 0.04 spk/s and 0.78 ± 0.02, respectively, while those in wild types (n = 79 from 14 animals) equalled 0.90 ± 0.03 spk/s and 0.81 ± 0.02 (p = 0.1 and p = 0.3, respectively; *t*-test). Autocorrelograms of complex spikes in *tg* mutants recorded during optokinetic stimulation (n = 25 cells from 10 animals) did not differ from those in wild types (n = 14 from 5 animals), neither with regard to the analyses using binned spike counts (Figures 6A and B) nor in the analyses of the individual interspike intervals (p = 0.4 and p = 0.1, respectively; ANOVA) (Figure 6C). To further assess the integrative properties of the olivary neurons and its inputs from the accessory optic system (Simpson et al., 1996), we also investigated the preference of their climbing fiber responses for particular axes of optokinetic stimulation, i.e. their spatial tuning (Figure 6D). In addition to the

neurons that modulated optimally by rotations about the vertical axis, we also identified numerous neurons preferring a horizontal axis directed 135° ipsilateral to the azimuth. The bimodal vector distribution and the resulting vectors of the spatial tuning curves in *tg* mutants did not differ from those in wild types and they resembled those described for other animals such as rabbits and pigeons (De Zeeuw et al., 1994; Graf et al., 1988; Wylie et al., 1995). Moreover, the complex spike activities were always in counter phase with the simple spike activities, independent of the spatial axis used for stimulation. We conclude that both the spontaneous activities and integrative properties of the olivary neurons that ultimately determine the complex spike output of the floccular Purkinje cells are not affected by the *tg* mutation, and that the simple spike irregularities are therefore not an indirect consequence of the expression of mutant P/Q channels in the inferior olive.

3.4.6 Acute blockade of P/Q-type channels in flocculus of wild types partially mimics behavioural phenotype of tg

The data presented above suggest that the connections downstream and upstream of the cerebellar cortex are sufficiently intact in the tg mutants to allow functional synaptic transmission, but it remains to be demonstrated whether dysfunctional P/Q-type channels in the cerebellar cortex alone are sufficient to induce the eye movement abnormalities. Based on electrophysiological observations gathered *in vitro*, abnormal Purkinje cell activities in mutants could potentially arise from direct effects of the CACNA1A mutation on dendritic and presynaptic P/Q calcium currents. In addition, the P/Q channel dysfunction could trigger compensatory changes (e.g., developmental changes in circuitry, channel distribution, channel accessory protein composition) that play out over longer time scales (Matsushita et al., 2002). We therefore examined whether the ocular motor abnormalities and simple spike irregularity in *tg* could be approximated by an acute pharmacologic manipulation in P/Q channel function in wild types (Figure 7). In wild types ($n = 6$) injections of 100 nM ω-Agatoxin IVA into the flocculus resulted in a significant decrease of the OKR gain values over the entire frequency range ($p < 0.001$; ANOVA) (Figure 7A). Injections of the vehicle solution alone ($n = 6$) had no significant effect. Injections of the P/Q-channel blocker also caused a significant reduction in the gain of the VVOR ($p < 0.02$; ANOVA), but the differences with the results following vehicle injections were relatively small at the higher frequencies. The phase lead during VVOR was significantly enhanced ($p < 0.001$, ANOVA; data not shown), whereas the phase lag during OKR was not affected ($p = 0.1$, ANOVA). In *tg* mutants ($n = 6$) the injections did not produce any significant effect ($p = 0.5$ and $p = 0.6$ for OKR and VVOR gain values, respectively; ANOVA). These data indicate that acute local application of P/Q-type channel blockers to the flocculus of wild type mice can be sufficient to mimic the eye movement performance of *tg* mice during OKR and VVOR.

Following juxtacellular application of ω-Agatoxin IVA in both the floccular and non-floccular regions of the cerebellar cortex, three categories of Purkinje cell activity were observed: a type in which simple spike activity intermittently ceased for long periods (seconds) while complex spike activity continued ($n = 14$); a second type in which both simple spike and complex spike activity continued throughout the recordings ($n = 13$); and a third type in which simple spikes continued but complex spike activity ceased ($n = 3$). All cells in the second category exhibited an unambiguous pause in simple spike activity following each complex spike throughout the recordings, confirming that the recording was obtained from a well-isolated, single unit Purkinje cell (Simpson et al., 1996) (Figure 7B). Analysis of this second response category showed that the average simple spike frequency increased from 59 ± 11 spk/s to 83 ± 16 spk/s and that their aver-

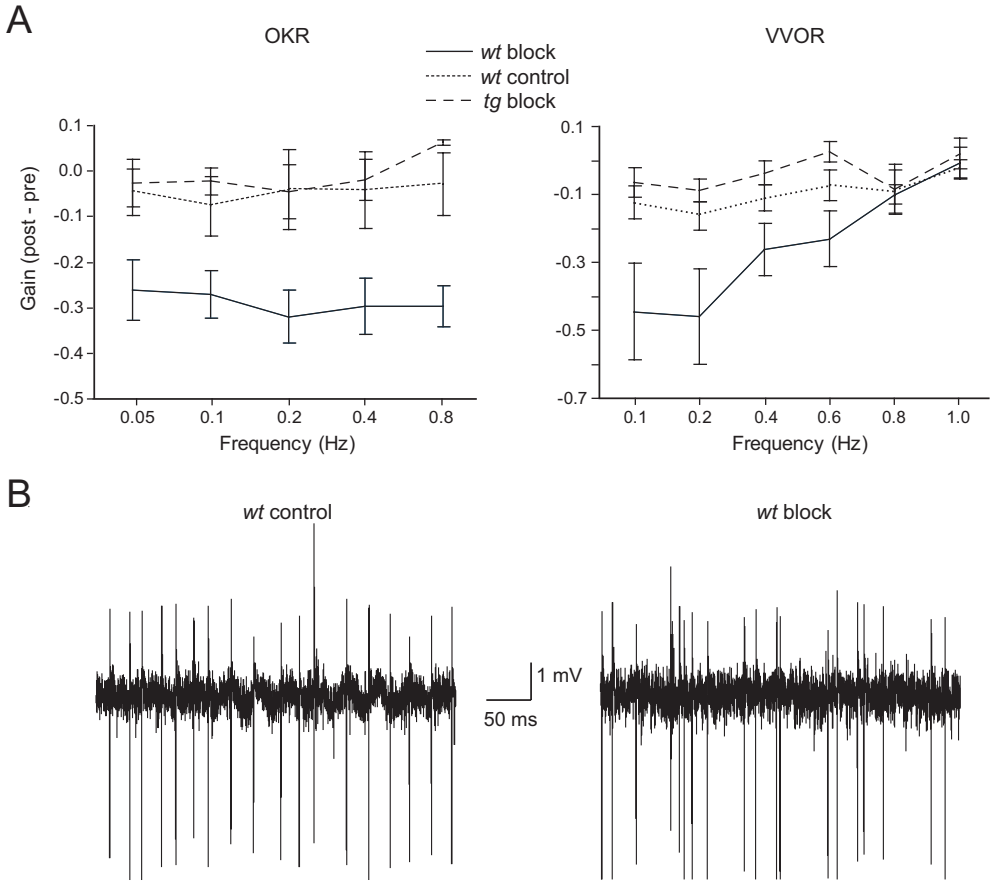

Figure 7. Phenotype of *tg* mutant can be largely mimicked by local injection of selective P/Q-type channel blocker. (A) OKR and VVOR gain values in *wt* (n = 6) decreased after injection of ω-Agatoxin IVA into the flocculus (solid line; *wt* block), but not after injection of the solution vehicle (dotted lines; *wt* control; n = 6). Injection of the blocker into the flocculus in *tg* mice (dashed line; *tg* block; n = 6) resulted in no change in OKR gain values. (B) Extracellular recordings of spontaneous Purkinje cell activities after juxtacellular application of the vehicle (control; left panel) or ω–Agatoxin (block; right panel) in the cerebellum of wild types. Note the increased irregularity of the simple spikes in the right panel (complex spikes go up, simple spikes go down). (C) Histograms showing the differences in CV and firing frequency of complex spikes and simple spikes in wild type mice (cvCS, ffCS, cvSS and ffSS, respectively) following juxtacellular application of ω-Agatoxin IVA as compared to the control injection with the vehicle alone.

age CV tended to change concomitantly following injection of ω-Agatoxin IVA as compared to injection of the vehicle alone (Figure 7C). However, none of the average changes in simple spike responses was statistically significant when we considered this category alone ($p = 0.1$ and $p = 0.3$ for differences in CV and firing frequency, respectively; ANOVA). Yet, when we pooled the simple spike data sets of all three categories of cells, the change in CV, but not in firing frequency, was significant ($p < 0.02$ and $p = 0.4$, respectively; ANOVA). In contrast, the average change in CV of the complex spike activities after application of ω-Agatoxin IVA was not significant ($p = 0.2$; ANOVA). Thus, even though one cannot rule out the possibility that some of the cells of the first and third category were not a pure single unit (for technical reasons see Simpson et al., 1996), the data are at least consistent with the possibility that reduction in P/Q calcium currents is sufficient to create the irregular firing rates, which our other data suggests are responsible for deficits in motor performance.

3.5 Discussion

The potential impact of noise on signal processing has been recognised by theoretical neuroscientists, but experimental evidence in which a change in noise results in altered sensorimotor behaviour has been lacking. Here we provide such evidence, showing that a mutation in P/Q-type voltage gated calcium channels leads to irregular simple spike activities of Purkinje cells without any change in other activity parameters (most importantly, the firing rate modulation) that could explain the deficiencies we observed in types of eye movement normally mediated by the cerebellum. The data supports the idea that regularity of firing influences sensorimotor processing. Because mutations in CACNA1A are associated with ataxia in humans, the current data raise the possibility that alterations in noise levels of neuronal signals could be sufficient to produce the behavioural manifestations of certain neurological diseases.

3.5.1 Cellular basis of irregular simple spike firing pattern

The increased irregularity of simple spike firing in *tg* could arise from several sources, including alterations of the signals carried by mossy fiber inputs, alterations of signals carried by climbing fibers, or abnormalities of neurons in the cerebellar cortex that alter their response to mossy and climbing fiber inputs. The first possibility appears unlikely in the case of the flocculus, since in situ hybridisation and immunocytochemical studies indicate that P/Q channels are relatively rare in regions of the brain giving rise to mossy fiber inputs to the vestibulocerebellum (Craig et al., 1998; Fletcher et al., 1996; Stea et al., 1994; Westenbroek et al., 1995). The second possibility is more plausible, as P/Q-type channels are expressed throughout the inferior olive. However, we were able to refute this possibility by demonstrating that complex spike activity was normal in terms of waveform, firing frequency, regularity, preferred stimulus axis, and phase with respect to simple spikes. Thus, simple spike irregularities in *tg* are most likely the result of the third source, i.e., an abnormal response due to the alteration of synaptic and dendritic properties of the cerebellar cortical neurons that are affected by the mutated P/Q channels.

Because P/Q-type channels are heavily expressed throughout all layers of the cerebellar cortex (Fletcher et al., 1996; Hillman et al., 1991; Stea et al., 1994; Westenbroek et al., 1995), there are several sites and types of electrophysiological abnormalities that could contribute to the simple spike irregularity. First, alterations in P/Q channel current density and micro- and macroscopic current magnitudes are likely to affect the intrinsic excitability of Purkinje cells and gran-

ule cells (Randall and Tsien, 1995; Wakamori et al., 1998; Zhang et al., 1993). Second, the synaptic connection between the granule and Purkinje cells is likely weakened in that the amplitude of their EPSC evoked by parallel fiber stimulation is smaller (Matsushita et al., 2002). Third, even though the climbing fiber-mediated EPSC is normal (Matsushita et al., 2002), our results indicated that the length of the climbing fiber pause is slightly reduced. Pause duration, which probably largely reflects the activity of calcium-dependent potassium channels (Schmolesky et al., 2002; Sausbier et al., 2004), may influence simple spike activities even after firing resumes (Sato et al., 1993). Fourth and finally, the P/Q alteration may trigger indirect, compensatory mechanisms in the cerebellar cortex of *tg*. For example, there may be a shift in the reliance of neurotransmitter release mechanisms from P/Q-type channels to N-type channels, as has been shown in the forebrain and hippocampus (Cao et al., 2004; Leenders et al., 2002; Qian and Noebels, 2000). Because an increased reliance on N-type channels leads to an increased susceptibility to inhibitory modulation by G protein-coupled receptors (Zhou et al., 2003), and because a single action potential of a cerebellar inhibitory interneuron is able to delay action potentials in Purkinje cells (Hausser and Clark, 1997), such an increased susceptibility would be predicted to prolong the silent periods that basket cells and stellate cells can impose on them. Thus, a combination of multiple defects of different natures at various synapses in the cerebellar cortex may well explain the enormously enhanced irregularity of the simple spike response in *tg* mutants.

3.5.2 Behavioural consequences of irregular simple spike activities

Our behavioural and neuronal observations that gain and phase values during compensatory eye movements under vision are affected and that simple spike activities in their flocculus are irregular while their modulation amplitude is normal raise the possibility that irregular simple spike activities of Purkinje cells are one of the prime causes of ataxia in *tg* mutants. This notion is in line with the fact that the density of P/Q-type channels in the cerebellum far exceeds that of other brain structures involved in ocular motor behaviour (Fletcher et al., 1996; Hillman et al., 1991; Stea et al., 1994; Westenbroek et al., 1995). However, these studies cannot exclude the possibility that P/Q-type channels play key roles at restricted sites in tissues with low overall expression levels, nor the possibility that the P/Q mutation had developmental effects on circuits outside the regions of high-density P/Q expression in the adult brain. It remained therefore possible that the alterations in visually driven eye movements reflect derangements of extrafloccular circuits. To overcome this potential caveat we addressed the integrity of signal flow from the flocculus to the eye muscle by investigating the dynamics of saccadic eye movements in *tg* mutants as well as their eye movements following electrical stimulation in the flocculus. The duration and velocity re amplitude profiles of their saccadic eye movements were normal, supporting the functional integrity of the neuromuscular junction. In this respect the phenotype of *tg* mutants diverges from that in *rocker* mice, which suffer from a different mutation in the same $\alpha 1_a$-subunit of the P/Q-type channel and show deficits in both slow and fast phases of their eye movements (Stahl, 2004). Thus, strengthened by the specificity of the behavioural phenotype, the experiments on saccadic eye movements indeed suggest that neurons of the oculomotor nuclei in *tg* mice operate functionally normally. In addition, we demonstrated that the eye movements evoked by prolonged floccular stimulation appeared normal in *tg* mice, indicating that rapid depletion of neurotransmitters at synapses or neuromuscular junctions from flocculus to eye muscles is unlikely to contribute to the ocular motor abnormalities, despite such depletion having been observed at selected synapses outside the ocular motor system (see also Plomp et al., 2000; Qian and Noebels, 2000; Cao et al., 2004). Finally, we also demonstrated that gain and phase values of the vestibulo-ocular re-

flex in the dark were hardly affected. Thus, all synaptic connections in the oculomotor pathway downstream of the flocculus appear at least at the systems level functionally intact. These data however do not allow us to conclude that there are no changes at these synaptic inputs at the cell physiological level (Qian and Noebels, 2000). In fact, the total of cellular compensations may be such that the overall synaptic strengths in these connections are sufficiently preserved despite the shift away from the P/Q-type predominance mentioned above (Cao et al., 2004).

If Purkinje cell irregularities in *tg* cause complete loss-of-function of the cerebellar cortex, ablations of their flocculus should not further decrease the gain values of their compensatory eye movements and their gain values with an intact flocculus should be similar to those of flocculectomized wild types. On the other hand, if aberrations in neurons downstream of the cerebellar cortex contribute to ataxia in *tg* mutants, ablations of their flocculus should further decrease the gain values of their compensatory eye movements and their gain values with an intact flocculus does not need to be similar to those of wild types with an ablated flocculus. The outcome of our flocculectomy experiments in wild types and *tg* mutants confirmed the first hypothesis and contradicted the second. Finally, if the abnormalities in the cerebellar cortex of the *tg* mutant are indeed sufficient to cause the ocular motor deficits, one expects that local blockade of P/Q-channels in some portion of the wild type flocculus might be sufficient to qualitatively reproduce some of the electrophysiological and behavioural abnormalities seen in *tg*. This prediction was also largely upheld. The relatively small, but present, discrepancies between the phenotype of *tg* mutants and that of wild types treated with the P/Q-type channel blocker ω-Agatoxin IVA could be due to the long-term secondary compensations that occur in the mutant, or to the more profound but also restricted region of the pharmacological blockade in wild types. Taken together, our eye movement recordings combined with our electrophysiological recordings, electrical stimulations, lesions and pharmacological blockage experiments are consistent with the speculation that the *tg* mutation in the $\alpha 1_a$-subunit of the P- and Q-type voltage-gated calcium channel leads to irregular simple spike firing patterns, and that these irregular simple spike activities are sufficient to contribute to deficits in motor performance.

3.5.3 Functional implications

Our data obtained in *tg* mutants suggest that the noise level of neuronal firing patterns can be altered without any impact on their modulation depth and that such a change is sufficient to alternate motor behaviour. This possibility is strengthened by our analyses of simple spike autocorrelations. Not only was the regularity of wild types greater than that of *tg* mice when recorded in the absence of eye movements, during compensatory eye movements the regularity increased in normal animals, while it remained unchanged in the mutants. Together the data suggest that the average firing rates and modulation amplitudes are not the sole determinants of cerebellar output. It has been recognized by information theoreticians that an optimal level of noise (i.e. irregularity) can be important for signal processing (Bialek et al., 1991; Mar et al., 1999; Steinmetz et al., 2001; Tiesinga et al., 2002). For example, models by Rieke and Chacron indicate that on the one hand noise will increase the trial-to-trial variability of a neural response to repeated presentations of a stimulus, but on the other hand it will also increase the variability of the spike train and thereby potentially lead to increased information capacity (Chacron et al., 2003; Rieke et al., 1997). Here, we showed that a mutation in the $\alpha 1_a$-subunit of the P/Q-type voltage-gated calcium channel can lead to an increased noise level in the simple spike activities of Purkinje cells in that both the variability in interspike intervals and the trial-to-trial variability are increased. In principle both forms of irregularity may affect signal transmission at the synaptic input from the

Purkinje cells to their target neurons in the cerebellar and vestibular nuclei and thereby contribute equally well to deficits in motor performance. In fact, one may hypothesize that due to both forms of irregularity too many of the simple spikes in *tg* mutants fall outside the temporal window that is formed by the effective range of spike frequencies that can be handled by the propagation properties of the Purkinje cell axons and/or by the properties of their synapses onto their target neurons. Such a notion would be in line with recent findings by Monsivais and Hausser (2003) who showed that propagation failures in Purkinje cell axons during climbing fiber bursts can be correlated with a frequency limit of about 252 Hz, which is less than the maximum frequency one can observe during a short burst of simple spikes in *tg* (even though the average firing frequency is not above normal). Moreover, the effective range of simple spike activities may not only be determined by properties of the Purkinje cell axons or their synaptic terminals onto their target neurons, but also by changes in intrinsic excitability of these neurons (Aizenman and Linden, 2000; Nelson et al., 2003).

The gross motor deficits of *tg* resemble those seen in human patients with the CACNA1A-related disorders familial hemiplegic migraine, episodic ataxia type II, and spinocerebellar ataxia type VI (Baloh et al., 1997; Ducros et al., 1999; Harno et al., 2003; Ophoff et al., 1996; Zuchenko et al., 1997). To the extent that the function of the flocculus in eye movements parallels the role of extra-floccular cerebellum in limb and trunk motor control, and assuming that attenuated compensatory eye movements are analogous to limb and truncal ataxia, then, irregular Purkinje cell activities could lie at the root of the ataxia found in these patients. It should be noted, however, that the most severe forms of ataxia in these patients appear in association with Purkinje cell degeneration (Gomez et al., 1997; Buttner et al., 1998), so irregular Purkinje cell activities could only explain the milder types of incoordination seen in the first years of the disease. By suggesting a physiological mechanism that could account for motor incoordination, the current data raise the possibility to design therapeutic neurostimulation protocols for patients with beginning forms of ataxia that result from similar mutations in their P/Q-type calcium channel (Ophoff et al., 1996). The fact that we were able to evoke eye movements by electrical stimulation in the flocculus of *tg* mutants further supports this possibility. The significance of noise levels in firing patterns is probably not restricted to the CACNA1A diseases, nor to the neurons of the cerebellum. For example, Huxter and colleagues (2003) recently showed in hippocampal pyramidal cells that the time of firing and firing rate are dissociable and can encode independent variables (respectively, the animal's location within, and speed of movement through, a place field). Thus, proper modulation of neuronal activities is essential, but the absolute moment in time at which individual spikes occur can also influence signal processing under both pathological and normal circumstances.

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Chapter 4

Calbindin is a critical determinant of the precision of eye and limb coordination in cerebellar Purkinje cells

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4.1 Abstract

Long-term depression (LTD) of Purkinje cell - parallel fiber synaptic transmission is a critical determinant of normal cerebellar function. Impairment of LTD, for example by disruption of the metabotropic glutamate receptor/IP_3/calcium signaling cascade, results in severe deficits of both synaptic transmission and cerebellar motor control. Here we demonstrate that selective genetic deletion of the calcium-binding protein calbindin D-28k in cerebellar Purkinje cells results in distinctly different cellular and behavioural alterations. Both compensatory eye movements and locomotion of these mutants display marked permanent performance deficits, while the amplitudes and time-courses of the fast calcium transients of their Purkinje cells following afferent stimulation are increased and decreased, respectively. Even so, the delayed mGluR-mediated calcium transients, as well as parallel fiber LTD, and the basal complex spike and simple spike discharge properties of their Purkinje cells are not affected. In conjunction, our results reveal a specific but permanent role of calbindin D-28k in rapid calcium buffering in Purkinje cells relevant for their signal transmission and thereby ultimately for motor performance.

4.2 Introduction

One of the hallmarks of cerebellar Purkinje cells is their ability to express a characteristic form of activity-dependent synaptic plasticity named long-term depression (LTD). LTD is induced by the joint activity of afferent climbing and parallel fibers (CFs; PFs), and represents a persistent reduction of the efficacy of parallel fiber-mediated excitatory postsynaptic potentials (EPSPs) (Ito, 1986). A key event in LTD induction is the activation of postsynaptic metabotropic glutamate receptors (mGluRs) (Rose and Konnerth, 2001; Coesmans et al., 2003). Null mutant mGluR1 mice, which lack a subtype of mGluRs that is expressed in Purkinje cells (Martin et al., 1992; Gorcs et al., 1993; Ryo et al., 1993), exhibit severe cerebellar symptoms such as ataxia and lack of LTD (Aiba et al., 1994; Conquet et al., 1994). The behavioural relevance of Purkinje cell mGluR1 for proper cerebellar function was elegantly and unequivocally confirmed by the selective rescue of mGluRs in Purkinje cells of mGluR1-null mutant mice (Ichise et al., 2000), which restored both LTD and locomotor activity.

Further confirmation and extension of these findings came from studies showing that a perturbation of the mGluR1-activated signaling cascade at virtually every level, e.g. G-protein Gαq (Offermanns et al., 1997), PLCβ (Kano et al., 1998) and IP_3 (Matsumoto et al., 1996; Miyata et al., 2000) results in impaired LTD and disturbed cerebellar motor control. IP_3 causes Ca^{2+}-release from intracellular stores (Mikoshiba et al., 1994; Finch and Augustine, 1998; Takechi et al., 1998) and together with synaptically-induced dendritic Ca^{2+}-signaling is known to be critical for LTD induction (Sakurai, 1990; Konnerth et al, 1992). Based on earlier evidence it seemed that not merely the lack of synaptically-mediated Ca^{2+}-signaling in Purkinje cells, but also a change in the temporal dynamics of postsynaptic Ca^{2+}-transients following general genetic deletion of the calcium-binding protein calbindin D-28k (calbindin) might cause impairment of motor coordination (Airaksinen et al., 1997). However, despite the suggestion of an involvement of Purkinje cells, it remained unclear, which cell types were responsible for the behavioural phenotype, because calbindin is expressed by many types of neurons throughout the brain (Celio, 1990). Even in the cerebellum, calbindin is expressed abundantly not only in Purkinje cells, but also in climbing fibers (Celio, 1990; Scotti, 1995). Previous studies demonstrated that Ca^{2+}-binding proteins influence presynaptic transmitter release (Chard et al., 1995; Klapstein et al., 1998; Caillard et al., 2000). Finally, it was not tested whether the calbindin-mediated change in Ca^{2+}-signaling affected

cerebellar LTD or basal Purkinje cell discharge. To study the specific role of calbindin in a single defined neuronal cell type, we generated a Purkinje cell-specific calbindin null mutant mouse strain and performed an analysis on the cellular and behavioural level.

4.3 Methods

4.3.1 Conditional null mutant mice

A mouse strain carrying a floxed calbindin allele was used (Barski et al., 2002). In brief, a pgk promoter-neo-pA-pgk promoter-TK-pA cassette flanked by loxP sites in the same orientation was inserted into the Eco47III site of a 6kb XhoI/SalI genomic fragment (Airaksinen et al., 1997) and a third loxP was ligated into the ClaI site upstream of the first coding exon. ES cell clones were generated in R1 embryonic stem cells, which are originally derived from 129/Sv mice (gift by A. Nagy), and correctly targeted clones underwent a second electroporation with the Cre expression plasmid pMCCre (gift of H. Gu and K. Rajewsky). Clones with the desired recombination were injected into C57BL/6 (C57BL/6NCrl BR, Charles River, Sulzbach, FRG) blastocysts. Resulting chimeras were crossed with C57BL/6 mice. Animals of heterozygote intercrosses were used for analysis. The Cre transgenic strain employed in this study has previously been characterized (Barski et al., 2000); here we used substrain L7Cre-2.

4.3.2 Western blot and immunohistochemistry

Western blotting was as previously described (Airaksinen et al., 1997) using a mouse monoclonal antibody to calbindin (1:10000, SWant). For immunohistochemistry (Airaksinen et al., 1997) on 30µm thick free floating sections we used rabbit antibodies to parvalbumin (1:500, SWant) and mouse monoclonal calbindin antibodies (1:500, SWant) together with fluorescein or Texas Red-coupled secondary antibodies (Jackson). Images were taken on a Leitz DM IRB confocal microscope (Leica) using the graphics program ImageSpace (Molecular Dynamics).

4.3.3 RT-PCR

The RT-reaction was performed on 1 µg of total RNA from the indicated brain regions as described earlier (Barski et al., 2002).

4.3.4 Locomotion Analysis

Open field analysis was performed on the TruScan activity monitor (Coulbourn Instruments) as described (Barski et al., 2000). The runway and horizontal bar test were performed essentially as before (Airaksinen et al., 1997). Number of assay days and width of the runway were as indicated. Maximal time on the horizontal bar was 120 sec. Data were analyzed with the ANOVA-single factor test.

4.3.5 Compensatory eye movements

Measurements of compensatory eye movements were performed essentially as described before (DeZeeuw et al., 1998a). The eye coil had an outside diameter of 1 mm and was made of 60 windings (resistance of 25 to 35 Ω). A servo-controlled turntable and drum (Benedict, NY, USA) delivered optokinetic and vestibular stimuli. The stimulus paradigms included sinusoidal optokinetic stimulation and sinusoidal table stimulation in the dark and light. Optokinetic stimulus frequencies ranged from 0.1 - 1.6 Hz and with constant peak velocity of 8°/s and varying amplitude ranging from 0.8 - 12.7 deg. Vestibular stimulus frequencies ranged from 0.2 - 1.6 Hz and the amplitude of was kept constant at 10 deg. All gain and phase values were analyzed off-line using Spike 2 (CED, Cambridge, UK) and custom-made Matlab programs (Mathworks Inc. Natick, MA, USA) according to standard procedures in our laboratory (Van Alphen et al., 2001). Eye movements were recorded from 11 recombined and 10 control mice.

4.3.6 Electrophysiology in slices and LTD

Whole-cell recordings were obtained from Purkinje cells of 300 µm thick cerebellar slices from 18 to 30-day-old mice. The animals were decapitated following anesthesia with CO2 and cerebella were rapidly removed and placed in ice-cold artificial cerebro-spinal fluid (ACSF) composed of (in mM): 125 NaCl, 2.5 KCl, 2 CaCl2, 1 MgCl2, 1.25 NaH2PO4, 26 NaHCO3 and 20 glucose, bubbled with 95% O2 and 5% CO2. After cutting, slices were kept for 45 – 60 min at 37°C and then for up to 8h at 25°C until they were used for experiments. Somatic whole-cell recordings were obtained with an EPC8 or EPC9 amplifier (HEKA). „PULSE“-software (HEKA) was used for data acquisition. Pipettes (2-4 MΩ resistance) were pulled from borosilicate glass (Hilgenberg) and coated with silicon (RTV 615, GE Silicons). The pipette solution contained (in mM): 148 potassium gluconate, 10 HEPES, 10 NaCl, 0.5 MgCl2, 4 Mg-ATP and 0.4 Na3-GTP, pH 7.3. For Ca^{2+}-imaging experiments 100 µM Oregon Green BAPTA-1 (Molecular Probes) was added to the pipette solution. During the experiments, the slices were continuously perfused with ACSF bubbled with 95% O2 and 5% CO2 (at 22-23°C except for LTD experiments) containing 10 µM bicuculline (Sigma). Synaptic stimulation was performed by using a standard patch-pipette filled with 1 mM NaCl (1 MΩ resistance) placed in the molecular layer. The stimulus pulse amplitude (150 µs duration) was 2-20 V for PF stimulation and 20–55 V for CF stimulation. PF-EPSCs were identified by their characteristic features (graded response amplitude, paired-pulse facilitation). Without moving the stimulus pipette, the intensity was increased until a climbing fiber response was observed distinguished by its all-or-none character and paired-pulse depression (Konnerth et al., 1990; Konnerth et al., 1992) and the CF stimulus threshold was determined. Parallel fibers were stimulated at 0.2 Hz and EPSCs were recorded in the voltage-clamp mode until stable baseline amplitude was obtained for at least 10 min. To induce LTD the stimulus intensity was raised to a value at least 20% over CF threshold and 240 stimuli were repeated at 1Hz in conjunction with a depolarizing pulse to the soma (200 ms, -60 mV – 0 mV). After pairing, the stimulus intensity was set to the initial value and the recording of PF-EPSCs at 0.2 Hz was resumed for 60 min. Passive membrane properties of Purkinje cells were monitored by applying 3 mV hyperpolarizing pulses. The series resistance was kept constant throughout the measurement at around 10 – 20 MΩ. The bath temperature was 32°C.

4.3.7 In vivo electrophysiology

Extracellular activity of Purkinje cells in awake mice was recorded as described in detail by Goossens et al. (2001). Single unit Purkinje cell activity was identified by the presence of a brief pause (climbing fiber pause; Simpson et al., 1996) in simple spike discharge following a complex spike, and the presence of this pause was carefully monitored during the course of the recording. Off-line spike analysis was done using custom software implemented in Matlab by means of amplitude threshold and principal component analysis. Firing frequency, climbing fiber pause, Inter Spike Interval histogram, and coefficients of variation were all derived from recording epochs (> 2 min) of spontaneous single unit simple spike and complex spike activity. All statistics were performed using Student's *t*-tests.

4.3.8 Calcium imaging

A confocal laser-scanning microscope (Odyssey, Noran), attached to an upright microscope (Zeiss Axioskop2, x63 water immersion objective, NA 0.9) was used to acquire fluorescence images at 30 Hz in parallel to the whole-cell recordings. Ca^{2+}-imaging was started at least 20 min after establishment of whole-cell configuration.

Full-frame images were recorded with 30 Hz on an optical disk (TQ-FH224, Panasonic) using the Image-1 software (Universal Image) and analyzed off-line with custom-made software based on LABVIEW (National Instruments). Ca^{2+}-transients (see fig. 5D) were recorded in regions of interest in active dendritic regions. For measurement of calcium transients in dendritic spines, two-photon imaging was performed in parallel to whole-cell patch clamp recordings. We used a custom-build two-photon laser-scanning microscope based on a mode-locked Ti:sapphire laser system operated at 790 nm center wave length, 80 MHz pulse repeat, < 100 fs pulse width (Tsunami and Millenia, Spectra Physics) and a laser-scanning system (MRC 1024, Bio-Rad) coupled to an upright microscope (BX50WI, Olympus) equipped with a 60x 0.9 NA water immersion objective (Olympus). To achieve high temporal resolution, fluorescence was acquired in the line scan mode at a sampling rate of 160 Hz (control animals) or 480 Hz (recombined). Background-corrected line scan images were analyzed off-line with a custom-written routine in LabView (National Instruments). The Ca^{2+}-dependent fluorescence signals were expressed as increases in fluorescence divided by the prestimulus fluorescence values ($\Delta F/F_0$) and further analyzed using Igor Pro (Wavemetrics) or Origin (Microcal) software.

4.4 Results

4.4.1 Characterization of Purkinje cell-specific null mutant mice

For orientation, the targeting strategy for the floxed calbindin allele (referred to as $Calb^{tm2}$) (Barski et al., 2002) is depicted in Fig. 1A. As the properties of the floxed and recombined alleles are critical to the work presented here, we performed additional experiments to this point and repeated some of the earlier work. Western blotting and RT-PCR experiments confirmed our previous conclusion that the introduced loxP sites are without effect on calbindin levels or distribution in homozygous $Calb^{tm2}$ mice (Fig. 1B). We had previously shown that $Calb^{tm2}/Calb^{tm2}$ mice are also indistinguishable from their wild type littermates in the open field and runway behavioural assays (Barski et al., 2002). The L7Cre-2 transgenic mouse strain which expresses Cre recombinase

under the control of the Purkinje cell-specific L7/pcp-2 minigene has previously been shown to allow highly selective and efficient recombination in Purkinje cells (Barski et al., 2000).

Intercrossing of double heterozygous mice gave offspring at the expected Mendelian ratios which was divided into two groups. The control group (not recombined) comprises all genotypes lacking either the Calb^{tm2} or the L7Cre-2 allele. The recombined group consists of all mice homozygous for the Calb^{tm2} allele combined with one or two L7Cre-2 alleles. Within each group behavioural, biochemical (besides genotypes), histological and immunocytochemical assays did not reveal differences between animals. Mice of both groups were similar with respect to growth, life span and fertility. Tissue-specific recombination was verified by RT-PCR (Fig. 1C) and Western blotting (Fig. 1D). In the cerebellum, calbindin transcripts and protein were readily detected in the control group but almost absent in recombined mice. Residual calbindin was estimated to be less than 5% of control levels. No differences were detected in cortex and hippocampus. Cell

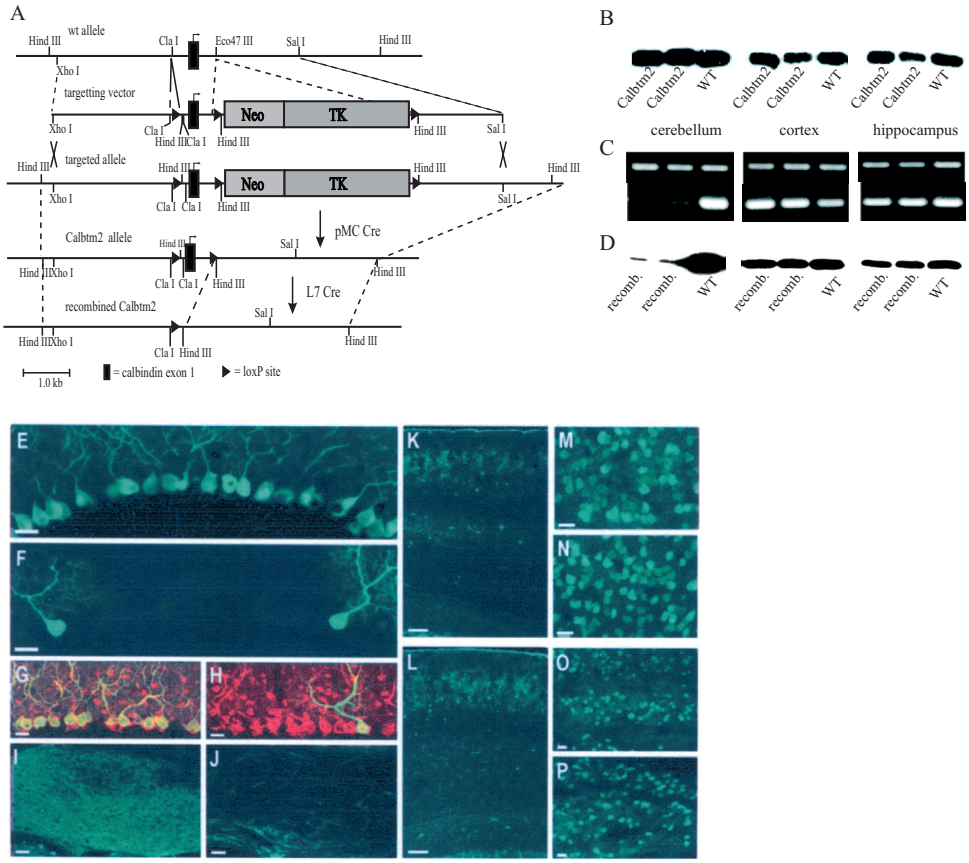


Figure 1. Generation and characterization of the conditional mutant. A, Targeting strategy. B, Western blot of equal amounts of protein from the indicated tissues from Calb^{tm2}/Calb^{tm2} and *wt* mice detected by a monoclonal calbindin antibody. C, RT-PCR of calbindin transcripts in cerebellum, cortex and hippocampus. GAPDH was amplified as an internal control. D, Western Blot analysis of calbindin expression (as in B) of recombined and control mice. Cb28kD indicates positions of calbindin protein or amplification product. E-P, Localization of calbindin in recombined (F,H,J,L,N,P) and not-recombined (E,G,I,K,M,O) mice by immunohistochemistry using a monoclonal calbindin antibody. E,F Cerebellar cortex; G,H cerebellar cortex double-stained for calbindin (green) and parvalbumin (red); I,J deep cerebellar nuclei; K,L cerebral cortex; M,N Striatum; O,P inferior olive. Bars: (E-H, M-P) 20 µm, (I,J) 40 µm, (K,L) 80 µm.

type-specificity was investigated by immunohistochemistry in sagittal brain sections. We found that less than 2% of Purkinje cells of recombined mice displayed residual calbindin immunoreactivity (Fig. 1E-H; fields selected for a maximum number of not recombined cells are shown). Purkinje cells of normal morphology could, however, be rendered visible by immunostaining for parvalbumin, another major cytosolic calcium-binding protein of these cells (Fig. 1G,H). Calbindin immunoreactivity was also reduced in basal parts of the cerebellum, where Purkinje cell axons terminate in the deep cerebellar nuclei (Fig. 1I,J). The presence of few calbindin-positive fibers is consistent with the low number of Purkinje cells in which recombination did not occur. No differences in calbindin immunoreactivity were observed for other brain regions (Fig. 1K-P; dentate gyrus and CA1 pyramidal cells; data not shown). Thus, efficient and specific deletion of calbindin from Purkinje cells has been achieved.

4.4.2 Limb coordination

Our previous work has revealed impaired motor coordination in the general calbindin null mutant

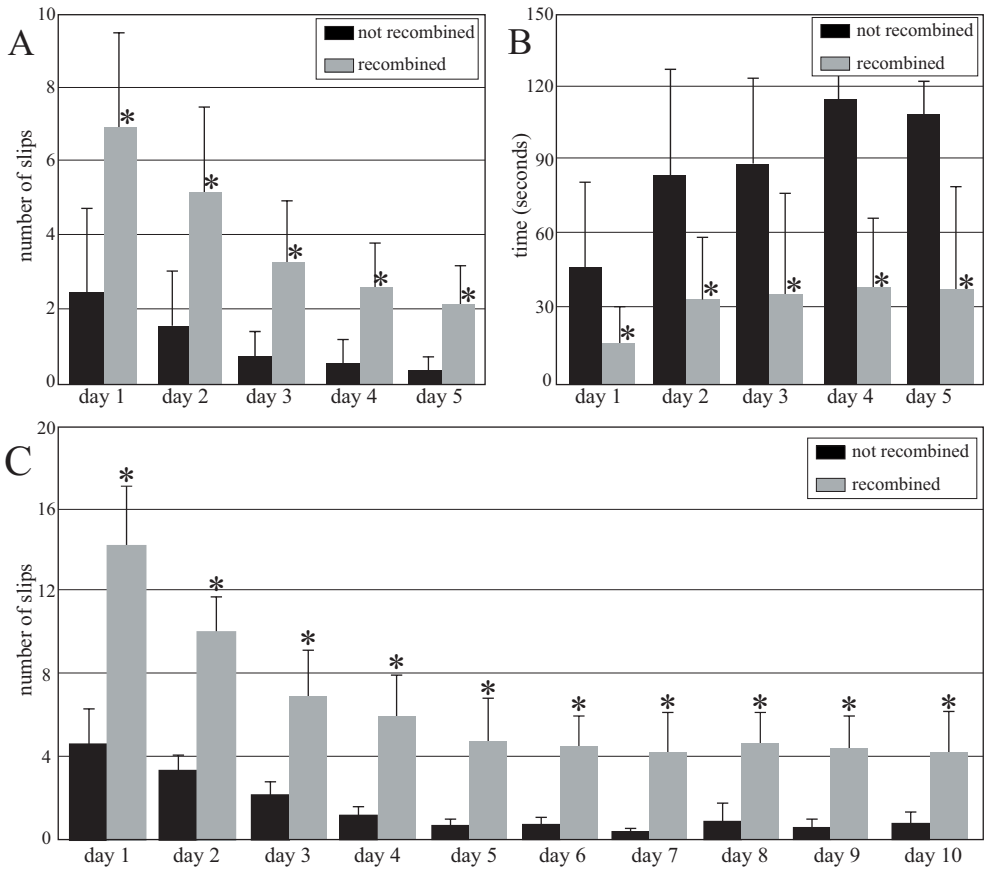


Figure 2. Limb coordination. A, 5-day runway task on 2 cm wide bar (not recombined n = 19, recombined n = 21, * p = 3.1E-06). B, Balance rod test (not recombined n = 9, recombined n = 7, * p = 3.2E-02). C, 10-day runway task on 1 cm wide bar (not recombined n = 9, recombined n = 10, * p = 9.2E-05). Error bars indicate standard error of the mean (s.e.m.)

mouse (Airaksinen et al., 1997). In that study, however, it could not be tested, whether changes in multiple normally calbindin-expressing populations caused these deficits, or whether calbindin is critically required within a single cell type. To resolve this question, we examined motor coordination of Purkinje cell-specific null mutants.

General locomotor abilities and explorative behaviour as tested in the open field did not differ between control and Purkinje cell-specific null mutant groups (not shown). However, severe locomotor deficits in recombined mice were revealed in a standardized limb coordination test in which foot slips are counted during passage over a narrow runway (Airaksinen et al., 1997). Recombined mice made significantly more errors throughout the entire testing period and during prolonged training (Fig. 2A). Although their slip rate decreased during the first days of the test, they never reached the performance levels of controls. Rather, their error rate leveled off asymptotically at values higher than those of the control group. Thus, lack of calbindin in Purkinje cells results in a permanent impairment of coordination that cannot be compensated even by prolonged training. Moreover, the deficit was correlated with the difficulty of the task (Fig. 2C). Within groups, decreasing the width of the runway by a factor of two (from 2 to 1 cm) resulted in two-fold higher initial slip rates with the recombined group making more errors on both runways than the not recombined animals. An interesting additional difference became apparent at the end

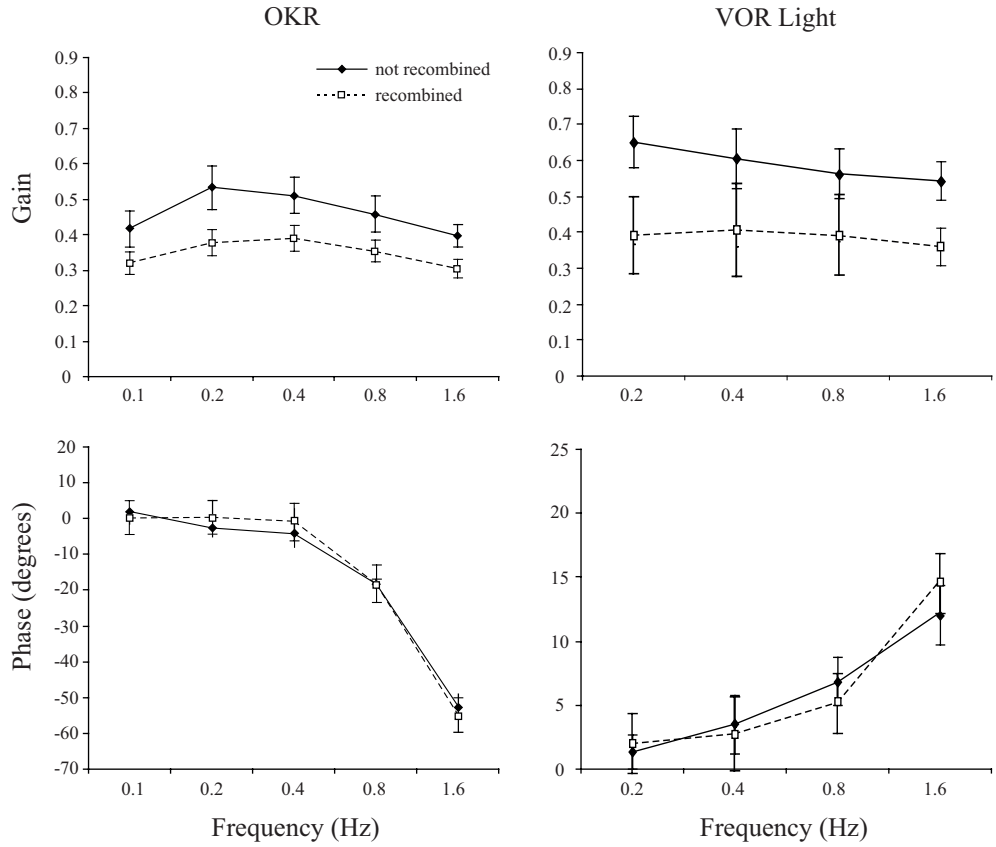


Figure 3. Compensatory eye movements. The gain values of recombined mice (n = 11) were significantly smaller than those of the control animals (n = 10) during both OKR (A) and VOR-L (C) at all tested frequencies. In contrast, the phase values (B, D) did not differ significantly at any of the frequencies. Error bars indicate s.e.m.

of testing. Control mice reached similar levels of performance independent of the width of the runway used, whereas recombined mice made two times more errors on the narrow than on the wide runway. Furthermore, groups differed in their ability to stay on a stationary horizontal bar (Fig. 2B). Whereas many mice of the control group reached the test criterion (120 sec on the horizontal bar) on the fourth day, for recombined animals only a small increase in the time spent on the bar occurred between day 1 and 3, after which no further improvement was observed. These experiments show that specific motor deficits are indeed induced by lack of calbindin in a single cell type, the cerebellar Purkinje cell.

4.4.3 Compensatory eye movements

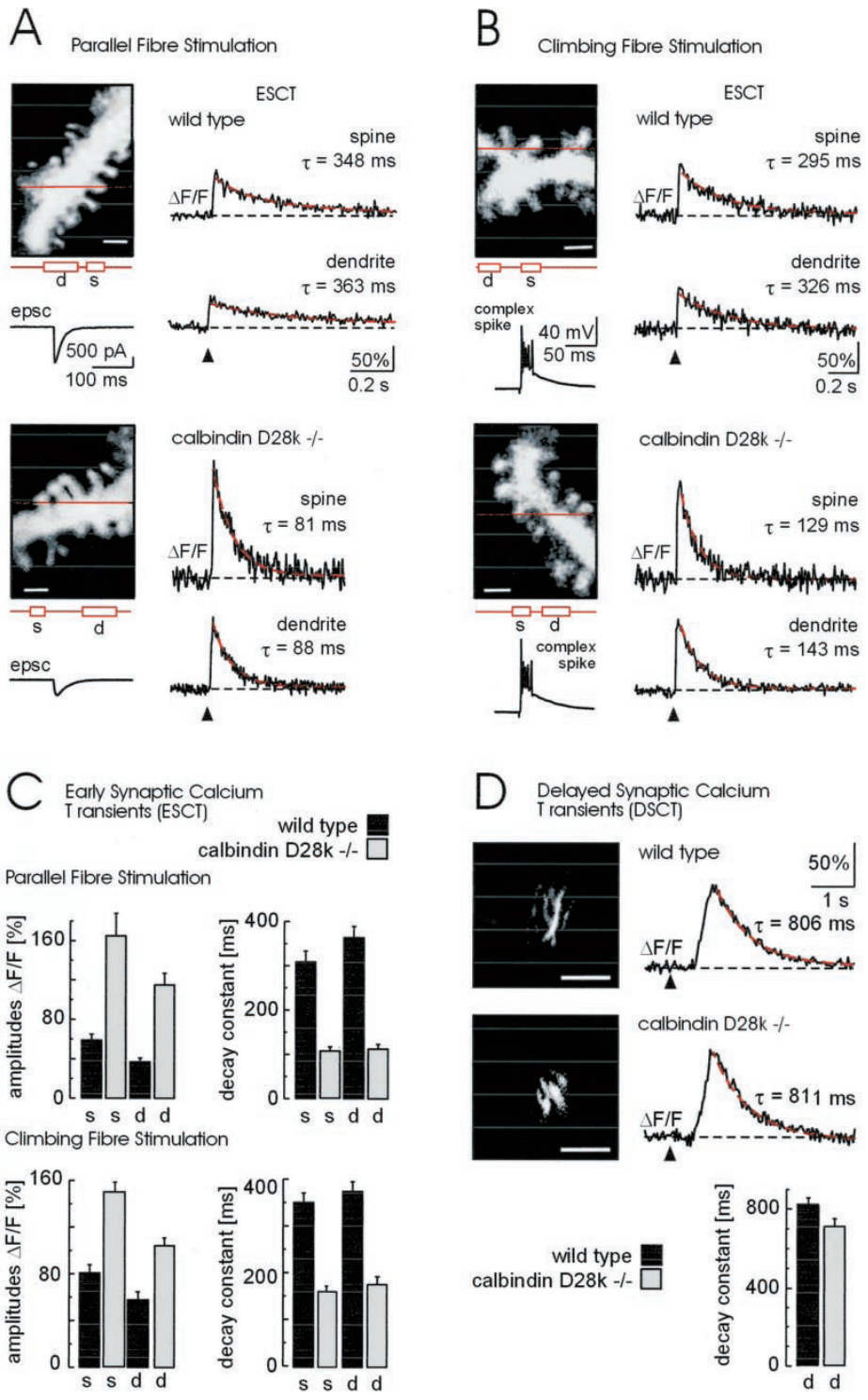
Two systems in which the functioning of the cerebellum is rigorously defined are the oculomotor and vestibular systems. By measuring the optokinetic reflex (OKR) and vestibulocular reflex (VOR) of recombined and control animals, we can study the function of calbindin in the cerebellar Purkinje cells and the effect of its absence on the compensatory eye movements. Gain and phase parameters were recorded as a measure for the amplitude and timing of eye movements relative to those of the visual scene or head (Van Alphen et al., 2001).

The gain of the OKR was significantly lower in recombined than control animals at all stimulus frequencies tested (0.1 Hz – 1.6 Hz; $p < 0.01$; repeated measures ANOVA), while the phase values were not significantly different (Fig. 3A, B). Similarly, gain but not phase of the VOR measured in the light (VOR-L) was significantly reduced at all frequencies (0.2 Hz – 1.6 Hz; $p = 0.02$; repeated measures ANOVA) (Fig. 3C, D). In contrast, VOR-D parameters were not altered; the average gain and phase values of both animal groups varied from 0.1 to 0.3 and a lead from 123 to 18 degrees, respectively (not shown). Thus, the OKR and the visual enhancement of the VOR, which are both dependent on transmission of visual signals through the cerebellar Purkinje cells of the floccular cortex, were both affected, whereas the VOR in the dark, which does not significantly depend on the flocculus (see chapter 2), appears not to be affected. One might argue that visual signal transmission is also impaired in the retina itself, because retinal bipolar cells express L7/pcp-2 and the Cre transgene used in this study (Barski et al., 2000). Yet, this possibility can be excluded as calbindin is not expressed in these cells in mice (Celio, 1990; Pochet et al., 1991). Therefore, the present data show that absence of calbindin in cerebellar Purkinje cells specifically affects the performance of movements that are dependent on transmission through Purkinje cells.

4.4.4 Calcium transients evoked by synaptic stimulation

To find out whether the behavioural deficits described above can be due to long-lasting changes in fast or delayed calcium transients, we measured synaptic calcium transients in Purkinje cells in cerebellar slices (Fig. 4A,B) using a combination of whole-cell patch-clamp recordings with two-photon imaging. As reported earlier, single-shock parallel fiber activation produced fast calcium transients in dendrites and spines due to an AMPA receptor-mediated local depolarization associated with Ca^{2+}-entry (Eilers et al., 1995). These early synaptic calcium transients (ESCTs) were 2-3 times larger in amplitude and their decay time constants 3-4 times shorter in recombined than in control animals.

We also investigated synaptic calcium transients elicited by climbing fiber stimulation that results in a standard all-or-none electrical response, the so-called “complex spike” (Llinás


A
Parallel Fibre Stimulation
ESCT
wild type
spine
τ = 348 ms
ΔF/F
dendrite
τ = 363 ms
d s
epsc
500 pA
100 ms
50%
0.2 s
calbindin D28k -/-
spine
τ = 81 ms
dendrite
τ = 88 ms
s d
epsc
B
Climbing Fibre Stimulation
ESCT
wild type
spine
τ = 295 ms
dendrite
τ = 326 ms
d s
complex spike
40 mV
50 ms
50%
0.2 s
calbindin D28k -/-
spine
τ = 129 ms
dendrite
τ = 143 ms
s d
complex spike
C
Early Synaptic Calcium Transients (ESCT)
wild type
calbindin D28k -/-
Parallel Fibre Stimulation
amplitudes ΔF/F [%]
160
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Delayed Synaptic Calcium Transients (DSCT)
wild type
50%
1 s
τ = 806 ms
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calbindin D28k -/-
τ = 811 ms
ΔF/F
wild type
calbindin D28k -/-
decay constant [ms]
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Figure 4. Synaptic Ca2+-signaling. A, Top: Line scan recording of early synaptic calcium transients evoked by single parallel fiber stimulation (ESCT) in a spine and the adjacent dendrite of a not recombined mouse. Left: Image of the spiny dendrite and the line and regions of interest chosen for the experiment (scale bar: 1 µm; d: dendrite; s: spine). Below: Current (EPSC) evoked by parallel fiber stimulation (stimulation intensity: 30 V). Right: ESCT in the spine and the dendrite. Broken red lines represent mono-exponential decay functions fitted to the data points. Time constants (τ) were 348 ms (spine) and 363 ms (dendrite). Bottom: ESCT in a spine and the adjacent dendrite of a recombined mouse. Left: cf. top, stimulation intensity was 10 V. Right: Despite the smaller EPSC amplitude, ESCT amplitudes were much larger than in not recombined animal. Time constants were significantly faster. B, Top: Line scan recording of early synaptic calcium transients evoked by climbing fiber stimulation in a spine and the adjacent dendrite of a not recombined mouse. Left: cf. A, below: Complex spike evoked by climbing fiber stimulation. Right: Calcium transients in the spine and the dendrite and time constants calculated from monoexponential decay functions (cf. A). A, Bottom: Calcium transients in a spine and the adjacent dendrite of a recombined mouse. Right: Despite the similar electrical response amplitudes of calcium transients were much larger than in not recombined animal. Time constants were significantly faster. Calcium transients represent averages of 3 consecutive trials, arrowhead indicates the time of synaptic stimulation. C: Histograms of ESCT amplitudes and decay time constants in spines (s) and dendrites (d) of not recombined and recombined animals (parallel fiber stimulation: black bars; n = 21 dendrites, 30 spines; grey bars; n = 17 dendrites, 44 spines; climbing fiber stimulation: black bars; n = 11 dendrites, 26 spines; grey bars; n = 18 dendrites, 35 spines;). Error bars represent s.e.m. D, mGluR-mediated synaptic calcium signaling. Right: Local dendritic Ca^{2+}-signals mediated by repetitive parallel fiber stimulation (marked by arrowheads, 5 pulses, 50 Hz) in the presence of 40 µM CNQX. Images demonstrate activated dendritic regions (scale bars: 20 µm). Traces show relative changes in fluorescence in regions of interest within these active regions. Broken red lines represent monoexponential decay functions fitted to the data points. Time constants were 806 ms (not recombined) and 811 ms (recombined). Below: Histogram of dendritic decay time constants of the delayed synaptic calcium transients evoked by repetitive parallel fiber stimulation (DSCT) (n = 5 for not recombined and n = 7 for recombined; averages are not significantly different (*t*-Test, $p < 0.01$)). Error bars represent s.e.m.

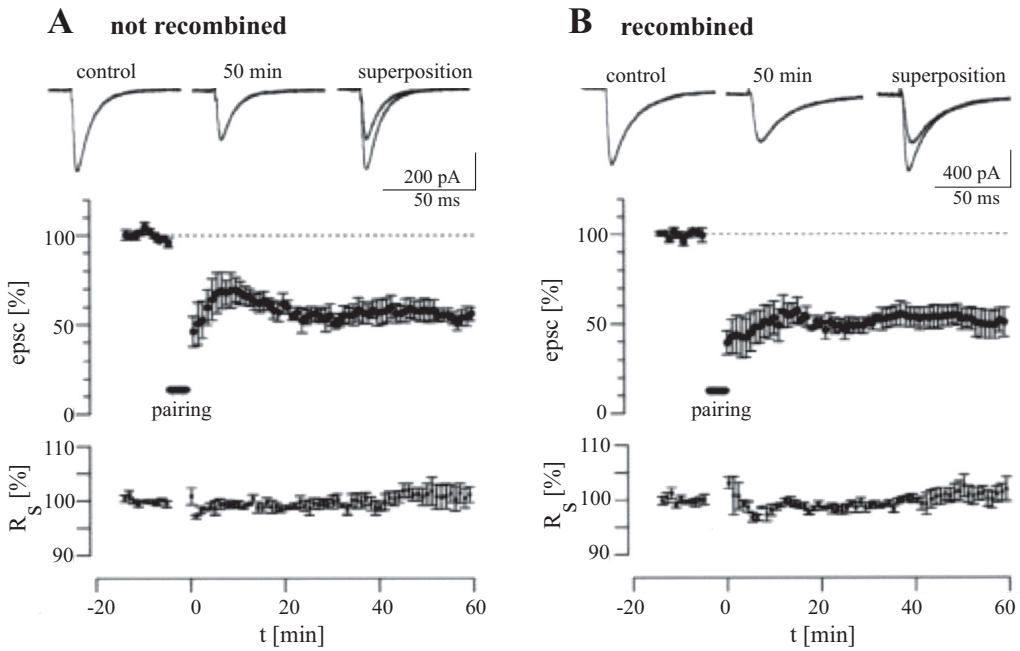

Fig.5. LTD in not recombined mice (A) and recombined mice (B). Upper panels: PF-EPSCs (average of 12 traces for A; a single experiment for B) recorded 5 min before start of LTD-induction (control) and 50 min after the end of LTD-induction (50 min). The superposition demonstrates the reduction in amplitude after pairing. On average, the pairing protocol induced a reduction of approximately 40% in the amplitude of PF-EPSCs in both groups (middle panels, summary of 4 experiments; error bars = s.e.m.). The series resistance (R_s; lower graphs) remained well within the range of 10% change compared to the baseline value in all experiments.

and Sugimori, 1980b). Single-shock activation of climbing fibers evoked a complex spike that consisted of a burst of 5-6 action potentials. As observed earlier (Airaksinen et al., 1997), the electrical responsiveness of the Purkinje neurons was indistinguishable between control and calbindin-deficient mice. Postsynaptic calcium transients were detected throughout the entire dendritic tree and in adjacent dendritic spines. The decay of climbing fiber evoked calcium transients was best fitted with a mono-exponential function (Fig. 4B, C). Although the electrical response was similar in control and mutant animals, striking differences between the amplitudes and time courses of fast synaptic calcium transients were observed. In mutant mice, peak amplitudes were enhanced by about 80% in both spines and dendrites, and decay time constants were reduced by more than 50% (Fig. 4B, C). These data clearly show that calbindin-deficiency of Purkinje neurons results in a considerable increase in amplitude and decrease in decay time constant of fast postsynaptic calcium transients.

To address the question of whether calbindin also affects the amplitude and kinetics of slow postsynaptic calcium signals, a third type of calcium transients that can be evoked by repetitive PF stimulation was investigated (Finch and Augustine, 1998; Takechi et al., 1998). These transients start only about 200 ms after stimulation and peak within 500 ms and were thus termed "delayed synaptic calcium transients" (DSCTs) (Takechi et al., 1998). DSCTs represent IP_3-mediated Ca^{2+}-release signals from intracellular stores following activation of mGluRs. Recent work suggests that these are essential for behaviourally relevant forms of LTD at the PF-Purkinje cell synapse (Wang et al., 2000). We analyzed dendritic DSCTs in the presence of the AMPAR antagonist CNQX by means of conventional confocal microscopy and whole-cell patch clamp. In contrast to ESCTs, DSCTs were not significantly altered in recombined mice (Fig. 4C,D). These data indicate that calbindin selectively buffers only fast synaptically-evoked Ca^{2+}-transients, and that it acts close to synaptic sites.

4.4.5 LTD at the parallel fiber-Purkinje cell synapse

To find out whether the performance deficits are indeed only due to changes in fast calcium transients and not to changes in induction of LTD at the parallel fiber – Purkinje cell synapse, we studied LTD using whole-cell patch-clamp in acute cerebellar slices in which postsynaptic effects of calbindin deficiency can be studied independently from presynaptic alterations. The stimulation protocol we applied produces reliable, nearly saturating depression. The same and similar protocols have revealed impaired LTD in other mutant mouse models (Aiba et al., 1994; Conquet et al., 1994; Kashiwabuchi et al., 1995; De Zeeuw et al., 1998b). In both control and Purkinje cell-specific calbindin null mutant mice, the amplitude of Purkinje cell EPSCs evoked by PF stimulation decreased by 40% after performing the LTD induction protocol, and remained at this level for at least 60 min (Fig. 5). There were no differences in the time course of depression of EPSCs. These results indicate that the buffering of calcium signals by calbindin is not harmfull for induction and maintenance of a form of LTD, which is often impaired in cerebellar mutants and necessary for the induction of cerebellar motor learning.

4.4.6 Basal firing properties of Purkinje cells in alert mice

The findings described above raise the possibility that the behavioural deficit in the Purkinje cell specific calbindin mutant is caused by changes in the regulation of fast calcium transients at the level of the dendrites and/or at the level of the axon terminals. If it is merely caused by changes

Table 1. Purkinje cell firing in awake mice

	FF SS	cv SS	FF CS	cv CS	CS pause	m.p. ISI SS
not	77.60±23.05	0.53±0.31	0.94±0.33	0.88±0.16	12.58±7.11	11.32±5.19
rec	67.31±23.11	0.62±0.48	1.02±0.31	0.80±0.15	12.41±5.53	13.26±4.42

not rec: not recombined, n = 42; rec: recombined, n = 38. All frequencies are in Hz; FF SS: simple spike firing frequency; FF CS: complexspike frequency; cv SS coefficient of variation of simple spike frequency; cv CS coefficient of variation of CS frequency; CS pause: complex spike pause in ms; m.p.ISI SS: most probable simple spike interspike intervan. Data indicates mean±std.

at the dendritic level, one expects that at least the simple spike activities of the Purkinje cells will be affected, while the impact at the axonal level must be dominant if the Purkinje cell activities recorded at the level of the cell body are unaffected. We therefore recorded extracellular single unit activities of Purkinje cells from the left cerebellar vermis, paramedian lobule and flocculus of alert restrained mice. Population averages of simple and complex spike frequency were similar in both groups (Table 1). The duration of the complex spike pause was almost identical in both groups. The regularity of firing of the simple spikes and complex spikes as indicated by the coefficient of variation was identical for both groups and agree with those recorded in other mouse strains (Goossens et al., 2001). The most probable interspike interval for simple spike activity (the inverse of the instantaneous firing frequency) did not show significant differences of increased variability in recombined re wild type animals. Finally, as far as can be determined with the use of extracellular recordings, the shape and size of both the simple spikes and complex spikes of the mutants were indistinguishable from those of the control animals. Thus, Purkinje cell specific calbindin deficiency does not result in marked alterations of their discharge properties when recorded at the level of their cell bodies.

4.5 Discussion

Our results demonstrate that calbindin expressed in Purkinje cells is an essential determinant of normal motor performance. The deficits in both locomotion and compensatory eye movements as observed in mutants lacking calbindin in Purkinje cells are permanent and apparently cannot be compensated by learning-related or other plasticity mechanisms.

The deficits in locomotion in Purkinje cell specific mutants resembled those of the global knock-outs of calbindin (Airaksinen et al., 1997) indicating that the role of calbindin in Purkinje cells is dominant for the locomotion system and that the role of calbindin in neurons upstream or downstream of Purkinje cells remains to be elucidated. Since the slip rate in the mutants during the runway and horizontal bar test remained significantly higher throughout the entire test period, it appears likely that lack of calbindin in Purkinje cells causes a permanent impairment of coordination that cannot be compensated for by prolonged training. The same experiments showed that the phenotype is in fact relatively subtle in that the mutants do show non-ataxic locomotion and that the differences in slip rate only become robust when the system is challenged profoundly. The compensatory eye movement tests showed comparable results. OKR and visual enhancement of the VOR, which are both dependent on transmission of visual signals through the cerebellar Purkinje cells of the floccular cortex, were both affected, whereas the VOR in the dark was not. Thus this test indicates that the deficit is restricted to the Purkinje cells and that we cannot detect any secondary aberrations at their postsynaptic neurons in the cerebellar and

vestibular nuclei neurons. In addition, we showed that the deficits of the OKR and VOR-L were only reflected in their gain values in contrast to the compensatory eye movements of for example lurcher mice, which are ataxic and show significant, phase aberrations (van Alphen et al., 2002), unlike L7-calbindin mutants. Yet, similar to the impact on locomotion, the deficits were not only subtle and restricted to specific parameters, but also permanent. It is in fact striking that the OKR and VOR-L gain values of the L7-calbindin mutants, which have normal LTD induction, do not reach normal levels over time. In this respect L7-calbindin mutants differ from L7-PKCi mutants, which lack LTD induction but show normal baseline gain and phase values during both OKR and VOR (De Zeeuw et al., 1998b). Apparently, calbindin in Purkinje cells serves some particular role(s) that cannot be compensated for over time.

Our cell physiological data confirm that calbindin operates as a rapid Ca^{2+}-buffering protein (Roberts et al., 1993; Airaksinen et al., 1997; Maeda et al., 1999) and suggest that at least four types of Ca^{2+}-signals are affected by calbindin deficiency. First, as shown above, the AMPAR-mediated Ca^{2+}-transients produced by parallel fiber stimulation in spines and dendrites are distorted. In spines and the adjacent dendrites, the amplitudes of the Ca^{2+}-transients are enhanced and the time constant of the decay is markedly faster. This distortion is predicted by the absence of a fast Ca^{2+}-buffer (Wagner and Keizer, 1994). Second, climbing fiber-mediated postsynaptic Ca^{2+}-signals exhibit a similar modification in amplitude and time course in the absence of calbindin (cf. Fig. 4B and C), while the electrical responsiveness of the Purkinje cells is apparently not changed. The distortion is observed in dendrites and interestingly also in spines. These results are similar to those described by Airaksinen et al. (1997), who reported amplitude changes that are in the same range. However, while the decay phase of synaptic calcium transients was fitted with bi-exponential functions in the foregoing study, the decay phase of the vast majority of calcium transients analyzed in the present study are best fitted with mono-exponential functions. Both the measurement in small compartments and the use of a low dye concentration implicated a reduced signal-to-noise ratio, which might have masked a small additional fast decay time constant in the results by Airaksinen et al. (1997). Moreover, Airaksinen et al. found that while the amplitude of the calcium transients was increased in calbindin-deficient mice, the time constants were unchanged (cf. Fig. 4 in Airaksinen et al., 1997). If the data of Airaksinen et al. are fitted with only one exponential, the resulting time constants are identical to those obtained in the present study. We believe that the measurements performed in the present paper are more accurate, since we monitored the calcium signals directly at the site of calcium entry. In contrast, the earlier study was performed in thick dendrites relatively close to the cell body.

A third type of Ca^{2+}-signals that might be distorted by calbindin deficiency are action potential associated Ca^{2+}-transients in the cell bodies (Llinás and Sugimori, 1980a), dendrites (Llinás and Sugimori, 1980b), and axons (Callewaert et al., 1996). Thus, changes in action potential-evoked Ca^{2+}-transients may alter signal integration in dendrites and spines, for example by a reduced efficacy of coincidence detection (Wang et al., 2000), and/or it may affect the frequency of action potentials traveling along the axon (Callewaert et al., 1996). Finally, transmitter release from Purkinje cell nerve terminals in the cerebellar and vestibular nuclei may be altered. It has been demonstrated that another calcium-binding protein, parvalbumin, regulates the paired-pulse ratio through modulation of transmitter release (Caillard et al., 2000). Furthermore, overexpression or deletion of calbindin in hippocampal neurons resulted in selective modification of post-tetanic potentiation (Chard et al., 1995; Klapstein et al., 1998), presumably by changing the global Ca^{2+}-concentration increase in presynaptic terminals necessary for mediating potentiation of neurotransmitter release on a second to minute time scale. Thus, expression of Ca^{2+}-binding proteins in presynaptic terminals does indeed play an important role in short-term synaptic plasticity. Taken together, we can conclude that even within a single, defined cell-type of the cerebel-

lum, i.e. the Purkinje cell, calbindin may serve a wide range of cellular actions involving rapid Ca^{2+}-signaling.

Which of the four potential mechanisms described above are probably predominantly responsible for the behavioural deficits in the L7-calbindin mutants? The fact that we find permanent deficits in motor performance without changes in simple spike or complex spike activities strongly suggests that the cellular alterations contributing most significantly must be situated beyond the initial segment of the Purkinje cells. We therefore argue that it appears likely that the transmitter release from its terminals must be the dominant factor(s) responsible for the behavioural deficits.

This notion does not imply that a lack of calbindin in the dendrites and spines does not have any impact. It may well affect the integrative processes in these structures, but at the same time these aberrations may be compensated for by other cellular processes that remain intact. In this respect it may be relevant to discuss the fact that LTD induction is preserved in the L7-calbindin mutant, because Ca^{2+}-signaling is essentially needed for the induction of LTD (Sakurai et al., 1990; Konnerth et al., 1992). This preservation of LTD is mostly likely a consequence of the fact that mGluR-mediated Ca^{2+}-signaling, known to be critical for LTD induction (Inoue et al., 1998; Daniel et al., 1998; Wang et al., 2000; Ito, 2001), appears to be normal in the mutant mice. Thus, at least under experimental conditions, the distortion of AMPAR-mediated Ca^{2+}-signals does not significantly hinder LTD induction, which agrees with the fact that initial rates of improvement of runway performance were similar in calbindin-deficient and control mice, suggesting that some forms of motor learning are intact in the Purkinje cell-specific null mutant. We cannot exclude that alterations might be seen with other protocols for the induction of LTD, or in other forms of cerebellar synaptic plasticity. Interestingly, a recent report reveals conditions under which postsynaptic, NO-dependent LTP is observed at the parallel fiber - Purkinje cell synapse (Lev-Ram et al., 2002). As this LTP is enhanced by postsynaptic calcium chelators with properties similar to calbindin, a decrease in its strength might be expected in the Purkinje cell-specific null mutants. It is, however, not yet established whether calcium-dependence of this form of LTP is a direct effect or the consequence of decreased LTD, and the nature of the calcium signals potentially involved in its regulation is unknown.

The explanation for the calbindin-independency of the slow mGluR-mediated Ca^{2+}-signaling is straightforward. Our recordings of ESCTs in calbindin-deficient mice reveal decay time constants of the Ca^{2+}-signals in the range of 80-100 ms. These decay time constants are a direct measure of the Ca^{2+}-clearing mechanisms in dendrites. Because the mGluR-mediated Ca^{2+}-signal has much slower kinetics, with a time-to-peak of more than 500 ms and decay time constants in the range of 800-900 ms, Ca^{2+}-clearance as well as Ca^{2+}-buffering by calbindin will not interfere with the waveforms of DSCTs.

In sum, we conclude that calbindin probably exerts its functions at multiple sites within the Purkinje cells, and that its function at the dendrites and spines may be compensated for in calbindin null mutants by local mechanisms that remain intact, while its functions at the axonal terminals cannot be fully compensated for, which in turn lead to permanent deficits in motor performance without affecting motor learning.

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Chapter 5

Simple spike and complex spike activity of floccular Purkinje cells during the optokinetic reflex in mice lacking cerebellar long term depression

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5.1 Abstract

Cerebellar long-term depression (LTD) at parallel fiber – Purkinje cell (P-cell) synapses is thought to embody neuronal information storage for motor learning. Transgenic L7-PKCI mice in which cerebellar LTD is selectively blocked indeed exhibit impaired adaptation in the vestibulo-ocular reflex (VOR) while their default oculomotor performance is unaffected. Although supportive, these data do not definitively establish a causal link between memory storage required for motor learning and cerebellar LTD. Because the L7-PKCI transgene is probably activated from the early stages of P-cell development, an alternative could be that P-cells develop abnormal signals in L7-PKCI mutants, disturbing mechanisms of motor learning that rely on proper P-cell outputs. To test this alternative hypothesis, we studied simple spike (SS) and complex spike (CS) activity of vertical axis (VA) P-cells in the flocculus of L7-PKCI mice and their wild-type littermates during sinusoidal optokinetic stimulation. Both SS and CS discharge dynamics appeared to be very similar in wild-type and transgenic P-cells at all stimulus frequencies (0.05 - 0.8 Hz). CS activity of all VA-cells increased with contralateral stimulus rotation and lagged ipsiversive eye velocity by 180–165 deg. SS modulation was roughly reciprocal to the CS modulation and lagged ipsiversive eye velocity ~ 15 deg. The baseline SS and CS discharge characteristics were indistinguishable between the two genotypes.

We conclude that the impaired VOR learning in L7-PKCI mutants does not reflect fundamental aberrations of the cerebellar circuitry. The data thus strengthen the evidence that cerebellar LTD is implicated in rapid VOR learning, but not in the development of normal default response patterns.

5.2 Introduction

Mechanisms of synaptic plasticity, such as long-term potentiation (LTP) and LTD, are candidate mechanisms for information storage in the brain (e.g., Linden & Conner, 1995). A major goal in neuroscience is to understand how these mechanisms function in neural circuits that control learning in animal behaviour. The flocculus of the vestibulo-cerebellum is a crucial part of the reflex circuitry involved in the control and learning of compensatory eye movements, including the optokinetic and vestibulo-ocular reflex (OKR and VOR). More specifically, it has been hypothesised that learning in the VOR relies on LTD of the parallel fiber-Purkinje cell synapses, driven by the coincidence of vestibular parallel fiber and visual climbing fiber signals (see e.g. Ito 1998, for review). This hypothesis represents a specific implementation of the general idea that synaptic plasticity in the cerebellar cortex is one of the major mechanisms of cerebellum-dependent motor learning.

Recent studies using various knock-out mice have supported this theory by showing correlations between deficits in LTD and behavioural learning (Aiba et al., 1994; Conquet et al., 1994; Funabika et al., 1995; Kashiwabuchi et al., 1995; Shibuki et al., 1996). However, the interpretation of this work has suffered from the limitations that the knock-out technique lacks anatomical and functional specificity of the genetically induced lesions. To overcome these limitations, we have previously created a transgenic mouse in which a protein kinase C inhibitory peptide, PKC[19-31], is selectively expressed in P-cells (De Zeeuw et al., 1998). Both cerebellar LTD and adaptation of the VOR is impaired in these L7-PKCI mice while their default eye movement performance is unaffected (De Zeeuw et al., 1998; Van Alphen & De Zeeuw, 2002; Gao et al., 2003).

Although these data are consistent with LTD playing a role in actual memory storage,

there is an alternative way of interpreting the results. It is possible that rapid motor learning is deficient in these mutants because the mutation disturbs the signal processing within the flocculus, which in turn could disturb the learning process (see also Lisberger, 1998). To explore this possibility, we recently studied the spontaneous discharge of P-cells in the vermis and paramedian lobule of alert L7-PKCI mice and their wild-type littermates (Goossens et al., 2001). It appeared that the SS and CS discharge properties of P-cells in L7-PKCI mice are normal, indicating that neither the activation of PKC nor the induction of LTD is essential for the normal baseline operation of the cerebellar circuitry.

Still, these results do not rule out the possibility that the neural signals in the flocculus of the cerebellum, which are known to be related to the control and learning of compensatory eye movements, are abnormal in LTD-deficient animals. In fact, very little is known about these signals even for wild-type mice. Because the L7-PKCI transgene is probably activated from the early stages of P-cell differentiation and maturation (De Zeeuw et al., 1998), it could well be that a normal development of P-cell responsiveness to parallel fiber and/or climbing fiber signals is not possible in the L7-PKCI mutant. Clearly, if the P-cell output were abnormal, it could disturb mechanisms of synaptic plasticity that rely either directly or indirectly on proper P-cell signals. This possibility complicates interpretation of the relation between LTD blockade and motor learning behaviour.

To test whether P-cell signals are disturbed in the LTD-deficient L7-PKCI mutant, we studied the SS and CS discharge properties of P-cells in the flocculus of L7-PKCI mice and their wild-type littermates during the optokinetic reflex.

5.3 Materials and Methods

5.3.1 Animal preparation

Data were collected from heterozygous transgenic L7-PKCI mice and their wild-type littermates (C57BL/6 mouse strain background; 3-12 months old). In the L7-PKCI mouse, the pseudo substrate PKC inhibitor, PKC[19-31], is selectively expressed in P-cells under the control of the pcp-2(L7) gene promoter. The animals were prepared for chronic neurophysiological experiments under inhalant anaesthesia using procedures described in a recent paper (Goossens et al., 2001). In short, a head-holder was implanted on the scull and a recording chamber was placed over a small hole in the cerebellar cranium. In addition, all mice received a search coil implant for recording of eye position (see Van Alphen et al., 2001, for details). During an experiment, the animal was immobilised in a custom restrainer by bolting the head holder to a head fixation post. Head orientation was such that the horizontal semicircular canals were in the horizontal plane (plane of nasal bone tipped approximately 35 deg down). All experiments were conducted in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) and were reviewed and approved by the local ethics committee of the Erasmus University Rotterdam.

5.3.2 Optokinetic stimulation

A panoramic stimulus was used for binocular visual stimulation. The stimulus consisted of a random-dot pattern that was back-projected on a translucent cone-shaped dome surrounding the mouse. To generate stimulus rotations about the vertical axis running through the centre of the animals' interaural line, the projection system was equipped with a servomotor that could rotate a

slide in front of the lens. Stimulus position was measured and controlled by a CED 1401plus unit (Cambridge Electronic Design Limited, Cambridge, UK). We used sinusoidal stimuli consisting of at least 10 continuous cycles, and lasting at least 120 sec. Stimulus amplitude was ± 5 deg and frequencies were 0.05, 0.1, 0.2, 0.4 and 0.8 Hz. In this manner, peak velocities ranged from 1.5 to 25 deg/s. In a few animals, the OKR was also tested at 0.025 and 1.6 Hz.

5.3.3 Eye movement recordings

The position of the left eye was measured with the magnetic induction method using miniature coils that were implanted on the lateral side of the eye (Van Alphen et al., 2001). Eye position signals were sampled at 500 Hz (1401plus, Cambridge Electronic Design (CED), Cambridge, UK) and stored on disk for off-line analysis using Spike 2 (CED). The horizontal component of the eye position signal was calibrated by rotating the magnetic field ± 10 deg about the vertical axis running through the centre of animals' interaural line.

5.3.4 Single cell recordings

Extracellular activity was recorded with glass micropipettes that were advanced into the left flocculus by a hydraulic micro-drive equipped with a stepping motor (see Goossens et al. 2001, for details). The raw electrode signal was amplified, filtered, digitised and stored on disk for off-line analysis. Single unit P-cell activity was identified by the presence of a brief pause in SS discharge after a CS, and was carefully monitored during the course of a recording. Once a P-cell was isolated, its preferred axis of rotation was determined by monitoring its CS activity while moving a random dot pattern in various directions (Simpson et al., 1988). Between recording sessions the brain was covered by a silastic sheet and the chamber was sealed.

5.3.5 Histology

The anatomical locations of the recording sites were marked either by methylene blue injections or by electrolytic lesions. The animals were deeply anaesthetised with pentobarbital sodium and transcardially perfused with saline followed by 4% Formalin. Sagital slices of the brainstem and cerebellum (50 μm thick) were prepared and stained according to standard histological procedures. Inspection of successive slices at the light microscopic level confirmed that all recording sites were located within in the flocculus (see Fig. 1).

5.3.6 Data analysis

Off-line analysis was performed in Matlab (Mathworks Inc., Natick, MA, USA). Eye velocity was calculated from the calibrated eye position data, and quick phases were removed. Gain and phase of the eye movements relative to the stimulus were determined by fitting sine functions to the slow-phase eye velocity traces. The response gain was defined as ratio between the (fitted) amplitudes of the eye velocity and stimulus velocity traces.

SS and CS were detected and discriminated with custom software that clustered groups of spikes by means of a linear discriminant analysis on the first four principal components of the

spike wave forms (see e.g., Eggermont, 1990). Histograms of SS triggered on the occurrence of a CS were made (bin width 1 ms) to verify that each isolated P-cell showed a clean climbing fibre pause (Simpson et al. 1996). Lack of a pause was taken to indicate that the cell was not a P-cell, or isolation was imperfect. Spontaneous activity of each P-cell was characterised by 1) the mean SS and CS firing rate, 2) the SS and CS coefficient of variance, and 3) the climbing fiber pause duration (see also Goossens et al., 2001).

P-cells that showed optimal CS modulation for stimulus rotations about the vertical axis were selected for further analysis. Peri stimulus time histograms (PSTH) were made to evaluate modulation of the SS and CS discharge (48 and 24 bins per cycle, respectively), where quick-phase epochs were discarded. The percentage of SS modulation amplitude, A, was determined using Fourier analysis: $A = a_1 / a_0$ x 100%, with a_0 the mean firing rate and a_1 the amplitude at fundamental frequency, f. To further assess whether wild-type and mutant P-cells generate different responses in relation to the actual OKR movements, the SS and CS firing rates (F) were both quantified as function of eye position (E) and eye velocity (E'):

$$\begin{array}{ll} \text{for } F(t) = kE(t) + rE'(t) + c & \text{for } F(t) > 0 \\ F(t) = 0 & \text{otherwise} \end{array}$$

where t denotes time in seconds, k denotes apparent eye position sensitivity in spk/sec per deg, and r is the apparent eye velocity in spk/sec per deg/sec (Stahl and Simpson, 1995). The least squares criterion was used to determine the best-fit parameters. Unlike the Fourier analysis, this procedure provides a convenient way to quantify the P-cell discharge because it can account for complete suppression of the discharge at a particular phase of the response (see Fig. 3 for illustration), and because it allows one to exclude quick-phase epochs. Note, however, that by correlating the P-cell activity with various components of the eye movement, we do not mean to imply a causal linkage. The sensitivity parameters merely serve as a tool to quantify possible differences between wild-type and mutant P-cell responses taking into account the animals' actual eye movement behaviour. Statistical evaluation of the fit results indicated significant SS and CS modulation for all identified VA-cells ($P < 0.01$; Pearson's correlation coefficients typically > 0.75). The phase of the firing rate in reference to ipsiversive eye velocity (θ) at each stimulus frequency (f) was obtained from $\theta = \arctan [k / 2\pi f \cdot r]$. Phase leads were taken as positive and phase lags as negative. The magnitude sensitivity (M), which is the ratio of the amplitudes of firing rate and eye

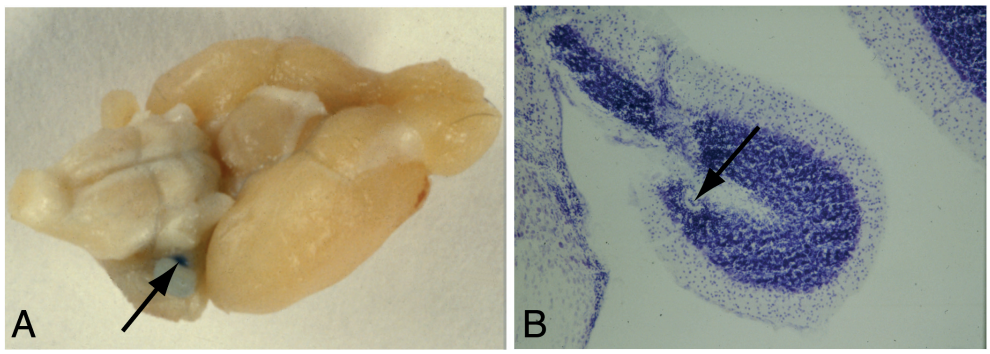

Figure 1: Histology of the recording sites confirmed that the recordings were from floccular P-cells. A: Injection of methylene blue at one of the recording sites in a C57BL/6 mouse. B: Sagital slice of brainstem and cerebellum of an L7-PKCI mouse with electrolytic lesions along an electrode track through the flocculus.

position, was calculated from the relation $M = \mathrm{sqrt}((2\pi f \cdot r)^2 + (k)^2)$.

Statistical analysis of the data included non-orthogonal (i.e., "unequal n") two-factor analysis of variance (ANOVA) and unpaired Student's *t*-tests (two-tailed) to evaluate differences in means as well as non-parametric Wilcoxon rank-sum tests for comparison of medians. Kolmogorov-Smirnov and Kuiper tests were used to evaluate differences in empirical distribution functions (see e.g., Press et al., 1992). Significance levels (*P*) are indicated in the text. To determine whether violations of the normality assumption and heteroscedasticity of the data affected the outcomes of the ANOVAs, we also used a two-factor ANOVA by ranks in an extension of the Kruskal-Wallis test called the Scheirer-Ray-Hare test (Scheirer et al, 1976). The results from these tests are not shown since the outcomes of the classical ANOVAs proved to be valid in all cases. To augment the traditional hypothesis testing approach, 95% confidence intervals (*CI*, two-sided) of the differences between the two genotypes are reported to indicate upper and lower limits of the observed differences. Since data were not normally distributed, these 95% confidence intervals were estimated with the use of a Monte Carlo bootstrap procedure in which the observations were resampled 10,000 times (with return).

5.4 Results

We recorded single unit activity of P-cells in the flocculus of L7-PKCI transgenic mice ($n = 7$) and their wild-type littermates ($n = 7$). Simultaneous records of SS and CS activity were obtained from 54 wild-type and 44 mutant P-cells. Histology of recording sites confirmed that the isolated P-cells were located within the flocculus (see Fig. 1).

5.4.1 Spontaneous Purkinje cell activity

Spontaneous activity of floccular P-cells was recorded for about 2-3 min in the light. Table 1 summarizes the results. Note that both the mean SS firing rates (~ 60 spk/s) and the mean CS firing rates (~ 0.9 spk/s) were very similar between wild-type and L7-PKCI P-cells. Statistical analysis indicated that neither the SS firing rates nor the CS firing rates were significantly different between the two P-cell populations (Student's *t*-test, $P > 0.2$; Wilcoxon rank-sum test, $P > 0.2$). The temporal jitter in the spontaneous SS and CS discharge, as quantified by the coefficient of variance, was also not significantly different between the two P-cell populations (Student's *t*-test, $P > 0.1$; Wilcoxon rank-sum test, $P > 0.3$). Average values for the SS coefficient of variance as well as the CS coefficient of variance were ~0.7 in both genotypes (Table 1). The average duration of the climbing fibre pause was ~13 ms in both wild-type and mutant P-cells (not significantly different; Student's *t*-test, $P > 0.3$; Wilcoxon rank-sum test, $P > 0.2$). The right-hand column of Table 1 lists the 95% confidence intervals for the differences between the two genotypes to indicate the sampling allowances for these observations. For each parameter, we also examined possible differences in its empirical distribution function using two-sample Kolmogorov-Smirnov and Kuiper tests, but no significant differences between the two genotypes were found ($P > 0.3$ for all six parameters and for both types of tests).

5.4.2 Eye movements

The gain and phase of the optokinetic eye movement responses of the wild-type and L7-PKCI

mice used in this study were in the same range as the ones previously observed by Van Alphen et al. (2001, 2002). With stimulus amplitude fixed at ± 5 deg, the average gain of the optokinetic reflex gradually decreased from about 0.35 at 0.025–0.05 Hz to approximately 0.02 at 1.6 Hz (Fig. 2). The phase of the eye velocity in reference to stimulus velocity (relative to the head) ranged from an average lead of about 10 deg at 0.025–0.05 Hz to an average lag of about 35 deg at 1.6 Hz (Fig. 2).

Table 1. Spontaneous P-cell activity

		C57BL/6 (n = 34)	L7-PKCI (n = 33)	95% CI difference
Simple Spikes	Mean firing rate (spk/s)	55 ± 20	59 ± 19	[-5, 13]
	Coefficient of variance	0.7 ± 0.3	0.7 ± 0.2	[-0.1, 0.1]
Complex Spikes	Mean firing rate (spk/s)	0.9 ± 0.4	0.8 ± 0.3	[-0.3, 0.1]
	Coefficient of variance	0.8 ± 0.2	0.7 ± 0.1	[-0.1, 0.1]
Climbing fibre pause (ms)		12 ± 9	14 ± 7	[-2, 6]

Parameters of spontaneous simple spike and complex spike activity of floccular Purkinje cells in C57BL/6 wild-type and L7-PKCI mutant mice (expressed as mean ± SD) and 95% confidence intervals for the differences between the two genotypes (estimated with Monte Carlo bootstrap).

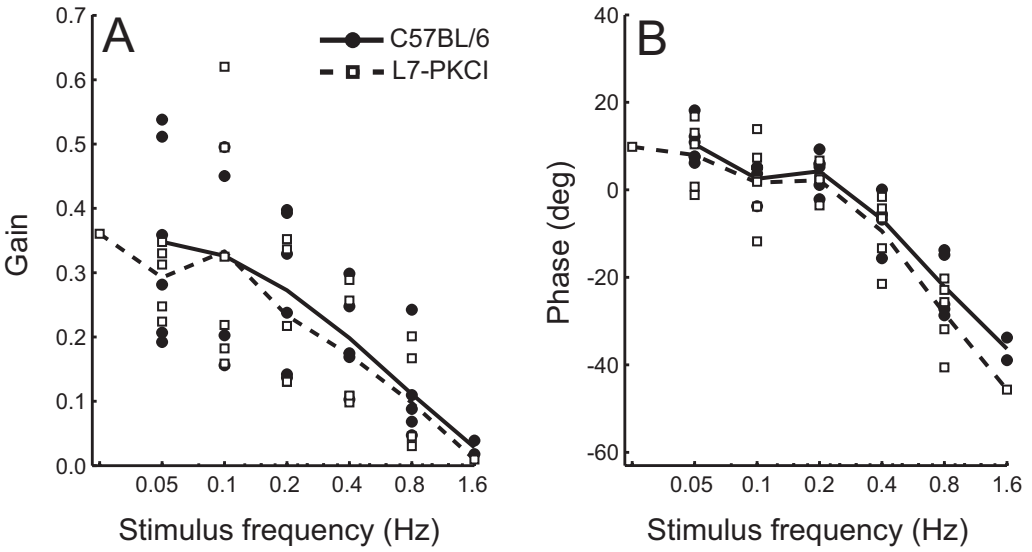

Figure 2: Gain (A) and phase (B) of the optokinetic reflex responses of the C57BL/6 wild-type mice (n = 7) and the L7-PKCI transgenic mice (n = 7) used in this study. Phase leads relative to the stimulus are positive. Each symbol represents the (mean) response of an individual animal. Solid lines are the average values for each genotype. Note very similar gain and phase relations for the two genotypes.

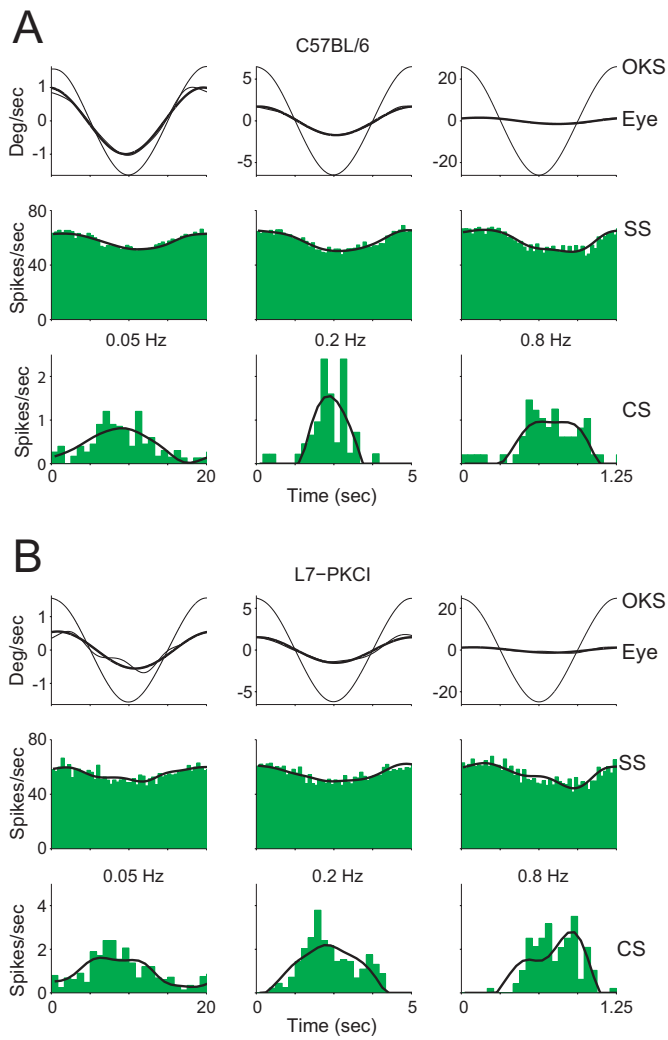

Figure 3: Simple spike (SS) and complex spike (CS) responses of a typical vertical axis (VA) Purkinje cell in the flocculus of a C57BL/6 wild-type (*A*) and an L7-PKCI mouse (*B*) to sinusoidal optokinetic stimulation (OKS) at three different frequencies (0.05, 0.2 and 0.8 Hz; ± 5 deg amplitude).

Top row: velocity of the optokinetic stimulus (OKS) and eye velocity (Eye) in world coordinates (ipsiversive motion is positive). Thick lines are sine fits to the eye movement data.

Middle and bottom row: Peri stimulus time histograms of SS and CS activity, respectively. Thick lines are data fits of the (conditional) firing rate as function of measured eye position and eye velocity (see Methods and Results).

5.4.3 Response pattern of vertical axis Purkinje cells

Of all 98 P-cells recorded in this study, 39 wild-type and 36 transgenic cells were identified as vertical axes P-cells (VA-cells for short) and could be tested in further detail during vertical axis optokinetic stimulation. The CS discharge of the remaining 23 P-cells showed optimal sensitivity for stimulus rotations about the horizontal axis oriented at 135˚ ipsilateral azimuth. In line with the modular organization of the cerebellum, the two types of P-cells were reproducibly encountered at distinctly different locations (as inferred from successive penetrations in the same animal) in both genotypes.

Figure 3 illustrates the SS and CS discharge of a typical wild-type (Fig. 3A) and mutant VA-cell (Fig. 3B) during optokinetic stimulation at three different frequencies (0.05, 0.2 and 0.8 Hz). Note that the response patterns of the two cells are qualitatively comparable. In line with the response patterns typically seen in floccular VA-cells from other species such as the rabbit (e.g.,

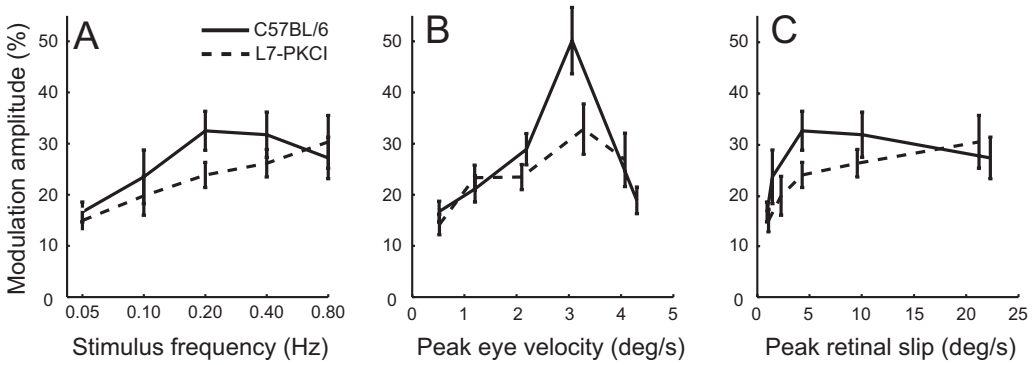

Figure 4: Mean percentage of SS modulation amplitude as function of stimulus frequency (*A*), peak eye velocity (*B*), and peak retinal slip velocity (*C*). In *A* and *C*, responses were averaged per stimulus frequency. 95% confidence intervals for the differences between C57BL/6 and L7-PKCI P-cells at 0.05-0.8 Hz are: $\Delta A_{0.05}$ = [-7, 4], $\Delta A_{0.1}$ = [-16, 8], $\Delta A_{0.2}$ = [-17, 1], $\Delta A_{0.4}$ = [-16, 4], and $\Delta A_{0.8}$ = [-9, 16] %, where the subscripts refer to stimulus frequency. In B, data were binned according to the measured peak eye velocity (bin width 1 deg/s). 95% confidence intervals for the differences between the two genotypes in each velocity bin are: $\Delta A_{0.5}$ = [-7, 3], $\Delta A_{1.2}$ = [-5, 9], $\Delta A_{2.1}$ = [-13, 2], $\Delta A_{3.2}$ = [-32, 2], and $\Delta A_{4.2}$ = [-2, 18] %, where the subscripts refer to peak eye velocity (deg/s, average between wild-type and mutant). Note very similar response curves for the two genotypes. Error bars: ±1 SEM.

De Zeeuw et al., 1995; Frens et al., 2001; Simpson et al., 1996), CS activity increased during contralateral stimulus rotation and was strongly inhibited during ipsilateral motion. SS activity, on the other hand, increased with ipsilateral stimulus rotation and decreased during contralateral rotation, resulting in a temporally reciprocal relation between SS and CS modulation. Both eye velocity and SS modulation were approximately in phase with stimulus velocity at 0.05 and 0.2 Hz, but clearly lagged stimulus velocity at 0.8 Hz. A similar phase shift can be observed in the CS activity of both cells. Complete suppression of CS activity during ipsilateral stimulus rotation was often observed at stimulus frequencies > 0.1 Hz. Complete cessation of SS activity was rare under all stimulus conditions.

Cells that showed an increase in SS firing for contralateral stimulus motion were frequently observed, but they could not be identified as VA-cells. Either they showed no pause in SS activity after the CS, or their CS activity was optimally modulated by visual world rotation about the ipsilateral 135˚ axis in the horizontal plane (Graf et al., 1988).

5.4.4 Simple spike discharge dynamics

Figure 4 quantifies the percent modulation amplitude (see Methods) of the SS discharge as function of stimulus frequency (Fig. 4A), maximum eye velocity (Fig. 4B) and maximum retinal slip velocity (Fig. 4C). As can be observed in Fig. 4A, the SS modulation amplitude for wild-type versus transgenic P-cells was very similar at each stimulus frequency (Wilcoxon rank-sum tests, $P > 0.1$, Kuiper tests, $P > 0.2$, at all frequencies). In both genotypes, the SS modulation amplitude tends to increase with increasing stimulus frequency from about 15 % at 0.05 Hz to about 30 % at 0.8 Hz. Further analysis of these data with a two-factor ANOVA confirmed that there was a significant main effect of stimulus frequency ($F_{4,175} = 3.60$, $P < 0.01$). In addition, genotype ($F_{1,175} = 1.35$, $P > 0.2$) and interaction terms ($F_{4,175} = 0.66$, $P > 0.6$) were not significant, indicating that

the two response curves in Fig. 4A are not significantly different. To augment these findings, 95% confidence intervals for the differences between the two genotypes at each stimulus frequency are indicated in the legend. As shown in Fig. 4B, also the relation between SS modulation amplitude and maximum eye velocity was comparable between the two genotypes (not significantly different; two-factor ANOVA, $F_{1,175} = 1.30$, $P > 0.2$; Wilcoxon rank-sum tests, $P > 0.05$, Kuiper tests, $P > 0.2$, at all velocity bins; minor differences in ordinate value per bin not taken into account). In both genotypes, the SS modulation amplitude tends to be greatest at about 3 deg/s (significant main effect of maximum eye velocity; $F_{4,175} = 11.01$, $P < 0.0001$). Fig. 4C re-plots the average SS modulation amplitudes from Fig. 4A as function of the average maximum retinal slip velocity at each frequency. Although the ordinate values are slightly different in this case (as in Fig. 4B), it can be inferred from the error bars (indicating ±1 SEM) that the values for mutants fall within the 95% confidence intervals estimated for the control curve (about 2 x SEM), indicating that these response curves are also not significantly different between wild-type and L7-PKCI P-cells (see analysis Fig. 4A for two-factor ANOVA).

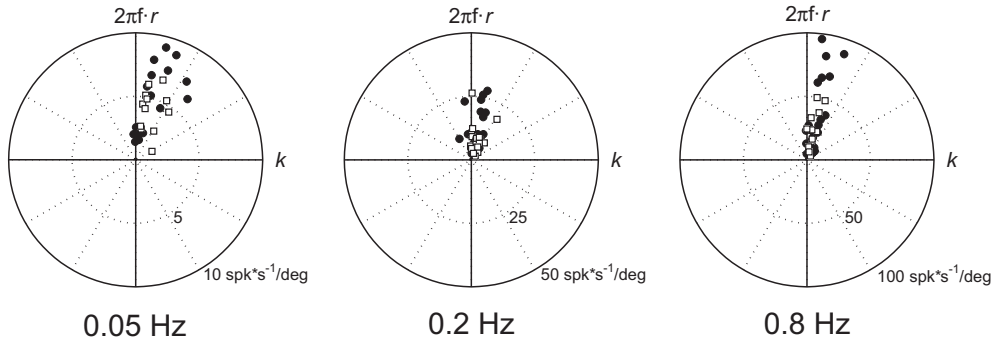

Figure 5: Polar plots of SS phase and SS magnitude sensitivity of individual P-cells in wild-type and mutant mice during optokinetic stimulation at 0.05, 0.2 and 0.8 Hz. Horizontal and vertical axis represent k and $2\pi f \cdot r$ values, respectively. The phase of SS modulation in reference to eye velocity is indicated by the angle with the vertical axis, whereas the magnitude sensitivity corresponds to the radial distance from the centre of the plot. Note scaling differences.

Table 2. Regression results simple spike modulation

	0.05 Hz	0.1 Hz	0.2 Hz	0.4 Hz	0.8 Hz
C57BL/6					
k (spk/s per deg)	1.41 ± 0.34	1.34 ± 0.34	1.89 ± 0.48	2.91 ± 0.54	7.58 ± 1.46
r (spk/s per deg/s)	14.6 ± 2.00	7.33 ± 1.72	7.96 ± 1.14	6.41 ± 0.84	6.51 ± 1.15
n	17	15	28	26	23
L7-PKCI					
k (spk/s per deg)	1.23 ± 0.22	0.56 ± 0.24	2.05 ± 0.46	3.67 ± 0.75	5.02 ± 1.19
r (spk/s per deg/s)	12.5 ±1.51	7.21 ± 1.26	6.09 ± 0.87	4.60 ± 0.61	4.74 ± 0.86
n	12	10	23	19	12

Apparent eye position sensitivity (*k*) and apparent eye velocitiy sensitivity (*r*) for simple spike responses of floccular Purkinje cells in C57BL/6 wild-type and L7-PKCI mutant mice. Values of *k* and *r* are expressed as mean ± SEM.

Interpretation of the above results is somewhat complicated by the variability in the eye movement responses, both within and across individual animals (see Fig. 2). To circumvent this problem, the SS modulation was analysed as function of eye position and eye velocity (see Methods). As is illustrated for the P-cell responses in Fig. 3, this analysis procedure yielded a good description of SS modulation (thick lines, middle rows in A and B; correlation between data and model > 0.75). Apparent eye position (k, in spk/s per deg) and eye velocity (r, in spk/s per deg/s) sensitivities fitted to the SS data from the wild-type VA-cell (Fig. 3A) were $[k,r]_{0.05}$ = [0.86, 5.16], $[k,r]_{0.2}$ = [0.92, 4.46] and $[k,r]_{0.8}$ = [1.35, 5.58], where the subscripts refer to stimulus frequency. For the L7-PKCI cell (Fig. 3B), these values were $[k,r]_{0.05}$ = [-0.59, 8.98], $[k,r]_{0.2}$ = [0.29, 3.95] and $[k,r]_{0.8}$ = [13.9, 5.05]. As indicated in the Methods, the r- and k-values were subsequently used to calculate the SS magnitude sensitivity, M, and the phase, θ, of the SS modulation in reference to eye velocity. A prerequisite for a valid comparison of SS responses in wild-type and mutant P-cells on the basis of these parameters is of course that the model describes the responses equally well in both genotypes. Analysis of Pearson's correlation between data and model fit indicated that the goodness of fit was indeed comparable between the two P-cell populations (not significantly different; ANOVA, $F_{1,175}$ = 1.28, P > 0.25; typically, correlations > 0.75). Note that this is not trivial, because in the mutant, the SS responses could have been much noisier or somehow more distorted due to possible aberrations of the cerebellar circuitry.

Figure 5 demonstrates polar scatter plots of SS phase and magnitude sensitivity of all individual VA-cells tested during optokinetic stimulation at 0.05, 0.2 and 0.8 Hz. In this type of plot, the horizontal and vertical axis represent k (eye position component) and $2\pi f \cdot r$ (eye velocity component), respectively. The phase of SS modulation in reference to eye velocity is therefore indicated by the angle with the vertical axis, whereas the magnitude sensitivity corresponds to the radial distance from the centre of the plot. Note that the SS responses of all VA-cells lie in the top two quadrants, signifying that their SS firing rate increased during ipsilateral eye rotation. Furthermore, the SS responses of wild-type and transgenic P-cells are largely overlapping. Statistical analysis of these scatter plots with a two dimensional Kolmogorov-Smirnov test indicated that the location and shape of the two-dimensional distributions of k- versus r-values were not significantly different between the two genotypes at all five stimulus frequencies (P > 0.4). To further scrutinize these results, subsequent analyses focused on the respective Cartesian (k- and r-values) and polar (M and θ) coordinates of the data.

Table 2 lists the average k- and r-values for the wild-type and transgenic P-cell population for all five stimulus frequencies. Note that for wild-type VA-cells, k gradually increases from 1.41 spk/s per deg at 0.05 Hz to 7.58

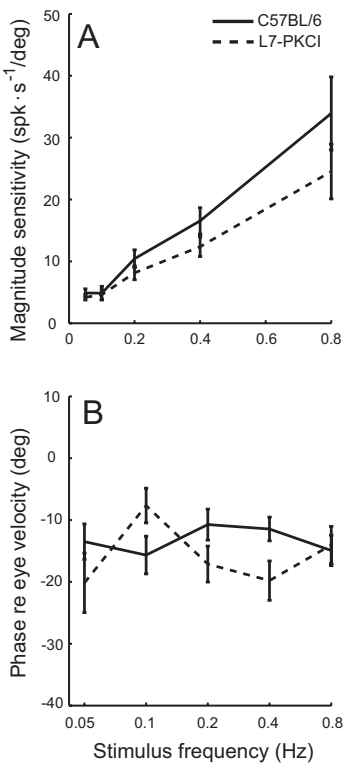

Figure 6: Magnitude sensitivity (A) and phase (B) of the SS discharge for wild-type and L7-PKCI P-cells as function of stimulus frequency. Error bars: ±1 SEM. Note very similar sensitivity and phase relations for the two genotypes. 95% confidence intervals for the difference between magnitude sensitivities in wild-type and L7-PKCI P-cells are: $\Delta M_{0.05}$ = [-2, 1], $\Delta M_{0.1}$ = [-3, 2], $\Delta M_{0.2}$ = [-6, 2], $\Delta M_{0.4}$ = [-9, 2], and $\Delta M_{0.8}$ = [23, 5] spk/s per deg. 95% confidence intervals for the phase differences between the two genotypes are: $\Delta\theta_{0.05}$ = [-17, 3], $\Delta\theta_{0.1}$ = [-2, 15], $\Delta\theta_{0.2}$ = [-13, 2], $\Delta\theta_{0.4}$ = [-15, 2], $\Delta\theta_{0.8}$ = [-7, 8] deg.

spk/s per deg at 0.8 Hz, whereas *r* drops from 14.6 spk/s per deg/s at 0.05 Hz to 6.51 spk/s per deg/s at 0.8 Hz. Very similar results were obtained in the L7-PKCI P-cell population. Two-factor ANOVA indicated that neither *k* nor *r* was significantly influenced by genotype ($F_{1,175} = 0.73$, $P > 0.3$ and $F_{1,175} = 1.57$, $P > 0.2$, respectively) and that there was a significant main effect of stimulus frequency on *k* ($F_{4,175} = 12.16$, $P < 0.0001$) as well as *r* ($F_{4,175} = 11.84$, $P < 0.0001$). Interaction terms were not significant ($F_{4,175} = 1.21$, $P > 0.3$ and $F_{4,175} = 0.28$, $P > 0.8$, respectively). Wilcoxon rank-sum tests and Kuiper tests further supported the conclusion that there were no significant differences in the *k*- and *r*-values between the two genotypes ($P > 0.2$, at all stimulus frequencies). The average baseline of the SS firing rate as quantified by the c-parameter (not listed), was ~ 60 spk/s in both genotypes (no significant differences; two-factor ANOVA, $F_{1,175} = 0.17$, $P > 0.6$) and did not vary as function of stimulus frequency ($F_{4,175} = 1.13$, $P > 0.3$).

Figure 6 quantifies the average magnitude sensitivity of the SS responses and the average phase of the SS discharge in reference to eye velocity as function of stimulus frequency as calculated from the *k*- and *r*-values (see Methods). 95% confidence intervals of the differences between the two genotypes are indicated in the legend. Note that the magnitude sensitivity of the SS responses increases from about 4 spk/s per deg at 0.05 Hz to about 30 spk/s per deg at 0.8 Hz in both P-cell populations (Fig. 6A). Accordingly, two-factor ANOVA of these data indicated a significant main effect of stimulus frequency ($F_{4,175} = 23.53$, $P < 0.0001$), and in line with the Kolmogorov-Smirnov analysis of the scatter plots in Fig. 5, differences between the two genotypes were not statistically significant ($F_{1,175} = 2.30$, $P > 0.1$). The latter conclusion was sustained also by Wilcoxon rank-sum tests and Kuiper tests comparing the SS magnitude sensitivities of wild-type and mutant P-cells at each stimulus frequency ($P > 0.2$, at all stimulus frequencies). As illustrated by the raw data in Fig. 3, the SS modulation was roughly in phase with eye velocity even though there were substantial changes in the phase of the OKR in reference to the stimulus. As shown in Fig 6B, this behaviour was consistent across the population of recorded P-cells; the average phase lag of the SS modulation in reference to eye velocity was only about –15 deg at all stimulus frequencies in both genotypes. Evaluation of these data with a two-factor ANOVA indicated that the SS phase curves in Fig. 6B were not significantly different between the two genotypes ($F_{4,175} = 0.50$, $P > 0.7$) and that stimulus frequency ($F_{1,175} = 2.12$, $P > 0.1$) as well as interaction terms ($F_{4,175} = 1.86$, $P > 0.1$) were also not significant. Comparing the phase of the SS modulation in wild-type and mutant P-cells with the use of Wilcoxon rank-sum tests and Kuiper

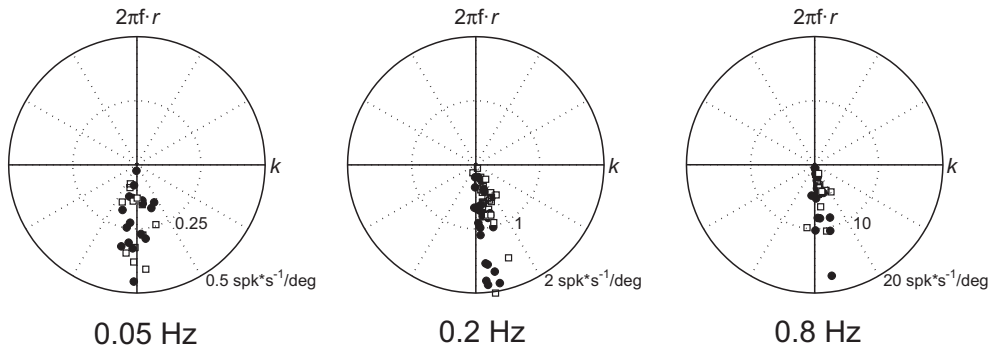

Figure 7: Polar plots of CS phase and CS magnitude sensitivity of individual wild-type and mutant P-cells during optokinetic stimulation at 0.05, 0.2 and 0.8 Hz. Note that CS responses of all cells lie in the bottom two quadrants, signifying that the CS firing rate of vertical axis P-cells increased during contralateral eye rotation.

Table 3. Regression results complex spike modulation

	0.05 Hz	0.1 Hz	0.2 Hz	0.4 Hz	0.8 Hz
C57BL/6					
k (spk/s per deg)	0.00 ± 0.01	0.05 ± 0.01	0.13 ± 0.02	0.33 ± 0.05	0.67 ± 0.17
r (spk/s per deg/s)	-0.66 ± 0.08	-0.55 ± 0.07	-0.71 ± 0.08	-0.78 ± 0.09	-1.01 ± 0.16
n	17	15	28	26	23
L7-PKCI					
k (spk/s per deg)	-0.01 ± 0.01	0.06 ± 0.03	0.17 ± 0.02	0.54 ± 0.08	0.84 ± 0.27
r (spk/s per deg/s)	-0.71 ± 0.11	-0.46 ± 0.10	-0.47 ± 0.07	-0.84 ± 0.15	-0.92 ± 0.17
n	12	10	23	19	12

Apparent eye position sensitivity (*k*) and apparent eye velocity sensitivity (*r*) for complex spike responses of floccular Purkinje cells in C57BL/6 wild-type and L7-PKCI mutant mice. Values of *k* and *r* are expressen as mean ± SEM.

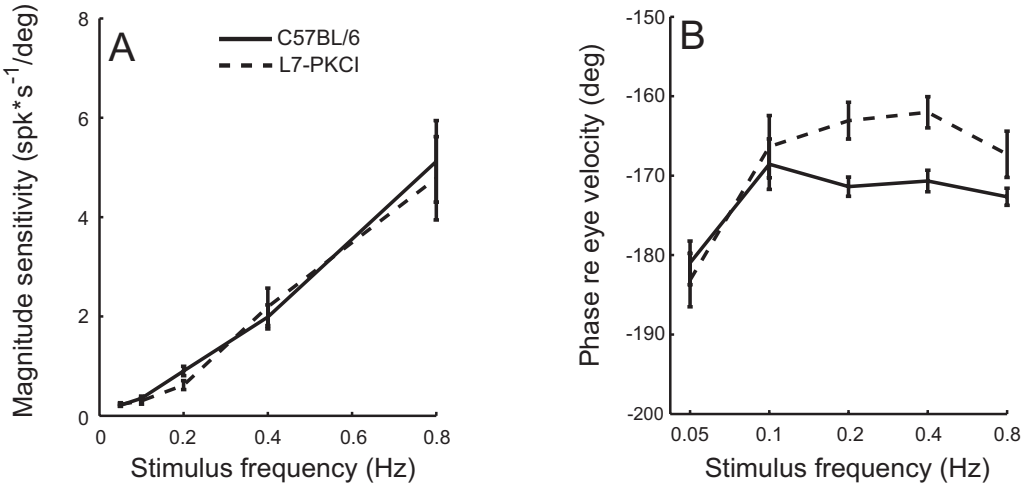

Figure 8: Magnitude sensitivity (A) and phase (B) of the CS discharge of wild-type and mutant P-cells as function of stimulus frequency. Error bars: ±1 SEM. Note very similar sensitivity and phase relations for P-cells in C57BL/6 and L7-PKCI mice. 95% confidence intervals for the difference between the magnitude sensitivities in the two genotypes are: $\Delta M_{0.05}$ = [-0.06, 0.09], $\Delta M_{0.1}$ = [0.20, 0.12], $\Delta M_{0.2}$ = [-0.53, 0.03], $\Delta M_{0.4}$ = [-0.63, 1.08] and $\Delta M_{0.8}$ = [-2.5, 1.8] spk/s per deg. 95% confidence intervals for the phase differences between the C57BL/6 and L7-PKCI P-cells are: $\Delta\theta_{0.05}$ = [-10, 6], $\Delta\theta_{0.1}$ = [-8, 11], $\Delta\theta_{0.2}$ = [3, 13], $\Delta\theta_{0.4}$ = [4 13], $\Delta\theta_{0.8}$ = [-1, 11] deg.

tests at each stimulus frequency also revealed no significant differences between the two P-cell populations ($P > 0.4$ at all stimulus frequencies).

5.4.5 Complex spike discharge dynamics

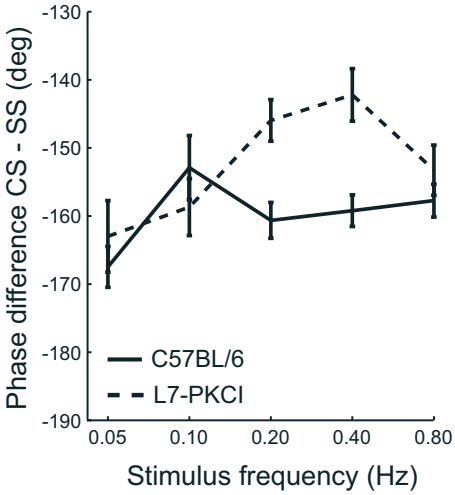

Figure 9: Average phase of the CS modulation in reference to the SS modulation as function of stimulus frequency. Error bars: ±1 SEM. 95% confidence intervals for the phase differences between the two genotypes are: $\Delta\theta_{0.05}$ = [-6, 16], $\Delta\theta_{0.1}$ = [-18, 5], $\Delta\theta_{0.2}$ = [7, 22], $\Delta\theta_{0.4}$ = [8, 25], $\Delta\theta_{0.8}$ = [-3, 13] deg.

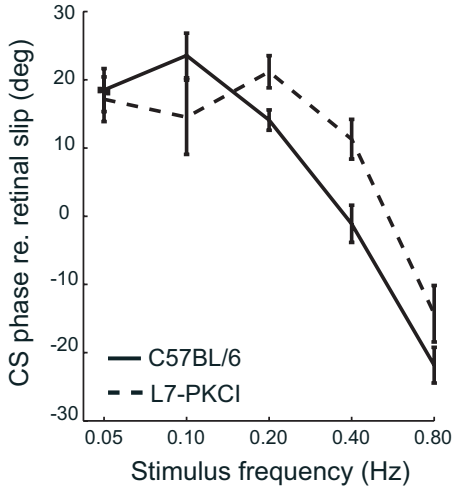

Figure 10: Average phase of the CS modulation in reference to contralaterally directed retinal slip velocity as function of stimulus frequency. Error bars: ±1 SEM. 95% confidence intervals for the phase differences between the two genotypes are: $\Delta\theta_{0.05}$ = [-10, 7], $\Delta\theta_{0.1}$ = [-20, 3], $\Delta\theta_{0.2}$ = [2, 12], $\Delta\theta_{0.4}$ = [5, 20], $\Delta\theta_{0.8}$ = [-2, 16] deg.

The CS firing rate modulation was analysed in a similar fashion as the SS discharge, i.e., as function of eye position and eye velocity. Typical results obtained with this procedure are shown in Fig. 3 (thick lines, bottom rows in A and B; correlation between data and fit > 0.77). *k*- and *r*-values fitted to the CS responses of the wild-type VA-cell (Fig. 3A) were $[k,r]_{0.05}$ = [0.04, -0.38], $[k,r]_{0.2}$ = [0.37, -1.34] and $[k,r]_{0.8}$ = [0.60, -0.48], and for the L7-PKCI cell (Fig. 3B) the values were $[k,r]_{0.05}$ = [0.32, -1.01], $[k,r]_{0.2}$ = [0.32, -0.85] and $[k,r]_{0.8}$ = [-1.17, -1.07]. Across all recordings, goodness of fit was comparable between the two P-cell populations (not significantly different; two-factor ANOVA, $F_{1,175} = 1.28$, $P > 0.35$; correlations between data and fit typically > 0.75). The scatter plots in figure 7 illustrate the fit results for all individual VA-cells tested during optokinetic stimulation at 0.05, 0.2 and 0.8 Hz. Note that the CS responses fall in the bottom two quadrants, signifying that the CS firing rate of all VA-cells increased during contralateral eye rotation. Analysis of these scatter plots with a two-dimensional Kolmogorov-Smirnov test indicated that the location and shape of the distributions of *k*- versus *r*-values were not significantly different between the two genotypes for all stimulus frequencies ($P > 0.05$), except at 0.2 Hz ($P = 0.03$) where the orientation of the distribution for CS responses in L7-PKCI cells is slightly shifted in counter clockwise direction with respect to the wild-type data.

Table 3 summarizes the average *k*- and *r*-values of the CS discharge for wild-type and transgenic P-cells. Note that both the *k*- and *r*-values were significantly influenced by stimulus frequency (two factor ANOVA; $F_{4,175} = 18.86$, $P < 0.0001$ and $F_{4,175} = 4.57$, $P < 0.002$, respectively). In both genotypes, the average *k*-value gradually increased with stimulus frequency from 0 spk/s per deg at 0.05 Hz to about 0.75 spk/s per deg at 0.8 Hz. The average *r*-value gradually changed from about -0.5 spk/s per deg/s at the two lowest frequencies to about -0.95 spk/s per deg/s at 0.8 Hz. However, neither *k*- nor *r*-values were significantly different between wild-type

and transgenic P-cells ($F_{1,175} = 1.38$, $P > 0.2$ and $F_{1,175} = 1.21$, $P > 0.2$, respectively) and interactions were absent ($F_{4,175} = 0.5$, $P > 0.7$ and $F_{4,175} = 0.69$, $P > 0.5$, respectively). Comparing the *k*- and *r*-values with the use of Wilcoxon rank-sum tests and Kuiper tests at each stimulus frequency also revealed no significant differences between the two P-cell populations ($P > 0.2$, at all stimulus frequencies).

Figure 8 quantifies the average magnitude sensitivity of the CS responses and the average phase of the CS discharge in reference to eye velocity as function of stimulus frequency. In both P-cell populations, the magnitude sensitivity of the CS discharge gradually increased from about 0.2 spk/s per deg at 0.05 Hz to about 4.5 spk/s per deg at 0.8 Hz (Fig. 8A; not significantly different between genotypes; two factor ANOVA, $F_{1,175} = 0.13$, $P > 0.7$; Wilcoxon rank-sum and Kuiper tests, $P > 0.3$, at all stimulus frequencies). Whereas the phase of the SS discharge in reference to eye velocity remained constant, the phase of the CS discharge showed a small, but significant change as function of stimulus frequency (Fig 8B; two factor ANOVA, main effect, $F_{4,175} = 13.91$, $P < 0.0001$). There were also some subtle differences between wild-type and mutant P-cells (significant main effect, $F_{1,175} = 12.86$, $P < 0.0005$; no significant interactions, $F_{4,175} = 1.56$, $P > 0.1$). 95% confidence intervals of these differences are listed in the legend. At 0.05 Hz, CS modulation was almost perfectly in counter phase with eye velocity in both genotypes (not significantly different; Student's *t*-test, $P = 0.6$; Wilcoxon rank-sum test, $P = 0.9$). At the higher frequencies, however, CS modulation in wild-type VA-cells lagged eye velocity by about -170 deg while the average phase lag in transgenic VA-cells was reduced to about -165 deg at 0.2 Hz and 0.4 Hz (Student's *t*-tests, $P < 0.004$; Wilcoxon rank-sum test, $P < 0.0002$).

The results presented above suggested that the phase of the CS modulation in reference to the SS discharge might be slightly different between wild-type and mutant P-cells. Figure 9 therefore quantifies the average phase difference between the CS and SS modulation as function of stimulus frequency. Note that the average phase lag of the CS modulation in reference to the SS modulation tends to decrease gradually from about -165 deg at 0.05 Hz to about -155 deg at 0.8 Hz. In mutant VA-cells, however, the average phase lag of the CS modulation reduced further to about -145 deg at 0.2 and 0.4 Hz. (Student's *t*-test, $P < 0.001$; Wilcoxon rank-sum test, $P < 0.002$). 95% confidence intervals of these differences are listed in the legend. Two-factor ANOVA of these data indicated that there was indeed a significant difference between the two P-cell populations (main effect, $F_{1,175} = 4.43$, $P < 0.002$) and a significant influence of stimulus frequency (main effect, $F_{4,175} = 12.85$, $P < 0.0005$). There were no significant interactions ($F_{4,175} = 1.86$, $P > 0.1$).

Because the OKR responses were variable between individual animals (see Fig. 2), the consequent differences in retinal slip across recording sessions might have biased the analysis in Figs. 8 and 9. To examine this possibility, we also analysed the phase of the CS modulation in reference to contralaterally-directed retinal slip velocity (Fig. 10). It appeared, however, that there were frequency-dependent differences between the two P-cell populations (i.e., significant interactions in two-factor ANOVA; $F_{4,175} = 3.44$, $P = 0.01$). As shown in Fig. 10, the phase of the CS discharge in reference to the retinal slip velocity gradually shifts from an average phase lead of approximately 20 deg at 0.05 Hz to an average phase lag of about –20 deg at 0.8 Hz in wild-type P-cells whereas these changes are less steep in mutant P-cells. If the phase curves in Fig. 10 had been identical, one could have argued that the timing differences observed in Figs. 8 and 9 were entirely due to differences in retinal slip rather than differences between the two genotypes. However, the argument cannot be easily reversed. Caution is warranted because non-linearities in the CS responses, such as complete suppression of the discharge during contralateral stimulus rotation (see Fig. 3 for illustration), suggest that changes in retinal slip could have a non-linear effect on the phase of the CS modulation as well.

5.5 Discussion

The main finding of this study is that P-cells in the flocculus of LTD-deficient mice can acquire virtually normal SS and CS discharge dynamics despite the P-cell-specific inhibition of PKC and the consequent blockage of cerebellar LTD from the early stages of development. This result provides a critical extension of our previous work in that it validates the implicit assumption that the normal default oculomotor performance of L7-PKCI mutants is indicative of a proper operation of the cerebellar circuitry (De Zeeuw et al., 1998; Goossens et al., 2001). As discussed below, this extension strongly reinforces the evidence for a causal relation between the lack of LTD induction and the impaired VOR learning in L7-PKCI mutants.

The L7-PKCI transgene is probably activated from the early stages of P-cell differentiation and maturation, resulting in an almost complete suppression of LTD induction throughout pre- and post-natal life of the animal (De Zeeuw et al., 1998; Goossens et al., 2001). Since the development of neural circuits is shaped in part by their activation patterns, these observations raised the interesting question whether P-cells in L7-PKCI mice possess abnormal response properties due to the lack of LTD, which may be one of the mechanisms through which activity-dependent modifications are mediated. Our current results indicate, however, that the SS output of P-cells in LTD-deficient mice is very similar to that in normal ones. First, it appeared that floccular P-cells in L7-PKCI mice exhibit a normal baseline discharge, which agrees well with our recent results obtained from P-cells in the vermis and paramedian lobule (Goossens et al., 2001). Second, we found that the SS discharge dynamics of vertical axis P-cells were unaffected. Both the magnitude sensitivity and phase relations were statistically indistinguishable between wild-type and mutant VA-cells. Finally, the CS discharge dynamics were virtually identical between wild-type and transgenic VA-cells, except for a subtle difference in the phase of the CS modulation at some of the applied stimulus frequencies.

Taken together, these findings warrant the conclusion that neither the inhibition of PKC nor the consequent blockage of LTD induction causes fundamental abnormalities in the cerebellar circuitry of L7-PKCI mutant mice. So far, this was merely conjectured on the basis of data showing that L7-PKCI mutants exhibit no deficits in their default oculomotor performance (De Zeeuw et al., 1998; Van Alphen & De Zeeuw 2002) and no general motor coordination deficits (De Zeeuw et al., 1998). The behavioural data alone, however, did not allow for any conclusive inferences regarding the integrity of cerebellar circuitry. In principle, aberrations in the circuit could have been fully masked by compensatory mechanisms in multiple areas of the brain. Clearly, if the discharge dynamics of floccular P-cells had been totally corrupted in the L7-PKCI mutant, there would have been ample reason to argue that the impaired VOR adaptation resulted from fundamental circuit abnormalities rather than from a lack of cerebellar LTD.

Two specific hypotheses have been proposed regarding the neural signals that guide motor learning in the VOR. One suggests that learning in the VOR could be guided by the coincidence of SS firing of P-cells and activity of vestibular inputs to premotor neurones in the vestibular nuclei (Miles & Lisberger, 1981). The other suggests that coincidence of visual climbing fiber and vestibular parallel fiber activity guides learning by inducing LTD of synapses from vestibular parallel fibres to floccular P-cells (Ito, 1982). Although the involved mechanism of synaptic plasticity in the deep cerebellar and vestibular nuclei remains yet to be uncovered, motor learning in the VOR probably occurs at both sites of plasticity. In view of these hypotheses, it is important to note that the SS signals transmitted to flocculus receiving neurones in the vestibular nuclei are apparently unaffected in the L7-PKCI mutants since the dynamics of the SS activity

of their floccular P-cells appears to be very similar to that in normal animals during the OKR. Of course, we cannot prove that the signals are truly identical; no one can. Nevertheless, it is clear from our present results that the impaired VOR learning in L7-PKCI mutants cannot be explained as a simple side effect of corrupted SS signals that would functionally disrupt SS guided motor learning in the brain stem.

Our present results provide no explanation as to why the phase of the CS modulation in reference to the SS modulation is somewhat different between wild-type and L7-PKCI mutant mice at some of the stimulus frequencies. It is known that an olivary subnucleus which provides the climbing fiber inputs to a particular zone of floccular P-cells is in turn innervated by the vestibular and/or cerebellar nucleus that is inhibited by that floccular zone (De Zeeuw et al., 1994). Even so, it appears difficult to explain how the activities in such an olivofloccular loop could account for the observed differences in olivary timing and thereby in CS timing, as the SS output of P-cells was unaltered. Perhaps, the small, but significant difference at 0.2 and 0.4 Hz merely reflect the fact that the CS modulation was non-linear at these frequencies and that our analysis was therefore more susceptible to differences in retinal slip between individual cell recordings. In any case, the observed changes seem to be too subtle (< 20 deg) to really account for the robust VOR learning deficits found in the L7-PKCI mutant, as they would have but a very limited impact on the probability of coincident parallel fiber and climbing fiber inputs.

The fact that the SS output of floccular P-cells is normal in L7-PKCI mutants indirectly supports our tenet that LTD is one of the major mechanisms underlying cerebellar motor learning because it demonstrates that the learning deficits observed in the mutants do not result from abnormal default response patterns. On the other hand, this finding raises the interesting question as to how LTD can have its permanent effect on learning. If LTD modifies the efficacy of the parallel fiber - Purkinje cell synapse to permit learning, it should ultimately influence motor behaviour via a change in SS activities. So how can both the SS activities and motor performance be normal in an LTD-deficient mutant? Presumably, SS activation patterns are shaped by a variety of slow and fast acting plastic processes that may interact with each other. One can imagine, for example, that parallel fiber LTD is counteracted by postsynaptic LTP at the same synapse (Lev-Ram et al., 2002) and that these processes collectively determine, via some slower auto-regulatory mechanism, the density of AMPA-receptors. In this way, the level of LTD induction alone does not determine the absolute efficacy of the synapse in the long run; it would do so only in conjunction with other forms of plasticity. In such a schema, LTD induction could support motor learning both through its immediate (fast acting) effects on AMPA-receptor properties as well as through its (slow acting) influence on the regulation of AMPA-receptor density, but with a different time course. This hypothesis would predict that an impairment of LTD-induction affects the speed of learning rather than the ultimate outcome of various training paradigms. This phenomenon was indeed observed when we subjected the L7-PKCI mutants to long periods of VOR-adaptation (Van Alphen & De Zeeuw, 2002).

In conclusion, the present findings are the first to show that inhibition of cerebellar LTD induction does not prevent the development of virtually normal P-cells responses during reflex eye movement behaviour in alert L7-PKCI mutants, indicating that their impaired VOR learning cannot be due to fundamental aberrations of the cerebellar circuitry. These results are in line with and re-emphasise our working hypothesis that cerebellar LTD is important for the information storage and retrieval in rapid VOR learning (De Zeeuw et al., 1998; Goossens et al., 2001; Van Alphen & De Zeeuw, 2002).

Acknowledgements

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Chapter 6

General Discussion

The present thesis describes the response of floccular Purkinje cells to oculomotor stimuli in awake, behaving mutant mice. We have been able to measure for the first time simultaneously activity patterns of floccular Purkinje cells and compensatory eye movements in mutant mice. Thereby, we have been able to make correlations between particular molecular, cell physiological, systems electrophysiological and behavioural parameters (Table 1). We have focused on three mutants, which all have a deficit in a calcium mediated process. We have shown that Purkinje cells of *tottering* (*tg*) mice, which suffer from dysfunctional voltage-gated P/Q type calcium channels, have disturbed, irregular simple spike activities, while the Purkinje cells in mutants with deficiencies in calcium buffering (L7-calbindin mutants) or LTD induction (L7-PKCi mutants) show normal activity patterns of both their simple spikes and complex spikes. Interestingly, the general eye movement performance was affected in both tg mutants and L7-calbindin mice, but not in L7-PKCi mutants, while motor learning appears to be specifically affected in the latter mutants. The details of the possible correlations will be discussed below for each of the three mutants investigated for this thesis.

	Motor performance	Motor learning	Modulation amplitude	Simple spike irregularity	Parallel fiber plasticity
Tottering	↓	↓	N	↑	?
L7-calbindin	↓	↓	N	N	N
L7-PKCI	N	↓	N	N	↓

Table 1: Various mutants and their characteristics as described in the present thesis. Upward and downward arrows indicate increase and decrease, respectively, compared to values found in wild type littermates. N indicates no difference between mutants and control littermates. Motor performance is tested by quantifying compensatory eye movements. Motor learning is tested by VOR adaptation protocols. Modulation amplitude is calculated during optokinetic stimulation by dividing the amplitude of the sine fitted to the simple spike response by its offset. Simple spike irregularity is calculated as the coefficient of variance. Parallel fiber plasticity is characterized by long-term depression of the parallel fiber - Purkinje cell synaptic strength (see text for details).

6.1 Purkinje cell firing patterns and motor behaviour in *tg* mice

Chapter 3 describes the spontaneous and compensatory eye movements and the Purkinje cell firing properties of *tg* mutants. *Tg* mice are characterized by a mutation in the gene coding for P/Q-type high voltage activated calcium channel, which gates ~ 90% of the calcium influx in cerebellar Purkinje cells. In *tg* Purkinje cells, calcium influx is decreased by ~ 45%, while in *leaner* Purkinje cells calcium influx is decreased by ~ 60% (Wakamori et al., 1998). Interestingly however, despite the decrease in the calcium current density, the Purkinje cell excitabilities of both leaner and *tg* mice are normal (Dove et al., 1998; Wakamori et al., 1998; Matsushita et al., 2002). In addition to a decreased influx of calcium, *leaner* mice show both a decreased functioning of calcium-uptake into internal calcium stores and a decreased calbindin D-28k and parvalbumin expression (Dove et al., 2000). It is possible that in *tg* Purkinje cells a similar reduction in calcium buffering capacity might occur (Murchison et al., 2002). To elucidate whether the calcium buffer-

ing capacity in *tg* mice show similar features as described for *leaner* mice, quantitative analysis of *in situ* hybridization for calbindin and parvalbumin expression should be conducted, as well as a quantitative assessment of calcium uptake by intracellular calcium stores.

A mutation in the gene coding for the $\alpha 1_a$-subunit of P/Q-type calcium channels is not cell-specific, as P/Q-type calcium channels are present in most synapses in the central nervous system. Still, we showed clear indications that decreased Purkinje cell calcium influx is causal to increased irregularity of simple spike firing, and that this aberration may ultimately lead to abnormal ocular motor behaviour. Below, the following issues will be discussed: 1) what are the possible causes of irregular simple spike firing; and 2) what are the possible consequences of the irregular simple spike firing.

6.1.1 Possible causes of increase in irregularity of simple spike firing patterns

Both spontaneous and modulated simple spike activities recorded from Purkinje cells throughout the whole *tg* cerebellum were severely irregular (Figure 1). Recently published results indicate various possible causes for this increased irregularity. Regularity of simple spike activity has been shown to be affected by both the parallel fiber – Purkinje cell interaction and the inhibitory input from interneurons. Neurotransmitter release from parallel fibers has been shown to be mediated by P/Q-type calcium channels (Mintz et al., 1995). Kulik et al. (2004) confirmed this finding by showing that the spatial distribution of P/Q-type calcium channels at the parallel fiber varicosities demonstrates a close relation between these calcium channels and the active zones of neurotransmitter release. Zhou et al. (2003) showed that glutamate release from parallel fiber nerve terminals did not depend on P/Q-type channels as much in *tg* mice as in controls. Instead, N-type channels are the most prominent type of calcium channel coupled to parallel fiber glutamate release in *tg* mice (Zhou et al., 2003). Similar coupling between calcium channels and neurotransmitter release has been shown for hippocampal CA3 and forebrain tissue (Qian and Noebels, 2000; Leenders et al., 2002). Since N-type channels are more susceptible to GABAB-receptor activation than P/Q-type channels, glutamate release from parallel fibers is more susceptible to GABA-ergic input in *tg* mice than in wild type littermates (Zhou et al., 2003). In addition to this increase in inhibitory input on the parallel fiber synaptic input, Matsushita et al. (2002) showed that parallel fiber-evoked EPSC amplitude is decreased in *tg* Purkinje cells. However, Rhyu et al. (1999) showed that in *tg* mice the number of synapses per dendritic spine between Purkinje cells and parallel fibers was significantly increased, which may compensate for the decreased transmission at parallel fiber – Purkinje cell synapses.

In addition to the altered parallel fiber – Purkinje cell synaptic contacts, the direct inhibitory synaptic input of cerebellar interneurons to the Purkinje cells is increased in *tg* mice. Synaptic input from inhibitory interneurons has a significant effect on the regularity of Purkinje cell action potential firing *in vitro* (Hausser and Clark, 1997). $GABA_B$-receptor activation reduces the calcium influx through P/Q-type channels in Purkinje cells (Mintz and Bean, 1993), which indicates that in *tg* mice the P/Q-type calcium influx is relatively more reduced by GABA-ergic input than in wild type mice. In addition to the increase of the direct effect of inhibitory synaptic input, an indirect inhibitory effect has been recently described. GABA release from inhibitory interneuron axon terminals is decreased by glutamate release from climbing fibers, which leads to the disinhibition of cerebellar Purkinje cells (Satake et al., 2000). Since the coupling between glutamate binding by AMPA-receptors on inhibitory interneuron terminals and GABA release is mediated by P/Q-type calcium channels (Stephens et al. 2001; Satake et al., 2004), this disinhibitory effect may be decreased in *tg* mice. This relative increase in GABA release from inhibitory

interneurons in *tg* would lead to an increase of Purkinje cell inhibition by inhibitory interneurons. Because single IPSPs delay Purkinje cell action potentials (Hausser and Clark, 1997), the increases in both direct and indirect inhibitory synaptic inputs could contribute to the increased irregularity of simple spike firing.

Another possible cause for increased irregularity of simple spike firing is that decreased P/Q-type calcium currents result in altered calcium-dependent potassium channel currents. Various studies have shown that large- and small-conductance calcium-dependent potassium channels regulate Purkinje cells firing (Raman and Bean, 1999; Womack and Khodakhah, 2003; Sausbier et al., 2004; Womack et al., 2004). When large-conductance calcium-dependent potassium channels are genetically removed, the knock-out mice exhibit ataxia, and their Purkinje cells show burst-like action potential firing in vitro (Sausbier et al., 2004). Womack et al. (2004) showed that small-conductance calcium-dependent potassium channels are exclusively activated by calcium entering the Purkinje cell through P/Q-type calcium channels. These data leads to the prediction that small-conductance calcium-dependent potassium channel currents are decreased in *tg* mice.

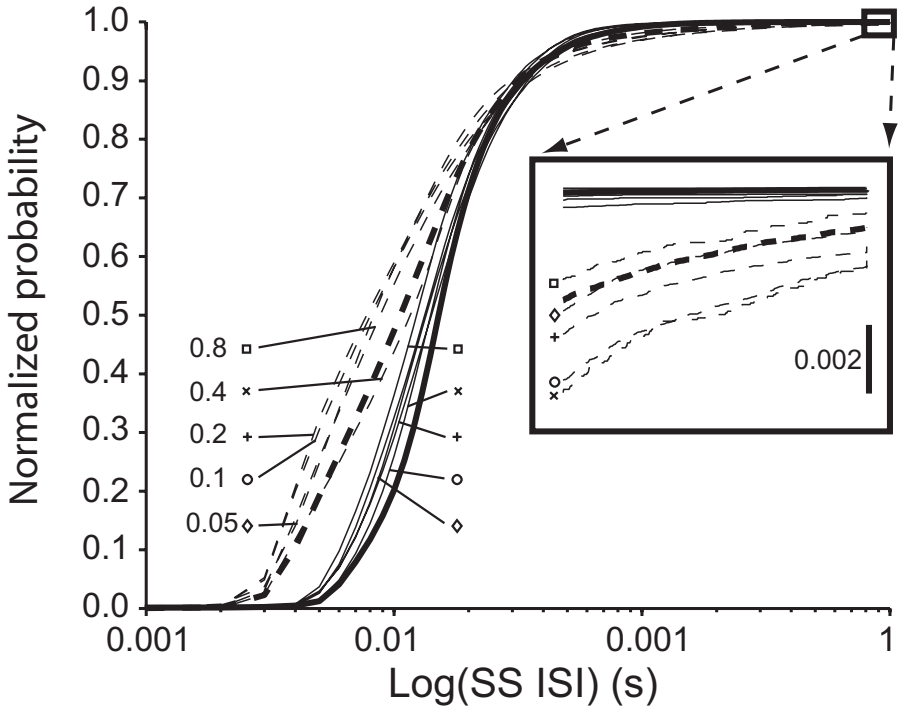

Figure 1: Both spontaneous and modulated simple spike interspike interval length spreading is increased in *tg* mice compared to wild type. The bold solid line indicates the cumulative distribution of the length of the simple spike interspike interval (SS ISI) for spontaneous Purkinje cell activity. The bold dashed line indicates the spontaneous SS ISI distribution for *tg* Purkinje cells. More than 300,000 SS ISI were used for both distributions. *Tg* Purkinje cells clearly show an increase in the amount of shorter and longer SS ISI than wild types (note the slope of the bold solid and bold dashed line). Thin solid and dashed lines represent modulated SS ISI of respectively wild type and *tg* Purkinje cells at a certain frequency of optokinetic stimulation (ranging from 0.05 – 0.8 Hz; see legend and chapter 3 for details). The amount of SS ISI used for these distributions ranged from 15,000 – 125,000. These modulated SS ISI distributions indicate that the increase in spreading of SS ISI length in *tg* mice compared to wild type mice, as is seen during spontaneous activity, persists during modulation. Inset: Distribution of SS ISI lengths of 500 - 1000 ms (vertical indication bar: 0.2%). All *tg* distributions (dashed lines) show an increase in the number of SS ISI of 500 – 1000 ms length compared to wild type distributions (solid lines).

In addition, Berridge (1998) proposed a link between calcium released from ryanodine-mediated calcium stores and calcium-dependent potassium channels (Berridge, 1998). Since the expression of the ryanodine receptor is significantly decreased in *tg* mice (Cicale et al., 2002), it seems highly likely that in *tg* mice the function of calcium dependent potassium-channels is affected.

Concerning the above described recent findings we hypothesize that the decrease in P/Q-type calcium currents as found in *tg* Purkinje cells is causal to: (1) an altered synaptical input from parallel fibers, (2) an increased influence of inhibitory interneurons, and (3) an altered calcium-dependent potassium channel function. The first hypothesis could be tested using inducible transgenic mutant mice, in which the *tg* mutation can be activated in adult mice, following which changes in morphology, connectivity and electrophysiology of the parallel fiber – Purkinje cell synaptic contact can be studied over time. In order to test the second hypothesis the firing patterns of wild type and *tg* interneurons should be determined in order to elucidate whether their firing pattern is affected by the *tg* mutation. Also, recordings of Purkinje cells combined with iontophoretic application of GABA-blockers could elucidate the effect of inhibitory input on Purkinje cells in *tg* mice. In addition, *in vivo* patch-clamp experiments should be conducted in which double patches are made on monosynaptically connected interneurons and Purkinje cells. These experiments quantify the inhibitory effect of these interneurons on Purkinje cell output. In order to test the third hypothesis, extracellular recordings of Purkinje cell activity combined with iontophoretic micro-injections of calcium-dependent potassium channel blockers should be conducted. These results may shed light on the potential effects of partial or complete blockade of these channels on simple spike activity.

6.1.2 Possible consequences of increase in irregularity of simple spike patterns

Simple spikes are transmitted by the Purkinje cell axon as single action potentials while complex spikes are transmitted as bursts of action potentials (Ito and Simpson, 1971; Ito, 1984). Recent findings show a low-pass cut-off filter function of the Purkinje cell axon on the Purkinje cell output (Monsivais and Hausser, 2003), which is of particular importance for *tg* mice, since the instantaneous firing frequency of simple spikes reaches higher values in *tg* Purkinje cells than in Purkinje cells of control mice (Figure 1). In addition, axonal propagation of *tg* Purkinje cells may be altered, because Callewaert et al. (1996) showed that Purkinje cell axonal calcium influx is controlled by P/Q-type calcium channels. Furthermore, preliminary electron microscopy results indicate morphological anomalies in *tg* Purkinje cell terminals (S. Khosravani, personal communication). This latter finding is in line with the presence of P/Q-type calcium channels in the Purkinje cell terminals. Moreover, since P/Q-type calcium channels mediate neurotransmitter release at other GABA-ergic cerebellar interneurons (Caillard et al., 2000; Stephens et al., 2001; Satake et al., 2004), it seems likely that neurotransmitter release is disturbed in *tg* Purkinje cells. Experiments presented in chapter 3 of the present thesis show that electrical stimulation of the flocculus using regular pulse trains elicits eye movements in *tg* mice comparable to those produced in wild type littermates. This indicates the functionality of the *tg* Purkinje cell axon and terminal. Thus, despite the possible electrophysiological and morphological aberrations, *tg* Purkinje cell axons and terminals seem relatively functional.

If we disregard the possible morphological aberrations on *tg* Purkinje cell axonal terminals and assume that their function is normal, the irregularity of simple spike firing is the prime candidate for the cause of the ineffectiveness of Purkinje cell modulation. This assumption is supported by data showing that inhibitory input of Purkinje cells to neurons in the cerebellar nuclei is both necessary and sufficient to control the spike timing (Gauck and Jaeger, 2000). A single

action potential generated by a single Purkinje cell is thought to be ineffective in eliciting an IPSP, but simultaneous activation of Purkinje cells can trigger an IPSP in the Purkinje cell target neuron (Aizenman and Linden, 1999). IPSPs in turn can elicit a rebound depolarization, which consists of several action potentials. Not only the number of simultaneously active Purkinje cells, but also the synchronicity of action potential firing of multiple Purkinje cells is important for induction of a rebound depolarization (Gauck and Jaeger, 2000). This indicates that there is a certain time window for the simple spikes in order to elicit a rebound depolarization; in *tg* mutants more simple spikes may fall outside the time window. The size of this time window may be determined not only by the properties of the synapses of the target neurons of the Purkinje cell terminals, but also by changes in intrinsic excitability of these neurons (Aizenman and Linden, 2000; Sekirnjak and du Lac, 2002; Nelson et al., 2003). A decrease in synchronicity between the incoming inhibitory inputs is likely to result in longer periods of inhibition, which could decrease the firing frequency of cerebellar nuclei neurons. In order to elucidate whether the temporal spreading between the action potential firing of Purkinje cells in *tg* mice is increased, three experiments should be conducted. First, the activity patterns of cerebellar nucleus neurons should be measured extracellularly in wild type and *tg* mice. Since the absence of synaptic input make cerebellar nucleus neurons fire regular *in vitro*, the firing pattern of these neurons *in vivo* could be more regular in *tg* mice than in wild type littermates. Second, extracellular recordings of multiple Purkinje cells that project to the same target neuron should be done. In order to do these experiments, the exact geometrical layout of the convergence pattern of Purkinje cells onto single DCN neurons needs to be clarified. A recent study indicates that Purkinje cells along a parallel fiber beam show no simple spike synchronicity in wild type mice (Cheron et al., 2004). Other studies indicate that temporal spreading of Purkinje cell action potential firing is minimal between co-activated patches of cerebellar cortex instead of all Purkinje cells that lie along a parallel fiber beam (Bower et al., 1997). Finally, *in vitro* measurements of synaptic efficacy of the Purkinje cell terminal – cerebellar nucleus neuron should be conducted, since electrical stimulations of the Purkinje cells cannot exclude that the *tg* Purkinje cell terminal does not function normally under physiological circumstances.

6.2 Influence of calcium buffers on motor behaviour and Purkinje cell output

Chapter 4 describes how mice that lack the calcium buffering protein calbindin D-28k in their cerebellar Purkinje cells exhibit motor dysfunction. Various behavioural tests demonstrate that the presence of calbindin D-28k in cerebellar Purkinje cells is essential for a certain form of fine-tuning of motor functions. One may expect that absence of fast high-affinity calcium buffering proteins affects spontaneous Purkinje cell activity, but extracellularly recorded simple and complex spike activity patterns from control and mutant mice showed no difference. We suggest that parvalbumin, the abundantly present calcium binding protein, which demonstrates binding kinetics and affinity similar to calbindin D-28k (Schmidt et al., 2003), partially compensates for the absence of calbindin, despite the evidence that parvalbumin expression is not upregulated in Purkinje cells of L7-calbindin D-28k mice. The idea that parvalbumin compensates for the loss of calcium buffering capacity is supported by recently published results showing that during climbing fiber activity-evoked calcium transients parvalbumin is not saturated (Schmidt et al., 2003). These data indicate that under physiological conditions the calcium buffering by parvalbumin can increase. Besides parvalbumin, other calcium binding proteins and calcium clearance mechanisms (Sabatini et al., 2002; Schmidt et al., 2003) may contribute significantly to rapid calcium buffering in L7-calbindin mice. Future studies need to elucidate what buffering mechanisms lead to the maintenance of normal spontaneous Purkinje cell activity in L7-calbindin D-28k mutant

mice. The use of viral constructs should help; viral constructs containing fluorescent proteins that form subunits of for instance, a calcium extrusion pump, could be used for transfection of Purkinje cells. When the gene construct of the pump-subunit contains a mutation that affects the function of the pump, infected Purkinje cells will show affected calcium clearance dynamics. Provided that the calcium extrusion pump function is increased in L7-calbindin D-28k Purkinje cells, the difference in amplitude and decay of calcium transients between infected and non-infected L7-calbindin D-28k Purkinje cells will differ from that between infected and non-infected wild type Purkinje cells. This approach enables one to distinguish the contributions of the various calcium clearance mechanisms to the regulation of the intracellular calcium concentration.

Calbindin D-28k is not only expressed abundantly in the dendritic tree and soma of the Purkinje cell, but also in its axonal terminal. We showed that the amplitude of parallel and climbing fiber evoked calcium transients is significantly increased in L7-calbindin D-28k Purkinje cells and thus it seems likely that calcium transients in Purkinje cell terminals are increased too. Neurotransmitter release from parallel fibers is shown to be controlled by the intracellular calcium concentration (Mintz et al., 1995) and thus the increase in calcium transient amplitude in L7-calbindin D-28k Purkinje cells is likely to increase the amount of neurotransmitter release. A study that uses parvalbumin knock-out mice provides indications of such an effect (Caillard et al., 2000). In addition to an effect on neurotransmitter release, this same study showed an effect of decreased calcium buffering capacity on short-term synaptic plasticity. In parvalbumin knock-out mice, paired-pulse *facilitation* of Purkinje cell IPSCs is elicited by a double stimulation of GABA-ergic interneurons with 30 – 300 ms intervals, while the same stimulation protocol elicits paired-pulse *depression* in wild type Purkinje cells. This altered polarity of short-term synaptic plasticity could results from the decreased calcium buffering capacity, because more residual (unbuffered) calcium is present after the first stimulus, which leads to more calcium during the second stimulus. These data indicate that the calcium buffering capacity mediates both neurotransmitter release and short-term synaptic plasticity at inhibitory cerebellar synapses, which suggests that similar effects of decreased calcium buffering capacity might occur in L7-calbindin D-28k mice. To find out whether the results found by Caillard et al. (2000) also hold true for the Purkinje cell terminal, *in vitro* experiments should be conducted on the synaptic efficacy of the Purkinje cell – cerebellar nucleus neuron synapse in L7-calbindin D-28k mutants.

The assumption that the increased amplitude of calcium transients increases the amount neurotransmitter released at the Purkinje cell terminal, leads to the prediction that in L7-calbindin D-28k mice the net inhibitory input from Purkinje cell on their target neurons is significantly increased. Preliminary results indicate that the amplitude of modulation (amplitude divided by offset of sine fitted to simple spike response during optokinetic stimulation) is not affected in L7-calbindin Purkinje cells (Hoebeek and De Zeeuw, unpublished observation). These data indicate that the amplitude of modulation in the Purkinje cell terminal, where synaptic transmission might be increased, could be lower (offset increases in contrast to modulation amplitude) in L7-calbindin D-28k mice. Tan and colleagues showed that an increase in Purkinje cell amplitude of modulation increases the amplitude of the compensatory eye movements (Tan et al., 1992; Van der Steen and Tan, 1997), which indicates that in L7-calbindin D-28k mice the increased neurotransmission at the Purkinje cell terminals could be causal to the decreased OKR and VVOR. This latter prediction is highly speculative, moreover since it remains to be elucidated what effect short term synaptic plasticity could have on the synaptic transmission in the Purkinje cell terminal during optokinetic behaviour. However, it seems reasonable to hypothesize that the predicted alteration of neurotransmission at the Purkinje cell terminal in L7-calbindin D-28k mice is likely to affect long term changes in synaptic strength at the Purkinje cell terminal.

6.3 Calcium mutants and motor learning

Two specific hypotheses have been proposed regarding the neural signals that guide motor learning in the VOR. In one, learning in the VOR is proposed to be guided by the coincidence of SS firing of Purkinje cells and activity of vestibular inputs to Purkinje cell target neurons in the vestibular nuclei (Miles and Lisberger, 1981). In the other, coincidence of visual climbing fiber and vestibular parallel fiber activity guides learning by inducing LTD at synapses of vestibular parallel fibres on floccular Purkinje cells (Ito, 1982). Both hypotheses have recently been challenged (as reviewed by Boyden et al., 2004). In response to VOR adaptation the sensitivity of Purkinje cells to vestibular input alters in the direction opposite to that predicted by the action of cerebellar LTD. Moreover, the Purkinje cell vestibular sensitivity alters in the direction opposite to that required to account for the change in the VOR gain (Lisberger et al., 1994). Raymond and Lisberger (1998) showed that Purkinje cells are not able to discriminate between VOR gain decrease or VOR gain increase at low- and high-frequencies; in other words, Purkinje cells had the same firing pattern during VOR gain increase stimulus protocols at 0.5 Hz as during VOR gain increase stimulus protocols at 5.0 Hz. This inability indicates that the Purkinje cell cannot provide an unambiguous teacher signal to guide plasticity under these particular circumstances. Raymond and Lisberger (1998) proposed that VOR motor learning is mediated by multiple mechanisms, each driven by different combinations of neural signals, such as coincidence detection of climbing fiber and vestibular mossy fiber input by the cerebellar Purkinje cell, or coincidence detection of Purkinje cell output and vestibular afferent input by floccular target neurons in the vestibular nucleus. The induction of these forms of synaptic plasticity is likely to be altered in *tg* and L7-calbindin D-28k mutants, since the polarity of synaptic plasticity of both the parallel fiber – Purkinje cell and Purkinje cell – cerebellar nuclei neuron synapses is dependent on the intracellular calcium concentration (Aizenman et al., 2000; Coesmans et al., 2004).

Chapter 3 demonstrates that calcium currents are decreased in *tg* mice, but whether the induction and maintenance of LTD in *tg* Purkinje cells is possible remains to be elucidated (see Table 1). Because calcium influx is decreased in *tg* Purkinje cells (Wakamori et al., 1998; Matsushita et al., 2002) one would predict a change in the induced form of plasticity (e.g., see Coesmans et al., 2004). Wang et al. (2000) showed that supralinear calcium currents trigger LTD. The induction of these supralinear calcium currents depends on a prominent temporal component: parallel fiber burst activity should precede climbing fiber activity by 50 - 200 ms in order to elicit supralinear calcium currents and the subsequent robust depression of the parallel fiber EPSC (Wang et al., 2000). Decreased calcium currents as found in *tg* Purkinje cells (Wakamori et al., 1998) might result in a narrower time window for the induction of LTD. Moreover, a decrease in calcium currents might result in a decreased spread of supralinear calcium influx in *tg* Purkinje cells, and hence LTD might be restricted to a smaller area of the dendritic tree. This decreased spreading of calcium transients holds especially true for the experiments in which a dense bundle of parallel fibers are stimulated, because the widely spread calcium influxes following such a stimulus rely solely on voltage-gated calcium channel currents, most likely of the P/Q-type (Wang et al., 2000). However, the decrease in P/Q-type calcium current amplitude in *tg* mice might be compensated for by a decrease in calcium buffering capacity, as is found in *leaner* mice (Dove et al., 2000; Murchison et al., 2002). Thus, we should elucidate whether the spreading of calcium currents in *tg* Purkinje cells is normal before we can answer whether the induction of LTD is affected by the lowered calcium current amplitude.

Tg Purkinje cells exhibit grossly irregular simple spike firing patterns, which indicates both more short and long interspike intervals (Figure 1). Aizenman and colleagues have shown

that plasticity at the Purkinje cell terminal is affected by the inhibitory synaptic input (Aizenman et al., 1998). When repetitive bursts of IPSPs (100 Hz, 10 pulses) are applied to hyperpolarized cerebellar nucleus neurons, the IPSP amplitude decreases, but when the target neurons are at resting membrane potential, the repetitive train stimulus induces IPSPs with increasing amplitude (Aizenman et al., 1998; Aizenman et al., 2000). In *tg* mice the decrease in simple spike firing frequency, which presumably results in Purkinje cell target neurons being less inhibited, is likely to cause LTP instead of LTD of the Purkinje cell - cerebellar nucleus neuron synapse. In contrast, when the temporal spreading between the simple spike firing of multiple Purkinje cells is increased in *tg* mice, the inhibitory input would be less focused in time, which may effectively increase the tonic inhibitory synaptic input. Which of these different effects dominates *in vivo* can only be resolved empirically, perhaps by recording activity of multiple Purkinje cells that all project to one single target neuron.

In summary, it seems likely that in *tg* mice the long-term changes in synaptic strength at the parallel fiber – Purkinje cell synapse, as well as at the Purkinje cell – target neuron synapse would be affected by the decrease in the P/Q-type calcium current. Since one current hypothesis on the location of the neuronal substrate for motor learning implicates both these synapses (Raymond and Lisberger, 1998), *tg* mice should exhibit decreased motor learning. Indeed, cross-axis adaptation (sinusoidal vestibular stimulation around vertical axis combined with optokinetic stimulation around the horizontal axis) in *tg* mice is absent (J. Stahl, personal communication). In contrast, the allelic mutant *rocker* does show cross axis adaptation, although less than wild type littermates (Stahl, 2004). The fact that *rocker* mice do cross-adapt to some extent is in line with the similar but milder aberrations in *tg* compensatory eye movements (Stahl, 2002, 2004).

In Chapter 4 it is shown that synaptically-activated calcium transients in L7-calbindin D-28k Purkinje cells are larger compared to control values. However, the induction of parallel fiber LTD was unchanged in L7-calbindin D-28k mice. This shows that deviations in the level of calcium buffering capacity as found in L7-calbindin D-28k do not affect the induction of LTD. Still, one could argue for an alteration of synaptic plasticity in L7-calbindin D-28k mice, since Coesmans et al. (2004) showed that only intracellular calcium concentrations below a certain threshold can elicit LTP. This means that due to the increased amplitude of calcium transients, conditions that normally induce LTP could result in LTD instead in Purkinje cells of L7-calbindin D-28k mice. In order to test this hypothesis, *in vitro* experiments should elucidate whether LTP-induction protocols elicit LTD in L7-calbindin D-28k Purkinje cells.

In addition to alteration of long-term changes in synaptic plasticity at the parallel fiber – Purkinje cell synapse, long-term synaptic plasticity at the Purkinje cell – cerebellar nucleus neuron is likely to be affected by a decrease in calcium buffering capacity too. Chapter 6.2 advances the hypothesis that in L7-calbindin the inhibitory input to cerebellar nucleus neurons is increased. One likely effect of this hypothesized increase in the neurotransmitter release from Purkinje cell terminals is that the target neurons are more hyperpolarized and thus a high-frequency burst of Purkinje cell action potentials would elicit LTD of the IPSP amplitude instead of LTP. In order to test this predicted effect of decreased calcium buffering capacity, extracellular activity patterns from cerebellar and vestibular nuclei cells in L7-calbindin D-28k mice should be recorded *in vivo*. A lower firing frequency of cerebellar nucleus neurons could indicate an increased Purkinje cell synaptic transmission. In addition, the electrophysiological characteristics of Purkinje cell terminals of L7-calbindin D-28k mice should be studied *in vitro*.

In conclusion, L7-calbindin mice are predicted to exhibit 1) more LTD than LTP at their parallel fiber - Purkinje cell synapses, 2) alterations in the polarity of short-term synaptic plasticity at the Purkinje cell terminal (chapter 6.2), and 3) alterations in the polarity of long-term synaptic plasticity at the Purkinje cell terminal synapses. Since synaptic plasticity at both these

synaptic connections have been linked to motor learning, it seems likely that adaptation of certain motor behaviour would be affected by the decrease in calcium buffering capacity in Purkinje cells. Chapter 4 indicates that the permanent impairment of coordination can be compensated for in L7-calbindin mutants by prolonged training, which shows that this particular form of motor learning is not affected. In contrast, preliminary results on VOR adaptation indicate that L7-calbindin mice show a decrease in the ability to decrease their VOR (F. Hoebeek, unpublished observation). These indications call for experiments in L7-calbindin mice, during which the ability of adapting oculomotor behaviour is explored.

A possible link between LTD and Purkinje cell firing patterns is: decreased LTD is caused by a decrease in the removal of AMPA receptors from the cellular membrane, which in turn increases the Purkinje cell response to parallel fiber glutamate release. Chapter 5 of the current thesis addresses the question of whether a decrease in LTD disrupts Purkinje cell firing patterns. The main finding is that Purkinje cells in the flocculus of LTD-deficient mice develop virtually normal SS and CS discharge dynamics. During sinusoidal vertical axis optokinetic stimulation, amplitude of modulation, magnitude sensitivity, and phase of the simple spike and complex spike activity patterns of mutant mice are indistinguishable from those of littermate controls. These data indicate that a decrease in LTD and the subsequent alteration of the number of AMPA receptors in the cell membrane as found in L7-PKCI mice, is not sufficient to cause an alteration of Purkinje cell firing patters.

The fact that the SS output of floccular P-cells is normal in L7-PKCI mutants raises the following interesting question: how does LTD have its permanent effect on learning? Compensatory eye movements of L7-PKCI mutants measured before the start of visuo-vestibular training are normal (Van Alphen and De Zeeuw, 2002). One can imagine, for example, that parallel fibre LTD is counteracted by postsynaptic LTP at the same synapse (Lev-Ram et al., 2002; Coesmans et al., 2004). Both LTD and LTP modulate the density of AMPA-receptors, which ultimately determines the synaptic strength. Recently, a model concerning how synaptic plasticity at the parallel fiber – Purkinje cell synapse encodes both the decrease and increase in VOR gain was proposed (Boyden and Raymond, 2003). This model proposes that postsynaptic LTD at the parallel fiber – Purkinje cell synapse mediates VOR gain increase, whereas pre- and postsynaptic LTP mediates VOR gain decrease. Parallel to this model, Van Alphen and De Zeeuw (2002) showed that L7-PKCI mutants decrease their VOR gain during long periods of VOR decrease training. Moreover, LTD-deficient mutant mice hardly increase their VOR gain during long periods of VOR increase training, in contrast to wild type littermates. In addition, preliminary results indicate that L7-PKCI mice hardly increase their gain following cross-axis adaptation (M. Schonewille, personal communication). These results indicate that calcium-dependent LTD-deficient mice are able to adapt certain forms of motor behaviour.

6.4 References

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Summary

The present thesis describes motor behaviour and Purkinje cell activity of three mutants in which either modulation of calcium homeostasis or a calcium-dependent process that regulates synaptic strength is disturbed. These include 1) the *tottering* (*tg*), a natural mouse mutant with a mutation in the P/Q type voltage gated calcium channel; 2) the L7-calbindin mouse mutant which lacks the calcium buffer calbindin D-28k specifically in its Purkinje cells; and 3) the L7-PKCI mouse mutant, which expresses a peptide that inhibits the function of various isoforms of protein kinase C (PKC) specifically in its Purkinje cells. The present thesis thus touches upon multiple aspects of calcium regulation or signalling in the cerebellar Purkinje cell and its effects on motor behaviour.

Tg mice have a mutation in the same gene that is mutated in humans that have episodic ataxia II, spinocerebellar ataxia VI and familial hemiplegic migraine. *Tg* mice are characterized by the occurrence of dystonic episodes that resemble human episodic attacks. In addition, these animals are severely ataxic. The present study used measurements of compensatory eye movements to characterize and quantify the cerebellar contribution to the ataxia. Visually-driven compensatory eye movements were severely impaired in *tg* animals. Extracellular recordings of their floccular Purkinje cells showed that both spontaneous and modulated simple spike activities are extremely irregular. When the flocculus was lesioned this resulted in the expected attenuation of visually-driven compensatory eye movements in wild type animals, but no significant differences were found in *tg* mice. In order to check whether the efferent floccular pathway is blocked in *tg* mice, we electrically stimulated their ipsilateral flocculus. These stimuli elicited similar eye movements in *tg* and control animals. In conclusion, we showed that the effects of the *tg* mutation were severe in the cerebellar Purkinje cell and that the affected simple spike firing pattern of these cells is likely to be the main cause of the ataxia. The cause of increased irregularity of simple spike firing is probably complex, but can be related to the decrease of calcium influx through the P/Q-type calcium channels.

When calcium enters the Purkinje cell it affects the internal calcium concentration, which in turn acts as a second messenger in numerous processes. In order to protect the Purkinje cells from cell death by excitotoxicity, they contain high concentrations of mobile high-affinity calcium buffers, including calbindin D-28k and parvalbumin. Using various behavioural tests we showed that the removal of calbindin from Purkinje cells results in motor dysfunction. Calcium homeostasis of Purkinje cells was also disturbed; excitatory synaptic input resulted in increased calcium transients. The increase in calcium transient amplitude did affect neither the induction nor the maintenance of long-term depression of the strength of the parallel fiber – Purkinje cell synapse, nor the spontaneous Purkinje cell firing patterns. We hypothesize that in L7-calbindin D-28k mice the synaptic transmission of floccular Purkinje cells to their target neurons is affected and is causal to the decreased amplitude of compensatory eye movements.

Both the *tg* and L7-calbindin D-28k mouse models have abnormal calcium homeostasis in their cerebellar Purkinje cells. The internal calcium concentration controls the induction of long term changes in the synaptic strength of the excitatory inputs to the Purkinje cell. To see whether a decrease in the ability to change synaptic strength results in altered Purkinje cell firing patterns during motor behaviour, we studied the floccular Purkinje cell activity during optokinetic reflex performance. L7-PKCI mice show decreased ability to adapt their motor behaviour, but their floccular Purkinje cells show normal output patterns. This result indicates that the decrease in long term depression is not enough to disturb firing rate modulation, just as it is not enough to prevent normal compensatory eye movement values prior to adaptation. In conclusion, alteration of calcium homeostasis is more powerful in altering motor behaviour than the decrease of long

term depression, even though all these animals had to ”learn” to make compensatory eye movements.

Samenvatting

De cerebellaire cortex is sterk georganiseerd en heeft een herkenbare cyto-architectuur. Purkinje-cellen vormen de output van de cerebellaire cortex en hebben een grote, platte dendrietboom, die grote aantallen synaptische contacten met verschillende soorten cellen bevat. Purkinje-cellen krijgen synaptische input vanuit de hersenstam en het cerebrum via zowel het klimvezel- als het mosvezel-systeem. Klimvezels worden gevormd door de axonen van neuronen in de inferiore olijfkern uit de hersenstam, die in de dendrietbomen van de Purkinje-cellen 'klimmen'. Neuronen in de inferiore olijfkern zijn excitatoir, wat wil zeggen dat de postsynaptische cel, in dit geval de Purkinje-cel, wordt gedepolarizeerd door activiteit van deze neuronen. In een volwassen muis wordt elke Purkinje cel geïnnerveerd door een enkele klimvezel. Doordat de klimvezel duizenden synapsen maakt met het proximale deel van de dendrietboom en al deze synapsen dus tegelijkertijd worden geactiveerd, vuurt de Purkinje-cel altijd een actiepotentiaal als het presynaptische neuron een actiepotentiaal vuurt (een 'all-or-none' effect). Simultane activatie van al deze klimvezel – Purkinje-cel-synapsen leidt tot een wijdverspreide depolarisatie die het ontstaan van een zogenaamde "complex spike" veroorzaakt. Een complex spike bestaat uit meerdere golven, in tegenstelling tot normale actiepotentialen. Neuronen in de inferiore olijf hebben een vuurfrequentie van ongeveer 1 Hz, maar die kan voor korte periodes oplopen tot 10 Hz. Neuronen van de inferiore olijfkern vuren simultaan door electrotone koppeling tussen deze cellen. Deze koppeling resulteerd in synchrone complex spike activiteit in Purkinje-cellen.

Purkinje-cellen hebben een pacemakerfunctie; zonder enige vorm van synaptische input genereert het cellichaam actiepotentialen. Deze activiteit wordt gemoduleerd door twee soorten synaptische inputs, namelijk input van excitatoire parallelvezels en van inhibitoire korf- en ster-interneuronen. Parallel vezels worden gevormd door korfcel-axonen. Elke parallelvezel maakt een of twee keer contact met de dendrietboom van de Purkinje-cel en deze vezels lopen loodrecht op de Purkinje-cel dendrietbomen. Per Purkinje-cel zijn er 150.000 synapsen met parallelvezels. Deze massieve excitatoire synaptische input wordt tegengewerkt door inhibitoire synaptische input van interneuronen. Deze inhibitoire synaptische input zorgt voor een hyperpolarisatie. Als de generatie van actiepotentialen wordt beïnvloed door synaptische input van de parallelvezels en interneuronen dan worden actiepotentialen "simple spikes" genoemd. Purkinje-cellen vuren simple spikes met een frequentie van ongeveer 60 Hz, maar deze vuurfrequentie varieert sterk. Het vuurpatroon van de simple spikes wordt onderbroken door een zogenaamde klimvezel-pauze. Deze pauze vindt plaats na elke complex spike en heeft een minimumduur van ongeveer 10 milliseconde.

Het mechanisme achter de generatie van spikes in Purkinje-cellen is al decennia onderwerp van intensief onderzoek. De in- en uitstroom van natrium-, kalium- en calcium-ionen door kanalen en poriën in het celmembraan speelt een essentiële rol bij de generatie van zowel simple spikes als complex spikes. Daarnaast is calcium een van de belangrijkste 'second messengers' in de Purkinje-cel; fluctuaties in de intracellulaire calciumconcentratie kunnen leiden tot genexpressie, excitotoxiciteit en veranderingen in synaptische plasticiteit. Purkinje-cellen zijn dan ook uitgerust met een calcium-buffersysteem dat een hoge capaciteit heeft. Verschillende calciumbindende eiwitten zoals calbindin D-28k en parvalbumine binden de vrije calciumionen en houden zo de intracellulaire calciumconcentratie op peil. Ook zijn er verscheidene membraan-kanalen en pompen die vrije calciumionen verwijderen uit het cytosol. Deze actieve mechanismen beperken de verspreiding van pieken in de intracellulaire calciumconcentratie door het cytosol. Verder zijn er sterke indicaties dat ook de morfologie van de Purkinje-celdendrietboom een sterk beperkend effect heeft op de verspreiding van stijgingen in de intracellulaire calciumconcentratie. Dus niet alleen actieve maar ook passieve vormen van calcium-buffering zorgen voor de calcium-homeo-

stase binnen een Purkinje-cel.

Een van de belangrijkste effecten van fluctuaties in de intracellulaire calciumconcentratie is de verandering van synaptische plasticiteit. Zowel de parallelvezel- als klimvezel-acitiviteit induceert calciuminstroom. Wanneer parallelvezel- en klimvezel-activiteit worden gecombineerd, stijgt de intracellulaire calciumconcentratie zodanig dat er verscheidene mechanismen in werking treden die leiden tot de internalisatie van AMPA-receptoren. Deze glutamaatreceptoren reguleren de postsynaptische respons op presynaptische input. De vermindering van het aantal AMPA-receptoren in het celmembraan leidt uiteindelijk tot een verminderde electrische repons van de Purkinje-cellen op parallelvezel activiteit. Dit fenomeen wordt beschreven als "long-term depression" (LTD) van de parallelvezel – Purkinje-cel-synaps. De gelijktijdige activering van parallelvezels en klimvezels werd als noodzakelijk gezien voor de inductie van adaptatie van de motoriek, maar het is recent gebleken dat ook muizen waarin parallelvezel LTD is aangedaan nog in staat zijn om hun motorisch gedrag aan te passen.

Het huidige proefschrift beschrijft hoe het cerebellum bijdraagt aan oogbewegingen. Compensatoire oogbewegingen, zoals de optokinetische reflex (OKR) en de vestibuloculaire reflex (VOR), zijn een simpele en goed kwantificeerbare vorm van motorisch gedrag. Het oog en de oogkas vormen samen het enige gewricht. De stimuli die nodig zijn om compensatoire oogbewegingen op te wekken zijn makkelijk te genereren en quantificeren. De relatie tussen stimulus-input en oogbeweging-output kan hierdoor nauwkeurig bestudeerd worden. In het huidige proefschrift is er voor gekozen om compensatoire oogbewegingen in combinatie met de activiteit van flocculaire Purkinje cellen te meten. In de afgelopen decennia is dit onderzoek al uitvoerig gedaan in verschillende diermodellen zoals aap, kat, konijn en rat. Het huidige proefschrift beschrijft voor het eerst deze experimenten in de muis. Er zijn verschillende redenen om muizen te kiezen voor dit onderzoek. Muizen zijn relatief makkelijk en goedkoop te onderhouden en het is ethisch verantwoord deze zoogdieren te gebruiken voor wetenschappelijke experimenten. En doordat de volledige DNA-sequentie van de muis bekend is, is het relatief eenvoudig om mutaties in het genoom van de muis vast te stellen of te genereren. De onderzoeken gepresenteerd in dit proefschrift behandelen drie muismodellen, waarvan er twee genetisch geproduceerd zijn. Al deze muismodellen zijn gekarakteriseerd door een mutatie die effect heeft op de calciumhomeostase en / of calcium-afhankelijke veranderingen van synaptische plasticiteit. Door het gebruik van deze muismodellen kan in detail worden bepaald hoe de interne calciumconcentratie het vuurpatroon van Purkinje-cellen kan bepalen en hoe dit vuurpatroon een effect heeft op het motorisch gedrag.

Het huidige proefschrift beschrijft hoe de calciumhuishouding een effect kan hebben op het vuurpatroon van Purkinje-cellen en hoe zulke effecten het motorisch gedrag kunnen beïnvloeden. Daar dit onderwerp erg specifiek is, wordt in hoofdstuk 1 van dit proefschrift allereerst ingegaan op de morfologie en de electrofysiologie van Purkinje-cellen. Verder wordt uiteengezet hoe de calciumhuishouding in Purkinje cellen wordt gereguleerd en wat voor effect de intracellulaire calciumconcentratie heeft op synaptische plasticiteit. Hoofdstuk 2 gaat verder in op de specifieke verbindingen van de flocculus en laat zien hoe de flocculaire afferente projecties van invloed zijn op de regulatie van de compensatoire oogebewegingen.

De intracellulaire calciumconcentratie beïnvloedt verschillende andere intracellulaire systemen die essentieel zijn voor het functioneren van de cel. Het huidige proefschrift gaat in op drie facetten van de calciumhuishouding van cerebellaire Purkinje-cellen. Allereerst wordt de influx van calcium besproken aan de hand van de *tottering* (*tg*) mutant. Deze mutant worden gekenmerkt door een mutatie in het gen dat codeert voor het P/Q-type calcium kanaal, dat meer dan 90% van de calcium-instroom in Purkinje-cellen van wild type muizen controleert (Llinas et al., 1989). Hoofdstuk 3 beschrijft wat voor effect deze mutatie heeft op het spontane en gemodu-

leerde vuurpatroon van Purkinje-cellen. Vervolgens wordt in dit proefschrift de functie van de calcium-buffercapaciteit van de Purkinje cellen bestudeerd aan de hand van Purkinje-cel-specifieke calbindin D-28k knock-out muizen. Calbindin D-28k is het meest voorkomende calcium bufferende eiwit in Purkinje-cellen. Hoofdstuk 4 beschrijft wat voor effect de absentie van calbindin D-28k heeft op de celfysiologie van Purkinje-cellen. Tot slot wordt er in hoofdstuk 5 ingegaan op de vraag of calcium-afhankelijke depressie van de synaptische kracht (LTD) een effect heeft op het vuurpatroon van Purkinje cellen tijdens oogbewegingsmotoriek.

Hoofdstuk 3 beschrijft de Purkinje-cellen van de *tg*-muizen. Deze muizen worden gekenmerkt door een mutatie in P/Q-type voltage-gated calciumkanaal, die wijd verspreid is in het centrale zenuwstelsel. Deze kanalen laten ongeveer 40% minder calcium door in de tottering muizen (Wakamori et al., 1998). De influx van calcium is van groot belang voor de generatie van spikes in Purkinje-cellen, want de *tg* Purkinje-cellen hebben een extreem onregelmatig spontaan simple spike vuurpatroon. Flocculaire Purkinje-cel-acitiviteit tijdens optokinetische stimulatie laat zien dat complex spikes en simple spikes in *tg*-muizen normaal moduleren. Daarintegen is de regelmaat van het simple spike vuurpatroon verstoord in dezelfde mate als tijdens spontane activiteit. *Tg* muizen hebben een significant slechtere OKR en visueel versterkte VOR. Wanneer in "wild type" muizen de flocculus wordt geledeerd dan verslechteren de OKR en VVOR dramatisch. De amplitude van de oogbewegingen in deze geledeerde wild type muizen is niet significant verschillend van de oogbewegingen van *tg* muizen waarin de flocculus nog intact is. Deze resultaten laten zien dat de functionele output van de flocculus in *tg* muizen nihil is. Daar P/Q-type calciumkanalen in vrijwel alle synapsen distaal van de flocculus aanwezig zijn, zou een van deze synapsen functioneel geblokkerd kunnen zijn. Daar het toedienen van korte en lange (200 – 1500 ms) electrische pulstreinen aan de flocculus vergelijkbare oogbewegingen opwekt in wild type- en *tg*-muizen kunnen we concluderen dat de synapsen distaal van de flocculus functioneel intact zijn. Verder zijn de karakteristieken van de spontane snelle nystagmus-oogbewegingen bestudeerd. Deze soort oogbewegingen erg intensief zijn voor de neuromusculaire verbindingen, omdat in korte tijd (milliseconden) een grote spierkracht moet worden opgewekt. Er zijn geen significant verschillende parameters gevonden voor *tg* en wild type muizen. Tezamen leidt dit tot de conclusie dat het hoogst waarschijnlijk is dat het onregelmatige simple spike vuurpatroon van de flocculaire Purkinje-cellen resulteert in een functionele lesie van het cerebellum. Wat het vuurpatroon van de simple spikes zo onregelmatig maakt en waarom deze onregelmatigheid leidt tot het verlies van kracht van modulatie, zal in de nabije toekomst worden onderzocht. Hiervoor wordt een Purkinje-cel-specifieke "rescue"-muis gemaakt, waarin alleen in de Purkinje-cellen het gen dat codeert voor het normale P/Q-type kanaal tot expressie komt.

Het verstoren van de instroom van calcium heeft grote gevolgen voor de Purkinje-cellen en het functioneren van het cerebellum en dus ook voor het functioneren van de muis. Niet alleen de instroom van calcium is van groot belang voor het functioneren van de Purkinje cellen. Ook als de instroom van calcium normaal is, maar er intracellulair iets verstoord is in de calcium-huishouding kunnen er problemen ontstaan in het functioneren van het cerebellum. Purkinje-cellen zijn overvloedig uitgerust met calciumbuffers die binnenkomende calciumionen snel binden en daardoor de calciumconcentratie onder controle houden. Calbindin D-28k en parvalbumine zijn calcium buffers die een hoge affiniteit hebben voor calcium. Verschillende voorgaande studies hebben het effect van de afwezigheid van deze twee calcium buffers bestudeerd (Airaksinen 1997, Caillard et al., 2000; Vecellio et al., 2000; Schmidt et al., 2003). Maar aangezien verschillende soorten cellen in het cerebellum deze calcium-bufferende eiwitten bevatten, is het effect dat deze studies beschrijven niet toegespitst op de functie van deze calcium-bindende eiwitten in de Purkinje cel. Hoofdstuk 4 beschrijft de motoriek en de Purkinje cel electrofysiologie van de L7-calbindin muizen. Ten gevolge van parallelvezel- en klimvezel-activiteit stijgt de intracellulaire

calciumconcentratie hoger dan in wild type Purkinje cellen. De daaropvolgende daling van de intracellulaire calciumconcentratie is sneller in de mutanten. Deze verandering van de calcium stromen leidt echter niet tot veranderingen in de inductie van parallelvezel LTD. Verder wordt in dit hoofdstuk beschreven dat de Purkinje-cellen een normaal spontaan simple spike en complex spike vuurgedrag vertonen. De OKR en de visueel versterkte VOR zijn daarentegen slechter in L7-calbindin muizen, evenals de prestatie tijdens de "run-way" test van deze muizen. Dit laat zien dat L7-calbindin muizen naast een verhoogde amplitude van calciumstromen een verstoorde motoriek hebben. Aangezien het Purkinje-cel-vuurpatroon niet veranderd lijkt, kan de volgende hypothese gesteld worden: In L7-calbindin muizen is de synaptische transmissie in de Purkinje-cel-terminal verstoord en deze verstoring ligt ten grondslag aan de verstoorde motoriek van deze mutante muizen.

Zowel *tg* als L7-calbindin D-28k muismodellen hebben abnormale calciumhomeostase in hun Purkinje-cellen. De interne calciumconcentratie controleert de polariteit van lange termijn veranderingen in synaptische plasticiteit in de Purkinje-cel. Om te zien of een defect in calcium-afhankelijke LTD in Purkinje cellen leidt tot een verandering in het vuurpatroon van Purkinje-cellen tijdens motoriek, bestudeerden wij de flocculaire Purkinje-cel-activiteit tijdens optokinetische stimulatie in muizen die geen PKC-functie hebben. PKC is een enzym dat de internalisatie van AMPA-receptoren reguleert. De activiteit van PKC wordt mede gereguleerd door de intracellulaire calciumconcentratie. In L7-PKCI muizen komt er in Purkinje-cellen een enzym tot expressie dat PKC inhibeert. Deze L7-PKCI muizen tonen verminderde capaciteit om hun motoriek aan te passen, maar hun Purkinje-cellen tonen normale vuurpatronen: Zowel de simple spike als de complex spike vuurpatronen zijn niet verschillend in L7-PKCI- en wild type-muizen. Deze resultaten wijzen erop dat LTD van de parallel vezel – Purkinje-cel-synapsen niet essentieel is voor de modulatie van het Purkinje-cel vuurpatroon, wat ook in de verwachting lag, gezien het feit dat de compensatoire oogbewegingen van de L7-PKCI muizen niet zijn aangedaan. Samenvattend hebben effecten op de calciumhomeostase in de Purkinje-cel grotere gevolgen voor zowel het vuurgedrag van deze cellen als het motor gedrag van de muis, dan effecten op de veranderingen in de synaptische plasticiteit. Hoofdstuk 6 bediscussieert hoe het komt dat een veranderde calciumhomeostase een effect heeft (of juist niet) op het Purkinje-cel vuurpatroon. Verder wordt ingegaan op de vraag wat voor effect het al dan niet veranderde Purkinje-cel vuurpatroon zal hebben op de synaptische transmissie van de Purkinje-cel naar de cerebellaire kern cellen.

Het huidige proefschrift beschrijft voor het eerst het Purkinje-cel vuurpatroon in de muis tijdens compensatoire oogbewegingen. Deze gecombineerde metingen van cellulaire activiteit en oogbewegingsmotoriek in genetisch gemuteerde muizen zijn erg informatief gebleken. Het is nu duidelijk dat niet alleen de modulatieamplitude, maar ook de regelmaat van het Purkinje-cel vuurpatroon indicatief kan zijn voor de motoriek van de muis. Het huidige onderzoek heeft geleid tot initiatie van vele vervolgonderzoeken, die duidelijkheid moeten scheppen in hoe de output van Purkinje cellen het gedrag van muizen kan veranderen.

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En ik moet ook zeker Hans bedanken. Handige Hans mag ik wel zeggen. Als ik iemand uit moet leggen wie Hans is, dan zeg ik dat hij van een doosje lucifers een wasmachine kan maken. Dat is misschien wat overdreven, maar het komt slechts zelden voor dat Hans geen oplossing weet en het woord 'jammer' of 'kapot' uit z'n mond komt. Altijd was er wel een oplossing. Je hebt me menigmaal uit de brand geholpen, waarvoor ik je dankbaar ben. Je bent echt een bron van inspiratie in de zin dat je me hebt geleerd dat je een probleem op een hele boel manieren kan benaderen en zo vrijwel altijd wel tot een oplossing komt. Ondanks dat ik in Hans z'n boekje een ongelofelijke hoeveelheid minpunten heb opgebouwd, heeft ie altijd voor me klaar gestaan en had hij altijd wel even tijd voor me. Ik hoop dat het de komende drie jaar niet anders zal zijn. Naar aanleiding van een stelling uit Bas z'n proefschrift ("Wanneer een geBaste opstelling niet meer verHanst kan worden is deze ofwel perfect ofwel onbruikbaar") wil ik nu ook gelijk Bas bedanken. Als er iemand bijna net zo handig is als Hans, dan ben jij het wel, Bas. Bovendien ben jij zo ongeveer het computer-wonder van de afdeling en mag ik mezelf dus ook gelukkig prijzen dat ik een plaats bij jou op de kamer heb gevonden. En jahaaa, ik ga binnenkort echt patchen! Die cellen komen er echt wel hoor. Je hebt je broodnodige ruimte echt niet voor niets afgestaan aan een niet-blinker! Ook Bjorn en Hester wil ik bedanken voor de morele steun, de sloten koffie die jullie maar bleven aanvoeren, het checken van manuscripten (waar een hoop koffie voor nodig was) en natuurlijk de gezelligheid.

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de kroon spant. Daar zou ik 20 muizen tegelijk op kwijt kunnen!!

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For three persons I'll switch to English. A big shout-out to Matthew! Matt, thank you so much for all your support. Too bad you had to leave, but I can understand your need for non-science work. I must say that I expect you to return to neuroscience, because I've seen no one so enthusiastic in attending the Monday-morning meetings, no matter the subject. You were always asking questions and were always trying to help out the speaker by asking good questions and making helpful comments. Hope to see you soon again! Have a ball in Washington DC.

And of course I'm grateful to Jerry. Jerry, thank you for the tons of discussions and laughs we had on the (mouse) flocculus and all the other interesting subjects. I must say that I've never met somebody that knows more about such a wide variety of subjects than you do. You're a 'wandelende bibliotheek'. I hope that we'll meet many more times. Plus that I've lined up some more good Dutch dishes you should try! In addition, we'll go the Now & Wow as you requested several times.

Last but not least, I would like to show my gratitude to John Stahl. Like you showed Arjan, you showed me that good data can only be gathered by hard work and that I should always be full of critique on my own data. Your endless knowledge on the oculomotor system has been priceless to me during my student time. Your comments were always extensive, but were always guiding me in the right direction. Thanks for supporting me through my Ph.D.-student-time and I hope that we can be co-authors on other projects.

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Curriculum Vitae

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