Cerebellar Plasticity in Health and Disease

Cerebellaire plasticiteit bij gezondheid en ziekte

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CHAPTER 1

GENERAL INTRODUCTION

1.1 General function of the cerebellum

The cerebellum has an important role in fine-tuning movements and posture, as was already demonstrated early in the nineteenth century by lesion studies in animals (Rolando, 1809; 1823; Flourens, 1824; Luciani 1891; 1915). Damage of the cerebellum results in distinctive motor symptoms, which can be summarized as a combination of ataxia, a decrease of muscle tone, and intention tremor (Babinski, 1899; 1906; Holmes, 1939). Ataxia, or lack of coordination, includes a variety of movement disorders. In general, the preparation, initiation, and coordination of movements are impaired, as well as balance. As opposed to lesions of other motor processing centers, damage or even removal of the cerebellum does not produce paralysis, involuntary movements, or altered sensory thresholds, but results in erroneous movements. This indicates that the cerebellum regulates rather than generates movements.

Theories on how the cerebellum would regulate motor coordination have been mainly inspired by the clear motor impairments resulting from lesions of the cerebellum, as well as by its anatomical organization. The cerebellum can be divided into distinct functional regions with their own specific connections (Groenewegen and Voogd 1977; Groenewegen et al., 1979; Voogd and Bigaré, 1980). Throughout these different cerebellar cortical regions, neurons are arranged in a highly regular manner as repeating units (Eccles et al., 1967), which form basic structural-functional modules (Oscarsson, 1979; Ito, 1984). Given this extremely regular organization, it is generally believed that the cerebellum performs a general computation, which is similar for its different target systems (Marr, 1969; Albus, 1971).

The cerebellum helps coordinate movements by evaluating disparities between intention and action, in order to adjust the operation of motor centers in the cerebral cortex and brain stem, while a movement is in progress as well as during repetitions of the same movement. It is hard to believe that the cerebellum would enable such accurate and smooth movement coordination without being capable of learning. Classic observations already suggested an adaptive role for the cerebellum (Flourens, 1842; Luciani, 1891), but it was not until a further understanding of the cerebellar circuitry arose that one could start to speculate on the nature of the underlying mechanisms of learning in the cerebellum. Several theories and lines of evidence have indicated that the cerebellum plays an important role in the recalibration and adaptive adjustment of movements, as well as in learning new motor skills and associative learning (see Houk et al., 1996; Raymond et al., 1996; Thach, 1996; Ohyama et al., 2003 for reviews). The modifiable synaptic transmission in the cerebellar circuit modules is thought to be the neuronal basis for the information storage underlying these forms of ‘motor learning' (see Raymond et al., 1996; Ito, 2001; Carey and Lisberger, 2002 for reviews).

Besides the classical view of the cerebellum as a ‘sensory analyzer and movement coordinator’, and the later emphasis on its role in motor and associative learning processes, more recent reports suggest this view on the cerebellum might still be too narrow. An increasing body of evidence links the cerebellum with more cognitive functions like imagined movements or the planning of movements (see Thach, 1996; Petrosini et al., 2003 for reviews), and even with non-motor functions (see Schmamann, 1997; 1998 for reviews). This thesis, however, will mainly focus on the more

established aspects of cerebellar motor behavior, motor learning, and the underlying cellular processes.

1.2 Cerebellar motor coordination and motor learning

The inputs to the cerebellum carry extensive internal feedback information about goals, commands and programming of movements, as well as external feedback information associated with the execution of movement, which allows the cerebellum to compare the intended movement with the reported actual movement. The cerebellar circuitry is essentially composed of a relay station in the deep cerebellar and vestibular nuclei (DCN and VN) and a cortical 'side-loop' (Fig. 1). Cerebellar output to premotor centers originates from the DCN and VN and is driven by direct excitatory input from the mossy fibers. The DCN and VN output is modulated by inhibitory input from Purkinje cell axons, which convey the computations and interactions in the Purkinje cell. Two main types of afferents, the mossy fiber afferent/parallel fiber system and the inferior olive/climbing fiber system, project to the cerebellar cortex, and produce different firing patterns in the Purkinje neurons, which provide the only output from the cerebellar cortex.

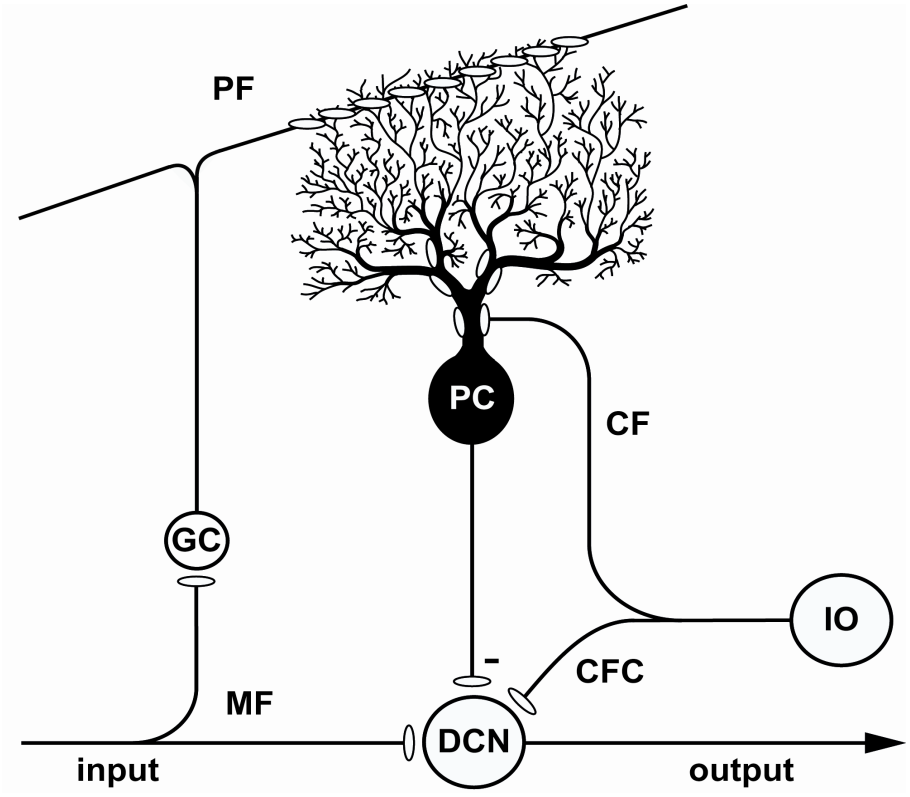

Figure 1: Simplified diagram of cerebellar circuitry. The main relay pathway: mossy fiber (MF) input from the precerebellar nuclei projects to the deep cerebellar nuclei (DCN), and DCN excitatory output targets premotor centers. Synapses are excitatory except when indicated (-). GC, granule cell; PF, parallel fiber; PC, Purkinje cell; IO, inferior olive; CF, climbing fiber; CFC, climbing fiber collateral

The mossy fibers originate from nuclei in the spinal cord and brain stem and transmit sensory information from the periphery as well as cerebral cortical information. They project to both DCN/VN neurons and to the cerebellar cortex, where they terminate on the dendrites of granule cells. The granule cell axons, called parallel fibers, travel long distances in the molecular layer, which allows them to excite large numbers of Purkinje neurons in the transverse plane. Parallel fiber activity produces a brief excitatory postsynaptic potential (EPSP) in the Purkinje neurons. The depolarization caused by the spatial and temporal summation of parallel fiber EPSPs increases the probability of the generation of a single action potential, the Purkinje cell 'simple spike' (Eccles et al., 1967). The information that is transmitted to the Purkinje neurons by the modulated discharge of the mossy fibers and parallel fibers is represented in the Purkinje neuron as a modulation in the simple spike frequency.

Climbing fibers originate from the inferior olivary nucleus and carry somatosensory, visual, or cerebral cortical information. Each climbing fiber projects to about 10 Purkinje cells, and each Purkinje cell is contacted by one climbing fiber, which wraps around the Purkinje cell soma and proximal dendrites, making numerous, extremely powerful synaptic contacts. In contrast to the parallel fibers, the climbing fiber can generate a prolonged depolarization of the Purkinje cell membrane that produces an initial large-amplitude spike followed by a burst of smaller action potentials, together called the 'complex spike' (Eccles et al., 1966). However powerful their connection, climbing fibers fire at very low rates (around 1 Hz, up to 8 Hz), which raises questions about their capacity to transmit moment-to-moment changes in sensory information. Despite their low firing frequency, climbing fibers may still have a great impact on cerebellar output, by influencing the Purkinje neuronal activity in two ways. First, climbing fiber activity acutely influences Purkinje neuronal simple spike firing (see Simpson et al., 1996 for review). Second, climbing fiber activity can induce selective long-term depression in the synaptic strength of the parallel fibers that are concurrently active (Ito et al., 1982a; Ekerot and Kano, 1985). This long-term depression may be an important information storage mechanism underlying cerebellar motor learning behavior (Ito, 1989).

The differences in connectivity, information content, firing behavior and postsynaptic effects are in line with the models of cerebellar function and plasticity, as proposed by Marr (1969), Albus (1971), and Ito (1972). In general, these models propose that the modulated discharge of the mossy fiber/parallel fiber system represents the conditions and context in which a movement is learned and performed, and the inferior olive/climbing fiber system transmits performance error signals. When such an error signal is conveyed by the climbing fiber, the strength of those parallel fiber-Purkinje cell synapses that are active at the same time (and therefore also support the erroneous movement), is reduced (Albus, 1971). The gradual inactivation of this subset of parallel fibers leaves the Purkinje cell with input from the parallel fibers whose action supports the correct movement, thus gradually improving performance. The long-term depression of parallel fiber-Purkinje cell synapses results in a decrease of Purkinje neuronal spike activity. This causes a disinhibition of the DCN, leading to an increased throughput of the direct mossy fiber-DCN-pathway.

Single-unit extracellular recordings from Purkinje cells gave results that were consistent with the Marr-Albus-Ito theory of long-term depression (LTD). Repetitive

conjoint electrical stimulation of climbing fibers and parallel fibers decreases the ability of this set of parallel fibers to induce spiking activity in a Purkinje neuron, while the synaptic strength of other, non-stimulated, parallel fibers is spared (Ito et al., 1982a; Ito 1984; Ekerot and Kano, 1985). However, this does not necessarily prove that LTD of the parallel fiber-Purkinje cell synapse is the (only) cellular substrate for cerebellar motor learning. In fact, there is evidence that information storage at the DCN and VN plays an important role in several types of motor learning (see Raymond et al., 1996; Mauk 1997; Bear and Linden, 2000; Hansel et al., 2001 for reviews).

The fact that many of the molecular mechanisms underlying LTD have been elucidated (see Bear and Linden, 2000; Ito, 2001 for reviews) has given an exciting new edge to the classical lesion-behavior correlation studies. Previous experiments that studied the effects of lesioning the cerebellar cortex on cerebellar motor learning were often contaminated by undesirable side effects. For instance, surgical or chemical ablation of cerebellar cortical tissue blocked motor learning (Robinson, 1976; Zee et al., 1981; Optican and Robinson, 1980; McCormick et al., 1982; Yeo et al., 1985a,b), but it also appeared to cause a retrograde degeneration of the afferent inferior olivary cells (Barmack and Simpson, 1980; Ito et al., 1980). Local application of chemicals that temporarily inactivated rather than destroyed cerebellar neurons dealt with this problem, indicating that inactivation of the cerebellar cortex directly blocks motor learning (Krupa et al., 1993; Krupa and Thompson, 1997; Garcia and Mauk, 1998; McElligott et al., 1998). However, these experiments still lacked the ability to target a specific type of cell or synapse. The unraveling of the molecular mechanisms that underlie LTD allowed for more refined chemical lesion experiments, by blocking specific signaling molecules or pathways that are involved in LTD induction, together with behavioral analysis of cerebellar learning tasks (Nagao and Ito, 1991; Li et al., 1995). The possibilities provided by developments in genetic technology set the stage for yet another experimental approach, by genetically disrupting the cellular processes leading to LTD (Aiba et al., 1994; Conquet et al., 1994; Shibuki et al., 1996; Chen and Tonegawa, 1997; De Zeeuw et al., 1998; Inoue et al., 1998; Chung et al., 2003; Feil et al., 2003). Although such a disturbance of LTD is generally accompanied by motor learning deficits of some kind, most of these genetically modified mouse models have their own specific issues, like developmental deficits or the presence of certain compensatory mechanisms (see Bear and Linden, 2000 for review).

Another important obstacle for investigating cerebellar learning is the fact that various kinds of manipulations of cerebellar function often induce a change or impairment in motor performance. If cerebellar damage affects performance, discussing its effect on motor learning might not be very meaningful, unless cerebellar motor coordination and learning can be dissociated in some way. Only few of the aforementioned mutant mice studies manage to combine affected cerebellar motor learning with otherwise healthy cerebellar motor coordination (Shibuki et al., 1996; De Zeeuw et al., 1998; Van Alphen and De Zeeuw, 2002; Koekkoek et al., 2003; Feil et al., 2003) and normal Purkinje neuronal excitability and firing behavior (Goossens et al., 2001; 2004). However, this raises the question as to how these animals managed to achieve normal motor performance, while lacking the ability to learn.

LTD at the parallel fiber-Purkinje cell synapse has been extensively studied and is one of the better-understood processes of plasticity in the brain. The evidence

gathered from the aforementioned range of experiments is definitively suggestive of a role for parallel fiber LTD in motor learning behavior, but it is still not conclusive. Several forms of plasticity have been described also at the Purkinje cell-DCN inhibitory synapse, including long-term potentiation (LTP) (Racine et al., 1986; Aizenman et al., 1998) and LTD (Morishita and Sastry 1993; 1996; Aizenman et al., 1998; 2000), as well as another form of plasticity in which the intrinsic excitability of DCN/VN neurons is persistently increased (Aizenman and Linden, 2000; Nelson et al., 2003).

Although the locus/loci of the memory trace underlying motor learning is still a matter of debate (see Ito, 1982; 1989; Raymond et al., 1996; Lisberger, 1988; 1998; Thach, 1992; 1996; Mauk, 1997; Bear and Linden, 2000; Carey and Lisberger, 2002 for reviews), the notion emerges that the information storage is distributed within both the cerebellar cortex and the brainstem. In view of a possible distribution of information storage, it is noteworthy that multiple use-dependent plasticity processes have been described at various other locations in the cerebellar cortex. Similarly to parallel fiber-LTD, LTD of the climbing fiber-Purkinje cell synapse, LTP of the inhibitory interneuron - Purkinje cell synapse, and LTP of the mossy fiber – granule cell synapse are triggered by presynaptic activity and/or CF activation, however their exact role in modulating cerebellar output remains unclear (see Hansel et al., 2001 for review).

Experimental evidence for a cerebellar role in motor learning has largely come from analysis of eyeblink conditioning and of adaptation of eye movements. In our behavioral experiments we focused on cerebellar coordination and adaptation of different forms of eye movements, which will be briefly introduced.

1.2.1 Eye movements: Compensatory eye movements

Eye movements have been widely studied by physiologists who investigate the neural control of movement. The oculomotor system is an attractive model, because of its relatively simple composition, while it covers the entire conversion from sensory input to the generation of movement. Both sensory input and behavioral output can be accurately measured, which makes the system amenable to detailed quantitative analysis. The fact that the cerebellum is generally assumed to act as a giant switchboard, performing similar computations for its different target (motor) systems, may enable us to extrapolate cerebellar influence on eye movements to cerebellar motor coordination in general.

The main function of the oculomotor system is to optimize vision. In humans this means that the fovea, the area in the center of retina with the highest density of photoreceptors and a 1:1 ratio to ganglion cells, has to be kept on its target. To this purpose, the human oculomotor system has a range of different voluntary gaze-directing, and involuntary gaze-stabilizing eye movements (Leigh and Zee, 1999). Mammals that lack a fovea, such as mice and rats, have relatively undeveloped gaze-directing eye movements, and rely mostly on gaze-stabilizing eye movements to prevent the projection of the visual surroundings to slip across the retina. In order to compensate for self-generated or imposed head movements, vestibular and visual information is used to generate compensatory eye movements to keep retinal slip to a minimum. The vestibulo-ocular reflex (VOR) generates eye movements in response to head movements,

which are detected by the labyrinth. The optokinetic reflex (OKR) generates eye movements in response to movement of images on the retina ('retinal slip'). Information about head movement and retinal slip signals are both sent to the VN, which send their eye movement commands directly to the oculomotor nuclei (Fig. 2). In natural behavior, the VOR and OKR act jointly to maintain a stable image on the retina. The fast-acting VOR is particularly sensitive to high-frequency head movements, and is supplemented by the OKR, which compensates for residual slow retinal slip, using visual feedback (Collewijn, 1969). The response to joint visual and vestibular stimulation is a linear sum of the VOR and the OKR response to residual slip (Baarsma and Collewijn, 1974; Batini et al., 1979), called visually enhanced VOR (VVOR), and is much more accurate over a wider range of stimulations than either of the two reflexes separately.

The cerebellum, the cerebellar flocculus in particular, is strongly involved in the coordination of compensatory eye movements (see Leigh and Zee, 1999 for review). Floccular Purkinje cells receive visual and vestibular information via mossy fiber and climbing fiber afferents, and project inhibitory output back to the vestibular nuclei, thus forming a cerebellar side loop onto the direct vestibular pathways that direct the VOR (Fig. 2). Therefore the flocculus is perfectly suited to modify VOR performance 'on-line' through visual feedback (Ito, 1972). When the flocculus is lesioned, this loss of on-line adjustment produces surpisingly little change in vestibular responses; the VOR

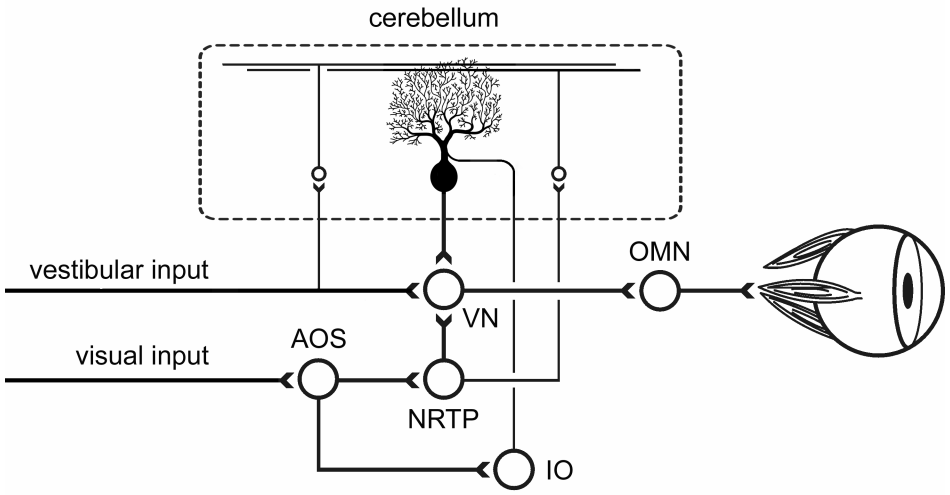

Figure 2: A schematic representation of the compensatory eye movement pathways illustrates parallel placement of the flocculus to direct oculomotor pathways. Information about linear and angular head acceleration from the labyrinth, and visual information from the retina are both projected to the vestibular nuclei (VN), which relays to the oculomotor nuclei (OMN), forming the 'direct pathway'. The cerebellar flocculus receives visual information and vestibular information via mossy fibers, as well as a 'performance error' signal via the climbing fibers, which signals a difference in the velocity of the eye movement and the stimulus that triggers it (Frens et al., 2001). Based on this error signal the Purkinje cell changes its contribution to floccular target neurons in the vestibular nuclei and thus acts to reduce the performance error. AOS, accessory optic system; IO, inferior olive; NRTP, nucleus reticularis tegmentum pontis.

gain may be slightly higher or lower. However, the OKR is much more severely impaired, especially at the higher velocities (Zee et al., 1981; Nagao, 1983; Waespe et al., 1983; Barmack and Pettorossi, 1985).

Apart from the rapid on-line modification of the VOR, the flocculus also uses visual information to recalibrate the VOR in a more permanent way in response to repeated errors. This visual calibration mechanism is necessary to maintain an accurate VOR throughout life. Experimentally, such long-term modifications to the VOR can be induced by altering visual feedback that accompanies head rotation (human: Gauthier and Robinson, 1975; Gonshor and Melvill-Jones, 1976a,b; monkey: Miles and Fuller, 1974; Miles and Eighmy, 1980; cat: Robinson, 1976; rabbit: Ito et al., 1979). Although lesioning the flocculus hardly affects the VOR itself, it does result in an inability to modify the VOR performance in response to this kind of visuo-vestibular training (Robinson, 1976; Zee et al., 1981; Ito et al., 1982b; Nagao, 1983; Lisberger et al., 1984; McElligott et al., 1998). Although this lack of VOR-adaptation could (partly) be due to the impaired visual OKR caused by the floccular lesion, lesioning the flocculus also removes previously established adaptation (Robinson, 1976), which indicates that the flocculus acts as a storage site for the newly adapted VOR. This finding is confirmed by experiments in which chemical inactivation of the flocculus returns the previously adapted VOR to pre-training values (goldfish: McElligott et al., 1998; monkey: Nagao and Kitazawa, 2003). This would suggest that modifications to the VOR are, at least initially, stored in the flocculus. Whether the memory trace remains there is less probable, in view of the fact that floccular lesions only slightly affect VOR performance.

Such evidence of memory storage in the flocculus supports the aforementioned theories of cerebellar function (Marr, 1968; Albus, 1971), and after finding experimental proof of synaptic plasticity in the flocculus (Ito et al., 1982a), Ito formulated his seminal hypothesis of floccular function (Ito, 1982). According to this hypothesis the climbing fibers, which carry retinal error signals (Simpson and Alley, 1974), relay the error signal that is induced by an inadequate VOR, to Purkinje cells in the flocculus. This climbing fiber activity causes LTD of the appropriate 'vestibular' parallel fiber inputs to these Purkinje cells, in order to keep the VOR calibrated (Ito, 1982; 1989) and thereby reduce retinal error.

Miles and Lisberger (1981) obtained evidence that VN neurons also undergo plastic modifications during VOR adaptation, which led to the 'multiple site hypothesis'. The multiple site hypothesis proposes that the cerebellum does 'guide' the learning, but the brain stem neurons targeted by the floccular Purkinje cells ('floccular target neurons': FTNs) are the primary site of plasticity. Comparative analyses of electrical activity of Purkinje cells and FTNs support a possible distribution of information storage within both brainstem and cerebellum (Lisberger, 1994; Partsalis et al., 1995; Lisberger, 1998; Hirata and Highstein, 2001). In addition, recent behavioral experiments indicate that changes in the VOR are implemented by different plasticity mechanisms with different characteristics (Boyden and Raymond, 2003).

The cellular mechanisms underlying plastic modifications in the VN remain to be elucidated, whereas the substrate of the cerebellar cortical component of VOR adaptation is generally assumed to be located at the parallel fiber-Purkinje cell synapse. LTD of this synapse is a well-described phenomenon, and specifically blocking LTD could therefore provide more insight on the role of the flocculus in VOR adaptation. Infusion

into the peri-floccular area of LTD-blocking chemicals indeed prevents adaptation of the VOR (Nagao and Ito, 1991; Li et al., 1995), but may have affected more processes than just LTD at the parallel fiber-Purkinje cell synapse. Studies in transgenic mice, in which a Purkinje cell-selective block of LTD was induced, provided evidence that LTD may indeed play a role in short-term VOR adaptation, but is not indispensable to recalibrate the VOR over the course of days (De Zeeuw et al., 1998; Van Alphen and De Zeeuw, 2002). The fact that these LTD-lacking mice show no detectable impairments in oculomotor or in general motor performance, nor any changes in Purkinje cell excitability and spike firing behavior (Goossens et al., 2001; 2004), which would be expected in case of a complete absence of a calibration mechanism, also makes the existence of additional sites of plasticity plausible.

1.2.2 Eye movements: Saccades

Saccades are quick jerky eye movements that enable rapid changes of gaze direction. Saccadic eye movements consist of a hierarchy of behavior, from the simple resetting of eye position during rotation in the dark, through reflexive saccades to appearing visual targets, to higher-level volitional behavior such as saccades to remembered target locations (Leigh and Zee, 1999; Leigh and Kennard, 2004). Similarly to compensatory eye movements, the lower-level saccades can be seen in afoveate animals, whereas primates also make higher-level voluntary saccades. The neuronal circuitry underlying saccade generation is much more complex than that of the VOR, and is still subject of debate (see Leigh and Zee, 1999; Scudder et al., 2002; Munoz, 2002; Pélisson et al., 2003 for reviews). However, the cerebellar side loop in the circuitry looks

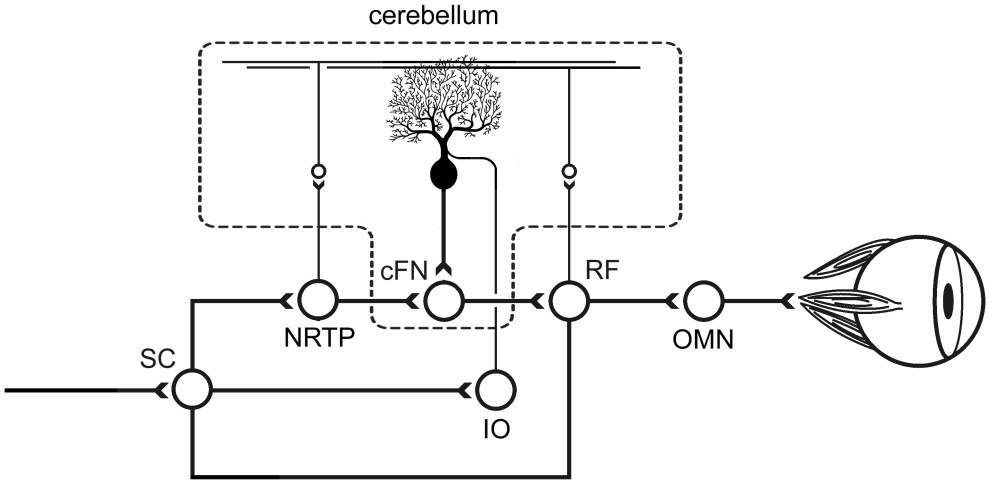

Figure 3: A schematic representation of the saccadic system illustrates parallel placement of the cerebellum to the 'direct' oculomotor pathway. Saccadic commands from the superior colliculus (SC) traverse both a direct path to the saccadic burst generator in the brainstem reticular formation (RF), and two 'indirect' pathways through the cerebellum. Note the similarity to the VOR circuitry in figure 2. cFN, caudal fastigial nucleus; IO, inferior olive; NRTP, nucleus reticularis tegmentum pontis; OMN, oculomotor nuclei

similar to that in the VOR circuitry (Fig. 3), and modulates the output of the main pathway in an analogous manner.

The direction and amplitude of saccades are coded by the superior colliculus (SC). This information is sent to the brainstem reticular formation, which generates the neuronal motor command that is sent to the oculomotor nuclei. Various cerebral cortical and subcortical areas project directly to the SC and the brainstem, generating higher-level saccadic behavior such as target-selective saccades, saccades to remembered targets, predictive saccades, or suppression of saccades (Leigh and Zee, 1999; Scudder et al., 2002; Munoz, 2002; Leigh and Kennard, 2004).

The accuracy of saccades is controlled by the cerebellum, especially lobules VI-VII of the dorsal cerebellar vermis and the underlying caudal fastigial nuclei (cFN). The dorsal vermis and cFN receive afferents from a variety of brain stem and thalamic structures that are concerned with the generation of saccades (see Leigh and Zee, 1999; Pélisson et al., 2003 for reviews). The cFN projects back to the brain stem, as well as to the SC and the thalamus (not shown in fig. 3), thus completing an anatomical cerebellar side loop, resembling that described in the VOR circuitry. Microstimulation of the dorsal vermis in monkeys during saccadic eye movements induces a dysmetria of the saccades, meaning the saccades over- or undershoot their target (Ron and Robinson, 1973; Noda and Fujikado, 1987). Experimental lesions or inactivation of the dorsal vermis (Sato and Noda, 1992; Takagi et al., 1998; Barash et al., 1999), the cFN (Vilis and Hore, 1981; Robinson et al., 1993) or both (Ritchie, 1976; Optican and Robinson, 1980) induce an enduring saccadic dysmetria without abnormalities of velocity or latency (Optican and Robinson, 1980). These findings suggest that the cerebellum is not crucially involved in the initiation or execution of saccadic eye movements, but rather modifies the saccade trajectory 'on-line', adding a signal to central commands executing a saccade (see Robinson and Fuchs, 2001; Scudder et al., 2002; Pélisson et al., 2003; Leigh and Kennard, 2004 for reviews). However, normal saccadic eye movements are so quick and short-lasting that there is not enough time for sensory feedback generated during that same saccade to guide it to its target. Therefore the saccade is dependent on accurate predictive control, much like the feedforward control of the VOR (Ito, 1989). Analogous to the VOR circuitry model, one may suppose that the cerebellum contributes to the modification of the major brain stem pathway for saccades, thus keeping sensory and motor components calibrated with each other. Such a recalibration would be needed in order to compensate for the changes that can occur in the oculomotor system throughout life, such as neuronal death or muscle weakness (Kommerell et al., 1976; Abel et al., 1978; Optican et al., 1985).

Experimentally, adaptation to saccadic dysmetria can be induced by surgically lesioning the extraocular muscles of monkeys (Optican and Robinson, 1980; Scudder et al., 1998). A second, noninvasive approach is to change the target location during a saccade, thus making the saccade seem inaccurate. After a number of such recurrent trials, subjects automatically begin to make saccades that are gradually bigger or smaller, depending upon the particular nature of the induced dysmetria (McLaughlin, 1967; Deubel et al., 1986; Frens and Van Opstal, 1994). This saccade adaptation is dependent on the cerebellum, as was shown in patients with syndromes associated with cerebellar dysfunction (Waespe and Baumgartner, 1992; Straube et al., 2001). Studies performed in monkeys show that saccade adaptation does not occur after lesioning the oculomotor

vermis (Takagi et al., 1998; Barash et al., 1999), lesioning or inactivating the cFN (Goldberg et al., 1993; Robinson et al., 2002) or lesioning both (Optican and Robinson, 1980). However, this once more rises the question as to what extent the induced performance deficits affect the cerebellar learning behavior. Cerebellar lesions invariably lead to a larger variability in saccade amplitudes and thus interfere with the assessment of a constant error signal. However, experiments in patients (Straube et al., 2001) and monkeys (Takagi et al., 1998; Barash et al., 1999) with posterior vermis lesions show a dissociation between the extent of the saccadic variability and the lack of adaptive capability, indicating that the lack of adaptation is not solely caused by the saccade dysmetria. More evidence for cerebellar involvement in saccade adaptation came from positron emission tomography (PET) studies performed in healthy human subjects. Metabolic changes in the medioposterior cerebellum indicate that neuronal activity of the dorsal vermis is increased during saccade adaptation (Desmurget et al., 1998; 2000).

Together the above results suggest that plastic modifications are made in the cerebellar side loop, analogous to the VOR circuitry. This would be in line with previous experiments showing that the locus of plasticity is neither upstream (Frens and Van Opstal, 1997), nor downstream of the SC (Melis and Van Gisbergen, 1996). As Frens and van Opstal (1997) point out, an adaptation site in parallel to the SC would be a parsimonious solution for these apparently conflicting results. However, some questions remain about the exact inputs into the cerebellum, and about its role in the modification of different kinds of saccades (Melis and Van Gisbergen, 1996). Additional sites of plasticity outside the cerebellum cannot be ruled out, although PET studies in healthy humans do only show marginal contributions of cerebral cortical areas and SC in fast saccade adaptation (Desmurget et al., 2000). It is noteworthy that, although lesioning the cerebellar cortex completely and permanently removes fast saccade adaptation, it does not completely abolish a slow recovery of dysmetria (Barash et al., 1999).

The exact location of the plastic modifications within the cerebellar side loop leading to saccade adaptation remains subject of debate. Recordings from the cFN show a change in neuronal discharge during saccade adaptation (Inaba et al., 2003; Scudder and McGee, 2003). Temporary inactivation of the cFN with muscimol blocks an immediate change of saccade amplitude in a paradigm that consistently changes the target location during the saccade. However, saccades appear to be at least partly adapted after the muscimol dissipates (Robinson et al., 2002), indicating that (at least part of the) plastic modifications underlying saccade adaptation occur upstream of the cFN. Plasticity in the cerebellar cortex, and more specifically long-term modification of the parallel fiber-Purkinje cell synapse, is a major candidate to serve such a function. Whereas the cellular-molecular mechanisms of VOR learning have been extensively studied, the mechanisms underlying saccade adaptation have not received this much attention. The fact that saccade adaptation can only be observed in humans and primates has limited the range of possible experiments. As genetically modifying molecular pathways is not an option in primates, future experiments should focus on neuronal recordings and/or studying the effects of acutely and selectively blocking molecular pathways of synaptic plasticity by infusion of chemicals in monkeys.

1.3 Cerebellar processing and plasticity

Purkinje neurons show a spontaneous rate of action potential firing, the pattern and frequency of which can be modulated by the various excitatory and inhibitory synaptic inputs. The Purkinje cells provide the sole output of the cerebellar cortex, which makes them an interesting site to study on a single-cell level. The previously mentioned in vivo experiments have mainly studied the information content of the cerebellar cortical output after integration of the various inputs, and its correlation with behavior and adaptation. In vitro studies have contributed to our understanding of how Purkinje neuronal firing behavior emerges on a cellular level, and how it can be modulated both acutely and in the long term.

1.3.1 Purkinje cell spikes and excitability

Cerebellar Purkinje neurons show spontaneous action potential firing (Granit and Phillips, 1956; Eccles et al., 1967), which is generated by intrinsic membrane conductances (Raman and Bean, 1997; 1999; Williams et al., 2002) and becomes very regular when synaptic inputs are blocked (Häusser and Clark, 1997). The various synaptic input signals are superimposed upon this tonic firing. Inhibitory input from interneurons can be detected as a delay in action potential firing, excitatory input from parallel fibers as an increase in spike rate, and climbing fiber excitatory input induces a powerful all-or-none complex spike response.

Purkinje cells receive input from numerous parallel fibers, which synapse onto Purkinje cell dendritic spines on secondary and tertiary branches. Parallel fiber activation results in glutamate release from these synapses, which activates glutamate receptors on the Purkinje cell dendrite. Two types of glutamate receptor are found at high concentrations in the dendritic spines where parallel fibers are received: ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, and metabotropic mGluR1 receptors which couple via G-proteins to intracellular messenger cascades. The AMPA receptors mediate a fast excitatory postsynaptic potential (EPSP) through Na^+ influx, whereas mGluR1 activation results in an additional slow EPSP under certain activity conditions.

As was mentioned in paragraph 1.2, each Purkinje cell also receives excitatory input from a single climbing fiber, which predominantly contacts the spines at the Purkinje cell's primary dendrite through approximately 1500 synapses that operate at a high release probability (Dittman and Regehr, 1998). Upon climbing fiber activity, glutamate is released and binds to postsynaptic AMPA and mGluR1 receptors, producing a strong postsynaptic depolarization. This massive depolarization evokes an all-or-none spike with multiple peaks: the complex spike. Complex spikes consist of an initial fast spike component followed by series of smaller spikes riding on top of a plateau, after which a fast repolarization and slow afterhyperpolarization bring the membrane potential back to resting levels (see Schmolesky et al., 2003 for review). It has been suggested that the fast component is a somatic Na^+ spike, whereas the following slow components are initiated by dendritic Ca^{2+} conductances (Llinás and Sugimori, 1980a,b) and a 'resurgent' Na^+ current (Raman and Bean, 1997). However, the exact 'anatomy' of the

complex spike is not completely clear (see Schmolesky et al., 2003 for review). The main cellular consequence of a complex spike is a large, widespread Ca^{2+} influx through voltage-gated Ca^{2+} channels (VGCCs) (Ross and Werman, 1987; Knöpfel et al., 1990; Miyakawa et al., 1992), which can trigger a variety of intracellular cascades, some of which for instance are involved in various types of synaptic plasticity.

1.3.2 Long-term changes of synaptic strength

Long-lasting synaptic gain changes in the cerebellar circuitry are generally thought to form the information storage mechanism involved in cerebellar motor learning. Various forms of synaptic and non-synaptic plasticity might interact to provide this 'cerebellar memory' (see Hansel et al., 2001 for review). As described previously, plastic modifications of parallel fiber (PF) to Purkinje cell (PC) synapses are believed to be crucial for certain forms of cerebellar motor learning (Marr, 1969; Albus, 1971; Ito, 1972).

Long-term depression of the PF-PC synapse (PF-LTD) was first described in the intact cerebellum (Ito et al., 1982a), and has since then been described in a variety of preparations. In slices or in situ, PF-LTD is typically induced by pairing PF and climbing fiber (CF) stimulation at low frequencies. This paired stimulation results in a selective attenuation of the PF-PC synapse (typically to about 50-80 % of its baseline synaptic strength), which reaches its full extent in about 10 minutes, and persists for the duration of the experiment, typically 1-2 hours. Repeated PF-LTD induction results in saturation of the response: a maximum depression of 50 % is typical. Originally, PF-LTD was reported to be strictly input-specific: PF-LTD only occurs in those PF synapses that are stimulated during CF activation (Ito et al., 1982a; Ekerot and Kano, 1985). However, recently PF-LTD has been reported to spread to neighboring synapses up to 100 μm from those involved in conjoint PF-CF stimulation (Hartell, 1996; Reynolds and Hartell, 2000; Wang et al., 2000b). It is generally thought that combined stimulation of PFs and CF is critical for PF-LTD induction (however see Hartell, 1996; Eilers et al., 1997 for reports on PF-LTD induced by very strong PF-only stimulation). The timing constraints on this PF-CF coactivation, which are important in order to correlate artificially induced PF-LTD and real-life behavioral learning, are still subject of debate (see Bear and Linden, 2000; Ito, 2001 for reviews).

Three initial signals are required for PF-LTD induction: activation of AMPA and mGluR1 receptors by glutamate release from the PF, and depolarization of the PC by CF activity (Fig. 4) (see Bear and Linden, 2000; Ito, 2001 for reviews). CF activation evokes a Ca^{2+} transient in PC dendrites, which is needed for PF-LTD induction (Sakurai, 1990; Konnerth et al., 1992; Shibuki and Okada, 1992). Glutamate release from the PF activates AMPA and mGluR1 receptors, both of which need to be activated in order for PF-LTD to occur (see Bear and Linden, 2000; Ito, 2001 for reviews). AMPA receptor activation causes a local depolarization in PF spines, leading to some influx of Ca^{2+} through VGCCs, which in turn enhances IP_3-mediated Ca^{2+} release from intracellular stores (Okubo et al., 2001). This local depolarization and Ca^{2+} rise combines with the massive complex spike-mediated depolarization to cause a supralinear Ca^{2+} influx

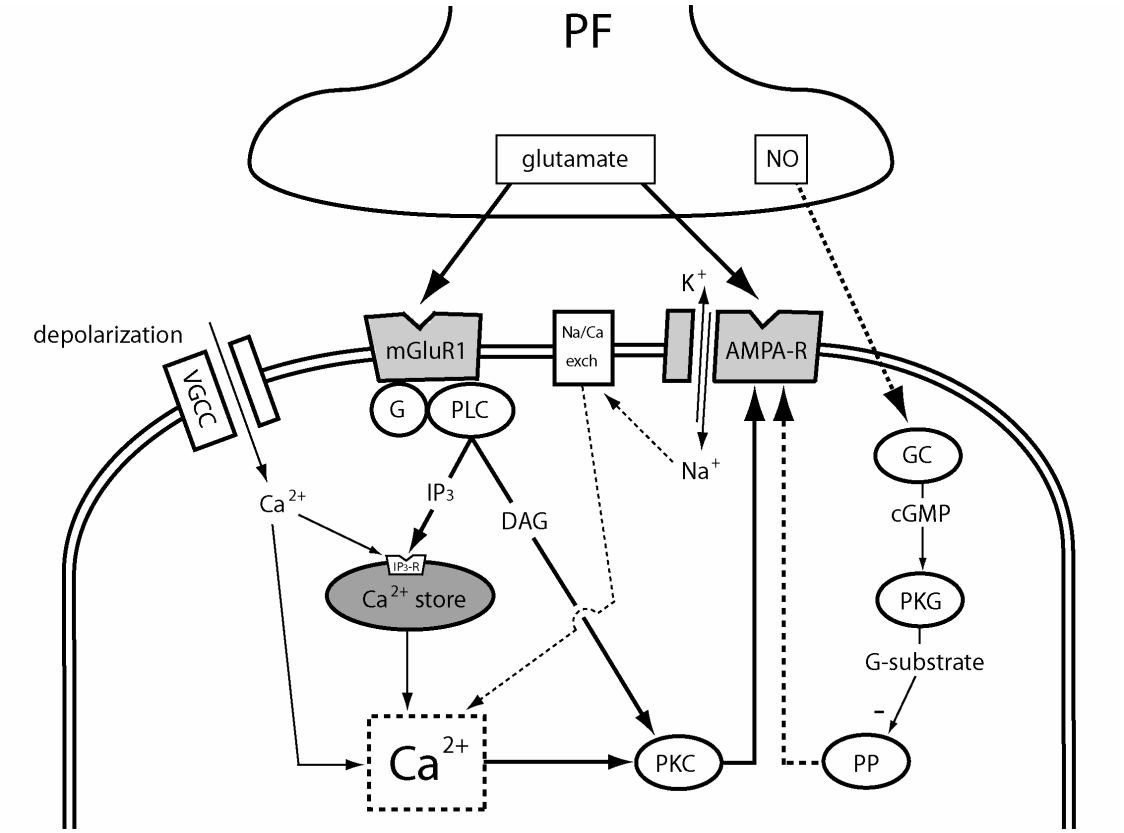

Figure 4: Model of LTD induction at the parallel fiber-Purkinje cell synapse. Phosphorylation of the AMPA receptor by PKC and its subsequent internalization leads to a reduction of synaptic strength. Well established mechanisms are represented as solid lines, whereas those that remain controversial are dotted. AMPA-R, AMPA receptor; DAG, 1,2,-diacylglycerol; G, G-protein; GC, guanylyl cyclase; IP_3, inositol-1,4,5-triphosphate; mGluR1, metabotropic glutamate receptor type 1; Na/Ca exch, plasma membrane Na^+/Ca^{2+} exchanger; NO, nitric oxide; PF, parallel fiber; PKC, protein kinase C; PKG, protein kinase G; PLC, phospholipase C; PP, phosphatase; VGCC, voltage-gated Ca^{2+} channel

(Wang et al., 2000a). Furthermore, the AMPA receptor appears to contribute to PF-LTD induction through a specific effect of Na^+ influx (Linden et al., 1993) which has not yet been fully elucidated (Bear and Linden, 2000). Activation of mGluR1 results in G-protein coupled activation of phospholipase C (PLC) and the consequent production of two second messengers: inositol-1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG). IP_3 binds to intracellular IP_3 receptors, resulting in a release of Ca^{2+} from intracellular stores (Takechi et al., 1998; Finch and Augustine, 1998). DAG and Ca^{2+} are both required for the activation of protein kinase C (PKC), which is thought to underlie the final pathway for expression of PF-LTD. Inhibition of PKC blocks PF-LTD induction (Crépel and Jaillard, 1990; Linden and Connor, 1991; De Zeeuw et al., 1998), while exogenous PKC activation induces PF-LTD (Crépel and Krupa, 1988; Linden and Connor, 1991). PKC acts on AMPA receptors, phosphorylating a serine-residue on the carboxy-terminus of the GluR2 subunit (Matsuda et al., 1999; 2000), which has been

shown to be critical for PF-LTD induction (Chung et al., 2003). This serine-residue is located in a PDZ domain-recognition site, by which AMPA receptors interact with the PDZ domain-containing proteins glutamate receptor interacting protein (GRIP) and protein interacting with c-kinase (PICK). GRIP stabilizes the AMPA receptor in the postsynaptic membrane, whereas binding to PICK triggers internalization (Xia et al., 2000). Phosphorylation of the PDZ domain by PKC disrupts the binding to GRIP (Matsuda et al., 1999; Chung et al., 2000) and promotes the binding to PICK (Chung et al., 2000), which finally results in clathrin-mediated endocytosis of the AMPA-receptor (Wang and Linden, 2000). Thus, internalization of AMPA receptors from the postsynaptic membrane enables the PC to reduce the contribution of selected PFs by reducing their synaptic weight.

In addition to PKC activation, a second pathway, mediated by release of nitric oxide (NO), has been implicated in PF-LTD induction (Fig. 4). NO release from PF terminals and/or interneurons activates a postsynaptic cascade consisting of soluble guanylyl cyclase, cGMP and cGMP-dependent protein kinase G (PKG). PKG activation results in phosporylation of G-substrate, which inhibits protein phosphatases, thus increasing the levels of phosphoproteins generated by the action of protein kinases. It has been hypothesized that the mGluR1-PKC and the NO-cGMP cascade converge on the same molecular alteration, such as an equilibrium between PKC and phosphatase activity (Daniel et al., 1998; Feil et al., 2003; Launey et al., 2004). Although many studies have shown an involvement of NO in PF-LTD, the reports on this pathway are rather contradictory, and its exact effector mechanisms remain unclear (see Daniel et al., 1998; Bear and Linden, 2000 for reviews).

PF-PC synapses cannot only be depressed, but can also show long-term potentiation (PF-LTP). The first reports on PF-LTP were merely side observations in studies focusing on PF-LTD (Sakurai, 1987; Sakurai, 1990; Hirano, 1990; Crépel and Jaillard, 1991; Shibuki and Okada, 1992). Systematic characterization revealed a presynaptic form of PF-LTP, induced by stimulating the PFs 120 times at 4-8 Hz. This form of PF-LTP depends on presynaptic influx of Ca^{2+}, activation of adenylyl cyclase I and the subsequent cAMP-dependent activation of protein kinase A (Salin et al., 1996; Storm et al., 1998). However, this presynaptic PF-LTP is not an appropriate mechanism for reversing postsynaptic PF-LTD: a downregulation of postsynaptic AMPA receptors cannot be balanced by an increase of transmitter release alone, without saturating the synapse. A recently described, different form of PF-LTP (Lev-Ram et al., 2002; 2003), provides a much better candidate for such a reversal mechanism. This cAMP-independent PF-LTP is induced by stimulating the PF alone for a prolonged period at a low frequency, and paired pulse facilitation (PPF) analysis suggests a postsynaptic expression site. PPF is a very short-term enhancement in synaptic efficacy attributed to residual presynaptic Ca^{2+} facilitating more transmitter release (Atluri and Regehr, 1996; Salin et al., 1996). Changes in PPF during PF-LTP would argue that PF-LTP is expressed presynaptically, whereas unchanged PPF would suggest a postsynaptic expression site. The molecular pathways of postsynaptic PF-LTP have been shown to differ from those of presynaptic PF-LTP (Lev-Ram et al., 2002), but remain to be characterized in detail.

The contribution of CF signals to PF-LTD induction has long been appreciated. However, the CF-PC synapse itself was generally considered invariant, although the

molecular machinery required for PF-LTD (mGluR1, AMPA-receptors, voltage-gated calcium channels, PKC) is also present at the CF-PC synapse. It was not until recently that LTD of the CF-PC synapse was observed after CF-only stimulation at 5 Hz for 30 seconds (Hansel and Linden, 2000; Weber et al., 2003). CF-EPSCs are typically reduced by approximately 20 %, and slow complex spike components, presumably reflecting Ca^{2+} conductances, are selectively reduced. This CF-LTD does not spread to neighboring PF synapses, is saturated after repeated tetanization, and does not show a change in PPF ratio, suggesting a postsynaptic expression site (Hansel and Linden, 2000; Shen et al., 2002). Similar to PF-LTD, CF-LTD induction depends on an increase of postsynaptic Ca^{2+}, activation of mGluR1 (Hansel and Linden, 2000) and PKC (Weber et al., 2003). The functional meaning of LTD at the CF-PC synapse is less apparent than at PF-PC synapses. CF-LTD might alter the probability of complex spike firing and/or the subsequent climbing fiber pause. In addition, the fact that CF-LTD results in a decrease of CF-evoked Ca^{2+} transients in the PC (Weber et al., 2003) might have several important functional implications (Hansel and Linden, 2000; Hansel et al., 2001; Weber et al., 2003). For instance, the CF-evoked Ca^{2+} transient has been shown to contribute to PF-LTD induction (Sakurai, 1990; Konnerth et al., 1992; Shibuki and Okada, 1992). Buffering of postsynaptic Ca^{2+} blocks PF-LTD induction (Sakurai, 1990; Linden and Connor, 1991; Konnerth et al., 1992; Shibuki and Okada, 1992), and facilitates postsynaptic PF-LTP induction (Lev-Ram et al., 2002). This suggests that the direction of the postsynaptic gain change might be determined by the amplitude of the postsynaptic Ca^{2+} transient: high Ca^{2+} for PF-LTD induction and low Ca^{2+} for PF-LTP induction. A reduction of CF-evoked Ca^{2+} transients might therefore affect normal PF-LTD induction. Similar Ca^{2+} threshold mechanisms controlling LTD vs. LTP induction have been described in the hippocampus and neocortex. However, in these areas the Ca^{2+} thresholds regulating LTD vs. LTP appear to be inversed: low postsynaptic Ca^{2+} concentrations induce LTD, whereas a higher Ca^{2+} influx leads to LTP (see Bear and Linden, 2000).

1.3.3 mGluR1

Both PF- and CF-PC synapses use glutamate as a neurotransmitter, which activates mGluR1 and AMPA receptors on the postsynaptic membrane. AMPA receptors are located at the center of the synaptic junction, whereas mGluRs are located at the periphery of the synapse. This spatial segregation may permit a differential activation of the AMPA receptor and mGluR1 according to the amount of glutamate released presynaptically (Nusser et al., 1994). The fact that transmission via mGluR1 is gradually recruited by increasing PF activity agrees with this hypothesis (Batchelor et al., 1994; Batchelor and Garthwaite, 1997; Tempia et al., 1998; 2001).

Activation of mGluR1 results in activation of a slow EPSC (Batchelor et al., 1994), which is carried by a mixed-cation conductance (Tempia et al., 1998; 2001; Knöpfel et al., 2000; Canepari et al., 2001; Kim et al., 2003). This inward current has been reported to increase the frequency of PC spontaneous action potential firing (Yamakawa and Hirano, 1999). A better understanding of the mechanisms underlying the mGluR1-mediated EPSC is needed in order to explain the interesting phenomenon

that a single activation of the CF can potentiate the mGluR1-mediated EPSC for about 2 minutes (Batchelor and Garthwaite, 1997). Although mGluR1 is also present at CF-PC synapses (Nusser et al., 1994; Petralia, 1998), mGluR1-mediated EPSCs have not been found at this synapse until recently, perhaps because of their size being limited by glutamate transporter activity (Dzubay and Otis, 2002).

Another direct effect of mGluR1 activation is an elevation of intracellular Ca^{2+}, caused by IP_3-dependent release from intracellular stores (Takechi et al., 1998; Finch and Augustine, 1998) and/or by Ca^{2+} influx from outside the PC (Wang et al., 2000a; Tempia et al., 2001). Similarly to the PF, increasing CF activity also gradually recruits mGluR1 activation, adding to the postsynaptic Ca^{2+} transient (Weber, personal communication).

Besides causing a slow EPSC and a rise in postsynaptic intracellular Ca^{2+}, mGluR1 activation has been reported to have a short-term retrograde inhibitory effect on presynaptic transmitter release from the PF and CF (Glaum et al., 1992). This mGluR1-mediated retrograde inhibition probably uses endogenous cannabinoids as a retrograde messenger (Maejima et al., 2001; Brown et al., 2003). These findings suggest that mGluR1 does not only play a role in PC excitability (Netzeband et al., 1997; Yamakawa and Hirano, 1999), but also dynamically modulates synaptic strength and fine-tunes the properties of synaptic integration in the short term (Brown et al., 2003).

In addition to these short-term effects, mGluR1 has been shown to play a role in several forms of long-term changes in synaptic transmission. First it was shown that induction of PF-LTD required activation of metabotropic glutamate receptors (Kano and Kato, 1987; Linden et al., 1991), which were later specified as mGluR1 (Shigemoto et al., 1994). Activation of mGluR1 results in production of the second messengers DAG and IP_3, both of which are thought to be essential for PF-LTD induction (Fig. 4). DAG activates PKC directly, whereas IP_3 is thought to contribute to PKC activation through release of Ca^{2+} from intracellular stores (see Bear and Linden, 2000; Ito, 2001 for reviews). The contribution of mGluR1 to PF-LTD was further confirmed and extended by studies in mGluR1-knockout mice (Aiba et al., 1994; Conquet et al., 1994). The fact that these mGluR1-lacking mice showed no PF-LTD made them interesting for studying the link between PF-LTD and cerebellar motor learning. However, several major problems complicated the behavioral analysis of these mice. First, mGluR1 is normally present in various cell types in the central nervous system, so effects on PF-LTD and motor behavior that were reported in mGluR1 knockout mice cannot be reliably linked to the specific absence of mGluR1 at the PF-PC synapse. Second, mGluR1 knockout mice show characteristic cerebellar symptoms such as ataxic gait, intention tremor, and motor discoordination (Aiba et al., 1994; Conquet et al., 1994), which complicates specifically testing their motor learning behavior. Third, these mice have PCs that fail to undergo the normal developmental conversion from multiple to mono climbing fiber innervation in early postnatal life (Kano et al., 1997; Levenes et al., 1997). The cell-specificity issue was largely resolved by the fact that Purkinje cell-specific reintroduction of mGluR1 in these mice rescues PF-LTD as well as the developmental and behavioral phenotype (Ichise et al., 2000). However, the fact remains that mGluR1-knockout mice have cerebellar developmental abnormalities as well as severe ataxia, which makes linking PF-LTD to motor learning problematic in these mice. In addition to its contribution to PF-PC synaptic transmission, mGluR1 is also present at CF-PC synapses, as well as the

rest of the molecular machinery thought to be necessary for PF-LTD induction. Like PF-LTD, CF-LTD induction has also been shown to be dependent on postsynaptic Ca^{2+} elevation, mGluR1 activation, and activation of PKC (Hansel and Linden, 2000). This suggests that mGluR1 might play a similar role in the induction of CF-LTD as it does in PF-LTD.

In the cerebellum, blocking mGluR1 function has not only been described to affect Purkinje neuronal spontaneous action potential firing (Yamakawa and Hirano, 1999; however see Neale et al., 2001) and induction of PF-LTD (Linden et al., 1991; Shigemoto et al., 1994) and CF-LTD (Hansel and Linden, 2000), a chronic mGluR1 block has also been reported to affect the development and survival of Purkinje cells (Catania et al., 2001).

1.4 Paraneoplastic cerebellar ataxia

During life, normal cerebellar functioning can be affected by a wide variety of processes that damage the cerebellum, either acutely or in a more subacute, degenerative manner. Subacute forms of cerebellar degeneration include a range of hereditary disorders as well as acquired kinds of ataxia. A rare acquired form of subacute cerebellar degeneration can be seen in some cancer patients. Cerebellar ataxia in cancer patients is often a 'direct' consequence of the tumor or side effects of treatment, but in rare cases the ataxia is associated with the presence of antineuronal autoantibodies in serum and/or cerebrospinal fluid (Henson and Urich, 1982). This syndrome, called paraneoplastic cerebellar ataxia (PCA), is thought to result from an immune response triggered by the expression in tumors of proteins that are normally expressed by cerebellar neurons only. It is assumed that the immune system recognizes these proteins as 'foreign' when ectopically expressed in tumor cells, which leads to an immune response not only directed against the tumor, but also against healthy neurons. The autoantibodies are generally thought to cause the ataxia, but since passive transfer of the antibodies into experimental animals fails to induce neurological damage, there is no direct evidence supporting this hypothesis (Graus et al., 1991; Sillevis Smitt et al., 1995). Considering the fact that nearly all forms of PCA are associated with autoantibodies directed against intracellular proteins, this lack of an immediate effect might be due to the inaccessibility of the neuronal 'target' proteins. Until now, anti-VGCC autoantibodies (VGCC-Ab) are the only PCA antibody type described to be directed against a neuronal membrane component. VGCC-Ab have been shown to directly affect neuronal function in Lambert-Eaton myasthenic syndrome (Lang and Newsom-Davis, 1995), but their exact role in PCA is not clear (Mason et al., 1997; Graus et al., 2002).

The most striking and consistent neuropathological finding in PCA is a severe, diffuse loss of Purkinje cells. Some neuronal loss may also be seen in the granule cell layer and DCN, and inflammatory infiltrates may be present in affected areas (Henson and Urich, 1982). A variety of paraneoplastic autoantibodies related to PCA have been described, showing different clinical features, prognosis and associated neoplasms (see Darnell and Posner, 2003 for review). Patients typically have an acute or subacute onset and progression of pancerebellar dysfunction. However, presenting symptoms may differ between cases since different cerebellar areas might not become damaged

simultaneously. Since PCA patients typically end up with a total loss of Purkinje cells, they effectively lose all cerebellar cortical output, resulting in severe disability.

Eye movement recordings in PCA patients reveal defects that are similar to those encountered in patients with other forms of cerebellar ataxia. Since in PCA the cerebellum is usually affected in its totality, the behavioral symptoms are comparable to the sum of the effects of lesions of the various oculomotor regions. Compensatory eye movement recordings show an impairment of smooth pursuit, OKR and VVOR, as well as problems with fixation and VOR cancellation (see Leigh and Zee, 1999 for review). The VOR usually remains intact, but VOR adaptation is impaired. Together, these symptoms indicate an inability of the smooth pursuit and OKR systems to complement, suppress or adapt the VOR. Analysis of the saccadic system reveals saccadic dysmetria (typically hypermetria), and postsaccadic drift (see Leigh and Zee, 1999; Leigh and Kennard, 2004 for reviews). Saccade adaptation is generally impaired, and this defect can not be attributed to an increase of saccade size variability only (Straube et al., 2001). These oculomotor defects are similar to those seen in cerebellectomized or focally lesioned cats (Robinson, 1976) and monkeys (Westheimer and Blair, 1973; Optican and Robinson, 1980; Takagi et al., 1998; 2000; Barash et al., 1999), or in mice that lack Purkinje cells and therefore cerebellar cortical output as a whole (Van Alphen et al., 2002).

Although PCA is a rare phenomenon, the study of these disorders could provide more insight into auto-immune diseases of the central nervous system, tumor immunology, and basic neurobiology in general (see Darnell, 1996 for review). An important step toward an increased understanding of PCA disease processes might be reached by establishing in vitro or in vivo animal models in which the effects of the autoantibodies can be mimicked or reproduced. Besides the clinical relevance, studying 'classical' as well as newly discovered PCA forms might also be interesting to the basic neurosciences. In some forms of PCA the autoimmune reaction targets specific cerebellar components, creating a very refined lesion, for instance through interfering with the function of a certain kind of receptor, or by destroying a specific type of neuron. Behavioral testing of such patients, as well as studying the potential effects of their autoantibodies in vitro and in vivo, could provide more insight into the deficits we see in the patients. Besides leading to an increased understanding of the pathophysiology of the patients' symptoms, further studies might also shed more light on the physiological function of the lesioned structure in normal behavior.

1.5 Scope of the thesis

This thesis addresses the (patho)physiology of cerebellar motor coordination and adaptation, as well as some of the putative underlying cellular mechanisms. An integrated approach of molecular biology, cellular physiology, and behavioral experiments is used to study various aspects of cerebellar function and dysfunction.

Cerebellar coordination and motor learning can be affected by degenerative processes, such as PCA. In order to understand the role of autoantibodies in the pathogenesis of PCA, it is important to establish models in which the effects of the autoantibodies on neuronal functioning can be studied directly. In chapter 2 we describe

two patients with Hodgkin's disease and PCA with a previously undescribed autoantibody. This autoantibody is characterized as directed against mGluR1, and is shown to interfere with receptor function in vitro. Potential direct effects of these autoantibodies on motor function are tested in mice. In chapter 3 we look further into the pathophysiological mechanisms that could underlie the patients' ataxia. Based on previous studies of mGluR1, a block of this receptor could affect neuronal excitability as well as synaptic plasticity, and even cause neuronal death. The effect of blocking mGluR1 with the patients' autoantibodies is studied on several levels: in vitro neuronal recordings, eye movements in mice, motor learning behavior in the patients, and degeneration as shown by pathological analysis of one patient's cerebellum. Apart from increasing our knowledge of their role in PCA, the anti-mGluR1 autoantibodies provide an interesting new edge in view of a possible role for mGluR1 in normal cerebellar motor coordination and adaptation. The autoantibodies enable us to study the effects of a block of mGluR1 on cerebellar motor coordination as well as motor learning, in both experimental animals and patients.

In humans, cerebellar motor learning can be evaluated using a saccade adaptation paradigm. Chapter 4 tries to examine the potential role of cerebellar synaptic plasticity processes in saccade adaptation. In order to study the motor learning rules governing saccade adaptation, experimental data recorded in humans are correlated to predictions from cerebellar plasticity. If synaptic plasticity would underlie saccade adaptation, the time course of induction and the error-based nature of both processes should be similar.

Various forms of plasticity have recently been described at every type of synaptic input onto PCs. In chapters 5 and 6 we describe experiments that investigate the requirements for induction of PF-LTD vs. PF-LTP in rat cerebellar slices. The effects of PF and CF activation and postsynaptic calcium concentrations on the polarity of PF-PC plasticity are studied, as well as the possibility of PF-LTD and PF-LTP reversing each other. Finally, we look into potential effects of CF-LTD on the probablility of PF-LTD induction.

In this thesis a variety of techniques and models is used to study from different angles the mechanisms that endow the cerebellum with the ability to adaptively coordinate movement, and to investigate what happens when these mechanisms fail.

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CHAPTER 2

PARANEOPLASTIC CEREBELLAR ATAXIA DUE TO AUTO-ANTIBODIES AGAINST A GLUTAMATE RECEPTOR

Abstract

There are many types of cerebellar ataxia, including ataxia due to congenital or metabolic disorders and a paraneoplastic form in patients with gynecologic cancer, breast cancer, lung cancer, or Hodgkin's disease (Henson and Urich, 1982). This paraneoplastic syndrome is the only type of cerebellar ataxia associated with autoantibodies against neuronal antigens. Often, the neuronal antigens are aberrantly expressed by the tumor cells (Furneaux et al., 1990; Luque et al., 1991; Hammack et al., 1992). The antineuronal autoantibodies are believed to cause cerebellar ataxia, but this is unproved (Graus et al., 1991; Sillevis Smitt et al., 1995). In Hodgkin's disease, the lymphoma precedes the ataxia by months to years in 80 percent of patients, and ataxia often occurs during a prolonged complete remission (Hammack et al., 1992). Among patients with this type of ataxia, 30 percent have anti–Purkinje-cell antibodies, some of which have the features of the neuronal antibody anti-Tr (Hammack et al., 1992; Graus et al., 1997).

We identified a new autoantibody in two patients with severe cerebellar ataxia that developed while they were in remission from Hodgkin's disease. The antibody reacts specifically with the metabotropic glutamate receptor mGluR1 in mouse brain. Metabotropic glutamate receptors belong to a large family of cell-surface receptors that transmit signals into the cell by coupling to guanine nucleotide-binding proteins (G-proteins) in the cytoplasm. Purified IgG from the serum of both patients blocked the glutamate-stimulated formation of inositol phosphates in Chinese-Hamster-Ovary (CHO) cells that expressed mGluR1α, and the injection of IgG from serum or cerebrospinal fluid into the cerebellar subarachnoid space of mice caused severe, reversible ataxia. These results indicate that antineuronal autoantibodies can cause disease of the central nervous system by blocking neuronal receptors.

Case reports

Patient 1

In 1995, when she was 19 years old, Patient 1 presented with subacute cerebellar ataxia. She had been treated with four cycles of mechlorethamine, vincristine, procarbazine, and prednisone plus doxorubicin, bleomycin, and vinblastine (MOPP-ABV) followed by subtotal nodal irradiation for stage IIA nodular sclerosing Hodgkin's disease. She had been in remission for two years when truncal ataxia, intention tremor, and gait ataxia developed. An examination of the brain with magnetic resonance imaging (MRI) was normal. The cerebrospinal fluid contained 28 mononuclear cells per cubic millimeter and had a protein concentration of 28 mg per deciliter. The IgG concentrations in cerebrospinal fluid and serum were 4.6 mg per deciliter (normal value, <8) and 0.89 g per deciliter, respectively. The albumin concentrations in cerebrospinal fluid and serum were 17 mg per deciliter and 4.8 g per deciliter, respectively. The calculated IgG index was 1.2 (an IgG index of more than 0.6 indicates intrathecal IgG synthesis) (Reiber and Felgenhauer, 1987). Cytologic examination showed no malignant cells. Serum and

cerebrospinal fluid contained IgG antineuronal antibodies of unknown specificity (Moll et al., 1995).

These findings strongly suggested a diagnosis of paraneoplastic cerebellar ataxia. The patient was treated with four plasma exchanges at intervals of two to three days, oral prednisone at a dose of 40 mg per day for six weeks, and two courses of intravenous immune globulin (total dose, 4 mg per kilogram of body weight). After the four plasma exchanges, the cerebrospinal fluid was acellular and the IgG concentration was less than 1 mg per deciliter. Over the following seven months, the ataxia slowly disappeared. An examination of serum for antineuronal antibodies when the patient was asymptomatic was negative. The Hodgkin's disease has remained in remission.

Patient 2

In 1996, at the age of 49 years, Patient 2 presented with severe cerebellar ataxia and short-term memory loss. She had been treated for stage II Hodgkin's disease (nodular sclerosing type) nine years earlier with carmustine, cyclophosphamide, vinblastine, procarbazine, and prednisone (BCVPP) and had been in remission since then. She also had polycystic renal disease and had required hemodialysis since 1991. On neurologic examination she was alert and oriented. She could repeat four words, but her recall after five minutes was limited to two words. Comprehension and naming were normal. She spoke with moderate cerebellar dysarthria. Severe appendicular and truncal ataxia with titubation of the head and trunk were present. She could walk only with support from another person. MRI examinations of the brain when ataxia was diagnosed and six months later were normal and did not show cerebellar atrophy. The serum contained IgG antineuronal antibodies that stained the cerebellum in a pattern identical to that of serum from Patient 1.

One year after the onset of ataxia, the patient received a diagnosis of probable paraneoplastic ataxia and was treated with 14 plasma exchanges, but there was no objective improvement of the truncal ataxia and she remained unable to walk without support. In 1998, after the 14 plasma exchanges, the cerebrospinal fluid was acellular and had an IgG concentration of 15 mg per deciliter. The serum IgG concentration was 0.84 g per deciliter. The IgG index was 0.62. High titers of antineuronal antibodies persisted in serum and cerebrospinal fluid. Hodgkin's disease remained in complete remission.

Methods

Samples

We analyzed samples of serum and cerebrospinal fluid from 3060 patients that had been sent to us for antineuronal-antibody testing. Of the 3060 patients, 26 had histologically proved Hodgkin's disease, including Patients 1 and 2. IgG was purified from specimens that were obtained from the first plasma exchange of Patients 1 and 2 and from normal serum with protein A Sepharose. The purified IgG was dialyzed against phosphate-

buffered saline containing 10 mM lithium chloride or artificial cerebrospinal fluid and used in assays for inositol phosphates or for in vivo transfer experiments. Frozen tumor tissues were provided by the pathology department of the Daniel den Hoed Cancer Center. We studied 10 samples of Hodgkin's tissue, including a lymph node from Patient 1, and 5 samples of non-Hodgkin's lymphoma.

Immunohistochemical Analysis

Normal C57BL/6 mice and mGluR1-deficient C57BL/6 mice (Aiba et al., 1994) were deeply anesthetized with pentobarbital and perfused through the heart with a fixative solution containing 4 percent paraformaldehyde, 0.2 percent picric acid, and 0.05 percent glutaraldehyde. Parasagittal sections (40 μm each) of brain from these mice were incubated overnight with a 1:1000 dilution of test serum or 0.5 μg of rabbit anti-mGluR1 G18 antibody per milliliter (Shigemoto et al., 1994) and were then incubated with biotinylated goat antihuman or antirabbit IgG (Vector). For confocal microscopy, sections underwent reaction with Texas red–avidin (Vector, Burlingame, Calif.) instead of avidin–biotin–peroxidase complex. To test for antineuronal antibodies, we incubated the 3060 samples of serum and cerebrospinal fluid with parasagittal frozen and acetone-fixed 6-μm sections of rat cerebellum and then with the addition of fluorescein-isothiocyanate–labeled goat anti-human IgG (Dako, Glostrup, the Netherlands). Anti–Purkinje-cell antibodies were classified as anti-Yo when reactive with the paraneoplastic Yo62 antigen, as anti-Tr when an additional characteristic dotted staining pattern was present in the cerebellar molecular layer, or as of unknown specificity (Moll et al., 1995).

Cell Labeling and Assay for Inositol Phosphates

Live CHO cells that expressed the mGluR1 isoform mGluR1α or the closely related receptor subtype mGluR5a were incubated in culture medium (Aramori and Nakanishi, 1995) with 1:1000 dilution of each patient's serum for one hour. After being washed with phosphate-buffered saline, the cells were fixed with 4 percent paraformaldehyde for 10 minutes, and serum antibodies that bound to the cells were detected by staining with fluorescent-labeled antihuman IgG (Vector). For measurement of the formation of inositol phosphates, the receptor-expressing CHO cells were labeled with [3H]inositol (1 μCi per milliliter) for 24 hours as described previously (Shigemoto et al., 1994).

The amino-terminal extracellular domain of mGluR1 is involved in glutamate binding (Okamoto et al., 1998). Antibodies raised against mGluR1 aminoterminal sequences inhibit the glutamate-stimulated formation of inositol phosphates in mGluR1α-expressing cells (Shigemoto et al., 1994). With this system, we assessed the effects of purified IgG on the activation of mGluR1 by analyzing the glutamate-stimulated formation of inositol phosphates in CHO cells that expressed mGluR1α. After we incubated the cells with phosphate-buffered saline for 20 minutes, we incubated them with phosphate-buffered saline that contained 10 mM lithium chloride in the absence or presence of the patient's purified IgG for 20 minutes at 37°C. Agonist stimulation was started by adding glutamate to a final concentration of 15 μM; this

caused an increase in the levels of inositol phosphates by a factor of two to three (Shigemoto et al., 1994). After incubation for 20 minutes at 37°C, the reaction was terminated by 5 percent trichloroacetic acid (wt/vol). [3H]Inositol phosphates (IP_1, IP_2, and IP_3) were separated by AG1-X8 chromatography (Bio-Rad, Hercules, Calif.), and the radioactivity was determined on a liquid scintillation spectrometer.

Absorption Experiments

The patient's IgG was incubated with mGluR1α - or mGluR5a expressing CHO cells ($1x10^8$ cells per milligram of IgG) in phosphate-buffered saline for one hour. After centrifugation, IgG that remained in the supernatant was purified again with protein A and dialyzed against artificial cerebrospinal fluid. Successful absorption of the anti-mGluR1 antibodies with mGluR1α-expressing CHO cells was confirmed by immunohistochemical assay, CHO cell labeling, and assay for inositol phosphates with the use of the mGluR1α-expressing CHO cells as described above. When the same assays were used, absorption with mGluR5a did not reduce the ability of the anti-mGluR1 antibodies to bind to mGluR1α

Transfer Experiments

A catheter was placed in the cisterna magna of C57BL/6 mice after they had received general anesthesia (Kobayashi et al., 1995). At least 24 hours later, artificial cerebrospinal fluid containing IgG either from the patient or from normal serum (20 µl [0.1 to 20 mg per milliliter]) was injected through the catheter over a period of 30 minutes. Footprints were made with ink applied to the hind paws of mice one hour after injection. For the rotorod test, animals were trained before injection. All animals managed to stay on a rod that was rolling at 20 rpm for longer than 60 seconds after several trials. Thirty minutes and every hour after the injection of IgG, each animal was tested in five trials, and the average time it remained on the rod was determined. The maximal time allowed was 60 seconds. In some experiments, aniline blue dye was injected concurrently to confirm the delivery of injected materials. For visualizing the penetration of human IgG into the cerebellum, the mice were perfused with the fixative solution 5 to 12 hours after injection, and transverse sections through the cerebellum were immunostained as described above with anti-human IgG primary antibody (Dako).

Expression of mGluR1 in Tumor Samples

Frozen sections (5 µm each) from Hodgkin's and non-Hodgkin's lymphoma tissues were fixed in acetone and then incubated with biotinylated IgG from Patients 1 and 2 and normal human serum or with G18. We extracted RNA from the same tumors using the triple-extract reagent (Tri-Reagent, MRC, Cincinnati) followed by reverse transcription. For reverse transcriptase–polymerase chain reaction (RT-PCR), we used three primer sets specific to both intracellular and extracellular coding sequences of mGluR1: primer

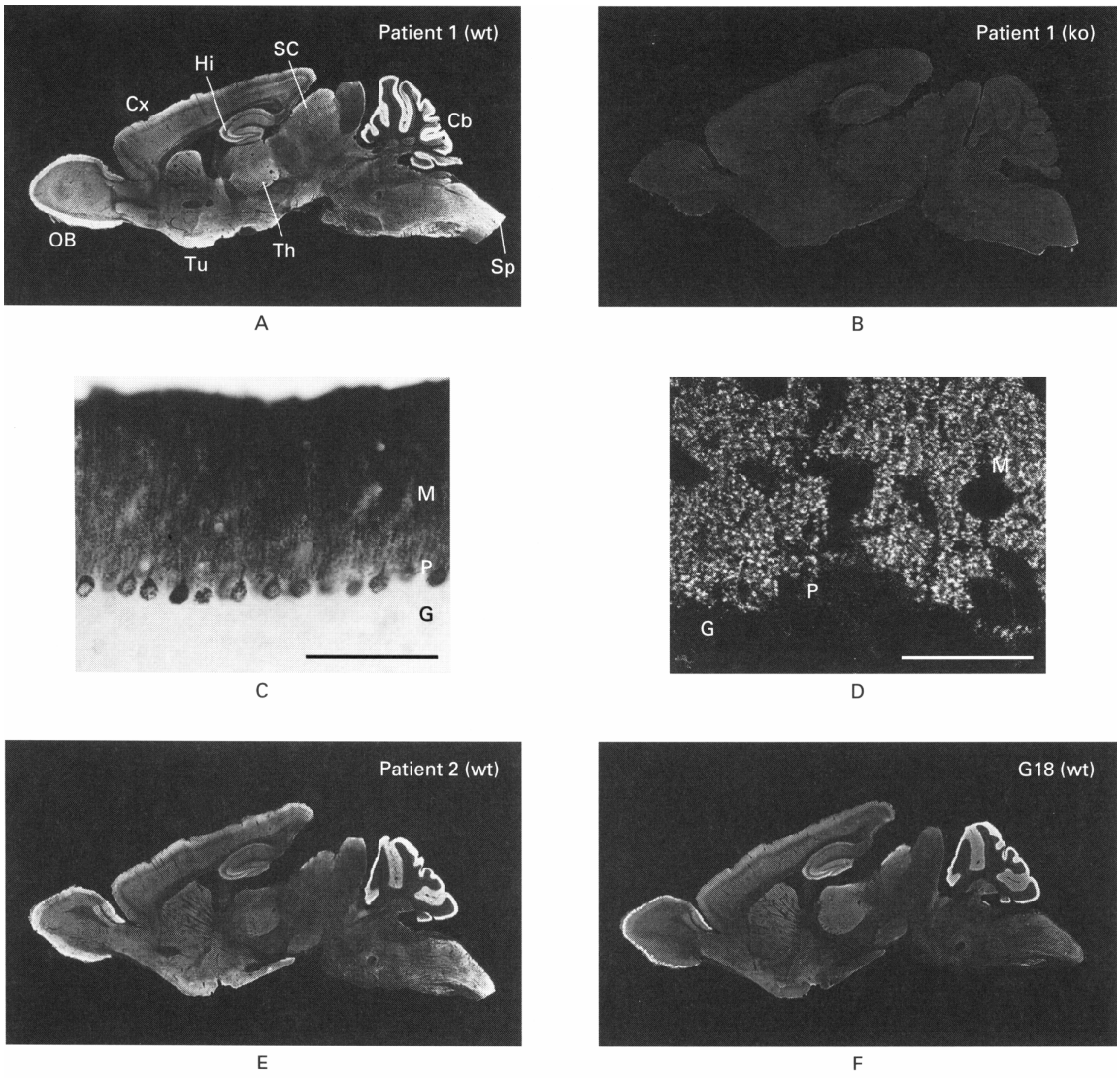

Figure 1: Immunohistochemical Analysis of Sections of Mouse Brain with Serum from Patients 1 and 2.
We performed an immunohistochemical analysis of parasagittal sections of mouse brain with the serum from Patient 1 (Panels A, B, C, and D) and Patient 2 (Panel E) and with a rabbit antibody (G18) to mGluR1 (Panel F). With brain tissue from mice (wild type [wt]), strong immunoreactivity was observed with both of the serum samples (Panels A, C, D, and E) in the Purkinje cells and the molecular layer (M) of the cerebellum (Cb), glomeruli of the olfactory bulb (OB), olfactory tubercle (Tu), CA3 area of the hippocampus (Hi), cerebral cortex (Cx), thalamus (Th), superior colliculus (SC), and spinal trigeminal nucleus (Sp). In the molecular layer, strong punctate labeling was observed with confocal microscopy (Panel D). These staining patterns resemble those found with a rabbit antibody against mGluR1 (Panel F) and were abolished (Panel B) in mGluR1-deficient mice (knockout [ko]). P denotes the layer of Purkinje cells, and G the layer of granule cells. The bar represents 100 μm for Panel C and 25 μm for Panel D.

set 1: 5'TCTGGGGTGCATGTTCACTCC3' and 5'AGGCCGTCTCATTGGTCTTCA3'; primer set 2: 5'CGAGAAAGTGCCCGAGAG3' and 5'GTGGCTGAATAAGCGATCT-G3'; and primer set 3: 5'TGAAGGCATAGTAGGTACAG3' and 5'GAGTGGAGCAAC-ATCGAAT3'. The primers used for our positive control glyceraldehyde-3-phosphate dehydrogenase were 5'CCGAGCCACATCTGCTCAGACAC3' and 5'GGCCATCCAC-AGTCTTCTGGGT3'.

Results

Samples of serum and cerebrospinal fluid from both patients had similar, specific immunohistochemical staining patterns on sections of mouse brain (Fig. 1A and 1E). Purkinje-cell bodies were strongly stained (Fig. 1C), and distinctive punctate staining, compatible with labeling of the Purkinje-cell spines, was observed in the molecular layer of the cerebellum (Fig. 1D). Strong staining of neurons and neuropil was also observed in the glomeruli of the olfactory bulb, the olfactory tubercle (including the islands of Calleja), the superficial layer of the cerebral cortex, the CA3 area of the hippocampus, the thalamus, the superior colliculus, and the spinal trigeminal nucleus. The immunohistochemical staining pattern appeared to be similar to the distribution of the metabotropic glutamate receptor mGluR1 (Fig. 1F) (Fotuhi et al., 1993; Shigemoto et al., 1992). To test whether these antibodies were indeed directed against mGluR1, we incubated the patients' serum and cerebrospinal fluid with sections obtained from mGluR1- knockout mice (Fig. 1B) (Aiba et al., 1994). These sections were not stained by either the serum or the cerebrospinal fluid (data not shown).

To confirm the specificity of the reactivity of the serum with native mGluR1 proteins, we incubated living CHO cells that expressed rat mGluR1α (Aramori and Nakanishi, 1992) or mGluR5a (Abe et al., 1992) with serum from both patients. The two serum samples strongly labeled CHO cells that expressed mGluR1α but not cells that expressed mGluR5a (Fig. 2A-D). The reactivity of the patients' IgG and cerebrospinal fluid with CHO cells that expressed human mGluR1 and with human cerebellar sections was also demonstrated (data not shown). These results indicate that IgG from both patients reacted specifically with the amino-terminal extracellular domain of native mGluR1α. Inhibition of the activation of mGluR1α in a dose-dependent manner was also found with the IgG from each of the patients (Fig. 2E). The mean (±SE) concentrations of IgG that caused 50 percent inhibition of the activation of mGluR1α (IC50) for IgG from Patient 1 and Patient 2 were 58±9 and 194±36 μg per milliliter, respectively. Normal human IgG had no effect. IgG from the two patients did not block the activation of mGluR5a, a finding that indicates its specificity.

We then examined the pathogenicity of the antimGluR1 autoantibodies by injecting purified IgG from the two patients (400 μg; 20 μl [20 mg per milliliter]) into the subarachnoid space of normal mice, near the cerebellum. Thirty minutes after the injection, the mice became increasingly ataxic, with a wide gait (Fig. 3A). They were unable to walk a straight line, and the distance between their steps was small and irregular, an indication of cerebellar dysfunction (Brunner and Altman, 1973). At the peak of the symptoms, the most strongly affected mice could hardly walk or stand up because of severe truncal ataxia. As assessed by the rotorod test, the ataxic behavior

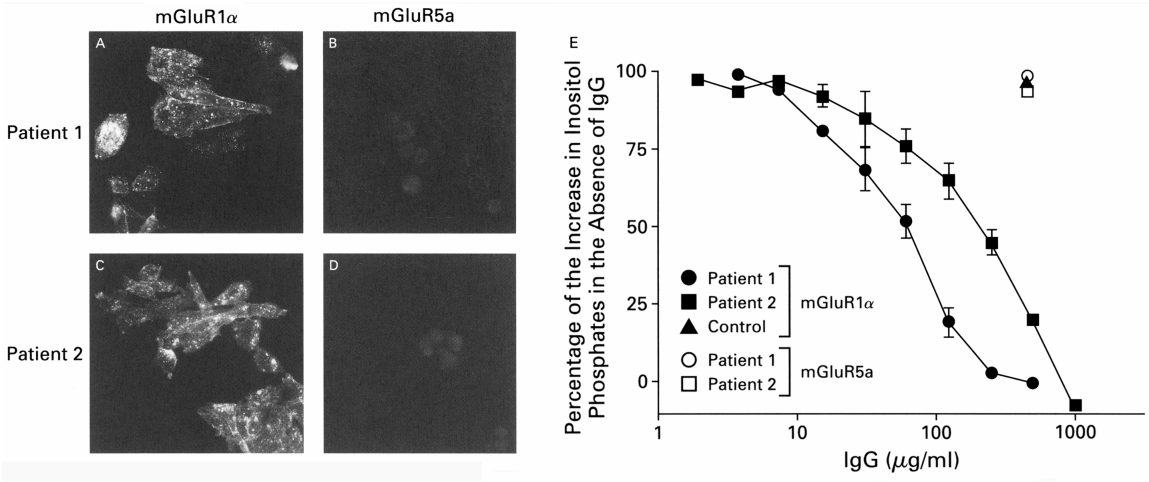

Figure 2: Functional Blocking of the mGluR1 Receptor by Autoantibodies. Autoantibodies to mGluR1 blocked the glutamate-stimulated formation of inositol phosphates in mGluR1a-expressing Chinese-hamster-ovary (CHO) cells. With the serum from Patient 1 (Panels A and B) and Patient 2 (Panels C and D), we incubated CHO cells that expressed either mGluR1a (Panels A and C) or mGluR5a (Panels B and D). The serum samples from both patients reacted strongly with the native mGluR1a protein on the cell surface but not with mGluR5a. Panel E shows the effects of purified IgG on the glutamate-stimulated formation of inositol phosphates in the CHO cells that expressed mGluR1a (solid symbols) and mGluR5a (open symbols). The formation of inositol phosphates was stimulated with 15 μM glutamate in the presence of IgG prepared from a control subject (triangles), Patient 1 (circles), and Patient 2 (squares). The values are the means (±SE) of three experiments performed in triplicate and are given as percentages of the increase in the formation of inositol phosphates stimulated with 15 μM glutamate in the absence of IgG. The IgG of both patients inhibited the glutamate-induced production of inositol phosphates in a dose-dependent manner in CHO cells that expressed mGluR1a but not mGluR5a. The mean (±SE) concentrations of IgG from Patients 1 and 2 that inhibited the activity of mGluR1a by 50 percent were 58±9.4 and 194±36 μg per milliliter, respectively. The IgG of the control subject had no effect at a concentration of 500 μg per milliliter.

peaked at about 2 to 4 hours after injection of IgG and subsided after 24 hours (Fig. 3B). Significant effects on the behavior of the mice ($P<0.05$) could be detected with as little as 10 μg of IgG (20 μl [0.5 mg per milliliter]) from Patient 1, whereas no effects were detected with normal human IgG (20 μl [20 mg per milliliter]).

To show that this in vivo effect of the IgG from the patients was caused by the anti-mGluR1 autoantibody, IgG was absorbed with CHO cells that expressed mGluR1α or mGluR5a. The IgG that was absorbed with mGluR1α completely lost its effect (Fig. 3B), but IgG that was absorbed with mGluR5a remained effective (the value on the rotorod test 2 hours after injection was 18±12 seconds — not significantly different from the values for nonabsorbed IgG; $P>0.3$). The injected IgG was restricted largely to the cerebellum (Fig. 3C-D); it had penetrated throughout various layers of the cerebellar cortex, as shown by immunohistochemical visualization of human IgG (Fig. 3D).

Furthermore, antibodies eluted from the cells that expressed mGluR1α caused similar ataxic behavior in mice at a low concentration (20 μl [about 0.15 mg per milliliter]) (Fig. 3B). These results clearly indicate that anti-mGluR1 autoantibodies

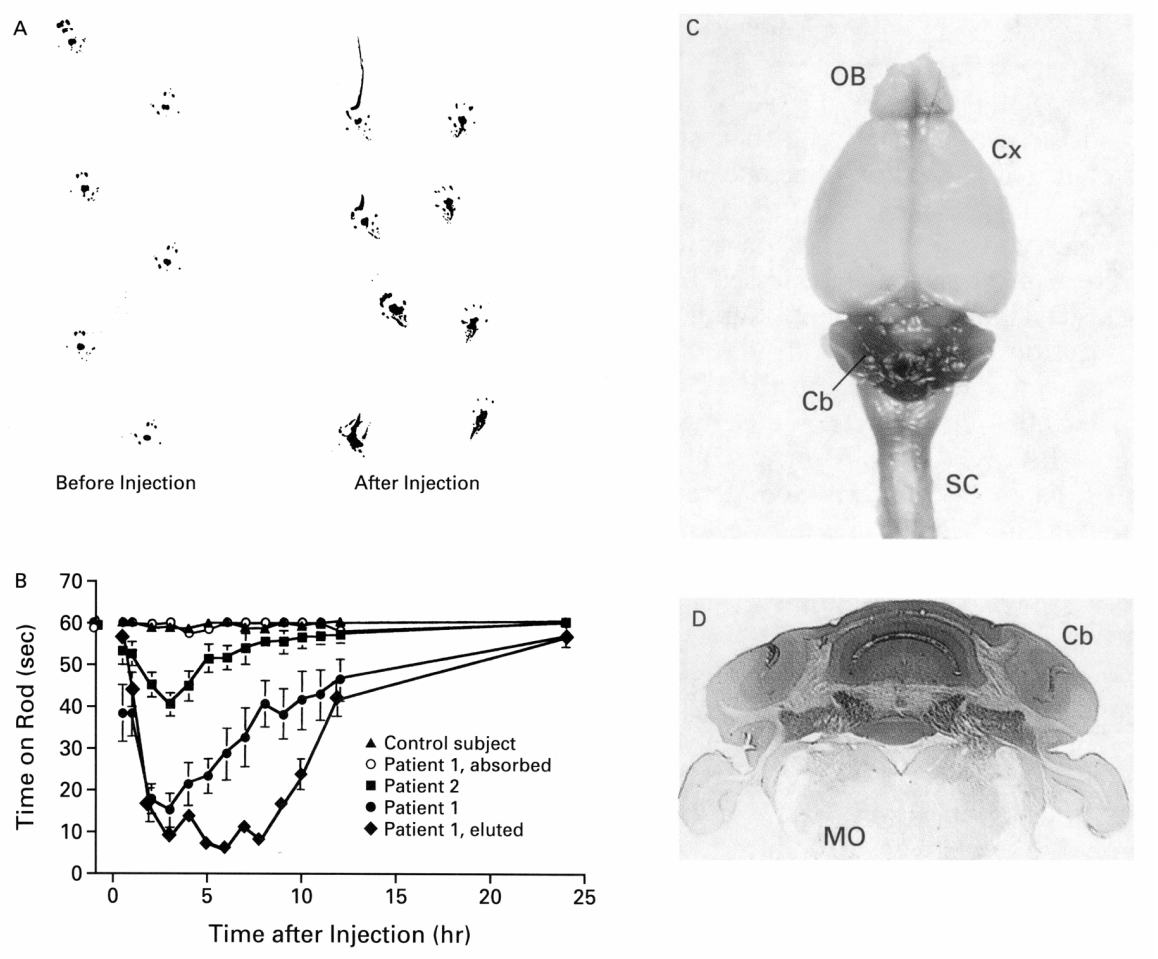

Figure 3: Impaired Motor Coordination Resulting from the Passive Transfer of IgG from Two Patients into the Cerebellar Subarachnoid Space of Mice. Panel A shows the hind footprint pattern of a mouse injected with IgG from the patients. After the injection, the gait was wide and the steps were short. The mice affected most strongly were unable to stand up because of severe truncal ataxia at the peak of the effect. Panel B shows the results of the rotorod test (at 20 rpm), which indicated the course of the ataxia caused by the injection of IgG (20 µl [20 mg per milliliter]) from Patient 1 (solid circles) and Patient 2 (squares). The ataxia peaked 3 hours after the injection, and a significant difference ($P<0.05$) between behavior before and after the injection was detected up to 12 and 6 hours after the injection of IgG from Patients 1 and 2, respectively. IgG from a control subject (triangles) and IgG from Patient 1 that was preabsorbed with the CHO cells that expressed mGluR1a (open circles) had no significant effects. Antibodies eluted from the cells that expressed mGluR1a caused similar ataxic behavior in mice at a much lower concentration (20 µl [approximately 0.15 mg per milliliter], diamonds). The values for numbers of seconds on the rod are means (±SE) of measurements in 3 to 10 mice. The maximal time allowed on the rotorod was 60 seconds. The delivery of the injected IgG was confirmed with dye injected concurrently (Panel C) and with immunolabeling of human IgG in transverse sections through the cerebellum fixed 12 hours after injection (Panel D). The injected material was seen mainly in the cerebellum (Cb) and penetrated the cerebellar cortex. OB denotes olfactory bulb, Cx cerebral cortex, SC spinal cord, and MO medulla oblongata.

from these patients caused cerebellar ataxia in mice by functional blocking of mGluR1 in the cerebellum.

Titers of the autoantibody in cerebrospinal fluid and serum samples from both patients were examined by end-point titration of immunohistochemical mGluR1 staining in sections of rat brain. Before Patient 1 received plasma exchanges, the titers in her cerebrospinal fluid and serum were 512 and 3200, respectively. When these values were normalized according to concentrations of IgG, the anti-mGluR1 titer per unit of IgG was 31 times as high in cerebrospinal fluid as in serum, an indication of intrathecal synthesis. Using these values, we found that the anti-mGluR1 antibody content in the cerebrospinal fluid was 25 times as high as that in the IC50 of serum IgG (46 μg per milliliter x 31÷58 μg per milliliter). In Patient 2, after she received plasma exchanges, the anti-mGluR1 titer in serum was 400 and the titer in cerebrospinal fluid was 256. Normalized according to IgG concentrations, the anti-mGluR1 IgG titer per unit of IgG was 36 times as high in cerebrospinal fluid as in serum. In Patient 2, the content of anti-mGluR1 antibody in cerebrospinal fluid was therefore 28 times that in the IC50 of serum IgG (150 μg per milliliter x 36÷194 μg per milliliter).

To investigate whether the anti-mGluR1 autoantibodies were related to Hodgkin's disease, we examined a frozen biopsy specimen of a lymph node from Patient 1 and lymph nodes from nine other patients with Hodgkin's disease and five patients with non-Hodgkin's lymphoma by RT-PCR and an immunohistochemical assay, using biotinylated IgG from the patients. We could not detect mGluR1 RNA in any of these samples, but we did detect it in positive controls (rat and human cerebellum; data not shown). Also, an immunohistochemical assay with biotinylated IgG from the patients did not provide evidence for the expression of mGluR1 or a cross-reactive epitope in any of the tumor samples (data not shown).

We also examined serum samples from more than 3060 patients for the presence of paraneoplastic antineuronal antibodies. These samples included serum samples from 26 patients with Hodgkin's disease. Only the serum from Patients 1 and 2 showed anti-mGluR1 immunoreactivity. Of the 24 serum samples from other patients with Hodgkin's disease, 5 reacted with Purkinje cells; the pattern was anti-Tr in 3 of these. These anti-Tr serum samples did not bind to CHO cells that expressed mGluR1 and showed the same immunolabeling pattern of Purkinje cells in wild-type and mGluR1-knockout mice (data not shown).

Discussion

IgG from serum and cerebrospinal fluid from two patients with cerebellar ataxia bound to mGluR1 receptors in the brain and caused ataxia in mice. In Patient 1, the most striking symptom at presentation was gait ataxia; she was unable to walk with a tandem gait. At that time, the titers of the anti-mGluR1 autoantibodies in serum and cerebrospinal fluid were 3200 and 512, respectively. After 25 days of treatment with prednisone and four plasma exchanges, her gait improved and the serum and cerebrospinal fluid titers of the autoantibodies had dropped to 200 and 64, respectively. Later, when she was asymptomatic, we could not detect anti-mGluR1 autoantibodies in her serum.

The serum of Patient 2 was first tested when she had had severe ataxia for almost one year. The titer of anti-mGluR1 autoantibodies in her serum was 3200 at that time. After 14 plasma exchanges, she continued to have severe ataxia and was unable to walk without support. Although the plasma anti-mGluR1 titer had dropped to 400 after the plasma exchanges, the cerebrospinal fluid titer remained high, an indication of ongoing intrathecal synthesis of anti-mGluR1 autoantibodies.

Several molecules have been identified as autoantigens associated with nervous system diseases. These include the acetylcholine receptor in myasthenia gravis (Toyka et al., 1975), voltage-gated calcium channels in the Lambert–Eaton syndrome (Lennon et al., 1995), presynaptic potassium channels in Isaacs' syndrome (neuromyotonia) (Lang and Vincent, 1996), GluR3 in Rasmussen's encephalitis (Rogers et al., 1994), and Hu antigens in paraneoplastic encephalomyelitis (Szabo et al., 1991). So far, functional effects of such autoantibodies have been found only in disorders of the peripheral nervous system, such as myasthenia gravis (blocking of acetylcholine receptors) (Toyka et al., 1975) and the Lambert–Eaton syndrome (blocking of presynaptic voltage-gated calcium channels at the neuromuscular junction) (Lang et al., 1981). Our results indicate that autoantibodies may also affect the central nervous system by blocking neuronal receptors.

We detected anti-mGluR1 autoantibodies in the serum of only two of 3060 patients with a variety of disorders. These two patients had Hodgkin's disease, but we were unable to show conclusively that the ataxia and the Hodgkin's disease were linked in a paraneoplastic syndrome. We did not detect expression of mGluR1 in the tumor-containing lymph node of Patient 1, a finding that would have tied the two disorders together. Nevertheless, in about 50 percent of cases, the cerebellar syndrome of nonhereditary subacute ataxia in adults is paraneoplastic and can occur when Hodgkin's disease is in remission (Henson and Urich, 1982; Hammack et al., 1992).

The mGluR1 receptors are located at the perisynaptic site of the Purkinje-cell dendritic spines, which form excitatory synapses with parallel fibers or climbing fibers (Nusser et a., 1994). The activation of mGluR1 receptors is necessary for the induction of cerebellar long-term depression, which is probably the mechanism of cerebellar motor learning (Aiba et al., 1994; Shigemoto et al., 1994; Conquet et al., 1994; De Zeeuw et al., 1998). Mice that lack the mGluR1 gene have ataxic gait and intention tremor and impaired cerebellar long-term depression and motor learning (Aiba et al., 1994). The ability of the anti-mGluR1 autoantibodies to cause ataxic behavior in mice by blocking mGluR1 in the cerebellum indicates that the activation of mGluR1 is necessary for normal cerebellar coordination.

Impaired cerebellar long-term depression and motor learning, which result from the blocking of mGluR1 (Aiba et al., 1994; Shigemoto et al., 1994; Conquet et al., 1994) are unlikely to be the cause of ataxia in our study, because the effects of the injected antibodies were evident in the short term. The activation of mGluR1 induces slow inward–outward currents as well as a depression of parallel fiber-mediated excitatory postsynaptic currents in Purkinje's cells (Conquet et al., 1994). The blocking of these mGluR1-mediated effects in the parallel fiber synapses may have a role in the ataxic behavior. Finally, we should consider the possibility that the short-term ataxic effect results partly from impaired mGluR1 activation at other locations, such as climbing fiber

synapses - activation that is necessary for normal motor coordination (Welsh et al., 1995).

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CHAPTER 3

MECHANISMS UNDERLYING CEREBELLAR MOTOR DEFICITS DUE TO ANTI-MGLUR1 AUTOANTIBODIES

Abstract

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

Introduction

Paraneoplastic cerebellar ataxia (PCA) occurs secondary to certain forms of cancer such as small cell lung cancer, ovarian cancer, breast cancer or Hodgkin's disease (Henson and Urich, 1982; Posner, 1995). The syndrome results from an immune response against components of cerebellar neurons that usually are homologous to antigens present in the tumor. Recently, we showed that PCA following Hodgkin's disease can be caused by auto-antibodies against the metabotropic glutamate receptor type I (mGluR1-Ab) (Sillevis Smitt et al., 2000). In the cerebellum this receptor is abundantly present in dendrites of Purkinje cells, at the perisynaptic site of both their parallel fiber and climbing fiber input (Masu et al., 1991; Shigemoto et al., 1992; Fotuhi et al., 1993; Nusser et al., 1994). However, the mechanisms by which mGluR1-Ab evoke cerebellar motor coordination deficits remain to be demonstrated.

Several potential pathophysiological mechanisms can be considered based upon recent mGluR1 agonist/antagonist studies. First, an in vitro block of mGluR1 has been shown to exert acute effects on the excitability (Netzeband et al., 1997; Yamakawa and Hirano, 1999; Neale et al., 2001) and firing rate of Purkinje cells (Yamakawa and Hirano, 1999; however see Neale et al., 2001). Second, an in vitro mGluR1 block has been shown to interfere with long-term depression (LTD) at the parallel fiber – Purkinje cell synapse (Linden et al., 1991; Hartell, 1994; Shigemoto et al., 1994), a form of synaptic plasticity commonly thought to play a role in cerebellar motor learning behavior. A third possible pathophysiological mechanism for which indications have recently emerged (Catania et al., 2001) could be a more chronic degenerative effect of an mGluR1-block on Purkinje cells, affecting dendritic arbor morphology or even killing the cells.

These potential pathophysiological mechanisms, which all may contribute to motor coordination deficits in PCA patients, are probably not mutually exclusive and may in fact interact during the various stages of the disease. To find out whether all

three mechanisms can indeed play a role in the cerebellar coordination deficits of PCA patients with Hodgkin's disease we investigated the motor behavior of these patients in more detail and we studied the impact of their mGluR1-Ab on the activity of Purkinje cells of mice in vitro as well as on their motor behavior in vivo. In addition, we analyzed post-mortem the brain of PCA patient A, who was subjected to mGluR1-Ab for at least five years.

Subjects and Methods

IgG purification

IgG was purified from the first plasma exchange of patients A and B and from normal control plasma using a protein A-sepharose column (Biorad). The purified IgG was dialyzed against phosphate-buffered saline and then concentrated using a centrifugal filter (Centricon, Millipore).

Basal activity recordings from Purkinje neurons in mouse cerebellar slices

The whole-cell patch-clamp recording in a cerebellar slice was carried out as previously described (Yamakawa and Hirano, 1999; Kashiwabuchi et al., 1995). Transverse slices cut from the cerebellum of a ICR mouse were perfused continuously with external solution saturated with O_2 and CO_2. A cell body of a Purkinje neuron was perforated-patch clamped with a patch electrode. The holding potential was –70 mV, and the current was low-pass filtered at 1 Hz. Anti-mGluR1 IgG from patient B was added to the slice bath.

Chronic mGluR1-Ab infusion in floccular region and eye movement recordings in mice

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

Sixteen C57Bl6 wild-type mice were prepared for eye movement recordings as previously described (Van Alphen et al., 2001). Optokinetic reflex (OKR) responses were tested using sinusoidal stimuli at peak velocities of 2, 4, 8, 16 and 32 °/s at a frequency of 0.4 Hz. Vestibulo-ocular reflex responses in dark (VOR) and light (VVOR) were evaluated during sinusoidal whole body rotation at 0.1, 0.2, 0.4, 0.8, and 1.6 Hz and stimulus amplitudes of 5°, in complete darkness or light. After 6 days of baseline recordings a second operation was done. Animals were anaesthetized with a mixture of O_2, N_2O and 1.5 –2% halothane. An incision was made behind the ear, the dura over the paraflocculus was opened and a small polyvinylchloride catheter was connected to the hole. The catheter was led under the skin and connected to a micro-osmotic pump (Alza Co, Mountainview, CA), which had been filled earlier according to the Alza instructions, and was then implanted under the skin on the back. Mice were allowed one day recovery before the eye movement recordings continued. The micro-osmotic pumps used in these

experiments deliver their contents at a rate of 0.5 µl/hr for 7 days, and were filled with purified and concentrated IgG (5-10 mg/ml) from either patient B or a healthy control person. In the first series of experiments the eye movements were recorded for another 7 days. Seven mice received mGluR1-Ab and 5 mice control IgG. In the second series of experiments (n=4, all receiving mGluR1-Ab) pump and catheter were removed 2 days after onset of the effects, and eye movements were recorded for an additional 5 days. After the eye movement recordings were finished, the mice were perfused transcardially with 4 % paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2). Brains were removed, postfixed, gelatin-embedded and coronal sections (40 µm) were cut on a freezing microtome. Free-floating slices were incubated with biotinylated anti-human IgG (Vector), then with avidine-biotine complex (Vector), DAB-reacted, and counterstained with thionin.

LTD-induction experiments in embryonic mouse cerebellar cultures

Mouse embryonic cerebellar cultures were prepared as previously described (Linden et al., 1991) and maintained in vitro for 9-16 days before their use in patch-clamp experiments. Patch electrodes were attached to Purkinje cell somata and contained CsCl (135 mM), HEPES (10 mM), EGTA (0.5 mM) and Na_2-ATP (4 mM). Iontophoresis electrodes were filled with 10 mM glutamate in 10 mM HEPES. Glutamate test pulses were delivered using negative current pulses (600-900 nA, 30-110 ms duration) applied at a frequency of 0.05 Hz. After acquisition of baseline responses, six conjunctive stimuli were applied at 0.05 Hz, each consisting of a glutamate test pulse combined with a 3s long depolarization step to 0 mV, timed so the depolarization onset preceded the glutamate pulse by 500 ms. Cells were bathed in a solution which flowed at a rate of 0.5 ml/min and contained NaCl (140 mM), KCl (5 mM), $CaCl_2$ (2mM), $MgCl_2$ (0.8mM), HEPES (10 mM), glucose (10 mM), 0.005 mM tetrodotoxin and picrotoxin (0.1 mM) and was adjusted to pH 7.35 with NaOH. Membrane currents were recorded with an Axopatch 200A amplifier (Axon Instruments) in resistive voltage-clamp mode, low-pass filtered at 5 kHz, and digitized at 10-20 kHz using an ITC-16 interface (Instrutech). Bis-fura-2 ratio imaging of intracellular free Ca^{2+} was accomplished by measuring the background corrected fluorescence ratio at 340 and 380 nm excitation using a cooled CCD camera system (Linden et al., 1995). Exposure times were 100-400 ms per wavelength image. Human IgG was either bath applied (extracellular application) or added to the internal saline in the patch electrode (intracellular application).

Saccade adaptation in humans

We performed a standard saccade adaptation paradigm (Frens and Van Opstal, 1994). During the experiment changes were imposed on the visuo-motor system by altering the visual input during saccades.

Subjects

Patient A (female, 49) still harbored mGluR1-Ab and was suffering from severe cerebellar symptoms. Patient B (female, 23) expressed mGluR1-Ab earlier, but no longer harbored mGluR1-Ab and had no remaining symptoms at the time of the experiment. Clinical details on Patients A and B can be found elsewhere (Sillevis Smitt et al., 2000). Patients C (female, 64) and D (male, 56) suffered from PCA with different well-characterized autoantibodies: anti-Yo (Fathallah-Shaykh et al., 1991) and anti-VGCC (Mason et al., 1997) autoantibodies, respectively. Four male healthy control persons, ranging in age from 24-34, also participated in the experiments. The study was performed with the approval of the ethics committee at our institution, and informed consent was obtained from the patients.

Experimental setup

Subjects were seated in a darkened room and their head was fixated using a biteboard. Visual targets were red dots on a monitor, which was positioned 50 cm from the subjects' head. Two-dimensional eye position was recorded with non-invasive infrared corneal reflection techniques. In patient A eye movements were measured using an Eyelink-system (SMI) (Van der Geest and Frens, 2002). In patient B eye movements were measured using a Ober2-system (Permobil meditech. Inc.). Controls were tested in both systems. The sampling rate of the Ober2-system was 500 Hz/channel, whereas the sampling rate of the Eyelink was 250 Hz. In the calibrated eye position signal the onset and offset of saccadic eye movements were detected by the computer on the basis of both velocity and mean acceleration criteria.

Experimental paradigm

Each trial, a red fixation spot appeared on the monitor at an eccentricity of 10 deg to the left of the straight-ahead position. After a random period of 1-2 sec, the target was displaced over 20 deg to the right. Subjects had to redirect their gaze as quickly as possible to the new target. However, triggered by the saccade to this new target, the red dot was again displaced over 6 deg to the left, thus making the first saccade appear too large. The timing of the saccade-triggered target displacement was about 30 ms after saccade onset, which is the expected moment of the saccade peak velocity. The simultaneous occurrence of target jump and saccade peak velocity is optimal for good adaptation (Frens and Van Opstal, 1994).

Anatomical and immunohistochemical analysis

Brains were obtained from patient A and 3 controls. Patient A (female, 49) died of a cardiac infarct, while Hodgkin's disease was still in remission. Control 1 (female, 49) died of cerebral contusion by metastased carcinoma of ovarian origin. Control 2 (male,

68) died of respiratory insufficiency and control 3 (male, 85) of cardiac decompensation. All control persons were without known cerebellar disease. Consent was obtained from the next of kin for autopsy. Brain samples were taken in order to obtain frozen material. The rest of the brain was fixed by suspension in 10 % formalin in PBS for approximately 6 weeks. Blocks of tissue were taken from the culmen of the lobus anterior, the nodulus and flocculus, and embedded in paraffin.

For examination of Purkinje cell dendritic morphology 20 µm sections were cut from tissue of all 4 subjects, incubated with rabbit anti-calbindin D28k antibodies (Swant 1:6000), processed using the avidin-biotin peroxidase diaminobenzidine tetrahydrochloride method (Vector, Burlingame, CA) and counterstained with Haematoxylin/Eosin (Sigma).

For Purkinje cell counts, sections were cut perpendicularly to the cerebellar folia at 7 µm thickness. Every tenth section was selected and counterstained with Haematoxylin/Eosin (Sigma). In order to obtain a reliable estimate of Purkinje cell density, the number of Purkinje cells, divided by the length of the Purkinje cell layer, was determined per section. From patient A and the two male control persons the flocculus, nodulus and lobus anterior of the vermis were analyzed, from the female control person only the nodulus and lobus anterior were analyzed.

We examined the possibility of immune-mediated degeneration of Purkinje cells with a range of immunological markers. We cut 5 µm sections from frozen non-fixed cerebellar tissue of patient B and incubated these with rabbit-anti-CD3 (DAKO 1:200), mouse-anti-CD4 (1:3000, TNO-PG), mouse-anti-CD8 (1:800, TNO-PG), mouse-anti-CD20 (1:25, Coulter), mouse-anti-human IgM (1:1000, Nordic), rabbit-anti-human IgG (1:400, TNO-PG), mouse-anti-CD68 (1:800, DAKO), mouse-anti-CD83 (1:150, Immunotech), rabbit-anti-iNOS (1:4000, Calbiochem) and acid phosphatase. Secundary antibodies used were biotine-conjugated donkey-anti-rabbit (Amersham) for CD3 and iNOS, rabbit-anti-mouse (DAKO) for CD8, CD20 and CD68, and peroxidase-conjugated rabbit-anti-mouse for CD4, swine-anti-rabbit (DAKO) for IgG. Slices were processed with avidine-peroxidase (Sigma) if applicable, and counterstained with Haematoxylin/Eosin (Sigma).

Results

Acute effects on currents evoked by exogenous mGluR1 agonist

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

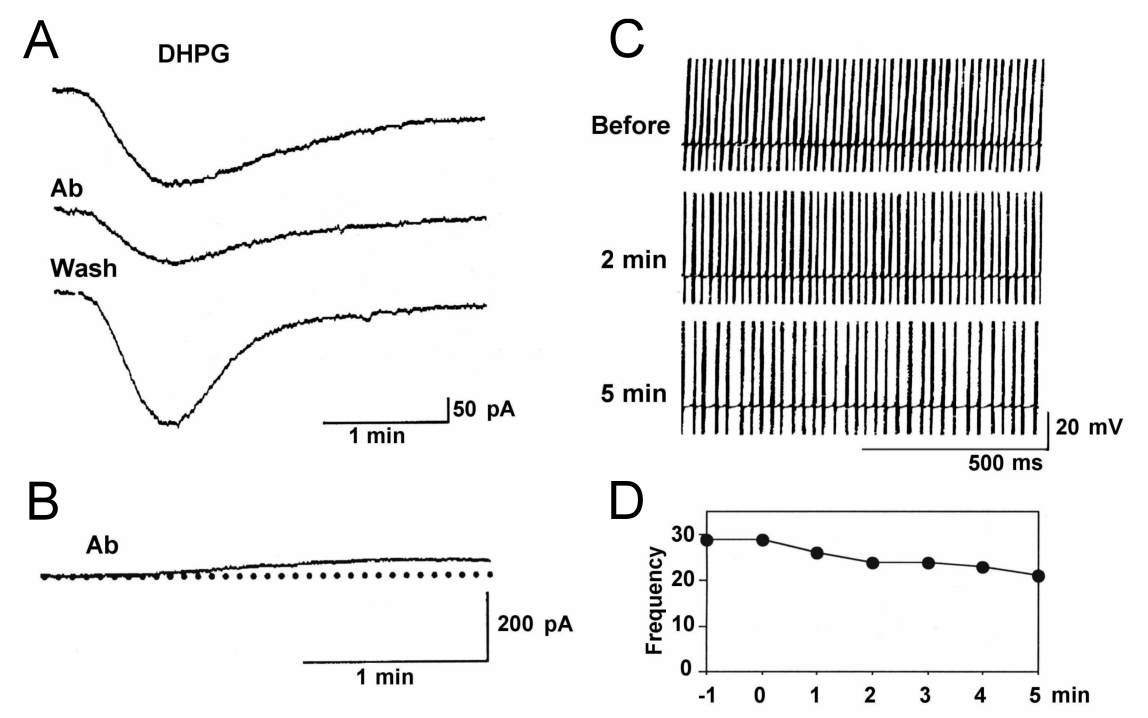


Figure 1: Acute effects of mGluR1-Ab on Purkinje neuronal activity. (A) DHPG-induced inward current before, during (Ab) and after (Wash) application mGluR1-Ab (patient B). 200µg IgG/ml suppressed the transient inward current induced by 50 µM DHPG to 72.3 ± 11.7 % and the steady current to 62.7 ± 56.7% (paired t-test; $p<0.001$) (middle trace). After a 20-40 minute wash the transient current increased again to 107 ± 25.6 % and the steady current increased to 94.6 ± 68.7%. (B) mGluR1-Ab without other ligands reduce the holding inward current by 5 ± 8 pA (n=5). (C) and (D): mGluR1Ab reduce Purkinje cell action potential frequency from 31.8 ± 10.4 Hz to 24.4 ± 11.1 Hz (paired Student's t-test: $p<0.02$). Application of mGluR1-Ab started at 0 min.

Acute effects on basal activity of Purkinje cells

This change in excitability induced by mGluR1-Ab suggests that these human antibodies can directly affect the spontaneous firing frequency of Purkinje neurons. We investigated this issue in current-clamp recordings. Bath application of mGluR1-Ab to spontaneously firing Purkinje neurons slightly hyperpolarized them (3 out of 5 neurons) and significantly reduced their action potential firing frequency over the course of 5 minutes (Fig. 1C-D). Together these data show that acutely blocking mGluR1 on Purkinje cells by human mGluR1-Ab reduces Purkinje neuronal excitability and firing rate in unstimulated conditions.

Acute impact on performance of compensatory eye movements

The observed effects of mGluR1-Ab on excitability and firing rate of Purkinje cells raise the possibility of an acute impact on motor performance. To test this possibility in a quantifiable manner we recorded the compensatory eye movements of 16 C57/Bl6 mice

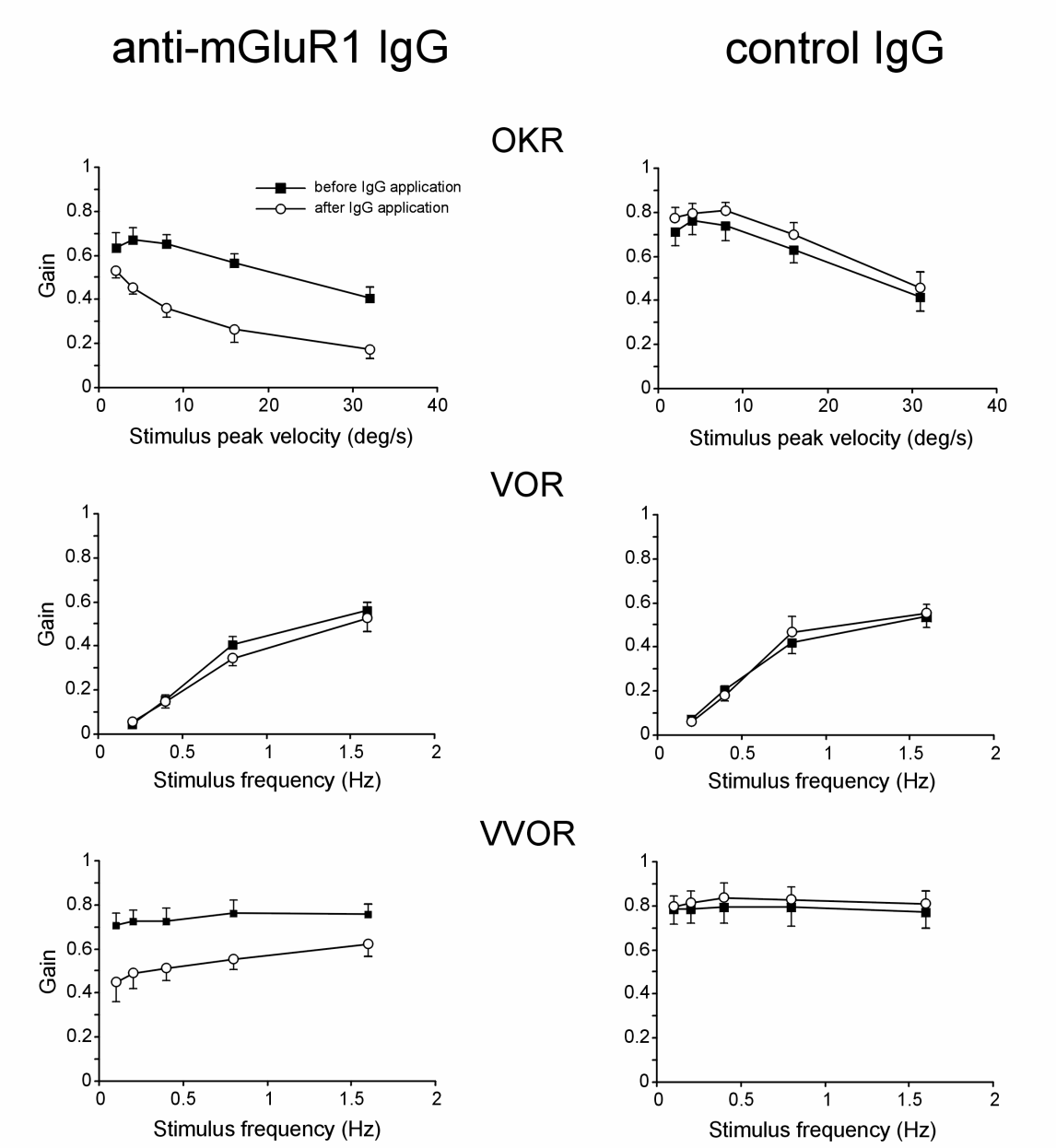


Figure 2: Effects on mice compensatory eye movements following mGluR1-Ab (patient B) infusion into the flocculus. Optokinetic reflex (OKR) gain values (eye velocity / stimulus velocity) shown were plotted against stimulus peak velocity at a frequency of 0.4 Hz. Vestibulo-ocular reflex (VOR) and visually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

following infusion of the human antibodies into their flocculus. After baseline recordings were done we implanted an osmotic minipump, which delivered either patients' mGluR1-Ab (n = 11) or control IgG (n = 5) to the flocculus. All mice that received mGluR1-Ab showed a strong effect on their eye movement behavior after implant of the minipump. OKR gain values (eye velocity / stimulus velocity) decreased significantly at all frequencies, whereas VOR gain values did not change in any of them (Fig. 2, mid panel). VVOR gain values were more prominently affected at the lower frequencies than at the higher frequencies (Fig. 2, bottom panel). Infusion of control IgG did not exert any effect on the gain nor on the phase of both OKR and VOR (Fig. 2, right column). Finally, in all cases tested (n=7 for mGluR1-Ab, n=5 for control IgG) immunohistochemical analysis showed the integrity of the floccular region including the injection site as well as the presence of human IgG in the flocculus. Removal of the pump resulted in increase of both the OKR and VVOR gain values in all cases tested (n=4, all receiving mGluR1-Ab) (data not shown). These results indicate that application of mGluR1-Ab to the flocculus reduces selectively, acutely and reversibly the amplitude of the visual component of compensatory eye movements.

Patients' mGluR1-Ab block induction of long-term depression

Apart from an acute role in the excitability of Purkinje cells, mGluR1 may also be involved in the induction of long-term depression (LTD) at the parallel fiber - Purkinje cell synapse (Bear and Linden, 2000). To find out whether this process is also impaired, we applied mGluR1-Ab to cultured embryonic Purkinje cells during LTD induction (Linden et al., 1991). Following acquisition of baseline responses to glutamate test pulses, glutamate/depolarization conjunctive stimulation was applied to cultured mouse Purkinje cells to induce LTD. Glutamate test pulses were then resumed (Fig. 3A). Previous experiments show a decrease in EPSC amplitude to about 60 % of the baseline (Linden et al., 1991; Linden et al., 1995). However, when this experiment was performed in the presence of patients' mGluR1-Ab in the extracellular medium, LTD was strongly attenuated. In contrast, when mGluR1-Ab were added intracellularly via the patch pipette, or when IgG obtained from a healthy control person was added extracellularly, the decrease in EPSC amplitude was similar to that previously seen when no IgG was added (Linden et al., 1991; Linden et al., 1995). These results indicate that patients' mGluR1-Ab are directed against the extracellular part of the receptor and that they can block LTD. To find out whether this blockage may be partly due to an effect on calcium currents, we measured the effect of mGluR1-Ab on Ca^{2+} influx and/or mobilization. Figure 3B illustrates that neither basal Ca^{2+} nor Ca^{2+} influx via voltage-gated calcium channels was significantly altered by adding mGluR1-Ab from either patient or IgG from a healthy control person. In contrast, mGluR1-mediated Ca^{2+} mobilization was significantly reduced after adding the patients' antibodies. This result is consistent with the reduction in mGluR1-mediated inward current shown in Figure 1A, and the reduction of phosphatidyl-inositol turnover measured in mGluR1-expressing CHO cells (Sillevis Smitt et al., 2000). These results indicate that patients' mGluR1-Ab can block mGluR1-evoked processes, such as diacylyglycerol production and IP3-mediated Ca^{2+} mobilization which both normally contribute to LTD induction.

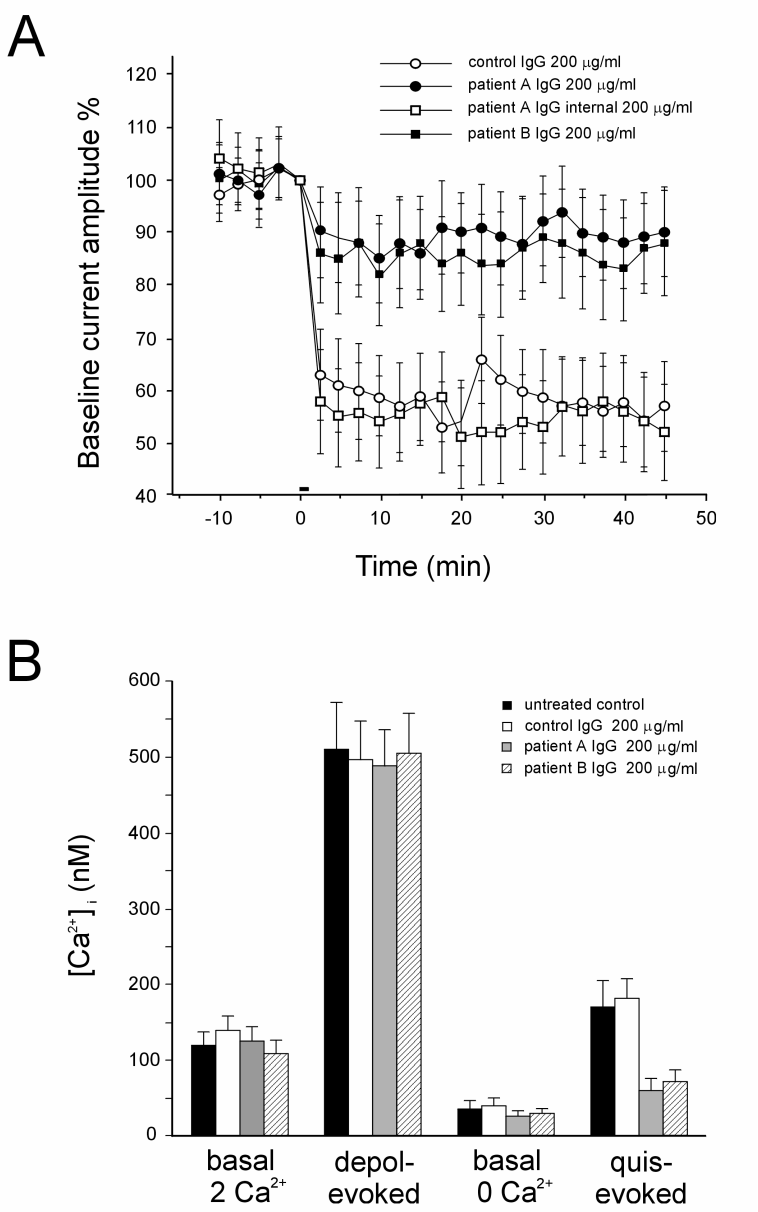


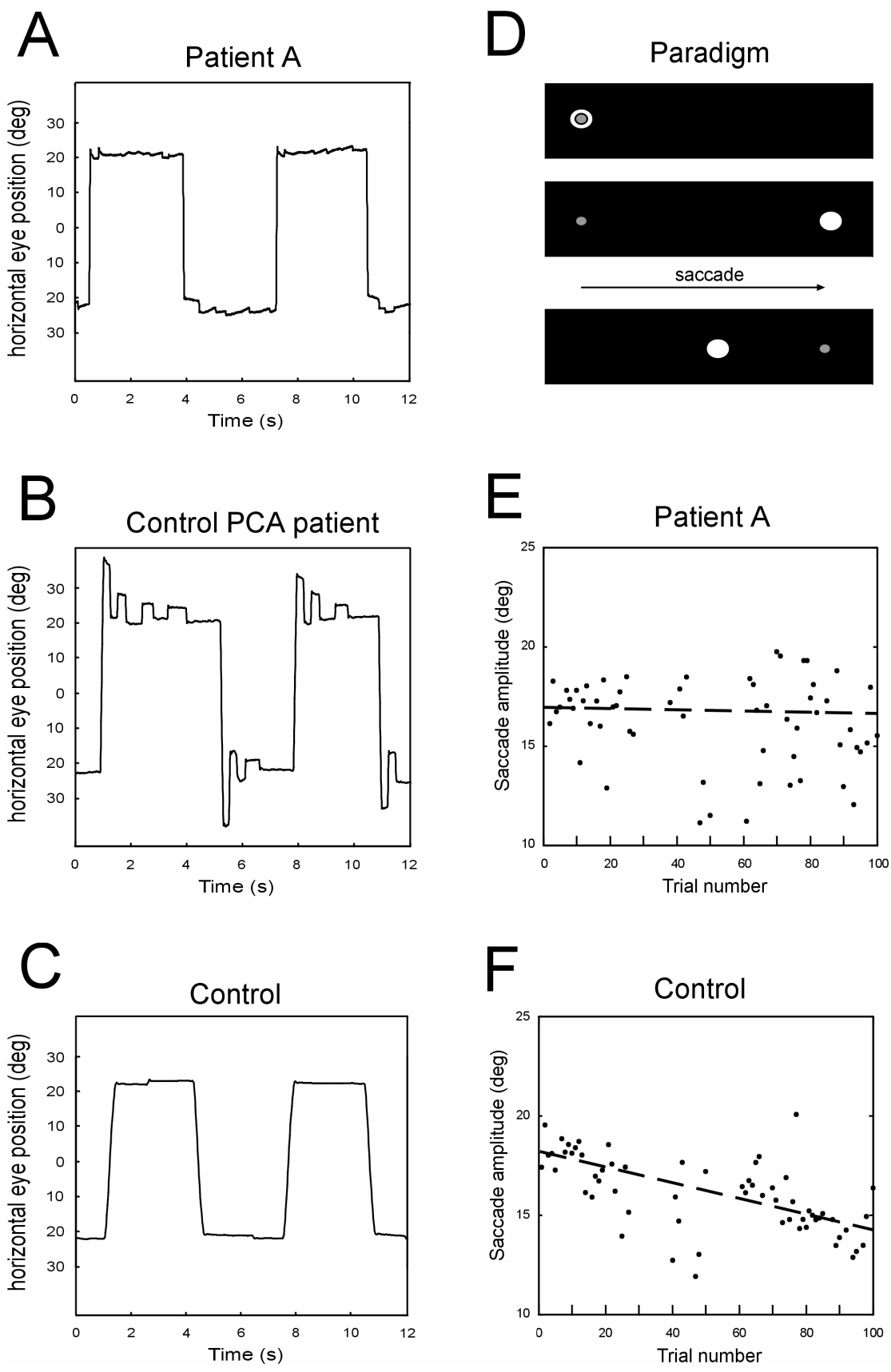
Figure 3: mGluR1-Ab block induction of long-term depression (LTD) and mGluR1-mediated Ca^{2+} mobilization. (A) After glutamate/depolarization conjunctive stimulation, the amplitudes of EPSCs induced by glutamate test pulses generally decrease to about 60% of their baseline values. However, this LTD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

Adaptation of saccadic eye movements is impaired in PCA patients

Since LTD may be a mechanism underlying certain forms of cerebellar motor learning (Ito et al., 1982; De Zeeuw et al., 1998), the data described above suggest that motor learning capabilities of PCA patients with mGluR1-Ab could be impaired. Yet, potential deficits in particular forms of cerebellar motor learning in these patients may be hard to ascribe specifically to a lack of LTD, because deficits in motor performance itself will also directly impair cerebellar motor learning (Koekkoek et al., 1997). We therefore investigated a cerebellar learning paradigm, which was only blurred by performance deficits during relatively small periods of the training process: adaptation of saccadic eye movements. In this paradigm (Fig. 4D), which is mediated by the oculomotor vermis (Optican and Robinson, 1980; Takagi et al., 1998; Desmurget et al., 1998), fast adaptation of saccade amplitude can be obtained by consistently changing the visual target during visually guided saccades (Frens and Van Opstal, 1994; Deubel et al., 1986).

Four patients were tested. Patient A had severe PCA and a positive titer for mGluR1-Ab at the time of testing; patient B had been successfully treated for PCA due to mGluR1-Ab (Sillevis Smitt et al., 2000). Patients C and D suffered from severe PCA associated with anti-Yo and anti-VGCC auto-antibodies, respectively (Fathallah-Shaykh et al., 1991; Mason et al., 1997). PCA associated with the latter two autoantibodies is characterized by massive Purkinje cell loss (Posner, 1995; Mason et al., 1997) and these patients served as positive controls. The general dynamic properties of the saccadic eye movements of both patients A and B were within the normal range (Collewijn et al., 1988) (Fig. 4A). Still, while patient B showed normal adaptation of her saccadic eye movements following amplitude reduction training (data not shown), patient A did not show adaptation (Fig. 4E). Thus, the lack of adaptation in patient A may be caused by impaired LTD-induction due to the activity of the mGluR1-Ab. However, since these auto-antibodies may also contribute to degeneration, this association does not need to be directly causal. We therefore compared the saccadic eye movements of patient A with patients C and D who were likely to suffer from severe Purkinje cell loss. Patients C and D also did not show any adaptation of the amplitude of their saccadic eye movements following the training process, but in contrast to patient A they showed typical cerebellar motor performance deficits such as saccadic dysmetria and postsaccadic drift (Fig. 4B). This difference suggests that possible degeneration in patient A must be insufficient to result in performance deficits as observed in patients C and D, and that the lack of adaptation in patient A is probably not predominantly due to massive Purkinje cell loss.

Even though patient A did not have the gross performance deficits of patients C and D, she still had a slight opsoclonus and she frequently made a small gaze step towards the right from the initial fixation spot before the presentation of the target (Fig. 4A). Thus, in some trials the stimulus was presented at different retinal positions, affecting the amplitude of the required saccade. Since saccade adaptation is amplitude specific (Frens and Van Opstal, 1994), this might interfere with the saccade adaptation process. To find out whether this failure could contribute to her lack of adaptation, we devised a second, slightly modified, adaptation paradigm for 4 healthy control subjects. In this paradigm, we presented the control persons with a fixation spot that was at the exact same position where patient A fixated at the onset of the target. Thus the sequence of presented motor errors was identical in the controls as in patient A. All four control


A
Patient A
horizontal eye position (deg)
Time (s)
D
Paradigm
saccade
B
Control PCA patient
horizontal eye position (deg)
Time (s)
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Patient A
Saccade amplitude (deg)
Trial number
C
Control
horizontal eye position (deg)
Time (s)
F
Control
Saccade amplitude (deg)
Trial number

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

persons subjected to this paradigm showed a considerable and gradual decrease of saccade amplitude (Fig. 4F), indicating that the lack of adaptation in patient A was not due to the presence of scatter in initial fixation position. Together, these data show that adaptation of the amplitude of saccadic eye movements is impaired in PCA patients with mGluR1-Ab, and that this deficit can not be explained by a deficit in motor performance.

mGluR1-Ab can cause Purkinje cell loss

Above we demonstrated that the motor coordination deficits in PCA patients with mGluR1-Ab can be caused by acute disturbances in the excitability and plasticity of Purkinje cells. Yet, although MRI-scans of these patients did not show robust atrophy or degeneration (data not shown), it remains possible that degeneration occurred and contributed to their motor deficits. We therefore performed post-mortem analysis on the cerebella of PCA patient A, described above, and of 3 control persons. Control 1 was of the same age and sex as patient A, and died from cancer without cerebellar disease. In general, the size of patient A's cerebellum including its granular layer, molecular layer and white matter appeared normal. The density of Purkinje cells, however, was affected in all parts of the cerebellar hemispheres and vermis (Fig. 5A, top panels). Purkinje cell morphology was also affected. As visualised with calbindin immunostaining it was evident that the dendritic trees of the remaining Purkinje cells were severely amputated (Fig. 5A). In the quantitative analysis special emphasis was put on the areas that are involved in the control of eye movements, i.e. the flocculus and nodulus of the vestibulocerebellum, which control compensatory eye movements, and the lobus anterior of the cerebellar vermis, which abuts the oculomotor vermis that controls saccadic eye movements. Figure 5B shows that the densities of Purkinje cells in these areas are approximately a third of those in three age-matched controls. In general, PCA is characterized by extensive inflammatory infiltrates consisting of cytotoxic T-lymphocytes (Mason et al., 1997; Giometto et al., 1997; Sillevis Smitt et al., 2000), that progressively disappear over time when the immune response fades away, leaving the patient severely disabled with almost complete loss of Purkinje cells. To examine immune-mediated degeneration of Purkinje cells in patient A, we performed immunohistochemical analysis of selected areas of the cerebellum. We did not observe any signs of an ongoing inflammatory reaction and no cytotoxic $CD8^+$ T-lymphocytes were detected. In areas with Purkinje cell loss, reactive Bergmann gliosis was noted. Although these findings do not support a role for a specific cellular immune reaction in the degeneration of the Purkinje cells, it cannot be excluded that such a process had occurred previously (Sillevis Smitt et al., 2000). However, in contrast to other reported

PCA autopsy cases, the loss of Purkinje cells in Patient A was much less prominent and may indicate, together with the absence of inflammatory infiltrates, that other mechanisms than a cytotoxic cellular immune response caused Purkinje cell degeneration.

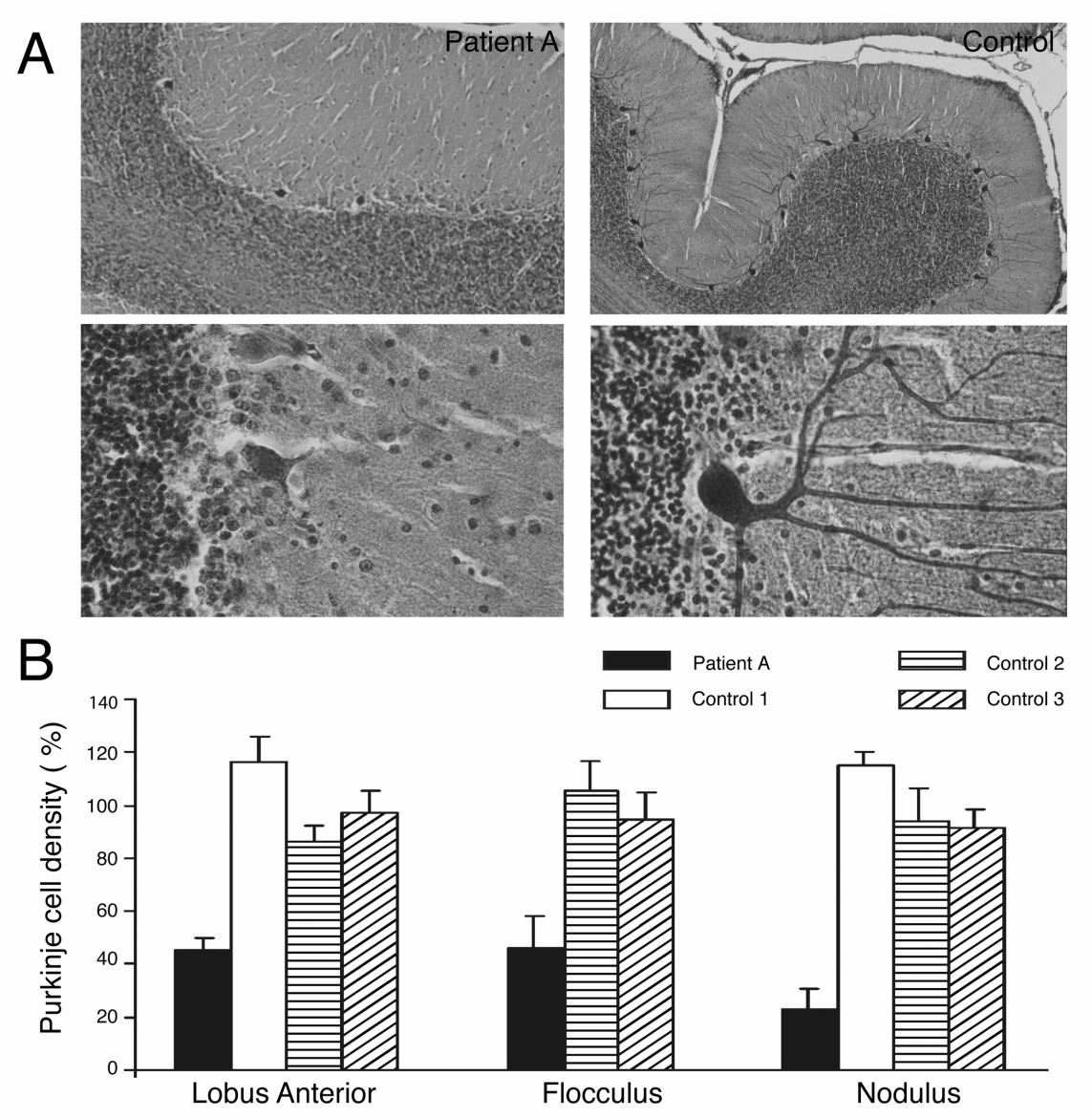

Figure 5: Effects of chronic exposition to mGluR1-Ab on Purkinje cell number and dendritic morphology. (A) Purkinje cell density in the cerebellum of patient A is significantly lower than in control cerebella (top panels). Dendritic morphology of the remaining Purkinje cells is severely affected in the cerebellum of patient A. (B) Purkinje cell loss in the cerebellum of patient A. The Purkinje cell density in the lobus anterior was 45.1 ± 4.5 %, in the flocculus 46.3 ± 11.9 % (no floccular tissue from control 1 was available), and in the nodulus 23.1 ± 7.9 % of the average density in the controls (two-sample unequal variance Student's t-test $p<0{,}0001$ for all analyzed regions).

Discussion

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

The acute effects of patients' mGluR1-Ab on Purkinje cells in the cerebellar slice preparation were a reduction of their excitation by mGluR1 agonists as well as their spontaneous firing rate, which is largely in line with the effects that have been shown at the in vitro level for antagonists such as MCPG (Netzeband et al., 1997), CPCCOEt (Yamakawa and Hirano, 1999; however see Neale et al., 2001) and LY367385 (Miniaci et al., 2001). The major difference was the relatively long time constant of the effect of the mGluR1-Ab as compared to the antagonists (Yamakawa and Hirano, 1999), which might be due to slower diffusion of the human IgG into the slice preparation.

The short-term effects of the mGluR1-Ab at the Purkinje cell level most likely explain the acute performance deficits of the compensatory eye movements that we observed following application of the mGluR1-Ab with the infusion pump into the flocculus of mice. The decreased spike activity we see after mGluR1-Ab application to a cerebellar slice suggests that mGluR1-Ab injection into the flocculus reduced floccular spike activity and thereby the gain of the OKR and VVOR. This is in line with the fact that an artificially increased floccular activity leads to an increased OKR gain (Van der Steen and Tan, 1997).

Other possible explanations such as a blockage of LTD or a degeneration of Purkinje cells do probably not contribute to this acute process. For example, a blockage of LTD induction at the parallel fiber - Purkinje cell synapse impairs adaptation of the VOR, but does not necessarily result in performance deficits or ataxia (De Zeeuw et al., 1998). Similarly, we showed in the autopsy study of PCA patients that chronic exposure to mGluR1-Ab can lead to a severe loss of Purkinje cells, but histological analysis of the floccular region of mice that were treated with mGluR1-Ab showed that this form of degeneration does not occur within a couple of days. Moreover, the infusion experiments in mice also demonstrated that the gain reducing effects were reversible indicating that no damage with permanent functional consequences was done. Thus, direct exposure to the patients' mGluR1-Ab can lead to acute changes in the excitability and firing rate of Purkinje cells and this effect in itself is sufficient to evoke acute deficits in cerebellar motor performance.

The learning deficits on the other hand are most likely due to a blockage of LTD. The experiments on cultured Purkinje cells indicated that the patients' mGluR1-Ab can block mGluR1-mediated Ca^{2+} mobilization as well as LTD induction, while the behavioral investigations of the patients showed that fast adaptation of the amplitude of their saccadic eye movements is impaired. The fact that the general performance of their saccades was relatively normal suggests that for this particular cerebellar paradigm the lack of motor learning was not due to such severe performance deficits as are seen in the PCA positive control patients with anti-Yo and anti-VGCC autoantibodies. Even so, for other cerebellar motor learning paradigms such as adaptation of the VOR we were

unable to determine whether impairment of LTD induction was the major cause underlying the learning deficit, since in both the patients and the mice treated with the infusion pumps the general motor performance of their compensatory eye movements was insufficiently normal (Koekkoek et al., 1997). In this respect it is noteworthy that the normal saccadic performance of patient B was relatively spared. Why the general performances of different cerebellar motor behaviors were affected differently eludes us.

The essential role of mGluR1 for LTD induction at the parallel fiber - Purkinje cells synapse has been demonstrated in various studies both in cultured Purkinje cells and in the slice preparation (Kano and Kato, 1987; Linden et al., 1991; Daniel et al., 1992; Hartell, 1994; Shigemoto et al., 1994). The role of LTD induction at the parallel fiber - Purkinje cell synapse in cerebellar motor learning was originally suggested by Marr (1969) and Albus (1971), and the first experimental evidence for the relation between LTD induction and cerebellar motor learning was provided by Ito (Ito et al., 1982; Ito, 1989). Both the role of mGluR1 in LTD induction and the role of LTD induction in cerebellar motor learning, have been recently supported by experiments in various mouse mutants. For example, a global knock out of mGluR1 shows a blockage of LTD induction and impaired eye blink conditioning (Conquet et al., 1994; Aiba et al., 1994). However, the investigations of these animals also showed the general caveats of global knock outs such as developmental deficits and a lack of cell specificity. The mGluR1 knock out showed a persistent multiple climbing fiber innervation of their Purkinje cells (Chen et al., 1995; Kano et al., 1997; Levenes et al., 1997), and since the expression of mGluR1 is not restricted to Purkinje cells or the cerebellar cortex (Masu et al., 1991; Shigemoto et al., 1992; Fotuhi et al., 1994) they showed a complex of several behavioral deficits which makes it difficult to specifically attribute the impaired eye blink conditioning to a lack of LTD induction at the parallel fiber - Purkinje cell synapse. The problem of cell specificity however has been largely solved by creating and testing a cell specific rescue of the mGluR1 knock out, in which LTD induction and the major cerebellar motor deficits were rescued (Ichise et al., 2000). The present study adds to the body of evidence that LTD is probably necessary for cerebellar motor learning, because with the use of the mGluR1-Ab it was possible to circumvent the developmental problems of the mutants and still observe the same relationship between LTD and motor learning.

Although we have in part successfully dissected here the various direct molecular, cellular and behavioral effects of the mGluR1-Ab, it should be acknowledged that in the patients all these direct effects act in concert with their chronic degenerative impact on both the morphology and number of Purkinje cells. Although this latter effect is not as severe as in many forms of PCA caused by other immune reactions (Brain and Wilkinson, 1965; Graus et al., 1991; Posner, 1995; Sillevis Smitt et al., 1995) it probably still was sufficiently prominent to contribute substantially to part of the symptoms. PCA associated with antineuronal antibodies is generally caused by a specific cytotoxic immune reaction involving $CD8^+$ lymphocytes (Posner, 1995; Mason et al., 1997; Giometto et al., 1997). The absence of inflammatory infiltrates combined with the relatively large number of spared Purkinje cells suggests that mGluR1-Ab may cause Purkinje cell degeneration by other mechanisms.

In addition, we have demonstrated that mGluR1-Ab can cause neurological symptoms in vivo upon transfer into experimental animals (Sillevis Smitt et al., 2000),

whereas other paraneoplastic autoantibodies do not cause neurological damage upon passive transfer into animals (Graus et al., 1991; Sillevis Smitt et al., 1995). With the exception of anti-VGCC, mGluR1-Ab is the only PCA associated auto-antibody directed against a cell surface epitope. The accessibility of the target antigen to mGluR1-Ab can explain the ability of mGluR1-Ab to directly interfere with Purkinje cell function. In the nervous system, autoimmune myasthenia gravis (MG) is caused by autoantibodies that are directed against the acetylcholine receptor, and cause degeneration of the neuromuscular junction. Due to the similarity in cellular localization of the antigen and demonstrated direct effects, similar mechanisms may apply to Purkinje cell degeneration caused by mGluR1-Ab. Such possible antibody-mediated mechanisms include accelerated degradation of receptors due to cross-linking and complement-mediated damage (Drachman, 1994). Interestingly, degeneration of Purkinje cells has recently been demonstrated in vitro and in vivo by application of the group 1 mGluR antagonist CPCCOEt (Catania et al., 2001). How this degeneration exactly comes about is still a matter of debate. Further studies of chronic application of mGluR1-Ab in vitro and in vivo may help elucidate the role of chronic mGluR1 blockade and antibody-mediated immune mechanisms involved in the chronic Purkinje cell degeneration in PCA caused by mGluR1-Ab.

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CHAPTER 4

INDEPENDENT ERRORS INDUCE SACCADIC MOTOR LEARNING

Abstract

In a saccade adaptation paradigm a target is displaced systematically during a series of consecutive saccadic eye movements. Each saccade therefore misses the target, which results in a visual error signal. The amplitude of the saccades gradually changes in the direction of the target displacement. In the present study we show that saccade adaptation strength depends on error signals, without a noticeable influence of consolidation by correct saccades. Furthermore, we show that each single adaptation trial has an effect on the adaptation process that does not abate even if long intervals between trials are introduced. Such intervals even facilitated the speed of adaptation. The amount of change in saccade amplitude per trial was more than twice as large when sufficiently long intervals (> 10 s) were introduced.

Introduction

The saccade system serves to direct the eyes to an object of interest. Saccades are very fast ballistic eye movements that allow for a rapid but detailed inspection of the visual world. In order to maximize the accuracy of saccades, plastic modification of saccade metrics is necessary. Saccade accuracy can be reduced in real life, for example due to weakening of the eye muscles related to ageing or disease (Optican et al., 1985). The behavioral plasticity of the saccade system, or saccade adaptation, is used extensively as a model for motor control and plasticity. It is commonly studied in the laboratory by means of the so-called "short-term saccade adaptation paradigm" (see, e.g., McLaughlin, 1967; Frens and Van Opstal, 1994). This paradigm experimentally induces a mismatch between actual visual input and required motor output by displacing the target in mid-flight of a saccadic eye movement (Fig. 1A). Recurrent but systematic mismatches evoke a gradual modification of saccade metrics (Fig. 1B), that is usually complete within 50 to 100 trials in human subjects (Deubel et al., 1986; Frens and van Opstal, 1994).

Saccade adaptation is a form of sensori-motor learning allegedly driven by post-saccadic visual error signals (Wallman and Fuchs, 1998, Noto et al., 2001; Seeberger et al., 2002). A single individual error is associated with each saccade (i.e. the distance between the end point of the saccade and the post-saccadic position of the target) that is available immediately after the movement through visual feedback. Throughout the saccade adaptation paradigm the position of the initial visual target remains the same, but the motor response toward that visual target is gradually modified during the adaptation phase. When non-adaptive trials are presented to the subject immediately after the adaptation phase, a proportion of the adaptation is usually abolished acutely, within one or two trials (e.g. Frens and van Opstal, 1994). Thereafter the saccadic system will slowly re-adapt to its original state.

The conventional view on saccade adaptation suggests that a sequence of systematically induced errors leads to the gradual change of saccade amplitude, so that after several successive trials, saccades will land on the displaced target (e.g. McLaughlin, 1967; Miller et al., 1981; Frens and Van Opstal, 1994; Desmurget et al., 2000). On the other hand, since each saccade has its individual post-saccadic visual

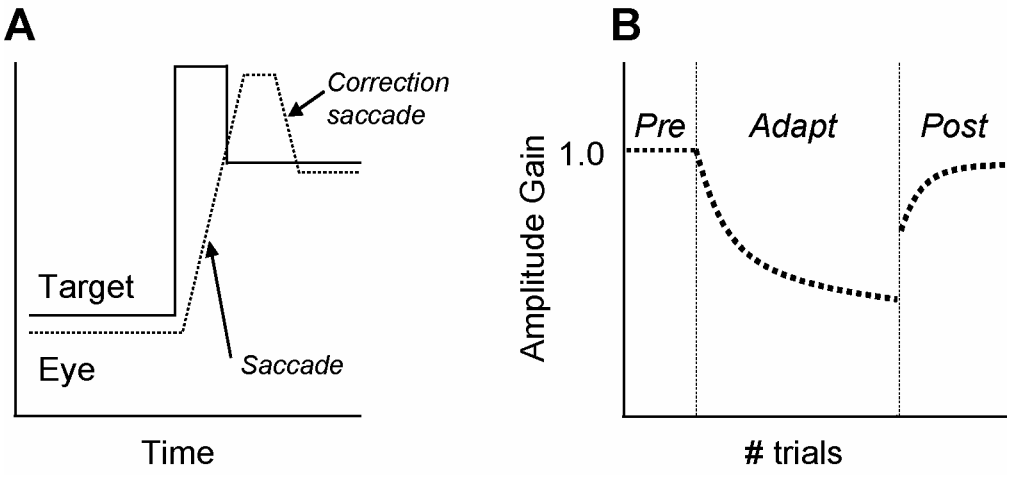

Figure 1: Schematic overview of saccade adaptation. A: A single trial. The positions of target (solid line) and hypothetical eye position (dotted line). For each trial the subject is instructed to look at the target (a small red dot) at a position of 12.5° on the left side of the screen and press button a button when ready. After a random interval between 0.5 and 1.5 seconds, the target jumps to the right, requiring a saccadic eye movement of 25° to foveate the target again. During this saccade the target is automatically displaced backward, and hence the saccade overshoots the target. This results in a correction saccade to foveate the target on its final position.
B: The adaptation process. In the pre-phase baseline measurements are taken. As the adaptation progresses the amplitude of the primary saccade gradually decreases, so that its endpoint comes increasingly nearer to the displaced target position, in response to the sensory information of the original target position. In the post adaptation phase the amplitude quickly resumes an intermediate value. Finally, re-adaptation to the original (pre-phase) values occurs gradually.

error associated with it, the saccade adaptation process can be a learning process that is based on discrete trials, unlike for instance adaptation of the vestibulo-ocular reflex. Discrete trials can be determined in forms of associative learning, with eyeblink conditioning as the most prominent example. However, the latter type of learning seems to involve not only error-based learning (Koekkoek et al., 2003), but also reinforcement (see Doya, 2000 for review). This type of learning involves reward signals rather than error signals and is typically associated with the basal ganglia (e.g. Schultz, 1998). Kawagoe et al. (1998) showed in a delayed saccade task that the saccadic system of primates can induce expected reward signals in the basal ganglia.

In the present paper we will investigate the hypothesis that saccade adaptation is indeed a learning process that is based on discrete error information. First we will test whether errors are indeed the only factor in the adaptation process. In order to do this we will look at the strength of adaptation when an extra set of adaptation trials is presented after the adaptation is already complete. Such additional trials could confirm that the new sensori-motor state is correct, but do not provide systematic error information. The hypothesis of error-based learning therefore predicts that once adaptation is complete these additional trials do not aid in maintaining the adapted state. Alternatively, involvement of non-error based learning processes in consolidation of the sensori-motor

state would predict that the newly learned amplitude is more robust after the additional trials. Secondly, we will investigate how the discrete trials interact with each other by introducing intervals between the successive trials of a classical saccade adaptation paradigm. The efficacy of single trials on the overall adaptation process might be facilitated and/or hindered when the interval between successive adaptation trials is too short or too long.

Methods

General Paradigm

We recorded saccadic eye movements in slight modifications of a classical saccade adaptation paradigm (Frens and Van Opstal, 1994). Each adaptation paradigm consisted of a series of trials. Subjects were seated in total darkness, straight in front of a computer monitor at a distance of 70 cm. The head was immobilized by means of a bite board. For each trial the subject was instructed to look at a target (a small red dot) that appeared at 12.5º on the left side of the screen. When the target appeared, the subject had to press a mouse button to continue with the trial. After a random period between 0.5 and 1.5 seconds after the button was pressed, the target jumped to a position of 12.5º right of the centre, requiring a saccadic eye movement response by the subject of 25º to foveate the target again. During that saccade however, the target was displaced 7.5º to the left. This jump of the target was triggered when the recorded horizontal gaze direction of the eye was 8º from the initial target position. The delay in the recording system was sufficiently small (<10 ms) to ensure that the target jump always occurred well within the saccade. Because of this target displacement, the saccade initially made an overshoot with respect to the final target position (Fig. 1). The displaced target remained visible for 1 second. Before and after these adaptation trials, subjects received 20 trials in which the intra-saccadic target jump did not occur (pre- and post-adaptation trials, respectively). Each individual trial lasted about three seconds.

Both studies described below required several sessions for each subject in which adaptation was evoked. For each subject these sessions were run on separate days. The different experimental designs were presented to the subjects in counterbalanced order in order to rule out effects of learning. Because the experiments could involve inactivity for long periods (20 minutes at a stretch as well as up to 20 times 180 seconds) in total darkness, music of the subject's choice was played in the background.

Study 1: Adaptation Strength

This study required the testing of each subject under 3 conditions. The conditions differed only in the adaptation phase. In the 'short' condition subjects were presented with 75 adaptation trials. After the adaptation phase the post-adaptation phase was started immediately. The 'long' condition differed from this only by the number of adaptation trials. This was set at 275. The 'pause' condition had 75 trials, i.e. the same number of trials as the short condition. However, before continuing with the post

adaptation phase, the subject was kept in total darkness for 20 min, which is by approximation equal to the time needed for the 200 extra trials in the long condition. Because of the lack of visual feedback in this period, eye movements could not provide feedback about the motor performance.

Study 2: Trial Interactions

In this study the experimental conditions were identical for each series, except for one parameter; the inter-stimulus interval (ISI) of the adaptation trials. The ISI was defined as the time between two consecutive trials. The ISI could be 0, 10, 30 or 180 seconds and was constant for the series. During this interval the subject sat in total darkness. Between trials, subjects could move their eyes freely but without any visual feedback. Post-hoc analysis showed that at trial onset the saccades toward the initial fixation point were randomly distributed with respect to their direction and amplitude. During an adaptation series in the ISI test 100 trials were performed. Only for the series with an ISI of 180 seconds the number of trials was limited to 20 in order to keep experimental time within reasonable limits.

Setup

Stimuli were presented on a 21-inch monitor (Nec XP21), covered by a red-filter to remove all possible light reflections from the monitor, using custom-made experimentation software that ran on a Pentium II. We recorded the gaze direction of the left eye using infrared videooculography (EyeLink, SMI, Berlin, Germany; see Van der Geest and Frens, 2002). Communication between the stimulus PC and the EyeLink PC was provided by a dedicated Ethernet connection. During each trial 2D eye gaze was continuously recorded at a sample frequency of 250 Hz. These recordings were stored on hard disk for offline analysis. The eye gaze data were also used to trigger the intra-saccadic target jump (see above).

Subjects

Subjects were 7 volunteers (25-35 yr) that were recruited from the department. All subject had no oculomotor abnormalities and (corrected-to) normal visual acuity. Informed consent was obtained prior to the experiments. The experiments were approved by the ethics committee of the Medical Faculty of the Erasmus University, in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. Five subjects participated in study 1 on adaptation strength, and four subjects participated in study 2 on trial interactions. Two subjects (MC and JN) participated in both studies.

Results

Experimental modification of saccadic amplitude

The saccade adaptation paradigm that we used in this paper was effective in all subjects, and under all experimental conditions that we employed. All adaptation curves showed the same trend: the amplitude of the saccades decreases gradually until it was adequate to land on the displaced target at 17.5º. The amplitudes of the saccades that were made in the last 5 trials of the adaptation were always significantly smaller than the amplitude in the last five pre-adaptation trials (average across all conditions and subjects 17.6º (±1.1º) vs. 22.8º (±1.4º), $p<0.0001$; individual analyses, all $p<0.01$).

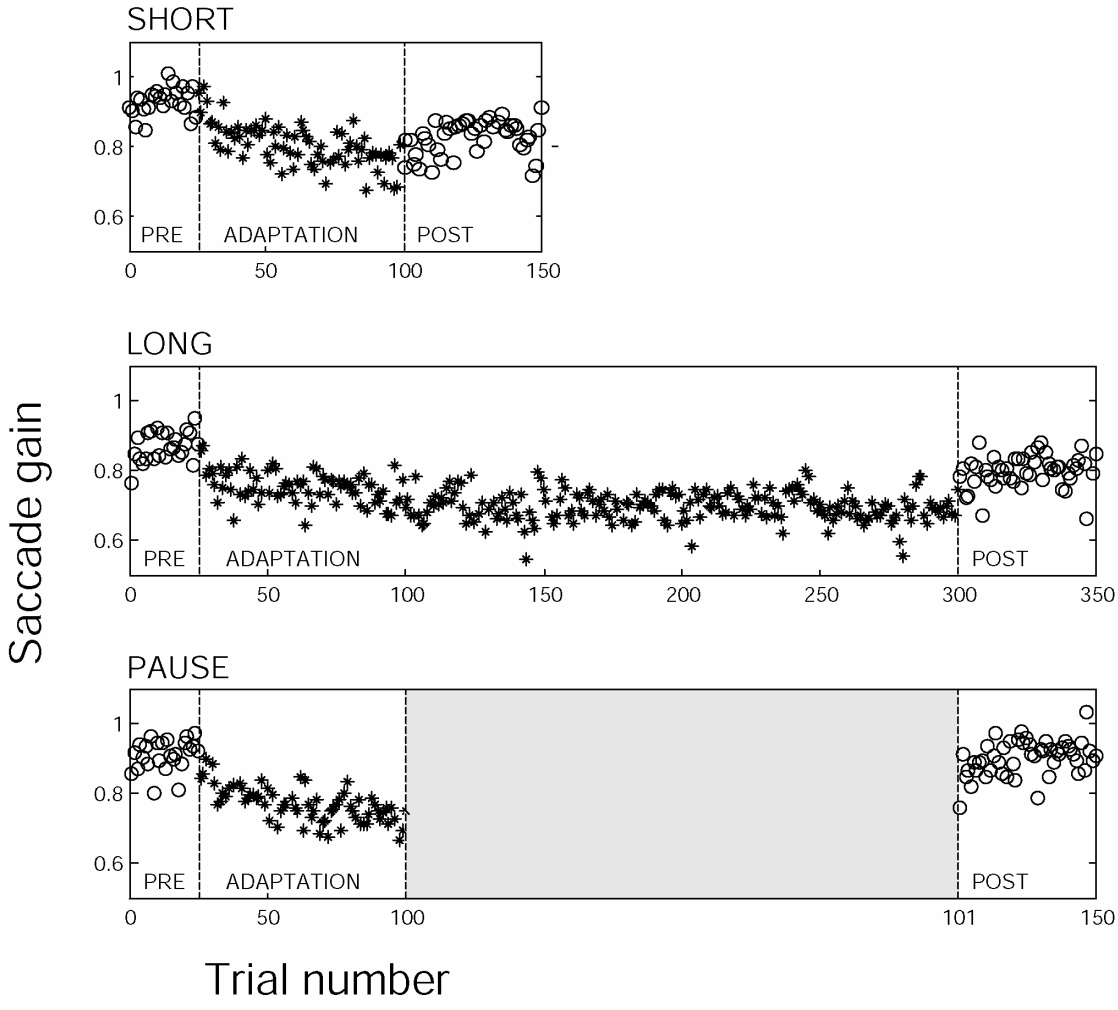

Figure 2: Saccade adaptation strength. This figure shows adaptation curves of one subject (BW) in the short, long, and pause series. Saccade gain is defined as the saccade amplitude divided by the amplitude of the primary target jump (25º). Note that adaptation is complete at trial 100 (after 75 adaptation trials) in all conditions. At the onset of the post adaptation phase a fraction of the adapted state is immediately abolished after which readaptation occurs gradually. The pause in the pause series lasted for 20 min, which is approximately equivalent to the duration of the 200 extra adaptation trials of the long series.

Adaptation Strength

In order to investigate whether the imposed changes in saccade amplitude are mainly due to error-based learning, we performed both short adaptation series (75 trials) and long adaptation series (275 trials) and compared the amplitude change between the pre- and post-adaptation saccades (i.e., the adaptation strength) between these two conditions (Fig. 2). Since adaptation was complete after 75 trials in all of our subjects, the extra 200 trials do not provide any additional error information. However, they could provide information that the newly adapted saccade amplitude is 'correct', i.e. fit to foveate the target. Thus, these trials could potentially consolidate the adaptation, yielding smaller post-adaptation amplitudes in the long series than in the short series. We determined the strength of adaptation by taking the difference between the average saccade gain before adaptation and trial 2-6 of the post adaptation series. Other measures of adaptation strength (e.g. including up to 10 post-adaptation trials, or calculating the ratio rather than the difference) resulted in qualitatively similar results. Figure 3A shows the adaptation strength in both conditions for all subjects. No systematic differences can be observed. The average values (short series: 0.16±0.05; long series: 0.16±0.06) did not differ significantly ($p>0.9$). Also when the long series (275 trials) is compared to a third condition in which the subject remained in darkness for 20 min after a series of 75 trials, no differences in post- adaptation amplitudes are observed ($p>0.6$; Fig. 3B).

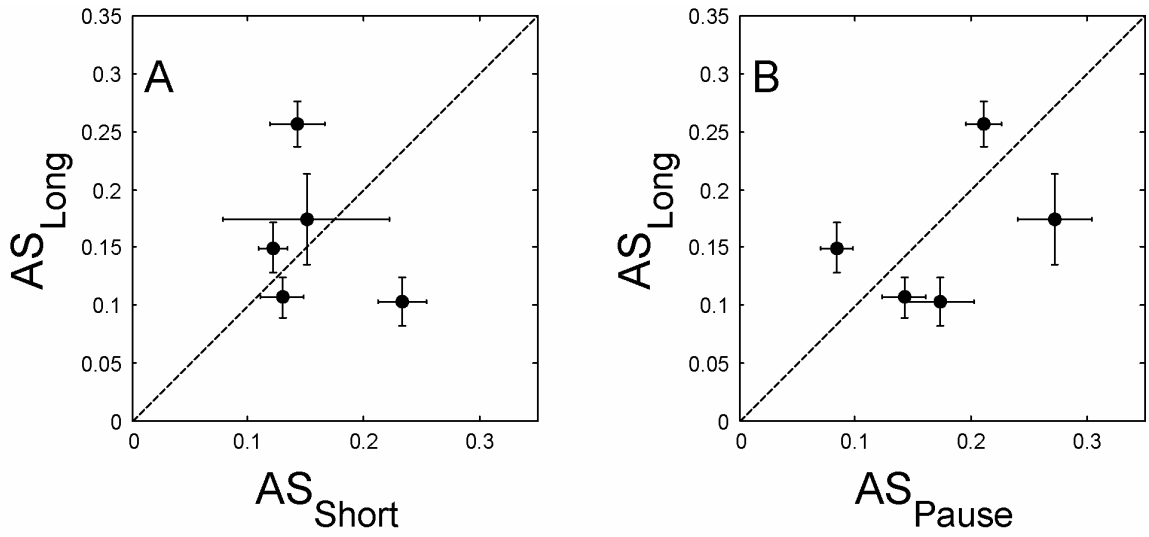

Figure 3: Comparison of adaptation strength. A: This panel shows the strength of the adaptation in the long condition as a function of the short condition. Adaptation strength (AS) is defined as the difference in saccade gain before and after the adaptation. Each dot represents a subject. Errorbars indicate SEM. The dotted line represents the unity line: points close to this line have similar values in both conditions. Note that the prolongation of the adaptation series in the Long condition does not increase the adaptation strength significantly in any of the subjects.
B: This graph compares the adaptation strength in the 'Long' and 'Pause' condition (same format as panel A). Also here are no systematic differences between the conditions: the 20 minutes pause does not decrease the adaptation strength.

Trial Interactions

We varied the interstimulus interval (ISI) between trials in a saccade adaptation paradigm. In different series the ISI could have a value of 0 s (identical to the 'short' paradigm discussed above) up to 180 s. During the ISI the subject sat in total darkness, in absence of any visual feedback. By increasing the ISI we separated the adaptation trials, disrupting putative interaction between the effects of consecutive errors.

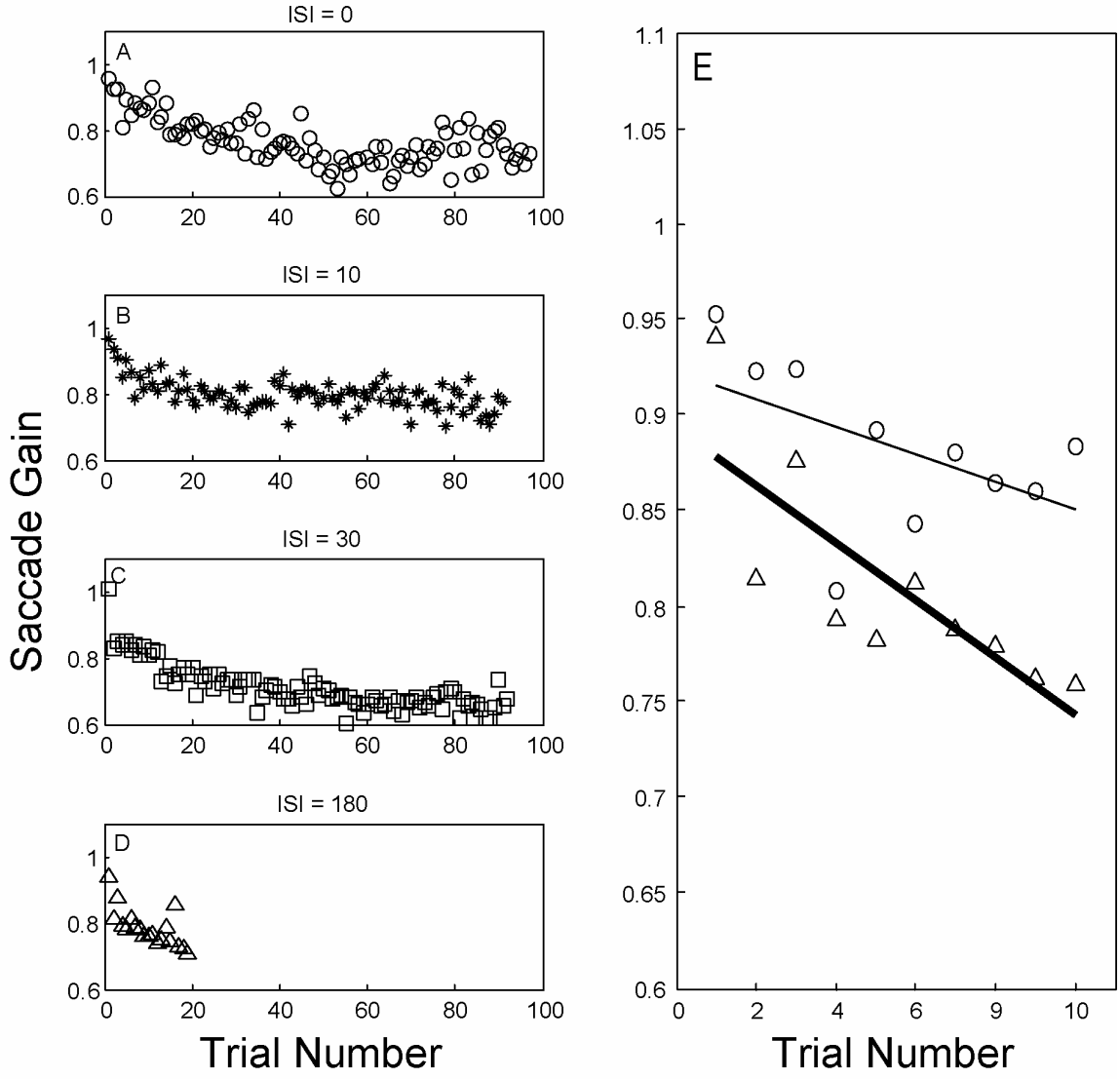

Figure 4: Adaptation Efficacy. A-D: The gradual decrease of saccadic amplitudes as a function of trial number for the four ISI conditions of a single subject. E: The saccade amplitude as a function of the first 10 trials in the 0 seconds ISI (circles) and the 180 seconds (triangles) ISI conditions for the same subject. The thin line is the regression line for the 0 seconds ISI condition. The thick line is the regression line for the 180 seconds ISI condition. The slope of this line was taken as a measure of the adaptive efficacy of a single trial.

Figure 4 shows the adaptation process. In panel A-D the adaptation curves for the four interval conditions are plotted for one subject. Note that saccade amplitudes do adapt fully under all conditions, as in all other subjects. This means that the introduction of an ISI does not disrupt the overall adaptation process. However, one can readily observe that the total number of trials needed to obtain full adaptation varies between the four conditions. In other words, the individual contribution of a single trial to the whole adaptation differs between the conditions.

In order to quantify the effect of a single trial on the adaptation process we fitted a straight line through the amplitudes of the first 10 trials (Fig. 4E), using a Monte-Carlo bootstrap method (Efron and Tibshirami, 1991; Manley, 1991) with 500 runs. For each of the four subjects and four conditions this method yields an average and standard deviations of the slope, as derived from the 500 slopes estimated in each bootstrap run. We consider the slope of this line to be a good first order approximation for the effect of a single trial on the adaptation process (see also Desmurget et al., 2000), which estimates the adaptive efficacy of a trial as the change in saccadic amplitude per single trial. The 16 adaptive efficacy estimations, i.e. the 16 slopes, are used for further analyses. In figure 5A-C we plot for each subject the adaptive efficacy in the classical paradigm (without an interval between the trials) as a function of the adaptive efficacy in the 10, 30 and 180 seconds interval conditions. In all graphs the points lie below the unity line, indicating that the adaptive efficacy in the 0 second interval condition is lower than in the other series, with the exception of one point in panel A where one subject did not adapt significantly during the first ten trials of the 10s interval adaptation. The adaptation efficacy in the three interval conditions (ISI 10, ISI 30, and ISI 180) was significantly increased in 10 of the 12 adaptation curves as compared to the 0 s ISI condition ($p<0.001$). This finding was not critically dependent on the measure that we chose for the adaptive efficacy. Fitting lines through less (down to 5) or more (up to 15) trials gave qualitatively similar results. This means that increasing the interval between trials facilitates the overall adaptation process. Figure 5D shows the mean adaptation efficacy as a function of ISI duration. The facilitation reaches its maximum at about 30s, and this remains even when the ISI is prolonged to 3 minutes. On average, the adaptation efficacy was doubled in the 30s and 180s interval condition compared to condition in which no interval was introduced (both $p < 0.02$). The 10s interval condition did not differ from the other conditions in this respect.

Strikingly, the magnitude of the ISI had no influence on the strength of the adaptation ($r=-0.18$; $p>0.05$), even when the adaptation was effectuated in very few trials (as in the 30 and 180 s condition). This underlines once more that consolidation plays no significant role in short-term saccadic plasticity.

Discussion

Adaptation strength

Increasing the number of trials in the adaptation phase had no effect on the consolidation of adaptation, since the post adaptation amplitudes were not different from the short condition. This means that only the error in the first part of the adaptation phase is likely

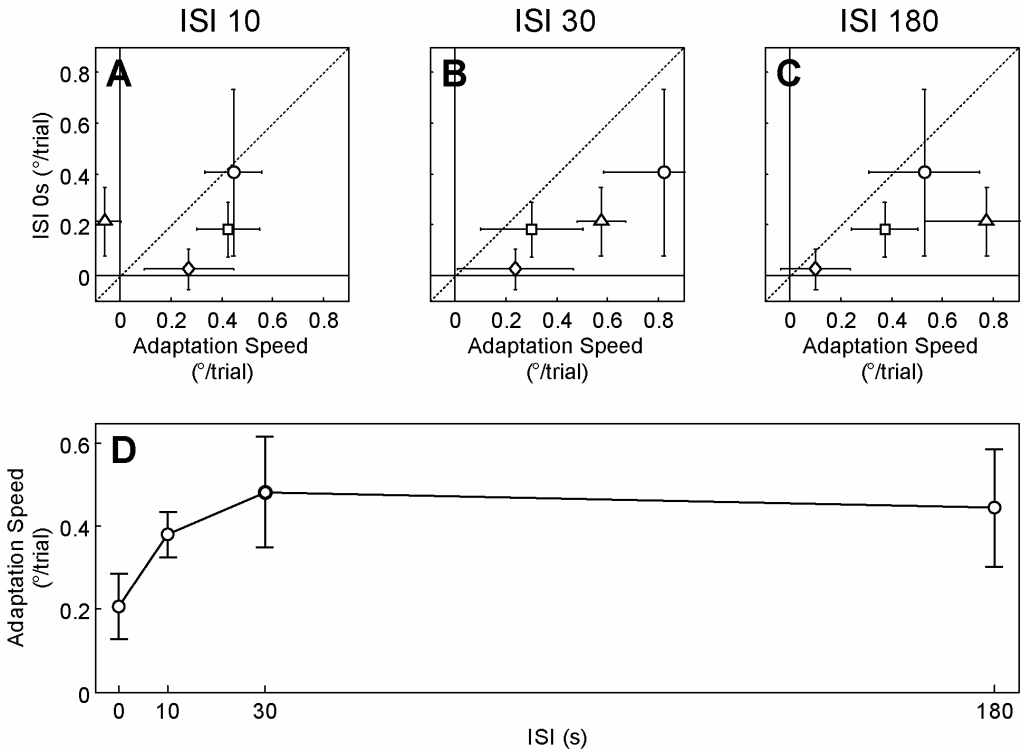

Figure 5: Adaptation efficacy increases with longer intervals. A) The adaptation efficacy in the shortest interval condition (ISI of 0 seconds) plotted against the adaptation efficacy in the 10 seconds interval condition (ISI 10). Each point represents one subject and corresponding symbols indicate the same subject in panel A, B, and C. The horizontal and vertical error bars are the standard deviations of the adaptation efficacy as estimated by the bootstrap procedure. The dotted line is the unity line. B) The adaptation efficacy in the shortest interval condition plotted against the adaptation efficacy in the 30 seconds interval condition (ISI 30). C) The adaptation efficacy in the shortest interval condition plotted against the adaptation efficacy in the 180 seconds interval condition (ISI 180). Points reflect the slopes of the lines in panel B-E of figure 2. D) Mean adaptation efficacy (±SEM) as function of interval between trials (ISI). For the ISI of 10 seconds, subject 3 who did not adapt in that condition was removed.

to have an effect on the new oculomotor response. Once the system has adjusted to its new amplitude the extra trials in the long condition do not establish an adaptation that is more resistant to a new change, i.e. readaptation to the original gain. It is likely that over a much longer period of consolidation, the strength of the adaptation does change. The sudden change of amplitude that we observe when the post-adaptation phase starts suggests that at least part of the original pre-adaptation status is actively maintained. After the adapted state is maintained for longer, this effect is likely to disappear, to be replaced by a chronic change in the direct motor pathways. However, it should be noted that human saccades are usually already completely adapted in about 75 trials, equivalent of about 7 min of stimulation (see however below). Therefore, if non-error based mechanisms influence the saccade gain they are likely to act substantially slower than the primary, error-based process.

Another finding is that the extra trials that were presented in the long series do not only lack the possibility to actively consolidate the adaptation, but that they are also

not necessary for preventing loss of adaptation strength during a period of inactivity (the pause condition). In other words, once an adapted state is reached, the system will keep this value, even in the absence of stimulation. This latter point was also shown in the monkey by Straube et al. (1997). In their study the pause lasted for 20 hours, after which adaptation was still present. These findings are consistent with the notion that saccade adaptation is mainly dependent on error-based learning. Neither continuous confirmatory feedback nor lack of sensori-motor feedback over a period of 20 min has an influence on the strength of the adaptation.

Trial Interaction

By varying the intervals between the trials in the adaptation phase we investigated the interaction between those trials. We found that the adaptation speed varies systematically with trial interval (ISI). The amplitude change/trial is optimal when the trials are separated at least several seconds. This suggests that each trial induces a form of plasticity that requires some time effectuate. At the same time the induced changes by a single trial do not abate over longer periods. Once the maximum adaptation speed is reached, this speed is kept also at extremely long intervals of 180 s. This suggests that the retention of adaptation is as solid during the actual learning phase (where the amplitude truly changes) as during the later stages of the adaptation phase (as we showed in the pause condition.

In conclusion, our findings suggest that the mechanism driving saccade adaptation turns an error signal derived from a single trial into a long-lasting change that takes some time to be completely effective. The single contribution of an individual trial is persistent throughout the following interval until another trial is presented and does not decay over long periods of time (up to 180 seconds). Furthermore, the increase of adaptation efficacy of single trials in the larger intervals suggests that the contribution of a single visual error increases its weight to the adaptation process when the interval between successive error signals is prolonged.

Neurophysiological basis

Since quite a lot is known about the neurophysiological basis of saccade adaptation in both humans and rhesus monkeys, we feel that it is worthwhile to speculate about the putative mechanisms that may underlie our behavioral data.

It has been shown extensively that the cerebellar cortex is critically involved in saccade adaptation (Robinson and Fuchs, 2001). Waespe and Baumgartner (1992) as well as Straube et al. (2001) showed an impairment of saccade adaptation capacities in patients suffering from syndromes associated with cerebellar dysfunction. PET studies show that during saccade adaptation the oculomotor vermis of the cerebellar cortex is active (Desmurget et al., 1998; Desmurget et al., 2000). Monkey studies have shown that lesioning the oculomotor vermis prevents rapid saccade adaptation (Takagi et al., 1998; Barash et al., 1999).

Several types of use-dependent plasticity processes that could underlie these behavioral changes have been described in the cerebellar cortex (Hansel et al., 2001) such as long-term depression (LTD) of the parallel fiber and climbing fiber to Purkinje cell synapses. Interestingly, LTD of both the parallel fiber (Shigemoto et al., 1994) and climbing fiber-Purkinje cell synapse (Hansel et al., 2000) depend on activation of the metabotropic glutamate receptor type 1, of which a block has recently been described to cause an impairment of saccade adaptation (Coesmans et al., 2003). LTD of the parallel fiber-Purkinje cell synapse is considered a major candidate to underlie forms of error-based cerebellar motor learning, such as saccade adaptation (Raymond, 1998).

During saccade adaptation, the climbing fiber signal in the oculomotor vermis of the cerebellar cortex has been reported to encode a form of the error associated with imprecise saccades (Catz et al., 2003). These error signals might in turn influence the amplitudes of subsequent saccades made to the same target by modifying the synaptic efficacies of cerebellar Purkinje neurons. The behavioral data we show are largely in line with what is known about the neurophysiology of cerebellar motor learning:

1. Synaptic plasticity processes in general, and changes of parallel fiber-Purkinje cell synaptic strength in particular, are generally assumed to underlie forms of cerebellar motor learning. PF – Purkinje cell synaptic strength is modified when a performance error signal (Frens et al., 2001) is given by a change in the CF firing rate. During either the retention of adaptation (the 'Long condition') or lack of sensori-motor feedback (the 'Pause condition') no change in CF activity is expected. Consequently one expects no modifications at the level of the cerebellar cortex under these conditions. This is compatible with our behavioral findings regarding the strength of the adaptation after either a long adaptation series or a pause (Figs 3 and 4). During these stages the oculomotor system is not modified with respect to saccadic amplitudes.

2 The time course of the saccade amplitude changes in humans is largely in line with that of in vitro changes in synaptic strength, such as LTD of the parallel fiber-Purkinje cell synapse (Daniel et al., 1998). The fact that synaptic gain changes take some time to be effectuated might account for our behavioral finding that the adaptation efficacy of single trials decreases when the ISIs are not sufficiently large (< 10 s; Figs 5 and 6). However, the available data on short term synaptic gain changes (< 1 minute) upon in vitro induction of synaptic plasticity remain too course to allow for an optimal comparison.

Thus, although our results by no means show a direct link between saccade adaptation and neuronal adaptation processes in the cerebellum, it seems promising to study the timing aspects of both forms of learning in greater detail to investigate whether processes of cerebellar plasticity at the neuronal level actually underlie saccade plasticity at the behavioral level.

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CHAPTER 5

THE USE OF PATCH-CLAMP RECORDINGS OF PURKINJE CELLS IN RAT CEREBELLAR SLICES AS A TOOL TO ASSESS SYNAPTIC PLASTICITY

Introduction

The patch-clamp technique is a powerful and versatile method for studying electrophysiological properties of neurons. It allows for the recording and manipulation of currents flowing through ion channels, enabling one to monitor physiological membrane potential changes such as action potentials (Sakmann and Stuart, 1995 for review). This chapter describes the techniques we used to study long-term synaptic gain changes in excitatory synapses onto Purkinje cells in rat cerebellar slices.

Studying long-term synaptic plasticity in Purkinje cells in rat cerebellar slices by use of patch-clamp recordings

The patch-clamp setup

In our experiments we used a standard patch-clamp setup consisting of a microscope (Zeiss Axioskop FS) on a vibration isolation table (Newport) within a Faraday cage (Fig. 1), a patch-clamp amplifier (HEKA EPC-9 or EPC-10), a peristaltic pump (Rainin Dynamax) to supply the slice chamber with artificial cerebrospinal fluid (ACSF), stimulus isolation units (Cygnus) for the stimulation electrodes, an oscilloscope (Hameg) and a computer running PULSE + PULSEFIT 8.53 (HEKA). Rat cerebellar slices were placed in a slice chamber, which was connected to a fixed stage that also carried the micromanipulator holding the amplifier probe and the attached patch pipette, micromanipulators for positioning the stimulation electrode(s) (Luigs and Neumann), a reference electrode, and two tubes supplying and retrieving artificial cerebrospinal fluid (ACSF) to and from the slice bath (Fig 2). In caged Ca^{2+} experiments photolysis was achieved with a 100 W HBO lamp (Zeiss), the light of which passed through a Uniblitz shutter (Vincent Associates) and an excitation filter with a maximal transmission at 365 nm.

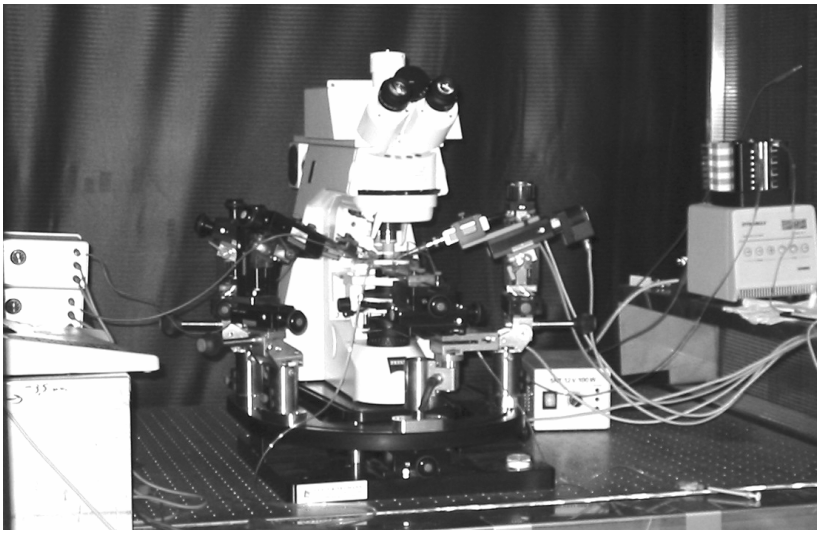

Figure 1: The patch-clamp setup used in our experiments.

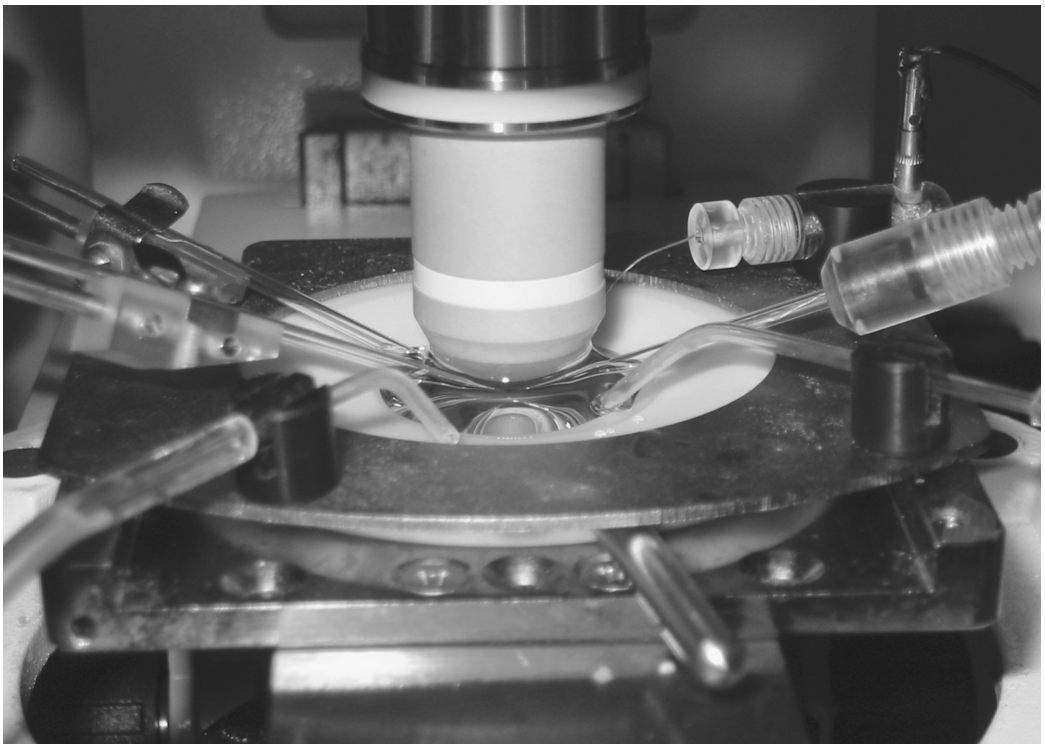

Figure 2: A close-up of the slice bath recording chamber, with brain slice, reference electrode, patch pipette and cannula for ACSF supply (right), stimulation pipettes and cannula for ACSF discharge (left), as seen during recording.

Borosilicate glass single barrel capillaries (World Precision Instruments PG52165-4) were used to pull patch-clamp recording and stimulation pipettes, on a Flaming/Brown pipette puller (Sutter). After fire polishing using a microforge (Narishige), the recording pipette tips were typically 1-2 μm in diameter. When filled with the recording saline their resistance was typically 2-5 MΩ.

Patch clamp recording from the soma of Purkinje cells in rat cerebellar slices

Sagittal slices of the cerebellar vermis (200 μm) were prepared from postnatal-day 18-27 Sprague-Dawley rats by using a vibratome (Leica) and ice-cold standard ACSF containing 124 mM NaCl, 5 mM KCl, 1.25 mM Na_2HPO_4, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM D-glucose, bubbled with 95% O_2/5% CO_2. After slicing, the slices were placed in an incubation chamber filled with ACSF, and bubbled with 95% O_2/5% CO_2 at room temperature. After a recovery period of at least 1 h, the slices were placed in the slice chamber that was perfused at a flow rate of 3 ml/min with room-temperature ACSF supplemented with 20 μM bicuculline methiodide to block γ-aminobutyric acid type A receptors. In some experiments, the low-affinity competitive AMPA receptor antagonist γ-D-glutamylglycine (γ-DGG, 0.5 mM) was added to the ACSF.

Under visual control, stimulation electrodes filled with ACSF were carefully placed in the molecular and/or granular cell layer around the Purkinje cell that was selected for recording (Fig. 3). For standard EPSC recordings, the recording electrode was filled with a K^+-based solution containing 9 mM KCl, 10 mM KOH, 120 mM K gluconate, 3.48 mM $MgCl_2$, 10 mM HEPES, 4 mM NaCl, 4 mM Na_2ATP, 0.4 mM Na_3GTP, and 17.5 mM sucrose (300 ± 5 mM ; pH 7.25 ± 0.3). In some experiments, the calcium chelator BAPTA (5-20 mM) or the caged Ca^{2+} compound 1-(4,5-dimethoxy-2-nitrophenyl)-1,2-diaminoethane- N,N,N',N'-tetraacetic acid (DMNP-EDTA, 8mM) was added to the patch pipette saline. All drugs used for ACSF or recording pipette solutions were purchased from Sigma, except for BAPTA, DMNP-EDTA (Molecular Probes) and γ-DGG (Tocris).

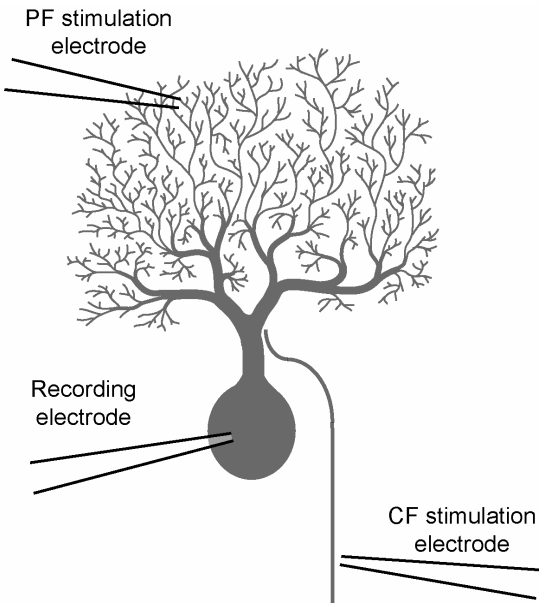

Figure 3: A schematic drawing of a Purkinje cell and the recording and stimulation electrodes. The recording electrode is attached to the Purkinje cell soma. The stimulation electrodes are placed in the granule cell layer, through which the climbing fiber ascends, and in the molecular layer, in which the parallel fibers are located.

When entering the bath, positive pressure was applied to the patch pipette, generating a pressure wave of pipette solution in front of the tip, in order to keep the tip clean and open and to clean the Purkinje cell membrane. Once the patch pipette tip touched the membrane (as seen by the dimpling of the membrane), the positive pressure was released, and slight negative pressure was applied until the membrane was tightly sealed to the electrode tip. Hyperpolarization of the patch pipette to -65 mV was used to aid seal formation. The forming of the seal could be registered as a large increase in resistance, typically reaching values over 10^9 Ω (1 GΩ: 'gigaseal'). Subsequently the patch membrane was ruptured by brief pulses of suction to the patch pipette, thereby creating a hole in the plasma membrane and gaining access to the Purkinje cell interior. This rupture leaves the seal between the pipette and plasma membrane intact, thus preventing leak currents flowing between the pipette and the reference electrode, and separating the Purkinje cell's constituents from the ACSF in the bath. This configuration is characterized by a low-resistance access to the cell interior through the pipette tip

(typically 2-5 MΩ), allowing a voltage-clamp of the whole cell ('whole-cell' configuration). In whole-cell 'voltage-clamp' mode the membrane potential of the neuron is controlled by the potential applied to the pipette electrode, enabling the recording of membrane currents. 'Current clamp' mode enables one to monitor the changes in membrane potential, which constitute the physiological response of a cell, such as action potentials. Currents were filtered at 2.9 kHz, digitized at 8 kHz, and acquired by using PULSE 8.53 software (HEKA). During voltage-clamp recordings, holding potentials were chosen in the range of -75 to -60 mV. During current-clamp recordings, small negative currents were passed to move the PC in the range of -75 to -60 mV, in order to prevent spontaneous spike activity.

Synaptic gain changes

In the majority of our experiments we investigated long-term changes in the strength of the PF-PC synapse (Fig 4). In cerebellar slices, PF-LTD can be induced by coactivation of PF and CF for 5 min at a rate of 1 Hz (Karachot et al., 1994), while PF-LTP can be induced by activation of the PF alone for 5 min at 1 Hz (Lev-Ram et al., 2002). After breaking the gigaseal, EPSCs in response to PF stimulation were recorded at 0.05 Hz in voltage-clamp mode. We recorded these PF-EPSPs, until a stable baseline of PF-EPSCs with only small variations in amplitude was recorded for 5-10 min. The subsequent PF-only (for PF-LTP induction) or PF+CF tetanus (for PF-LTD induction) was recorded in current-clamp mode, in order to allow the PC to fire complex spikes. After the tetanus we resumed the 0.05 Hz PF-stimulation in voltage-clamp mode, and, if possible, we recorded the EPSCs for 30 more minutes. When PF-LTD was successfully induced, PF-EPSCs were reduced in size after PF+CF coactivation, whereas PF-EPSCs amplitudes were larger after successful PF-LTP induction by PF-only stimulation.

In order to establish the localization of the synaptic gain changes, we used a paired-pulse facilitation (PPF) paradigm (Fig. 4). PPF is a very short-term enhancement in synaptic efficacy attributed to residual presynaptic Ca^{2+} facilitating more transmitter release (Atluri and Regehr, 1996; Salin et al., 1996). Changes in PPF during PF-LTP would argue that PF-LTP is expressed presynaptically, whereas unchanged PPF would suggest a postsynaptic expression site. In addition to PPF-analysis, we used the low-affinity competitive AMPA receptor antagonist γ-DGG as a reporter for changes in glutamate release (Wadiche and Jahr, 2001). γ-DGG unbinds rapidly from AMPA receptors and released glutamate will replace it at some binding sites. Thus, the degree of EPSC blockade by γ-DGG can be used as an indicator of changes in glutamate release after LTD or LTP induction (Shen et al., 2002). If PF-LTP results from an enhanced release of glutamate, the degree of blockade after LTP induction should be smaller.

PF-LTD induction has been reported to be Ca^{2+} dependent (Sakurai, 1990; Konnerth et al., 1992; Shibuki and Okada, 1992), whereas PF-LTP induction is not (Lev-Ram et al., 2002). A potential role of postsynaptic Ca^{2+} transients in determining the polarity of PF-PC synaptic gain change was studied in a second set of experiments. We studied the effects of the calcium buffer BAPTA at different concentrations in the recording pipette solution on PF-LTD induction. In a second set of experiments we

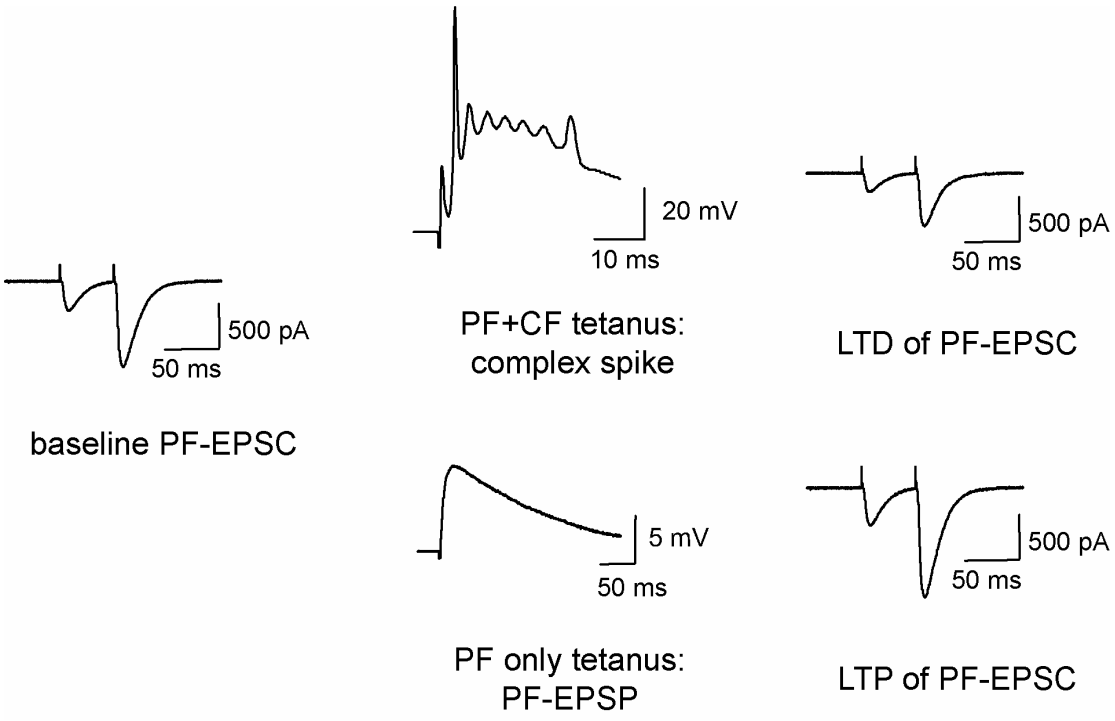

Figure 4: Induction of long-term PF-PC synaptic plasticity. The amplitude of the PF-evoked EPSC (left panel) is changed after PF+CF coativation or PF-only stimulation, respectively (mid panel). PF+CF coactivation at 1 Hz for 5 minutes leads to a reduction of PF-PC synaptic strength, which is expressed as a decreased PF-EPSC amplitude (top right panel). PF-only stimulation induces an increase of PF-PC synaptic strength, expressed as an increase of PF-EPSC amplitude (bottom right panel).

studied the effect of an artificial postsynaptic Ca^{2+} rise on PF-LTP induction by photolytically releasing Ca^{2+} from the caged calcium compound DMNP-EDTA, which was added to the pipette saline.

A third way to manipulate postsynaptic Ca^{2+} transients is by changing the CF-evoked Ca^{2+} transient. CF-LTD, which can be monitored as a decrease in CF-EPSC amplitude or as a reduction of the amplitude of the complex spike slow spikelets, can be induced by tetanization of the CF at 5 Hz for 30 s (Hansel and Linden, 2000), and has been shown to reduce the CF-evoked Ca^{2+} transient (Weber et al., 2003). In order to study a potential effect of this reduction of CF-evoked Ca^{2+} transients on PF-LTD induction, we did a last set of experiments in which we first induced CF-LTD, and applied the regular PF+CF tetanus 15 minutes later. The results of the experiments mentioned in this paragraph will be described in detail in chapter 6.

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CHAPTER 6

BIDIRECTIONAL PARALLEL FIBER PLASTICITY IN THE CEREBELLUM UNDER CLIMBING FIBER CONTROL

Abstract

Cerebellar parallel fiber (PF)-Purkinje cell (PC) synapses can undergo postsynaptically expressed long-term depression (LTD) or long-term potentiation (LTP) depending on whether or not the climbing fiber (CF) input is coactivated during tetanization. Here, we show that modifications of the postsynaptic calcium load using the calcium chelator BAPTA and photolytic calcium uncaging, respectively, result in a reversal of the expected polarity of synaptic gain change. At higher concentrations, BAPTA blocks PF-LTP. These data indicate that PF-LTD requires a higher calcium threshold amplitude than PF-LTP induction and suggest that CF activity acts as a polarity switch by providing dendritic calcium transients. Moreover, previous CF-LTD induction changes the relative PF-LTD versus -LTP induction probability. These findings suggest that bidirectional cerebellar learning is governed by a calcium threshold rule operating 'inverse' to the mechanism previously described at other glutamatergic synapses (BCM rule) and that the LTD/LTP induction probability is under heterosynaptic climbing fiber control.

Introduction

Perhaps the most fundamental feature of the brain is its ability to process and store large amounts of information. Long-lasting synaptic gain changes present the most likely correlates of learning and memory at the neuronal circuit level. LTD at cerebellar PF-PC synapses, for example, is believed to underlie certain types of motor learning that involve the cerebellum, such as adaptation of the vestibulo-ocular reflex (VOR) or associative eyeblink conditioning (Marr-Albus-Ito models; for review see Ito, 2001). PF-LTD can be observed after paired PF and CF stimulation at low frequencies. CF activation leads to an all-or-none complex spike (for review see Schmolesky et al., 2002) and concurrently to a widespread calcium transient in PC dendrites (Ross and Werman, 1987; Knöpfel et al., 1991; Konnerth et al., 1992; Miyakawa et al., 1992), which reaches supralinear levels when the PF is coactivated (Wang et al., 2000). These CF-evoked calcium transients are required for PF-LTD induction (Sakurai, 1990; Konnerth et al., 1992; Augustine et al., 2003). At the PF synapses, there is a requirement for the activation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors and type 1 metabotropic glutamate receptors (mGluR1). These signals converge on the activation of protein kinase C (PKC). The involvement of the nitric oxide (NO) pathway remains disputed (for review see Daniel et al., 1998; Bear and Linden, 2000; Hansel et al., 2001; Ito, 2001).

PF-PC synapses cannot only be depressed, but also potentiated (Sakurai, 1987; 1990; Hirano, 1990; Crépel and Jaillard, 1991; Shibuki and Okada, 1992). This potentiation can occur pre- or postsynaptically. Induction of presynaptic PF-LTP depends on presynaptic calcium influx, the activation of adenylyl cyclase I and the subsequent activation of cAMP-dependent kinase (PKA; Salin et al., 1996; Chen and Regehr, 1997; Storm et al., 1998; Jacoby et al., 2001; Hartell, 2002; Lonart et al., 2003). However, this presynaptic form of LTP does not provide an efficient candidate mechanism for reversing postsynaptic PF-LTD, because continuous synaptic activity would result in a downregulation of postsynaptic AMPA receptors while increasing

transmitter release. A better candidate for a true reversal mechanism is provided by a recently described, postsynaptically induced and expressed form of PF-LTP, which has been shown to be NO-dependent in a cGMP-independent way (Lev-Ram et al., 2002; 2003).

In brain areas other than the cerebellum, LTP and LTD can be induced at the same synapse as well. For example, bidirectional synaptic plasticity has been studied in detail at excitatory synaptic inputs to hippocampal and neocortical pyramidal cells. At these types of synapses, the direction of postsynaptic gain change is determined by the amplitude of the postsynaptic calcium signal in that there is a higher threshold for LTP than for LTD induction (Bienenstock et al., 1982; Bear et al., 1987; Artola et al., 1990; Singer, 1995; Cummings et al., 1996; Hansel et al., 1997; Yang et al., 1999; Zucker, 1999; Cormier et al., 2001). Interestingly, it seems that there are different calcium signaling requirements for cerebellar synaptic plasticity. PF-LTD induction depends on postsynaptic calcium transients, which are largely contributed by CF activity (Sakurai, 1990; Konnerth et al., 1992; Augustine et al., 2003). However, in contrast to the cortical mechanisms described above, PF-LTP seems to require less calcium than LTD, as LTD induced by PF stimulation paired with either CF stimulation (Sakurai, 1990), or application of 8-bromo cyclic guanosine monophosphate (Br-cGMP; Shibuki and Okada, 1992), a membrane-permeable analog of cGMP, could be reversed towards LTP when a calcium chelator was added to the internal saline. PF-LTP induction was described to be enhanced when BAPTA (5mM) was added to the pipette saline and it was concluded that PF-LTP is not calcium-dependent (Lev-Ram et al., 2002). These data point towards unique calcium-related induction conditions for cerebellar synaptic plasticity, but the available evidence remains fragmentary as earlier studies failed to examine whether the LTP studied was postsynaptically expressed (Sakurai, 1990; Shibuki and Okada, 1992), and in the more recent studies on postsynaptically expressed LTP, no attempt was made to directly test whether the polarity switch between depression and potentiation was calcium-dependent (Lev-Ram et al., 2002, 2003).

We have now systematically analyzed the calcium signaling requirements for PF-LTD and -LTP induction as well as the role of the heterosynaptic CF input. More specifically, we a) find that paired CF activity determines whether LTD or LTP is induced at the PF input, b) provide additional, complementary evidence that the described form of PF-LTP is indeed postsynaptically expressed and can reverse previously induced LTD, c) demonstrate that the polarity of synaptic gain change can be entirely determined by solely manipulating the postsynaptic calcium load using calcium chelators and caged calcium compounds, respectively, while leaving PF and CF stimulation patterns unaltered, d) show that, despite of its lower calcium threshold, PF-LTP induction is also calcium-dependent (in contrast to Lev-Ram et al., 2002) and e) show that previous CF-LTD induction (Hansel and Linden, 2000), which is accompanied by a reduction of complex spike-evoked calcium transients (Weber et al., 2003), changes the probability for subsequent PF-LTD induction.

Results

To characterize calcium signaling requirements for PF-LTD and -LTP induction, we performed whole-cell patch-clamp recordings from PCs in rat cerebellar slices. PF-LTD was observed after paired PF and CF stimulation at 1Hz for 5 min in current-clamp mode (Fig. 1A). Test responses were recorded in voltage-clamp mode and the depression of excitatory postsynaptic current (EPSC) amplitudes amounted to 82.2 ± 8.5% of baseline (n=15; t=30-34 min). We applied two pulses at an interval of 50ms to measure the paired-pulse facilitation ratio (PPF). This parameter did not change after tetanization (102.7 ± 3.1%; n=12; Fig. 1B), confirming that PF-LTD is postsynaptically expressed.

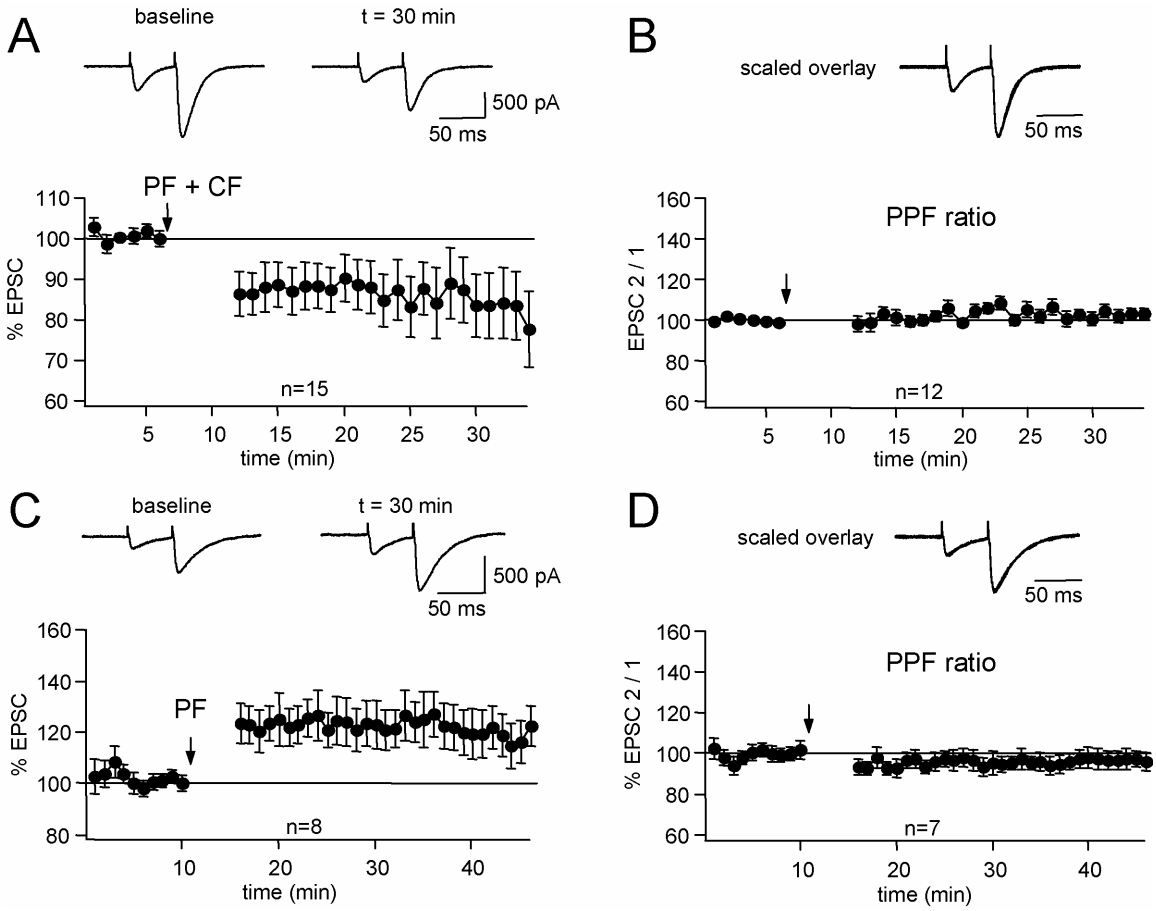

Figure 1: CF activity determines the polarity of postsynaptic PF plasticity. (A) PF-LTD can be induced after paired PF+CF stimulation (n=15). Each data point represents the average of three successive test responses evoked at 0.05Hz. The traces above show EPSCs before and after LTD induction. (B) Paired-pulse facilitation ratio (EPSC 2 / EPSC 1) from the LTD group shown in (A). Traces above show the overlay of traces shown in (A) after scaling to the same amplitude of EPSC 1. (C) LTP is obtained when the LTD protocol described in (A) is applied in the absence of CF stimulation (n=8). (D) Paired-pulse facilitation ratio from the LTP group shown in (C). Traces above (C) and (D) show EPSC pairs before and after LTP induction (left) and scaled to the same amplitude of EPSC 1 (right).

To obtain PF-LTP, we applied the same PF stimulation as for PF-LTD induction, but in the absence of CF activation (Fig 1C). This 'PF alone' stimulation resulted in LTP induction (117.5 ± 8.6%; n=8; t=40-45min). For this study, it is crucial to demonstrate that PF-LTP is postsynaptically expressed and thus shares the same expression site with PF-LTD. To do so, we measured the PPF ratio before and after LTP induction. PF-LTP was not associated with a change in the PPF ratio (96.6 ± 4.7%; n=7; Fig. 1D), which is a first indication for a postsynaptic expression site. It cannot be excluded that the relatively small changes in the PPF ratio that accompany PF-LTD (+2.7%) and -LTP (-3.4%) might result from minor presynaptic plasticity components, but the data nevertheless suggest that the major modifications underlying the depression or potentiation occur postsynaptically.

It has been sufficiently demonstrated that PF-LTD is a postsynaptic phenomenon. In contrast, only paired-pulse data are availabe to support the claim that the form of PF-LTP described here is postsynaptic as well. To obtain further evidence, we used the low-affinity competitive AMPA receptor antagonist γ-D-glutamylglycine (γ-DGG; 0.5mM) as a reporter for changes in glutamate release (Wadiche and Jahr, 2001). γ-DGG unbinds rapidly from AMPA receptors and released glutamate will replace it at some binding

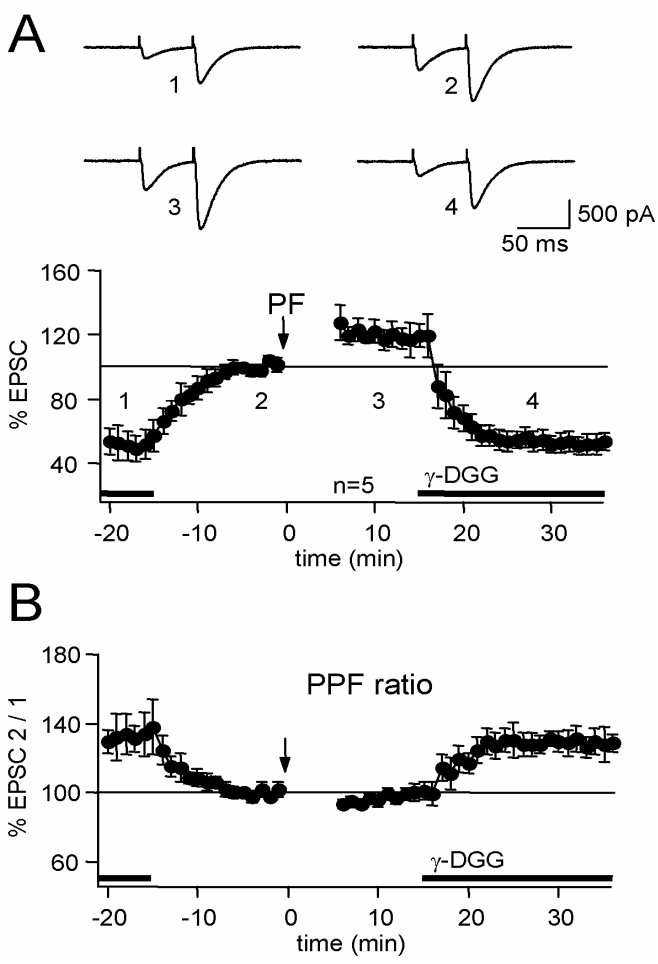

Figure 2: γ-DGG reveals that PF-LTP is postsynaptically expressed.
A) γ-DGG application (0.5mM) before and after LTP induction (n=5).
B) Paired-pulse facilitation ratio from the group shown in (A). Traces in (A) show EPSC pairs taken from the time points indicated in the time graph.

sites. Thus, the degree of EPSC blockade by γ-DGG can be used as an indicator of changes in glutamate release after LTD or LTP induction (Shen et al., 2002). If PF-LTP results from an enhanced release of glutamate, the degree of blockade after LTP induction should be smaller. Instead, the degree of blockade observed after LTP induction at t=30-35min (43.9 ± 5.2% of the 'potentiated' baseline) was not smaller than the degree of blockade measured before at t= -20 to –15min (52.2 ± 9.3% of pre-tetanization baseline; n=5; Fig. 2A). γ-DGG enhances the PPF ratio (133.8 ± 12.3%; t= -20 to –15min; n=5; Fig. 2B) indicating that the drug indeed detects differences in the glutamate transient (the second, larger EPSC results from a larger glutamate transient). These PPF data therefore underline previous claims of the sensitivity of the γ-DGG approach for changes in the glutamate transient (Wadiche and Jahr, 2001).

Our results demonstrate that PF-LTP predominantly is a postsynaptic phenomenon. When PF-LTD and -LTP share the same expression site, they should be able to reverse each other. This has been demonstrated using extracellular spike probability measurements (Lev-Ram et al., 2003). This technique allows for long-lasting recordings without wash-out effects, which is advantageous, because reversibility experiments optimally require, for example, saturation of LTD first, then induction of LTP and then again application of the LTD protocol. If further LTD can be induced, the

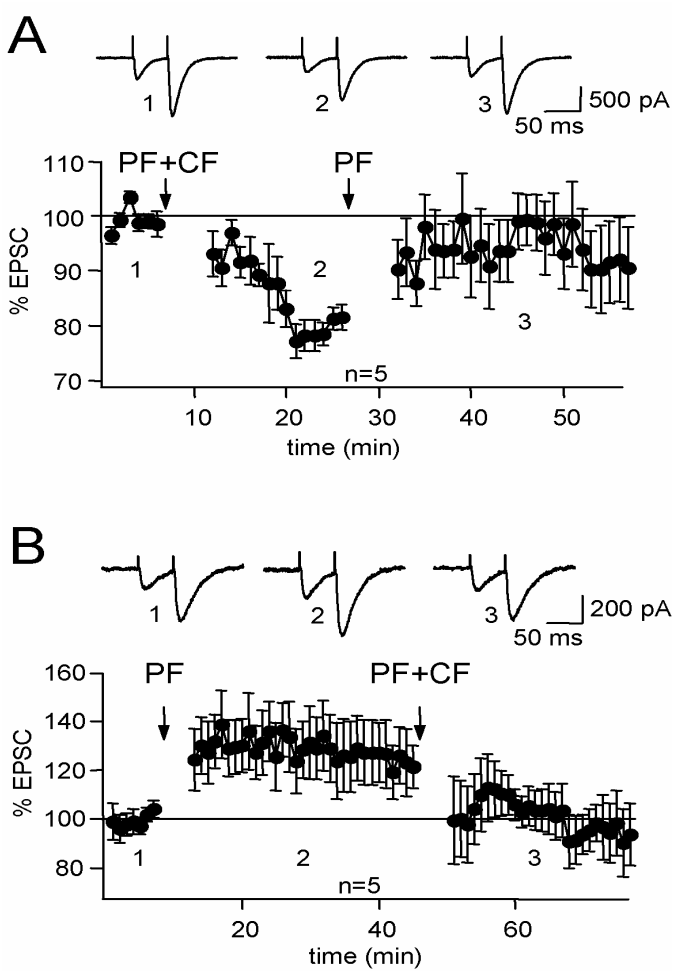

Figure 3: PF-LTD and -LTP can reverse each other.
(A) Application of the LTP protocol can reverse previously established LTD (n=5).
(B) Application of the LTD protocol can reverse previously established LTP (n=5). In (A) and (B), traces on top show EPSCs from the time points indicated.

previously saturated LTD has been reversed by the potentiation. The extracellular spike probability recordings allow for these long measurements, but there is a severe disadvantage. The spike probability changes could result from synaptic or intrinsic plasticity (for review see Zhang and Linden, 2003) or a combination of both and thus they do not allow a true characterization of the reversal of LTD and LTP. To test whether LTD and LTP can reverse each other under whole-cell patch-clamp conditions, we first applied the PF-LTD protocol (79.5 ± 2.4%; t= 20-25min) and subsequently applied the LTP protocol (Fig. 3A). This manipulation reversed the previously established LTD (97.2 ± 5.7% at t=45-50min, but note the drop to 93.0 ± 7.5% at t=50-55min; n=5). Next, we induced LTP first (123.4 ± 11.8%, t=40-45min) and following 33min of LTP expression, we applied the LTD protocol (Fig. 3B). This second stimulation period resulted in a reversal of LTP (94.3 ± 11.1%; t=68-77min; n=5). These data demonstrate that PF-LTD and –LTP do not only share the same expression site, but that they can also reverse each other. Our observations complement the results of Lev-Ram et al. (2003), which demonstrate reversibility of spike probability changes at the substrate level, whereas our data focus on synaptic plasticity, but merely show phenomenological reversibility.

The stimulation protocols for LTD and LTP induction described above differ only in the presence or absence of paired CF stimulation, respectively, which is known to initiate a dendritic calcium influx. Therefore, we wanted to examine the possibility that LTD induction requires a larger calcium signal than LTP induction and that this additional calcium transient is the switch factor determining the polarity of synaptic gain change. We applied the LTD protocol, but reduced the postsynaptic calcium signal amplitude by adding 20mM of the calcium chelator BAPTA to the internal saline. In the presence of BAPTA, the otherwise LTD inducing protocol caused LTP (112.9 ± 7.2%; at t=36-39min; n=9; Fig. 4A). The diagram in Fig. 4B shows LTD / LTP amplitudes obtained with paired PF and CF stimulation in the absence (empty dots) and presence of BAPTA (filled dots) and demonstrates that a reduction in the postsynaptic calcium signal determines whether LTD or LTP is induced. If the calcium load is the switch factor, we should be able to reverse the polarity of synaptic gain change in the opposite direction, namely to induce LTD when the LTP protocol is applied under conditions that lead to a larger calcium transient. To address this possibility, we applied the LTP protocol and photolytically released calcium from the caged calcium compound 1-(4,5-dimethoxy-2-nitrophenyl)-EDTA (DMNP-EDTA; 8mM), which was added to the pipette saline. Photolytic uncaging experiments have been used to describe the calcium signaling requirements of PF-LTD (Lev-Ram et al., 1997; Finch and Augustine, 1998), but not to examine relative calcium thresholds for LTD versus LTP induction. As described above, the LTP protocol consists of a 1Hz, 5min PF stimulation. In addition to the synaptic stimulation, we applied five light exposure periods to photolytically uncage calcium, which lasted 5 s each and were applied at the beginning of each minute of synaptic stimulation. Under these activation conditions, the otherwise LTP inducing protocol resulted in LTD induction (61.5 ± 14.3%; at t=30-35min; n=5; Fig. 4C). When PCs filled with DMNP-EDTA were exposed to the same photolysis protocol in the absence of

synaptic stimulation, no LTD, but rather a potentiation was observed (114.6 ± 10.5%; n=5; Fig. 4C). The diagram in Fig. 4D shows LTP / LTD amplitudes obtained with PF stimulation when photolysis of caged calcium was not (empty dots) and when it was applied (filled dots) and demonstrates that the higher calcium signal amplitude reached with uncaging promoted LTD instead of LTP induction.

These experiments show that a higher calcium signal amplitude is required for LTD than for LTP induction, but they allow no conclusion as to whether or not there is a calcium threshold for PF-LTP induction. The experiments described above show that

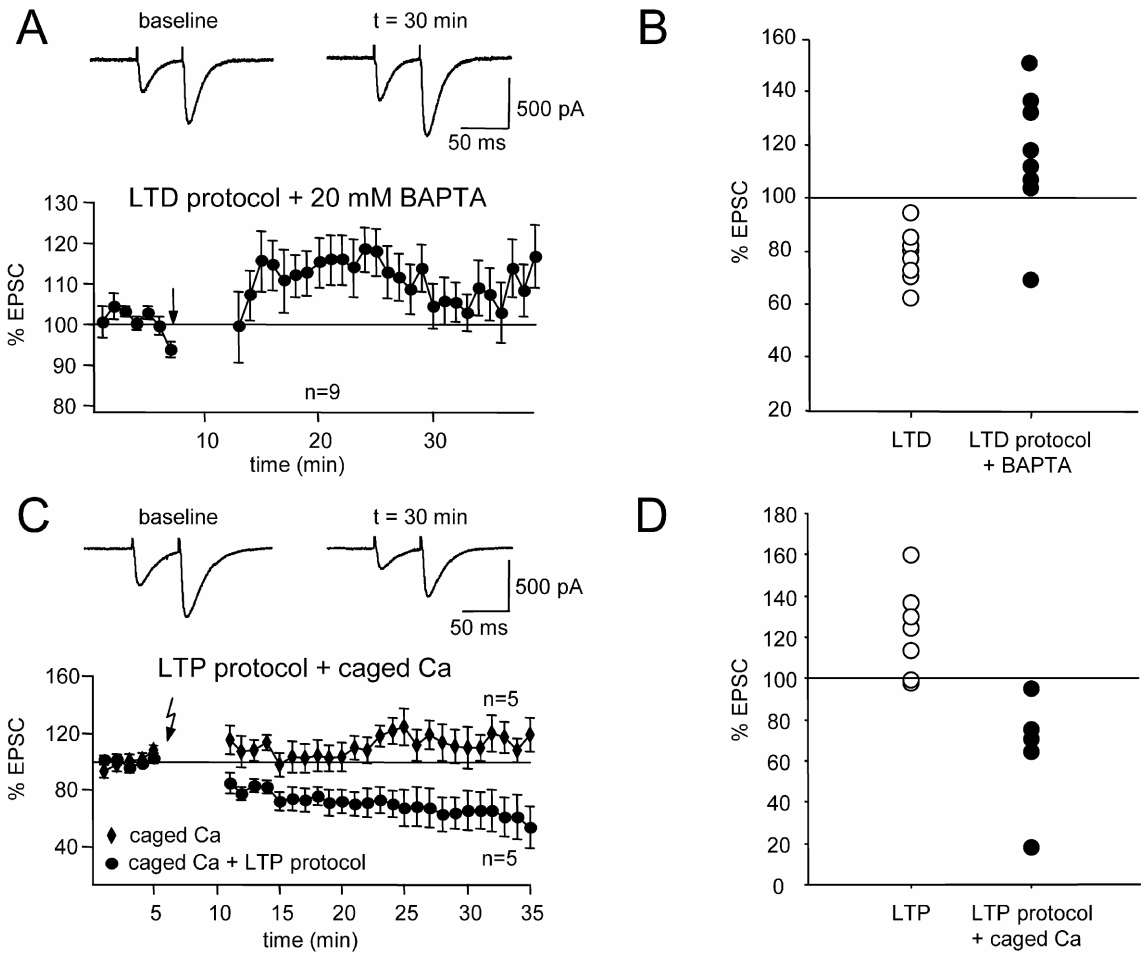

Figure 4: The calcium signal amplitude determines whether LTD or LTP is induced. (A) Application of the LTD protocol results in LTP induction when BAPTA (20mM) is added to the pipette saline (n=9; n=11 up to t=23min). Traces on top show EPSCs before and after tetanization. (B) Plot of individual cell data (at t=28min) obtained from the original LTD group (n=8) and the BAPTA group. As in the BAPTA group 700µs PF pulses were applied, we restricted the LTD group to those cells that were stimulated using the same pulse duration. (C) Application of the LTP protocol (compare to Fig. 1C) results in LTD induction when the synaptic stimulation is paired with photolysis of DMNP-EDTA (8mM; n=5). Photolysis alone does not induce LTD (n=5). Traces above depict EPSCs before and after LTD induction. (D) Plot of individual cell data (at t=28min) obtained from the original LTP group (Fig. 1C) and the photolysis group.

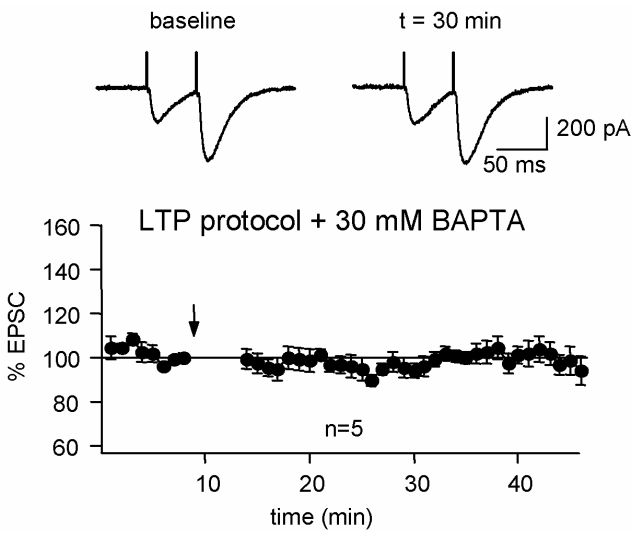

Figure 5: PF-LTP induction is calcium dependent. The application of the LTP protocol does not lead to a potentiation when BAPTA (30mM) is added to the pipette saline (n=5). Traces above depict EPSCs before and after tetanization.

adding 20mM BAPTA to the internal saline leads to the induction of LTP when the otherwise LTD-inducing protocol is applied. To test for calcium-dependence of LTP, it was thus necessary to use a higher BAPTA concentration. It has previously been shown that high BAPTA concentrations in the range of 30-40mM are needed to block a calcium-dependent form of short-term plasticity in Purkinje cells, namely depolarization-induced suppression of inhibition (DSI; Llano et al., 1991). Concentrations up to 50mM have been used to demonstrate the dependence of LTP at mossy fiber-CA3 synapses on postsynaptic calcium signaling (Yeckel et al., 1999). When 30mM BAPTA was added to the internal saline and the PF-LTP protocol was applied, no potentiation could be observed (101.3 ± 5.1% at t=40-45min; n=5; Fig.5), indicating that indeed there is a calcium threshold amplitude that has to be reached for LTP induction.

Under physiological conditions, the polarity-reversing calcium signal is provided by CF activity. Recently, we have shown that LTD can be induced at the CF input as well (Hansel and Linden, 2000) and that CF-LTD is associated with a long-term reduction in the amplitude of CF-evoked dendritic calcium transients (Weber et al., 2003). To address the possibility that CF-LTD might modify the probability for subsequent PF-LTD induction, we performed experiments in which CF-LTD was induced, followed by the application of the PF-LTD protocol. Throughout the experiments, cells were held in current-clamp mode as we chose to record complex spikes to monitor CF-LTD. We first performed control experiments in which LTD of PF-excitatory postsynaptic potentials (EPSPs) was established. In current-clamp mode, PF-LTD amounted to 82.0 ± 6.0% (at t= 45-50min; n=6; Fig. 6A). As described earlier (Hansel and Linden, 2000), 5Hz CF tetanization for 30s resulted in CF-LTD. This effect was measured as a reduction in the first slow complex spike component (Hansel and Linden, 2000; Weber et al., 2003) and amounted to -8.5 ± 4.4% at t=19-21min and -17.3 ± 8.8% at t=45-50min (Fig. 6B; n=5). Following 15min of CF-LTD stabilization, the PF-LTD protocol was applied. In contrast to the control experiments, no PF-LTD was observed, but rather a potentiation was seen after previous CF-LTD induction (125.1 ±

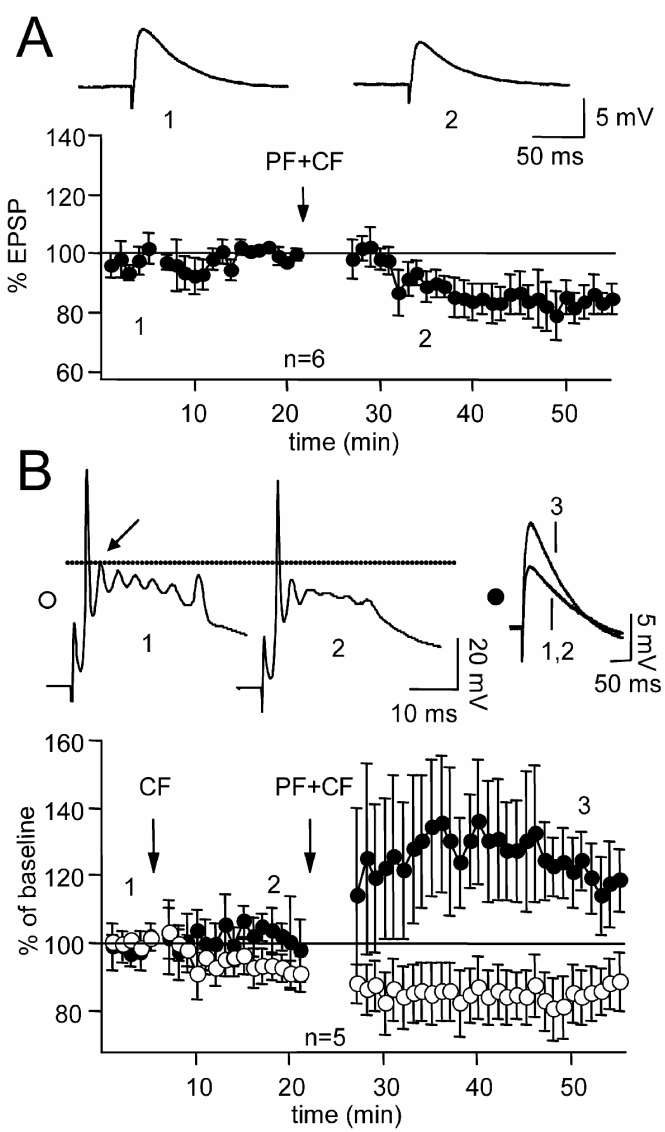

Figure 6: CF-LTD lowers the probability for subsequent PF-LTD induction.
(A) Under control conditions, LTD can be induced by paired PF+CF activation (n=6). In three cells, the baseline started at t=-15min. Traces on top show EPSPs at the time points indicated.
(B) CF tetanization at 5Hz for 30s results in CF-LTD induction, which was measured as a reduction in the first slow spike component (arrow; open circles). Subsequent application of the PF-LTD protocol used in (A) results in LTP instead of LTD induction (n=5; closed circles). Traces above show CF-evoked complex spikes (left and middle) and EPSPs (right) at the time points indicated.

12.3%; at t=45-50min; n=5; Fig. 6B). These results indicate that CF-LTD can shift the relative induction probability of PF-LTD and -LTP.

Discussion

The data presented here show that bidirectional PF long-term plasticity is governed by a calcium threshold mechanism, which is characterized by a high calcium threshold for LTD, and a lower calcium threshold for LTP induction (Fig. 7). It has been shown that PF-LTD induction requires postsynaptic calcium influx, the activation of mGluR1 receptors and the activation of PKC (for review see Daniel et al., 1998; Bear and Linden, 2000; Hansel et al., 2001; Ito, 2001). The central role of PKC activation in PF-LTD induction is emphasized by transgenic mouse studies, in which a PKC peptide inhibitor is selectively expressed in PCs. In these mice, PF-LTD and cerebellar motor learning are

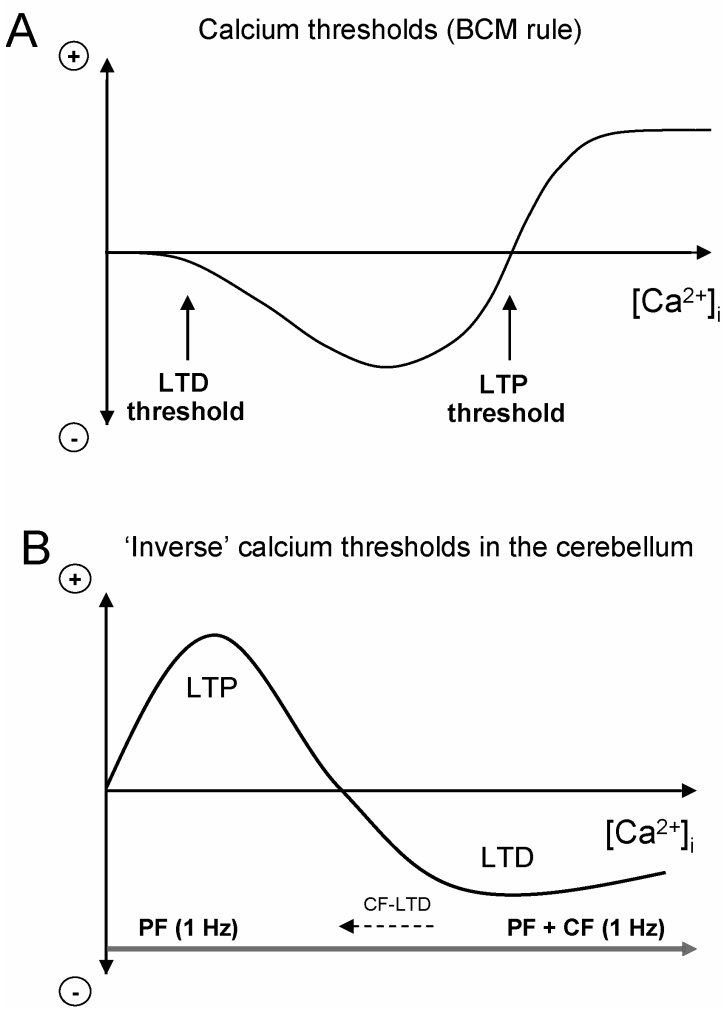

Figure 7: Calcium threshold models for LTP and LTD induction.
(A) At excitatory inputs to cortical pyramidal cells, there is a higher calcium threshold amplitude for LTP than for LTD induction (BCM rule). (B) As demonstrated in this paper, bidirectional PF plasticity is governed by a calcium threshold mechanism 'inverse' to the one illustrated in (A). PF-LTD has a higher calcium threshold than LTP. CF activity contributes sufficient calcium to increase sub-stantially the probability that the LTD threshold is reached. This probability can be modified by CF-LTD.

impaired (De Zeeuw et al., 1998; Koekkoek et al., 2003). In contrast, the induction conditions and key signaling cascades for postsynaptic PF-LTP are not well characterized. PF-LTP has been shown to be NO-dependent in a cGMP-independent way (Lev-Ram et al., 2002), but it is not known how this NO dependence relates to that suggested for PF-LTD. Recent studies have demonstrated a role of phosphatase inhibition in PF-LTD induction (Eto et al., 2002; Launey et al., 2003), suggesting that phosphatase activation might promote PF-LTP. In this scenario, a LTP-associated dephosphorylation event could counteract the GluR2 phosphorylation by PKC that is known to mediate PF-LTD induction (Chung et al., 2003).

PF plasticity under heterosynaptic CF control

Bidirectional synaptic plasticity controlled by a kinase / phosphatase antagonism requires that LTD- and LTP-inducing stimuli result in distinct patterns of calcium transients. While the involvement of phosphatases in PF-LTP induction is at this point merely speculative, we here provide a detailed description of the role of calcium signaling in

bidirectional PF synaptic plasticity, which could form a useful basis for a further characterization of signaling cascades involved in PF-LTD and -LTP induction, respectively. Previously, it has been shown that adding 5mM BAPTA to the pipette saline enhances PF-LTP and it was concluded that LTP induction is not calcium-dependent (Lev-Ram et al., 2002). In contrast, our study shows that PF-LTP induction is calcium-dependent, but that a more efficient calcium chelation is required to block LTP. Using 20mM BAPTA, application of the LTD protocol induced LTP instead. However, when adding 30mM BAPTA, LTP induction was blocked. This observation is in line with previous studies showing that some calcium-dependent forms of synaptic plasticity in PCs (Llano et al., 1991), or CA3 pyramidal cells (Yeckel et al., 1999) can only be blocked using high chelator concentrations. Our findings demonstrate that the induction cascades of both, PF-LTD and -LTP are dependent on calcium threshold amplitudes, but that the LTD induction threshold is higher than that for LTP induction. This pattern of calcium-dependence allows the CF input to act as a polarity switch factor. CF activation results in an all-or-none electrical response in PCs, the complex spike (for review see Schmolesky et al., 2002), which causes a widespread calcium transient in PC dendrites. As indicated in Fig. 7B, this additional calcium shifts the total calcium concentration into the range at which PF-LTD is induced. Under these conditions, modifications of the CF-evoked calcium signal might alter the PF-LTD induction probability. It is likely that this is the reason why we observed PF-LTP instead of -LTD following previous CF-LTD induction as CF-LTD is associated with a reduction in the amplitude of CF-evoked calcium transients (Weber et al., 2003). These observations assign a unique polarity switch function to the heterosynaptic CF input and suggest that at PF-PC synapses the term 'activity-dependent synaptic plasticity' cannot be applied in a strictly homosynaptic sense. However, it should be noted that previous studies have documented PF plasticity that depends on the activity level of the PF itself rather than that of the CF. For example, it has been shown that strong PF activation can induce PF-LTD in the absence of CF stimulation (Hartell, 1996; Eilers et al., 1997) and that, when using paired PF stimulation and depolarization for induction, PF-LTD or -LTP can be obtained depending on the amplitude of the PF responses (Reynolds and Hartell, 2000). LTD was more readily obtained with large responses and LTP with smaller responses. This observation certainly fits the 'inverse calcium threshold' model, because larger EPSCs can be expected to result in larger calcium transients. It thus seems likely that, while CF activity provides the major polarity switch, especially in a behaviorally relevant context (the PF and CF pairing resulting in PF-LTD closely matches the conditioned and unconditioned stimulus application resulting in learning of a conditioned response; for further discussion see below), it is not the only factor that can influence the probability for PF-LTD or -LTP induction.

'Inverse' calcium thresholds for PF-LTD and -LTP

Calcium threshold mechanisms controlling LTD vs. LTP induction have been studied extensively in the hippocampus and neocortex. Early papers suggested a high calcium threshold for LTP and a lower threshold for LTD induction (Bienenstock et al., 1982; Bear et al., 1987; Lisman, 1989). This hypothesis, which is best known as the

Bienenstock, Cooper and Munro (BCM) model (Bienenstock et al., 1982), was verified by experiments in which the postsynaptic calcium load was modified (Cummings et al., 1996; Hansel et al., 1997), or in which the calcium signal amplitude was measured using fluorescence imaging techniques (Hansel et al., 1997; Connor et al., 1999; Cormier et al., 2001). Experiments, in which LTP and LTD induction were achieved by photolytic release of calcium from caged calcium compounds, confirmed and extended the two-threshold-model by showing that, in hippocampal pyramidal cells, LTP was best induced by brief, high amplitude calcium transients, whereas LTD was more easily obtained by prolonged, lower amplitude calcium signals (Yang et al., 1999; Zucker, 1999). The need for larger calcium signals for LTP than for LTD induction explains why, under more physiological conditions, LTP is induced when the synaptic event shortly preceeds the occurence of dendritic spike activity (e.g. a backpropagating spike), whereas LTD is induced if the synaptic event occurs too early, or follows the spike (Debanne et al., 1994; Magee and Johnston, 1997; Markram et al., 1997; Feldman, 2000; Golding et al., 2002; for review see Linden, 1999). The reason for this observation is that dendritic calcium transients are larger, when the backpropagating spike follows an EPSP than when it preceeds an EPSP (Koester and Sakmann, 1998). A similar observation of spike-timing dependent bidirectional plasticity, but in reverse order, has been made in the electrosensory lobe (a cerebellum-like structure) of mormyrid electric fish, where LTD resulted when EPSPs preceded dendritic spikes, but LTP resulted when EPSPs followed the spikes (Bell et al., 1997). Although tempting, the comparability to our study is limited, because the electrosensory lobe of these fish does not receive CF input. Here, we demonstrate a novel type of calcium threshold rule, characterized by a higher calcium threshold for LTD than for LTP induction (Fig. 7). In addition, our data demonstrate a novel form of heterosynaptic interaction, in which the activity of a qualitatively different, heterosynaptic input provides a polarity switch for PF synaptic plasticity, and LTD at one type of synaptic input (the CF) changes the probability for LTD induction at another type of synaptic input (the PF). This type of interaction shares some features with the 'heterosynaptic metaplasticity' that was previously described in the hippocampus (Abraham et al., 2001), but does not seem to involve a shift of the threshold values, but rather a shift in the probability to reach a given calcium threshold amplitude.

LTP as a reversal mechanism

PF-LTD is widely viewed as a cellular correlate of cerebellar motor learning. LTP might provide a cellular basis for a reversal mechanism. An example for such a role of LTP might be extinction of conditioned eyelid responses after repeated application of the conditioned stimulus alone. Under physiological conditions, this is most likely to occur when CF activity is reduced through inhibitory projections from the cerebellar nuclei to the inferior olive (Medina et al., 2002; Ohyama et al., 2002). PF-LTD and -LTP can also mediate bidirectional modifications such as decreases and increases in the PC receptive field size (Jörntell and Ekerot, 2002), or bidirectional changes in the VOR gain (Boyden and Raymond, 2003). The latter example is particularely interesting, because the authors demonstrate an asymmetric reversibility. This has been interpreted as resulting from a combination of true reversal and masking effects. It seems likely that the masking

component is caused by presynaptic LTP (Salin et al., 1996; Chen and Regehr, 1997; Storm et al., 1998; Jacoby et al., 2001; Hartell, 2002; Lonart et al., 2003), which cannot directly affect the postsynaptic LTD substrate(s), whereas the reversal component is related to postsynaptic LTP. This example illustrates that, while postsynaptic LTP might provide the most efficient reversal mechanism for postsynaptic LTD (another possibility is a decline of the depression level over time), in vivo pre- and postsynaptic modifications most likely act in concert.

Experimental Procedures

Slice preparation

Sagittal slices of the cerebellar vermis (200 μm thick) were prepared from P18-27 Sprague-Dawley rats. Slices were kept in artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 5 KCl, 1.25 Na_2HPO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, and 10 D-glucose bubbled with 95% O_2 and 5% CO_2. The ACSF used for perfusion was supplemented with 20 μM bicuculline methiodide to block $GABA_A$ receptors. Whole-cell patch-clamp recordings were performed at room temperature using either an EPC-9 or an EPC-10 amplifier (HEKA Electronics, Germany). The recording electrodes were filled with a solution containing (in mM): 9 KCl, 10 KOH, 120 K gluconate, 3.48 $MgCl_2$, 10 HEPES, 4 NaCl, 4 Na_2ATP, 0.4 Na_3GTP, and 17.5 sucrose (pH 7.25). In two group of experiments, 20 and 30mM bis(2-amino-phenoxy)ethane-N,N,N',N'-tetraacetate (BAPTA), respectively, was added to the internal saline. The tetrapotassium salt of BAPTA was used and the K gluconate concentration was reduced accordingly to maintain the desired osmolarity and ionic strength. In the BAPTA experiments, $CaCl_2$ was added (10 and 15mM, respectively) to maintain the resting calcium concentration (see Dzubay and Otis, 2002). In another group, 0.5mM γ-D-glutamylglycine (γ-DGG) was added to the ACSF. All drugs were purchased from Sigma, except for BAPTA (Molecular Probes) and γ-DGG (Tocris).

Neurophysiology

Currents were filtered at 3kHz, digitized at 8kHz, and acquired using PULSE software. During voltage-clamp experiments, holding potentials in the range of –60 to –75mV were chosen to prevent spontaneous spike activity that might escape voltage-clamp due to the poor space clamp characteristics that is typcial for recordings in PCs. For extracellular stimulation, standard patch pipettes were used that were filled with external saline. CFs were stimulated in the granule cell layer and PFs in the molecular layer. Test responses were evoked at a frequency of 0.05 Hz using ~3 μA pulses that were applied for 500-700 μs (within this range of pulse durations the results were not significantly different). In all experiments, cells were switched to current-clamp mode for tetanization. Recordings were excluded from the study if the series or the input resistance varied by >15% over the course of the experiment. All values are shown as %

of baseline ± SEM. For the photolysis experiments, patch pipettes were filled with internal saline supplemented with 8mM DMNP-EDTA (Molecular Probes), 50% loaded with calcium and pH-adjusted. Photolysis was achieved with a 100-W HBO lamp (Zeiss), the light of which passed through a Uniblitz shutter (Vincent Associates) and an excitation filter (maximal transmission at 365 nm). In a set of preceeding calibration experiments using micro-cuvettes (VitroCom) and the fluorescent calcium indicator Oregon Green BAPTA-2 (Molecular Probes), we determined that a 5s photolysis period leads to an increase in $[Ca^{2+}]_i$ in the range of 100 to 300 nM. This value presumably is an underestimation, because of the delay of the fluorescence measurement caused by the switch of excitation filters.

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CHAPTER 7

GENERAL DISCUSSION

In the past chapters we have described several aspects of cerebellar function. The relation between synaptic transmission, synaptic plasticity, motor behavior and adaptation was studied using various models and techniques. We describe a newly discovered autoantibody against mGluR1 (mGluR1-Ab), which affects Purkinje neuronal excitability, plasticity of the parallel fiber-Purkinje cell synapse and Purkinje neuronal survival. The behavioral consequences of these cellular effects are ataxia and impaired cerebellar motor learning. Analysis of saccade adaptation in humans indicates that the characteristics of this form of cerebellar motor learning are in line with the learning rules of cerebellar synaptic plasticity processes. Next we show that not only long-term depression (PF-LTD), but also long-term potentiation (PF-LTP) can be induced at parallel fiber-Purkinje cell synapses, and that climbing-fiber induced calcium transients plays a key role in their respective induction. Long-term depression of the climbing fiber (CF-LTD) is shown to reduce the probability of PF-LTD induction. In this chapter these results, their correlation, and some functional implications will be evaluated.

7.1 Anti-mGluR1 autoantibodies cause PCA

In chapters 2 and 3 a new autoantibody associated with PCA is described. This anti-mGluR1 autoantibody acutely affects motor behavior in experimental animals upon infusion into their cerebrospinal fluid, which makes it the first autoantibody described to directly interfere with cerebellar function. Previous studies of PCA-associated autoantibodies did not reveal such effects (Graus et al., 1991; Sillevis Smitt et al., 1995), which suggests that they might have a different effector mechanism in the patients. It appears that in PCA forms associated with autoantibodies other than mGluR1-Ab, cellular immunity plays a role in the cerebellar degeneration (Posner, 1995; Mason et al., 1997; Giometto et al., 1997). Pathological analysis of the cerebellum of one of our patients with mGluR1-Ab shows no signs of any ongoing inflammatory reaction. Previous occurrence of a cellular immune reaction cannot be excluded, although this does not seem probable given the facts that the patient still harbored high concentrations of mGluR1-Ab and had severe cerebellar symptoms, while still having a fairly high amount of spared Purkinje cells. The full recovery of our other patient and the reversibility of the symptoms seen in the acutely and subacutely injected mice indicate that the ataxia is not exclusively due to Purkinje cell loss. Immunohistochemical analysis of the flocculus of mice that were infused with mGluR1-Ab for 7 days revealed no visible signs of neuronal degeneration, which suggests that a longer exposition to mGluR1-Ab is needed to cause Purkinje cell death. Long-term chronic application of mGluR1-Ab to cultured Purkinje cells in vitro and chronic infusion into experimental animals could help elucidate the process leading to neuronal death.

Considering the fact that most forms of PCA have autoantibodies targeting intracellular components, the discrepancy between the direct effect of mGluR1-Ab infusion and the absence of effects in studies using different PCA autoantibodies (Graus et al., 1991; Sillevis Smitt et al., 1995) might very well be due to the accessibility of the target antigen. The only other known PCA-associated autoantibody directed against a membrane component is anti-VGCC, which targets voltage-gated calcium channels of the P/Q type, and is also associated with Lambert Eaton myasthenic syndrome (LEMS),

(Mason et al., 1997). In LEMS, the anti-VGCC autoantibodies target presynaptic VGCCs at the neuromuscular junction, interfering with neurotransmitter release (Lang and Newsom-Davis, 1995). Incubation of cultured Purkinje cells with VGCC-Ab from patients with LEMS leads to a reduction in P/Q type current (Pinto et al., 1998), but this effect is readily compensated for by upregulation of VGCCs of the R-type (Lang et al., 2003). This compensatory mechanism might account for the fact that, using a similar method as described in chapter 3, no direct effects of VGCC-Ab infusion on cerebellar function in mice can be observed in vivo (Hoebeek, personal communication). PCA with VGCC-Ab might therefore very well be due to a cellular immune reaction.

In future research of PCA pathogenesis, newly found and characterized autoantibodies directed against membrane components are candidates to be tested for potential direct effects on neuronal function. For PCA forms targeting intracellular components, future in vitro experiments will mainly focus on unraveling the immunological mechanisms leading to neuronal degeneration. Differentiating between cellular or purely humoral immunological mechanisms leading to different forms of PCA could be of great importance in the choice of immunosuppressive therapy. In order to evaluate therapeutic trials, detailed quantitative analysis of cerebellar function, for instance by recording patients' eye movements, can be of great value.

7.2 Direct effects of blocking mGluR1

7.2.1 Neuronal activity and motor performance

In vitro, mGluR1 plays a role in Purkinje neuronal excitability (Yamakawa and Hirano, 1999) and in short-term dynamic modulation of synaptic strength (Brown et al., 2003). The decreased firing rate we observed in the mouse cerebellar slices suggests that mGluR1-Ab infusion into the flocculus reduces floccular spike activity, and thereby affects the OKR and VVOR gain. Various studies confirm a correlation between floccular activity and oculomotor performance (Miles et al., 1980; Van der Steen et al., 1994; De Zeeuw et al., 1995). The fact that an artificial increase of floccular spike activity by carbachol enhances compensatory eye movement gains (Van der Steen and Tan, 1997) is in line with the hypothesis that a decrease of spike activity by mGluR1-Ab can cause a reduction of OKR/VVOR gains. Interestingly, floccular mGluR1 expression is changed upon labyrinthectomy in rats, suggesting that a change of mGluR1 expression might be used to alter Purkinje cell firing as a means for vestibular compensation (Horii et al., 2001).

The impairments of compensatory eye movements we observed in the mGluR1-Ab treated mice are similar to those in animals with a lesioned flocculus (Barmack and Pettorossi, 1985) or with a total absence of Purkinje cells (Van Alphen et al., 2002). It is unclear if a decrease in floccular simple spike frequency alone would be sufficient to mimick a reversible total shutdown of floccular output, or that blocking mGluR1 has additional effects on the Purkinje cell spike firing, for instance by affecting the modulation of simple spike frequency. In order to address this question, in vivo recordings of the effect of mGluR1-Ab on Purkinje cell simple spike modulation to optokinetic stimulation would have to be made. We should also consider the possibility

that the short-term ataxic effect results partly from effects of blocking mGluR1 at other locations, such as the CF-PC synapse or other mGluR1-expressing cell types in the cerebellar cortex (Shigemoto et al., 1992; Knöpfel and Grandes, 2002).

mGluR1-Ab are the first autoantibodies to cause PCA by directly and reversibly interfering with neuronal function, as was indicated by the reversibility of one patient's ataxia. However, the reduction of the amount of Purkinje cells in the other patient's cerebellum shows that chronic exposition causes irreversible neuronal damage, which probably contributes to the symptoms in the longer term.

7.2.2 Synaptic plasticity and motor learning

An acute block of mGluR1 has been reported to interfere with PF-LTD induction in vitro (Shigemoto et al., 1994) as well as in vivo (Gao et al., 2003). The PCA patients' mGluR1-Ab also block LTD induction and can be used in high concentrations at physiological pH levels, providing a new tool to study the link between mGluR1 activation, synaptic plasticity and cerebellar motor learning. Previous studies of the role of mGluR1 in cerebellar motor learning using mGluR1 mutant mice (Aiba et al., 1994; Conquet et al., 1994; Ichise et al., 2000) suggest that a lack of mGluR1 function leads to a block of PF-LTD and learning deficits. However these mice are reported to have developmental abnormalities (Kano et al., 1997; Levenes et al., 1997), which might have certain effects on motor learning as well. Besides, the occurrence of certain mechanisms compensating for a lifelong total absence of mGluR1 cannot be excluded either. These issues are addressed by the acute injections of mGluR1-Ab in mice in chapters 2 and 3. The acute mGluR1 block induces similar behavioral effects as seen in the mGluR1 mutant mice, thus making a direct role for mGluR1 in motor coordination more plausible.

However, studying the link between mGluR1, PF-LTD, and motor learning in these mice remains problematic. Similarly to the mGluR1 mutant mice, our mGluR1-Ab injected mice show severe signs of ataxia, which makes specifically assessing potential motor learning problems difficult. For instance, the impaired OKR and VVOR we see in mGluR1-Ab treated mice will unquestionably lead to VOR adaptation deficits, but this lack of learning cannot be attributed to a lack of synaptic plasticity. Unless the effects on cerebellar motor coordination and learning can be dissociated in some way, using an mGluR1-block to study the role of PF-LTD in motor learning is not very meaningful.

Behavioral testing of the PCA patients appeared to be more promising in this respect. When tested in a saccade adaptation paradigm, saccade performance of the PCA patient with mGluR1-Ab appeared to be within the normal range, whereas saccadic adaptation was absent. It could be argued that the increased variability of saccade amplitude interferes with saccade adaptation. However, saccade variability and adaptive capability have been reported to be dissociated after posterior vermis lesions in patients (Straube et al., 2001) and monkeys (Takagi et al., 1998; Barash et al., 1999), and healthy control subjects showed normal saccadic adaptation in a specially devised control paradigm that simulated the variation in start position of the patient's saccades. Therefore the lack of saccade adaptation seen in the PCA patient with mGluR1-Ab is

unlikely to be (solely) due to a change in saccade performance. Hence, the saccade adaptation deficit is likely at least partly due to a block of mGluR1.

This raises the question as to how the saccadic system manages to keep itself properly calibrated in the absence of saccade adaptation mechanisms. Previous experiments in humans (Straube et al., 2001) and monkeys (Takagi et al., 1998; Barash et al., 1999) have indicated that the saccadic system is capable of slow recovery of saccadic dysmetria after cerebellar cortical lesions, while still being unable to rapidly adjust saccade amplitudes in a saccade adaptation paradigm. Together, these results suggest that synaptic plasticity processes in the cerebellar cortex underlie rapid saccade recalibration, whereas in the absence of cerebellar cortical plasticity residual mechanisms are still able to keep saccades calibrated in the long term. A similar compensation mechanism has been described for the mouse VOR, in which a block of PF-LTD impairs rapid VOR adaptation, but additional form(s) of plasticity still manage to change VOR gains over days (De Zeeuw et al., 1998; Van Alphen and De Zeeuw, 2002).

The effect of an mGluR1 block on saccade adaptation adds to the body of evidence suggesting a role for PF-LTD in motor learning. However, we should still consider the possibility that the learning deficits result partly from impaired mGluR1 function at other locations than PF-PC synapses. mGluR1 has been reported to be involved in LTD of CF-PC synapses (Hansel and Linden, 2000) and is also present in low concentrations in the DCN and IO (Shigemoto et al., 1992).

7.3 Role of cerebellar plasticity in saccade adaptation

Saccade adaptation has been shown to depend on plasticity processes in the cerebellar cortex and cFN (see Robinson and Fuchs, 2001 for review). The exact nature of the processes underlying the behavioral changes has not been unraveled, but synaptic plasticity mechanisms such as PF-LTD and PF-LTP are plausible candidates to play a role in saccade adaptation. The effects of mGluR1-Ab on saccade adaptation described in chapter 3 might suggest a relation between cerebellar synaptic plasticity and saccade adaptation, but a direct link remains to be established.

In order to be able to attribute changes in behavior to neural plasticity mechanisms, analogous learning rules should be shown to apply to both neuronal and behavioral changes. The time course of the saccade amplitude changes in humans as shown in chapter 4 is largely in line with that of in vitro changes in synaptic strength, such as PF-LTD (Daniel et al., 1998). So far, however, in vitro studies have hardly focused on the very short-term (< 1 minute) changes in PF-LTD, and even less so at other sites of cerebellar plasticity. Since most other forms of cerebellar plasticity have also been described to occur within minutes, their potential contribution to saccade adaptation cannot be ruled out.

In addition, saccade adaptation is shown in chapter 4 to be an error-based process, in which saccade amplitudes are adjusted as a consequence of performance errors. PF-LTD would be well-suited to underlie such error-based learning. It is very well possible that inaccurate saccades cause CF 'performance error' signals in a similar fashion as has been reported for compensatory eye movements (Frens et al., 2001).

However, the exact information content of CF signals during (adaptation of) saccadic eye movements has not yet been elucidated.

The data shown in chapter 4 agree with a potential role of cerebellar synaptic plasticity processes in saccade adaptation. In order to directly prove such a relation, additional experiments are needed. It has been shown that although Purkinje cells in the vermis show a great variation in firing behavior, they encode saccade amplitude by a population burst response (Thier et al., 2000). In such a system, adjusting the weights of the contributions of subpopulations of Purkinje cells changes the population burst response, which leads to a change in saccade amplitude (Thier et al., 2003). Although these results indicate that Purkinje cell activity is changed during saccade adaptation, the form(s) of plasticity underlying these changes remains to be characterized. Experiments investigating a potential role of CF activity in changing the contribution of individual Purkinje cells to the population burst response could provide more information about a role for PF-LTD in saccade adaptation.

7.4 Plasticity of the PF-PC synapse

In theory, postsynaptic rather than presynaptic PF-LTP provides a perfect reversal or compensation mechanism for PF-LTD. Postsynaptic PF-LTP might work in a more non-specific manner than PF-LTD, keeping the total effect of PFs in each PC constant (Schweighofer et al., 1998), but it may also be used to actively erase memory stored by PF-LTD (Fujita, 1982). This would enable PF-LTD and PF-LTP to work in synergy, actively shaping PC output: 'incorrectly active' PFs are attenuated, whereas 'correctly active' PFs are potentiated. In Marr-Albus-Ito learning models, PF activity provides contextual information, whereas CF activity codes for errors, such as compensatory eye movement performance errors (Frens et al., 2001) or the unanticipated air puff in associative eyeblink conditioning (see Mauk, 1997; Ohyama et al., 2003 for reviews). In eyeblink conditioning for example, repeated pairing of a shock, activating CFs (the so-called unconditioned stimulus: US) and a tone, activating PFs (the conditioned stimulus: CS), is thought to induce PF-LTD and subsequent disinhibition of the interpositus nucleus by Purkinje cells. This means that tone-evoked PF-activity can then act via the interpositus nucleus to evoke conditioned blinking. Recent experiments and models of eyeblink conditioning suggest that extinction of this conditioned blinking response is not a passive process of 'forgetting', but rather an active 'unlearning' process, that is driven by inhibition of climbing fibers (Medina et al., 2002). Postsynaptic PF-LTP, favored by a decreased CF firing frequency or reduced CF synaptic strength, could very well underlie this extinction.

The results described in chapter 6 indicate that the postsynaptic Ca^{2+} concentration determines the polarity of synaptic gain change at the PF-PC synapse: high Ca^{2+} leads to PF-LTD, whereas low or no Ca^{2+} influx leads to PF-LTP. Similar Ca^{2+} threshold mechanisms controlling LTD vs. LTP induction have been studied extensively at excitatory synapses onto hippocampal and neocortical pyramidal cells. However, at these synapses there is a higher Ca^{2+} threshold for LTP than for LTD induction (Bienenstock et al., 1982; Bear et al., 1987; Lisman, 1989). This model, the so-called Bienenstock, Cooper and Munro (BCM) model, was confirmed by experiments in which

postsynaptic Ca^{2+} transients were monitored using fluorescence imaging techniques (Hansel et al., 1997; Yang et al., 1999; Cormier et al., 2003). In addition, decreasing postsynaptic Ca^{2+} concentrations by limiting Ca^{2+} influx (Cummings et al., 1996) or buffering Ca^{2+} transients (Hansel et al., 1997) increased the probability of induction of LTD vs. LTP. Experiments in which postsynaptic Ca^{2+} was released by photolysis further indicated that in hippocampal pyramidal cells LTP brief, large Ca^{2+} transients induce LTP, whereas prolonged, lower amplitude Ca^{2+} signals rather induce LTD (Yang et al., 1999; Zucker, 1999). Under more physiological conditions, a large Ca^{2+} transient and subsequent LTP occur when a synaptic event shortly precedes the occurrence of a dendritic, backpropagating spike, whereas the Ca^{2+} is much smaller when the synaptic event occurs too early, or follows the spike, resulting in LTD (see Linden, 1999 for review). Similarly, in the cerebellar cortex, PF+CF coactivation leads to a supralinear Ca^{2+} response (Wang et al., 2000). Postsynaptic application of Ca^{2+} buffers inhibits PF-LTD induction, and at sufficiently high concentrations even favors induction of PF-LTP (Sakurai, 1990; Konnerth et al., 1992; Shibuki and Okada, 1992; chapter 6 of this thesis). A physiological way to reduce the size of the CF-mediated Ca^{2+} transient is CF-LTD (Weber et al., 2003), which renders subsequent PF+CF coactivation unable to induce PF-LTD. On the other hand, combining PF activity with an artificial Ca^{2+} rise induced by uncaging Ca^{2+} leads to PF-LTD. It should be noted that merely altering postsynaptic Ca^{2+} is not enough to induce a change of PF-PC synaptic strength: both PF-LTD and PF-LTP induction need concurrent PF activation as well.

Although the molecular mechanisms leading to PF-LTD induction have been largely elucidated, much less is known about the molecular events underlying the recently described postsynaptic PF-LTP. This PF-LTP has been reported to be dependent on NO, but not cAMP or cGMP, and is enhanced by chelating postsynaptic Ca^{2+} (Lev-Ram et al., 2002). This indicates that PF-LTP uses different expression pathways from those leading to presynaptic PF-LTP, which is cAMP-dependent (Salin et al., 1996), and also from PF-LTD, in which NO reportedly exerts its effect through cGMP elevation (see Daniel et al., 1998; Bear and Linden, 2000 for reviews). Further details about the effector mechanisms of induction and expression of postsynaptic PF-LTP remain to be characterized.

PF-LTP is induced by activation of the PF alone, and PF-LTD results from coactivation of PF and CF, which raises the point of the timing constraints of PF+CF coactivation. In a system of constant PF and CF activity, when exactly does the PF contribute to potentiation, and when to depression? In other words: how wide can the time window of PF-CF coactivity be in order to still induce PF-LTD, and when are PF and CF activity dissociated enough in order to induce PF-LTP? Several studies have addressed this issue by changing the interval between CF and PF activation. However, various experimental approaches to this question have led to different results. PF-LTD has been reported to be induced optimally with PF activity preceding (Chen and Thompson, 1995), following (Ekerot and Kano, 1985; 1989), or being simultaneous (Ito and Kano, 1982; Ito, 1984; Sakurai, 1987; Hirano, 1990; Linden et al., 1991) with CF activity. This somewhat confusing variation in results can largely be attributed to differences in stimulus parameters, preparation and/or environmental conditions (see Bear and Linden, 2000; Ito, 2001 for reviews). Since studying the timing constraints of PF-CF coactivation is especially interesting from a behavioral perspective, in vitro

experiments focusing on this issue should aim to mimick the natural situation as realistically as possible. However, it is presently still quite unclear what patterns of artificial stimulation would best mimick in vivo PF and CF inputs under different behavioral conditions.

Postsynaptic PF-LTP provides yet another form of plasticity to the cerebellar circuitry, which in recent years has been shown to contain a rich variety of plasticity processes other than the 'classical' PF-LTD (see Hansel et al., 2001 for review). An example of how these various forms of plasticity might interact is given in chapter 6, where CF-LTD is shown to affect PF-LTD induction. Although its in vivo functional relevance still has to be examined, CF-LTD could in fact very well be another fine-tuning mechanism for cerebellar motor learning. Continuous CF activity might make subsequent PF-CF association less behaviorally relevant, and could therefore make a block of PF-LTD induction desirable. CF-LTD might also affect other cellular processes that occur upon CF-mediated Ca^{2+} influx, such as modulation of dendritic excitability through effects on ion channels, or heterosynaptic processes such as short-term potentiation of mGluR-mediated responses at PF-PC synapses (Batchelor and Garthwaite, 1997) or rebound potentiation of inhibitory interneuron-Purkinje cell synapses (Kano et al., 1992). In addition, CF-LTD might also function as a neuroprotective mechanism to delimit Ca^{2+}-triggered neurodegeneration.

The notion emerges that the cerebellar network uses multiple mechanisms to calibrate its output. More experiments will be needed to increase our understanding of the molecular mechanisms underlying PF-LTP, CF-LTD, and the various other forms of cerebellar plasticity, of their relative interactions, and of their potential role in motor learning.

The (patho)physiology of cerebellar function has been widely studied for decades. In recent years, much progress has been made in elucidating the mechanisms underlying the different aspects of cerebellar motor coordination and adaptation, as well as some of the disease processes affecting these. However, much has not yet been fully elucidated. Only an integrated approach of various disciplines, such as molecular biology, genetics, neurophysiology, immunology, pathology, and behavioral studies, will in the end enable us to tackle the major questions involving cerebellar function and its diseases.

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LIST OF ABBREVIATIONS

ACSF	artificial cerebrospinal fluid
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AOS	accessory optic system
BAPTA	bis-(o-aminophenoxy)-N,N,N',N'-tetraacetic acid
cAMP	cyclic adenosine monophosphate
CF	climbing fiber
CFC	climbing fiber collateral
CF-LTD	long-term depression of the climbing fiber – Purkinje cell synapse
cFN	caudal fastigial nucleus
cGMP	cyclic guanosine monophosphate
CHO	Chinese hamster oocyte
DAG	diacylglycerol
DCN	deep cerebellar nuclei
DHPG	(RS)-3,5-dihydroxyphenylglycine
DMNP-EDTA	1-(4,5-dimethoxy-2-nitrophenyl)-1,2-diaminoethane-N,N,N',N'-tetraacetic acid
EGTA	ethylene glycol-bis(beta-amino-ethyl ether)-N,N,N',N'-tetraacetic acid
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
FTN	floccular target neuron
γ-DGG	γ-D-glutamylglycine
GC	guanylate cyclase
GRIP	glutamate receptor interacting protein
HEPES	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
IgG	immunoglobulin type G
IO	inferior olive
IP_3	inositol trisphosphate
ISI	inter stimulus interval
LEMS	Lambert-Eaton myasthenic syndrome
LTD	long-term depression
LTP	long-term potentiation
MF	mossy fiber
MG	myasthenia gravis
mGluR1	metabotropic glutamate receptor type 1
mGluR1-Ab	anti-mGluR1 autoantibody
mGluR5	metabotropic glutamate receptor type 5
NO	nitric oxide
NRTP	nucleus reticularis tegmentum pontis
OKR	optokinetic reflex
OMN	oculomotor nuclei
PC	Purkinje cell
PCA	paraneoplastic cerebellar ataxia
PDZ	PSD95/SAP-90, Discs-large, ZO-1 homologous domain

PF	parallel fiber
PF-LTD	long-term depression of the parallel fiber-Purkinje cell synapse
PF-LTP	long-term potentiation of the parallel fiber-Purkinje cell synapse
PICK	protein interacting with C-kinase
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PLC	phospholipase C
PPF	paired-pulse facilitation
RF	brainstem reticular formation
SC	superior colliculus
VGCC	voltage-gated calcium channel
VGCC-Ab	anti-voltage-gated calcium channel autoantibody
VN	vestibular nuclei
VOR	vestibulo-ocular reflex
VVOR	visually enhanced vestibulo-ocular reflex

SUMMARY

The cerebellum helps fine-tuning movements by evaluating disparities between intention and action, in order to adjust the execution of movements 'online', and to keep movements calibrated in the long term. The cerebellar capacity to store information, which provides the 'memory' needed for the recalibration of movements, the learning of new motor skills, and associative learning, is provided by modifications in the strength of synaptic couplings between neurons in the cerebellar circuitry ('synaptic plasticity').

Cerebellar coordination and motor learning can be affected by degenerative processes, such as paraneoplastic cerebellar ataxia (PCA). This is a severe side effect of certain forms of cancer, usually characterized by the degeneration of Purkinje cells, which provide the sole output of the cerebellar cortex. PCA is associated with the expression of antineuronal autoantibodies. In chapters 2 and 3 we describe two patients with Hodgkin's disease and PCA associated with a previously undescribed autoantibody against the metabotropic glutamate receptor type 1 (mGluR1). This autoantibody directly interferes with receptor function in vitro, affecting synaptic transmission and spontaneous Purkinje neuronal firing behavior, as well as inhibiting the induction of long-term depression of the parallel fiber to Purkinje cell synapse (PF-LTD), a form of synaptic plasticity widely associated with motor learning. Infusion of these anti-mGluR1 autoantibodies into the cerebellum of mice causes severe, reversible ataxia, indicating that PCA autoantibodies can directly affect Purkinje neuronal function by blocking receptors. Post-mortem analysis of one mGluR1-PCA patient's cerebellum reveals a reduction in the number of Purkinje cells after chronic exposition to anti-mGluR1 autoantibodies. Together these results indicate that the anti-mGluR1 autoantibodies can cause ataxia by acutely interfering with neuronal function and synaptic plasticity, as well as through a chronic degenerative effect on cerebellar Purkinje cells.

The block of PF-LTD by the anti-mGluR1 autoantibodies was also shown to affect the patients' ability to recalibrate motor output. Cerebellar motor learning in the patients was assessed using a saccade adaptation paradigm, in which the amplitude of voluntary fast eye movements ('saccades') is gradually changed by systematically displacing a target during a series of consecutive saccades. Although their saccade performance was within the normal range, the capability to gradually adapt saccade amplitude was impaired in the patients with anti-mGluR1 autoantibodies, adding to the body of evidence that PF-LTD underlies forms of motor learning. In chapter 4, further analysis of saccade adaptation characteristics in humans indicates that this form of motor learning conforms to learning rules similar to those of cerebellar synaptic plasticity processes. The time course of induction and the error-based character of saccade adaptation are in line with the properties of cerebellar synaptic plasticity forms, such as PF-LTD.

In chapters 5 and 6, the cellular mechanisms putatively underlying cerebellar motor learning were further explored by studying synaptic plasticity in vitro. PF-LTD can be induced by coactivation of parallel fiber (PF) and climbing fiber (CF) input at low frequencies, and is expressed as a reduction in AMPA glutamate receptors on the postsynaptic membrane. This selectively decreases the effect of glutamate released by the PFs that were concurrently active with the CF. In order to prevent synapse saturation and to allow reversal of motor learning, this reduction of PF-Purkinje cell synaptic

strength must be counterbalanced by a form of potentiation that is also expressed postsynaptically. This modification, called long-term potentiation of the PF – Purkinje cell synapse (PF-LTP), can be induced by tetanizing only the PF at low frequencies. CF-evoked calcium transients into the Purkinje cell are shown to be the polarity switch factor making the difference between PF-LTD and PF-LTP induction. Long-term depression of synaptic strength can also be induced at the CF – Purkinje cell synapse (CF-LTD), by high-frequent CF activity. CF-LTD reduces the amplitude of the CF-evoked calcium transient, which is shown to inhibit the induction of PF-LTD. The concept arises that the cerebellar circuitry uses multiple interacting mechanisms to calibrate its output.

SAMENVATTING

Het cerebellum is betrokken bij de coördinatie en de calibratie van bewegingen. Door voortdurend te evalueren of gemaakte bewegingen wel worden uitgevoerd zoals ze waren bedoeld, is het cerebellum in staat bewegingen 'bij te schaven'. Dit gebeurt door een fijnregulatie van de 'ruwe' motorische signalen vanuit de grote hersenen en hersenstam, zowel tijdens het maken van een beweging als bij herhalingen van dezelfde beweging. Het cerebellum speelt ook een rol in het aanleren van nieuwe bewegingen en bewegingspatronen. Dit 'motorisch leergedrag' zorgt ervoor dat we onze bewegingen snel kunnen aanpassen in nieuwe of veranderde situaties.

De geheugenopslag die nodig is voor dit motorisch leren wordt verondersteld plaats te vinden door het versterken of verzwakken van de verbindingen tussen neuronen, de zogenoemde synapsen. De biochemische processen die de informatieoverdracht door de synaps kunnen veranderen worden samengevat onder de noemer 'synaptische plasticiteit'. In het cerebellum is synaptische plasticiteit beschreven in diverse soorten synapsen, waarvan de mogelijke rol in motorisch leren niet altijd even duidelijk is. Echter, de synapsen tussen de granulaire cellen en de Purkinjecellen in de cerebellaire schors worden op grond van hun vuurgedrag en hun plaats in het neuronale netwerk algemeen verondersteld een cruciale rol te spelen in vormen van motorisch leren. Ongeveer 100.000 granulaire cellen maken via hun parallelvezels synaptisch contact met één enkele Purkinjecel. De informatie uit deze granulaire cellen wordt geïntegreerd in het vuurgedrag van de Purkinjecellen, dat de enige output van de cerebellaire schors vormt. Behalve van de parallelvezels ontvangt een Purkinjecel ook informatie van één klimvezel, die signalen doorgeeft vanuit een neuron in de oliva inferior, een kern in de hersenstam. De meest gangbare hypothese stelt dat de klimvezel foutmeldingen doorgeeft aan de Purkinjecel. Het gevolg van deze foutmelding is dat specifiek die parallelvezels die op het moment van de foutmelding ook actief waren, langdurig worden verzwakt. Dit proces, 'long-term depression' van de parallelvezel-Purkinjecel synaps (PF-LTD) genoemd, kan experimenteel worden opgewekt door de parallelvezels en de klimvezel gelijktijdig elektrisch te stimuleren. Inductie van PF-LTD heeft op korte termijn tot gevolg dat de parallelvezels die actief zijn bij correct uitgevoerde bewegingen relatief worden versterkt, terwijl parallelvezels die bijdroegen aan de 'foute' beweging worden verzwakt. Op gedragsniveau leiden deze aanpassingen in synapssterkte tot een verbetering van de uitvoering van de beweging.

Schade aan het cerebellum, bijvoorbeeld door letsel of degeneratie, veroorzaakt duidelijke symptomen, die wel worden samengevat als 'ataxie': het voorbereiden, in gang zetten en uitvoeren van bewegingen is aangedaan. Degeneratie van het cerebellum kan optreden als een zeldzaam bijverschijnsel bij bepaalde soorten kanker. In deze vorm van cerebellaire degeneratie, die paraneoplastische cerebellaire ataxie (PCA) wordt genoemd, maakt het immuunsysteem van de patiënt antilichamen die zijn gericht tegen componenten van neuronen. Dit wordt hoogstwaarschijnlijk veroorzaakt doordat de kankercellen eiwitten tot expressie brengen die normaliter alleen voorkomen in neuronen. Het immuunsysteem van de patiënt 'herkent' deze eiwitten in combinatie met de kankercellen vervolgens als lichaamsvreemd, waarop een immuunreactie in werking wordt gezet. De auto-antilichamen worden verondersteld de ataxie te veroorzaken, maar

hun exacte rol in het ziekteproces is niet geheel duidelijk. In de hoofdstukken 2 en 3 van dit proefschrift wordt een vorm van PCA beschreven met een niet eerder beschreven soort auto-antilichamen. Deze auto-antilichamen zijn gericht tegen een type glutamaat-receptor die voorkomt op de celmembraan rond synapsen van parallel- en klimvezels met Purkinjecellen: de metabotrope glutamaatreceptor type 1 (mGluR1). Deze receptor is zowel betrokken bij de synaptische prikkeloverdracht als bij synaptische plasticiteit. In gekweekte Purkinjecellen en in plakken van het cerebellum van ratten blijken de auto-antilichamen van de PCA-patiënten mGluR1 functioneel te blokkeren. Op cellulair niveau heeft dit directe gevolgen: de spontane vuurfrequentie van actiepotentialen door de Purkinjecellen wordt lager én de inductie van PF-LTD wordt geblokkeerd.

Om te onderzoeken of deze effecten op de synaptische transmissie en plasticiteit de onderliggende oorzaak kunnen zijn van de symptomen die we zien in de patiënten, werden de mogelijke effecten van de auto-antilichamen op het gedrag van muizen bestudeerd. Wanneer de anti-mGluR1 auto-antilichamen (mGluR1-Ab) worden ingespoten in de cerebrospinale vloeistof van muizen treedt er vrijwel direct een ernstige ataxie op, die vervolgens langzaam wegtrekt. Dit geeft aan dat de mGluR1-Ab direct ataxie kunnen veroorzaken, maar niet direct onomkeerbare schade aan het cerebellum aanrichten. Dit wordt bevestigd door microscopisch-anatomische analyse van het cerebellum van deze muizen, dat geen Purkinjecel-degeneratie laat zien. Post-mortem analyse van het cerebellum van een PCA-patiënt met mGluR1-Ab laat echter zien dat het aantal Purkinjecellen drastisch is afgenomen, wat aangeeft dat chronische blootstelling aan de mGluR1-Ab op langere termijn wel kan leiden tot celdegeneratie.

De effecten op de coördinatie en het leergedrag van bewegingen werden meer gedetailleerd bestudeerd met behulp van oogbewegings-metingen, in zowel muizen als in de patiënten. Oogbewegingen zijn relatief simpel en goed quantificeerbaar, waardoor ze uitermate geschikt zijn om neurologische processen die ten grondslag liggen aan de sturing van bewegingen te bestuderen. In muizen werden de compensatoire oogbewegingen gemeten. Dit zijn reflexmatige oogbewegingen, die ervoor zorgen dat het beeld op het netvlies zo stabiel mogelijk wordt gehouden, zodat het zicht zo scherp mogelijk blijft. Compensatoire oogbewegingen treden op als reflex op beweging van het visuele veld (de optokinetische reflex: OKR) en op basis van informatie uit het evenwichtsorgaan over beweging van het hoofd (de vestibulo-oculaire reflex: VOR). In praktijk werken deze twee compensatoire reflexen voortdurend samen. Aangezien het cerebellum gebruik maakt van visuele feedback om de compensatoire oogbewegingen accuraat te houden, kan de OKR voortdurend ‘online’ worden aangepast, terwijl de VOR afhankelijk is van visuele input om de vestibulaire respons te calibreren.

PF-LTD wordt verondersteld (mede) verantwoordelijk te zijn voor de informatieopslag in de cerebellaire schors, die nodig is voor de recalibratie van de VOR. In theorie zou het experimenteel blokkeren van mGluR1 dus gevolgen kunnen hebben voor zowel de coördinatie als de adaptatie van compensatoire oogbewegingen. Wanneer het deel van het cerebellum dat verantwoordelijk is voor de coördinatie van compensatoire oogbewegingen, de flocculus, voortdurend wordt blootgesteld aan mGluR1-Ab, leidt dit in muizen tot duidelijke problemen in de coördinatie van de compensatoire oogbewegingen. Deze directe effecten kunnen hoogstwaarschijnlijk worden verklaard door een aantasting van de synaptische prikkeloverdracht. De inadequate OKR zal echter direct gevolgen hebben voor het recalibreren van de VOR,

zodat het op grond van dit experiment niet goed mogelijk is om uitspraken te doen over een directe rol van mGluR1-gemedieerde synaptische plasticiteit in motorisch leren.

Cerebellair motorisch leergedrag kan in mensen snel en nauwkeurig worden beoordeeld door het adapteren van snelle, doelgerichte oogbewegingen, ofwel saccades. De amplitude van saccades kan worden aangepast door de proefpersoon een saccade te laten maken naar een doel, dat tijdens het maken van de saccade verplaatst wordt. Dit geeft de proefpersoon onbewust de indruk dat de zojuist gemaakte saccade incorrect was. Wanneer dit consequent wordt herhaald, zal het cerebellum de amplitude van de saccade geleidelijk aanpassen. Aangezien in de PCA-patiënten met mGluR1-Ab de saccades zelf normaal zijn, kan een eventuele invloed van de mGluR1-Ab op het cerebellair motorisch leergedrag getest worden zonder dat een gestoorde coördinatie van de saccades het leerproces beïnvloedt. In een patiënt met mGluR1-Ab bleek het niet mogelijk om saccade-adaptatie op te wekken, wat aangeeft dat de mGluR1-Ab specifiek het cerebellair motorisch leergedrag van saccades blokkeren. Een andere patiënt, die na behandeling niet langer mGluR1-Ab in haar bloed en cerebrospinale vloeistof had, vertoonde daarentegen een volledig normale saccade-adaptatie. Dit toont aan dat de effecten van de mGluR1-Ab ook in de PCA-patiënten aanvankelijk een reversibel karakter hebben en bij een tijdige succesvolle behandeling kunnen verdwijnen.

Hoewel algemeen wordt aangenomen dat saccade-adaptatie wordt veroorzaakt door aanpassingen in het cerebellum, zijn de exacte onderliggende processen nog niet bekend. In hoofdstuk 4 worden een aantal karakteristieken van saccade-adaptatie nader bestudeerd. Door saccade-adaptatie op te wekken met intervallen met verschillende duur tussen de foutieve saccades, kunnen we zien dat het een aantal seconden duurt voordat informatie over een foutieve saccade optimaal is verwerkt. Verder blijken slechts de foutieve saccades bij te dragen aan het leerproces. Wanneer de saccades reeds maximaal zijn geadapteerd, leidt een verdere bevestiging van de geadapteerde staat door correcte saccades niet tot een toename van de sterkte van de adaptatie. Deze beide eigenschappen, zowel het tijdsbeloop van de saccade-adaptatie als het feit dat de adaptatie uitsluitend wordt veroorzaakt door ‘foutsignalen’, zijn vergelijkbaar met de karakteristieken van cerebellaire synaptische plasticiteits-mechanismen zoals PF-LTD. Hoewel deze experimenten geen direct verband aantonen tussen cerebellaire synaptische plasticiteit en saccade-adaptatie, lijken deze resultaten, in combinatie met het feit dat mGluR1-Ab saccade-adaptatie blokkeren, toch te suggereren dat synaptische plasticiteits-mechanismen als PF-LTD het cerebellum in staat stellen saccades te recalibreren.

De PF-Purkinjecel synaps gebruikt glutamaat als neurotransmitter. Bij PF-activiteit wordt er ‘presynaptisch’ glutamaat vrijgemaakt uit de PF, wat ‘postsynaptisch’ AMPA-receptoren op de Purkinjecel activeert. Activatie van deze AMPA-receptoren zorgt ervoor dat er door het openen van ionkanaaltjes een elektrisch signaal wordt opgewekt in de Purkinjecel. De moleculaire basis van PF-LTD is een verlaging van de hoeveelheid AMPA-receptoren op de Purkinje-celmembraan, waardoor de Purkinjecel ongevoeliger wordt voor het glutamaat uit de PF. Om verzadiging van synaptische plasticiteit te voorkomen (wanneer alle AMPA-receptoren worden weggehaald uit de synaps zou er niets meer aangepast kunnen worden) en om veranderingen desgewenst weer ongedaan te maken, is het belangrijk dat PF-LTD kan worden gecompenseerd door een

tegengesteld mechanisme. De PF-Purkinjecel synaps kan daarom niet alleen worden verzwakt, maar ook worden versterkt: long-term potentiation, ofwel PF-LTP. Een eerder beschreven vorm van PF-LTP zorgt voor een hogere glutamaat-release bij PF-activatie. Hoewel deze 'presynaptische' vorm van PF-LTP tot op zekere hoogte kan compenseren voor de 'postsynaptische' PF-LTD, zou de synaps op een zeker moment toch verzadigd raken: een maximale hoeveelheid glutamaat wordt vrijgemaakt, terwijl er (vrijwel) geen AMPA-receptoren meer over zijn om te activeren. Logischerwijze zou er dus ook een vorm van PF-LTP moeten bestaan die ervoor zorgt dat de synaps ook postsynaptisch kan worden versterkt. In hoofdstuk 6 wordt een dergelijk proces beschreven.

Opvallend aan deze vorm van PF-LTP is dat hij kan worden opgewekt door een laagfrequente stimulatie van de PF, terwijl een dergelijke laagfrequente stimulatie van zowel PF als de klimvezel (climbing fiber: CF) leidt tot inductie van PF-LTD. Blijkbaar is de aan- of afwezigheid van CF-activiteit de factor die bepaalt of er PF-LTD of PF-LTP wordt opgewekt. CF-activatie leidt tot een sterke depolarisatie van de Purkinje-celmembraan, die ervoor zorgt dat een grote hoeveelheid calcium-ionen (Ca^{2+}) de cel instroomt. Wanneer deze CF-gemedieerde toename van de Ca^{2+}-concentratie wordt gebufferd, treedt er bij gelijktijdige PF+CF activatie geen PF-LTD op, maar PF-LTP. Aan de andere kant leidt een kunstmatige verhoging van de Ca^{2+}-concentratie in de Purkinjecel tijdens PF-activatie niet tot inductie van PF-LTP, maar van PF-LTD. Kortom: wanneer de PF gestimuleerd wordt, bepaalt de Ca^{2+}-concentratie in de Purkinjecel of er PF-LTD of PF-LTP wordt opgewekt. Recent is gebleken dat ook de CF een vorm van LTD kan vertonen. Wanneer CF-LTD is opgewekt, stroomt veel minder Ca^{2+} de Purkinjecel in bij CF-activatie. In een laatste experiment wordt aangetoond dat deze afname van de Ca^{2+}-instroom voldoende is om de inductie van PF-LTD te blokkeren. Uit dit experiment blijkt dat verschillende vormen van synaptische plasticiteit elkaar sterk kunnen beïnvloeden. Het beeld ontstaat dat het cerebellum een scala aan plasticiteitsmechanismen gebruikt, die elkaar beïnvloeden en samenwerken om zo de output optimaal te calibreren.

In dit proefschrift wordt met een multidisciplinaire benadering geprobeerd op verschillende niveaus inzicht te krijgen in cerebellaire (patho)fysiologie. Een uitgebreid scala aan technieken wordt gebruikt om de mechanismen te analyseren die het cerebellum in staat stellen bewegingen te coördineren en te calibreren, en om te bestuderen wat de gevolgen kunnen zijn wanneer deze mechanismen niet goed functioneren.

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CURRICULUM VITAE

The author of this work was born in Waalwijk on November 6 1974. After elementary school he attended gymnasium at the Dr Moller College in Waalwijk from 1987-1991 and at BC Schöndeln in Roermond from 1991-1993, from which he graduated cum laude. In September 1993 he started his medical studies at the Erasmus University in Rotterdam. After fours years of medical school, a research project at the department of anatomy drew his interest toward neuroscience. He spent one year in the laboratory of Dr De Zeeuw, working with Bas Koekkoek and Arjan van Alphen on eye movements in mutant mice. After this year he spent 6 months in the laboratory of Prof Dr Simpson at New York University, investigating the correlation between the firing behavior of Purkinje cells in the rabbit flocculus and various kinds of eye movements. In September 1998 he returned to Rotterdam to receive his 'Geneeskunde doctoraal diploma' from the Erasmus University. The author suspended his medical training and accepted a PhD position in the 'neurobreedtestrategie' project, working in the departments of anatomy, physiology and neuro-oncology of the Erasmus MC, investigating a rare form of cerebellar ataxia, under the leadership of Prof Dr De Zeeuw, Dr Frens and Dr Sillevis Smitt. The departments of anatomy and physiology later merged into the department of neuroscience, where the author continued his investigations of the cerebellum in the laboratories of Prof Dr De Zeeuw, Dr Frens and Dr Hansel. On May 1st 2004 he will complete his research and return to his medical studies at the Erasmus MC.

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