# The influence of protein kinases and microtubule binding proteins on cerebellar motor learning

# De invloed van eiwit kinases en microtubuli bindende eiwitten op motorische leerprocessen van het cerebellum

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# Influence of Protein Kinases and Microtubule Binding Proteins in Cerebellar Motor Learning

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# Contents

# Chapter I

# Introduction

# 1. The cerebellum

## 1.1. Anatomy of the cerebellum

The cerebellum is divided into two major symmetrical lateral extensions, the cerebellar hemispheres, which are separated by a medial "worm like" tubular shaped structure called the vermis (Bourret and Louis, 1986). Major fissures in the cortex define the anterior, posterior and flocculo-nodular lobes, which are further subdivided into lobules (Sarna and Hawkes, 2003). Functionally, the cerebellum is divided into the medial (the vermis and its projections), intermediate (the paravermal areas), lateral

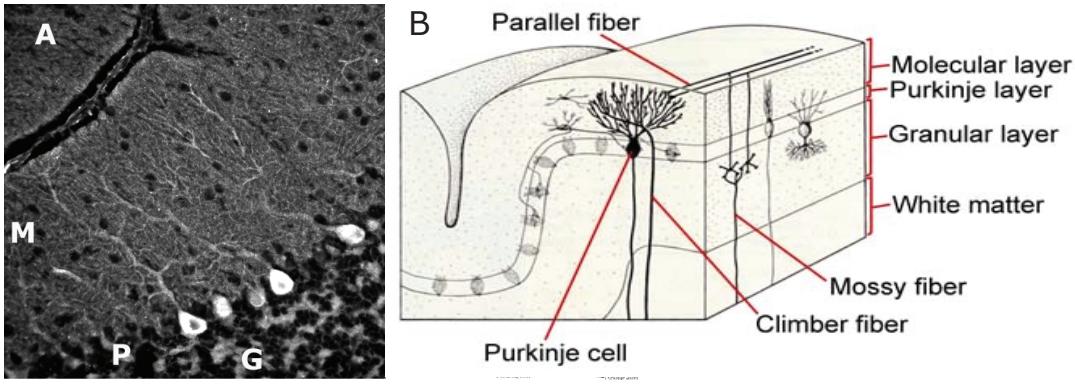



Figure 1 – Cerebellar cortex: A) cerebellar slice stained with an anti CLIP-115 antibody showing the layer organization of the cortex. The outermost region is the molecular layer (M) occupied by the dendritic trees of Purkinje cells, whose cells bodies are found in regular alignment in the Purkinje cell layer (P). The innermost granule cell layer (G) contains the cell bodies of granule cells, here seen as dark rounded structures (Photo courtesy of Dr C.C. Hoogenraad).
B) An illustration of the cortex layers with the most prominent connections to the Purkinje cells, such as the parallel fibers, the climbing fibers, Golgi and, stellate and basket cells
(http://www.astralgia.com/webportfolio/omnimoment/live_science/purkwrld/index.html)

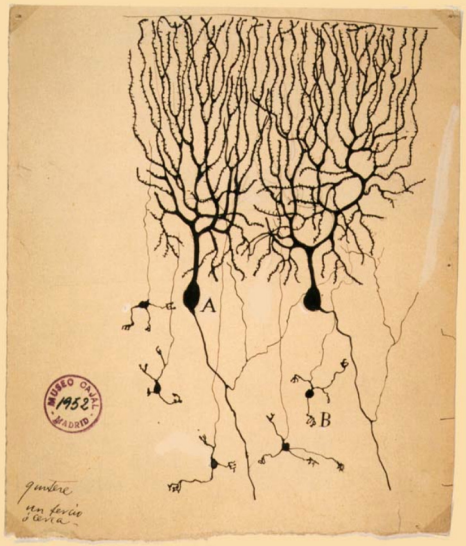
(the outermost part of the hemispheres) and flocculo-nodular lobe (Ito, 1984).

The entire cerebellar cortex is organized in three layers (depicted in Figure 1), called the molecular layer, the Purkinje cell (PC) layer and the granule cell layer. The outermost layer of the cerebellum, the molecular layer, is made up of the axons of granule cells, which contact the dendrites of PCs; big inhibitory neurons that use γ-amino-butyric-acid (GABA) as neurotransmitter (depicted in Figure 2). The molecular layer also contains inhibitory interneurons (basket and stellate cells). The second layer is the PC layer. It contains the cell bodies of the PCs, aligned in a single layer following the involutions of the folia. The third and innermost cortical layer is the granule cell layer, which consists mostly of granule cells. Various types of neurons are present in the cerebellar cortex, including granule cells, Golgi cells, basket and

stellate cells and PCs, among others (Ito, 2001, Bower, 2002).

Within the cerebellum are the deep nuclei that receive projections from other brain regions (e.g. the pontine and olivary nuclei) as well as from the cerebellar cortex. For example, PCs project onto the deep cerebellar nuclei and brain stem vestibular nuclei, exerting an inhibitory signal (Sekirnjak et al., 2003). The deep nuclei in turn send projections, among others, to the red nucleus, the premotor nuclei and the inferior olive (Ito, 1984).

PCs, which are characterized by their intricate dendritic trees, form the sole output of the cerebellar cortex (Ito, 1989, 2001). They are controlled by two major inputs. First, the axons of granule cells form parallel fibers, which bifurcate in the molecular layer, extending up to 3 mm in either direction, parallel to the long axis of the folia (Ito, 1984, 2001). Parallel fibers (PFs) have an excitatory synaptic effect on Purkinje cells, releasing glutamate and nitric oxide (NO) as neurotransmitters (Ito, 2001). They relay information coming from mossy fibers (Figure 3) onto PCs. Mossy fibers originate from pontine nuclei and other precerebellar nuclei like the reticular, spinal and trigeminal nuclei (Ito, 1984, Pijpers et al., 2006, Lalonde and Strazielle, 2007a). Sensori-motor information is also relayed by mossy fibers to the deep cerebellar nuclei (Medina et al., 2001, Ohyama et al., 2002) (Figure 3).




"Pigeon cerebellum. A, Purkinje cell, B, granule cell Modified from a photograph taken from the original (14X15.5 cm). Drawn on sheet/paper. P.Y. 1899. S.R. y Cajal Institute - CSIC - Madrid, Spain.

Figure 2- reproduction of an Original drawing from Santiago Ramon-y-Cajal depicting pigeon cerebellar Purkinje cells http://www.psu.edu/nasa/images/cajal8.jpg

The second major input on PCs comes from the inferior olive, which is located in the brainstem at the level of the pons, sends powerful excitatory projections to the PCs via so-called climbing fibers (De Zeeuw et al., 1998b). Vice versa, PCs contact the inferior olive, albeit indirectly, via the red nucleus and the motor nuclei (Bagnall and du Lac, 2006).

## 1.2. Organization of the cerebellum

The output of the cerebellar cortex is organized in parasagittal zones (Voogd and Glickstein, 1998) that strictly correlate with the cerebellar nuclei. PCs of a particular parasagittal zone all project to a specific cerebellar or vestibular nucleus. Furthermore, climbing fibers (CFs) of a particular olivary subnucleus

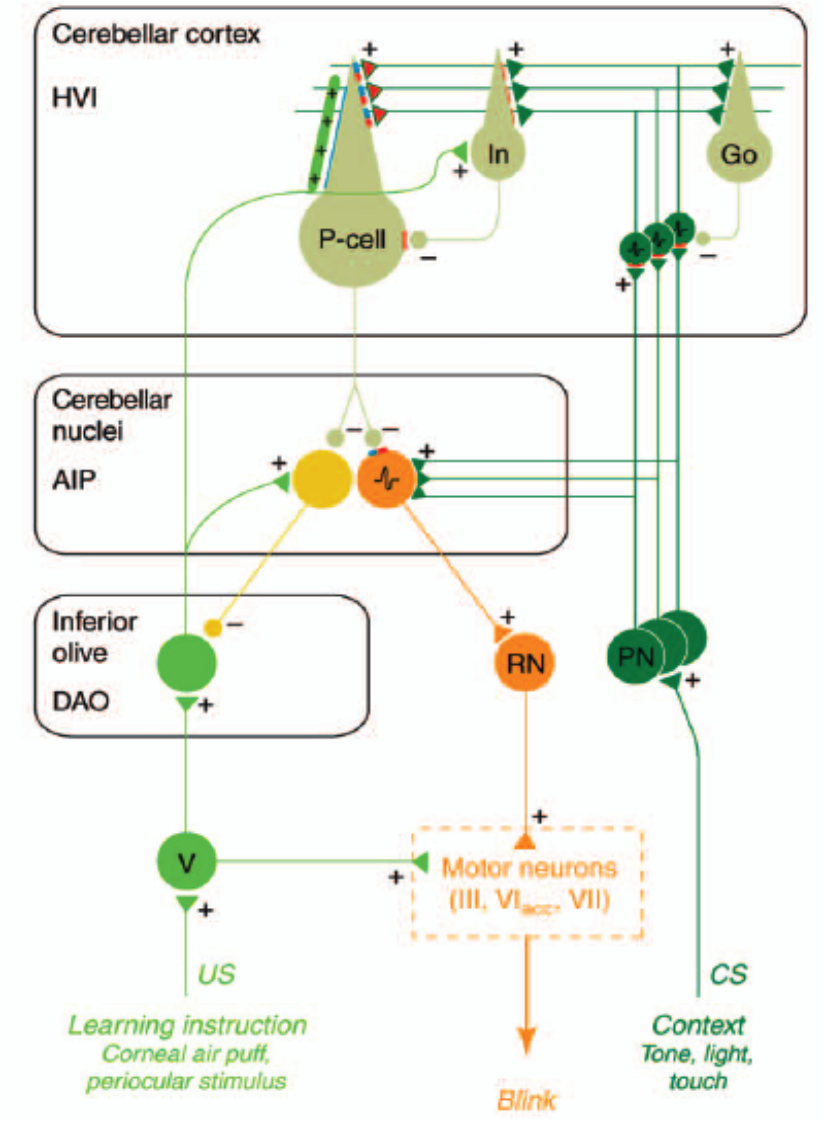



Figure 3– cerebellar circuitry involved in eye-blink conditioning (from deZeeuw and Yeo, 2005). Information about the air puff is conveyed via the VIIth cranial nerve reaches the inferior olive and is conveyed to the cerebellar cortex via climbing fibers; these have strong excitatory inputs onto Purkinje Cells (PC) but also onto the cells of the deep cerebellar nucleus, like the anterior interpositus (AIP). Information about the sound and other cortical information is conveyed to the pontine nuclei (PN) where in the form of mossy fibers it is relayed also the cortex and the nuclear cells. Mossy fibers relay information to granule cells (Go) that form parallel fibers and discharge also onto PCs, Golgi cells and Basket cells, and stellate cells are inhibitory neurons (In). PCs integrate the two signals and send inhibitory signals to the deep cerebellar nuclei and indirectly to the same olivary neurons that project to them. The AIN influences the Red Nucleus (RN) to effect on the motor neurons therefore originating a blink. Blinks are automatically originated to nociceptive stimuli conveyed to the motor neurons. The cortical area where stimuli related to the eye-blink converge is called HVI, located in the lobule simplex.

project to a single PC zone or to a pair of zones that share the same target nucleus. These target nuclei in turn provide an inhibitory projection to the corresponding olivary cells. Zones that receive peripheral input have been demonstrated to show a somatotopical CF microzonation (Andersson and Oscarsson, 1978, Ekerot et al., 1991, Garwicz et al., 1992).

This microzonation divides the cerebellum into modules, each having its own connectiona with the inferior olive. Each microzone, or ensemble of microzones, controls a set of muscles related to a specific function, and the CFs convey signals about particular aspects of activities of this set of muscles (Garwicz et al., 1998). The organization of corticonuclear projections of the intermediate cerebellum in cats is compatible with this hypothesis (Ekerot et al., 1991). Such a scheme would also be compatible with hypotheses on the potential roles of the cerebellar circuitry in eye-blink conditioning and locomotion, subjects that are dealt with below.

## 2. Cerebellar motor learning

### 2.1. Motor learning and memory

Learning is defined as the acquisition of information or knowledge that allows the subject to respond appropriately to an environmental stimulus, or to a sequence of events, from the perception of a stimulus to its entrance into long-term storage. Memory is defined as the capacity to recall the response to a specific stimulus (Salamon, 2002). Short-term memory involves the retention of information for brief moments while long-term memory is associated with the recall of events that did not occur recently (usually >24 hr). Long term memory is accompanied by *de novo* protein synthesis (Touzani et al., 2007) and is thought to be mediated by the transfer of short-term memory to a more permanent storage (Hebb, 1949, Medina et al., 2002b, Inda et al., 2005). This concept involves trigger cells, which are responsible for the establishment of memory, and storage cells, which are responsible for storing memories formed in the trigger cells. Such systems are postulated for the hippocampus, the cerebellum, and the amygdala (Medina et al., 2002b).

Motor memory is a type of non-declarative memory, characterized by acquisition and retention of complex knowledge of skills, habits or procedures through practice and irrespective of the subject's awareness. Common examples are riding a bike or learning how to position the feet in order to walk (Boje, 2002, Ito 2000). Current theories postulate that the cerebellar cortex is crucial for the acquisition of motor memories and that the deep cerebellar nuclei might play a more important role in the maintenance of these memories (Medina et al., 2002a, Krakauer and Shadmehr,

2006), consistent with the trigger/storage model.

The striking organization of the cerebellar cortex has inspired hypotheses that implicate the cerebellum as a pattern-learning machine that could guide the learning of specific motor tasks (Marr, 1969, Albus, 1971, Gilbert, 1974). The general idea of the Marr-Albus hypothesis is that activity in one system (CFs) alters the responsiveness of the PC to activity in the other system (mossy fibers or PFs). Because a CF has a one on one relation with a Purkinje cell, while the same Purkinje cell receives input from thousands of PFs, this system is suitable for associating a large variety of signals with a single error event. Feedback information about ongoing movement is conveyed to the Purkinje cell via the mossy fibers and PFs. If the movement is incorrect, the error will be translated into CF activity. The effectiveness of the active PFs will then be weakened by the co-active CF. This system appears to be very suitable for the representation of timing (Figure 3).

In the following sections I will describe forms of cerebellar-related motor performance that are related to relatively simple reflexes. In section 3 of this introduction, I will describe locomotion learning, which is more complex as it involves not only reflexes, but a whole scale of voluntary and non-voluntary interactions.

### 2.2. Eye blink conditioning

Classical or Pavlovian conditioning of motor reflexes, like the eye-blink reflex or the vestibulo-ocular reflex (VOR), induces a cerebellar-dependent type of long-term motor memory to the stimuli used (Thompson, 1986, Yeo, 1991). Conditioning involves evoking a learned or conditioned response (often abbreviated as CR) to a previously neutral stimulus (conditioned stimulus or CS) after a period of training in which this stimulus is presented paired to noxious or non-neutral stimulus (unconditioned stimulus or US). Classical conditioning was first described by Ivan Pavlov (Pavlov, 1927) and has been widely used ever since as a model for studying associative learning.

In the 1960s, a very powerful *in vivo* system to study conditioned eye-blink responses was developed (Gormezano et al., 1962), paving the way for future research. In this model, an air-puff aimed at the cornea of a rabbit is the US and a neutral tone is the CS. The US always causes a reflexive fast closure of the eyelid and a passive sweep of the nictitating membrane (NM) over the cornea. This reflexive response is called the unconditioned response (UR). Repetitive presentation of the CS temporally paired with the US results in the generation of a conditioned response (CR), a closure of the eyelid after presentation of the CS.

With the use of this or similar setups several researchers started to look for

the engram, or the site of convergence responsible for classical conditioning of somatic muscle responses to an aversive stimulus. In this search, two basic types of conditioning procedures were used. The first and simplest is delay conditioning, where the onset of the CS precedes the US. As both stimuli terminate together, the CS is still active at the moment of US presentation. The second type is called trace conditioning and is somewhat more demanding than delay conditioning. It requires the memory of the CS to be retained because the CS is followed by a brief stimulus free interval before the US is presented. Association can only be made when the CS event is held in the memory until presentation of the US (see also Thompson, 1986, Thompson and Kim, 1996). In the following sections I will review studies on stimuli and responses and present evidence that the engram is in fact the cerebellum.

### 2.3. The unconditioned stimulus and response

In classical conditioning of reflexes the unconditioned stimulus (US) should always cause a reflexive unconditioned response (UR). The UR was initially described in 1896 by the British physiologist Overend (Overend, 1896). He described that tapping the forehead skin above the eyes with a stethoscope (the US) caused ipsilateral eyelid twitching, while tapping the midline caused bilateral eyelid twitching. In 1901, McCarthy redefined the reflex by noting that tapping the skin overlying the supraorbital (SO) nerve with a reflex hammer (the US) elicits a usually bilateral response of the orbicularis oculi (OO) muscles (McCarthy, 1901). To date, in eye-blink conditioning research the UR is a fast closure of the eyelid, most frequently induced by electrical stimulation of the SO nerve or an air-puff aimed at the cornea.

The underlying mechanisms of the reflex were further elucidated in the 1950s with the analysis of electrically evoked blink reflexes in humans (Kugelberg, 1952). From this study it became apparent that the blink reflex consists of two components. The first response ($R_1$), is ipsilateral to the stimulus side with a latency of about 10 ms (Kugelberg, 1952). $R_1$ is mediated by non-nociceptive fiber inputs relaying signals to pontine low-threshold mechanoreceptive (LTM) neurons located in the principal sensory nucleus. The second response ($R_2$) is bilateral, has a latency of about 30 ms and is mediated by both nociceptive and no-nociceptive fiber inputs to wide dynamic range neurons (WDR) of the spinal trigeminal nucleus (Ellrich and Treede, 1998). Similar latencies of $R_1$ and $R_2$ have been observed in cats and rabbits (Gruart et al., 1995). In humans the total duration of the electrically stimulated blink reflex lasted about 200 ms and consisted of a downward phase of about 50 ms and an upward phase of about 150 ms (VanderWerf et al., 2003). In contrast with electrically induced blinks, air-puff induced blinks lack an ipsilateral $R_1$ component. The air-puff

induced UR lasts about 300 ms in humans (VanderWerf et al., 2003).

### 2.4. Unconditioned response pathways

The UR circuit has been extensively studied in rabbit (van Ham and Yeo, 1996b, a), guinea pig (Pellegrini et al., 1995), cat (Holstege et al., 1986a, Holstege et al., 1986b) and rat (Gormezano et al., 1962, Morcuende et al., 2002), but not in mice. The neuroanatomical circuitries that have been proposed to underlie the $R_2$ component may vary among species, i.e. the circuitry proposed for cats differs from that proposed for rabbits and guinea pigs.

Eye-blinks result from the conjunctive action of 3 elements: 1) excitation of the musculus orbicularis oculi (MOO), which is ultimately responsible for the eyelid closure; 2) inhibition of the levator palpebrae muscle (LP), that elevates the eyelid; and 3) activation of the accessory abducens nucleus that causes the retraction of the eyeball via the retractor bulbi muscle (RB).

Motor neurons responsible for the concerted action of these muscles, receive inputs from neurons in the trigeminal nuclei and rostral levels of the spinal cord (van Ham and Yeo, 1996b).

The $R_1$ component involves the trigeminal neurons and the intermediate subarea of the facial nucleus. The $R_2$ component involves a circuit from the periocular and corneal receptors to the trigeminal nuclei, reinforced by a projection from the spinal cord that does not project directly to the motor neurons (van Ham and Yeo, 1996a).

In cats a more complex $R_2$ circuitry has been proposed. In the medulla oblongata and in the pons, blink premotor areas receive indirect input from the trigeminal nuclei. The trigeminal nuclei project via the red nucleus to the pons and via the superior coliculus to the medulla oblongata. Both these blink premotor areas send strong projections to the motor neurons controlling the RB and MOO (Holstege et al., 1986a, Holstege et al., 1986b).

The essential blink circuit is probably the same across mammalian species, but may be differently utilized. In rodents the $R_1$ component contributes substantially to eyelid closure (Pellegrini et al., 1995). As in the cat it has been shown in rat that specific areas of medullary, pontine and mesencephalic reticular formations project onto MOO motoneurons. These projections were frequently monosynaptic and larger in number than those from the trigeminal nuclei. Some of those pathways may be involved in the genesis of premotor signals related to the expression of internal emotional states, because limbic structures project to these areas through the central amygdala and hypothalamus (Holstege et al., 1986b). Finally, rats display a strong monosynaptic input from auditory pathways to blink motoneurons, which may explain

the much more noticeable eyelid component in the auditory startle response in these animals (Morcuende et al., 2002).

### 2.5. The conditioned stimulus and response

The most frequently used conditioned stimulus (CS) is an auditory cue. However, as long as the CS is strong, well timed and neutral at all levels from the US, other stimulus modalities can be used as well (e.g. flashes of light or electrical paw stimulation). Only the auditory CS will be discussed here. Initially in naive subjects a CS has to be neutral, i.e. not to evoke a response by itself. However, it is important to note that even though an auditory CS can appear to be neutral on the behavioral level, this does not mean that it is neutral at the cellular level. Excitatory postsynaptic potentials (EPSPs) have been recorded in identified mooVII neurons, evoked by a CS-like tone in animals with no noticeable eye-blink responses (Trigo et al., 1999).

Essential parts of the anatomical pathway that conveys the auditory CS are the cochlear and pontine nuclei. CS can be mimicked by microstimulation of the neurons in the pontine nuclei (Steinmetz et al., 1986, Steinmetz et al., 1989).

In 1975 it was demonstrated that eye-blink conditioning after a tone was paired with an air-puff to the eye was possible in decerebrated rats (Lovick and Zebrozyna, 1975), even though twice the number of training sessions were needed compared to control rats and the peak of percentage of correct CRs was twice as low.

Several other studies have been performed where the animals have been decerebrated before training (Kelly et al., 1990) or after training (Mauk and Thompson, 1987). In one case, the auditory cortex was inhibited during training (Case et al., 2002). Even though these studies cannot be directly compared, it is obvious that almost all researchers had difficulties conditioning decerebrated animals with a tone. The CRs of decerebrated animals were worse compared to intact animals, the decerebrated animals varied more from block to block and the training sessions of the decerebrated animals were usually longer. However, conditioning was possible.

### 2.6. Role of the cerebellum in eye blink conditioning

As described above, electrical stimulation of the pontine nuclei can mediate the CS. In addition similar studies performed using electrical stimulation of the inferior olive (IO) have shown that such stimulations can substitute the US (Mauk et al., 1986). Both structures project to the cerebellum. Therefore the cerebellum could play an important role in the mechanisms underlying eye-blink conditioning. Conditioned responses (CRs) not only need to be acquired, to be effective they also have to peak at the right moment (i.e. just before the US will arrive). Lesion studies of the cerebellum, the anatomy of the cerebellum and electrophysiological studies of cerebellar neurons

have provided evidence that the cerebellum is essential for some or all aspects of the CR.

McCormick and Thompson were the first to report that eye-blink conditioning was not possible without a cerebellum (McCormick and Thompson, 1984). Lesions of the ipsilateral interposed nucleus abolished the CR but not the UR. To pinpoint the essential region, others (Clark et al., 1984, Lavond et al., 1985) have made very small lesions in the cerebellum and reported the importance of the dorsal region of the anterior interposed nucleus. However, many other groups argue that the site of the engram is the cerebellar cortex rather than the anterior interposed nucleus (for reviews see Yeo, 1991, Bloedel and Bracha, 1995, Thompson and Krupa, 1994). The importance of the cerebellum for eye-blink conditioning was further strengthened by studies showing that lesions in the pathway between the interposed nuclei and the motor neurons innervating the eyelid muscles caused deficits similar to lesions of the nuclei itself (Desmond et al., 1983, Rosenfield and Moore, 1983, 1985).

An alternative view has been presented by Welsh and colleagues. They reported that after lesions in the cerebellum were made, only two rabbits showed no CRs, thirteen were severely impaired, six were impaired, fourteen recovered and fifteen rabbits had no deficits. They found an inverse relation between response strength and cerebellar damage and therefore concluded that the cerebellar lesions primarily induce loss of neural and muscle tone and that the reduction in eye-blink conditioning is merely secondary to this loss (Welsh and Harvey, 1989, Welsh, 1992). It has also been reported that conditioning is possible without a cerebellum (Kelly et al., 1990). It was hypothesized that this is possible because cerebellar lesions dramatically modify the excitability of a variety of brainstem nuclei. This points to the possibility that learning is accomplished at least partially outside the cerebellum.

Finally, Koekkoek and colleagues showed that the CR in mice consisted of a cerebellar component and an extracerebellar component (Koekkoek et al., 2002, Koekkoek et al., 2003). After lesioning of the Anterior Interpositus Nucleus (AIN), mice still showed CRs, but these were differently shaped than CRs of intact animals.

## 3. Locomotion

### 3.1. Locomotion learning

In order to produce normal patterns of locomotion, the following prerequisites need to be met: 1) a rhythmic pattern of activity of flexor and extensor muscles, 2) the control of equilibrium and 3) the capacity to learn and adapt the basic movement pattern according to novel contexts (Grillner and Wallen, 1985). Although the precise

mechanism of control of walking is not known, the cerebellum contributes directly to balance and locomotion (Morton and Bastian, 2004, Lalonde and Strazielle, 2007a).

### 3.2. Lesion studies and mutants

The involvement of the whole cerebellum and of different cerebellar regions in the control of movement became apparent from studies in humans with cerebellar lesions (Holmes, 1917, 1939) and lesion studies in rats (Schneiderman and Isaacson, 1976, Joyal et al., 1996), mice (Caston et al., 1995), cats or monkeys (Chambers and Sprague, 1955b, a, Thach et al., 1992). For example, cerebellectomized adult mice perform less well than control littermates in the rotarod (Caston et al., 1995), a setup which is very often used in the laboratory and which tests locomotor performance (see also chapter 3). This type of lesions can produce variable effects, according to the age at which the lesion is made, possibly due to compensation from other cerebellar regions.

Unilateral cerebellar hemispherectomy in rats at postnatal day 1 (P1) revealed recovery of most of their locomotor control, except for tasks requiring fine-tuning of movement, like the hanging wire test and the stationary rod. If lesions were applied at later time points, rats had more pronounced locomotion defects and showed a lower rate of recovery, except for hindlimb usage in which case the effect was greater for lesions inflicted at earlier stages (Molinari et al., 1990,Petrosini et al., 1990). It is believed that the cerebellum can develop to compensate most lesions suffered at early ages but that tasks like the hanging wire require both hemispheres. It can also be inferred that hindlimb control is particularly sensitive to lesions at early stages of cerebellar development perhaps because the control of stance and gait begins at an early age.

Bilateral cerebellectomy, sparing the flocculo-nodular lobe, was performed in rats at P10, P20 and P24. Animals were trained daily in a rotarod setup before and after the surgical procedure and their coordination was monitored. Cerebellectomy severely impaired the motor performance of the rats although partial recovery could be observed with post-operative training, indicating functional compensation from other brain areas or the flocculo-nodular lobe, which did not fully compensate for a missing cerebellum (Auvray et al., 1989). Lesions of the vestibular or fastigial nuclei result in abnormal posture tone and difficulties in walking in cats and monkeys (Sprague and Chambers, 1953, Chambers and Sprague, 1955a). Lesions of the vestibular/ fastigial nuclei in cats also induced abnormal timing of relative limb movements and decreased stride lengths (Yu and Eidelberg, 1983). Rats with lesions in the fastigial or dentate nuclei performed poorly in motor coordination setups (Fish et al., 1979).

Lesions of the vermis/fastigial nucleus, the lateral hemispheres/dentate nucleus or the fastigial nuclei alone also caused motor impairment (Joyal et al., 1996). These studies point to the lack of localized foci of cerebellar control of locomotion but to a general role of the cerebellum.

Naturally occurring mutant mice displaying motor coordination problems have been extensively studied (Zuo et al., 1997, Lalouette et al., 2001, Hirotsune et al., 1995, Hamilton et al., 1996, Fernandez-Gonzalez et al., 2002). Most of these mice have mutated glutamate receptor (GluR) subunits (for review see (Lalonde and Strazielle, 2007b). Examples of mouse mutants are the *lurcher*, *weaver* and *hot-foot* mice. Other mutations affect the retinoic orphan receptor A (*Rora*, this mouse mutant is also commonly known as *staggerer*), the AGTP binding protein (*Agtpbp1*, commonly known as *Purkinje Cell degeneration* mouse), and Reelin (*Reln*, also known as the *reeler* mouse). The mutant known as *nervous* mouse has an unidentified mutation (Lalonde and Strazielle, 2007b). These mutations result in PC loss, associated with granule cell and deep cerebellar nuclei cell loss. Locomotion studies performed with these naturally occurring mutants have helped to elucidate the role of the cerebellar cortex, mainly the PCs in the control of locomotion.

Transgenic mice deficient for several proteins of interest have also been generated and analyzed for their performance in motor coordination tasks (see section 7 for an explanation of the generation of transgenic mice). Some of the mice that showed deficits in motor performance (as tested by the rotarod test) are listed below. mGluR2 mutant mice (Zuo et al., 1997, Lalouette et al., 1998) show deficits in both rotarod coordination and cerebellar long-term depression, or LTD (Kashiwabuchi et al., 1995) (see section 4 for an explanation on LTD). Protein kinase C-$\gamma$ null mutants also displayed motor coordination deficiencies but not impaired LTD (Chen et al., 1995). This is curiously in contrast with the lack of motor coordination defects and strong LTD impairment in the L7-PKCi mice, in which an inhibitory peptide is expressed (De Zeeuw et al., 1998a). These mice will be described in more detail in section 4.2. Mutant mice in which genes were deleted (so-called "null" mutants, see section 7) and that have motor coordination effects are *Cntn6*, encoding a NB-3 neural recognition molecule (Takeda et al., 2003), *Klf9*, a basic transcription element binding protein (Morita et al., 2003), *Syt4*, coding for Synaptogamin IV (Ferguson et al., 2000), and *En2*, coding for Engrailed2 (Gerlai et al., 1996). All these genes are expressed in the cerebellum and absence of the encoded protein products causes motor coordination deficits. Together these results strengthen the link between cerebellum and motor coordination but do not clarify the mechanisms involved.

### 3.3. Cerebellar regions involved in locomotion

The control of posture and locomotion (and motor learning) involves known anatomical pathways. Important information related to movement originates directly from the limbs and the spinal cord, the visual inputs, the vestibular and reticular nuclei, the pontine nuclei and the cerebral cortex (for review see (Morton and Bastian, 2004, Lalonde and Strazielle, 2007a). Here, I will deal only with cerebellar regions involved in locomotion.

The cerebral cortex (primary motor, premotor, somatosensory, posterior and parietal and prefrontal cortices) sends projections to the pontine nuclei. Projections from the pontine nuclei reach the cerebellar cortex as mossy fibers. Cortical information (via the pontine nuclei) is received in all areas of the cerebellum (Matsushita and Okado, 1981, Middleton and Strick, 2001). The vestibular and reticular nuclei send information directly to the vermis, the intermediate and the flocculo-nodular lobes (Clendenin et al., 1974, Kotchabhakdi and Walberg, 1978). The spinal cord projects directly to the vermis and intermediate cerebellum (Bosco and Poppele, 2001, Matsushita et al., 1981). These spino-vermal projections convey information about the sensory state of the limbs (dorsal spinocerebellar tracts) and about motor commands (ventral spinocerebellar tracts) (Bosco and Poppele, 2001).

The vermis projects back to the spinal cord. It also sends strong projections directly to the fastigial nucleus, and indirectly back to the vestibular and reticular nuclei. Vermal control of the thalamus occurs via direct weak projections and indirectly via the fastigial nucleus. Vermal control of cortical inputs occurs via the thalamus. The vermis can influence locomotion by acting on the vestibulo-spinal tracts.

In humans, the intermediate cerebellum projects via the globose and emboliform deep cerebellar nuclei to the red nucleus and the thalamus/cortex, affecting motor-cortical areas. The lateral cerebellum projects via the dentate deep cerebellar nucleus to the red nucleus and indirectly also to the thalamus/cortex, regulating cortical interactions involved in voluntary movements (Dum and Strick, 2003, Middleton and Strick, 2001, Clower et al., 2001). The flocculo-nodular lobe projects directly back to the vestibular nuclei regulating balance and eye movements (Akaike, 1983).

Lesion studies combined with direct electrophysiological measurements indicate that the medial cerebellum (vermis and fastigial nucleus) as well as the flocculo-nodular lobe are the most import regions of the cerebellum controlling posture and locomotion. The paravermal or intermediate regions are thought to be necessary for adjusting movements in circumstances when extra accuracy is needed. The lateral zones are thought to be recruited when motor learning occurs (see Morton and

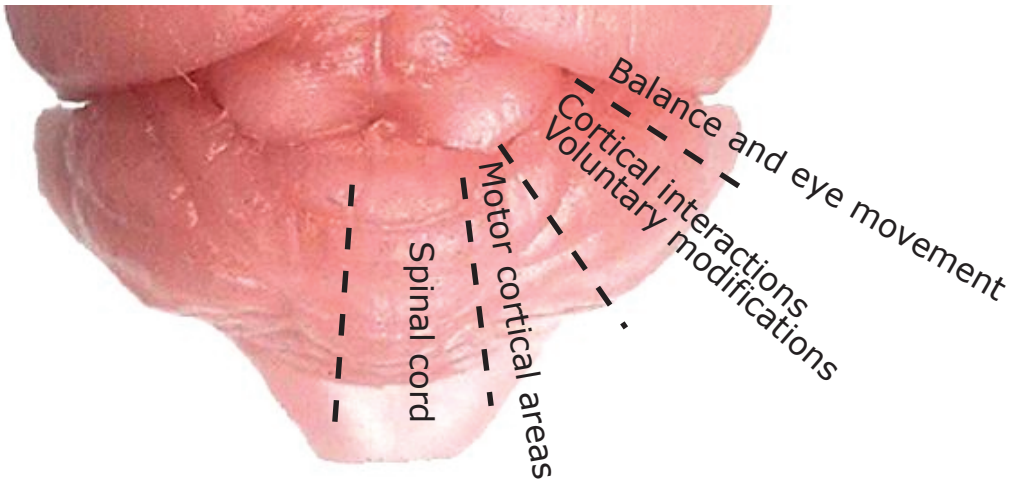



Figure 4 – simplified view of the contribution of different areas of the cerebellum to different aspects of the locomotion process. The vermis receives and sends projections directly to the spinal cord. The paravermis receives inputs from the cortex and pontine/reticular formations. It projects back to the cortex via rubro/thalamic projections and influences motor cortical areas. The lateral cerebellum receives extensive projections from the cortex via pontine nuclei and influences the voluntary aspects of locomotion. The Flocculus and Nodulus (rostrally involuted) receive visual inputs and play a role in the balance and eye movements (Morton and Bastian, 2004).

Bastian 2004). Figure 4 is a simplified representation of the cerebellar areas involved in the control of locomotion.

As an example of the complexity of the motor circuits involved, I describe locomotion in the awake moving cat. Information from the forelimbs is conveyed to areas C1 and C3 of the ipsilateral intermediate lobule V. The associated CF responses exhibit the highest excitability during the swing phase (the limb is raised from the floor) and the lowest excitability during the stance phase (the limb contacts the floor) (Lidierth and Apps, 1990, Apps et al., 1995, Apps and Lee, 1999). This pattern of alternating excitability of neurons in the lobule V is thought to represent a mechanism of preparation for the correct positioning of the limb. Neurons in the vestibular nucleus are active during the stance phase while neurons in the reticular nuclei are active during the swing phase. This cyclic activation of vestibular/reticular neurons is correlated with increased flexor/extensor muscle activity, respectively (Morton and Bastian, 2004). The firing rates of these neurons are controlled by the cerebellum, as demonstrated by cerebellectomy before and after training. Neurons in the red nucleus (or the cerebello-rubrospinal tract) are active during the flexion phase of the walking cycle (Arshavsky et al., 1988) and thus involved in the establishment of walking rhythm (Muir and Whishaw, 2000). The thalamus also plays a role in the control of locomotion in less clear manner; thalamic nuclei lesions have been

shown to affect rotarod performance without affecting performance in other motor coordination paradigms (Jeljeli et al., 2000, 2003).

### 3.4. Locomotion versus eye-blink conditioning

The most widely used paradigms to study motor coordination are the accelerating rotarod, the stationary beam and the suspended wire. The accelerating rotarod provides a sensitive test to assess cerebellar dysfunction, probably because the coordination of locomotion necessary to perform this task relies on multiple areas of the cerebellum (Lalonde and Strazielle 2007a). Despite the fact that tests are available, the brain circuitry involved in each motor coordination or locomotion paradigm has not been completely elucidated. In eye-blink conditioning, for example, many more details are known of the anatomical circuits involved.

Cerebellar control of locomotion and posture is mediated differently from the control of eye-blink conditioning. Evidence supports a thalamus-dependent anatomical pathway in the regulation of the former (Lorincz and Fabre-Thorpe, 1997) and thalamus-independent modulation of the latter (Sears et al., 1996). It also seems that a vast extension of the cerebellar cortex as well as many cerebellar nuclei are involved in the control of locomotion, making it difficult to pinpoint specific contributions of individual areas to the locomotion effects. Areas of the cerebellar cortex such as lobule HVI (Yeo et al., 1985, Attwell et al., 2001) and the interpositus nuclei (Chen et al., 1999, Delgado-Garcia and Gruart, 2002, Jimenez-Diaz et al., 2004) have been mapped as sites of convergence for the eye-blink conditioning.

In locomotion, there is a strong voluntary component, depending on cerebral cortical input, which is not so pronounced in eye-blink conditioning. Eye-blink conditioning also has an element of fear, which is probably absent in basic locomotion. Despite the differences mentioned above, the cellular mechanisms of cerebellar control are thought to be similar, and to rely on activity-dependent cerebellar plasticity (see section 4). In the experiments described in this thesis we used rotarod, openfield and runway (catwalk) setups to investigate the contribution of several mutations in mice to cerebellum-related motor coordination deficits (See chapters 2 and 3). We also used eye-blink conditioning to assess the contribution of specific protein kinase pathways in PCs to cerebellar associative learning outputs (see chapter 2).

## 4. Purkinje cell plasticity

### 4.1. Plasticity and long term depression

According to Hebb's postulates (Hebb, 1949), synapses firing together will be

strengthened together and this change in the relative strength of the synapses accounts for the storage of memories. Apart from the strengthening of the synapses, other changes can occur, such as the selective weakening of synaptic transmission that characterizes cerebellar long-term depression. These alterations in synaptic strength are collectively termed synaptic plasticity and indicate the capacity of the brain to change and adapt the nature of its interconnections based on experience, i.e. learning. Memories are thought to be harbored in a network of related neurons, displaying a pattern of alternating stronger and weaker synapses.

Within the cerebellum, the synaptic efficacy of the PF to PC synapse can be selectively decreased. This decrease is termed long-term depression (LTD). Cerebellar parallel fiber-long term depression (PF-LTD) is a long lasting reduction in synaptic

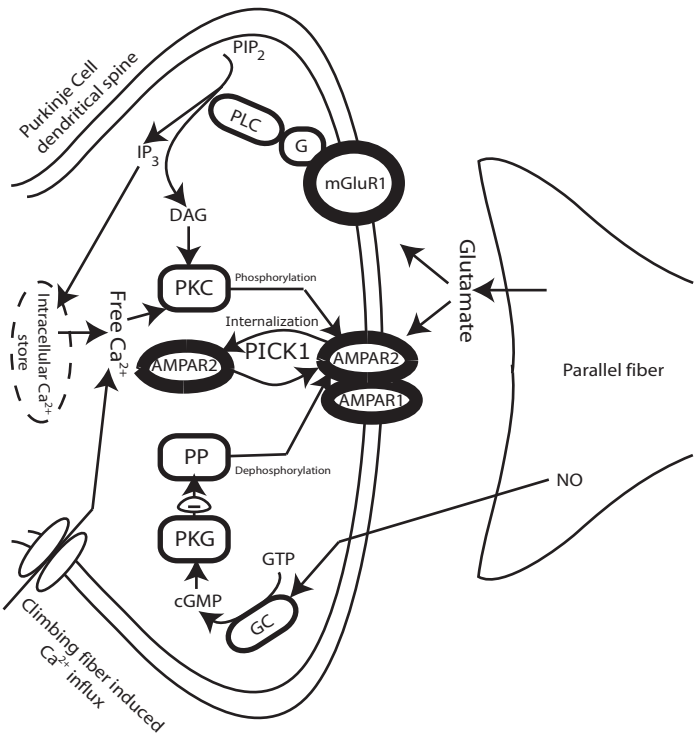



Figure 5 – schematic representation of cerebellar LTD. Climbing fiber inputs depolarize the Purkinje cell (PC) they contact and cause Ca2+ influx. Parallel Fibers release Glutamate that will activate metabotropic glutamate receptors (mGluR) as well as AMPA-type glutamate receptors (AMPAR). mGluR are coupled to g-proteins and upon glutamate binding, activate phospholipase C (PLC) that will give rise to diacyl glycerol and inositol tri-phosphate (IP3) from the membrane phospholipid phosphoinositol bi- phosphate (PIP2). Via IP3 receptors Ca2+ will be released from internal stores. Together with the supra elevated Ca2+ pool, DAG will activate Protein kinase C (PKC) that phosphorylates the AMPAR2 moiety at Ser 880. Nitric Oxide (NO), also released from the parallel fibers will diffuse into the PC and activate the guanylyl cyclase (GC) that hydrolyzes GTP into cyclic-GMP (cGMP) that will in turn activate the cyclic-GMP dependent Protein Kinase (PKG). Acting on one of its downstream targets, the G-substrate, PKG will cause the inhibition (marked with the minus sign) of Protein Phophatases (PP). This results in retention of the phosphorylation state of the AMPAR2, causing a loss of interaction with the scaffolding Protein that Interacts with C Kinase 1 (PICK1) and a disruption of the AMPAR clusters and subsequent internalization of the AMPAR2 moieties. On a reversal mechanism, PICK1 will be able to re-attach the AMPAR2 moieties and restore the AMPA receptor clusters. When the AMPAR are internalized, the PC is in a state of non-responsiveness to parallel fiber Glutamate (Adapted from Koekkoek, 2004).

efficacy and can be induced by pairing PF activity with that of the climbing fiber (CF) coming from the inferior olive (Linden and Connor, 1995, Ito, 2001). The process of LTD is summarized in Figure 5.

### 4.2. Kinase-dependent long term depression

Evidence that *in vivo* LTD at the PF-PC synapse might be responsible for parts of the learning process arose from the work of Schreurs and colleagues who, using both *in vivo* and *in vitro* techniques, showed in rabbits that: 1) LTD occurred when CS and US where temporally paired, 2) membrane bound protein kinase C (PKC) is upregulated after training, and 3) LTD induced by natural stimulations could be blocked by blocking PKC or intracellular $Ca^{2+}$ (Freeman et al., 1998a, Freeman et al., 1998b, Schreurs et al., 1996). These results highlight the importance of PKC in LTD. Work in this thesis focuses on protein kinases, some of which are dealt with in more detail in the following sections. However, one should bear in mind that many factors regulate LTD.

#### 4.2.1. Protein kinase C

Protein kinase C is one of the most ubiquitous kinases and is present (sometimes in multiple isoforms) in all cell types. It regulates cell size and shape, cytoskeletal remodeling, cell-cell communication, receptor desensitization, transcription, and many other processes (Parker, 1999, Way et al., 2000). The PKC family of kinases are signal transduction proteins that have been shown to also be involved in learning and memory (De Zeeuw et al., 1998a, Goossens et al., 2001).

There are at least 11 known isozymes of PKC (Liu and Heckman, 1998), all of which consisting of a single peptide chain. The classical PKC isozymes ($\alpha$, $\beta$ and $\gamma$ - the latter is a neuron specific isozyme) bind $Ca^{2+}$ and diacyl glycerol (DAG). The novel PKC isozymes ($\delta$, $\varepsilon$, $\eta$, and $\theta$) do not have a $Ca^{2+}$ binding site and are $Ca^{2+}$ insensitive. Atypical PKC isozymes ($\tau$,$\lambda$ and $\zeta$) do not respond to $Ca^{2+}$ or DAG. Finally, a nuclear form of PKC has been described that corresponds to the free catalytic subunit and has transcription factor activity (Shea et al., 1994). A novel DAG binding protein was originally considered a member of the PKC family (Wang, 2006), but is nowadays known as protein kinase D (PKD) (Stafford et al., 2003).

PKC contains a pseudo-substrate inhibitory domain at its N-terminus, that covers the active site and thereby inhibits PKC when the enzyme is not properly phosphorylated (Newton, 1995). This pseudo-substrate can be synthesized on its own and has been successfully used to block PKC activity *in vivo* (De Zeeuw et al., 1998a). In its inactive state, PKC is membrane- or cytoskeleton-bound. It is modified by a

trans-phosphorylation and two autophosphorylation events. These relieve pseudo-substrate inhibitor binding to the active site, in conjunction with a preferential high affinity binding of the two regulatory domains to membrane phospholipids and DAG, and a conformational change, induced by $Ca^{2+}$ binding. The pseudo-substrate becomes proteolytically labile and can be cleaved (Newton, 1995, 1996).

Acitvated PKC has the capacity to phosphorylate multiple targets. Its specificity is dependent on receptors for inactive C kinase (RICKs) or receptors for active C kinase (RACKs), as well as other cell type and isoform specific anchoring proteins that restrict the contact between active PKC and potential substrates. The binding site of PKC for anchoring proteins is isozyme-specific meaning that each isoform of PKC has different anchors (Newton, 1996).

As described above, long-term depression (LTD) occurs when parallel fiber (PF) and climbing fibers (CF) concurrently signal on a single PC. The molecular mechanisms underlying LTD have been studied in great detail and involve PKC. PF activation leads to the activation of the ionotropic, $\alpha$-amino-3-hydroxy-5-methylisoxazolproprionate-type glutamate receptors (AMPAR) and the metabotropic glutamate receptors (mGluR) on the dendritic synapse of PC. Concomitantly, CF activation leads to a massive depolarization of the Purkinje cell and subsequently to a large increase of intracellular $Ca^{2+}$. Activation of mGluR1 induces a G-protein coupled activation of phospholipase C (PLC), which converts the membrane phospholipid phosphatidyl-inositol-phosphate ($PIP_2$) into diacyl-glycerol (DAG) and inositol-tri-phosphate ($IP_3$). $IP_3$ mediates release of intracellular $Ca^{2+}$. Increased intracellular $Ca^{2+}$ and DAG activate protein kinase C (PKC), which acts on AMPAR by phosphorylating a serine residue (Ser 880, (Matsuda et al., 2000, Chung et al., 2003) on the intracellular C-terminal end of the AMPAR2 subunits. Subsequently, AMPAR with phosphorylated serine residues are internalized by clathrin-mediated endocytosis. In short, LTD causes a reduction in selected PF synapse efficacy by down-regulating the number of AMPAR on the postsynaptic membrane.

In order to study the contribution of the PKC pathway in more detail *in vivo*, transgenic mice (L7-PKCi mice, see also chapter 2) have been generated that selectively express a PKC peptide inhibitor in PCs. Since PKC is required for the induction of LTD (Figure 5) of the PF-PC synapse, this main cellular candidate mechanism for cerebellum-dependent motor learning is blocked. It has been reported that L7-PKCi mice show cerebellum-dependent CRs that lack any form of temporal adaptive capacity (Koekkoek et al., 2003). Other evidence supports this view. For example, PF-LTD of these transgenic mice is blocked *in vitro* (De Zeeuw et al., 1998a) and *in vivo* (Gao et al., 2003). Interestingly, they had no other impairments and the simple spike

and complex spike-firing properties (such as mean firing rate, interspike interval, and spike count variability), oscillations, and CF pause were indistinguishable from those in wild-type mice (Goossens et al., 2001). However, contradicting results have also been reported (Welsh et al., 2005), which indicate that pharmacological blockage of LTD in rats did not seem to affect conditioned responses. In light of these contradictory results the role of LTD to cerebellar behavioral outputs is not entirely clear what is.

### 4.2.2. Protein kinase G

A second pathway involved in LTD is mediated by nitric oxide (NO) (Daniel et al., 1993,Lev-Ram et al., 1995). NO is a short-lived gas that can diffuse into (and influence) an estimated 4000 synapses in 10 ms *in vivo* (Ito, 2001). NO acts on guanylyl-cylase (GC), which converts guanosine-tri-phosphate (GTP) into cyclic guanosine-mono-phosphate (cGMP). cGMP then activates a cGMP-dependent protein kinase, also known as (and further referred to as) protein kinase G, or PKG (Wu et al., 1998, Hofmann et al., 2000). In synapses, PKG activation will ultimately result in blocking the dephosphorylation of AMPA receptors. PKG is also involved in muscle relaxation and platelet activation. It is thought to contribute to the regulation of relatively few biological processes compared to other kinases, as only about 10 substrates for this kinase are known (Hofmann et al., 2006). Due to its effect on insect behavior, PKG has also been described as the "foraging gene" (Ben-Shahar et al., 2002).

Structurally, PKG is a dimer of two identical peptide chains. Each peptide chain contains a catalytic domain, recognizing the sequence Arg-Lys-Arg-Ser-Arg-Lys-Glu (Dostmann et al., 1999), and a regulatory domain. Like PKC, PKG has an autoinihibitory pseudo-substrate sequence at its amino terminus. PKG "matures" via autophosphorylation, and is activated via cGMP binding. There are two main forms of PKG, called PKGI and II. PKGI is soluble and interacts with cytoplasmic targets, while PKGII is membrane bound and is expressed in several brain nuclei. PKGI appears in two alternative spliced isoforms, $\alpha$ and $\beta$, of which PKGI$\alpha$ is especially abundant in cerebellar PCs.

The similarity between the catalytic site of protein kinase A (PKA) and PKG is so high that it has been difficult to specifically inhibit PKG, even pharmacologically (Mitchell et al., 1995). However, screening of a synthetic peptide library yielded a peptide sequence capable of inhibiting PKG with over 100x selective affinity over PKA (Dostmann et al., 1999, Dostmann et al., 2000).

Mice that specifically lack PKG in cerebellar PCs have been generated and tested and shown to have deficits in LTD. From their impaired adaptation of the vestibulo-ocular reflex (Feil et al., 2003a) it was concluded that they also have deficits in

cerebellar learning. These mice were further analyzed in the eye-blink conditioning paradigm described in chapter 2. In conclusion, PKG contributes to LTD and the cerebellar behavioral outputs.

### 4.2.3. Calcium- and calmodulin-dependent kinase alpha

Although I focus in this thesis on the effect of the PKG pathway in eye-blink conditioning (see chapter 2), a new pathway leading to cerebellar LTD has recently been elucidated and is worth mentioning. This pathway involves the $Ca^{2+}$/calmodulin-dependent Protein Kinase II (CaMKII), of which types $\alpha$ and $\beta$ are present in the cerebellum (Walaas et al., 1988). $\alpha$CaMKII is a "molecular switch" that can be present in an active autophosphorylated state as well as an inactive dephosphorylated state (Miller et al., 2005). $Ca^{2+}$ activates CaMKII and this is known to be associated with the establishment of hippocampal long-term potentiation, or LTP (Hayashi et al., 2000, Lledo et al., 1995, Silva et al., 1992).

CaMKII can directly phosphorylate mGluR1 on serine residue 831 (S831, Barria et al., 1997a, Mammen et al., 1997). A potential role for $\alpha$CaMKII in cerebellar LTD had been proposed before (Ito, 2001) but could only recently be demonstrated (Hansel et al., 2006). $\alpha$CaMKII null mutants (Elgersma et al., 2002) display altered LTD and motor learning as assessed by the vestibulo-ocular reflex adaptation (Hansel et al., 2006). CaMKII phosphorylation of anchoring proteins regulates synaptic targeting of AMPAR subunits in the hippocampus (Nicoll et al., 2006) but the precise site and role of CaMKII phosphorylation in cerebellar LTD still has to be elucidated (Jorntell and Hansel, 2006). The mechanism by which $\alpha$CaMKII regulates LTD in the cerebellum seems to be the inverse of the mechanism seen in the hippocampus. An increase in intracellular $Ca^{2+}$ promotes $\alpha$CaMKII induced LTD in the cerebellum but $\alpha$CaMKII induced LTP in the hippocampus.

## 4.3. Long term potentiation

Long-term potentiation (LTP) is a physiological process first described in studies of the hippocampus (Bliss and Lomo, 1973), which is believed to engage cellular mechanisms similar to those that underlie learning (Bliss and Collingridge, 1993). The most extensively studied form of LTP occurs in the CA1 region of the hippocampus and involves the interaction between pre-synaptic glutamate and two classes of postsynaptic receptors. First, glutamate binds to AMPA receptors and depolarizes the postsynaptic cell. This depolarization allows glutamate to bind to the N-methyl-D-aspartate (NMDA) class of receptors. NMDA-channel mediated $Ca^{2+}$ influx then triggers a host of intracellular events that ultimately result in gene induction and

synthesis of new proteins (Kandel, 1997). The newly synthesized proteins, in turn, help to maintain an increased excitability of the neurons over long periods of time.

To retain modulatory capacity and allow for the reversal of motor learning, LTD at cerebellar PF-PC synapses needs to be balanced by other processes. A candidate for this role is cerebellar LTP. A form of postsynaptic LTP has been described (Lev-Ram et al., 2002) that is enhanced by chelating postsynaptic calcium and depends on NO but not on 3'5'-cyclic adenosine monophosphate (cAMP) or cGMP. PF long-term plasticity (both LTD and LTP) is regulated by calcium (Coesmans et al., 2004), and is characterized by a high calcium threshold for LTD induction and a lower calcium threshold for LTP induction.

Because activation of neighboring PFs would lead to an increase of $Ca^{2+}$, LTD will be favored over LTP upon PC activation. Therefore, PF-PC LTP might work more locally. This would enable PF-LTD and PF-LTP to work in synergy, actively shaping Purkinje cell output: 'correctly activated' PFs without CF activity are potentiated (LTP), whereas 'correctly activated' PFs with CF activity are depressed (LTD) (Coesmans et al., 2004). This is in line with a role of LTP in the active erasure of memory stored by PF-LTD as has been proposed earlier (Fujita, 1982).

Alternative sites of plasticity that regulate conditioned eye-blink responses have been proposed. Mossy-fiber (MF) inputs have an excitatory action in deep cerebellar nuclei as well as conveying information to cortical areas via PFs. Therefore, information about the eye-blink might be processed in parallel. PCs send strong inhibitory inputs to the deep cerebellar nuclei, regulating the MF induced excitation. When PCs are silenced or tuned down, they no longer exert their inhibitory action, allowing nuclear cells to respond to the excitatory inputs from MFs. This possible regulation of the MF-deep nuclei input led to the postulation of its role in eye-blink conditioning (Chen et al., 1999, Medina et al., 2000a, Medina et al., 2000b, Ohyama et al., 2002, Park et al., 2006).

## 5. The microtubule network and cerebellar motor learning

### 5.1. Cytoskeleton

The cytoskeleton is a dynamic network present in every cell. It plays an important role in the maintenance of cell structure, but also in the adaptation of cells to their environment. The cytoskeleton is composed of microtubules, actin, and intermediate filaments. Actin networks form from the cytoplasmic pool of unpolymerized or globular actin (G-actin). ATP-bound G-actin can polymerize into linearized single chains of polymeric filamentous actin (F-actin), upon which ATP is hydrolyzed. Polymerization

occurs head to tail, generating polarized filaments (De La Cruz et al., 2000). F-actin forms a meshwork or bundles that are mainly located at the cell periphery. The actin cytoskeleton is important for maintaining cell motility and polarity (Feldner and Brandt, 2002, Furukawa and Fechheimer, 1997). In neurons, actin filaments play a crucial role in endo- and exocytosis and also help to maintain synapse structure.

Intermediate filaments (IFs) have a diameter of 8-10 nm, in between the diameter of actin filaments and microtubules. The building blocks of IFs are coiled-coil dimers of two parallel chains. Two such dimers associate in an antiparallel manner forming a helical structure. Further polymerization creates an elongated structure with no polarity (Woll et al., 2005). This makes IFs different from microtubules and actin, which are polar filamentous networks. Several classes of proteins, including the keratins in the epithelium, and neurofilaments (light, medium and heavy) in neurons, can form IF networks (Steinert and Liem, 1990). IFs display a highly dynamic behavior during cell division and axon outgrowth in neurons (Eriksson et al., 1992, Eriksson et al., 2004). IFs are not involved in transport of organelles or other intracellular cargo but provide cells with mechanical resistance.

Microtubules (MTs) are composed of $\alpha$ and $\beta$ subunits of tubulin that form heterodimers. These heterodimers connect head-to-tail to form protofilaments. The elongation of MTs occurs by addition of tubulin dimers. Protofilaments associate laterally to form a cylindrical structure. Thirteen protofilaments assemble around a hollow core to make a polarized MT with 25 nm diameter (Raff, 1979, Chretien and Wade, 1991). Upon polymerization, the GTP bound to the $\beta$-tubulin subunit is hydrolyzed. The $\beta$-tubulin subunit is exposed at one of the two MT extremities, the plus end, which is more dynamic and grows faster than the other end, the MT minus end. In many cells the minus ends of MTs are embedded in the Microtubule Organizing Centre (MTOC). When attached to the MTOC MTs spread radially across the cell with plus ends directed to the periphery. However, other sites of MT nucleation and embedding have been described (Bornens, 2002), which must play a crucial role in neurons, where the MTOC remains in the cell body, and minus ends of MTs are located in the dendrites and axons and are somehow stabilized (Baas et al., 1988).

MTs display a characteristic pattern of dynamic behavior including treadmilling and dynamic instability. Treadmilling is characterized by a simultaneous growth at the plus end and shrinkage at the minus end (Margolis and Wilson, 1998). Obviously, treadmilling can only occur when the minus end is not embedded in the MTOC. Dynamic instability is characterized by alternating periods of growth and depolymerization of the MT at the plus end (Desai and Mitchison, 1997). This dynamic behavior of MTs is

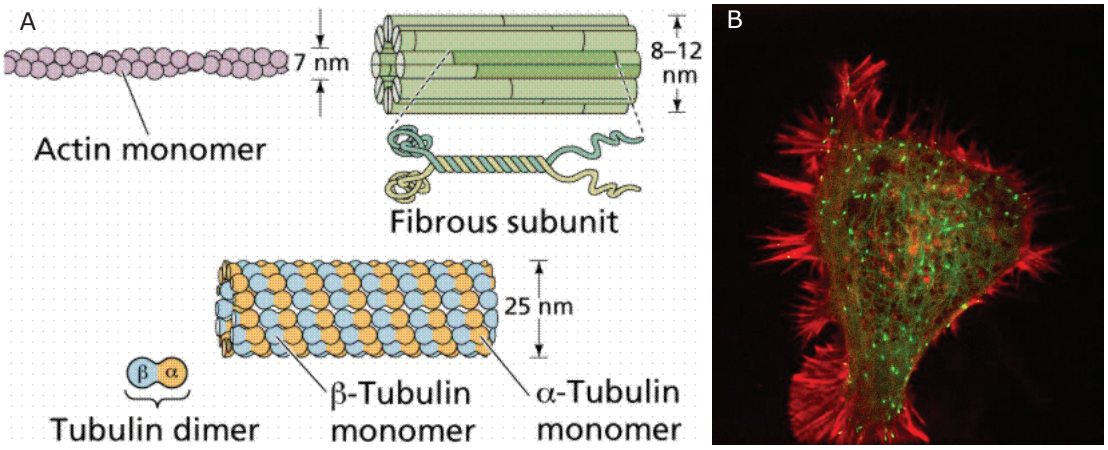



Figure 6 - A) Schematic representation of the three major components of the cytoskeleton, actin filaments (upper left), intermediate filaments (upper right) and microtubules (lower). Actin filaments are made of from unpolymerized or globular actin (G actin), present as a pool in the cytoplasm. ATP-bound G actin can associate to form a linearized single chain of polymeric filamentous actin (F actin). Upon polymerization ATP is hydrolyzed. Polymerization occurs head to tail, generating polarized filaments. Intermediate filaments (IFs) have a diameter of 8-10 nm, in between the diameter of actin filaments and microtubules. At the base of the filament is a coiled-coil dimer of two parallel chains. Two such dimers associate in an antiparallel manner forming a helical structure here depicted as "fibrous subunit". Upon elongation , a structure with no polarity is formed. Microtubules (MTs) are composed of a and b subunits of tubulin that form heterodimers. Tubulin heterodimers connect head to tail to form protofilaments. The elongation of MTs occurs by addition of tubulin dimers. Protofilaments associate laterally to form a cylindrical structure. Thirteen protofilaments assemble around a hollow core to make a polarized MT of 25 nm in diameter. Microtubules are hollow tubes made of intercalating repeats of two different subunits of tubulin and therefore intrinsic polarized (From Purves et al., Life: The Science of Biology, 4th Edition, Sinauer) B ) Photograph depicting the actin cytoskeleton (red) and the one of the +TIPS, EB1 of microtubules (green) (http://ijm2.ijm.jussieu.fr/ijm/research/research-groups/macromolecular-complexes-in-live-cells)

thought to be crucial for many of their functions in cells (Kirschner and Mitchison, 1986, Liao et al., 1995, Tanaka et al., 1995, Sharp et al., 2000), including changes in cell shape. Figure 6A depicts the three types of cytoskeletal elements mentioned.

### 5.2. Microtubule function in neurons

Neurons are highly polarized cells, with long dendrites and axons. Among the many functions of the neuronal MT cytoskeleton is the establishment of cell polarity. In axons, MTs are uniformly polarized with their plus ends oriented towards the axon terminal and their minus ends towards the cell body, while in dendrites, MTs display a mixed orientation (Baas et al., 1988). This MT arrangement helps neurons to selectively transport cargo destined for the axon and dendrites. As MTs are the only means by which cells carry out long-distance transport, the MT network is extremely important and needs to function optimally in neurons where very long distances need to be bridged.

MTs play a crucial role in the transport of necessary components for appropriate synaptic function from the cell body to the neurite extremity (Zakharenko and Popov, 1998, Winckler and Mellman, 1999). Inside growth cones, both MTs and actin filaments are involved in axon and growth cone guidance (Kalil and Dent, 2005).

Furthermore, MTs help to control axon branching (Kornack and Giger, 2005). A neuron specific regulation of MTs (and their dynamics) is suggested by neuron-specific MAPs like TAU and MAP2 (Wunderlich et al., 2006. See also (Houtman et al., 2007).

While MTs are abundant in axons and dendrites of PCs, they are not observed at the PF-PC synapses, sites where actin filaments abound (Brenman et al., 1998, Ito, 1984). Actin filaments anchor a structure visible with electron microscopy, called the post-synaptic density (PSD), which was described as a dense web of proteins underneath the postsynaptic membrane of synapses (Boeckers, 2006). Proteins present in the PSDs are cytoskeletal proteins, membrane bound receptors and channels, scaffold proteins and protein kinase/phophatases (Ziff, 1997, Walikonis et al., 2000). These proteins influence each other's localization and function. Signaling at the synapse can cause remodeling of the PSD.

Fast rearrangement of the PSD seems to be crucial for the alterations required for LTP and LTD (Hering and Sheng, 2001, Carlisle and Kennedy, 2005). As mentioned before, LTD involves the internalization of AMPA-type glutamate receptors (AMPAR), which are clustered at the PDS before internalization (Mayer and Armstrong, 2004, Collins et al., 2006). AMPAR can be inserted into and removed from the cell membrane in a very rapid subunit-dependent manner (Shi et al., 2001). Clustering is regulated by a scaffolding protein called glutamate receptor interacting protein (GRIP) (Dong et al., 1997) as well as the protein interacting with protein kinase C –1 (PICK-1) (Xia et al., 1999, Kim and Sheng, 2004). These proteins are part of a AMPAR complex (ARC), which helps in the recycling of AMPAR clusters (Boeckers, 2006) depending on the phosphorylation state of the receptor. Internalization via clathrin coated vesicles (Wang and Linden, 2000) is thought to occur concomitantly with actin depolymerization, and is helped by actin-binding motor proteins like myosin Va and myosin VI (Boeckers, 2006). A role for MT-mediated transport in the recycling of AMPAR clusters has also been reported (Zhou et al., 2001, Chen et al., 2007)

GRIP1 establishes a link between the actin and the MT cytoskeleton. On the one hand, it binds the vesicles containing AMPAR clusters while on the other hand it helps the MT-motor protein KIF5A to steer towards the dendrites (Chen et al., 2007). Scaffold proteins of the transmembrane AMPAR regulatory protein (TARP) category, that help to stabilize AMPAR clusters, are also associated with MTs (Tomita et al., 2004) and

are able to interact with MT-associated proteins (Shen et al., 2000). In addition to MT-related transport of AMPAR vesicles, lateral diffusion of AMPAR subunits has also been described as a regulatory mechanism (Adesnik et al., 2005). These examples illustrate roles for the MT and actin cytoskeleton in tasks related to learning. Both networks are involved in organizing synapses, each with its distinct function. It can therefore be expected that deregulation of the MT network leads to altered synaptic transmission and neuronal dysfunction.

The MT based transport network has also been implicated in transport of mRNA to the dendrites (Hirokawa, 2006, Chen et al., 2007), where a local protein translation machinery is present (Steward and Levy, 1982, Martin and Zukin, 2006). mRNAs known to be sorted to the synapse include glutamate receptor subunits, and the protein kinases αCaMKII and PKC (Steward and Schuman, 2003, Grooms et al., 2006, Mayford et al., 1996, Moriya and Tanaka, 1994). These studies have focused on hippocampal areas but dendritic protein synthesis is also believed to be involved in cerebellar plasticity (Schuman et al., 2006). Dendritic mRNA targeting and local protein translation provide a mechanism for fast, transcription-independent protein synthesis required for LTD (Huang et al., 2005, Karachot et al., 2001). Both the AMPAR trafficking and localized protein synthesis at the post-synaptic dendrite indicate a crucial role for the MT cytoskeleton in synaptic plasticity and cerebellar function. Figure 7 depicts the functions of the microtubule transport network in neurons

### 5.3. Microtubule plus end tracking proteins

MT dynamics are regulated by a vast array of MAPs. Plus end tracking proteins (+TIPS, Figure 6B) are a special class of MAPs that bind preferentially to the growing plus ends of MTs (Schuyler and Pellman, 2001). Examples of +TIPS are the cytoplasmic linker proteins of 115 and 170 kDa (CLIP-115 and CLIP-170, respectively) and the CLIP associated proteins (CLASPs) (Perez et al., 1999, Hoogenraad et al., 2000, Akhmanova et al., 2001). Binding of CLIPs to MTs occurs via so-called CAP_GLY motifs, which recognize the C-terminus of alpha-tubulin (Honnappa et al., 2006). The process leading to a specific association with the ends of growing MTs is still unclear.

CLIPs are redundant MT-rescue factors in non-neuronal cells (Komarova et al., 2002). CLIP-170, product of the *Clip1* gene, is also involved in binding endocytotic vesicles to MTs (Pierre et al., 1992) and helps to localize dynactin, an accessory factor of the dynein MT-motor complex, to the ends of growing MTs (Lansbergen et al., 2004). Another important characteristic of CLIP-170 is that it interacts with other +TIPs, possibly functioning as a recruitment factor. Besides the dynactin complex, CLASP1 and CLASP2 (Akhmanova et al., 2001), and LIS1 (Coquelle et al., 2002) might

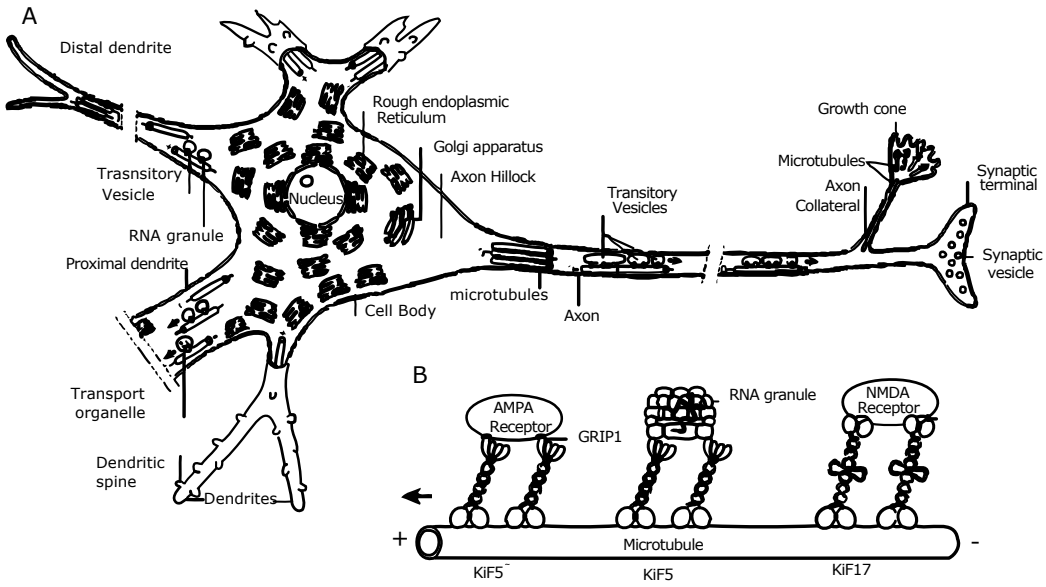



Figure 7– representation of the transport and structural roles of microtubules in neurons. The polarity of microtubules is an important factor in the establishment of the polarity of axons. In axons, microtubules are oriented in parallel with their + end towards the axon terminal. In dendrites, there is a mixed population of microtubules present in both possible orientations. Microtubules also play a role in the guidance of the growth cones. Typical transported vesicles are depicted, containing glutamate receptor moieties as well as mRNA destined for local translation. KIF5 and KIF17 are microtubule associated motor proteins. (Adapted from Hirokawa, 2006).

be recruited in this manner.

CLIP-115 and CLIP-170 are highly homologous. Interestingly, the gene encoding CLIP-115 (*Clip2*) is part of a region on human chromosome 11 that is hemizygously deleted in patients with a neurodevelopmental disorder called Williams Syndrome (Meyer-Lindenberg et al., 2006). The deleted region encompasses nearly 30 genes. A mouse model was made, in which the *Clip2* gene is deleted (Hoogenraad et al., 2002). The knockout animals mimic some of the neurological abnormalities in Williams Syndrome patients, including deficits related to the cerebellum (Hoogenraad et al., 2002, van Hagen et al., 2007). These results establish a link between cytoskeletal regulation and cerebellar function.

CLASPs have been identified in a search for CLIP-binding proteins (Akhmanova et al., 2001). They exist in several isoforms and bind to CLIP-115, CLIP-170 and to MTs directly. CLASPs are involved in the stabilization of microtubules at the leading edge of motile fibroblasts (Akhmanova et al., 2001). CLASPs stabilize MTs by regulating their growth speed and favoring rescue (Mimori-Kiyosue et al., 2005). CLASP2 is involved in the establishment of cell polarity necessary for fibroblast migration (Drabek et al., 2006). CLASPs are also involved in the regulation of mitosis and meiosis by influencing chromosome alignment, attachment of microtubules to kinetochores and

maintaining spindle polarity (Hannak and Heald, 2006, Inoue et al., 2000, Maiato et al., 2002). Thus, both CLIPs and CLASPs are positive regulators of MT dynamics. Both protein families are expressed in the brain and cerebellum (Hoogenraad et al., 2002, Akhmanova et al., 2005, Drabek et al., 2006).

Mice deficient in CLIP-170, CLIP-115 and CLASP2 have been generated in our group. The phenotypes of both CLIP mutants have been described (Akhmanova et al., 2005, Hoogenraad et al., 2002). Recently, a double knockout mouse model was obtained by crossing *Clip1* with *Clip2* knockout mice. The double knockout mice displayed a progressive hydrocephalus, which might be linked to abnormal neuronal migration (Miedema, 2007). *Clasp2* null mutants are about one third smaller than their wild type littermates and have hematopoietic defects. Like the *Clip1* and *Clip1/2* knockouts, they are severely impaired in male germ cell development (Drabek, 2005). As +TIPs are important for MT regulation, we became interested in testing the above mentioned mouse mutants for locomotor defects (see chapter 3). In this manner we wanted to analyze the contribution of the MT cytoskeleton to cerebellar plasticity and motor coordination.

## 6. Scope of the thesis

The work described in this thesis was aimed at elucidating two main questions. First, we wanted to establish the contribution of the nitric oxide-PKG pathway in PCs to cerebellar motor learning. The hypothesis was that PKG is crucial for LTD and that inhibition or ablation of PKG in the cerebellum would correspond to decreased learning of the eye-blink conditioning. To address this hypothesis, studies of locomotion and eye-blink conditioning were performed. Two mouse models of PC-specific PKG depletion were used. The first was a transgenic mouse model expressing a peptide inhibitor of PKG (PKGi) under the control of the L7-pcp2 promoter. The L7-pcp2 promoter drives expression exclusively in cerebellar PCs and retinal bipolar neurons. The second was a PC specific knockout of PKG (Feil et al., 2003a).

The second aim was to establish the contribution of the MT cytoskeleton to cerebellar function. The hypothesis was that a well regulated MT network is essential for the transport of vesicles and the regulation of intracellular processes required for LTD and that lack of +TIPs would correspond to abnormal locomotion or motor learning because of defects in the MT network. To address this question, locomotion studies were performed in mouse models lacking the +TIPs CLIP-115, CLIP-170 or CLASP2.

Before describing results obtained with respect to these two main questions,

I will introduce some of the technical approaches and learning paradigms in more detail.

## 7. Technical approach

### 7.1. Genetically modified mice

Many behavioral studies can be performed with naturally occurring mouse mutants that display cerebellar abnormalities. Similarly, lesion studies have produced extensive information about cerebellar function. Although this led to a better understanding of the cerebellum, tailor-made modifications are needed to pinpoint the effect of specific genes on a particular aspect of cerebellar function. The possibility of generating genetically modified mice has provided unprecedented opportunities to establish genotype-phenotype correlations. Many different transgenic mice have been generated to study behavioral outcomes (for review see Gaveriaux-Ruff and Kieffer, 2007, Lalonde and Strazielle, 2007b). In our studies, several type of mutant mice are used and the next sections aim at explaining briefly how they are generated.

#### 7.1.1. Classic transgenesis

Transgenic mice are defined as mice that have “extra” or “foreign” genes or DNA sequences added to their genetic content (Hogan et al., 1994, Auerbach, 2004). The foreign DNA often is a linear DNA molecule containing a fully functional transcription unit, which is injected into the male pronucleus of a fertilized mouse oocyte with a microscope-assisted microinjection setup (Hogan et al., 1994, Auerbach, 2004). The foreign DNA will integrate into the mouse chromosomal DNA at a random locations, often in concatemeric (head-to-tail) arrays. The number of integrated genes varies per oocyte.

Oocytes containing transgenic DNA are then introduced into the womb of pseudo-pregnant surrogate mothers. Founders (first generation progeny) bearing the transgene are either bred further to obtain a transgenic mouse line, or analyzed at the founder stage for specific phenotypes. Compared to other methods, transgenic mice are obtained relatively fast and easily. A major disadvantage of this technique is the lack of control over expression of the transgene due to random integration into the genome. The influence of regulatory elements nearby the insertion site and by epigenetic phenomena such as DNA methylation can reduce or completely shut off transgene expression (see Aronoff and Petersen, 2006 for review).

### 7.1.2. Gene targeting

A more subtle way of manipulating the genome and gene expression is homologous recombination in mouse Embryonic Stem (ES) cells and subsequent generation of mice from these ES cells (Thomas and Capecchi, 1987,Capecchi, 1989). The gene of interest is manipulated, for example, by replacing it with a bacterial marker gene, or by surrounding it with so-called loxP sites. In the first case the normal gene is not present anymore (knocked out). Because it is replaced by another gene this strategy is also called a "knock-in". In the second case the gene is surrounded by loxP sites (see section 7.2.1), but is still intact. Only upon a second recombination event will the gene be knocked out. This strategy is called "conditional knockout".

The mutated gene is electroporated into ES cells. If the DNA used has normal regions of homology flanking the mutant sequences, homologous recombination will occur, albeit at low efficiency. As a result, the transgene or foreign DNA can replace the endogenous gene. Clones of positively recombined ES cells are then inserted into a blastocyst. Recombined ES cells will divide along with the other "wild type" ES cells in the blastocyst to give rise to a mouse embryo. This mouse is chimeric in that it contains cells derived from the manipulated ES cells, as well as cells derived from non-manipulated ES cells. If the manipulated ES cells come from a strain with a different fur color than the non-manipulated ES cells in the recipient blastocyst, the chimeric mouse will have mixed fur colors (Hogan et al., 1994). The chimeric mouse is bred further and if the mutant ES cells contributed to the germline of the mouse, the mutated allele is passed on to the progeny. This event is called "germ line transmission".

As mentioned above, homologous recombination is used to create subtle mutations and is very precise. One problem associated with this technique is that mutations will be present in every cell of the mouse. Thus mutations are present throughout the whole development and in all areas of the body. These mutations can be lethal or cause developmental related problems that complicate the analysis of adult phenotypes (Aronoff and Petersen, 2006). Examples of mouse models in which the total ablation of the gene compromised the analysis of the phenotype are the PKCγ (Abeliovich et al., 1993) and the PKG type I (Pfeifer et al., 1998) knockout mice.

A second problem associated with ES cells is that (random) mutations might be introduced by culturing these cells. As these mutations are not planned, they are not obvious to the researcher. They segregate with the targeted mutation and can affect mouse behavior as well. The only way to lose these unwanted mutations is to cross the knockout mice back to inbred strains for many generations.

### 7.2. Spatio-temporal control of gene expression

#### 7.2.1. The Cre-lox system

Cre (Cyclization Recombination) is a DNA recombinase, an enzyme that has the capacity to excise and integrate DNA. Cre is part of the strategy used by bacteriohage P1 to integrate its genome into the *E. coli* host genome. Cre can excise DNA flanked by specific sequences, the loxP sites (Nagy, 2000), if these sites are inserted in the same orientation. LoxP site are small sequences of DNA (34 basepairs) bearing a palindromic sequence with two 13 bp inverted repeats and an 8 bp asymmetric core sequence (Sauer and Henderson, 1988, Sauer, 2002). Cre recombinase will bind to loxP sites and align two of them. By exchanging DNA strands between the two sites, DNA that was in between the two sites will be removed as a circular molecule containing one loxP site. The other loxP site remains in the genomic DNA.

Enzymatic activity of Cre can be controlled by fusing it to the ligand-binding domain (LBD) of the estrogen or progesterone receptors, members of the superfamily of steroid hormone receptors. These receptors will remain cytoplasmic until cognate hormone (or hormone analogs) binds to the LBD, upon which translocation of the receptor to the nucleus occurs. Fusion proteins that carry the LBD should show the same cytoplasmic retention in absence of hormone. The LBDs will confer theses properties to Cre as well. Mutated estrogen and progesterone LBD moieties have been developed, which are only responsive to synthetic hormone analogs and not to the naturally occurring molecules (Kellendonk et al., 1996, Feil et al., 1997, Kellendonk et al., 1999). Further engineering increased the levels of inducibility of the system and reduced its leaky background activity (Indra et al., 1999). The so-called CreERt2 LBD contains a mutated version of the human estrogen receptor LBD (Imai et al., 2001) that shows relatively good induction levels and was used in the work described in this thesis.

Both estrogen and progesterone analogs have been shown to cross the blood-brain barrier. Thus, the inducible Cre-lox system appears attractive for use in inducible recombination events in the brain. However, the rates of recombination of Cre fused to LBD in the brain seem lower than in other parts of the body (Vooijs et al., 2001, Casanova et al., 2002).

#### 7.2.2. The Tet-on/Tet-off system

Another system promising the possibility of spatio-temporal controlled regulation of transgene expression, is the Tet system (Baron and Bujard, 2000), which is based on the bacterial tetracycline-resistance operon. In this system the Tet-repressor binds

tetracycline responsive element (TRE) sequences in the DNA with very high affinity. Addition of tetracycline relieves DNA binding.

Improved versions of the Tet-system (Tet-On/Tet-Off) allow for better control over transgene expression. In Tet-regulated gene expression systems the gene of interest is under control of a minimal Cytomegalovirus (minCMV) promoter (which lacks sequences necessary to drive gene expression), in combination with TREs. The expression of genes placed downstream of this recombinant promoter is now dependent on the binding of an activator. In these systems, a mutated Tet-repressor has been fused to a potent transcriptional activation domain, namely that of the VP16 protein, thus promoting transcription upon binding. The nature of the repressor protein is the key difference between Tet-On and Tet-Off. In the Tet-Off system, the fusion protein is called tetracycline-controlled trans-activator (tTA) and upon tetracycline administration, it will unbind the DNA and stop transcription. In the Tet-ON system, the fusion protein is called reverse tetracycline-controlled trans-activator (rtTA) and will bind and promote gene expression upon tetracycline administration (Mansuy and Bujard, 2000, Corbel and Rossi, 2002, Mizuguchi and Hayakawa, 2002). The tetracycline analog doxycycline has been chosen as the most efficient activating moiety for both systems.

The Tet-On system has received special attention because it alleviated the need to keep mice under antibiotics (Doxycycline) for long periods of time. This system has been used to drive brain specific expression of a calcineurin inhibitor (Malleret et al., 2001). More recent reports appeared a few years later describing the use of modified versions of the rtTA (Yamamoto et al., 2003, Michalon et al., 2005, Uchida et al., 2006).

### 7.2.3. The L7/Pcp-2 promoter

Purkinje cells express the Purkinje cell specific protein 2, also referred to as L7 (L7/pcp-2, Oberdick et al., 1990), that is also expressed in retinal bipolar neurons (Berrebi et al., 1991). L7/pcp-2 is thought to be play a role in the regulation of voltage gated calcium channels (Kinoshita-Kawada et al., 2004) and seems to be dispensable for the functioning of the PCs (Vassileva et al., 1997).

The L7 gene and its promoter have been cloned as a ~3 kb DNA fragment, and have been characterized in detail (Oberdick et al., 1990). Other genes have been cloned into the L7 locus, once introduced as transgenes these were expressed specifically in PCs. The translation start site (ATG) of the L7 gene at the beginning of exon 2 has been removed, allowing the transcription of transgenes bearing a start codon cloned into exon 4 (Smeyne et al., 1995). This L7 cassette has been used to drive expression

of several genes of interest in PCs (De Zeeuw et al., 1998a, Oberdick et al., 1998, Barski et al., 2000, Tomomura et al., 2001, Zhang et al., 2001). Indeed, mouse lines expressing the Cre recombinase specifically in PCs were announced as early as 1996, Cre being driven by the L7 promoter (Tsien et al., 1996).

Other L7-driven Cre mice generated a few years later (Barski et al., 2000), were at the basis of the successful specific inactivation of PKG (Feil et al., 2003b) or Calbindin (Barski et al., 2002, Barski et al., 2003) in PCs. Using a bacterial artificial chromosome (BAC), an improved L7-Cre mouse has been generated, showing tight control of spatial expression of the transgene (Zhang et al., 2004). Finally, Cre recombinase was targeted into the endogenous L7 locus using a knock-in approach and a new mouse model, expressing Cre under the control of the endogenous L7 promoter, was obtained (Saito et al., 2005).

## 7.3. Testing mouse behavior

### 7.3.1. Measuring the vestibulo ocular reflex

A moving object or visual field can cause a blurred image on the retina due to "slip" in respect to retinal photoreceptors. Avoiding this visual disturbance is a constant operation of the visuo-vestibular system. Eye movements are used to stabilize moving images on the retina, or to stabilize the gaze in response to head movements. This reaction is called vestibulo ocular reflex (VOR) and is a reflex that can be trained. The adaptation is a classical setup used to study cerebellar learning. Just like in the eye-blink conditioning, the neuronal circuits and mechanisms controlling VOR have been extensively studied.

A VOR assay was developed at the Department of Neuroscience in the Erasmus MC (de Zeeuw et al., 1998, van Alphen et al., 2001). In this assay animals are completely immobilized on a rotating platform, which is placed within an independently rotating drum. The rotating drum contains a non-neutral visual field, for example, a bar code, which serves to guide eye movements. A copper wire coil is surgically implanted into the eye, allowing monitoring of the eye position by magnetic field coils mounted onto the restrain assembly.

The table with the immobilized animal is rotated in one direction and the drum with the visual stimulus in the other. In order to prevent retinal slip, animals need to produce eye movements of appropriate amplitude to include the rotation angle of the platform plus the angle of rotation of the visual stimulus. After an appropriate number of trials, the eye movements generated in response to an induced head rotation are altered from the naïve situation (i.e. increased). This alteration will still be present when animals are tested without visual stimulus (in the dark). This is called VOR

adaptation, because the angle of rotation of the eyes in response to a rotation of the head will change from the untrained situation, creating a learned response of "adapted" amplitude.

### 7.3.2. Measuring eye-blink conditioning

The neural basis for eye-blink conditioning has been discussed in section 2.2. The experimental setup used was the Magnetic Distance Measurement Technique (MDMT), which was developed at the Department of Neuroscience in the ErasmusMC (Koekkoek et al., 2002). Air puffs to the mouse eye are delivered via a hollow tube that is controlled by a variable pressure control system. This tube is held in close proximity to the eye with the help of a custom made pedestal that is surgically attached to the head of the mouse. A magnetic current detector on the pedestal continuously measures the movements of a small magnet inserted in the skin of the lower eyelid. The output of the magnetic current detector is recorded by a computer that also controls the delivery of the air puffs. Mice can move freely during the measurements.

### 7.3.3. The Rotarod

The rotarod consists of a rotating cylinder (usually 3 cm in diameter) that is held in place approximately 15 cm from the floor plane. The cylinder can rotate at constant or accelerating speed, usually up to 40 rpm. In order to stay on rotating rod, mice need to move constantly. This is a task that requires balance and the capacity to learn new movements and is considered to be a sensitive measure for cerebellar dysfunction (Crawley, 1999, Lalonde and Strazielle, 2007a). The parameter measured with the rotarod is the latency before falling off.

A commercial rotarod setup is available, but other setups have also been devised (e.g. (de Zeeuw et al., 1998, van Hagen et al., 2007). The setup commonly used at the Neuroscience Institute at the ErasmusMC, has a smooth surface and the diameter of the cylinder is wider. Results obtained with this setup are difficult to compare to those obtained with standard, commercially available rotarods. It could be that the non-commercially available setups pose a greater challenge to the mice and therefore increases voluntary jumping or accentuates cerebellar defects (Lalonde and Strazielle 2007a).

#### 7.3.4. The Erasmus Ladder

The Erasmus Ladder is another setup that is currently being developed at the ErasmusMC. This setup will be briefly described as a possible tool for future

experiments.

The Erasmus Ladder is a fully conFigureurable horizontal ladder with a shelter at both ends. Each shelter contains two small air nozzles for pressurized air delivery. The horizontal ladder has 37 rungs. Each rung consists of a left and a right pressure sensor attached to rods that are 2 mm apart. The distance between the individual rungs is 15 mm and each alternating rung is descended 13 mm. On the left side the even rungs and on the right side the odd rungs are descended thereby creating an alternated stepping pattern with 30 mm gaps. Each rung is fixed to a high-speed pneumatic slide, which provides vertical position control of the rung. A computer system records sensor data, adjusts air pressure, predicts future touches, calculates interventions, repositions slides and stores data every 2 ms.

According to the current experimental protocol, a mouse is placed in one the shelters. Activation of the bottom air nozzles in this shelter usually causes the mouse to leave the shelter immediately. Airflow of 15 km/h in the tunnel encourages the mouse to walk quickly over the ladder to the opposite shelter. Mice that do not learn to cross the ladder with a constant speed in the first session are excluded.

After the training sessions, mice are conditioned to avoid an ascending rung as

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# Chapter II

# Role of protein kinase G-dependent long-term depression in motor learning

# Role of protein kinase G-dependent long-term depression in motor learning

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## Summary

Nitric oxide is a short-lived gas that can diffuse into synapses, acting on guanylyl-cylase, and activating a cyclic GMP-dependent protein kinase, called PKG. PKG is thought to be involved in long-term depression (LTD) in Purkinje cells of the cerebellum by blocking the dephosphorylation of AMPA receptors. In this study, we studied the effect of inactivation of PKG in the cerebellum on eye-blink conditioning, a paradigm of cerebellar-dependent motor learning. We established that a PKG inhibitory peptide (PKGi) successfully blocks LTD in vitro. We subsequently generated transgenic mice expressing this inhibitor, but the level of PKGi in the cerebellum was not high enough to warrant a full *in vivo* analysis. We therefore analyzed mice with a conditional PKG knockout allele, which lack PKG in Purkinje cells ($PKG^{pko}$). Using these mice, we report an effect of PKG in the amplitude and percentage of conditioned eye-blink responses but not in the timing of the responses.

## Introduction

The cerebellum is involved in the adaptation or learning of movements (Mauk, 1997), (Kassardjian et al., 2005), among others to allow animals to carry out smooth and accurate movements, even at high speed and without the need for visual feedback (Ito, 2000). The eye-blink conditioning paradigm is an example of cerebellar-dependent associative learning. In this assay a puff of air is directed at the cornea of the eye, which causes a fast closure of the eyelid. If the puff is coupled to a sound animals learn to respond to the sound and close their eyelids even in the absence of the puff (Schneiderman et al., 1962).

Purkinje Cells (PCs), the sole output neurons of the cerebellar cortex, are thought to be a major site of convergence for associative learning. The concerted action of parallel fibers (PF) and climbing fibers (CF), the main excitatory inputs to PCs, causes an input-dependent reduction of the synaptic efficacy of PF to PC synapses, which is called long-term depression, or LTD (Ito, 2001). LTD is prominently post-synaptic, taking place in PCs. Strong links between cerebellar LTD and associative learning have been obtained, among others in our group (Koekkoek et al., 2003), although

controversy remains about the exact mechanism of cerebellar learning (Kassardjian et al., 2005; Welsh et al., 2005).

Protein kinase C (PKC) and cyclic GMP –dependent protein kinase (PKG), are serine-threonine protein kinases, at the basis of distinct signal transduction pathways. Both kinases are thought to be central to the establishment of LTD (Ito, 2001, 2002; Lev-Ram et al., 1997a; Lev-Ram et al., 1995; Levenes et al., 1998; Linden and Connor, 1991; Reynolds and Hartell, 2001). The PKG pathway was considered to be an accessory pathway for the establishment of LTD (Ito, 2001) as studies in cell culture and with pharmacological inhibitors provided contradictory data about the participation of PKG in LTD (Hartell et al., 2001; Lev-Ram et al., 1997a; Linden and Connor, 1992; Reynolds and Hartell, 2001). PKG acts downstream of nitric oxide (NO), which is released from PFs and readily diffuses into the PCs, after which it activates soluble guanylyl cyclase and enables production of cGMP (Daniel et al., 1998). One of the targets of cGMP is PKG; by phosphorylating G-substrate (Endo et al., 1999; Hall et al., 1999), PKG causes the inhibition of protein phosphatases. A prominent target of protein phosphatases are the $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors (AMPAR). By blocking protein phophatases, PKG tilts intracellular equilibrium towards the phosphorylated state of AMPAR. As phosphorylated receptors are more prone to removal from the plasma membrane by internalization, LTD is induced by the action of PKG (Wang and Linden, 2000). Interestingly, serine 880 of AMPAR subunit 2 is a phosphorylation target of PKC (Chung et al., 2003). So, on this subunit the PKC and PKG signaling pathways converge.

Lack of PKC activity in PCs, and its effect on LTD (De Zeeuw et al., 1998; Gao et al., 2003; Goossens et al., 2001) as well as on eye-blink conditioning (Koekkoek et al., 2003) has been studied in great detail. Activation of the NO-PKG pathway is necessary for LTD establishment, as mutant mice lacking neuronal NO synthase failed to show LTD (Lev-Ram et al., 1997b). Furthermore, PKG-dependent LTD has been shown to be associated with vestibulo-ocular reflex (VOR) adaptation in mice (Feil et al., 2003a). However, the role of PKG in eye-blink conditioning has not been tested yet.

The enzymatic activities of PKC and PKG are inhibited by pseudo-substrate domains, located in the N-terminal regions of the respective proteins ((Busch et al., 2002; House and Kemp, 1990). In the case of PKC a sequence coding for the auto-inhibitory peptide has been cloned into a Purkinje Cell specific promoter sequence to generate transgenic mice (De Zeeuw et al., 1998). To study the effect of PKG, a synthetic peptide was prepared, based on the sequence of an inhibitory substrate, obtained by screening a combinatorial library (Dostmann et al., 1999), and used *in vivo* to block enzymatic activity. The main aim of this study was to further clarify the contribution of PKG to eye-blink conditioned responses. We followed different approaches. First, we aimed at generating novel transgenic mice in which the expression of an inhibitory peptide to PKG (PKGi) could be manipulated in a spatio-temporally controlled manner. We confirmed that a synthetically produced PKGi peptide affected PCs *in vitro*. Subsequently, we made three types of transgenic mice: mice in which PKGi was under control of the L7 gene, which is expressed specifically in cerebellar PCs (Oberdick et al., 1990). The L7 promoter has been used to drive PC-specific expression in several other studies (Barski et al., 2000; De Zeeuw et al., 1998; Oberdick et al., 1993; Paradies et al., 1996; Zhang et al., 2001). Second, we

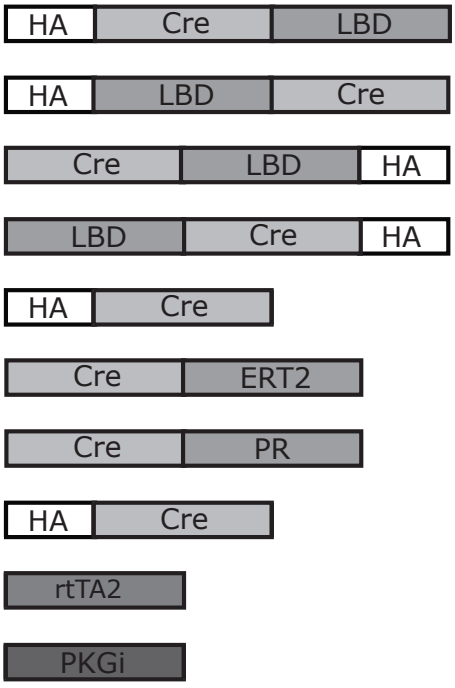


Figure 1 – Scheme of the different Cre-LBD constructs. The Cre recombinase (Cre) was cloned together with the estrogen receptor ligand-binding domain (LBD) in different relative positions, with a triple hemaglutinin (HA) tag. Constructs with Cre fused to newer versions of the estrogen receptor LBD (ERT2) or the progesterone receptor LBD (PR) are also shown.

generated mice in which the PKGi peptide was under control of other regulatory sequences (see below). Third, we made transgenic mice expressing the regulators under control of the L7 promoter. By crossing mice we aimed to obtain PC-specific ablation and re-expression of PKGi. The efficiency of the strategy was compromised due to low levels of expression of the transgenes.

Mice lacking PKG type I selectively in Purkinje cells have been developed by crossing PKGI floxed mice (Wegener et al., 2002) with L7Cre mice (Barski et al., 2000). PKGi conditional knockout mice display normal cerebellar morphology, normal calcium signaling, normal motor coordination and normal eye movement performance. They do however show impaired LTD and impaired adaptation of the VOR (Feil et al., 2003a). We used this established PC-specific conditional PKGI knockout mouse model to study eye blnk conditioning. Our results show a marked difference in performance of these mice in the eye-blink conditioning. This study shows that PKG in cerebellar Purkinje Cells is necessary for correct conditioned responses but nor for the precise timing of the responses.

## Materials and Methods

*Constructs*

Plasmids encoding Cre recombinase and the estrogen receptor (ER) ligand binding domain (LBD) were kind gifts of Dr Pierre Chambon. These constructs were used to generate new fusion proteins (Cre 1-5; see Figure 1), in which the positions of Cre and LBD were switched in order to determine the optimal placement of Cre and LBD. A triple hemaglutinin (HA) epitope tag was added to each fusion protein to allow detection with anti-HA antibodies. A second version of Cre, fused with the

human estrogen receptor LBD (CreERT2) was also obtained from Dr Pierre Chambon. A construct containing Cre fused to the human progesterone receptor (CrePR) was obtained from Dr Masaioto Mishina. The PUHD-rtTA2$^{s}$-M2 plasmid (further referred to as rtTA2) encodes the reverse tetracyclin transactivator protein (Urlinger et al., 2000) and was obtained from Dr Hermann Bujard.

A modified version of the L7 promoter, called L7ΔAUG (kind gift of Dr. John Oberdick) has a BamHI cloning site in exon 4 and lacks the endogenous translation start site (Smeyne et al., 1995), thus allowing expression and translation of transgenes inserted at the BamHI cloning site and bearing their own ATG codon. All Cre constructs and the rtTA2 encoding cDNA were cloned into the BamHI site of the L7ΔAUG construct. Oligonucleotides (see Table I) encoding PKGi (MTQAKRKKALAMA, see (Dostmann et al., 1999)) were annealed and ligated into the BamHI site of L7ΔAUG.

*Transgenic mice and treatments of mice*

The constructs bearing HaCre, HaCreLBD, CreERT2, CrePR, rtTA2 and PKGi under the control of the L7 promoter were used to produce transgenic mice. DNA from each construct was digested with HindIII/EcoRI, purified with QiaQuick columns (Qiagen), and used for pronuclear microinjection in FVB/N fertilized oocytes at a concentration of 1 μg/ml in 8mM Tris (pH: 7.5), 0,1 mM EDTA (Hogan et al., 1994).

The c*GKI*$^{PKO}$ mice have been described previously (Feil et al., 2003a; Wegener et al., 2002) and are here referred to as *PKGI*$^{pko}$. L7HaCre transgenic mice were crossed with ROSA26-LacZ reporter mice (Soriano, 1999). L7CreERT2 or L7CrePR transgenic mice were crossed with *Clip2*$^{fl/fl}$ (Hoogenraad et al., 2002). L7rtTA2 transgenic mice were crossed with a TRE-GFP reporter mouse (obtained from Dr. Rini de Crom).

L7CreERT2 and L7CrePR mice were treated with tamoxifen or Mifepristone (Ru486; Sigma), respectively. Injections were done once per day, intraperitoneally (i.p.). Tamoxifen (10 ng/μl; free base) was dissolved in 100 μl of sunflower seed oil (Casanova et al., 2002; Imai et al., 2001; Weber et al., 2001). RU486 (25 μg/μl) was dissolved in 100 μl of carboxymethyl cellulose/tween 80 (Kellendonk et al., 1999). As recombination is expected to occur after 5 days for CreERT2 (Feil et al., 1997) and 4 days for CrePR (Kitayama et al., 2001), treatments were stopped after these time periods. RtTA2 -GFP mice were fed doxycycline in the drinking water (1mg/ml) as previously described (Gallagher et al., 2003). Doxycycline was refreshed 3 times a week and administration was continued for 3 weeks.

*DNA and RNA detection*

Genomic DNA was isolated from tail snips of mouse pups, digested with EcoRI or HindIII and analyzed by Southern blot according to standard procedures (Sambrook et al., 1989). Transgenic mice were screened by PCR (see Table I) or Southern blot with a L7 specific probe (Sambrook et al., 1989).

Total RNA was extracted from adult mouse tissues (cerebrum, cerebellum, kidney, liver) using RNAzol (Campro Scientific) and treated with RNAse guard (Pharmacia). RNA was analyzed by RT-PCR (see Table I for primers) or northern blot.

Quantitative PCR (real-time PCR) was performed according to standard methods (BioRad) in a BioRad I-cycler, using the L7 and PKGi primer combinations and HPRT as control. Briefly: 40 μl reaction tubes were prepared with 250 nM primers, 0,5 μl cDNA each sample and a 1:1 dilution (20 μl) of the SYBR green Buffer (Eurogentech).

Samples were run in triplicate and results are presented as mean values. Cycle conditions were an initial denaturing cycle of 95oC, 15 min followed by 45 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s. Calculations were performed as described before (Pfaffl, 2001). Values were normalized to HPRT expression.

*Immunofluorescence and immunohistochemistry*

COS-1 cells were transfected using Superfect (Qiagen). Cells were allowed to recover for 24 hr. Tamoxifen (4-OHT) was added at a concentration of 100nM for 24 hr (Feil et al., 1997). For immunofluorescence studies cells were washed with PBS and fixed in 2% paraformaldehyde (20 min at room temperature), followed by 5 min in 0.1% Triton-X100/PBS. Samples were blocked in 0.5% BSA/0.02 glycine/PBS. The slides were labeled with mouse monoclonal antibodies against HA (1:500) (BabCo) for 1 hour. After washing in 0.05% Tween-20/PBS slides were incubated with sheep-antimouse rhodamine-conjugated secondary antibody (1:25, Boehringer), washed again, and mounted in Vectashield (Vector laboratories) containing 4',6 Diamidine-2-phenylindole-dihydrochloride (DAPI) and 1,4-diazobicyclo[2,2,2]-octane (DABCO). Fluorescence analysis was performed as previously described (Hoogenraad et al., 2000).

*Western blot analysis*

COS-1 cells grown on 12 cm plates were transfected using the DEAE-Dextran method (Sambrook et al., 1989). After 40 hr cells were split in two samples. One of the samples was treated with 4-hydroxytamoxifen (100 nM), the sample was not treated. Cytoplasmic and nuclear protein extracts were prepared as previously described (Schreiber et al., 1989). Nuclear fractions were frozen and thawed three times and sonicated. All samples were sonicated and boiled in SDS sample buffer and analyzed on 12% SDS - polyacrylamide gels (Sambrook et al., 1989). Western blots were prepared as previously described (Hoogenraad et al., 2000) and analyzed with mouse anti HA antibodies (1:1000) and a Fab goat anti-mouse conjugated with alkaline phosphatase (1:5000, Sigma) secondary antibody.

*In vitro electrophysiology experiments*

Sagittal slices from male FVB/N mice were prepared from the cerebellar vermis as previously described (Conquet et al., 1994). Briefly, the mice were stunned and decapitated, and cerebellar slices (200 mm thick) were cut with a vibroslicer (Campden Instruments LTD). Whole-cell patch-clamp recordings of PCs were performed with an Axopatch-1D amplifier (Axon instruments). As reported previously (Goossens et al., 2001), PCs were clamped at -70 mV and PFs were stimulated at 0.33 Hz. Throughout the recording, PF-mediated EPSCs were elicited on a 10 mV hyperpolarizing voltage step.

In pairing experiments, PF-mediated EPSCs were first evoked in PCs during a control period of at least 5 min. The first pairing was performed 10 min. after the beginning of the session. Five min. later, a second pairing was performed. For analysis, electrophysiological data were filtered at 2kHz and digitized at 20 kHz, PF-mediated responses were analyzed on-line and off-line by using the Acquis1 computer program (Biologic).

A PKG inhibitory peptide with the sequence MTQAKRKKALAMA (Dostmann et al.,

1999; Dostmann et al., 2000) was synthesized using an Applied Biosystems Model 432A "Synergy" peptide synthesizer (Applied Biosystems). The peptide was lyophilized and frozen until needed. Rehydration was performed in water, 1M stock solutions were used.

*Rotarod studies*

L7PKGi mice were analyzed in the accelerating rotarod setup (UgoBasile). Mice were placed in individual segments of the Rotarod. The rotating cylinder was constantly accelerated from 2 to 40 rpm. The latency of each animal to fall was registered. The exercise ended either when the mice fell, or after 300 sec. The training scheme was composed of 5 daily session in consecutive days. Data was analyzed using the Prism software (GraphPad).

*Eye-blink conditioning*

Mice were analyzed in their response to the eye-blink conditioning using the Magnetic Distance Measurement Technique (MDMT) (Koekkoek et al., 2002). Briefly, a pedestal containing a magnetic current detector put in place via a premade adapter that holds the end of a hollow tube directly onto the proximity of the eye, is surgically attached to the head of the animals. The air tube is linked to a variable air pressure controller system, thus allowing a puff of air to be directed to the animal's (left) eye.

Surgery was performed under anesthesia with an oxygenated mixture of isofluorane and nitrous oxide. The pedestal (both the air tube and the wires) is connected via a swivel to a computer setup that allows the continuous measurement of the movements of a mini magnet inserted in a pocket under the eyelid. The swivel held in place with the help of a specially made "harness" adjusted around the ribcage and the front legs. This allows a broad range of movements inside the measuring cage while constantly monitoring the movements of the animal and the eyelid and providing the opportunity to deliver precise air puffs.

*PKGI$^{pk0}$* mice (n=10) and control littermates (n=11), housed in groups, were used at ages between 14 and 18 weeks. Animals were kept on a 12h light/dark cycle with unrestricted access to fresh water and food. Mice were trained in a schedule of 5 days. Day 1 consisted of habituation, i.e. mice were allowed to adjust to the new environment of the measuring cages. From days 2 to day 5 a pairing protocol was used. A tone of 1 kHz, 78 dB was followed by an air puff, with an ISI of 350 ms. Each day of measurement consisted of 8 sessions of 8 trials. The first trial on every session was a conditioned event (sound only) and the last trial of every session an unconditioned event (air puff only). The other 6 were paired events (air puff and sound). Data was gathered as previously described (Koekkoek et al., 2002) using MATLAB (The MathWorks, inc) software and analyzed statistically with SPSS software (SPSS, Inc.).

## Results

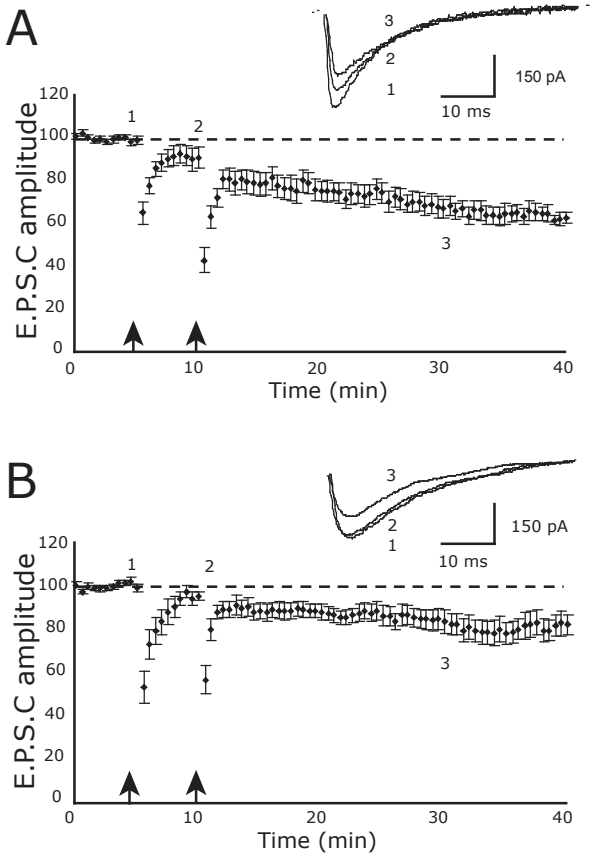


Figure 2 – EPSCs evoked by pairing stimulation of parallel fibers and depolarization of Purkinje Cells in slices, as measured by patchclamp. In (A) non-treated slices from wildtype juvenile FVB/N mice were tested. In (B) slices microiontophoretical application of a PKG inhibitor peptide was performed. 1, 2 and 3 indicate the different time points, i.e. 5, 10 and 30 minutes, respectively. Block arrows indicate a paired stimulation. The curves at the upper right corner of each image (A and B) represent the actual shape of the measured EPSCc, while the plot with error bars represents the average of the peak of several measurements.

Table I. Primers used for cDNA constructs and PCR-based analysis.

| name | sequence (5' to 3') | function |
|---|---|---|
| PKGi sense | GATCCATCATGACTCAGGCTAAGCGTAAGAAG GCTTTGGCAATGGCTTAAG | Generating PKGi coding sequence |
| PKGi antisense | GATCCTTAAGCCATTGCCAAAGCCTTCTTACGC TTAGCCTGAGTCATGATG | |
| P26 | TTCTTCAAGCTGCCCAGCAGAGCTC | PCR screen of L7PKGi transgenes |
| P33 | AATGCAGAAACGACCTGGGA | |
| P35 | AAGGCTTCTTCAACCTGCTGACC | L7 RT-PCR and/or real time PCR |
| P27 | GGGCTGCTGTTCCTGCGGAAGC | L7 RT-PCR and/or real time PCR (with P35) |
| P36 | TTGCCAAAGCCTTCTTACGCT | L7-PKGi RT-PCR and/or real time PCR (with P35) |
| P12 | GGACAGAAGCATTTTCCAGGTATG | |
| HPRT sense | AGTGATAGTCCATTCCTATGACTGTAG | Real time PCR control |
| HPRT antisense | GTTAAAGAGAGATCATCTCCACC | |

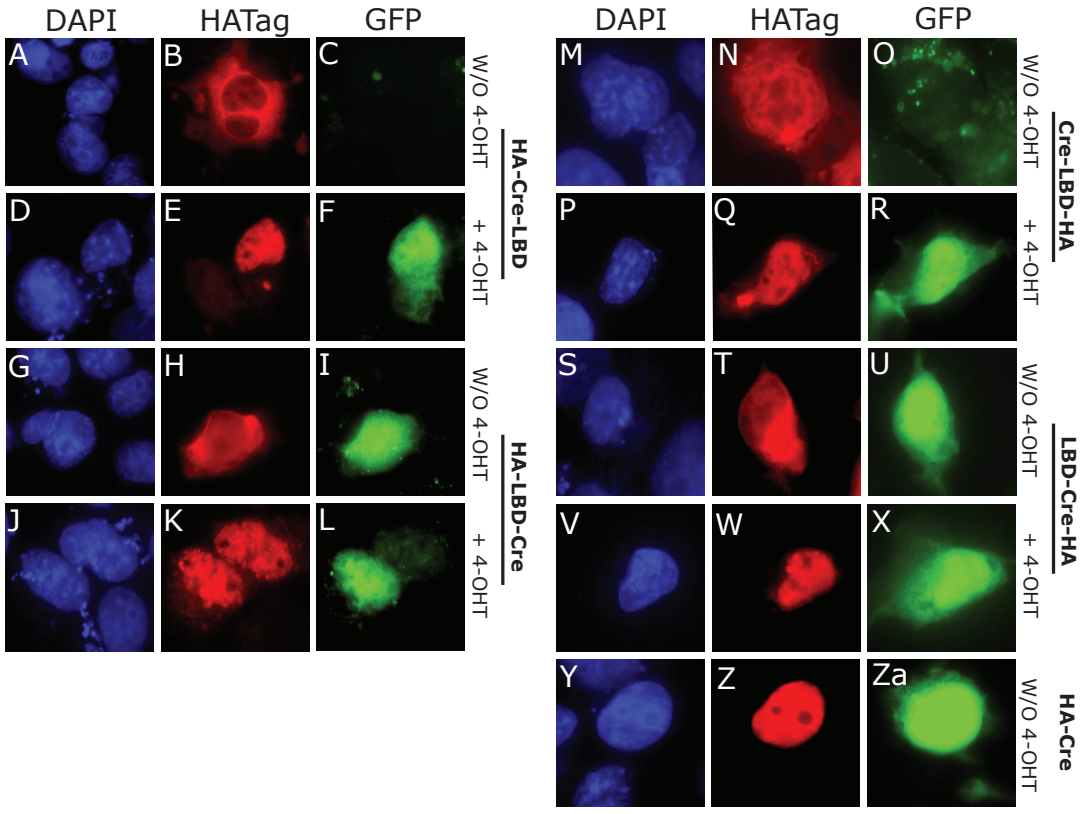


Figure 3 - Immunofluorescence analysis of transfected cells. COS-1 cells were co-transfected with Cre1-5 (described in Fig 1) and a "tester" plasmid containing a Lox-Puro-Lox-GFP cassette. Cells were either not treated, or treated with 4-hydroxy tamoxifen (4-OHT), 100 mM. After treatment cells were fixed and stained with anti-HA antibodies (red, middle panels) to detect Cre fusion proteins. DAPI staining (in blue, left panels) shows the nucleus of the cells. GFP signals (green, right panel) indicate recombination events.

*Effect of in vitro inhibition of PKGI in LTD of cerebellar PCs*

A synthetic peptide inhibitor of PKG (PKGi) was obtained after screening a combinational library, showing 100-fold better inhibition of PKG as compared to PKA (Dostmann et al., 1999). We used this peptide to assay inhibition of LTD induction in *in vitro* assays in cerebellar slices of wildtype mice. From this slices, in 9 PCs obtained from 4 wild-type mice, the pairing protocols of PF-mediated EPSCs with Ca2+ spikes induced a clear-cut LTD of PF-mediated EPSCs, which lasted for at least 30 minutes. The mean amplitude of the PF-mediated EPSCs was 68.38% ± 4.77% of control (mean ± SE) 20 min after the pairing period (Figure 2A). In another group of 9 PCs prepared from 7 wild-type mice, we applied 20 μM of the PKG inhibitory peptide to the internal solution of the recording electrode. In these conditions, PF-mediated EPSCs remained constant in amplitude during the control period. The presence of the inhibitor in the recording pipette significantly reduced (Student's t test ; $P < 0.05$) the amplitude of the LTD as compared to the control group. The mean amplitude of the responses was 84.95% ± 4.63% of the control value, 20 min after the pairing period (Figure 2B). Therefore, the present results show that inhibition of PKG partially blocks induction of LTD.

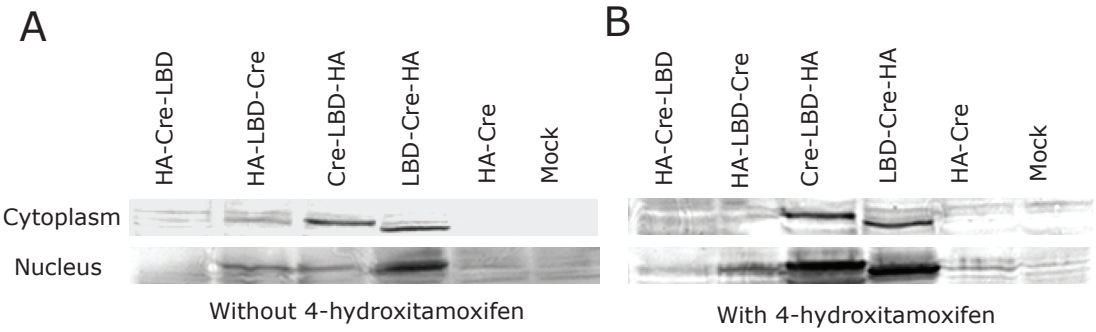


Figure 4 – Analysis of nuclear translocation of CreLBD. Western blots contain cytoplasmic and nuclear fractions of transfected COS-1 cells. COS cells were transfected with Cre1-5 (see Fig 1). Extracts were prepared before treatment with 100 mM 4-OHT (A) or after 4-OHT treatment (B). Blots were incubated with anti-HA antibodies, allowing detection of the CreLBD fusion proteins (78 kDa).

Table II – transgenic mouse lines with L7 constructs

| Transgene | L7CreLBD | L7CreERT2 | L7CrePR | L7HACre | L7rtTA2 |
|---|---|---|---|---|---|
| Founders | 9 | 2 | 3 | 2 | 8 |
| Lines | 6 | 1 | 2 | 2 | 3 |

*Regulation of Cre activity by LBD*

Cre recombinase removes any DNA sequence that is present between two loxP sites, if these sites are in the same orientation (Sauer, 2002). Cre has been widely and successfully used to modify the mouse genome in vivo. Brain specific recombination has been reported but is often incomplete (Seibler et al., 2003; Tsien et al., 1996). We wanted to use the Cre recombinase system to manipulate the expression in the cerebellum.

When fused to the ligand binding domain (LBD) of the estrogen, or progesterone, steroid hormone receptors, the activity of Cre becomes hormone-regulatable (Kellendonk et al., 1999; Zhang et al., 1996). In trying to optimize efficiency and regulatability of Cre, we tested whether the position of the LBD with respect to Cre was important (Casanova et al., 2002; Kellendonk et al., 1999; Zhang et al., 1996). We made 5 different constructs with in which the LBD was either put N-terminal to Cre or C-terminal (Figureure 2). A triple HA-epitope tag was inserted to enable detection of fusion proteins.

Cre-LBD constructs were co-transfected with a “tester” (lox-Puro-lox-GFP) construct in COS-1 cells (Figure 3). In the “tester” GFP is not expressed when the lox-Puro-lox cassette is present upstream of it, but is expressed after Cre has removed the sequence. Half of the transfected cells were treated with 4-hydroxy tamoxifen (4-OHT), which binds the LBD, and causes nuclear localization (and thus activation) of Cre. GFP expression allowed the identification of positive “tester” recombination events (and thus the activity of Cre).

The results show that in the absence of 4-OH-T, Cre1 (HA-CreLBD) and Cre3

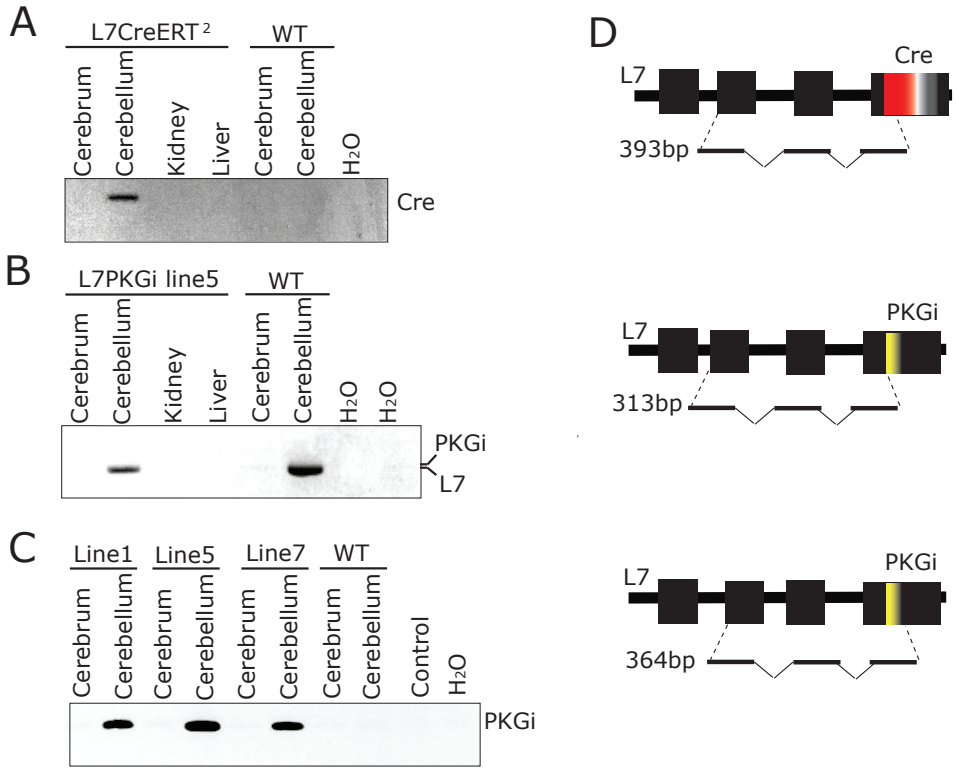


Figure 5 - RT-PCR analysis of PKGi expression. Total mRNA, extracted from different tissues of transgenic and wildtype mice, was tested for L7, PKGi and/or Cre expression. A) L7CreERT2 transgenic line1 tested for expression of Cre RNA. B) L7PKGi transgenic line 5 was analyzed for expression of PKGi mRNA. Wild type animals were analyzed for endogenous L7 expression. C) Different lines of PKGi transgenic mice were analyzed for cerebellar-specific PKGi mRNA expression. D) Schematic representation of the PCR products obtained with the different primer combinations in A, B and C.

(CreLBD-HA) appear to be strictly cytoplasmic (Figure 3B and N), whereas Cre2 (HA-LBDCre) and Cre4 (LBDCre-HA) seem to produce a more diffuse pattern of staining which is also present, albeit at low levels, in the nucleus (Figure 3H and T). Thus, Cre2 and -4 are not absolutely retained in the cytoplasm. Consistently, recombination could be seen in cells transfected with Cre2 and -4, in the absence of 4-OHT (Figure 3I and U). Cre5 (HA-Cre) is also ubiquitously distributed (Figure 3Z) and, as expected, recombination is also detected in cells without tamoxifen (Figure 3Za).

Upon addition of 4-OHT there is a marked increase in nuclear amounts of Cre for all the constructs (Figure 3E, K, Q, W) and recombination is observed in cells transfected with Cre1 and -3 (Figure 3F and R).

Western blot analysis of nuclear and cytoplasmic pools of Cre transfected cells showed that proteins are expressed at variable levels (Figure 4). Cre1 displays a complete cytoplasmic localization in the absence of 4-OHT (Figure 4A). Upon addition of 4-OHT all the constructs display increased nuclear localization, but only for Ha-Cre-LBD and Ha-LBD-Cre is this associated with a full cytoplasmic depletion (Figure 4B). These results support the data obtained with immunofluorescence. Taken together,

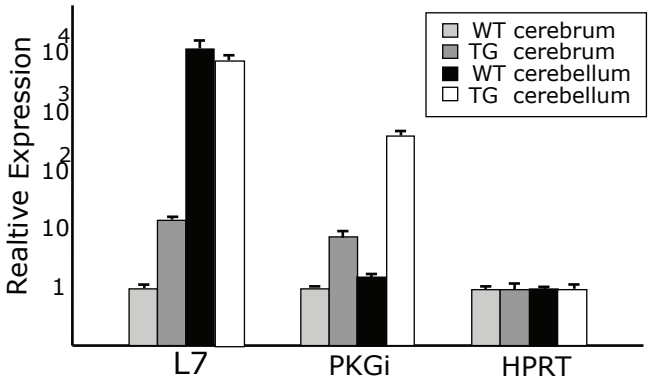


Figure 6 – Real-time PCR analysis of PKGi expression. Total RNA from cerebrum and cerebellum of wild type and PKGi line 5 mice was reverse transcribed and tested for PKGi expression in a quantitative manner. L7 and HPRT served as controls. Results are shown relative to HPRT.

our results indicate that in cultured cells, Cre1 is regulated most tightly with respect to 4-OHT.

*In vivo tests of Cre and LBD activities*

Based on the transfection experiments, constructs Cre1 (HA-CreLBD) and Cre5 (HA-Cre) were cloned into the modified L7 vector and transgenic mice were generated. Concurrently, we generated mice with improved versions of the estrogen LBD fused to Cre (Cre-ERT2). Furthermore, after a report that the progesterone receptor LBD (PR) works best for recombination in the brain (Kellendonk et al., 1999), we also made transgenic mice expressing Cre-PR under the control of L7. A list of the transgenic mice that were made is shown in Table II .

We tested for expression of CreLBD by RT-PCR or by northern blot. RT-PCR analysis of total cerebellar RNA of line 1 of the L7CreERT2 transgenic mice showed a band of the expected size (393 bp), which was not present in other mRNA samples (Figure 5A). These results indicate specific expression of CreERT2 in transgenic cerebella. However, this result could not be confirmed by northern blot analysis (not shown), indicating that CreERT2 expression is very low. There was no detectable expression in line 2 (not shown).

To assess the usability of the L7CreERT2 and L7CrePR mice, animals were crossed with *Clip2*$^{fl/fl}$ reporter mice, which express bacterial beta-galactosidase (LacZ) in PC but only after Cre recombination (Hoogenraad et al., 2002). LacZ is easily detected with a blue-coloring substrate. Offspring of the L7CreERT2 or L7CrePR and *Clip2*$^{fl/fl}$ reporter mice were injected with tamoxifen (CreERT2) or RU486 (CrePR) and brains were collected after one week. Cerebellar preparations were made and stained for LacZ. However, no recombination could be observed, except in control mice (not shown).

To test the *in vivo* activity of HA-Cre in the cerebellum, L7HaCre transgenic mice were crossed with ROSA26-LacZ reporter mice, in which the gene encoding lacZ is placed in the ROSA26 locus, downstream of a sequence surrounded by loxP-sites. This sequence prevents expression of LacZ, in the same manner that the "tester" construct prevented GFP expression in COS-1 cells. Active Cre will delete the sequence and allow expression of LacZ. Brains of double transgenic mice were examined by immunohistochemistry, but lacZ activity (and thus Cre recombination) could only be observed in a highly limited number (less than 1%) of cells in transgenic cerebella (not

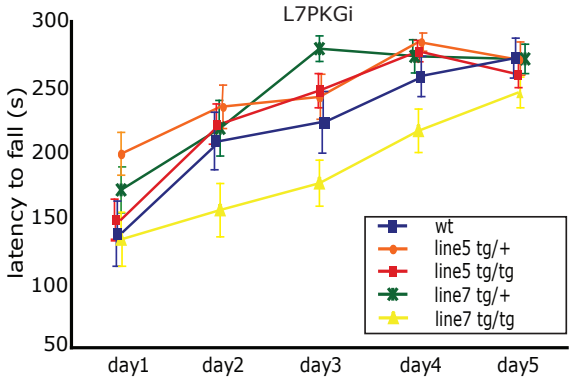


Figure 7 – rotarod analysis of L7PKGi mice L7PKGi mice (lines 5 and 7) and wild type littermates were tested for their performance in the accelerating rotarod setup. The population was as follows: L7PKGi tg/+ (line 5) n= 25, L7PKGi tg/tg (line 5) n=28, L7PKGi tg/+ (line 7) n= 20, L7PKGi tg/tg (Line 7) n= 17 and wild type littermates n=17. Tests were repeated every day, 5 days in a row.

shown). As a positive control for lacZ activity, we used *Clip2* knockout mice, which express LacZ instead of CLIP-115 (Hoogenraad et al., 2002), and which revealed blue staining in the complete PC layer (not shown). We conclude that none of the L7Cre mice contained a satisfactory level of Cre activity for further analysis.

*Generation and behavioral analysis of L7PKGi mice*

A second in vivo approach to study the contribution of the PKG pathway to cerebellar LTD and adaptation of the eye-blink conditioning was attempted by generating transgenic mice bearing PKGi under the control of the L7 promoter. A total of 7 transgenic lines were obtained. The expression of the PKGi mRNA was determined by RT-PCR and quantitative PCR on total RNA samples (Figure 5 and 6). RT-PCR analysis showed that PKGi was expressed in transgenic lines 1, 5 and 7 (Figure 5). Real time PCR on RNA from transgenic line 5 showed expression of PKGi in the cerebellum (and to a lower extend in the cerebrum). PKGi expression was approximately 10-fold lower than endogenous L7 levels (Figure 6).

In order to assess the importance of PKG to general cerebellar function locomotion tests were performed. PKGi transgenic mice (lines 5 and 7) were analyzed on the rotarod setup. Line 7 showed a marked difference in performance from day 2 onward (Figure 7). This difference was maintained until day 5. The means were statistically significantly different for days 3, 4 and 5 between line 7 homozygous animals and wild-type littermates ($P<0,05$ t-test and $P<0,01$, MANOVA). To examine if the coordination difficulties in the rotarod are specific to a cerebellar transgene or due to muscle problems, we analyzed the same group of mice for their grip strength. This analysis revealed no differences between the groups (not shown). Combined the data suggest that lines 5 and 7 express PKGi and that this expression affects locomotor function of the transgenic animals.

Homozygous PKGi line 5 and 7 animals were subsequently tested for their performance in the eye-blink conditioning paradigm. No differences were found in the groups (MANOVA with Bonferroni correction for multiple measurements) for any of the parameters analyzed (data not shown).

*Eye-blink conditioned responses in conditional PKG knockout mice*

To address the role of PKG in eye-blink conditioning and to confirm the results obtained with the L7PKGi mice we tested the conditional PKGI knockout mice, *PKGI$^{pko}$*

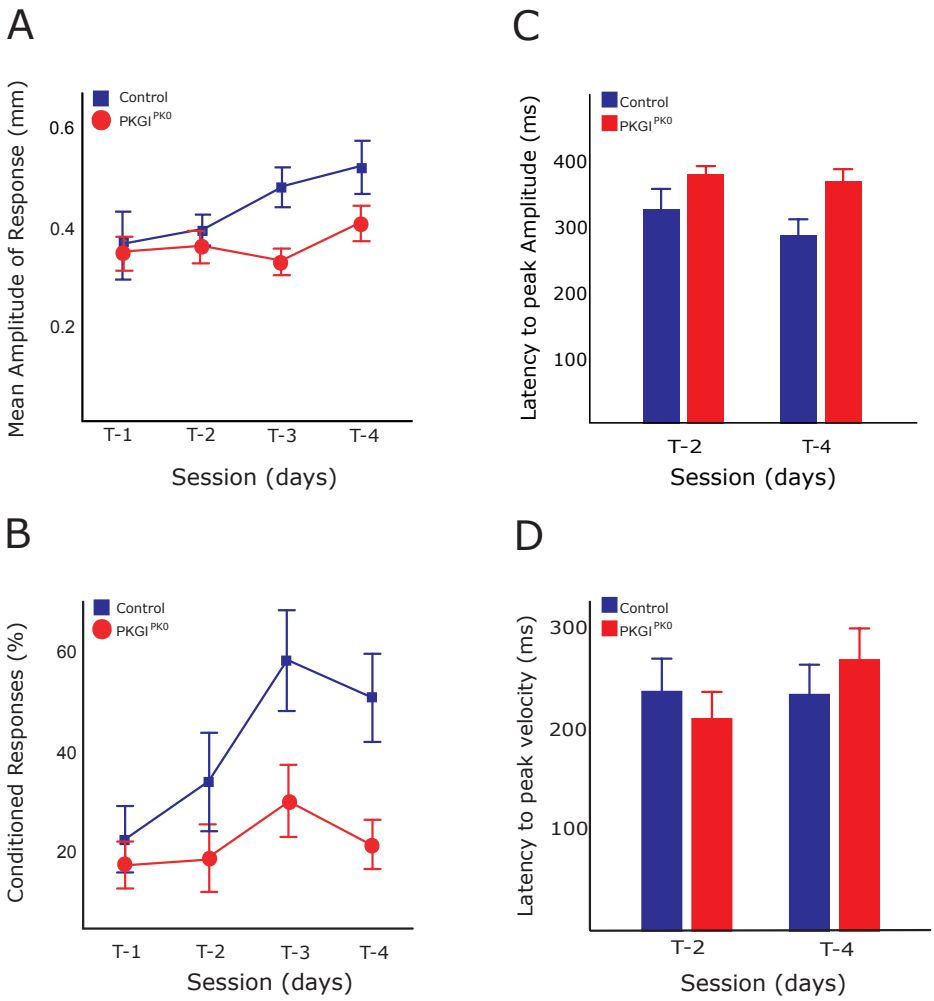


Figure 8 – Eye-blink conditioning of PKGpko mice. Measurement of the number of eye-blinks induced by a sound during training with a paired presentation of a sound/puff of air and the timing of the conditioned responses. PKGpko animals (n=10) are represented by (red) circles and control animals (n=11) are represented by (blue) squaresA) mean amplitude of positive responses. B) Percentage of positive conditioned responses over the training period C) Latency to the maximum amplitude of the eye-blink.

D) Latency for the maximum velocity of the eye-blink

(Feil et al., 2003) in the eye-blink conditioning paradigm. In the *PKGI*$^{pko}$ mice PKGI is specifically ablated in cerebellar PCs.

We found that the mean amplitude of the eye-blinks induced by the puff of air was reduced in *PKGI*$^{pko}$ animals as compared to controls ($p=0.043$; MANOVA with Bonferroni correction for multiple measurements). The differences were most obvious on day 3 and 4 of measurements (for *PKGI*$^{pko}$ mice the amplitudes are $0.3336 \pm 0.0855$ mm and $0.4099 \pm 1.317$ mm respectively , Figure 8). Similarly, the total percentage of positive responses (responses where an eye-blink can be observed) were higher in control mice at day 3 and 4 as compared to the *PKGI*$^{pko}$ mice ($p < 0.01$, MANOVA with Bonferroni correction for multiple measurements).

We also measured the time necessary for animals to engage in the full speed of the eye-blink (i.e.: time to peak velocity: tPV) and time to reach the maximum amplitude of the blink (i.e. time to peak amplitude: tPA). Time to peak amplitude varied for wild type animals from 325.670 ± 32.752 ms (day 2) to 285.334 ± 30.879 ms (day 4), while the *PKGI$^{pko}$* animals displayed values that varied from 378.386 ± 30.879 ms( day 2) to 368.550 ± 30.879 ms (day 4) (Figure8C). As for time to peak velocity, control animals displayed values that ranged from 235.958 ± 34.441 ms (day 2) to 233.206 ± 32.472 ms (day 4) while *PKGI$^{pko}$* animals displayed values that ranged from 208.975 ±32.472 ms (day 2) to 267.639 ± 32.472 ms (day 4) (Figure 8D). There were no significant differences between the two groups of mice ($p>0.05$; MANOVA with Bonferroni correction for multiple measurements). These results indicate that PKGI is important for the percentage and amplitude of eye-blink conditioned responses but not for timing.

## Discussion

In this study we wished to investigate the effect of PKG inactivation on learning of the eye-blink conditioned response in mice. Different strategies were attempted to achieve this aim. Previous studies have reported the capacity of pharmacological PKG (or cGK) inhibitors, like H89 or KT5823 (Reynolds and Hartell, 2001), to influence LTD in slice preparations. As previously stated (Feil et al., 2003b), pharmacological protein kinase inhibitors are not very selective (Davies et al., 2000). We therefore used a peptide (PKGi), which is over 100 times more potent in blocking PKG over PKA activity (Dostmann et al., 1999) (Dostmann et al., 2000). We first used PKGi in a microiontophoresis set-up to confirm the influence of the blockade of PKG in the cerebellum for the establishment of LTD. Our results show an obvious but partial inhibition of LTD caused by the application of the inhibitory peptide. With an average reduction of the EPSCs down to approximately 85%, the results indicate that the effect of the peptide in cerebellar LTD is less extensive than the effect of the PKCi peptide in LTD induction tested in a similar setup. (Goossens et al., 2001). There is however a robust lack of LTD in the *PKG$^{PKO}$* mice (Feil et al., 2003a).

Based on our in vitro results, we attempted to generate transgenic mice in which PKGi was expressed in a spatio-temporally controlled manner in PCs. We followed this strategy despite reports that PKGI and -II knockout mice did not have a measurable cerebellar phenotype (Pfeifer et al., 1998; Ruth, 1999). We used the Cre-lox and rtTA systems to manipulate PKGi expression. Using transiently transfected COS-1 cells, we confirmed previous results that point to an effect of the placement of the LBD in controlling Cre activity (Kellendonk et al., 1999). Placing the LBD at the C-terminus of Cre seems to be the most efficient arrangement. This might be due to the fact that this fusion protein is less susceptible to proteases that release Cre from the LBD (Wunderlich et al., 2001).

To make transgenic mice, several Cre fusion proteins were placed under control of the L7 promoter as this element has been shown to be specific for PCs and retinal bipolar neurons (Berrebi et al., 1991; Oberdick et al., 1990) and has been used in other transgenic approaches (Barski et al., 2000; De Zeeuw et al., 1998; Oberdick et al., 1998; Tomomura et al., 2001; Zhang et al., 2001). However, our results indicate that the level of Cre activity in the cerebellum of transgenic mice is too low to be

effective. Even with the two L7CrePR transgenic lines, in which copy-numbers were estimated to be 8 and 20, respectively, Cre activity was undetectable. As the levels of expression of the Cre and rtTA proteins in our transgenic mouse models were too low this strategy was abandoned.

In a second set of experiments we generated transgenic animals expressing PKGi under control of the L7 promoter. In this strategy expression of PKGi is not inducible anymore. We detected expression of the PKGi mRNA in the cerebellum, specifically in PCs but again at low levels. The reasons for this low expression are not clear. A cryptic splice site in the sequence of the L7 promoter might affect transgene expression (Oberdick, personal communication). It should be noted that in other studies many more transgenic lines were made (Tsujita et al., 1999; Vandaele et al., 1991), perhaps this is a prerequisite for obtaining one good transgenic line. For example, Vandaele et al (1991) report the generation of multiple limes each with different anatomical expression patters. Lines with higher copy number seemed to expand the expression of a LacZ element to a wider number of PCs (Vandaele et al., 1991). In addition, there were at least 31 founders generated in another study (De Zeeuw et al., 1998). Thus, it is possible that if we had made extra transgenic mouse lines with the same constructs, we would have obtained a line with appropriate Cre and/or PKGi expression.

Despite the low expression levels of PKGi, we detected a phenotype in the accelerating rotarod for L7PKGi mice (line 7). The rotarod is considered a sensitive method to measure cerebellar dysfunction (Crawley, 1999, 2000; Lalonde and Strazielle, 2007). However, rotarod performance and LTD are not necessarily associated (Feil et al 2003, deZeeuw et al 1998). Because *PKG$^{pko}$* mice do not display an alteration in their performance in the rotarod setup (Feil et al 2003), it is not clear what the contribution is of PKGi to the L7PKGi line 7 phenotype. One possibility is that it is an "insertional phenotype" caused by disruption of another gene by the random integration of the transgene. This idea is strengthened by the fact that only one of the transgenic lines displays a phenotype in the rotarod and only in the homozygous situation. Based on these results, the expectation is that with low levels of expression, an L7 driven PKGi might not be sufficient to inhibit LTD in vivo. We therefore did not test this hypothesis, also because the conditional PKGI knockout (*PKG$^{pko}$*) became available and studies with this mice established the necessity of PKG to the establishment of LTD (Feil et al 2003).

We subjected a population of *PKG$^{pko}$* mice to the eye-blink conditioning paradigm. We used the powerful MDMT protocol (Koekkoek et al., 2002) to assess the involvement of the NO-PKG pathway in the amplitude, percentage and timing of conditioned eye-blink responses. We conclude that the lack of PKG is detrimental to the amplitude and percentage of conditioned responses but the occurring responses are in general perfectly timed. We therefore propose that LTD has no effect on timing of responses.

One drawback of the *PKG$^{pko}$* approach is that the percentage of PCs exhibiting Cre-induced deletion of the PKG gene is less than 100% (Feil et al., 2003b). For this reason we cannot exclude the possibility that a small set of normal PCs is contributing to efficient eye-blink responses. A small number of cells in the simplex lobule could account for perfectly timed responses despite a virtually undetectable. LTD (Kotani et al., 2003). This is, however, not a totally plausible explanation since previously

obtained single cell recordings show that the studied PCs do have restricted LTD. The distribution of PKG-containing PCs in the *PKG$^{pk0}$* animals seems to be a random event (Feil et al., 2003b).

Koekkoek et al (2003) have demonstrated that mutant mice in which LTD has been impaired by the selective inhibition of PKC show a marked inability to learn coordinated timing of the eyelid response, suggesting that timing of the cerebellum response is mediated by an input specific mechanism involving LTD (Koekkoek et al., 2003). The *PKG$^{pko}$* show no effect in the timing of the responses. One fundamental difference between the PKC and the PKG pathways is the way they are activated. The PKC pathway requires activation of mGluR by glutamate released onto the synapse by PFs, requiring the cells to be within synaptic distance from each other (Crepel et al., 1996; De Zeeuw et al., 1998; Eto et al., 2002; Goossens et al., 2001; Ito, 2001; Levenes et al., 1998). This is consistent with the idea of input specificity, where the active synapse will be tuned down (Ohyama et al., 2003). PKG on the other hand, is activated by membrane-diffusible NO that can be released by other types of non contacting cells (Crepel et al., 1996). Possible sources are PFs but also basket cells (Vincent, 1996). Yet another difference is that PKC acts on the target, the AMPAR moieties, directly (Chung et al., 2003) while PKG exerts its action indirectly via phosphorylation of G-substrate (Endo et al., 1999) and protein phosphatases. It seems clear that the timing information is conveyed by the cortical connections, in a manner of state-dependent changes in network dynamics (Mauk and Buonomano, 2004). Lesions to the cortex can cause disrupted timing (Garcia et al., 1999) possibly by disrupting the actual connections, even if more unspecific or widespread lesions do not contribute to impairment of the timing of events in the cerebellum (Harrington et al., 2004). PKC seems a more likely candidate than PKG to process time-related information intracellularly.

PKG has been demonstrated to be essential for the establishment and/or maintenance of LTD *in vitro* (Reynolds and Hartell, 2001) and *in vivo* (Feil et al., 2003c) but it seems not to be necessary for LTD in cultured cells (Linden, 2001). Based on the results we postulate that the action of the mGluR-PKC pathway is more important for the maintenance LTD and that the role of the PKG pathway is to facilitate LTD and prevent it from reverting too quickly. Mechanisms for the reversal of LTD have been proposed (Lev-Ram et al., 2003). However, we should bear in mind that LTD might not be sufficient to explain the whole array of cerebellar dependent behavioral outputs (Welsh et al., 2005) ; (Bagnall and du Lac, 2006).

The action of NO has been show to increase the endogenous firing rate of PCs. The basal firing rate of PCs can be from 5 to 80 spontaneous discharges per minute even without stimulatory inputs. It has been shown that cGMP and NO donors cause a long lasting increase in the spontaneous firing rate of PCs. NO signals to groups of active neurons to adjust their baseline firing rates and this system is thought to be dependent on PKG (Smith and Otis, 2003). NO will increase the spontaneous activity of Purkinje neurons by 15%, thus causing high modulation of the output of the cerebellum. NO seems to be an important modulator of PC activity (Ito, 2001) but also to have hardly any temporal specificity of action. In *PKG$^{pko}$* mutants, the lack of PKG could contribute to a diminished response to the native activation caused by NO either to the basal firing rates (Smith and Otis, 2003) or to the reversal of LTD (Lev-Ram et al., 1997a; Lev-Ram et al., 2003).

A new hypothesis is that the cerebellar control of outputs might be mediated by pattern recognition. In this model, the number of synchronously activated PF synapses will determine the alteration of the firing pattern of PCs. An increased pause in the firing pattern is associated with PKC-dependent LTD deficient mice. As this mechanism has been shown to be dependent on Ca2+ (Steuber et al., 2007), it makes a stronger point in favor of the PKC-dependent control of timed responses and allows us to infer that these type of temporal processing is independent of PKG. It would be interesting to analyze the *PKG$^{pko}$* mice for their PC-firing pauses in the same system.

Our results point to a dissociation between signaling and LTD or to multiple mechanisms in LTD. Because PKC phosphorylates the AMPAR directly, compensation of function is more difficult in PKC deficient mutants than in the PKG mutants for which the action is indirect. Partial compensation of function (e.g. by exogenous activation of the Protein Phosphatases) in the *PKG$^{pko}$* mice could explain a reduction (but not complete elimination) of the number of responses and also that the actual responses are perfectly timed. We also propose that *PKG$^{pko}$* mice have an unaltered PKC and Ca2+ dependent PC firing rate and pause timing, which might be independent of LTD. To confirm our proposals, further studies are needed in which PKC and PKG mutants are directly compared.

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## Chapter III

# Comparative behavioral analysis of *Clip1*, *Clip2* and *Clasp2* knockout mice

Manuscript in preparation

# Comparative behavioral analysis of *Clip1*, *Clip2* and *Clasp2* knockout mice

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## Summary

Plus-end tracking proteins, or +TIPs, specifically bind to the ends of growing microtubules thereby regulating the dynamic behavior of this network. CLIP-115, CLIP-170 and CLASP2 are +TIPs, which positively influence microtubule dynamics, i.e. CLIPs act as microtubule rescue factors in mammalian cells, whereas CLASP2 enhances microtubule stability under specific conditions. CLIPs and CLASPs interact with each other, indicating they act in overlapping pathways. All three proteins are expressed in the central nervous system, including the cerebellum. We have generated knockout mice for CLIP-115, CLIP-170 and CLASP2. As the microtubule cytoskeleton is extremely important for the functioning of neuronal circuitries we have examined simple behavioral parameters of the different mouse lines. Here we show that a lack of CLIP-115 results in motor coordination problems on the rotarod. A deficiency of CLIP-170 results in aberrant spontaneous activity, whereas lack of CLASP2 leads to highly increased spontaneous activity. These results point to an important role for +TIPs in behavior and in cerebellar-mediated functions.

## Introduction

The microtubule (MT) cytoskeleton has numerous functions inside cells, including the maintenance of cell structure and shape, cell motility, growth and division. The MT cytoskeleton also provides a scaffold system to permit transport (and anchoring) of organelles, vesicles, proteins and mRNA populations to specific locations. This is particularly obvious and important in neurons, where the establishment and maintenance of axons and dendrites, which are involved in neurotransmission, requires the careful regulation of long distance transport. It is therefore understandable that even subtle defects in the MT network may lead to neuronal dysfunction and, hence, behavioral abnormalities. Several studies have shown the effect of cytoskeletal abnormalities in neurodegenerative disease (Ramaekers and Bosman, 2004) and other conditions, for example schizophrenia (Andrieux et al., 2006; Andrieux et al., 2004).

MTs display dynamic instability, which is exemplified by the alternate periods of growth and shrinkage occurring at their plus ends (Desai and Mitchison, 1997). The dynamic behavior of MTs is regulated by MT-associated proteins (MAPs). Some of these are the so-called plus-end tracking proteins, or +TIPs (Schuyler and Pellman, 2001), that bind specifically to the end of growing MTs (for review, see (Galjart and Perez, 2003). The first discovered +TIP was CLIP170 (Perez et al., 1999). Since then the group of +TIPs has expanded rapidly and most +TIPs are actually structurally unrelated.

CLIP-170, which is the product of the *Clip1* gene (Akhmanova et al., 2005), is similar to CLIP115, which is the product of the *Clip2* gene (Hoogenraad et al., 1998). Although CLIP170 was initially shown to be involved in binding endocytic vesicles to MTs (Pierre et al., 1992), later analysis emphasized a role in the regulation of the dynamic MT cytoskeleton. In fact, CLIPs act as MT rescue factors in mammalian cells (Komarova et al., 2002). Furthermore, both CLIPs bind directly to CLASP1 and -2 (Akhmanova et al., 2001). CLASPs are also +TIPs, and are involved in the establishment of cell polarity and in cell motility. These data underscore a role for CLIPs in MT-mediated processes. Finally, CLIP170 binds directly to dynactin (Lansbergen et al., 2004). As dynactin controls the behavior of molecular motors, these results point to a function for CLIP-170 in intracellular transport.

Mutant mice lacking the *Clip1* gene (Akhmanova et al., 2005), the *Clip2* gene (Hoogenraad et al., 2002), and the *Clasp2* gene (Drabek et al., 2006) have been generated in our group. More recently, a *Clip1* and *-2* double knockout (DKO) line was made (Miedema, 2007). From each mouse line embryonic fibroblasts (MEFs) were derived. We could show that a single CLIP deficiency in MEFs does not lead to obvious

abnormal effects on the MT network, suggesting redundant roles for CLIP-115 and -170 in cultured cells. However, in Clip DKO MEFs MT dynamics was perturbed, in line with a role of CLIPs as rescue factors (Komarova et al., 2002). In *Clasp2* knockout fibroblasts alterations in the stability of MTs and in directed cell motility were visible (Drabek et al., 2006), consistent with a function for CLASP2 in local regulation of MT dynamics.

A deletion of CLIPs and CLASP2 leads to different phenotypes in mice. A marked defect in spermatid manchette formation, detrimental to spermatogenesis, was observed in *Clip1* knockout mice (Akhmanova et al., 2005), indicating a unique function for CLIP-170 in MT regulation in the testis, where CLIP-115 is not expressed. In *Clip2* knockouts behavioral deficits were linked to mild brain abnormalities (Hoogenraad et al., 2002). *Clip2* deficient mice are very interesting to study, because they might represent a mouse model for some of the behavioral or locomotor defects present in Williams Syndrome patients (van Hagen et al., 2007). In *Clip* DKO mice we observed a much more severe phenotype than in the single knockout strains, i.e. progressive hydrocephalus and heterotopia (Miedema, 2007). Finally, *Clasp2* knockout mice have multiple defects and die because of internal bleeding (Drabek, 2005). The results in cell lines and mice show that CLIPs and CLASP2 are not essential proteins, but they are required to maintain normal phenotype.

The cerebellum is essential for the fine-tuning and control of coordinated movement, such as hand-eye coordination, walking, or timely controlled motor responses (Ito, 2000). For example, in cerebellar Purkinje cells, Long Term Depression (LTD) is a cellular mechanism thought to be responsible for adaptive timing of conditioned responses (Ito, 2002). One of the hallmarks of LTD is the internalization of AMPA-type glutamate receptors as clathrin coated vesicles (Chung et al., 2003; Wang and Linden, 2000) an operation that requires MT transport (Zhou et al., 2001). Other examples of MT function in the cerebellum are the targeting and anchoring of specific proteins and mRNA to the dendrites (Wu et al., 2007) or the anchoring of proteins to (e.g.) the membrane (Shao and Hochmuth, 1999).

CLIP-115 expression is highly enriched in the brain, including the cerebellum. CLIP-170 is more ubiquitously expressed, but a brain-specific splice form has been described. Alternative splicing has also been described for CLASP1 and -2 (Akhmanova et al., 2001), and of the three alternative spliced CLASP-isoforms, the CLASP-2ß transcript appears to be brain-specific. Immunohistochemistry studies have confirmed that CLASP-2 is expressed throughout the brain. High levels of expression can be observed in hippocampal CA pyramidal neurons and in the dentate gyrus, as well as in the molecular and Purkinje cell layers of the cerebellum (Drabek, 2005).

The cerebellar expression of CLIPs and CLASPs sparked interest in the behavioral and locomotor phenotype of the *Clip1* and *-2* and *Clasp2* mutant mice. It was expected that cytoskeletal deficits in the cerebellum would have a phenotype in locomotion or motor coordination. Previous studies from our group indeed pointed to the necessity of a well-regulated MT cytoskeleton for motor coordination in *Clip1* single and *Clip1/2* DKO knockout mice (Hoogenraad et al., 2002; Miedema, 2007). In this study we report performance in several behavior/locomotion paradigms such as the open field test, the rotarod and the catwalk, and we compare the effects of the lack of each +TIP.

## Materials and methods

*Transgenic mice*

The *Clip1* and *-2* and *Clasp2* knockout, and the GFP-Clip170 knock-in (further referred to as *Clip1*$^{ki}$) alleles have been described (Akhmanova et al., 2005; Drabek, 2005; Hoogenraad et al., 2002). Briefly, the *Clip1* and *Clasp2* knockouts were made by inserting a cassette containing green fluorescent protein (GFP) and a neomycin resistance gene (*neo*$^{r}$) flanked by loxP sites, at the respective ATG translation initiation sites. This abrogates expression and leads to a knockout allele. In the case of the *Clip1* gene we subsequently removed the *neo*$^{r}$ gene using Cre recombinase, which yielded an in-frame GFP-CLIP170 fusion and resulted in the *Clip*$^{ki}$ allele. The *Clip2* knockout allele was generated by deleting most of the gene using loxP sites and Cre recombinase. Recently, we generated a *Clip1/Clip2* double knockout (DKO) (Miedema, 2007). Prior to these studies, *Clip* $^{-/-}$ mice were backcrossed into Bl/6 or 129/SvJ backgrounds for 9 or 2 generations respectively.

*Behavioral analysis*

*Open field*

Spontaneous locomotion activity was measured using an open field cage (50 x 50 cm, Coulbourn Instruments) as described previously (Hoogenraad et al., 2002). Briefly, mice were placed in a computer-monitored plexiglas cage. Mice were allowed to move freely for 5 minutes and all movements were recorded with the help of the TruScan software (Coulburn Instruments). The cage was carefully cleaned before and after each mouse was tested. The parameters tested were: movement episodes – the number of times that a set of movement is initiated, also a measurement of the number of pauses; movement time – time spent engaged in movement; the amount of time spent in the center of the arena (center time) or at the margin (margin time), the number of entries in the center of the arena (center entries), time spent not

moving (rest time), the total distance walked in the arena (distance), walked in the margin of the arena (margin distance) or in the center of the arena (center distance), the average velocity of the movement in cm/sec (velocity), the number of entries in the vertical plane (head raised above the detection level or standing on the hind paws, vp entries) and the amount of time spent in the vertical plane (vp time), the number of jumps (less than 2 paws on the floor plate) and stereotypic movement episodes were also recorded. Results were analyzed statistically using GraphPad Prism or SPSS software. Results were analyzed with t-tests, to compare homozygous mutant and wild type animals, and with one-way ANOVA to compare multiple genotypes.

*Rotarod*

The accelerating rotarod paradigm (UgoBasile 7650) was used as previously described (Miedema, 2007). Adult mice 3 or 8 months old were used. Briefly, up to 5 mice are placed in the rotating rod in individual divisions. The rotarod was accelerated from 4 to 40 rpm. For each mouse, the training stopped when it fell from the rod or when 300 s of exercise had been reached. Mice that stayed on the rod by clinging on to the base and rotated passively were removed after three full rounds. The training was repeated daily for 5 days. Latency to fall was registered. Results were analyzed statistically with two-way ANOVA and Bonferroni correction for multiple measurements using SPSS software.

*Catwalk*

To assess locomotion and posture deficits, the Catwalk setup (Hamers et al., 2001) was used, as described before (Miedema, 2007). Briefly, a glass plate is illuminated from the side. A camera set-up under the plate detects no light, until pressure is exerted on the plate, allowing light diffusion. This way, mouse paws become visible when the mouse walks on the glass plate. Each mouse is placed on the plate and allowed to cross the runway, 50 cm of which is monitored and recorded. The movement is recorded and analyzed with the help of the catwalk software (Hamers, FT). The parameters analyzed were: time taken to cross the 50 cm are of the glass runway (Duration of crossing), the percentage of normal step sequence patterns (Normal Patterns) (see also (Basso et al., 1996)), the distance between left and right forepaws or left and right hind paws (base of support forepaws and base of support hind paws) and the stride length of each paw (step length left forelimb, right forelimb, left hindlimb, right hindlimb). Results were analyzed statistically with t-tests and ANOVA using GraphPad Prism and SPSS software.

## Results

*Mouse models for behavioral analysis*

CLIPs and CLASPs have been implicated in the dynamic regulation of the MT network. In man, the gene encoding CLIP-115 (*Clip2*) is hemizygously deleted in most patients with Williams Syndrome (Meyer-Lindenberg et al., 2006), making the *Clip2* knockout mice an interesting mouse model for behavioral analysis. Previous work performed with these mice revealed striking deficits on the rotarod performance, as well as other defects (Hoogenraad et al., 2002). However, later analyses indicated that some of the behavioral phenotypes in the *Clip2* knockout mice might have been enhanced by the mixed genetic background of the mice (data not shown). Previous work done with *Clip1*/*Clip2* double knockouts revealed that the phenotypes are age-dependent and that severe manifestations appear at around 15 months (Miedema, 2007).

In order to attribute behavioral abnormalities to a specific +TIP deficiency, we performed simple behavioral experiments with defined groups of knock-in and knockout mice generated in our laboratory, at different ages (for an overview of gene nomenclature and mice used, see Tables I and II, respectively). *Clip1* mutant mice display mild behavioral phenotypes that became more noticeable with increasing age. At 3 months the same pattern of differences that can be found at 8 months between *Clip1* mutants and wildtype littermates is present but the differences are too small to be statistically significant. For this reason, all results shown for *Clip1* mutant mice were obtained at 8 months.

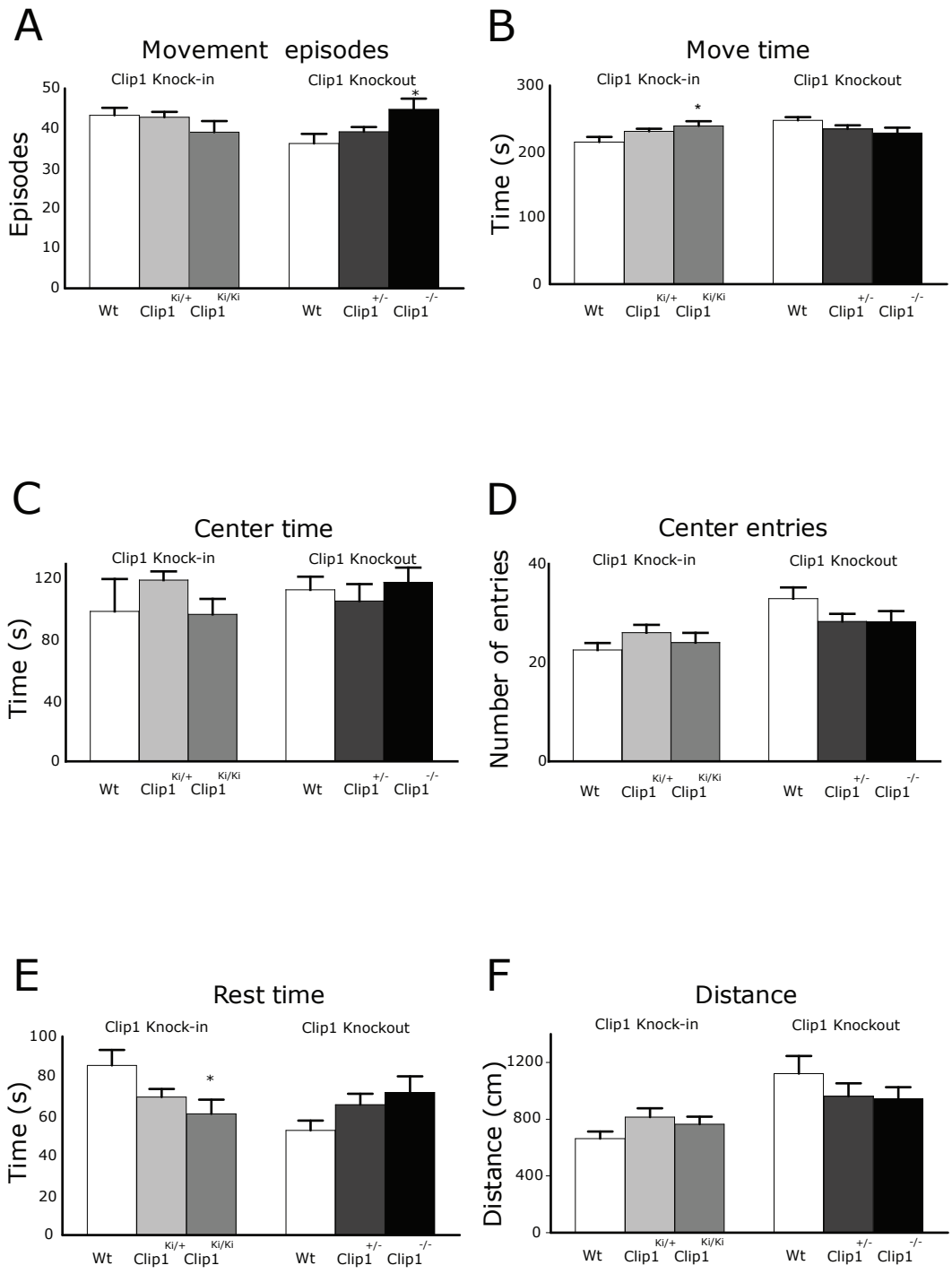
Table I – Gene nomenclature

| Gene name | Protein product | Modified allele | Protein product from modified allele | Genotype |
|---|---|---|---|---|
| *Clip1* | CLIP-170 | Knockout | - * | $Clip1^{-/-}$ |
| *Clip1* | CLIP-170 | Knock-In | GFP-CLIP170 | $Clip1^{ki/ki}$ |
| *Clip2* | CLIP-115 | Knockout | - | $Clip2^{-/-}$ |
| *Clip2* | CLIP-115 | Floxed** | CLIP-115 | $Clip2^{fl/fl}$ |
| *Clip1/2* | CLIP-170 / CLIP-115 | Double Knockout (DKO) | - * | $Clip^{-/-;-/-}$ |
| *Clasp2* | CLASP2 | Knockout | - | $Clasp2^{-/-}$ |

*: hypomorphic for CLIP-170 in lung and embryo (Akhmanova et al., 2005)
**: Gene is flanked by loxP-sites (floxed), expression of protein is not influenced (Hoogenraad et al., 2002)

Table II –Mice used in behavioral assays

| Assay | Gene | Genotype | Age (months) | n | trials |
|---|---|---|---|---|---|
| Openfield | *Clip1* knockout | wt | 3 /6 / 8 | 11 | 2/1 |
| | | +/- | 3 /6 / 8 | 11 | 2/1 |
| | | -/- | 3 /6 / 8 | 11 | 2/1 |
| | *Clip1* knock-In | wt | 3/6 / 8 | 6 | 2/1 |
| | | +/ki | 3/ 6 / 8 | 10 | 2/1 |
| | | ki/ki | 3/6 / 8 | 9 | 2/1 |
| | *Clasp2* knockout | wt | 3 | 10 | 2 |
| | | +/- | 3 | 13 | 2 |
| | | -/- | 3 | 6 | 2 |
| Rotarod | *Clip2* knockout | wt | 4 | 20 | 2 |
| | | +/- | 4 | 20 | 2 |
| | | -/- | 4 | 20 | 2 |
| | | fl/fl | 4 | 20 | 2 |
| | *Clip1* knockout | wt | 3 / 8 | 11 | 2/1 |
| | | +/- | 3 / 8 | 11 | 2/1 |
| | | -/- | 3 / 8 | 11 | 2/1 |
| | *Clip1* knock-in | wt | 3 / 8 | 7 | 2/1 |
| | | +/ki | 3 / 8 | 10 | 2/1 |
| | | ki/ki | 3 / 8 | 9 | 2/1 |
| | *Clasp2* knockout | wt | 3 | 10 | 2 |
| | | +/- | 3 | 13 | 2 |
| | | -/- | 3 | 6 | 2 |
| Catwalk | *Clip2* knockout | wt | 4 | 20 | 2 |
| | | +/- | 4 | 20 | 2 |
| | | -/- | 4 | 20 | 2 |
| | | fl/fl | 4 | 20 | 2 |
| | *Clip1* knockout | wt | 3/6/ 8 | 11 | 1 |
| | | +/- | 3/6/ 8 | 11 | 1 |
| | | -/- | 3/6/ 8 | 11 | 1 |
| | *Clip1* knock-in | wt | 3/6/ 8 | 6 | 1 |
| | | +/ki | 3/6/9 | 10 | 1 |
| | | ki/ki | 3/ 6/8 | 9 | 1 |
| | *Clasp2* | wt | 4 | 11 | 1 |
| | | +/- | 4 | 13 | 1 |
| | | -/- | 4 | 6 | 1 |
| | Double knockout *Clip1/2* | wt | 3,6,9,12,15,18,21,24 | 7 | 1 |
| | | -/- ; -/- | 3,6,9,12,15,18.21,24 | 6 | 1 |


A
Movement episodes
Clip1 Knock-in
Clip1 Knockout
Episodes
50
40
30
20
10
0
*
Wt
Clip1 Ki/+
Clip1 Ki/Ki
Wt
Clip1 +/-
Clip1 -/-
B
Move time
Clip1 Knock-in
Clip1 Knockout
Time (s)
300
200
100
0
*
Wt
Clip1 Ki/+
Clip1 Ki/Ki
Wt
Clip1 +/-
Clip1 -/-
C
Center time
Clip1 Knock-in
Clip1 Knockout
Time (s)
120
80
40
0
Wt
Clip1 Ki/+
Clip1 Ki/Ki
Wt
Clip1 +/-
Clip1 -/-
D
Center entries
Clip1 Knock-in
Clip1 Knockout
Number of entries
40
20
0
Wt
Clip1 Ki/+
Clip1 Ki/Ki
Wt
Clip1 +/-
Clip1 -/-
E
Rest time
Clip1 Knock-in
Clip1 Knockout
Time (s)
100
80
60
40
20
0
*
Wt
Clip1 Ki/+
Clip1 Ki/Ki
Wt
Clip1 +/-
Clip1 -/-
F
Distance
Clip1 Knock-in
Clip1 Knockout
Distance (cm)
1200
800
400
0
Wt
Clip1 Ki/+
Clip1 Ki/Ki
Wt
Clip1 +/-
Clip1 -/-

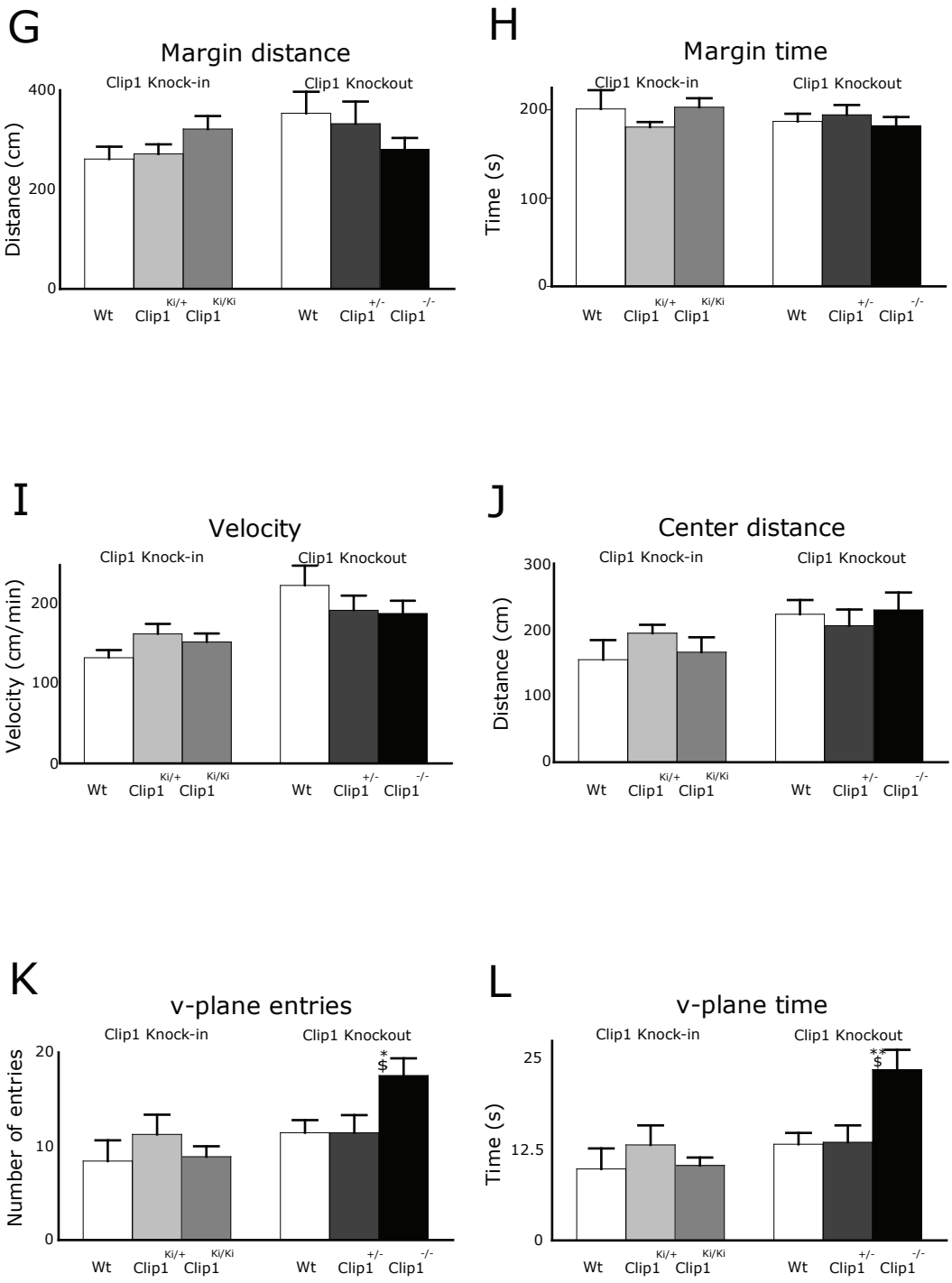

G
Margin distance
Clip1 Knock-in
Clip1 Knockout
Distance (cm)
400
200
0
Wt
Clip1 Ki/+
Clip1 Ki/Ki
Wt
Clip1 +/-
Clip1 -/-
H
Margin time
Clip1 Knock-in
Clip1 Knockout
Time (s)
200
100
0
Wt
Clip1 Ki/+
Clip1 Ki/Ki
Wt
Clip1 +/-
Clip1 -/-
I
Velocity
Clip1 Knock-in
Clip1 Knockout
Velocity (cm/min)
200
100
0
Wt
Clip1 Ki/+
Clip1 Ki/Ki
Wt
Clip1 +/-
Clip1 -/-
J
Center distance
Clip1 Knock-in
Clip1 Knockout
Distance (cm)
300
200
100
0
Wt
Clip1 Ki/+
Clip1 Ki/Ki
Wt
Clip1 +/-
Clip1 -/-
K
v-plane entries
Clip1 Knock-in
Clip1 Knockout
Number of entries
20
10
0
*
$
Wt
Clip1 Ki/+
Clip1 Ki/Ki
Wt
Clip1 +/-
Clip1 -/-
L
v-plane time
Clip1 Knock-in
Clip1 Knockout
Time (s)
25
12.5
0
**
$
Wt
Clip1 Ki/+
Clip1 Ki/Ki
Wt
Clip1 +/-
Clip1 -/-

Figure 1 (previous pages – Openfield analysis of *Clip1* mutants. *Clip1* $^{ki/+}$ (n =10) *Clip1* $^{ki/ki}$ (n = 9) and wt litter mates (n=7) as well as *Clip1* $^{+/-}$ (n=11), *Clip1* $^{-/-}$ (n=11) and Wt littermates (n=11) were tested for their pattern of spontaneous locomotor activity in the openfield setup. Animals were placed individually at the center of the 50cmx50cm arena and allowed to move for 5 min, in which time all the movements were recorded with an automated system. Parameters analyzed are described in the Materials and Methods section. Results were gathered and statistical analysis was performed with the Prism or SPSS software. Groups were compared to their respective control (Wt) littermates with t-student tests. Significant values ($p<0,05$) are noted with the symbols * (as compared to Wt) or $ (as compared to +/-)

*Openfield behavior of +TIP knockout and knock-in mice*

The open field test is used to measure and record spontaneous locomotor activity. A small plexiglas cage allows a focus on impulsive movement rather than fear-related behavior. A total of 12-15 parameters from the vast possible array (CoulbournInstruments, 1998-2003) have been measured (Figures 1 and 2, respectively). Indications of fear-related behavior can be inferred, for example from the amount of time the animals spend in the center of the arena versus the time spent in the margin (Figure 1C, G, respectively).

As mentioned above, the spontaneous locomotor activity of *Clip2* mutants has already been published (Hoogenraad et al., 2002). These mice did not show an altered spontaneous activity. We compared data to the behavior of *Clip1* and *Clasp2* mutants in the openfield setup. For each mutant genotype, wild-type littermates were used as controls. For *Clip1* deficient mice, a few parameters show significant differences between knockout and control animals (Fig. 1), including the number of move episodes (Fig. 1A; ($P<0.05$, t-test), and the number of vertical plane entries (Fig 1 K; significant differences are found between knockout and either wild type or heterozygous littermates ($P<0.05$, t-test)). Strikingly, the time spent in the vertical plane was much higher for the *Clip1*$^{-/-}$ mice when compared to wild type ($P<0.01$, t-test) or heterozygous mice ($P<0.05$, t-test; Fig. 1K). Thus, *Clip1*$^{-/-}$ mice make more movements than wild type animals, and display higher interest in the vertical plane. However, the total moving time (Fig 1 B) of these mice is decreased, whereas resting time (Fig 1 E) is increased (both $P<0.05$, t-test).

The *Clip1*$^{ki/ki}$ mice express GFP-CLIP170 instead of CLIP-170 (Akhmanova et al., 2005). This fusion protein behaves identical to CLIP-170 in cultured cells, and in contrast to the *Clip1*$^{-/-}$ animals, the *Clip1*$^{ki/ki}$ mice display no obvious phenotype in the male germ line, showing that GFP-CLIP170 functions *in vivo* as well. It was therefore somewhat surprising to see statistically significant differences between *Clip1*$^{ki/ki}$ mice and heterozygous and wild type littermates in some of the parameters of the openfield assay (Fig. 1). However, the number of vertical plane entries and time

spent in the vertical plane were significantly less for all types of *Clip1* mice, except homozygous *Clip1*$^{-/-}$ (Fig. 1K, L). We conclude that the latter phenotypes are truly due to a deficiency of CLIP-170.

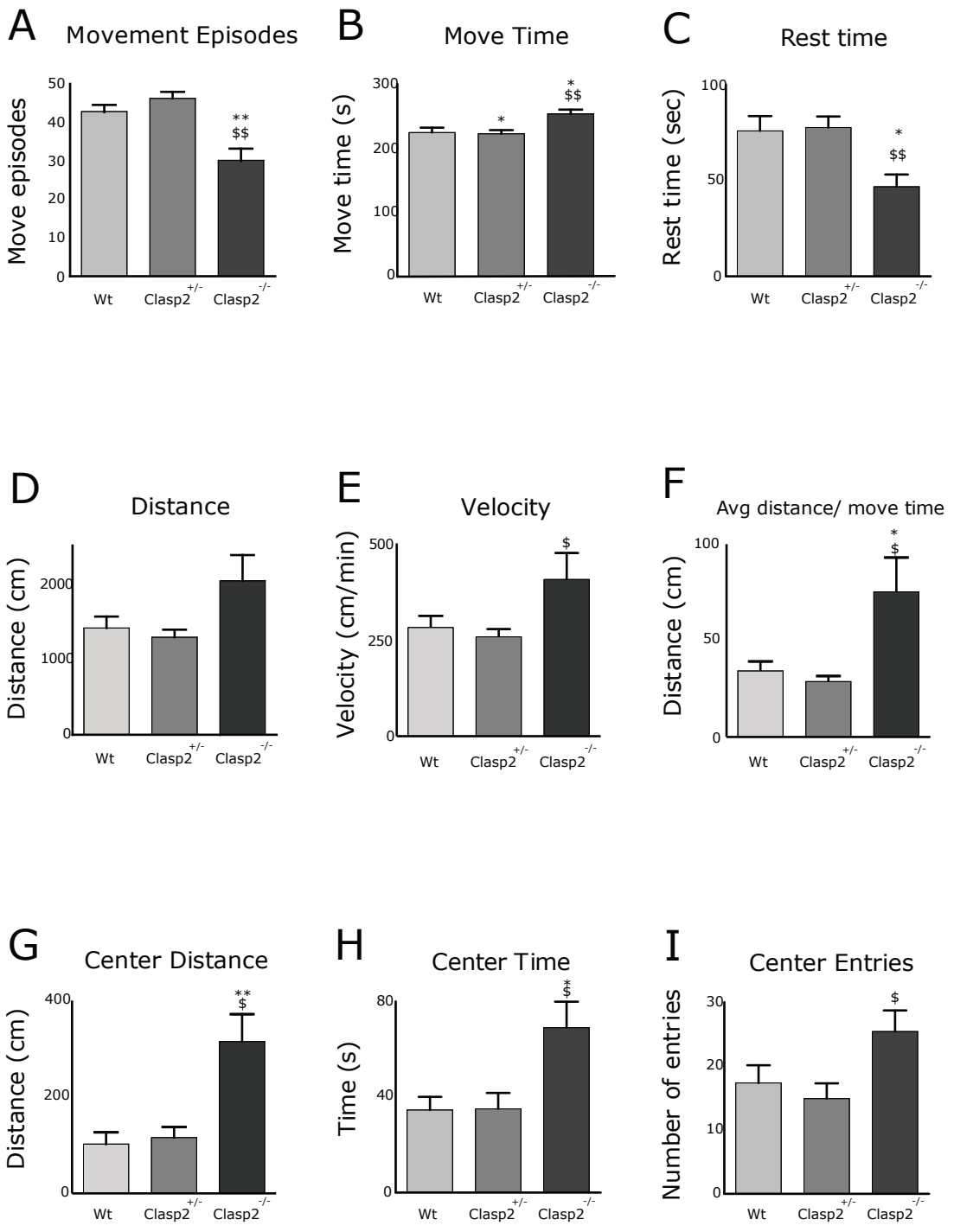
Compared to the *Clip1*$^{-/-}$ animals, the *Clasp2*$^{-/-}$ mice displayed a completely different pattern of behavior in the openfield test (Fig 2). The total number of movement episodes was significantly lower than either wild type ($P<0.01$, t-test) or heterozygous littermates ($P<0.01$, t-test), indicating a reduced number of pauses. Accordingly, moving time was higher (Fig 2 B, $P<0.05$ (t-test), compared to wild type, and $P<0.01$ (t-test) compared to heterozygous littermates) and especially the total distance walked (Fig 2D) and the velocity of mice while moving (Fig. 2E, $p<0.05$ (t-test), compared to wild type and heterozygous littermates) were significantly increased in *Clasp2*$^{-/-}$ mice. Resting time (Fig 2 C) was instead significantly reduced in knockout animals when compared to wild type ($P<0.05$, t-test) or heterozygous ($P<0.01$, t-test) littermates. Combined these data strongly suggest that *Clasp2*$^{-/-}$ mice are more active than wild type or heterozygous littermates.

Interestingly, the total time spent in the center of the arena (Fig 2 H) was also significantly higher in knockout mice as compared to either wild type ($P<0.05$, t-test) or heterozygous littermates ($P<0.05$, t-test). Accordingly the total distance walked in the center of the arena was much higher for knockout mice as compared to wild type ($P<0.01$) or heterozygous ($P<0.05$) littermates. As mice normally avoid open areas, these data indicate that the *Clasp2*$^{-/-}$ mice are rather fearless. They are also easy to handle and will voluntarily seek contact with the experimenter (unpublished observation). Still, even if the *Clasp2*$^{-/-}$ mice spend less time in the margin of the openfield box (Fig 1 K) compared to both wild type ($P<0.05$, t-test) and heterozygous littermates ($P<0.05$), the actual distance walked in the margin was significantly higher (Fig 1 J; $P<0.05$ (t-test), compared to wild type and heterozygous littermates). These results reinforce the notion that *Clasp2*$^{-/-}$ mice are very active.

The number of entries into the vertical plane (standing on the hind limbs or raising the head above the sensors) was significantly reduced in *Clasp2*$^{-/-}$ mice as compared to wild type littermates ($P<0.01$, t-test) and accordingly, the time spent in the vertical plane was reduced ($P<0.01$, t-test). These data suggest that *Clasp2*$^{-/-}$ mice are more active but are less interested in exploring the arena in the vertical direction, which could be an indicator of mental retardation.

*Rotarod behavior of +TIP knockout and knock-in mice*

The rotarod test is widely used as a simple measure of cerebellar functionality. The acceleration of the rotating rod requires balance and coordination and also that mice


A
Movement Episodes
Move episodes
50
40
30
20
10
0
**
$$
Wt
Clasp2+/-
Clasp2-/-
B
Move Time
Move time (s)
300
200
100
0
*
*
$$
Wt
Clasp2+/-
Clasp2-/-
C
Rest time
Rest time (sec)
100
50
0
*
$$
Wt
Clasp2+/-
Clasp2-/-
D
Distance
Distance (cm)
2000
1000
0
Wt
Clasp2+/-
Clasp2-/-
E
Velocity
Velocity (cm/min)
500
250
0
$
Wt
Clasp2+/-
Clasp2-/-
F
Avg distance/ move time
Distance (cm)
100
50
0
*
$
Wt
Clasp2+/-
Clasp2-/-
G
Center Distance
Distance (cm)
400
200
0
**
$
Wt
Clasp2+/-
Clasp2-/-
H
Center Time
Time (s)
80
40
0
**
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Wt
Clasp2+/-
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Center Entries
Number of entries
30
20
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Clasp2-/-

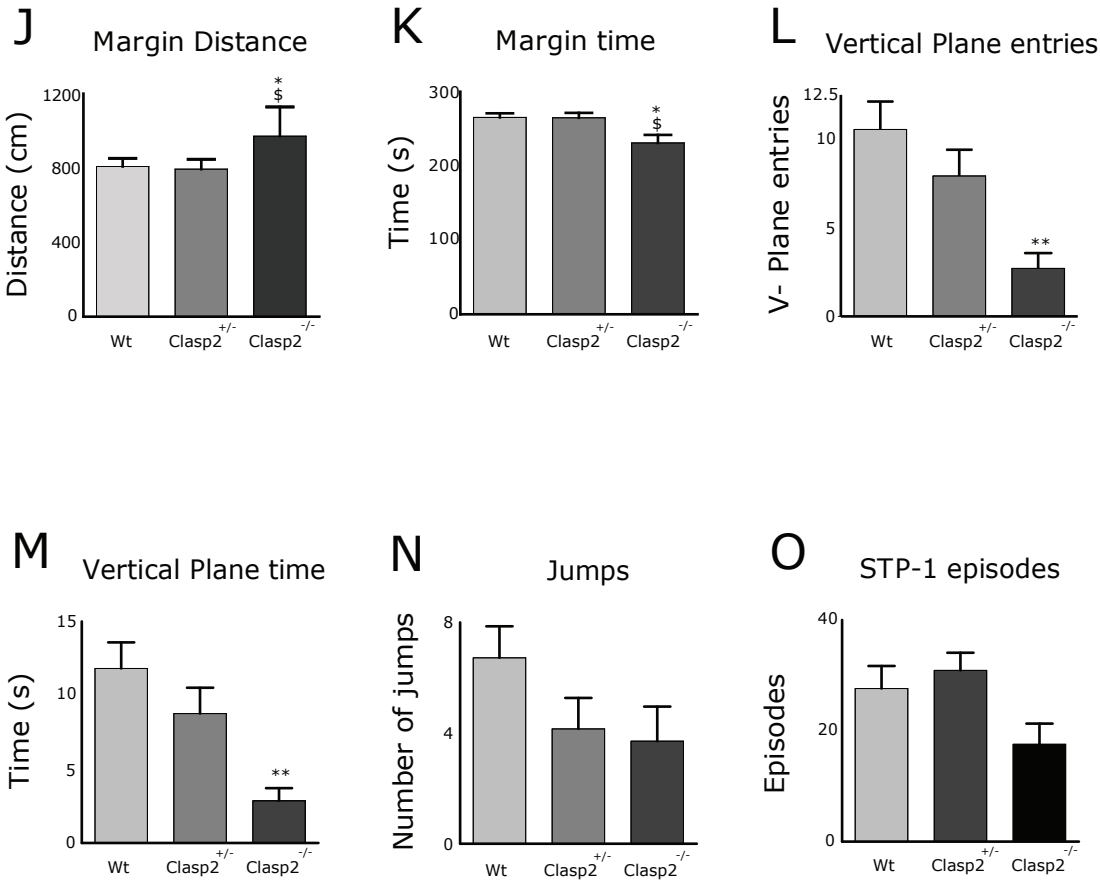


Figure –2 - Openfield analysis of *Clasp2* mutants. *Clasp2* $^{+/-}$ (n =13) *Clasp2* $^{-/-}$ (n = 6) and Wt litter mates (n=10) were tested for their pattern of spontaneous locomotor activity in the openfield setup. Animals were placed individually at the centre of the 50cmx50cm arena and allowed to move for 5 min, in which time all the movements were recorded with an automated system. Parameters analysed are described in the Materials and Methods section. Results were gathered and statistical analysis was performed with the Prism or SPSS software. Groups were compared to their respective control (Wt) littermates with t-student tests. Significant values ($p<0,05$) are noted with the symbols * (as compared to Wt) or $ (as compared to +/-). Double symbols (** or $$) represent a higher statistical significance ($p<0.01$).

learn to adjust their movement in order to remain on the rod; functions typically associated with the cerebellum. In order to assess if +TIP depletion has an effect on motor coordination, we tested the performance of *Clip1*, *Clip2* and *Clasp2* mutant mice in the accelerating rotarod setup. All of the *Clip1* genotypes tested produced comparable results with no observable statistic difference ($P>0,05$; Two-way ANOVA with Bonferroni correction), when tested on the rotarod (Fig. 3 A and B). Interestingly, even though the *Clasp2*$^{-/-}$ mice appear to be hyperactive and walk in a wobbly pattern

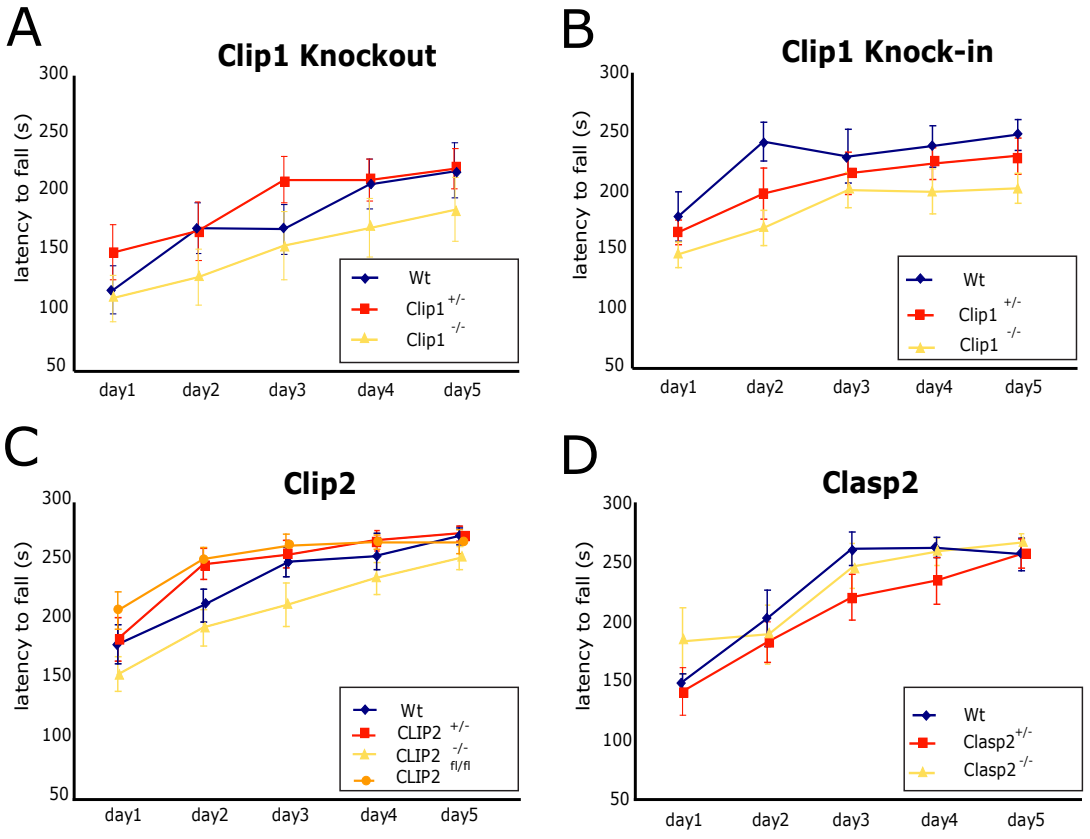


Figure 3 – rotarod analysis. Mice were placed on the rotating platform of the UgoBasile rotarod. The rod is accelerated from 4 to 40 rpm in 5 minutes. The sessions are repeated daily for 5 days. Latency to fall from the rod is measured (in s). A- *Clip1* $^{+/-}$ and *Clip1* $^{-/-}$ compared to Wt littermates. B – *Clip1* $^{ki/+}$ and *Clip1* $^{ki/ki}$ compared to Wt littermates. C – *Clip2* $^{+/-}$ and *Clip2* $^{-/-}$ compared to *Clip2* $^{fl/fl}$ and Wt littermates. D – *Clasp2* $^{+/-}$ and *Clasp2* $^{-/-}$ compared to Wt littermates

(data not shown), no differences (P>0,05: two-way ANOVA) were observed among groups in the rotarod paradigm (Fig 3 D).

*Clip2* mutants have previously been tested in the rotarod paradigm (Hoogenraad et al., 2002; van Hagen et al., 2007). The results showed that *Clip2* $^{-/-}$ mice performed much worse than wild type littermates and significant differences in performance were also observable for *Clip2* $^{+/-}$ mice. However, these experiments were done with an atypical rotarod set-up (see de Zeeuw et al 1998) and we therefore repeated this test with a standard rotarod (UgoBasile) to allow a better comparison with other mouse mutants in this study. Furthermore, the *Clip2* mutants used in the present study were obtained by backcrossing into the c57Bl/6 background for

nine generations, unlike the previously reported mice. We found that the overall performance of the *Clip2*$^{-/-}$ mice was worse than that of control littermates (Fig 3 C; p=0.021, two-way ANOVA), compared to wild type littermates; and compared to heterozygous *Clip2* mutants or to *Clip2*$^{fl/fl}$ mutants (P=0.000). However, in contrast to our previous results (Hoogenraad et al., 2002), which were again reported in a more recent publication (van Hagen et al., 2007), differences in rotarod performance were not observable for the heterozygous *Clip2*$^{+/-}$ mice when compared to wildtype mice (Fig. 3C, p>0,05; two-way ANOVA). Based on these results we conclude that a complete lack of CLIP-115 is required to cause impaired rotarod performance in mice.

*Catwalk behavior of +TIP knockout and knock-in mice*

Previous reports on *Clip* mutant mice from our group have pointed to differences in posture (Hoogenraad et al., 2002; Miedema, 2007). In order to assess the contribution of CLIPs and CLASP2 to posture, we tested mice in the catwalk setup (Hamers et al., 2006; Hamers et al., 2001; Muir and Webb, 2000), which provides a digital version of the paw print or runway tests. The catwalk is more accurate than the paw print tests, allowing inferences on both coordination and posture. In our study we measured the time that each mouse took to cross a fixed length of the glass plate (duration of crossing), the percentage of steps that follow a normal pattern, as defined by the sequence of paw placements (normal patterns); the average distance between left and right forepaws (base of support forepaws) as well as the average distance between left and right hindpaws (base of support hindpaws) (Hamers et al., 2001) see also Ma et al., 2001) for an example on base of support) and the step length for each of the four limbs (step length left forelimb, right forelimb, left hindlimb, right hindlimb). Results of this analysis are depicted in Fig. 4 (*Clip1* mutants), Fig. 5 (*Clip2* mutants), Fig. 6 (*Clasp2* mutants) and Fig. 7 (*Clip* DKO mutants).

The *Clip1* mutant mice were divided into two groups, i.e. knockout and knock-in mice (Fig. 4). We tested differences within groups and among groups. Of all parameters tested, only the base of support for the hind limbs showed significant differences (Fig. 4D, p<0.05, ANOVA). However, in this test the two wild type groups showed the most significant differences when compared to each other. Based on these observations we cannot conclude that a deficiency of CLIP-170 contributes to abnormal posture or walking.

The *Clip2* mutant mice were previously shown to have a mild growth deficit (Hoogenraad et al., 2002), which could result in different posture and catwalk behavior. We found that the base of support for the hind paws of *Clip2*$^{-/-}$ mice (Fig 5 D)

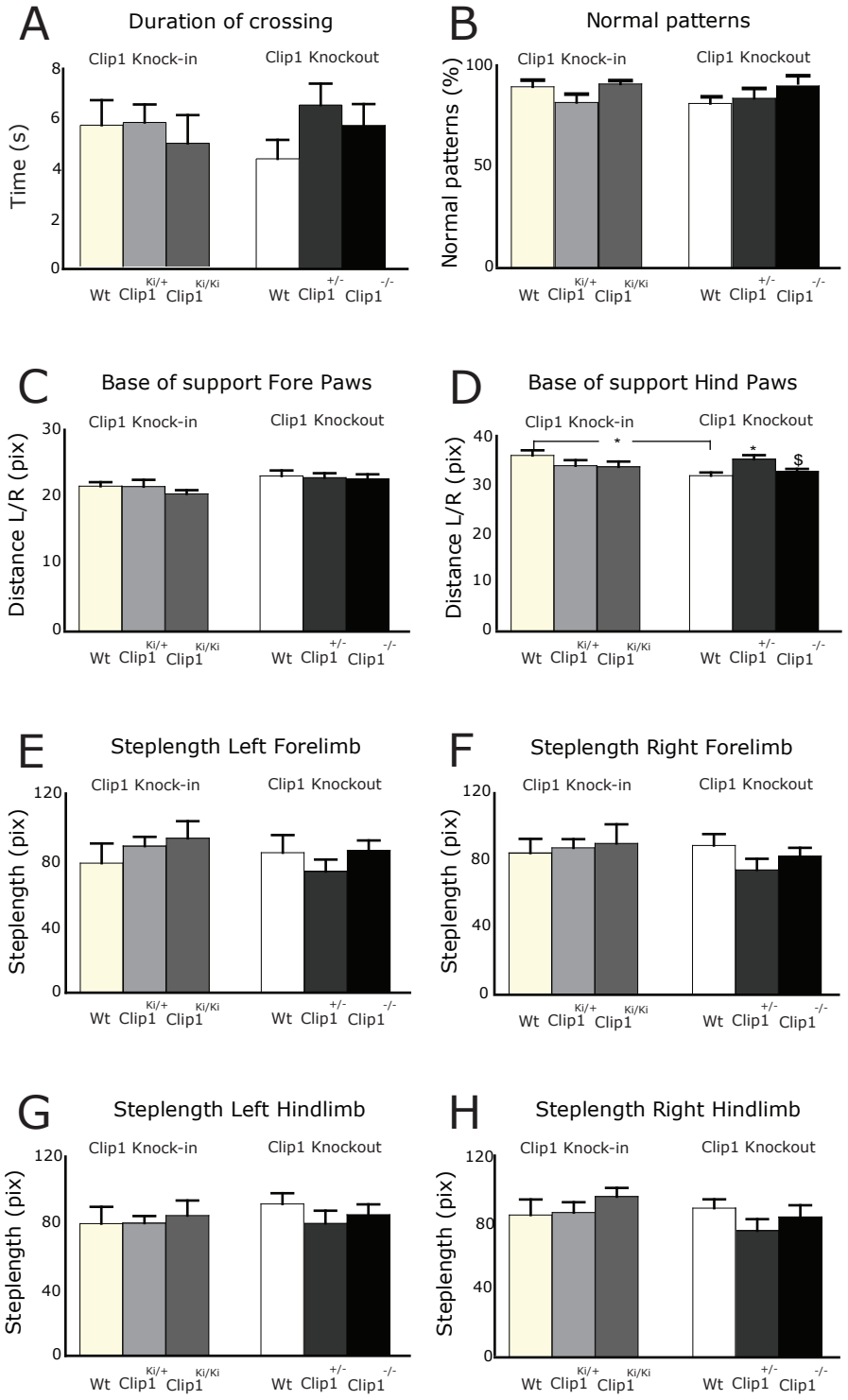
A
Duration of crossing
Clip1 Knock-in
Clip1 Knockout
Time (s)
0
2
4
6
8
Wt
Clip1 Ki/+
Clip1 Ki/Ki
Wt
Clip1 +/-
Clip1 -/-
B
Normal patterns
Clip1 Knock-in
Clip1 Knockout
Normal patterns (%)
0
50
100
C
Base of support Fore Paws
Distance L/R (pix)
0
10
20
30
D
Base of support Hind Paws
Distance L/R (pix)
0
10
20
30
40
*
*
$
E
Steplength Left Forelimb
Steplength (pix)
0
40
80
120
F
Steplength Right Forelimb
Steplength (pix)
G
Steplength Left Hindlimb
Steplength (pix)
H
Steplength Right Hindlimb
Steplength (pix)

Figure 4 (previous page) – Catwalk analysis of *Clip1* mutant mice. *Clip1* mutant mice were divided in two groups (see table 1). Knockout corresponds to *Clip1* $^{+/-}$ and *Clip1* $^{-/-}$ and Wt littermates. Knock-in corresponds to *Clip1* $^{ki/+}$ and *Clip1* $^{ki/ki}$ and Wt littermates. The base of support for the hindpaws of the mice is statistically significantly different between *Clip1* $^{+/-}$ and *Clip1*$^{-/-}$ (P<0,05, t-test) (marked with the asterisk) and between *Clip1*$^{+/-}$ and Wt (p<0,05, t-test)(marked with the dollar sign) but not between *Clip1* $^{-/-}$ and Wt.

was markedly narrowed when compared to wild type (P<0.01, t-test) or *Clip2*$^{fl/fl}$ mice (P<0.05, t-test). We also detected differences in the duration of crossing of the *Clip2*$^{fl/fl}$ mice (Fig 5 A), as well as a reduction in the number of normal walking patterns (Fig 5 B) when compared to wild type controls (both P<0.05, t-test). Combined, these data indicate that a deficiency of CLIP-115 influences walking patterns. Since in the *Clip2*$^{fl/fl}$ mice CLIP-115 is normally expressed, our results also suggest a *Clip2*-independent influence on locomotion.

When we analyzed the locomotion of *Clasp2* mutant mice, we detected significant deviations in the knockout versus control mice (Fig. 6). *Clasp2* mutants seem to be faster crossing the catwalk glass plate (Fig 4A) than control littermates (P<0.05, t-test). Furthermore, the average distance between the left and the right hind paws was significantly reduced in the *Clasp2* knockout mice as compared to wild type littermates (Fig. 6D, P<0.05, t-test). The length of the steps taken by the left forelimb (Fig 4 E, P<0.05, t-test) and the right forelimb (Fig 4 F, P<0.01, t-test) was significantly longer than in control littermates, but since differences in step length were also visible for the heterozygous animals (P<0.05, t-test), we considered this aspect of less relevance. We conclude that a CLASP2-deficiency results in altered walking behavior.

Recently, *Clip1* and *-2* double knockout (*Clip* DKO) mice were generated, displaying very obvious posture defects (Miedema, 2007). These mice were analyzed in the catwalk setup to examine if a combined deficiency of CLIP-115 and -170 also leads to a phenotype of altered walking patterns. The locomotive posture of the hind limbs of *Clip* DKO mice is indeed affected, but in an age-dependent manner (Fig 7 A). Wild type control mice show a reasonably stable base of support for their hindlimbs (aproximately 30-32 pix) throughout the studied lifespan (from 3 to 24 months). *Clip* DKO mice show an apparently reduced base of support for their hind limbs up to the age of 15 months. This trend is consistent with what we observed in the *Clip2* knockouts (Fig. 4), however, it is not statistically significant. Strikingly, the base of support for the hindpaws of Clip DKO increased at 18 months, remaining increased up to the age of 24 months, at which time statistically significant differences were measured (p<0.05, t-test). These data indicate that a deficiency of CLIP-115 and -170 leads to a complex but measurable phenotype in the catwalk.

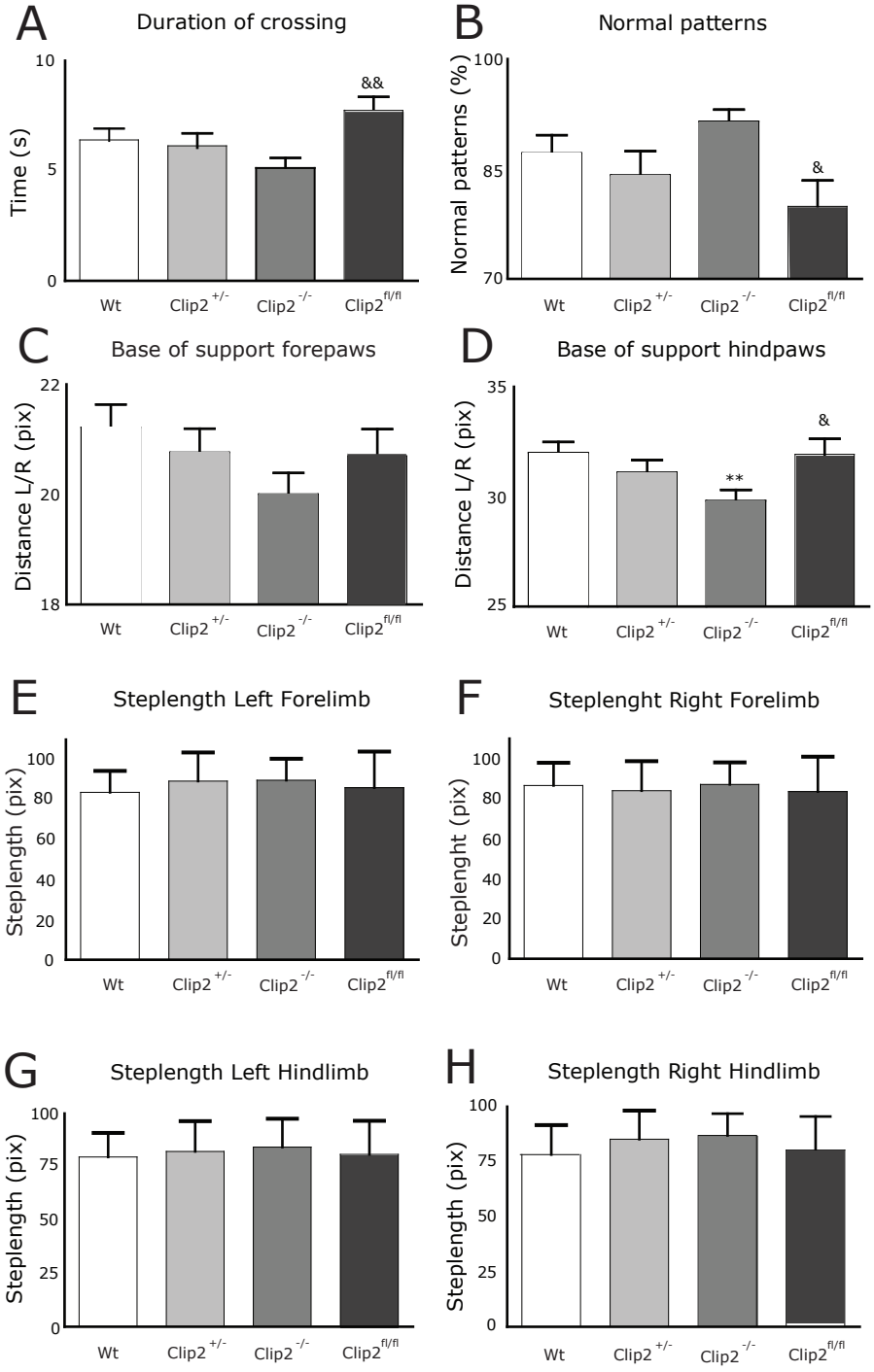
A
Duration of crossing
Time (s)
10
5
0
&&
Wt
Clip2 +/-
Clip2 -/-
Clip2 fl/fl
B
Normal patterns
Normal patterns (%)
100
85
70
&
C
Base of support forepaws
Distance L/R (pix)
22
20
18
D
Base of support hindpaws
35
30
25
**
E
Steplength Left Forelimb
Steplength (pix)
100
80
60
40
20
F
Steplenght Right Forelimb
Steplenght (pix)
G
Steplength Left Hindlimb
75
50
25
H
Steplength Right Hindlimb

Figure 5 (previous page) – Catwalk analysis of *Clip2* mutants. *Clip2* $^{+/-}$ and *Clip2* $^{-/-}$ mutant mice were analyzed for their performance in the Catwalk setup, compared to *Clip2* $^{fl/fl}$ and Wt littermates. Parameters analyzed are described in materials and methods, Statistically significant differences are mentioned by the symbols: * comparison between Wt and *Clip2* $^{-/-}$ ; & comparison between *Clip2* $^{-/-}$ and *Clip2* $^{fl/fl}$. One symbol (* or &) represents a statistically significant difference (p<0,05, t-test) and two symbols (** or &&) represent a more significant difference (P<0,01, t-test).

An effect of age can also be observed for the duration of crossing (Fig 7 B). At 15 months, the crossing times for *Clip* DKO mice stops decreasing, unlike what happens for wild type mice (P<0.001, t-test). A clearly observable difference in performance up to the end of the tested time points (age 24 months) is maintained. These results show a tendency for divergence between the groups. Statistically, the significance is not always visible because the number of tested animals was quite small, due to the difficulties inherent to the generation of the double knockouts ((Miedema, 2007).

*Comparison of behavior of wild type mice*

While performing the different tests we noted that groups of wild type mice tended to behave differently from each other. This could mean that the tests used might be sensitive to genetic background variation and that the phenotypes observed might not be due to the specific lack of +TIPs. We therefore compared the performance of the wildtype littermates of all genotypes used in this study. We also extended this test to include wild type mice of inbred strains such as FVB, 129/Svj and Bl/6. Just like +TIP mutants, wildtype mice were tested in the openfield, rotarod and catwalk setups.

Comparison of wild type mice in the openfield test indicates highly significant differences, particularly when wild type littermates of the *Clip1* knockout mice are included in the analysis (Appendix Fig. 1). These differences are noticeable for multiple parameters (movement episodes, movement time, rest time, velocity, margin distance, margin time, center distance, center time, center entries). These data indicate that these parameters of the openfield test are influenced by genetic background. However, parameters like Vertical-plane entries and Vertical-lane time are more robust.

While most wild type groups behaved rather similar on the rotarod, the wild type littermates of the *Clip1* knockout mice showed an aberrant behavior (Appendix Fig. 2). As this group of animals behaves differently in two tests, we speculate that while generating the *Clip1* $^{-/-}$ mutant mice another mutation was introduced, perhaps in the embryonic stem (ES) cells, and that this mutation contributes significantly to behavioral output.

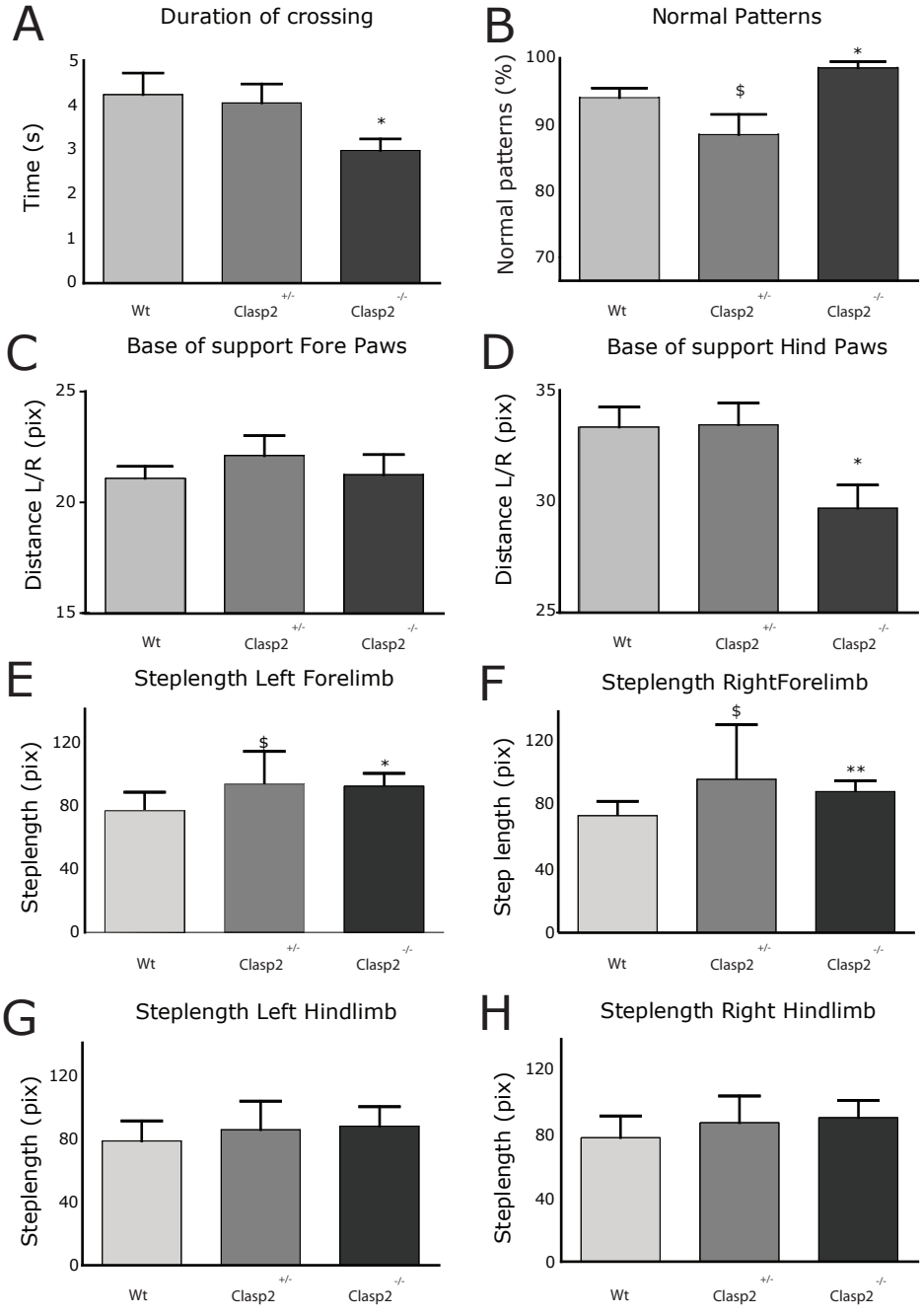
A
Duration of crossing
Time (s)
5
4
3
2
1
0
*
Wt
Clasp2 +/-
Clasp2 -/-
B
Normal Patterns
Normal patterns (%)
100
90
80
70
$
*
Wt
Clasp2 +/-
Clasp2 -/-
C
Base of support Fore Paws
Distance L/R (pix)
25
20
15
Wt
Clasp2 +/-
Clasp2 -/-
D
Base of support Hind Paws
Distance L/R (pix)
35
30
25
*
Wt
Clasp2 +/-
Clasp2 -/-
E
Steplength Left Forelimb
Steplength (pix)
120
80
40
0
$
*
Wt
Clasp2 +/-
Clasp2 -/-
F
Steplength RightForelimb
Step length (pix)
120
80
40
0
$
**
Wt
Clasp2 +/-
Clasp2 -/-
G
Steplength Left Hindlimb
Steplength (pix)
120
80
40
0
Wt
Clasp2 +/-
Clasp2 -/-
H
Steplength Right Hindlimb
Steplength (pix)
120
80
40
0
Wt
Clasp2 +/-
Clasp2 -/-

Figure 6 (previous page) – catwalk analysis of *Clasp2* mutants. *Clasp2* $^{+/-}$ and *Clasp2* $^{-/-}$ mutant mice were compared to their Wt littermates in the catwalk setup. Each group was compared to the Wt group (* or $). Single signs (* or $) represent a statistically significant difference (P<0,05, t-test) and double signs (** and $$) represent a higher statistically significant difference (P<0,01, t-test).

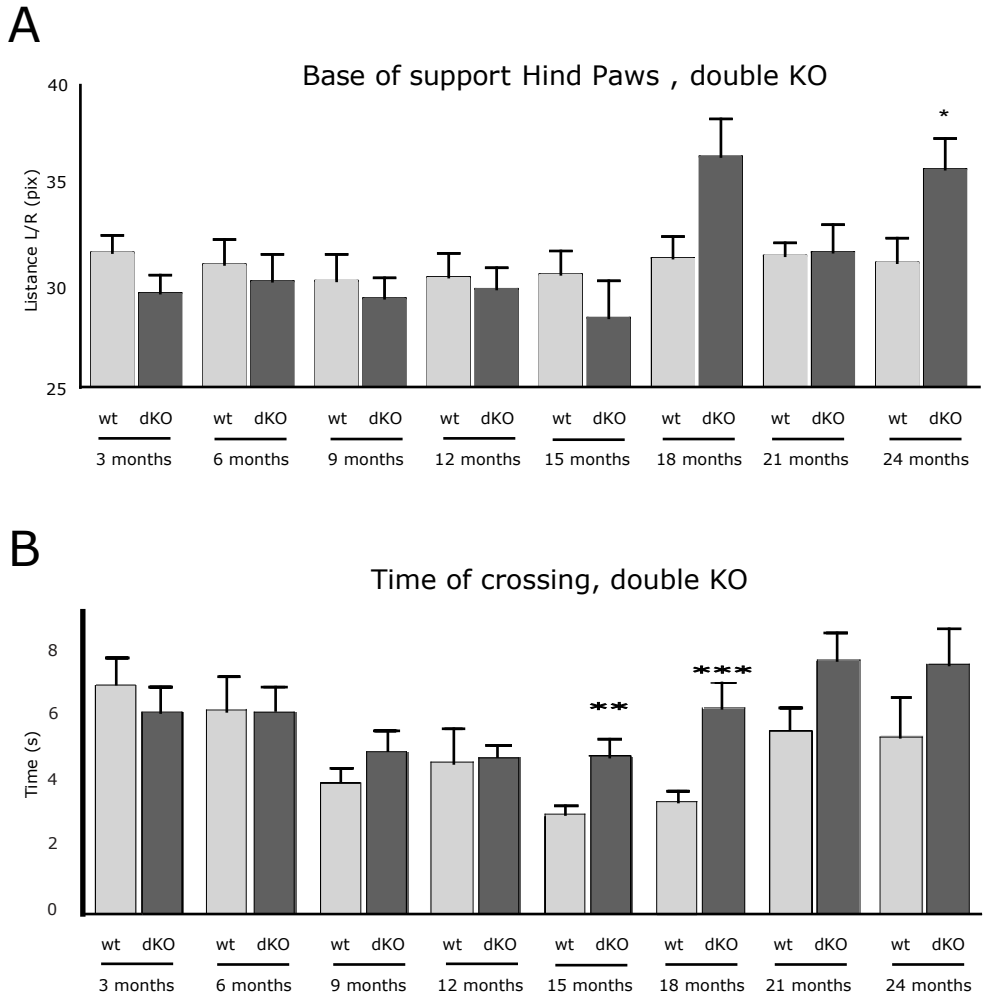


Figure 7 – Catwalk analysis of double (*Clip1* & *Clip2* KO). Double *Clip1*/*Clip2* knockout mice were analyzed in the catwalk setup, compared to wild type littermates. This analysis was repeated every three months up to the age of 24 months. Differences between the groups were analyzed statistically (students t-test) and the differences are marked with asterisk. * - p<0,05; ** - p<0,01; *** - < 0,005. Panel A depicts the base of support for the hindpaws and panel B the duration of crossing of the runway.

For the catwalk analysis, significant differences between the wild type groups can be observed for the duration of crossing and the base of support of the forepaws (Appendix Fig 3). Other parameters like the percentage of normal patterns, the base of support for the hindpaws and the step length of left or right forepaws do not show any difference between groups. As with the other tests, these data start to indicate which aspects of the catwalk assay are easily influenced by genetic background and which parameters are more robust.

## Discussion

In this study we have compared several behavioral parameters of *Clip1*, *Clip2*, *Clip-dko* and *Clasp2* mutant mice. We focused on locomotor behavior in well-established paradigms. The aims of this study were to document the type of locomotor abnormalities in the different mouse models, to compare results with published studies and to explore the relevance of CLIPs and CLASP2 for behavior, in particular for cerebellar function.

The influence of genetic background on mouse behavior has been extensively discussed (Crawley, 1999, 2000). To minimize the chance that a behavioral output is influenced by differences in genetic background, mice are backcrossed to an inbred strain (e.g. C57/BL6) for as many generations as possible. To test the contribution of genetic background to the outcomes in the selected paradigms we therefore compared six different groups of wild type mice, including inbred FVB, Bl/6 and 129/Svj strains, as well as the wild type littermates of the knockout mice produced. Results of the openfield test revealed variations in all parameters tested ($p<0.05$, ANOVA) except for the vertical plane entries and time. This indicates that most openfield results should be carefully interpreted. However, as the hyperactivity phenotype, related to CLASP2-deficiency, is supported by other results (see below), we consider this a reliable output.

A comparative analysis of the wildtype groups in the rotarod indicates a very significant difference between the Bl/6 mice and the wildtype littermates of the *Clip1* knockout mice ($p<0.01$, two-way ANOVA with Bonferroni correction for multiple measurements), FVB or 129/Svj mice ($p<0.05$, two-way ANOVA with Bonferroni correction for multiple measurements). Only punctual differences are found between other genotypes. This is somewhat surprising due to the expectations that Bl/6 mice are generally good learners (Crawley, 2000). These results emphasize the importance of laboratory conditions and intra-experiment variation (Crabbe et al., 1999).

The comparison of wildtype strains in the catwalk analysis shows statistically significant differences in the duration of crossing ($p=0.047$, ANOVA). Big differences

can be seen in the base of support for the forepaws (p=0.001, ANOVA) but not for the hindpaws (p=0.122, ANOVA). In conclusion, the differences in behavior in the various tests between wild type groups of mice suggest an influence due to genetic background. By appropriate inter-group comparisons this influence can be measured.

It has recently been reported that high levels of Cre recombinase in the brain may lead to neuronal loss and hydrocephaly (Forni et al., 2006). As Cre recombinase was used in ES cells to generate the *Clip1* knock-in and *Clip2* knockout mice, cryptic Cre activity may have resulted in unwanted and unnoticed mutations in ES cells. An appropriate number of back crossings would be expected to eliminate such unspecific mutations. This highlights the importance of backcrossing even more.

In the present study, we find that homozygous *Clip2* knockout mice show a statistically significant difference in their performance in the accelerating rotarod when compared to wild type and heterozygous littermates. Thus, CLIP-115 is important for cerebellar output. Previously, we also reported significant deficits in the rotarod for *Clip2*$^{-/-}$ mice. At that time defects were already noticeable at the heterozygous level (Hoogenraad et al., 2002). More recently, the rotarod and MRI data that were obtained in 2002 with the *Clip2*$^{-/-}$ mice (Hoogenraad et al., 2002) were reported again, and used to compare behavior in those assays with *Gtf2ird1*$^{-/-}$ mice (van Hagen et al., 2007). Thus, the phenotype obtained in heterozygous *Clip2* knockout animals all come from a group of mice generated in 2001-2002. One marked difference between the presently used group of animals and the mice in the previous reports is a higher number of backcrossing into the C57Bl6 background for the present study. Moreover, in our previous studies on motor coordination of *Clip2* mutant mice (Hoogenraad et al., 2002), we performed experiments on a homemade rotarod, which was situated outside of the mouse house. In the present study we used a standardized (UgoBasile) rotarod placed inside the mouse house. Both factors may have contributed to differences in results with the rotarod.

In a separate experiment *Clip2* knockout mice were crossed back to the 129/Svj background for two generations and tested on the rotarod. No differences were found between the groups in this experiment (M. Miedema et al, unpublished). Taken together these data suggest that the effect of a CLIP-115 deficiency is background dependent and becomes more obvious in the C57Bl6 background. Based on all considerations we conclude that CLIP-115 is important for motor coordination but only when the protein is completely absent. Thus, our previous study (Hoogenraad et al., 2002) may have yielded an exaggerated effect because: 1) a different genetic background of the animals; 2) different laboratory conditions; 3) a different rotarod

set-up.

Recently, a Williams Syndrome (WS) patient has been described with an atypical deletion. This patient did not display the coordination problems associated with WS. In this patient, the *Clip2* gene was not mutated, (van Hagen et al., 2007) indicating that a deficiency of CLIP-115 underlies aspects of locomotion deficits in WS patients (Meyer-Lindenberg et al., 2006). This is consistent with the data on the *Clip2* knockout mice, but we stress that in mice a complete absence of CLIP-115 is needed for a reliably observable phenotype, whereas in most WS patients only one copy of the gene is deleted.

*Clip1* mutants have extensively been documented at the level of spermatogenesis (Akhmanova et al., 2005) but no behavioral studies have been reported yet. CLIP-170 has an essential role in the targeting of dynactin to MT plus ends (Lansbergen et al., 2004); the protein is therefore likely to regulate dynein function and vesicle transport over MTs (Pierre et al., 1992). Furthermore, this protein is expressed in the brain as a brain-specific isoform, fueling the expectations that CLIP170 be necessary for the fine tuning of microtubule related functions and that its deficiency would translate in altered patterns of locomotion. However, except for differences in the number vertical plane entries and time spent in the vertical plane by *Clip1* knockout mice in the openfield assay, which are two robust and striking phenotypes, we did not find behavioral abnormalities in any of the assays in mice lacking CLIP-170. These results suggest that in the cerebellum CLIP-170 function is either not essential or redundant. Because of the high level of similarity between the CLIPs, CLIP115 is the most logical candidate to take over the function of CLIP-170 (Hoogenraad et al., 2000; Komarova et al., 2002). A comparison of *Clip1* and *Clip2* knockout mice indicates that CLIP-170 is less relevant for motor coordination than CLIP-115.

To investigate the influence of the genetic background, the *Clip1* knockout mice were analyzed together with *Clip1* knock-in mice. Since the GFP-CLIP170 fusion protein appears to behave similarly to the endogenous CLIP170, an effect of the knock-in construct would not be expected. However, there are some visible differences in behavior. As the knock-in mice are derived from ES cells that were treated with the Cre recombinase (Akhmanova et al., 2005), it could be that in these ES cells mutations are present that contribute to behavioral deficits.

To test the effect of a double CLIP deficiency, we generated *Clip1/2* DKO mice (Miedema, 2007). When tested in the rotarod set up, these mice showed a surprising age-dependent phenotype. At young ages (3-6 months) the *Clip1/2* DKO mice performed significantly worse than wild type littermates. Between 9-21 months this deficit was overcome, but at 24 months a phenotype was detected again (Miedema,

2007). These data are consistent with our present results and point to an important role for the CLIPs in behavior.

The *Clip1/2* DKO mice display an accentuated change in posture, as measured by their base of support for the hindlimbs, which does increase with age. This altered posture of the hindlimbs was observed in some degree in all of the (homozygous) mutants analyzed in this study. Reasons for this are still unknown, since all the mice displayed normal muscle strength (data not shown). Combined, the data indicate that lack of CLIPs may result in locomotion deficits associated with aging.

CLASP2 is another +TIP that binds both CLIP-115 and CLIP-170 (Akhmanova et al., 2001). CLASP2 plays a very important role in the stabilization of MTs and in directed cell motility (Drabek et al., 2006). Others have shown an important role for CLASP2 in neuronal function (Lee et al., 2004). CLASP2-deficient mice have growth abnormalities, develop an anemic phenotype, and die at a relatively young age due to hemorrhages (Drabek et al., 2007). No behavioral studies for these mice have been published yet. *Clasp2* mutant mice displayed striking particularities in their pattern of movement in the openfield. An increase in time spent and distance walked in the center of the open field arena points to lack of fear. Accordingly, these mice are mostly docile and easy to handle (unpublished results). Their relative lack of interest in the vertical plane could be an indication of lack of curiosity, possibly related to mental retardation or, alternatively, a deficit related to their altered hind paw posture.

*Clasp2* knockout mice are smaller and do not seem to walk smoothly, so it was somewhat surprising to observe that they did not show any difference in motor coordination in the rotarod and that they generally moved faster in both the open field and the catwalk. It was also surprising to see that these mice stride with bigger steps (taken with the forepaws) than wild type littermates. Our analysis of these mice suggests severe defects that are not necessarily locomotion related and that should be investigated further in other paradigms, such as fear conditioning or memory tests. Combined our results indicate that +TIPs have an effect on cerebellar-related tasks, and on hindlimb and general posture. Further tests are needed to understand the exact nature of the MT-related effect on posture, which appears to be muscle-tone independent.

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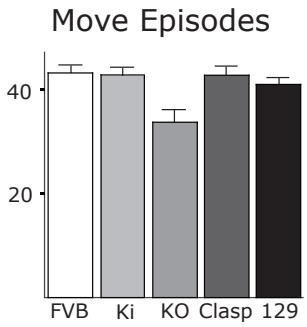
# Appendix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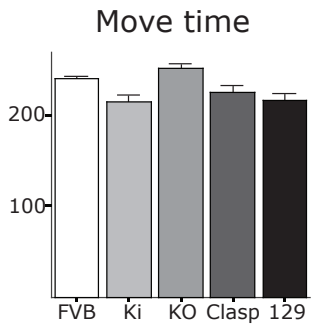
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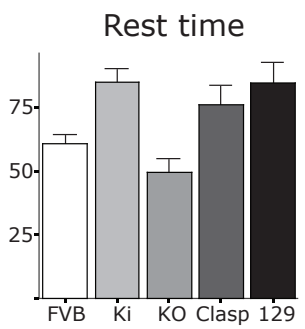
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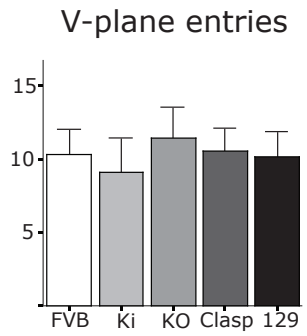
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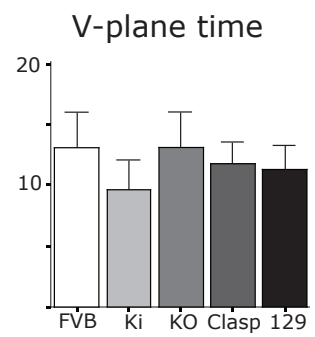
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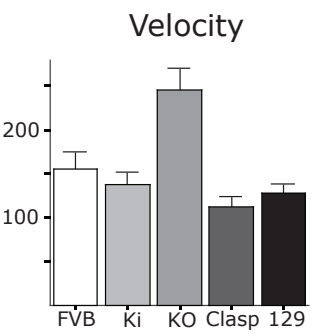
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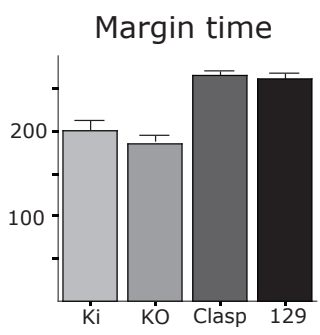
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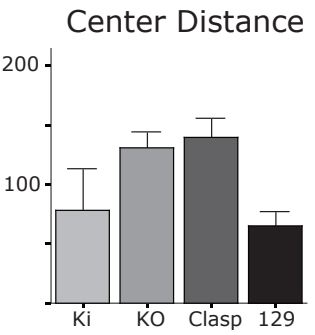
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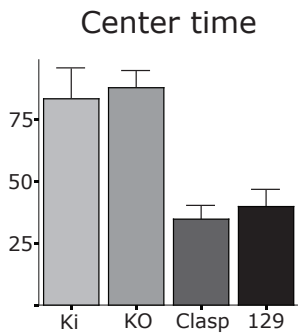
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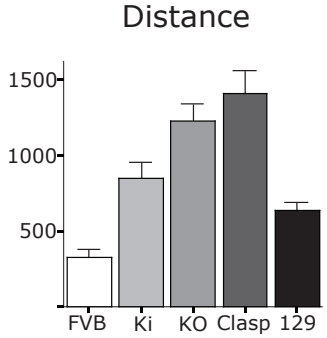
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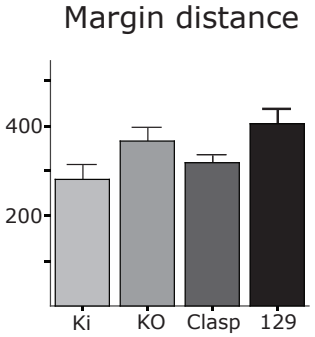
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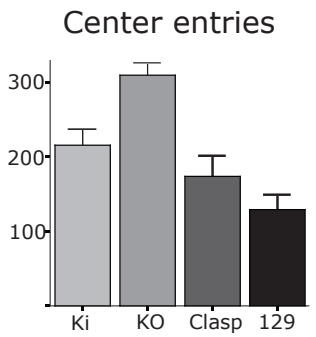
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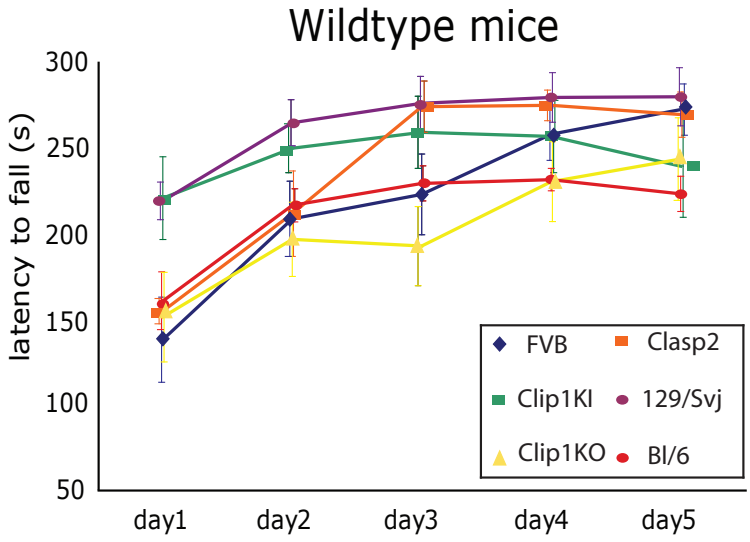
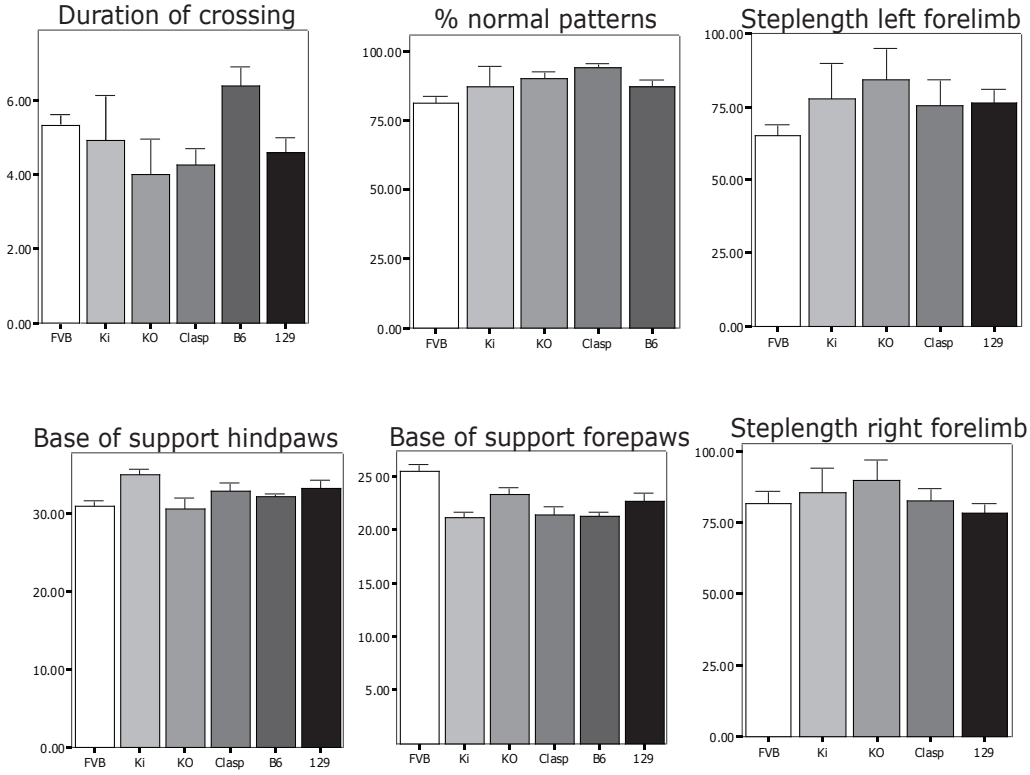
Appendix Fig A (previous page) – Openfield analysis of wildtype mice. FVB mice (n=8), 129/Svj mice (n=20), Wildtype littermates of the Clip170 Knock-in (Ki, n=7) and knockout (KO, n=11) and *Clasp2* KO (n=11) were analyzed. Groups were analyzed with one-way ANOVA. Only the parameters corresponding to the number of entries into the vertical plane (VP entries) and the time spent in the vertical plane (VP time) did not show a statistically significant difference (P>0.05, ANOVA). All the other parameters show a statistically significant difference between the groups




Appendix Fig B - Rotarod analysis of wildtype mice. FVB mice (n=8), 129/Svj mice (n=20), Bl/6 mice (n=20). Wildtype littermates of the *Clip1* Knock-in (Ki, n=7) and knockout (KO, n=11) and *Clasp2* ko (n=11) were analyzed. Data was analyzed statistically with two-way ANOVA with Bonferroni correction for multiple measurements. Big statistically significant differences (P<0.001) are observed when comparing Bl/6 mice with FVB, 129/Svj and the wildtype littermates of the *Clip1* knockout mice.

Appendix Fig C – catwalk analysis of wildtype mice. FVB mice (n=8), 129/Svj mice (n=20), Bl/6 mice (n=20). Wildtype littermates of the *Clip1* Knock-in (Ki, n=7) and knockout (KO, n=11) and *Clasp2* (KO, n=11) were tested. Data was compared statistically by one-way ANOVA. Significant differences were observed for the duration of crossing (P=0.047) and for the base of support of the forepaws (p = 0.001).




Appendix Fig C – catwalk analysis of wildtype mice. FVB mice (n=8), 129/Svj mice (n=20), Bl/6 mice (n=20). Wildtype littermates of the *Clip1* Knock-in (Ki, n=7) and knockout (KO, n=11) and *Clasp2* ko (n=11) were tested. Data was compared statistically by one-way ANOVA. Significant differences were observed for the duration of crossing (P=0.047) and for the base of support of the forepaws (p = 0.001).

# Chapter IV

# General Discussion

## General Discussion

In this thesis I have described work aimed at addressing the contribution of protein kinase G (PKG) to cerebellar-dependent motor learning/conditioning and the role of microtubule plus-end tracking proteins (+TIPs) in locomotion. In my studies I regularly made use of genetically modified mice (transgenes and knockouts). I have also generated transgenic mice myself. Hence I will first discuss this technical aspect of my work before I discuss the actual results on PKG and +TIPs.

### *Transgenic mice*

The aim of the transgenic mice generated in this study was to specifically express PKGi in cerebellar Purkinje cells (PCs). For this I used the L7/pcp-2 promoter (Oberdick, Smeyne et al. 1990). At the time a transgenic mouse model was preferred over making knockout mice because in the latter a gene is knocked out in the whole animal and developmental defects (and/or compensation by other proteins) may make interpretations more difficult. For example, protein kinase c (PKC) g null mutants have been produced to study cerebellar function (Abeliovich, Paylor et al. 1993). The defects were quite different from what was expected and surprisingly mild (Abeliovich, Chen et al. 1993; Chen, Kano et al. 1995). Although mutant mice did display cerebellar developmental defects, the function of PKC-g was possibly compensated by other members of this kinase family (Chen, Kano et al. 1995; Kano, Hashimoto et al. 1995). Using a transgenic approach on which we later based ours, De Zeeuw et al (1998) have shown very different results of PKC inactivation compared to the knockout studies. In this experiment, the L7 promoter was used to drive the expression the PKC inhibitory peptide (De Zeeuw, Hansel et al. 1998). The data showed the necessity of PKC for the establishment of long-term depression (LTD) in vivo. The transgenic mice had very mild secondary phenotypes, which did not cause any further complications (De Zeeuw, Hansel et al. 1998; Goossens, Daniel et al. 2001). It remains to be investigated why in the knockout situation compensation by other PKC members did occur and in the transgenic approach not.

PKGI and PKGII null mutants have been generated (Pfeifer, Klatt et al. 1998) (Kleppisch, Pfeifer et al. 1999) and both were tested for their effect on hippocampal long-term potentiation (LTP). No effects were observed and it was concluded that PKG is not necessary for LTP. It was also stated that these mutants do not show defects in cerebellar LTD even though it was not clearly stated how the studies were performed (Ruth 1999). Subsequently, a specific ablation of PKGI in PCs was generated (Feil, Hartmann et al. 2003), using a floxed allele of the Prkg1 gene, coding for PKG type I (Wegener, Nawrath et al. 2002) and a PC specific Cre expressing mouse (Barski, Dethleffsen et al.

2000). These mice displayed normal cerebellar structure but they had impaired LTD as well as an impaired adaptation of the vestibulo ocular reflex (VOR)(Feil, Hartmann et al. 2003). The expectation is that in both PKGI or PKGII full knockouts developmental related compensation of the phenotype might have occurred. It is possible that other kinases will take over the function of PKG. The inducible knockout presents a much more anatomically restricted mutation and is less sensitive to compensation mechanisms. Ideal, though, would be a temporally controlled system to ablate PKG in the adult cerebellum.

Based on the succesful results with the PKCi transgenic mice (De Zeeuw, Hansel et al. 1998; Goossens, Daniel et al. 2001):(van Alphen and De Zeeuw 2002) , we aimed at generating transgenic mice expressing a PKG inhibitory peptide in PCs. However, the expression levels of the different "foreign mRNAs" in the transgenic lines were too low. One possible explanation is the presence of a cryptic alternative splice site in the L7 casette that could compromise the expression of a transgene. However, other studies using the same L7 promoter did obtain high expression transgenes (e.g. (De Zeeuw, Hansel et al. 1998; Zhang, Baader et al. 2001). In these studies a higher number of transgenic lines was made. Pronuclear microinjection of DNA sequences results in random integration of the foreign DNA in the host genome, which can cause many possible effects on expression, including silencing of the transgene (Gordon and Ruddle 1986; Melton 1994). As the L7ΔAUG casette is small it is unlikely to contain elements that protect against influences from the surrounding host genome. Increasing the number of transgenic lines made with the L7 constructs might have originated one or a few lines with appropriate expression levels.

***Effect of PKC and PKG in timing of the eye-blink conditioning***

The results with the PKGpko mice in the eye-blink conditioning experiments are interesting. Apparently, a lack of PKG in Purkinje cells results in altered percentage and amplitude of conditioned responses without affecting the timing of the response. By contrast, the timing of the responses was affected by mutations blocking PKC activity in Purkinje cells (Koekkoek, Hulscher et al. 2003). These data indicate different functions in LTD for two different signalling pathways. Inside the Purkinje cells there are many targets for PKG, such as the G-substrate (Endo, Suzuki et al. 1999) and phosphodiesterases (Shimizu-Albergine, Rybalkin et al. 2003). PKG can also induce the posphorylation of the inositol 1,4,5-trisphosphate (IP3) receptor (Haug, Jensen et al. 1999) contributing to the release Ca2+ from internal stores (Miyata et al 2000; Wang et al 2000).

The action of PKG on the a-amino-3-hydroxy-5-methyl-4-isoxazolepropri-onic acid glutamate receptors (AMPAR) is indirect, involving the inactivation

of the protein phosphatases 1/2A (Endo, Suzuki et al. 1999). On the other hand, the phosphorylation of AMPAR by PKC is direct (McDonald, Chung et al. 2001); (Xia, Chung et al. 2000). PKC obviously has many other targets, including also protein phosphatases (Eto, Bock et al. 2002). PKC activation is Ca2+ and diacyl glycerol (DAG) dependent (Newton 1995), and driven by metabotropic glutamate receptors (mGluR) activity, while PKG is activated by diffusible nictric oxide (NO) (Hofmann, Ammendola et al. 2000).

The representation of timing in the cerebellum is thought to be accounted for by the differential activation of specific parallel fibers in a timely manner (Medina, Garcia et al. 2000; Mauk and Buonomano 2004). PKC or elements of the PKC pathway are more likely candidates to process timing information (Koekkoek, Hulscher et al. 2003). mGluR activation has since long been proposed to be a cellular mechanism for respone timing (Fiala, Grossberg et al. 1996). A recent report showed that Ca2+ signaling has strong timing properties, with two distinct components (Canepari and Ogden 2006): a transient, phospholipase C (PLC) dependent Ca2+ release from internal stores with precisely reproducible timing and a slower Ca2+ influx via cell surface Ca2+ channels. In PKGpko mutants synaptic Ca2+ signaling was studied (Feil, Hartmann et al. 2003). In these mice, neither the early synaptic Ca2+ transient, that reflects Ca2+ influx through voltage-gated Ca2+ channels after AMPA receptor activation, nor a delayed second component that reflects mGluR-mediated Ca2+ release from intracellular stores (Finch and Augustine 1998); (Takechi, Eilers et al. 1998) were affected (Feil, Hartmann et al. 2003).

The precise timing of the release of Ca2+ from internal stores might contribute to the timing control of Purkinje Cell responses. PKC is activated directly by this increase in the cytosolic Ca2+ (Liu and Heckman 1998) thus being itself activated in timed intervals. PKG will have little effect on the timely release of Ca2+ due to the nature of its activation by diffusible NO and supposedly not play a role in timed responses since it is Ca2+ independent.

Another possible clue on the different action of PKC and PKG lies in the mechanism proposed by Nick Hartell (2001). According to this view, different mechanisms exist for LTD: homosynaptic and heterosynaptic LTD. In homosynaptic LTD, localized influx of calcium to dendritic branchelets of PC (via PF activation) will cause (when sustained) a reduction in the synaptic transmission at the site of induction (PF-induced LTD) that does not spread out to other sites. In heterosynaptic LTD the effects are seen arising at sites far from the site of stimulation. This “spread of LTD” seems to be calcium independent and selectively blocked by NO-cGMP inhibition (Hartell 2001; Reynolds and Hartell 2001). In this model, the effect of inhibition of PKG (or of the NO-cGMP pathway) prevents the spread of LTD to synapses distant from the immediately depressed synapse, which in normal circumstances occurs (Hartell, 2001). Being involved in “diffusion of LTD”, makes the action of PKG potentially more

inaccurate in terms of timing of response and allows us to conceive a mechanism which involves several degrees of completeness. This is in accordance with what we have seen in PKGpko mice. The PKGpko mice could be deficient in the "spreading" of LTD. In this scenario, PKC mediated LTD could still occur and account for perfectly timed eye-blinks, while the abolishment of the response would need this LTD to spread. This could partially explain that the number of responses measured in PKGpko mice is lower but well timed.

We cannot exclude the possibility that yet another mechanism is important for learning and timing in the eye-blink conditioning. Apart from a whole palette of synaptic plasticity elements (Hansel et al., 2001) such mechanisms have not been convincingly demonstrated to be involved in eye-blink conditioning. Previously hypothesized sites of plasticity have been experimentally reported recently: newly discovered LTD and LTP at the mossy fiber -deep nuclei synapse (Pugh and Raman, 2006; Zhang and Linden, 2006).

On a speculative note, a few mechanisms might be considered.

LTP has been described in the cerebellum (Lev-Ram, Mehta et al. 2003; Coesmans, Weber et al. 2004; Jorntell and Hansel 2006). This infers that the cerebellar cortex has other possible forms of response to the stimuli conveyed by parallel and climbing fibers. It is not clear how or if PKG influences LTP (Kleppisch, Pfeifer et al. 1999).

Another potential site of plasticity might be at the inhibitory interneurons to PC synapses (Hansel, Linden et al. 2001).

Finally a different type of mechanism comes to mind : the so-called intrinsic plasticity, in which the mechanism for plasticity lies in changes in the membrane excitability associated with downregulation of K+ channels (Gittis and du Lac 2006) resulting in changes of the spike frequency of the PCs. Many common elements exist between the mechanisms of LTD and intrinsic plasticity in PCs such as the involvement of PKC and the Ca2+/calmodullin dependent protein kinase II (CaMKII) and protein phosphatases (Daoudal and Debanne 2003) making this system an attractive model to consider when trying to distinguish the roles of PKC and PKG in cerebellar function.

***A role for +TIPs in cerebellar LTD?***

In chapter 3, I describe a series of behavioral and locomotion studies performed with +TIP mutant mice generated in our group. These were aimed at understanding the role of +TIPs in cerebellar function. The microtubule cytoskeleton plays an important role in the establishment of neuronal polarity and forms a "railroad track" system that allows long range transport of organelles, vesicles, and other intracellular cargo (Dotti and Banker 1991; Guzik and Goldstein 2004). Most of the components necessary for synapse function are synthesized in the cell body and they therefore need to be transported over long distances by the microtubule cytoskeleton towards their site of aciton.

The anchoring of the receptors at their membrane sites is also dependent on scaffolding action of the cytoskeleton (Guzik and Goldstein 2004).

Neurotransmitter release is partly regulated by actin filaments (Doussau and Augustine 2000; Morales, Colicos et al. 2000). Endocytosis such as in the event of LTD, also requires the cytoskeleton (Zhou, Xiao et al. 2001). For local mRNA translation found in late phases of LTD microtubule transport is essential (Huang, Chotiner et al. 2005). A reasonable expectation is that proper regulation of the microtubule cytoskeleton is essential for cerebellar function even if this link has not yet been fully established. Indications that such claims are justified arise from the behavioral analysis of mice lacking CLIP-115 (Hoogenraad, Koekkoek et al. 2002; Hoogenraad, Akhmanova et al. 2004).

+TIPs might play a role in cerebellar LTD by regulating microtubule dynamics. Defects in the microtubule network could alter the rate of neurotransmitter release, the rate of trafficking of receptors at the membrane, and the deposition of components for local mRNA translation. Interestingly, recent reports point to an essential and direct role of the +TIP EB1 in the targeting of voltage-gate channels (Gu, Zhou et al. 2006) and of connexins (Shaw, Fay et al. 2007), which are components of gap-junctions. Connexins are important (but not indispensable) for the function of the inferior olive (De Zeeuw, Chorev et al. 2003) These data directly link +TIPs to cerebellar output and, perhaps, to LTD. In the hippocampus, LTD is associated with the shrinkage of dendritic spines (Zhou, Homma et al. 2004) where mostly actin is present. Actin binding proteins might therefore play a very direct role in LTD (Chen, Bourne et al. 2004). Interestingly one of these proteins is CaMKII, whose role in the maintenance of the shape of the dendritic spines has recently been identified (Okamoto, Narayanan et al. 2007).as mentioned before, microtubule transport is necessary for the targeting of CaMKII (Hirokawa 2006).

Other actin binding proteins known to be involved in LTD are Homer and Cupidin which interact with IP3 receptors (Tu, Xiao et al. 1998; Shiraishi, Mizutani et al. 1999; Yuan, Kiselyov et al. 2003), and GRIP or PICK1 that regulate the anchoring and internalization of AMPAR (Wyszynski, Kim et al. 2002).

Both the CLIPs and the CLASPs are positive regulators of the microtubule cytoskeleton. Moreover, CLIP-170 binds dynactin, an accessory factor of the dynein motor, and targets dynactin to microtubule ends (Lansbergen, Komarova et al. 2004). CLIP-170 might therefore play a role in retrogade microtubule–dependent vesicle transport (Pierre, 1992). Brain-specific isoforms of CLASP2 and CLIP-170 have been detected (Akhmanova, Hoogenraad et al. 2001; Akhmanova, Mausset-Bonnefont et al. 2005). CLIP 115 is predominantly expressed in the brain (De Zeeuw, Hoogenraad et al. 1997). While LTD was not studied in CLIP-115 knockout mice, an effect on LTP was ob-

served (Hoogenraad, Koekkoek et al. 2002). A CLIP double knockout mouse model showed progresisve hydrocephalus in combination with heterotopia (Miedema 2007). Finally, lack of CLASP2 results in smaller brains of knockout mice, just like the rest of the body of these animals are smaller (Drabek et al., submitted). Combined these results point to an important role for CLIPs and CLASP2 in brain development and neuronal function.

***Behavioral studies of mice deficient in +TIPs***

General behavioral paradigms such as the rotarod, the open field and the catwalk give hints on (among others) coordination of movements and on posture, which are associated with cerebellar function (Crawley 2000). It has already been discussed that genetic background can influence the outcome of behavioral experiments (Crawley 2000)). Other groups have taken this into consideration, devising strategies to minimize background influences in their transgenic models (Mishina and Sakimura 2007). Another source of bias in behavioral results lies in the effect of the laboratory conditions (Crabbe, Wahlsten et al. 1999). To determine the influence of genetic background we compared the performance of groups of wild type littermates, derived from the different crosses of heterozygous knockout mice, as well as some inbred strains. In the different paradigms all wild types should behave identical, however, this was not the case. Phenotypes that differed between groups of wild type mice were treated with caution. Those phenotypes that were only present in knockout mice and not in wild type mice were considered strong and highly reliable. This way we concluded that hindleg position, a posture related phenotype, is a phenotype inherent to the depletion of the +TIPs. Similarly, and possibly related, CLIP-170 defficiency seems to contribute to an an altered posture of the animals , resulting in an increase in time standing on the hindpaws.

The level of hyperactivity of CLASP2 knockout mice appears to be a robust phenotype. This phenotype is associated with a seemingly fearless pattern of locomotion that also indicates lack of curiosity for the vertical plane. Strikingly, these smaller mice take bigger steps with their forelimbs (Chapter 3). These phenotypes might not all be cerbellar related but become apparent in the absence of specific +TIPs in vivo.

One of the +TIP mutants used in this study were the CLIP-115-deficient mice. These mice are a model for the locomotion problems associated with Williams Syndrome in human patients. Williams Syndrome (WS) is a neurodevelopmental disorder caused by a hemyzygous deletion of about 28 genes in chromosome 7q11.23 (Meyer-Lindenberg, Mervis et al. 2006; van Hagen, van der Geest et al. 2007). In recent years, genotype-phenotype correlations using knockout mouse models has pointed to genes that might play a role in specific aspects of the condition. An Elastin deficiency, for example, is re-

sponsible for the cardiac and facio-cranial abnormalities in WS. Deficiency of the Limk1 gene is thought to influence fear-related behaviors of WS patients and altered brain morphology. Deficiency of the *Clip2* gene (encoding CLIP-115) might be important for locomotor defects (see Meyer-Lindenberg, Mervis et al. 2007) for review). Results from patients with smaller deletions allow to exclude some of the genes or to attribute relatively greater importance to others in generating a WS-related phenotype. A newly described patient has the *Clip2* gene spared in the mutated region on chromosome 7 and concomitantly shows none or negligible locomotor defects (van Hagen, van der Geest et al. 2007).

*Clip2* has been proposed as the candidate gene for the locomotion effects associated with WS also because CLIP-115 deficient mice show strong abnormalities in motor coordination in the rotarod or running wheel setups ( Hoogenraad, Koekkoek et al. 2002). Just like in WS patients the phenotype is observed at the heterozygous level in *Clip2* knockout mice. We recently tested the *Clip2* mutant mice in a more defined genetic background using a more standardized rotarod set-up, to try and understand the effect of genetic background to the behavioral outputs. In contrast to previous reports (Hoogenraad, Koekkoek et al. 2002; van Hagen, van der Geest et al. 2007), we could not reproduce the rotarod phenotype in heterozygous mice. We could, however, reproduce the results in homozygous mutant mice (chapter 3). We therefore support the conclusion that depletion of CLIP-115 produces behavior phenotypes that mimic the locomotion problems associated with WS.

It should be noted that the recent results on heterozygous *Clip2* knockout mice (van Hagen, van der Geest et al. 2007) were taken from our previous publication (Hoogenraad, Koekkoek et al. 2002). Thus the effect seen in heterozygous *Clip2* knockout mice was observed in a population of mice generated in 2001-2002. The strategy used to generate *Clip2* knockout mice first included the generation of clones of 129/Svj ES cells and later crossings into the C57/BL6 background, creating mixed backgrounds. A high number of back-crossings into C57/Bl6 eventually eliminates the effect on behavior of the 129/Svj ES cell background and crosses out any undesired mutations in the ES cells that might interfere with behavior. One of the main differences between the mice tested in 2001-2002 and those tested in chapter 3 was indeed the number of back-crossings into the C57/Bl6 background.

In light of our results, we hypothesize that mice differ from humans in the amount of CLIP-115 needed for proper motor coordination. Reasons for this might be related to the different size of (Purkinje) neurons in the cerebellum (Friede 1963). Longer axons must have more microtubules and might need higher levels of microtubule associated proteins. This would explain why a hemizygous phenotype would be seen in man but not in mouse.

Our results (Chapter 3) reiterate the moderate phenotypes observed in

+TIP mutant mice and indicate that CLIP-115 is more important for motor coordination than CLIP-170. This is surprising taking into account the generally accepted view that CLIP-170 has a more important role than CLIP-115 in the regulation of microtubule dynamics.

***Future perspectives***

The idea of generating a genetic switch to turn on and off Purkinje cell-specific and/or cerebellar-specific gene expression is still very useful. We know now that the strategy we chose was not optimal. But taking into account new reports, for example, a mouse model expressing the Cre recombinase in Purkinje cells in the context of a bacterial artificial chromosome (Zhang, Ng et al. 2004) or targeted into the endogenous L7 locus (Saito, Tsumura et al. 2005), including temporal control of the Cre recombinase (by hormone receptor moieties) still seems a logical step to take.

New reports of positive results using the Tet-On system in the brain have also appeared (Gimenez, Lavado et al. 2004; Michalon, Koshibu et al. 2005) indicating the possibility of timely inducing gene expression in the brain.

In the meantime, LTD has positively been established as a mechanism for learning and memory in the cerebellum, but new sites and forms of plasticity emerge (Hansel 2005; Bagnall and du Lac 2006; Boyden, Katoh et al. 2006; Jorntell and Hansel 2006; Mapelli and D'Angelo 2007). Future focus should therefore be directed towards dissecting the contribution of each form of cerebellar plasticity to learning and memory and at dissecting their molecular basis.

The contribution of PKC, PKG or CaMKII to any of the newly characterized and emergent forms of cerebellar plasticity can be individually addressed by using the existing mouse mutants.

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# List of Abbreviations

AIN - Anterior Interpositus Nucleus
AMPA - alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Arg - arginine
ATP - adenosine tri-phosphate
BAC- bacterial artificial chromosome
bp – basepairs
aCaMKII - Calcium-calmodulin dependent protein kinase II alpha
CF- Climbing Fibre
cGK - cyclic-GMP dependent protein kinase
cGMP - cyclic guanosine-mono-phosphate
CLASP - CLIP associated protein
CLIP - Cytoplasmic Linker Protein
minCMV - minimal Cytomegalovirus promoter
COS - cell line derived from the african green monkey (Cercopithecus aethiops)
CPI-17 protein-kinase C-potentiated myosin phosphatase inhibitor of 17 kDa
Cre - Cyclization Recombination
CR - conditioned response
CS - conditioned stimulus
DKO - double knockout
DNA - desoxyribonucleic acid
DAG - diacyl glycerol
EPSC - excitatory post-synaptic current
EPSP- excitatory post-synaptic potential
ER - estrogen receptor
GABA - gamma amino-butyric acid
GC - guanylyl cyclase
GFP - green fluorescent protein
Glu - glutamate
GluR - Glutamate receptor
GRIP - glutamate recpetor interacting protein
HA - hemaglutinin
HPRT - Hypoxanthine-guanine phosphoribosyltransferase
IF - intermediate filament
IO - inferior olive
ip - intra-peritoneal
ISI - inter-stimulus interval
kb - kilo basepairs
kDa - kilo Dalton
kHz - kilo Hertz
KI - knock-in
KIF - kinesin family member
KO - knockout
LBD - Ligand Binding Domain
loxP -locus of cross-over in P1
LTD - long-term depression
LTM - low threshold mechanoreceptive neurons
LTP - long-term potentiation
Lys - lysine
MAP - microtubule associated protein
MDTM - magnetic distance measurement technique
MEF - murine embryonic fibroblast
MF - mossy fibre
mGluR - metabotropic Glutamate Receptor
MOO - musculus orbicularis oculi
mRNA - messenger ribonucleic acid
MT - microtubule
MTOC - microtubule organizing centre NMDA - N-methyl-D-aspartate
4-OHT - 4-hydroxy-tamoxifen
PBS - phosphate buffer saline
PC - Purkinje Cell
pcd - Purkinje Cell Degeneration
pcp2 -Purkinje Cell specific protein 2
PCR - polymerase chain reaction
PDE - phosphodiesterase
PSD - Postsynaptic density
PF - parallel fibre
PICK-1 - protein interacting with protein kinase C 1
PIP2 - phosphatidyl-inositol-biphosphate
PKA - protein kinase A
PKC - protein kinase C
PKC - protein kinase G
PKCi - PKC inhibitory peptide
PKD - protein kinase D
PKGi - PKG inhibitory peptide
PLC - phospholipase C
PP - protein phosphatases
PR - progesterone receptor
RACKs - receptors for active C kinase
RB - retractor bulbi muscle
rpm - revolutions per minute
rtTA - reverse tetracycline trans activator
Ser - serine
TARP - transmembrane AMPAR regulatory protein
Tet - tetracycline
tg - transgenic
+TIPS - Plus-end tracking proteins
tPA - time to peak amplitude
tPV - time to peak velocity
TRE - tetracycline responsive element
US - unconditioned stimulus
UR - unconditioned response
VOR - vestibulo-ocular reflex
WDR - wide dynamic range neurons
WS - Williams Syndrome
wt - wildtype

# Summary

## Summary

The cerebellum (from the latin - little brain) is located at the posterior end of the brain. It is known to be involved in vital functions like the control of heart beat and respiration and also in motor coordination, a function involving balance and equilibrium, which also requires the capacity to learn and adapt. Cerebellar motor learning and memory are at the base of the studies described in this thesis. The structure of the cerebellar cortex (the outer layers) is remarkably conserved, consisting of many equivalent modules. These modules, called micro-zones, are based around Purkinje cells. Purkinje cells are big neurons in the cerebellum with flamboyantly arborized dendrites which receive hundreds of thousands of inputs, and which transmit information of various types. Purkinje cells are very important in cerebellar function because they integrate signals and respond to them by selectively changing the strength of responses. This phenomenon is called plasticity. It involves a long-lasting reduction of currents evoked at synapses (or neuronal contacts) at the Purkinje dendrites, termed long-term depression or LTD. Purkinje cells are the only neurons in the cerebellar cortex that exert a direct action on targets outside the cortex and LTD in this system is thought to permit a rapid adjustment of responses (i.e learning) to ongoing movements.

In this thesis two stimuli are mainly studied, eye blink conditioning and locomotion. Specific areas of the cerebellum (area HVI in the lobule simplex as well as its related interposed deep nuclei), have been identified as sites of convergence for stimuli related to eye-blink conditioning. For the control of locomotion, the cerebellar areas involved are less well defined. In eye blink conditioning paradigms the stimulus is an air puff to the eye, which evokes a reaction, namely eye closure. If an air puff is preceded by a sound (like a warning), the animal will learn to interpret the sound; the result will be a reduction of the activity of the involved PCs in a perfectly timed manner to ensure appropriate closure of the eye when the air puff is expected. Like eye blink conditioning, locomotion paradigms often contain a learning element as well. Animals are trained and evaluated in sessions that may take several days.

Purkinje cells perceive external signals and react to them via complex signal transduction cascades, in which each protein will act on the following in a chain reaction that will lead to an effect according to the nature of the stimulus. Many players are involved in these cascades and the most important involve PKC and PKG, two protein kinases, a special type of enzymes that work by adding phosphate groups to their targets. In Chapter 2 of this thesis I describe the attempts at generating mouse mutants lacking PKG activity specifically in cerebellar Purkinje cells. I further describe the effect of

PKG ablation specifically in Purkinje cells in eye-blink conditioning. We have shown that unlike PKC, PKG has little of no effect on the control of timing of conditioned eye-blinks, but contributes to the number of blinks. This result was somewhat surprising since the lack of both kinases results in a similar perturbation of LTD. We confirmed the effect of PKG in LTD ex vivo, by applying a PKG-specific inhibitory peptide to cerebellar slice preparations.

Another very important component of Purkinje cells, as well as any other cell, is the cytoskeleton. Inside every cell, a very dynamic structure composed of actin fibers, intermediate filaments and microtubules, helps cells to maintain their structure and shape, and plays an important role in cell migration and cell division. In neurons, microtubules are crucial in the establishment of cell polarity. Because all the long-range transport of organelles and even mRNA necessary for proper synaptic function as well as internalization/externalization of membrane receptors relies on microtubules, the assumption is that a properly regulated microtubule network is essential for adequate neuronal and cerebellar function.

Regulation of microtubule dynamics is carried out , among others, by a special class of proteins called +TIPs or 'plus-end tracking proteins". These proteins bind specifically to the ends of growing microtubules (termed +ends), which, in axons, is virtually always oriented towards the growth cone. Examples of this type of proteins are CLIP 170, CLIP 115 and CLASP 2. These +TIPs help to stabilize microtubules by promoting growth over shrinkage. All these proteins are abundantly expressed in the brain (and cerebellum) leading us to question their role in normal cerebellar function. Furthermore, CLIP 115 has been linked to locomotion and cognitive defects in Williams Syndrome patients. So-called knockout mouse mutants, lacking each of the +TIPS mentioned above, had previously been generated in the lab. In chapter 3 these were used to study the contribution of each protein to locomotion in standard paradigms like the rotarod, openfield or catwalk setups, as described. Results presented in this thesis point to altered locomotion patterns of mice lacking each of the +TIPs studied. As reported in other studies CLIP115 knockout mice performed less well on the rotarod than normal mice. All mutant mice studied displayed an abnormal posture of their hind-limbs. This was shown to be age dependent. Combined the results presented in this thesis point to the necessity of having a well regulated microtubule cytoskeleton and PKG for normal cerebellar function.

# Sammenvatting

## SAMMENVATTING

Het cerebellum (kleine hersenen) is het achterste deel van de hersenen. Het controleert vitale functies als hartslag, ademhaling, evenwicht en motorische coordinatie en cognitieve functies als specifieke soorten van leren en geheugen. In dit proefschrift richten wij ons op leerprocessen door het cerebellum. De structuur van de buitenlagen van het cerebellum, de zogenaamde cortex, is opvallend regelmatig en bestaat uit een serie gelijke modules, de microzones. Het centrum van deze microzones is de Purkinjecel. Het is de enige neuron van de cortex die verbinding maakt met structuren buiten de cortex. Purkinje cellen hebben buitengewoon wijdvertakte dendriten die tienduizenden verschillende soorten informatie verwerken. Purkinje cellen zijn van groot belang, omdat zij deze impulsen integreren en op ze reageren door de sterkte van hun reactie aan te passen. Dit verschijnsel noemen wij plasticiteit. Er zijn andere plekken in het cerebellum die verschillende vormen van plasticiteit vertonen, maar wij hebben ons gericht op de meest geaccepteerde, de Long-Term Depression of LTD. Bij LTD treedt er een langdurige reductie op van de reactie op stimuli in de Purkinje cel. Hierdoor kunnen de gerelateerde stimuli de motorische neuronen direct beinvloeden. Deze reductie is een leerproces die een snelle aanpassing op bewegingen mogelijk maakt. Een van de methoden om deze functie van het cerebellum te bestuderen is de zogenaamde eye-blink conditioning, waarbij een herhaaldelijk toedienen van een luchtpufje op het oog, gepaard gaat met een waarschuwingstoon. Het leerproces maakt dat uiteindelijk op de toon wordt gereageerd. Het is aangetoond dat een specifiek deel van het cerebellum (het HVI in lobule simplex en de daarmee verbonden interpositus diepe nuclei) de controle uitoefent op deze eye-blink conditioning. We vermoeden een oorzaakelijk verband tussen LTD en dit leerproces in het cerebellum. Purkinje cellen ontvangen, net als andere neuronen en de meeste cellen, externe signalen en reageren daarop via complexe kettingreacties. Het resultaat is afhankelijk van de stimulus die de reactie heeft veroorzaakt. Als de stimulus een luchtpufje op de oogbol en een waarschuwingstoon is, is het resultaat een afname van de activiteit van de betrokken Purkinje cellen, en een oogknip als reactie op de waarschuwingstoon op het perfecte tijdstip. De belangerijkste proteinen in dit proces zijn PKC en PKG. Er is veel informatie over de activiteit van PKC.

In dit proefschrift beschrijf ik de pogingen genetisch gemanipuleerde muizen te maken zonder PKG activiteit in Purkinje cellen. Voorts beschrijf ik het effect van totale afwezigheid van PKG, met name bij eye-blink conditioning. We tonen aan dat, in tegenstelling tot PKC, PKG wekiswaar niet

of nauwelijks van invloed is op de controle of timing, maar wel bijdraagt in het aantal van de geconditioneerde oogknippen. Dat is opvallend aangezien beiden op gelijke wijze LTD verstoren. We hebben ook het effect van PKG in LTD ex vivo bevestigd, door een peptide (een deeltje van een eiwit) toe te voegen aan de cerebellum-preparaten die PKG remt. Een ander belangerijk component van de Purkinjecel, zoals iedere andere cel, is het cytoskelet. In iedere cel helpt een zeer dynamische structuur die bestaat uit zogenaamde intermediaire filamenten, actine en microtubuli, zijn structuur en vorm intact te houden. Ze spelen een belangerijke rol in cel beweging en celdeling. Microtubuli zijn van cruciaal belang voor het functioneren van neuronen. Alle transport binnen de cel hangt af van microtubuli. Bij LTD neemt de hoeveelheid receptoren af, waardoor de Purkinje cel niet reageert. Deze afname wordt mogelijk gemaakt door de microtubuli, die deze receptoren vervoeren. Dat geldt ook voor mRNA en andere celdelen, nodig voor het goed functioneren van het contactpunt tussen neuronen (synapse). Het gedrag van microtubuli wordt onder andere geregeld door een speciaal soort eiwitten, de zogenaamde +TIPS. Van dit type eiwitten gebruikten we CLIP 170, CLIP 115 en CLASP 2. Deze +TIPS helpen de stabilisatie van microtubuli. Al deze eiwitten zijn duidelijk aanwezig in het cerebellum. Dat roept de vraag op naar hun betekenis voor het functioneren van het cerebellum. We weten bijvoorbeeld dat het gebrek aan CLIP 115 te maken heeft met locomotie en cognitieve deffecten bij patienten met het Williams-syndroom. In het laboratorium zijn al eerder muizen zonder de boven genoemde +TIPS (knockout) gemaakt. Ze zijn gebruikt om de invloed van ieder van de bovengenoemde eiwitten op locomotie bij standaard methoden zoals de rotarod, openfield of catwalk te bestuderen, zoals we in hoofdstuk 3 beschrijven. De resultaten in dit proefschrift geven een duidelijke indicatie dat de locomotie-patronen van +TIP knockout muizen anders zijn dan de controle muizen. CLIP 115 knockout muizen presteren slechter op de rotarod (homozygoten). Alle gemanipuleerde muizen vertonen een abnormale stand van de achterpoten, wat met de leeftijd nog toeneemt, een bewijs dat microtubuli en +TIPS van belang zijn voor een goed functioneren van het cerebellum.

## Curriculum Vitae

| | |
|---|---|
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