Diseases of the nervous system associated with calcium channelopathies

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GENERAL INTRODUCTION

PREFACE

Neurological brain disorders form a major burden on modern society, because they often have devastating effects on the social life of patients and their family members, and because they are associated with high economic costs[1]. Still, for most of them, the pathological mechanism is largely unknown. Dedicated research aimed at elucidating these mechanisms is dearly needed to better understand the etiology of the diseases, and may ultimately lead to the development of better treatments for patients. Often, the clinical presentation of a brain disease is not isolated to the brain itself, but it also involves other organs. Examples include disorders such as Parkinson's disease or cerebellar ataxia, in which total body motor coordination is severely disturbed.

Movement is an essential function of virtually all organisms. Whether voluntary or reflex, movement requires complex patterns of muscular activity that is controlled by motor neurons. Located in the ventral horn of the spinal cord, somatic motor neurons reach the muscles in the periphery via their motor axons. In a functional sense, motor neurons execute commands from higher centers of the nervous system, such as motor centers in the brain stem and the cerebral cortex. Integration of sensory information occurs in the brain stem, which, among other things, is involved in the control of body posture, and eye and head motion. At the top of motor control hierarchy is the cerebral cortex that is involved in the selection of movement planning and the programming of sequences of movements (for review see *Calton & Taube, 2009*). Motor activity is also modulated by the action of basal ganglia and the cerebellum. While the basal ganglia are involved in the planning and initiation of movements, the cerebellum aims to coordinate movement by bringing intention to move in line with motor performance (for review see *Ito, 2002*).

This functional hierarchy in motor control is evolutionarily conserved across species, and is, for instance, very similar between humans and mice. This similarity holds the promise that knowledge obtained from experiments in mice will help to understand the pathophysiology in humans with the same disease. An increasingly important tool to study movement and associated disorders is the use of genetically sensitized animals that either are naturally occurring or are transgenic mouse models with mutations in genes known to cause disease in humans. Such models will further our knowledge of pathophysiological mechanisms of, for instance, cerebellar ataxia (one of the main topics of this thesis) and thus enable the development of novel treatment strategies for patients.

MOTOR CONTROL IN THE PERIPHERAL NERVOUS SYSTEM

The axon of each motor neuron innervates up to several hundred muscle fibers, forming a *motor unit*. The combined action of motor units allows contraction of muscles through a complex cascade of electrical and biochemical events. Much of the

action is centered around important structures at the distal end of motor neurons: the neuromuscular junctions (NMJs). NMJs are highly specialized peripheral synapses, at which neuronal activity is transduced upon muscle fibers. An NMJ consists of a presynaptic motor nerve terminal, a synaptic cleft, and a postsynaptic muscle fiber membrane that is enclosed by a Schwann cell (*Fig. 1*).

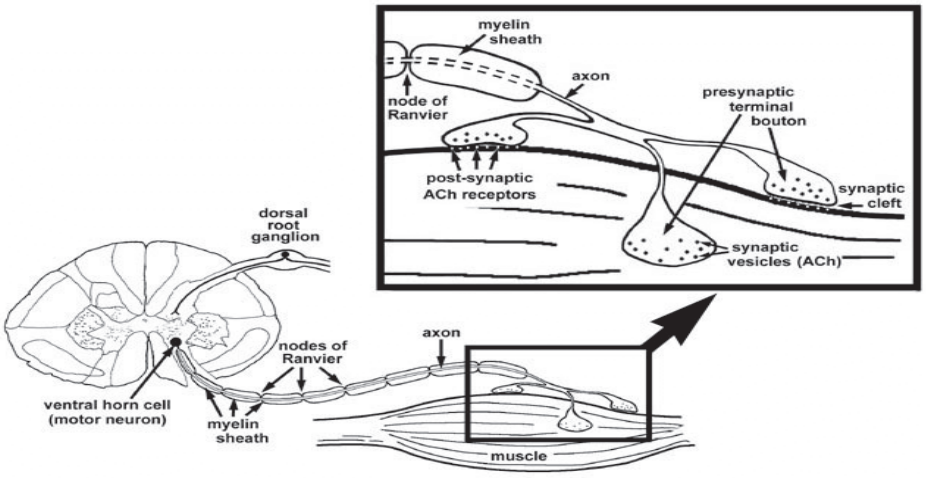

Figure 1. Schematic drawing of a neuromuscular junction (NMJ). An NMJ is characterized by a presynaptic motor nerve terminal, a synaptic cleft, and a postsynaptic muscle fiber membrane that is enclosed by a Schwann cell. The axon of a motor neuron enters the muscle and is split into many unmyelinated branches. These terminal fibers run along the myocytes and end at NMJs. At an NMJ, an action potential is transduced upon the muscle. Each motor neuron innervates a few to several hundred muscle fibers, but a particular muscle fiber receives input from only a single neuron. Adapted from *Plomp et al., 2003*.

The structure and function of NMJs have been well characterized and thus making NMJs a popular target to study the main characteristics of a single synapse (for review, see *Sanes & Lichtman, 1999*). The sequence of events is as follows. Electrical stimulation of the motor neuron will result in an action potential. When the action potential arrives at the motor nerve terminal, Ca^{2+} enters the neuron through specialized structures called voltage-gated Ca^{2+} channels. At the terminal, presynaptic active zones are present that contain synaptic vesicles filled with neurotransmitter; in the case of an NMJ the neurotransmitter is acetylcholine (ACh). Synaptic vesicles contain about equal amounts of ACh, also called quanta. As a result of Ca^{2+} entry, synaptic vesicles will fuse with the plasma membrane and ACh is released into the synaptic cleft; an approximately 50-nm-wide space between the nerve terminal and the muscle fiber. Massive release of quanta occurs as a result of an action potential (i.e., *evoked* release). Release of single quanta into the synaptic cleft is also possible (i.e., *spontaneous* release), but does not require an action potential. At the postsynaptic, i.e. muscle, side of the junction, the muscle fiber membrane is organized in postsynaptic junction folds

that contain acetylcholine receptors (AChRs) and voltage-gated Na^+ channels. When ACh crosses the synaptic cleft and binds AChRs, the receptors open and an inward sodium current is generated. As a consequence, the postsynaptic muscle membrane becomes depolarized and a postsynaptic action potential is generated. This action potential will be propagated along the muscle fiber (i.e., *all-or-nothing response*), and will ultimately cause contraction of the muscle. Normally, more ACh will be released than is required to surpass the depolarization threshold for the generation of an action potential (i.e., the *safety factor of the NMJ*). This ensures that the contraction of muscles occurs with high fidelity, which is important for any organism (for review, see *Wood & Slater, 2001*). Finally, access acetylcholine in the synaptic cleft is broken down by acetylcholinesterase to choline.

Experimentally, the characteristics of NMJs can be studied with relative ease in diaphragm preparations that contain muscle and an innervating phrenic nerve. A microelectrode is placed in the muscle to measure postsynaptic voltage changes that are the result of depolarizations of the plasma membrane after the binding of ACh to AChRs. In the case of *spontaneous* release, single quanta cause small depolarizations or miniature end-plate potentials (MEPPs). MEPPs normally occur at a rate of about 0.5 - 1.5 per second. *Evoked* release after a single action potential causes proportionally larger depolarizations or end-plate potentials (EPPs). The total number of vesicles released, can also be determined and is called quantal content. Analysis of the parameters of spontaneous and evoked release provides important information on the functioning of synapses in the peripheral motor system[4, 7].

MOTOR CONTROL BY THE CENTRAL NERVOUS SYSTEM

Various structures in the central nervous system (CNS) contribute to motor control, including the cerebral (motor) cortex, the basal ganglia, the cerebellum, the brain stem, and the spinal cord. Most relevant for this thesis is the role of the cerebellum in the fine-tuning of ongoing movement. Therefore its structure and function will be discussed below.

Anatomy and morphology of the cerebellum

The cerebellum is a symmetric, highly foliated structure of the hind brain. In brief, deep horizontal fissures separate the anterior, the large posterior, and the smaller flocculo-nodular lobes of the cerebellum. Two longitudinal grooves running through the posterior lobes demarcate a mid-line vermis and two hemispheres, each containing ten smaller lobules (for review, see *Voogd & Glickstein, 1998*).

The morphology of the cerebellum is well described and has two main components: the cerebellar cortex and the various nuclei embedded in the underlying white matter. In humans, the cerebellum has four deep cerebellar nuclei (DCN). From lateral to

medial, these nuclei are known as dentate, emboliform, globose, and fastigial nuclei. Mice do not have distinct emboliform and globose nuclei, but instead have a single, fused interposed nucleus. In addition, closely related to the cerebellum, there are the hind brain structures: inferior olivary nuclei, consisting of neurons part of medulla oblongata, and the vestibular nuclei, formed by the neurons of the vestibular nerve. There are three sources that provide input to the cerebellum: (1) the pontine nuclei, which carry information from the contralateral side of the cerebral cortex; (2) the spinocerebellar tract, delivering information from the ipsilateral side of the spinal cord; and (3) the inferior olivary nuclei, providing input from the contralateral side of the body.

The cerebellar cortex consists of three layers: the *Purkinje cell layer*, the inner *granular cell layer*, and the outer *molecular layer*.

The Purkinje cell layer consists of a monolayer of Purkinje cells (PCs) that is located between the molecular and granule cell layers. PCs are large neurons with a characteristic beet-shaped morphology[9]. In addition, PCs have very characteristic extensive and heavily branched dendrite trees with an extremely flat structure (average PC dimensions are 250 μm × 250 μm × 6 μm). Notably, PCs form the sole output of the cerebellar cortex with their axons projecting to cerebellar and vestibular nuclei.

The granule cell layer contains the cell bodies of small granule cells and several types of large neurons. The latter include the Golgi cells and less known neurons, such as the neuron of Lugaro, and the unipolar brush neuron (for reviews, see *Ambrosi et al., 2007; Simat et al., 2007*). Granule cells are excitatory, glutamatergic, neurons and are by far the most abundant type of neurons in this layer. Axons of granule cells are unmyelinated and ascend towards the molecular layer[11], where they bifurcate into so-called parallel fibers[8].

Finally, the molecular layer contains the dendritic trees of PCs (and Golgi cells), the parallel fiber tracts originating from the granule cells, and climbing fiber endings originating from the inferior olive nucleus in the brainstem. Although not cell dense, the molecular layer also contains two types of inhibitory interneurons: the stellate and the basket cells. These interneurons form GABAergic synapses onto PC dendrites[13].

In mammals, the cerebellar cortex is functionally organized in a pattern of parallel longitudinal zones[14]. PCs of a specific zone receive input only from a particular region of the inferior olive, and in turn send output to a particular region of the DCN, thereby forming discrete olivo-cerebellar complexes (for review, see *Voogd & Glickstein, 1998*).

Function of the cerebellum

The cerebellum coordinates movement by integrating afferent information into electrical signals guiding the precise execution and timing of motor tasks. To this end, the cerebellum continuously compares objectives (i.e., motor cortex input) and outcomes (i.e., proprioceptive feedback) (for review, see *Ito, 1984*). In addition

to its role in the coordination of movements, the cerebellum is also involved in reflex adaptation, motor learning, and possibly cognition (for review, see *Glickstein & Doron, 2008*). Investigating the cerebellum is specifically interesting because this structure has an unique way of processing information that requires action and feedback from various neurons: *the cerebellar circuitry* (*Fig. 2*). The characteristics of the cerebellar circuitry are being unraveled rapidly and will further our understanding of cerebellar function and its role in motor coordination dysfunction (for reviews see, *Houk et al., 1997; D'Angelo & De Zeeuw, 2009*).

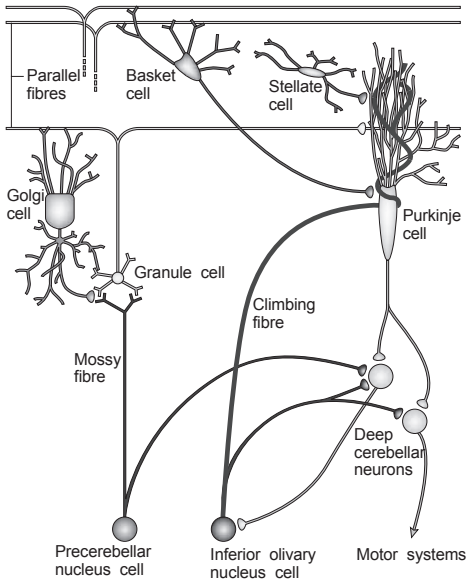

Figure 2. Schematic representation of the cerebellar circuitry. The climbing fiber and mossy fiber pathways are the main types of afferents of the cerebellum. Climbing fibers originate from the inferior olivary complex and directly innervate the Purkinje cells (PCs). Mossy fibers originate from precerebellar nuclei (i.e. the pontine nuclei and the spinocerebellar tract) and project to neurons at the deep cerebellar nuclei (DCN) with axon collaterals innervating cerebellar granule cells. These granule cells project further to PCs via ascending axons (known as parallel fibers) and through inhibitory interneurons of the molecular layer. PCs form the sole output of the cerebellar cortex and project to neurons of the DCN and the vestibular nuclei. Adapted from *Wang and Zoghbi, 2001*.

The cerebellar circuitry

The cerebellum receives sensory and cortical input from the mossy fibers (for review, see *Glickstein, 1997*). Mossy fibers deliver their information to DCN neurons, but also to granule and Golgi cell neurons of the granule layer[21]. Granule cells, which form the sole output of the granule layer, deliver the information further through their ascending axons (directly and via the parallel fibers) to interneurons of the molecular layer[22] and to many tiny distal branchlets of PC dendrites[23]. While a parallel fiber forms only a few synapses with a given PC, each PC is innervated by no less than 150,000 to 200,000 parallel fibers. The efficiency of a given parallel fiber-to-PC (PF-PC) synapse is low[24]. However, when between 30 and 150 parallel fibers are simultaneously activated, an action potential, i.e. *simple spike*, can be generated in a PC. The frequency and pattern of these simple spikes is important for motor behavior, since they comprise the output of the cerebellar cortex[25, 26, 27].

Next to the "mossy-fiber–granule-cell–parallel-fiber" pathway, PCs receive input from climbing fibers. Climbing fibers originate from the inferior olivary complex and their input is believed to signal motor error or discoordination (for review, see *Gibson et al.,*

2004). The primary projections of a climbing fiber are multiple, powerful synapses that dock close to the proximal end of a PC dendrite (for review, see *Sugihara, 2006*). In adult brain, each climbing fiber makes contact with several PCs, but each PC receives input from only a single climbing fiber. Approximately 1,500 synaptic contacts are activated simultaneously, thus triggering extremely powerful postsynaptic responses that override all other synaptic input[30]. A PC action potential generated in response to climbing fiber activation is called a *complex spike*. A complex spike is followed by a slow after-hyperpolarization of the PC plasma membrane, interrupting the simple spike activity (i.e. climbing fiber pause). Both simple and complex spike activity determine the PC firing pattern.

In summary, the cerebellar circuitry receives sensory and cortical information, processes this information, and provides feedback to DCN neurons by changing the firing pattern of the (inhibitory) PCs (for review, see *D'Angelo & De Zeeuw, 2009*). The information content is then transmitted further downstream to, for instance, the thalamus and motor centers in the brain stem and the spinal cord, ultimately resulting in adaptations of motor behavior.

Experimental methods investigating cerebellar function

Neuronal activity in the cerebellar circuitry is constantly adapting during movement. For instance, PCs, which are tonically active at rest change their firing frequency upon sensory input. By recording PC activity, such as recording simple and complex spike frequency during various motor tasks, one can obtain information about the function of the cerebellum in relation to the timing and the coordination of movement. The analysis of *compensatory eye movements* is a popular test paradigm to study cerebellar control of movement[31]. Compensatory eye movements occur, for instance, when one looks out of a train window and tries to lock on a nearby object; to perform the task, the motor system tries to minimize the movement of the image on the retina retina (i.e., to minimize the retinal slip). As a consequence, the eye follows the object as long as possible until it quickly returns to its original position and the sequence restarts. What happens is that the optokinetic reflex (OKR) aims to constrain the moving image on a part of the retina. Similarly, the vestibulo-ocular reflex (VOR) moves the eye in response to head movement in an attempt to reduce retinal slip. Performance in OKR and VOR (and the combined visually-enhanced VOR, VVOR) tests is presented as *gain* and *phase values* (*Fig. 3*). While gain values represent the ratio between the amplitude of the eye and the stimulus velocity, phase values correspond to the time difference between eye and stimulus expressed in degrees. Both gain and phase are important indicators for the function of the cerebellum and its ability to integrate the vestibular and/or optokinetic sensory information needed for controlling movement[32].

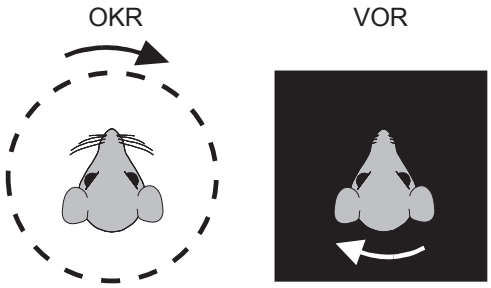

Figure 3. Schematic drawing of the training paradigm used to study optokinetic reflex (OKR) and vestibulo-ocular reflex (VOR) in mice. While the OKR allows the eye to follow objects in motion when the head remains stationary, VOR preserves the image on the center of the visual field during head movement by moving the eyes in the direction opposite to head movement. The "gain" of the VOR for example is defined as the change in the eye angle divided by the change in the head angle during the head turn. "Phase" is a parameter that describes the timing relationship between head movement and the reflexive eye response. When the head and eyes move at exactly the same velocity in opposite directions, they are said to be completely out of phase, or 180°. If the reflex eye movement leads the head movement, a phase lead is present. Likewise, if the compensatory eye movement trails the head movement, a phase lag is present.

NEURONAL CALCIUM INFLUX

Ca^{2+} is important for the functioning of the motor system, both at the periphery and in the CNS. Most relevant to this thesis is the entry of Ca^{2+} into the neurons through voltage-gated Ca^{2+} channels, which results in the release of neurotransmitters, the generation of postsynaptic currents, and, thereby, the regulation of synaptic activity[33].

Voltage-gated Ca^{2+} channels

Voltage-gated Ca^{2+} channels (VGCC) are responsible for Ca^{2+} influx into many excitable cells[34]. It has been known since the 1980's[35] that Ca^{2+} channels are multimeric protein complexes, with a pore-forming α_1 subunit and auxiliary subunits β and $\alpha_2\delta$. In some cases, a γ subunit makes up part of the protein complex (*Fig. 4*).

VGCCs differ with respect to protein composition, location of expression, as well as pharmacological and electrophysiological properties (for review, see *Doering & Zamponi, 2003*) (*Table 1*). The nomenclature of VGCCs is based on the presence of a specific α_1 subunit[37].

At the genetic level, four genes encoding β subunits (i.e., β_1 to β_4),

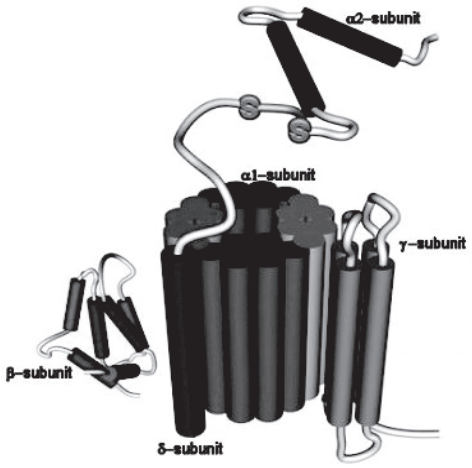

Figure 4. Schematic structure of voltage-gated Ca^{2+} channels. A voltage-gated Ca^{2+} channel consists of a pore-forming α_1 subunit, a dimeric $\alpha_2\delta$ subunit, an intracellular β subunit, and in some cases a γ subunit.

Table 1. Nomenclature according to *Ertel et al., 2000* and certain properties of voltage-gated calcium channels

Channel Type	α1 Subunit	α1 Gene	Ca^{2+} current	Specific blocker(s)	Primary localization of channel
$Ca_V1.1$	α_{1S}	CACNA1S	L-type	Dihydropyridines Phenylalkylamines Benzothiazepines	Skeletal muscle; transverse tubules
$Ca_V1.2$	α_{1C}	CACNA1C	L-type		Cardiac & smooth muscle myocytes; endocrine and neuronal cells
$Ca_V1.3$	α_{1D}	CACNA1D	L-type		Endocrine cells and neurons; atrial myocytes & pacemaker cells
$Ca_V1.4$	α_{1F}	CACNA1F	L-type		Retina & spinal cord, glands
$Ca_V2.1$	α_{1A}	CACNA1A	P/Q-type	ω-Agatoxin IVA ω-Conotoxin-MVIIC	Nerve terminals and dendrites
$Ca_V2.2$	α_{1B}	CACNA1B	N-type	ω-Conotoxin-GVIA ω-Conotoxin-MVIIC	Nerve terminals and dendrites
$Ca_V2.3$	α_{1E}	CACNA1E	R-type	SNX-482	Neuronal cell bodies and dendrites
$Ca_V3.1$	α_{1G}	CACNA1G	T-type	Mibefradil Kurtoxin Amiloride	Neuronal cell bodies and dendrites; cardiac and smooth muscle myocytes
$Ca_V3.2$	α_{1H}	CACNA1H	T-type		
$Ca_V3.3$	α_{1I}	CACNA1I	T-type		

four genes encoding $\alpha_2\delta$ subunits (i.e., $\alpha_2\delta$-1 to $\alpha_2\delta$-4), and eight genes encoding γ subunits (i.e., γ1 to γ8) have been identified (for review, see *Arikkath & Campbell, 2003*). $\alpha_2\delta$ and γ subunits are membrane-associated proteins, while the β subunit is localized entirely in the cytoplasm. The function of auxiliary subunits is to modify the biophysical properties of their respective channel (for review, see *Dolphin et al., 2009*). These subunits can also effect the expression of Ca^{2+} channels in the plasma membrane[39]. Since there are many possibilities of, for instance, β and α2δ to associate with α1 subunits, there is an enormous diversity of VGCCs that are expressed in a cell type-specific and tissue-specific manner[40].

$Ca_V2.1$ channels

The main focus of this thesis is on a particular type of VGCC, namely $Ca_V2.1$ channels. The α_1 subunit of $Ca_V2.1$ channel is encoded by the *CACNA1A* gene. Many $Ca_V2.1$ channels contain β_4 and $\alpha_2\delta$-2 auxiliary subunits, but combinations with other auxiliary subunits have been also be found. Alternative splicing of the *CACNA1A* gene results in further diversity in the composition of $Ca_V2.1$ channels. Depending on their composition, the $Ca_V2.1$ channels exhibit (sometimes subtle) differences in their electrophysiological properties. For instance, alternative splicing can result in $Ca_V2.1$ α_1 subunits that produce either P- or Q-type Ca^{2+} currents as can be deduced from their pharmacological profile in response to specific $Ca_V2.1$ channel blockers[41].

Although $Ca_V2.1$ channels are broadly expressed throughout the central nervous system, their expression is particularly high in the cerebellum[42, 43]. Most neurons of the cerebellar circuitry are to a considerable extent dependent on $Ca_V2.1$ channel

function: in PCs, over 90% of Ca^{2+} current density is $Ca_V2.1$ channel-dependent[44]; and in granule cell neurons this figure is about 50%[45]. Outside the CNS, $Ca_V2.1$ channels are crucial for synaptic transmission at NMJs[46].

DISEASES OF THE MOTOR SYSTEM IN WHICH $CA_V2.1$ CHANNELS PLAY A ROLE

Normal $Ca_V2.1$ channel function is necessary for proper motor coordination. Most relevant to this thesis, is the *dysfunction* of these calcium channels, which can result in impairment of the sensorimotor system and cerebellar ataxia. This impairment is characterized by gait disturbances, poor coordination of movement, and a wide-based, unsteady gait. During ataxic movements, motor behavior is typically characterized by abnormal timing with delayed muscle activation and/or sudden interruptions of movement, followed by exaggerated corrections[47]. Cerebellar ataxia can occur during attacks, can be present chronically, and, depending on the type of ataxia, can progress during the course of the disease (for review, see *Manto & Marmolino, 2009*).

$Ca_V2.1$-dependent diseases with cerebellar ataxia signs

Cerebellar ataxia associated with $Ca_V2.1$ channel dysfunction can be either the main clinical feature as in *Episodic Ataxia type 2* (EA2) and *Spinocerebellar Ataxia type 6* (SCA6), or it can be part of a more complex clinical syndrome such as in *Familial Hemiplegic Migraine (FHM) with ataxia* (with or without additional clinical symptoms of epilepsy and/or mild head trauma-induced edema), or in the autoimmune diseases *Lambert Eaton Myasthenic Syndrome* (LEMS) and *Guillain-Barré Syndrome* (GBS).

- Episodic Ataxia type 2

EA2 manifests as recurrent attacks of cerebellar ataxia lasting from hours to days that can differ widely in severity (for review, see *Jen et al., 2004*). Clinical symptoms include gait disturbances, nystagmus, vertigo, and generalized weakness. Migraine occurs in as many as half of the patients. Interictal cerebellar symptoms are not uncommon and include gaze-holding deficits, saccadic smooth pursuit, and impaired visual suppression of the vestibulo-ocular reflex, especially downbeat nystagmus. Cerebellar atrophy, especially of the anterior vermis, has been observed in many cases (for review, see *Strupp et al., 2007*). EA2 is an autosomal dominant genetic disorder that is caused by mutations in the *CACNA1A* gene[51]. EA2 mutations include truncation and certain missense mutations that result in a severely dysfunctional or non-functional $Ca_V2.1$-α_1 protein[52, 53]. EA2 mutations are loss-of-function mutations and are predicted to decrease neurotransmitter release[54]. In cellular assays, EA2 mutations were shown to exert a dominant-negative effect, thereby leading to inactivity of co-expressed normal α_1 protein[54, 55].

- Spinocerebellar ataxia type 6

SCA6 is a late-onset, permanent, slowly progressive autosomal dominant type of ataxia with nystagmus, dysarthria, and sensory loss, and exhibits considerable phenotypic overlap with EA2 (for review, see *Frontali, 2001*). SCA6 is caused by moderate expansions of a polyglutamine (CAG) stretch in the part of *CACNA1A* gene that encodes the carboxyl terminus of the $Ca_V2.1$-α_1 protein[57]. Healthy individuals have between 4 and 18 CAG-repeats, while SCA6 patients have 20 to 30 repeats. It is not entirely clear what the consequences are of the SCA6 $Ca_V2.1$-α_1 protein, but evidence suggests that toxic protein aggregates can be formed[58]. Investigating the electrophysiological properties of the SCA6 protein in transfected cells yielded contradictory results: both an increased $Ca_V2.1$ current density[59] and normal $Ca_V2.1$ current densities accompanied by a negative shift in the voltage dependence of inactivation[60] have been reported.

- Familial hemiplegic migraine with cerebellar ataxia

FHM is a rare subtype of migraine with aura that is characterized by hemiparesis during the aura phase. The aura precedes the headache and is caused by a phenomenon called cortical spreading depression (CSD), a wave of neuronal and glial cell depolarization that starts in the occipital cortex and slowly progresses to more frontal regions of the brain[61]. The headache is caused by dysfunction of the trigeminovascular system (for review, see *Goadsby, 2005*). Experiments in rats have suggested that CSD may activate brain stem nuclei, and thereby trigger headache mechanisms[63], indicating the relevance of CSD in rare FHM and common migraine. Three genes have been identified in FHM (i.e., FHM1, FHM2, and FHM3), all encoding ion transporters (for review, see *van den Maagdenberg et al., 2007*). FHM1 is caused by certain heterozygous missense mutations in the *CACNA1A* gene, which encodes the α_1 subunit of $Ca_V2.1$ channels[51]. In about 20% of FHM1 patients, cerebellar ataxia is observed. Depending on the mutation, epilepsy, mental retardation, and mild head trauma-triggered delayed brain edema (that can even result in the death of a patient) can also be part of the clinical spectrum[65]. Detailed electrophysiological investigation of the functional consequences of *CACNA1A* mutations has revealed that, , due to a combination of increased channel open probability and/or a rather dramatic left-shift in channel activation, specifically at lower voltages, all eight studied FHM1 mutations (including mutations R192Q, S218L, and T666M that are specifically relevant for this thesis) show enhanced single-channel Ca^{2+} influx over a broad voltage range[66, 67, 68]. Consequently, FHM1 mutations are portrayed as *gain-of-function* mutations. Notably, whole-cell experiments pointed to a *loss-of-function* effect of FHM1 mutations[66, 67, 69], and resulted in considerable debate on the consequences of FHM1 mutations. Clearly, FHM seems to be the result of a disturbed ionic (and neurotransmitter) balance in the brain, with single-channel data pointing to an increased release of the excitatory neurotransmitter glutamate (for review, see *Moskowitz et al., 2004*).

- Lambert-Eaton Myasthenic Syndrome and Guillain-Barré Syndrome

Finally, there are acquired immune-mediated motor coordination problems in patients with LEMS[71] and GBS[72] that are caused by auto-antibodies that target $Ca_V2.1$ channels. LEMS is characterized by muscle weakness, impaired tendon reflexes, and autonomic dysfunction and is associated with small-cell lung cancer of endocrine origin in approximately 60% of patients[73]. GBS is an acquired peripheral neuropathy that is characterized by an acute motor axonal neuropathy, and acute inflammatory demyelinating polyneuropathy[74]. Both LEMS and GBS are, at least partly, caused by $Ca_V2.1$ auto-antibodies blocking and/or causing the removal of $Ca_V2.1$ channels from the plasma membrane, resulting in the severe impairment of neurotransmission and the clinical symptoms[75].

MOUSE MODELS OF $CA_V2.1$ CHANNELS

The discovery of $Ca_V2.1$ gene mutations (*Fig. 5*) that result in motor coordination dysfunction, has stimulated the search and development of suitable experimental animal models. Over the last decade, several mouse strains were shown to have spontaneously occurred mutations in the $Ca_V2.1$ channel genes (see *Naturally occurring $Ca_V2.1$ mutant mice*). In the same time attempts were also made to generate transgenic mice in which the genome has been manipulated (see *Cacna1a transgenic mice*). Both types of mouse models have been a very useful in the study of disease mechanisms, not only of motor coordination dysfunction, but also of the other $Ca_V2.1$-associated diseases such as epilepsy, dystonia, and in particular, migraine. A brief overview is given below of the phenotypes and main findings in these mouse models that were already available for investigation before the start of the research discussed in this thesis. For more detailed reviews on naturally occurring and transgenic $Ca_V2.1$ channel mutant mice see *Fletcher & Frankel, 1999*, and *van de Ven et al., 2007*.

Naturally occurring $Ca_V2.1$ mutant mice

Tottering mice exhibit highly stereotyped episodes of motor dysfunction and, between episodes, a mild ataxic gait, beginning after four weeks of age[79, 80]. In addition, the mice exhibit absence seizures[81]. *Tottering* is caused by a recessive P601L missense mutation in the *Cacna1a* gene[82]. At their NMJs, *tottering* mice show an increased spontaneous acetylcholine release[83] and a decreased $Ca_V2.1$-dependent neurotransmission[84]. Cellular studies of transfected and dissociated PCs have revealed a reduction in $Ca_V2.1$ current density that is not associated with changes in single channel conductance[85]. Relevant to this thesis, PF-PC synapses of *tottering* mice show a decreased synaptic efficacy[86], although there is controversy on the subject as compensation from other Ca^{2+} channel types can occur[87]. The *tottering* mutation causes increased irregularity of PC firing, offering a likely explanation for the motor discoordination in these mice[25, 88]. With respect to (ultra)structural abnormalities in *tottering* mice, mild PC loss[89]

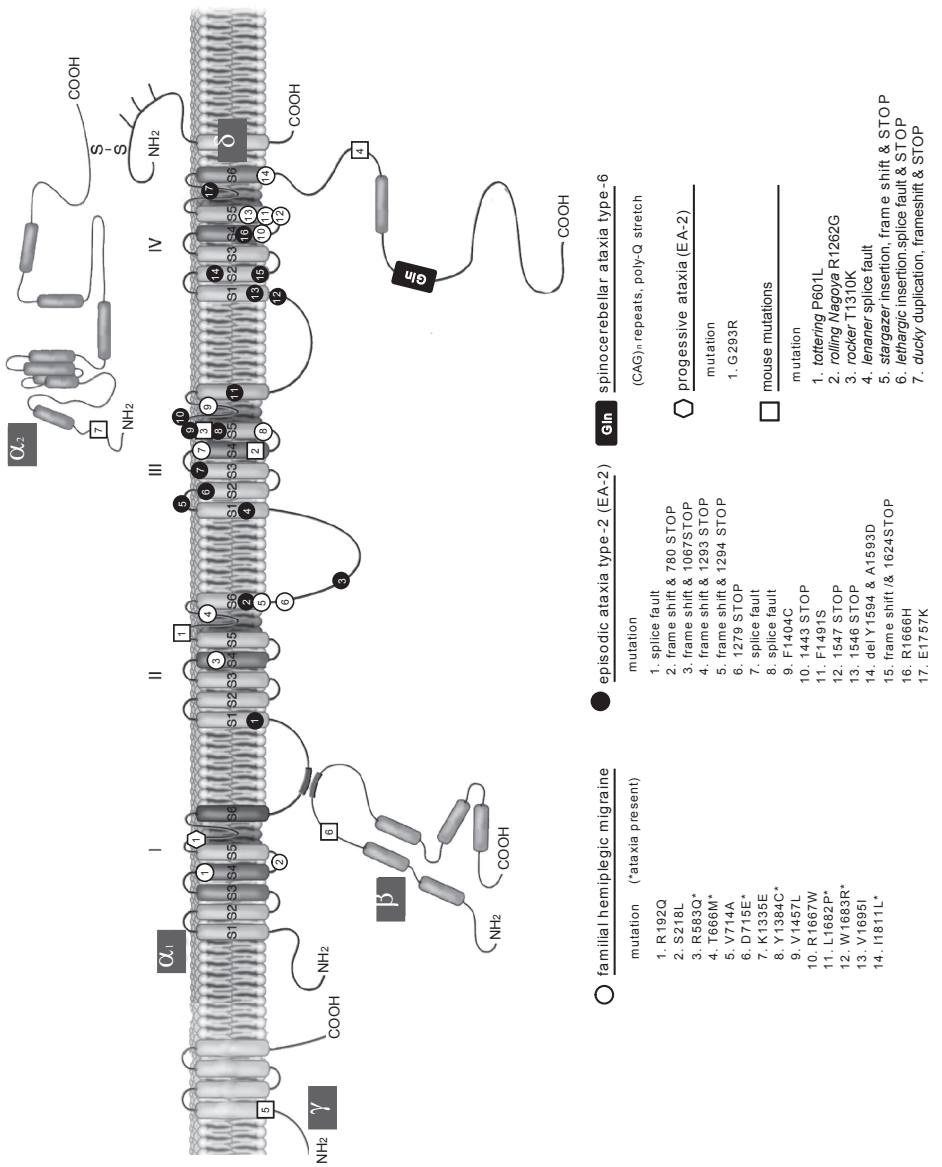

Figure 5. Schematic representation of $Ca_v2.1$ channels and their subunits with the position of disease-causing mutations in mice and humans that are relevant to this thesis. The α_1 subunit of $Ca_v2.1$ channels has four repeats (I-IV), each consisting of six (S1-S6) transmembrane domains. N - amino terminus; C - carboxyl terminus. Naturally occurring mouse mutations and human mutations introduced in knockin mouse models are depicted. *Ducky*, *lethargic*, and *stargazer* mice are knockouts of the subunits $\alpha_2\delta$-2, β_4, and γ_2, respectively. Adapted from *van de Ven et al.* (*2007*) and *Barrett et at.* (*2008*).

and an increase in the multisynaptic index of the PF contacts, somatic inclusions, and axonal swellings[90] have been reported.

Leaner mice are severely affected: they remain much smaller than their wild-type littermates and develop progressive ataxia, absence seizures, and dystonia starting at P10[79]. If left unaided, *leaner* mice will die between P20 and P28. The phenotype is caused by exon-skipping and the inclusion of intronic sequences in the aberrantly spliced C-terminus, resulting in shorter and longer *Cacna1a* transcripts[82]. Electrophysiological analysis showed a nearly 50% reduction in the neurotransmitter release at *leaner* NMJs[91]. $Ca_V2.1$ channels of *leaner* PCs revealed a right shift in voltage dependence of activation and inactivation as well as a reduction in channel open probability[92, 93]. Reduced $Ca_V2.1$ channel function was associated with a decrease in excitatory neurotransmission at PF-PC synapses[94] and, like in *tottering*, an increased irregularity of intrinsic PC firing[26]. Cerebellar granule cell loss in *leaner* mice, starts at P10, but Purkinje and Golgi cell loss is not detected until P40[95]. Other PC abnormalities include multiple dendritic spines contacting single parallel fiber varicosities[90].

Rolling Nagoya mice exhibit severe dyscoordination of the hind limbs and an ataxic gait[96, 97]. This phenotype is the result of a recessive R1262G missense mutation[98]. Muscle weakness and fatigue have been reported for this strain of mutant mice. Evoked neurotransmission release at NMJs is 50-75% reduced in *rolling Nagoya*[99]. Dissociated *rolling Nagoya* PCs revealed a reduced voltage-sensitivity and a $Ca_V2.1$ Ca^{2+} current density[98]. Dysfunctions in the parallel and climbing fiber systems, but also basal ganglia, have been suggested to underlie the ataxic phenotype in *rolling Nagoya* mice[86, 100]. At the (ultra)structural level, *rolling Nagoya* mice exhibit a reduced cerebellar weight, a decreased number of granule cells and PCs, and PC abnormalities, such as multiple dendritic spines synapsing on single parallel fiber varicosities and axonal swelling[101, 102]. Notably, an increased expression of $Ca_v2.1$ channels was reported for deep cerebellar nuclei of *rolling Nagoya* mice[103].

Finally, *Rocker* mice display absence seizures as well as cerebellar ataxia. This phenotype is caused by a recessive T1310K missense mutation in the *Cacna1a* gene[104]. Abnormal PC morphology without their loss has been reported, likely underlying the motor and cerebellar dysfunction in this strain of natural mutants[104].

There are also natural mutants relevant to this thesis that have mutations in a $Ca_V2.1$ channel subunit other than the α_1 subunit. These include *ducky*, *lethargic*, and *stargazer* that lack functionally auxiliary subunits $\alpha_2\delta$-2, β_4, and γ_2, respectively. Their main characteristics are described below.

Ducky mice exhibit a wide-open gait, severe ataxia, spike-wave discharges, and paroxysmal dyskinesia[105]. The *ducky* phenotype results from a homozygous partial genomic duplication giving rise to two possible transcripts of the *Cacna2d2* gene, only one of which is actually translated into a protein, but lacks most of the α_2 domain. As a consequence, the *ducky* mutation is a functional $\alpha_2\delta$-2 knockout[106]. Dissociated

ducky PCs show approximately 35% reduction in $Ca_V2.1$ Ca^{2+} current density[105], that is associated with increased irregularity of intrinsic PC firing[26]. At the (ultra)structural level, mutant PCs present with severely reduced dendritic arborization, thickening of the dendrites, and a so-called "weeping willow"-like structure[106].

Lethargic mice exhibit a phenotype of severe ataxia and slow (lethargic) movement[107]. The onset of the phenotype is around P10, and mice pass through a critical period between P15 and P60, during which they show increased mortality rate, weight loss and severe lymphocytopenia[108]. The *lethargic* mutation is a homozygous four nucleotide insertion of a splice site of the *Cchb4* gene, which results in the translation of a truncated, non-functional β_4 protein[39]. $Ca_V2.1$ Ca^{2+} current density in *lethargic* PCs is not affected[39]. At the (ultra)structural level, no abnormalities in the cerebellum were reported.

Finally, *stargazer* mice display ataxia, paroxysmal dyskinesia, spike-wave discharges (indicative of absence epilepsy), and typical head-tossing movements[109]. The *stargazer* mutation is a homozygous insertion in the *Cacng2* gene, leading to a non-functional γ_2 protein product[110, 111]. The cerebellar morphology of *stargazer* mice appears normal however, loss and/or reduction of the receptors in cerebellar granule cells has been noted[112].

Cacna1a transgenic mice

Various transgenic knockout (KO) models (see Section 6.4; *Generation of transgenic mice*) have been generated that target the *Cacna1a* gene[91, 113, 114]. By disrupting the *Cacna1a* gene sequence, the KO mice lack functional $Ca_V2.1$ channels. Homozygous KO mice exhibit a severe phenotype of ataxia and dystonia that starts around P10 and is very similar to what is seen in *leaner* mice. KO mice die around 3 weeks after birth. Heterozygous KO mice have been considered a possible model for EA2, but they do not exhibit the (relevant) phenotype. Notably, only one strain of KO mice shows decrease in $Ca_V2.1$ Ca^{2+} current density in cerebellar granule neurons of heterozygous mice[114], whereas in the other strain $Ca_V2.1$ Ca^{2+} current density is normal[113], possibly due to the fact that, in the latter strain, truncated $\alpha 1$ proteins may have been produced that prevent transcriptional compensation of the wild-type allele. A mouse model has been generated also for SCA6. Transgenic knockin mice expressing a "hyperexpanded" (i.e., far beyond the expansion seen in SCA6 patients)' glutamine repeat in the α_1 protein developed progressive motor impairment and aggregation of mutant $Ca_V2.1$ channels, but no changes in intrinsic electrophysiological properties of the mutant channels were observed[115].

Finally, knockin mice were generated that harbor the human pathogenic R192Q mutation in the *Cacna1a* gene[116]. In humans, mutation R192Q causes FHM *without* cerebellar ataxia[51]. R192Q mutant mice did not exhibit an overt phenotype. At the molecular level, several *gain-of-function* effects were found in homozygous mutants

that include increased $Ca_V2.1$ current density in cerebellar granule cell neurons, enhanced spontaneous and evoked neurotransmission at NMJs, and, in the intact animal, an increased susceptibility for CSD[116]. A detailed analysis of NMJs revealed a gene dosage-dependent increase in neurotransmitter release that was not accompanied by overt structural adaptations[118].

Limitations of the existing mouse models

Attempts to unravel the neuronal basis of motor coordination dysfunction using natural mutants and transgenic knockout and knockin $Ca_v2.1$ mice have been hampered by the widespread expression of $Ca_V2.1$ channels throughout the brain. That is, the relative contribution of a cell type cannot be assessed in the existing mice. This is especially relevant since a lack of $Ca_V2.1$ channel expression during development has been shown to cause cell-specific compensatory upregulations of other Ca^{2+} channel types (for review, see *Urbano et al.., 2002*). Expression or lack of expression of mutant and/or normal $Ca_V2.1$ channels in *specific cell types* is needed to address the problem. Notwithstanding, it would also be very interesting to compare knockin mouse models harboring FHM1 mutations that are (i.e., S218L) or are not (i.e., R192Q) associated with cerebellar ataxia.

Generation of transgenic mice

There are two main strategies to generate transgenic mice: *conventional transgenesis* and *gene targeting*.

In the conventional transgenesis strategy, one or more copies of a transgene (often a cDNA with a specific promoter or a piece of genomic DNA) gets randomly integrated into the mouse genome after the microinjection of DNA into the male pronucleus of a fertilized oocyte. Relevant to this thesis, a modified promoter of the *L7* gene can be used to obtain expression of the cDNA of a gene of interest exclusively in cerebellar Purkinje cells[118].

In the gene targeting strategy, a specific part of the mouse genome of embryonic stem cells is replaced by sequences from the targeting construct by a process called homologous recombination. There are three frequently used types of gene targeting: *knockout*, *conditional knockout*, and *knockin*. The use of knockouts to obtain mice that lack, for instance, $Ca_V2.1$ channels was discussed above. This can be achieved by either introducing the resistance cassette (mostly neomycine that is used to select for targeted embryonic stem cells) into an exonic sequence or by using a deletion construct in such a way that the expression of the target gene is abnormal and can no longer lead to translation of a functional protein. In a conditional knockout mice, the resistance cassette is placed in an intron. The cassette is flanked by two so-called loxP sites. A third loxP site is placed in another intron in such a way that the floxed resistance cassette and the third loxP site flank one or more exons. Sequences between loxP sites are deleted at the genomic level in cells where the enzyme Cre recombinase is expressed. Crossing of

gene-targeted mice containing the three loxP sites with conventional transgenic mice expressing Cre recombinase driven by the *EIIA* promoter (i.e., Cre-deleter mice)[119], can result in mice that contain two loxP sites and no longer the resistance cassette. These mice are called *conditional knockout* mice. In the subsequent crossing of the conditional knockout mice with Cre-transgenic mice that express the recombinase in a specific spatial and temporal manner, novel mutant mice can be generated in which the target gene product is deleted *only* in these cells in which Cre is expressed. For example, transgenic L7-driven Cre mice can be used to specifically ablate a gene product in PCs[120]. The third type of gene targeting yields knockin mice. In knockin mice, a floxed resistance cassette is placed in an intron close to the exon in which, for instance, a mutation is introduced. Using the Cre-deleter mice, the cassette is removed, yielding knockin mice that only contain the mutation and essentially one remaining intronic loxP site. Heterozygous, homozygous, and wild-type control mice are usually compared in experimental designs.

OUTLINE OF THE THESIS

The aim of the studies described in this thesis was to investigate how abnormal $Ca_V2.1$ channel function can cause disease, in particular motor coordination dysfunction. The chapters illustrate how various neuronal cell types in the periphery (peripheral nervous system: PNS) and the central nervous system (CNS) are affected by mutations in subunits of $Ca_V2.1$ channels. Using existing and newly generated mouse models, the consequences of such mutations were investigated at the molecular, cellular, and systems level so as to unravel pathways involved in motor coordination.

Using the peripheral neuromuscular junction (NMJ) synapse as a model, *Chapters 2* and *3* describe the role of various subunits of $Ca_V2.1$ channels on neurotransmission. Naturally occurring mouse models, i.e., *ducky*, *lethargic,* and *stargazer*, with mutations in Ca_V subunits $\alpha_2\delta$-2, β_4, and $\gamma_{2,}$ respectively, were the subject of detailed investigations (*Chapter 2*). As NMJ functioning did not seem to be affected in these mutants, either these subunits are not expressed at the mouse NMJ or adequate compensation by other subunit proteins occurs. The generation of a conditional mouse model that carries a so-called "floxed" *Cacna1a* allele is described in *Chapter 3*. This model allows for the spatial and temporal ablation of the *Cacna1a*-encoded $Ca_V2.1$-α_1 protein, and thereby the specific ablation of $Ca_V2.1$ channels. Crossing the floxed mice with *EIIA* promoter-driven Cre-deleter mice resulted in KO mice that had a severe, early postnatal lethal phenotype that is identical to that seen in conventional $Ca_V2.1$ KO mice.

The consequences of $Ca_V2.1$-α_1 mutations in various neuronal cell types of the CNS are described in *Chapters 4* and *5*, focusing on cell types in the cerebellar cortex where $Ca_V2.1$ channels are highly expressed. We used cell-specific Cre recombinase–expressing transgenic mice to study the effect of specific ablation of $Ca_V2.1$ channels in either Purkinje (i.e., Purkinje $Ca_V2.1$ KO or $L7^{Cre}$*Cacna1a* KO) (*Chapter 4*) or granule

(i.e., granule $Ca_V2.1$ KO or $\alpha6^{Cre}$*Cacna1a* KO) (*Chapter 5*) cell neurons to dissect their $Ca_V2.1$-mediated role on cerebellar motor coordination. It was shown that the lack of $Ca_V2.1$ channels in PCs, but not in granule cells is sufficient to cause cerebellar ataxia. Moreover, it could be demonstrated that $Ca_V2.1$ channels in granule cells exert an as yet undiscovered role in the consolidation of newly acquired motor learning. *Chapter 6* describes transgenic mice expressing mutant human $Ca_V2.1$-α_1 from a transgene, driven by a Purkinje cell-specific promoter, that contained a *CACNA1A* cDNA construct with the T666M mutation. In humans, this T666M mutation causes familial hemiplegic migraine type 1 (FHM1) and in most patients with this mutation also cerebellar ataxia. The transgenic mice did not, however, exhibit a noticeable phenotype, which may be due to the relatively low expression level of the transgene.

Chapter 7 elucidates several phenotypic and neurobiological features in transgenic mouse models that are associated with mutant $Ca_V2.1$ channels when expressed in the PNS and CNS. Two knockin mouse models were compared that carry human pathogenic $Ca_v2.1$-α_1 mutations that are at both ends of the clinical spectrum of FHM1. While the FHM1 R192Q mutation in patients causes pure FHM without additional neurological symptoms, a much more severe phenotype of FHM with cerebellar ataxia, seizures, and severe, sometimes lethal, head trauma-triggered edema is observed in patients with the FHM1 S218L mutation. The molecular consequences of the S218L mutation can explain why S218L KI mice, and not R192Q KI mice, exhibit permanent ataxia and an increased susceptibility to epilepsy. In *Chapter 8,* the consequences of the S218L mutation in the cerebellum of S218L KI are expolred in much greater detail and reveal that abnormal synapse organization leads to a relevant disturbance of the amount of Ca^{2+} influx in neurons, which in turn causes irregular firing of Purkinje cells and thereby to cerebellar ataxia.

A general discussion on the experimental findings presented in this thesis is presented in *Chapter 9*, and suggestions for future research are included.

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