Phenotypes and mechanisms in myoclonus-dystonia
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Summary

Myoclonus-dystonia (M-D) is a rare hyperkinetic movement disorder. Patients generally present with sudden, brief, shock-like jerks called myoclonus and with dystonic symptoms, which are repetitive twisting movements or abnormal postures. Symptoms in M-D mainly affect the upper part of the body and manifest in the first or second decade of life. Alcohol responsiveness of motor symptoms is a common feature in M-D as well as psychiatric abnormalities such as depression and anxiety syndromes. Mutations in the epsilon-sarcoglycan (SGCE) gene have been identified in several M-D patients and families. M-D inheritance follows an autosomal dominant pattern with a reduced penetrance owing to imprinting. The SGCE gene is maternally imprinted meaning that symptoms only manifest upon paternal transmission. The SGCE protein is a ubiquitously expressed transmembrane glycoprotein located at the plasma membrane. The function of SGCE is unknown. Also, M-D pathomechanisms remain largely elusive; there is evidence for involvement of the basal ganglia as well as the cerebellum. This thesis comprises different studies aiming at understanding the role of SGCE in M-D by studying the phenotypic spectrum of SGCE mutations, the SGCE mRNA expression and splicing pattern throughout the human brain and the function of the SGCE protein.

Chapter 1 reviews current knowledge on M-D, its genetic basis and pathophysiology and elaborates the main open questions.

In 43% of reported M-D families dystonia manifested as writer’s cramp. In order to elucidate the phenotypic spectrum of M-D, we investigated whether writer’s cramp as presenting symptom is associated with SGCE mutations in chapter 2. In a large cohort of writer’s cramp patients it became apparent that testing for SGCE mutations should only be considered if writer’s cramp is accompanied by myoclonic jerks. We did not find evidence for involvement of other dystonia genes, DYT1 (TOR1A) and DYT16 (PRKRA), in this patient group. However, intronic and regulatory regions have not been investigated. With the current knowledge, we cannot conclude which dystonia genes need to be tested in patients presenting with isolated writer’s cramp. Course of disease and disease spread in the patient and family members may provide an indication for which genes need to be examined.

In chapter 3 the need for strict clinical criteria for genetic testing in M-D is discussed and corroborated by studying the phenotypic spectrum of SGCE mutations in a large Dutch M-D cohort. In literature, SGCE mutations were identified in only about 23% of M-D patients. This low frequency can partly be attributed to the lack of screening for large SGCE deletions or insertions and to the lack of comprehensive clinical criteria; many patients that do not present with the definite M-D symptoms are tested for SGCE mutations. Therefore, we applied recently introduced clinical criteria to our large
M-D cohort and grouped them into definite, probable, and possible M-D. We showed that only definite patients (early-onset myoclonus and dystonia or isolated myoclonus predominantly in the upper body half and a positive family history for myoclonus and/or dystonia) should be considered for genetic testing. Exceptions should be made for patients with the typical phenotype and a young onset with a negative family history as well as for patients with the typical phenotype, late onset and a positive family history. Several different types of mutations were identified in our definite M-D cohort such as nonsense, missense, and splice site mutations and one multi-exonic deletion. There were no genotype-phenotype correlations; all reported mutations are thought to lead to loss of function explaining this lack of association. In our cohort, large exonic deletions played a minor role, but several exonic rearrangements have been reported in literature, and testing for large exonic deletions should be performed in standard diagnostic settings for definite M-D patients. Alcohol responsiveness, psychiatric abnormalities, and a paternal transmission were common features in our SGCE-associated patient group. Patients showing dystonic features only did not belong to the SGCE mutation spectrum. Despite implementation of the strict inclusion criteria, we did identify a SGCE mutation in only 50% of definite patients, suggesting genetic heterogeneity or unknown factors or mutations affecting the SGCE gene or its expression.

Chapter 4 addresses an important question in M-D pathomechanisms: why do loss-of-function mutations in the widely expressed SGCE gene lead to exclusively neurological symptoms? We hypothesised that a brain-specific SGCE isoform plays a role in disease pathomechanisms, whereas the function of ubiquitous SGCE is redundant. Therefore, we systematically investigated the tissue-specific SGCE mRNA exon structure, and conducted an ultra-deep amplicon sequencing study. Using this approach we showed that exon 11b is the major brain-specific alternatively spliced exon. Inclusion of this alternatively spliced exon results in an alternative intracellular C-terminus containing a potential binding site for proteins. We showed that transcripts containing exon 11b are differentially expressed throughout the human brain with moderate to low expression in the basal ganglia and notably high levels in the cerebellum. By isoform-specific in situ hybridizations, we confirmed cerebellar expression of the brain-specific SGCE isoform in Purkinje cells and neurons of the dentate nucleus. We hypothesised that loss of function of brain-specific SGCE leads to the specific M-D symptoms and provided evidence for a role of the cerebellum in M-D pathogenesis.

Based on these findings, we proceeded with the investigation of SGCE protein interactions in the cerebellum and focused on interactions with the brain-specific intracellular SGCE C-terminus in chapter 5. We performed immunoprecipitation and isoform-specific pull-down experiments using biotinylated SGCE peptides encompassing the ubiquitous or the brain-specific C-terminus. As SGCE was enriched in synaptosomal fractions, we used this fraction from mouse and human cerebellum for our assays and performed mass spectrometric analysis to identify precipitated proteins.
Two binding candidates emerged from immunoprecipitations as well as isoform-specific SGCE peptide pull-down experiments, synapsin I and synapsin II. Both proteins were detected in independent experiments by both approaches, for both species, and only with the brain-specific and not ubiquitous SGCE C-terminal peptide. To validate these two candidates, we performed (1) co-immunoprecipitations in HEK293 cells and in the motor neuron-like cell line NSC-34 and (2) immunofluorescence and confocal microscopy to study the (co-) localisation of both candidates in differentiated NSC-34 cells. We confirmed binding of synapsin I and II with brain-specific and not ubiquitous Sgce by immunoprecipitating the complex using a synapsin antibody in differentiated NSC-34 cells. Synapsin immunoprecipitation in HEK293 cells gave variable results, which implies that both proteins are not direct binding partners, but may be part of the same complex and/or that certain neuronal factors are required for the interaction. Confocal microscopy revealed co-localisation of the synapsins with Sgce at the plasma membrane and tips of dendrites of differentiated NSC-34 cells. Synapsins are involved in the modulation of neurotransmitter release and play a role in synaptic function and plasticity. Synapsins anchor synaptic vesicles to the extracellular matrix and regulate their release upon an action potential. SGCE may play a modulatory role in this pathway and loss of this modulatory function may explain the abnormal, hyperkinetic movements in M-D by “lack of tuning” of neurotransmitter release in the cerebellum.

\section*{Discussion}

The aim of the studies presented in this thesis was to characterise the role of \( SGCE \) in M-D and to get insights into pathophysiological mechanisms. We performed clinical studies to define the phenotypic spectrum caused by \( SGCE \) mutations, studied tissue-specific \( SGCE \) mRNA isoform expression patterns to identify disease-associated brain regions and investigated SGCE protein interactions to get insights into SGCE function.

\section*{Myoclonus-dystonia: a cerebellar disorder?}

Little is known about M-D disease mechanisms and affected brain regions, but recent evidence suggests that the cerebellum plays a crucial role in \( SGCE \)-associated M-D. In this thesis, we have shown that the major brain-specific \( SGCE \) isoform is differentially expressed throughout the human brain with highest expression in the cerebellum and provided evidence that may indicate a function for brain-specific SGCE in synaptic transmission. Our findings have been supported by two clinical studies. An impaired saccadic adaptation has been reported in \( SGCE \) mutation carriers, providing neurophysiological evidence of cerebellar dysfunction.\(^1\) An fMRI study in \( SGCE \)
mutation-positive and negative M-D patients revealed a different activation pattern between both patient groups with a notable difference in the cerebellum. Both findings support our hypothesis that loss of function of brain-specific SGCE, which is highly expressed in the cerebellum, is causative for the disease. The function of ubiquitous SGCE is presumably redundant. We proposed that reported striatal changes in an M-D animal model and in M-D patients are secondary to cerebellar pathology, which can be attributed to a highly interconnected network. The cerebellum and basal ganglia are interconnected by projections from the dentate nucleus to the striatum and from the subthalamic nucleus to the cerebellum, and the dentate nucleus projects to the ventrolateral thalamus. Brain-specific SGCE was highly expressed in the dentate nucleus and Purkinje cells, which project to the dentate nucleus and can thereby influence signalling to the basal ganglia.

Subcortical myoclonus is the predominant symptom in M-D. Our results suggest involvement of the cerebellum in the generation of the myoclonic symptoms. In literature, some cases were reported that developed myoclonic symptoms associated with cerebellar dysfunction owing to selective degeneration of the dentate nucleus or nondegenerative lesions that were limited to the cerebellum. The clinical description of these cases resembles cortical myoclonus with stimulus-sensitive and action-associated myoclonus, but the myoclonic symptoms were classified as subcortical based on normal EEG recordings. The lack of abnormal EEG activity associated with the myoclonic jerks does, however, not rule out the cortical involvement in the myoclonus. More sophisticated electrophysiology, such as somatosensory evoked potentials (SSEP) and EEG back-averaging might be required to detect cortical involvement. Pathologic abnormalities in the cerebellum have more often been described associated with electrophysiologically confirmed cortical myoclonus, suggesting that enhanced excitability of the sensorimotor cortex may arise as a distant effect of cerebellar pathology. It remains unclear if cortical abnormalities lead to myoclonic symptoms in these reports. M-D appears to find its origin also in the cerebellum, but does not change the cortical excitability, suggesting that cerebellar dysfunction can cause myoclonic symptoms via a different mechanism.

Also in dystonia pathogenesis, there is emerging evidence for involvement of the cerebellum as discussed in chapter 4. Evidence comes from animal studies, patients with cerebellar lesions, and fMRI studies in dystonia patients. A recent study revealed morphological changes in the cerebellum in Dyt1 (Tor1a) ΔGAG knock-in mice, which is a model for early-onset primary dystonia. A decrease in the number of spines on the distal dendrites of Purkinje cells and a reduction in the length of primary dendrites were observed, suggesting an important regulatory role for torsinA in the cerebellum. Thus, dystonic symptoms in M-D may be secondary as a response to abnormal cerebellar
output affecting basal ganglia function or they may be attributed to abnormal neuronal function in the cerebellum.

**Myoclonus-dystonia: a synaptopathy?**

The work described in this thesis provides first insights into SGCE function in the brain. We have shown that the brain-specific SGCE isoform interacts with the presynaptic proteins synapsin I and II. Synapsins are modulators of synapse formation and neurotransmitter release. Synapsin I and II are predominantly expressed at nerve terminals in mature neurons, suggesting a synapse-associated function for brain-specific SGCE. Figure 1 shows a model of presumed functions of synapsin proteins.

The predominant and most studied function of synapsin is the maintenance of the synaptic vesicle pool and the modulation of neurotransmitter release. Synapsins link synaptic vesicles to the actin cytoskeleton creating a reserve pool of vesicles and regulate their availability for exocytosis after an action potential. Release of synaptic vesicles is achieved by a conformational change induced by calcium influx and subsequent phosphorylation of the synapsins. Synapsins may also be involved in docking, priming, and fusion of vesicles with the presynaptic membrane (Figure 1). As SGCE is a transmembrane protein, it may act as a stabilising factor and/or as a link between synapsin and the cytoskeleton to form the vesicle reserve pool, or to facilitate synaptic vesicle docking, priming, or fusion. SGCE contains a cadherin domain homologue in the extracellular domain. Cadherins comprise a superfamily of proteins that mediate calcium-dependent cell-cell adhesion. In neurons they play a role during outgrowth of neurites and axonal patterning, and they are thought to influence synaptic activity and regulate synaptic plasticity. Cadherins are associated with vesicle-release zones and probably involved in the formation of synaptic adhesions. In view of these findings, the extracellular SGCE domain may be involved in synapse formation and its intracellular domain may interact with synapsins to modulate vesicle docking, post-docking or fusion events. To get a better understanding of the synapsin-SGCE interaction, the precise site of SGCE action needs to be investigated; it may modulate synapsin function at different steps or one specific step of the life cycle of synapsin-associated synaptic vesicles.

In humans, synapsin I and II have been associated with epilepsy, autism spectrum disorders, and schizophrenia. Synapsin knockout mice exhibited epileptic seizures without remarkable alterations in brain morphology and connectivity. Expression pattern and functional studies indicate that the different synapsin isoforms accomplish distinct functions in different neuronal subtypes. At excitatory synapses, loss of synapsin leads to an increase in synaptic depression, which is reflected by a reduced number of synaptic vesicles in the reserve pool. At inhibitory synapses, loss of synaptic...
Figure 1. Schematic model of the synaptic vesicle life cycle and the role of synapsins in the presynaptic compartment. (1) Synaptic vesicles in the reserve pool (RP) are thought to be reversibly linked to the actin-cytoskeleton by dephosphorylated synapsin. Upon an action potential, synapsins are phosphorylated and undergo a conformational change affecting their binding to the synaptic vesicles. (2) Synaptic vesicles are released and move close to the active zone to form the readily releasable pool (RRP). (3) Synaptic vesicle tethering or docking describes the process of line-up of vesicles at the presynaptic membrane. (4) Priming of synaptic vesicles is achieved by the formation of partially assembled SNARE complexes and ATP-dependent protein and lipid modifications. (5) Primed vesicles fuse with the presynaptic membrane upon an increase in cytoplasmic calcium and fusion is mediated by the SNARE complex (not shown). (6) After fusion, vesicles undergo clathrin-mediated endocytosis, followed by clathrin-uncoating (7), neurotransmitter uptake (8), and the recycling and reclustering of synaptic vesicles in the RP or RRP. Evidence suggests that synapsins not only play a role in maintenance of the RP, but may also modulate synaptic vesicle docking, post-docking, and fusion events as well as take part in endocytosis of vesicles. This figure was adapted from Cesca and colleagues.18
vesicles were observed at the reserve and also at the readily releasable pool, a pool in which synaptic vesicles are not anchored to the cytoskeleton and free to fuse with the presynaptic active zone upon stimulation (Figure 1).\textsuperscript{32,33} Especially inhibitory synapses are sensitive to the depletion of the reserve pool, as GABAergic interneurons often experience high frequency firing.\textsuperscript{31} Therefore, it has been proposed that epileptic seizures can be evoked by an unbalance of excitatory and inhibitory transmission.\textsuperscript{31,34,35} In view of the confined phenotype of M-D, it is likely that SGCE accomplishes a modulatory role in synaptic function that is restricted to certain neuronal subpopulations and/or to the cerebellum as indicated by its expression pattern. The uncontrolled movements in M-D may hint towards a deficit in synaptic transmission triggered by loss of the synapsin-SGCE interaction. As mentioned earlier, synapsins undergo a conformational change after an action potential resulting in the release of synaptic vesicles. Loss of SGCE may hinder or promote this conformational change resulting in reduced or enhanced neurotransmitter release. In the same way, docking or fusion of vesicles with the presynaptic membrane may be affected. However, this is currently purely theoretical and remains to be investigated.

Motor symptoms in M-D are alcohol-responsive. Alcohol is known to interfere with the olivo-cerebellar-olivary circuit, where it interacts with different neurotransmitters and ion channel receptors with impact on motor control and coordination.\textsuperscript{36} It has been shown that alcohol enhances GABA-mediated transmission and impairs glutamatergic transmission.\textsuperscript{37,38} As possible disease mechanism, we therefore suggest that loss of the synapsin-SGCE interaction affects synapsin function and thereby (GABA-mediated and/or glutamatergic) neurotransmission resulting in an altered cerebellar synaptic homeostasis. This defect may be reversed temporarily by alcohol ingestion (Figure 2).

The role of the synapsins in the modulation of neurotransmitter release implies its involvement in neuronal plasticity, which has been confirmed in several studies.\textsuperscript{21} Interestingly, an abnormal plasticity, a defect in the fine-regulation of synaptic strength, has been implicated in dystonia pathophysiology. It is thought that an altered plasticity may predispose to develop dystonia and that a “second hit” (environmental factors such as trauma, peripheral injury, repetitive training or genetic factors) may trigger dystonia.\textsuperscript{39} Assuming that SGCE interacts with the synapsin protein complex, loss of SGCE may affect neurotransmitter release, resulting in an abnormal synaptic plasticity over time. In M-D, an abnormal plasticity may therefore be the “second hit” causing the dystonic symptoms, which develops owing to defective SGCE function (Figure 2). This theory is supported by clinical observations. Myoclonus is the starting symptom in most cases and dystonia can develop several years after disease onset.\textsuperscript{40-43}

To conclude, our findings provide evidence for SGCE to function at the presynapse in a complex with synapsin and may thereby modulate neurotransmitter release.
Myoclonus-dystonia: a myoclonus-plus rather than dystonia-plus syndrome?

Initially, different terms were used to describe the typical M-D phenotype (lightning jerks, dramatic response to alcohol, and additional dystonic symptoms in some cases) such as “essential myoclonus”, “hereditary essential myoclonus”, “alcohol-responsive myoclonic dystonia”, and “dominantly inherited myoclonic dystonia”. In 1996, it has been proposed that these distinct reports describe the same disorder and suggested the term “essential myoclonus”. However, “myoclonus-dystonia” became the accepted term for the $SGCE$-associated disorder and it was classified as the...
dystonia-plus syndrome DYT11. Dystonia-plus syndromes refer to conditions in which dystonia presents with additional neurological features. On the basis of the previous proposal and with growing knowledge about the disease, we suggest re-evaluation of the classification of M-D: (1) The majority of patients present with myoclonus at onset, dystonic symptoms develop in most cases after several years. (2) In line with this clinical observation, there is evidence for dystonic symptoms to be secondary due to abnormal cerebellar output. Also, the association of SGCE with synapsin indicates that dystonic symptoms may develop secondary due to an abnormal plasticity. (3) Sgce knockout mice exhibited myoclonic features and no dystonic symptoms. In line with these observations, it has been recently hypothesised that dystonia may be a relatively common downstream manifestation of various hereditary or environmental insults and not owing to a common upstream pathway. In view of these facts, we think the classification of M-D as a dystonia-plus syndrome is misleading as it rather represents a myoclonus-plus syndrome.

Conclusion and future research

The work described in this thesis is aimed at getting insights into pathophysiological mechanisms of SGCE-associated M-D. Our findings implicate that loss of the major brain-specific SGCE isoform leads to M-D. We provided evidence for this isoform to play a role in the cerebellum and more precisely in synaptic function via its interaction with the synapsins, which are key proteins in the modulation of neurotransmitter release. As disease mechanism, we hypothesise that loss of the synapsin-SGCE interaction results in altered synapsin function. This yields a presynaptic cerebellar deficit in the modulation or processing of neurotransmitter release, which leads to an abnormal cerebellar output to striatal-thalamo-cortical and/or cerebello-thalamic motor circuits and to the abnormal motor symptoms in M-D.

Our findings raise several new questions and different studies are needed to elucidate the role of SGCE in the cerebellum, its presumed function in the modulation of neurotransmitter release and in M-D pathomechanisms.

Myoclonus-dystonia pathology

No signs of neurodegeneration have been reported in brain imaging studies and no data on M-D brain pathology are available, neither from patients nor the Sgce knockout mouse model, suggesting that M-D is a neurofunctional rather than a neurodegenerative disorder. SGCE is highly expressed in several tissues during embryonic development with high levels in the cerebellar cortex. This observation hints towards a role for SGCE during cerebellar development, and thus loss of SGCE may result in functional and possibly microstructural abnormalities. To learn more about affected brain regions
in M-D, it will be crucial to investigate, whether there are morphological changes, particularly in the cerebellum.

**Cerebellar dysfunction and myoclonus-dystonia**

There is growing evidence for cerebellar dysfunction in M-D. An abnormal saccadic adaptation has been observed in *SGCE* mutation-positive patients (14 patients). An fMRI study revealed that cerebellar alterations distinguishes between *SGCE* mutation-positive and negative M-D patients suggesting different disease etiologies. Future research should focus on studying cerebellar dysfunction in *SGCE*-associated M-D patients to validate previous findings. Eye movement recordings in *SGCE* mutation-positive versus negative patients can be performed to validate findings of the fMRI study, which may help to distinguish between both groups and to give a more accurate diagnosis.

Cerebellar dysfunction is associated with abnormal motor learning. *Sgce* knockout mice exhibited deficits in motor learning, which, to our knowledge, has not been investigated in M-D patients and should be addressed in future research.

**Epsilon-sarcoglycan and synaptic vesicle turnover**

Identification of protein interactions of brain-specific and also ubiquitous SGCE are crucial to study their function and pathways involved in M-D. We provided evidence for brain-specific SGCE to interact with synapsins. Synapsins are important modulators of the synaptic reserve vesicle pool and the regulated release of vesicles after an action potential. We speculated that loss of SGCE function leads to abnormal synapsin function and thus to an abnormal neurotransmitter release and possibly to loss of inhibitory transmission as seen in synapsin knockout mice. Monitoring synaptic vesicle recycling as well as trafficking and its kinetics in living neurons from *Sgce* knockout animals, will be valuable tools to test this hypothesis. Also, the question whether the synaptic vesicle reserve or readily releasable pools are affected upon *Sgce* knockout needs to be answered.

Synapsin function is strongly regulated by phosphorylation/dephosphorylation of nine potential phosphorylation sites. Phosphorylation of specific sites causes conformational changes, resulting in a reduced binding to actin and synaptic vesicles. Its effect on the interaction with SGCE should be investigated to narrow SGCE function. Electron microscopy may help to further determine the subcellular localisation of the identified interaction.

**Identification of novel myoclonus-dystonia associated genes**

There are still many *SGCE* mutation-negative patients with the typical M-D phenotype suggesting genetic heterogeneity. There is evidence for a different disease etiology in
the $SGCE$ mutation-positive and negative patient groups, indicating that a different mechanism and thus a different gene is involved. The identification of novel disease-associated genes will bring new insights into pathophysiological mechanisms in M-D, and efforts should be taken to study the genetic background of large $SGCE$ mutation-negative M-D families.

References