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## The molecular neuropathology of spinocerebellar ataxia type 23

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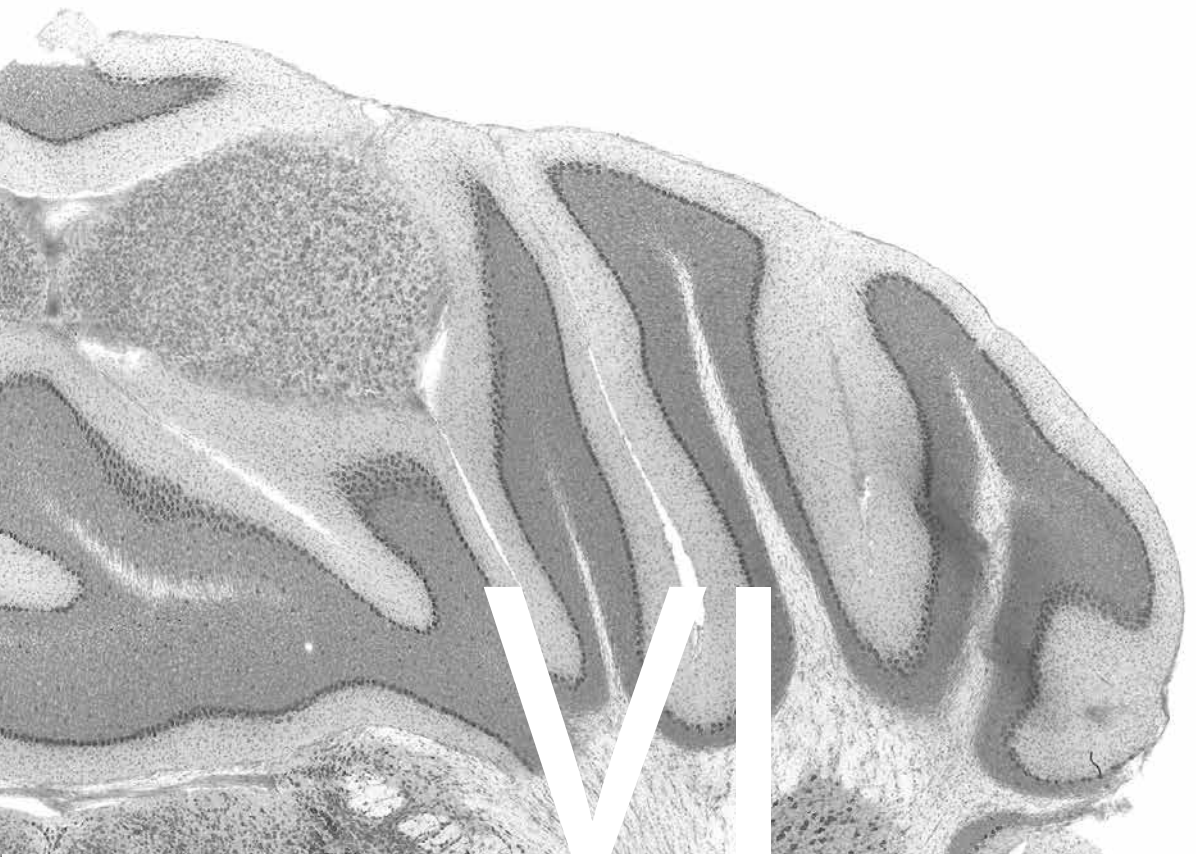
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## Discussion

During the last 25 years of SCA research, 44 different spinocerebellar ataxia (SCA) types have been described. With the implementation of next generation sequencing (NGS), SCA diagnostics have improved greatly, and the rate of mutation identification has increased dramatically. While it is beneficial to identify novel disease-causing variants, studies reporting novel variants often lack functional validation confirming pathological changes on protein level. Furthermore, researchers have been working to pinpoint a shared mechanism among the many SCAs, however, no such single mechanism has been identified. We believe that altered glutamate/Ca<sup>2+</sup> signalling is very promising as a common mechanism. We hypothesize that unbalanced glutamate/Ca<sup>2+</sup> underlies the SCA23 pathology, which is caused by mutations in prodynorphin (*PDYN*). In addition, we have implicated *PDYN* in cerebellar neurodevelopment, as we observed developmental abnormalities in the neurodegenerative disorder SCA23.

## Genetic diagnosis of SCA

Currently, approximately 70% of Dutch dominant SCA cases are confirmed genetically via mutation analysis for the common SCA types in the Netherlands: SCA1, 2, 3, 6, and 7. An additional 5% of patients suffer from a less frequent SCA type, and can be diagnosed genetically when tested on a second panel of SCA types prevalent in the Netherlands: SCA12, SCA13, SCA14, SCA17, SCA19, SCA23, and SCA27. However, this still renders approximately 25% of patients without a genetic diagnosis. Identifying novel causal mutations in these patients would not only improve the diagnostic possibilities for this group, which can be beneficial for the patient and for their carers' psychological states and would aid in counselling, it would also provide more insight into the SCA pathology.

Despite the wide-spread implementation of next generation sequencing (NGS) in research, identifying novel SCA mutations is still not an easy task. There are currently 44 known types of SCA (for an overview, see Chapter I, Table 1), for 11 of which, disease genes have been identified using NGS. Since most of the genetically undiagnosed SCA cases are single cases or come from small families with only a few affected individuals, it is difficult to define the causal variant among the hundreds to thousands of variants identified. Next generation sequencing offers several platforms on which to sequence genetic material, one of which is targeted resequencing (TRS), which we used in Chapter V. In this study, we identified novel variations by sequencing target candidate genes assembled on a gene panel, bringing down the cost and time required for a sequence run and for data analysis. Although effective, TRS has its limitations. First, only a pre-defined set of genes is sequenced and only exonic regions and their respective splice-site regions are sequenced. This is problematic because, while most disease-causing mutations can be found in exons, deep-intronic mutations also occur with some regularity. Second, repeat expansions, large deletions, and duplications cannot be detected, and will therefore be missed. Third, while TRS is a useful tool in identifying novel disease variants, it still yields a large number of rare variants per case that

need to be properly interpreted. Currently, we are heavily dependent upon *in silico* pathogenicity predictions of these variations, often leading to inconclusive outcomes from the various prediction programs. Additionally, rare single nucleotide polymorphisms (SNPs) can be wrongly interpreted as novel mutations. Functional validation would be the best alternative method to validate whether a rare variation is benign or damaging. However, functional validation of these rare variations ties up resources, and slows down work on disease-causing variants.

Functional validation of novel variants is an important step in determining whether a variant is causal. When a novel variant is reported, it should be accompanied by a preliminary functional analysis<sup>1</sup>. These can be simple assays, for example transfection of a relevant cell line (like N2A, NG108, or SH-SY5Y cells) with a plasmid expressing the mutated gene, and determining expression and localization of the protein, as changes in protein expression and/or localization indicate a variant is very likely not benign. Unfortunately, when novel mutations are reported, these types of analyses are most often not included, leading to questions about whether the reported mutations are indeed causal. A preliminary functional analysis on the novel mutations in *GRIN3B* and *GRIK1* described in Chapter V revealed that both novel variants affect surface expression of the receptor channels, suggesting these are indeed disease-causing mutations. However, follow-up studies are needed to determine how these novel mutations affect surface expression of the receptors. This underlines the need to perform more specific functional assays, determining alterations in protein function and electrophysiology, and for *in vivo* models to fully determine the causality and effect of any newly identified variant. The work described in this thesis demonstrates the benefits of studying a mutation extensively and in several models, as the deviations between our *in vitro* and *in vivo* findings proffer additional insights into the disease mechanism.

## Altered glutamate/Ca<sup>2+</sup> signalling as a common pathology in SCA

While the SCAs are genetically highly heterogeneous, their symptoms are surprisingly homogeneous. Guided by this homogeneity, researchers have been searching for a common pathological mechanism underlying Purkinje cell (PC) dysfunction and death. However, despite more than two decades of research, no such singular mechanism could be identified. Using a gene-gene interaction network analysis, we showed that numerous SCA/ataxia-causing genes function and interact with each other, and could therefore highlight the shared biological mechanisms among the SCAs<sup>2</sup>. These mechanisms include neurogenesis, synaptic transmission, cell cycle and proliferation, protein synthesis and folding, regulation of transcription, and mRNA transport<sup>2</sup>. Overlapping with these mechanisms, Matilla-Dueñas *et al.*<sup>3</sup> describe a number of shared mechanisms that have been studied extensively in SCAs, including dark cell degeneration, dysregulation of gene transcription, autophagy, and RNA toxicity. These are all mechanisms to which PCs seem more vulnerable than other neuron populations, making them good candidates for a common mechanism. Interestingly,

for some SCAs several such suggested commonalities have been implied, indicating there may be more than one common route to pervasive PC death.

The last 25 years of SCA research has yielded one particularly promising shared mechanism, namely altered glutamate/Ca<sup>2+</sup> signalling. Several SCA types display climbing fibre (CF) regression, a possible consequence of increased glutamate/Ca<sup>2+</sup> signalling. Excitotoxicity has long been implicated in neurodegeneration<sup>4,5</sup>, and has been suggested as the underlying mechanism for SCA5<sup>6</sup>. Increased intracellular Ca<sup>2+</sup> levels in SCA1, 2, 3, 7, and 28 may be responsible for dark cell degeneration (DCD), a type of cell death to which PCs seem particularly sensitive<sup>3,7</sup>, further substantiating that disrupted glutamate/Ca<sup>2+</sup> signalling could be a common pathology among SCAs. Additional evidence supporting this hypothesis is found in observed reduced expression of glutamate transporters on PCs and the resident cerebellar astrocytes, Bergman glia, in SCA1, 5, and 7<sup>6,8,9</sup>. However, as described in Chapter III, PCs in SCA23 most likely suffer from reduced Ca<sup>2+</sup> levels due to loss of glutamatergic signalling induced by lost CF innervation. Following this reasoning, CF regression in SCA1, 7, and 14 could also lead to decreased intracellular Ca<sup>2+</sup> levels in PCs. Yet, SCA1 and 7 mouse PCs have been suggested to have increased Ca<sup>2+</sup> levels<sup>8-10</sup>. As in SCA1 and SCA7, the CF deficits do not occur until 6 and 40 weeks of age, respectively, thus CF regression could be a consequence of elevated Ca<sup>2+</sup> signalling in these SCA types. Nonetheless, the CF/parallel fibre (PF) synaptic balance deficits are likely caused by a disturbance in glutamate/Ca<sup>2+</sup> signalling, whether it be increased or decreased. Therefore, a likely common mechanism could be unbalanced glutamate/Ca<sup>2+</sup> signalling, leading to altered proportions of synapse types, changes in cerebellar plasticity, and eventually, ataxia.

As described in Chapter V, we suspect that mutations in genes encoding components of the glutamatergic signalling system are associated more with intellectual disability than ataxia, challenging the hypothesis of unbalanced glutamate/Ca<sup>2+</sup> as a shared mechanism for the SCAs. However, we have identified two novel mutations in genes within the glutamatergic system causing ataxia, and mutations in *GRID2* leading to ataxia have also been reported<sup>11</sup>. A possible explanation for this divergence is that some glutamatergic components may have a more prominent function in the cerebellum, and mutations in these genes could induce ataxia, while other glutamatergic components may be more important in regions affected in intellectual disability, like the frontal cortices. In any case, this indicates that further research into involvement of glutamate/Ca<sup>2+</sup> signalling in ataxia, should focus on the proteomics of glutamate signalling components, more specifically electrophysiology. We have investigated the role of altered glutamate/Ca<sup>2+</sup> signalling in the molecular pathomechanism of SCA23.

## Mechanisms underlying PC death in SCA23

One of the SCA types in which unbalanced glutamate/Ca<sup>2+</sup> signalling plays an important role, is SCA23. Mutations in *PDYN* cause SCA23, a mild, slowly progressive form of ataxia<sup>12,13</sup>. To date, the

cerebellar function of Dyn A, or its precursor prodynorphin (PDYN), was largely unknown. Previous studies mainly focused on the physiological functioning in pain signalling, stress, and addiction control via opioid signalling<sup>14–16</sup>, and pathological features of the peptide, including induction of excitotoxicity and cell death via direct *N*-methyl-D-aspartate receptor (NMDA-R) interaction<sup>4,15,17</sup>.

In order to understand the molecular pathology of SCA23, we generated a transgenic mouse model and demonstrated that mice expressing PDYN-R212W recapitulate the symptoms of SCA23 patients<sup>12</sup>. *PDYN*<sup>R212W</sup> mice displayed elevated Dyn A levels, subtle gait abnormalities at 3 months of age, and a general loss of motor function at 12 months of age. This confirmed our previous genetic and cellular findings<sup>13</sup>, and demonstrated that expression of PDYN-R212W results in elevated mutant Dyn A levels *in vivo* and causes cerebellar ataxia. As PDYN levels are low while peptide cleavage product levels are unchanged in *PDYN*<sup>R212W</sup> mice, and the R212W mutation led to reduced peptide degradation *in vitro*, elevated Dyn A levels in *PDYN*<sup>R212W</sup> mice are likely due to enhanced PDYN-R212W processing, and increased peptide stability.

How the increased levels of mutant Dyn A induce ataxia is not yet clear. Initially, we hypothesized that elevated levels of mutant Dyn A mimic glutamate and potentiate NMDA currents on PCs, causing increased intracellular Ca<sup>2+</sup> levels, leading to dysregulated gene transcription, PC dysfunction, and altered synaptic plasticity (Chapter II, Fig. 8). However, after we identified developmental CF abnormalities, early loss of vGlut2, and reduced expression of voltage-gated calcium channel (VGCC) subunits in the vermis of *PDYN*<sup>R212W</sup> mice, we inverted our hypothesis. We now believe that reduced Ca<sup>2+</sup> signalling induces PC dysfunction in SCA23, as has been described for SCA6<sup>18</sup>.

## Climbing fibres in SCA pathology with focus on SCA23

Another shared characteristic we observed is CF pathology. CFs are the major excitatory input of PCs, and therefore the largest contributor to glutamate/Ca<sup>2+</sup> signalling in PCs, implicating CF pathology in the SCA phenotype as a major contributor. As described in Chapter I, CFs exert much control over the induction of cerebellar synaptic plasticity, which in turn is crucial for maintaining motor learning and performance<sup>19–25</sup>. CF deficits have been described in SCA1, 7, 14 and 23 (Chapter I), and disrupted cerebellar plasticity has previously been demonstrated in several SCA types, including SCA3, 5, 6, 14, and 27<sup>26–31</sup>, and indirectly for SCA1<sup>10</sup>.

The reduced Ca<sup>2+</sup> signalling in SCA23 is likely caused by the loss of vesicular glutamate transporter 2 (vGlut2), as measured by reduced CF-PC synapse numbers. In the hippocampus, vGlut2 has been shown to have a neurodevelopmental role<sup>32</sup>. We propose that vGlut2 plays a similar developmental role in the cerebellum, as we observed loss of vGlut2 in *PDYN*<sup>R212W</sup> vermis at an early stage. In addition, the observed reduction in CF-PC synapses in the *PDYN*<sup>R212W</sup> vermis may lead to disturbances in glutamatergic signalling and synaptic plasticity, as was seen in vGlut2 knockout

mice<sup>32</sup>, implicating CF deficits in SCA23 pathology. Consequently, due to the loss of CFs, PF-PC synapses are likely increased, as vesicular glutamate transporter 1 (vGlut1) levels are elevated in *PDYN*<sup>R212W</sup> vermis. CF-PC and PF-PC synapses balance each other within their respective territories (Chapter I, Fig. 1), and a disruption of this balance affects motor learning and performance (Chapter I)<sup>19-25</sup>, which could underlie SCA23 pathology. Notably, the developmental anomalies indicate a neurodevelopmental role for PDYN in the cerebellum not previously recognized.

In essence, we hypothesize that the expression of PDYN-R212W leads disruption of cerebellar plasticity. It would be interesting to examine the state of plasticity in the SCA23 vermis, in order to determine whether synaptic plasticity is indeed disrupted. Electrophysiological experiments could reveal a lot about the changes induced by PDYN-R212W, most importantly whether glutamatergic/ $\text{Ca}^{2+}$  signalling is increased or reduced. This could be a crucial piece of information, en route to developing a therapy. Although we have compelling evidence that CF deficits could contribute to SCA23 pathology, a causal relation has yet to be determined. Cerebellar examination and assessment of an ataxic phenotype of a vGlut2 knockout mouse model could provide insights into the causality of CF pathology.

## Therapeutic strategies in SCA

Currently, there are no therapeutic strategies to treat SCA. Here, identification of a common pathology could be instrumental in developing a therapy useful for many, if not all, SCA types. As discussed in the previous paragraph, a promising common mechanism is disturbed glutamate/ $\text{Ca}^{2+}$  signalling. Two studies have already demonstrated a beneficial effect by restoring physiological  $\text{Ca}^{2+}$  transients. First, in SCA28,  $\text{Ca}^{2+}$  peaks are inefficiently buffered, leading to elevated intracellular  $\text{Ca}^{2+}$  levels. Administration of the  $\beta$ -lactam antibiotic ceftriaxone, which promotes synaptic glutamate clearance, prior to or after onset of symptoms, ameliorated the SCA28 phenotype in mice<sup>7</sup>. Second, mGluR1 dysfunction leading to reduced  $\text{Ca}^{2+}$  levels is linked to SCA1 pathology. Pharmacological enhancement of the mGluR1 receptor with the mGluR1 positive allosteric modulator Ro0711401 resulted in robust and sustained improvement of the SCA1 phenotype in mice<sup>10</sup>. Another therapeutic strategy could be increasing glutamate signalling by augmenting NMDA-R functioning via prolonged administration of D-serine (an NMDA-R co-activator) and a D-amino acid oxidase inhibitor to SCA23 mice, as this has been shown to ameliorate the effects of early loss of vGlut2 in the hippocampus in adult mice<sup>32</sup>.

These above mentioned strategies are promising and further examination developing them into effective therapies should be a point of focus within the SCA field. Covering both elevated and reduced  $\text{Ca}^{2+}$  levels, these therapies could be beneficial for a large number of SCAs, and possibly other neurodegenerative disorders with altered  $\text{Ca}^{2+}$  signalling, including Alzheimer's disease, Parkinson's disease, epilepsy, ischemic stroke, and major depressive disorder<sup>33</sup>. However, the effectiveness of any strategy in other SCA types should be determined *in vivo*, and developing transgenic mouse

models for each SCA type would be lengthy and costly. Expressing a specific SCA gene lentivirally in primary cerebellar neurons is a practical and relevant model for electrophysiological studies in order to identify Ca<sup>2+</sup> imbalances. A lentiviral mouse model, as used in SCA3 and SCA14<sup>29,34</sup>, could then be used to test the appropriate therapy *in vivo*.

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