The molecular neuropathology of spinocerebellar ataxia type 23
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Altered secondary structure of Dynorphin A associates with loss of opioid signalling and NMDA-mediated excitotoxicity in SCA23

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Spinocerebellar ataxia type 23 (SCA23) is caused by missense mutations in prodynorphin (PDYN), encoding the precursor protein for the opioid neuropeptides α-neoendorphin, Dynorphin (Dyn) A, and Dyn B, leading to neurotoxic elevated mutant Dyn A levels. Dyn A acts on opioid receptors to reduce pain in the spinal cord, but its cerebellar function remains largely unknown. Increased concentration of or prolonged exposure to Dyn A is neurotoxic and these deleterious effects are very likely caused by an NMDA-mediated non-opioid mechanism as Dyn A peptides were shown to bind NMDA receptors and potentiate their glutamate-evoked currents. In the present study, we investigated the cellular mechanisms underlying SCA23-mutant Dyn A neurotoxicity. We show that SCA23 mutations in the Dyn A-coding region disrupted peptide secondary structure leading to a loss of the N-terminal α-helix associated with decreased kappa-opioid receptor affinity. Additionally, the altered secondary structure led to increased peptide stability of R6W and R9C Dyn A, as these peptides showed marked degradation resistance, which coincided with increased peptide aggregation. Notably, L5S Dyn A displayed increased degradation and no aggregation. R6W and wt Dyn A peptides were most toxic to primary cerebellar neurons, likely due to a switch from opioid to NMDA-R signalling. We propose that the pathology of SCA23 results from converging mechanisms of loss of opioid signaling neuroprotection and NMDA-mediated excitotoxicity.

Keywords: Dynorphin A, spinocerebellar ataxia type 23, peptide structure, receptor affinity, peptide toxicity
Introduction

Missense mutations in prodynorphin (PDYN) cause spinocerebellar ataxia type 23 (SCA23), an autosomal dominant disorder in which patients suffer from relatively slowly progressive motor coordination impairment due to loss of Purkinje cells in the cerebellum\textsuperscript{1,2}. PDYN is the precursor protein for the opioid neuropeptides α-neoendorphin, Dynorphin (Dyn) A, and Dyn B, which are inhibitory neurotransmitters and function in pain processing, stress-induced responses and addiction control\textsuperscript{3-6}. Dyn A acts on opioid receptors and preferentially binds to the κ-opioid receptor (KOR)\textsuperscript{3}. While the role of Dyn A has been extensively studied in pain, stress, and addiction, the cerebellar function of this peptide, by which SCA23-mutant Dyn A can mediate an ataxic phenotype, is largely unknown.

Evidence is accumulating that pathophysiological changes in Dyn A production, concentration or processing can contribute to maladaptive neuroplastic changes and neurodegeneration (reviewed in Hauser et al.\textsuperscript{4}). The deleterious effect of Dyn A seen in secondary neuronal injury is mediated through non-opioid mechanisms as it cannot be blocked by opioid antagonists\textsuperscript{2}. The finding that MK801, a specific blocker of the N-methyl-D-aspartate (NMDA) receptor, is able to prevent the negative consequences of elevated concentrations of or prolonged exposure to Dyn A, indicates that non-opioid mechanisms of Dyn A are mediated via this receptor\textsuperscript{8}. Dyn A peptides were shown to bind NMDA receptors and potentiate their glutamate currents\textsuperscript{9}. The dichotomous effects of Dyn A peptides were proposed by Hauser et al. to include neuroprotection through opioid signaling and neurotoxicity via NMDA receptor activation\textsuperscript{8}. The neuroprotection mediated via Dyn A interaction with KOR relies on reduction of intracellular calcium concentrations upon KOR activation by Dyn A\textsuperscript{10,11}, while the neurodegenerative actions mediated by NMDA receptor activation involve excitotoxic mechanisms and pathological calcium increase in neurons upon stimulation.

Structure-activity analyses of Dyn A, consisting of 17 amino acids, indicate that Tyr\textsuperscript{1}, Leu\textsuperscript{5} and Arg\textsuperscript{6}-Lys\textsuperscript{11} are required for KOR binding efficiency\textsuperscript{12,13}. Interestingly, the SCA23 mutations that are localized to the Dyn A-coding region (L5S, R6W, and R9C) alter these amino acids\textsuperscript{2}. Receptor binding requires a properly structured Dyn A peptide containing a stable N-terminal α-helical structure, from Phe\textsuperscript{4} to Pro\textsuperscript{10}, and a relatively unstable C-terminal β-turn, formed by amino acids Trp\textsuperscript{14} to Gln\textsuperscript{17} \textsuperscript{14}. However, Dyn A is unfolded in an aqueous environment, and only folds when in a membrane environment\textsuperscript{15}. Therefore, we suggest that membrane interaction of Dyn A precedes and facilitates receptor binding.

Previously, we showed that mutant PDYN-L211S and -R212W expressed in a cell model, and PDYN-R212W in mice, lead to enhanced levels of mutant Dyn A peptide\textsuperscript{2,16}. This work points to a crucial role for mutant Dyn A in the underlying mechanism of SCA23. Additionally, the R6W and R9C Dyn A peptides induced toxicity above that of wild type Dyn A in cultured mouse striatal neurons, in contrast to the L5S mutant, which did not exhibit increased neurotoxic activity\textsuperscript{2}. Here, we have studied the impact of the SCA23 mutations on Dyn A to reveal the molecular mechanisms underlying SCA23-mutant Dyn A toxicity.
Material and Methods

Modelling of secondary structure and interaction of Dyn A with POPC membranes

The interaction of Dyn A mutants with palmitoyl-oleyl phosphatidylcholine (POPC) membranes was simulated using the GROMACS software package version 4.5, the GROMOS43A1 force field, and parameters for the phospholipids adapted from Berger et al.\textsuperscript{17,18}. Simulations were carried out for at least 200 ns. The systems were composed by a single peptide molecule, a 104 POPC membrane patch, 3428 water molecules and either 3 or 4 countering chloride ions depending on the formal charge of the mutant. Prior to production runs, the system with the Dyn A-wt peptide was equilibrated for 5µs using the MARTINI coarse-grain force field\textsuperscript{19}. In the coarse-grain simulations, the secondary structure of wt Dyn A was restrained to reproduce the structural propensities reported by Tessmer and Kallic\textsuperscript{20}. The mutations/deletion were also performed at the (more robust) coarse-grain level, after which the system was again briefly equilibrated for 2ns before being converted to a fine-grained representation following the procedure described in Rzepiela et al.\textsuperscript{21}. At the fine-grain level, the systems were energy-minimized then equilibrated for 0.5ns at a 0.5fs time step and pressure coupling time of 0.5ps, and then again for 1ns at 1fs time step with a pressure coupling time of 0.05ps. Secondary structure analysis was done using the DSSP tool (CMBI version of April 4, 2000\textsuperscript{22}). Visualization was done using the VMD package\textsuperscript{23}.

Peptide synthesis

SCA23-mutant and wt Dyn A peptides were synthesized by solid phase strategies using an automated multiple peptide synthesizer (Syroll, MultiSyntech). For the quenched Dyn A peptides, a Cystein was inserted into the Dyn A sequence in between two glycines at the N-terminus and a fluorescein (Fl) was introduced by covalent coupling of fluorescein-5-iodoacetamide (5-IAF, Fluka) to the cysteine. Quenching of Fl fluorescence was performed by a dabcyl group that had been introduced in the peptide by coupling of Fmoc-L-Lys (Dabcyl)-OH (NeoMPS). Peptides were purified by size exclusion chromatography and Reversed phase -HPLC (>95% pure) and showed the expected molecular mass as determined by mass spectrometry (Maldi Tof, Voyager, ABI). Peptides were dissolved in 100% 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP; Sigma), aliquoted, dried under vacuum in a SpeedVac (Eppendorf) and stored at -20°C.

KOR competitive binding assay

The competitive binding assay of Dyn A peptides to KOR was performed as described previously\textsuperscript{24}. Briefly, the Dyn A competitive binding was assessed in the presence of radioactively labeled U50,488 as a competitor in the cerebellar membrane fraction. Different concentrations of Dyn A peptide were tested. Unlabeled U50,488 was used to decrease unspecific binding and generate standards. Bound radioactivity was counted on a Wallac Beta-plate Liquid Scintillation Counter (Piscataway, NJ, USA). Non-linear regression analysis of the competition curves were best fit by using a two sites analysis, of which the high affinity site represents the KOR.
Quenched Dyn A peptide degradation assay
The degradation assay was performed as described previously\textsuperscript{25}. Briefly, HFIP-treated aliquots of Dyn A peptides were resuspended in DMSO followed by sonication for 10 minutes, immediately before addition to 5µg protein from the mouse cerebellum extracts or human liquor in KMH buffer (110 mM KAc, 2 mM MgAc and 20 mM Hepes-KOH, pH 7.2) to a total volume of 50µl. Degradation of the peptide was analyzed at 37°C using a fluorescence plate reader (FLUOstar OPTIMA, BMG Labtec).

Electron microscopy
Dyn A peptide (150ng/ml in water) preparations were adsorbed on 300-mesh formar/copper grids for 2 minutes and excess of fluid was filtered off. Upon staining with 2.5% uranyl acetate for 2 minutes, grids were analyzed with a transmission electron microscope (Tecnai-12 G2, FEI).

Primary cell culturing
Cerebella were dissected from 4-6-day-old C57Bl6 mouse pups, and kept in ice cold sterile PBS, supplemented with 0.3% bovine serum albumin and 0.6% glucose. After dissection, the meninges were removed without rupturing the tissue. Cerebella were then incubated in warm trypsin for 10 minutes and mechanically dissociated in DMEM medium, supplemented with 10% fetal bovine serum, 0.25% penicillin/streptomycin, 0.25% glutamine, and DNase I (Sigma). After at least two dissociation cycles, the neurons were plated in dissociation medium without DNAse I for one hour to remove glia from the cell suspension. Neurons were then plated into 24-wells plates coated with poly-d-lysine, with 200,000 cells per well in Neurobasal culture medium (Gibco, Life Technologies), supplemented with B-27 (Gibco, Life Technologies), 0.25% penicillin/streptomycin, 0.25% glutamine, and 0.5% fetal bovine serum. After 24 hours of culturing, Ara-C was added to the cultures to inhibit growth of any remaining glial cells. Cultures were then allowed to mature for 7-9 days \textit{in vitro}.

Peptide-induced lactate dehydrogenase activity assay
Mature cerebellar cultures were stimulated with 100nM Dyn A (wt, L5S, R6W, or R9C) in sterile saline, and 25µl of medium was collected at each time point, spun down for 10 minutes at 13,000rpm and 4°C, and stored at -80°C for analysis. The time points were t0 (before addition of peptide), t1 (1 hour after addition of peptide), t3, t6, t12, t24, t48, and t72. Negative controls consisted of no stimulation and stimulation with saline, and cell dysfunction and death was induced by stimulation with 10µM EDTA as a positive control. After collection of all samples, the lactate dehydrogenase (LDH) activity was measured with an LDH Activity Kit (Sigma), performed according to the manufacturer’s protocol. Blocking of peptide-induced toxicity was achieved via 30 minutes of pretreatment with 10µM of either naloxone (opioid receptor block) or MK-801 (NMDA receptor block).
Chapter IV

Animals
All animal experiments were performed according to the ethical guidelines of the Animal Welfare Committee of the University of Groningen, the Netherlands. C57Bl/6N mice were purchased from Charles River (Leiden, the Netherlands). Mice were anaesthetized and placed in a stereotaxic frame, after which the skull was exposed. A hole was drilled in the skull at Bregma – 6.64mm on the brain midline, to place a stainless-steel guide cannula and dummy (26GA; PlasticsOne) into the cerebellar ventricle, penetrating the cerebellum 3mm. The cannula was then anchored to the skull with dental cement, and the wound was sutured. Mice were allowed to recover from surgery for 7 days, after which either 10 or 100nM of wt or SCA23-mutant Dyn A was injected. Immediately after injection, the righting reflex was tested, and a tail-clip task was performed. Mice were filmed for 30 minutes after injection, then terminated. The films were analyzed on a number of ataxic phenotypes, including ataxic gait, dragging, shaking, paralysis, and cramping.

Statistical analysis
All data are expressed as mean ± SEM of at least three independent experiments. Statistical significance was tested using unpaired Student’s t-test, unless stated otherwise.

Results
SCA23 mutations disrupt the secondary structure of Dyn A peptides
To gain insight into the mechanism underlying SCA23 mutations in the Dyn A coding region, we first examined the impact of these mutations - including L5S, R6W, and R9C - on the secondary structure of Dyn A peptides by computational modelling of the peptides in the presence of membrane. The simulation was carried out for at least 200ns, enabling us to follow the stability of peptide secondary structures in time. Upon interaction with POPC membranes, the wt Dyn A peptide kept a stable N-terminal α-helix configuration from residues Phe4 to Arg9 (Fig. 1, Suppl. Fig. 1A). This is in agreement with different previous structural characterizations of the peptide\textsuperscript{20,26,27}, and validates our modelling approach. The wt Dyn A secondary structure was stable across the entire simulation time. The SCA23 mutations in Dyn A peptides resulted in a loss of the N-terminal α-helical structure (Fig. 1, Suppl. Fig. 1B-D), even during the equilibration procedure, suggesting that amino acids Lys\textsuperscript{5}, Arg\textsuperscript{6}, and Arg\textsuperscript{9} are essential for the stabilization of the α-helix. While the mutants had a mostly poorly-defined secondary structure, R6W Dyn A did display a stable turn configuration (Fig. 1). This structure however, was not brought about by a helix but by a β-bridge between amino acids Arg\textsuperscript{9} and Lys\textsuperscript{13} (Suppl. Fig. 1D). The in-depth membrane profile of each peptide upon interaction with POPC was also monitored. R6W Dyn A underwent the largest change in in-depth positioning relative to wt Dyn A, with a deeper positioning of Gly\textsuperscript{2}-Arg\textsuperscript{6} and a shallower positioning of Arg\textsuperscript{9}-Gln\textsuperscript{17} in the membrane (Fig. 2A). In-depth positioning of both L5S and R9C Dyn A relative to wt Dyn A showed only minor differences (Fig. 2A). Additionally, flexibility of the peptides was determined.
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While wt and L5S Dyn A displayed a similar low flexibility, R6W and R9C Dyn A showed increased flexibility between Gly3 and Leu12 and Tyr1 and Pro10, respectively (Fig. 2B).

Altogether, these data show that SCA23 mutations disrupt the native conformation of Dyn A peptides, R6W Dyn A positions higher in the membrane, and R6W and R9C Dyn A display increased flexibility. These factors can be expected to affect in-membrane binding to the κ-opioid receptor, either through a lower affinity of the receptor due to a destructured N-terminal signal sequence and increased peptide flexibility, or by in-depth misalignment of the two.

SCA23-mutant Dyn A peptides exhibit reduced KOR affinities

Since the SCA23 mutations affect amino-acids Lys5, Arg6, and Arg9, which are important for KOR binding12,13, we hypothesized that SCA23-mutant Dyn A peptides may exhibit decreased receptor affinity. To test this hypothesis, we analyzed the KOR binding of the various mutant Dyn A peptides in vitro in a competitive binding assay using selective KOR antagonist (U50,488) as a competitor. The competition analysis showed that SCA23-mutant Dyn A peptides have significantly lower KOR affinity than wt Dyn A (IC50 values: L5S: 2.59nM, R6W: 4.91nM, and R9C: 3.12nM versus wt: 0.63nM, respectively; Fig. 3). The loss of KOR affinity is likely caused by the increased flexibility of the mutant peptides, making interaction with the KOR binding site more difficult. These data indicate that partial loss of KOR signalling might contribute to the SCA23 pathology.

As the SCA23-mutant Dyn A peptides displayed lower KOR affinity, which associated with disruption of the α-helical secondary structure, we hypothesized that the α-helix in Dyn A may be important for KOR interaction. Therefore we performed secondary structure modelling of the Y1del Dyn A mutant, lacking Tyr1, that is known to have no affinity to opioid receptors, including KOR, and exhibits only non-opioid functions28. Our results showed that Y1del Dyn A does not display the α-helical structure at the N-terminus either (Suppl. Fig. 2A and B), as was observed for the various SCA23-mutant Dyn A peptides (Fig. 1, Suppl. Fig. 1). Additionally, a shift in Y1del Dyn A positioning in the membrane was seen between Ile6 and Trp14 (Suppl. Fig. 2C), which was similar to the shift seen in R6W Dyn A positioning (Fig. 2). However, while SCA23-mutant Dyn A peptides and Y1del Dyn A

Figure 1. SCA23 mutant peptides lose helix structure

Histogram of the percentage of α- + 3-helix and helix + turn within each peptide. While wt Dyn A displayed an α-helical structure in approximately 10% of the peptide, and helix + turn in approximately 45% of the peptide, the SCA23 mutant Dyn A peptides did not show any α-helical structure, and only small amounts of helix + turn. R6W Dyn A demonstrated the highest helix + turn content, most likely due to β-bridge stabilization of these structures.
displayed disruption of the N-terminal α-helix, the SCA23-mutant Dyn A still bound KOR much more efficiently than Y1del Dyn A, indicating that the α-helical structure in Dyn A is important for KOR interaction, but is not the sole determinant for this interaction.

**SCA23 mutations affect the degradation efficiency and aggregation of Dyn A peptides**

Since SCA23 mutations disrupt the native conformation of Dyn A, we hypothesized that alterations in the secondary structure of SCA23-mutant Dyn A affect proper peptide degradation. To test this, we determined the breakdown of the mutant Dyn A peptides in mouse cerebellar extracts and human liquor in real time. For this purpose, we generated Dyn A peptides with a small fluorescent group at the N-terminus and a quenching dabcyl group introduced to medial/C-terminal part of Dyn A peptides. The quenched Dyn A peptides become fluorescent upon separation of the fluorophore and quencher due to degradation of the peptide. In mouse cerebellar extracts, rapidly increasing fluorescence levels were observed for wt Dyn A and L5S Dyn A, but not for R6W Dyn A and R9C Dyn A (Fig. 4A), reflecting markedly reduced degradation of these mutant peptides. Notably, LSS Dyn A was more rapidly degraded than wt Dyn A. We also determined peptide stability in human liquor, which contains many metabolic enzymes, including hydrolases and peptidases. Under these conditions, the degradation of wt Dyn A and LSS Dyn A was less rapid than in cerebellar extracts.

**Figure 2. Altered SCA23-mutant Dyn A peptide membrane positioning and flexibility**

Modelling of the membrane positioning and flexibility of the various Dyn A peptides. (A) Membrane positioning is displayed as the average distance of Ca carbons of each residue of wild type and SCA23-mutant Dyn A peptides to the center of the POPC bilayer. The simulation was carried out for 80 ns. The gray line indicates the average depth of the phosphate groups. The insertion depth of LSS and R9C Dyn A peptides seems to be little affected during the studied time scale. R6W Dyn A is positioned higher in the membrane, specifically between Arg7-Gln17. (B) Peptide flexibility is displayed as the movement of the various Dyn A peptides compared to wt Dyn A during the time scale studied. Wt and LSS Dyn A display little flexibility, while R6W and R9C Dyn A both show increased peptide flexibility, with R9C Dyn A demonstrating most flexibility.
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**Figure 3. SCA23-mutant peptides display reduced affinity to KOR**
Quantification graph of competitive binding assay between the various Dyn A peptides and the highly selective KOR antagonist U50,488. All SCA23-mutant Dyn A peptides exhibited lower potential to displace the antagonist from the high affinity binding site as compared to wt Dyn A. Data is shown as a mean of four independent experiments (± SEM). **** p < 0.0001

extract, but similar profiles were generated for all peptides, validating our previous findings (Fig. 4B). These data clearly showed that the R6W and R9C mutations impair Dyn A degradation, whereas the L5S mutation seems to increase Dyn A breakdown. Impaired peptide degradation, combined with increased precursor processing, is a likely cause of increased peptide levels. Furthermore, the more efficient degradation of wt Dyn A and L5S Dyn A peptides in cerebellar extract compared to human liquor, suggests the presence of neuronal-tissue-specific peptidases in cerebellum that degrade this peptide with higher efficiency.

As the SCA23 mutations markedly disrupt the wt Dyn A secondary structure, desegregating hydrophobic and cationic residues (Suppl. Fig. 3A and B), it is admissible that the resulting “denatured” structures have lower solubility. Therefore, we investigated the *in vitro* oligomerization of SCA23-mutant Dyn A peptides in time by electron microscopy. After 4 hours of incubation at 37°C, wt Dyn A formed small oligomeric structures in an aqueous solution compared to wt Dyn A before incubation (Fig. 5A-H). An increased number of oligomeric structures was observed for R6W and R9C Dyn A after 4 hours of incubation as compared to wt Dyn A, which was not observed for L5S Dyn A.

Altogether, these data indicate that R6W and R9C Dyn A have an increased half-life due to decreased degradation. These stable R6W and R9C Dyn A peptides display a lower solubility than wt and L5S Dyn A, likely affecting membrane solubility and self-interaction. L5S Dyn A is characterized by rapid degradation and absence of aggregation, which may underlie its lower neurotoxicity compared to R6W and R9C Dyn A, observed in striatal neurons.

**SCA23-mutant Dyn A display increased neurotoxic effects via NMDA receptor**
Recent work has shown that Dyn A non-covalently binds NMDA receptors and can potentiate their excitatory currents. Additionally, high concentrations of or prolonged exposure to Dyn A has been shown to cause neuronal cell death via non-opioid mechanisms. Given the elevated mutant Dyn A levels in SCA23 pathology, we hypothesized that the mutant peptides may induce neurotoxicity through NMDA receptor activation, and subsequent cell death. In order to reveal the neurotoxicity
Figure 4. Altered degradation rates of SCA23-mutant Dyn A peptides
Degradation curves of wild type and SCA23-mutant Dyn A peptides over time as measured by increases in fluorescence intensity upon separation of the quencher and fluorophore. The assay was carried out in mouse cerebellar extract (A) and human liquor (B) for 200 cycles of 5 minutes. L5S Dyn A displays increased degradation in both mouse cerebellar extract and human liquor, while both R6W and R9C Dyn A show reduced degradation in mouse cerebellar extract and human liquor.

Figure 5. Changes in oligomerization of SCA23-mutant Dyn A peptides in time
Electron microscopy images showing increased oligomerization of R6W Dyn A and R9C Dyn A compared with wt Dyn A at 37°C in time (0 and 4 hours). In contrast, no large oligomeric structures were observed for L5S Dyn A. Scale bars upper panel 1μm (L5S and R6W Dyn A) and 5μm (wt and R9C Dyn A), lower panel 500nm.

of the SCA23-mutant peptides, primary mouse cerebellar cultures of 7-9 DIV (days in vitro) were treated with exogenously added peptides (100nM), and lactase dehydrogenase (LDH) activity was measured in time up to 72 hours post treatment. Notably, R9C Dyn A was already significantly more toxic to primary neurons at 100nM after 3 hours of treatment than all other peptides (Fig. 6A and B). However, after 6, and up to 72 hours, all peptides were toxic as shown by significantly increased LDH activity, and wt and R6W Dyn A increased LDH activity most at 72 hours (Fig. 6A and B).

To determine which receptor system mediates the neurotoxicity caused by Dyn A, we pre-
treated primary cerebellar neurons with 10µM of either naloxone or MK801 for 30 minutes in order to block opioid-mediated or NMDA-mediated toxicity, respectively. Wt Dyn A toxicity was not affected differently by either pre-treatment over the full time course, with the exception that naloxone pre-treatment led to higher LDH activity at 24 hours, compared with MK801 pre-treatment (Fig. 7A). MK801 pre-treatment significantly reduced R6W Dyn A toxicity up to 6 hours, and at 72 hours, as compared to naloxone pre-treatment (Fig. 7B), and R9C Dyn A toxicity was reduced by both pre-treatments at different time points (Fig. 7C). In contrast, naloxone pre-treatment decreased toxicity of L5S Dyn A at 12, 48 and 72 hours as compared to MK801 pre-treatment (Fig. 7D).

Overall, in our model, all Dyn A peptides cause cellular dysfunction at 100nM, with R6W and wt Dyn A being most potent, and L5S Dyn A being least efficient. Additionally, the SCA-mutant Dyn A peptides likely interact with both opioid and NMDA receptors, and our data suggest that R6W Dyn A toxicity is mainly mediated via NMDA receptors, while that of L5S Dyn A is mainly opioid mediated. Furthermore, blocking of the opioid receptors seems to inhibit opioid-induced neuroprotection.

Injection of SCA23 mutant Dyn A peptides into the cerebellum did not cause ataxia in mice

Intracerebroventricular administration of Dyn A has been shown to induce characteristic behaviour, including barrel rolling and limb rigidity in mice. In this work, we aimed to establish whether R6W and R9C Dyn A peptides cause a similar behavior, and to determine which receptor pathway mediates these behavioral effects. Therefore, we injected 10 or 100nM wt or mutant Dyn A directly into the cerebellar ventricle via a pre-implanted cannula, and observed the mouse behaviour for 30 minutes after peptide injection. The main phenotypes scored included ataxic gait, inactivity, and the
lasting effect was timed by two blinded independent researchers. For all parameters, no significant
differences were found between the mice injected with the various Dyn A peptides (Suppl Fig. 4A-
C).

Additionally, to determine whether the SCA23-mutant peptides alter pain perception, we
performed a tail-clip task upon peptide injection. In this assay, a small clip is placed on the tail of the
mouse, and the reaction time and intensity of the reaction of the animal was scored. No significant
differences were found in the reaction time, or sensitivity to the tail-clip between the mice injected
with the various Dyn A peptides (Suppl Fig. 4D-E).

Based on these experiments, we conclude that wt, R6W or R9C Dyn A peptides at 100nM do not
induce an ataxic phenotype or changes in pain perception when injected into the fourth ventricle.

Figure 7. The toxic effects of Dyn A are mediated via both opioid and NMDA receptor interaction
Quantification graphs of LDH activity in the presence of 100nM of wt (A), L5S (B), R6W (C), and R9C (D) Dyn A
peptides in time, with 30 minutes of pre-treatment with either naloxone (10 μM) or MK801 (10 μM). (A) Naloxone
pre-treated wt Dyn A only displayed increased toxicity at t24, as compared to MK801 pre-treated wt Dyn A. (B)
R6W Dyn A toxicity was significantly reduced by MK801 pre-treatment at t0, t1, t3, t6, and t72. (C) Naloxone
pre-treatment reduced R9C Dyn A toxicity at t3, t12, t48, while it increased R9C Dyn A toxicity at t6, and t72. (D)
Naloxone pretreatment increased toxicity of L5S Dyn A at t0, t1, and t3, while it decreased L5S Dyn A toxicity at
t12, t48, and t72. The data are presented as mean ± SEM. Significant differences (Student’s t-test) are indicated as
# 0.10 > p > 0.05, * p < 0.05, ** p < 0.01, and *** p < 0.001
Discussion

In the present study, we showed that both loss of opioid signaling and increased NMDA receptor activation underlies neuronal cell death in SCA23. The secondary structure of the SCA23 mutant peptides showed marked changes, and R6W Dyn A displayed similarities with the secondary structure of Ydel1 Dyn A, lacking KOR binding and opioid activity\(^2\)\(^8\). Likewise, the SCA23 mutant peptides showed reduced KOR binding affinity, and increased peptide toxicity, in part, mediated via the NMDA receptor leading to enhanced cellular dysfunction and cell death. Additionally, increased Dyn A peptide stability and subsequent reduced peptide solubility very likely underlies a large part of the elevated mutant Dyn A levels previously observed in our SCA23 disease models\(^2\),\(^1\)\(^6\), in addition to increased precursor processing\(^1\)\(^6\). An overview of the changes can be found in Table 1.

The loss of the N-terminal α-helix in the Dyn A secondary structure caused by the SCA23 mutations and deletion of Tyr\(^1\) demonstrates that these amino acids are crucial for α-helix formation. Since the SCA23 mutant Dyn A peptides exhibit reduced KOR affinity, whereas Y1del Dyn A has no remaining opioid activity, we suggest that the N-terminal α-helix plays a crucial role in the positioning of the peptide for interaction with the opioid receptors, but that it is not the sole determinant of proper receptor binding. Moreover, the altered secondary structures may contribute to the formation of stable oligomeric structures in vitro, as the R6W and R9C mutations in particular desegregate hydrophobic and cationic residues, lowering their solubility. Yet physiological relevance of these findings is hard to assess, as the oligomerization assays were performed in an aqueous solution, i.e. without the presence of membranes. Notably, no oligomeric structures were observed in the cerebella of PDYN-R212W mice that exhibit elevated R6W Dyn A levels (unpublished data). As these mutant peptides may not oligomerize in physiological conditions, these data suggest that R6W and R9C Dyn A peptides may form additional pathological interactions, either accumulating in higher molecular weight species or interacting with unknown partners. All these effects on Dyn A induced by the SCA23 mutations may contribute to increased neurotoxic actions.

Surprisingly, we did not observe the increased toxicity for R6W and R9C Dyn A compared to wt Dyn A that has been seen before\(^2\) in our current cellular model containing several different cell types, namely mainly granule cells, basket cells, Golgi cells, and Purkinje cells. Therefore, we cannot exclude that Dyn A toxicity in SCA23 is specific for specific populations of neurons, and different effects could be seen for the different cell types, and combinations thereof. Dyn A toxicity was, at

| Table 1. Summary of the effects of SCA23 mutations on peptide characteristics |
|-----------------------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| vs wt Dyn A                | α-helix Membrane | Flexibility | KOR         | Degradation | Solubility | Toxicity  |
|                            | positioning    |             | affinity    |             |            | (100 nM)    |
| L55 Dyn A                  | No            | ≈           | ≈           | ↑           | ≈           | ↓           |
| R6W Dyn A                  | No            | ↑           | ↑           | ↓↓          | ↓↓          | ≈           |
| R9C Dyn A                  | No            | ≈           | ↑↑          | ↓↓          | ↓↓          | ↓           |

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least in part, mediated via the NMDA receptor, as blocking the NMDA receptor with MK801 did not completely prevent the cellular dysfunction caused by the various Dyn A peptides. This data validated the work reported previously by others\textsuperscript{7,8}, and suggests that part of the toxicity is non-NMDA-R mediated. In addition to NMDA signalling, the opioid route contributed to the neuronal cell loss induced by SCA23-mutant Dyn A as well. We speculate that the strong oligomeric properties of R9C Dyn A underlie its relatively mild toxic effects, which may also contribute to the inconsistent responses under a specific pre-treatment. In contrast, R9C Dyn A may exert its neurotoxicity via alternative routes such as inducing membrane leakage via membrane penetration leading to cellular dysfunction\textsuperscript{14,33,34}. Furthermore, the observation that R6W Dyn A toxicity was increased during opioid blockade, supports the hypothesis of opioid-signaling-induced neuroprotection\textsuperscript{4}. These data indicate that partial loss of neuroprotection through opioid signaling may contribute to the pathogenesis of SCA23.

Unfortunately, we were unable to reproduce the motor dysfunction effects seen by others using intrathecal or intracerebroventricular administration of low doses of wt Dyn A\textsuperscript{30–32,35,36} by direct intracerebroventricular injection of Dyn A peptides into the fourth ventricle. This could be due to the deviation in administration techniques, however, these techniques result in peptide infusion into the cerebrospinal fluid, from where it can diffuse into the tissue. Therefore, we could not further assess the underlying neurotoxic mechanisms of the SCA23-mutant Dyn A peptides \textit{in vivo}.

In conclusion, the R6W and R9C Dyn A peptides reflect gain-of-function mutations, leading to increased peptide stability and decreased peptide solubility, whereas the L5S mutation seemingly induced a loss of function, indicated by the enhanced peptide degradation. This work further supports our previous data, in which L5S Dyn A did not display increased toxicity in primary neuronal cultures, while R6W and R9C Dyn A showed high toxicity. Additionally, the patient carrying the L5S mutation exhibited an age of onset of 73 years, and only a mild ataxic phenotype\textsuperscript{2}, further strengthening our finding. Overall, decreased affinity to KOR, loss of opioid receptor-mediated neuroprotection, and potentiation of the NMDA pathway most likely contribute to the pathological actions of SCA23-mutant Dyn A peptides. Interfering with both opioid and NMDA signalling routes should be considered as potential therapeutic strategies for SCA23.

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References

Supplementary Figure 1. Secondary structures of SCA23-mutant Dyn A peptides
Computational modeling of the various Dyn A peptides interacting with a POPC membrane. (A) Wt Dyn A exhibits an α-helical conformation between amino acids 4 and 9, which was relatively stable over time. (B-D) Conversely, the mutations L5S, R6W, and R9C caused loss of the α-helix and the resulting mutant structures were overall extended, although β-bridge stabilization was occasionally observed in R6W Dyn A (C). The graphs show adopted secondary structures per amino acid residue (Y axis) over time (X axis). Computational modeling of the various Dyn A peptides interacting with a POPC membrane was conducted for at least 200ns after the different mutations were performed.
Supplementary Figure 2. Modelling of Y1del Dyn A upon interaction with POPC membranes
Modelling of the secondary structure, membrane positioning and flexibility of Y1del Dyn A. (A) Deletion of the first tyrosine in Dyn A peptide also leads to loss of the α-helical structure. For complete legend see Fig. 1 and Suppl. Fig. 1. (B) Computational modeling of the various Dyn A peptides interacting with a POPC membrane was conducted for at least 200ns after the mutation was performed. Y1del Dyn A demonstrates loss of structures similar to that seen in SCA23 mutant peptides. The graph shows adopted secondary structures per amino acid residue (Y axis) in time (X axis). (C) Y1del Dyn A is positioned higher in the membrane between Ile8 and Trp14, similar to R6W Dyn A (Fig. 2).
Supplementary Figure 3. Cationic and hydrophobic residues are separated in wt Dyn A by the α-helix
Front (A) and side view (B) of residues 3-9 of wt Dyn A with explicit side chains around a cartoon representation of the backbone secondary structure. The helical structure separates the cationic Arg6,7,9 residues (blue) from the hydrophobic Phe4, Leu5, and Ile8 residues (orange). Images were taken aligned with the plane of the membrane.

Supplementary Figure 4. Injection of wt, R6W or R9C Dyn A does not induce an ataxic phenotype or alterations in pain perception
Intracerebroventricular administration of 10nM or 100nM of the various Dyn A peptides into the fourth ventricle of awake mice. No differences were detected in induction of ataxic gait (A), increased inactivity (B), the lasting effect of the peptides (C), the time to respond to the tail-clip task (D), or the sensitivity to the tail-clip task (E).