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ATXN1 repeat expansions confer risk for amyotrophic lateral sclerosis and contribute to TDP-43 mislocalization

Running Title:

ATXN1 repeat expansions in ALS

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Abstract:

Increasingly, repeat expansions are being identified as part of the complex genetic architecture of amyotrophic lateral sclerosis. To date, several repeat expansions have been genetically associated with the disease: intronic repeat expansions in *C9orf72*, polyglutamine expansions in *ATXN2* and polyalanine expansions in *NIPA1*. Together with previously published data, the identification of an amyotrophic lateral sclerosis patient with a family history of spinocerebellar ataxia type 1, caused by polyglutamine expansions in *ATXN1*, suggested a similar disease association for the repeat expansion in *ATXN1*. We, therefore, performed a large-scale international study in 11,700 individuals, in which we showed a significant association between intermediate *ATXN1* repeat expansions and amyotrophic lateral sclerosis ($P = 3.33 \times 10^{-7}$). Subsequent functional experiments have shown that ATXN1 reduces the nucleocytoplasmic ratio of TDP-43 and enhances amyotrophic lateral sclerosis phenotypes in Drosophila, further emphasizing the role of polyglutamine repeat expansions in the pathophysiology of amyotrophic lateral sclerosis.

Keywords: amyotrophic lateral sclerosis; trinucleotide repeat expansions; DNA repeat expansion; genetic association study.

Abbreviations: ALS = amyotrophic lateral sclerosis; FTD = frontotemporal dementia; GL(M)M = generalized linear (mixed) model; polyQ = polyglutamine; SCA1 = spinocerebellar ataxia type 1; SCA2 = spinocerebellar ataxia type 2; WGS = whole-genome sequencing

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Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by the loss of motor neurones leading to progressive weakness and spasticity (Brown and Al-Chalabi, 2017; van Es *et al.*, 2017). Genetically, ALS is a highly heterogeneous disease with many underpinning factors (Al-Chalabi *et al.*, 2017). In 5-15% of patients, there is a positive family history and it is assumed that there is a single causal mutation (Andersen and Al-Chalabi, 2011; Byrne *et al.*, 2011). However, familial ALS mutations have also been identified in patients without a clear family history and multiple studies show that the genetic contribution to the risk of developing sporadic ALS is considerable (±40-60%) (Al-Chalabi *et al.*, 2010; Wingo *et al.*, 2011; Ryan *et al.*, 2019). To date, over 40 different genes have been linked to ALS, mostly containing (rare) point mutations that significantly increase the risk of disease (Al-Chalabi *et al.*, 2017). However, over the last few years, repeat expansions in several genes have also been implicated in ALS, including *C9orf72*, *NIPA1* and *ATXN2* (Blauw et al., 2010; Elden et al., 2010; DeJesus-Hernandez et al., 2011; Ajroud-Driss et al., 2015). *ATXN2,* for instance, contains trinucleotide repeat motif of CAG repeats, coding for a stretch polyglutamine (polyQ) and was first implicated as a risk factor in ALS after the discovery of it being a potent modifier of TDP-43 toxicity, an important step in ALS pathogenesis (Elden *et al.*, 2010). A large expansion (>34) of the number of CAG repeats in *ATXN2* (normally 22 or 23) is known to cause spinocerebellar ataxia type 2 (SCA2), whereas intermediate-length (29 - 33) repeats are associated with ALS (Elden *et al.*, 2010; Rub *et al.*, 2013).

In our outpatient clinic, we came across an ALS patient who had a positive family history for spinocerebellar ataxia type 1 (SCA1) (Fig. 1), a neurodegenerative disease caused by a polyQ repeat expansion in the *ATXN1* gene (Banfi *et al.*, 1994; Rub *et al.*, 2012). There are some interesting similarities between *ATXN2* and *ATXN1*, most importantly the presence

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of the coding CAG repeat motif. SCA1 patients may also have upper motor neurone signs and autopsy studies show prominent loss of Betz cells, suggesting phenotypic overlap with ALS (Seidel *et al.*, 2012; Rub *et al.*, 2013; Saberi *et al.*, 2015; Genc *et al.*, 2017). Interestingly, a similar Italian ALS-SCA1 pedigree was reported a few years ago (Spataro and La Bella, 2014). This phenotypic overlap, as well as the co-occurrence of ALS and SCA1 in two unlinked pedigrees, makes *ATXN1* a plausible candidate gene for ALS.

Three previous studies have already explored this possible association between *ATXN1* expansions and ALS (Lee *et al.*, 2011; Conforti *et al.*, 2012; Lattante *et al.*, 2017). However, these studies have produced conflicting results which are difficult to compare, due to the use of different repeat size cut-offs for expanded alleles; their conclusions mostly rely on nominal significance. Therefore, we set out to perform a large-scale genetic association study using data from 11,700 individuals and explore the possible role of *ATXN1* in ALS.

Materials and methods

Subjects

All participants gave written informed consent and approval was obtained from the local, relevant ethical committees for medical research. Genotyping experiments were performed on a total of 5,088 DNA samples from four populations. All patients were diagnosed according to the revised El Escorial criteria. Control subjects were from ongoing population-based studies on risk factors in ALS(Huisman *et al.*, 2011). All related individuals were excluded from further analysis.

PCR, sequencing and genotyping

Samples were analyzed using PCR according to protocols described previously and results were analyzed in a blinded and automated fashion. To confirm PCR fragment length, 850

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samples were additionally analyzed with Sanger sequencing. Primers: 5'-CAGTCTGAGCCAGACGCCGGGACACAAG-3' (forward) and 5'-CGGTGTTCTGCGGAGAACTGGAAATGTGG-3' (reverse).

To further increase sample size, we analyzed *ATXN1* repeat size in whole-genome sequencing (WGS) data, available to us through Project MinE using ExpansionHunter (Dolzhenko *et al.*, 2017; Van Rheenen *et al.*, 2017). There was a 1,129 sample overlap in genotypes obtained from ExpansionHunter and PCR/Sanger sequencing, showing a 97.7% concordance in allele genotypes (2,207/2,258). In 30 of the 51 discordant alleles, there was only a single repeat unit difference between PCR and WGS, and of the remaining 21, at least 16 could simply be explained by mix-up of eight samples. Considering this high percentage of concordance between ExpansionHunter and Sanger/PCR results, we did not perform additional validation experiments on the WGS samples and proceeded with the ExpansionHunter calls. *C9orf72* status had been determined previously for 4,530 ALS samples.

To identify the number and position of CAT interruptions in the CAG trinucleotide repeat of *ATXN1*, we analyzed the Dutch WGS data of 353 control and 547 ALS cases sequenced using the HiseqX Sequencing System (resulting in 150bp reads, able to span the entire repeat). All 150bp reads mapped to the genomic region of *ATXN1* (chr6:16,327,000 - 16,329,000; hg19) were isolated and spanning reads were genotyped after the recognition of both the start and end-motif within a single read. Two samples (one ALS, one control) did not contain any spanning reads and were, therefore, excluded. We only included repeat genotypes with two or more supporting reads and found repeat size predictions in 95% of alleles (1345/1418) comparable to genotypes determined with PCR and/or ExpansionHunter.

Cell culture and immunohistochemistry

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mCherry-Ataxin-1 constructs were synthesized by Genscript (Piscataway, USA). HeLa cells expressing mNeongreen fusion to the endogeneous KPNA2 were created by CRISPR-mediated non-homologous endjoining of an mNeongreen-P2A-puromycin PCR product at the last codon of the KPNA2 CDS in its genomic locus. HeLa cells (ATCC) were cultured in high glucose DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Greiner), 4 mM Glutamax (Invitrogen), penicillin (100 U/mL), streptomycin (100 µg/mL) and non-essential amino acids (1%). Cells were grown at 37°C in a humidified atmosphere with 5% CO_2. Cells were transiently transfected using Lipofectamine F3000 (Invitrogen) according to manufacturer's instructions. Cells were fixed 24h after transfection in 4% formaldehyde in PBS and stained according to standard protocols (including methanol fixation and permeabilization by PBS-T 0.04%). Rabbit anti-TDP-43 (12892-1-AP, Proteintech) was used to stain for TDP-43. AlexaFluor 488 secondary antibodies (Life Technologies) were used. Nuclei were visualized using NucBlue counterstaining (Thermo Scientific). Slides were mounted using ProLong Gold antifade reagent (Life Technologies).
Confocal images were obtained using a Zeiss LSM 510 Meta NLO confocal microscope. Images were analyzed, formatted and quantified with FIJI software.

In brief, transfected cells from three independent transfections were analyzed for their nuclear cytoplasmic ratio of TDP-43 or KPNA2, and scored for the presence of cytoplasmic inclusion bodies (only observed in ATXN1, but not mCherry transfected cells). All data were aggregated and statistical analyses were carried out using Prism software.

Fly strains

Drosophila were maintained on a 12:12 light/dark cycle on a standard sugar-yeast medium (15 g/l agar, 50 g/l sugar, 100 g/l autolyzed yeast, 30 ml/l nipag and 3 ml/l propionic acid) at 25°C. The following transgenic *Drosophila* strains were used in this study: *GMR-TARDBP*

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(#51370)*, UAS-GR36* (#58692). All fly strains used, were obtained from the Bloomington *Drosophila* Stock Center at Indiana University (BDSC) or the Vienna *Drosophila* RNAi Center (VDRC). The *UAS-GR36* strain was crossed with balancer CyO and driver GMR, to obtain a balanced fly stock expressing the DPR construct in the eye.

Drosophila eye phenotype analysis

To assess the effect of ataxin-1 (ATXN1) repeat length, we crossed the *GMR-TARDBP* and the *GMR-GR36* stocks with fly lines carrying UAS constructs expressing various sizes of the ATXN1 polyQ repeat.

Following strains were used: *UAS-ATX1.2Q* (#39738), *UAS-ATX1.30Q* (#39739), *UAS-ATX1.82Q* (#37940) and *UAS-eGFP (#5428)*. For each cross, the collected offspring were divided by sex and the genotypes counted according to the balancers. We used a slightly modified eye phenotype analysis protocol as described in Boeynaems et al., 2016. Briefly, each fly was individually scored in a blinded fashion for the presence of necrotic spots using the following scoring scale (not affected = 0, mild = 1, medium =2, heavy = 3, extreme = 4). We crossed each line at least three times independently to validate the specific phenotype. Eye phenotypes were imaged by light microscopy (Zeiss imager.M1) and the made Z-stacks were processed with ImageJ with the extended depth of field algorithm.

Statistical analysis

All statistical procedures were carried out in R 3.3.0 (http://www.r-project.org). Mantel-Haenszel method meta-analysis of odds ratios was performed on subgroup and pooled data using ‘metafor’ 2.0 package. For the joint analysis on individual data, a generalized linear model (GLM) was used with fixed-effects covariates: method of genotyping and country of

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origin. We additionally applied generalized linear mixed model (GLMM) to account for possible random effects which gave similar results as the GLM.

The effect on disease survival after onset and age at onset of the disease and were tested using multivariate Cox regression with sex at birth, site of onset, age at onset (for survival only) and *C9orf72* status as covariates. To calculate the expected frequency of co-occurring variants, we used the frequency of one variant in the unaffected population and multiplied this with the number of carriers of the other variant in the affected population. A binomial test was performed to compare the observed frequency of co-occurring variants in ALS patients with the calculated expected frequency.

The orthogonal data of the *Drosphila* eye images were analyzed with the lbl_test of the coin package in R. This linear by linear association test takes into account the gradual scoring scale, where a score of 4 impacts the p-value more than a score of 1.

Data and materials availability

Genetic data generated from these cohorts has been placed for public access on the Project MinE Data Browser (http://databrowser.projectmine.com). DNA tissue samples can be obtained by contacting the corresponding author (M.A.v.E) or Project MinE ALS Sequencing Consortium. Fly strains can be obtained by contacting P.V.D.

Results

Genetic association of increased *ATXN1* repeat size (≥ 33) with ALS

In our analysis, we included data from three different sources. First, we genotyped the repeat expansion in *ATXN1* using PCR in 2,672 ALS patients and 2,416 geographically matched control samples from four different cohorts (Belgium, France, Ireland and The Netherlands)(Supplementary Table 1). In this sample series, we found the most frequent

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alleles contained 29 or 30 trinucleotide repeats in both cases and controls (69.8% and 71.3%, respectively). In SCA1, *ATXN1* repeat sizes ≥39 CAG/CAT are considered "expanded" (Rub *et al.*, 2013). We hypothesized that, similar to previous findings in *ATXN2*, "intermediate" repeat sizes (between normal and expanded) could be associated with an increased risk of motor neurone disease. We determined the cut-off for these intermediate size expansions to be 33 or more CAG/CAT repeats using receiver operating characteristics and allele distribution analysis (with 94.7% of control alleles being within the "normal" range) (Fig. 2). In this sample series, 12.2% of ALS patients (328/2,672) and 10.1% of controls (244/2,416) carried at least one *ATXN1*-allele with an expanded repeat size (i.e. above the ≥33 CAG/CAT cut-off). The fixed-effect meta-analysis of these four cohorts indicated an association between the presence of at least one expanded allele in *ATXN1* and ALS status with an odds ratio (OR) = 1.37 (95% CI = 1.18-1.60, $P = 1.21 \times 10^{-5}$)(Fig. 3).

Secondly, we investigated the association with *ATXN1* repeat expansions in an independent cohort of 2,048 ALS cases and 891 controls using whole-genome sequencing (WGS) (Van Rheenen *et al.*, 2017). *ATXN1* repeat sizes were estimated from WGS data using ExpansionHunter (Dolzhenko *et al.*, 2017). We confirmed a subset (n = 1,129) of the ExpansionHunter genotypes using PCR and found 98% concordance between the two methods. Using the same cut-off for (intermediate) expanded alleles as in the PCR cohort (≥33 CAG/CAT), we found the direction of effect and allele frequency to be similar in all cohorts; expanded alleles were observed in 12.0% of cases (248/2,048) compared to 8.8% in controls (78/891), resulting in an OR = 1.38 (95% CI = 1.02-1.88, P = 0.037) (Fig. 3).

Lastly, we performed a fixed-effects meta-analysis on all available data, in which we also included the data from all three studies that previously reported on *ATXN1* in ALS (totalling 2,346 cases and 1,327 controls). Using a Mantel-Haenszel meta-analysis, we found improved evidence of an association with *ATXN1* expansions and ALS status with P =

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$3.55x10^{-6}$; and OR = 1.38, 95% CI = 1.20-1.57 (Fig. 3). We additionally applied a generalized linear model with correction for country of origin and method of genotyping on the pooled data of 7,066 ALS patients and 4,634 controls and found our results to be essentially unchanged (OR = 1.41, 95% CI = 1.24-1.61, $P = 3.33x10^{-7}$).

No differences in CAT interruptions

In SCA1, the presence or absence of CAT interruptions in the CAG repeat can influence disease risk and/or phenotype (Menon *et al.*, 2013). We explored the possibility that differences between cases and controls could be attributed to differences in CAT interruptions by analyzing the WGS sequencing data in a subset of 352 control and 546 ALS cases. Almost all repeats contained one or more CAT interruptions, with only one affected and one non-affected individual carrying an uninterrupted repeat (13 and 30 CAG repeats resp.). The majority of the *ATXN1* repeats in both cases and controls contained two CAT interruptions (Fig. 4A), with 99.9% (1,267/1,268) having a $(CAG)_{n1}(CAT)(CAG)(CAT)(CAG)_{n2}$ interruption pattern. Because of this minimal variation in the interruption number and position, we found a similar correlation and distribution of uninterrupted CAG repeat size compared to that of the full length repeat (Fig. 4B).

No effect on age at onset or survival

Several ALS associated risk factors also affect the clinical phenotype. We investigated the effect of *ATXN1* repeat expansions on survival and age at onset in a subset of 1,890 ALS patients for whom clinical data were available, but found no significant effects (Fig. 5A-B).

Ataxin-1 overexpression perturbs nucleocytoplasmic transport of TDP-43.

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The pathological hallmark of ALS is the aggregation and cytoplasmic mislocalization of the RNA binding protein, TDP-43(Neumann *et al.*, 2006). It is thought that the mislocalization of TDP-43 leads to both a nuclear loss-of-function as well as a cytoplasmic toxic gain-of-function. However, the exact mechanisms underpinning TDP-43 mediated neurodegeneration have not yet been fully elucidated. Considering the large number of genes that have been implicated in ALS to date, it seems likely that TDP-43 pathology may arise through multiple pathways. Recent evidence shows that ataxin-2 drives localization of TDP-43 to cytoplasmic stress granules; this process, because of the subsequent incapacity to disassemble these stress granules, has been proposed as the first stepping stone towards the formation of pathological aggregates (Ramaswami *et al.*, 2013; Becker *et al.*, 2017). Given that ataxin-1 has similarities with ataxin-2, we initially considered that similar mechanisms would be involved.

Ataxin-2 is, however, a cytoplasmic stress granule protein known to interact with TDP-43 (Elden *et al.*, 2010), whereas this is not the case for ataxin-1. We, therefore, explored other disease mechanisms for ataxin-1 in ALS and started by performing simple overexpression studies of wildtype/normal-length ataxin-1 in HeLa cells. Overexpression of ataxin-1 did not alter endogenous TDP-43 expression (Fig. 6A-B) and resulted in the formation of nuclear and cytoplasmic ataxin-1 inclusion bodies, negative for TDP-43 (Fig. 6C, top panel). We also observed that some cells overexpressing ataxin-1 showed cytoplasmic mislocalization of TDP-43 (Figure 6C, bottom panel). Interestingly, this TDP-43 mislocalization significantly correlated with the presence of the cytoplasmic ataxin-1 inclusion bodies (Fig. 6D).

A possible mechanism for mislocalization of TDP-43, recently implicated in ALS pathogenesis, is that of misregulation of nucleocytoplasmic transport, making TDP-43 unable to (re)enter the nucleus and as a result become trapped in the cytoplasm (Woerner *et al.*, 2016). We hypothesized that ataxin-1 cytoplasmic accumulation could perturb the nuclear

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import system and subsequently investigated importin-α2 (KPNA2; karyopherin subunit alpha 2), which is involved in importing TDP-43 into the nucleus (Nishimura *et al.*, 2010). Similar to TDP-43, we indeed found significant mislocalization of endogenous KPNA2 in HeLa cells containing ataxin-1 inclusion bodies (Fig. 6E-F).

Co-expression of human TDP-43 with Ataxin-1 aggravates the phenotype in *Drosophila*

Considering the modest effect of intermediate *ATXN1* expansions in our genetic analysis, we do not presume that they have a directly pathogenic effect, but rather that they are a contributing factor in the multi-step process towards developing the disease. Based on this hypothesis, we postulate that expanded *ATXN1* CAG repeats would aggravate the phenotype in an in vivo model of TDP-43 pathology. We, therefore, turned to *Drosophila*, a suitable model organism for genetic experiments, the fly eye being widely used to evaluate neurodegeneration (Fig. 7A) (Freibaum *et al.*, 2015; Zhang *et al.*, 2015; Boeynaems *et al.*, 2016). Expression of the human TDP-43 gene in the *Drosophila* eye using *GMR-GAL4* results in a "rough eye" phenotype (Choksi *et al.*, 2014). This rough eye phenotype is mainly characterized by a progressive, age-dependent degeneration of the structure which ultimately results in depigmentation by retinal degeneration. To increase the chance of an observable effect, we tested with *ATXN1* containing either an exaggerated normal (2Q) and expanded (82Q) polyQ repeat length. Co-expression of human TDP-43 with ataxin-1 polyQ constructs with a repeat length of 82 aggravated the phenotype, with the formation of necrotic spots (Fig. 7B), whereas expressing ataxin-1 2Q or 82Q alone did not result in an eye phenotype. Scoring the severity of the eye abnormalities via a graduated scoring table showed a significant increase in the score in TDP-43-expressing flies that jointly expressed the 82Q

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repeat, indicating a synergistic effect of ataxin-1 on TDP-43 toxicity in *Drosophila* ($P = 2.65x10^{-4}$); (Fig. 7C).

ATXN1 polyQ also aggravates the phenotype in a *Drosophila* model for *C9orf72*

The co-occurrence of variants in multiple ALS genes within a single case is observed frequently (van Blitterswijk *et al.*, 2012; Bury *et al.*, 2016). In particular, this co-occurrence of multiple variants has been reported for patients carrying repeat expansions in *C9orf72* (van Blitterswijk et al., 2014a; van Blitterswijk et al., 2014b; Dekker et al., 2016), which to some degree might also explain the phenotypic heterogeneity associated with this gene (including ALS, frontotemporal dementia (FTD), parkinsonism and psychosis)(Cooper-Knock *et al.*, 2015). A previous study on *ATXN1* in ALS reported the co-occurrence of *ATXN1* and *C9orf72* expansions (Lattante *et al.*, 2017). In our cohort, we identified a total of 23 patients carrying both expansions (6.4% of all *C9orf72*-positive patients also had an *ATXN1* expansion ≥33 CAG/CAT) and also came across a familial ALS pedigree in which two ALS-affected first degree relatives carried both repeat expansions (Supplementary Fig. 2). We, therefore, explored whether co-expression of *ATXN1* polyQ constructs in a *Drosophila* model for *C9orf72* (expressing toxic glycine-arginine (GR36) dipeptide repeats) would aggravate the rough eye phenotype (Mizielinska *et al.*, 2014). Indeed, these flies show a strong eye phenotype, characterized by eye depigmentation and necrotic spots (Fig. 7B). When ATXN1 82Q, but not 2Q, was co-expressed in the eye, we observed a significant enrichment of the severely affected eyes ($P < 2.0 \times 10^{-16}$) (Fig. 7C). Almost 50% of the scored flies showed a harsh degenerated eye with numerous necrotic spots, indicating that ataxin-1 polyglutamine expansions also aggravate the GR-mediated neurodegeneration. These findings suggest an interaction of expanded ataxin-1 polyQ with pathological events in the disease.

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Discussion

In this study we demonstrate an association between intermediate polyglutamine expansions in *ATXN1* and risk of ALS. We observed similar allele frequencies and direction of effect across international cohorts and the increase in sample size resulted in stronger statistical evidence compared to previous reports, indicating a robust association.

Using a generalized linear model with correction for country of origin and method of genotyping on the pooled data of 7,066 ALS patients and 4,634 controls, we found a p-value of 3.33×10^{-7}. Empirical significance thresholds have been set for studies analyzing common genetic polymorphisms across the genome, such as genome-wide significance ($P = 5.0 \times 10^{-8}$) for genome-wide studies and exome-wide significance ($P = 5.0 \times 10^{-7}$) for studies that only focus on coding single nucleotide variants. However, no such thresholds have been set for genetic studies looking at repeat expansions on a genome/exome-wide level. We, therefore, considered 3 different cut-off values for significance; (1) Bonferroni correction for the number of previously reported polymorphic polyQ stretches of 6 and longer in the genome ($P = 0.05 / 85 = 5.9 \times 10^{-4}$) (Kozlowski *et al.*, 2010), (2) correcting for the total number of genes in the genome containing a homo-amino acid stretch ($P = 0.05 / 878 = P = 5.6 \times 10^{-5}$) (Kozlowski *et al.*, 2010), or (3) simply applying the level for exome-wide significance, as polyQ repeats are a coding form of genetic variation. A valid argument can be made for all 3 thresholds and as more association studies on structural variation on a genome-wide level become available, it seems likely that empirical significance thresholds will be determined. For now, our findings are significant regardless of which threshold is applied.

It is still unclear as to how *ATXN1* polyQ expansions could have a contributing effect on ALS development. We sought to provide the first steps by performing functional experiments investigating the effect of *ATXN1* polyQ on the cellular processing of the nuclear RNA binding protein TDP-43, the pathological hallmark of ALS. Ataxin-2 plays an

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important role in stress granule formation and in ALS these stress granules fail to disassemble, hereby forming the precursors of TDP-43 aggregates(Elden *et al.*, 2010; Hart *et al.*, 2012). As *ATXN1* is largely homologous to *ATXN2* and both contain an expanded polyQ stretch, this was our initial hypothesis. There is, however, no literature implicating ATXN1 in stress granule formation and in our *in vitro* model, we did not observe co-localization with TDP-43. We did, however, observe a cytoplasmic mislocalization that seemed to be dependent on the disruption of ataxin-1. Since mislocalization was observed in both expanded as well as wild-type (Q27) ATXN1 HeLa cell models, disruption is possibly due to overexpression itself (Supplementary Fig. 3); this is similar to observations in ATXN2, where the effects of wild-type overexpression on TDP-43 was an important first step for further investigation (Elden *et al.*, 2010). Although a HeLa cell overexpression model is far from representative for ALS, the current consensus that both a nuclear loss- and cytoplasmic gain-of-function of TDP-43 play a key role in ALS pathogenesis led us to shift our focus to nucleocytoplasmic transport, another mechanism that has recently been implicated in ALS (Neumann *et al.*, 2006; Ling *et al.*, 2013). *In-vitro* studies have shown that disruption of the classical nuclear import pathway (which includes KPNA2) in neurones leads to the cytoplasmic accumulation of TDP-43, and also in post-mortem studies of ALS and FTD cases, KPNA2 levels were found to be decreased in both brain and spinal cord (Nishimura *et al.*, 2010). Similarly, our *in vitro* results show that overexpression of normal-length ataxin-1 can cause mislocalization of TDP-43 and KPNA2. This suggests that pathological ataxin-1 effects could be mediated via perturbed nucleocytoplasmic transport.

Given the multifactorial aetiology of ALS, the modest genetic effect and a possible pathological effect through TDP-43, we lastly explored whether *ATXN1* polyQ would aggravate the phenotype in an in-vivo model of TDP-43 pathology. For this we used existing *Drosophila* models, that indeed show an aggravated phenotype when expanded ATXN1 is

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co-expressed with human TDP-43. Since there is only a relatively small difference in the size of the polyQ tract between normal and intermediate expansions, we deliberately chose two extreme values (Q2 and Q82) to maximize the possible phenotypic effect of ATXN1 polyQ on TDP-43 pathology. Despite this exaggeration, the absence of a necrotic eye phenotype in ATXN1 Q82 alone suggests a neurotoxic effect via TDP-43 and since co-expression of ATXN1.82Q, but not ATXN1.2Q, dramatically enhanced the degenerative eye phenotype, this suggest that TDP-43 or GR36 overexpression-induced toxicity by ATXN1 occurs in a repeat-length dependent manner.

As the co-occurrence of *C9orf72* and *ATXN1* expansions was observed in multiple ALS patients, we performed a similar *Drosophila* experiment in which we co-expressed ATXN1 polyQ with GR36 (toxic dipeptide repeat associated with *C9orf72*) and again found synergistic toxic effect in these flies. There is high phenotypic variability amongst individuals carrying repeat expansions in *C9orf72*, which includes ALS, FTD, parkinsonism and psychosis (Cooper-Knock *et al.*, 2015). It has been proposed that additional genetic factors influence the *C9orf72*-phenotype. For instance, there is evidence suggesting that SNPs in *TMEM106b* protect against dementia (Nicholson and Rademakers, 2016), whereas other variants in other genes may give rise to ALS (van Blitterswijk *et al.*, 2014a; van Blitterswijk *et al.*, 2014b; Dekker *et al.*, 2016). Our data suggest that expanded *ATXN1* polyQ alleles influence the phenotype associated with *C9orf72*.

In conclusion, we demonstrate a robust genetic association between *ATXN1* repeat expansions with risk of ALS and provide evidence suggesting that this contributes to ALS pathophysiology through perturbed nucleocytoplasmic transport. In line with the multistep and oligogenic hypothesis for ALS, we show *ATXN1* polyQ aggravates the phenotype in multiple transgenic fly models (hTDP-43 and GR36). As the *ATXN1* polyQ expansion is likely to result in a gain of function, silencing the expanded allele and perhaps thereby

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(partially) restoring nucleocytoplasmic transport could prove to be an interesting therapeutic approach.

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Competing interests

The authors declare no competing financial interests.

Supplementary materials

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Supplementary materialis available at Brain Communications online.

Supplementary List 1. Members and their affiliations of the Project MinE ALS Sequencing Consortium.

Supplementary Table 1. Baseline characteristics of study participants.

Supplementary Fig. 1. Uncropped Western blot of TDP-43 levels and loading control GAPDH (Glyceraldehyde 3-phosphate dehydrogenase).

Supplementary Fig. 2. Pedigree with co-occurrence of C9orf72 and ATXN1 expansions

Supplementary Fig. 3. TDP-43 mislocalization in HeLa ATXN1 overexpression models

Appendix I: Project MinE ALS Sequencing Consortium

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Members are listed in alphabetical order, a full list of members with affiliations can be found in Supplementary List 1.

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Figure legends

Figure 1. Pedigree with co-occurrence of SCA1 and ALS. The index patient (arrow) was diagnosed with ALS and reported a positive family history for spinocerebellar ataxia type 1 (SCA1) in four other family members. No DNA samples from family members diagnosed with SCA1 were available for analysis.

Figure 2. Distribution of *ATXN1* CAG/CAT repeat length. Proportion of total alleles grouped per *ATXN1* repeat length determined via PCR-analysis in a cohort of 2,672 individuals affected with ALS (grey) and 2,416 geographically matched controls (black) from four different cohorts (Belgium, France, Ireland and The Netherlands).

Figure 3. ATXN1 polyglutamine repeat expansion meta-analysis. Forest plot for the fixed-effect Mantel-Henzel meta-analysis of the effect of expanded (≥33) ATXN1 CAG/CAT repeats on ALS risk in three different datasets grouped per country of origin: Previous reports, PCR genotyped cohort and whole genome sequencing (WGS) genotyped cohort. Additionally, individual level data of all three datasets were combined in a single logistic regression analysis (Joint analysis), which was corrected for country of origin and method of genotyping. Weights depending on number of participants. CI, confidence interval. *Conforti et al. used a different cut-off for expanded/non-expanded status (≥32 CAG/CAT repeats). However, since the most frequent alleles in their data [28/29] seem to also have shifted one repeat unit compared to the Italian population in Lattante et al. and our data [29/30], we did not alter the expansion status.

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Figure 4. Presence and number of CAT interruptions in *ATXN1* CAG repeat expansion. (**A** and **B**) Plots show the results after genotyping 1418 repeat alleles (849 ALS; 569 Control) from 150bp WGS reads that span the full repeat. (**A**) Number of CAT interruptions per repeat allele (**B**) Correlation between the total repeat size, including both CAG and CAT, and the longest stretch of uninterrupted CAG per allele for both ALS affected (blue) and unaffected (orange). CAT interruptions usually and exclusively appear after the first 12 to 17 CAG repeats, resulting in a significant correlation between the total and uninterrupted CAG repeat size (Kendall's tau cor., $p < 2.2e\text{-}16$ for both ALS and controls,) and therefore a similar distribution (margin panels; prop.tot = proportion of total alleles). There were two exceptions (red border): one ALS affected allele had no interruptions, probably because of its short length (13), and one unaffected sample seemed to carry an uninterrupted stretch of 30 CAG.

Figure 5. Effect of ATXN1 repeat expansion on survival and age at onset in ALS. (**A** and **B**) Plots of time-dependent probabilities in 1,890 ALS patients with either ATXN1 normal (<33, orange) or expanded (≥ 33, blue) CAG/CAT repeat expansion. (**A**) Survival after onset of disease in months, corrected for: sex, age at onset, bulbar site of onset and presence of C9ORF72 expansion. (**B**) Age at onset of the disease in years corrected for: sex, site of onset and the presence of a C9orf72 repeat expansion. No significant effects were found.

Figure 6. HeLA cells were transfected with mCherry-tagged ataxin-1 containing 27 polyglutamine repeats (mCherry-Atx227Q) or control vector (mCherry). (**A**) TDP-43 protein levels are not altered in ataxin-1 expressing cells (Uncropped blot image in Supplementary Fig. 1). (**B**) Quantification of TDP-43 levels normalized to loading control GAPDH (Glyceraldehyde 3-phosphate dehydrogenase). Unpaired t-test, two-sided, p-value: 0.1312 (**C**) The presence of cytoplasmic inclusion bodies correlates with TDP-43

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mislocalization in ataxin-1-expressing cells. TDP-43 does not accumulate in nuclear or cytoplasmic ataxin-1 inclusion bodies (IB), but does mislocalize to the cytoplasm in cells with inclusion bodies. (**D**) Quantification of TDP-43 mislocalization in controls cells (mCherry) and cells without ($-IB_{cyto}$) or with cytoplasmic ataxin-1 inclusion bodies ($+IB_{cyto}$). (**E**) Cytoplasmic inclusion bodies also correlate with GFP-tagged KNPA2 mislocalization to the cytoplasm (**F**) Quantification of KPNA2 mislocalization. (**D,F**) 1-way ANOVA, **** p-value < 0.0001.

Figure 7. Ataxin-1 polyQ modifies eye phenotypes in Drosophila. (**A**) Scheme indicating assessment of genetic modifiers. (**B**) Effect of eye phenotype after co-expression of eGFP, 2Q ataxin-1 and 82Q ataxin-1 in wildtype (top), TDP-43(middle) and GR36 (bottom)-expressing flies. (**C**) Fraction of flies per necrotic eye score rank (darker shading equals higher score). Right panel: Flies overexpressing ATXN1.82Q only show a clear degenerative phenotype characterized by a moderate rough eye phenotype, but only very small necrotic spots. Middle panel: Flies co-expressing TDP-43 and ATXN1 polyQ with a repeat length of 82 with a severe eye phenotype are significantly enriched compared to flies expressing TDP-43 and ATXN1 with a polyQ repeat length of 2 ($P = 2.65 \times 10^{-4}$); there was no significant difference with eGFP and 2 polyQ. Left panel: Flies co-expressing GR(36) and ATXN1 polyQ with a repeat length of 82 with a severe eye phenotype are significantly enriched compared to flies expressing GR(36) and ATXN1 with a polyQ repeat length of 2 ($P < 2.0 \times 10^{-16}$); there was no significant difference with eGFP and 2 polyQ. Statistical analysis using linear by linear association test, n > 50 per genotype.

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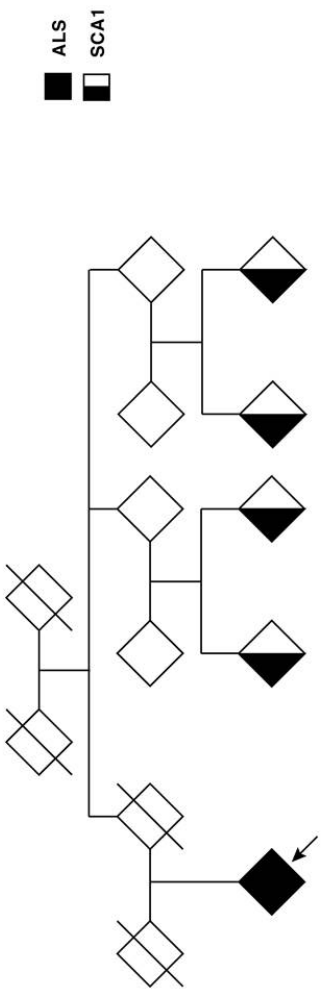

Figure 1

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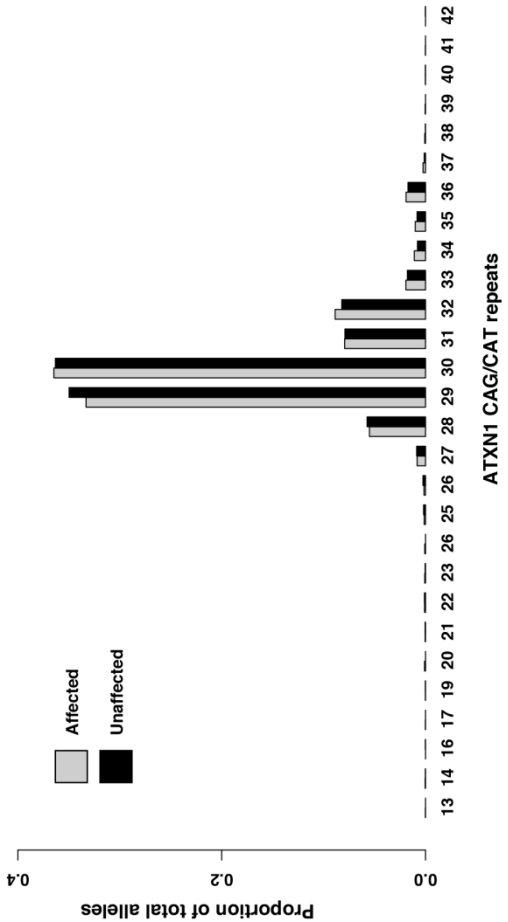

Figure 2

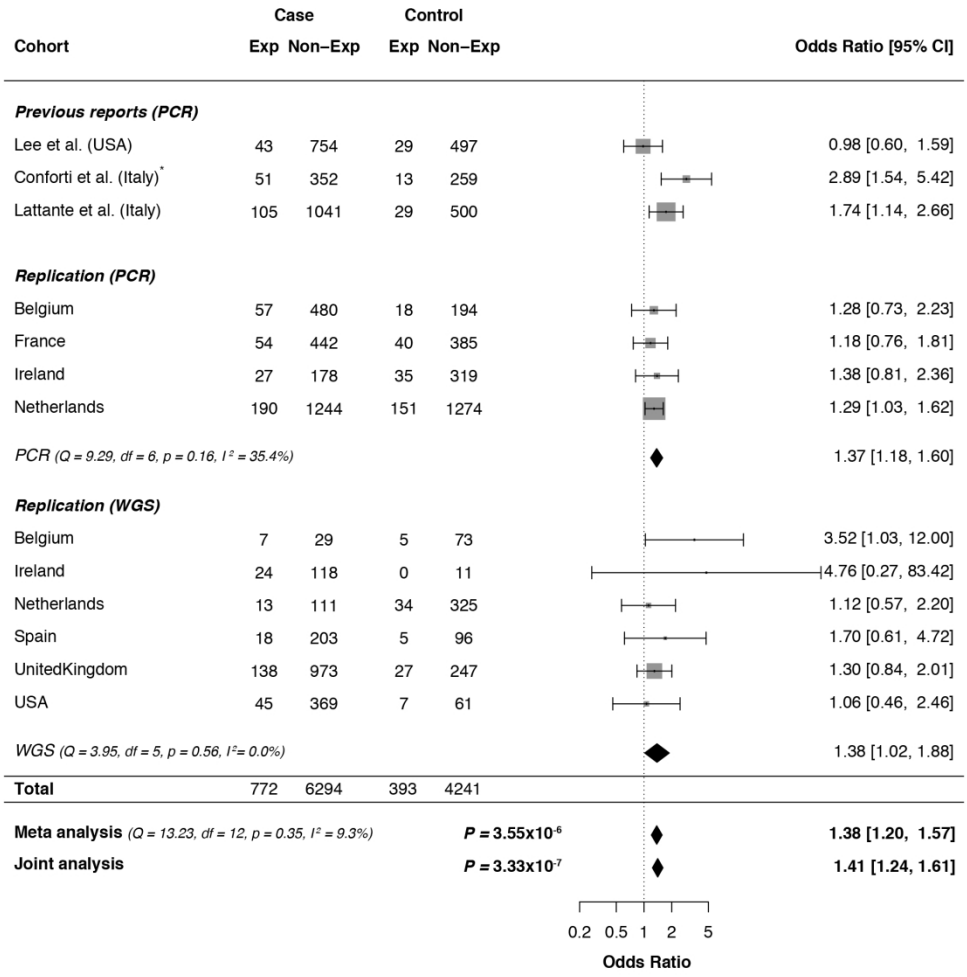

Figure 3

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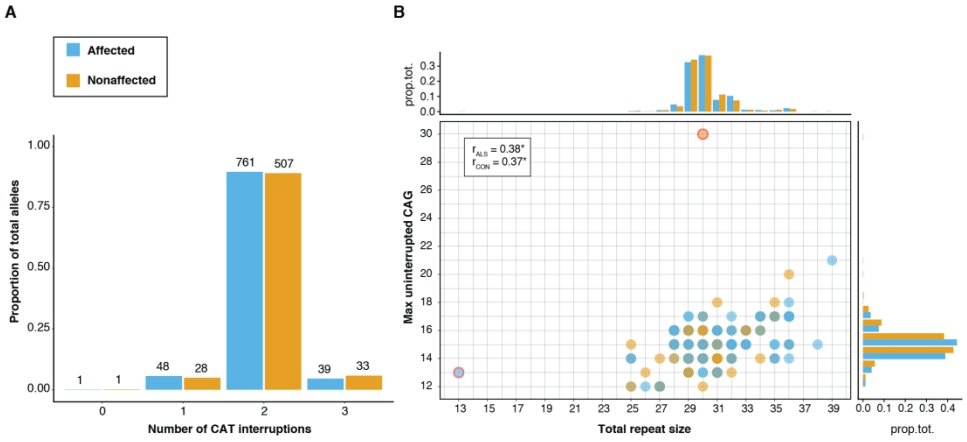

Figure 4

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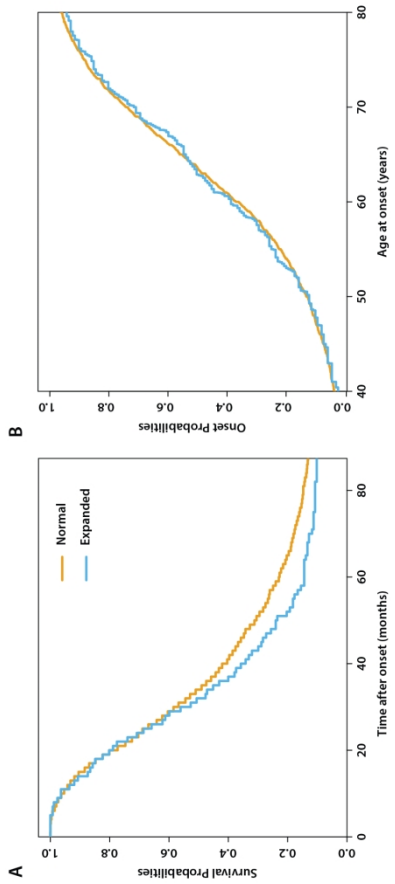

Figure 5

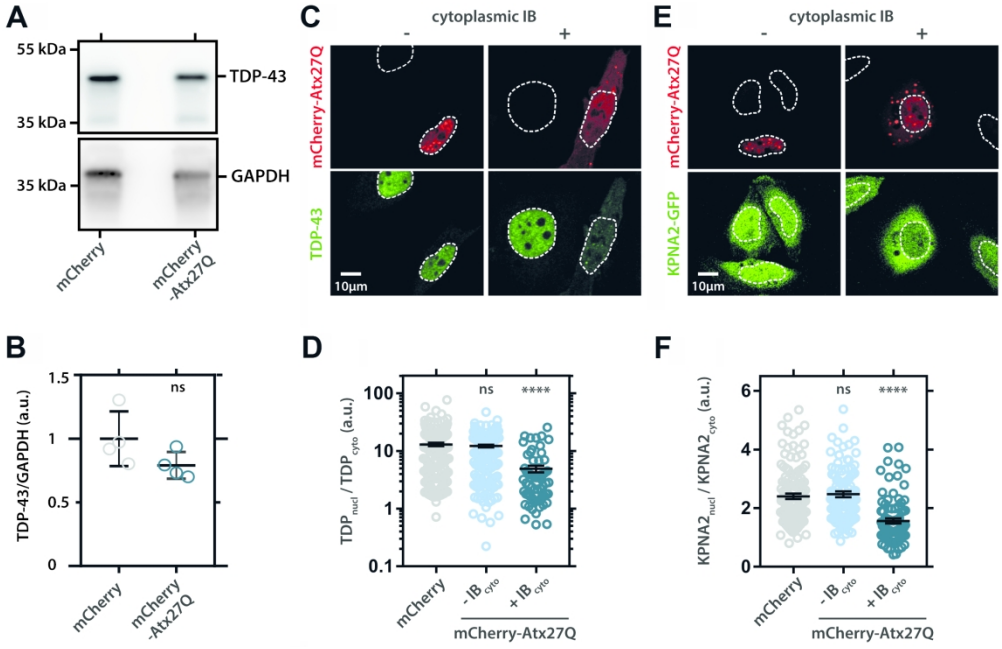

Figure 6

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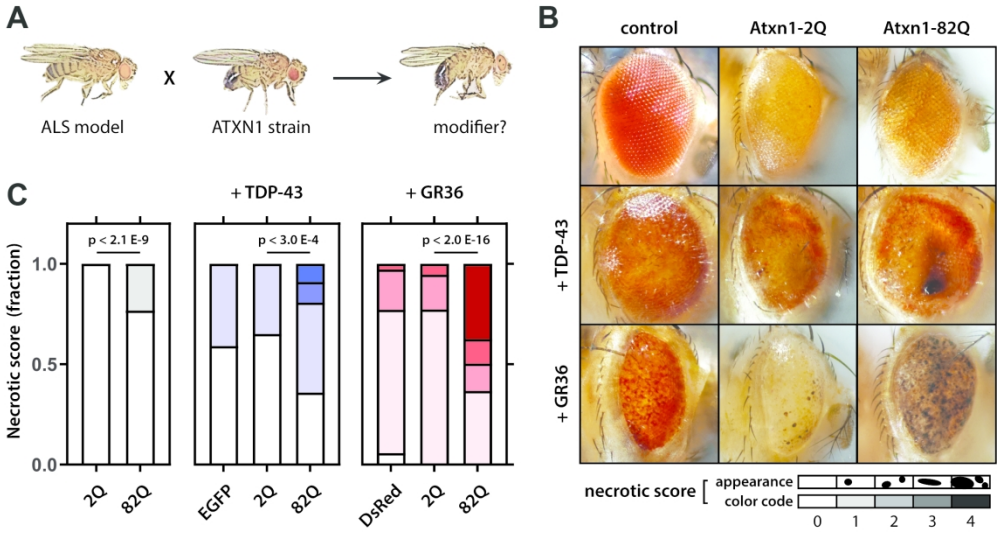

Figure 7

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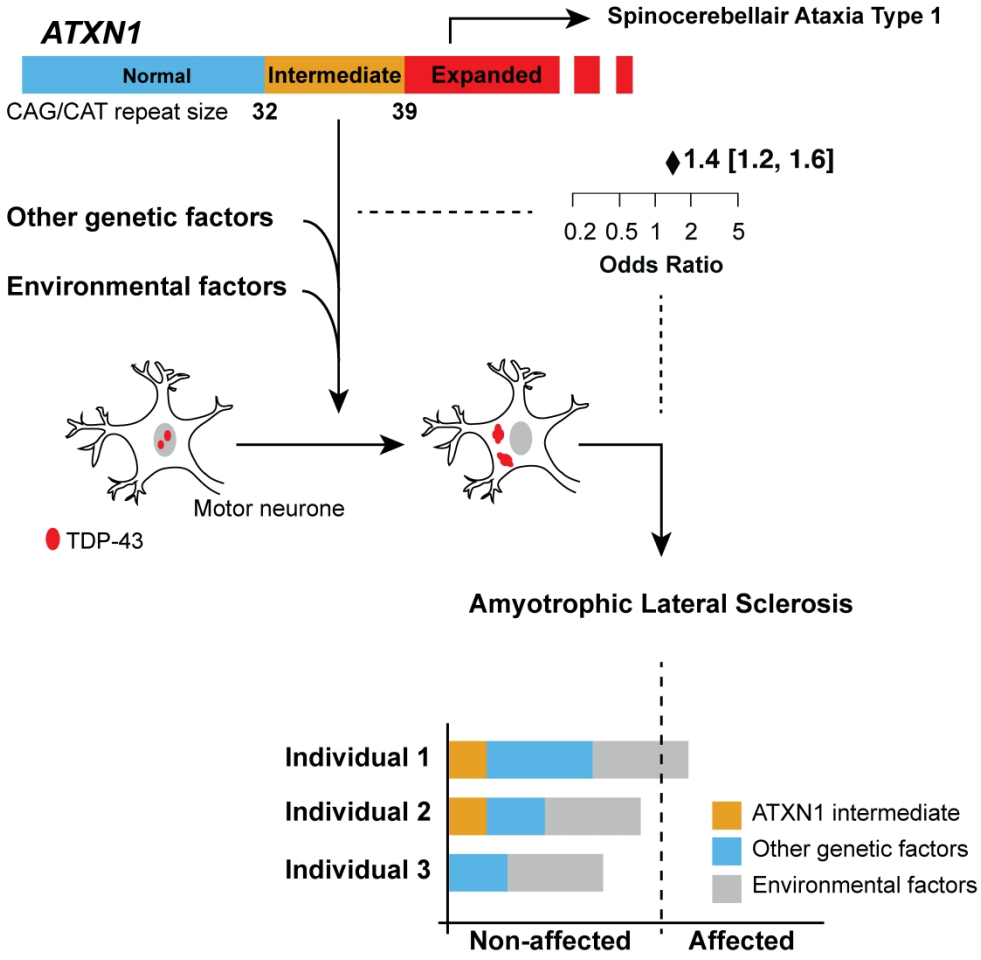

Graphical abstract

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Abbreviated summary:

Repeat expansions are being identified as part of the complex genetic architecture of amyotrophic lateral sclerosis. This study shows a significant association between intermediate *ATXN1* repeat expansions and amyotrophic lateral sclerosis, possibly via mislocalization of TDP-43, further emphasizing the role of polyglutamine expansions in the pathophysiology of amyotrophic lateral sclerosis.

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