Protein interacting with C kinase (PICK1) is a suppressor of spinocerebellar ataxia 3-associated neurodegeneration in *Drosophila*

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Received May 16, 2011; Revised September 12, 2011; Accepted September 20, 2011

Spinocerebellar ataxia 3 (SCA3) is the most common autosomal dominant ataxia. The disease is caused by an expansion of a CAG-trinucleotide repeat region within the coding sequence of the *ATXN3* gene, and this results in an expanded polyglutamine (polyQ) tract within the Ataxin-3 protein. The polyQ expansion leads to neuronal dysfunction and cell death. Here, we tested the ability of a number of proteins that interact with Ataxin-3 to modulate SCA3 pathogenicity using *Drosophila*. Of 10 candidates, we found four novel enhancers and one suppressor. The suppressor, *PICK1* (*Protein interacting with C kinase 1*), is a transport protein that regulates the trafficking of ion channel subunits involved in calcium homeostasis to and from the plasma membrane. In line with calcium homeostasis being a potential pathway mis-regulated in SCA3, we also found that down-regulation of *Nach*, an acid sensing ion channel, mitigates SCA3 pathogenesis in flies. Modulation of *PICK1* could be targeted in other neurodegenerative diseases, as the toxicity of SCA1 and tau was also suppressed when *PICK1* was down-regulated. These findings indicate that interaction proteins may define a rich source of modifier pathways to target in disease situations.

INTRODUCTION

The most common dominantly inherited ataxia is spinocerebellar ataxia 3 (SCA3). The mutation is the result of a CAG-trinucleotide expansion in the coding region of the *ATXN3* gene, which leads to the expression of a large stretch of glutamines (polyQ) within the protein. There are eight other polyQ diseases, including several of the SCAs (SCA1, 2, 6, 7 and 17) and Huntington’s disease. Although the genes responsible for the polyQ diseases appear to be different in amino acid sequence and function, they share pathological hallmarks. For example, this group of diseases is characterized by the formation of polyQ protein aggregates in the nucleus or cytoplasm (1–4). Studies show that the polyQ accumulations sequester proteins involved in the ubiquitin proteasome system (UPS) (5–8). In addition to UPS impairment, it has been proposed that the toxic polyQ protein may impair transcription, mitochondrial function, cytoskeletal transport, genome stability and calcium homeostasis (9). Several therapeutic compounds have been proposed that target protein mis-folding and aggregation, excitotoxic mechanisms and oxidative stress (10,11).

It has become apparent that the polyQ proteins can interact with each other. For example, loss of the *Drosophila ATXN2* homologue, *dAtx2*, mitigates SCA1 and SCA3 pathogenesis, suggesting the possibility of mis-regulation of common molecular pathways (12,13). In addition, a human protein–protein interaction network for 23 different ataxia-causing proteins and 31 ataxia-interacting proteins (14) revealed an interconnected protein network that may prove critical to pathogenesis in humans. Indeed, novel disease interactors were found in the ataxia network, including Puratrophin-1 (pleckstrin homology domain containing, family G), which was independently identified as an interactor of SCA1 (15). One of the genes in the ataxia-interactome, *AFG3L2*, was found to be responsible for SCA28 (16). These findings highlight that the effects observed in neurodegenerative diseases characterized by ataxia may in part be due to disruption of shared or interacting networks.

The use of model organisms has revealed great insight into the key genes that mediate polyQ-associated neurodegeneration, particularly *Drosophila* (8,17). To identify novel and
common interactors of ataxia, we tested a number of proteins that have been shown to interact with and are predicted to be one to two proteins away from direct interaction with the Ataxin-3 protein in the ataxia interaction network (14). Our studies reveal that the network could be used to successfully predict genetic modifiers of pathogenesis. Our data define PICK1 (Protein interacting with C Kinase 1) as a modifier of SCA3 pathogenesis, as well as Ataxin-1 and tau-associated toxicity. These data suggest that PICK1-associated pathways may be neuroprotective and a potential target for neurodegenerative diseases.

RESULTS

The SCA3–Friedreich’s ataxia network

The ataxia interactome was a project based on 54 proteins, of which 23 are ataxia-causing genes and 31 are genes that were interactors of or paralogues of ataxia-causing genes. Yeast two-hybrid analysis using these 54 genes as bait revealed 561 interacting proteins. Further database mining with the 615 proteins extended the network to 3607 that could be involved in the pathogenesis of ataxia (14). We concentrated on the proteins identified as direct interactors of Ataxin-3. Of the 10 proteins, 9 have clear sequence orthologues in the Drosophila genome: Rad23A and Rad23B both share sequence similarity to Rad23, and Caspase 1 and Caspase 2 share sequence homology with Ice (Fig. 1). We excluded VCP from our analysis because it is an established modulator of SCA3 pathogenesis (18).

One proposed mechanism of polyQ toxicity is mitochondrial dysfunction (19–21). Oxidative stress has been shown to cause an increase in the nuclear accumulation of both pathogenic and normal Ataxin-3 protein, indicating that oxidative stress may be an important factor that influences the pathogenesis of SCA3 (22). One of the interactors, PICK1, connects Ataxin-3 with the mitochondrial protein Frataxin, which is responsible for Friedreich’s ataxia (FA) (23). FA and SCA3 share pathology in that the purkinje cells are preserved, unlike SCA1 and SCA2, and both SCA3 and FA affect the dentate nuclei and the dorsal nuclei of Clarke (24). We therefore included the FA network in the analysis of SCA3 pathogenesis.

The effect of the FA network on SCA3 pathogenesis

To investigate whether the FA network could genetically interact with SCA3 toxicity, we first tested the effect of down-regulating the frataxin homolog (fh) gene. Expression of the polyQ domain of mutant Ataxin-3 (SCA3trQ78) in all tissues of the fly eye using the eye-specific gmr-GAL4 driver results in degeneration of the external eye (Fig. 2A). To test the effect of fh, and additional FA-network genes on SCA3-associated toxicity, we co-expressed SCA3trQ78 with RNAi lines directed to fh, Aconitase (Acon), CG3731 and α-actinin (Actn) in the fly eye. We consistently saw an enhancement with two independent RNAi lines directed to fh, with RNAi lines directed to Acon (a protein involved in the tricarboxylic acid cycle) and with CG3731 (which in mammals cleaves the leader peptides from proteins transported into the
mitochondria) (Fig. 2B–D). The modulation was specific as the expression of the fh and Acon RNAi transgenes with gmr-GAL4 alone had no effect, whereas expression of the CG3731 RNAi transgene alone produced a very mild disruption of the external eye (Supplementary Material, Fig. S1). Down-regulation of Actn, an actin-binding protein, had no effect on SCA3trQ78 degenerative eye phenotype (Fig. 2E). These data suggest that the down-regulation of components of the mitochondria can enhance SCA3 pathogenesis.

To determine whether these modifiers are dosage-sensitive regulators, we tested whether up-regulation of components of the frataxin network could mitigate SCA3trQ78 pathogenesis. UAS-fh and UAS-Acon were co-expressed with SCA3trQ78. We found no modification of the external or internal retinal morphology (Supplementary Material, Fig. S2). These data suggest that the down-regulation of components of the mitochondria can enhance SCA3 pathogenesis.

To identify additional genes involved in the pathogenesis of SCA3, we concentrated on the proteins that had been identified to directly interact with the Ataxin-3 protein (14) and knocked-down the expression of those genes by RNAi. This revealed that the reduction in RhoGAP68F expression enhanced the eye phenotype of SCA3trQ78 (Fig. 2F). RhoGAP68F, the Drosophila counterpart of the human gene ARHGAP, is a GTPase activating protein that negatively regulates Rho GTPase (25). No phenotype was observed when the RhoGAP68F transgene was expressed with gmr-GAL4 alone (Supplementary Material, Fig. S1).

We identified one gene, PICK1 (protein interacting with C kinase 1), that, when knocked-down, suppressed the external eye degeneration of SCA3trQ78 (Fig. 2G). Real-time polymerase chain reaction (PCR) analysis was performed on flies globally reducing PICK1 with the daughterless-GAL4 (da-GAL4) driver. The da-GAL4 driver is expressed in all tissues; this allowed us to determine the efficiency of the knock-down without dilution of wild-type gene levels from tissues not expressing the RNAi transgene. Real-time PCR showed that the PICK1 RNAi line reduced the PICK1 mRNA level to below 20% of the control (Fig. 3C). The internal retinal structure of flies co-expressing SCA3trQ78 and the RNAi transgene to PICK1 also revealed mitigated degeneration (Fig. 3A). Suppression was confirmed with an independent PICK1 RNAi line, a deficiency line and heterozygous loss of PICK1 (Supplementary Material, Fig. S3).

To further test the suppression of SCA3trQ78 upon down-regulation of PICK1, we also examined the loss of photoreceptor neuronal integrity with knockdown of PICK1 by RNAi or heterozygous loss of endogenous PICK1 (26) using an adult-onset assay directing the pathogenic polyQ protein to the photoreceptor neurons. Both of these situations significantly suppressed the degeneration of photoreceptor neurons (Fig. 3D), indicating that the reduction in PICK1 is neuroprotective in the SCA3trQ78 fly model both with developmental toxicity and in the adult stages.

Figure 2. SCA3 candidate modifiers. (A) Expression of strong SCA3trQ78 causes degeneration of the external fly eye. Genotype: w; gmr-GAL4, UAS-SCA3trQ78/UAS-mCD8-GFP. (B–E) Genes of the frataxin network. Genotypes: w; gmr-GAL4, UAS-SCA3trQ78 in trans to the alleles indicated. (B–D) Genes of the frataxin network that enhance the SCA3trQ78 degenerate eye phenotype. (E) Reduction of Actn, a frataxin network protein, has no effect on the SCA3trQ78 degenerate eye phenotype. (F–J) Genes of the SCA3 network. Genotypes: w; gmr-GAL4, UAS-SCA3trQ78 in trans to the alleles indicated. (F) RhoGAP68F when knocked-down enhances the SCA3trQ78 degenerate eye phenotype. (G) Reduction in PICK1 suppresses the SCA3trQ78 eye phenotype. (H–J) Genes of the SCA3 network, when knocked-down, that have no effect on the SCA3trQ78 degenerate eye phenotype.
Loss of PICK1 expression has some effect to reduce insoluble SCA3trQ78 protein accumulation

The formation of polyQ aggregates is a hallmark of the polyQ diseases. In SCA3, the aggregates form insoluble nuclear inclusions (NIs). Expression of SCA3trQ78 in differentiated photoreceptor neurons with the adult-onset photoreceptor driver, *rhodopsin-1-GAL4* (*rh1-GAL4*), allowed us to examine degeneration slowly over time in the adult, and is more sensitive for analysis of changes in protein levels. Expression of SCA3trQ78 in adult photoreceptor neurons results in the detection of insoluble SCA3trQ78 by d3 (Fig. 4A). Co-expression of the RNAi transgene to PICK1 or heterozygous loss of endogenous PICK1 reduced the amount of insoluble SCA3trQ78 and increased the amount of soluble protein, such that the ratio of soluble protein (presented as amount of soluble protein relative to tubulin loading control) was significantly increased (Fig. 4B). At d3, the formation of NIs can be observed in the retina of flies expressing SCA3trQ78, with the *rh1-GAL4* driver (Fig. 4C). Reduction in PICK1 with the RNAi transgene caused a change in the appearance of the accumulations, such that they appeared less compact, and a trend for fewer inclusions was observed (Fig. 4C–E).

To test the broader role of PICK1 to modulate neurodegenerative-associated proteins, we then tested the ability of PICK1 to affect Tau toxicity. Mutation in the microtubule-binding protein, Tau, leads to the formation of insoluble, hyperphosphorylated-Tau positive neurofibrillary tangles in Alzheimer’s disease (27–29). We expressed a mutant form of human Tau, *hTau.R406W*, with either the PICK1 RNAi transgene or with heterozygous loss of endogenous PICK1. These experiments showed that the reduction in PICK1 suppressed the toxicity of hTau.R406W (Fig. 4F). Heterozygous loss of PICK1 did not suppress the Tau.R406W as well as the RNAi line (Fig. 4E); however, PICK1 in the heterozygous condition is anticipated to be ≏50%, whereas the RNAi line directed to PICK1 reduced the levels of PICK1 to 20% of the wild-type (Fig. 3C). These data indicate that down-regulation of PICK1 activity may be protective in a number of different neurodegenerative situations.

A reduction in Nach expression mitigates SCA3 pathogenicity in flies

PICK1 is known to act as a transport protein that traffics the GluR2 subunit away from the plasma membrane and the acid sensing ion channels (ASICs) to the plasma membrane.
In both of these situations, there is an increase in the influx of calcium ions, and under conditions of traumatic neuronal injury and ischemia, this leads to an increase in cell death (33–37). Interestingly, calcium excitotoxicity has also been proposed to be involved in the pathogenesis of a number of neurodegenerative diseases, including SCA3, SCA1 and Huntington’s disease, and suppression of the mammalian ACCN2 gene (an ASIC) suppresses Huntington’s disease-associated pathology in cells (38–43). These data raised the hypothesis that a reduction in ion channels, and their associated effects on excitability, may also suppress SCA3trQ78 toxicity in flies. PICK1-mediated transport of ion channels involved in glutamatergic signaling is dependent upon PKCα (30,31,32). We found that inhibition of or a reduction in PKCα failed to suppress SCA3trQ78 toxicity (Supplementary Material, Fig. S4). In addition, the main neurotransmitter in the fly eye is histamine. Together, this suggests that modulation of glutamatergic signaling in the SCA3trQ78 fly would have no effect. We therefore focused on the ASICs. Intriguingly, knockdown of one ASIC gene in the fly eye, Nach, suppressed the eye degeneration of SCA3trQ78 (Fig. 5A and B), whereas knockdown of a second ASIC, pickpocket (ppk), only partially suppressed the retinal degeneration (Supplementary Material, Fig. S5). There are 16 different ASIC genes in the fly genome. We tested all RNAi lines available to these genes (14 genes) but did not see suppression upon reduction in any additional genes (data not shown). It could be that the reduction in a combination of two or more of these other ASIC proteins may be required to offer protection in the SCA3 model, or that Nach is the primary ASIC mediating the suppression of SCA3trQ78 in the retina.

SCA3 shares common pathogenic pathways to SCA1

Several lines of evidence suggest that ataxia proteins share common molecular pathways. For example, in flies reduction in dAtx2 suppresses SCA3- and SCA1-induced pathogenesis and up-regulation of the normal Ataxin-3 protein can suppress Atx1Q82 (SCA1) degenerative phenotypes (12,13,44).
proteins revealed an interconnected network, suggesting that the ataxia-causing proteins, which share phenotypic features, also share common molecular pathways (14). Using an RNAi approach, we disrupted some of the major molecular players proposed to be involved in interactions with Ataxin-3 or this polyQ protein network: mitochondrial dysfunction, cytoskeletal transport, genome stability or calcium homeostasis (9). Our studies support the idea that mitochondrial dysfunction and calcium homeostasis may be critical. Moreover, although this network is based on the normal proteins, and not the mutant proteins, our data suggest the network can modulate pathogenicity of the proteins associated with human disease in vivo.

Disrupting mitochondrial function by reducing the expression of three distinct mitochondrial genes, fh, Acon and CG3731, enhanced SCA3 pathogenesis. These genes, however, do not appear to be rate-limiting because up-regulation of fh and Acon did not suppress degeneration. Thus, neurons expressing pathogenic SCA3 are susceptible to oxidative stress; however, up-regulation of components of the frataxin network with drugs such as erythropoietin may not be of benefit to SCA3 patients as the processes influenced by these genes is not dose-sensitive and rate-limiting in an up-regulation manner. The key nodes of these networks that are dose-sensitive would be important to reveal and target.

Two of the six proteins predicted to interact with the Ataxin-3 protein also genetically modified SCA3 disease pathogenesis in the fly. Reduction in the gene encoding cdc42 GTPase activating protein, RhoGAP68F, enhanced pathogenesis, whereas loss of PICK1 suppressed toxicity, indicating that reduction in PICK1 gene expression is neuroprotective. Recently, a small molecule inhibitor targeted to the PDZ (post synaptic density protein-95, Drosophila disc large tumor suppressor, and zonula occludens-1 protein) domain of PICK1 was identified (45,46). It is therefore plausible to test the effect of inhibiting PICK1 function with small molecule inhibitors in the treatment of SCA3 and in other SCA3 model systems (39).

PICK1 is known to transport subunits of ion channels to and from the plasma membrane (30–33). The reduction in mammalian PICK1 function reduces excitotoxicity which has led to great interest into therapeutically targeting PICK1 for isch-emia (36). The mutant Ataxin-3 protein has also been found to associate and activate calcium release from the calcium trans-porter InsP₃R1 (39). With this in mind, we investigated whether other proteins involved in calcium homeostasis could suppress SCA3 toxicity. This approach revealed that reduction in Nach, an ASIC, recapitulated the suppression observed with the reduction in PICK1, suggesting that this channel may be involved in the toxicity of the pathogenic SCA3 protein.

Our work also revealed that a reduction in the expression of the Drosophila homologue of ATXN1, Atx-1, in the SCA3 fly model mitigated disease pathogenesis, suggesting that SCA1 and SCA3 pathogenesis may share more molecular pathways than previously appreciated. These studies indicate that therapeutics directed at SCA1 may be of benefit to SCA3 due to overlapping functional pathways. We also tested whether PICK1 reduction could mitigate hAtx1Q82 toxicity. This result was unanticipated, as PICK1 had not been predicted to bind to the Ataxin-1 protein in the ataxia interactome.
Studies in flies have revealed that the ataxia-causing proteins share molecular and pathogenic activities, since Drosophila dAtx-2 interacts genetically with both hAtx-Q82 and SCA3 (12,13). Given that we have found that reduction in PICK1 can suppress both SCA3 and hAtx1-Q82 pathogenesis in flies, PICK1 may be involved in the shared pathogenic pathways.

Our data suggest that protein interaction networks may be rich sources of genomic modification pathways for pathogenic proteins, and vice versa—such modifiers may be key candidates to test for direct interactions with pathogenic proteins of interest. Finally, our studies suggest that excitotoxic pathways may be of interest to attack not only in ischemic situations, but also in neurodegenerative situations.

MATERIALS AND METHODS

Drosophila stocks and crosses

Fly lines were grown in standard cornmeal molasses agar with dry yeast at 25°C. Transgenic lines for SCA3trQ78 have been described previously (44,47,48). We used UAS-mCD8-GFP as a control transgene to co-express a second transgenic line, when comparing any experimental UAS-transgenic lines to the normal degeneration induced by the SCA3 toxic protein. RNAi lines included: PICK1 [P(KK109273)VIE-260B], RhoGAP68F [P(KK102738)VIE-260B], Rad23 [P(KK107826)VIE-260B], Ice [w1118; P(GD12284)v28065 and w1118; P(GD12284)v28064/TM3], CG7083 [P(KK107820)VIE-260B], CG3731 [P(KK108539)VIE-260B], Acon [P(KK100999)VIE-260B] and Actn [w1118; P(GD1354)v7760 and w1118; P(GD1354)v7762] from the VDRC stock center (49). RNAi lines were also obtained to PICK1 (line TRiP.JF01199), Atx-1 (line TRiP.HM05022), Nach (line TRiP.JF02566) and ppp (line TRiP.JF03250) from TRiP at the Harvard Medical School. The RNAi lines directed to frataxin homolog (fh) have been described (50). All deficiency strains were obtained from the Bloomington stock center, as was UAS-PK Ci (51).

The PICK1 mutant flies PICK1 delEP147 and PICK1 delEP197 (referred to here as delEP147 and delEP197) have been described (26) and were a kind gift from Ole Kjaerulff (The Panum Institute University of Copenhagen). The rh1-GAL4 driver was a gift of C. Desplan (New York University, New York, USA) and UAS-htau.R406W a gift of M. Feany (Harvard Medical School, Boston, MA, USA).

Histochemistry

Paraffin sections and cryosections were performed as described (47,48). To examine the internal structure of the retina 7 μm paraffin, sections were cut and mounted onto glass slides. Tissue was visualized using the auto-fluorescent property of the fly brain, and are presented in reverse black and white images. For immunohistochemistry, primary antibodies used were mouse anti-HA primary antibody (5B1D10, 1:100, Invitrogen) and mouse anti-Myc (9E10, 1:100, Santa Cruz Biotechnology). Secondary antibodies were anti-mouse conjugated to Alexa Fluor 594 or 488 (1:200 or 1:100, Molecular Probes). Western immunoblots were performed as described (52). Protein was extracted in Laemmli sample buffer (Bio-Rad) from 10 heads of each
genotype, the protein was heat denatured and electrophoresed under denaturing conditions. Each experiment was performed six independent times. Antibodies used were anti-HA antibody conjugated to horseradish peroxidase (3F10, 1:500, Roche), mouse anti-tubulin (E7, 1:2,000, Developmental Studies Hybridoma Bank) and goat anti-mouse IgG (1:2,500, Jackson Laboratories). Signal was detected using Amersham™ ECL Plus and chemiluminescence was detected and quantified on the Fujifilm LAS3000. One-way analysis of variance (ANOVA) followed by a Tukey’s test was performed to calculate statistically significant changes in the ratio between soluble SCA3trQ78 protein and tubulin loading control.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at *HMG* online.

**ACKNOWLEDGEMENTS**

We thank L. Hao, S. Shieh and Z. Yu for critical comments. We thank C. Desplan, M. Feany, Ole Kjaerullff, the Bloomington Stock Center, the VDRC, the TRiP at Harvard Medical School (NIH/NIGMS RO1-GM084947) and the Developmental Studies Hybridoma Bank (funding from NICHD) for fly lines, reagents or advice.

Conflict of Interest statement. None declared.

**FUNDING**

This work was supported by the National Ataxia Foundation (L.M.) and the Howard Hughes Medical Institute (N.M.B.). Funding to pay the Open Access publication charges for this article was provided by the Howard Hughes Medical Institute.

**REFERENCES**