Cellular/Molecular

Progressive Degeneration of Dopaminergic Neurons through TRP Channel-Induced Cell Death

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Progressive neurodegenerative diseases are among the most frequently occurring aging-associated human pathologies. In a screen for *Caenorhabditis elegans* mutant animals that lack their normal complement of dopaminergic neurons, we identified two strains with progressive loss of dopaminergic neurons during postembryonic life. Through whole-genome sequencing we show that both strains harbor dominant (d), gain-of-function mutations in the Transient Receptor Potential (TRP) mechanosensory channel *trp-4*, a member of the invertebrate and vertebrate TRPN-type of the TRP family channels. Gain-of-function mutations in TRP channels have not been previously implicated in dopaminergic neuronal degeneration. We show that *trp-4(d)* induces cell death in dopamine neurons through a defined, calcium-related downstream pathway.

Key words: C. elegans; **calcium; cell death; dopaminergic neurons; neurodegeneration; TRP channels**

Introduction

In an ever-aging population, neurodegenerative disorders, characterized by progressive loss of structure or function in various neuronal cell types, are bound to increase. Although several genetic causes of neurodegenerative diseases have been discovered, for the majority of them the causes as well as the underlying molecular mechanisms remain elusive. Nevertheless, family histories and lessons from GWAS suggest a substantial contribution of still undiscovered genetic susceptibility factors to disease etiology (Lesage and Brice, 2009; Lill and Bertram, 2011). Studies using various animal models of neurodegenerative disease have collectively illuminated some aspects of the molecular pathology of such conditions (Harrington et al., 2010; Lee et al., 2012). In addition, these studies uncovered novel triggers of neuronal cell death, which resulted in the discovery of downstream molecular mechanisms contributing to the process (Driscoll and Chalfie, 1991; Hong and Driscoll, 1994; Yoon et al., 2000; Kim et al., 2007; Ni et al., 2008). Thus, identifying new genetic causes of neuronal degeneration in model organisms can enhance our mechanistic insights into neurodegenerative diseases in humans.

Despite their differences, most major neurodegenerative diseases share several clinical, pathological, and molecular characteristics, such as the occurrence of oxidative stress, protein misfolding, and calcium dyshomeostasis (Gorman, 2008). Specifically, Ca^{2+} dysregulation has been suggested to contribute to the etiology as well as progression of various neurological diseases (Bezprozvanny, 2009; Nikoletopoulou and Tavernarakis, 2012), including Parkinson's disease (Chan et al., 2007; Gandhi et al., 2009; Surmeier et al., 2010), Alzheimer's disease (Bojarski et al., 2008), amyotrophic lateral sclerosis (Grosskreutz et al., 2010), and Huntington's disease (Giacomello et al., 2011; Wu et al., 2011). Particularly relevant to intracellular Ca^{2+} homeostasis is the family of Transient Receptor Potential (TRP) channels, known to be involved in initiating Ca^{2+} entry pathways as well as maintaining cytosolic, endoplasmic reticulum (ER), and mitochondrial Ca^{2+} levels (Gees et al., 2010). Because of their role in ion homeostasis and their expression in the nervous system, TRP channels have been increasingly considered as contributing factors to neuronal degeneration (Selvaraj et al., 2010; Vennekens et al., 2012), but their precise role in degenerative conditions is largely unexplored. Furthermore, mutations in TRP channel family have not been previously implicated as causal to dopaminergic degeneration.

Given the importance of studying the genetic basis of neurodegenerative conditions, it is perhaps surprising that only a few model system studies have attempted to screen in an unbiased manner for mutants in which neurons initially develop but progressively degenerate. We have undertaken such an approach and

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describe here a novel *Caenorhabditis elegans* mutant with robust and progressive degeneration of dopaminergic neurons during postembryonic development. We show that a single amino acid substitution in a TRP channel is responsible for the phenotype, implicating mutations in TRP family channels as a direct cause of dopaminergic degeneration for the first time. We provide insights on the mode of cell death and the downstream mechanisms of action, revealing the involvement of intracellular Ca^{2+} homeostasis in the process of dopaminergic cell death.

Materials and Methods

C. elegans *strains.* Strains were maintained as previously described (Brenner, 1974). *C. elegans* hermaphrodites were used in all experiments, unless otherwise stated. Strains used were as follows: BY200: *vtIs1[dat-1::gfp;rol-6(d)]*; MDH26: *trp-4(ot337);vtIs1*; MDH41: *vtIs1;norEx38[dat-1::trp-4(d);ttx-3::cherry];vtIs1*; MDH43: *norEx40[dat-1::trp-4(d);ttx-3::cherry];vtIs1*, OH6071: *trp-4(ot337);vtIs1,vsIs33[dop-3::rfp]*; MDH233: *trp-4(ot337);eri-1(mg366);lin15B(n744);vtIs1*; MDH123: *trp-4(ot337); unc-79(e1068),ced-4(n1162);vtIs1,vsIs33*; MDH86: *unc-79(e1068)ced-4(n1162); vtIs1,vsIs33*; MDH124: *trp-4(ot337); ced-3*(n717);*vtIs1,vsIs33*; MDH51: ced-3(n717); vtIs1,vsIs33. OH9051: *otIs259[dat-1::gfp]*; MDH28: *trp-4(ot337);otIs259*; MDH22: *crt-1(bz29);otIs259*; MDH23: *trp-4(ot337);crt-1(bz29);otIs259*; MDH20: *crt-1(ok948);otIs259*; MDH21: *trp-4(ot337);crt-1(ok948);otIs259*; MDH90: *itr-1(sa73);vtIs1*; MDH89: *itr-1(sa73);trp-4(ot337);vtIs1*; MDH126: *cnx-1(ok2234);vtIs1*; MDH127: *trp-4(ot337); cnx-1(ok2234); vtIs1*; MDH128: *cnx-1(ok2234);crt-1(bz29); trp-4(ot337);otIs259. bxIs19[Ptrp-4::gfp,ttx-3::gfp]*; MDH84: *crt-1(ok948);bxIs19*; MDH141: *dat-1(ok157);trp-4(ot337);vtIs1*; MDH112: *cat-2(e1112);vtIs1*; MDH129: *cat-2(e1112);trp-4(ot337);vtIs1*; MDH66: *norEx7[dat-1::hcalbindin-1;ttx-3::cherry];trp-4(ot337);vtIs1*; MDH119*: norEx34[dat-1::hcalbindin-1;ttx-3::cherry];trp-4(ot337);vtIs1*; MDH121*: norEx36[dat-1::hcalbindin-1;ttx-3::cherry]; trp-4(ot337);vtIs1*; MDH97: *norEx24[dat-1::mdh-1;ttx-3::mcherry];trp-4(ot337);vtIs1*; MDH98: *norEx25[dat-1::mdh-1;ttx-3::mcherry];trp-4(ot337);vtIs1*; MDH99: *norEx26[dat-1::mdh-1;ttx-3::mcherry];trp-4(ot337);vtIs1*; MDH138: *bxIs19[trp-4::gfp;ttx-3::gfp]; otIs181[dat-1::mcherry;ttx-3::mcherry]*; MDH139: *dpy-11(e224) unc-68(e450);trp-4(ot337);otIs259*; MDH140: *dpy-11(e224),unc-68((e450);otIs259*; MDH150*: norEx47 [dat-1::GFP-TRP-4;rol-6]*; MDH169: *crt-1(ok948);norEx47[dat-1::GFP-TRP-4;rol-6]*; and MDH225: *norEx82[Posm-6::trp-4(d);rol-6;ttx-3];oyIs59[Posm-6::gfp]*.

Mapping and cloning of trp-4(d) *mutants.* The *ot337* allele was mapped on the right arm of CHRI (+14 to +26 cM) using high-throughput SNP mapping (Davis et al., 2005). Both *ot337* and *ot477* alleles were whole-genome sequenced using an Illumina platform followed by data analysis as previously described (Bigelow et al., 2009). Only one locus, *trp-4*, was affected in common in the two alleles. The mutation was confirmed by Sanger sequencing. We phenocopied the effect of the *ot337* mutation by introducing a *trp-4(d)* transgene in a wild-type background. To this end, a plasmid containing the *trp-4* cDNA under the control of *dat-1* promoter (kindly provided by Shawn Xu) was used to introduce the *ot337* mutation by site-directed mutagenesis.

Ectopic expression of *trp-4(d)*: *trp-4(d)* cDNA followed by *unc-54* 3′UTR was cloned under the osm-6 promoter (Collet et al., 1998) and injected into *oyIs59* worms expressing osm-6::GFP at 30 ng/μl, along with 60 ng/μl *rol-6* and 50 ng/μl *ttx-3::mcherry*. Worms containing the *ttx-3::mcherry* coinjection marker were scored for degeneration of ADE, PHA, PHB, and PQR neurons.

Suppressor screen on ot337 *mutants.* For the suppressor screen, *trp-4(ot337)* mutant worms were mutagenized using ethyl methanesulfonate (EMS) and standard protocols (Brenner, 1974; Doitsidou et al., 2008). An automated screen was performed using the COPAS Biosorter (Union Biometrica) as previously described (Doitsidou et al., 2008) with the sorting region set to select worms that had gained GFP signal.

Degeneration assays. Well-fed worms raised at 20°C for at least two generations were scored as freshly hatched L1s and/or mid L4 stage. Neurons were scored for cell body presence and morphology as well as axodentritic integrity.

RNAi assays. RNAi assays were performed using a bacterial feeding protocol (Kamath and Ahringer, 2003) and a *C. elegans* strain with a sensitized genetic background: eri-1;lin15b (Wang et al., 2005).

Fluorescence intensity quantification. Fluorescence intensity measurements were done as previously described (Gavet and Pines, 2010) using ImageJ (Schneider et al., 2012). The fluorescence was represented as Corrected Total Cell Fluorescence (CTCF) where, CTCF = Integrated Density − (Area of the selected cell × Mean fluorescence of background readings). Worms in lateral orientation were selected for analysis and fluorescent intensity was separately determined for proximal and distal CEPDs or CEPVs.

Chemical treatments. For the dantrolene and EGTA assays, dantrolene (10 μM) in DMSO or EGTA (10 mM) from Sigma was added to NGM plates (prepared without $CaCl_2$). For the BAPTA-AM assays, BAPTA-AM (Life Technologies) in DMSO was added to NGM plates (minus $CaCl_2$) at a final concentration of 100 μM. Progeny of worms grown on these plates were scored.

Behavioral assay. The basal slowing response was measured according to Sawin et al. (2000) with the following modifications: worms were video recorded and their velocities were calculated using WormLab 2.0 software (MBF Biosciences).

Statistical analysis. Prism6 (GraphPad) was used for statistical analysis. The sample size was ≥50 for all data points. For comparison of two means, the unpaired two-tailed *t* test was used. For more than two means, one-way ANOVA, and for group comparisons, two-way ANOVA were used. Multiple comparisons of the means were done by either Tukey's or Sidak's *post hoc* test; p values <0.05 were considered to be statistically significant.

Results

Isolation of *C. elegans* mutants with progressive degeneration of dopaminergic neurons

Dopaminergic neurons are critically involved in various human behaviors and pathologies and it has been a long-standing goal to understand how these neurons develop and maintain their differentiated properties. The 302-cell nervous system of a *C. elegans* hermaphrodite contains exactly eight dopaminergic neurons (a pair each of CEPVs, CEPDs, ADEs, and PDEs), making it easy to monitor the presence and integrity of these neurons in living animals with fluorescent protein labeling. Using a semi-automated selection scheme implemented by the COPAS Biosorter technology (Pulak, 2006; Doitsidou et al., 2008), we screened for EMS-mutagenized animals in which dopaminergic neurons were lost (Doitsidou et al., 2008). This phenotype could be caused either by a failure to execute the appropriate developmental program or by a failure to maintain the integrity of dopaminergic neurons after they are formed. This screen did indeed result in the identification of gene regulatory factors involved in dopaminergic neuron development (Doitsidou et al., 2008, 2013; Flames and Hobert, 2009). However, two viable mutants that we have not previously described, *ot337* and *ot477*, showed normal development of these neurons, i.e., in late stage embryos, the complete set of dopaminergic neurons is present (Fig. 1*A*,*B*). Yet, in adult animals, the majority of dopaminergic neurons are lost (which is the phenotype the mutants were selected for; Fig. 1*A*,*B*). Analysis of animals from different stages showed that in both alleles the loss of dopaminergic neurons is progressive (Fig. 1*B*; data not shown). Moreover, the progressive neurodegeneration phenotype of the *ot337* and *ot477* alleles is semidominant, with heterozygous animals showing degeneration of their dopaminergic neurons, albeit to a lesser degree than the homozygous animals (Fig. 1*C*). Furthermore, we observed that only the head dopaminergic neurons were affected in these mutants whereas no degeneration was observed in the postembryonically generated

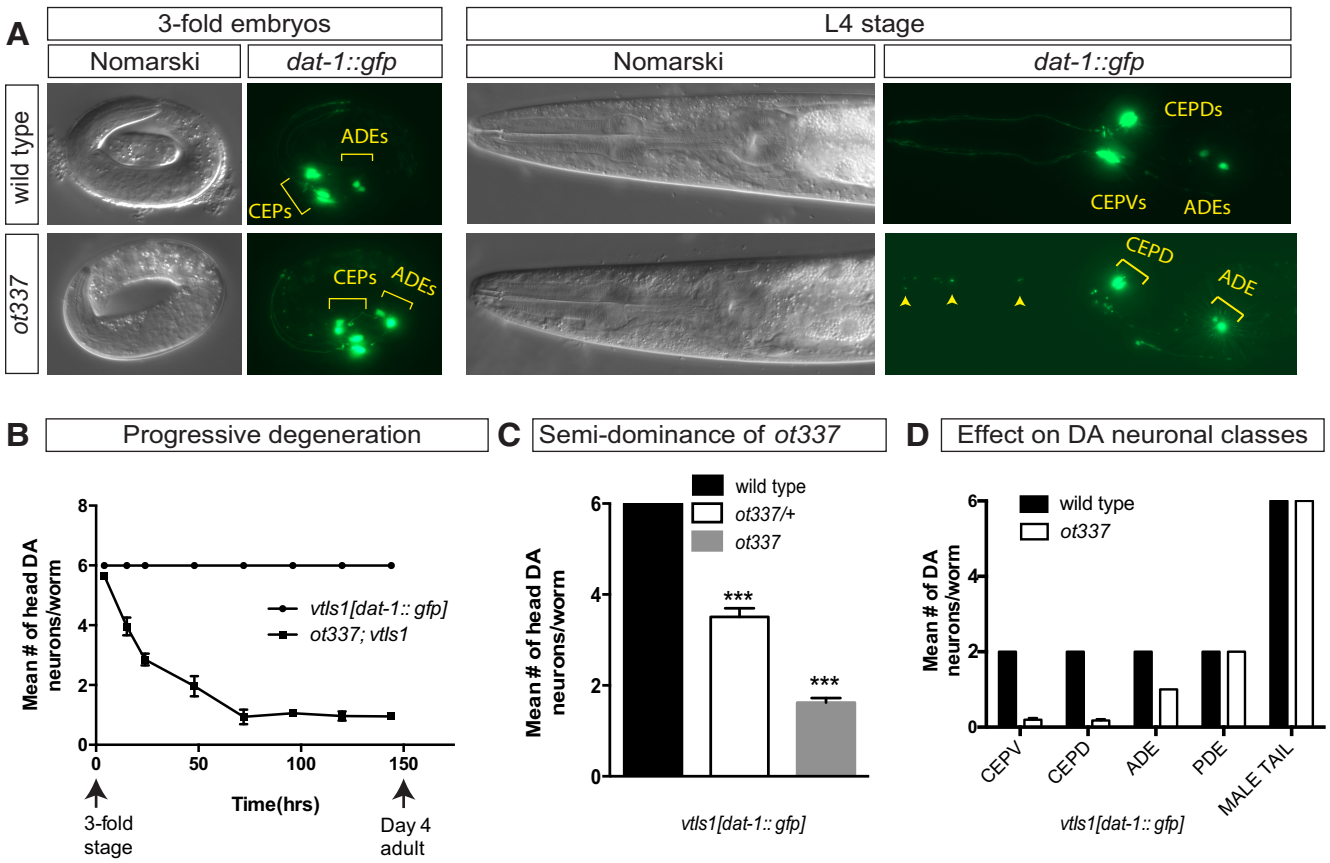

Figure 1. Selective loss of dopaminergic neurons in *ot337* mutants. ***A***, ***B***, Progressive loss of dopaminergic (DA) neurons labeled with *dat-1::gfp (vtIs1)* in *ot337* mutants. Arrowheads indicate degenerating CEPD dendrite. ***C***, *ot337* mutants are semidominant. ***D***, Cell-type specificity of dopaminergic neuron loss. Error bars represent ± SEM around the mean, ***$p < 0.001$.

mid-body PDE neurons and the male tail dopaminergic neurons (Fig. 1*D*).

A gain-of-function mutation in the TRP channel *trp-4* causes degeneration

We mapped both *ot337* and *ot477* on the right arm of chromosome I and identified the molecular lesions through whole-genome sequencing. Both alleles, which were independently isolated, showed a small number of variants in protein-coding genes on chromosome I, yet only one locus was affected in both strains (Fig. 2*A*). This locus, termed *trp-4* (Li et al., 2006), encodes a TRPN-type channel of the TRP six-transmembrane channel family. TRP-4 protein was previously described as a cell-surface mechanoreceptor expressed in dopaminergic neurons (Li et al., 2006; Kang et al., 2010; Li et al., 2011). Both of the mutations that we isolated affect the same amino acid in the pore-forming sixth transmembrane helix of TRP-4 (Fig. 2*B*,*C*). Substitutions in adjacent amino acids in TRP channel orthologs in yeast were previously shown to be gain-of-function mutations resulting in gate destabilization and a higher probability of the channel being in an open state (Su et al., 2007; Zhou et al., 2007).

To confirm that the identified dominant *trp-4* mutations in *ot337* and *ot477* are indeed responsible for the degeneration phenotype, we conducted reversion-of-phenotype screens, reasoning that the neurodegeneration phenotype should be suppressed by loss-of-function mutations in *trp-4*. We mutagenized *ot337* animals that express GFP in their dopaminergic neurons and screened using the semi-automated selection scheme. We identified 16 mutants in which the GFP signal in adult animals is restored, thus indicating that the normally susceptible dopaminergic neurons do not degenerate. All 16 suppressor mutants contain presumptive loss-of-function mutations in *trp-4*, including several premature stop codons, deletions, and splice site mutations (Fig. 2*C*) indicating that *ot337* allele harbors a gain-of-function mutation. Apart from confirming that the *trp-4(ot337)* substitution is indeed the phenotype-causing mutation, the retrieved suppressor mutants may also provide some insights about functionally important residues of the TRP-4 protein. Specifically, *trp-4(ot749)* results in an alanine to valine substitution (A1064V) of a highly conserved alanine of the 21st ankyrin repeat, *trp-4(ot756)* in a substitution of a highly conserved glycine (G1735R) in the pore-forming region, *trp-4(ot755)* in a proline to leucine substitution (P1749L) between the pore-forming region and the sixth transmembrane domain, and *trp-4(ot757)* carries two mutations in the cytosolic C-terminal tail of TRP-4 (R1845C+L1876H).

To further confirm the degeneration effects of the *trp-4(ot337)* locus, a *trp-4* cDNA that carries the *ot337* mutation was expressed under the control of a dopamine neuron-specific promoter from the *dat-1* (dopamine transporter) locus. Transgenic animals expressing this construct recapitulated the dopaminergic neuron degeneration phenotype (Fig. 2*D*). Moreover, in these transgenic animals we observed robust degeneration of all dopaminergic neuronal classes, including PDEs and male tail dopaminergic neurons, which do not typically degenerate in the *ot337* mutants. Since transgenic arrays are usually multicopy in *C. elegans*, we interpret this finding as an indication of the importance of gene dosage.

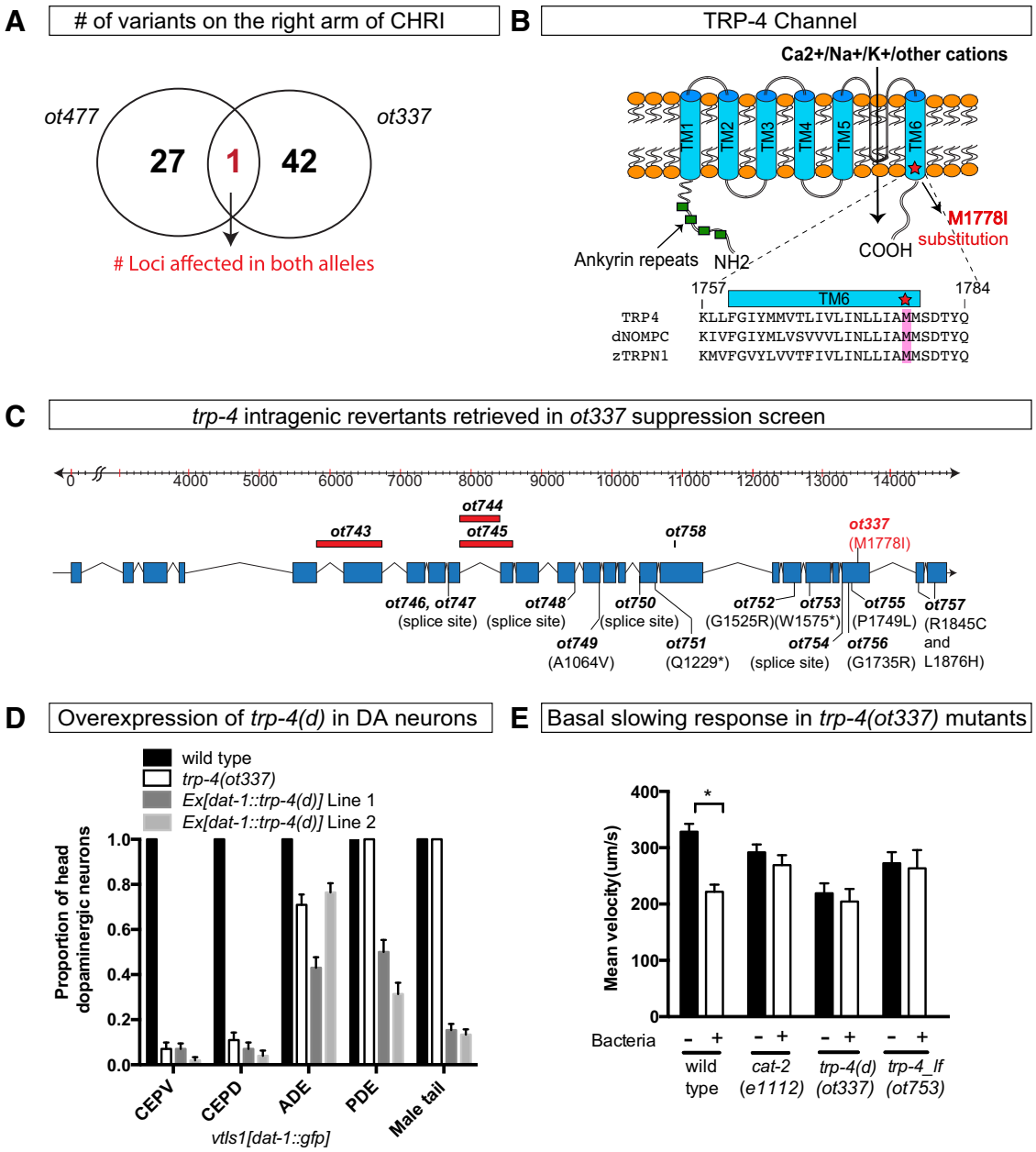

Figure 2. Molecular characterization of *trp-4*. ***A***, Venn diagram depicting the number of molecular lesions identified in two whole-genome sequenced alleles, *ot477* and *ot337*. Only one locus within the mapping region was affected in both strains (*trp-4*). ***B***, Schematic drawing of the predicted structure of TRP-4 channel (based on alignment with the *Drosophila* ortholog NOMPC; Walker et al., 2000). Location of the gain-of-function allele is depicted (star) and alignment of the region adjacent to the sixth transmembrane domain, bearing the *ot337* mutation. dNOMPC and zTRPN are, respectively, the *Drosophila* and zebrafish orthologs of *trp-4*. ***C***, *trp-4* locus with gain-of-function mutation *ot337* and intragenic suppressor mutations. ***D***, Transgenic animals containing *trp-4(d)* cDNA expressed under control of the *dat-1* promoter show degeneration phenotype in their dopaminergic (DA) neurons. ***E***, Basal slowing response in *trp-4(ot337)* mutants is defective (lf: loss of function). Error bars represent ± SEM around the mean, ***$p < 0.001$.

To assess the behavioral consequences of the *ot337* mutation in the TRP-4 channel, we measured the basal slowing response in the *trp-4(d)* mutants. This is a previously characterized dopamine-dependent behavioral response mediated redundantly by the various classes of dopamine neurons as a result of mechanosensory stimuli presented by food (Sawin et al., 2000). Well-fed, wild-type worms showed a distinctive slowing behavior in the presence of food as assessed by locomotion velocity (Fig. 2*E*), which was abolished in dopamine-deficient *cat-2* mutants. *trp-4(d)* mutants were equally defective in basal slowing response, despite the fact that not all dopaminergic neurons degenerate in these mutants. Similar defects in basal slowing response are observed in *trp-4* loss-of-function mutants (Kang et al., 2010; Figure 2*E*), indicating that the *ot337* gain-of-function allele is defective for at least some aspects of the TRP-4 channel function.

trp-4(d) degeneration is dopamine independent

trp-4 is expressed in all dopaminergic neurons, as previously reported (Li et al., 2006), and as assessed by us using a previously described reporter gene construct (Barrios et al., 2008; data not shown). *trp-4* is also expressed in 14 additional neurons in the head (data not shown) and in the DVA and DVC tail neurons (Li et al., 2006). In addition to dopaminergic neurons, a degeneration phenotype was observed in other *trp-*

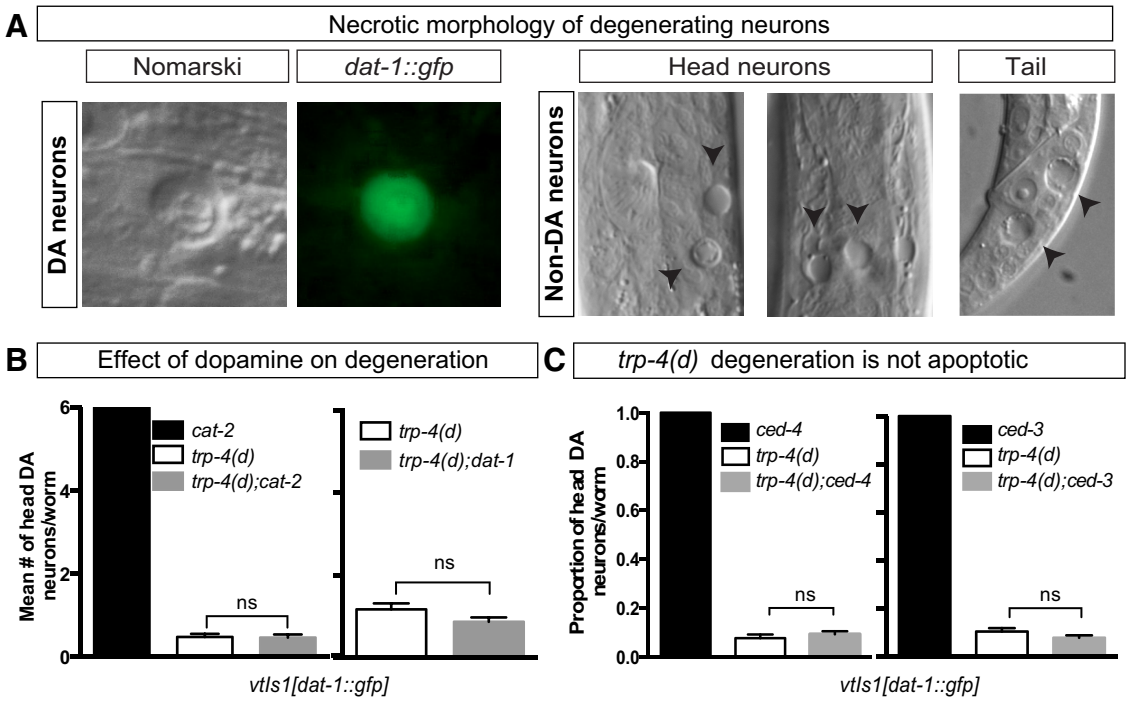

Figure 3. Characteristics of cell death in *trp-4(d)* mutants. ***A***, Necrotic morphology of degenerating dopaminergic (DA) and non-dopaminergic neuronal types in *trp-4(d)* animals (cell swelling). Eventually the cell bodies disappear. Arrowheads point to cell bodies with necrotic morphology. ***B***, Degeneration does not depend on dopamine. *cat-2* and *dat-1* mutations do not suppress *trp-4(d)* dopaminergic degeneration. *trp-4(d)*-induced degeneration does not depend on intracellular dopamine levels. ***C***, The *trp-4(d)* phenotype is not suppressed by apoptotic mutants, *ced-4* and *ced-3*. Error bars represent ± SEM around the mean. ns: $p > 0.05$.

4-expressing neuronal cell types including the DVA and DVC neurons (Fig. 3*A*). Consistent with the observation that degeneration is not dopaminergic neuron specific, we find that the degree of degeneration does not depend on dopamine levels: neither removal of dopamine in *cat-2* mutants (the *C. elegans* ortholog of tyrosine hydroxylase, catalyzing the rate-limiting step in dopamine synthesis) nor interfering with dopamine reuptake from the synapse in *dat-1* mutants (dopamine transporter) had any effect on the severity of dopaminergic degeneration (Fig. 3*B*).

TRP-4(d) channel does not cause degeneration in all neuronal types when ectopically expressed

Since degeneration in *trp-4(d)* mutants is not dependent on dopamine or dopaminergic neuron specific, we tested whether the mutated TRP-4(d) channel can induce degeneration in other neuronal subtypes where it is normally not expressed. We ectopically expressed the mutated channel under the control of *osm-6* promoter and used an *osm-6::gfp* transgenic background to visualize the relevant neurons. *osm-6* is expressed in the majority of ciliated neurons (56 neurons, including the dopaminergic neuronal classes; Collet et al., 1998). To bypass quantification difficulties due to the high density of *osm-6*-expressing neurons near the nerve ring, we focused our analysis on the more posterior *osm-6*-expressing neuronal classes: the dopaminergic ADEs that normally express TRP-4, and the tail neurons PHA (L and R), PHB (L and R), and PQR that do not normally express TRP-4. In *Posm-6::trp-4(d)* transgenic worms, PHA, PHB, and PQR neuronal classes remained unaffected, in contrast to the ADEs that degenerated in >80% of the worms. Furthermore, we did not observe any obvious degeneration phenotype in the *osm-6*-expressing neurons around the nerve ring (data not shown). Thus, TRP-4(d) was not able to induce degeneration ectopically in all neuronal subtypes.

trp-4(d) degeneration of dopaminergic neurons is independent of apoptosis and exhibits characteristics of necrosis

Even though progressive dopaminergic neurodegeneration has not yet been directly associated with TRP-type ion channels, a few cases of TRP channelopathies affecting other neuronal subtypes have been reported in humans (Kremeyer et al., 2010; Nilius and Owsianik, 2010). In addition, at least three *in vivo* animal models of gain-of-function mutations in TRP channels have been identified: one in flies, affecting photoreceptors (Yoon et al., 2000); two in mice, affecting sensory hair cells (Waddler mouse) and Purkinje cells (moonwalker mouse; Kim et al., 2007; Becker et al., 2009). These TRP gain-of-function models result in degenerative phenotypes, but the nature of cell death has not been characterized. We examined *trp-4(ot337)* mutant animals in more detail and noted that dopaminergic neurons show features characteristic of necrosis, which in addition to apoptosis and autophagy, is one of the three best characterized cell death pathways (Edinger and Thompson, 2004). In contrast to apoptotic cells that show characteristic shrinking and blebbing morphology, necrotic cells swell before they disintegrate, a phenotype that we observed in *trp-4(d)* mutants (Fig. 3*A*). Furthermore, mutations that eliminate two executioners of apoptosis, *ced-3* and *ced-4*, have no effect on *trp-4(ot337)*-induced degeneration (Fig. 3*C*), ruling out involvement of the apoptotic cell death pathway.

Involvement of intracellular Ca^{2+} and exit of Ca^{2+} from the ER in *trp-4(d)* neurodegeneration

Members of the TRP channel family are mostly nonspecific cation channels, able to conduct monovalent as well as bivalent

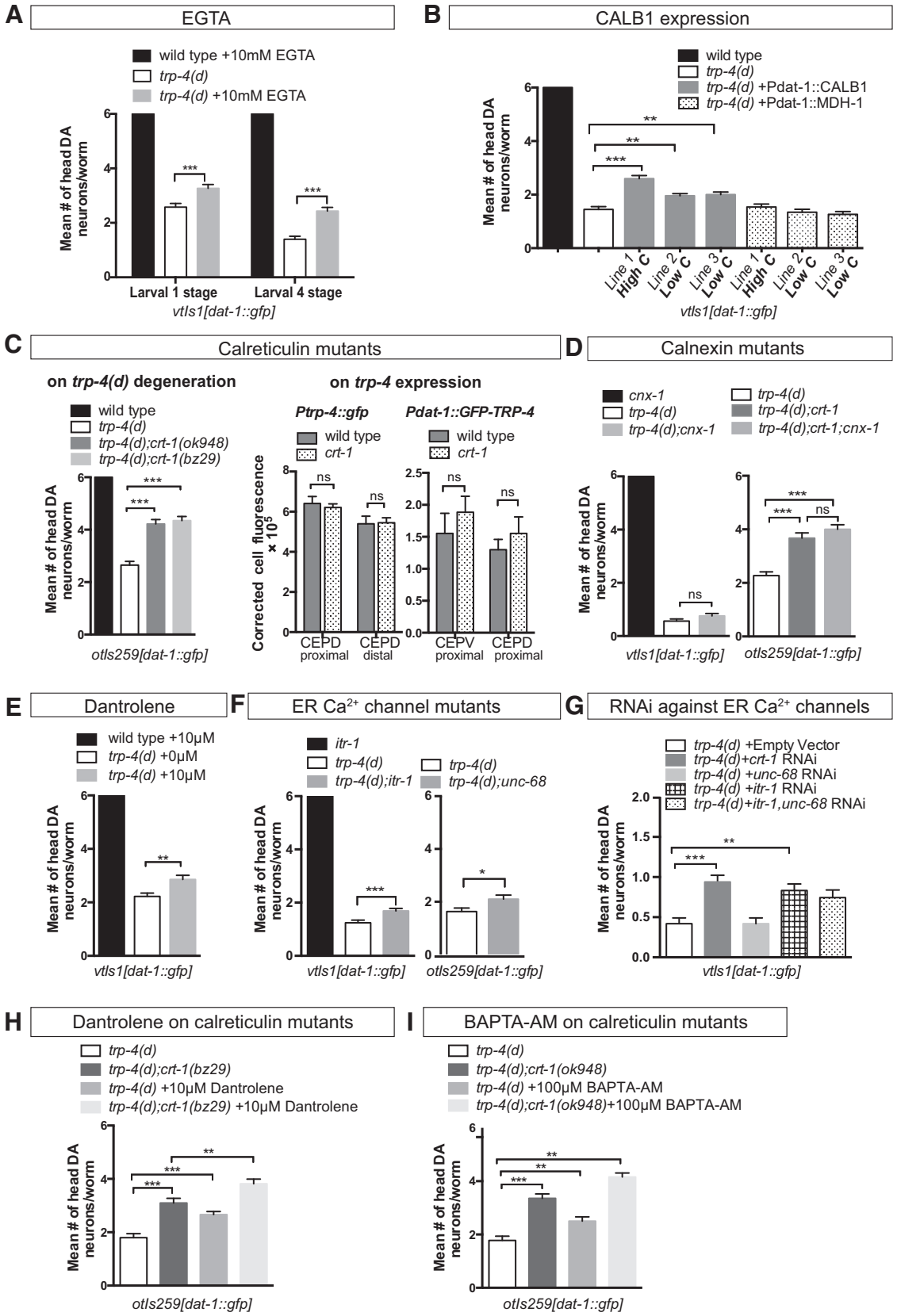

Figure 4. Involvement of intracellular Ca^{2+} and exit of Ca^{2+} from the ER in *trp-4(d)* neurodegeneration. ***A***, Chelating cytoplasmic calcium with EGTA suppresses *trp-4(d)*-induced neuronal degeneration. ***B***, Expression of human calbindin (CALB1) suppresses *trp-4(d)*-induced neuronal degeneration (injected concentrations: Line 1 = 25 ng.μl, lines 2,3 = 7.5 ng/μl). Corresponding negative control, expression of malate dehydrogenase (*mdh-1b*), had no effect. ***C***, Left, Calreticulin (*crt-1*) mutation suppresses *trp-4(d)*-induced dopaminergic (DA) degeneration. Middle and right, Calreticulin mutation does not affect *Ptrp-4::gfp* or Pdat-1::GFP-TRP-4 expression levels. Average fluorescent intensities of the indicated CEPs were calculated (see Materials and Methods). ***D***, Calnexin does not affect *trp-4(d)*-induced neuronal degeneration. ***E***, Dantrolene suppresses *trp-4(d)*-induced neuronal degeneration. ***F***, Mutations in the ER inositol triphosphate and ryanodine receptors partially suppress *trp-4(d)*-induced dopaminergic neuronal degeneration. ***G***, RNAi against ryanodine receptors on *trp-4(d)* mutants. The RNAi "sensitized" strain (*Figure legend continues*.)

cations (Venkatachalam and Montell, 2007). With the exceptions of TRPM4 and TRPM5, which are only permeable to monovalent cations, all functionally characterized TRP channels are permeable to Ca^{2+}. To explore the possibility that intracellular Ca^{2+} dyshomeostasis is a contributing factor in *trp-4(d)*-induced degeneration, cytoplasmic Ca^{2+} was pharmacologically and genetically chelated in these mutants. Treatment of *trp-4(d)* mutants with EGTA significantly suppressed dopaminergic degeneration (Fig. 4*A*). Another way to chelate cytoplasmic Ca^{2+} is to overexpress an intracellular "calcium sink," such as calbindin, a Ca^{2+} binding protein (Mattson et al., 1991). *trp-4(d)* worms expressing human calbindin (CALB1) under the control of the *dat-1* promoter exhibited significantly lower levels of neuronal loss, whereas no such effect was observed when a non-calcium binding gene was expressed (Fig. 4*B*).

Other types of channelopathies involving DEG/ENaC-type ion channels also result in necrotic cell death and involve specific downstream components that regulate Ca^{2+} storage in the ER (Xu et al., 2001; Barbagallo et al., 2010). Exit of Ca^{2+} from the ER as a response to hyperactivated DEG/ENaC channels was shown to contribute to neuronal cell death (Xu et al., 2001; Bianchi et al., 2004; Barbagallo et al., 2010). We tested the involvement of these components in *trp-4(d)*-induced neurodegeneration. Loss-of-function mutants of the Ca^{2+} binding ER chaperone calreticulin (*crt-1*), the main ER Ca^{2+} store, significantly suppressed dopaminergic degeneration (Fig. 4*C*). *trp-4* transcript and TRP-4 protein levels were unaffected in *crt-1* mutants (Fig. 4*C*). Mutations in another ER chaperone, calnexin (*cnx-1*), which has comparatively less Ca^{2+}-binding capacity than CRT-1 (Groenendyk et al., 2006), neither suppressed degeneration nor enhanced the effects of *crt-1* mutants (Fig. 4*D*).

In agreement with a role for ER Ca^{2+} exit in *trp-4(d)*-induced neurodegeneration, pharmacological inhibition of Ca^{2+} exit from the ER in *trp-4(d)* mutants using the ryanodine receptor antagonist dantrolene (Zhao et al., 2001) partially suppressed dopaminergic neurodegeneration (Fig. 4*E*). Similar results were obtained using mutations in channels responsible for Ca^{2+} exit from the ER, in particular inositol triphosphate receptor (*itr-1*) and *unc-68* (a ryanodine receptor; Fig. 4*F*), as well as with RNAi treatment against *itr-1* in *trp-4(d)* mutants (Fig. 4*G*).

Combining the genetic depletion of Ca^{2+} store in the ER with the pharmacological inhibition of ryanodine receptors resulted in a stronger suppression of *trp-4(d)* degeneration than each treatment alone (Fig. 4*H*). Furthermore, when double mutants for *crt-1* and *trp-4(d)* were treated with BAPTA-AM, a membrane-permeable calcium chelator, the result was an additive effect in the suppression levels of dopaminergic degeneration (Fig. 4*I*).

Discussion

Here we introduce a novel model for a neurodegenerative channelopathy, showing that gain-of-function mutations in a TRP ion channel cause progressive loss of dopaminergic neurons as well as other neuronal types. We have shown that dopaminergic neurons die through mechanisms independent from classical apoptosis pathways, and that the dying cells exhibit morphological characteristics of necrotic cell death. Furthermore, we provide evidence that intracellular calcium homeostasis as well as calcium exit from the ER contribute to the process of *trp-4(d)*-induced neuronal cell death.

The many ways in which Ca^{2+} influx can occur across the plasma membrane include voltage-gated, ligand-gated, receptor-operated, and store-operated Ca^{2+} channels (Brini et al., 2014). TRP channel family members are not only highly permeable to Ca^{2+}, but also have been shown to participate in the above mechanisms of Ca^{2+} signaling and influx (Gees et al., 2010). In relevance to degeneration, cases of gain-of-function mutations in TRP channels have been reported that cause an increased calcium influx (Reiser et al., 2005; Grimm et al., 2007; Kim et al., 2007; Klein et al., 2011). Furthermore, members of TRPC channel family were shown to contribute to the mechanism of Huntington's disease, through interaction with the store-operated Ca^{2+} entry pathway (Wu et al., 2011) and to mediate glutamate-induced excitotoxic neuronal death by promoting cellular Ca^{2+} overload (Narayanan et al., 2008). Whether the mutated TRP-4(d) channel conducts toxic levels of Ca^{2+} directly, or causes Ca^{2+} dysregulation through indirect mechanisms, remains to be determined. Consistent with a role for membrane depolarization and voltage-gated calcium channel (VGCC) activation in our model, we observed partial suppression of *trp-4(d)* degeneration in knockdown experiments of some (*cca-1*) but not other (*egl-19* and *unc-2*) VGCC α-subunits (M. Doitsidou and A. Nagarajan, unpublished data). *In vivo* experiments that systematically address the involvement of different types of calcium influx pathways (mentioned above) as well as redundancy issues between them will further illuminate the mechanisms of calcium toxicity in TRP-4(d)-mediated dopaminergic degeneration.

According to our observations, the various classes of dopamine neurons are not equally susceptible to *trp-4(d)* degeneration: CEPVs and CEPDs show nearly complete degeneration by adult stage, whereas ADEs are only partially affected and PDEs are completely unaffected. Different levels of TRP-4 protein could be partly responsible for their differential susceptibility to degeneration. Indeed, overexpression of the *trp-4(d)* cDNA in dopamine neurons caused partial degeneration of PDEs. Nevertheless, even through overexpression we were unable to cause PDEs to degenerate as frequently as the head dopaminergic neurons, indicating that there might be intrinsic differences between neuronal classes that render them differentially susceptible to degeneration, for example, availability of channel subunits or downstream effectors of degeneration. In agreement with this hypothesis, introducing TRP-4(d) channel in neurons that normally do not express it was not sufficient to induce degeneration.

Despite the fact that some dopaminergic neurons are spared in *trp-4(d)* mutants, their function appears to be defective. Previous ablation studies (Sawin et al., 2000) have shown that various classes of dopaminergic neurons act redundantly to mediate basal slowing response and the presence of PDEs alone is sufficient to elicit this behavior. *trp-4(d)* mutants were, in contrast, completely defective for basal slowing response, similar to the *trp-4*-null or dopamine-deficient mutants, indicating that the presence of the mutated channel in the remaining dopamine neurons has functional consequences.

Mutations in TRP family channels have not been previously implicated in direct degeneration of dopaminergic neurons. Interestingly, an association has been reported between a variant in TRPM7 channels and environmentally triggered Parkinsonism dementia in a population of Guam (Hermosura et al., 2005).

←

(*Figure legend continued.*) *trp-4(ot337);eri-1;lin15b;vtIs1* was used. ***H***, Combining genetic deletion of *crt-1* and pharmacologic inhibition of ryanodine receptors results in stronger suppression of degeneration. ***I***, Combining genetic deletion of *crt-1* and intracellular Ca^{2+} chelation with BAPTA-AM results in stronger suppression of degeneration. In all parts, we refer to the *trp-4(ot337)* allele as *trp-4(d)*. Error bars represent ± SEM around the mean. ns: $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

However, direct evidence that TRPM7 variants are causal to this condition is yet to be obtained. There are 27 members of the TRP channel family in humans (Venkatachalam and Montell, 2007), several of which are known to be expressed in the substantia nigra dopaminergic neurons (Riccio et al., 2002; Tozzi et al., 2003; Guatteo et al., 2005). Among those, TRPC channels have received special attention for their protective role in MPP+ models of dopaminergic neurotoxicity (Selvaraj et al., 2009). With the advent of next generation sequencing technologies it would be very pertinent to examine whether TRP channel loci harbor risk-modifying variants for Parkinson's disease.

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