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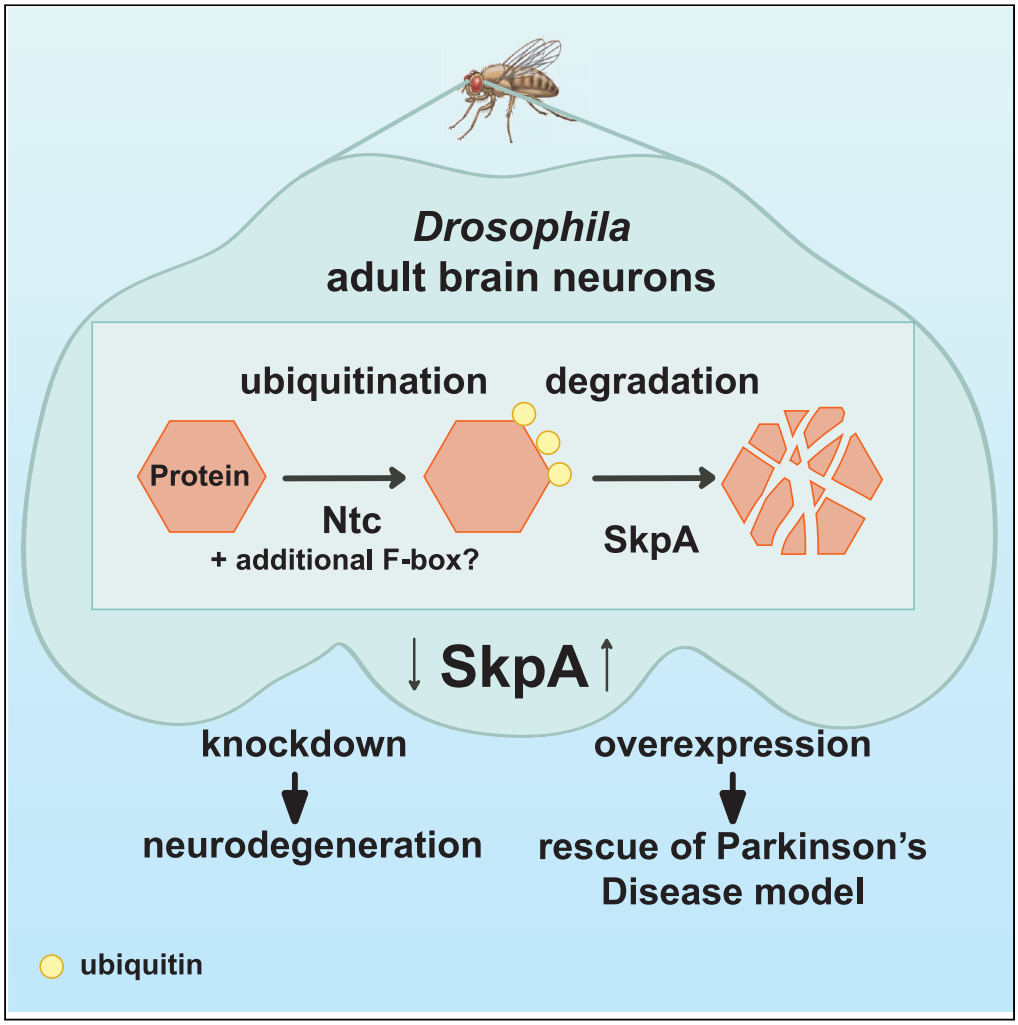

Lital Dabool, Ketty Hakim-Mishnaevski, Liza Juravlev, Naama Flint-Brodsly, Silvia Mandel, Estee Kurant

ekurant@univ.haifa.ac.il

HIGHLIGHTS

SkpA-mediated protein degradation is required for normal function of the adult brain

SkpA overexpression rescues neurodegeneration in α-synuclein-induced fly PD model

SkpA and Ntc work in the same pathway of protein degradation in adult brain neurons

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Drosophila Skp1 Homologue SkpA Plays a Neuroprotective Role in Adult Brain

Lital Dabool,[1,2] Ketty Hakim-Mishnaevski,[1] Liza Juravlev,[1] Naama Flint-Brodsly,[1] Silvia Mandel,[2] and Estee Kurant[1,2,3,*]

SUMMARY

Skp1, a component of the ubiquitin E3 ligases, was found to be decreased in the brains of sporadic Parkinson's disease (PD) patients, and its overexpression prevented death of murine neurons in culture. Here we expose the neuroprotective role of the *Drosophila skp1* homolog, *skpA*, in the adult brain. Neuronal knockdown of *skpA* leads to accumulation of ubiquitinated protein aggregates and loss of dopaminergic neurons accompanied by motor dysfunction and reduced lifespan. Conversely, neuronal overexpression of *skpA* reduces aggregate load, improves age-related motor decline, and prolongs lifespan. Moreover, SkpA rescues neurodegeneration in a *Drosophila* model of PD. We also show that a *Drosophila* homolog of FBXO7, the F Box protein, Nutcracker (Ntc), works in the same pathway with SkpA. However, *skpA* overexpression rescues *ntc* knockdown phenotype, suggesting that SkpA interacts with additional F box proteins in the adult brain neurons. Collectively, our study discloses Skp1/SkpA as a potential therapeutic target in neurodegenerative diseases.

INTRODUCTION

Parkinson's disease (PD) is the second most common heterogenic degenerative brain disorder involving multiple etiologies. Various mechanisms have been implicated in PD neurodegeneration. The majority of cases are sporadic but certain cases are familial, driven by both genetic and environmental factors (Mandel et al., 2007; Shulman et al., 2011). It is primarily characterized by the specific degeneration of dopaminergic (DA) neurons in the substantia nigra (SN), where intraneuronal aggregates named Lewy bodies, are typically found. The major component of these protein-rich aggregates is the presynaptic protein α-Synuclein (α-Syn) (Chen and Feany, 2005; Mandel et al., 2007; Warner and Schapira, 2003).

The ubiquitin/proteasome system (UPS) plays a central role in maintaining cellular homeostasis by degrading and recycling most intracellular proteins. A set of three different enzymes, E1 activating enzyme, E2 conjugating enzyme, and E3 ligase enzyme, act in a sequential manner to tag proteins with ubiquitin moieties and target them for proteasomal degradation (Chen and Dou, 2010). Because UPS dysfunction leads to protein aggregation, and because protein aggregates in various neurodegenerative diseases (ND) frequently appear ubiquitin conjugated, it is assumed that the UPS function might be compromised in ND (Zheng et al., 2016). E3 ubiquitin ligases are responsible for substrate specificity, providing selectivity of target proteins for proteasomal degradation (Chen and Dou, 2010). A large class of ubiquitin ligases is the E3 Skp1/Cullin1/F box protein (SCF). Whereas the S-phase kinase-associated protein 1 (Skp1) and Cullin1 are essentially invariable components of the SCF complexes, several dozens of distinct F box proteins exist, determining substrate specificity by binding to different protein substrates through protein-protein interaction domains, and to Skp1 through the F box domain, Skp1 in turn binds to Cullin1 (Lee and Diehl, 2014). Importantly, a robust decrement in the Skp1 gene and protein expression was detected in the SN of postmortem parkinsonian brains (Grunblatt et al., 2004; Mandel et al., 2007), and Skp1 protein has been listed among the predictive blood signatures with high discriminating power to categorize early PD patients (Molochnikov et al., 2012). Furthermore, mutations in an F box protein, FBXO7, were associated with autosomal recessive early onset of PD (Di Fonzo et al., 2009). Whereas these findings highlight Skp1 as a potential important factor in PD, its function in both healthy and diseased human brains remains unclear.

Drosophila is a powerful model for studying the molecular and cell biological mechanisms of human diseases *in vivo* (Dionne and Schneider, 2008; Lee and Sun, 2015; McGurk et al., 2015; Michno et al., 2005;

[1]Department of Human Biology, Faculty of Natural Sciences, University of Haifa, 199 Aba Khoushy Avenue, Mount Carmel, Haifa 34988-38, Israel

[2]The Rappaport Family Institute for Research in the Medical Sciences, Faculty of Medicine, Technion - Israel Institute of Technology, Haifa 31096, Israel

[3]Lead Contact

*Correspondence: ekurant@univ.haifa.ac.il

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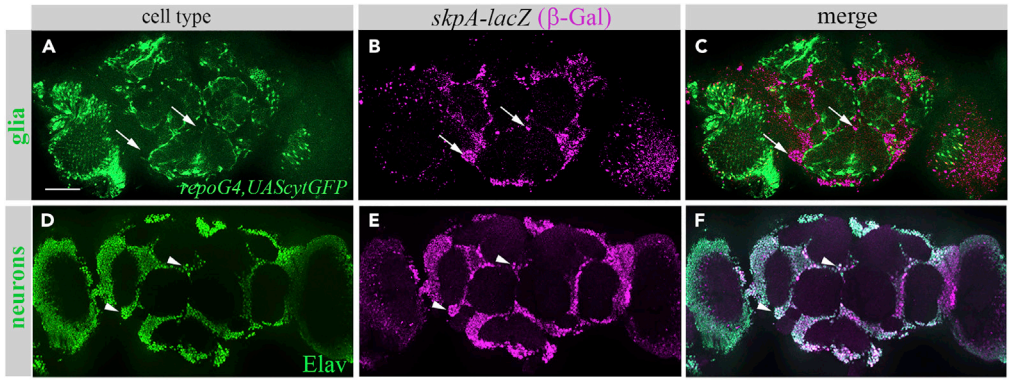

Figure 1. *skPA* Is Specifically Expressed in Adult Brain Neurons

Projections from apotome stacks of whole-mount entire brains of 5-day-old females developed at 29°C. (A–C) *skpA-lacZ/+;tubGal80^{ts}; repoGal4,UAScytGFP*.

(A) Glial cells are labeled with GFP in green.

(B) *skpA* expression is marked in magenta by an anti-β-Gal antibody.

(C) No overlap is detected between glial cells and β-Gal expression (arrows).

(D–F) *skpA-lacZ/FM7*. (D) Neurons are labeled with an anti-Elav antibody in green. (E) *skpA* expression is marked by the anti-β-Gal antibody in magenta. (F) An overlap between β-Gal and Elav immunostaining indicates *skpA* expression in neurons (arrowheads). Bar: 50 μm.

Pandey and Nichols, 2011). The *Drosophila* genome contains six *skp1* homologs (*skpA-F*), of which *skpA* shares the highest similarity with human *skp1* (76%) and is the most widely expressed. It was found to be necessary for larval growth and viability (Murphy, 2003). Different studies have implicated *skpA* in apoptosis regulation through ubiquitination of pro- and anti-apoptotic proteins (Bader et al., 2010; Fereres et al., 2013), negative regulation of innate immunity (Aparicio et al., 2013; Khush et al., 2002), and guiding dendritic and axonal pruning during metamorphosis (Wong et al., 2013). In fly models of polyQ neurodegenerative diseases, SkpA was reported to modify neurodegeneration in the eye, increasing aggregate load and toxicity upon eye-specific knockdown, implying possible involvement in pathogenesis of these ailments (Bhutani et al., 2012). SkpA was also found to bind to the F box protein Nutcracker (Ntc), which was discovered in a screen for regulators of non-lethal caspase activation and sperm terminal differentiation in *Drosophila* (Arama et al., 2007; Bader et al., 2010). Ntc and its closest mammalian homolog, the PD-linked protein FBXO7, share 27% amino acid sequence similarity, which is much higher in the conserved active protein domains (Merzetti et al., 2017). Consistent with the PD linkage of its human homolog, Ntc was recently shown to partially rescue climbing defects and precocious death in α-Syn-expressing flies proposing a similar role in neurodegeneration (Merzetti et al., 2017).

Here we demonstrate that *skpA* is required for normal function of the adult brain; *skpA* knockdown in adult stage neurons increases aggregate load and causes loss of DA neurons accompanied by motor decline and shortened lifespan. Furthermore, we show that *skpA* overexpression significantly rescues neurodegeneration in α-Syn-induced fly PD model, as well as prevents accumulation of protein aggregates, improves motor ability and survival rate of wild-type flies, therefore uncovering a neuroprotective role of SkpA in the adult brain. We further reveal that SkpA interacts with Ntc and likely with alternative F box proteins emphasizing its central role in the degradation of neuronal proteins. Taken together, these findings implicate Skp1/SkpA as an important potential target for diagnosis and therapy in ND. Given that the human genome contains only one functional Skp1 isoform (Global Variome shared LOVD), our *in vivo* data place Skp1 at a strategic point for possible intervention in neurodegenerative processes.

RESULTS

skpA Is Specifically Expressed in Adult Brain Neurons

To start exploring possible roles for SkpA in the adult brain, we first learned about its expression pattern in this tissue. For this, we examined flies carrying a *lacZ* exon trap insertion into the *skpA* locus (*skpA-lacZ*) and stained the dissected brains with anti-β-Gal antibodies. The glial and neuronal cells were respectively labeled with cytoplasmic GFP driven by the pan-glial *repoGal4* (*repoGal4;UAScytGFP*) (Figures 1A and 1C) and by staining with the neuron-specific anti-Elav antibody (Figures 1D and 1F). β-Gal, which

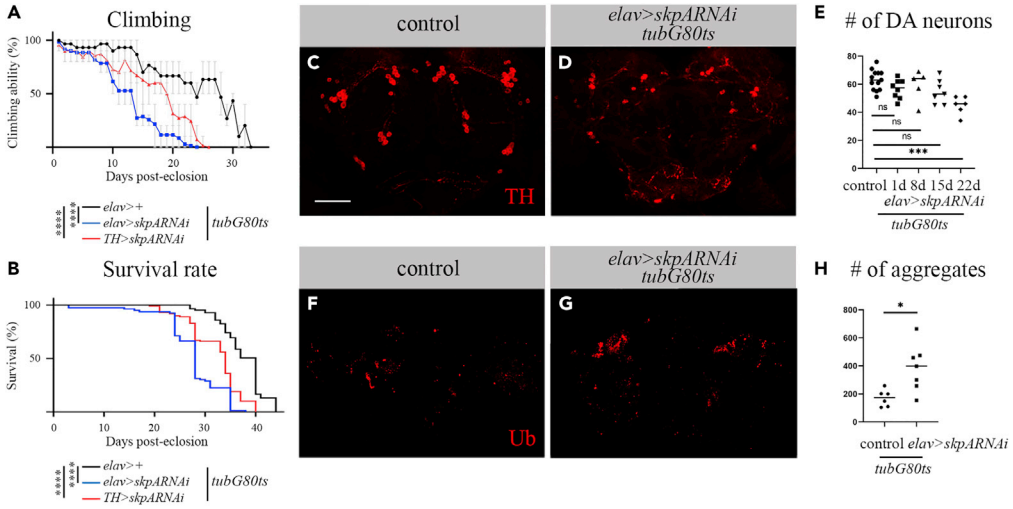

Figure 2. *skpA* Knockdown in Adult Brain Neurons Causes Accumulation of Ubiquitin-Tagged Protein Aggregates and Neuronal Loss Accompanied by Reduced Climbing Ability and Survival Rate

(A and B) (A) Climbing ability and (B) survival rate of flies expressing *skpARNAi* in a pan-neural manner (*elavGal4/+;tubGal80^{ts}/UASskpARNAi*) (blue) or in DA neurons alone (*THGal4/+;tubGal80^{ts}/UASskpARNAi*) (red) compared with control flies (*elavGal4/+;tubGal80^{ts}/+*) (black) at 29°C from 1 DPE. (A) Transgenic flies expressing *skpARNAi* in all neurons display a higher decline in climbing ability as compared with flies expressing *skpARNAi* in DA neurons. (B) Transgenic flies expressing *skpARNAi* in a pan-neural pattern present a shorter lifespan compared with flies expressing *skpARNAi* in DA neurons alone. n = 12 represents the number of vials per genotype.

(C and D) Projections from apotome stacks of the posterior part (~45μm) of whole-mount female brains 22 DPE. (C) Control (*elavGal4/+;tubGal80^{ts}/+*). (D) Transgenic fly expressing *skpARNAi* in a pan-neural pattern (*elavGal4/+;tubGal80^{ts}/UASskpARNAi*). DA neurons are labeled with an anti-TH antibody (red). Bar: 50 μm.

(E) The mean total numbers of DA neurons in transgenic female flies at different ages expressing *skpARNAi* specifically in adult brain neurons (*elavGal4/+;tubGal80^{ts}/UASskpARNAi*); n = 8 for 1 DPE, n = 5 for 8 DPE, n = 7 for 15 DPE, and n = 6 for 22 DPE. n = 13 for control (*elavGal4/+;tubGal80^{ts}/+*).

(F and G) Projections from apotome stacks of whole-mount entire female brains 25 DPE. (F) Control (*elavGal4/+;tubGal80^{ts}/+*). (G) Transgenic fly expressing *skpARNAi* in a pan-neural pattern (*elavGal4/+;tubGal80^{ts}/UASskpARNAi*). Ubiquitinated proteins in the adult brains (posterior view) are detected with an anti-Ub antibody (red). Bar: 50 μm.

(H) The mean total number of aggregates within apotome stacks of adult brains. n = 6 for control, n = 7 for *skpA* knockdown fly brains. Data are represented as mean ± SEM. Statistical significance was analyzed employing two-way ANOVA or students' t test, ****p < 0.0001, ***p < 0.001, *p < 0.05, n.s. = non-significant.

designates the expression pattern of *skpA* (Figures 1B, 1C, 1E, and 1F), did not overlap with glial GFP (Figure 1C), but colocalized with Elav, indicating that in the adult fly brain *skpA* is specifically expressed in neurons but not in glial cells (Figure 1F).

Lack of *skpA* in Adult Brain Neurons Leads to Neurodegeneration

skpA is required for normal fly development and its null mutants are lethal (Murphy, 2003). Therefore, to study *skpA* role in the adult brain, we knocked down its expression in neurons using the pan-neural driver *elavGal4* or the DA neuron-specific driver *Tyrosine Hydroxylase* (*TH*)*Gal4*. To restrict *skpRNAi* expression to the adult stage, we used a ubiquitous temperature-sensitive allele of the Gal80 repressor, which can be inhibited at 29°C. We kept crosses at 18°C to repress Gal4 expression by Gal80 and transferred the descendants to 29°C during the first day post-eclosion (DPE) to allow Gal4-induced RNAi expression. We then evaluated brain function following pan-neural *skpA* knockdown or DA neuron-specific knockdown by testing climbing ability and survival rate (Figure 2). Both genotypes exhibited strong motor deficit (Figure 2A) and shortened lifespan (Figure 2B), as compared with control flies (Figures 2A and 2B), indicating a critical role of SkpA in the adult fly brain. It is noteworthy that the effects caused by the pan-neural knockdown were more pronounced than those caused by the DA neuron-specific knockdown, as the flies exhibited steeper decline in climbing performance and a more severe reduction in lifespan (Figures 2A and 2B), indicating that other neuronal types are also involved in motor activity and that SkpA functions in different neuronal populations.

Pan-neural *skpA* Knockdown in the Adult Brain Reduces the Number of DA Neurons

To better understand how *skpA* inactivation affects motor functions and lifespan, we quantified the number of DA neurons in the adult brain following pan-neural *skpA* knockdown. The DA neurons were labeled with an anti-TH antibody, and the brains were monitored at different time points following *skpARNAi* expression (Figures 2C–2E). Both our and other's previous work showed that the number of DA neurons does not change during adult stages (Dabool et al., 2019; White et al., 2010); therefore, we included only one control age for clarity (Figure 2E). Although the number of DA neurons in the knockdown was similar to that in control brains during the first two weeks of *skpARNAi* expression, their number significantly declined as comparted with the control at 22 DPE (Figure 2E). Interestingly, the impaired climbing ability was observed earlier than the reduction in the number of DA neurons (Figures 2A and 2E), suggesting that other neuronal cell types might be involved in locomotor function or that the DA neurons become dysfunctional prior to their complete loss.

Pan-neural *skpA* Knockdown Increases Accumulation of Ubiquitin-Conjugated Proteins

SkpA is a core component of E3 ligase, playing a major role in protein ubiquitination followed by proteasomal degradation. Aggregation of ubiquitinated proteins is a landmark of many ND. We therefore hypothesized that the neuronal loss observed in the *skpA* knockdown could be linked to increased aggregate load in the brain. To test this idea, we detected ubiquitin-conjugated proteins using an anti-Ubiquitin (Ub) antibody and quantified labeled aggregates. A significantly higher number of aggregates was found in brains of *skpA* knockdown flies compared with control fly brains (Figures 2F–2H), indicating that SkpA function is required for proper degradation of ubiquitin-conjugated proteins in adult brain neurons.

skpA Overexpression in the Adult Fly Brain Improves Locomotor Performance and Prolongs Lifespan

Our findings that *skpA* is required for healthy brain function prompted us to examine the effect of increased *skpA* expression specifically in the adult brain. For this, we used the *elavGal4* driver and *tubGal80* repressor to drive overexpression of *skpA* specifically in the adult stage neurons by transferring the flies to the Gal80 restrictive temperature after eclosion. Strikingly, locomotor performance and lifespan of these flies were significantly increased compared with control flies (Figures 3A and 3B), supporting the important role of SkpA in the adult brain neurons and suggesting that its higher amount could be beneficial for adult brain function.

skpA Overexpression in the Adult Fly Brain Prevents Accumulation of Protein Aggregates

To examine the connections between the improved climbing ability, increased survival rate, DA neurons number, and protein aggregate load, we quantified DA neurons and aggregate numbers using the anti-TH and anti-Ub antibodies, respectively (Figures 3C and 3D). Interestingly, although no significant difference was found in the number of DA neurons in fly brains overexpressing *skpA* compared with control brains (Figure 3C), the number of aggregates was significantly lower in the flies overexpressing *skpA* (Figure 3D). This suggests that the improved locomotion and survival rate are not the result of changes in the numbers of the DA neurons but are rather attributed to the enhancement in the degradation of ubiquitinated proteins.

skpA Overexpression Recovers Neurodegeneration in the Adult Fly PD Model

Many studies have suggested and demonstrated the relevance of Skp1 to both development and progression of PD in humans (Grunblatt et al., 2004; Mandel et al., 2007; Molochnikov et al., 2012). This prompts us to study the role of SkpA in a *Drosophila* model mimicking PD. For this, we used transgenic flies that represent an adult α-Syn-induced PD model (Dabool et al., 2019). To study the effect of SkpA on the PD model, we examined flies simultaneously expressing human *α-syn* with *skpA* in the adult brain neurons. We found that both climbing ability (Figure 4A) and survival rate (Figure 4B) were significantly improved as a result of *skpA* overexpression, indicating that SkpA rescues PD-model-associated neurodegeneration phenotype. Moreover, consistent with these results, the number of ubiquitin-conjugated protein aggregates and DA neurons were reverted to the control numbers (Figures 4C and 4D). These data demonstrate that *skpA* overexpression rescues α-Syn-induced neurodegeneration phenotype, revealing again the protective role of SkpA in the adult brain.

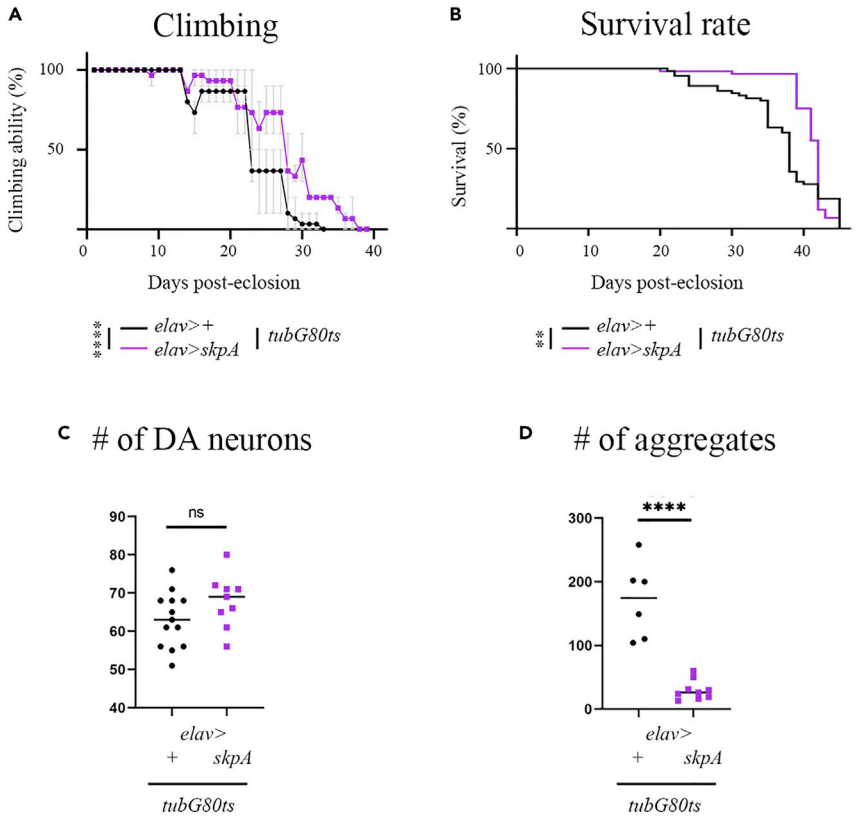

Figure 3. Overexpression of *skpA* in Adult Brain Neurons Reduces Amount of Ubiquitin-Tagged Protein Aggregates and Improves Motor Ability and Survival Rate

(A and B) (A) Climbing ability and (B) survival rate of flies overexpressing *skpA* in all brain neurons (*elavGal4/+;tubGal80^{ts}/UASskpA*) (violet) compared with control flies (*elavGal4/+;tubGal80^{ts}/+*) (black) at 29°C from 1 DPE. n = 6 represents the number of vials.

(C and D) The mean total numbers of (C) DA neurons and (D) ubiquitin-labeled protein aggregates within apotome stacks of adult female brains. (C) Flies overexpressing *skpA* (n = 9) exhibit no significant difference in the number of DA neurons as compared with control flies (n = 13) at 22 DPE. (D) Flies overexpressing *skpA* (n = 9) exhibit a significantly lower number of protein aggregates compared with control (n = 6). Data are represented as mean ± SEM. Statistical significance was analyzed employing students' t test, ****$p < 0.0001$, **$p < 0.01$, n.s. = non-significant.

Lack of the Ntc Causes Neurodegeneration

SkpA/Skp1 interacts with various F Box proteins in different tissues. One of *Drosophila* SkpA-interacting F Box proteins is Ntc, which was previously shown to affect α-Syn-expressing flies (Merzetti et al., 2017). To test the role of Ntc in the fly brain, we first evaluated neurodegeneration in *ntc^{ms771}* mutant flies that carry a premature stop codon in the *ntc* sequence that deletes the entire F box domain (Bader et al., 2010). These flies were barely able to climb and completely lost this ability in a few days (Figure 5A), whereas wild-type flies continued climbing for more than 40 DPE (Figure 5A). Moreover, the mutants did not survive more than 10 days compared with wild-type flies that live over 2 months at 25°C (Figure 5B). Thus, these results show that the F Box protein Ntc contributes to adult brain maintenance as SkpA.

The Level of *ntc* Expression Is Critical for Adult Brain Function

To further understand the role of *ntc* in the adult brain we knocked it down by RNAi or increased its expression specifically in all brain neurons at the adult stage. Interestingly, both manipulations, *ntc* knockdown and overexpression, resulted in reduced locomotor ability and shortened lifespan (Figures 5C and 5D), whereas the knockdown (blue curve) caused a steeper decline in both climbing ability and survival rate compared with the overexpression (pink curve). Remarkably, in the *ntc* knockdown fly brains, a lower number of DA neurons was detected than in control (Figure 5E); however, the number of protein aggregates was not different (Figure 5F). Conversely, *ntc* overexpression did not change the number of DA neurons

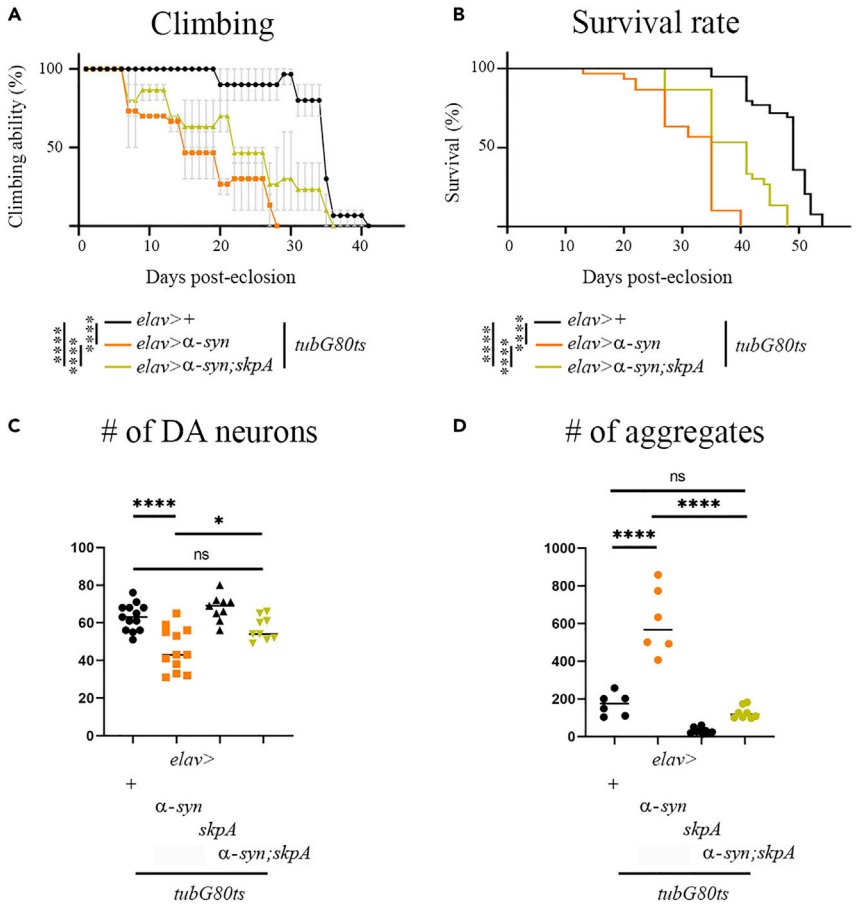

Figure 4. *skpA* Neuronal Overexpression Inhibits Neurodegeneration in the Adult Fly PD Model

(A and B) (A) Climbing ability and (B) survival rate of adult PD model flies, expressing human α-*syn* in all brain neurons (*elavGal4/UAS*α*-syn*; *tubGal80^{ts}/+*) (orange) or PD model flies simultaneously overexpressing *skpA* (*elavGal4/UAS*α*-syn;tubGal80^{ts}/UASskpA*) (green) in all brain neurons. Control flies (*elavGal4/+;tubGal80^{ts}/+*) (black). *skpA* overexpression improves climbing ability and increases lifespan compared with α-*syn* transgenic flies. n = 3 represents the number of vials.

(C and D) The mean total numbers of (C) DA neurons and (D) Ub-labeled protein aggregates within apotome stacks of adult female brains. Control (*elavGal4/+;tubGal80^{ts}/+*) (n = 13 and n = 6); flies overexpressing *skpA* (*elavGal4/+;tubGal80^{ts}/UASskpA*) (n = 9 and n = 8); adult fly PD model (*elavGal4/UAS*α*-syn;tubGal80^{ts}/+*) (n = 12 and n = 6); adult fly PD model carrying neuronal expression of *skpA* (*elavGal4/UAS*α*-syn;tubGal80^{ts}/UASskpA*) (n = 9 and n = 8). Data are represented as mean ± SEM. Statistical significance was analyzed employing two-way ANOVA, ****p < 0.0001, **p < 0.01, *p < 0.05, n.s. = non-significant.

(Figure 5E) but significantly increased the number of protein aggregates (Figure 5F). These data demonstrate that either the lack or the excess of *ntc* is detrimental to the adult brain, indicating that the level of *ntc* expression must be tightly regulated for proper brain function.

skpA and *ntc* Genetically Interact in the Adult Brain Neurons

Neuron-specific knockdown of *skpA* or *ntc* causes a comparable phenotype, suggesting that the two proteins could work in the same pathway. To test whether *skpA* and *ntc* genetically interact, we simultaneously knocked them down in adult brain neurons and examined the climbing ability (Figure 6A) and survival rate of the flies (Figure 6B) in comparison to each single gene knockdown (Figures 6A and 6B). The concurrent knockdown of both genes led to a similar phenotype (Figures 6A and 6B). Moreover, no significant difference in the number of DA neurons and ubiquitinated protein aggregates was observed between *skpA* knockdown alone and the double knockdown (Figures 6C and 6D), revealing no additive effect. These results suggest that the two proteins likely work together.

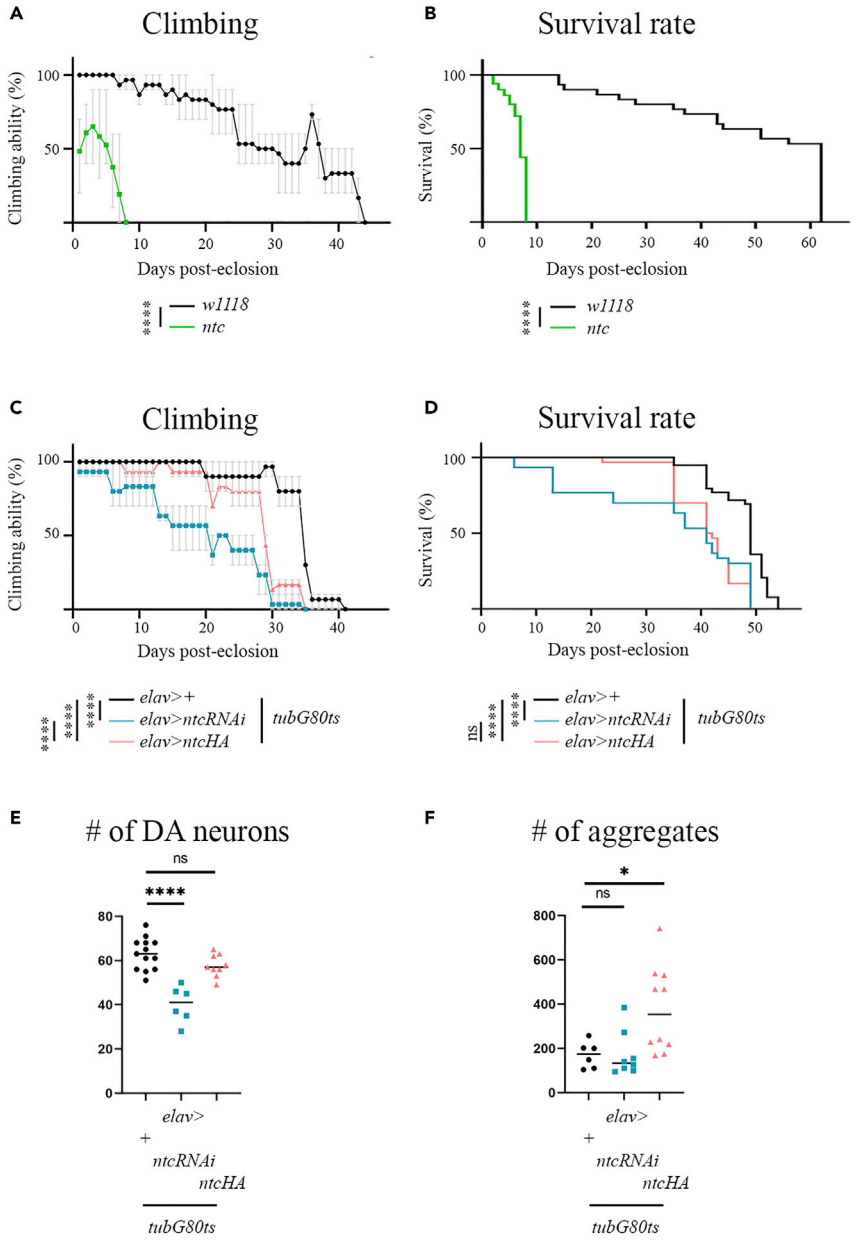

Figure 5. Altered Levels of *ntc* in Adult Brain Neurons Cause Neurodegeneration

(A and B) (A) Climbing ability and (B) survival rate of control flies (w^{1118}) (black) and *ntc* mutant flies (ntc^{ms771}/ntc^{ms771}) (green) from 1 DPE at room temperature. n = 3 represents the number of vials.

(C and D) (C) Climbing ability and (D) survival rate of flies expressing *ntcRNAi* (*elavGal4/+;tubGal80^{ts}/UASntcRNAi*) (blue) or overexpressing *ntc* (*elavGal4/+;tubGal80^{ts}/UASntcHA*) (pink) in a pan-neural manner compared with control flies (*elavGal4/+;tubGal80^{ts}/+*) (black) at 29°C from 1 DPE. n = 6 represents the number of vials.

(E and F) The mean total numbers of (E) DA neurons and (F) Ub-labeled protein aggregates within apotome stacks of adult female brains. Control (*elavGal4/+;tubGal80^{ts}/+*) (n = 13 and n = 6); (*elavGal4/UASntcRNAi;tubGal80^{ts}/+*) (n = 6 and n = 8); (*elavGal4/+;tubGal80^{ts}/UASntcHA*) (n = 9 and n = 10). Data are represented as mean ± SEM. Statistical significance was analyzed employing students' t test or two-way ANOVA, ****p < 0.0001, *p < 0.05, n.s. = non-significant.

skpA and *ntc* Mutually Rescue Neurodegeneration Phenotype of Each Other

To further explore the genetic interactions between *skpA* and *ntc* in the adult brain, we carried out rescue experiments of *ntc* knockdown by overexpression of *skpA* and vice versa. As shown in Figure 6, the over-expression of *skpA* in adult brain neurons rescued the phenotype of *ntc* knockdown. The climbing activity

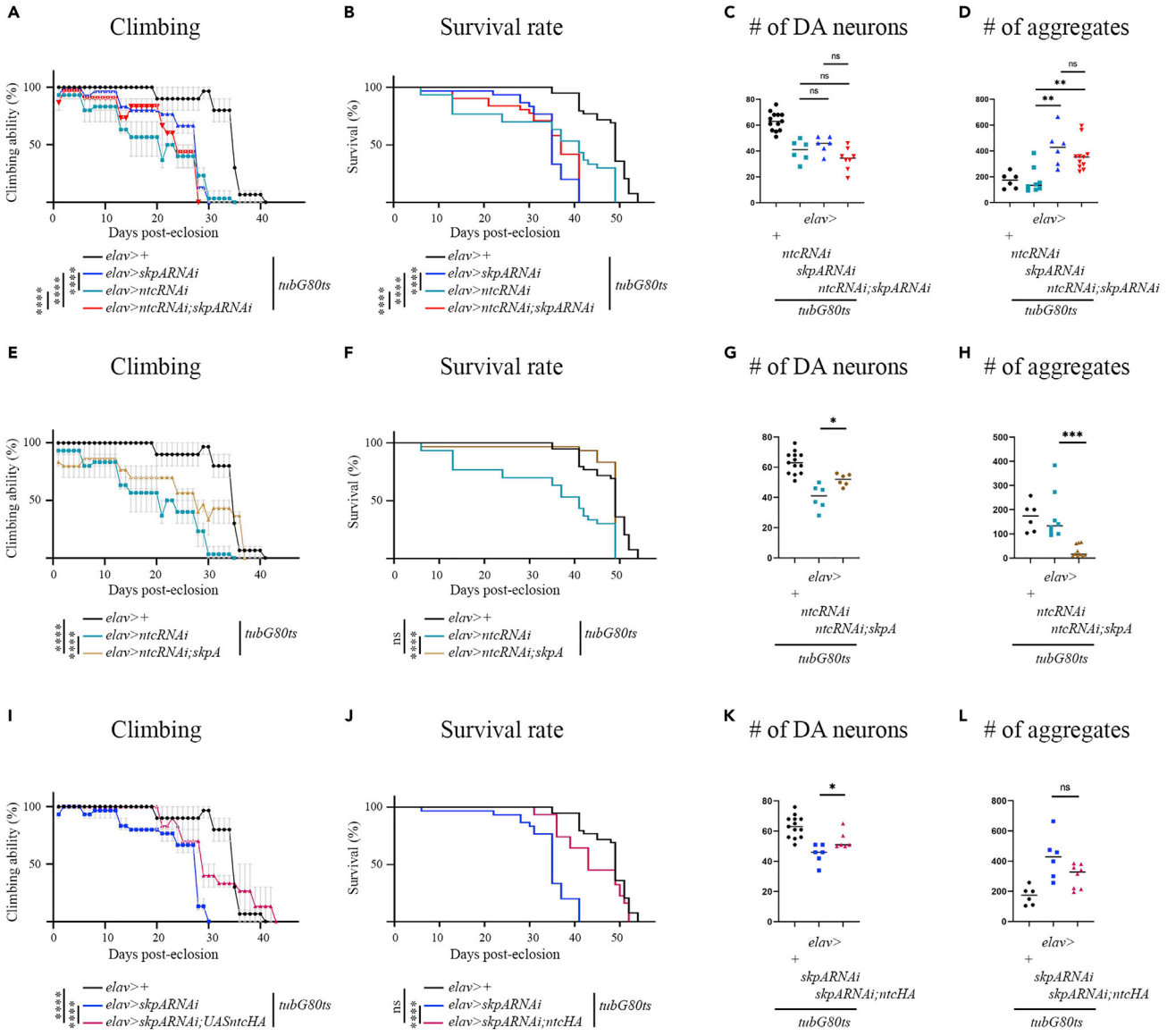

Figure 6. *skpA* and *ntc* Genetically Interact in Adult Brain Neurons and Rescue Each Other's Neurodegeneration Phenotype

(A and B) (A) Climbing ability and (B) survival rate of flies carrying *skpA* knockdown (*elavGal4/+;tubGal80^{ts}/UASskpARNAi*) (dark blue) or *ntc* knockdown (*elavGal4/UASntcRNAi;tubGal80^{ts}/+*) (light blue) or both (*elavGal4/UASntcRNAi;tubGal80^{ts}/UASskpARNAi*) (red) simultaneously. Control (*elavGal4/+;tubGal80^{ts}/+*) (black). n = 6 represents the number of vials.

(C and D) The mean total numbers of (C) DA neurons and (D) Ub-labeled protein aggregates within apotome stacks of adult female brains. Control (n = 13 and n = 6); (*elavGal4/UASntcRNAi;tubGal80^{ts}/+*) (n = 8 and n = 6); (*elavGal4/+;tubGal80^{ts}/UASskpARNAi*) (n = 6 and n = 6); (*elavGal4/UASntcRNAi;tubGal80^{ts}/UASskpARNAi*) (n = 8 and n = 11).

(E and F) (E) Climbing ability and (F) survival rate of flies carrying *ntc* knockdown (*elavGal4/+;tubGal80^{ts}/UASntcARNAi*) (light blue) or *ntc* knockdown and *skpA* overexpression (*elavGal4/UASntcRNAi;tubGal80^{ts}/UASskpA*) (brown). Control (*elavGal4/+;tubGal80^{ts}/+*) (black). n = 6 represents the number of vials.

(G and H) The mean total numbers of (G) DA neurons and (H) Ub-labeled protein aggregates within apotome stacks of adult female brains. Control (n = 13 and n = 6); (*elavGal4/UASntcRNAi;tubGal80^{ts}/+*) (n = 6 and n = 8); (*elavGal4/UASntcRNAi;tubGal80^{ts}/UASskpA*) (n = 6 and n = 10).

(I and J) (I) Climbing ability and (J) survival rate of flies carrying *skpA* knockdown (*elavGal4/+;tubGal80^{ts}/UASskpARNAi*) (dark blue) or *skpA* knockdown and *ntc* overexpression (*elavGal4/UASntcHA;tubGal80^{ts}/UASskpARNAi*) (pink). Control (*elavGal4/+;tubGal80^{ts}/+*) (black). n = 6 represents the number of vials.

(K and L) The mean total numbers of (K) DA neurons and (L) Ub-labeled protein aggregates within apotome stacks of adult female brains. Control (n = 13 and n = 6); (*elavGal4/UASskpARNAi;tubGal80^{ts}/+*) (n = 6 and n = 6); (*elavGal4/UASntcHA;tubGal80^{ts}/UASskpARNAi*) (n = 6 and n = 8).

Data are represented as mean ± SEM. Statistical significance was analyzed employing two-way ANOVA, ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05, n.s. = non-significant.

and lifespan were significantly improved in flies overexpressing *skpA*, compared with flies carrying only *ntc* RNAi (Figures 6E and 6F). Moreover, *skpA* overexpression significantly increased the number of DA neurons (Figure 6G) and reduced the amount of ubiquitin-conjugated protein aggregates (Figure 6H) in flies expressing *ntcRNAi* compared with *ntc* knockdown alone (Figures 6G and 6H). These data strongly suggest that SkpA may act through an additional pathway(s) comprising another F box protein(s).

Specific overexpression of *ntc* in adult brain neurons carrying *skpARNAi* significantly rescued *skpA* knockdown phenotype, namely, climbing ability and survival rate of these flies were significantly improved compared with transgenic flies expressing *skpARNAi* alone (Figures 6I and 6J, pink curve compared with dark blue curve). Consistent with these data, the number of DA neurons in brains of *skpA* knockdown flies overexpressing *ntc* was significantly higher than in *skpA* knockdown alone (Figure 6K). Importantly, no significant change in aggregate load was detected (Figure 6L). These results further support our notion that SkpA and Ntc act in the same pathway.

DISCUSSION

The molecular mechanisms underlying ND are mainly unknown. Finding biomarkers for timely diagnostics of these diseases as well as potential targets for therapeutic intervention requires identifying critical players in the processes leading to neurodegeneration. Our study uncovers the central role of the *Drosophila* Skp1 homolog, SkpA, in adult brain function and depicts it as a potential biomarker and therapeutic target in neurodegenerative processes.

In this work, we show that *skpA* is specifically expressed in the adult brain neurons and that its targeted knockdown in the adult brain leads to accumulation of ubiquitinated protein aggregates and loss of DA neurons accompanied by motor dysfunction and shortened lifespan. This neurodegeneration phenotype demonstrates that SkpA is required for protein degradation in neurons, a process essential for brain health. In consistence with this, we discovered that overexpression of *skpA* specifically in adult brain neurons prevents accumulation of protein aggregates, prolongs fly lifespan, and hinders the age-related motor decline. These exciting data place SkpA in a strategic position regulating neuroprotective pathways. Interestingly, no significant difference was found in the number of DA neurons between flies overexpressing *skpA* in the adult brain and control flies, suggesting that the gain of survival rate and motor ability is not specifically related to the number of DA neurons in the brain but rather to their proper function. It can also suggest that other types of neurons are involved in age-related motor decline. In accordance with this, we have recently shown that climbing ability of flies can be improved regardless of change in the number of DA neurons (Hakim-Mishnaevski et al., 2019). Because SkpA is widely expressed in the adult brain neurons, we suggest that it functions in diverse neuronal cell types.

Previous studies describing association of *skp1* and parkinsonism and the potential implication of *skp1* in proper function and viability of DA neurons (Fishman-Jacob et al., 2009; Mandel et al., 2012) prompted us to examine the role of SkpA in the fly model of PD (Dabool et al., 2019). We found that *skpA* overexpression partially rescued neurodegeneration in PD-like flies, demonstrating that increased levels of *skpA* accelerate evacuation of ubiquitinated protein aggregates. The protection afforded by *skpA* overexpression supports the conclusion regarding its potential to serve as a target for therapeutic intervention.

To gain a deeper insight into the molecular mechanisms of SkpA function in the adult brain, we investigated the role of the F box protein Ntc in neurodegeneration. Although Ntc and SkpA interaction has been implicated in the sperm differentiation process (Bader et al., 2010) and Ntc was recently shown to affect α-Syn-expressing flies (Merzetti et al., 2017), no studies have analyzed whether these proteins interact/cooperate in the adult brain. Interestingly, reduced or increased levels of *ntc* affect fly motor ability and survival rate, suggesting that the amount of Ntc is critical in the adult brain. However, *ntc* knockdown, which causes a strong neurodegeneration phenotype, does not affect the number of ubiquitin-tagged protein aggregates. These results suggest that Ntc might be required for protein ubiquitination and, therefore, the anti-Ub antibodies do not recognize unlabeled protein aggregates in the *ntc* knockdown brains. Consistently with this suggestion, *ntc* overexpression probably leads to surplus ubiquitination, which is pronounced in slightly higher number of Ub-labeled protein aggregates. This off-target ubiquitination may cause excess protein degradation and affect other neurons than DA (the number of DA neurons remains normal under *ntc* overexpression), leading to the reduced climbing and survival phenotype.

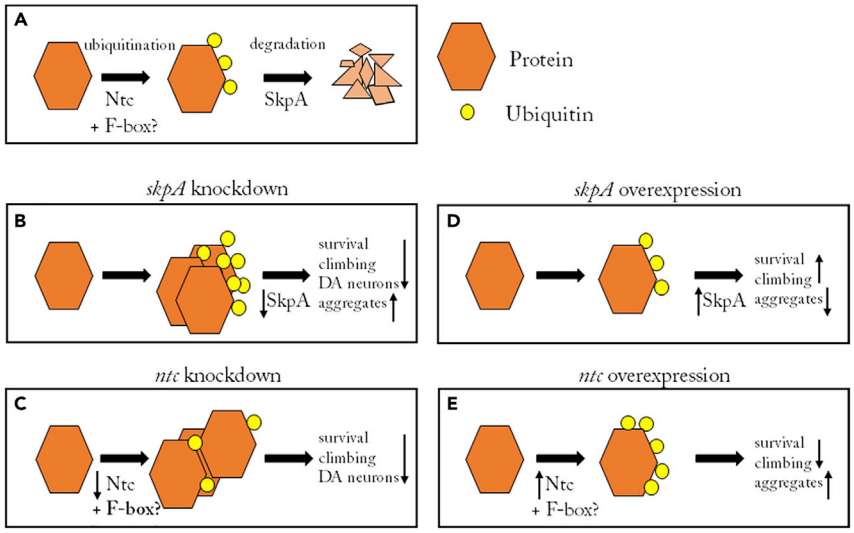

Figure 7. Model Summarizing SkpA and Ntc Interactions in the Adult Fly Brain

(A) A suggested model for ubiquitin-mediated protein degradation in the adult fly brain. Our data propose that Ntc and SkpA act in the same pathway. Ntc is mostly involved in substrate ubiquitination, whereas SkpA likely sends already ubiquitinated proteins to proteasomal degradation.

(B) *skpA* knockdown leads to accumulation of ubiquitinated protein aggregates, which are detected with the anti-Ub antibody. It also reduces the number of DA neurons, climbing ability, and survival rate.

(C) *ntc* knockdown reduces the number of DA neurons accompanied by declined climbing ability and shortened lifespan; however, no increase in the number of the ubiquitin-tagged protein aggregates is detected. We suggest that Ntc might be involved in protein ubiquitination, and therefore, its knockdown causes an accumulation of unubiquitinated proteins, which are not detected with the anti-Ub antibody.

(D) *skpA* overexpression accelerates degradation of ubiquitinated proteins resulting in significantly lower number of Ub-labeled aggregates than in control, accompanied by improved climbing ability and increased survival rate.

(E) Overexpression of *ntc* outcomes slightly increased number of Ub-labeled protein aggregates but does not affect the number of DA neurons (see Figure 5E) because SkpA normally degrades the ubiquitinated substrates. Weak reduction in climbing ability and shortened lifespan may result from degradation of off-target substrates or sequestration of SkpA by the elevated Ntc. The excess of Ntc may engage SkpA and prevent its function in additional pathway(s), leading to weak neurodegeneration phenotype.

Based on our data we suggest a working model (Figure 7) in which SkpA and Ntc are required for proper protein degradation in adult brain neurons. The genetic interaction and rescue experiments reveal that SkpA and Ntc likely act in the same pathway, where Ntc is mostly involved in ubiquitination of substrates and SkpA is subsequently required for their proteasomal degradation (Figure 7A). Such a model might explain why in *skpA* knockdown flies we detect an accumulation of Ub-labeled aggregates (Figure 7B), which is not seen in *ntc* knockdown (Figure 7C). Because *ntc* overexpression partially rescues *skpA* knockdown, it might occur due to increased ubiquitination, which allows degradation of tagged substrates even when SkpA levels are reduced. Conversely, *skpA* overexpression partially rescues *ntc* knockdown phenotype, suggesting that SkpA interacts with additional F box proteins in the adult brain ("F box?", Figure 7). It will be interesting to test potential candidates in future experiments. Our study reveals the critical role of SkpA in neuronal maintenance, which likely functions as a bottleneck in the protein degradation processes, whose dysregulation could lead to formation of aggregates. Collectively, this discloses a substantial potential of SkpA/Skp1 as a diagnostic marker and therapeutic target in ND. The parallel role of SkpA/Skp1 in the brain function of flies and mammals indicates that modeling neurodegeneration in *Drosophila* continues to provide paradigms that can guide clinical research.

Limitations of the Study

Our data suggest that Ntc is required for ubiquitination of neuronal proteins in the adult brain; however, because there are no available antibodies/reagents that recognize non-ubiquitinated aggregates we could not demonstrate their accumulation in the brains of *ntc* knockdown. Our results also suggest that SkpA interacts with additional F box protein(s) that are yet to be identified and beyond the scope of this research.

Resource Availability

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Estee Kurant (ekurant@univ.haifa.ac.il)

Materials Availability

All reagents generated in this study will be made available on request to the Lead Contact; however, requestor will cover shipping costs. This study did not generate new unique reagents.

Data and Code Availability

The original/source data are available from the lead contact on request.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101375.

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AUTHOR CONTRIBUTIONS

L.D., K.HM., L.J., and N.FB. conducted the experiments; L.D. and E.K. designed and analyzed the experiments; S.M. advised on Skp1; and E.K. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

Drosophila Skp1 Homologue SkpA Plays a Neuroprotective Role in Adult Brain

Lital Dabool, Ketty Hakim-Mishnaevski, Liza Juravlev, Naama Flint-Brodsly, Silvia Mandel, and Estee Kurant

Transparent Methods

Fly strains

All stocks were maintained on standard *Drosophila* media at 25°C unless differently stated. For adult specific expression, relevant crosses were placed at 18°C and adult flies were transferred to 29°C during the first day after eclosion. *skpAlacz/FM7* (#11523), *UAScytGFP* (#1521), *UASα-syn/CyO* (#51375), *UASskpARNAi* (#32991) and *tubGal80^{ts}* (#7018) were obtained from Bloomington Stock Center. *elavGal4/CyO* (O. Schuldiner); *repoGal4* (U. Gaul); *THGal4* (B. Mollereau); *UASntcHA* (H. Steller); *ntc^{ms771}*/TM6B (E. Arama); *UASskpA* (FlyORF collection, Zurich); *UASntcRNAi* (#104761KK, VDRC). The *w^{1118}* strain was used for outcross as a wild-type control.

Climbing assay

After eclosion at 18°C, triplicates of 10 female flies per vial of each genotype were placed at 29°C. Their climbing ability was tested from the following day by counting the number of flies climbing 7 cm distance within 10 seconds, after tapping them all to the bottom. We provided flies with new food every other day and performed this procedure consecutively until all examined flies lost locomotive skills. We repeated the experiment at least 3 times with independently derived transgenic flies for each genotype.

Survival rate measurement

Triplicates of 10 female flies per vial of each genotype were transferred to new food every other day. The number of living flies out of 10 was recorded every day from the first day after eclosion. The experiment continued until all flies were dead.

Immunohistochemistry

For immunohistochemistry, adult fly brains were dissected, fixed and stained according to standard procedures. Mouse anti-TH (Millipore) and rat anti-Elav (Developmental Studies Hybridoma Bank) were used at 1:500 and 1:100 dilutions respectively. Mouse anti-Ub (Enzo Life Sciences) and mouse anti-GFP (Roche) were used at 1:100 dilution. Mouse anti-βGal (Promega) was used at 1:1000 dilution. Fluorescent secondary antibodies Cy3/488, from Jackson ImmunoResearch, were used at 1:200 dilution. Dako solution was used as imaging medium. All images were acquired on a Zeiss Axio Observer microscope equipped with an Apotome system using the AxioVision software. To count the number of TH-positive neurons, confocal stacks were acquired from the posterior part of the brains

and traced through confocal Z-stacks. The cells were counted manually through each Z-stack using the point selection tool in the data visualization software IMARIS (Bitplane). The number of TH labeled cells was recorded per brain and the mean number of cells was calculated per each genotype. To quantitate the number of ubiquitin-conjugated aggregates, apotome stacks were acquired from the posterior part of the brains and aggregates were quantified using the IMARIS software (1 µm as the minimal diameter for ubiqitinated aggregates in a 1000x1000 µm square). For immunohistochemistry and quantification of DA neurons and protein aggregates, flies were dissected after they stopped climbing. Therefore, flies of appropriate ages were selected in the different experiments.

Statistical analysis

Each experiment was repeated independently a minimum of three times, error bars represent the standard error of replicate experiments. Statistical significance of climbing, survival and cell numbers data was calculated with Two-way ANOVA followed by Dunnett's multiple comparisons test using GraphPad Prism version 8.1.2 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com. Students' *t*-test was used to compare only two groups. Asterisks indicate statistical significance, as determined by students' *t*-test or two-way ANOVA, P values of < 0.05 = *, < 0.01 = **, < 0.001 = ***, < 0.0001=**** were considered significant, P values of >0.05 were considered non-significant (n.s.). Error bars = SEM.

KEY RESOURCES TABLE

REAGENT or RESOURCE	**SOURCE**	**IDENTIFIER**
Antibodies		
Mouse monoclonal anti-TH	Millipore	Cat# MAB318; RRID: AB_2313764
Rat monoclonal anti-Elav	DSHB	Cat# 7E8A10; RRID: AB_528218
Mouse monoclonal anti-Ub (FK2)	Enzo Life Sciences	Cat# BML-PW8805; RRID: AB_10541434
Rabbit monoclonal anti-GFP	Cell Signaling	Cat# #2956; RRID: AB_1196615
Mouse monoclonal anti-βGal	Promega	Cat# Z3781; RRID: AB_ 430877
Alexa Fluor® 488 AffiniPure Donkey Anti-Rat IgG (H+L)	Jackson ImmunoResearch Labs	Cat# 712-545-150; RRID: AB_2340683

DyLight™488-conjugated AffiniPure Donkey Anti-Rabbit IgG (H+L)	Jackson ImmunoResearch Labs	Cat# 711-485-152; RRID: AB_2492289
Cy™3-conjugated AffiniPure Donkey Anti-Mouse IgG (H+L)	Jackson ImmunoResearch Labs	Cat# 715-165-151; RRID: AB_2315777
Experimental Models: Organisms/Strains		
D. melanogaster: skpAlacz; P{w[+mC]=lacW}SkpA[G0037] w[67c23]/FM7c	Bloomington Stock center	BDSC: 11523; RRID:BDSC_11523
D. melanogaster: : UAScytGFP; w[*]; P{w[+mC] = UAS-GFP.S65T}Myo31DF[T2]	Bloomington Stock center	BDSC:1521; RRID:BDSC_1521
D. melanogaster: UASα-syn; w[1118]; P{w[+mC]=UAS-SNCA.J}1/CyO	Bloomington Stock center	BDSC: 51375; RRID:BDSC_51375
D. melanogaster: UASskpARNAi; y[1] sc[*] v[1] sev[21]; P{y[+t7.7]v[+t1.8]=TRiP.HMS00791}attP2	Bloomington Stock center	BDSC:32991; RRID:BDSC_32991
D. melanogaster: tubP-Gal80; w[*]; sna[Sco]/CyO; P{w[+mC] = tubP-Gal80[ts]}ncd[Gal80ts-7]	Bloomington Stock center	BDSC:7018; RRID:BDSC_7018
D. melanogaster: elav-Gal4	Laboratory of O. Schuldiner	N/A
D. melanogaster: repo-Gal4	Laboratory of U. Gaul	N/A
D. melanogaster: TH-Gal4	Laboratory of B. Mollereau	N/A
D. melanogaster: UASntcHA	Laboratory of H. Steller	N/A
D. melanogaster: ntcms771/TM6B	Laboratory of E. Arama	N/A
D. melanogaster: UASskpA	FlyORF collection, Zurich	F001570; CG16983
D. melanogaster: UASntcRNAi	VDRC Stock center	104761KK
Software and Algorithms		
AxioVision	ZEISS	https://zeiss.com/
IMARIS	Bitplane	https://imaris.oxinst.com/packages
Prism 8.1.2	GraphPad	https://www.graphpad.com/