Drosophila Skp1 Homologue SkpA Plays a Neuroprotective Role in Adult Brain

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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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 invariant 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;
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;UAScrtGFP) (Figures 1A and 1C) and by staining with the neuron-specific anti-Elav antibody (Figures 1D and 1F). β-Gal, which
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.

**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+/UASskpARNAi) (blue) or in DA neurons alone (THGal4+/;tubGal80+/UASskpARNAi) (red) compared with control flies (elavGal4+/;tubGal80+/+) (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+/+). (D) Transgenic fly expressing skpARNAi in a pan-neural pattern (elavGal4+/;tubGal80+/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+/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+/+).

(F and G) Projections from apotome stacks of whole-mount entire female brains 25 DPE. (F) Control (elavGal4+/;tubGal80+/+). (G) Transgenic fly expressing skpARNAi in a pan-neural pattern (elavGal4+/;tubGal80+/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 < 0.001, **p < 0.01, *p < 0.05, n.s. = non-significant.
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 compared 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-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; Molochniov 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.
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 ntcms771 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.
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.
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 overexpression of skpA in adult brain neurons rescued the phenotype of ntc knockdown. The climbing activity

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 (w1118) (black) and ntc mutant flies (ntc<sup>ma771</sup>/ntc<sup>ma771</sup>) (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°/+; UASntcRNAi) (blue) or overexpressing ntc (elavGal4/+; tubGal80°/+; UASntcHA) (pink) in a pan-neural manner compared with control flies (elavGal4/+; tubGal80°/+; UASntcRNAi) (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°/+; UASntcRNAi) (n = 13 and n = 6), (elavGal4/+; tubGal80°/+; UASntcHA) (n = 6 and n = 8), (elavGal4/+; tubGal80°/+; UASntcRNAi) (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.
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/UASntcRNAi) (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 = 6).

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.
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. 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.

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*

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**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), *UASa-syn/Cyo* (#51375), *UASSkpARNAi* (#32991) and *ubGal80*° (#7018) were obtained from Bloomington Stock Center. *elavGal4/Cyo* (O. Schuldiner); *repoGal4* (U. Gaul); *THGal4* (B. Mollereau); *UASntcHA* (H. Steller); *ntc-ms77*/TM6B (E. Arama); *UASskpA* (FlyORF collection, Zurich); *UASntcRNAi* (#104761KK, VDRC). The w1118 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 Axios 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 ubiquitinated 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 |
| Experimental Models: Organisms/Strains |
|----------------------------------------|
| **D. melanogaster:** skpAlacz; P{w[+mC]=lacW}SkpA[0037] 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[*]; snai/[CyO; P{w[+mC] = tubP-Gal80[ts]}ncd[Gal80[ts-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/ |