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Evaluation of type 1 diabetes mellitus as a risk factor of Parkinson's disease in a *Drosophila* model

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Abstract

Diabetes mellitus (DM) is a chronic metabolic disease characterized by high blood glucose levels, resulting from insulin dysregulation. Parkinson's disease (PD) is the most common neurodegenerative motor disorder caused by the selective loss of dopaminergic (DA) neurons in the *substantia nigra pars compacta*. DM and PD are both age-associated diseases that are turning into epidemics worldwide. Previous studies have indicated that type 2 DM might be a risk factor of developing PD. However, scarce information about the link between type 1 DM (T1DM) and PD does exist. In this work, we have generated a *Drosophila* model of T1DM based on insulin deficiency to evaluate if T1DM could be a risk factor to trigger PD onset. As expected, model flies exhibited T1DM-related phenotypes such as insulin deficiency, increased content of carbohydrates and glycogen, and reduced activity of insulin signaling. Interestingly, our results also demonstrated that T1DM model flies presented locomotor defects as well as reduced levels of tyrosine hydroxylase (a marker of DA neurons) in brains, which are typical PD-related phenotypes. In addition, T1DM model flies showed elevated oxidative stress levels, which could be causative of DA neurodegeneration. Therefore, our results indicate that T1DM might be a risk factor of developing PD, and encourage further studies to shed light into the exact link between both diseases.

KEYWORDS

Drosophila, insulin signaling, motor deficits, neurodegeneration, Parkinson's disease, type 1 diabetes mellitus

1 | INTRODUCTION

Diabetes mellitus (DM) is the most common metabolic disease (Gaspar et al., 2016), with a prevalence of 10.5% in 2021, which is expected to increase in the future (Sun et al., 2022). DM is a chronic disorder characterized by high blood glucose levels and caused by insulin dysregulation (Animaw & Seyoum, 2017; Tan et al., 2019). Currently, DM is classified into different types, being type 1 DM (T1DM) and type 2 DM (T2DM) the most common. T1DM results from insulin deficiency due to the autoimmune loss of pancreatic

Abbreviations: DA, dopaminergic; DM, diabetes mellitus; ER, endoplasmic reticulum; ILP, insulin-like peptide; ISP, insulin signaling pathway; OS, oxidative stress; PD, Parkinson's disease; ROS, reactive oxygen species; T1DM, type 1 Diabetes mellitus; T2DM, type 2 Diabetes mellitus; TH, tyrosine hydroxylase.

β-cells (the main insulin-producer cells), whereas T2DM is characterized by the onset of insulin resistance (American Diabetes Association, 2013; Deshmukh & Jain, 2015; Tan et al., 2019). DM is a multisystemic disorder able to affect different tissues in the organism, as well as to increase the risk of several health complications such as cardiovascular alterations, stroke, nephropathies, blindness, and even neuropathies (Deshmukh & Jain, 2015). Moreover, DM has been related to neurodegenerative diseases like Parkinson's disease (PD), Huntington disease, amyotrophic lateral sclerosis, or Alzheimer's disease (Muddapu et al., 2020).

PD is the most common neurodegenerative movement disorder, affecting approximately 10 million people in the world (Balestrino & Schapira, 2020; Church, 2021; Gouda et al., 2022), a number that is expected to increase due to the growing aging population (Church, 2021; Gouda et al., 2022). The pathological hallmark of PD is the selective loss of dopaminergic (DA) neurons in the *substantia nigra pars compacta*; however, the exact cause of this neurodegeneration remains elusive (Avazzadeh et al., 2021; Koszła et al., 2021; Vázquez-Vélez & Zoghbi, 2021). It has been proposed that DA neuron loss has a multifactorial ethiology, in which mitochondrial alterations, protein missfolding, autophagy defects, epigenetic modifications, high oxidative stress (OS) levels, endoplasmic reticulum (ER) stress, inflammation and calcium dyshomeostasis are involved (Anandhan et al., 2017; Groenendyk et al., 2021; Mahmood et al., 2021; Poewe et al., 2017; Solana-Manrique et al., 2021). In addition, metabolic alterations might also play a key role in PD onset/progression (Anandhan et al., 2017; Solana-Manrique et al., 2022).

DM and PD are both age-associated diseases that are turning into epidemics worldwide. To date, several association studies have been conducted to determine whether DM might be a risk factor of developing PD (Labandeira et al., 2022). Most of them are focused on T2DM and predominantly demonstrate that this disease might be involved in PD onset (Cheong et al., 2020; Fiory et al., 2019; Reed et al., 2021). Besides, it has been reported that PD patients with T2DM show deterioration of motor and cognitive deficits (de Iuliis et al., 2022). Therefore, this disease can also determine the prognosis and progression of PD symptoms (Labandeira et al., 2022). Interestingly, both diseases share several molecular phenotypes, such as mitochondrial alterations, increased OS levels, ER stress, or inflammation (Hassan et al., 2020). The cause of this link is still unknown; however, several authors point to micro and macrovascular defects, hyperglycemia, and insulin dysregulation as plausible reasons (Álvarez-Rendón et al., 2018; Deshmukh & Jain, 2015; Murillo-Maldonado et al., 2011). It is worth to mention that our group recently demonstrated a link between T2DM and PD using *Drosophila* as a model organism (Sanz et al., 2022). We found that T2DM model flies exhibited PD-related phenotypes, such as reduced lifespan, elevated OS levels, and locomotor defects. They also showed DA neurodegeneration, a result validated in a T2DM mice model (Sanz et al., 2022). Overall, these results allowed us to confirm the link between both diseases. Unfortunately, the number of studies investigating the connection between T1DM and PD are scarce. To our knowledge, only one epidemiological study has been carried out, in which an association between both diseases was found (Klimek et al., 2015). In such a scenario, we decided to study if T1DM might be a risk factor of developing PD by using a *Drosophila* model of this disease. *Drosophila* has emerged as an outstanding model to study DM due to similarities in its carbohydrate metabolism to that of humans. In addition, glucose levels are regulated in flies through insulin-like peptides (ILPs), which are mainly produced and secreted by specialized neurons known as insulin-producing cells (IPCs), and glial cells in the brain (Álvarez-Rendón et al., 2018; Chatterjee & Perrimon, 2021; Liguori et al., 2021; Murillo-Maldonado et al., 2011). These fly peptides are also involved in growth control (Rulifson et al., 2002), and exert their function through the activation of the insulin signaling pathway (ISP), which has similar components and regulatory interactions in flies and humans (Chatterjee & Perrimon, 2021; Graham & Pick, 2017; Liguori et al., 2021; Teleman et al., 2012).

In this work, we demonstrated that T1DM model flies exhibited typical T1DM phenotypes such as increased carbohydrate levels, reduced expression of genes encoding ILPs as well as a reduction of ISP activity, thus confirming their potential to study this metabolic disease. Interestingly, we also found that T1DM model flies displayed increased OS levels, locomotor deficits, and reduced levels of tyrosine hydroxylase (a marker of DA neurons), which are typical PD-related phenotypes. These results confirm for the first time the existence of a link between both diseases in an animal model and suggest that T1DM might be a risk factor of developing PD as shown for T2DM.

2 | MATERIAL AND METHODS

2.1 | Drosophila stocks

Fly stocks used in this study were obtained from the *Bloomington Drosophila Stock Center* and are the following: *w**; *P{Ilp2-GAL4.R}2/CyO* (#37516, hereafter called *Ilp2*-GAL4), $w^{[1118]}$; *P{w[+mC]=UAS-rpr.C}14* (#5824, hereafter called UAS-*rpr*) and y^{1},w^{1118} (#6598, hereafter called *y,w*). Flies were cultured on standard *Drosophila* food at 25°C. T1DM model flies were the progeny of a cross between *Ilp2*-GAL4 and UAS-*rpr*, while control flies were the progeny of a cross between *Ilp2*-GAL4 and *y,w*.

2.2 | RT-qPCR analyses

Total RNA from 10 15-day-old female flies of each genotype was extracted and 1 μg of RNA of each sample was reverse-transcribed as described in Solana-Manrique et al. (2020). Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) assays were performed as in Solana-Manrique et al. (2020). Primers used in these

analyses were the following: *ilp2* direct primer (5′-CTGGGAG CTATCTTGGGGGT-3′); *ilp2* reverse primer (5′-ACTCCCGCAGAGC CTTCATA-3′); *ilp3* direct primer (5′-TTATGATCGGCGGTGTCCAG-3′); *ilp3* reverse primer (5′-TTGGTCATTGCGTTGAAGCC-3′); *ilp5* direct primer (5′-GCGGATTTGGATAGCTCCGA-3′); *ilp5* reverse primer (5′-AAAGGAACACGATTTGCGGC-3′); *InR* direct primer (5′-TTTCACGGAAGTCGAACATA-3′); *InR* reverse primer (5′-GACCTT AGCATAGCTCGG-3′); *chico* direct primer (5′-TATGCACAACAC GATACTGAG-3′); *chico* reverse primer (5′-GACTCTGTTTTGGC TGACA-3′); *PI3K* direct primer (5′-ATTTGGACTACCTACGGGAA-3′); *PI3K* reverse primer (5′-TGCTTTTTCCTCCGTGTAG-3′). *tubulin* direct primer (5′-GATTACCGCCTCTCTGCGAT-3′); *tubulin* reverse primer (5′-ACCAGAGGGAAGTGAATACGTG-3′). *tubulin* levels were measured and used as an internal control for RNA amount in each sample. All experiments were performed in quadruplicate. Data analysis was performed using the comparative C_t method ($2^{-\Delta\Delta C_t}$ method) with the StepOnePlus™ v2.3 software (Livak & Schmittgen, 2001). Results are shown as fold change expression in T1DM flies compared with controls.

2.3 | Quantification of glycogen and soluble carbohydrates

Levels of glycogen and soluble carbohydrates in 15-day-old female flies of each genotype were calculated by using the anthrone reagent as previously described in Sanz et al. (2022). All experiments were carried out using three biological replicates and three technical replicates for each sample.

2.4 | Weight estimation

Groups of five 15-day-old female flies of each genotype were weighed in a microbalance to estimate their total weight. At least, six replicates per each group of flies were studied.

2.5 | Climbing assays

Locomotor ability of flies was analyzed by performing a climbing assay as previously described in Sanz et al. (2017). At least 100 7 and 15-day-old female flies of each genotype were analyzed and the climbing ability was calculated as the average of the height reached by each fly in 10 s.

2.6 | Western blot analysis

Protein extracts were obtained from fly heads of 15-day-old females of each genotype. Western blots were performed as described in Sanz et al. (2022). Primary antibodies used were anti-tyrosine hydroxylase (TH) (1:1000, Sigma; #AB152) and anti-α-tubulin (1:5000; Hybridoma Bank; #12G10). Secondary antibodies used were anti-rabbit or anti-mouse HRP-conjugated (1:5000, Sigma). Protein levels were quantified using an ImageQuantTM LAS 4000mini Biomolecular Imager (GE Healthcare), and images were analyzed with ImageJ software (NIH). All experiments were performed in quadruplicate.

2.7 | Quantification of H_2O_2 levels

H_2O_2 levels were measured in 15-day-old female flies of each genotype using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen) as previously described in Sanz et al. (2017). All experiments were carried out using three biological replicates and three technical replicates for each sample.

2.8 | Statistical analyses

The significance of differences between means was assessed using a *t* test when two experimental groups were analyzed. Differences were considered significant when $p < 0.05$. Data are expressed as means ± standard deviation (SD).

3 | RESULTS

3.1 | T1DM model flies exhibit alterations in carbohydrate metabolism

In this study, T1DM model flies were obtained by using the GAL4-UAS system, which allows the expression of different transgenes in specific tissues (Osterwalder et al., 2001). In such flies, the proapoptotic protein reaper (White et al., 1996) was specifically expressed in IPCs, thus leading to their death. Due to IPC ablation, ILPs production should be inhibited hence mimicking T1DM pathology (Broughton et al., 2005; Haselton et al., 2010; Rulifson et al., 2002). To confirm this assumption, we first checked whether T1DM model flies presented a reduction of *Ilp* gene expression, as expected in the absence of IPCs. There are eight different *Ilp* genes in the *Drosophila* genome, which have redundant functions in metabolism. However, we focused on *Ilp2*, *Ilp3*, and *Ilp5* since they are predominantly expressed in IPCs, and have a role in controlling glucose levels in the fly hemolymph (Gáliková & Klepsatel, 2018; Graham & Pick, 2017). Indeed, we found that 15-day-old T1DM model flies presented a significant reduction of *Ilp2*, *Ilp3*, and *Ilp5* expression levels when compared with control flies (Figure 1a). Subsequently, we evaluated carbohydrate content in model flies since T1DM is characterized by high carbohydrate levels due to insulin dysregulation. Our results showed that T1DM model flies presented increased levels of soluble carbohydrates as well as of glycogen when compared with controls (Figure 1b,c), in agreement with previous studies

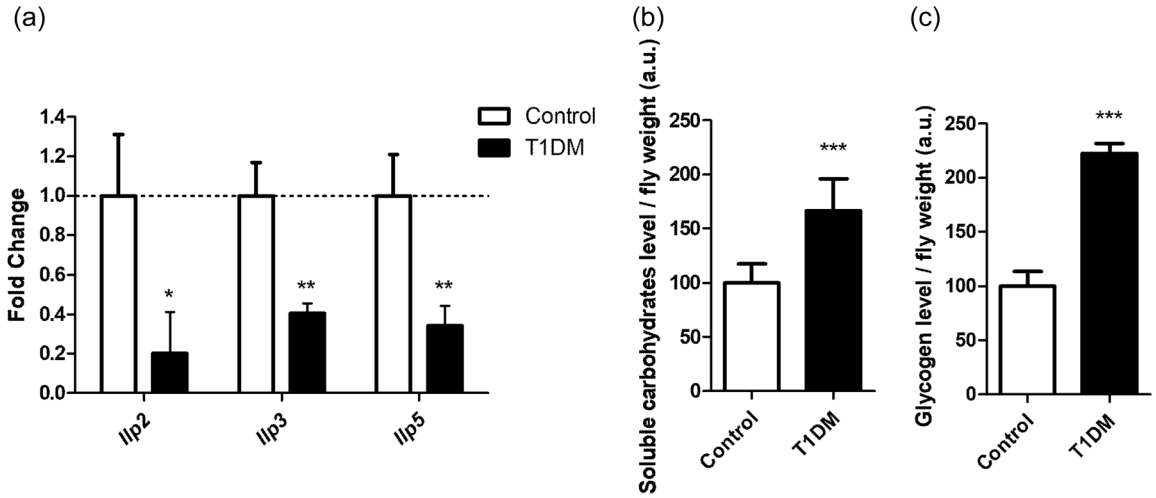

FIGURE 1 Metabolic alterations in type 1 diabetes mellitus (T1DM) model flies. (a) Expression levels of *Ilp2*, *Ilp3*, and *Ilp5* genes in 15-day-old T1DM model flies and aged controls were analyzed by reverse-transcription quantitative polymerase chain reaction (RT-qPCR). Results are expressed as fold change compared with controls. (b) Soluble carbohydrate and (c) glycogen levels in 15-day-old T1DM model flies were analyzed by absorbance using the anthrone method. In all cases, results are expressed as arbitrary units (a.u.) and are normalized to data obtained in control flies. Error bars show SD from four independent experiments in (a) and from three replicates and three independent experiments in (b) and (c) (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

performed in larvae (Rulifson et al., 2002). Overall, these results confirmed that these flies displayed typical T1DM phenotypes.

3.2 | T1DM model flies present a reduction in the ISP activity

Insulin is a hormone that exerts its functions through the activation of ISP signaling. In turn, ISP might activate two different pathways: the mitogen-activated protein kinase (MAPK) pathway and phosphatidylinositol 3 kinase (PI3K) signaling. In *Drosophila*, the MAPK pathway is involved in cell growth and differentiation, while the PI3K pathway has a role in metabolism (Świderska et al., 2020). Given that *Ilp*s gene expression was reduced in T1DM model flies, we analyzed whether this could lead to alterations in the expression of genes encoding ISP components in *Drosophila* like *InR*, *chico* (which encodes the only substrate of InR), and *PI3K* (Muñoz-Soriano et al., 2018). Accordingly, we found that 15-day-old T1DM model flies presented a significant reduction of *chico* and *PI3K* expression levels (Figure 2), thus indicating that ISP activity was reduced. Although no significant changes in *Inr* expression levels were evident in T1DM model flies compared with controls, a decreasing trend was observed (Figure 2).

Several studies have shown that low ISP activity results in growth retardation in several T1DM models, thus causing a reduction in weight (Matsumoto et al., 2011; Pasco & Léopold, 2012; Rulifson et al., 2002). Therefore, we evaluated whether 15-day-old T1DM model flies were smaller than controls, by comparing their body weight with that of aged controls. Our results are consistent with previous observations, since we found that body weight was significantly reduced in model flies (Figure 3).

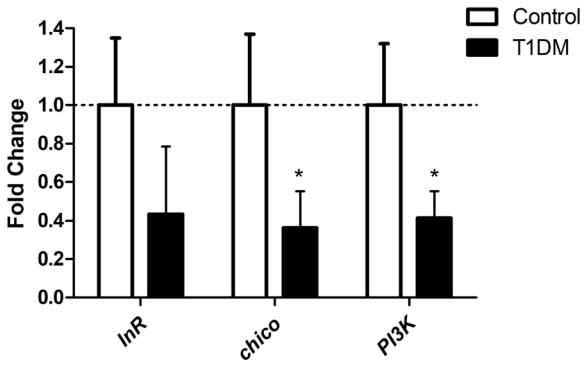

FIGURE 2 Insulin signaling pathway activity in type 1 diabetes mellitus (T1DM) model flies. Expression levels of genes encoding insulin signaling pathway (ISP) components like *InR*, *chico*, and *PI3K* were analyzed by reverse-transcription quantitative polymerase chain reaction (RT-qPCR) in 15-day-old T1DM model flies and aged controls. Results are expressed as fold change compared with control flies. Error bars show SD from four independent (*$p < 0.05$).

3.3 | T1DM model flies exhibit PD-related phenotypes

As previously indicated, there are several evidence showing that DM and PD present similar molecular phenotypes, despite being very different diseases (Álvarez-Rendón et al., 2018; Deshmukh & Jain, 2015; Murillo-Maldonado et al., 2011). Moreover, numerous studies have demonstrated that DM might be a risk factor of developing PD (de Iuliis et al., 2022; Fiory et al., 2019), and that it might worsen the progression of PD symptoms (de Iuliis et al., 2022). Most of them are focused on T2DM, but little information about the possible relationship between T1DM and PD is available. Therefore,

we decided to study if T1DM model flies exhibited common PD-related phenotypes. Interestingly, climbing assays performed with 15-day-old T1DM model flies showed that they exhibited motor defects when compared with aged controls (Figure 4a). It has been demonstrated that such defects appear in PD patients when there is a significant loss of DA neurons (Mahlknecht et al., 2022). However, other studies performed in animal models have shown that PD-related phenotypes might also appear due to impairment of DA neuron function, instead of neuron loss, which leads to a reduction of TH levels (a specific marker of DA neurons) (González-Rodríguez et al., 2021; Navarro et al., 2014; Rose et al., 2017). Therefore, to analyze possible alterations in DA neuron function, we quantified TH levels in the heads of T1DM model flies. We found that 15-day-old T1DM model flies presented a reduction of TH levels when compared with controls (Figure 4b), a clear indication of DA neurodegeneration. Taken together, our results demonstrated that T1DM model flies also exhibited typical PD-related phenotypes thus supporting that T1DM might be a risk factor of developing PD. It has been reported that hyperglycemia may contribute to DA neuron loss by increasing ROS levels (Murillo-Maldonado et al., 2011; Renaud et al., 2014). Therefore, we decided to quantify levels of H_2O_2 (a component of the total ROS pool) in 15-day-old T1DM model flies, finding that they exhibited a significant increase of H_2O_2 levels when compared with aged control controls (Figure 5).

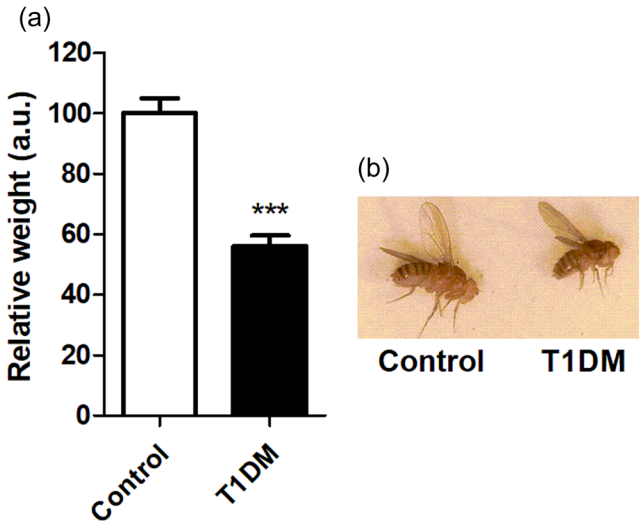

FIGURE 3 Type 1 diabetes mellitus (T1DM) model flies display growth impairment. (a) Relative body weight of T1DM model flies and controls. Results were normalized to data obtained in control flies. Error bars show SD from six independent experiments in (***$p < 0.001$). (b) A representative image of size differences between T1DM model flies and controls is shown.

4 | DISCUSSION

Current evidence support the negative effects of metabolic disturbances in the onset and/or development of neurodegenerative diseases (Onaolapo et al., 2023). According to this, it has been suggested that metabolic alterations could be the origin of those disorders (Muddapu et al., 2020). Furthermore, the existence of cellular and molecular connections between DM and Alzheimer's disease has led to term this disease as type 3 DM (Kandimalla et al., 2017). To date, DM has been widely proposed as a risk factor of developing PD. Several studies have demonstrated the existence of a link between PD and T2DM; however, very little evidence do exist regarding its connection to T1DM (de Iuliis et al., 2022; Fiory et al., 2019; Reed et al., 2021). In fact, there is only one epidemiological study carried out in Austria that reported a likely association between T1DM and PD (Klimek et al., 2015). Despite this,

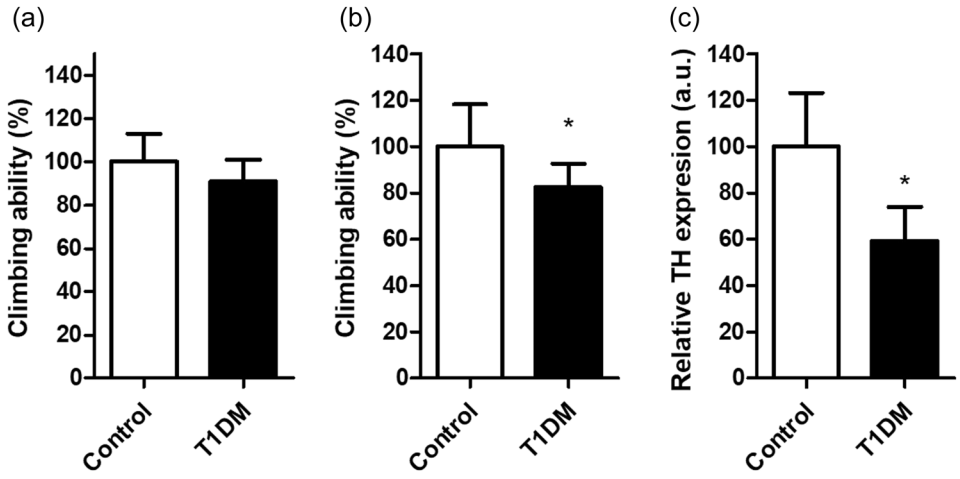

FIGURE 4 Parkinson's disease (PD)-related phenotypes in type 1 diabetes mellitus (T1DM) model flies. Motor performance of (a) 7 and (b) 15-day-old T1DM model flies and controls was evaluated by performing a climbing assay, and results were referred to data obtained in control flies. (b) Graphical representation of tyrosine hydroxylase (TH) protein levels in 15-day-old T1DM model flies and controls. α-tubulin was used as the loading control. Results are referred to data obtained in control flies and are expressed as arbitrary units (a.u.). Error bars show SD from three replicates and three independent experiments in (a), and from four independent experiments in (b) (*$p < 0.05$).

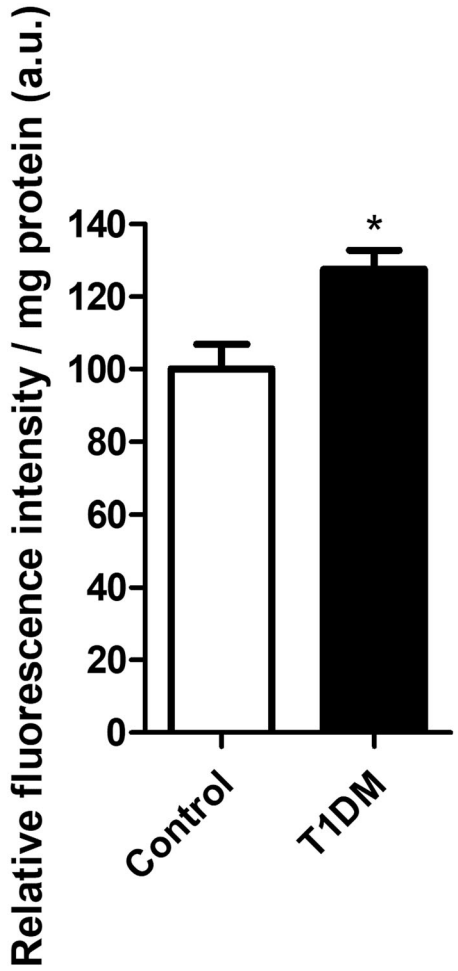

FIGURE 5 Oxidative stress levels in type 1 diabetes mellitus (T1DM) model flies. H_2O_2 levels in 15-day-old T1DM model flies and controls were analyzed by fluorescence using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit. Data were expressed as arbitrary units (a.u.) per mg of protein, and results were referred to data obtained in control flies. Error bars show SD from three replicates and three independent experiments ($^*p < 0.05$).

several findings already suggested that T1DM might be a potential cause of developing PD. For instance, it was found that some T1DM patients displayed spontaneous movements caused by brain dysfunction (Moheet et al., 2015). In addition, induction of T1DM by streptozotocin injection in α-synuclein-overexpressing PD model mice aggravated motor deficits and increased DA degeneration as well as α-synuclein accumulation (Lv et al., 2022).

In this scenario, we aimed to confirm the potential link between T1DM and PD by using a *Drosophila* model of the disease based on IPCs ablation. A reduction of *Ilp* gene expression and a dysregulation of carbohydrate homeostasis was observed in model flies, as previously reported in other *Drosophila* models of T1DM (Broughton et al., 2005; Haselton et al., 2010; Rulifson et al., 2002). Moreover, T1DM model flies also exhibited a reduction in size, a phenotype associated with low ISP activity (Broughton et al., 2005; H. Zhang et al., 2009). Thus, we confirmed that T1DM model flies exhibited typical features of this metabolic disease. However, what about the link between T1DM and PD? It has been reported that insulin participates in neuronal homeostasis, playing a key role in mitochondrial activity (Chomova, 2022; Lee et al., 2016). Indeed, insulin deficiency was shown to cause alterations in the electronic transport chain, leading to a reduction in ATP production (Chomova, 2022; Karakelides et al., 2007). DA neurons have elevated energetic demands but low respiratory capacity, which makes them vulnerable to low ATP levels (Gerhold et al., 2021; Renaud et al., 2018). All these observations suggested that DA neurons might be more susceptible to degenerate in T1DM conditions due to insulin deficiency. Supporting this assumption, we found a reduction in TH levels in T1DM model flies heads together with the presence of locomotor deficits. Interestingly, motor defects in T1DM flies appeared earlier than in a Drosophila model of T2DM in which flies were cultured in a high sugar medium (Sanz et al., 2022). This suggests that T1DM alterations might specifically accelerate neurodegeneration, which could lead to PD onset. Since insulin deficiency has detrimental effects in neurons, this molecule was studied as a potential treatment for PD. According to this, insulin pretreatment was shown to reduce 1-Methyl-4-phenyl pyridinium-induced death in human neuroblastoma cells (Ramalingam & Kim, 2016). Besides, insulin administration was able to ameliorate motor and cognitive alterations in PD patients (reviewed in Sharma et al., 2021). Moreover, an increase of ISP activity by using GLP-1 analogs reduced locomotor defects and attenuated DA loss in PD model mice (Zhang et al., 2018). All these results supported the existence of a link between T1DM and neurodegeneration/PD as demonstrated in the present study. To date, the cause by which DM may trigger PD onset is still unknown. Among the possible explanations, macrovascular and microvascular complications might play a key role (Álvarez-Rendón et al., 2018). In addition, hyperglycemia was proposed as a potential cause of neurodegeneration by increasing OS levels (Murillo-Maldonado et al., 2011; Renaud et al., 2014). According to this, we have demonstrated that T1DM model flies exhibited elevated H_2O_2 levels compared with controls. However, other alterations such as neuroinflammation or increased methylglyoxal levels have been described as possible links between DM and PD (de Iuliis et al., 2022; Hassan et al., 2020; Rocha et al., 2021). Further studies will be required to confirm these assumptions.

5 | CONCLUSIONS

In summary, we have demonstrated that T1DM might trigger PD onset using *Drosophila* as a model. Excitingly, our results showed that T1DM model flies based on IPCs ablation exhibited DA neurodegeneration and locomotor defects, the most common PD phenotypes, together with alterations associated with the metabolic disease. Therefore, we show for the first time that T1DM might be a risk factor of developing PD. These results encourage further experiments to validate these findings in other models and to investigate the exact causes by which T1DM might trigger DA neurodegeneration.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data supporting the findings of the present study are available from the corresponding author upon reasonable request.

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