Drosophila Insulin Pathway Mutants Affect Visual Physiology and Brain Function Besides Growth, Lipid, and Carbohydrate Metabolism

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OBJECTIVE—Type 2 diabetes is the most common form of diabetes worldwide. Some of its complications, such as retinopathy and neuropathy, are long-term and protracted, with an unclear etiology. Given this problem, genetic model systems, such as in flies where type 2 diabetes can be modeled and studied, offer distinct advantages.

RESEARCH DESIGN AND METHODS—We used individual flies in experiments: control and mutant individuals with partial loss-of-function insulin pathway genes. We measured wing size and tested body weight for growth phenotypes, the latter by means of a microbalance. We studied total lipid and carbohydrate content, lipids by a reaction in single fly homogenates with vanillin-phosphoric acid, and carbohydrates with an anthrone-sulfuric acid reaction. Cholinesterase activity was measured using the Ellman method in head homogenates from pooled fly heads, and electroretinograms with glass capillary microelectrodes to assess performance of central brain activity and retinal function.

RESULTS—Flies with partial loss-of-function of insulin pathway genes have significantly reduced body weight, higher total lipid content, and sometimes elevated carbohydrate levels. Brain function is impaired, as is retinal function, but no clear correlation can be drawn from nervous system function and metabolic state.

CONCLUSIONS—These studies show that flies can be models of type 2 diabetes. They weigh less but have significant lipid gains (obese); some also have carbohydrate gains and compromised brain and retinal functions. This is significant because flies have an open circulatory system without microvasculature and can be studied without the complications of vascular defects. *Diabetes* **60:1632–1636, 2011**

I nsulin stimulates cell growth and regulates lipid and carbohydrate metabolism (1). Defects in insulin signaling lead to diabetes in humans, a chronic degenerative disease characterized by metabolic disturbances diagnosed by elevated glucose plasma levels. Complications include retinopathy, neuropathy, and nephropathy. Diabetic retinopathy courses with abnormal retinal angiogenesis and vascular permeability that lead to blindness. Diabetic retinopathy was linked recently to neuronal damage independent of vascular defects (2) where diabetes symptoms such as hyperglycemia are thought to act as triggers (3). However, molecular mechanisms underlying these processes are not clear. It is obvious that a model where a clean separation can be made from complications dependent and independent of vascular damage is highly desirable.

Molecular and genetic studies in *Drosophila* have documented evolutionary conservation of insulin signaling (4,5). The fly genome encodes seven insulin–like peptides (termed dilp1-7), homologs of mammalian insulin, and insulin-like growth factors. Dilps bind and activate the insulin receptor (InR), which autophosphorylates, allowing the binding and phosphorylation of Chico (fly insulin receptor substrate homolog). The fly phosphatidylinositol 3 kinase (PI3K) homolog then binds Chico and InR. The PI3K catalytic subunit (Dp110) phosphorylates phosphatidylinositol (4,5) bisphosphate (PIP_2) to phosphatidylinositol (3–5) trisphosphate (PIP_3). PIP_3 recruits the fly PDK1 and PKB kinase homologs to the membrane. Activated PKB regulates growth and metabolism via diverse protein targets, including the fly homologs of Rheb, a G protein required for target of rapamycin (TOR) kinase activation, TOR, S6K, a kinase that phosphorylates the S6 ribosomal protein, glycogen synthase kinase 3 (GSK-3), and the transcriptional factor forkhead box class O (5).

Insulin pathway conservation also occurs at the physiologic level. Flies with reduced dilps show developmental delays and combined elevated hemolymph glucose and trehalose levels (trehalose being the primary circulating sugar in flies, a nonreducing disaccharide) besides higher whole-body glycogen and lipid content (6,7). Fly glucose metabolism is similar to that in mammals, with the exception that trehalose is also used. Trehalose is synthesized in the fat body (a rough equivalent of liver/adipose tissue of mammals), circulated in hemolymph, and taken up by muscle and target tissues, where it is metabolized to glucose. Trehalose fits the need for a quickly metabolizable, nontoxic intermediate energy storage molecule, especially for indirect flight muscles (8). Overall, and because of evolutionary conservation, *Drosophila* has been key for genetic and molecular elucidation of metabolism and diabetes (9).

The adult *chico* and *InR* mutant flies that emerge after metamorphosis are significantly smaller, with a higher lipid content per milligram of protein. They are obese and diabetic, with stunted growth (10,11). It is unknown, as is true in humans, whether aging diabetic flies will show diminishing lipid and carbohydrate levels (12). Here, we studied weight, metabolic alterations in young adult flies with partial loss-of-function mutations for the insulin pathway and brain and retinal function.

RESEARCH DESIGN AND METHODS

***Drosophila* stocks.** Flies were cultured under standard conditions (25°C, 50% relative humidity, 12/12-h light/dark cycles). Fly stocks used were InR^{E19}/TM3 (chemically induced hypomorphic mutation not in the coding sequence), InR^{O5545}/TM3 (transposable element insertion in the first coding exon), InR^{3T5}/TM3, $chico^1$/CyO (transposable element insertion in the first coding exon), $Dp110^{2H1}$/TM6B, $Dp110^{5W3}$/TM6B, $Dp110^A$/TM6 (deletion of the 3′ end of the coding sequence), PKB^1/TM3 (point mutation in the kinase catalytic domain F327I), PKB^3/TM3 (point mutation in the coding sequence G99S), $dRheb^{7A1}$/TM6B (point mutation in the coding sequence V71 K), $dRheb^{2D1}$/TM6B, $dRheb^{44.1}$/TM6B (0.6-Kb insertion in the 5′−coding sequence), $dS6K^{I-1}$/TM3 (5′−deletion in the coding sequence), and $dS6K^{P1713}$/TM3 (transposable element insertion) obtained from E. Hafen (ETH, Zurich, Switzerland). The mutant alleles and balancer chromosomes (CyO, TM3, and TM6B) used in this study are described in FlyBase (http://flybase.org) and the references therein, and with the exception of InR^{3T5} and $dS6K^{P1713}$, all are characterized and published bona fide mutants.

Genetics. Homozygous mutant flies for *chico*, *Dp110*, and *Rheb* were made by crossing heterozygous $chico^1$/CyO, $Dp110^{2H1}$/TM6B, $Dp110^{5W3}$/TM6B, and $dRheb^{7A1}$/TM6B inter se, respectively, and selecting progeny flies without balancer chromosomes. Because mutant alleles of other insulin pathway loci are homozygous embryonic lethal, we used flies with the following viable heteroallelic combinations: InR^{E19}/InR^{5545}, InR^{5545}/InR^{3T5}, InR^{E19}/InR^{3T5}, $Dp110^{2H1}$/$Dp110^{5W3}$, $Dp110^A$/$Dp110^{5W3}$, PKB^1/PKB^3, $Rheb^{2D1}$/$Rheb^{44.1}$, and $S6K^{I-1}$/$S6K^{P1713}$. These last were obtained by crossing heterozygous flies for both mutant alleles in the heteroallelic combination desired and selecting flies without balancer chromosomes in the progeny. These viable alleles and heteroallelic combinations represent unique and different fly models of type 2 diabetes. Most insulin pathway alleles and heteroallelic combinations are lethal and so cannot be studied. Among rare viable insulin pathway mutant combinations, these were chosen to represent a cross section of the pathway at different levels, and to our knowledge, are the sole known *Drosophila* models of diabetes type 2. To account for differences in genetic backgrounds, pairwise comparisons were always done between mutant combinations and sibling controls.

Body weight, wing size, lipid, and carbohydrate content. To determine body weight, wing size, and lipid and carbohydrate content, flies were aged for 2 days after eclosion before use. We measured body weight in cold-killed individual females placed in a microbalance (Cahn C-31; Manasquan, NJ) with 0.1 μg of sensitivity and a range of 0.1 μg to 25 mg. For wing analysis, flies were anesthetized with CO_2, and wings were dissected, placed in absolute ethanol, and mounted in a 6:5 mixture of lactic acid/ethanol (13). Measurements were made directly on digitized images of mounted wings using iVision software (Calgary, AB, Canada).

Lipid determination was as described previously (14). Total lipids were calculated against a calibration curve using vegetable oil solutions as the lipid standard. Carbohydrate content was determined as described previously (15) and was estimated against a calibration curve of standard glucose solutions. Estimates may not reflect total carbohydrate content, because no test was done to ensure complete digestion and reaction of cuticular carbohydrates; nevertheless, all flies were treated in the same way. Results are expressed as the percentage change.

Determination of cholinesterase activity. Groups of 10 adult flies (aged 2 days) were decapitated, and heads were frozen for at least 24 h. Heads were homogenized in Tris-HCl saline buffer containing protease inhibitors (1 mol/L NaCl, 50 mmol/L $MgCl_2$, 1 mmol/L EGTA, 1 mg/mL bacitracin, 2 mmol/L benzamidine, 0.1 mg/mL soybean trypsin inhibitor, 10 μg/mL pepstatin, 20 units/mL aprotinin, 20 μg/mL leupeptin, and 10 mmol/L Tris buffer, pH 7.0).

Acetylcholinesterase activity was measured by the Ellman method (16) using 1 mmol/L acetylthiocholine as the substrate and 50 μmol/L iso-OMPA
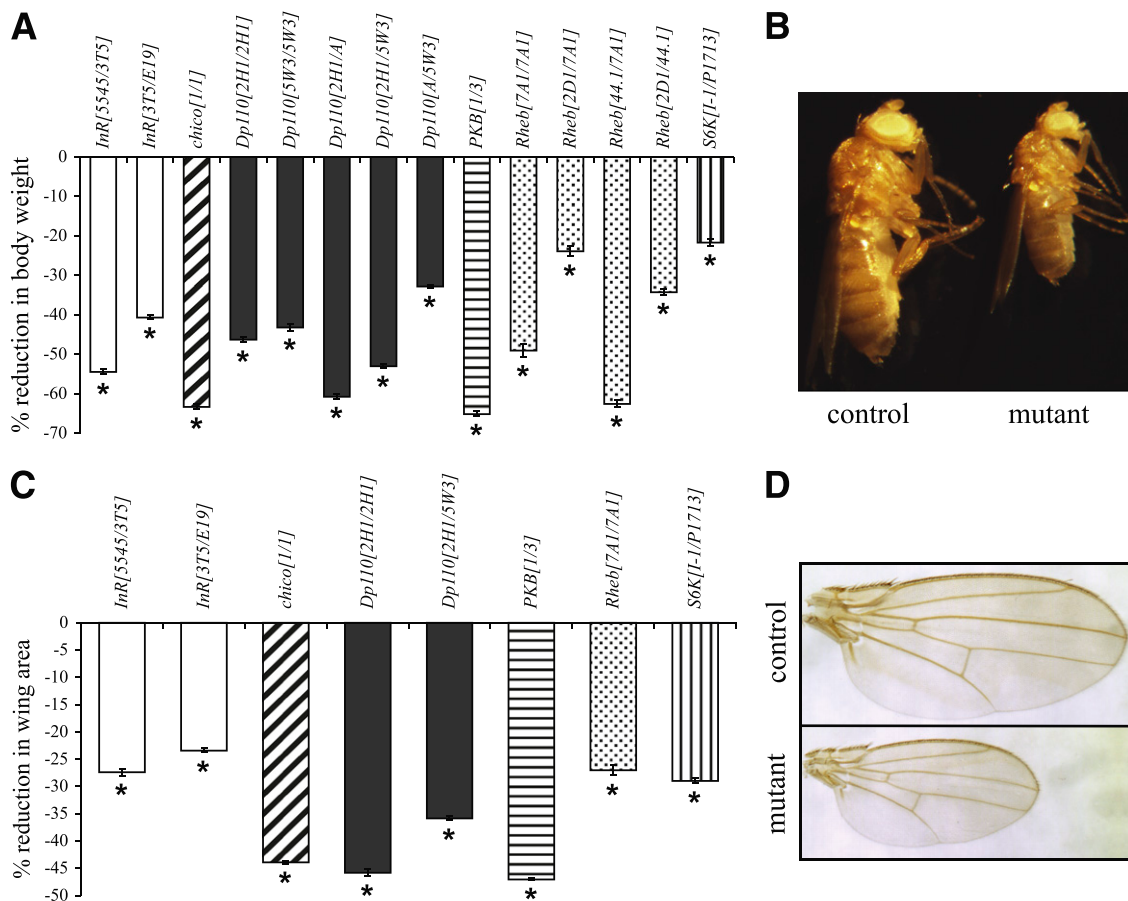
FIG. 1. Mutant flies for insulin signaling show defective growth. *A*: Histogram shows the percentage of reduction in body weight of heteroallelic and homozygous mutants of the insulin pathway compared with their respective paired heterozygous sibling controls ($n = 16$). *$P < 0.01$. The error bars represent the SEM. *B*: Heteroallelic $PKB^{1/3}$ mutant flies are smaller than $PKB^{1/+}$ or $PKB^{3/+}$ heterozygous flies (controls). *C*: The wing area of insulin pathway mutant flies is reduced compared with paired sibling controls ($n = 30$). *$P < 0.01$. The error bars represent SEM. *D*: Heteroallelic mutant $PKB^{1/3}$ wings are smaller than heterozygous $PKB^{1/+}$ or $PKB^{3/+}$ wings (controls). (A high-quality color representation of this figure is available in the online issue.)

(tetraisopropyl pyrophosphoramide). Iso-OMPA is a cholinesterase inhibitor with relative specificity for butyrylcholinesterase (17). The assay mixture contained 0.05% (w/v) Triton X-100. Acetylcholinesterase activity was determined as described previously (18). One unit of enzymatic activity is equal to 1 μmol of substrate hydrolyzed per hour per milliliter at pH 7.5 and 25°C. Protein content was determined according to Lowry (19) using BSA as the standard.

Electroretinogram recordings. Electroretinogram (ERG) recordings were performed as described previously (20), with the exception that the recording electrode made electrical contact with the compound eye. The ground microelectrode was placed in the thorax. Flies were adapted to the dark for at least 5 min before recordings, and all recordings were performed in the dark. Stimulating white light was 427 lux for 500 ms. Electrical responses were amplified, visualized, and photographed in a TDS210 oscilloscope (Tektronix, Beaverton, OR). Each fly was tested five times, and individual results were averaged per fly. For each genotype, 10 flies were tested.

Statistical analysis. Student t test was used for pairwise comparisons between mutant and sibling controls. Data are presented as mean ± SEM.

RESULTS

Most insulin pathway mutant alleles are homozygous lethal. To circumvent lethality and obtain varying degrees of reduction in insulin signaling, we used viable heteroallelic and viable homozygous hypomorphic mutant alleles ($Dp110^{2H1}$, $Dp110^{5W3}$, and $Rheb^{7A1}$). The allele $chico^1$ is viable, despite being a null (partly because the *Drosophila* InR has an intracellular extension homologous to insulin receptor substrate genes, allowing the *Drosophila* InR to function as an insulin receptor substrate) (21).

These flies have some insulin pathway functionality left, typical of type 2 diabetic patients. This allows escape from lethality; because of this, alterations in the parameters studied, although not necessarily present in all mutant combinations tested for the same gene, if seen in at least one mutant combination, is treated to mean that the gene affects the particular function studied.

As a way to quantify insulin-signaling effects on growth, we measured body weight and wing size of mutant flies and control siblings and calculated percentage reductions. All of the mutant combinations had different, but significant, degrees of weight reduction (range 22% for $S6K^{I-1/P1713}$ to 65% for $PKB^{1/3}$). Wings were similarly significantly reduced (Fig. 1).

To examine effects on metabolism, we determined lipid content in individual flies. We found that mutants generally have a significant increase in lipid content, which was close to 100% in PKB mutants and in $Dp110^{2H1/2H1}$. Less dramatic but significant increases were found for the insulin receptor, $chico$, $S6K$, and three heteroallelic combinations of $Dp110$. No significant differences were found for $Dp110^{5W3/5W3}$ or for three of four mutant combinations for $Rheb$. From these results we conclude that all tested proteins are involved in lipid metabolism (Fig. 2A).

We also determined carbohydrate content. Of the mutants tested, fewer showed significant changes. The three heteroallelic combinations of $Dp110$, $InR^{3T5/E19}$, and $Rheb^{7A1/7A1}$ exhibited significant increases, whereas $Rheb^{44.1/7A1}$ had a discrete decrease (Fig. 2B). Other mutants, such as $Dp110^{2H1/2H1}$, $PKB^{1/3}$, and $chico^{1/1}$ had no significant changes. We interpret these data to mean that at least InR, $Dp110$, and $Rheb$ are involved in carbohydrate metabolism. Two ways to rationalize that other genes had no effect ($chico$, PKB, and $S6K$) is that lipid metabolism may be affected more than carbohydrates by insulin signaling, and/or that the particular combinations tested retained more carbohydrate function.

Taken together, these results argue that growth and both lipid and carbohydrate metabolism is partially regulated by
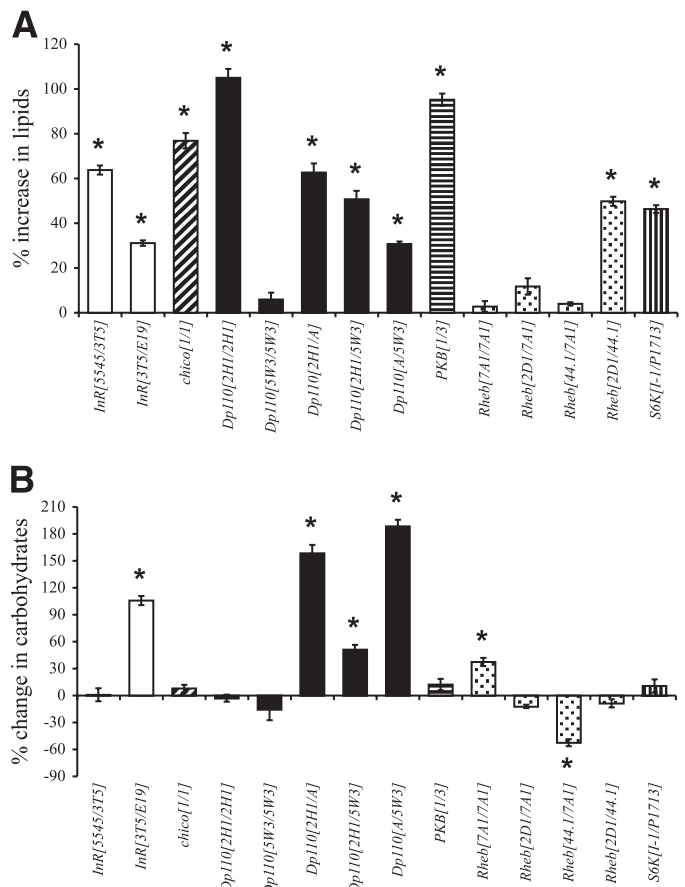
FIG. 2. Insulin signaling is required for lipid and carbohydrate metabolism. *A*: Histogram shows the percentage increases in lipids of heteroallelic and homozygous mutant flies for the insulin pathway compared with paired heterozygous sibling controls. *B*: Histogram shows the percentage change in body carbohydrates of heteroallelic and homozygous mutant flies for the insulin pathway compared with paired heterozygous siblings ($n = 8$). *P < 0.01. The error bars represent the SEM.

insulin. This has been shown in mammals and diabetic patients, making the fly model similar.

Insulin signaling has been implicated in retinal (22) and brain function (23), so we looked for alterations in the fly nervous system. We assessed retinal physiology using ERGs. Most mutant genotypes tested, but not all, showed a decrease in amplitude of the ERG-sustained component (Fig. 3A). The off transient, a consequence of light-evoked synaptic activity at the brain lamina (24), also showed amplitude reductions (Fig. 3B).

ERG decreases do not correlate with the extent of metabolic alterations. $Dp110^{5W3/5W3}$ shows severe ERG defects but normal lipid and carbohydrate content. $PKB^{1/3}$ has defective ERGs (Fig. 3B), a dramatic increase in body lipids, but no change in carbohydrates. $InR^{5545/3T5}$ exhibits no change in ERG and carbohydrate levels but an increase in lipid content. $Dp110^{2H1/A}$ has no ERG defect but does have severe lipid and carbohydrate alterations. Finally, $Rheb^{7A1/7A1}$ has normal ERG and lipid levels but a significant reduction in carbohydrates. As suggested for diabetes, retinal defects here arise independently of microvasculature defects.

A general and global way to assess nervous system function is to use an enzymatic activity common in nervous tissue as an activity reporter. We measured cholinesterase activity from fly head extracts because acetylcholine is the

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B

C
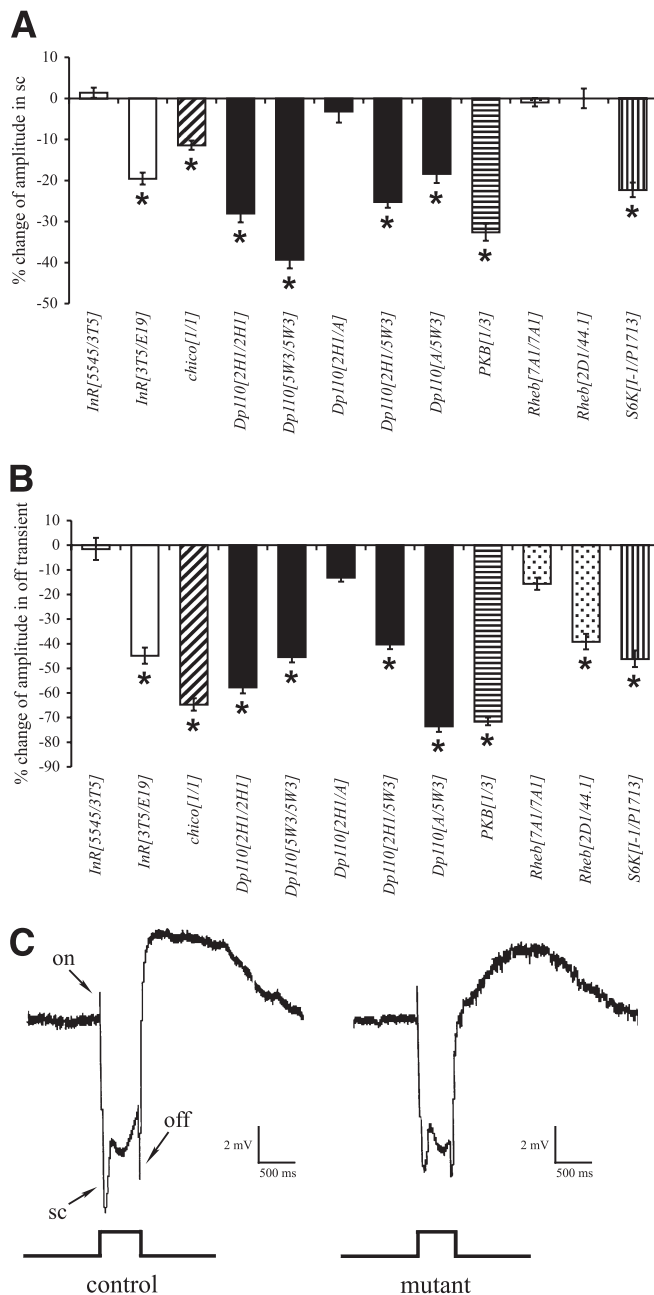
FIG. 3. Insulin pathway mutant flies show defective retinal function. Histograms show the percentage reduction in the amplitude of the sustained component (*A*) and off transient (*B*) of the ERG of heteroallelic and homozygous mutants compared with paired heterozygous sibling controls (*n* = 10). *P < 0.01. The error bars represent the SEM. *C*: ERGs from heteroallelic *PKB*[1/3] mutant flies and heterozygous sibling controls. The mutant condition shows a significant amplitude reduction of the receptor potential (sc) and the off transient (off) in the ERG. The on transient (on) is also marked.

main excitatory neurotransmitter in fly brains (25). Most mutant phenotypes studied have significantly diminished cholinesterase activity, implying faulty brain acetylcholine metabolism (Fig. 4). Diabetic retinas in rats also show significant changes in cholinesterase activity (18).

DISCUSSION

Insulin signaling affects lipid and carbohydrate metabolism and size in flies, showing evidence of the pathway's two-prong control over metabolism and growth. Moreover, nervous
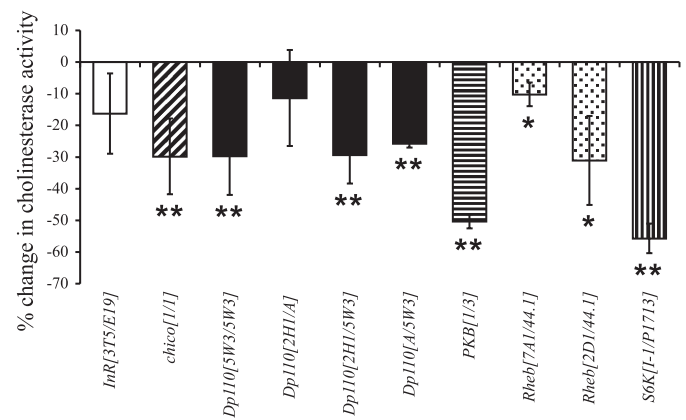
FIG. 4. Cholinesterase activity is defective in insulin mutant flies. Histogram shows percentage reduction in cholinesterase of the insulin pathway heteroallelic and homozygous mutants compared with paired heterozygous siblings (*n* = 3–8). *P < 0.1, **P < 0.05. The error bars represent the SEM.

system defects exhibit alterations on at least two neurotransmitter systems: acetylcholine and histamine. Histamine is the fly photoreceptor neurotransmitter (25). Reduced photoreceptor depolarization (amplitude reductions of sustained ERG component) leads to reduced neurotransmitter release, and whether this solely explains reduced off transients (brain electrical responses to histamine) does not belie the fact that neuronal communication is compromised. Reduced cholinesterase activity, as a general brain readout, implies widespread nervous system effects.

Our results, in general, argue that nervous system phenotypes are partially independent of metabolic and growth phenotypes, strongly implying an independent origin. This has the unforeseen benefit of allowing the study of insulin-signaling defects in relative isolation: mutant conditions occur without other effects in nervous system physiology in $Dp110^{5W3/5W3}$, lipid metabolism in $InR^{5545/3T5}$, and carbohydrate metabolism in $Rheb^{7A1/7A1}$.

In summary, partial loss-of-function insulin pathway fly mutants have reduced growth, lipid and carbohydrate alterations, and abnormal nervous system function. They represent viable type 2 diabetes models, allowing study of several typical insulin-signaling defects of patients with diabetes, some in relative isolation.

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