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SLOB, a SLOWPOKE Channel Binding Protein, Regulates Insulin Pathway Signaling and Metabolism in Drosophila

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Abstract

There is ample evidence that ion channel modulation by accessory proteins within a macromolecular complex can regulate channel activity and thereby impact neuronal excitability. However, the downstream consequences of ion channel modulation remain largely undetermined. The Drosophila melanogaster large conductance calcium-activated potassium channel SLOWPOKE (SLO) undergoes modulation via its binding partner SLO-binding protein (SLOB). Regulation of SLO by SLOB influences the voltage dependence of SLO activation and modulates synaptic transmission. SLO and SLOB are expressed especially prominently in median neurosecretory cells (mNSCs) in the pars intercerebralis (PI) region of the brain; these cells also express and secrete Drosophila insulin-like peptides (dILPs). Previously, we found that flies lacking SLOB exhibit increased resistance to starvation, and we reasoned that SLOB may regulate aspects of insulin signaling and metabolism. Here we investigate the role of SLOB in metabolism and find that slob null flies exhibit changes in energy storage and insulin pathway signaling. In addition, slob null flies have decreased levels of dilp3 and increased levels of takeout, a gene known to be involved in feeding and metabolism. Targeted expression of SLOB to mNSCs rescues these alterations in gene expression, as well as the metabolic phenotypes. Analysis of fly lines mutant for both slob and sio indicate that the effect of SLOB on metabolism and gene expression is via SLO. We propose that modulation of SLO by SLOB regulates neurotransmission in mNSCs, influencing downstream insulin pathway signaling and metabolism.

Introduction

Large conductance, calcium- and voltage-sensitive potassium channels (BK channels) play a critical role in the regulation of neuronal excitability and neurotransmitter release. The Drosophila melanogaster BK channel is encoded by the slowpoke (slo) gene [1,2]. SLO binds to and is regulated by several different protein kinases [3,4], as well as by a novel protein named SLOB (Slo-binding) that we isolated by a yeast two-hybrid screen using the extended carboxy-terminal tail domain of SLO as bait [5,6]. SLOB is expressed especially prominently in median neurosecretory cells (mNSCs) in the pars intercerebralis (PI) region of the fly brain [7]. SLOB expression level in these neurons modulates whole-cell potassium current and properties of single SLO channels [8]. Because these neurons also express and secrete Drosophila insulin-like peptides (dILPs) [7,9], we hypothesized that SLOB might influence behavior related to feeding or metabolism. Indeed, slob mutant fly lines with dramatically decreased SLOB expression show prolonged survival under conditions of complete food-deprivation [8]; such a change in survival under starvation conditions is thought to reflect differences in feeding behavior and/or metabolism during the period prior to food-deprivation [10]. In addition, slob null flies exhibit altered locomotor activity during starvation (Reddy and Levitan, unpublished results). Wild-type flies typically exhibit an extended period of activity under starvation conditions; such hyperactivity is thought to reflect an adaptive foraging strategy in response to diminished food availability [11]. Slob null flies lack this hyperactive response, suggesting a role for the SLO/SLOB complex in mNSCs in integrating food stimuli and coordinating a response to nutrient conditions.

Altered insulin/insulin-like growth factor signaling (IIS) has also been implicated in increased resistance to starvation [12,13]. The IIS pathway is conserved throughout evolution and is a critical regulator of growth, development, and lifespan (reviewed in [14]). Seven insulin-like peptides are expressed in Drosophila melanogaster: dilp1–7 [15]. Of these, dilp2, dilp3, and dilp5 are expressed in mNSCs of adult flies [9,12], which project to the corpora cardiaca (CC), a pair of neurohemal glands located on the walls of the aorta [16]. CC cells express adipokinetic hormone (AKH), which is similar to mammalian glucagon; the PI-CC system in fruit flies is functionally homologous to the hypothalamic-pituitary axis in mammals [17]. MNSCs also project to the dorsal blood vessel, allowing for direct release of dILPs into the circulating hemolymph [16]. Together, AKH and dILPs regulate the levels of circulating sugars [16,18,19]. Disruption of the insulin receptor (InR) or mNSC ablation causes developmental delay, growth retardation, elevated levels of triglycerides, and increased levels of circulating glucose and trehalose [15,16,20,21].

Interestingly, slob and sio are both regulated in a circadian manner [7,22]. Other circadian genes have also been implicated in metabolism. For example, takeout (to) encodes a protein similar to...
juvenile hormone binding protein and also cycles with a daily rhythm [10,23]. To is expressed in structures related to feeding, such as the cardia, crop, antennae, and head fat body [10,23,24]. Similar to the mammalian liver, the fat body is the storage site for lipids and glycogen in insects [25]. To mutant flies are hyperphagic and exhibit alterations in energy storage [24]. Furthermore, they are more sensitive to starvation [10].

Here we sought to determine how SLOB expression in mNSCs influences insulin pathway signaling and metabolism. We find that slb mutant flies exhibit alterations in downstream measures of insulin signaling, as well as differences in energy storage. In addition, we present evidence that lack of SLOB in mNSCs results in dramatic changes in gene expression of to and dilp3.

Interestingly, the effect of SLOB on metabolism appears to depend on SLOB’s effect on to expression level. Importantly, intact SLO function is necessary for changes manifested in the slb null phenotype, implying that the modulation of SLO by SLOB mediates the alterations in gene expression and metabolism.

Results

SLOB levels regulate expression of to

The circadian protein TO is involved in regulation of feeding and energy storage; in addition, to/ mutant flies exhibit decreased resistance to starvation [10,23,24]. Since slb null flies also exhibit a starvation phenotype, surviving significantly longer than wild-type control flies during starvation stress [8], we sought to determine whether expression is altered in slb null flies. To this end, to transcript levels were measured in control (WT) and slb null (slbPP1) fly heads, as well as in rescue lines expressing SLOB specifically in mNSCs. Two rescue lines in the slbPP1 background were examined. The ma301-GAL4 driver targets expression to mNSCs, as well as some additional neurons [26], whereas the dilp2-GAL4 driver line is specific for the dilp-expressing mNSCs [16,27]. SLOB expression is restored with either driver (Fig. 1A).

Interestingly, to transcript levels are significantly upregulated in slbPP1 fly heads, and this effect is rescued by expression of SLOB in mNSCs, regardless of the driver (Fig. 1B, C). The slbPP1, dilp2>slb line expresses the least amount of to and this correlates with the highest levels of SLOB expression (Fig. 1A, C), suggesting a role for SLOB in regulating to levels. SLOB expression is also regulated in a circadian manner [7,22]; therefore, we next investigated whether cycling of to transcripts is disrupted in flies lacking SLOB. As previously reported, to levels peak around ZT 17–21 (Fig. 1D) [10]. To transcript levels are elevated at all time points in slbPP1 fly heads; however, cycling of to remains intact in slbPP1 fly heads under LD conditions. To determine if upregulation of to persists at the protein level, fly head lysates were run on Western blots and probed for TO.

To protein levels still cycle in slbPP1 fly heads (Fig. 2B).

In order to confirm that SLOB levels in mNSCs regulate the expression level of to, we used a different method to decrease SLOB expression, namely slb-RNAi (Fig. 3A). Ubiquitous knockdown of SLOB results in significantly increased levels of to in fly heads (Fig. 3B). Furthermore, knockdown of SLOB in mNSCs is also sufficient to increase expression of to (Fig. 3C). Conversely, ubiquitous overexpression of SLOB results in significantly decreased transcript levels of to (Fig. 3D), and overexpression of SLOB in mNSCs only is sufficient to decrease to transcript levels (Fig. 3E). Likewise, ubiquitous knockdown of SLOB results in increased levels of TO protein (Fig. 4A), and targeted knockdown of SLOB in mNSCs also causes elevated levels of TO (Fig. 4B). Unsurprisingly, total SLOB protein levels are not decreased in ma301>slb RNAi fly heads because SLOB is expressed in other cell types such as photoreceptors, which are not targeted by the ma301 driver [7]. Therefore it appears that SLOB levels in mNSCs influence expression levels of TO, although the mechanism underlying this effect is unclear.

SLOB levels regulate expression of dilp3

Since SLOB is expressed especially prominently in mNSCs [7], we hypothesized that SLOB may influence IIS by modifying dILP expression or release. DILP2 is the most abundant dILP expressed by mNSCs and has the greatest effect on carbohydrate metabolism [12]. Although dilp2 transcript levels are slightly decreased in slbPP1 fly heads, we found no significant difference in dilp2 levels between WT and slbPP1 fly heads (Fig. 3A, Table 1). Likewise, levels of dilp5 transcript are not significantly different between WT and slbPP1 fly heads (Fig. 5B). However, there is a dramatic reduction in dilp3 levels in slbPP1 fly heads compared to WT fly heads (Fig. 5C, Table 1), and this effect is rescued by expression of SLOB in mNSCs only (Fig. 5C). Conversely, overexpression of SLOB in mNSCs results in a striking upregulation of dilp3 (Fig. 5D), suggesting that expression levels of SLOB in mNSCs regulate dilp3 levels. The slight decreases in dilp2 and dilp5 transcript levels are consistent with a role for dilp3 as a positive regulator of dilp2 and dilp5 expression [28]. Since TO is significantly increased in slbPP1 fly heads, and TO is expressed in the head fat body, which has been shown to signal to mNSCs [29,30], we sought to determine whether TO can influence dilp3 expression. To this end, we measured dilp3 transcripts in the double mutant line slbPP1, to1. Interestingly, expression of dilp3 remains low in slbPP1, to1 fly heads; hence TO is not required for downregulation of dilp3 in slbPP1 fly heads (Fig. 5C). Alternatively, dilp3 levels may influence to expression; in order to determine if the effect of SLOB on dilp3 expression is upstream of the effect on to expression, we generated flies lacking dilp3 in the WTPP1 or slbPP1 background. We find that to is expressed at equivalent levels in WTPP1, WTPP1, dilp3, and slbPP1,dilp3 fly heads, indicating that dilp3 is not required for basal expression of to (Fig. 5E). However, the upregulation of to exhibited by slbPP1 fly heads is abolished in slbPP1, dilp3 fly heads; therefore, even though dilp3 is greatly reduced in slbPP1 flies, it appears that the minimal residual amount of dilp3 is required for the control of to expression by SLOB levels.

Slob null flies exhibit alterations in energy metabolism and insulin pathway signaling

SLOB binds to and modulates SLO activity in mNSCs [8]; in addition, we recently demonstrated that flies lacking SLOB exhibit enhanced neurotransmission at the neuromuscular junction, and this is due to modulation of SLO by SLOB in the presynaptic nerve terminal [31]. We therefore hypothesized that slbPP1 flies might exhibit altered excitability in mNSCs, resulting in differences in dILP release and IIS pathway signaling. Altered IIS is associated with changes in energy storage; mNSC ablation or mutation of genes encoding key components of the IIS pathway results in fasting hyperglycemia and altered triglyceride storage [12,15,16,20,21,32]. In addition, to mutant flies exhibit altered energy metabolism [24], therefore we investigated levels of sugars and triglycerides in slbPP1 flies. Circulating carbohydrates in the fly consist of trehalose, the main homeostatic sugar, and glucose from the diet [33]. SlbPP1 flies exhibit significantly decreased levels of circulating trehalose and glucose (Fig. 6A and Table 1), indicative of elevated IIS in slb null flies. To assess activation of the IIS pathway more directly, phosphorylated AKT (P-AKT) was measured in WT and slbPP1 fly heads. InR activation results in downstream
phosphorylation of AKT at Ser 505 in Drosophila, which is homologous to phosphorylation of AKT at Ser 473 in mammals [34]. P-AKT is significantly upregulated in slob flies (Fig. 6B), indicating increased activation of IIS, while total AKT levels remain the same; both PAKT and AKT levels were normalized to levels of the loading control MAPK in these experiments. Although activation of MAPK is altered by IIS, total MAPK levels remain the same [35–37]; therefore normalizing P-AKT to MAPK is

Figure 1. to mRNA levels are increased in slob flies but still cycle. A, Western blot demonstrating rescue of SLOB expression in fly heads using two separate drivers for mNSCs in the slob background. B, C, to mRNA levels in fly heads were measured by qPCR. to relative transcript levels are increased in slob flies and rescued by targeted expression of slob in mNSCs. D, to transcript levels cycle in WT and slob flies. Zeitgeber time (ZT) is plotted on the X axis; the white and black bars represent "lights on" and "lights off", respectively. Each graph is a summary of a minimum of three independent experiments (mean ± SEM). For comparisons between fly lines, * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001; for comparisons between ZT points within one fly line, ## indicates p<0.01, One-way ANOVA with Bonferroni post-test. doi:10.1371/journal.pone.0023343.g001
appropriate. We also normalized P-AKT levels to ACTIN levels, and this analysis yielded results similar to those obtained by normalizing P-AKT to MAPK (data not shown). Measuring circulating sugars in adult flies is difficult due to the small volume of hemolymph present. Therefore we sought to determine if measures of stored sugars in WTP41 and slobIP1 flies reflect those of circulating sugars. Indeed, whole body trehalose and glucose levels are also decreased in slobIP1 flies compared to WTP41 flies (Fig. 6C, Table 1), again suggesting enhanced IIS in slobIP1 flies. Levels of stored trehalose and glucose are rescued in transgenic flies expressing SLOB under the control of mNSC-targeted drivers, mai301-GAL4 or dilp2-GAL4, indicating that expression of SLOB in mNSCs is sufficient to restore whole body trehalose levels. Stored trehalose is also restored in slobIP1,to1 flies, as well as in single to mutants (to1) (Fig. 6D). Therefore it appears that one mechanism underlying the effect of SLOB on whole body sugars is through SLOB’s regulation of to expression. In support, both fly lines lacking dilp3: WTP41, dilp3 and slobIP1, dilp3, express equivalent levels of to and exhibit whole body sugar levels similar to those of WTP41 flies (Fig. 5E, 6D). Whole body trehalose levels are unchanged in single dilp3 mutants [28]; similarly, we find that WTP41, dilp3 flies have levels of whole body trehalose and glucose comparable to those of WTP41 flies. However, the decrease in stored sugars exhibited by slobIP1 is abolished in slobIP1, dilp3 flies; this result is consistent with lack of upregulation of to in slobIP1, dilp3 flies.

Triglyceride levels are altered in to1 flies, as well as in IIS pathway mutant flies [24,38]. Also, flies which exhibit increased resistance to starvation often display increased storage of triglycerides [39–41]. Likewise, slobIP1 flies exhibit increased storage of triglycerides compared to WTP41 flies, and this effect is rescued by expression of SLOB in mNSCs (Fig. 6D). Interestingly, triglyceride levels are also rescued in slobIP1, to1 flies, indicating that the effect on lipid metabolism requires TO.

SLO is required for the effects on gene expression and metabolism in slob null flies

We have established that modulation of SLO activity by SLOB influences neuronal excitability and neurotransmitter release [8,31]. In order to determine if the effects of SLOB on gene expression and metabolism are dependent on modulation of SLO by SLOB, we
Figure 3. *to* mRNA levels are dependent on expression of SLOB. Slob and *to* levels in fly heads were measured by qPCR. 

A. Relative slob transcript levels are decreased in heads of flies in which SLOB expression was knocked down ubiquitously using RNAi.

B. Relative *to* transcript levels are increased in heads of flies in which SLOB expression was knocked down ubiquitously using RNAi.

C. Relative *to* transcript levels are increased in heads of flies in which SLOB expression was knocked down in mNSCs using RNAi.

D. Relative *to* transcript levels are decreased in heads of flies in which SLOB expression was knocked down in mNSCs using RNAi.
which there is ubiquitous overexpression of SLOB. E, Relative to transcript levels are decreased in heads of flies in which SLOB was overexpressed exclusively in mNSCs. Each graph is a summary of a minimum of three independent experiments (mean ± SEM). * indicates p<0.05, ** indicates p<0.01, Student’s t-test.

Figure 4. TO protein levels are dependent on expression of SLOB. TO protein levels in fly heads were measured by Western blot analysis and normalized to MAPK levels. A, Representative Western blot showing increased TO in heads of flies in which SLOB expression was knocked down ubiquitously using RNAi. The graph is a summary of five independent experiments (mean ± SEM). * indicates p<0.05, One-way ANOVA with Bonferroni post-test.

B, Representative Western blot showing increased TO in heads of flies in which SLOB expression was knocked down in mNSCs using RNAi. The graph is a summary of six independent experiments (mean ± SEM). * indicates p<0.05, ** indicates p<0.01, Student’s t-test.

E, Relative to transcript levels are decreased in heads of flies in which SLOB was overexpressed exclusively in mNSCs. Each graph is a summary of a minimum of three independent experiments (mean ± SEM). * indicates p<0.05, ** indicates p<0.01, Student’s t-test. doi:10.1371/journal.pone.0023343.g003

crossed the mutant slo' line [1] into the WTP41 and slobPI1 fly lines to generate WTP41,slo' and slobPI1,slo' lines. WTP41,slo' and slobPI1,slo' flies express approximately equivalent amounts of to in fly heads at levels intermediate to those in WTP41 and slobPI1 flies (Fig. 7A). Compared to slobPI1, there is a trend towards decreased levels of to in these slo' mutant lines, suggesting that the effect of SLOB on to expression may be due at least in part to modulation of SLO. Interestingly, intact SLO function is not a requirement for to expression, as demonstrated by expression of to in WTP41,slo' flies. However, SLO function is necessary for the upregulation of to due to the lack of SLOB, as there is no increase in to in slobPI1,slo' fly heads compared to WTP41,slo' fly heads. Similarly, WTP41,slo' and slobPI1,slo' flies exhibit increased amounts of whole body sugars compared to slobPI1, supporting a role for modulation of SLO in SLOB’s effect on metabolism (Fig. 7B).

We also measured dilp3 levels in WTP41,slo' fly heads, and found dilp3 levels unchanged in WTP41,slo' fly heads compared to WTP41 fly heads. Since the SLOB present in WTP41 fly heads acts to inhibit SLO function [8,42], and SLO activity is not intact in WTP41,slo' flies due to the slo' mutation, these results suggest that decreased SLO activity results in greater levels of dilp3. Conversely, SLO activity is elevated in slobPI1 flies [8], and dilp3 expression is much reduced (Fig. 7C). Although lack of SLOB still results in decreased expression of dilp3 in the slo' mutant line (compare WTP41,slo' and slobPI1,slo'), the effect is significantly attenuated compared to dilp3 downregulation in slobPI1 heads. Therefore, the effect of SLOB on dilp3 expression functions at least in part through modulation of SLO.

Discussion

Previously, we found that slob null flies live significantly longer than control flies under conditions of complete food deprivation [8]. Increased resistance to starvation is often accompanied by
Figure 5. Effect of SLOB on expression of dilps. Relative dilp2, -3, and -5 transcript levels in fly heads were measured by qPCR. **A, B**, Relative dilp2 or dilp5 transcript levels are unchanged in slob<sup>IP1</sup> fly heads. **C**, Relative dilp3 transcript levels are reduced in slob<sup>IP1</sup> fly heads and rescued by expression of SLOB in mNSCs, but not by mutation of to. **D**, Relative dilp3 transcript levels are increased in heads of flies overexpressing SLOB in mNSCs. **E**, The increase in to levels is abolished in slob<sup>IP1</sup>, dilp3 fly heads. Each graph is a summary of a minimum of three independent experiments (mean ± SEM). *** indicates p<0.001, One-way ANOVA with Bonferroni post-test (C, E) or Student’s t-test (D). doi:10.1371/journal.pone.0023343.g005
changes in energy storage or alterations in genes involved in metabolism. We find that levels of the metabolic gene to are upregulated almost five-fold in slb null flies, and this effect is rescued by expression of SLOB targeted specifically to mNSCs. Using RNAi to decrease SLOB levels results in a similar increase, even when SLOB knockdown is targeted to mNSCs. Furthermore, overexpression of SLOB either ubiquitously or specifically in mNSCs downregulates to levels. We confirmed that TO protein levels are similarly upregulated in slb null flies. The mechanism underlying regulation of to in response to SLOB levels in mNSCs is unclear. Since mNSCs project to the CC and the dorsal blood vessel, it is likely that there are several steps between SLOB regulation of mNSCs and its downstream effects on to expression in areas of the head such as the fat body. Like slb, levels of to are regulated by the circadian clock [10,23]. Although to levels are elevated at all time points in slb null flies, to still cycles in the absence of SLOB, suggesting that factors other than SLOB influence circadian regulation of to. PAR domain protein 1 (PDP1) is a circadian transcription factor reported to indirectly regulate to expression [43]; however, pdp1e transcript levels are not significantly different between control and slb null flies (data not shown). TO protein levels also cycle in slb null flies. Expression levels were measured under LD conditions however, and TO protein expression is directly regulated by light [43]; therefore it is not surprising that TO still cycles in the absence of SLOB. It would be interesting to examine whether TO protein still cycles in constant darkness in slb null flies.

It has been proposed that TO may link circadian and feeding behaviors. Of note, to mutant flies die faster during starvation [10]; in combination with our results showing that SLOB expression level regulates to expression, we conclude that upregulation of TO in slb null flies mediates starvation resistance. In addition, slb null flies exhibit increased storage of triglycerides, which may enable them to withstand longer durations of starvation. To mutant flies are hyperphagic and also exhibit increased energy storage; however they cannot harness this energy during periods of starvation, resulting in early death [10,24]. Decreasing TO levels in the slb null background, either through SLOB rescue in mNSCs or through mutation of to, restores triglyceride levels to those of control flies, suggesting that metabolic changes due to the lack of SLOB are mediated by TO. Likewise, fly lines which exhibit low levels of to relative to slb null flies also exhibit increased levels of trehalose compared to slb null flies, suggesting that TO regulates sugar levels as well. The mechanism underlying TO-mediated changes in metabolism is still not entirely clear; TO is secreted into the hemolymph and shares sequence similarity with juvenile hormone binding protein (JHBP) [10]. However it is unknown if TO is also a carrier for juvenile hormone (JH); in addition the receptor for JH remains poorly understood.

Since SLOB is co-expressed with dILPs in mNSCs [7], we reasoned that SLOB may influence the IIS pathway, perhaps by regulating expression or release of dILPs. Dilp2 has the most profound effect on IIS pathway activation and metabolism; however there is no significant change in dilp2 expression in slb null flies. Surprisingly, we discovered a striking decrease in dilp3 levels in slb null flies. This effect is due to SLOB in mNSCs, since it is rescued by specific expression of SLOB in mNSCs. Conversely, overexpression of SLOB in mNSCs results in dramatically upregulated levels of dilp3, suggesting that SLOB in mNSCs positively regulates dilp3 expression. How might changes in SLOB expression regulate dilp3 transcript levels? Dilp3 transcription undergoes autocrine regulation, whereby dILP signaling through InRs expressed by mNSCs results in downstream sequestration of the transcription factor dFOXO to negatively regulate transcription of dilp3, but not dilp2 or dilp5 [44]. It follows that dilp3 expression will be downregulated by elevated IIS resulting from increased release of dILPs from mNSCs (Fig. 8). Several lines of evidence suggest that IIS activity is indeed enhanced in slb null flies. First, circulating sugars in slb null flies are less than half those of control flies. In addition, whole body trehalose and glucose levels are similarly decreased in slb null flies. The effect of SLOB on carbohydrate metabolism is mediated by SLOB in mNSCs, since two independent drivers for expression of SLOB in mNSCs restore sugar levels to those of control flies. Finally, phosphorylation of AKT is increased in slb null flies, indicating activation of InRs by circulating dILPs.

There is a high degree of compensation among dILPs, and mutation of dilp3 alone has no effect on varied measures of the IIS pathway [28]. As levels of dilp2 and dilp5 are not significantly altered, it is unlikely that downregulation of dilp3 alone in slb null flies can account for changes in circulating and stored sugars; rather our data suggest that release of dILPs from mNSCs is increased in slb null flies, resulting in enhanced IIS pathway activation. This conclusion is consistent with reports that dilp3 transcription is under control of an autocrine feedback loop [28,44]. Although disrupted IIS is often associated with increased storage of triglycerides [12,13,16,21], studies demonstrating elevated IIS resulting in increased levels of triglycerides have also been reported [32,45,46]. Therefore, increased triglyceride storage in slb null flies is not at odds with elevated IIS in these flies.

Expression levels of dilp2, dilp3, and dilp5 are differentially regulated by nutrition, stress, and genetic manipulations [9,12,47–49]. For instance, starvation suppresses expression of dilp3 and dilp5, but not dilp2 [9], while altering the nutrient composition of food by lowering the concentration of yeast reduces expression of dilp5 alone [47]. On the other hand, dilp2 expression is downregulated by the stress-activated Jun-N-terminal kinase pathway [48]. Although we have evidence for increased IIS pathway signaling in slb null flies, it is unknown which DILP is mediating downstream effects on metabolism. Dilp2 and dilp5 are both abundant in slb null flies, and it would be interesting to examine individual production or release of dilp2 vs. dilp5.

It is clear that SLOB expression level greatly influences expression of to and dilp3, as well as metabolic measures. The fat body signals to mNSCs via an unknown secreted factor to influence dILP release [29]; in addition, the fat body is a target of circulating dILPs released by mNSCs, so we wondered if alterations in slb mutant flies were dependent on either to or dilp3. Whole body trehalose and glucose levels are restored to wild-type
levels by mutation of to in the slob null background. Although to is necessary for the metabolic alterations manifested in slob null flies, to is not required for the downregulation of dilp3. Similarly, flies lacking dilp3 alone exhibit levels of to equivalent to those of wild-type flies. On the other hand, dilp3 is required for the upregulation of to expression displayed by flies lacking SLOB. Therefore, although to and dilp3 are independently regulated by SLOB, dilp3 modulates the effect of SLOB on to expression and resultant downstream metabolic measures (Fig. 8). In addition to dilps and to, other genes involved in regulation of metabolism have been identified, including adipokinetic hormone, target of brain insulin, slimfast, and pumplins. We hypothesize that expression of some of these genes will also be altered in slob null flies; therefore, expression of key metabolic genes in slob null flies will be investigated in future experiments.

In mammals, glucose-induced insulin secretion is primarily regulated by ATP sensitive K+ channels (KATP) in pancreatic islet beta cells [reviewed in [55]]. Increased ATP due to glucose metabolism inhibits KATP channels, resulting in beta cell depolarization and insulin secretion. Adult fruit flies express the slob gene; they express no SLOB. The WTP41; slob null fly line was described in detail previously [8,31]. In brief, slob flies were obtained from the imprecise excision of a P-element insertion in the slob gene; they express no SLOB. The WTP41; slob null flies were obtained from precise excision of the P-element and serves as a control for the slob flies. slob flies do not exhibit any gross growth or developmental defects. Multiple isoforms of slob exist [59], but slob is the most prominent isoform. In these studies, slob was used for expression or RNAi knockdown and is referred to simply as slob in these lines. Actin-GAL4, ma301-GAL4, and ry306, to lines were provided by Dr. Amita Schäg (University of Pennsylvania), and dlp2-GAL4 by Dr. Eric Rulifson (University of California, San Francisco). To flies have a deletion in the 3' region of to genomic DNA, resulting in very low levels of basal expression and rendering mutants incapable of regulating to expression in response to starvation [10]. Slob to flies and dilp3 [28] lines were provided by the Bloomington Stock Center. The molecular characterization of to is available, but it has been used extensively as a slob mutant line and exhibits electrophysiological and circadian phenotypes [1,22,60–62]. Dilp3 is undetectable by qPCR in dilp3 mutant flies (data not shown). Dilp2-GAL4 and dilp3 flies were outcrossed into the yw background seven times and a series of crosses were then conducted to generate the following lines: WTP41; dilp3, slob; and slob, a second rescue line expressing slob specifically in insulin producing neurons in the PI region (mNSCs). Additional crosses were conducted to create the following lines: WTP41; slob; and slob, lines. UAS-slob and UAS-slobRNAi lines were maintained as homozygous lines and crossed to GAL4 driver lines prior to experimentation.

### Quantitative RT-PCR (qPCR)

Total RNA was extracted from a minimum of 30 fly heads using the UltraSpec RNA isolation system following the manufacturer’s recommendations (Biotecx Laboratories). 2 μg of RNA was reverse transcribed using the High Capacity RNA-cDNA kit (Applied Biosystems). QPCR was performed on an Applied Biosystems 7000 detection system using Power SYBR green master mix and 1 ng template cDNA. Primers were designed using the Primer Express software (Applied Biosystems). Primer sequences are available upon request. Results were calculated for a minimum of three independent RNA extractions using the standard curve method and normalized to the ribosomal gene, RLS2.

### Western blots

At least 30 fly heads were homogenized in lysis buffer containing 1% CHAPS, 20 mM Tris-Hcl pH 7.5, 10 mM EDTA, 12 mM NaCl, 50 mM KCl, protase inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktail (Sigma-
Aldrich) at 4°C. Flies were starved with 1% agar for 30 min prior to protein extraction in order to establish a baseline regardless of food ingested immediately before protein extraction. Equivalent amounts of protein were separated on 4–12% Tris-Bis gradient gels and transferred to nitrocellulose blots. Blots were blocked with 5% nonfat milk in TBST (0.1% Tween in Tris-buffered saline) and probed with primary antibodies overnight. The following primary antibodies were used: rabbit polyclonal anti-SLOB [8], rat polyclonal anti-TO (kind gift of Dr. Michael Rosbash, Brandeis University [10]), rabbit polyclonal anti-Drosophila P-AKT (Cell Signaling Technology), rabbit polyclonal anti-pan AKT (Cell Signaling Technology), rabbit polyclonal anti-MAPK (Sigma-Aldrich), and rabbit polyclonal anti-β-actin (Cell Signaling). Following washes with TBST, blots were incubated with horseradish peroxidase-conjugated donkey anti-rabbit or anti-rat secondary antibody, washed again with TBST, and visualized using the Enhanced Chemiluminescence Detection System (GE Healthcare). The optical densities for proteins of interest were quantitated using NIH Image and normalized to the loading control MAPK. Furthermore, in 6 of 8 experiments in the PAKT data set, proteins were also normalized to β-actin.

**Figure 7. The effects of SLOB on gene expression and metabolism require SLO.** A. Relative transcript levels in fly heads were measured by qPCR. WTP41, slo1 and slobIP1, slo1 express equivalent amount of to in fly heads. B, Whole body trehalose plus glucose levels were measured in flies after fasting. Stored trehalose plus glucose levels are significantly decreased in slobIP flies compared to WTP41, slo1 or slobIP, slo1 flies. C, The reduction in dilp3 levels is attenuated in slobIP, slo1 fly heads. Results are a summary of a minimum of three (A, C) or 13 samples (B) (mean ± SEM). * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001, One-way ANOVA with Bonferroni post-test.

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Trehalose and glucose measurements

To measure circulating trehalose and glucose, hemolymph was extracted by decapitation and centrifugation from adult female flies after a 5 hr starvation with 1% agar as previously described [12,44]. 0.3 mL of hemolymph was added to 75 mL hexokinase reagent, pH 6.8 (ThermoElectron) in 96-well plates and incubated with 0.1 mL porcine trehalase (Sigma-Aldrich) at 37°C to convert trehalose to glucose. Trehalose standards were similarly incubated with trehalase. Samples were measured in duplicate at 340 nm and compared to a standard curve. Whole body trehalose plus glucose was measured in adult female flies after an 18 hr starvation with 1% agar as previously described [63,64]. Briefly, 10 flies per sample were weighed, crushed in 250 mL 0.24 M sodium carbonate, and incubated at 95°C for 2 hr to denature proteins. Samples were then mixed with 150 mL 1 M acetic acid and 600 mL 0.25 M sodium acetate, pH 5.2 and spun down at 12,500 rpm to pellet debris. 100 mL aliquot samples were incubated with 1 mL trehalase each overnight at 37°C. Trehalose standards of known concentrations underwent identical treatment. The following day, 10 mL of each sample was incubated with 90 uL hexokinase reagent (ThermoElectron) in triplicate in a 96-well plate at 37°C, measured at 340 nm, and compared to the standard curve. Sugars were normalized to the total mg of fly tissue in each sample.

Lipid measurement

Triacylglycerides (TAG) were measured in adult female flies as described previously with slight modifications [45]. Briefly, 4 female flies were homogenized in 250 mL lysis buffer (140 mM NaCl, 50 mM Tris-HCl, pH 7.4, and 0.1% Triton-X) containing protease inhibitor cocktail (Sigma-Aldrich), sonicated, and then centrifuged at 12,500 rpm, 4°C. Protein and TAG were measured in supernatants using the BCA Protein Assay (Pierce) and Triglyceride Liquicolor (Stanbio) kits respectively, per manufacturer instructions. TAG was normalized to the amount of protein in each sample.

Author Contributions

Conceived and designed the experiments: ALS IBL. Performed the experiments: ALS JZ. Analyzed the data: ALS. Contributed reagents/materials/analysis tools: HF. Wrote the paper: ALS IBL.

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