Abstract

The Inositol 1,4,5- trisphosphate receptor (InsP3R) is an intracellular ligand gated channel that releases calcium from intracellular stores in response to extracellular signals. To identify and understand physiological processes and behavior that depends on the InsP3 signaling pathway at a systemic level, we are studying Drosophila mutants for the InsP3R (itpr) gene. Here, we show that growth defects precede larval lethality and both are a consequence of the inability to feed normally. Moreover, restoring InsP3R function in insulin producing cells (IPCs) in the larval brain rescues the feeding deficit, growth and lethality in the itpr mutants to a significant extent. We have previously demonstrated a critical requirement for InsP3R activity in neuronal cells, specifically in aminergic interneurons, for larval viability. Processes from the IPCs and aminergic domain are closely apposed in the third instar larval brain with no visible cellular overlap. Ubiquitous depletion of itpr by dsRNA results in feeding deficits leading to larval lethality similar to the itpr mutant phenotype. However, when itpr is depleted specifically in IPCs or aminergic neurons, the larvae are viable. These data support a model where InsP3R activity in non-overlapping neuronal domains independently rescues larval itpr phenotypes by non-cell autonomous mechanisms.

Introduction

Calcium is a versatile signaling molecule that has been found to regulate a multitude of processes, from fertilization to cell death. The regulation of such diverse processes depends on the intricate regulation of calcium levels by an extensive toolkit that consists of calcium channels and pumps on the plasma membrane and the membrane of intracellular stores that help in assembling signaling systems with very different temporal and spatial dynamics [1]. An important component of this toolkit is the Inositol 1,4,5- trisphosphate receptor (InsP3R), a ligand gated calcium channel, which releases calcium from intracellular stores into the cytoplasm upon cell surface receptor stimulation. It is known that InsP3R is widely expressed and its role in various cellular processes has been identified using in vitro studies [2]. However, InsP3R function in the context of whole organism physiology is not well understood.

Drosophila melanogaster, a model system amenable to genetic and physiological manipulations, has therefore been utilized to understand both systemic and cellular requirements for the InsP3R [3–5]. Genetic analysis that ascribes genes to physiological processes however needs to be further complemented by an elucidation of the cells where these genes are functionally required. Experiments where the wild-type gene is expressed in different cellular subsets in an otherwise mutant animal to rescue mutant phenotypes help in identifying cellular components where InsP3R activity could underlie a physiological output. By this process, we have previously demonstrated that InsP3R expression in the neuronal domain and specifically the aminergic interneurons (with the DdcGAL4) rescues larval viability [4].

In this study, we show that larval itpr mutant phenotypes can be significantly rescued by restoring InsP3R activity in insulin producing cells (IPCs) with use of the Dilp2GAL4 [6]. Moreover, we find that growth defects and associated larval lethality in itpr mutants arise as a consequence of disrupted feeding behavior. An independent requirement of InsP3R activity in the prothoracic gland cells that synthesize and secrete the insect molting hormone ecdysone also exists. The Dilp2GAL4 and DdcGAL4 expression domains do not exhibit any obvious overlap suggesting that the Dilp2GAL4 rescue is mediated by a non-cell autonomous mechanism.

Results

Rescue of larval growth and viability in itpr mutants by restoring itpr function in insulin producing cells

Mutants in the Drosophila itpr gene exhibit larval and adult phenotypes based on the strength of the heteroallelic combination. Stronger mutant combinations are larval lethal while adult viable combinations exhibit defective wing posture with reduced flight ability and altered flight physiology [4,5]. Amongst the stronger allelic combinations, lethality in itpr<sup>ns5/ug3</sup> has been well characterized; a majority of these larvae die as second instars with a slightly extended lethality profile as compared with itpr null organisms [4]. Itpr<sup>ns5</sup> is a hypomorph in which the single point mutation lies in the N-terminal ligand binding domain while itpr<sup>ns3</sup> is a null allele with a stop codon in the modulatory domain [4]. itpr<sup>ns5/ug3</sup> larvae are smaller in size as compared to wild-type controls (Figure 1). As growth in Drosophila is largely regulated by...
the insulin signaling pathway [7], the effect of restoring itpr function in IPCs in the brain was assessed on the growth of itprsv35/ug3 animals. The Dlp2GAL4 strain that expresses in larval and adult IPCs [6] was utilized for expressing the wild-type itpr transgene (UASitpr) in the background of itprsv35/ug3. A significant rescue of larval size was observed (Figure 1A). About half the surviving larvae could pupate and emerge as adults in the Dlp2GAL4 rescued condition unlike itprsv35/ug3 (Figure 1B). Growth and lethality in itpr mutant larvae can thus be partially but significantly rescued by expression of UASitpr in the Dlp2GAL4 domain.

A comparable rescue of size was also observed in DdcGAL4 rescued animals, in agreement with previous observations where lethality of itprsv35/ug3 could be rescued by UASitpr expression in aminergic cells [4]. The extent of rescue of lethality in itprsv35/ug3 with Dlp2GAL4::UASitpr was less compared to DdcGAL4::UASitpr (Figure 1B). An independent requirement of itpr activity in the two neuronal subgroups predicts that the level of rescue observed by simultaneously expressing UASitpr in both Ddc and Dlp2GAL4 domains should be enhanced as compared with rescue by expression in individual domains. However, rescue of pupae and adults was not significantly improved by UASitpr expression in both DdcGAL4 and Dlp2GAL4 domains as compared with rescue from the DdcGAL4 domain alone (Figure 1B). This shows that the rescue of lethality is not a simplistic summation of restoring itpr activity in two independent cellular domains and suggests that aminergic neurons and IPCs might communicate with each other.

**Reduced growth of itprsv35/ug3 arises from defective feeding**

The smaller body size observed in itpr mutant larva could be either due to reduced insulin signaling, (as suggested by the rescue of the mutant phenotype by Dlp2GAL4::UASitpr) or due to a...
feeding defect in these mutants or a combination of both. The feeding ability of itpr mutants was determined quantitatively by measuring ingestion of colored food (Figure 2). Wild-type larvae in all cases had significant red food in their guts and consequently homogenates derived from these animals show a high absorbance at 520 nm. However, a majority of itpr<sup>UASitpr</sup> mutant larvae had no or very little food in their gut and thus exhibit reduced absorbance values indicating that they were unable to feed normally (Figure 2A, B, 60 hrs after egg laying (AEL) in Figure 2C, D, 108 hrs AEL). The feeding defect appeared progressive, as many more itpr<sup>UASitpr</sup> larvae had no food in their gut at 108 hrs than at 60 hrs AEL. Defective feeding behavior could be rescued by expressing UASitpr<sup>+</sup> transgene in either the Dlp2GAL4 or DdcGAL4 domains (Figure 2A-D). Smaller larvae in itpr<sup>UASitpr</sup> could also arise as a consequence of fewer cells. However, there was no significant difference in the total number cells in salivary glands from itpr<sup>UASitpr</sup> larvae as compared to wild-type larvae at 60 hrs AEL (Figure 2E, F and G).

Starving Drosophila larvae up-regulate several molecular markers including d4E-BP, a translation repressor and dLipase-3, an acidic lipase [8,9]. Unlike d4E-BP which is up-regulated by either reduced insulin signaling or starvation, dLipase-3 is specifically up-regulated upon starvation and not in insulin signaling pathway mutants [10]. Transcript levels of both d4E-BP and dLipase-3 were up-regulated in itpr<sup>UASitpr</sup> at 60 hrs AEL as determined by reverse-transcriptase-mediated polymerase chain reaction (RT-PCR) analysis (Figure 2H) and quantitative real-time PCR (Figure 2I and J). Expression of the UASitpr<sup>+</sup> transgene with either Dlp2 or DdcGAL4 reduced this up-regulation (Figure 2H, I and J). The reduction of transcript levels was significantly better with DdcGAL4 than with Dlp2GAL4 (Figure 2I and J), similar to the differential rescue of viability shown in Figure 1B. These data strongly suggest that the primary cause of the observed growth defect and lethality in itpr<sup>UASitpr</sup> larvae is reduced food intake.

**Larger body size in rescued itpr mutants is due to a delay in pupation**

Since DdcGAL4 and Dlp2GAL4 driven expression of UASitpr<sup>+</sup> in itpr<sup>UASitpr</sup> rescues feeding defects and lethality, we expected that larval, pupal and adult size of rescued animals to be similar to wild-type. Analysis of larval size was not possible since it was complicated by the presence of a few larvae in the rescued genotypes, smaller in size than controls. It is very likely that these correspond to animals in which the feeding defect is not completely rescued and which do not pupate finally. Surprisingly, we found a significant increase in pupal size in the Dlp2GAL4 rescued condition when compared to wild-type animals (Figure 3A and E). Over-expression of the UASitpr<sup>+</sup> transgene with Dlp2GAL4 in wild-type animals did not lead to bigger sized pupae indicating that the larger size is not a consequence of over-active insulin signaling by UASitpr<sup>+</sup> expression in IPCs (Figure 3E). A similar increase in body size of individual Dlp2GAL4 rescued itpr mutant flies was also observed (Figure 3B and F). Increased pupal length, body size and adult fly weights were also observed in the DdcGAL4 rescue of itpr<sup>UASitpr</sup> (Figure 3C, D, G and H).

A possible reason for the increase in body size and weight of the rescued animals could be a prolonged feeding period as third instar larvae, due to delayed pupation. Increased body size due to a prolonged feeding period is also observed when Prothoracotropic Hormone (PTTH) (which stimulates ecysode synthesis in the prothoracic gland of the ring gland) producing neurons are ablated [11]. In fact, the time taken to pupate by the UASitpr<sup>+</sup>:Dlp2GAL4 or UASitpr<sup>+</sup>:DdcGAL4 rescued animals is much more than wild-type animals (Figure 3J). While 50% of wild-type pupae formed by ~130 hrs AEL, 50% pupation for the Ddc rescued larvae was at ~170 hours AEL and among Dlp2 rescued larvae it was ~195 hrs AEL (Figure 3J). Delays in molting and pupation of itpr mutants, independent of their nutritional status, have been reported earlier [3,12]. These delays could be rescued by feeding 20-hydroxyecdysone to the mutant animals indicating a defect in prothoracic gland function and ecysode release in itpr mutants [3]. To rescue the pupation delay, we expressed the UASitpr<sup>+</sup> transgene with a prothoracic gland driver (P0163GAL4) [13]. Expression of the UASitpr<sup>+</sup> transgene with P0163GAL4 does not rescue the lethality of itpr<sup>UASitpr</sup> [4]. Animals expressing the UASitpr<sup>+</sup> transgene simultaneously with P0163 and either the Dlp2 or DdcGAL4 had more surviving pupae (Figure 3I) and pupated ~12–20 hours earlier than when expression was driven only by Ddc or Dlp2GAL4 (Figure 3J). Pupal and adult fly sizes of the double GAL4 rescued animals were comparable to that observed for wild-type animals (Figure 3A, C, D, E, G and H). The time to reach 50% pupation in DdcGAL4 rescued animals was significantly reduced when they were fed with 20-hydroxyecdysone (Figure 3K). Ecdysone feeding of Dlp2GAL4 rescued animals caused significant lethality and hence the time taken to pupate could not be measured accurately. Feeding ecdysone to wild-type larvae does not cause a similar speed-up of pupation but is known to reduce viability [14]. These results support an independent requirement of InsP3.R activity in Drosophila prothoracic glands for the synthesis and/or release of ecysode [3]. A role for intracellular Ca<sup>2+</sup> release in ecysode and steroid biogenesis has been previously proposed for Manduca [15] and the mammalian adrenal glands [16,17] respectively.

**Relation between the Dlp2GAL4 and Ddc domains**

The simplest explanation for rescue of itpr mutant phenotypes by restoring itpr function in the IPCs and aminergic neurons is that an overlap exists between the two domains. In order to determine this, a membrane bound GFP (UASmCD8GFP) was expressed with Dlp2GAL4 and larval brains of these animals were stained with an anti-Ddc antibody [18]. In the third instar larval brain, the IPCs consist of two bilaterally symmetric clusters of neurosecretory cells in the pars intercerebralis region of the protocerebrum (green arrowheads in Figure 4C and G) [19]. These did not stain with the anti-Ddc antibody (white arrowheads in Figure 4D and H). The IPC clusters extend processes that terminate at the lateral protocerebrum and sub-esophageal ganglion (green arrow in Figure 4C and G) [6]. We observed a pair of anti-Ddc stained cell clusters, each consisting of about four cells, located medially in the sub-esophageal region (red arrowheads in Figure 4B and F). Ddc labeled processes that emerge from these cells lie in close proximity to the processes originating from the IPCs (red arrows in Figure 4B and F). Though DdcGAL4 expresses in both serotonergic and dopaminergic neurons, itpr mutant phenotypes are not rescued by expression of UASitpr<sup>+</sup> in the dopaminergic domain (with the THGAL4) [20], unpublished data) suggesting that the aminergic domain rescue of itpr mutants is through serotonergic neurons in the context of the phenotypes under study. In order to determine whether anti-Ddc stained cell clusters (red arrows in Figure 4B and F) produce serotonin or dopamine, these brains were stained with an anti-serotonin antibody. A previous report has found strict segregation of serotonin and dopamine producing cells [18]. However, we observed differential levels of serotonin in cell bodies of larval brains. The cluster of Ddc labeled cells in the sub-esophageal ganglion seemed to contain lower levels of serotonin as compared with other cells that had higher serotonin staining (compare cells indicated with blue arrowhead vs asterisk in Figure 4E). Additional segmentally organized cells that were
Figure 2. Feeding defects in itpr<sup>sv35ug3</sup> can be rescued by UASitpr<sup>+</sup> expression in Dilp2GAL4 and DdcGAL4 domains. (A, C) At 60 hrs and 108 hrs AEL, itpr<sup>sv35ug3</sup> have much less red food in their guts in comparison to UASitpr<sup>+/+</sup>;DdcGAL4/+;itpr<sup>sv35ug3</sup>, UASitpr<sup>+/+</sup>;Dilp2GAL4/+;itpr<sup>sv35ug3</sup> and wild-type larvae. (B, D) Spectrophotometric quantification of homogenates from larvae fed yeast paste containing a red dye. Control, Dilp2GAL4 or DdcGAL4 rescued larvae ingest significantly more dye than itpr<sup>sv35ug3</sup> (itpr mutant) larvae at 60 hrs AEL (*p<0.05; Student’s t-test) and at 108 hrs AEL (*p<0.005; Student’s t-test). The following number of larvae (n) in batches (N) were assayed for each genotype: At 60 hrs AEL: n = 95 or more, N = 4 for all genotypes; at 108 hrs AEL: for UASitpr<sup>+/+</sup>;itpr<sup>sv35ug3</sup> L3 n = 46, N = 3; L2 n = 87, N = 3; for all other genotypes n = 100, N = 4 or more. Quantification of cell number in salivary glands from larvae at 60 hrs AEL stained with DAPI to visualize nuclei. itpr<sup>sv35ug3</sup> (itpr mutant) in (E) and wild-type are shown in (F). No significant difference (G) was observed in the number of nuclei in itpr mutant and wild-type salivary glands. n = 10 salivary glands for each genotype. RT-PCR analysis (H) and quantitative real-time PCR analysis (I, J) revealed significant up-regulation of transcript levels of d4E-BP and dLipase-3 in itpr<sup>sv35ug3</sup> at 60 hrs AEL that can be significantly rescued by Dilp2GAL4 or DdcGAL4 driven expression of UASitpr<sup>+</sup> (*p<0.005; Student’s t-test). Real-time PCR analysis was repeated three times with independently isolated RNA samples for each genotype. Results are expressed as mean±SEM.

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serotonin and Ddc positive were observed just posterior to this cluster of cells (small red arrowheads in Figure 4B) and these also gave rise to processes (red arrows in Figure 4B) that terminated in the sub-esophageal ganglion, once again in close proximity to the IPC clusters (green arrows in Figure 4C).

Numerous serotonergic varicosities were present on the processes emanating from the IPC clusters that terminate at the lateral protocerebrum and sub-esophageal ganglion. Interestingly, the sub-esophageal ganglion region has been implicated in feeding and taste responses, as gustatory sensory neurons and hugin neurons (that are known to modulate feeding behavior) project to this region [21]. We find that both IPCs and Ddc positive neurons also project to the sub-esophageal ganglion, suggesting the possibility of neuronal communication with the gustatory and hugin neurons to regulate feeding. Moreover, in each brain lobe, many serotonergic varicosities were observed in close proximity to the main cell bodies of the IPCs, as has been observed earlier [22]. No overlap of Ddc labeled cells and the GFP marked IPCs was observed in the ventral ganglia.

To confirm that the Ddc antibody being used provides an accurate representation of the DdcGAL4 domain, DdcGAL4::UASmcCD8GFP brains were stained with the Ddc antibody in
the larval stages (Figure 5A). These experiments showed that though a majority of DdcGAL4 labeled cells (Figure 5B) stained with the Ddc antibody, there are some cells that do not overlap (green arrows in Figure 5B). However, these do not appear in the region of the IPCs. Strong expression of the DdcGAL4 was observed in the cluster of anti-Ddc labeled cells in the sub-esophageal ganglion (green arrowhead in Figure 5B). These experiments indicate the absence of any detectable overlap between the Dilp and the DdcGAL4 domains in larval brains.

Figure 4. Ddc and serotonin labeled cells in larval brains do not overlap with IPCs. (A) A schematic drawing depicting a third instar larval brain with the relative positions of the IPCs and their processes (in green) and the Ddc labeled cells and their processes (in red). The cellular processes from the two domains seem to intermingle in the sub-esophageal ganglia region. (B–H) Three-dimensional projections of confocal Z-stacks of a wild-type Drosophila larval brain from a wandering third instar larva expressing mCD8GFP with Dilp2GAL4 and immunostained with anti-serotonin antibody (E), anti-Ddc antibody (B, F) and anti-GFP antibody, (C, G). (D) is a merge of (B) and (C) while (H) is a merge of (E), (F) and (G). In (D) and (H), anti-Ddc staining is in red and anti-GFP in green while anti-serotonin is blue in (H). Red arrowheads in (B, F) indicate Ddc stained cells in the sub-esophageal ganglia that lie in close proximity to IPC projections (bottom green arrows in C, G). Smaller red arrowheads indicate cells which send out processes (marked with red arrows) that seem to intermingle with these IPC projections. Green arrowheads in (C, G) mark the IPCs in the two brain lobes. Green arrows indicate the projections of the IPCs towards the lateral protocerebrum (top green arrows) and sub-esophageal ganglion (bottom green arrows). Ddc marked cells (indicated by big red arrowheads in B, F) stain with the anti-serotonin antibody (E, marked by blue arrowheads), but have lesser serotonin staining than some neighboring cells (for example, cells in the lateral protocerebrum indicated by blue asterisk in E). Scale bars B–H 20 µm.

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Figure 5. Overlap of DdcGAL4 and Ddc labeled cells in larval brains. Three-dimensional projections of confocal Z-stacks of a wild-type Drosophila 3rd instar larval brain (A–C) expressing mCD8GFP with DdcGAL4 and immunostained with an anti-Ddc antibody (A) and anti-GFP antibody (B). A majority of DdcGAL4:UASmCD8GFP labeled cells overlap with those stained with the Ddc antibody, though there are some cells in both cases that do not overlap (green arrows in B). These do not appear in the region of the IPCs. Green arrowhead in (B) indicates DdcGAL4:UASmCD8GFP expression in Ddc stained cells in the sub-esophageal ganglia that lie in close proximity to IPC projections. Scale bars A–C, 50 µm.

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Ubiquitous and tissue specific knockdown of itpr by RNA interference

Absence of a visible overlap between the Dilp and Ddc domains raises the question of the extent and mode of contribution of each domain to the rescue of itpr mutant phenotypes. To assess individual contributions, we obtained several RNA interference (RNAi) lines for the itpr gene and measured their effect on larval viability by ubiquitous expression with an Actin5cGAL4. Amongst the dsitpr lines tested, one line referred to as UASdsitpr1063, does not survive beyond the larval stages on expression with the Actin5cGAL4 line (Figure 6A). Larvae of the genotype Actin5cGAL4/UASdsitpr1063 were significantly smaller in size than controls of the genotype Actin5cGAL4 or UASdsitpr1063/CyoGFP at ~120 hrs AEL (Figure 6B). They appeared similar in size to controls at an earlier time point (~ 85 hrs AEL) when they had significantly higher levels of dLipase-3 transcripts, indicating that feeding defects preceded changes in size and subsequent lethality (Figure 6C). There is a near complete absence of the InsP3R in protein lysates of Actin5cGAL4/UASdsitpr1063 3rd instar larvae (Figure 6D). These larval phenotypes are analogous to those observed in itprsv35/ug3 reporter and re-emphasize the importance of InsP3R activity for feeding and larval viability. Expression of dsitpr1063 with the pan-neuronal GAL4 (Elavc155) or with either Dilp2GAL4 or DdcGAL4 had no significant effect on larval viability or size (data not shown) as judged by the number and size of pupae formed (Figure 6A). This was despite enhancing RNAi by introducing a UASdicer2 transgene [23] in the background. These results suggest that itpr knockdown in neuronal or sub-neuronal domains is insufficient for phenocopying larval itpr mutant phenotypes. More complex interpretations are also possible (see discussion).

Discussion

In this study we find that the loss of viability in itpr mutant larvae is preceded by feeding deficits. Viability, size and feeding deficits can all be rescued significantly by itpr expression in the IPCs as well as in aminergic neurons. The two cellular domains do not exhibit a visible overlap. Thus the rescues are not mediated by a shared neuronal subset. However, the two domains are closely apposed suggesting that they could interact. While ubiquitous depletion of itpr by dsRNA can phenocopy strong itpr mutants, depletion of itpr in IPCs or aminergic neurons produces no obvious phenotype, indicating that InsP3R-mediated calcium release has a modulatory role in these neuronal domains.

InsP3 signaling in energy metabolism, growth and viability

A role for InsP3 signaling in regulating metabolism and growth in mammalian systems has been previously suggested from studies of InsP3R mutant mice [24,25]. Body mass and overall brain sizes were found to be reduced by half in weight in homozygous InsP3R type 1 knock out mice as compared to control mice [24]. These mice gradually become emaciated and died by postnatal day 25 or 26 [26]. Interestingly, homozygous opishthosous pups, that have a functionally altered InsP3R type 1, are also smaller than their littermates [25]. Moreover, InsP3R type 2 and type 3 double mutants appeared similar to their control littermates at birth, but subsequently started losing weight and died within the 4th week of age when fed on dry food due to a defect in the secretion of saliva [27]. These results mirror the growth defects and lethality we observe in itpr−/− organs and by the ubiquitous knockdown of itpr. However, neither pan-neuronal

![Figure 6. Ubiquitous but not tissue specific knockdown of itpr recapitulates itpr−/− phenotypes.](image-url)
knockdown of itpr nor specific knockdown in IPCs or aminergic cells results in larval lethality though expression of itpr in the same domains is able to rescue lethality observed in itpr<sup>35/ug3</sup> ([4] and this study). This difference in the expected phenotypes probably arises due to a difference in the nature of rescue experiments compared to RNAi experiments. Expression of the itpr gene occurs in multiple larval tissues including the central nervous system [12]. Therefore larval lethality in itpr<sup>35/ug3</sup> is possibly a combination of both neuronal and non-neuronal perturbations. This is supported by the strong lethality observed on ubiquitous knockdown of itpr. Reduction of InsP<sub>3</sub>R in either the neuronal or a sub-neuronal domain would then be insufficient for inducing lethality. Restoration of itpr in the neuronal domain or specifically in IPCs or aminergic neurons might rescue lethality by non-cell autonomous mechanisms, such as modulating the release of either DILPs or serotonin. In this condition the system may not restore to a wild-type state at every level but instead achieve a new stable state in which a wild-type output is preserved [28]. Similar circuit outputs can be generated by multiple mechanisms [29], making it plausible that different stable states are achieved in the DcGAL4 and Dilp2GAL4 rescue conditions. It is also conceivable that the native function of the InsP<sub>3</sub>R in IPCs and aminergic cells can take place with extremely low levels of protein that persists in the RNAi knockdown condition. The reduced sensitivity of the anti-dInsP<sub>3</sub>R for immuno-histochemistry prevents a direct assessment of this last possibility.

InsP<sub>3</sub>Rs are present in mammalian pancreatic beta cells that release insulin and InsP<sub>3</sub>R has been shown to cause release of calcium from intracellular stores in these cells [30,31]. InsP<sub>3</sub>R is postulated to participate in the calcium oscillatory capacity of these cells in response to glucose which is required for insulin vesicle secretion [32]. Stimulation of mouse primary beta cells or MIN6 insulinoma cells with glucose led to oscillatory InsP<sub>3</sub> generation that was tightly correlated with calcium increase, but was found not to be the driving force for the calcium oscillations that led to insulin release [33,34]. In addition to glucose, insulin secretion from the beta cells is also modulated by coordinated inputs from several gut hormones and neurotransmitters [35]. Among these, acetylcholine plays a prominent role by binding to the muscarinic cholinergic receptors which activate the PLC-InsP<sub>3</sub> pathway to elevate cytosolic calcium and facilitate insulin vesicle exocytosis [36]. Interestingly, 5-8 mutations from mutant mice selectively lacking the M<sub>1</sub> muscarinic receptor in pancreatic beta cells have a dramatic decrease in agonist induced inositol phosphate production and insulin secretion [37]. These studies suggest a modulatory role for InsP<sub>3</sub>R activity in regulating insulin secretion form mammalian pancreatic beta cells. In this study, we find that restoring InsP<sub>3</sub>R activity in the IPCs of itpr mutant larvae rescues larval lethality, growth and feeding to a significant extent. However, itpr knockdown specifically in IPCs does not result in the converse phenotypes suggesting that reduced InsP<sub>3</sub>R activity does not impair DILP secretion and argues for a modulatory role for InsP<sub>3</sub>R in Drosophila IPCs, similar to the scenario in mammalian pancreatic beta cells.

Regulation of feeding and growth by InsP<sub>3</sub> insulin and serotonin signaling

In Drosophila, hyperactivation of the Insulin Receptor /PI3 Kinase signaling as well as over-expression of dFOXO, a direct mediator of insulin signaling, alters larval feeding behavior [38–40]. Serotonergic innervation is found in the Drosophila larval feeding apparatus [41] and decreased feeding behavior is observed in null mutants of neuronal Trptophan hydroxylase gene [42], the rate limiting enzyme in serotonin synthesis. Since, expression of the InsP<sub>3</sub>R in either IPCs or Ddc cells restores normal feeding behavior in Drosophila, the existence of an evolutionarily conserved system of energy intake and utilization involving insulin and serotonin is likely [43,44]. A link between InsP<sub>3</sub>R function and the control of feeding has also been suggested in Caenorhabditis elegans [45].

The absence of any cellular overlap between aminergic and DILP producing neurons suggests that these domains regulate feeding and growth through secreted serotonin and DILPs and thus communicate with each other or influence a common subset of downstream cells by binding of serotonin and DILP to their cognate receptors. High levels of Drosophila Insulin Receptor (DIR) mRNA are present in the larval and adult nervous system [46] and dIR protein has been localized to the larval brain [47] and in the fat body surrounding the adult brain [48]. Serotonergic varicosities are thought to engage primarily in volumetric type neurotransmission in which neurotransmitter is released for distribution over a region of neuropil containing many target synapses and therefore serotonergic varicosities often do not have post-synaptic partners [49,50]. Drosophila serotonin receptors 5-HT1B Dro (d5-HT1B) and 5-HT2Dro have been observed in larval and adult brains [51,52]. Unlike mammalian systems, ATP-sensitive K<sup>+</sup> channels that respond directly to glucose levels and signal insulin release are not present on Drosophila IPCs [6]. This implies that there might be other signaling mechanisms that integrate environmental, nutritional and physiological information to modulate DILP secretion from the IPCs and serotonin signaling working through the Gq/InsP<sub>3</sub> pathway could be one such mechanism [22,54].

Materials and Methods

Drosophila Strains

Itpr<sup>35/ug3</sup> is a heteroallelic combination of single point mutants in the itpr gene that were generated in an EMS (ethyl methanesulpho- nate) screen. Detailed molecular information on these alleles has been published [4]. The embryonic wild-type itpr cDNA (UASitpr<sup>+</sup>) [12] was used for rescue experiments. Itpr RNAi experiments were done with the UAS<i>itpr</i>(1063R-2) line from the National Institute of Genetics Fly Stock Center, Japan. The Dilp2GAL4 strain was from Dr. E. Rulifson [6]; DdcGAL4 [55], P0163GAL4 [56], Actin5cGAL4 (4414), Elav<sub>1063</sub>GAL4 and UAS<i>shibire</i>(III) (24651) were obtained from the Bloomington Stock Centre. The other fly strains used were generated by standard genetic methods using individual mutant and transgenic fly lines described above.

Larval staging and lethality measurements

To obtain molting profiles, staging experiments were performed with minor modifications as described previously [4]. Timed and synchronized egg collections were done for a period of 8 hrs at 25°C and the cultures were allowed to grow further at this temperature. Larvae of the desired genotype were selected at 56–64 h AEL and transferred into vials of cornmeal medium lacking agar. These larvae were grown at 25°C and screened at appropriate time points, for number of survivors and their stage of development. For each time interval, a minimum of 75 animals were screened in batches of 25 each.

Feeding Assay

Yeast paste containing red dye (Carmoisine Red; Anand Dyes and Co. Ltd., Mumbai, India) was placed centrally on 90mm petri dishes plated with 2% agar in Phosphate Buffered Saline (PBS). Larvae of the appropriate age and genotype were placed on red yeast paste and allowed to feed for 4 hrs (at 60 hrs AEL) or 2 hrs
(at 108 hrs AEL). After feeding, each group of larvae were washed in distilled water, dried on blotting paper and placed in 1.5 ml tubes and immediately frozen in liquid nitrogen. Larvae were then homogenized in PBS, centrifuged at 14 g for 5 minutes and the supernatant was transferred to a fresh tube. The supernatant was mixed with PBS and the Abs520 read.

RT-PCR and real-time analysis

2nd instar larvae of the indicated genotypes were selected at 56–64 h AEL and snap frozen in liquid nitrogen. Total RNA was extracted with Trizol Reagent (Invitrogen) according to the manufacturer’s protocol. Approximately 1 μg of purified total RNA was used for reverse transcription reactions. cDNA was generated using gene specific primers and MMLV reverse transcriptase (Invitrogen) at 42°C for 1 hour. Polymerase chain reactions (PCRs) were performed using cDNA as template in a 25 μl reaction. rp49 gene primers were used for internal normalization of every batch of RNA. The same sense and antisense primers were used for RT-PCR and Realtime PCR. Quantitative realtime PCRs were performed on the Rotor-Gene 3000 (Corbett Research, Australia) operated with Rotor Gene software version 6.0.34 using SYBR Green JumpStartTM Taq ReadyMix (Sigma). Experiments were performed with rp49 and the gene of interest, using serial dilutions (1:100, 1:1000 and 1:10,000) of the cDNA preparation. The experiment was repeated three times with independently isolated RNA samples. Cycling parameters were 95°C for 10 min, 45 cycles of 95°C for 20 s and 55°C (for rp49) and 55°C (for 4E-BP and Lipse-3) for 30 s, 72°C for 30 s, then 1 cycle of 72°C for 5 min and hold at 5°C for 1 min. The fluorescent signal produced from the amplicon was acquired at the end of the polymerization step at 72°C. A melt curve was also performed after the assay to check for specificity of the gene of interest, using serial dilutions (1:100, 1:1000 and 1:10,000) or FV10-ASW 1.3 viewer.

Saliary glands derived from 60 hr AEL larvae were dissected in PBS, fixed in 4% paraformaldehyde, and stained with DAPI to visualize nuclei. Images were acquired at different focal planes and the total number of nuclei per salivary gland was counted.

Western Blots

Protein extracts from 3rd instar larval of the indicated genotype were run on a 5% SDS-polyacrylamide gel and transferred to nitrocellulose membrane by standard western blotting protocols. The affinity purified anti-DInsP3R rabbit polyclonal antibody (IB-9075) raised against KLH-conjugated peptide CEQRKQK-QRLGLLTANTSLPPQ derived from the DInsP3R sequence [58] was used at a dilution of 1:300. The mouse α-spectrin antibody (1:50 dilution, DSHB) was used as a loading control. Total protein estimation using the BCA (Bicinchoninic Acid) Kit (Sigma Aldrich) was performed to confirm that equal quantity of protein was loaded. Secondary antibodies conjugated to horseradish peroxidase were used, and the detection of protein in the blot was done by addition of a chemiluminescence substrate from Pierce (catalog #34075; Rockford, IL).

Immunohistochemistry

Immunohistochemistry was performed on Drosophila larval brains expressing a membrane bound GFP (UASmCD8GFP) with the Dilp2GAL4 or DilcGAL4 that were fixed in 4% paraformaldehyde for 30 minutes. The following primary antibodies were used - rat anti-Déc (1:100; provided by Dr. J. Hirsh), rabbit anti-GFP antibody (1:10,000; Molecular Probes) and monoclonal anti-3-HT antibody (1:50; NeoMarkers, Fremont, CA). The following fluorescent secondary antibodies were used at a dilution of 1:400 - anti-rabbit Alexa Fluor 488 and anti-rat Alexa Fluor 633 (Molecular Probes, Eugene, OR) and anti-mouse Rhodamine Red X (Jackson Laboratories). Confocal analysis was performed on a Zeiss LSM 510 Meta microscope (Carl Zeiss Micro Imaging, Inc.) or an Olympus Confocal FV1000 microscope using 20X 0.9 N.A. or 63X 1.4 N.A. objectives. Confocal data were acquired as image stacks of separate channels and combined and visualized as three-dimensional projections using the LSM5 version 3.2/SP2 software or FV10-ASW 1.3 viewer.

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Author Contributions

Conceived and designed the experiments: NA NP GH. Performed the experiments: NA NP GH. Analyzed the data: NA NP GH. Contributed reagents/materials/analysis tools: NA NP GH. Wrote the paper: NA NP GH.

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