Surviving nutritional deprivation during development: neuronal intracellular calcium signaling is critical

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ABSTRACT Developing cells and tissues in a growing animal need to sense food quality and integrate this information with on-going time-bound developmental programs. The integration of metabolism with development requires cellular and systemic coordination. Work in our laboratory has focused on Ca\textsuperscript{2+} signaling arising from the release of Ca\textsuperscript{2+} stored in the endoplasmic reticulum (ER), which triggers store-operated Ca\textsuperscript{2+} entry. We describe a role for ER-store Ca\textsuperscript{2+} that operates at the cellular level in various classes of neurons, and eventually drives the systemic coordination required to survive and complete development under conditions of nutritional deprivation. In the model system Drosophila melanogaster, we have developed a paradigm to induce nutritional stress during the larval stage and used pupariation as a read-out for development. Applying the vast genetic tool kit available in Drosophila to this paradigm, we have uncovered novel roles for intracellular Ca\textsuperscript{2+} signaling in regulating neuronal activity, at the level of transcription in glutamatergic neurons, and translation in neuropeptidergic neurons. We find that such regulation of cellular processes is critical for integrating information across a neural circuit at multiple levels, starting from the point of sensing systemic and environmental levels of amino acids to finally connecting with neuropeptide secreting neurons, that communicate with the prothoracic gland, an organ that makes the key developmental hormone, ecdysone. This work underscores the importance of ER-store Ca\textsuperscript{2+} for neuronal health, with consequences for animal development.

KEY WORDS: ER-Ca\textsuperscript{2+}, IP\textsubscript{3}R, neuropeptide, malnutrition, glutamatergic, Drosophila

Introduction

Nutritious food is indispensable for proper animal development and growth. Two major processes, metabolism and development, need to converge at the systemic as well as cellular level, to complete the formation of a fully mature adult animal. Metabolic pathways are tuned by developmental cues to supply the necessary nutrients, while developmental pathways rely on metabolic pathways to fuel cellular differentiation and build organ systems. The two pathways are thus deeply entwined, and understanding how they are coordinated would help us understand how developing animals cope with sub-optimal nutrition. This is particularly relevant in a world where fetal and early childhood malnutrition is still rife. To investigate biological mechanisms that underpin nutrition and development, we need model systems. Drosophila melanogaster (abbreviated herein as Drosophila), the vinegar fly, has emerged as one such powerful model system for studying how dietary input is sensed and integrated with the developmental program (Boulan et al., 2015; Edgar, 2006; Mirth and Shingleton, 2012). We discuss our work in this area in the context of intracellular Ca\textsuperscript{2+} signaling.

Drosophila as a model system to study how nutrition and development are linked

A holometabolous insect, Drosophila exhibits four distinct life stages beginning from the egg (~24h), followed by three larval stages, L1, L2 and L3 (~96 h), pupa (~120 h) and adult (Fig.1). The egg and pupa are non-feeding stages during which development is fueled by stored nutrients. In contrast, the larva and adult are feeding stages, acutely sensitive to the availability of nutritious food. However, feeding patterns of the larva and adult differ significantly: larvae appear to feed continuously, except for brief periods of

Abbreviations used in this paper: Drosophila, Drosophila melanogaster; ER, endoplasmic reticulum; InR, insulin receptor; IPC, insulin-producing cell; IP\textsubscript{R}, inositol 1,4,5-trisphosphate receptor; STIM, stromal interacting molecule; NR, nutrient restriction; NP, neuropeptide; NE, neuroendocrine; PG prothoracic gland.

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moulting, whereas adults feed intermittently. Importantly, all growth occurs during the larval stages. Consequently, adult organismal size is determined by the final larval size. Because the systemic output in larvae is growth and maturation, whereas in adults it is survival and reproduction, it is likely that nutrient sensing pathways are tuned differently in the two stages. Thus, mechanisms derived from studying the effect of nutrition on adult flies may not necessarily be relevant or similarly important in larvae.

Laboratory fly food typically consists of the three major macronutrients: proteins, carbohydrates and lipids. In our laboratory, yeast serves as the major source of proteins, in addition to supplying precursors for sterol and lipid biosynthesis, whereas sugars and corn flour provide the bulk of the carbohydrates. While there are descriptions of completely synthetic fly media composed of amino acids, sugars and select micronutrients (Piper et al., 2013), in general, nutrient content can be altered by changing the proportion of yeast and sugar in the fly media. Thus, protein deficiency is easily accomplished by reducing the amount of yeast.

Holometabolous insect larvae feed and reach an established critical weight after which starvation does not affect their ability to complete development (Robertson, 1963). For the laboratory grown w1118 strain of Drosophila this happens ~8 h after transition to L3, with a weight checkpoint of ~0.8mg (Koyama et al., 2014; Stieper et al., 2008). Starvation after reaching critical weight does not prevent development to pupae and further, to adults, except that a major cost is imposed on the size of the resulting adult. Changing the nutrient value of fly food after critical weight and before wandering stage, during which larvae crawl up the vial and stop feeding, yields a time window where integration of nutritional deficiency with development can be studied, in the absence of survival being a major issue (Fig. 1).

**Signaling mechanisms that integrate growth and development in Drosophila**

The key hormonal event in Drosophila that triggers development to the next stage and ultimately metamorphosis, is release of the steroid hormone ecdysone, from the prothoracic gland (PG) (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). The PG senses growth and coordinated development of various larval parts through multiple ways. Importantly, these pathways ultimately regulate pupariation timing by regulating ecdysone biosynthesis. Signaling pathways known to regulate PG function include insulin receptor (InR) signaling (Colombani et al., 2005; Mirth et al., 2005), TOR signaling (Layalle et al., 2008; Ohhara et al., 2017), TGFβ/activin pathway (McBrayer et al., 2007), PTTH peptide secreted by a pair of neurons in the brain (Rewitz et al., 2009), circulating hedgehog released by enterocytes (Rodenfels et al., 2014), dILP8 secreted by damaged imaginal discs (Colombani et al., 2012; Garelli et al., 2015) and more recently, circulating Dpp from peripheral tissues (Setiawan et al., 2018). Of these, the InR and TOR signaling pathways are perhaps the major ones that link nutrient availability to cellular growth (Britton et al., 2002; Layalle et al., 2008). Both pathways respond to local and systemic nutrient levels to promote protein synthesis and therefore, increase in cellular size and proliferation. Importantly, the molecular components of these signaling systems are conserved genetically and functionally between mammals and Drosophila (Das and Dobens, 2015).

InR activation is reliant on the availability of its ligand, insulin. Drosophila possess several insulin-like peptides (dILPs 1-8) of which the transcription and release of dILPs 2, 3 and 5 are known to be regulated by nutrient availability (Broughton et al., 2005; Kim and Neufeld, 2015; Okamoto and Nishimura, 2015; Slaidina et al., 2009). Unlike mammals though, insulin-like peptides in Drosophila are made in the central nervous system, in neurons called insulin-producing cells (IPCs) (Rulifson et al., 2002). IPCs release insulin peptides into circulation and also directly innervate the PG, thereby regulating ecdysone synthesis through non-cell autonomous as well as cell-autonomous mechanisms (Mirth et al., 2005). IPCs themselves appear to sense nutrient availability through a number of secreted factors that include, Neuronal Lazarillo (Pasco and Léopold, 2012), Upd2 (Rajan and Perrimon, 2012), CCHamide 2 (Sano et al., 2015), Eiger (Agrawal et al., 2016), Stunted (Delanoue et al., 2016) and Growth-Blocking Peptides (Koyama and Mirth, 2016), with the number of factors regulating IPCs set to grow. Together, the InR and TOR pathways are essential for integrating nutritional inputs such as sub-optimal protein conditions with growth and development. They operate at the level of peripheral tissues as well as on the PG. Such growth signaling pathways explain how nutritional input is converted to optimal cellular proliferation and growth. However they fail to explain how the animal is able to complete development in the absence of nutritional inputs, as in the case of Drosophila, where after reaching critical weight, larval to pupal development continues even in the absence of food. It is in this niche that our studies on ER-Store Ca²⁺ signaling have unexpectedly made a contribution.

**IP₃R regulated Ca²⁺ signaling in development and metabolism**

In the early 1990s, it became apparent that Ca²⁺ released from the ER is utilized by cells in order to respond to external stimuli...
Basal cytosolic Ca\(^{2+}\) levels are tightly maintained, at concentrations that are 100-500 fold lower than in the ER, as well as the extracellular milieu. This maintenance is enabled in part by an ATP-dependent channel on the endoplasmic reticulum (ER) called SERCA, which continuously pumps Ca\(^{2+}\) from the cytosol into the ER (Clapham et al., 2007). Dynamic changes in the levels of cytosolic Ca\(^{2+}\) ions, either via release of Ca\(^{2+}\) from the ER or entry from the extracellular milieu, thus provides metazoan cells a mechanism to utilize Ca\(^{2+}\) as a second messenger to connect external stimuli to cellular function (Berridge et al., 2003; Clapham et al., 2007). The two known ligand-gated receptors on the ER that release ER-Store Ca\(^{2+}\) are the ryanodine receptor and the inositol 1,4,5-trisphosphate receptor (IP\(_3\)). Homologs of both intracellular Ca\(^{2+}\) release channels were identified in Drosophila by reverse genetic methods (Hasan and Rosbash, 1992). Subsequently, studies from our lab have focused exclusively on the IP\(_3\)R, whose activation lies downstream of G-protein coupled receptors (GPCRs) of the G\(_{q}\) subtype. The IP\(_3\)R is present in all metazoan cell types and given its pivotal role in gating ER-Store Ca\(^{2+}\) it is not surprising that it plays an important role in many different cellular functions. The temporality and amplitude of the stimuli, as well as the presence of cell-type specific proteins deliver outputs of intracellular Ca\(^{2+}\) signaling that vary according to cell-type. Thus there exists a breadth of cell signaling events where the IP\(_3\)R is important: fertilization, muscle contraction, neurotransmitter release, vesicle secretion, transcription and mitochondrial dynamics, amongst others (Mikoshiba, 2015). Here we focus on the participation of IP\(_3\)R in cellular mechanisms that culminate in development and metabolic homeostasis.

Complete loss of the single IP\(_3\)R gene (itpr) in Drosophila resulted in early larval lethality. Hence, to investigate IP\(_3\)R function at later stages of development and in adults, an EMS-based mutagenesis screen was performed. This screen yielded several single itpr point mutants which in homoallelic combinations resulted in larval lethality, while in some heteroallelic combinations yielded animals with varying levels of survival to adulthood (Joshi et al., 2004). The latter provided a genetic tool kit to study IP\(_3\)R function at various stages of development. Genetic analysis showed that IP\(_3\)-mediated Ca\(^{2+}\) release in the PG lies upstream of either ecysdyme synthesis and/or release, as feeding ecysdyme could rescue development of IP\(_3\)R mutants (Venkatesh and Hasan, 1997). This finding was validated independently in 2015, when a role for IP\(_3\)R in releasing ecysdyme from the PG was shown (Yamanaka et al., 2015).

Mammals have three isoforms of the IP\(_3\)R whose expression varies across tissue types. Mice lacking IP\(_3\)R1 are mostly embryonic lethal (Matsumoto et al., 1996) with survivors ultimately succumbing to ataxia and seizures, while mice lacking IP\(_3\)R2/3 die early due to poor secretion of digestive enzymes (Futatsugi et al., 2005). In humans, a number of mutations in the IP\(_3\)R genes have been associated with disease (Terry et al., 2018) of which two that have parallels with mice studies are spino cerebellar ataxia (SCA), that affects motor coordination (van de Leemput et al., 2007), and Sjögren’s Syndrome where salivary secretion is compromised (Teos et al., 2015). In regards to development, in mammals the earliest role for IP\(_3\)R is in fertilization (Miyazaki et al., 1992; Miyazaki et al., 1993) followed by a role in specifying the dorso-ventral axis (Kume et al., 1997). While there is no single hormone responsible for coordinating overall development in vertebrates akin to ecysdyme, the oscillatory pattern of Ca\(^{2+}\) release induced by IP\(_3\)R activation is necessary for the secretion of hormones such as the gonadotropes follicle-stimulating hormone and luteinising hormone (Durán-Pastén and Fiordelisi, 2013), as well as oxytocin (Ludwig et al., 2002), whose downstream targets ultimately control sexual maturation, reproduction and social behavior. Thus, while the details vary from species to species, IP\(_3\)R mediated Ca\(^{2+}\) release is important for the release of certain hormones and overall animal development.

In so far as nervous system development is concerned, an early study showed that IP\(_3\)R is enriched in growth cones of neurons in the chick dorsal root ganglion and required there for neurite extension (Takei et al., 1998). In Drosophila, our heteroallelic IP\(_3\)R mutants were useful in demonstrating a neuronal requirement for IP\(_3\)R in viability (Joshi et al., 2004) and flight circuit maturation (Banerjee et al., 2006). These studies established physiological consequences of a loss in IP\(_3\)R function in neurons during development. At that time, this line of investigation was particularly novel. A major focus of Ca\(^{2+}\) signaling in neurons was the characterization of activity-dependent Ca\(^{2+}\) channels on the cell surface. That ER-Store Ca\(^{2+}\) too can contribute to, and modulate neuronal activity, was less well studied. Our group has pioneered this aspect of intracellular Ca\(^{2+}\) signaling and neuronal function to a significant extent.

At the cellular level, there is evidence that IP\(_3\)R regulates metabolism most notably by regulating the transfer of Ca\(^{2+}\) from the ER to the mitochondria (Cardenas et al., 2010). The IP\(_3\)R is enriched at ER-Mitochondria contact sites and this coupling is important to allow for efficient transfer of Ca\(^{2+}\) from the ER to the mitochondria via the mitochondrial Ca\(^{2+}\) uniporter (Rizzuto et al., 2012). Ca\(^{2+}\) thus imported under basal conditions is required for the proper functioning of Ca\(^{2+}\) dependent enzymes of the TCA cycle and in cancerous cells, is crucial for cell survival (Cardenas et al., 2016). At the systemic level, in mice hepatocytes, there is evidence that IP\(_3\)R, phosphorylated upon glucagon induction, leads to glucogenic gene expression, which ultimately contributes to systemic glucose homeostasis during fasting (Wang et al., 2012). This process is dysregulated in diabetes. Additionally a role for IP\(_3\)R in regulating lipid droplet formation in mice hepatocytes in the context of fatty liver disease has been described (Period et al., 2017). The contribution of IP\(_3\)R to metabolism in neuronal cells, in tissue or primary culture, has been poorly explored. Our explorations in this area are detailed in the next section.

Store-operated Ca\(^{2+}\) entry and neuronal function

In 2005, the discovery of Stromal Interacting Molecule (STIM) an ER-Ca\(^{2+}\) sensor and in 2006, of Orai, a calcium selective ion channel on the plasma membrane, ushered in the molecular study of intracellular Ca\(^{2+}\) signaling characterized as Store-operated Ca\(^{2+}\) entry (SOCE) (Hogan and Rao, 2015). When GPCRs activate the IP\(_3\)R, it leads to a release of ER-Ca\(^{2+}\) and concomitant decrease in ER-Ca\(^{2+}\) levels that is sensed by STIM (Fig. 2). Loss of ER-Ca\(^{2+}\) triggers a conformational change in STIM followed by STIM dimerization and the exposure of Orai-binding sites. Bound by STIM, the channel pore of Orai opens, leading to an influx of Ca\(^{2+}\) ions from the extracellular milieu into the cytosol. Increased cytosolic Ca\(^{2+}\) then leads to the activation of varied cell specific processes. Thus, SOCE offers cells yet another means to modulate the frequency and amplitude of Ca\(^{2+}\) signaling in the cytosol. Functional studies have shown a role for SOCE in cell types as different as immune cells (Shaw and Feske, 2012), various cancer cells (Vashisht et
and neural progenitor cells (Gopurappilly et al., 2018; Somasundaram et al., 2014; Toth et al., 2016), with the list growing as new functions for STIM and Orai are discovered. Of note, both STIM and Orai have been implicated in systemic metabolism in the maintenance of lipid homeostasis; STIM in fat body of flies (Baumbach et al., 2014; Xu et al., 2019), and both STIM and Orai in mammals (Maus et al., 2017).

Capitalizing on single gene homologs of STIM and Orai in the Drosophila genome, our lab demonstrated that IP$_3$-mediated Ca$^{2+}$ release is followed by SOCE in Drosophila neurons (Venkiteswaran and Hasan, 2009). Importantly, by using flight as a behavioral readout, we showed that SOCE in neurons is required for maturation of the flight neuronal circuit during pupal development (Agrawal et al., 2010; Venkiteswaran and Hasan, 2009). Thus, not only do Drosophila neurons display SOCE signaling, but also, this signaling has functional consequences during neural development. Next we delved into the molecular basis of how neuronal function is regulated by SOCE. A transcriptomic screen in pupal brains identified many genes that are regulated by STIM and are required for flight circuit maturation (Richhariya et al., 2017). We are now exploring how these molecular changes differ in various neuronal subtypes, and how they affect cellular output both at the level of the neuron, and in the neural circuit required for flight behavior.

Unlike the investigations on flight circuit maturation, our investigations on SOCE in the context of nutrition and development were somewhat serendipitous. Adult flies carrying hypomorphic mutations for the IP$_3$R exhibit hyperphagia and altered lipid metabolism, with systemic consequences such as increased weight and higher levels of Triacylglycerides (TAGs) (Subramanian et al., 2013). To understand if adult metabolism could be reset by manipulating the nutritional input of larvae, IP$_3$R mutants were subjected to nutrient restriction (NR) in the late larval stage. We devised an NR paradigm that involved shifting mid to late stage 3rd instar larvae to a protein deficient diet consisting of 100mM Sucrose, up until pupariation (Fig. 1). While control larvae can successfully complete development in the NR paradigm, surprisingly, IP$_3$R mutant larvae cannot. This despite the fact that both control and mutant larvae were placed under NR after crossing critical weight, and much after critical time, beyond which the development of Drosophila typically becomes independent of food quality. Why do itpr mutants fail to pupariate under NR conditions? It is this question that led to insights on the neuronal requirement of intracellular Ca$^{2+}$ signaling and SOCE for development, in the NR paradigm.

**Neuronal control of development under conditions of nutritional stress**

Because key components of IP$_3$ signaling and SOCE, like the IP$_3$R, STIM and Orai, are expressed ubiquitously in multiple cell types, the cellular basis of pupariation needed further investigation. The GAL4-UAS system (Brand and Perrimon, 1993) was used to perform a genetic screen in which GAL4s specific to various organs and cell types were employed to reduce IP$_3$R expression using a transgenic RNAi construct referred to as UAS-itprIR (Agrawal et al., 2010). The resulting larvae were tested for pupariation on NR. This screen identified a strong requirement for IP$_3$R in neuronal tissues and as expected in the PG, for larval to pupal development on an NR diet (Jayakumar et al., 2016; Megha and Hasan, 2017). Based on the strength of the NR phenotype observed we focused on two neuronal subtypes, the neuroendocrine (NE) cells and a subset of glutamatergic neurons of the ventral nerve cord referred to as VGN6341 neurons, for further study.

Neuroendocrine (NE) cells produce neuropeptides (NPs), a class of signaling agents, which can act either locally on neural circuits or systemically on other organs, to ultimately regulate a large number of behaviors and metabolism (Nassel and Winther, 2010). The broad role played by NPs and the poor characterization of SOCE molecules in modulating NE cell activity, fueled our interest in this neuronal subtype. We observed that reducing SOCE activity by manipulating IP$_3$R, STIM or Orai in NE cells reduced pupariation on NR (Megha and Hasan, 2017). A parallel observation at the systemic level was that IP$_3$R mutants were sensitive to levels of dietary protein and displayed lower protein/TAG ratio suggesting a protein metabolism defect. Because InR and TOR signaling pathways are known regulators of protein metabolism we combined the two observations to design a genetic experiment to test if molecules of the InR and/or TOR pathway compensated...
for IP$_R$ function. Indeed, over-expression of either InR or TOR signaling in NE cells was sufficient to rescue pupariation of IP$_R$ mutants on NR, as well as systemic protein/TAG ratio. Conversely, over-expression of IP$_R$ could overcome loss of InR signaling in NE cells. These experiments led to the hypothesis that the InR and IP$_R$ pathways are functionally complementary in NE cells.

To test this, we measured levels of protein translation in NE cells in various genetic conditions. IP$_R$ mutant NE cells displayed decreased protein translation and this could be restored to normal levels by the over-expression of wild type IP$_R$. Together, these experiments describe a novel role for IP$_R$ in protein translation in NE cells and hence as an important determinant of development on NR (Megha and Hasan, 2017). Unlike InR or TOR pathways, the IP$_R$ signaling pathway is nutrient-independent. We speculate that this type of nutrient-independent control of protein translation is required in NE cells, perhaps because peptides made by these neurons are required in NR conditions to coordinate development. Reduced nutrient availability during NR likely reduces the activity of InR and TOR pathways in NE cells and a positive nutrient-independent regulator of protein translation, such as IP$_R$, is required to maintain a certain level of protein translation and therefore, peptide levels. This speculation was based on a study of how neural stem cells (NSCs), which usually rely on InR and TOR signaling to exit quiescence and begin proliferation, continue to do so under NR conditions by switching to another ligand-receptor system that helps to maintain active signaling downstream of InR and TOR (Cheng et al., 2011).

Each NE neuron secretes one or more distinct neuropeptide (NP) and together the NE subset tested in the previous project make at least 20 known NPs. To understand which neuropeptide(s) made in these cells is required in the context of SOCE to survive NR a curated genetic screen was performed. This screen yielded several candidate NPs, of which we decided to pursue Corazonin (Crz) and short Neuropeptide F (sNPF). Crz and sNPF are co-expressed in small set of neurons (∼6; Dorsolateropeptidergic; DLPs) in the larval brain, providing a system where STIM’s role in cellular neuropeptide biology could be easily studied. Immunohistochemistry and mass spectrometry revealed that loss of STIM perturbed peptide levels in DLP neurons: in the fed state, peptide levels were increased, while upon starvation, they remained unaltered while in the control they increased. Additional genetic experiments suggest two possible points at which STIM acts on neuropeptides – secretion and synthesis. Ultimately, loss of STIM in DLPs, resulted in reduced systemic Crz signaling which correlated with an inability of larvae to survive nutritional stress (Megha et al., 2018).

**Nutrient restriction stimulates a neuropeptide dependent glutamatergic neuron–insulin-producing cell circuit for stimulating ecdysone synthesis**

In addition to regulating the synthesis and secretion of neuropeptides, experiments on a set of glutamatergic interneurons (the VGN6341 neurons) in the context of development on NR have established a role for neuropeptide and acetylcholine stimulated intracellular Ca$^{2+}$ signaling through the IP$_R$ in regulating neuronal gene transcription as well as neuronal activity (Jayakumar et al., 2016). VGN6341 neurons are located in the mid-ventral ganglion and IP$_R$ activity in this glutamatergic interneuron subset was found to be necessary for survival on NR. Because IP$_R$ operates downstream of GPCRs, a genetic screen was conducted to identify receptors on VGN6341 neurons required in the NR paradigm. Interestingly, amongst the receptors identified, the largest class belonged to neuropeptide receptors, further underscoring the importance of neuropeptides to animal development under NR conditions. In VGN6341 neurons neuropeptides stimulate specific aspects of intracellular Ca$^{2+}$ signaling (see next paragraph). Additionally, the identification of muscarinic acetylcholine receptor (mAChR) suggested that cholinergic neurons were involved in transmitting NR information to VGN6341 neurons. Here, the genetic toolkit available to Drosophila researchers came handy: by testing a series of GAL4 lines known to mark various subsets of cholinergic neurons, we identified multidendritic sensory cholinergic neurons (MSNs) located on the larval body wall as the source of NR sensing. Subsequently, we discovered that MSNs can sense nutrients directly through the amino acid transporter slimfast and also respond to the loss of essential amino acids, including arginine, in the food media (Jayakumar et al., 2018). The MSNs convey this information to the glutamatergic neuronal subset covered by VGN6341-GAL4. A role for glutamatergic neurons in nutrient sensing is novel and required better understanding. Mapping of neuronal projections from the VGN6341 neurons identified projections from the VNC that travel up to the larval brain lobes and reach the medial neurosecretory cells (mNSCs), a set of NE cells that make and secrete a number of NPs, and includes the IPCs. Others have demonstrated that ILPs secreted from the IPCs stimulates ecdysone synthesis from the PG (Colombani et al., 2005; Mirth et al., 2005). Thus, glutamatergic neurons in the ventral ganglion receive cholinergic inputs from the MSNs present on the larval body wall and transmit this information to the mNSCs in the brain. Signals received by the mNSCs stimulate ILP synthesis and/or release under protein-deprived conditions. Overall, this neural circuit up-regulates the transcription of ecdysone synthesizing genes in the PG (Jayakumar et al., 2016).

In addition to the circuit, we examined how IP$_R$ regulates activity in the VGN6341 neurons using ex-vivo brain preparations (Jayakumar et al., 2018). Oscillating and long lasting Ca$^{2+}$ signals were produced in the glutamatergic neurons upon loss of arginine that were dependent on intracellular Ca$^{2+}$ signaling. Whereas initiation of Ca$^{2+}$ signals required cholinergic stimulation of the mAChR, subsequent oscillations depended on NP receptor activation. To understand how neuronal properties were altered in IP$_R$ mutants, differentially regulated genes were identified by an RNAseq and validated in fluorescent activator-sorted VGN6341 neurons. Many genes coding for voltage-gated ion channels including NaCP60E (Sodium channel), H$_K$, eag (Potassium channel), cacophony, Caapisha1D (Calcium channels) were down-regulated in the IP$_R$ mutants as well as upon dSTIM and IP3R knockdown. Changes in intracellular Ca$^{2+}$ signaling thus modulate neuronal receptivity to acetylcholine and neuropeptides, and also leads to chronic changes in gene expression that can alter neuronal properties of excitability.

Together, these studies show that intracellular Ca$^{2+}$ signaling plays an important and necessary role in regulating neuronal activity at the level of transcription as well as translation (Fig 2). Importantly, this regulation appears to be a fail-safe mechanism because it does not affect development when sufficient food resources are present. In times of nutritional stress, after the animal has sufficient internal resources to reach adult hood, this neuronal regulation takes on an important role. It ensures that nutrient-sensitive circuits and neuropeptidergic outputs continue to...
adequately provide measures to over-ride loss of nutrition signals and allow development to proceed.

Future studies

There are two main directions we see emerging from these studies: further identification of molecular changes that are brought about by intracellular Ca\(^{2+}\) signaling in neurons during neural circuit maturation, and adapting the NR paradigm to ask questions that resonate with public health investigations on malnutrition.

In psychiatric disorders with developmental origins such as autism, no single gene correlates with major disease risk. Instead, the emerging hypothesis is that a change in balance of excitatory and inhibitory stimuli experienced by developing neural circuits might underlie disease manifestation (Nelson and Valakh, 2015). We propose that intracellular Ca\(^{2+}\) signaling can be contributory in maintaining a balance for such activation and silencing of neurons. Cumulatively, our work in *Drosophila* shows that intracellular Ca\(^{2+}\) signaling can perturb basic cellular processes in neurons, such as translation and transcription, providing broad mechanisms by which this balance may be controlled. Transcriptomic analysis of *Drosophila* pupal neurons has already provided us a list of genes whose expression is sensitive to intracellular Ca\(^{2+}\) signaling (Richhariya et al., 2017). Interestingly, mutations in *rala*, a modulator of synaptic vesicle release validated from this screen (Richhariya et al., 2018), were recently identified as a genetic cause of intellectual disability (Hiatt et al., 2018). Transcriptional regulation by intracellular calcium signaling also sets the stage to uncover how such gene expression changes are regulated: either directly perhaps by a common transcription factor whose activity is controlled by cytosolic Ca\(^{2+}\) levels, or other mechanisms of gene expressions, such as chromatin remodeling. The latter is suggested by another gene identified in the transcriptomic screen for SOCE-regulated genes, *dSet2*, a gene encoding a histone methyltransferase.

Scaling from these molecular studies to understanding how nutrition can re-program neurons and therefore behavior, requires a whole organism approach for which *Drosophila* is ideally suited. The molecular players involved in development as well as metabolism are strongly conserved between humans and animals, including *Drosophila*. There exist multiple classes of genetic tools that allow for scaling from molecules to organ systems to individuals, in *Drosophila*. Moreover, it is relatively cost-effective as compared to mammalian model organisms, easy to breed and its dietary input can be easily manipulated. We are at present characterizing the locomotory behavior (flight, walking, climbing, daily activity rhythms) in flies that have been subject to early life protein deprivation as well as investigating their metabolism in response to a variety of diets in adulthood. These experiments will hopefully help identify adult phenotypes that correlate with early life nutritional stress, whose molecular basis can then be investigated by working on neural circuits described for these phenotypes. In humans, severe to moderate protein deficiency during childhood is associated with growth retardation, along with long-term consequences for mental health and metabolism. The molecular basis of how early life nutrition manifests into disease and health risks later in adult life is not well understood. Given the high prevalence of protein malnutrition in India, model systems to pursue this biology are therefore, the need of the hour, a niche that *Drosophila* may fulfil.

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