



The enigma of store-operated Ca^{2+} -entry in neurons: answers from the *Drosophila* flight circuit

Gaiti Hasan* and Gayatri Venkiteswaran

National Centre for Biological Sciences, Tata Institute of Fundamental Research, Bangalore, India

Edited by:

Eve Marder,
Brandeis University, USA

Reviewed by:

Michael Nitabach,
Yale University School of Medicine,
USA
Bing Zhang,
University of Oklahoma, USA

***Correspondence:**

Gaiti Hasan,
National Centre for Biological Sciences,
Tata Institute of Fundamental
Research, GKVK Campus, Bellary
Road, Bangalore 560065, India
e-mail: gaiti@ncbs.res.in

In neurons a well-defined source of signaling Ca^{2+} is the extracellular medium. However, as in all metazoan cells, Ca^{2+} is also stored in endoplasmic reticular compartments inside neurons. The relevance of these stores in neuronal function has been debatable. The Orai gene encodes a channel that helps refill these stores from the extracellular medium in non-excitatory cells through a process called store-operated Ca^{2+} entry or SOCE. Recent findings have shown that raising the level of Orai or its activator STIM, and consequently SOCE in neurons, can restore flight to varying extents to *Drosophila* mutants for an intracellular Ca^{2+} -release channel – the inositol 1,4,5-trisphosphate receptor (InsP_3R). Both intracellular Ca^{2+} -release and SOCE appear to function in neuro-modulatory domains of the flight circuit during development and acute flight. These findings raise exciting new possibilities for the role of SOCE in vertebrate motor circuit function and the treatment of neurodegenerative disorders where intracellular Ca^{2+} signaling has been implicated as causative.

Keywords: Orai, STIM, neuromodulators, inositol, 1,4,5-trisphosphate receptor, central pattern generator

Living forms maintain a complex relationship with ionic calcium (Ca^{2+}). High concentrations of this ion are incompatible with several cellular processes, and lead to the precipitation of phosphates and aggregation of proteins. Since cells evolved in sea water with millimolar quantities of Ca^{2+} , they developed multiple means of maintaining calcium homeostasis including sequestering excess Ca^{2+} in intracellular membrane bound stores. In parallel, cells also evolved signaling mechanisms which used changes in cytosolic Ca^{2+} , either by entry from extracellular medium or release from intracellular stores, to regulate multiple aspects of cellular function.

In non-excitatory cells Ca^{2+} signals are generated using an extensive 'Ca²⁺ 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 varying temporal and spatial dynamics (Berridge et al., 2003). Cell surface receptor stimulation activates two closely coupled components of this toolkit; the inositol 1,4,5-trisphosphate receptors (InsP_3R) followed by store-operated Ca^{2+} (SOC) channels. InsP_3R s rapidly release Ca^{2+} from intracellular stores such as the endoplasmic reticulum (ER) while SOC channels function to replenish ER stores from the extracellular milieu and contribute to a longer term Ca^{2+} signal. The plasma membrane four-trans-membrane spanning Orai protein was recently identified as the long elusive SOC channel (Feske et al., 2006; Vig et al., 2006; Zhang et al., 2006), responsible for the Ca^{2+} -release activated Ca^{2+} current (I_{CRAC} ; Hoth and Penner, 1992). The precise mechanism of SOC entry (SOCE) is still under investigation but several reports have established that Ca^{2+} depletion of ER stores is sensed by (stromal interaction molecule) STIM, an ER membrane protein, which upon store Ca^{2+} depletion oligomerizes in the ER, translocates close to the plasma membrane and organizes the Orai channel into clusters to bring about SOCE (Liou et al.,

2005; Zhang et al., 2005; Cahalan, 2009). Recent findings strongly suggest that Orai and STIM together are necessary and sufficient for SOCE (Hewavitharana et al., 2007 and references therein). Here we discuss recent evidence for the function of these molecules in excitable cells like neurons and propose a general role for SOCE in neural circuits that co-ordinate rhythmic motor patterns.

Ca^{2+} signals play an important role in neural activity and its modulation, as well as the development and maintenance of neural circuits (Berridge, 1998; Spitzer, 2002; Borodinsky et al., 2004). In most cases these signals are generated by rapid entry of extracellular Ca^{2+} through plasma membrane channels activated directly by voltage changes, ligands or sensory stimuli. The possible role of intracellular Ca^{2+} release in modifying neuronal excitability is much less understood. The majority of such studies have been at the cellular level where they implicate the ryanodine receptor as the primary source of Ca^{2+} -release from the ER store leading to amplification and modulation of membrane conductances and/or neurotransmitter release. Similar studies of InsP_3 -mediated Ca^{2+} release in neurons are far fewer (Verkhatsky, 2005 and references therein). The impact of Ca^{2+} -release from neuronal ER stores on physiological function has been best understood from study of mouse and *Drosophila* mutant phenotypes (Matsumoto et al., 1996; Street et al., 1997; Banerjee et al., 2004). These studies with InsP_3R mutants support the idea that InsP_3 mediated Ca^{2+} release is required in specific classes of neurons for co-ordination of rhythmic movement such as walking or flying. Further support for this idea comes from a recent study of the genetic basis for human SCA15 which has been attributed to heterozygosity of the Type 1 InsP_3R (van de Leemput et al., 2007).

What has remained controversial is the means by which the ER store is refilled in neurons after intracellular Ca^{2+} -release. Given the range of plasma-membrane Ca^{2+} entry mechanisms present

on neurons it has been widely assumed that one or more of these could contribute to store-refilling. Despite this prevailing idea, the existence of SOCE in neuronal cells was originally demonstrated in the PC12 cell line (Clementi et al., 1992) and later in *Aplysia* bag cell neurons (Kachoei et al., 2006). Its relevance for development and plasticity of the nervous system has also been demonstrated *ex vivo* (Nishiyama et al., 2000; Emptage et al., 2001). Direct measurement of I_{CRAC} in neurons has however been confounded by the long-held idea in the field that the Transient Receptor Potential class of membrane channels (TRPs) are the source of SOCE in both excitable and non-excitable cells. This conjecture originally arose from the conformational coupling model which proposed that Ca^{2+} release through the ER channels (RyR and $InsP_3R$) induces a conformational change in which the ER and PM are closely apposed, leading to activation of the SOC channel on the PM (Irvine, 1990). Genetic studies in *Drosophila* visual transduction (where TRP channels were first discovered), demonstrated that mutant alleles of Gq and PLC β have strong deficits in Ca^{2+} entry through TRP channels. These results initially suggested that $InsP_3$ generated by PLC β activates ER Ca^{2+} -release through the $InsP_3R$ leading to extracellular Ca^{2+} -entry via TRP channels (Hardie and Minke, 1995). The observation that both partial and null alleles of the $InsP_3R$ have no effect upon Ca^{2+} entry through TRP channels in *Drosophila* photoreceptors essentially put the idea of TRPs as SOC channels to rest in *Drosophila* neurons (Acharya et al., 1997; Raghu et al., 2000). Consequently, until recently the possible source and role of SOCE in neuronal function remained doubtful.

With the discovery of STIM and Orai, their role in SOCE downstream of $InsP_3$ -mediated Ca^{2+} release has been investigated in *Drosophila* neurons (Venkiteswaran and Hasan, 2009) in the context of the flight circuit. The importance of the $InsP_3R$ and intracellular Ca^{2+} release in flight circuit development has been demonstrated earlier. *itpr* gene mutants, in the only *Drosophila* $InsP_3R$ gene, are flightless and unable to generate normal rhythmic action potentials from flight motor neurons despite no observable defects in general synaptic function (Banerjee et al., 2004). Moreover, $InsP_3R$ mediated Ca^{2+} signaling is required at multiple steps for generating the neural circuit responsible for air-puff stimulated *Drosophila* flight (Banerjee et al., 2006). Adult *Drosophila* with RNAi knock-down of either STIM or Orai in neurons exhibit phenotypes that are indistinguishable from each other or that of $InsP_3$ receptor mutants. Significantly, the over-expression of either STIM or Orai in $InsP_3$ receptor mutant neurons can suppress flight defects to various extents. These experiments provide the first functional link between Ca^{2+} -release through the $InsP_3$ receptor and store-operated Ca^{2+} entry through the STIM/Orai pathway in neurons. The temporal and spatial analysis of this mode of intracellular Ca^{2+} signaling has provided more interesting insights.

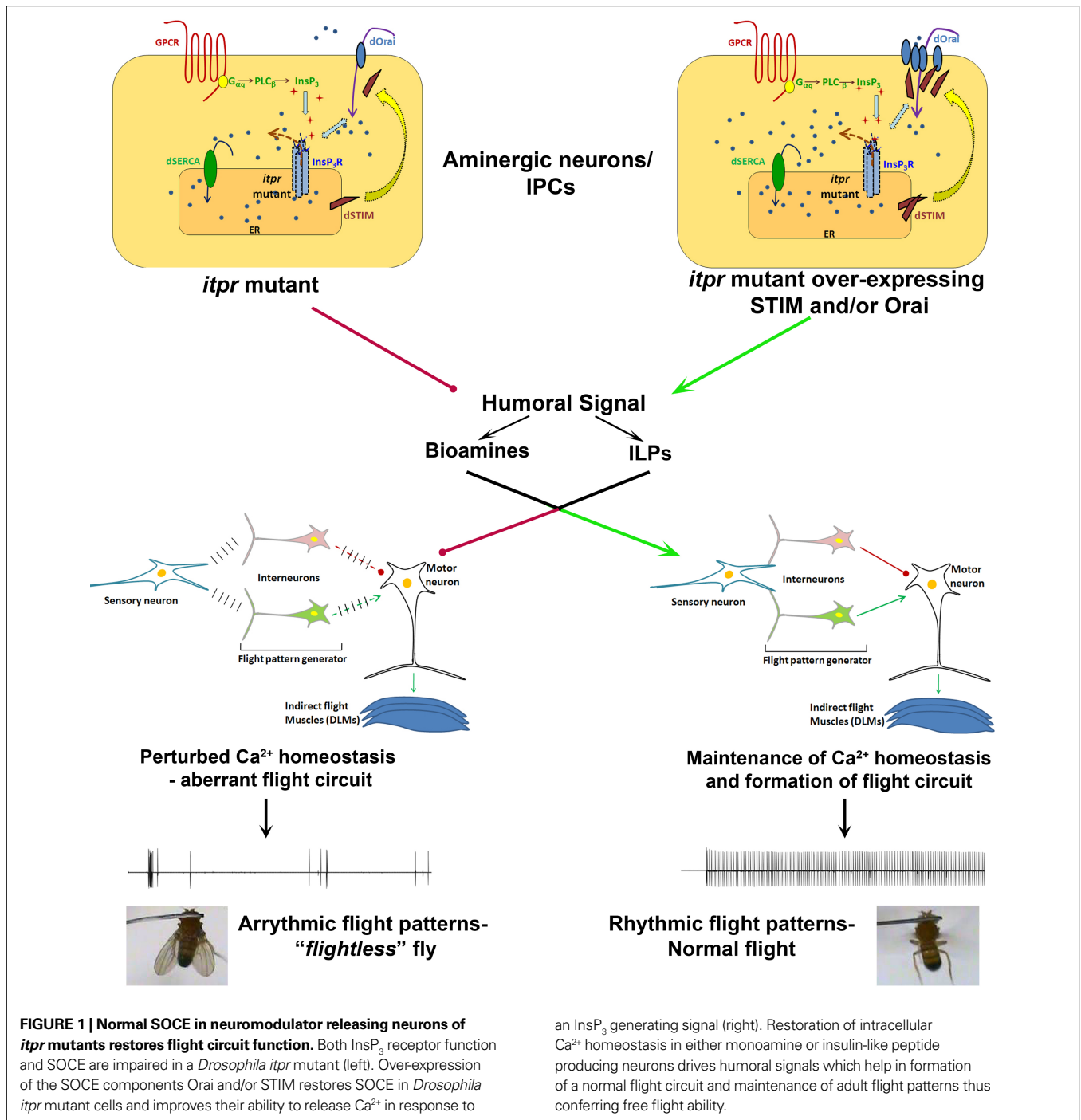
A range of mutant phenotypes related to flight are observed upon reduced $InsP_3$ mediated Ca^{2+} -release and SOCE in *Drosophila* neurons. Through measurements of intracellular Ca^{2+} signals in primary cultures of neurons from the appropriate genotypes it has been possible to correlate alterations of cellular Ca^{2+} homeostasis and signaling with systemic changes in flight ability. Amongst these, an abnormal wing position, high frequency of spontaneous firing

from the dorsal longitudinal muscles and the inability to initiate flight patterns arise from reduced intracellular Ca^{2+} release during flight circuit development. The temporal nature of Ca^{2+} signals that contribute to all of the above phenotypes are such that they can be suppressed by slowing down uptake of cytosolic Ca^{2+} to the ER store by a dominant mutant in the SERCA pump (Banerjee et al., 2006). This is not so for signals required for maintenance of flight after initiation. Maintenance of flight requires robust SOCE which can be restored in $InsP_3$ receptor mutant neurons by reducing SERCA and increasing Orai function. Through experiments that drive Orai over-expression in adults it appears that the requirement for SOCE is during flight though this needs more rigorous testing (Venkiteswaran and Hasan, 2009).

The ability to express specific transgenes in restricted neuronal domains in *Drosophila* has helped narrow down the range of neuronal classes where intracellular Ca^{2+} release and SOCE is important in the context of flight. At present, apart from sensory neurons and flight motoneurons, other neurons that constitute the air-puff induced flight circuit are poorly defined. Flight defects in *itpr* mutants are rescued by expression of either *itpr⁺* or *dSTIM⁺* (and to a lesser extent *dOrai⁺*) transgenes in aminergic and insulin-like peptide (ILP) secreting neurons (Banerjee et al., 2004; Agrawal et al., 2010). These domains are non-overlapping and both are neuro-secretory suggesting that they impact flight circuit formation and function through neuro-modulatory mechanisms that are non-cell autonomous. The relatively mild-phenotypes observed upon knock-down of *itpr* gene function in either one or both these domains supports this idea and suggests the existence of neuro-modulatory inputs in addition to monoamines and ILPs (Agrawal et al., 2010).

A better understanding of how $InsP_3$ mediated intracellular Ca^{2+} -release and SOCE control the signaling properties of neuro-modulatory neurons will be relevant for understanding the precise role of intracellular Ca^{2+} signaling in motor circuit function. Neuro-modulation of motor circuits can modify both cellular and synaptic properties of neurons in the circuit (Marder and Bucher, 2001). This is re-enforced by the *Drosophila* work, where deficits in the flight neural circuit can be rectified by what appears to be an altered balance of neuro-modulators like ILPs and monoamines. Measurements of intracellular Ca^{2+} at rest and upon cell surface receptor stimulation in *Drosophila* neurons support the idea that changes in resting Ca^{2+} levels in *itpr* mutants (Ca^{2+} homeostasis) strongly influence the Ca^{2+} signals generated upon stimulation. Altered Ca^{2+} homeostasis in *itpr* mutants is reverted back to normal to a significant extent by over-expression of dOrai and dSTIM (Figure 1). The effect of these cellular properties on the release of neuro-modulators needs to be assessed. Moreover experiments, which distinguish between the need for $InsP_3R$, STIM and Orai in the functioning of single neurons of the flight circuit, as compared with their role in maintaining overall health of the flight circuit, are required. In *Drosophila*, the use of genetic strategies that allow controlled spatial and temporal expression of siRNAs in smaller subsets of neurons will be informative.

The close coupling observed between $InsP_3$ mediated Ca^{2+} release and SOCE via the Orai/STIM pathway for maintenance of intracellular Ca^{2+} homeostasis in neurons and the proposed role of



neuromodulators in this process, suggests novel potential strategies for the treatment of diseases where intracellular Ca²⁺ dyshomeostasis may be causative or exacerbated. Perturbation of neuronal Ca²⁺ homeostasis is believed to be one of the underlying causes for several neurodegenerative disorders including Alzheimer's disease (Bouron et al., 2004), Parkinson's disease (Chan et al., 2009), Huntington's disease (Tang et al., 2003), and amyotrophic lateral sclerosis (ALS; Appel et al., 2001). It has also been implicated in the progression of several spino-cerebellar ataxia syndromes (Inoue et al., 2001; van de Leemput et al., 2007; Chen et al., 2008).

It is thought that dysregulation of Ca²⁺ homeostasis synergizes with accumulation of mutant proteins in promoting the symptoms and pathology associated with the diseases. The *Drosophila* work raises the possibility of using drugs that directly modulate Orai function on the cell membrane or providing specific neuromodulatory compounds to re-set Ca²⁺ homeostasis in diseased neurons. The hope is that these interventions could slow down the neurodegenerative process. Much needs doing in vertebrate model systems to address these possibilities before application in human disease conditions.

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