Intracellular Ca^{2+} signaling and store-operated Ca^{2+} entry are required in *Drosophila* neurons for flight

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Neuronal Ca^{2+} signals can affect excitability and neural circuit formation. Ca^{2+} signals are modified by Ca^{2+} flux from intracellular stores as well as the extracellular milieu. However, the contribution of intracellular Ca^{2+} stores and their release to neuronal processes is poorly understood. Here, we show by neuron-specific siRNA depletion that activity of the recently identified store-operated channel encoded by *dOrai* and the endoplasmic reticulum Ca^{2+} store sensor encoded by *dSTIM* are necessary for normal flight and associated patterns of rhythmic firing of the flight motoneurons of *Drosophila melanogaster*. Also, *dOrai* overexpression in flightless mutants for the *Drosophila* inositol 1,4,5-trisphosphate receptor ($InsP_3R$) can partially compensate for their loss of flight. Ca^{2+} measurements show that Orai gain-of-function contributes to the quanta of Ca^{2+}-release through mutant $InsP_3Rs$ and elevates store-operated Ca^{2+} entry in *Drosophila* neurons. Our data show that replenishment of intracellular store Ca^{2+} in neurons is required for *Drosophila* flight.

calcium homeostasis | flight patterns | inositol 1,4,5-trisphosphate receptor | sarco-endoplasmic reticulum-associated Ca^{2+} ATPase | STIM

Several aspects of neuronal function are regulated by ionic calcium (Ca^{2+}). Specific attributes of a Ca^{2+}"signature" such as amplitude, duration, and frequency of the signal can determine the morphology of a neural circuit by affecting the outcome of cell migration, the direction taken by a growth-cone, dendritic development, and synaptogenesis (1). Ca^{2+} signals also determine the nature and strength of neural connections in a circuit by specifying neurotransmitters and receptors (2). Most of these Ca^{2+} signals have been attributed to the entry of extracellular Ca^{2+} through voltage-operated channels or ionotropic receptors. However, other components of the "Ca^{2+} tool-kit" coupled to Ca^{2+} release from intracellular Ca^{2+} stores are also present in neurons. These molecules include the store-operated Ca^{2+} (SOC) channel, encoded by the *Orai* gene, identified recently in siRNA screens for molecules that reduce or abolish Ca^{2+} influx from the extracellular milieu after intracellular Ca^{2+} store depletion (3–5). Several reports have confirmed its identity as the pore forming subunit of the Ca^{2+}-release activated Ca^{2+} (CRAC) channel (6–8). Activation of this CRAC channel is mediated by the endoplasmic reticulum (ER) resident protein STIM (stromal interaction molecule), also identified in an RNAi screen for molecules that regulate SOC influx (9, 10). STIM senses the drop in ER Ca^{2+} levels, and interacts with Orai by a mechanism which is only just being understood (11). Orai and STIM function in conjunction with the sarco-endoplasmic reticular Ca^{2+}-ATPase pump (SERCA) to maintain ER store Ca^{2+} and basal Ca^{2+}. The importance of intracellular Ca^{2+} homeostasis and SOC entry (E) in neural circuit formation and in neuronal function and physiology remains to be elucidated.

Here, we report how Orai and STIM mediated Ca^{2+} influx and Ca^{2+} homeostasis in *Drosophila* neurons contribute to cellular and systemic phenotypes. Reduced SOCE measured in primary neuronal cultures is accompanied by a range of defects in adults, including altered wing posture, increased spontaneous firing, and loss of rhythmic flight patterns. These phenotypes mirror the spontaneous hyperexcitability of flight neuro-muscular junctions and loss of rhythmic flight patterns observed in *Drosophila* mutants of the inositol 1,4,5-trisphosphate receptor ($InsP_3R$, *itpr*, CG1063)

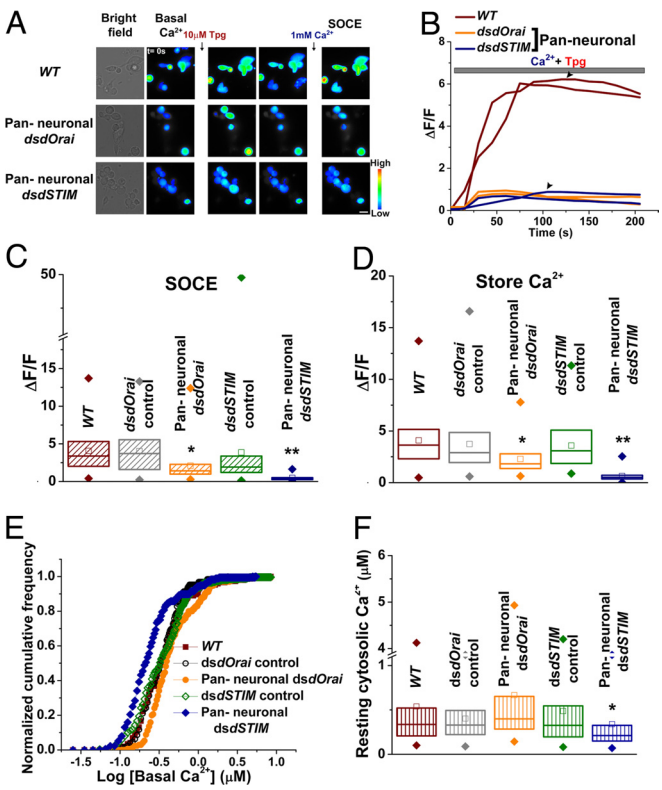

Fig. 1. Intracellular Ca^{2+} homeostasis in larval neurons is altered on expression of ds*dOrai* and ds*dSTIM*. (*A*) pseudocolor images represent $[Ca^{2+}]_{ER}$ and SOCE in primary cultures of neurons loaded with Fluo-4 from WT larvae and those expressing ds*dOrai* or ds*dSTIM*. (Scale bar, 10 μm.) (*B*) Single cell traces of SOCE by Ca^{2+} add-back after store depletion. Two cells of each genotype are shown. Arrow heads represent peak values of response, which have been plotted as a box chart in *C*. (*C*) Box plots of Δ*F*/*F* values of SOCE. The bigger boxes represent the data spread, smaller squares represent mean, and the diamonds on either side represent outlier values. (*D*) Box plot comparison of $[Ca^{2+}]_{ER}$ between neurons of indicated genotypes. (*E*) Kolmogorov–Smirnov (K-S) plot analyzing the distribution of $[Ca^{2+}]_i$ in neurons loaded with Indo-1. The frequency distribution is significantly shifted to the left for cells with *dSTIM* RNAi (ds*dSTIM*), indicating a higher frequency of cells with reduced $[Ca^{2+}]_i$ ($P_{K\text{-}S}<0.05$). (*F*) Box plot representation of $[Ca^{2+}]_i$ in neurons with *dsdOrai* or *dsdSTIM*. ($n \geq 150$ cells; *, $P_{ANOVA} < 0.05$; **, $P_{ANOVA} < 0.01$).

gene (12). The $InsP_3R$ is a ligand gated Ca^{2+}-channel present on the membranes of intracellular Ca^{2+} stores. It is thought to be critical for various aspects of neuronal function (1, 13). Mutants in the gene

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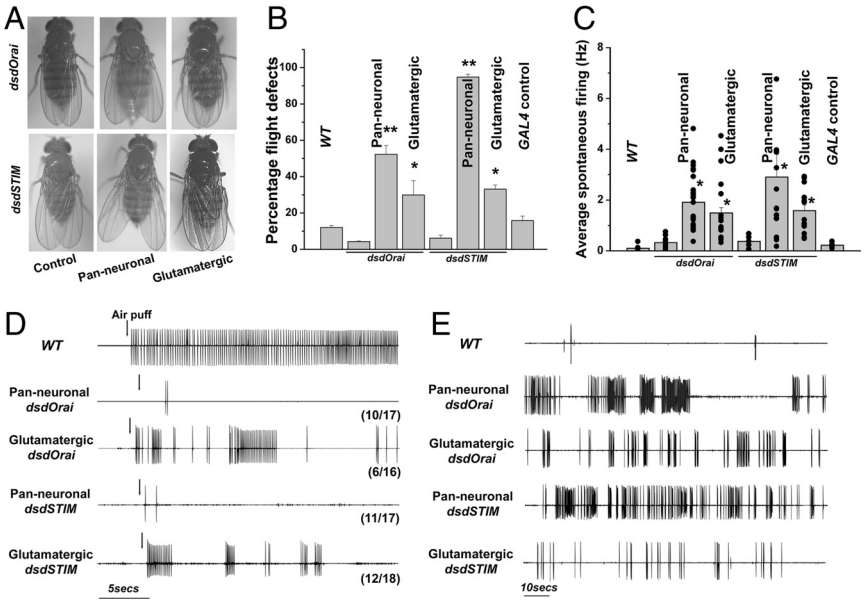

Fig. 2. RNAi knockdown of *dOrai* or *dSTIM* in subsets of neurons gives rise to flight motor defects. Pan-neuronal knockdown of *dOrai* and *dSTIM* induces (*A*) change in wing posture, (*B*) flight defects ($n \geq 100$ flies), (*C*) higher levels of spontaneous firing ($n \geq 15$ flies), and (*D*) defects in air-puff-induced flight patterns. (*E*) Representative traces of spontaneous firing activity from the DLMs of the indicated genotypes. Histograms represent mean ± SE; (*, $P < 0.05$; Student's *t* test). Both *GAL4* control strains were tested and found to be similar to WT. Data shown are for glutamatergic *GAL4* flies.

coding for the mouse $InsP_3R1$ are ataxic (14). Cerebellar slices from $InsP_3R1$ knockout mice show reduced long-term depression, indicating that altered synaptic plasticity of the cognate neural circuits could underlie the observed ataxia (15).

To understand the temporal and spatial nature of intracellular Ca^{2+} signals required during flight circuit development and function, *dOrai* (CG11430) and *dSERCA* (encoded by *CaP-60A* gene, CG3725) function was modulated by genetic means in *itpr* mutants. This modulation can restore flight to flightless adults, by altering several parameters of intracellular Ca^{2+} homeostasis including SOCE. Our results suggest that components of the central pattern generator (CPG) required for maintenance of normal rhythmic flight in adults have a stringent requirement for SOCE after $InsP_3R$ stimulation.

Results

SOC Entry in Neurons of *Drosophila* Requires Orai and STIM. Genes encoding the SOC channel (*Orai1*) and the store Ca^{2+} sensor (*Stim1*) are known to maintain intracellular Ca^{2+} store levels ($[Ca^{2+}]_{ER}$) in stimulated T cells. The replenishment of $[Ca^{2+}]_{ER}$ in T cells is required for their prolonged activation (16). Homologs of mammalian *Orai* and *Stim* exist in *Drosophila* as single genes, and perform similar cellular functions in S2 cells, where their depletion by gene specific double-stranded (ds)RNA leads to abrogation of SOCE (3–5). To investigate SOC channel activity in *Drosophila* neurons, we reduced levels of *dOrai* transcripts using dsRNA in primary neuronal cultures derived from larval brains. SOCE was monitored by Ca^{2+} imaging of cultured neurons in Ca^{2+} add-back experiments, after depletion of ER stores with thapsigargin in very low external Ca^{2+} (Fig. 1*A*). SOCE was significantly reduced in neurons expressing dsRNA for *dOrai* (*UASdOraiRNAi*^{221} denoted as ds*dOrai*; Fig. 1 *B* and *C*). Also, the level of intracellular store Ca^{2+} ($[Ca^{2+}]_{ER}$) was significantly lower in these cells (Fig. 1*D*), suggesting that Ca^{2+} entry through *Drosophila* Orai channels contributes to the maintenance of store Ca^{2+} in neurons. To ascertain that the reduced SOCE observed in cells expressing *dOrai* dsRNA is gene specific, SOCE was measured in 2 alternate conditions. Double-stranded RNA for the ER Ca^{2+}-sensor *dSTIM* (CG9126), (*UASdSTIMRNAi*^{073} denoted as ds*dSTIM*), and a ligand-gated extracellular Ca^{2+} channel, NMDAR1 (*UASdNR1RNAi*^{333} denoted as ds*dNR1*; CG2902) were expressed in all neurons. Normal function of STIM is considered essential for Orai channel activity, whereas SOCE is not predicted to change when levels of a plasma membrane localized ligand-gated Ca^{2+}-channel are reduced. Pan-neural expression of ds*dStim* followed by Ca^{2+} imaging revealed significant reduction of SOCE, $[Ca^{2+}]_{ER}$ (Fig. 1 *B–D*), and resting cytosolic Ca^{2+} ($[Ca^{2+}]_i$; Fig. 1 *E* and *F*). A significantly higher frequency of cells with lower $[Ca^{2+}]_i$ were present among the neuronal population with *dsdSTIM*. However, ds*dOrai* expression had no effect on $[Ca^{2+}]_i$. The efficacy of the dsRNA strains used was ascertained by semiquantitative RT-PCR, which showed a consistent reduction in the levels of the appropriate transcripts (Fig. S1 *A–D*). As expected, reduction in the level of *dNR1* transcripts did not affect store Ca^{2+} or SOCE (Fig. S1 *E* and *F*). These results demonstrate that Ca^{2+} influx, leading to replenishment of ER stores through the STIM–Orai pathway, is conserved in *Drosophila* neurons. Also, the single STIM-encoding gene in *Drosophila* appears to regulate both $[Ca^{2+}]_{ER}$ and $[Ca^{2+}]_i$. In mammalian systems, these cellular properties are regulated independently by *STIM1* and *STIM2*, respectively (17).

Reduced SOCE in *Drosophila* Neurons Causes Flight Defects. To determine whether reduced SOCE in *Drosophila* neurons affects neuronal function, motor coordination defects were measured in the appropriate genotypes. No obvious changes were visible in larvae expressing dsRNA for either *dOrai* or *dSTIM*. The larvae were viable and pupated normally. However, adult flies with pan-neural expression of ds*dOrai* and ds*dSTIM* had defective wing posture with significant loss of flight as seen in the "cylinder drop" test assay (Fig. 2 *A* and *B*) (18). Whereas >50% flies with *dOrai* knockdown were flightless, *dSTIM* knockdown resulted in a complete loss of flight (Fig. 2*B*). Expression of ds*dOrai* and ds*dSTIM* in glutamatergic neurons, which include the flight motor neurons, reduced flight ability in ≈35% of adult *Drosophila*, suggesting that the requirement for SOCE in flight extends beyond the glutamatergic domain. This observation is unlikely to be due to a difference in expression levels of the pan-neural and glutamatergic *GAL4* strains, because the latter appears to be the stronger driver, as judged by reporter gene expression in pupae and adults (30).

To understand how neuronal store Ca^{2+} and SOCE reduce flight ability, postsynaptic responses from the dorsal longitudinal indirect flight muscles (DLMs) that power flight were measured. Electrophysiological recordings were obtained during tethered flight (initiated in response to an air-puff stimulus) and at rest (Fig. 2 *C–E*). Nonfliers with pan-neural expression of *dsdOrai* and *dsdSTIM*, selected from the cylinder drop test were either unable to initiate

rhythmic action potentials in response to an air-puff stimulus or exhibited unsustained (<5 s) and arrhythmic flight patterns (Fig. 2*D*); the control flies were normal (Fig. S1*G*). Knockdown by ds*dOrai* and ds*dSTIM* in glutamatergic neurons lead to a milder change in flight patterns compared with pan-neural knockdown, consistent with a role for SOCE in nonglutamatergic interneurons in addition to the glutamatergic flight motor neurons. Recordings from resting DLMs of these flies revealed a high arrhythmic spontaneous firing rate of action potentials, the frequency of which was significantly higher than WT (Fig. 2 *C* and *E*) or other control flies (Fig. S1*H*).

Overexpressing *dOrai*$^+$ in Neurons Suppresses Flight Defects in *Drosophila itpr* Mutants. SOCE activation through Orai and STIM in vivo requires a signal for depletion of intracellular Ca^{2+} stores. Based on the similar phenotypes of *Drosophila itpr* mutants (12), we hypothesized that, in the context of flight, intracellular Ca^{2+} store depletion probably occurs through $InsP_3$ signaling and the $InsP_3R$. Therefore, the effect of overexpressing *dOrai* in the genetic background of *itpr* mutants was tested. For this purpose, *UASdOrai*$^+$ (expressing WT *dOrai*) transgenic strains were generated and expressed in selected neuronal subdomains. These subdomains include the glutamatergic domain tested above, aminergic domain, and the *Drosophila* insulin-like peptide 2 producing neurons (*Dilp2* neurons). Expression of a *UASitpr*$^+$ (expressing WT $InsP_3R$) transgene in the aminergic domain has been previously demonstrated to rescue *itpr* mutant phenotypes (12, 19), and more recently a similar rescue has been observed by *UASitpr*$^+$ expression in *Dilp2* neurons (N. Agrawal and G. Hasan, personal communication). Expression of 2 copies of *UASdOrai*$^+$ in *Dilp2* neurons and aminergic domain could partially suppress the altered wing posture of *itpr*$^{ka1091/ug3}$ (hereafter referred to as *itpr*^{ku}; Fig. S2*A*). Although flight ability was not restored, there was initiation of flight patterns on air-puff delivery, normally completely lacking in *itpr*^{ku} animals (Fig. 3*A*). Also, spontaneous hyperactivity of the DLMs in *itpr*^{ku} was suppressed to a significant extent by expressing *UASdOrai*$^+$ either ubiquitously (*hsGAL4-leaky* or *hsGAL4*^{L} at 25 °C) (12); or in the aminergic, *Dilp2*, and glutamatergic subneuronal domains (Fig. 3*B*; Fig. S2*C*).

To ascertain whether *dOrai*$^+$ expression is required during flight circuit formation in pupae and/or during acute flight in adults, ubiquitous expression of *dOrai*$^+$ in *UASdOrai*$^+$/*hsGAL4*^{L}; *itpr*^{ku} organisms was up-regulated by a heat shock (HS) either in 24-h pupae or in 1-day-old adults. In both conditions, a significant number of flies could initiate flight in response to an air puff. Thus, levels of *dOrai*$^+$ can modulate flight circuit activity both during its development and in adult function (Fig. 3*A*; Fig. S2*B*). However, the flight patterns obtained were not sustained and appeared arrhythmic (Fig. 3*A*), indicating that although *dOrai*$^+$ overexpression can suppress the flight defects and associated physiology of *itpr* mutants to a significant extent, it is insufficient to regain complete flight (see *Discussion*).

Pan-Neuronal Expression of *dOrai*$^+$ Restores Intracellular Calcium Homeostasis in *itpr*^{ku}. To understand the cellular basis of *dOrai*$^+$ suppression of *itpr* mutant phenotypes, SOCE and $[Ca^{2+}]_{ER}$ were measured in primary neurons from *itpr*^{ku} larval brains. SOC influx was greatly diminished (Fig. 4 *A* and *B*), whereas $[Ca^{2+}]_{ER}$ was significantly elevated in neurons derived from *itpr*^{ku} larvae grown at 25 °C, as compared with the WT. The mean $[Ca^{2+}]_{ER}$ in *itpr* mutant appeared twice as much as WT (Fig. 4*B*). The percentage of cells with detectable SOC was ≈3–5%, as compared with 70–80% in WT. Pan-neural overexpression of *dOrai*$^+$ in *itpr*^{ku} neurons restored detectable SOCE to 70% of *itpr*^{ku} neurons. Also, $[Ca^{2+}]_{ER}$ went back to WT levels, indicating that reduced SOCE and elevated $[Ca^{2+}]_{ER}$ in *itpr*^{ku} are linked homeostatic processes. Overexpression of *dOrai*$^+$ in WT neurons did not effect SOCE and $[Ca^{2+}]_{ER}$,

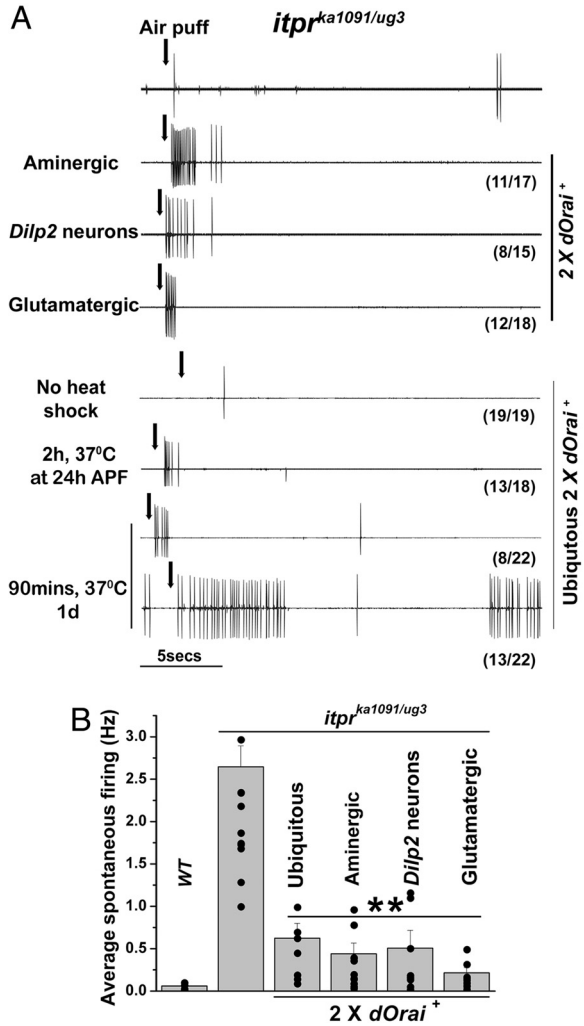

Fig. 3. Overexpression of *dOrai*$^+$ in neuronal subsets partially suppresses flight defects in an *itpr* mutant. (*A*) Flies overexpressing *dOrai*$^+$ in aminergic, *Dilp2*, and glutamatergic neurons or ubiquitously under a HS promoter and subjected to HS initiate unsustained (<5 s) flight patterns in response to air puff. (*B*) Overexpression of *dOrai*$^+$ (2 copies) either ubiquitously (by a leaky HS *GAL4* at 25 °C) or in the indicated subneuronal domains suppresses the elevated spontaneous firing of *itpr*^{ku} flies. (**, $P < 0.01$; Student's *t* test; $n \geq 20$ flies).

although it did elevate $[Ca^{2+}]_i$ to ≈1 μM in WT and *itpr*^{ku} backgrounds (Fig. 4 *C* and *D*).

The significance of deranged SOCE and $[Ca^{2+}]_{ER}$ in *itpr*^{ku} neurons was determined by measuring these parameters in cells of *itpr*^{ku} derived from second instar larvae maintained at 17.5 °C. The *itpr*^{ku} is a cold-sensitive allelic combination, and is lethal during the third instar larval stage at 17.5 °C (19). Interestingly, SOCE and $[Ca^{2+}]_{ER}$ in these conditions were similar to WT neurons grown under identical conditions at 17.5 °C (Fig. S3 *A* and *B*). These data suggest that *itpr*^{ku} organisms up-regulate store Ca^{2+} at 25 °C as a compensatory mechanism to allow for survival at that temperature, and that reduced SOCE may be a result of elevated store Ca^{2+}. The observation that return of $[Ca^{2+}]_{ER}$ and SOCE to normal by *dOrai*$^+$ overexpression at 25 °C is insufficient for restoration of complete flight in *itpr*^{ku} suggests that additional aspects of intracellular Ca^{2+} signaling are essential for flight in these organisms.

Restoration of Flight in *itpr*^{ku} by Dominant Alleles of *dOrai* and *dSERCA*. To investigate the additional properties of intracellular Ca^{2+} signaling required for flight, genetic interactions between *itpr* and

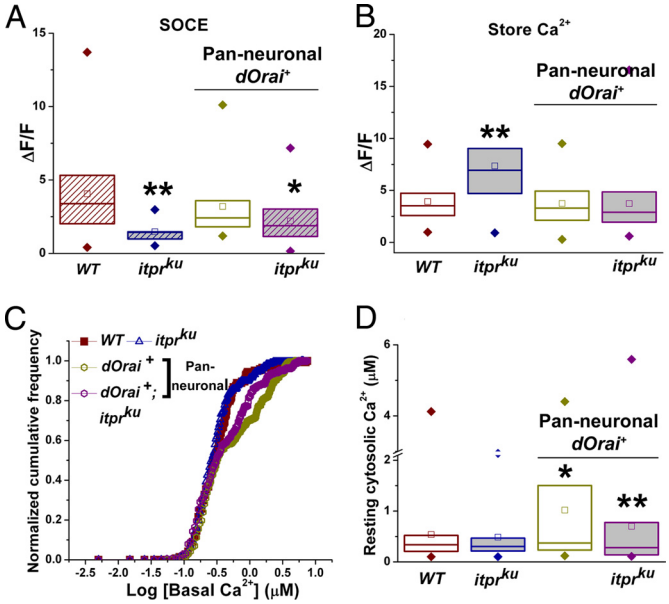

Fig. 4. The *dOrai*$^{+}$ overexpression in *itpr*^{ku} neurons restores intracellular Ca^{2+} homeostasis. (*A*) SOCE measurements in the indicated genotypes (*, $P_{ANOVA} < 0.05$, compared with *itpr*^{ku}; and **, $P < 0.01$, compared with WT). (*B*) $[Ca^{2+}]_{ER}$ in the indicated genotypes (**, $P_{ANOVA} < 0.01$, compared with WT). (*C*) K-S plot for $[Ca^{2+}]_i$ in neurons of the indicated genotypes ($P_{K\text{-}S} < 0.05$ for genotypes expressing *dOrai*$^{+}$ compared with WT). (*D*) Box plot representation of $[Ca^{2+}]_i$ (*, $P_{ANOVA} < 0.05$; **, $P_{ANOVA} < 0.01$; $n \geq 170$ cells).

dOrai were further probed. For this purpose, mutant alleles with P-inserts in the *dOrai* gene were obtained. The 2 alleles obtained, and referred to as *dOrai*^{1} and *dOrai*^{2}, both contain an *EP{gy2}* construct (enhancer P-element) (20) at a distance of 13 bps from each other in the 5′ UTR of the *dOrai* gene (Fig. S4). The 2 *dOrai* alleles were initially tested for their interaction with *itpr*^{ku} by measuring viability at 17.5 °C. Introduction of a single copy of either *dOrai* mutant allele could suppress cold-sensitive lethality of *itpr*^{ku} (Fig. S5*D*). A single copy of either *dOrai* allele also suppressed the wing posture defect of *itpr*^{ku} grown at 25 °C to a significant extent (Fig. S5*A*), suggesting that both *dOrai*^{1} and *dOrai*^{2} are hypermorphs. Subsequent observations support this conclusion further. The presence of a single copy of either *dOrai* allele in the background of *itpr*^{ku} restored flight initiation in response to an air puff (Fig. 5*A*; Fig. S5*B*), and suppressed hyperactivity of flight neuromuscular junctions (NMJs) (Fig. 5*D*; Fig. S5*C*). As expected for a hypermorph, the *dOrai*$^{2/+}$ mutant allele can also partially suppress flight-related defects and reduced SOCE and $[Ca^{2+}]_{ER}$ arising from pan-neuronal expression of ds*dSTIM* (Figs. 5 *A*, *D*, and *E*, and 6 *D* and *E*; Fig. S5*C*). Store Ca^{2+} and SOCE in neurons heterozygous for *dOrai*$^{2/+}$ are not significantly different from WT (Fig. S5*E*).

The partial suppression of *itpr*^{ku} phenotypes by hypermorphic *dOrai* mutant alleles is reminiscent of the recently demonstrated interaction between a dominant mutant allele of *dSERCA* (21) called *Ca-P60A*^{Kum170} (referred to here as *Kum*^{170}) and *itpr* mutants. This allele has been shown to delay cytoplasmic Ca^{2+} clearance after neuronal depolarization (22). Therefore, we tested the effect of introducing *Kum*^{170} in *dOrai*$^{1or2/+}$; *itpr*^{ku} organisms. Flies of the genotype *dOrai*^{2}/*Kum*^{170}; *itpr*^{ku} exhibited normal wings (Fig. S5*A*) and normal levels of spontaneous electrical activity in DLM recordings, consistent with the previously demonstrated dominant effect of *Kum*^{170} (22). Strikingly, flight ability was restored in a significant number of these triple mutant flies. This observation is in contrast to the complete loss of flight ability in *itpr* mutants and *itpr*, *dOrai* or *itpr*, *dSERCA* double mutant combinations; >60% of *dOrai*^{1}/*Kum*^{170}; *itpr*^{ku} adults and ≈50% of *dOrai*^{2}/*Kum*^{170}; *itpr*^{ku} adults passed as "fliers" in the cylinder drop test assay (Fig. 5*B*). Air-puff delivery elicited sustainable rhythmic flight patterns similar to WT in a high proportion of these flies (Fig. 5*A*; Fig. S5*B*, and Movie S1). Thus, down-regulating SERCA function restores or compensates for the additional intracellular Ca^{2+} signaling deficits required for free flight, which are lacking in *dOrai*$^{1or2/+}$; *itpr*^{ku} organisms. The nature of these Ca^{2+} signals was investigated next.

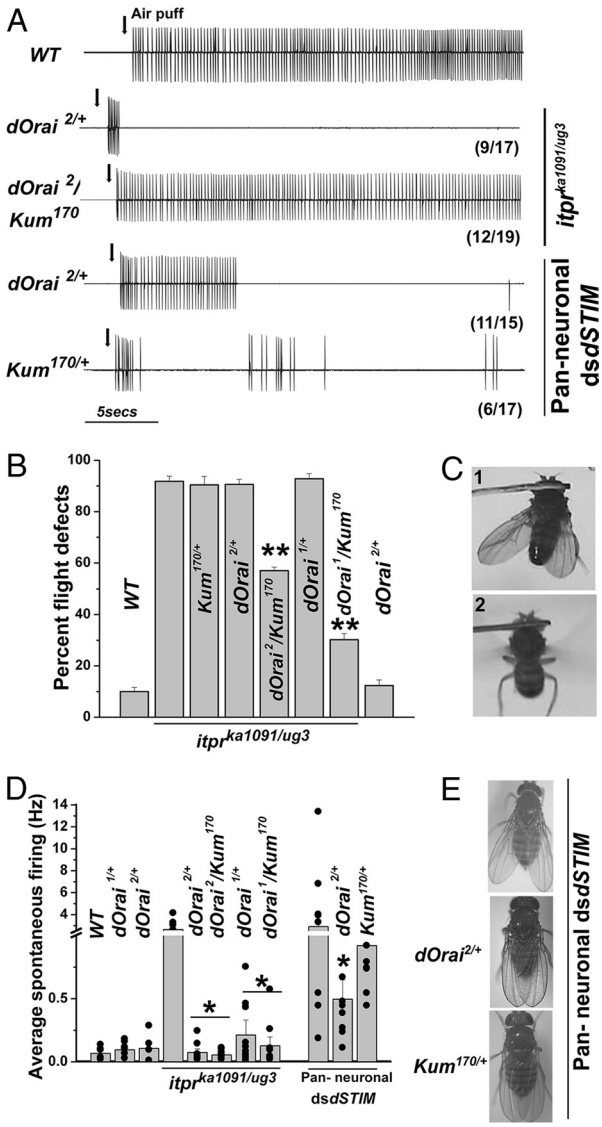

Fig. 5. Suppression of flight and related physiological defects by dominant mutants of *dOrai* and *dSERCA*. (*A*) Air-puff-induced flight patterns in the indicated genotypes. (*B*) Flight defects in *itpr*^{ku} are suppressed by the presence of both *Kum*^{170} and *dOrai*^{2} or *dOrai*^{1}, but not with *dOrai* mutants or *Kum*$^{170/+}$ on their own. (*C*) Snapshots taken within the first 5 s of air-puff-induced flight initiation in (*i*) *itpr*^{ku}; (*ii*) *Kum*^{170}/*dOrai*^{2}; *itpr*^{ku} (Movie S1). (*D*) Spontaneous hyperactivity in DLMs of indicated genotypes; $n \geq 15$. (*E*) Wing posture defects induced by ds*dSTIM* are suppressed by *dOrai*^{2} (50%) or *Kum*^{170} (10%). Histograms represent mean ± SE; (*, $P < 0.05$; **, $P < 0.01$, compared with *itpr*^{ku}; Student's *t* test).

Ca^{2+} Release Through $InsP_3$ Receptor and SOCE Together Contribute to Maintenance of Flight. Ca^{2+} release through the $InsP_3R$ was measured by stimulating neurons ectopically expressing the *Drosophila* muscarinic acetylcholine receptor (mAChR) with increasing concentrations of the agonist carbachol (23). For the WT $InsP_3R$ Ca^{2+}, release increased as a function of carbachol concentration (Fig.

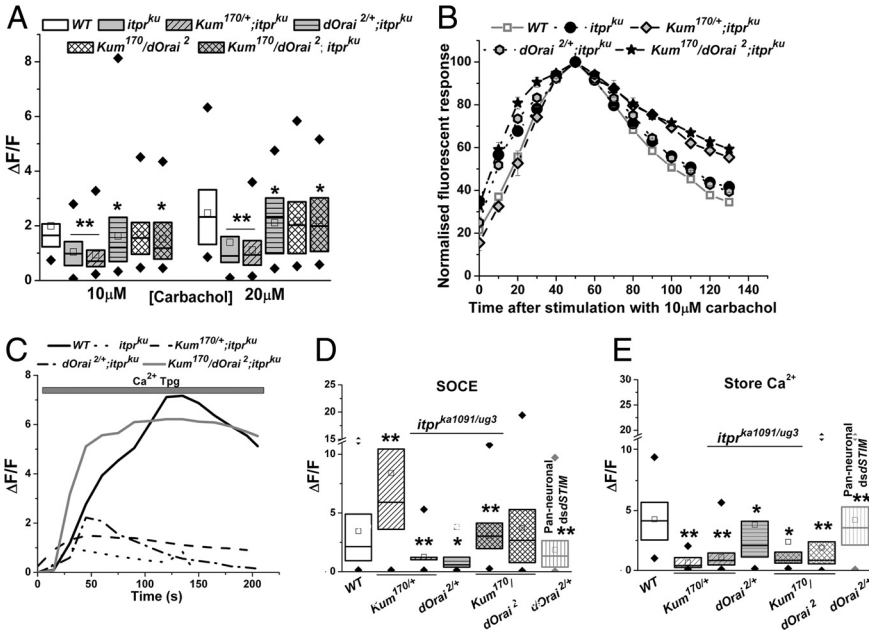

Fig. 6. Effects of *dOrai* and *dSERCA* mutants on different aspects of intracellular Ca^{2+} release. (*A*) Changes in stimulated Ca^{2+} release through $InsP_3R$ (measured as $\Delta F/F$) (**, $P_{ANOVA} < 0.01$, compared with WT; *, $P_{ANOVA} < 0.05$, compared with $itpr^{ku}$; $n \geq 150$ cells). (*B*) Effect of Kum^{170} and $dOrai^2$ on perdurance of $InsP_3R$ mediated Ca^{2+}-release signals; $n \geq 40$ cells, with similar peak response times. (*C*) Single cell traces of SOCE by Ca^{2+} add-back after store depletion. (*D*) SOCE measured in cultured neurons of indicated genotypes (**, $P_{ANOVA} < 0.01$, compared with WT). SOCE in $dOrai^{2/+}$; $itpr^{ku}$ is significantly higher than $itpr^{ku}$ (*, $P_{ANOVA} < 0.05$), and is normal in cells of $Kum^{170}/dOrai^2$; $itpr^{ku}$ (**, $P_{ANOVA} < 0.01$, compared with $itpr^{ku}$). Heterozygous $dOrai^{2/+}$ partially restores SOCE in ds*dSTIM* expressing neurons (**, $P_{ANOVA} < 0.01$). (*E*) $[Ca^{2+}]_{ER}$ measurements (**, $P_{ANOVA} < 0.01$; *, $P_{ANOVA} < 0.05$ in $Kum^{170/+}$ genotypes, compared with WT); $dOrai^{2/+}$ restores $[Ca^{2+}]_{ER}$ in $itpr^{ku}$ double mutants (*, $P_{ANOVA} < 0.05$). Presence of $dOrai^2$ restores $[Ca^{2+}]_{ER}$ in neurons expressing ds*dSTIM* (**, $P_{ANOVA} < 0.01$; $n \geq 170$ cells).

S3*D*); it was greatly attenuated in $itpr^{ku}$ (Fig. 6*A*; Fig. S3 *D–F*). Expression of *mAChR* transcripts, as determined by semiquantitative RT-PCR, was similar in mutant and WT (Fig. S3*C*).

Next, carbachol-stimulated Ca^{2+} release in $itpr^{ku}$ was measured in the presence of $dOrai^2$ and Kum^{170} double and triple mutant combinations. Kum^{170} had no direct effect on Ca^{2+}-release through the $InsP_3R$ on carbachol stimulation. The presence of $dOrai^2$ in either $dOrai^{2/+}$; $itpr^{ku}$ or in $dOrai^2/Kum^{170}$; $itpr^{ku}$ organisms restored carbachol-stimulated Ca^{2+} release to WT levels (Fig. 6*A*; Fig. S3*F*). However, this restoration is clearly not the only factor in flight maintenance, because $dOrai^{2/+}$; $itpr^{ku}$ organisms are flightless. Therefore, we measured additional parameters that are likely to contribute to the flight rescue in triple mutants. These measurements include perdurance of the carbachol-stimulated Ca^{2+} peak, SOCE, $[Ca^{2+}]_{ER}$, and $[Ca^{2+}]_i$.

The presence of a single copy of Kum^{170} delayed Ca^{2+} sequestration after carbachol-stimulated release, and led to greater perdurance of the Ca^{2+} peak; this effect of Kum^{170} was also present in cells derived from $dOrai^2/Kum^{170}$; $itpr^{ku}$ organisms (Fig. 6*B*; Fig. S3*F*). Consistent with the known function of SERCA, Kum^{170} had a dominant effect and reduced levels of store Ca^{2+} in all genotypes tested including $Kum^{170/+}$; $itpr^{ku}$ and $dOrai^2/Kum^{170}$; $itpr^{ku}$ (Fig. 6*E*). Concurrent with the lower store, SOCE was greatly elevated in Kum^{170} heterozygotes (Fig. 6*D*). Significantly, SOCE was normal in neurons derived from $dOrai^2/Kum^{170}$; $itpr^{ku}$ larvae, as compared with $itpr^{ku}$, $dOrai^{2/+}$; $itpr^{ku}$ and $Kum^{170/+}$; $itpr^{ku}$ (Fig. 6 *C* and *D*). Thus, the combined effect of $Orai^2$ and Kum^{170} on $itpr^{ku}$ is to restore near WT levels of $InsP_3$-stimulated Ca^{2+}-release, followed by a broader curve of Ca^{2+} persistence and normal SOCE. In $dOrai^{2/+}$; $itpr^{ku}$ organisms SOCE improved over $itpr^{ku}$, but remained low as compared with WT, similar to the observation with pan-neuronal expression of $dOrai^+$ in $itpr^{ku}$ (Figs. 4*B* and 6 *C* and *D*). Importantly, in the triple mutants, $[Ca^{2+}]_{ER}$ remained low (Fig. 6*E*), indicating that steady store Ca^{2+} levels do not effect flight directly, but perhaps contribute to driving the higher level of SOCE observed. Larval neurons heterozygous for $dOrai^2$ or $Kum^{170/+}$ had elevated levels of basal cytosolic Ca^{2+} with or without $itpr^{ku}$ in the background (Fig. S5 *F* and *G*). Higher $[Ca^{2+}]_i$ is unlikely to contribute directly to flight rescue, because *itpr* mutants with high $[Ca^{2+}]_i$ also exhibit flight defects.

Discussion

We have shown that SOC entry through the Orai/STIM pathway and the rate of clearance of cytoplasmic Ca^{2+} by SERCA together shape intracellular Ca^{2+} response curves in *Drosophila* larval neurons. The phenotypic changes associated with altering Orai/STIM function on their own and in *itpr* mutant combinations suggest that these Ca^{2+} dynamics are conserved through development among neurons in pupae and adults. The development and function of the flight circuit appears most sensitive to these cellular Ca^{2+} dynamics, changes in which have a profound effect on its physiological and behavioral outputs. Direct measurements of Ca^{2+} in flight circuit neurons are necessary in future to understand why these cells are more sensitive to changes in intracellular Ca^{2+} signaling. Other circuits such as those required for walking, climbing and jumping remain unaffected. Possible effects of altering intracellular Ca^{2+} homeostasis on higher order neural functions have yet to be determined.

The flow of information in a neural circuit goes through multiple steps within and between cells. Suppression experiments, such as the ones described here, present a powerful genetic tool for understanding the mechanisms underlying both the formation of such circuits and their function. The correlation observed between adult phenotypes and Ca^{2+} dynamics in populations of larval neurons from the various genotypes supports the following conclusions. Out-spread wings, higher spontaneous firing, and initiation of rhythmic firing on air-puff delivery in $itpr^{ku}$ are suppressed by either increasing the quanta (through hypermorphic alleles of *dOrai* and by $dOrai^+$ overexpression) or by increasing the perdurance (through mutant Kum^{170}) of the intracellular Ca^{2+} signal (Fig. S6 *Center*). Flight ability and maintenance of flight patterns requires SOCE in addition to increased quanta and perdurance of the Ca^{2+} signals, suggesting that SOCE in neurons contributes to recurring Ca^{2+} signals essential for flight maintenance (Fig. S6 *Right*).

The signals that trigger $InsP_3$ generation in *Drosophila* neurons and the nature of the downstream cellular response remain to be investigated. Previous work has shown that rescue of flight and related physiological phenotypes in *itpr* mutants require $UASitpr^+$ expression in early to midpupal stages, indicating the $InsP_3R$ activity is necessary during development of the flight circuit (12). Due to perdurance of the $InsP_3R$, its requirement in adults was not established. We now find that a basal level of $dOrai^+$ expression

through development followed by ubiquitous overexpression in adults can help initiate flight in $itpr^{ku}$, indicating a requirement for SOCE in adult neurons that probably constitute the CPG for flight. The precise neuronal circuit and neurons in the flight CPG are under investigation (24). Aminergic, glutamatergic, and insulin producing neurons could assist in development and/or directly constitute the circuit. Similar patterns of neuronal activity in the flight circuit of *itpr* mutants, either by generating different combinations of Ca^{2+} fluxes (as shown here), or by $UASitpr^{+}$ expression in nonoverlapping neuronal domains (N. Agrawal and G. Hasan, personal communication) supports the idea that different aspects of neuronal activity can compensate for each other to maintain constant network output.

Precisely how hypermorphic *dOrai* alleles modify $itpr^{ku}$ function to increase the quanta of Ca^{2+} release remains to be investigated. The ability of $itpr^{ku}$ to maintain elevated $[Ca^{2+}]_{ER}$ at 25 °C suggests a possible interaction between this heteroallelic combination and Orai/STIM. The mutated residue in $itpr^{ka1091}$ (Gly to Ser at 1891) lies in the modulatory domain, whereas in $itpr^{ug3}$, it lies in the ligand binding domain (Ser to Phe at 224); both residues are conserved in mammalian $InsP_3R$ isoforms (25). The mutant residues could directly affect $InsP_3R$ interactions with a store Ca^{2+} regulating molecule like STIM (26). Recent reports also demonstrate the formation of macromolecular assemblies of $InsP_3R$, SERCA, and SOC channels, suggesting specific functional interactions between them (27).

Last, our results suggest new ways of treating diseases where altered intracellular Ca^{2+} signaling or homeostasis has been suggested as a causative agent. Perhaps, the best documented of these diseases are spino-cerebellar ataxia 15, which arises by heterozygosity of the mammalian *IP_3R1* gene (28), severe combined immunodeficiency due to a mutation in *Orai1* (3), and Darier's disease from a mutation in *SERCA2* (29). Based on the underlying changes in intracellular Ca^{2+} properties in these genetic diseases, our study suggests ways of deciding appropriate combination of drugs that might target the causative gene products and their functionally interacting partners.

Materials and Methods

***Drosophila melanogaster* Strains.** The WT *Drosophila* strain used throughout is Canton-S. *UASdOrai* (S. Ziegenhorn), *UASmAChR* (from *Dm mAChR* cDNA clone) (23), $Ca\text{-}P60A^{Kum170ts}$ (21), $dOrai^{11042}$ and $dOrai^{20119}$ (referred as $dOrai^{1}$ and $dOrai^{2}$, respectively), Pan-neuronal *GAL4* ($Elav^{C155}$), and Ubiquitous *GAL4* ($hsp70^{L}$, $Heat\ shock^{Leaky}$) from Bloomington Stock Center. *UASRNAi* strains (VDRC). Glutamatergic *GAL4* (*OK371*) (30), aminergic (*Ddc*) (31), *GAL4* expressing in ILP2 producing neurons is *Dilp2GAL4* (32) .

Flight Assay and Electrophysiology. Flight tests, recording techniques, and data analysis have been published previously (12), and are described in detail in *SI Materials and Methods*.

Primary Neuronal Cultures, Calcium Imaging, and Data Analysis. Primary neuronal cultures were generated according to previously published protocols (22, 33). Calcium imaging for Fluo-4 was performed as described previously (2). Measurements for $[Ca^{2+}]_i$ were performed using the ratiometric dye Indo-1 (Invitrogen Technologies). Changes in fluorescence were quantified using the ImagePro plus software, V1.33. Detailed protocols are included in *SI Materials and Methods*.

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