Store-Operated Calcium Entry through Orai Is Required for Transcriptional Maturation of the Flight Circuit in *Drosophila*

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Store operated calcium entry (SOCE) is thought to primarily regulate calcium homeostasis in neurons. Subsequent to identification of Orai as the SOCE channel in nonexcitable cells, investigation of Orai function in neurons demonstrated a requirement for SOCE in *Drosophila* flight. Here, by analysis of an *Orai* mutant and by controlled expression of a dominant-negative *Drosophila* *Orai* transgene, we show that Orai-mediated SOCE is required in dopaminergic interneurons of the flight circuit during pupal development. Expression of dominant-negative *Orai* in dopaminergic neurons of pupae abolished flight. The loss of Orai-mediated SOCE alters transcriptional regulation of dopaminergic neurons, leading to downregulation of the enzyme tyrosine hydroxylase, which is essential for dopamine synthesis, and the dopamine transporter, which is required for dopamine uptake after synaptic release. These studies suggest that modulation of SOCE could serve as a novel mechanism for restoring dopamine levels in dopaminergic neurons.

Key words: dopamine transporter; dopaminergic neurons; IP3R; STIM; tyrosine hydroxylase

Significance Statement

The specificity of an animal's response to an environmental stimulus is determined in part by the release of neurotransmitters, which are sensed by responding neurons through cognate receptors on their surface. One way by which neurons respond is through release of calcium from intracellular stores followed by store refilling from extracellular calcium sources. This mechanism is called store-operated calcium entry (SOCE). The function of SOCE in neurons has been debated. Here we describe a new function for SOCE in the regulation of neurotransmitter levels in *Drosophila* flight neurons. This cell-signaling mechanism is required to maintain optimal levels of a key enzyme for dopamine synthesis and may serve as a mechanism for restoring dopamine levels in relevant pathological conditions.

Introduction

Neural circuits arrive at their pattern of adult connections through genetically encoded developmental programs, which define the identity of individual neurons, and neuronal activity during circuit maturation (Berridge, 1998; Spitzer, 2002; Borodinsky

et al., 2004). In vertebrates, neuronal activity followed by entry of extracellular Ca^{2+} has been shown to influence multiple aspects of neural function by activating target genes that play important roles in cell survival, dendritic and axonal growth, synaptic development, and neuronal plasticity (Berridge, 1998). In addition to activity-dependent mechanisms of Ca^{2+} entry channels, vertebrate neurons also express STIM 1 and 2 (Skibinska-Kijek et al., 2009) and Orai (Somasundaram et al., 2014), the recently identified components of store-operated Ca^{2+} entry (SOCE; Feske et al., 2006; Vig et al., 2006). Knockdown of STIM and Orai in cultured neural progenitor cells suggests that SOCE-dependent transcriptional changes could regulate mammalian neurogenesis

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(Somasundaram et al., 2014). In the mature brain, SOCE is thought to be required for the maintenance of neuronal Ca^{2+} homeostasis, which in turn can influence synaptic transmission and plasticity (Hartmann et al., 2014). Whereas some of these changes arise from a direct effect on endoplasmic reticular (ER) store Ca^{2+} (Hartmann et al., 2014), other changes might arise through regulation of transcription in resting neurons (Lalonde et al., 2014) and thus possible modulation of presynaptic and postsynaptic function. Neuronal activity also affects dendritic and synaptic growth and function in *Drosophila* (Miller et al., 2012; Timmerman et al., 2013; Vonhoff et al., 2013; Ryglewski et al., 2014). To identify molecular mechanisms that underlie the changes brought about by reduced SOCE in neural development, we investigated *Drosophila Orai* (*dOrai*) mutants for behavioral, cellular, and molecular deficits.

Previous work has shown that loss of inositol 1,4,5-trisphosphate receptor (IP_3R) function and knockdown of either dSTIM or dOrai in *Drosophila* neurons specifically affects the ability to initiate and maintain flight (Banerjee et al., 2004; Venkiteswaran and Hasan, 2009; Agrawal et al., 2010). Moreover, flight deficits in IP_3R mutant flies could be rescued by hypermorphic alleles of *dOrai* and by overexpression of *dSTIM* in neurons (Venkiteswaran and Hasan, 2009). These observations support the idea that in *Drosophila* neurons, Ca^{2+} release from ER stores through the IP_3R triggers SOCE through STIM and Orai (Venkiteswaran and Hasan, 2009). A similar signaling mechanism exists in mammalian Purkinje neurons (Wu et al., 2011; Hartmann et al., 2014). Here we investigated the spatiotemporal requirement of SOCE for flight in *Drosophila* neurons and have identified a requirement for SOCE in dopaminergic neurons during pupal development. To investigate mechanism(s) by which SOCE affects flight, we tested an existing loss-of-function mutant for *dOrai* (Cuttell et al., 2008), which enabled us to confirm our previous findings with pan-neuronal knockdown of *dOrai* (Venkiteswaran and Hasan, 2009). To identify neurons that require SOCE for flight, we developed an inducible dominant-negative *dOrai* transgene (Yeromin et al., 2006). Expression of the dominant-negative *dOrai* transgene, *dOrai*^{E180A} (*E180A*), specifically in dopaminergic neurons during pupal stages, abolished flight. Molecular analysis of *E180A*-expressing dopaminergic neurons indicates that SOCE helps establish and maintain their specific transcriptional profile during flight-circuit maturation.

Materials and Methods

Fly rearing and stocks. *Drosophila* strains were grown in corn flour agar media, which was supplemented with yeast. All strains were grown at 25°C, unless otherwise specified in the text. The *Drosophila* strain Canton S was used as the wild-type (WT) strain. A muscle-specific GAL4, *Dmef2* (BL27390); a pan-neuronal GAL4 for expression in pupal and adult stages, *nSyb*³⁰⁵⁸⁹ (BL39171; Pfeiffer et al., 2008); a glutamatergic neuron GAL4, *OK371* (BL26160); and the *orai*³ mutant strains (BL17538) were obtained from the Bloomington *Drosophila* Stock Center (BDSC; Department of Biology, Indiana University). The aminergic GAL4s, *Ddc*, *TH*, and *TRH* have been published previously and were obtained from their published sources (Lundell and Hirsh, 1994; Friggi-Grelín et al., 2003; Huser et al., 2012). The *THGAL4*, *UASH2BmRFP*, and *THGAL4*, *UASmGFP* strains were generated by recombining *UASH2BmRFP* (Languevin et al., 2005) and *UASmGFP* (Lee et al., 1999) with *THGAL4* respectively. The *UAS dOrai* strain (referred to as *dOrai*⁺) was generated by cloning of the publicly available full-length cDNA (RE30427) of *dOrai* (corresponding to the B transcript), in the *pUAST* vector (Venkiteswaran and Hasan, 2009), followed by microinjection to obtain a transgenic fly strain. A *dOrai* cDNA with the *E180A* mutation was a gift from Shenyan L. Zhang (Yeromin et al., 2006). The cDNA for *dOrai*^{E180A} was subcloned in the *pUASTattb* vector between *EcoRI* and *XhoI* sites and microinjected in embryos to generate the *UASE180A* strain. A *GAL80^{ts}* strain

with two insertions on the second chromosome was generated by Albert Chiang (National Centre for Biological Sciences, Bangalore, India). *TH* subdomain GAL4 strains (*THD1*, *THD'*, *THD4*, and *THC'*) were kindly provided by Mark Wu, Johns Hopkins University, Baltimore, Maryland (Liu et al., 2012). *UASRNAi* strains *dsdSTIM* (v47073), *dsDTH* (v108879), *dsdDAT* (v12082), and *dsdOrai* (v12221) were obtained from the Vienna *Drosophila* Resource Center (Campus Science Support Facilities, Vienna, Austria). *dsitpr* (1063-R2) was obtained from the National Institute of Genetics (Shizuoka, Japan). UAS effector strain for *Cac1* (BL8581) was obtained from the BDSC and for *DTHg* and *DTH1* from Serge Birman (Friggi-Grelín et al., 2003). *UASdicer* strains were obtained from the BDSC.

Flight assays and electrophysiological recordings. Single fly tests were performed with flies of either sex for measuring the time of flight bouts during air puff-stimulated tethered flight were performed as described previously (Agrawal et al., 2013). Flies from individual genotypes were collected 3–4 d after eclosion and tested for flight for ≤ 30 s. All videos were recorded for 30 s after flight initiation at 24 frames per second. The 30 s time period was based on our observation that flies exhibiting 30 s flight bouts usually continued flying for longer periods. Significant differences between flight performances of various genotypes were obtained by creating box plots of flight times, obtained from ≥ 30 flies of each genotype, using Origin 8 software (Micro Cal). Flight-duration box plots show the range of flight times of the genotypes tested, where the box represents 25 and 75% of the data points, the horizontal line is the median, and the small square-shaped solid box represents the mean. Flight times of individual flies appear as diamonds. Electrophysiological recordings for flight were obtained by inserting a sharpened tungsten electrode in fiber “a” of the indirect dorsal longitudinal muscles (DLMs). The recordings were performed as described previously (Banerjee et al., 2004; Agrawal et al., 2013). Average durations of DLM firing from ≥ 15 individuals are shown as histograms and diamonds represent the duration of DLM firing recorded from individual flies.

Climbing assays. Climbing assay was performed using a glass cylinder with a diameter of 2.5 cm. Flies of the appropriate genotype (3–4 d after eclosion) were dropped in the cylinder in batches of 10. A gentle tap was given three times so that all the flies reach the bottom of the cylinder. Then the number of flies that crossed an 8 cm mark in 12 s were counted manually. Each batch of flies was tested three times. Means and SEMs were calculated from ≥ 3 batches of 10 flies each using Origin 8 software (Micro Cal).

FACS of dopaminergic neurons. Approximately 15–20 CNSs were dissected in cold Schneider's medium (#21720-024, Life Technologies) from 48 h pupae of each genotype. The dissected CNSs were incubated in cysteine-activated papain (50 U/ml) for 30 min at room temperature (25°C) and then spun at 3000 rpm for 3 min. After discarding the supernatant, the brains were resuspended in cold Schneider's medium and gently triturated to obtain single-cell suspensions. This suspension was passed through a 40 μm mesh filter and kept on ice until sorting. Flow cytometry was performed on a FACS Aria cell sorter (BD Biosciences) equipped with a 488 nm laser to detect GFP. The threshold for GFP-positive cells was set using dissociated neurons from a non-GFP-expressing wild-type strain, Canton S. The same gating parameters were used to sort all genotypes in the experiment. GFP-positive and GFP-negative cells ($\sim 10,000$ each) were collected directly in TRIzol Reagent (Life Technologies) for further processing. Three independent sets of FACS-sorted neurons were used for RNA isolation.

RNA isolation, semiquantitative PCR, and qPCR. For RNA isolation, the CNS of either third-instar larvae, pupae of specific ages, or adult flies were dissected and homogenized in TRIzol Reagent (Life Technologies) and processed further according to the manufacturer's protocol. RNA integrity was determined on a 1% Tris-acetate-EDTA agarose gel. At least three independently isolated RNA samples were obtained for each genotype. Total RNA (~ 500 ng) was treated with 0.5 U of DNase I (amplification grade) in a reaction mixture (22.1 μl) containing 1 mM DTT and 20 U of RNase inhibitor. The reaction mixture was kept at 37°C for 30 min followed by heat inactivation at 70°C for 10 min. To this, 200 U of Moloney murine leukemia virus reverse transcriptase, 50 μM random hexamers, and 1 mM deoxyribonucleotide triphosphate were added in a

Figure 1. The *orai3* mutant exhibits reduced SOCE in primary neurons and flight deficits in adult *Drosophila*. **A**, Schematic representation of transcript A and B of *dOrai*. The thin line represents introns, the thick dark lines represent noncoding regions of the exon, and the light gray lines represent coding regions of the transcripts. The P element is inserted at position 1374265 (Cuttell et al., 2008). The arrows represent positions of primers used to perform semiquantitative PCR. **B**, Image of an agarose gel with RT-PCR products identifying transcripts A and B in RNA isolated from adult heads of the indicated genotypes. **C**, A representative Western blot of protein lysates from heads of *orai3* mutants, *elav^{C155}>dsdOrai*, and a wild-type strain (CS), probed with the dOrai antibody. The relative intensity of dOrai bands normalized to tubulin from ≥ 3 such blots was quantified with ImageJ (1.48V Java 1.6.0_20) and is shown in the bar graph. Diamonds represent relative intensity of dOrai bands from individual blots. The level of dOrai in lysates from *orai3* and *elav^{C155}>dsdOrai* heads was significantly different compared with wild type ($N \geq 3$; $*p_{t\text{-test}} < 0.05$). A *UASDicer* transgene was included to enhance the effect of *dsdOrai*. **D**, Images of primary neurons loaded with the calcium reporter dye Fluo-4 to measure store Ca^{2+} release after thapsigargin (Tg) treatment and SOCE upon addition of calcium (CaCl_2). **E**, Average traces of store Ca^{2+} release and SOCE in primary neurons from the indicated genotypes ($N \geq 100$). Iono, Ionomycin. **F**, The range of maximum peak intensity is lowest in *orai3* compared with the indicated genotypes shown as box plots. The midline represents the median and the solid box represents the mean of the (Figure legend continues.)

final volume of 25 μ l for cDNA synthesis. The reaction mixture was kept at 25°C for 10 min, then 42°C for 60 min, and finally heat inactivated at 70°C for 10 min. All reagents were from Invitrogen (Life Technologies). RT-PCR was performed in a reaction mixture of 25 μ l with 1 μ l of the cDNA.

Real-time quantitative PCRs (qPCR) were performed on an ABI 7500 Fast machine (Applied Biosystems) operated with ABI 7500 software version 2 in a total volume of 10 μ l using the KAPA SYBR FAST qPCR kit (#KK4601, Kapa Biosystems). *rp49* was used as an internal control. In the developmental profile of *TH*, *Act5c* was used as an additional internal control. A melt curve was performed at the end of the reaction to ensure a single peak. Sequences of the primers used (5' to 3') are as follows: *rp49*: forward, CGGATCGATATGCTAAGCTGT; reverse, GCGCTTGTTTCGATCCGTA; transcript A: forward, GCACCACAGCAACAGTCCCA; reverse, GCATCCAGCAAGACGTCCG; transcript B: forward, GTGT-TGGTGTATGATGCGATGG; reverse, GTCGTGGTGAAGGTCGTG C; *Act5c*: forward, GTCCACCTTCCAGCAGATG; reverse, CCTCCTC-CAGCAGAATCAAG; *TH*: forward, GTTGACAGCAGCCCCAAAAGAAC; reverse, GAGACCGTAATCATTGCTTGC; *dCac1*: forward, TTTCG TTGTAGTTACGGGAGCC; reverse, ATTTGCAGTAAAGGTGCCATC G; *dSlo*: forward, GCGGTGAAGTAAAAGAGAAG; reverse, CAAAT CGGGTGGTTTACGATGG; *dDAT*: forward, CATGCCACACACT GGT; reverse, GATCTTGGGAACTCGTCGCTC; *dOrai*: forward, GAGATAGCCATCCTGTGCTGG; reverse, CGGATGCCCGAGAC TGTC.

The fold change of gene expression in the mutant relative to wild type was determined by the comparative $\Delta\Delta C_t$ method (Lorentzos et al., 2003). Fold change equals $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t$ equals $(C_{t(\text{target gene})} - C_{t(\text{rp49})})_{\text{mutant2}} - (C_{t(\text{target gene})} - C_{t(\text{rp49})})_{\text{wild type}}$.

Immunohistochemistry. Immunostaining of adult *Drosophila* brains was performed as described previously (Sadaf et al., 2015). The following primary antibodies were used: rabbit anti-GFP antibody (1:10,000; A6455, Life Technologies), mouse anti-TH antibody (1:40; Immunos-tar), mouse anti-bruchpilot (anti-brp) antibody (1:50; kindly provided by Eric Buchner, University of Würzburg, Germany). Secondary antibodies were used at a dilution of 1:400 as follows: anti-rabbit Alexa Fluor 488 (#A1108, Life Technologies) and anti-mouse Alexa Fluor 568 (#A1104, Life Technologies). Confocal images were obtained on the Olympus Confocal FV1000 microscope (Olympus). For comparative studies of control and experimental samples, identical confocal settings were used and experiments were performed in parallel on the same day. Image visualization and analysis was done using an FV10-ASW 1.3 viewer (Olympus).

Primary neuronal cultures from *Drosophila* third-instar larvae and pupae. The culturing of *Drosophila* primary neurons was performed as described previously (Venkiteswaran and Hasan, 2009; Agrawal et al., 2010; Chakraborty and Hasan, 2012) with minor modifications. Dissociated neurons obtained from either third-instar larval or 32–48 h pupal brains were cultured in 200 μ l of DMEM/nutrient mixture F12 supplemented with 50 U/ml Pen Strep (Life Technologies) and 10 μ g/ml amphotericin B. All reagents for culturing primary neurons

were obtained from Life Technologies. Cultured neurons were kept at 25°C with 5% CO₂ for 14–16 h before imaging. This protocol for culturing primary neurons does not support the growth of glial cells, which remain at <1% of the total cell population (Wu et al., 1983).

Calcium imaging in primary neurons. After 14–16 h of growth, cells were washed with *Drosophila* M1 media (Agrawal et al., 2010) twice and loaded with 2.5 μ M fluo-4 acetoxymethyl ester (AM; Life Technologies) plus 0.002% Pluronic-F127 (Sigma-Aldrich) in M1 medium by incubation for 30 min in the dark at room temperature. After dye loading, the cells were washed with M1 three times and covered with 100 μ l of calcium-free M1 containing 0.5 mM EGTA. The cells were imaged within 40 min of dye loading. Imaging and quantification were performed as described previously (Agrawal et al., 2010). Briefly, store depletion was achieved by adding 10 μ M thapsigargin (Life Technologies; Venkiteswaran and Hasan, 2009) and SOCE was measured by the addition of 2 mM CaCl₂. Ionomycin (10 μ M; Calbiochem-407950, Merck Millipore) was added after 40 frames or 600 s so as to confirm that fluo-4 levels were not at saturation values during the experiment. Addition of thapsigargin, CaCl₂, and ionomycin was done manually at the indicated time points. Images were acquired automatically every 15 s over a period of 11 min.

Data analysis. For measuring changes in fluorescence, images were processed using the ImagePro plus software, V1.33 (Media Cybernetics). Fluorescence intensity at various time points was determined. The data were plotted with Origin 8.0 software as follows: $\Delta F/F = (F_t - F_{\text{basal}})/F_{\text{basal}}$ for each time point where F_t is the fluorescence at the time point and F_{basal} is the fluorescence of the cell when starting the experiment. Peak values of $\Delta F/F$ were obtained for every cell that responded and a box chart representing the data spread was plotted. The rectangular boxes represent the spread of data points between 25 and 75% of cells and the horizontal line is the median. Significant differences between multiple groups of data were analyzed by the Mann–Whitney test for nonparametric datasets and significant differences were taken at $p < 0.05$.

Generation of an antibody to dOrai in rats. The *dOrai*⁺ cDNA was subcloned in the pGEX6P1 expression vector (GE Healthcare Life Sciences) between restriction sites for EcoRI and XhoI, so as to tag the protein with GST at the N terminus. The *Escherichia coli* strain BL21 *codon*⁺ was transformed with the tagged *dOrai*⁺ cDNA clone. After overnight induction of the protein at 18°C and 180 rpm by addition of 0.3% isopropyl- β -D-thiogalactoside, cells were harvested from 5 L of culture, washed with PBS, and resuspended in 100 ml of lysis buffer (25 mM HEPES, pH 7.4, 250 mM NaCl, 5% glycerol, 1 mM DTT, 1% Triton X-100, 1 mM PMSF, and protease inhibitor mixture from Roche). Harvested cells were sonicated and centrifuged at 12,000 rpm at 4°C for 30 min. The filtered supernatant (100 ml) was loaded on a GST column (5 ml; GE Healthcare). After washing with five column volumes of washing buffer A (25 mM HEPES, pH 7.4, 250 mM NaCl, 5% glycerol, 1 mM DTT, 1% Triton X-100) and five column volumes of washing buffer B (25 mM HEPES, pH 7.4, 1 M NaCl, 5% glycerol, 1 mM DTT, 1% Triton X-100), the GST-tagged protein was eluted from the column by addition of 15 ml of 15 mM reduced glutathione (Sigma-Aldrich). The eluted protein was concentrated to 2 ml with a 100 kDa centricon filter (Amicon Ultra, Millipore) and further purified on a Sephadex-200 gel filtration column (GE Healthcare). The fraction corresponding to tagged dOrai (75 kDa) was reconcentrated in a 100 kDa centricon filter and 3 mg of purified protein was obtained. The purified protein was given to a company (Abexosome Biosciences) for generating an antibody in rats.

Western blots. Larval, pupal, and adult CNSs of appropriate genotypes were dissected in cold PBS. Between 5 and 10 brains were homogenized in 50 μ l of homogenizing buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 5% glycerol, 1 mM DTT, 1% Triton X-100, and 1 mM PMSF) and 10–15 μ l of the homogenate was run on an 8% SDS-polyacrylamide gel. The protein was transferred to a nitrocellulose membrane by standard protocols and the membrane was incubated in the primary antibody overnight at 4°C. Primary antibodies were used at the following dilutions: rat anti-dOrai, 1:1500; rabbit anti-TH (MAB318, Merck Millipore), 1:1000; mouse anti- β -tubulin mono-

←

(Figure legend continued.) distribution ($N \geq 100$; $**p < 0.001$, Mann–Whitney test). **G**, Images of adult *Drosophila* of the indicated genotypes. The *orai*² mutant exhibits expanded wing posture and reduced body size compared with controls. Scale bars, 0.5 mm. **H**, Flight time obtained in response to an air puff. The box represents the data range from 25 to 75% and diamonds represent the flight time of individual flies. The mean (solid square box) and median (solid horizontal line) of the data are shown ($N = 30$; $**p < 0.001$, Mann–Whitney test). *orai*² homozygotes are flightless and can be partially rescued by overexpression of *dOrai*⁺ in neurons. **I**, Duration of DLM firing, calculated from electrophysiological recordings (shown in **J**), are reduced in *orai*² mutants. Overexpression of *dOrai*⁺ in either pan-neuronal (*nSyb*), aminergic (*Ddc*), or dopaminergic (*TH*) neuronal domains can partially rescue the duration of DLM firings ($N = 15$; $**p < 0.001$ Mann–Whitney test). **J**, Representative electrophysiological recordings obtained from the dorsal longitudinal indirect flight muscles (DLMs) of tethered flies during an air puff-simulated flight bout. Normal flight patterns were absent in *orai*² mutants. Pan-neuronal expression of *dOrai*⁺ as well as overexpression of *dOrai*⁺ in either aminergic or dopaminergic neuronal domains could partially rescue the flight patterns.

Figure 2. Overexpression of a dominant-negative *dOrai* transgene, *dOrai*^{E180A}, in dopaminergic neurons reduces SOCE. **A**, Schematic representation of a monomer of the dOrai channel. The single point mutation E180A (glutamic acid to alanine at position 180) is present in the first transmembrane domain. **B**, Representative images of cultured single neurons from third-instar larval brains of the indicated genotypes. Overexpression of *E180A* in dopaminergic neurons (RFP cells in *TH>E180A;mRFP*) leads to reduced SOCE. **C**, The traces show mean store release and SOCE. The first peak represents passive store Ca^{2+} release upon addition of thapsigargin, whereas the second peak represents SOCE upon addition of extracellular Ca^{2+} ($N \geq 30$). **D**, The range of maximum $\Delta F/F$ values for individual cells from the indicated genotypes are shown as box plots. The midline represents the median and the solid box represents the mean of the distribution ($N \geq 30$; $*p < 0.01$ Mann–Whitney test). **E**, Values of $\Delta F/F \pm \text{SEM}$ calculated from primary neurons of 48 h pupae of the indicated genotypes ($N \geq 30$). SOCE was reduced upon expression of *E180A* in pupal dopaminergic neurons (RFP cells in *TH>E180A;mRFP*). **F**, Box plots with distribution of peak $\Delta F/F$ values from individual cells after thapsigargin and Ca^{2+} addition. SOCE was reduced in *E180A*-expressing pupal dopaminergic neurons (RFP cells in *TH>E180A;mRFP*) compared with controls (non-RFP cells in *TH>E180A;mRFP* or RFP cells in *TH>E180A*; $N \geq 30$, $**p < 0.001$, Mann–Whitney test).

clonal (E7; Developmental Studies Hybridoma Bank, University of Iowa, Iowa), 1:5000. Secondary antibodies conjugated with horseradish peroxidase were used at dilution of 1:3000 (anti-mouse HRP; 7076S, Cell Signaling Technology), 1:3000 (anti-rabbit HRP; 32260, Thermo Scientific), and 1:10,000 (anti-rat HRP; 012030003, Jackson Immuno Research). The protein was detected on the blot by a chemiluminescent detection solution from Thermo Scientific.

Results

Flight deficits and reduced SOCE are observed in the *orai*³ mutant

The *Drosophila* *Orai* gene *dOrai* encodes two major transcripts, A and B, which can both be translated to give the full-length dOrai protein (Fig. 1A). We characterized neuronal phenotypes of a

Figure 3. SOCE is required in dopaminergic neurons for flight. **A**, Flight time of flies of the indicated genotypes ($N \geq 30$; ** $p < 0.001$ Mann–Whitney test). **B**, Snapshots of flight videos at the indicated times during air puff-stimulated flight bouts. **C**, Average duration of DLM firing trains. DLM recordings from flies of the indicated genotypes were obtained during an air puff-induced flight bout ($N = 15$; * $p < 0.01$, ** $p < 0.001$, Mann–Whitney test). **D**, Average number of flies of the indicated genotypes that passed the climbing test. No significant difference was observed between various genotypes tested. **E**, Flight time of flies of indicated genotypes ($N \geq 30$; * $p < 0.01$, ** $p < 0.001$, Mann–Whitney test). **F**, Snapshots of flight videos at the indicated time intervals of air puff-stimulated flight bouts. *THD1GAL4*-driven overexpression of *E180A* resulted in complete loss of flight (extreme left). A *UASDicer* transgene was included with all the RNAi strains tested.

hypomorphic allele of *dOrai*, *olf186-FY09167* (Cuttell et al., 2008), henceforth referred to as *orai³*, which is a publicly available P-element insertion line. The position of the P-element insertion within the fourth intron (2R-1374265; Fig. 1A) of *dOrai* was also

confirmed by sequencing. Transcript A was significantly reduced in the CNS of *orai³/orai³* adults, whereas expression of transcript B was not affected significantly (Fig. 1B). These data suggest complex regulation of the *dOrai* gene in the nervous system, which

Table 1. PPM3 and PPL1 clusters and the a–a' dopaminergic neuron pair are marked by *TH*, *THD1*, *THD'*, and *THD4* GAL4s, but not by *THC* GAL4

	<i>TH</i>	<i>THD1</i>	<i>THD4</i>	<i>THD'</i>	<i>THC'</i>	<i>THD1>E180A</i>
TH-positive neurons per hemisphere (<i>n</i> = 12)						
PAM	11–13	0–2	1–2	0	0–1	0–1
Protocerebral anterior lateral	4–7	4	0	0	3–4	4
PPM1	1	0	0	0	0	0
PPM2	5–6	2–3	2–3	0	5–6	2–3
PPM3	6–8	6–7	6–7	6–7	0	6–7
PPM4	0–1	0	0	0	0	0
PPL1	12	10–11	7–9	10	0	10–11
PPL2	7–8	4–5	0	5–6	5–6	3–5
PPL3	0–1	0	0	0	0	0
PPL4	0–1	0	0	0	0	0
Subesophageal ventral posterior	1	0	0	0	1	0
Ventral unpaired medial 1	1	1	0	0	1	1
Ventral unpaired medial 2	2	2	0	0	2	2
TH-positive neurons in ventral ganglia (<i>n</i> = 6)						
T1	5–6	2–3	0	0	5	2–3
T2						
a–a'	2	2	2	2	0	2
b–b', ventral unpaired medial	2–3	0	0	0	3	0
T3	6–7	0–1	0	2	0	0–1
Abdominal	23–25	23–25	0	24–25	4–5	23–25

requires further characterization. Loss of transcript A was accompanied by reduction of dOrai level in the heads of *orai³/orai³* adults to a similar extent as observed by RNAi knockdown of *dOrai* transcripts (Fig. 1C).

dOrai functions as a channel for extracellular calcium entry after depletion of intracellular calcium stores (SOCE; Feske et al., 2006; Vig et al., 2006). In primary neurons derived from homozygous *orai³* larval brains, a significant reduction in SOCE was observed, whereas release of store calcium, after inhibition of the sarco-ER Ca^{2+} -ATPase by thapsigargin treatment, appeared similar to wild type (Fig. 1D–F). Neurons derived from *orai³* heterozygotes exhibit normal store release and SOCE (Fig. 1D–F). Thus both molecular and cellular phenotypes of *orai³* mutants confirm that it is a hypomorphic and recessive allele of *dOrai*.

To begin understanding the functional significance of reduced SOCE in neuronal function, adult viability of *orai³* mutants was determined and found to be ~20%. Their lethality is very likely in the larval stages because all pupae eclosed as adults. Adults that eclosed appeared smaller than *orai³* heterozygotes and had outspread wings (Fig. 1G). Previous results have established that knockdown of *dOrai* in *Drosophila* neurons reduces flight (Venkiteswaran and Hasan, 2009). In agreement with these published data, *orai³* mutant adults were flightless (Fig. 1H). Therefore, we tested flight in *orai³* mutants after expression of a *dOrai⁺* cDNA in either neurons or muscles with the help of the *UAS-GAL4* system of tissue-specific and cell-specific expression (Brand and Perrimon, 1993). A small but significant rescue of the flight deficit in *orai³* mutants was obtained by neuronal expression of a *dOrai⁺* cDNA. Expression was driven by *nsybGAL4³⁰⁵⁸⁹* with higher expression in pupae and adults compared with other neuronal drivers (Pfeiffer et al., 2008; Fig. 1H). However, *orai³* flies with expression of *dOrai⁺* in muscles (*Dmef*; Fig. 1H) continue to be flightless, thus supporting a neuronal focus for dOrai function in flight. Previous findings also suggest that *Drosophila* flight requires intracellular Ca^{2+} release and SOCE in glutamatergic and aminergic neurons. Flight was rescued significantly by *dOrai⁺* expression in aminergic neurons (*Ddc*;

Fig. 1H) but not by expression in glutamatergic neurons (*OK371*; Fig. 1H). Moreover, significant rescue of flight was observed by *dOrai⁺* expression specifically in dopaminergic neurons (*TH*; Fig. 1H). Tyrosine hydroxylase (TH) is a rate-limiting enzyme in dopamine synthesis and *THGAL4* is expressed in a subset of *DdcGAL4*-expressing neurons (Friggeri-Grelin et al., 2003). From the 30 flies of *THGAL4*-driven *dOrai⁺* tested, all but four could initiate flight. Their mean flight time was 7.1 ± 1.2 s (Fig. 1H). These data suggest that *GAL4*-driven expression of *dOrai⁺* in dopaminergic neurons contributes to the rescue of flight in *orai³* mutants. Flight deficits in *orai³* mutants and their rescue were further corroborated by electrophysiological recordings from the DLMs. Normal pattern of action potentials in response to an air puff were absent in *orai³* flies (Fig. 1I,J). Both *nsybGAL4*-driven and *DdcGAL4*-driven overexpression of *dOrai⁺* rescued the reduced duration of DLM firing in *orai³* homozygous flies to a significant extent (Fig. 1I,J). With *DdcGAL4*, characteristic tonic firing from the DLMs was observed in 1 of 15 randomly selected flies for 20–25 s, 5 of 15 for 10–15 s, and 9 of 15 for 3–5 s (Fig. 1I). Flies with *THGAL4*-driven *dOrai⁺* overexpression in dopaminergic neurons all initiated similar firing patterns, though these were not sustained beyond 10 s in a majority of flies (Fig. 1I,J). Thus overexpression of *dOrai⁺* in dopaminergic neurons of *orai³* mutants is sufficient for initiation of *Drosophila* flight.

SOCE in dopaminergic neurons is abrogated by expression of E180A, a dominant-negative form of dOrai

dOrai function in dopaminergic neurons was further investigated by cell-specific and stage-specific reduction of SOCE. For this purpose, we generated a *Drosophila* strain with an inducible dominant-negative form of *dOrai* in which the glutamate at residue 180 has been substituted with an alanine (Fig. 2A; Yeromin et al., 2006). Presence of dOrai^{E180A} (or E180A) subunits in dOrai tetramers abrogates channel function (Yeromin et al., 2006). We measured SOCE in TH-expressing neurons from *TH>E180A* larvae and pupae by marking TH neurons with H2BmRFP (Lan-gevin et al., 2005). SOCE was significantly reduced in both larval

Figure 4. *THD1* and *THC' GAL4s* express in different subsets of dopaminergic neurons. **A**, *THD1 GAL4*-driven expression of mGFP in the posterior central brain region. Prominent dopaminergic neuron clusters of this region are marked. Dopaminergic neurons that express mGFP (PPL1 and PPM3) appear yellow. **B**, Expression pattern of *THD1 GAL4* in the ventral ganglia of the adult CNS. *THD1 GAL4* marks the a–a' neurons but does not mark ventral unpaired medial (VUM) and b–b' neurons in the T2 segment of the ventral ganglia. **C**, *THD1 GAL4*-driven mGFP expression shown in the context of defined neuropil regions in the protocerebrum of an adult *Drosophila* brain. *THD1* neurons are marked by anti-GFP and neuropil regions are marked by anti-brp. **D**, Magnified image of part of the protocerebrum with cell bodies and their projections from the PPL1 cluster to the MB and from the PPM3 cluster [located near the ventromedial protocerebrum (VMP)] toward the central complex. LH, Lateral horn; CCP, caudal central protocerebrum; PB, protocerebral bridge. **E**, *THC' GAL4*-driven mGFP expression overlaps with anti-TH immunostaining of the PPL2 and PPM1/2 clusters (yellow) but not in the PPL1 and PPM3 clusters. **F**, *THC' GAL4* marks the VUM and b–b' neurons but does not mark the a–a' neurons in the T2 segment.

and pupal dopaminergic neurons compared either with non-TH neurons (non-RFP cells from *TH>E180A; mRFP*) or with dopaminergic neurons obtained from control animals expressing only mRFP (*TH>mRFP*; Fig. 2B–F). Expression of *E180A* did not

affect store calcium release in dopaminergic neurons (Fig. 2B–F). Interestingly, the peak amplitude of SOCE in pupal neurons was significantly lower compared with larval neurons, suggesting stage-specific control of SOCE during pupal stages of neural cir-

Figure 5. Reduction of SOCE in pupal dopaminergic neurons abolishes flight. **A**, Flight time of adult flies was measured after expression of either the appropriate RNAi strain (with *UASDicer*) or *E180A* by transfer to 29°C at the indicated stages of development (larval, pupal, and adult; $N = 30$). For adult expression, the flies were transferred to 29°C immediately after eclosion. As controls, the strains were also grown at either 29 or 18°C throughout development. Flight times were compared with corresponding genetic controls grown at 18°C ($N = 30$; $**p < 0.001$, Mann–Whitney test). **B**, Duration of DLM firing trains. DLM recordings from flies of the indicated genotypes were obtained during an air puff-induced flight bout ($N = 15$; $**p < 0.001$, Mann–Whitney test). **C**, Flight times were measured in *THD1>E180A; GAL80^{ts}* adult organisms by expressing *E180A* at either larval, pupal, or adult stages. **D**, Quantitative measurement (qPCR) of *dOrai* transcripts from dissected adult brains. Flies of the indicated genotypes were either maintained at 18°C throughout development and as adults or transferred to 29°C as adults immediately after eclosion for 48 h. Expression of *dOrai* transcripts was significantly higher ($P_{ANOVA} < 0.001$) in brains from *THD1>E180A, GAL80^{ts}* flies after 48 h at 29°C compared with controls and flies grown at 18°C. **E**, Snapshots obtained at the indicated time intervals from flight videos of air puff-stimulated flight bouts.

cuit maturation. Pupal neurons in *Drosophila* also exhibit spontaneous Ca^{2+} transients (Jiang et al., 2005). These data confirm that *E180A* expression results in a cell-autonomous dominant-negative effect on SOCE in *Drosophila* neurons. The consequence of *E180A* expression in dopaminergic neurons on *Drosophila* flight was investigated next.

Dopaminergic neurons of the *Drosophila* flight circuit require calcium release through the IP_3R and SOCE

SOCE is normally initiated in response to a physiological signal that first stimulates intracellular Ca^{2+} release from ER stores (Hogan et al., 2010; Smyth et al., 2010). To test whether *Drosophila* flight requires intracellular calcium release followed by SOCE in dopaminergic neurons, inducible RNAi strains for the IP_3R (*dsitpr*), the ER Ca^{2+} sensor *dSTIM* (*dsdSTIM*), and the dominant-negative form of *dOrai* *E180A* were specifically expressed in dopaminergic neurons (Fig. 3). The *dsitpr* and *dsdSTIM* strains have been validated in previous publications (Agrawal et al., 2009; Venkiteswaran and Hasan, 2009). A partial flight deficit (19 ± 2.2 s mean flight time) was observed upon knockdown of the IP_3R in dopaminergic neurons (*TH>dsitpr*), whereas a stronger flight deficit was observed upon *dSTIM*

knockdown (7.7 ± 0.8 s; *TH>dsdSTIM*), when compared with control flies (28.2 ± 0.6 s; *TH>dicer*; Fig. 3A,B). The flight deficits observed corresponded to the variable durations of action potentials recorded from the DLMs during air puff-induced flight bouts. Duration of DLM firing during the short flight bouts was no different from the spike frequency of 6–10 Hz observed in controls (Fig. 3C). Variable flight durations observed among the knockdown flies are very likely due to differential efficacy of the RNAi strains in individual flies (Agrawal et al., 2009, 2010; Venkiteswaran and Hasan, 2009).

Whereas knockdown of intracellular Ca^{2+} signaling genes by RNAi resulted in partial flight deficits, expression of *E180A* in either aminergic neurons (*Ddc>E180A*) or dopaminergic neurons (*TH>E180A*) resulted in complete loss of flight and flight-induced action potentials from the DLMs (Fig. 3A–C). Expression of *E180A* in serotonergic neurons (*TRH>E180A*), which are also marked by the aminergic GAL4 (*Ddc*; Huser et al., 2012; Sadaf et al., 2012; Casar et al., 2015), did not affect flight. Moreover, general motor function was not impaired by expression of *E180A* in dopaminergic neurons, as evident by their ability to climb normally (Fig. 3D). Together, these data support the idea that *Orai* function is specifically required in dopaminergic neurons of the flight circuit. Because

Figure 6. Loss of SOCE does not affect the pattern and number of dopaminergic neurons. **A**, Dopaminergic neuron clusters marked by anti-TH and anti-GFP immunostaining in the protocerebrum region of an adult *Drosophila* brain. Arrowheads indicate the PPL1 and PPM3 clusters of dopaminergic neurons. **B**, Dopaminergic neuron clusters in the protocerebrum of *THD1>E180A;mGFP* adults exhibit weak anti-TH immunostaining. GFP expression in cell bodies and their neurites appears similar to the control. **C**, Quantification of TH immunostaining in PPL1 ($n \geq 83$ cells), PPL2 ($n \geq 38$ cells), PPM2 ($n \geq 55$) and PPM3 ($n \geq 35$) neurons from *THD1>mGFP* (black) and *THD1>E180A;mGFP* (green) adult *Drosophila* brains ($N \geq 6$ brains). The ΔF values indicate $F - F_{\text{Basal}}$, where the basal fluorescence (F_{Basal}) of individual brain samples was subtracted from the total fluorescence (F) of the individual neurons. One-way ANOVA was performed for each cell cluster followed by *post hoc* Tukey's test ($*P_{\text{ANOVA}} < 0.05$).

TH is also expressed in the cuticle, where it is required for melanization (Wright, 1987), we tested flight after expression of *E180A* in multiple subdopaminergic neuronal domains that do not express in the cuticle (Liu et al., 2012). An essential requirement for SOCE in a

subset of dopaminergic neurons was confirmed by loss of flight in flies with *E180A* expression in dopaminergic neurons marked by *THD1GAL4*, *THD'GAL4*, and *THD4GAL4* (Fig. 3E,F). Partial flight deficits were also obtained by RNAi-mediated knockdown of the *IP₃R*, *dSTIM*, and *dOrai* in *THD1*-marked neurons (Fig. 3E,F). Interestingly, flies with *E180A* expression in the *THC'* dopaminergic neuron sub-domain flew normally (*THC'>E180A*; Fig. 3E,F). Therefore, we compared GFP-positive cells in the CNSs of flies with *THGAL4*-driven, *THD1GAL4*-driven, *THD'GAL4*-driven, *THD4GAL4*-driven, or *THC'GAL4*-driven *UASmGFP* expression (Table 1). This analysis suggests a requirement for SOCE in dopaminergic neurons of the protocerebrum posterior medial 3 (PPM3) and protocerebrum posterior lateral 1 (PPL1) clusters and a pair of neurons (a–a') in the T2 segment of the ventral ganglion, all of which are marked by *THD1GAL4* but not by *THC'GAL4* (Fig. 4). These data do not distinguish between the requirement for either one or all of these neuronal subsets for flight. Analysis of *THD1>mGFP* brains suggested that the PPM3 cluster projects to the central complex in the central brain, whereas the PPL1 cluster projects to the mushroom body (MB) and the lateral horn (Fig. 4C,D). Projections of the PPM3 and PPL1 cluster to these regions of the central brain have been described earlier (Mao and Davis, 2009). Previous work has also confirmed that the *THC'GAL4* does not mark a–a' cells of the ventral ganglion (Sadaf et al., 2015). Instead it marks three cells in the T2 segment, referred to as the b–b' pair and a single ventral unpaired medial neuron (Fig. 4F).

Maturation of the flight circuit during pupal development requires SOCE

Next, we asked whether loss of SOCE in dopaminergic neurons affects flight-circuit development and, if so, at what stage of development is SOCE required. To delineate the developmental stage for *IP₃R*, *dSTIM*, and *dOrai* requirement in dopaminergic neurons, we used the temporal and regional gene expression targeting system, in which a temperature shift to 29°C inactivates a mutant *GAL80^{ts}* repressor protein, thereby activating *GAL4* function (McGuire et al., 2004). Stage-specific knockdown of the *IP₃R* and *dSTIM*, as well as expression of *E180A* in dopaminergic neurons, followed by flight

tests in adults, revealed a requirement for intracellular calcium release and SOCE during pupal development (Fig. 5A–D). Pupal expression of *E180A* in dopaminergic neurons (*TH>E180A*; *GAL80^{ts}* and *THD1>E180A*; *GAL80^{ts}*) resulted in complete loss of

flight (Fig. 4*A–D*). Pupal knockdown of either the *IP₃R* or *dSTIM* in all dopaminergic neurons (*THGAL4*) led to partial flight deficits with mean flight times of 17.8 ± 1.9 s and 7.5 ± 0.5 s respectively (Fig. 5*A*). Normal flight bouts were observed upon restricting the RNAi knockdowns and *E180A* overexpression to larval stages, supporting SOCE function during pupal maturation of the flight circuit (Fig. 5*A–C,E*). As controls, we tested RNAi and *E180A*-containing flies with the *GAL80^{ts}* transgenes, grown at either 18°C (inactive GAL4) or 29°C (active GAL4) throughout development. Flight was not affected at 18°C, and was significantly reduced (knockdowns) or absent (*E180A*) at 29°C (Fig. 5*A–C,E*). Expression of *E180A* was also induced in adults for 48 h after eclosion. Normal flight durations were observed in these flies (Fig. 5*A, C,E*), indicating that *E180A* expression does not affect dopaminergic neuron function during acute flight. Expression of the *E180A* transgene in dopaminergic neurons was confirmed in adults after 48 h of induction by measuring *dOrai* transcripts (Fig. 5*D*). Electrophysiological recordings from the DLMs of *IP₃R* and *dSTIM* knockdowns and *E180A*-expressing flies, obtained after air puff stimulation of flight bouts, corroborated results from single-flight assays (Fig. 5*B*). From these data, we conclude that dopaminergic neurons of the *Drosophila* flight circuit require intracellular Ca^{2+} release through the *IP₃R*-mediated and *dSTIM*/*dOrai*-mediated SOCE during pupal development, which enables their normal function in adult flight. These data do not exclude possible effects of *E180A* expression over longer periods on adult dopaminergic neurons. These are currently under investigation. Moreover, a requirement for *IP₃*-mediated Ca^{2+} release and SOCE in dopaminergic neurons in adults is suggested by a significant difference between the flight times of *TH>dsitpr* flies with pupal knockdown compared with constant knockdown at 29°C (Fig. 5*A, B*; $p < 0.01$, Mann–Whitney test).

SOCE controls the scale of TH upregulation in pupal dopaminergic neurons

To investigate possible underlying causes for loss of flight by reduced intracellular calcium signaling in dopaminergic neurons, the status of dopaminergic neurons in *THD1>E180A* fly brains was examined. Immunostaining with anti-TH revealed reduced immunoreactivity in the CNS of *THD1>E180A* adults compared

Figure 7. Comparison of projections from the dopaminergic PPL1 and PPM3 clusters in wild-type and SOCE-deficient brains. *A*, The PPL1 neuronal cluster and their projections in a *THD1>mGFP* adult brain (confocal stacks 74–98). *B*, Projection from the PPL1 neurons enter the MB neuropil. The projections run from the posterior to the anterior (confocal section stacks 12–88). *C*, PPM3 cluster of dopaminergic neurons and their projections in a *THD1>mGFP* adult brain (confocal stacks 63–100). *D*, Projections from the PPM3 cluster (arrowheads) go toward the fan-shaped body (FB) and the EB (confocal stacks 41–52). *E*, Three PPM3 projections (arrowheads) enter the EB neuropil (confocal stacks 44–49). *F*, PPL1 cluster and its projection in a *THD1>E180A;mGFP* brain. The image is a Z-stack of confocal sections 69–93 running from posterior to anterior. *G*, Projections of PPL1 neurons enter the MB neuropil (stacks 15–80). *H*, PPM3 neurons in a *THD1>E180A;mGFP* brain (stacks 52–100). *I*, Three projections (arrowheads) of the PPM3 neurons head toward the EB. As in *D*, one projection goes through the FB (stacks 37–55). *J*, Three major projections of the PPM3 neurons enter the EB neuropil (stacks 36–48). Asterisks refer to contiguous positions in the series of confocal stacks of 0.3 μ m each. Scale bars, 50 μ m. $N = 5$ brains for each genotype.

Figure 8. SOCE in pupae is required for normal levels of TH mRNA and protein in adult neurons. **A**, TH transcript levels in pupal CNS of the indicated genotypes dissected at the indicated time points after puparium formation (APF). TH transcripts were normalized to *rp49* and are expressed as a Log₂-fold change from the wild-type strain (CS, gray) at 24 h APF ($N \geq 3$, one-way ANOVA was performed for each time point followed by a *post hoc* Tukey's test; ** $p < 0.05$ and * $p < 0.1$). **B**, TH protein levels are upregulated in parallel with TH transcripts in the CNS of developing pupae. TH protein levels were detected in Western blots with CNS lysates from control (TH/+) and experimental animals (TH>E180A). The experiment was repeated twice. **C**, A representative Western blot of dissected adult CNSs from animals with E180A expression in THD1-marked neurons. E180A expression was allowed either throughout development (29°C) or only during pupal stages (pupal) or prevented throughout development (18°C). Quantification of three such blots where TH levels were normalized to that of tubulin are shown below ($N = 3$, * $p_{t \text{ test}} < 0.05$). **D**, Quantitative measurement (qPCR) of the indicated transcripts from GFP-positive and GFP-negative (non-GFP) cells. Significant differences were observed in GFP-positive cells obtained from the CNSs of TH>E180A; mGFP (gray) animals compared with the control genotype, TH>mGFP (black). Transcript levels appear unchanged between non-GFP cells from TH>E180A; mGFP (light gray) and control TH>mGFP (dark gray) genotypes. Log₂-fold changes were obtained as described in the methods. ($N = 3$, * $p_{\text{ANOVA}} < 0.05$, ** $p_{\text{ANOVA}} < 0.01$). The inset contains a representative dot plot of FACS analysis of single-cell suspensions from the dissected CNSs (48 h APF) of animals expressing GFP under the THD1 promoter. Approximately 1% of the cells were GFP positive. Both GFP-positive and GFP-negative cells were collected for transcript analysis.

with controls (Fig. 6A,B). Reduced immunoreactivity in individual neurons from THD1-marked clusters was quantified from multiple samples (Fig. 6C). A significant reduction was observed in cells of the PPL1, PPM3, and PPL2 clusters, where THD1 marks either all or a majority of neurons (Table 1; Fig. 4A). The PPL2 cluster is also marked by *THC'* (Fig. 4E). Hence, it is unlikely to contribute to the flight deficits observed with THD1-driven expression of E180A. In contrast, only a few PPM1/2-cluster neurons are marked by THD1 (Fig. 4A) and here the TH immunoreactivity was not reduced significantly (Fig. 6C). Despite reduced TH immunoreactivity, the number of TH-positive cells in the CNS of THD1>E180A; mGFP animals was no different from that of THD1>mGFP controls. Specifically, the numbers of TH-positive neurons in CNSs of THD1>E180A; mGFP

animals in the PPM3 cluster (6–7), in the PPL1 cluster (10–11), and in the T2 segment of the ventral ganglion (a–a') were unchanged and were similar to the number in THD1>mGFP animals (Table 1). Next we investigated more carefully the status of neurite projections from the PPM3 clusters, the PPL1 clusters (Fig. 7), and the a–a' neurons (data not shown) in controls (Fig. 7A–E) and THD1>E180A animals (Fig. 7F–J). PPL1 cluster neurons project to the MB (Kong et al., 2010). These appeared no different between control and THD1>E180A flies (Fig. 7, compare A,B, F,G). In the 6–7 brains analyzed from controls and THD1>E180A flies, neurite extensions from the PPL1 to the MB varied between two and four in each brain hemisphere. On quantification, the average number of projections obtained from ≥ 12 brain hemispheres was 3 ± 0.96 for controls and 3 ± 1 for

Figure 9. TH and dDAT are required for *Drosophila* flight. **A**, Western blot of TH levels in the dissected CNSs of flies with TH RNAi expression (*THD1>dsdTH*) and controls (*dsdTH/+*). Quantification of the mean relative intensity ($\pm$ SEM) of TH bands from the indicated genotypes with respect to tubulin is shown on the right ($N = 3$, $*p_{t\text{-test}} < 0.05$). **B**, Flight time of adults with overexpression of the indicated transgenes under control of either *Ddc*, *TH*, or *THD1 GAL4*s. Flight times were compared with their corresponding controls ($N = 30$, $*p < 0.01$, $**p < 0.001$, Mann–Whitney test). Significant flight deficits were obtained by knockdown of either *TH* (*dsdTH*) or *DAT* (*dsdDAT*) and overexpression of *Cac1*. **C**, Flight times of flies of the indicated genotypes ($N = 30$; $**p < 0.001$, Mann–Whitney test).

TH>E180A flies. Projections from the PPM3 cluster are more complex and lead toward different regions of the ellipsoid body (EB) in the central complex (Kong et al., 2010; Fig. 7C–E,H–J). One neurite projection goes through the fan-shaped body to the central region of the EB (Fig. 7D,I), whereas a second and third go directly to the anterior and posterior parts of the EB. All three projections were also observed in *TH>E180A* adults (Fig. 7, compare C–E, H–J). Thus loss of SOCE by *E180A* expression does not alter projection patterns of the PPL1 and PPM3 neurons. However, these data do not rule out more subtle changes in synapse structure that might arise within the MB and/or central complex neuropil regions.

Based on the reduced TH immunoreactivity observed and the absence of any visible anatomical change, we hypothesized that intracellular calcium signaling might affect *TH* mRNA and TH protein levels during pupal development. Indeed, levels of TH

transcripts increase dramatically in the CNS of 24–72 h pupae from control and *Canton S* organisms (nearly a 70-fold increase; Fig. 8A). This increase is dampened significantly (~ 20 fold) in CNS dissected from *TH>E180A* and *TH>dsdSTIM* pupae (Fig. 8A). Quantification of TH protein from brain lysates revealed similar results where intensity of TH-specific bands at 96 h after puparium formation (APF) and in adults was significantly reduced in brains of *TH>E180A* organisms compared with controls (*TH/+*; Fig. 8B). TH levels were also reduced in the CNS of adult flies when expression of *E180A* was restricted to pupal stages (*THD1>E180A;GAL80^{ts}*; Fig. 8C). Thus, SOCE in pupal dopaminergic neurons is required for upregulation of *TH* mRNA and protein, and reduction in this upregulation correlates with loss of flight.

To test whether SOCE affects transcription of genes other than *TH* in dopaminergic neurons, we obtained GFP-marked neurons as well as non-GFP-marked neurons from brains of *E180A*; *mGFP*-expressing flies (*THD1>E180A;mGFP*) and controls (*THD1>mGFP*) by FACS (Fig. 8D). RNA was isolated from sorted cells for measurement of transcript levels. In dopaminergic neurons with reduced SOCE, downregulation of *TH* was accompanied by significant downregulation of dopamine transporter (*dDAT*) transcript levels, whereas transcripts encoding a voltage-gated calcium channel *Cac1* were upregulated (Fig. 8D). As a positive control, we measured *dOral* transcript levels, which exhibit significant upregulation upon *E180A* expression in GFP-marked neurons (*THD1>E180A, mGFP*), but remain constant in non-GFP-marked cells (Fig. 8D). Moreover, *TH*, *dDAT*, and *Cac1* transcripts remained constant in non-GFP-marked cells. Transcripts for a Ca^{2+} -gated K^{+} channel, *Slowpoke* (*Slo*), were unchanged by *E180A* expression in dopaminergic neurons (Fig. 8D).

Altered expression of TH, dDAT, and Cac1 in dopaminergic neurons affects *Drosophila* flight

To independently test the effect of altered TH levels on adult flight, we obtained an RNAi strain for *Drosophila TH* (*dsDTH*) and validated it by measuring TH protein in the CNS of *THD1>dsDTH* adults (Fig. 9A). We measured flight in flies with knockdown of *TH* in neurons marked by *DdcGAL4*, *THGAL4*, and *THD1GAL4*. All three knockdown strains exhibit significant flight deficits albeit to varying levels (Fig. 9B). Moreover, significant flight deficits were obtained upon knockdown of the dopamine transporter (*dDAT*) and overexpression of *Cac1* in *THD1*-marked neurons (Fig. 9B). Next, we tested whether overexpression of TH could rescue flight deficits of SOCE-compromised flies. Overexpression of the complete genomic region of *Drosophila TH* (*DTHg*) (Friggi-Grelín et al., 2003) could rescue the flight deficit of *TH>dsitpr* flies (compare Figs. 3A, 9C). Overexpression of a DTH cDNA (*DTH1*) encoding the neuronal isoform (Friggi-Grelín et al., 2003) either through development, or in pupae alone, could rescue flight initiation of *THD1>E180A* animals to a significant extent (compare Figs. 3E, 9C). The rescue by *DTH1* argues against changes in synaptic structure (that might affect dopamine release) as a primary cause of the flight deficits observed; rather, it supports reduced TH transcription as a key feature of neurons with compromised SOCE, and normal TH levels as an essential requirement for flight initiation. However, this rescue was insufficient to maintain flight beyond 7–8 s (Fig. 9C). Thus TH overexpression is probably insufficient to restore expression of other SOCE-regulated genes. Upregulation of the voltage-gated Ca^{2+} channel subunit, encoded by *Cac1* (Fig. 8D),

is one such factor that would alter membrane excitability and therefore neural function during adult flight.

Discussion

Null mutants in Ca^{2+} -signaling genes, such as *Orai*, *STIM*, and *IP₃R*, have pleiotropic effects during development, which frequently result in either defective development or early lethality of the organism (Venkatesh and Hasan, 1997; Joshi et al., 2004). Consequently, identification of molecular and cellular mechanisms regulated by intracellular Ca^{2+} signaling and SOCE in differentiated neurons requires genetic tools that can be restricted in their spatiotemporal expression. We addressed this problem by first studying a hypomorphic allele of the single *Drosophila* *Orai* gene followed by developing a dominant-negative *Orai* transgene whose expression could be manipulated in a stage-specific and tissue-specific manner. Temporal expression of the dominant-negative *E180A* transgene established that SOCE requirement in dopaminergic neurons is during pupal maturation of the flight circuit. Moreover, loss of SOCE does not affect the number of dopaminergic neurons; rather, it alters their transcriptional profile by affecting key molecules, such as TH, DAT, and *Cac1* or *Dmca1A/Ca_v2*. Whereas downregulation of TH and DAT expression would reduce synaptic neurotransmitter release, upregulated *Cac1* levels are likely to affect synaptic homeostasis (Lee et al., 2014). It is likely that SOCE regulates expression of other genes in addition to TH, DAT, and *Cac1*. Thus SOCE is required for attaining the final functional state of dopaminergic flight neurons in the adult brain.

Further analysis of dopaminergic subdomains that require SOCE support one or both of two central brain clusters (PPM3 and PPL1) and a pair of neurons in the second thoracic segment (T2) of the ventral ganglion (a–a') as necessary for flight. Interestingly, recent work has demonstrated a role for the a–a' pair of neurons in modulation of bilateral wing-coordinating motor neurons during initiation and cessation of *Drosophila* flight (Sadaf et al., 2015). The PPM3 and PPL1 clusters have not been implicated in flight control earlier. Cell bodies of the PPM3 cluster lie in the vicinity of the ventromedial protocerebrum neuropil and project to the EB of the central complex. As demonstrated recently, these regions form part of the auditory/mechanosensory module in the fly brain and might thus constitute centers for control of voluntary flight initiation (Shih et al., 2015). Maintenance of flight bouts over several minutes also requires synaptic activity in another cluster of central brain dopaminergic neurons, the protocerebral anteriomedial (PAM) cluster (Agrawal and Hasan, 2015). Both the PPL1 and PAM clusters project to a higher brain center, the MB (Mao and Davis, 2009; Aso et al., 2012), which is required for reinforcement of aversive and appetitive responses to olfactory stimuli (Waddell, 2013). In the natural environment, the behavioral response to such olfactory stimuli is likely to be flight. Thus regions of the MB may function as a center for integration of flight behavior with other relevant sensory inputs, such as olfaction.

TH levels appear reduced in all dopaminergic neurons with compromised SOCE. Dopaminergic interneurons in *Drosophila* are known to modulate several behaviors, including climbing (Riemensperger et al., 2013), sleep bouts (Liu et al., 2012), olfactory learning (Waddell, 2013), water reward (Lin et al., 2014), aversive memory formation (Schwaerzel et al., 2003), and amino acid sensing in larvae (Bjordal et al., 2014). The absence of climbing deficits in *TH>E180A* adults (Fig.

3D) suggests that dopaminergic flight-circuit neurons may be more sensitive to changes in dopamine signaling compared with other neural circuits. Moreover, the absence of flight deficits by either knockdown of dSTIM (Sadaf et al., 2012) or expression of E180A in serotonergic neurons (Fig. 3A) supports SOCE as an integral signaling component of maturing dopaminergic flight interneurons. Sensitivity of dopaminergic flight interneurons to E180A expression may in part be due to reduced dOrai RNA/protein in these neurons and needs further investigation. Other behavioral deficits in *TH>E180A* flies also need investigation.

In a previous study, flight deficits were obtained upon knockdown of *dOrai* in glutamatergic neurons of *Drosophila* (Venkiteswaran and Hasan, 2009). However, *orai³* mutants are not rescued by *dOrai⁺* expression in glutamatergic neurons (Fig. 1H). It is likely that both dopaminergic and glutamatergic neurons of the flight circuit require Orai function, but rescue of SOCE in glutamatergic neurons may not be sufficient to restore flight if their function is downstream of dopaminergic neurons. Simultaneous rescue from dopaminergic and glutamatergic neurons has not been attempted but should help resolve this issue. Moreover, partial rescue of flight in *orai³* mutants by *dOrai⁺* expression in dopaminergic neurons supports the existence of additional SOCE-requiring neuronal subsets for flight-circuit development and/or function.

At this stage, we cannot state conclusively whether 48 h of E180A induction in adults is sufficient to alter TH levels in dopaminergic neurons. Thus it will also be of interest to understand the role of SOCE for adult flight-circuit function over longer periods, such as days or weeks. During pupal development, upregulation of *Dmca1a/Cac1* in dopaminergic neurons with reduced SOCE (Fig. 8D) might serve as a compensatory mechanism for entry of extracellular calcium and consequent refilling of intracellular stores. Moreover, viability of SOCE-compromised neurons in pupae may depend on such a compensatory mechanism. In adults, however, *Cac1* overexpression is likely to affect membrane excitability and synaptic strength, thus altering the response from dopaminergic neurons of the flight circuit (Lee et al., 2014). Collectively, our data show SOCE-dependent transcriptional regulation of dopaminergic neurons during development with subsequent consequences on the function of an adult neural circuit. Normal function of dopaminergic neurons is essential for motor coordination in vertebrates and their malfunction can lead to neurodegenerative disorders, such as Parkinson's disease (Chan et al., 2009). Work on treatments for such conditions might benefit from a better understanding of the signaling mechanism(s) through which SOCE regulates TH transcription in development and the function of differentiated adult dopaminergic neurons.

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