2. Neuronal function of Orai in *Drosophila*

2.1 Introduction

In order to understand role of dOrai in the central nervous system of *Drosophila*, an existing mutant strain of dOrai (*olf-F^{EY09167}* also referred as *orai*³) was used (Cuttell et al., 2008). It is known that upon activation by IP₃, the Inositol 1, 4, 5 trisphosphate receptor (IP₃R) depletes the intracellular Ca²⁺ store (ER) and releases Ca²⁺ into the cytoplasm. This decrease in [Ca²⁺]ᵣₑᵣ is sensed by ER membrane resident protein dSTIM (Stromal Interaction Molecule) which in turn activates dOrai to bring Ca²⁺ into the cytoplasm from outside the cell (Feske et al., 2006; Prakriya et al., 2006; Vig et al., 2006). All three molecules are in the same signalling pathway and down regulation of any of these molecules resulted in reduced SOCE (Venkiteswaran and Hasan, 2009). It has been shown that knock down of either the IP₃R, dSTIM or dOrai in *Drosophila* neurons leads to a strong flight defect (Banerjee et al., 2004; Venkiteswaran and Hasan, 2009; Agrawal et al., 2010). The flight deficit of IP₃R mutant was suppressed by the hypermorphic alleles *orai*¹ and *orai*². Both are generated by P-element insertion in *dOrai*. Over expression of *dSTIM*⁺ or *dOrai*⁺ in neurons can also rescue flight deficit of IP₃R mutants (Venkiteswaran and Hasan, 2009). Previous work has shown that the IP₃R mutants show larval lethality and this lethality could be rescued by either Pan-neuronal or Aminergic expression of *IP₃R*⁺ and *dSTIM*⁺ (Joshi et al., 2004; Agrawal et al., 2009).

In this chapter I have characterized the lethality and flight defects of a hypomorphic allele of dOrai referred as *orai*³.
2.2 Results

2.2.1 Molecular characterization of orai\(^3\) mutation

orai\(^3\) is a hypomorphic allele of dOrai also known as olf186\(\text{-}F^{\text{EYO9167}}\) (Cuttell et al., 2008). It is a publicly available line in which a P-element is inserted in the 4\(^{\text{th}}\) intron of the dOrai gene located on the right arm of the 2\(^{\text{nd}}\) chromosome at position 1374265 (Fig 2.1A). The P-element insertion was confirmed by sequencing a PCR amplified product of the desired genomic DNA from orai\(^3\) (Fig 2.1B). To investigate the effect of the P-element insertion, levels of different transcripts of dOrai were checked. The dOrai gene encodes two major transcripts A and B which can be translated into the complete dOrai protein. It also has two other transcripts C and E. The transcript C can be translated into the N terminus of the protein and transcript E can translate into complete dOrai protein except N terminus. To check the levels of different transcripts in the central nervous system (CNS), transcript specific primers were designed and semi quantitative reverse transcription polymerase chain reaction (RT PCR) was performed using the RNA from adult heads (Fig 2.2A). The P-element insertion affected the levels of transcription of transcript A, C and E but not B in the Central Nervous System of
orai\textsuperscript{3}/orai\textsuperscript{3} organisms compared to wild type controls. Transcript A was not

![Diagram A](image1.png)

**Fig.2.2. Differential regulation of transcripts of dOrai** (A) Schematic representation of transcript A, B, C and E. The arrows represent the primer binding sites for particular transcripts. (B) The Agarose gel image with RT PCR products represents the amount of different transcripts in the adult heads of indicated genotypes. rp49 is used as a loading control. Transcript A, C and E was reduced in orai\textsuperscript{3} and rescued by pan neuronal expression of dOrai\textsuperscript{+}. The RNA was isolated from the larval brain of the given genotype. (C) Level of different transcript in larval brain, adult head and adult abdomen. Transcript A was very low in adult head and abdomen and transcript E was not affected in abdomen of orai\textsuperscript{3}.
detected in the CNS of adult orai\textsuperscript{3}/orai\textsuperscript{3} organisms. To rescue this phenotype transcript B (further referred to as \textit{dOrai}+) was used. \textit{dOrai}+ was over-expressed with a Pan-neuronal driver, \textit{Elav}\textsuperscript{C155} in orai\textsuperscript{3}/orai\textsuperscript{3} organisms. Surprisingly it rescued the level of all three transcripts A, C and E (Fig 2.2B). These results confirmed that the P-element insertion in \textit{dOrai} affects transcripts A, C and E but not B. (Fig 2.2B). Each transcript level was also determined in adult heads and abdomens of appropriate genotypes. Transcript A and C were reduced in the adult heads and abdomen of orai\textsuperscript{3} as compared to wild type organisms. Level of transcript E was reduced in the adult head but remained unaffected in the abdomen of orai\textsuperscript{3} organisms (Fig 2.2C). These results suggest complex and differential regulation of \textit{dOrai} transcripts in different tissues of \textit{Drosophila}.

**Fig.2.3.** Reduced SOCE in primary neurons of orai\textsuperscript{3} 3\textsuperscript{rd} instar larval brain (A) Images of primary neurons of CS, orai\textsuperscript{3} and \textit{Elav}\textsuperscript{C155} driving \textit{dOrai}+ in orai\textsuperscript{3} background. The neurons are loaded with the calcium reporter dye Fluo-4 in order to measure store Ca\textsuperscript{2+} release (Tg) and SOCE (CaCl\textsubscript{2}). (B) Average traces of store Ca\textsuperscript{2+} release and SOCE in primary neurons from the indicated genotypes (N\geq100). (C) The box plot represents distribution from 25-75\% of indicated genotypes. The midline represents the median and the solid box represents the mean of the distribution (N\geq100; *P\textsubscript{ANOVA}<0.05).
2.2.2 SOCE was reduced in primary neurons of orai3 larval brain

dOrai is a CRAC channel which is responsible for calcium entry in the cell on store depletion (Feske et al., 2006; Prakriya et al., 2006; Vig et al., 2006). Therefore, to test the functional effect of the P-element insertion in the dOrai gene Store Operated Calcium Entry (SOCE) was measured in cultured primary neurons from the CNS of orai3/orai3 larvae. Primary neurons from Drosophila 3rd instar larvae of the desired genotype were cultured (Chakraborty and Hasan, 2012). The neurons were loaded with the Fluo-4 AM (Ca2+ sensitive dye) and kept in 0 Ca2+ medium and then Thapsigargin (Tg) was added for store release. Thapsigargin was used to inhibit Sarco-endoplasmic reticular Ca2+-ATPase (SERCA), which prevents Ca2+ reuptake in to the ER store. Because of the difference in concentration of store Ca2+ (300-400µM) and cytosolic Ca2+ (>50-100nM; Solovyova N et al 2002) Ca2+ is released constantly in the cytosol by leak channels. Thus in the absence of SERCA function emptying of the ER store takes place by Tg treatment. After store release 2mM Ca2+ was added in the medium. On addition of Ca2+ SOCE will start and Ca2+ enters through dOrai (Feske et al., 2006; Prakriya et al., 2006; Vig et al., 2006). Thapsigargin induced store release was normal in orai3 neurons and similar to control neurons (Fig 2.3C). However, SOCE was reduced in primary neurons of orai3 larvae as compared to control (Fig 2.3). The relative change in peak fluorescence intensity (ΔF/F) after addition of Ca2+ was 2.3±0.15 in control neurons and 0.95±0.09 in orai3 neurons (Fig 2.3B). Reduced SOCE in orai3 neurons was rescued by Pan-neuronal over expression of dOrai+ using ElavC155 GAL4 (Pan-neuronal GAL4, (Lin and Goodman, 1994). The relative change in peak ΔF/F after addition of Ca2+ was found to be 2.03±0.2 (mean of peak ΔF/F) in the rescue condition (Fig 2.3C). These results show that the orai3 mutant exhibits reduced SOCE, primarily because of change in level of dOrai. This reduction in dOrai may be
transcriptional (transcript A) or translational (transcript B). Thus, orai<sup>ii</sup> is a hypomorphic allele of dOrai.

### 2.2.3 Physiological characterization of orai<sup>ii</sup>

To begin understanding the functional significance of reduced SOCE in neurons, adult viability of orai<sup>ii</sup> mutants was determined. orai<sup>ii</sup> homozygotes were found to be 80% lethal. Their lethality was at the larval stage, specifically at third instar larval stage. There was no lethality at the pupal stage. All pupae eclosed as adults (Fig. 2.4A). The larval size and

![Fig.2.4. orai<sup>ii</sup> mutant strain is significantly lethal at larval stage](image)

(A) The bar graph represents number of viable organism at the indicated time in hours after egg laying. Each bar represents number of viable organisms (out of 25 organisms) and their stage of life cycle. L2 stands for 2<sup>nd</sup> and L3 for 3<sup>rd</sup> instar larval stage. orai<sup>ii</sup> lags behind in development and starts dying at 3<sup>rd</sup> instar larval stage. Pan neuronal over expression of dOrai (Elav<sup>155</sup> > dOrai<sup>+</sup>; orai<sup>ii</sup>) rescued both larval lethality and slow development of orai<sup>ii</sup>. (B) Larval images represents size and stage at indicated time in hours after egg laying (AEL). (C) Line graph represents 3<sup>rd</sup> instar larval size at particular time point. Larvae of orai<sup>ii</sup> mutant are smaller compare to control and the reduced size was rescued by pan neuronal dOrai<sup>+</sup> expression. (D) The box plot represents the size of adult Drosophila of indicated genotype. Each diamond shaped points represents size of individual organism. The orai<sup>ii</sup> flies were significantly smaller than the control.
Development was also determined. Larval size was reduced in orai\textsuperscript{3} (4.7±0.11mm, at 176-182hr) compared to control (5.6±0.09mm, at 176-182hr) and their development was also slow (Fig 2.4C, D). Adults that eclosed also appeared smaller in size (orai\textsuperscript{3} 1.9±0.04mm) compared to control (CS 2.7±0.02mm, Fig 2.4C, 2.5A). Further to confirm that lethality is because of the mutation in dOrai gene, a complementation test was performed. The orai\textsuperscript{3} allele...
was put over a deficiency strain from which the coding region of the \textit{dOrai} gene was deleted. Two deficiency line BL9066 (\textit{Df(2R)ED3610}) and BL24362 (\textit{Df(2R)BSC338}) were used for complementation analysis. Both the lines were homozygous lethal. Heteroallelic combination of \textit{orai}^{3}/\textit{Df(2R)ED3610} was completely lethal however, \textit{orai}^{3}/\textit{Df(2R)BSC338} was partially lethal. These genetic data further suggested that lethality of \textit{orai}^{3} homozygous organisms is because of mutation in the \textit{dOrai} gene. Lethality of \textit{orai}^{3} mutants was rescued significantly (~80% adults) by \textit{dOrai}^{+} expression in neurons using \textit{Elav}^{\text{C155}}\text{GAL4} (Fig 2.4C). Pan-neuronal expression of \textit{dOrai}^{+} rescued the slow development and reduced size of larvae (5.4±0.07mm at 176-182hr) and adults (2.68±0.03mm) of \textit{orai}^{3} (Fig 2.4A, B and C). Further to investigate possible contribution of muscles in \textit{orai}^{3} lethality, \textit{dOrai}^{+} was over expressed in muscle (\textit{Dmef2GAL4}) in the \textit{orai}^{3} background. Over expression of \textit{dOrai}^{+} in muscle could not rescue the lethality or body size or slow development phenotype (Fig 2.5A and B). These results confirmed that the lethality, slow development and smaller body size of \textit{orai}^{3} organisms arises from reduced SOCE through \textit{dOrai} in neurons.

To identify the class of neurons that require SOCE function for viability and development we tested several neuronal sub-domains
and found significant rescue (~60% adults) by dOrai+ expression in the Aminergic domain (DdcGAL4; Fig 2.5B). Over expression of dOrai+ in Dopaminergic neurons (THGAL4) also rescued the lethality by ~50%. Further it rescued the slow development and reduced size of larvae and adults of orai3 (Fig 2.5 A, B). However, over expression of dOrai+ in either Glutamatergic (Ok371GAL4) or Peptidergic (c929GAL4) neurons in orai3 mutant background did not rescue either the lethality or slow development and reduced body size of orai3 (Fig 2.5B). From these results, I concluded that SOCE through dOrai is required primarily in Aminergic neurons for survival and development of Drosophila.

### 2.2.4 dOrai+ expression in neurons can rescue adult flight defect of orai3 allele

Previous results had suggested that neuronal knockdown of dOrai leads to flight deficits in adult Drosophila (Venkiteswaran and Hasan, 2009). orai3 adult flies were unable to initiate flight (Fig 2.7A, B). They also had wing posture defects with out-held wings (Fig 2.5A). Pan neuronal over expression of dOrai+ using ElavC155 GAL4 could not rescue the flight defect of orai3 adults but on using another Pan neuronal driver (nSybGAL4) the flies were able to initiate flight to a significant extent (Fig 2.7A, B). The mean flight time of nSyb30589>dOrai+;orai3 was found to be 4.9±0.9sec.
compared to the flight less phenotype of \textit{orai}^3 allele (Fig 2.7).

To identify subsets of neurons in which SOCE is required for flight \textit{dOrai}^+ was over expressed in different neuronal sub domains. Either Glutamatergic, Peptidergic or muscle over expression of \textit{dOrai}^+ was unable to rescue flight of the \textit{orai}^3 allele. However, Aminergic (\textit{DdcGAL4}) over expression of \textit{dOrai}^+ was able to rescue the flight defect of \textit{orai}^3 to a significant extent. All the flies of \textit{Ddc}>\textit{dOrai}^+;\textit{orai}^3 initiated flight. The mean flight time was 13.6±1.8sec (Fig 2.7A and B). To further narrow down the number of neurons, \textit{dOrai}^+ was over expressed in an Aminergic subset, dopaminergic neurons. Adults that eclosed from \textit{TH>\textit{dOrai}^+};\textit{orai}^3 initiated flight and the mean flight time of these organisms was 7±1.2sec (Fig 2.7). Thus, over expression of \textit{dOrai}^+ in dopaminergic neurons of the \textit{orai}^3 mutant was sufficient for flight initiation.

2.3 Discussion

Store Operated Calcium Entry (SOCE) is a major pathway for Ca$^{2+}$ entry in non-excitable cells. Its role in excitable cells such as neurons is less understood. Mutation in the \textit{dOrai} gene, which altered the channel responsible for SOCE, resulted in phenotypes at the level of the whole organism. The larval lethality at 3$^{rd}$ instar stage in \textit{orai}^3 suggested that \textit{dOrai} is required for viability of \textit{Drosophila} larvae. The reduced body size and slow development and flightless adults of \textit{orai}^3 suggest that SOCE has an important role in development of \textit{Drosophila}. Analysis of neuronal domains shows that \textit{dOrai} is required in Dopaminergic neurons for survival of 3$^{rd}$ instar larvae, its development and adult flight.

2.3.1 Differential regulation of \textit{dOrai} transcripts

The \textit{dOrai}^+ gene has two major transcripts A and B which can be translated into the complete \textit{dOrai} protein. Two other transcript C and E can be translated into the N terminus and complete protein without N terminus respectively. It was observed that transcript B was unaffected but transcript A was reduced in all
tissues of orai$^3$ mutant. Pan-neuronal expression of transcript B was able to rescue the level of transcript A, suggesting that transcript B is the major transcript present in all tissues and can compensate for other transcripts. orai$^3$ mutation also affected transcript levels of C and E. Transcript C was down in all tissues but E was down only in the brain of larvae and adults, suggesting complex regulation of these transcripts at the tissue level. However, pan neuronal over expression of transcript B rescued the levels of transcript C and E both, suggesting that transcript B increases the level of dOrai, probably leading to enhanced SOCE which in turn increases overall transcription resulting in regulation of all transcripts. It was observed that in spite of not forming a functional dOrai channel, transcripts C and E were present in all tissues. The function of these transcripts is not clearly understood. It was also observed that transcripts A and B are present in all tissues. If both forms were translated and lead to complete channels, then regulation of their expression by each other remains an open question. Transcript level of dOrai was quantified using semi-quantitative PCR because the difference in band intensity of each transcript between control and orai$^3$ allele was clearly distinguishable. qPCR would have resulted in a similar conclusion. Detailed study is required to understand the tissue specific and functional regulation of the multiple transcripts of dOrai.

2.3.2 Dopaminergic over expression of dOrai$^+$ significantly rescued the larval lethality and adult flight defect of orai$^3$ mutant

Pan-neuronal over expression of dOrai$^+$ significantly rescued larval lethality and development. It was surprising that aminergic over expression of dOrai$^+$ also rescued larval lethality of orai$^3$ to the same extent as pan-neuronal rescue. Neuronal requirement of SOCE in larval lethality was identified further. Dopaminergic rescue of orai$^3$ larval lethality was significant, suggesting that the downregulation of SOCE in dopaminergic neurons leads to larval lethality in the
orai\textsuperscript{3} mutant. Further investigation is required to identify the precise cause or causes of lethality in orai\textsuperscript{3} homozygotes.

Elav\textsuperscript{C155} driven dOrai\textsuperscript{+} expression rescued SOCE and lethality in orai\textsuperscript{3} larvae, but it could not rescue the adult flight defect. Rescue of flight required dOrai\textsuperscript{+} expression under control of nSybGAL4\textsuperscript{30589}. nSybGAL4\textsuperscript{30589} expression is observed in larvae and strong expression is observed in the adult CNS (Fig. 1.6). It is known that the flight circuit develops in the pupal stages. It possible that Elav\textsuperscript{C155}GAL4 has reduced expression compared to nSybGAL4\textsuperscript{30589} in flight circuit neurons at the pupal stage. This may be a likely cause of flight rescue of orai\textsuperscript{3} by nSybGAL4\textsuperscript{30589} but not by Elav\textsuperscript{C155}GAL4. For a complete understanding of these results, a careful analysis of the spatiotemporal expression of both GAL4 strains is required, coupled with their overlap with sub-domains of dopaminergic neurons.

2.4 Materials and Methods

2.4.1 Fly Strains Used

<table>
<thead>
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<th>Strain</th>
<th>Genotype</th>
<th>Remarks</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>Canton S</td>
<td>Used as wild type in all experiments</td>
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<tr>
<td>Mutant</td>
<td>orai\textsuperscript{3} (BL17538)</td>
<td>Hypomorphic allele of dOrai also referred to as olf-F\textsuperscript{EY09167} (Cuttell et al., 2008)</td>
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<td>Deficiency lines</td>
<td>BL9066</td>
<td>Deficiency line for dOrai (Ryder et al., 2007)</td>
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<td>Df(2R)ED3610</td>
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<td>Deficiency lines</td>
<td>BL24362</td>
<td>Deficiency line for dOrai (Christensen et al., 2007)</td>
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<td>Df(2R)BSC338</td>
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<td>UASdOrai/Tb</td>
<td>UAS line of dOrai of transcript B (Venkiteswaran and Hasan, 2009)</td>
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<tr>
<td><strong>GAL4 lines</strong></td>
<td><strong>Description</strong></td>
<td></td>
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<tr>
<td><strong>UASmGFP</strong> (BL5130)</td>
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<td>Pan-neuronal expression driver strain (Lin and Goodman, 1994)</td>
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<td>Pan-neuronal GAL4 driver strain</td>
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<td><strong>nSyb GAL4</strong> (BL51635)</td>
<td>Pan-neuronal GAL4 driver strain (Venken et al., 2011)</td>
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<td><strong>Ddc GAL4</strong></td>
<td>Aminergic expression driver strain (Lundell and Hirsh, 1994)</td>
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### 2.4.2 RNA isolation and Semi quantitative PCR

For RNA isolation, the CNS of either 3<sup>rd</sup> instar larvae of specific ages or adult flies were dissected and homogenized in TRIzol Reagent (Life Technologies, USA) and processed further according to the manufacturer’s protocol. RNA integrity was determined on a 1% TAE agarose gel. At least three independently isolated RNA samples were obtained for each genotype. Total RNA (~500ng) was treated with 0.5 units of DNase I (Amplification grade) in a reaction mixture (22.1 µl) containing 1mM DTT and 20u of RNase inhibitor. The reaction mixture was kept at 37° for 30min followed by heat inactivation at 70°
for 10min. To this, 200 units of Moloney murine leukemia virus (M-MLV) reverse transcriptase, 100ng random hexamers and 1mM dNTP were added in a final volume of 25µl for cDNA synthesis. The reaction mixture was kept at 25° for 10min, then 42° for 60min and finally heat inactivated at 70° for 10min. All reagents were from Invitrogen, Life Technologies, Carlsbad, USA. RT- PCR was performed in a reaction mixture of 25µl with 1µl of the cDNA. 2µl cDNA was use to perform PCR using standard program.

*rp49* Forward CGGATCGATATGCTAAGCTGT

*rp49* Reverse GCGCTTGTTTCGATCCGTA

Transcript A Forward GCACCACAGCAACAGTCCCA

Transcript A Reverse GCATCCAGC AAGACGTCCG

Transcript B Forward GTGTTGGTGTTATGATGCGATGG

Transcript B Reverse GTCGTGGTGAAGGTCGTGC

Transcript C Forward CGCCGATCGTACTTAATTAGCAC

Transcript C Reverse CGGTAGCGGCTTCCTTGTTCTC

Transcript E Forward GCCTGATGCTGAGCCACCA

Transcript E Reverse CTGTCAGAGCCCGTAGGC

**2.4.3 Primary neuronal culture from 3**<sup>rd</sup> **in star larval brain**

Primary cultures of dissociated *Drosophila* larval neurons were plated in 200µl of DMEM. Briefly, brain and the ventral ganglia were dissected from *Drosophila* third instar larvae of the appropriate genotypes. The brain tissue was mechanically dissociated in Schneider’s medium containing Collagenase (0.75 µg/µl, Invitrogen Technologies, USA) and Dispase (0.4 µg/µl, Roche) for 40min. The lysate was spun down at 4000rpm for 6min at room temperature, resuspended in DMEM medium and plated onto polylysine-coated coverslips which formed the base of 35 mm culture dishes. The cells were incubated at 25°C in CO<sub>2</sub> incubator for 14–16hr before imaging.
2.4.4 Calcium imaging

After 14-16hr of growth, cells were washed with M1 media (Agrawal et al., 2010) twice and loaded with 2.5µM fluo-4 acetoxyethyl ester (AM; invitrogen) plus 0.002% pluronic (Sigma p-2443) in M1 medium. The cells were incubated for 30min in the dark at room temperature. After dye-loading the cells were washed with M1 three times and kept in 100µl of calcium-free M1 containing 0.5mM EGTA. The cells were imaged within 40min of dye-loading. Briefly, store depletion was achieved by adding 10µM of thapsigargin (Invitrogen-T7458) and Store operated calcium entry was measured by addition of 2mM CaCl₂. 10µM Ionomycin was added after 40 frames or 600sec to confirm that the dye is not saturated. Images were acquired automatically every 15sec over a period of 11min (Agrawal et al., 2010).

2.4.5 Data Analysis

To measure fluorescence changes with time, images were processed using Image Pro plus software, V1.33. A region of interest (ROI) was marked along the cell boundary to measure fluorescence intensity inside the cell. Fluorescence intensity before \( F_{t0} \) and at various time points after addition of thapsigargin or CaCl₂ \( (F_t) \) were determined. The data were plotted using Origin 8.0 software as follows: \( \Delta F/F = \frac{F_t - F_{t0}}{F_{t0}} \) at every time point. The maximum value of \( \Delta F/F \) was obtained for every cell and a box plot representing the data spread of the DF/F max was plotted. The rectangular boxes represent the spread of data points between 25-75% of cells, the horizontal line is the median and the small square within the box represents the mean. Significant differences between multiple groups of data were analysed by one-way ANOVA with post-hoc tukey test.

2.4.6 Staging

Staging experiments were performed to obtain lethality profiles of orai\(^3\) allele and its rescue as described previously (Joshi et al., 2004). Timed and
synchronized egg laying was done for 8hr at 25°C. The larvae were collected after 60-66 hr after egg laying in batches of 3×25 and transferred in to agar less media and kept at 25°C. Heteroallelic and heterozygous larvae were identified using dominant markers (TM6Tb and CyoGFP). The larvae were screened at indicated time points for number of survivors and stage of development, determined by the morphology of the anterior spiracles (Ashburner, 1989). Origin 8.0 software (MicroCal, Northampton, MA) was used to plot the data as histograms representing the mean survivors of each stage at every time point with the error bars representing standard error of mean (SEM). Significance of data was calculated using Student`s t-test, P-value of <0.05 was regarded as significant.

2.4.7 Single Flight assay

Flies of appropriate genotype were tested for flight in the single flight assay. Briefly, flies were anesthetized on ice for 5-10 min and glued to a metal wire between the neck and thorax using nail polish. As soon as the flies recovered, a mouth-blown air puff was delivered to the flies and flight was monitored up to 30sec.