

# Genetic Dissection of *itpr* Gene Function Reveals a Vital Requirement in Aminergic Cells of *Drosophila* Larvae

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## ABSTRACT

Signaling by the second messenger inositol 1,4,5-trisphosphate is thought to affect several developmental and physiological processes. Mutants in the inositol 1,4,5-trisphosphate receptor (*itpr*) gene of *Drosophila* exhibit delays in molting while stronger alleles are also larval lethal. In a freshly generated set of EMS alleles for the *itpr* locus we have sequenced and identified single point mutations in seven mutant chromosomes. The predicted allelic strength of these mutants matches the observed levels of lethality. They range from weak hypomorphs to complete nulls. Interestingly, lethality in three heteroallelic combinations has a component of cold sensitivity. The temporal focus of cold sensitivity lies in the larval stages, predominantly at second instar. Coupled with our earlier observation that an *itpr* homozygous null allele dies at the second instar stage, it appears that there is a critical period for *itpr* gene function in second instar larvae. Here we show that the focus of this critical function lies in aminergic cells by rescue with *UAS-itpr* and *DdCGAL4*. However, this function does not require synaptic activity, suggesting that  $\text{InsP}_3$ -mediated  $\text{Ca}^{2+}$  release regulates the neurohormonal action of serotonin.

THE second messenger inositol 1,4,5-trisphosphate ( $\text{InsP}_3$ ) is generated in response to a variety of extracellular signals such as hormones, neurotransmitters, and growth factors (BERRIDGE 1993). Its target within the cell is the  $\text{InsP}_3$  receptor that functions as a ligand-gated calcium-release channel on the membranes of intracellular calcium stores (PATEL *et al.* 1999). The physiological role of  $\text{InsP}_3$ -mediated intracellular calcium release is only just beginning to be understood, although, given the widespread tissue expression of the  $\text{InsP}_3$  receptor, it is likely to regulate many developmental, physiological, and neuronal signaling pathways (TAYLOR *et al.* 1999). Mutants in genes for the  $\text{InsP}_3$  receptor ( $\text{InsP}_3\text{R}$ ) have been generated in mouse, *Drosophila*, and *Caenorhabditis elegans* (MATSUMOTO *et al.* 1996; ACHARYA *et al.* 1997; VENKATESH and HASAN 1997; CLANDININ *et al.* 1998). The mouse  $\text{InsP}_3\text{RI}$  knock-out strain is lethal soon after birth (MATSUMOTO *et al.* 1996). The reason for this lethality is not understood. In *Drosophila*, a single gene, referred to as *itpr*, codes for the  $\text{InsP}_3$  receptor. In previous work we have established a null mutation, consisting of a deficiency for the gene, as well as insertional mutants in regulatory regions of the gene, of which several are larval lethals (VENKATESH and HASAN

1997). The cause(s) of larval lethality has not been investigated so far.

In this study we undertook to generate a set of *itpr* alleles with single point mutations. Our aim was to obtain a set of mutant alleles, some of which might disrupt  $\text{InsP}_3$  receptor functions differentially and consequently allow the study of a wider range of  $\text{InsP}_3$  signaling phenotypes. Moreover, we were interested in investigating the physiological cause of larval lethality observed in  $\text{InsP}_3$  receptor mutants. The  $\text{InsP}_3$  receptor is a large protein of >2500 residues (PATEL *et al.* 1999) with three major domains consisting of an N-terminal ligand-binding region, a central modulatory domain, and a C-terminal calcium channel domain with six membrane-spanning regions. From sequence analyses of the ethyl methane-sulfonate (EMS) alleles obtained we have identified mutants in all three domains and compared these molecular changes with the allelic strength of each mutant. Furthermore, from studies of lethal and cold-sensitive combinations among these alleles, we show that there is a critical requirement for the  $\text{InsP}_3$  receptor, which is most likely in serotonin-containing cells of second instar larvae.

## MATERIALS AND METHODS

***Drosophila* stocks:** The existing *itpr* mutant alleles (*itpr*<sup>90B0</sup>, *itpr*<sup>B4</sup>, *itpr*<sup>XR12</sup>, and *itpr*<sup>1664</sup>) used in this study have been described earlier (VENKATESH and HASAN 1997). *itpr*<sup>90B0</sup> is a small deficiency uncovering the *itpr* locus. The *UAS-itpr* transgenic strain used for rescuing *itpr* mutant phenotypes has also been described previously (VENKATESH *et al.* 2001). A *UAS-itpr* transgene on the first chromosome was crossed into a background

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of *itpr<sup>EMS</sup>* and *armGAL4* to generate the strains *UAS-itpr; armGAL4*; *itpr<sup>EMS</sup>/TM6Tb* and *UAS-itpr; +; itpr<sup>EMS</sup>/TM6Tb*. The *armGAL4* line used for this purpose was obtained from J.-P. Vincent (SANSON *et al.* 1996). Third chromosome GAL4 lines like *neurGAL4* (JHAVERI *et al.* 2000), *prosGAL4* (from Chris Doe's lab), and *P0163GAL4* (SUSTER and BATE 2002) were recombined with the *itpr<sup>ug3</sup>* chromosome and maintained over a *TM6Tb* balancer. Second chromosome lines like *elavGAL4* (from Kalpana White's lab), *DdCGAL4* (LI *et al.* 2000), *c929* (HEWES *et al.* 2003), and *chaGAL4* (from P. Salvaterra) were crossed into the background of *itpr<sup>ug3</sup>*. All rescue crosses were set up with *itpr<sup>EMS</sup>* alleles, carrying the first chromosome *UAS-itpr* transgene. Control crosses with *itpr<sup>EMS</sup>* alleles in combination with each *GAL4* strain, and no rescue transgene, were checked for lethality in parallel in all cases. Transgenic flies homozygous for the inserts *UAS-TNT* (SWEENEY *et al.* 1995) and *UAS-hid* (ZHOU *et al.* 1997) were crossed to homozygous *GAL4* lines to obtain embryos heterozygous for *GAL4* and *UAS* inserts.

**Generation of EMS alleles and complementation analysis:** Canton-S (*CS*) males aged 2–3 days were fed 25 mM EMS for 12 hr and then mated with *w; TM3Sb/TM6Tb* virgin females. The *TM6Tb* balanced F<sub>1</sub> males were used to set up single male lines with virgin females of the genotype *itpr<sup>90B0</sup>/TM6Tb*. Mutagenized third chromosomes were screened for noncomplementation with *itpr<sup>90B0</sup>* by scoring for absence of *Tubby<sup>+</sup>* (*Tb<sup>+</sup>*) pupae. Putative EMS alleles for the *itpr* locus were mated with previously generated *itpr* alleles balanced over a *TM6Tb* chromosome. The progeny were screened for absence of *Tb<sup>+</sup>* pupae and adults. Intra-allelic complementation analyses for *itpr<sup>EMS</sup>* alleles were carried out in a similar manner at 18° and 29°. All *itpr<sup>EMS</sup>* alleles obtained have been maintained over the *TM6Tb* balancer chromosome.

**Larval staging and lethality measurements:** To obtain molting profiles of heteroallelic mutant larvae, staging experiments were carried out with minor modifications of previously published procedures (VENKATESH and HASAN 1997; VENKATESH *et al.* 2001). Heteroallelic and heterozygous larvae were identified on the basis of the *Tubby* phenotype, which is visible in larvae 60 hr after egg laying (AEL). Timed and synchronized egg collections were done for a period of 6 hr at 25°. The cultures were then allowed to grow at 17.5° or 25°, depending on the experiment. The temperature of 17.5° was chosen since larval development takes precisely double the time at this temperature compared with development at 25°. Heteroallelic mutant larvae were selected at 56–64 hr AEL in the experiments performed at 25° and at an interval of 116–124 hr AEL in experiments performed at 17.5°. Larvae were picked by selecting against the dominant marker *Tubby* and transferred into vials of cornmeal medium lacking agar. These larvae were grown at the desired temperatures of 17.5° or 25° and screened at appropriate time points for number of survivors and their stage of development. Larval stages were determined by appearance of the anterior spiracles (ASHBURNER 1989). For each time interval, a minimum of 150 larvae were screened in batches of 50 larvae each.

**Sequencing of *itpr<sup>EMS</sup>* alleles:** Genomic DNA, specific for the EMS allele to be sequenced, was isolated from second instar larvae of the genotype *itpr<sup>EMS</sup>/90B0* using standard DNA isolation protocols. *90B0* is a complete deficiency for the *itpr* locus (VENKATESH and HASAN 1997). The region of the *itpr* gene encompassing the complete open reading frame (11,509 bp) was amplified from this genomic DNA by PCR, using four sets of overlapping primers that gave four overlapping fragments. The primers were designed to facilitate cloning of the PCR-amplified fragments into the multiple cloning sites of the plasmid pBluescript (Stratagene, La Jolla, CA). Cloned fragments were sequenced using the Big Dye terminator cycle

sequencing kit on the 301 Genetic Analyser sequencer (ABI Prism). The obtained sequence was compared with the published *Drosophila* genomic sequence (ADAMS *et al.* 2000). At least three independent clones were sequenced when nucleotide changes, which affect the open reading frame, were observed. Single base changes observed consistently in an EMS allele were reconfirmed by amplification and direct sequencing of a PCR fragment from the appropriate region of genomic DNA.

**X-gal staining:** Brain and ring gland complexes of appropriately staged larvae were dissected and fixed at room temperature (RT) for 5 min in phosphate-buffered saline (PBS) containing 4% paraformaldehyde. Tissues were washed three times in PBS and incubated with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining solution (BELLEN *et al.* 1989). Staining was done for 15–20 min at RT and the stained tissues mounted in 60% glycerol.

**Immunohistochemistry:** Dissection and fixation were as described above for X-gal staining, except that fixation was for 40 min at 4° and tissues were processed according to published protocols (GOULD *et al.* 1990). Antiserotonin antibody (Sigma, St. Louis) was used at a dilution of 1/5000. Visualization was done by a standard HRP reaction carried out according to the manufacturer's specifications (Vector Labs, Burlingame, CA) in the case of nonfluorescent staining. Anti-rabbit antibody conjugated to Alexa-568 (Molecular Probes, Eugene, OR) was used for the fluorescent visualization of serotonin using a laser scanning confocal microscope (Bio-Rad MRC 1024).

## RESULTS

**Isolation of EMS-induced *itpr* alleles:** To generate alleles with point mutations in the *itpr* locus, we selected lines derived from EMS-fed wild-type males that were lethal when heterozygous with an existing *itpr* null (*itpr<sup>90B0</sup>*; VENKATESH and HASAN 1997). From a total of 12,304 mutagenized chromosomes, 14 lines that did not complement *itpr<sup>90B0</sup>*, as judged by the absence of *Tubby<sup>+</sup>* pupae, were obtained (Figure 1; for details see MATERIALS AND METHODS). These 14 lines were tested for complementation with *itpr<sup>90B0</sup>* and three existing hypomorphic *itpr* alleles, *itpr<sup>B4</sup>*, *itpr<sup>XR12</sup>*, and *itpr<sup>1664</sup>*, at 18° and 29° (Figure 1). None of the EMS-induced lines produced *Tubby<sup>+</sup>* pupae *in trans* with *itpr<sup>90B0</sup>*, *itpr<sup>B4</sup>*, or *itpr<sup>XR12</sup>*, indicating that all 14 lines are alleles of the *itpr* locus. However, 2 lines appeared to complement *itpr<sup>1664</sup>*, since a few *Tubby<sup>+</sup>* pupae were visible in test bottles of *itpr<sup>1664</sup>* mated with either *ug3* or *wc361* (Figure 1; DESHPANDE *et al.* 2000). Earlier studies have shown that *itpr<sup>1664</sup>* is a partially viable weak mutant allele of the *itpr* locus (VENKATESH and HASAN 1997).

Next, the EMS-induced alleles were tested for complementation among themselves by searching for *Tubby<sup>+</sup>* pupae in all possible homozygous and heterozygous combinations. It should be noted, however, that homozygous viable alleles would probably go undetected since the EMS concentration used during the mutagenesis is likely to result in greater than one lethal hit per haploid genome. This intra-allelic complementation analysis was carried out at 18° and 29° with the intention of identifying thermosensitive combinations. All, except seven

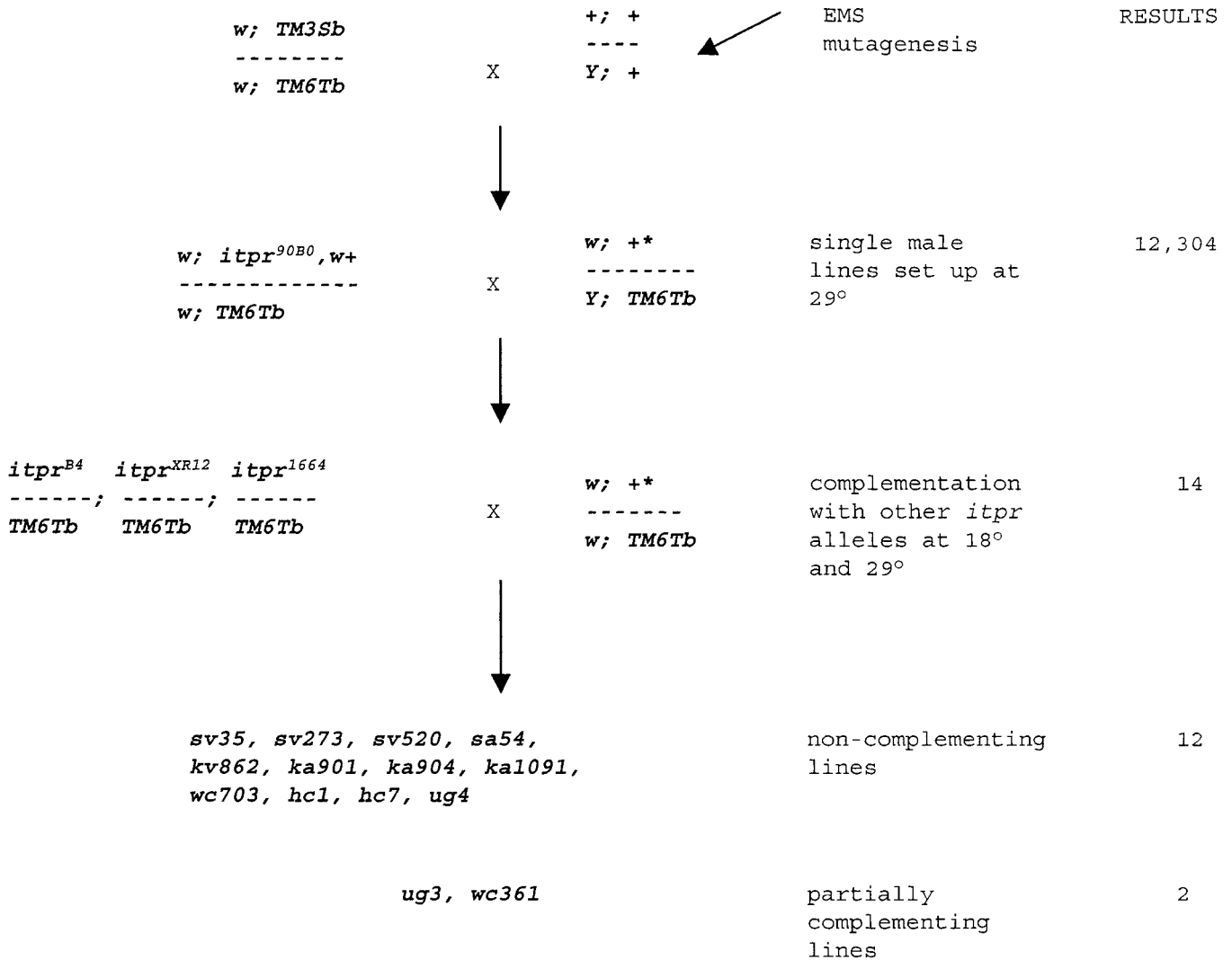


FIGURE 1.—Scheme for isolating EMS-induced lethal alleles for the *itpr* locus. Putative alleles were screened by noncomplementation of a mutagenized third chromosome with *itpr<sup>90B0</sup>*. *itpr<sup>EMS</sup>* alleles obtained at this step were further tested for noncomplementation with a previously generated class of *itpr* alleles (VENKATESH and HASAN 1997).

combinations, were lethal at both temperatures tested, supporting the idea that the majority of alleles obtained belong to the same lethal complementation group (data not shown). *Tubby<sup>+</sup>* pupae were observed in the case of three alleles, *wc703*, *sa54*, and *ka1091*, in combination with *ug3* and *wc361*, suggesting the existence of adult viable heteroallelic combinations. Adult viability in these seven combinations was confirmed by counting numbers of heteroallelic adults that eclosed at 18° and 29° (Table 1). Interestingly, heteroallelic combinations of *ug3* appear to be cold sensitive (Table 1). None of the combinations appear to be sensitive to higher temperatures. The functional form of the InsP<sub>3</sub>R is known to be a tetramer (MIGNERY *et al.* 1990; MAEDA *et al.* 1991). Presumably, adult viable combinations arise from functional compensation among mutant monomers derived from the two alleles of a heteroallelic combination.

**Allelic strength of EMS-induced alleles and their rescue by an *itpr* cDNA transgene:** Among the 14 new EMS-induced alleles obtained we were interested in identifying alleles with differing allelic strength. This was done in the context of one of the most consistent phenotypes seen in *itpr* gene mutants, which is lethality of second instar larvae at varying times AEL, with stronger alleles dying sooner (VENKATESH and HASAN 1997). Homozygotes of all the EMS alleles obtained in the EMS screen also die between 72 and 160 hr after egg laying at 25° (data not shown). From this analysis, homozygotes of two alleles, *sv35* and *ka901*, appeared to die the earliest, as late first or early second instar larvae, suggesting that they may be equivalent in phenotype to the *itpr* null allele *90B0*. However, as stated earlier, the concentrations of EMS used in our mutagenesis protocol could well lead to more than one mutation per haploid ge-

**TABLE 1**  
**Intra-allelic complementation of EMS-induced *itpr***  
**alleles at two temperatures**

Genotype	Survivors at 18° (total no. counted)	Survivors at 29° (total no. counted)
<i>ug3/ug3</i>	0 (461)	0 (520)
<i>ug3/ka1091</i>	2 (578)	164 (713)
<i>ug3/wc703</i>	1 (496)	116 (753)
<i>ug3/sa54</i>	4 (538)	24 (555)
<i>ug3/wc361</i>	154 (503)	207 (704)
<i>wc361/wc361</i>	0 (550)	0 (197)
<i>wc361/ka1091</i>	49 (588)	111 (489)
<i>wc361/wc703</i>	84 (535)	108 (597)
<i>wc361/sa54</i>	207 (704)	132 (591)
<i>ka1091/ka1091</i>	0 (430)	0 (481)
<i>wc703/wc703</i>	0 (507)	0 (571)
<i>sa54/sa54</i>	0 (540)	0 (732)

nome. Therefore, allelic strength was determined by counting the numbers of *itpr<sup>EMS</sup>/itpr<sup>90B0</sup>* larvae alive at 80–88 hr AEL (Figure 2A). The number of larvae found in *sv35/90B0* and *ka901/90B0* genotypes (<10) is comparable to the number of homozygous *90B0* larvae found at this stage (VENKATESH and HASAN 1997), confirming that both these alleles are equivalent to the null allele. The five *itpr* alleles that yield adult viable heteroallelic combinations were tested similarly and in all cases were found to have more viable larvae at this time point, indicating that they are hypomorphic alleles (Figure 2A). From this analysis the allelic strength of the *itpr* alleles tested is *sv35* ≥ *ka901* > *sa54* ≥ *ka1091* > *wc703* > *ug3* > *wc361*. Heterozygotes of all these alleles were tested for molting and lethality in parallel. Except for *sv35* all other alleles appear to be fully recessive (data not shown). Consistent with the observation that *ka901* and *sv35* behave like the null allele *90B0*, the lethality profiles of these two alleles with a weaker allele, *ug3*, are similar to each other (Figure 2, B and C) and to *ug3/90B0* (data not shown).

To confirm that lethality associated with the EMS-induced lethal complementation group described here is indeed due to mutation of the *InsP<sub>3</sub>* receptor, we looked at rescue of a specific heteroallelic combination (*ug3/ka901*) by a cDNA transgene for the *Drosophila* *InsP<sub>3</sub>R* (SINHA and HASAN 1999; VENKATESH *et al.* 2001). As shown in Figure 2C, the majority of *ug3/ka901* larvae die as second instars by 176–184 hr after egg laying. Introduction of an *itpr* gene cDNA expressed under the control of a ubiquitous promoter, however, can rescue the lethality in *ug3/ka901* larvae (Figure 2D). The rescued larvae are fully viable, exhibit no delays at any stage of larval development, and emerge as normal-looking adults. These data confirm that the lethal complementation group defined by the EMS alleles is in the *itpr* locus. Further confirmation was obtained by

sequencing of genomic DNA obtained from the relevant alleles used in this study.

#### Sequence analysis of EMS alleles for the *itpr* locus:

To ascertain the precise molecular change in *itpr<sup>EMS</sup>* alleles, the genomic region containing the complete open reading frame of the *itpr* gene from larvae of the genotype *itpr<sup>EMS</sup>/itpr<sup>90B0</sup>* was sequenced. Alleles that are either viable as heteroallelic combinations or equivalent to the null in their lethal phenotype were chosen for sequencing. Sequences obtained were compared with the sequence of the parent strain Canton-S and the published genomic sequence for this region. A C → T transition was observed in DNA from the *ug3* strain. This transition would result in a missense mutation leading to the presence of a phenylalanine at residue 224 instead of the wild-type residue serine (TCC → TTC; Figure 3A). The mutation in *ug3* lies in the N-terminal ligand-binding domain as judged by comparing the sequence with mammalian *InsP<sub>3</sub>R*s, but not in the proposed ligand-binding pocket. Interestingly, the serine residue at position 224 along with the flanking amino acids at positions 222 (T), 223 (W), 225 (K), and 226 (I), are all conserved between mammalian *InsP<sub>3</sub>R*s and the *Drosophila* protein (Figure 3B). The alleles *ka1091* and *wc703* exhibit changes in two conserved glycine residues, which lie in the modulatory domain. In *ka1091*, glycine 1891 is mutated to a serine (GGC → AGC), while in *wc703*, glycine 2117 is mutated to glutamate (GGA → GAA). As shown in Figure 3B, in both cases several flanking residues are conserved between the *Drosophila* and mammalian *InsP<sub>3</sub>R*s. The allele *itpr<sup>ka901</sup>* also has a mutation of a conserved glycine at position 2630, which is altered to give a serine (GGT → AGT). This change is located within the Ca<sup>2+</sup> channel domain of the protein, and, as in other alleles, the region containing glycine 2630 is well conserved (Figure 3B). The lethal phenotype of *ka901* is close to that of an *itpr* null, suggesting that this mutation may have a strong effect on Ca<sup>2+</sup>-release properties of the *InsP<sub>3</sub>* receptor. In the allele *sv35*, there is a stop codon at position 1572. *sv35* thus encodes a truncated protein predicted to have no Ca<sup>2+</sup>-release activity, consistent with its phenotype of a null allele. Finally, in the allele *sa54*, the mutation results in a proline-to-leucine change (CCC → CTC) at position 1510, while in *wc361* the change is from an arginine (residue no. 2814) to a stop codon (CGA → TGA). The *InsP<sub>3</sub>* receptor encoded by *wc361* would thus be smaller by just 15 residues. To begin understanding how each of these mutations affects the functional properties of the encoded protein, we are studying their Ca<sup>2+</sup>-release properties in microsomal vesicles and artificial lipid bilayers (S. SRIKANTH, unpublished results). Information from such experiments will prove useful in correlating *in vivo* phenotypes with biophysical properties of the Ca<sup>2+</sup> channel. In subsequent experiments we have used a cold-sensitive allelic combination (*ka1091/ug3*) and a



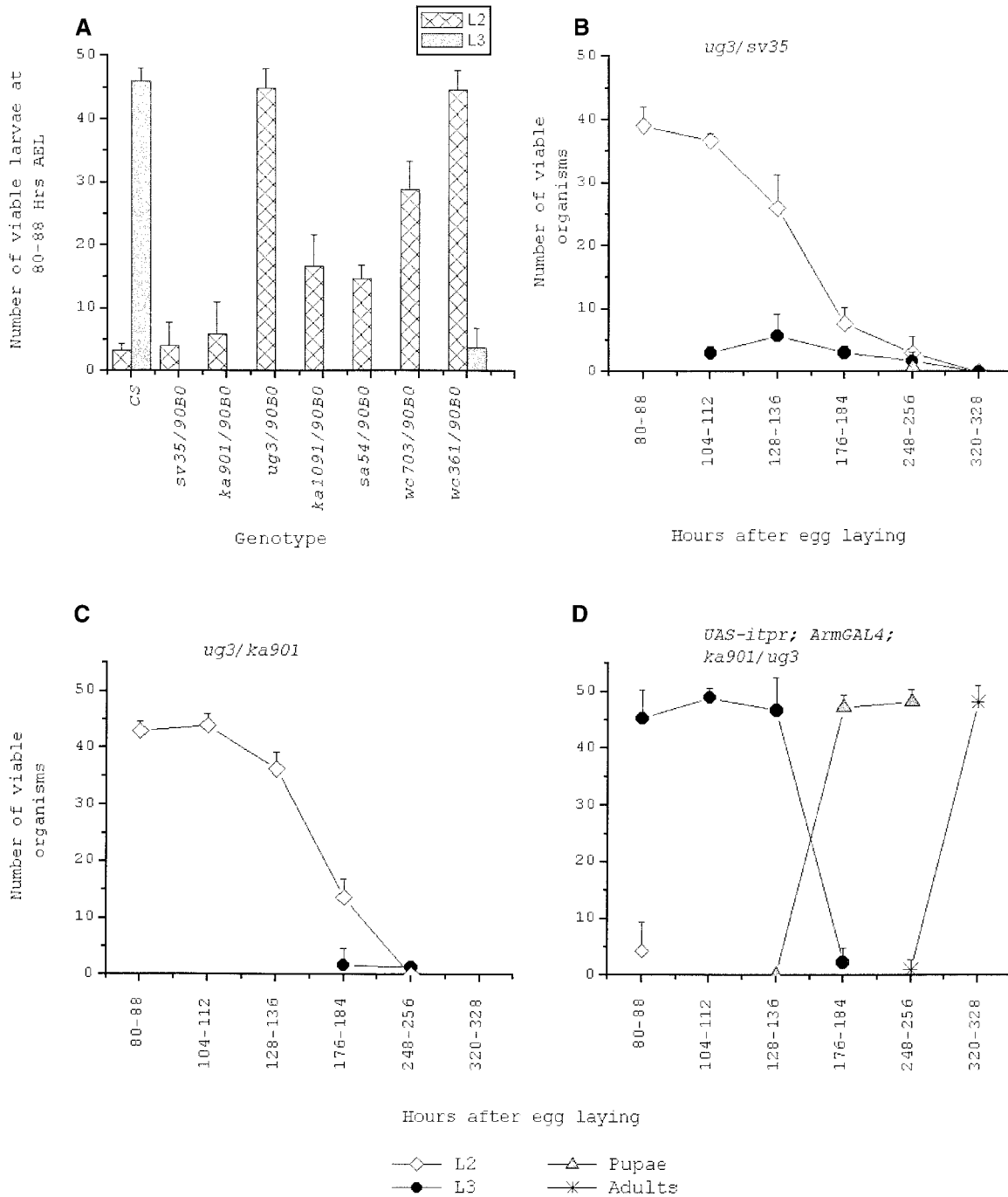


FIGURE 2.—Allelic strength and rescue of larval phenotypes in *itpr*<sup>EMS</sup> heteroallelic combinations. Heteroallelic mutant larvae of the genotype *itpr*<sup>EMS</sup>/*itpr*<sup>90B0</sup> were separated at 56–64 hr AEL and maintained at 25°. (A) Allelic strength of selected *itpr* alleles was determined by counting the number and stage of the larvae at 80–88 hr AEL. Larval staging profile is shown for (B) *itpr*<sup>ug3</sup>/*itpr*<sup>sv35</sup> and (C) *itpr*<sup>ug3</sup>/*itpr*<sup>ka901</sup> allelic combinations. (D) Larval staging profile is shown for the *itpr*<sup>ug3</sup>/*itpr*<sup>ka901</sup> heteroallelic combination in the presence of the *UAS-itpr* transgene and *armGAL4*. Lethality is rescued by the transgene. The error bars denote standard deviation.

strong allelic combination (*sv35/ug3*) for analysis of the *itpr* lethal phenotype.

**Cold sensitivity in *itpr* heteroallelic combinations and its rescue by an *itpr* transgene:** The isolation of EMS alleles that appeared to be cold sensitive suggested that these may be useful in carrying out a temporal analysis

of *itpr* gene function. We have therefore analyzed this phenotype in greater detail. To quantify the level of cold sensitivity in viable heteroallelic combinations of *ug3*, larvae of each genotype were selected and transferred to vials and maintained at either 18° or 25° as described in MATERIALS AND METHODS. Adult survivors

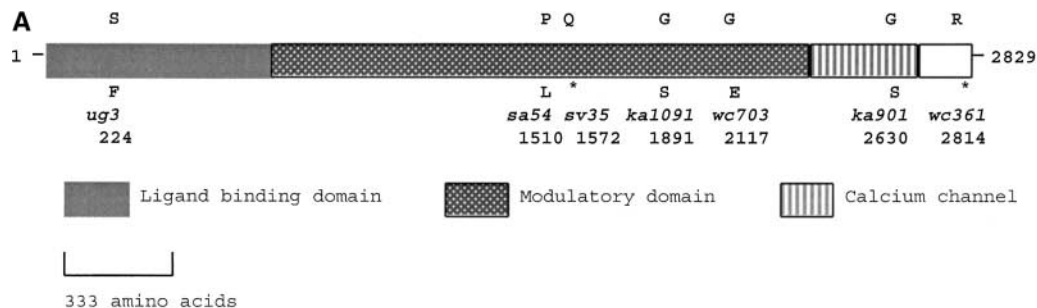


FIGURE 3.—Identification of single base-pair changes in the *itpr<sup>EMS</sup>* alleles.



from each of the vials were counted. Of the three combinations analyzed, *ug3/ka1091* exhibits maximum difference in viability between 18° (0.5%) and 25° (87%). Two other allelic combinations (*ug3/wc703* and *ug3/sa54*) also exhibit smaller but significant levels of cold lethality (data not shown). The cold-sensitive phenotype of *ug3/ka1091* organisms was analyzed further at 25° and 17.5° (Figure 4, A and B). While development appears normal with low levels of pupal lethality at 25° (Figure 4A), the opposite is seen at 17.5° (Figure 4B). In wild-type larvae, complete larval and pupal development takes approximately twice the time at 17.5° as at 25° (Figure 4C). At 17.5°, the second to third instar larval transition begins at 164–172 hr AEL in wild-type larvae. At this time point, *ug3/ka1091* larvae are all second instars. A few of these do molt into third instars at the next time point of 212–220 hr AEL, but the numbers remain small. Occasionally, a few late pupae were also observed. The majority of *ug3/ka1091* organisms die as second instar larvae at 17.5°, mirroring the lethality shown by *itpr<sup>90B0</sup>* homozygotes at 25° (VENKATESH and HASAN 1997).

Next, we asked if second instar larval lethality, observed at 17.5°, can be rescued by expression of the *UAS-itpr* cDNA transgene. Near-complete rescue of lethality in the cold-sensitive combination was obtained,

as obvious from the numbers of adults that eclosed at the 572- to 580-hr time point (see Figure 4D). The transitions from second to third instar (164–172 hr) and from third instar to pupae (356–364 hr) were rescued only partially (compare molting profile in Figure 4D with Figure 4C). Possible reasons for this partial rescue are discussed later.

**Temporal focus of cold lethality lies at the second instar larval stage:** The temporal focus of cold lethality in *ug3/ka1091* larvae was investigated by a series of temperature-shift experiments. Preliminary experiments, in which *ug3/ka1091* organisms were transferred to 18° as embryos, larvae, or pupae, established that lethality was associated with larval stages (data not shown). A detailed analysis of the period of cold lethality during larval stages is given in Table 2. *ug3/ka1091* larvae exhibit lethality when shifted from 25° to 17.5° as second instars at 48–56 hr AEL, but not when they are shifted as third instars at 80–88 hr AEL. From these data we conclude that the cold-sensitive period in *ug3/ka1091* larvae is during the second instar larval stage. Furthermore, cold lethality is irreversible, since shifting the larvae back to 25° as late second instars at 164–172 hr AEL does not improve viability (Table 2, third row). From the experiments described above, exposure to 17.5° during the second instar stage is apparently necessary for lethality

TABLE 2

Cold-sensitive period of *ug3/ka1091* larvae occurs primarily during the first and second instar stage

Hours after egg laying when shifted to 17.5°	Stage of development when shifted to 17.5°	No. of adult survivors (±SD)
48–56 to eclosion	Early second instar onward	2 (±1)
80–88 to eclosion	Early third instar onward	39 (±2)
0–8 to 164–172	Embryo to second instar only	2 (±2)
21–27 to 69–75	First instar only	31 (±5.9)
48–56 to 96–104	Second instar only	21.3 (±6.2)
No cold treatment	—	39.3 (±2.5)

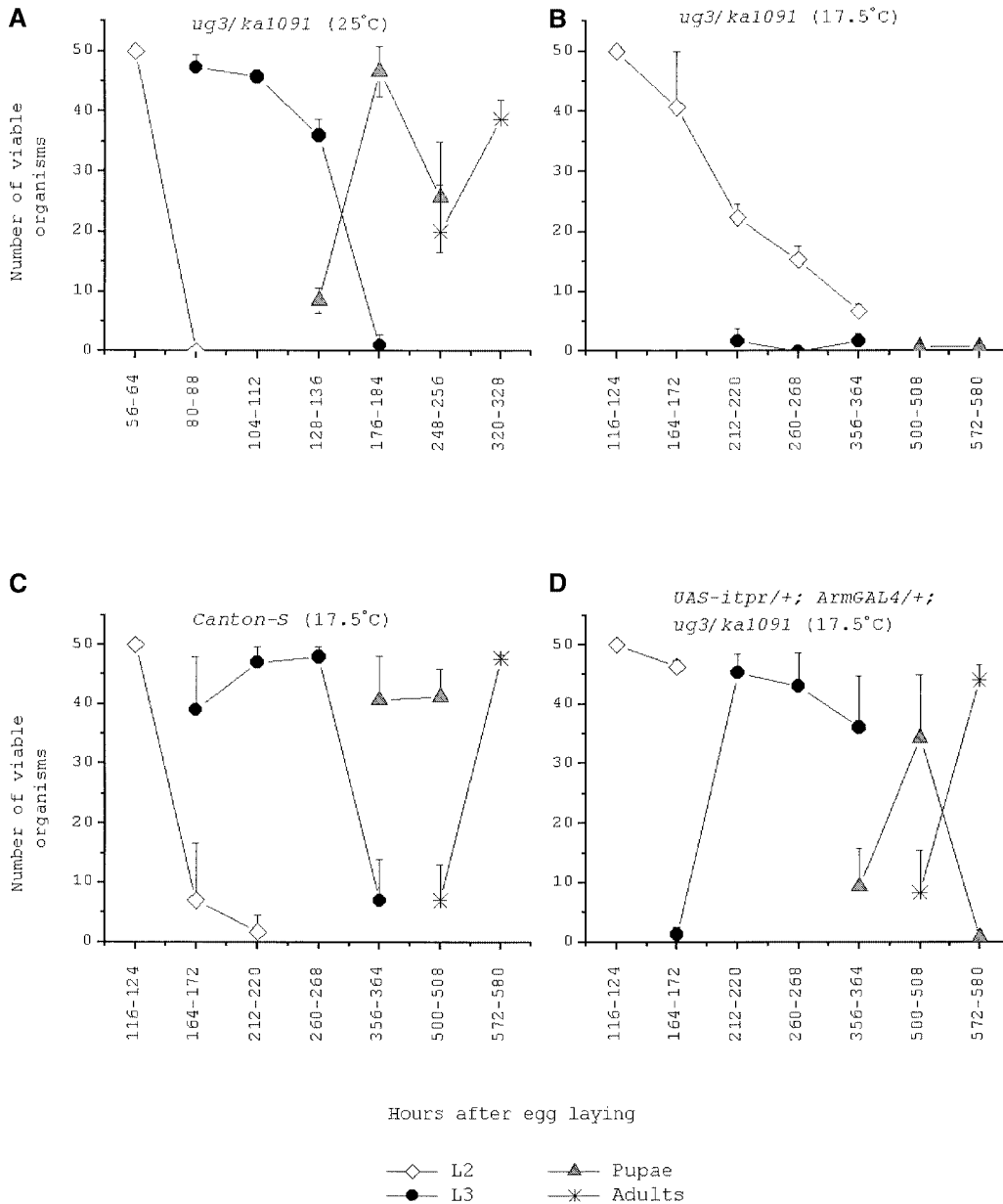


FIGURE 4.—Rescue of cold sensitivity in *itpr<sup>ug3</sup>/itpr<sup>ka1091</sup>* by *UAS-itpr* expression. (A) The staging profile of *itpr<sup>ug3</sup>/itpr<sup>ka1091</sup>* at 25° shows no molting delay and slight lethality at the pupal stage. (B) The staging profile of *itpr<sup>ug3</sup>/itpr<sup>ka1091</sup>* at 17.5° shows almost complete lethality. (C) Wild-type staging profile at 17.5°. (D) The staging profile of *itpr<sup>ug3</sup>/itpr<sup>ka1091</sup>* at 17.5° with an *UAS-itpr* transgene and *armGAL4* shows partial rescue of the molting delays and near-complete rescue of lethality.

in *ug3/ka1091* larvae. However, it is not sufficient for complete cold lethality, since transfer to 17.5° exclusively for a time period corresponding to the second instar stage from 48–56 hr to 96–104 hr AEL results in partial viability (Table 2, fifth row). Thus, while the critical step for cold lethality is in the second instar stage, it is compounded by prolonged exposure at 17.5° during either the first or third instar stages.

Interestingly, while lethality in *ug3/ka1091* organisms is cold sensitive, adult phenotypes seen in other viable heteroallelic combinations, such as the inability to fly and poor fertility, are not cold sensitive. These phenotypes are also seen in *ug3/ka1091* adults raised at 25° (S. BANERJEE and K. VENKATESH, unpublished results). Taken together with the observation that cold exposure

at times other than first or second instar larval stages had no effect on *ug3/ka1091* organisms, it is possible that cold lethality is not due to the effect of lower temperatures on the mutant *InsP<sub>3</sub>* receptor. Rather, lethality is caused by the cumulative deleterious effects of the *itpr* mutation and lower temperature on a cellular process in second instar larvae. An alternate explanation for the stage-specific cold sensitivity could be that certain mutant forms of the *InsP<sub>3</sub>*R (which is a tetrameric membrane protein), assemble slowly at lower temperatures. If *InsP<sub>3</sub>*R function in second instar larvae is required for a process with critical timing, it is conceivable that insufficient levels of functional *InsP<sub>3</sub>*R affect such a process irreversibly. For its function at later stages, the levels of the *InsP<sub>3</sub>*R may not be limiting. In either case, the

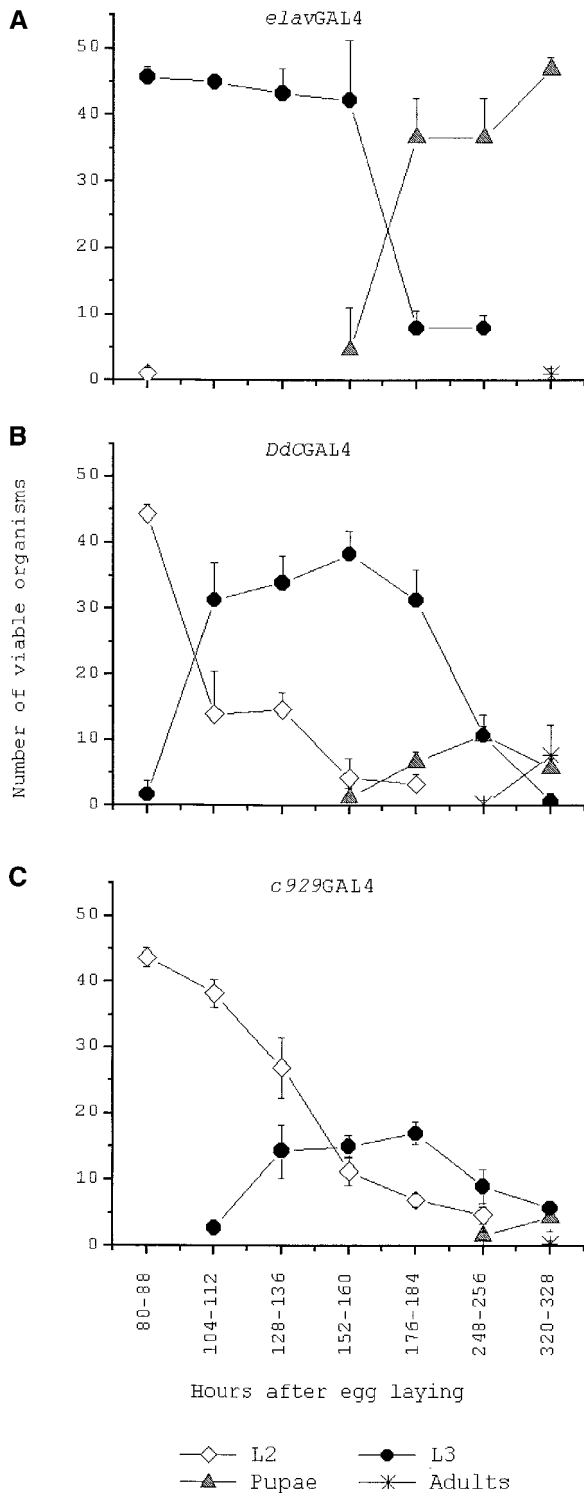


FIGURE 5.—Rescue of the larval lethality in *ug3/sv35* organisms by tissue-specific expression of *UAS-itpr*. (A) *elavGAL4* and (B) *DdCGAL4* could rescue second instar larval lethality. (C) *c929GAL4* rescues larval lethality weakly.

specific timing of cold sensitivity observed in larvae suggests a requirement for the  $\text{InsP}_3$  receptor in a critical cellular or physiological process. In subsequent experi-

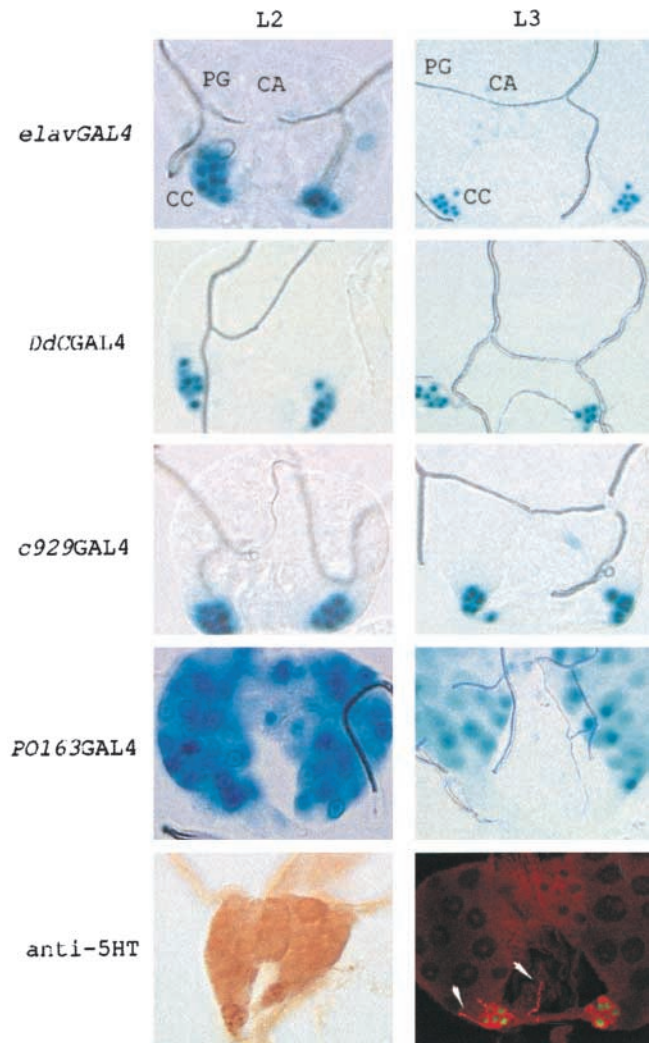


FIGURE 6.—Expression of tissue-specific GAL4 lines and serotonin staining in ring glands of second (L2) and third instar (L3) larvae. Ring glands of *elav*, *DdC*, *c929*, and *P0163* GAL4 lines were obtained from organisms of the genotype *GAL4/UAS-βgal*.  $\beta\text{gal}$  was visualized by a standard color reaction with X-gal as a substrate. The bottom panels of each column show anti-5-hydroxytryptamine (5-HT) staining of ring glands. The third instar ring gland was obtained from an organism of the genotype *DdCGAL4/UAS-GFP* and imaged on a confocal microscope for green fluorescent protein (GFP; 488 nm) and Alexa-568 (568 nm). 5-HT immunoreactivity (red) is seen in GFP-positive cells of the corpora cardiaca (green). Arrowheads show serotonin-containing processes from the corpora cardiaca extending toward the ring gland. CC, PG, and CA indicate cells of the corpora cardiaca, prothoracic gland, and corpora allata, respectively.

ments we attempted to identify this process by first establishing the tissue-specific focus of second instar lethality.

**The tissue-specific focus of second instar lethality:** To identify the tissue/cells that contribute to lethality observed in *itpr* mutants, tissue- and cell-specific GAL4 strains were used to drive expression of the *UAS-itpr* transgene in *ug3/sv35* organisms, the majority of



**TABLE 3**  
**Summary of expression pattern and rescue observed with different *GAL4* strains**

<i>GAL4</i>	Expression in L2	Rescue of lethality	
		L2	P
<i>neur</i>	Neuroblasts, CC, PG	++	++
<i>arm</i>	Ubiquitous	++	++
<i>elav</i>	CNS, PNS, CC, PG	++	±
<i>DdC</i>	Dopamine and serotonin neurons, CC	++	±
<i>pros</i>	Neuroblasts, neurons and glial cells, CC, PG	++	+
<i>c929</i>	Peptidergic neurons and CC	±	–
<i>P0163</i>	PNS, few neuroblasts, CA, PG	–	–
<i>cha</i>	Cholinergic neurons	–	–

L2, second instars; P, pupae; CC, corpora cardiaca; PG, prothoracic gland; CNS, central nervous system; PNS, peripheral nervous system; CA, corpora allata; “++,” complete rescue; “+,” moderate rescue; “±” weak rescue; “–,” no rescue.

which die as second instars (Figure 2B). Expression of the  $InsP_3$  receptor in larvae is known to be in the brain and ring gland complex, among other tissues (VENKATESH and HASAN 1997; VENKATESH *et al.* 2001). Consequently, we attempted to rescue lethality with lines that express in the central nervous system, peripheral nervous system, and ring gland. Complete rescue of second instar lethality was obtained with *elavGAL4*, including a normal transition from second to third instar larvae (Figure 5). *elavGAL4*-rescued organisms also pupate but are unable to eclose as adults. Expression of the *elav* gene is known to occur in all postmitotic neurons (ROBINOW and WHITE 1988, 1991). In addition, significant expression of *elavGAL4* is seen in cells of the ring gland, including the corpora cardiaca in second and third instar larvae (Figure 6). Next we tested several *GAL4* lines that are known to express in subsets of larval neurons and looked for their expression in cells of the ring gland (Table 3 and Figure 6). Among the *GAL4* lines, which could rescue second to third instar lethality to varying levels, were *DdCGAL4* ( $38.3 \pm 3.5$  third instar larvae at 152–160 hr AEL; Figure 5) and *c929* ( $15 \pm 1.7$  third instar larvae at 152–160 hr AEL; Figure 5). In the *ug3/sv35* strain,  $6.3 \pm 0.6$  third instar larvae are seen at 152–160 hr AEL. *GAL4* expression in these strains occurs in all dopamine and serotonin neurons (*DdCGAL4*; LI *et al.* 2000) and in all peptidergic neurons (*c929*; HEWES *et al.* 2003). Similar to *elavGAL4*, both strains also have significant *GAL4* expression in the corpora cardiaca region of second and third instar larval ring glands (Figure 6). Since the corpora cardiaca is the only tissue of overlap between *DdCGAL4* and *C929*, we attribute the modest rescue observed with *c929* to these cells. The higher level of rescue observed in *DdCGAL4*-containing organisms indicates that in addition to the corpora cardiaca, dopamine and serotonin neurons in the larval brain also require *itpr* gene function. Of these

two neurotransmitters, serotonin immunoreactivity has also been reported in the corpora cardiaca (VALLES and WHITE 1988). We confirmed this by immunostaining second and third instar ring glands with a polyclonal antiserum to serotonin (Figure 6). Taken together, these data point toward a critical requirement for the  $InsP_3$  receptor in serotonin-containing cells of second instar larvae.

**$InsP_3$  receptor function in *DdCGAL4*-expressing cells is probably independent of their synaptic function:** To obtain an understanding of the cellular function performed by the  $InsP_3$  receptor in *DdCGAL4* positive cells, we inhibited synaptic function in these cells by expression of the *UAS-tetanus toxin* transgene (*UAS-TNT*; SWEENEY *et al.* 1995). Expression of tetanus toxin in *Drosophila* neurons is known to specifically block evoked neurotransmitter release and to reduce the frequency of spontaneous quantal release (SWEENEY *et al.* 1995). *DdCGAL4*-driven expression of *UAS-TNT* did not affect larval viability or molting (Figure 7). In contrast, when TNT expression is under control of *c929* in peptidergic neurons, there is a significant loss of viability in second instar larvae (Figure 7B). Therefore, the TNT transgene used in this work is functional and capable of affecting viability, which is dependent on the neurons where it is expressed. Complete loss of *DdCGAL4*-expressing cells, however, is critical for larval viability, since expression of a cell death gene (*UAS-hid*, ZHOU *et al.* 1997) in these cells resulted in a high level of larval lethality (Figure 7C).

## DISCUSSION

**Multiple roles for the  $InsP_3$  receptor during larval and pupal development:** Cell-cell communication is crucial for the development and subsequent survival of multicellular organisms. Release of  $Ca^{2+}$  from intracellular stores, in response to extracellular signals that gen-

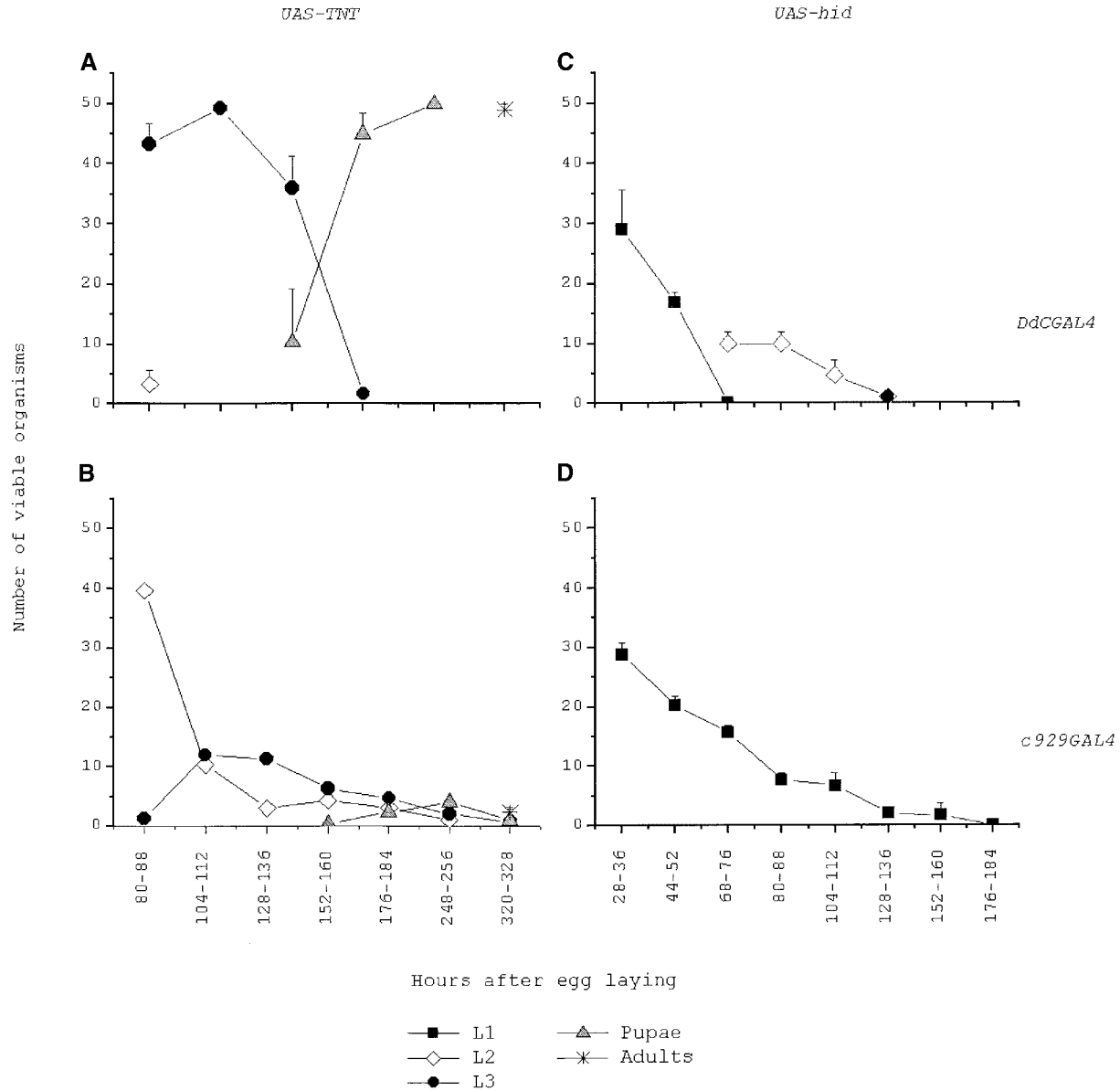


FIGURE 7.—Aminergic cells, but not their synaptic function, are critically required in larvae. Larval development proceeds normally in organisms where synaptic transmission is compromised in (A) the *DdCGAL4* domain by expression of *UAS-TNT*. However, expression of *UAS-TNT* by (B) *c929GAL4* in peptidergic neurons has lethal consequences. Ablation of (C) the *DdCGAL4* domain by expression of *UAS-hid* results in lethality, indicating that these cells are not redundant for larval viability. (D) *UAS-hid* expression by *c929GAL4* is also lethal for larvae.

erate  $\text{InsP}_3$ , appears to have evolved as one such signaling mechanism in metazoans. Our goal has been to manipulate the activity of a crucial component of this pathway, the  $\text{InsP}_3$  receptor, in a genetically tractable organism such as *Drosophila*. This has allowed us to begin understanding the critical role of  $\text{InsP}_3$ -mediated  $\text{Ca}^{2+}$  release during development. Hypomorphic mutants for the *itpr* locus described earlier were generated by either transposon hopping or X-irradiation and have consequently been fairly gross genetic defects affecting the regulatory regions of the *itpr* gene (ACHARYA *et al.* 1997; VENKATESH and HASAN 1997). To initiate an anal-

ysis of  $\text{InsP}_3$  receptor functions at different stages of development, more mutant alleles with specific lesions in the protein have now been generated. Analysis of larval lethality with these alleles described here has provided new insights into  $\text{InsP}_3$  receptor function in *Drosophila*. Our observations are consistent with the existence of a physiological process in second instar larvae, which requires a critical level of activity from the zygotically derived  $\text{InsP}_3$  receptor, the absence of which leads to lethality. This requirement occurs prior to the  $\text{InsP}_3$  receptor's role in regulating larval molting as suggested by the following observation. While feeding of 20-hydroxy-

ecdysone can rescue molting delays in a nonlethal allele (*itpr*<sup>1664</sup>; VENKATESH and HASAN 1997), it is not able to rescue lethality of any allelic combination (our unpublished data). However, the focus of both these defects in *itpr* mutants could lie in serotonin cells. This idea is supported by the fact that we and others (Figure 6; VALLES and WHITE 1988) have observed 5-HT immunoreactive fibers extending to the prothoracic gland and corpora allata. A role for serotonin in larval molting has been proposed in other insects (SHIRAI *et al.* 1995; AIZONO *et al.* 1997).

From the rescue profiles obtained with *elavGAL4* and *DdCGAL4* it is also clear that there is a pupal phase of lethality, which is rescued effectively by *UAS-itpr* expression in the domains of *neurGAL4* and *prosGAL4* (Table 3). To understand the cause of pupal lethality, the expression of these strains in pupae needs further investigation.

**Function of the InsP<sub>3</sub> receptor in serotonin and dopamine cells:** Both serotonin and dopamine are best known in their roles as neurotransmitters. However, serotonin is also known to have an essential role during gastrulation of *Drosophila* embryos when it is thought to trigger changes in cell adhesiveness by as-yet-unknown cellular mechanisms (COLAS *et al.* 1999a,b). In the context of neurons, serotonin can act as a neurotransmitter or as a neurohormone. On the basis of the results of our experiments with expression of *UAS-TNT* in *DdCGAL4* positive cells, we propose that serotonin's action as a neurohormone is critical for larval viability. The partial rescue of second instar lethality by *UAS-itpr* expression in serotonin-positive neurohemal cells of the corpora cardiaca (see Figure 5, *c929GAL4*), supports this idea. Neurohemal cells have no synaptic activity and their function is to secrete either stored or freshly synthesized neurohormones. Serotonin-positive fibers and varicosities extend from the corpora cardiaca to other regions of the ring gland (Figure 6), the aorta wall, and the surface of the gut (SIEGMUND and KORGE 2001). The effect of serotonin release from these fibers thus could be on the physiological function of any of these tissues. A direct test of this hypothesis would be to see the effect of inhibiting secretory pathways required for neurohormonal release (unrelated to neurotransmission) in serotonin cells, such as the one described recently in *Drosophila* neurons (MURTHY *et al.* 2003). However, at present we cannot rule out the possibility that a neurohormone other than serotonin is secreted by *DdCGAL4* expressing cells, which might be the critical factor in the observed lethality.

Our observations related to cold sensitivity in *ug3/ka1091* also support the idea of the InsP<sub>3</sub> receptor's role in regulating neurohormonal release from aminergic cells. Exposure to cold temperatures is known to have an inhibitory effect on secretory pathways described in CHO cells (TANUDJI *et al.* 2002) and in chromaffin cells

(BITTNER and HOLZ 1992). Moreover, certain exocytosis mutants in yeast are cold sensitive (LEHMAN *et al.* 1999).

Further investigation is required to understand the nature of extracellular signals that activate the InsP<sub>3</sub> receptor in *DdCGAL4*-positive cells. It is known that several neurosecretory neurons send their processes to the corpora cardiaca (SIEGMUND and KORGE 2001). Serotonin and dopamine cells of the larval brain and ventral ganglion are mostly interneurons and probably receive inputs from many types of neurons. A combination of cellular and genetic studies will be required to understand the nature of these signals.

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