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Multiple roles of a PLCβ regulator in olfaction, pupal development and synaptic vesicle recycling in *Drosophila melanogaster*

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Abstract

G-protein coupled Phospholipase-C-β (PLCβ) signaling and its various components are vital to the regulation of development and behaviour of *Drosophila*. The gene *stmA* (CG8739) was first characterized as a temperature sensitive paralytic mutant and recently identified as a regulator of PLCβ in G-protein signaling. It alters membrane phospholipid levels and affects visual transduction. The present study establishes the role of *stmA* in olfaction, pupal development and synaptic vesicle recycling in *Drosophila*. Interaction between *stmA* and genes for the *inositol triphosphate receptor* (*itpr*) and for endocytosis and exocytosis in synaptic vesicles is also shown.

Key words: *Drosophila*, olfaction, pupation, G-protein, itpr

Introduction

G-protein coupled Phospholipase-C-β (PLCβ) signaling and its various components are vital to the regulation of development and behaviour of *Drosophila* [1]. G-proteins transduce signals from a variety of receptors to a variety of targets. The Gq class of G-protein activates PLCβ and cleaves phosphatidyl inositol 4, 5 biphsophate ($P1P_2$) into inositol 1,4,5 triphosphate (IP_3) and diacylglycerol (DAG), which is further cleaved by DAG lipase to produce Poly Unsaturated Fatty Acids (PUFAs) [2]. Both IP_3 and DAG can function as second messengers.

The significant role of the components of G-protein signaling pathway in olfaction and pupation of *Drosophila* was reported by several researchers. Effect of mutations in PLCβ and inositol 1, 4, 5-triphosphate (IP_3) receptor on olfactory response of *Drosophila* was reported earlier [1, 3]. Recent studies showed that hypomorphic mutants of heterotrimeric G-protein, *dgq;* PLCβ ortholog *plcβ21c* and a DAG kinase, *rdgA* reduce olfactory responses [4]. Earlier, hypomorphic mutations in itpr were shown to be involved in larval growth and larval to pupal transition [5]. The chemical synapse is a highly evolved apparatus that rapidly translates excitatory electrical signals into neurotransmitter release and requires elaborate synaptic vesicle (SV) recycling at pre-synaptic boutons where protein–protein, protein–lipid interactions and lipid-modification would be expected to play a sizeable role. Geneticists have used the 'forward genetics' approach to identify genes for several of the molecular players in the SV recycling process in *Caenorhabditis elegans* and *Drosophila*. For example, *shibire*, a temperature sensitive paralytic mutation in the Dynamin family of GTPases is essential for neurotransmission SV endocytosis [6] and the *N* ethylmaleimide sensitive factor (NSF) ATPase encoding *comatose* gene in Drosophila, is essential for exocytosis. While several of the molecules and their genes are identified, forward genetics approaches of phenotype to genotype continue to reveal novel genes that are involved in these processes.

The gene *stmA*, a regulator of PLCβ, was discovered [7] while searching for temperature sensitive (ts) adult paralytic mutants. Mutants of *stmA* gene were found to alter the phospholipid level in the PIP_2-DAG pathway and affects visual transduction [8]. Being an integral part of the G protein signaling cascade and given the myriad downstream effectors of this cascade in a variety of organisms, it is expected that *stmA* will be required for various developmental and behavioural pathways. Knowledge on the interaction of this gene with other components of G-protein coupled PLCβ

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signaling is scanty. We report here, evidence for involvement of this membrane bound lipase and regulator of PLCβ- *stmA*, in multiple functions of olfaction, larval to pupal transition and in synaptic vesicle recycling in *Drosophila melanogaster*.

Materials and methods

Drosophila stocks

stambhA mutants : The following four recessive, temperature sensitive paralytic alleles of *stmA* were used *stambhA*^{1}, *stambhA*^{2} (both are recessive EMS induced mutants); *stambhA*$^{P\Delta1}$ and *stambhA*$^{P\Delta6}$ (P element excision mutants).

Inositol triphosphate receptor (itpr) mutants: *itpr*^{ka1091} and *itpr*^{ug3} (hereafter written as *ug3* and *ka1091*) homozygotes are recessive lethal, but the *ug3/ka1091* trans heterozygote is viable at 25°C, and lethal at 17°C. Double mutant flies of the genotype *stmA*^{2}/*stmA*^{2}; *ug3/ka1091* were generated through standard chromosome manipulations involving mutants balanced against the respective second and third chromosome balancers.

Synaptic Vesicle (SV) recycling mutants: Two mutants involved in endocytosis (*shibire*) and exocytosis (*comatose*) were used. Their double mutant combinations with *stmA* were also generated. *stmA-eGFP fusion transgene stock*: This transgenic stock (a gift from Kendal Broadie, Vander bilt, USA) expresses eGFP~stmA fusion under the *stmA* promoter [8]. An *eGFP* coding sequence fused with *stmA* genomic DNA by overlap extension PCR was ligated into pCaSpeR4 and the plasmid containing genomic *stmA-eGFP* DNA was injected into *w*^{1118} embryos with *p*$^{\Delta2\text{-}3}$ helper plasmid. The resultant transgenic flies show GFP reporter expression driven by the *stmA* promoter.

Odorant response evaluation : Response of flies towards ethyl acetate and benzaldehyde was studied by Park's method [9]. Pasteur pipettes (sealed at the smaller end) were stuck at opposite ends of the inner periphery of beakers (two litre) and each was filled with distilled water or odorant. Pre-starved flies were released into the beakers and sealed with cling film for 24 hours. Observations were taken on number of flies that had chosen to go into the odorant filled pipette, distilled water filled (DW) pipette or remained outside (i.e. in neither pipette). Odorant Response Index (ORI) = [(No. of flies in the odorant pipette - No. in the DW pipette)/ Total number of flies]. A standard curve for odor response in *CS* (wild type flies) was first determined at concentrations of 1, 10^{-1}, 10^{-2}, $2x10^{-3}$, 10^{-3} and $5x10^{-3}$ v/v aqueous solution of odorant. Five replications were carried out for each observation point.

Study of pupation pattern: Two hour egg collections were made from the following crosses/ stocks:

a) (♀) *stmA*^{2}, *ug3/Tm6Tb* × *stmA*^{2}, *ka1091/Tm6Tb* (♂) and its reciprocal

b) (♀) *+/+,ug3/Tm6Tb* × *+/+,ka1091/Tm6Tb* (♂) and its reciprocal

c) *stmA*^{2}

d) *CS*

Eggs of (a) and (b) were incubated separately at 17°C as well as 25°C while (c) and (d) were incubated only at 25°C. At the onset of pupation, 24 hourly counts of pupae were made till no new pupae were seen. In the case of (a) and (b), counts on the number of *Tb* and *Tb*$^{+}$ pupae were taken separately.

Calculation of survival value : For *stmA*^{2}, *ug3/Tm6Tb* × *stmA*^{2}, *ka1091/Tm6Tb* and +/+, *ug3/Tm6Tb* × *+/+, ka1091/Tm6Tb* crosses, count of total number of Tubby and non-Tubby pupae was taken. Survival of Tubby vs non-Tubby was calculated. Percentage Survival of particular genotype was calculated as, (Observed number of pupae/Expected number of pupae) × 100.

Assaying temperature-sensitive paralysis : Temperature-sensitive paralysis was assayed in a double-jacketed glass-walled container, commonly known as 'Sushi cooker' [10] in which circulating water was maintained at the desired temperature (with a precision of at least 5°C). Five to six batches of five male fly each were added and tested for paralysis at each temperature for three min. to obtain a paralysis profile. Paralysis was defined as the condition in which flies were on their backs with little or no movement of wings and legs. Double mutants and corresponding single mutants were tested at the same time. The temperature of 100% paralysis was strictly defined as that at which 100% of the flies paralyze in 3 min. This implies that at 0.5°C below the restrictive temperature several flies remain standing after the 3-min duration.

Results and discussion

Olfactory response of stmA mutant alleles : Optimal concentrations for testing olfactory response of benzaldehyde and ethyl acetate were selected after determining the standard Odorant Response Index (ORI) curve of *CS* (wild type flies) towards increasing

concentrations of the odorant. The best positive response for benzaldehyde was obtained at 0.1% with mean ORI value of 0.76 ± 0.05 from five replications. The best response for ethyl acetate was obtained at 1% concentration and the ORI value was 0.79 ± 0.07.

Olfactory response was studied for three *stmA* alleles - $stmA^{2}$, $stmA^{P\Delta1}$ and $stmA^{P\Delta6}$ towards 0.1% benzaldehyde and 1% ethyl acetate (Table 1). For 0.1% benzaldehyde, the mean ORIs of $stmA^{2}$, $stmA^{P\Delta1}$ and $stmA^{P\Delta6}$ were –0.51, +0.10 and –0.32, respectively as against 0.76 of wild type. Statistical analysis ('t' test) revealed significant differences in the ORI value for these three mutants as compared to ORI value of *CS* (P=0.01). Similarly at 1% of ethyl acetate, the mean ORI value for $stmA^{2}$, $stmA^{P\Delta1}$ and $stmAP^{\Delta6}$ were 0.44, 0.52 and 0.63, respectively as against 0.79 for wild type. $stmA^{P\Delta1}$ and $stmAP^{\Delta6}$ showed significantly reduced ORIs as compared to *CS* (P = 0.01). However, the reduction was found to be non-significant in case of $stmA^{2}$. Antennae of $stmA^{1}$ flies carrying the *stmA-eGFP* fusion construct observed under a confocal microscope displayed GFP reporter gene expression in all the olfactory neurons at the base of the antennal bristles (Fig. 2a). This showed that the *stmA* gene is expressed in the olfactory neurons. The above studies on the odor responses of various *stmA* mutant genotypes combined with the expression in the antenna revealed that the *stmA* gene is essential for processing olfactory signals.

Table 1. Odorant response of different *stmA mutants and CS*-wild type flies against two standard odorants

Genotypes	Total no. of flies analyzed	Mean odorant response index*	Standard deviation
Benzaldehyde (10^{-3} dilution)			
$stmA^{2}$	655	-0.51#	0.35
$stm^{P\Delta1}$	656	0.10#	0.56
$stm^{P\Delta6}$	658	-0.32#	0.18
CS	602	0.76	0.05
Ethyl Acetate (10^{-2} dilution)			
$stmA^{2}$	548	$0.44^{@}$	0.36
$stm^{P\Delta1}$	888	$0.52^{\#}$	0.12
$stm^{P\Delta6}$	469	$0.63^{\#}$	0.09
CS	622	0.79	0.07

*Based on values from five replications
@Significantly different from *CS* at P=0.05 for one tail t-test.
#Significantly different from *CS* at P=0.05 for both one tail and two tail t-tests.

Study of pupation pattern : Pupation in CS wild type flies (at 25°C) started at six days after egg laying (AEL) and peaked at seven days AEL (mean value of

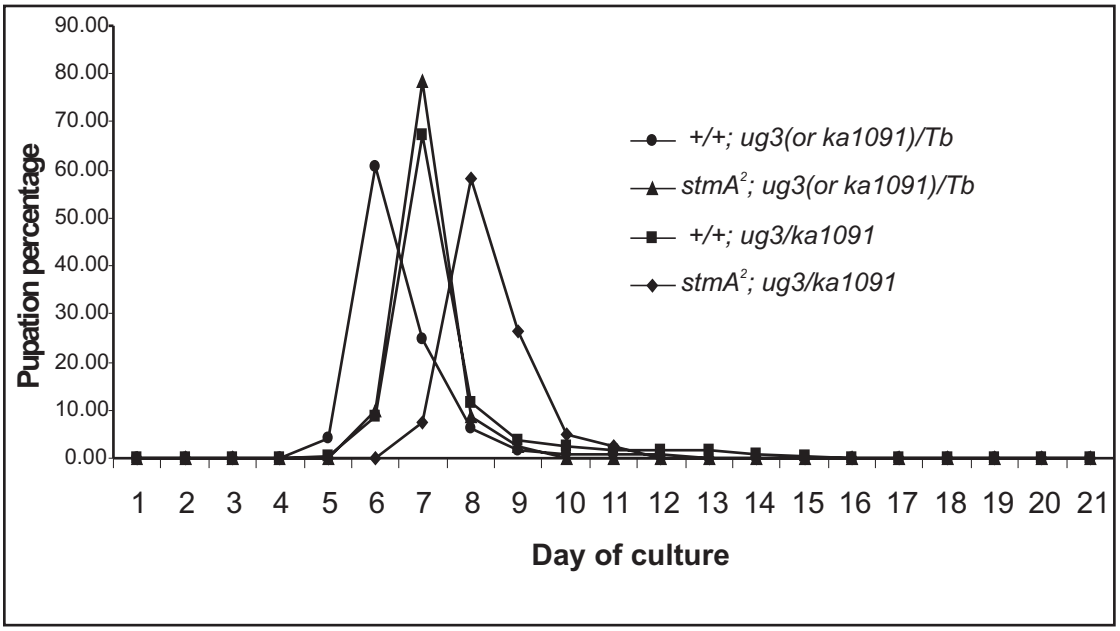

Fig. 1. ***$stmA^{2}$* and *itpr* double mutant combination show delay of one day for peak pupation as compared to *itpr heterozygote* (+/+; *ug3/ka1091*) and $stmA^{2}$ [$stmA^{2}$; (*ug3* or *ka1091*)/*Tb*]. But this delay is by two days as compared to wild type [(*ug3* or *ka1091*)/*Tb*].**

40.5 % from the total number of pupae developed, averaged over three replications). In *stmA*^{2} pupation started at six days AEL, but peaked 10 days AEL (26.25%, mean over three replications). Thus at a temperature of 25^{0}C peak pupation was delayed by three days in *stmA*^{2} as compared to CS.

Pupation pattern in viable *itpr* heterozygotes *ug3/ka1091* was determined from *ug3/TM6Tb* × *ka1091 / TM6Tb* mating using the dominant *Tubby* (*Tb*) marker on the T*M6Tb* chromosome to distinguish between heterozygous and homozygous individuals. Tubby [*ug3* (or *ka1091)/Tb*] and non-tubby [*ug3 /ka1091*] pupae were expected to develop in a 2:1 ratio. Both genotypes entered pupal development at five days AEL, but at this stage, *Tb* pupae were obtained at higher frequency than the expected (>2/3rd of the total pupae population) as compared to the *Tb+* type. This indicates that the rate of pupae development in *itpr/TM6Tb* is faster than *ug3/ka1091.* The development of tubby pupae peaked at six days AEL (60.56%, mean value over six replications) while pupation in non-tubby types peaked at seven days AEL (67.25%; mean over six replications). No significant difference was observed between the direct and reciprocal crosses. Thus *ug3/ka1091* showed a delay of one day in peak pupation as compared to its corresponding heterozygote but was not different from CS.

Pupation in double mutants (*stmA*^{2}; *ug3/ka1091*) was studied in *stmA*^{2}*, ug3*/*TM6Tb* × *stmA*^{2}*, ka1091*/ *TM6Tb* and its reciprocal cross. In this cross, tubby [*stmA*^{2}; (*ug3* or *ka1091*)*/Tb*] and non-tubby [*stmA*^{2}; *ug3 / ka1091*] pupae are expected. Pupation in *Tb* types started at six days AEL and peaked at seven days AEL (78.45%, mean over six replications), whereas in *Tb*$^+$ types, pupation started one day late at seven days AEL and peaked at eight days AEL (58.24%, mean over six replications). Reciprocal crosses did not show any significant difference. This demonstrated that peak pupation is delayed by one day in the double mutant *stmA*^{2}; *ug3/ka1091* as compared to individual mutants *stmA*^{2} and *ug3/ka1091* (Fig. 1).

Results obtained from 't'-test indicate that the number of pupae in single mutant of *stmA* [*stmA*^{2}*, ug3* (*or ka1091*)*/ TM6Tb*] was significantly higher than double mutants of *stmA* and *itpr* (*stmA*^{2}; *ug3/ka1091*) at P=0.01 on seven days AEL. However, at eight days AEL, the double mutants revealed significantly greater number of pupae as compared to single mutant of *stmA* (P=0.01).

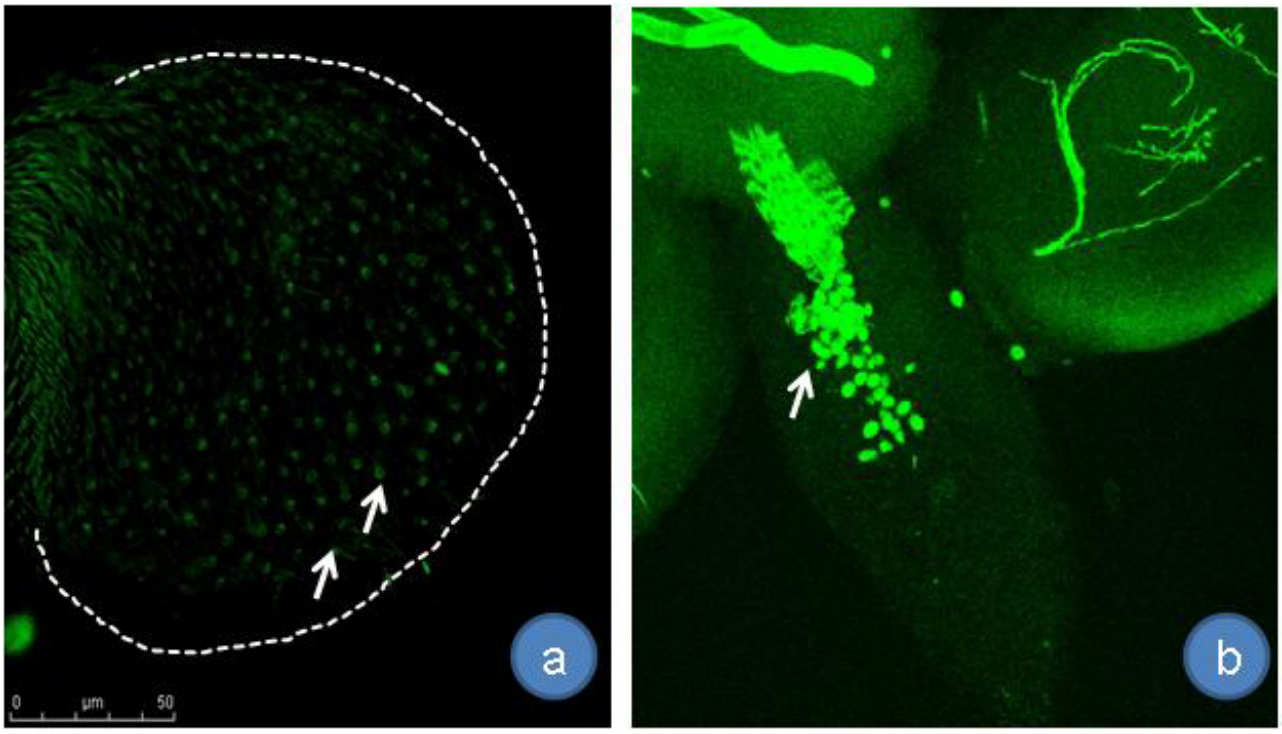

Fig. 2. Expression of GFP reporter from a *stmA*$^+$-eGFP fusion transgene in *Drosophila.* a. expression in neurons (arrows mark neurons at the base of sensory hairs) of adult antenna (white dashed outline) and b. Ring gland cells (arrow) of larval brain.

In summary, the series of experiments carried out on pupation behaviour show that both *stmA* and *itpr* mutants displayed delayed pupation as compared to CS wild type flies. Pupation was further delayed in the *stmA and itpr* double mutant. The larval ring gland plays a key role in transition from larvae to pupae stage, which is mediated by ecdysone release. We examined the ring gland of 3rd instar *stmA-eGFP* larvae and found that *stmA* was expressed strongly in the ring gland (Fig. 2b).

Analysis of lethality in stmA-itpr mutant combination : In the crosses *ug3/TM6Tb* × *ka1091/ TM6Tb* and $stmA^2$; *ug3 / TM6Tb* × $stmA^2$; *ka1019 / TM 6Tb*, tubby and non-tubby pupae are expected to segregate in 2:1 ratio. Lethality due to interaction of $stmA^2$ and *itpr* mutations can be predicted if they do not show the expected segregation ratio of 2:1 (tubby: non-tubby) in case of $stmA^2$; *ug3/TM6Tb* × $stmA^2$; *ka1091/ TM 6Tb* and its reciprocal crosses) but shows normal expected segregation ratio of 2:1 in the *ug3 / TM6 Tb* × *ka1091/ TM6Tb* and its reciprocal cross. Possible segregation distortion was confirmed by performing chi-square test for goodness of fit. Total number of pupae of two category (Tubby and non-tubby) over 6 replications showed chi-square value at 1 degree of freedom which is greater than the tabulated value, at 5% level of significance. So, the alternative hypothesis (i.e. presence of segregation distortion) was accepted. The (*ug3/TM6Tb* x *ka1091 / TM6Tb*) showed normal expected segregation ratio of 2:1 for Tubby and non-Tubby pupae.

Calculation of survival value (at 25°C) showed that the $stmA^2$; *ug3 (or ka1091) /TM6Tb* genotype has the mean survival value of 112.50, whereas $stmA^2$, *ug3/ ka1091* recorded a survival value of 75.00. Mean survival value of *ug3* (or *ka1091*)/*Tb* genotype was calculated as 99.30 whereas for *ug3/ka1091* it was 101.30 (survival value of 100 for two genotypes depicts equal survival ability of both under the defined conditions). Therefore, the interaction of $stmA^2$ with *ug3/ ka1091* heteroallelic combination leads to partial lethality (or reducing the fitness or the genotype) to the extent of 25% and this lethality occurs before the pupation stage is reached (possibly at the embryonic or larval stage).

Interaction of stmA with shi (Dynamin GTPase) and com (NSF ATPase) : To study the possible mechanisms that underlie the requirement of *stmA* in synaptic transmission we tested the paralysis profiles of *stmA* mutant alleles in combination with mutations in genes necessary for synaptic vescicle (SV) cycling. Homozygous flies of $stmA^2$ and $stmA^1$ begin to paralyse at 37°C and show 100% paralysis (by our definition of 100% paralysis within 3 minutes) at 38° and 38.5°C respectively (Figs. 3&4). Hundred percent paralysis temperatures for com^{tp7} was 35°C (Fig. 3) while for shi^{ts1} and shi^{ts4} it was 26.5°C and 29°C, respectively (Fig. 4). The double mutants were tested for paralysis from temperatures ranging from 30.5°C to 38°C for com^{tp7}; *stmA* double mutants (Fig. 3) and from 25°C to 38°C (at 0.5°C intervals) for *shi; stmA* double mutants (Fig. 4). Earlier it was shown that *stmA* does not interact with *shi* and *com* [11]. Their studies were carried out at only one temperature of 33°C and was chosen by them because $stmA^1$ flies are very slow to paralyse at 33°C (taking close to 30-40 minutes) and therefore was considered to be a sensitized condition to study any synergism. Our studies have been more exhaustive by examining paralysis over wide ranges of temperatures that embrace the paralysis temperatures of the individual single mutants and all temperatures points in between. Our studies have confirmed that there was no interaction between $stmA^1$ and *com* detectable at 33°C (Fig 3), thus confirming the earlier findings [11]. At 33.5°C 20% stmA; *com* double mutants were paralyzed while the *stmA* flies were still standing. Between 35.5 and 37°C 100% of the double mutants were paralysed while 100% of the single mutant flies remained standing.

Flies of *shi* genotype shows 100% paralysis at 26.5°C at which temperature ~80% of the double mutant flies of shi^{ts1}; $stmA^1$ and shi^{ts1}; $stmA^2$ were standing. The double mutants paralysed faster than the respective *stmA* allele but slower than the respective *shi* single mutants. The interaction of *stmA* with *com* (NSF ATPase, required for SV exocytosis) and *shi* (Dynamin GTPase, required for SV endocytosis) reveal that *stmA* is needed both for synaptic vescicle exocytosis as well as endocytosis. This is expected if *stmA* alters the membrane lipid composition.

The phenotypic effect of *stmA* gene was already reported for various biological processes like temperature dependent paralysis, photo transduction, neurotransmission, resistance to veratridine etc. [7, 8]. Reduced odorant response in *stmA* mutants was first observed in the year 2006 [12]. Reduced magnitude of electroantennogram signals in *stmA* homozygotes as compared to its heterozygotes with wild type flies was also reported [4]. In the present study, reduced odorant response index in *stmA* mutants coupled with expression analysis of the gene in the antennae confirmed the role of *stmA* in olfactory responses of *Drosophila melanogaster*. The possible interaction of

stmA gene with downstream genes (*itpr* mutants) of G-protein coupled *PLCβ* signaling was confirmed through study of pupation timing, expression analysis of *stmA* in the ring glands and partial lethality of double mutants. The gene works together with IP_3 and cause delayed peak pupation and partial lethality. This possibly happens due to its effects on ecdysone induced genes leading to developmental abnormalities.

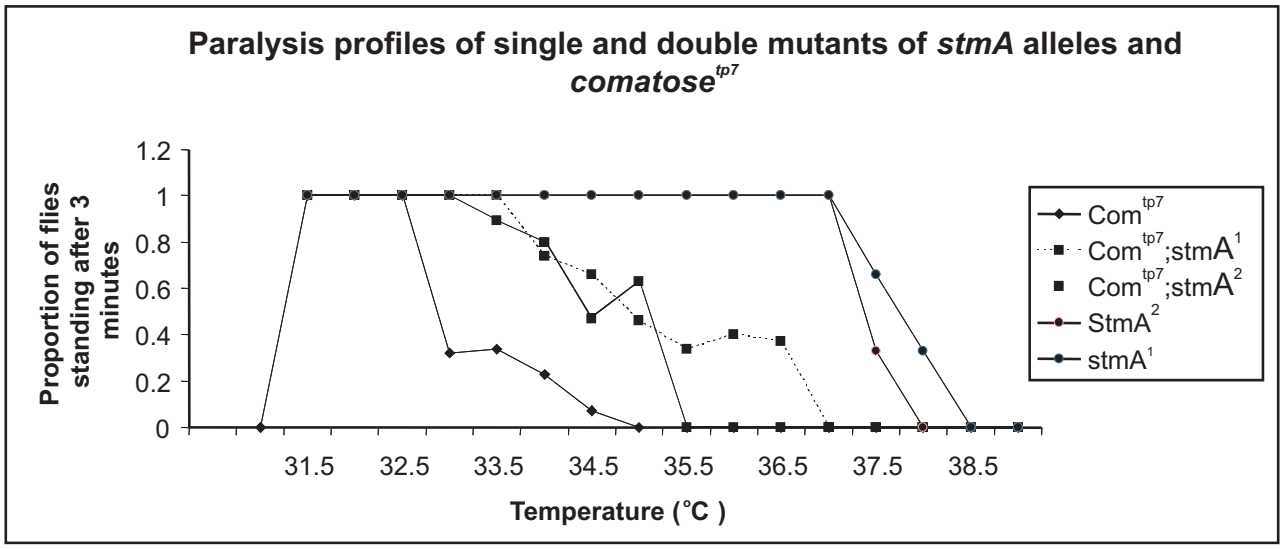

Fig. 3. Synergistic interaction between *stambhA* and *comatose*. Paralysis profile of single and double mutant genotypes of *stmA* and *comatose*.

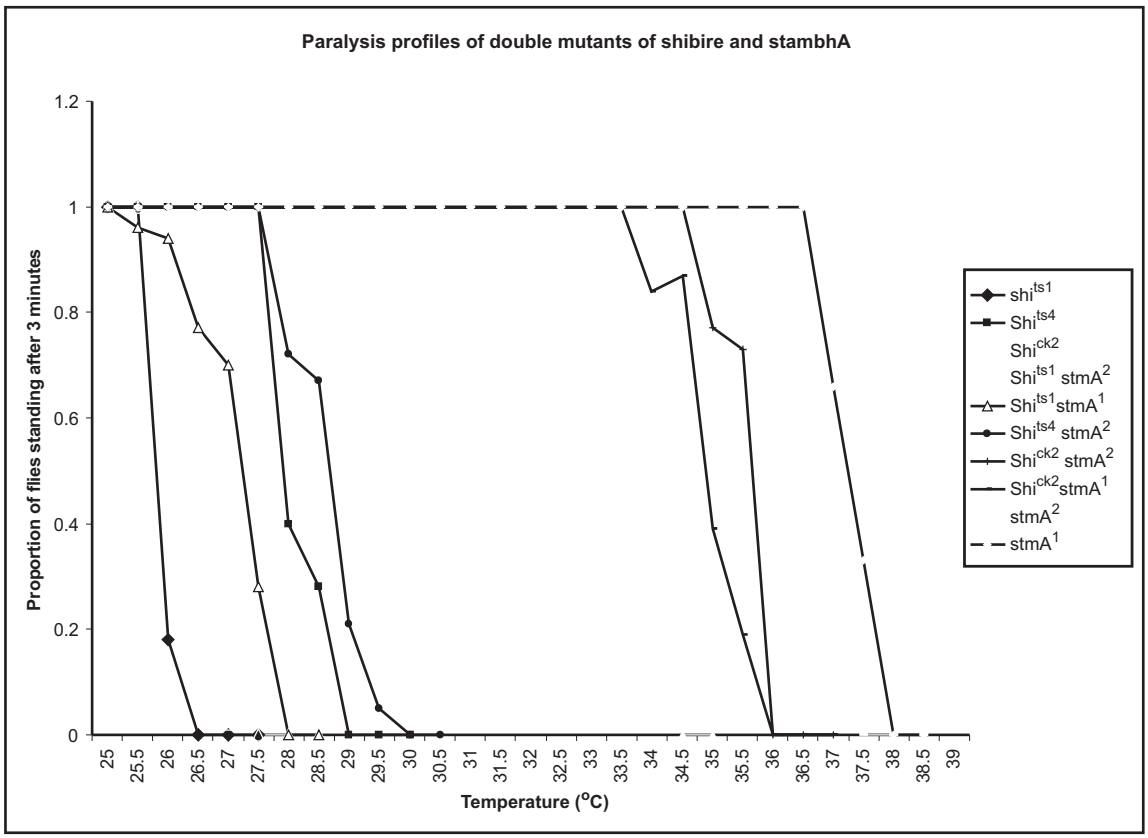

Fig. 4. Synergistic interaction between *stambhA* and *shibire*. Paralysis profiles of single and double mutants of *stmA* and *shi*.

The *stmA* gene also interacts with the genes involved in exocytosis (comatose) and endocytosis (shibire) leading to basic membrane alteration. It has been shown that *stmA* has a role in regulating neurotransmission strength through Synaptic Vesicle (SV) exocytosis and is expressed in synaptic boutons at neuromuscular junction [11, 13]. *shibire*^{ts1} and *shi*^{ts4} are mutations in a Dynamin GTPase that is essential for SV endocytosis in *Drosophila*. *comatose*^{tp7} is a mutant allele of the NSF ATPase that is necessary for SV exocytosis. Both *shi* and *com* flies show temperature sensitive neurotransmission blocks (as a result of a block in SV cycling) and this is manifested as paralysis at the "restrictive temperature" with recovery from the SV cycling block and consequent recovery from paralysis at a "permissive temperature" that is usually 23-24^{o}C. So *stmA* possibly affects both exocytosis and endocytosis in synaptic vesicle recycling.

Thus from three different sets of experiments, we have shown that a membrane bound PIP_2-DAG lipase is required in multiple processes of olfaction, in larvae to pupae transition and in synaptic vesicle recycling in *Drosophila melanogaster*.

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