

Published in final edited form as:

Eur J Neurosci. 2013 May ; 37(9): 1383–1393. doi:10.1111/ejn.12144.

Suppression of inhibitory GABAergic transmission by cAMP signaling pathway: alterations in learning and memory mutants

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Abstract

The cAMP signaling pathway mediates synaptic plasticity and is essential for memory formation in both vertebrate and invertebrates. In the fruit fly *Drosophila melanogaster*, mutations in the cAMP pathway lead to impaired olfactory learning. These mutant genes are preferentially expressed in the mushroom body (MB), an anatomical structure essential for learning. While cAMP-mediated synaptic plasticity is known to be involved in facilitation at the excitatory synapses, little is known about its function in GABAergic synaptic plasticity and learning. In this study, using whole-cell patch clamp technique on *Drosophila* primary neuronal cultures, we demonstrate that focal application of an adenylate cyclase activator forskolin (FSK) suppresses inhibitory GABAergic postsynaptic currents (IPSCs). We observed a dual regulatory role of FSK on GABAergic transmission, where it increases overall excitability at GABAergic synapses, while simultaneously acting on postsynaptic GABA receptors to suppress GABAergic IPSCs. Further we show that cAMP decreases GABAergic IPSCs in a PKA-dependent manner through a postsynaptic mechanism. PKA acts through the modulation of ionotropic GABA receptor sensitivity to the neurotransmitter GABA. This regulation of GABAergic IPSCs is altered in the cAMP pathway and short-term memory mutants *dunce* and *rutabaga*, with both showing altered GABA receptor sensitivity. Interestingly, this effect is also conserved in the MB neurons of both these mutants. Thus, our study suggests that alterations of cAMP-mediated GABAergic plasticity, particularly in the MB neurons of cAMP mutants, account for their defects in olfactory learning.

Keywords

cAMP-PKA signaling pathway; GABAergic IPSCs; fruit fly; forskolin; learning and memory

INTRODUCTION

Cyclic AMP signaling is the most well known second messenger system mediating synaptic plasticity, learning and memory in both vertebrates and invertebrates (Milner *et al.*, 1998; Kandel, 2001). In *Drosophila*, a number of short-term memory genes are essential molecular components of the cAMP signaling cascade. For example, *dunce* encodes a phosphodiesterase which breaks down cytoplasmic cAMP (Dudai *et al.*, 1976) and *rutabaga* codes for the cAMP synthesizing enzyme adenylate cyclase (Livingstone *et al.*, 1984). These genes are expressed preferentially in the mushroom body (MB), a brain region involved in olfactory learning (Keene & Waddell, 2007). cAMP signaling has been implicated in regulating synaptic plasticity at glutamatergic neuromuscular junctions and at excitatory cholinergic synapses in *Drosophila*. Interestingly, the memory mutants show alterations in

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facilitation and/or post-tetanic potentiation (Zhong & Wu, 1991; Lee & O'Dowd, 2000; Rohrbough & Broadie, 2002). These findings demonstrate that cAMP signaling regulates plasticity at excitatory glutamate and cholinergic synapses and thus mediates learning and memory in *Drosophila*.

γ -aminobutyric acid (GABA), a major inhibitory neurotransmitter (Bormann, 1988), plays an important role in neuronal communication (Paulsen & Moser, 1998). In mammals, GABAergic neurons innervate the memory center hippocampus (Freund & Buzsaki, 1996) and GABA is known to be critical for higher brain functions such as learning/memory and coordinated behaviors (Floyer-Lea *et al.*, 2006; Fernandez *et al.*, 2007). Indeed, inhibitory synaptic plasticity was observed at GABAergic synapses in rodent hippocampus CA1 neurons (Lu *et al.*, 2000; Chevaleyre & Castillo, 2003). In contrast to the excitatory synaptic plasticity (e.g. LTP), inhibitory GABAergic plasticity is relatively unexplored in the hippocampus. In particular, few studies explore inhibitory plasticity mediated by the cAMP-PKA pathway largely due to the complexity of hippocampal GABAergic circuits (Castillo *et al.*, 2011) and GABA_A receptor subunit composition (Nusser *et al.*, 1999).

Similar to the hippocampus, the *Drosophila* mushroom body (MB) is extensively innervated by inhibitory GABAergic neurons (Yasuyama *et al.*, 2002). The GABAergic neurons projecting on to the MB have also been implicated in olfactory learning (Liu & Davis, 2009). Further, it has been demonstrated that a *Drosophila* GABA receptor RDL (resistance to dieldrin) inhibits olfactory associative learning (Liu *et al.*, 2007). RDL containing GABA receptors have been shown to regulate GABAergic synaptic currents at *Drosophila* central synapses (Lee *et al.*, 2003; Su & O'Dowd, 2003). All these studies indicate an important role of GABAergic transmission in learning and memory. However, it remains unclear whether and how cAMP-dependent plasticity at inhibitory GABAergic synapses mediates learning and memory.

In the present study, we use whole-cell patch-clamp to examine physiological mechanisms underlying regulation of cAMP-mediated GABAergic plasticity in *Drosophila* primary neuronal cultures. Our results show that inhibitory GABAergic transmission is suppressed by the cAMP-PKA signaling pathway. We also examine alterations of GABAergic plasticity in the MB neurons of cAMP mutants *dunce* and *rutabaga*, and demonstrate that cAMP alters the sensitivity of ionotropic GABA receptors to modulate GABAergic transmission in the MB neurons of both learning mutants.

METHODS

Fly strains

The w¹¹¹⁸ (a “Cantonized” white eye stock) and Canton-S flies were used as wild-type flies in all experiments. *dunce* (*dnc1*), *rutabaga* (*rut1*), UAS-GFP(T10) and *Rdl1* null mutant (*Rdl1*/TM6B, ubi-GFP) strains were obtained from Bloomington stock center and maintained on standard fly food medium.

Drosophila embryonic neuronal cultures

Neuronal cultures were prepared from midgastrula stage embryos and grown in the culture medium (DDM1) as previously described (Lee & O'Dowd, 1999; Park & Lee, 2006). Cultures were maintained in an incubator supplied with 5% CO₂ at 24–25°C for 3–6 days *in vitro*.

***Drosophila* pupal neuronal cultures**

The pupal culture protocol was adapted from the one described earlier (Su & O'Dowd, 2003). The dissecting saline solution was composed of (in mM): 126 NaCl, 5.4 KCl, 0.17 NaH₂PO₄, 0.22 KH₂PO₄, 33.3 glucose, 43.8 sucrose, and 9.9 HEPES, pH 7.4. The enzyme solution in which the brains were incubated consisted of 5 U/ml papain, L-cysteine 5.5mM, EDTA (1.1mM) and 2-mercaptoethanol (0.067mM) in dissecting saline solution. Glass coverslips used to plate the neurons were coated with 0.01% of Poly-L-Lysine. 3–6 days *in vitro* pupal cultures were used for all electrophysiology experiments.

Pharmacology

Forskolin (Sigma), an activator of adenylate cyclase, the cAMP analog db-cAMP (Calbiochem) and GABA (Sigma) were focally applied to the patched neurons using Picospritzer III (Parker Hannifin Corp.). H-89 (Sigma) used to inhibit PKA was added to the external recording solution 10–15 min before recording. The membrane impermeable PKA blocker PKI (6–22) amide (Biomol) was added to the internal recording solution contained in the recording pipette. For perfusion experiments, forskolin was freshly made and added to the recording chamber by a perfusion system with vacuum/solution flow control valves (FR50, Warner Instruments Inc.) at a speed of 1 mL/min.

Electrophysiology

Each coverslip containing *Drosophila* neuronal cultures was transferred to a recording chamber with the external solution (mM): 140 NaCl, 1 CaCl₂, 4 MgCl₂, 3 KCl, and 5 HEPES, pH 7.2. Postsynaptic currents (PSCs) were recorded with whole-cell pipettes (tip resistance 4–6 M Ω for embryonic and 8–10 M Ω for pupal neuronal cultures) filled with internal solutions consisting of (mM): 120 CsOH, 120 D-gluconic acid, 0.1 CaCl₂, 2 MgCl₂, 20 NaCl, 1.1 EGTA, and 10 HEPES, pH 7.2. ATP (4 mM) was added to the internal solution to prevent rundown of the currents. For recording GABAergic IPSCs the voltage was held at 0 mV to prevent interference by cholinergic EPSCs (Lee *et al.*, 2003). GABA-evoked currents were recorded in the whole-cell configuration, at a holding potential of 0 mV, in response to puffer application of GABA and forskolin. Axopatch 200B amplifier (Axon Instruments Inc.) was used to measure PSCs. Action potentials (APs) were recorded in the cell-attached mode, and were hence extracellular.

Data Analysis

Individual PSCs were analyzed using the Minianalysis detection software (Synaptosoft) with threshold criteria for individual events at 5pA and 5pF for GABAergic PSCs (two-fold greater than the 2.5 pA root mean square noise level). Events were accepted for kinetic analysis only if the shape was asymmetrical with a fast-rising and slowly decaying falling phase. Current traces were filtered at 2 kHz and digitized at 20 kHz using pClamp9 software. For each acquired data trace, all events were detected and the frequency was calculated every 5 sec. If the PSC frequency declined continuously over 20 sec before drug application, a rundown of PSCs was suspected and the data were not analyzed further. For the frequency analysis, only PSCs (before drug application) with a stable frequency longer than 20 sec were used.

All statistical analyses were carried out using Origin 7 (Origin Lab, MA). When comparing multiple genotypes we used One-way Anova analysis with appropriate post-hoc tests to compare groups. For all other experiments two tailed Student's T-test was performed, unless otherwise noted in the text.

RESULTS

***Drosophila* inhibitory GABAergic synaptic transmission is suppressed by the adenylyl cyclase activator forskolin**

We examined modulatory effects of cAMP signaling on inhibitory GABAergic synaptic transmission in *Drosophila* primary cultured neurons. For this, GABAergic inhibitory postsynaptic currents (IPSCs) were recorded in cultured neurons using whole-cell patch technique as previously described (Lee *et al.*, 2003). These spontaneous IPSCs are comprised of both action potential (AP)-dependent and -independent (miniature IPSC) synaptic currents that are almost completely Ca²⁺-dependent (Lee *et al.*, 2003).

In order to eliminate interference by cholinergic synaptic currents neurons were held at 0 mV, the reversal potential of nicotinic acetylcholine receptors, as previously described (Lee & O'Dowd, 1999). The adenylyl cyclase (AC) activator forskolin (FSK; 20μM) was focally applied to a neuron showing GABAergic IPSCs. FSK strongly reduced the frequency of GABAergic IPSCs during its application and this suppression continued even after the end of FSK application (Figure 1A). Figure 1B shows the average reduction in the frequency of GABAergic IPSCs (n=9). To account for the variation in GABAergic IPSC frequency between different neurons, the frequency of IPSCs was calculated every 5 sec and normalized to the average frequency for 30–60 sec before FSK application (Yuan & Lee, 2007). The maximum reduction in the frequency of IPSCs was observed about 20 sec from the onset of FSK application as its effect was gradual for the first 20 sec. The time lag observed is probably due to the time taken by FSK to act on AC and to then increase the intracellular cAMP levels. We further observed a prolonged suppression of GABAergic IPSCs after the end of FSK application.

Since protein kinase A (PKA) is a known effector of cAMP, we examined its involvement in FSK-mediated suppression of GABAergic IPSCs using the PKA inhibitor H-89. Cultured neurons were incubated in the recording solution with H-89 for 10–15 minutes before whole-cell recordings of GABAergic IPSCs. In the presence of H-89, the focal application of 20μM FSK showed no decrease in the frequency of GABAergic IPSCs (n=5; Figure 1B). This attenuation of FSK-mediated GABAergic IPSCs by H-89 demonstrates that PKA is a key player in this process.

Dibutyryc cAMP down-regulates *Drosophila* GABAergic IPSCs in a dose-dependent manner

Our results with H-89 strongly indicate that GABAergic suppression by FSK is through the cAMP-PKA signaling pathway. However some studies have shown that FSK can also act through cAMP-independent mechanisms (Hoshi *et al.*, 1988; Harris-Warrick, 1989; Gandia *et al.*, 1997). We thus used a membrane-permeable cAMP analog (db-cAMP) to rule out such cAMP-independent effects of FSK. GABAergic IPSC frequency was markedly decreased by db-cAMP (n=6; Figure 2A) whereas it increased cholinergic EPSCs (data not shown, refer to Yuan & Lee, 2007). We observed no lag time for this suppression unlike that seen earlier with FSK application (Figure 1B).

In order to examine the lag time seen during FSK application we compared the decrease in IPSC frequency by both FSK and db-cAMP. For this, we plotted the average frequency of IPSCs (n=9 for FSK; n=6 for db-cAMP) and fitted the curves of both the plots to determine their decay constants (Figure 2B). The FSK plot had a decay constant (τ_1) of 12.2 sec, while the cAMP action was much faster (τ_1 =5.1 sec). This instantaneous action of db-cAMP is probably because it does not require the activation of intermediates such as AC. We also noted a faster rate of recovery of GABAergic IPSCs (Figure 2A) at the end of the db-cAMP puffing. This suggests that AC is active for a long duration even after the termination of

FSK application while the effect of db-cAMP lasts only as long as the external signal is present. We then examined the dose-dependent effects of forskolin and db-cAMP on GABAergic IPSCs. The comparison of average frequencies between different db-cAMP concentration groups shows a significant effect on GABAergic IPSCs (One-way ANOVA $F_{3,108}=9.579$, $P<0.001$). The lower concentrations of db-cAMP, 100 μ M (106.26 \pm 9.20, n=4) and 500 μ M (85.78 \pm 8.38, n=5) showed a small reduction in the IPSC frequency. This was significantly reduced to 56.3 \pm 8.99 ($P<0.01$, n=4) by 1mM and to 10.2 \pm 4.7% ($P<0.01$, n=6) by 10mM db-cAMP (Figure 2C). Similarly, we noted that increasing the concentration of FSK had a significant effect on GABAergic IPSCs (One-way ANOVA $F_{2,93}=7.798$, $P<0.001$). While 2 μ M FSK did not show any reduction in GABAergic IPSCs, 20 μ M FSK strongly suppressed them (11.6 \pm 4.7%, $P<0.01$, n=9). This effect was more pronounced with 100 μ M FSK (1.1 \pm 0.9%, $P<0.01$, n=5). Thus 20 μ M FSK was used in all our experiments with embryonic neuronal cultures.

Suppression of GABAergic IPSCs by cAMP-PKA signaling is postsynaptic

Previous reports have shown that the cAMP-dependent plasticity at excitatory cholinergic synapses in *Drosophila* is presynaptic (Lee & O'Dowd, 2000). In this study, we examined the locus of cAMP actions on GABAergic synaptic transmission. First, a PKA inhibitor H-89 was added to the whole-cell patch pipette in order to block possible postsynaptic actions of cAMP signaling. In the presence of 50 μ M H-89 we saw only 47% suppression of GABAergic IPSCs to 52.95 \pm 5.11% ($P<0.01$, n=4) by FSK, compared to 89% by FSK alone, suggesting that the locus of action may be postsynaptic (Figure 3). However this experiment did not rule out the possibility of presynaptic origin because H-89 in the pipette can diffuse to the presynaptic terminal. We thus included a membrane-impermeable PKA blocker PKI (6–22) amide (200 and 400 μ M) in the internal recording pipette. This resulted in a significant attenuation of FSK-mediated GABAergic IPSCs by 200 μ M (79.97 \pm 23.12%, $P<0.001$, n=4) and 400 μ M (175.76 \pm 21.82%, $P<0.001$, n=4) PKI (6–22) amide as compared to FSK alone. This indicates that the locus of PKA action is postsynaptic (Figures 3A & B).

Interestingly, we observed that a high concentration of PKI (400 μ M) not only rescued the phenotype, but resulted in a significant increase in the frequency of sIPSCs as compared to our normalized control values (Figure 3B). This suggests that once all postsynaptic actions of PKA are blocked by PKI, the focally applied FSK can still act on presynaptic targets and lead to an increase in frequency of GABAergic IPSCs. To examine this possibility, we looked at the excitability of GABAergic neurons in the presence of FSK. GABAergic neurons were labeled with red fluorescent protein (RFP) driven by the GABA specific Gad1 promoter (i.e. Gad1-RFP), allowing us to visualize live cells (Figure 4A). Most RFP(+) neurons also stained positive with a GABA-specific antibody (data not shown). Extracellular action potentials (APs) were recorded from RFP(+) neurons (Figure 4B) in cell-attached mode since it is known to not interfere with the intracellular milieu and is a good indicator of cellular excitability (Hodges *et al.*, 2002). Focal application of FSK significantly increased the frequency of APs in GABAergic neurons (4.28 \pm 0.98Hz, $P<0.001$, n=5) compared to controls (1.29 \pm 0.65Hz, n=5), indicating an increase of GABAergic neuronal excitability by FSK. However, it may be possible that cAMP indirectly increases GABAergic excitability through activation of excitatory synaptic inputs to GABAergic neurons. Since the cholinergic currents are a major excitatory synaptic input in *Drosophila*, we repeated the same AP experiments using a specific cholinergic blocker, curare. We were able to record APs from RFP(+) GABAergic neurons in the presence of 1 μ M curare although the number of GABA neurons showing APs were very few. In the presence of curare, FSK still increased AP frequency to about 2.3 fold (4.2 \pm 1.6Hz, $P<0.001$, n=4) compared to controls (1.8 \pm 0.4Hz, n=4), demonstrating that cAMP signaling directly increases GABAergic excitability. Taken together, our results clearly show that cAMP-PKA

signaling directly enhanced the excitability of not only inhibitory GABAergic (Figure 4) but also excitatory cholinergic neurons (Lee & O'Dowd, 2000).

Evoked I_{GABA} is suppressed by forskolin

Our results in Figure 4 have shown that although FSK increases GABAergic excitability, it also decreases the frequency of IPSCs. GABAergic IPSCs result from the activation of ionotropic GABA receptors expressed in a postsynaptic density. Therefore, it was of interest to directly examine the effects of FSK on synaptic GABA receptors. However, it is still technically challenging to study evoked synaptic IPSCs in *Drosophila* neuronal cultures, which are mediated by activation of synaptic GABA receptors. Instead, we examined GABA-evoked currents (I_{GABA}) in the presence of FSK. GABA-evoked currents in whole-cell mode were first recorded from a single neuron by focal application of 100 μ M GABA for 2 seconds (Figure 5A). Following this, 20 μ M FSK was bath-perfused and then 100 μ M GABA was puffed again on the same neuron (Figure 5B). We noted that GABA-evoked currents were strongly reduced after FSK perfusion ($19.5 \pm 2.1\%$, $P < 0.001$, $n=5$) compared to control ($101.24 \pm 4.16\%$, $n=5$) I_{GABA} (Figure 5B & C). These results support the notion that FSK-mediated suppression of GABAergic transmission is due to reduced response of the postsynaptic GABA receptors and not due to reduced presynaptic GABA release.

Frequency of mIPSCs is reduced by forskolin, but mIPSC amplitude remains unchanged

To further confirm the postsynaptic mode of suppression by cAMP signaling, we measured miniature IPSCs in the presence of 1 μ M TTX. The frequency of mIPSCs was quantified as described earlier for sIPSCs (Figure 1). Then, 20 μ M FSK was focally applied for 30 seconds. Figure 6 shows that mIPSC frequency was significantly reduced to $17.3 \pm 3.3\%$ ($P < 0.001$, $n=8$) during FSK application compared to frequency before FSK application. However there was no change in the amplitude of mIPSCs before (9.15 ± 0.64 pA, $n=8$) and during (8.38 ± 0.71 pA; $P=0.43$, $n=8$) FSK application. A comparison of the cumulative distribution of mIPSC amplitude before ($n=1170$) and during ($n=154$) FSK application revealed no significant change (Kolmogorov-Smirnov test; $D=0.33$; $P=0.30$). The identical effect of FSK on mIPSCs and sIPSCs indicates that both AP-dependent and -independent mechanisms at the inhibitory GABAergic synapses are regulated similarly by FSK. No detectable change in mIPSC amplitude by FSK possibly rules out an exclusively postsynaptic mode of action at GABAergic synapses. However our results on the effect of FSK perfusion on GABA evoked currents (Figure 5) and the increase in frequency of sIPSCs on inclusion of a membrane-impermeable PKA blocker in the postsynaptic neuron (Figure 3) strongly support postsynaptic suppression of GABAergic transmission by FSK.

Majority of IPSCs is mediated by Rdl-containing GABA receptors

We next wanted to examine why there was no change observed in mIPSC amplitude during FSK application. It is possible that there may be two types of synaptic GABA receptors: PKA-sensitive versus -insensitive. If so, PKA-insensitive synaptic GABA receptors would still contribute to mIPSCs that show no change in the amplitude during FSK application. In *Drosophila*, there are three ionotropic GABA receptor subunits cloned so far (Rdl, LCCH2, and GRD; Hosie et al, 1997). Of these three known GABA receptor subunits, the Rdl subunit is best characterized and also widely expressed in several regions of the *Drosophila* brain (Harrison et al., 1996). Our previous study has shown that Rdl-containing GABA receptor plays a role in inhibitory synaptic transmission (Lee et al, 2003). However, the exact subunit composition of *Drosophila* synaptic GABA receptors is not known.

In this study, we examined whether there are different types of GABA receptors mediating synaptic currents using a *Rdl* null mutant line. Since the homozygous *Rdl* null mutant is lethal, this mutant fly line was maintained over a balancer chromosome (Goldstein &

Fyrberg, 1994; Greenspan, 2004). For this we used a TM6B balancer carrying Ubi-GFP, in which GFP expression is controlled by the ubiquitin promoter. Three different genotypes (1:2:1 ratio) of embryos are expected from the heterozygous *Rdl1*/TM6B, Ubi-GFP fly: *Rdl1/Rdl1*, *Rdl1*/TM6B, Ubi-GFP, and TM6B, Ubi-GFP/ TM6B, Ubi-GFP. Therefore, each neuronal culture was prepared from a single embryo to avoid any intermingling of genotypes. Accordingly, homozygous *Rdl1* null mutant neuronal cultures were identified as GFP-negative (refer to Darya et al., 2009 for further experimental details). Functional properties of *Rdl1* null GABA synapses were examined using whole-cell recording technique (Table 1). Incidence rate (5.8%, n=3 out of 52) of GABAergic sIPSCs was markedly lower than that of wild type (29.4%, n= 10 out of 34). In contrast, the incidence rate (67%, n=42/63) of cholinergic EPSCs was increased in *Rdl1* null mutant compared to that of wild type (~50%; Lee & O'Dowd, 2009). Interestingly, we also observed that the frequency of sIPSCs in the *Rdl* null mutant was at least six times lower than wild-type strains (Table 1). This suggests that there are at least two functionally different types of synaptic GABA receptors, one with and the other without *Rdl* subunits. Since the majority of functional synaptic GABA receptors contain *Rdl* subunit, it is plausible that *Rdl* is a molecular target of PKA-signaling. In contrast, synaptic GABA receptors without the *Rdl* subunit would be resistant to PKA-dependent suppression and thus contribute to GABAergic IPSC currents with no change in amplitude in the presence of PKA (Table 1).

Memory mutants *dunce* and *rutabaga* show impaired cAMP-dependent GABAergic plasticity

Many known olfactory learning and memory mutants in *Drosophila* have defective cAMP signaling (Keene & Waddell, 2007; Davis, 2011). We thus examined if the suppression of GABAergic IPSCs by cAMP signaling is altered in these learning and memory mutants. For this, two well established olfactory memory mutants, *dunce1* (*dnc1*) (Dudai et al., 1976) and *rutabaga1* (*rut1*) (Livingstone et al., 1984) were used. The *dunce* gene encodes the cAMP phosphodiesterase (PDE) enzyme (Byers et al., 1981) and this mutation results in an increase in the cytoplasmic cAMP levels. On the other hand, *rut1* harbors a mutation in adenylyl cyclase (AC) enzyme (Levin et al., 1992), which results in a decrease in the cytoplasmic cAMP levels. Thus these two mutants provide an ideal background of intrinsically high and low levels of intracellular cAMP. 20 μ M FSK was applied on 3–6 days old cultured neurons of each of these two strains. FSK was focally applied for 30 seconds from 62.5–92.5 seconds and the frequency of events was plotted at 5 sec intervals as described earlier (Figure 1B). This resulted in only a modest decrease in GABAergic IPSC frequency in both *dnc1* (n=6; Figure 7A) and *rut1* neurons (n=7; Figure 7B) as compared to the wild type controls (Figure 7C). In addition, the rate of suppression of IPSCs by FSK had slowed down in the mutants as compared to the wild-type. To quantify this effect of FSK, we averaged the IPSC frequency from 80–95 sec. This showed that both mutants *dnc1* (53.40 \pm 1.45%, P < 0.001, n=5) and *rut1* (56.05 \pm 9.29%, P < 0.001, n=7) had similar levels of reduction in the IPSC frequencies when compared to the wild-type controls (Wt=11.63 \pm 2.70%, n=9). In contrast, the amplitude in *dnc1* and *rut1* was not significantly different before and after FSK application, although it was slightly reduced (Figure 7E).

We further examined whether the alteration in GABAergic IPSCs seen in both *dnc1* and *rut1* mutants is due to reduced ionotropic GABA receptor sensitivity to FSK as noted earlier (Figure 5B). Cultured embryonic neurons from *dnc1* and *rut1* were whole-cell patched and GABA (100 μ M) was focally puffed for 2 sec to measure I_{GABA} . Subsequently, FSK (20 μ M) was perfused in to the recording chamber and then GABA was puffed again. We noted that I_{GABA} was reduced in neurons derived from *dnc1* and *rut1* after FSK perfusion (Figure 8A & B). However, the amount of suppression was significantly less in *dnc1* (43.66 \pm 3.88%, P <

0.001, n=8) and *rut1* ($45.91 \pm 3.85\%$, $P < 0.001$, n=8) mutants (Figure 8C) when compared to wild-type neurons ($19.54 \pm 2.10\%$, n=5).

Embryonic and late-stage pupal mushroom body (MB) neurons from memory mutants show altered GABA receptor response to forskolin

The cAMP signaling pathway is essential for memory formation in *Drosophila*. It is also known that the *Drosophila* mushroom body (MB) is the center for olfactory learning and memory (Davis, 2011). We thus examined whether GABA receptors in MB neurons are also modulated by cAMP signaling. In order to identify MB neurons for electrophysiology, the MB specific c309-Gal4 driver with strong expression in α , β and γ lobes (Joiner *et al.*, 2006; Krashes *et al.*, 2007) was used to drive GFP expression in MB neurons (i.e. c309-Gal4×UAS-GFP). GABA-evoked currents (I_{GABA}) in embryonic MB neurons were reduced to $19.33 \pm 1.95\%$ (n=8) after 20 μ M FSK perfusion (Figure 9). Interestingly, this reduction in MB neurons by FSK was very similar to that seen in the total population of neurons (Figure 5C).

Since embryonic MB neurons are known to differentiate in culture, we examined already differentiated MB neurons to rule out the possibility that GABA receptors in the differentiated brain behaved differently from the embryonic cultures. Though there are studies showing electrophysiological recordings from intact fly brains, *in vivo* recording in *Drosophila* is very laborious and challenging. Thus for our experiments, we dissociated late stage c309-Gal4×UAS-GFP (T10) pupal brain neurons and cultured them as described earlier (Su & O'Dowd, 2003). GABA-evoked currents were successfully recorded from pupal MB neurons. When 20 μ M FSK was perfused, I_{GABA} reduced to $2.65 \pm 1.44\%$, which was significant when compared to embryonic neurons ($P < 0.001$, n=4). Pupal brain neuronal cultures were also prepared from fly lines carrying c309-Gal4×UAS-GFP in both the *dnc1* or *rut1* background. 20 μ M FSK perfusion greatly reduced I_{GABA} to 7.95 ± 2.05 (n=4) in *dnc1* and to 7.13 ± 2.87 (n=7) in *rut1* pupal MB neurons (Figure 9). This number was slightly higher than wild type neurons but not significantly different ($P=0.08$ for *dnc1* and $P=0.26$ for *rut1*). These results showed that GABA receptors in pupal MB neurons were more sensitive than embryonic MB neurons. Since 20 μ M FSK appeared to be too strong for pupal MB neurons, lower concentrations of FSK were tested. We found that 2 μ M FSK reduced I_{GABA} in wild type pupal MB neurons to $25.87 \pm 2.09\%$ (n=6), which is less suppressive than 20 μ M FSK (Figure 9). Subsequently, 2 μ M FSK was tested on *dnc1* and *rut1* pupal MB neurons and a significantly lesser reduction to $50.52 \pm 3.5\%$ ($P < 0.001$, n=6) and $47.19 \pm 3.39\%$ ($P < 0.001$, n=6), for *dnc1* and *rut1* respectively, was noted (Figure 10). These results confirm that GABA receptors in *dnc1* and *rut1* pupal MB neurons are significantly less sensitive to FSK. We also examined the effect of 2 μ M FSK on I_{GABA} in embryonic MB neurons. Figure 10C shows that while FSK strongly suppressed wild type I_{GABA} ($34.45 \pm 1.97\%$, n=5). Its effect on *dnc1* ($53.19 \pm 3.55\%$, $P < 0.01$, n=5) and *rut1* ($56.78 \pm 5.00\%$, $P < 0.01$, n=5) embryonic MB neurons was significantly less pronounced. Thus, the effects of FSK on the embryonic MB neurons were very similar to those observed in pupal MB neurons, indicating that there is no stage-specific or developmental difference in FSK-mediated suppression of GABA receptors.

DISCUSSION

cAMP signaling inhibits frequency of GABAergic IPSCs

Ca^{2+} /CaM dependent adenylate cyclase (AC) produces cAMP and is also known to function as a co-incidence detector during learning in both *Drosophila* (Tomchik & Davis, 2009; Gervasi *et al.*, 2010) and *Aplysia* (Abrams *et al.*, 1991). In addition, AC-dependent cAMP activation changes the strength of *Drosophila* excitatory synapses (Zhong & Wu, 1991; Lee

& O'Dowd, 2000) which may be the cellular mechanism underlying learning and memory. Although inhibitory synaptic transmission is equally important for proper neuronal communication, the effects of cAMP at the inhibitory GABAergic synapses have remained unexplored. In this study we show that forskolin (FSK), an activator of cAMP, suppresses the frequency of inhibitory GABAergic IPSCs in *Drosophila* primary neuronal cultures (Figure 1). We observed a concentration dependent effect of FSK on GABAergic IPSCs (Figure 2) in the same physiological range as described in recent imaging studies in intact fly brains (Tomchik & Davis, 2009; Gervasi *et al.*, 2010). Further we show that cAMP decreases GABAergic IPSCs in a PKA-dependent manner through a postsynaptic mechanism.

Sparsening of odor representation through GABAergic inhibition in the mushroom body (MB) neurons is thought to be a possible mechanism for information storage in locusts (Perez-Orive *et al.*, 2002). GABAergic local neurons are known to be involved in olfactory information processing in *Drosophila* (Wilson & Laurent, 2005; Olsen & Wilson, 2008) indicating that GABAergic transmission plays a crucial role in shaping odor response. The MB shows extensive GABAergic innervation in both locusts (Perez-Orive *et al.*, 2002) and *Drosophila* (Yasuyama *et al.*, 2002). This, along with the observation that cAMP pathway genes like *dunce* and *rutabaga* are preferentially expressed in the MB (Davis, 2011), indicates that cAMP mediated GABAergic plasticity may be important for learning in *Drosophila*. Consistent with this hypothesis, we observe altered cAMP mediated GABAergic IPSCs in the cAMP mutants *dncl* and *rut1* (Figure 6). The effect of cAMP on suppression of GABAergic currents was less pronounced in the mutants. This suggests that the altered inhibition contributes to their observed learning defects. In fact, recent studies have shown that GABA_A RDL receptors expressed in the MB and GABAergic neurons projecting to the MB are essential for olfactory learning (Liu *et al.*, 2007; Liu & Davis, 2009). It is thus possible that altered cAMP mediated GABAergic plasticity at the MB neurons may account for some forms of the learning defects in *Drosophila*.

Postsynaptic GABA receptors mediate the effect of cAMP on GABAergic IPSCs

GABAergic IPSCs are known to act through picrotoxin-sensitive postsynaptic GABA receptors in both *Drosophila* embryonic and pupal neuronal cultures (Lee *et al.*, 2003; Su & O'Dowd, 2003). We observe that the suppression of GABAergic IPSCs by FSK is completely abolished in the presence of a membrane impermeable PKA inhibitor restricted to the postsynaptic neuron (Figure 3). This indicates that PKA may modulate GABAergic IPSCs by regulating GABA receptor sensitivity by phosphorylation, similar to what has been suggested in the mammalian hippocampus (Nusser *et al.*, 1999).

There are three known ionotropic GABA receptor gene homologs in *Drosophila* – RDL, LCCH3 and GRD (Harvey *et al.*, 1994; Hosie *et al.*, 1997). Amongst them, the GABA RDL subunit is widely expressed in several regions of the *Drosophila* brain (Harrison *et al.*, 1996) and its expression in the MB is inversely correlated to olfactory learning (Liu *et al.*, 2007). Therefore, RDL-containing GABA receptors may play an important role in cAMP-dependent synaptic plasticity and thus be involved in learning and memory. Our data suggests that the majority of synaptic GABA receptors contain the RDL subunit while a small fraction of synaptic GABA receptors lack RDL (Table 1), providing evidence of heterogeneous synaptic GABA receptors in *Drosophila* for the first time. However, it is still not known what particular subunit of GABA receptors is involved in regulation of cAMP-dependent GABAergic plasticity. Based on the observation that RDL containing GABA receptors mediate the majority of GABAergic IPSCs in *Drosophila* primary neuronal cultures, the action of FSK on GABAergic IPSCs is probably through the GABA RDL subunit. While the detailed molecular mechanism remains to be explored, we propose that PKA-mediated phosphorylation of RDL subunits and subsequent GABA receptor

internalization may occur in the postsynaptic region (Mou *et al.*, 2011; Vithlani *et al.*, 2011). In this scenario, the only functional synaptic GABA receptors will be those lacking the RDL subunit at the postsynaptic regions. This will account for a decrease in mIPSC frequency with response to FSK, while leaving mIPSC amplitude almost unchanged (Figure 6).

Although GABA receptor subunits would be the target of cAMP-PKA signaling we still cannot rule out the possibility that other molecules can be phosphorylated and then indirectly regulate GABA receptor subunits. Future work using heterologous expression systems for GABA subunits will help to determine whether GABA receptors are directly phosphorylated.

Alterations in ionotropic GABA receptor sensitivity in learning mutants

Our recordings from embryonic and pupal MB neurons of both *dunce* and *rutabaga* mutants show a defect in ionotropic GABA receptor response in the presence of FSK (Figures 7, 9 & 10). Interestingly, this response to FSK is similar in both the mutants despite their contrasting levels of cellular cAMP. Recent imaging studies in the *rutabaga* mutant have shown that AC is required for co-incidence detection in the MB neurons (Tomchik & Davis, 2009; Gervasi *et al.*, 2010). FSK application also fails to increase PKA to wild-type levels in the MB neurons of *rutabaga* (Gervasi *et al.*, 2010). Thus in our experiments, the changes in receptor response in *rutabaga* can be explained by a lack of increase in cAMP/PKA levels due to defects in FSK-mediated AC activation.

The *dunce* mutants with high levels of cAMP also show defects in short-term memory due to alterations in the spatiotemporal restriction of *dunce* phosphodiesterase to the *Drosophila* MB (Gervasi *et al.*, 2010). Further, the *dunce* MB neurons show an increase in PKA levels on FSK application similar to the wild-type strains. These findings suggest that FSK-mediated inhibition of GABA receptor should be greater in *dunce* neurons. However, in our results (Figure 7) the *dunce* and *rutabaga* mutants, despite having opposing effects on cellular cAMP levels, showed very similar FSK mediated effects on GABAergic IPSCs. Several other studies have also shown that *dunce* and *rutabaga* have similar defects in growth cone motility, excitatory synaptic plasticity and more importantly, short-term memory (Kim & Wu, 1996; Gasque *et al.*, 2006). Even though the effect of FSK on GABAergic IPSCs in *dunce* and *rutabaga* mutants is similar, it is very likely that the molecular mechanisms underlying these responses differ in the two mutants. It has been shown that elevated cAMP signaling reduces phosphorylation in rat kidney cells through activation of protein phosphatase 2A (Feschenko *et al.*, 2002). In addition, increased PKA activity in mouse hippocampus hyper-phosphorylates several downstream molecular targets including a tyrosine phosphatase (STEP), correlates with decreased phosphodiesterase protein (PDE4) levels and results in memory defects (Giralt *et al.*, 2011). Therefore, it is tempting to speculate that high levels of cAMP due to the *dunce* mutation leads to the activation of phosphatase(s) and thus reduces the effects of FSK as seen in our study (Figure 8). Taken together, all these findings strongly suggest that the disruption of cellular cAMP homeostasis can alter inhibitory GABAergic synaptic plasticity and hence cause defects in olfactory learning, although the underlying mechanisms leading to this effect can be different (e.g. reduced PKA activity in *rut1* versus increased phosphatase activity in *dnc1*).

Dual regulation of synaptic excitation and inhibition by cAMP

Strengthening in the efficacy of excitatory transmission underlies enhanced synaptic plasticity such as hippocampal long-term potentiation (LTP) and facilitation (LTF) in *Aplysia* (Milner *et al.*, 1998). It is thus possible that the suppression of inhibitory transmission by a common second messenger like cAMP, which can enhance excitatory synaptic transmission, may lead to synaptic strengthening. Previous work has shown that the

cAMP activator FSK increases excitability at the cholinergic synapses in *Drosophila* primary neuronal cultures (Yuan & Lee, 2007). However the effect of FSK on other synapses like the GABAergic synapses has not been explored. We show that FSK elevates overall cellular excitability at GABAergic synapses as demonstrated by the increase in spontaneous AP frequency (Figure 4). Moreover, when PKA in the postsynaptic neuron is completely blocked by an inhibitor, we see an increase in the frequency of GABAergic IPSCs (Figure 3A&B). Together with previous studies (Yuan & Lee, 2007), our results indicate that FSK/cAMP act as common molecules regulating globally presynaptic excitability at both the cholinergic as well as GABAergic synapses. We also note that FSK inhibits the response of postsynaptic GABA receptors in a specific manner leading to a decrease in GABAergic synaptic strength. Our studies demonstrate a novel dual regulatory role of cAMP by showing that it increases overall presynaptic function on one hand; and, acts specifically on postsynaptic GABA receptors to decrease GABAergic plasticity on the other. This action of cAMP could result in global increases in excitability and learning.

Acknowledgments

This work was partially supported by NIH grant NS050260. We thank Dr. R.A. Colvin for valuable inputs on previous versions of this manuscript.

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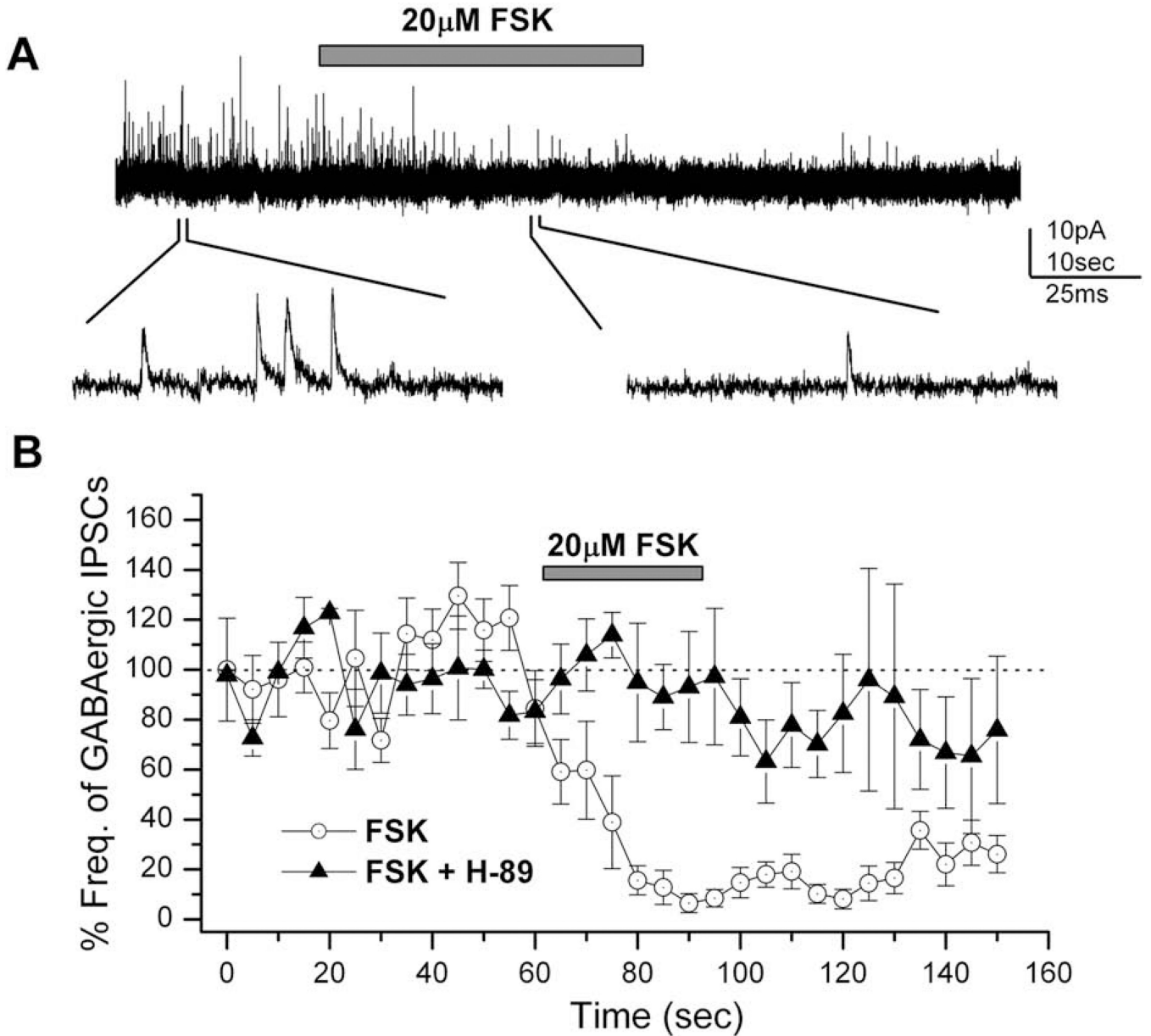


Figure 1. Suppression of inhibitory GABAergic postsynaptic currents (IPSCs) by forskolin (FSK)

A, FSK (20 μ M) was focally applied to a wild type neuron (3 days in culture) after obtaining a stable GABAergic IPSC trace. GABAergic IPSCs were almost completely suppressed by FSK. Example IPSC traces are shown on an expanded time scale below the complete recording trace. Holding potential (V_H) = 0 mV. **B**, The graph shows the reduction in GABAergic IPSC frequency by the application of 20 μ M FSK in wild-type neurons in the absence (n=9; circles) and presence (n=4) of 50 μ M H-89 (a membrane-permeable PKA inhibitor). The reduction seen in IPSCs in the presence of H-89 is almost negligible as compared to the change in the absence of H-89. FSK was applied for 30 seconds (indicated by bar) at 62.5 seconds after the initiation of the IPSC recording. The IPSC frequency was calculated every 5 seconds and normalized to that of controls as described in materials and methods. Bars indicate SEM.

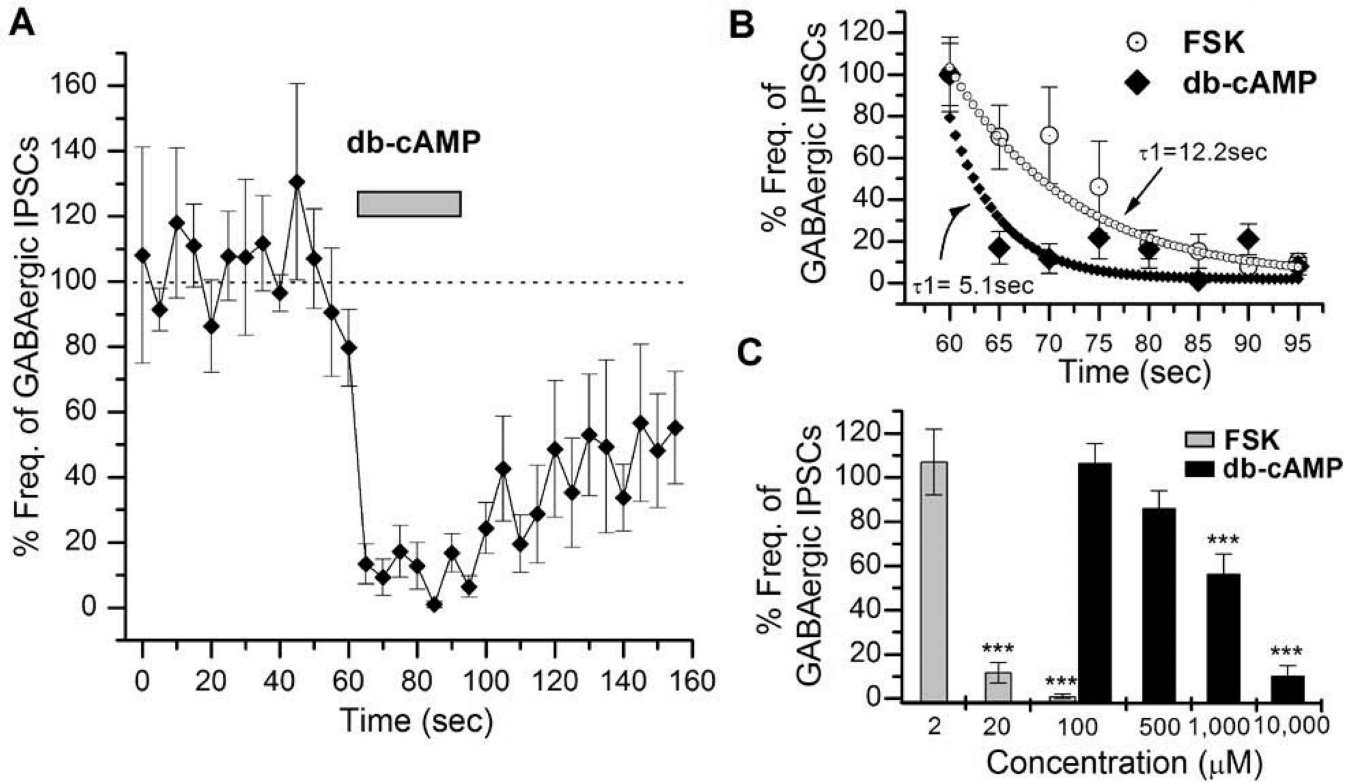


Figure 2. Inhibitory actions of cAMP on GABAergic synaptic transmission

A, The graph shows the reduction in GABAergic IPSCs ($V_H = 0mV$) on the application of 10mM db-cAMP ($n=6$) in wild type neurons ($n=9$). Near complete suppression was seen during 30 seconds of db-cAMP application. In each experiment, db-cAMP was applied at 62.5 sec after initiation of IPSC recording. Bars indicate SEM. **B**, The plot shows a comparison of the suppression rate of GABAergic IPSCs by FSK (20 μ M) and db-cAMP (10mM). This rate was calculated using a double exponential fit. The rate of suppression for FSK action was two-fold slower than that of db-cAMP. **C**, The graph shows effects of different concentrations of db-cAMP (100 μ M - 10mM) and FSK (2 - 100 μ M) on GABAergic IPSCs. The suppression potency was determined by averaging percent frequencies for 20 sec from 70–90 sec in a graph similar to **A**. Error bars indicate SEM. ANOVA test, *** $P < 0.001$. Number of replicates: FSK - 2 μ M ($n=2$), 20 μ M ($n=9$), 100 μ M ($n=5$); cAMP - 100 μ M ($n=4$), 500 μ M ($n=5$), 1,000 μ M ($n=4$), 10,000 μ M ($n=6$).

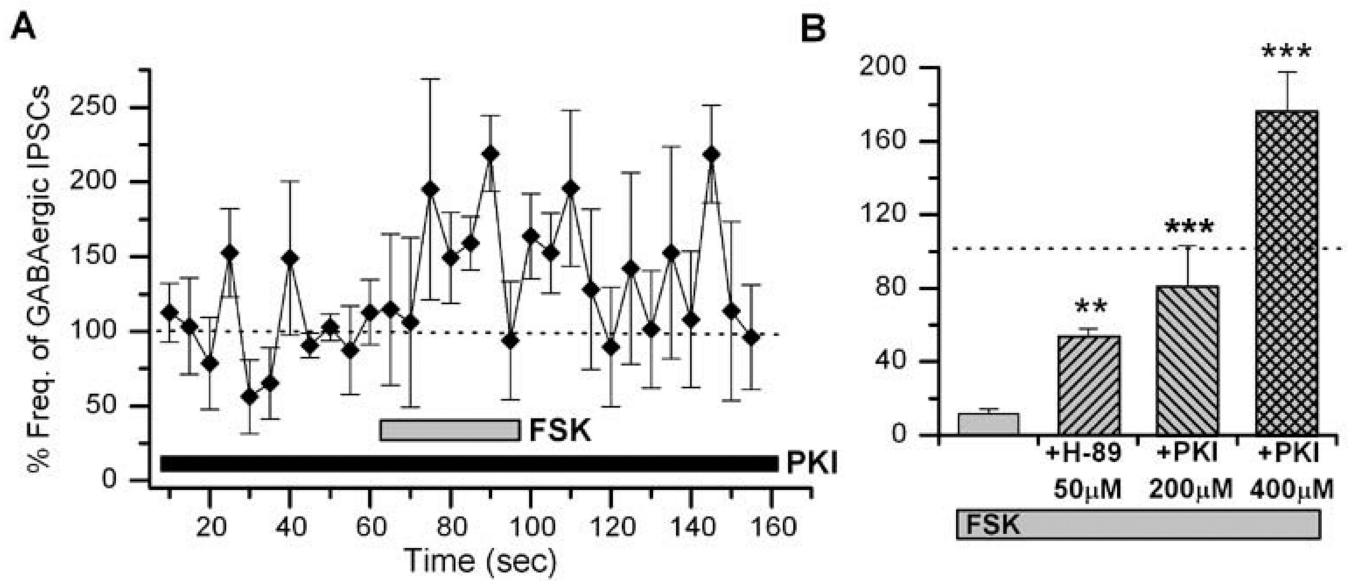


Figure 3. Postsynaptic addition of PKA blockers suppress the effect of forskolin (FSK) effects on GABAergic transmission

A, FSK action on GABAergic transmission was suppressed by a membrane-impermeable PKA inhibitor PKI (6–22) amide added to the postsynaptic neurons through the whole-cell patch pipette. FSK (20 μ M) was focally applied as indicated. **B**, The graph shows suppression of the modulatory action of FSK by PKI amide and H-89. In all cases, 20 μ M FSK was applied for 30 sec. Student t-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Number of replicates: 50 μ M H-89 (n=3), 200 μ M PKI (n=4), 400 μ M PKI (n=4).

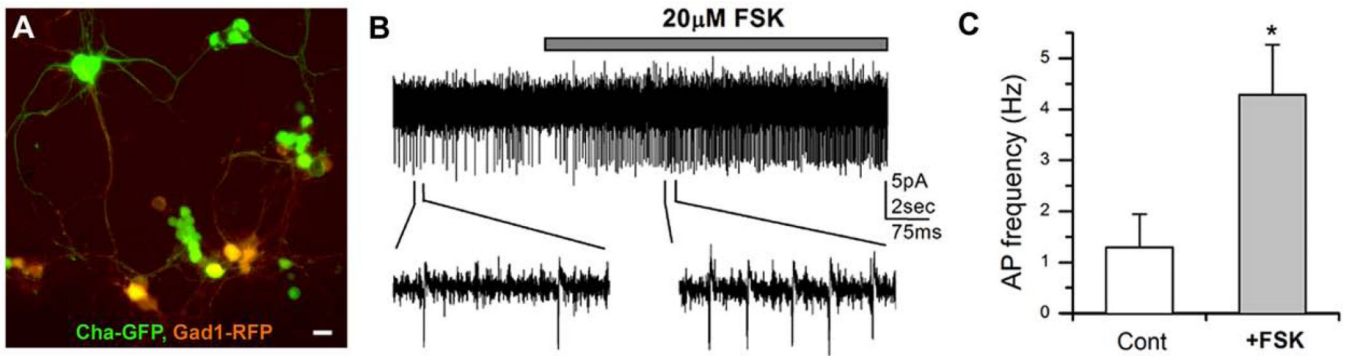


Figure 4. Increased excitability of GABAergic neurons by forskolin (FSK)

A, A fluorescent image showing GFP-labeled cholinergic and RFP-labeled GABAergic neurons. Neuronal cultures were prepared from a fly line carrying Cha-Gal4, UAS-GFP and Gad1-RFP transgenes (Cha-GFP; Gad1-RFP). Scale bar = 10 μm **B**, An extracellular action potential (AP) recording from a GABAergic neuron marked with Gad1-RFP. The AP frequency increased in the presence of FSK (20 μM). **C**, Graph shows the average AP frequency before (control) or during focal application of FSK. Student t-test, * $P < 0.05$. Number of replicates: 20 μM FSK (n=5).

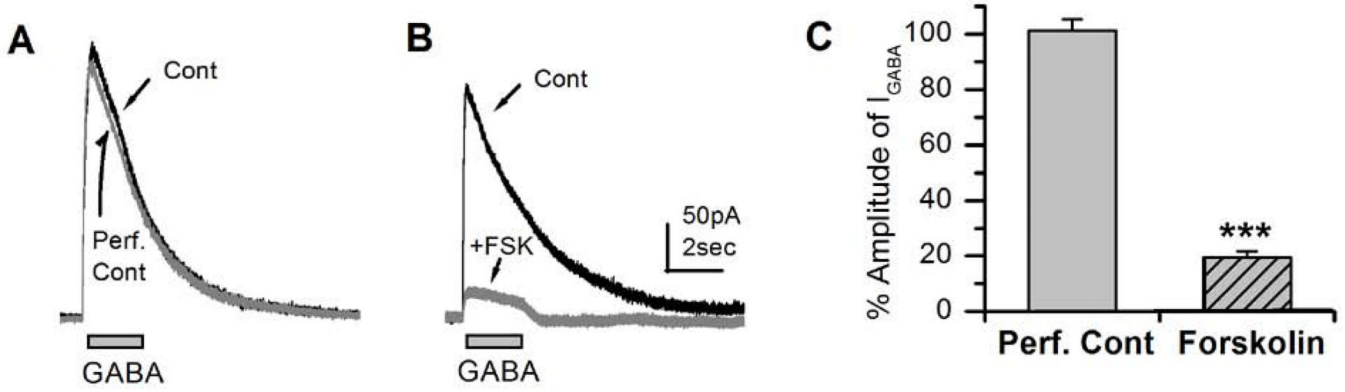


Figure 5. Suppression of GABA-evoked currents (I_{GABA}) by forskolin (FSK)

A, GABA-evoked currents (I_{GABA}) before and after perfusion of the control recording solution. 100 μ M GABA was focally applied for 2 sec as indicated. **B**, Perfusion of FSK (20 μ M) markedly suppressed I_{GABA} . **C**, Percent amplitude of I_{GABA} after perfusion of control versus FSK-containing solution. Student t-test, *** P < 0.001. Number of replicates: 20 μ M FSK (n=5).

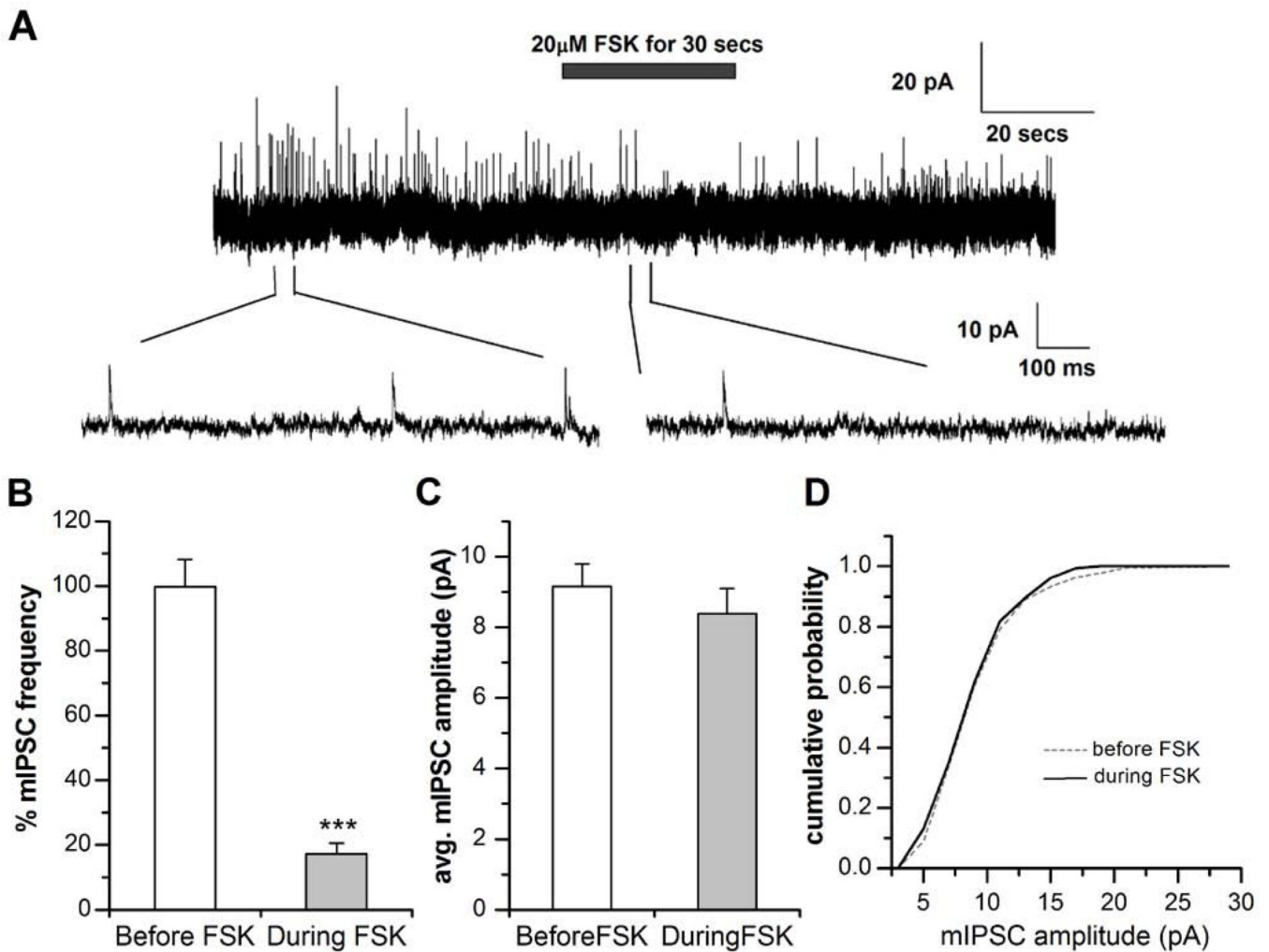


Figure 6. Suppression of miniature inhibitory GABAergic postsynaptic currents (mIPSCs) by forskolin (FSK)

A, FSK (20 μ M) was focally applied to a wild type neuron (3 days in culture) in the presence of 1 μ M tetrodotoxin (TTX) and 1 μ M curare after obtaining a stable GABAergic IPSC trace. Miniature IPSCs (mIPSCs) were recorded at holding potential of 0 mV. The frequency of GABAergic mIPSCs were significantly suppressed during the focal application of FSK. Example IPSC traces are shown on an expanded time scale below the complete recording trace. Holding potential (V_H) = 0 mV. **B**, The graph shows the reduction in GABAergic mIPSC frequency by the application of 20 μ M FSK as compared to the % frequency of mIPSCs before the application of FSK (***) $P < 0.001$, $n=8$). **C**, The graph shows no reduction in the average GABAergic mIPSC amplitude during the application of 20 μ M FSK as compared to the amplitude of mIPSCs before the application of FSK ($n=8$). Paired T-test was used to distinguish between groups. Bars indicate SEM. **D**, Kolmogorov-Smirnov test was carried out to distinguish any difference in the cumulative probability of mIPSC amplitude between before and after FSK treatment. We observed no change in the cumulative probability distribution of mIPSCs before FSK ($n=1170$ events) and after FSK treatment ($n=154$ events) binned at 5pA for both the groups ($P=0.308$).

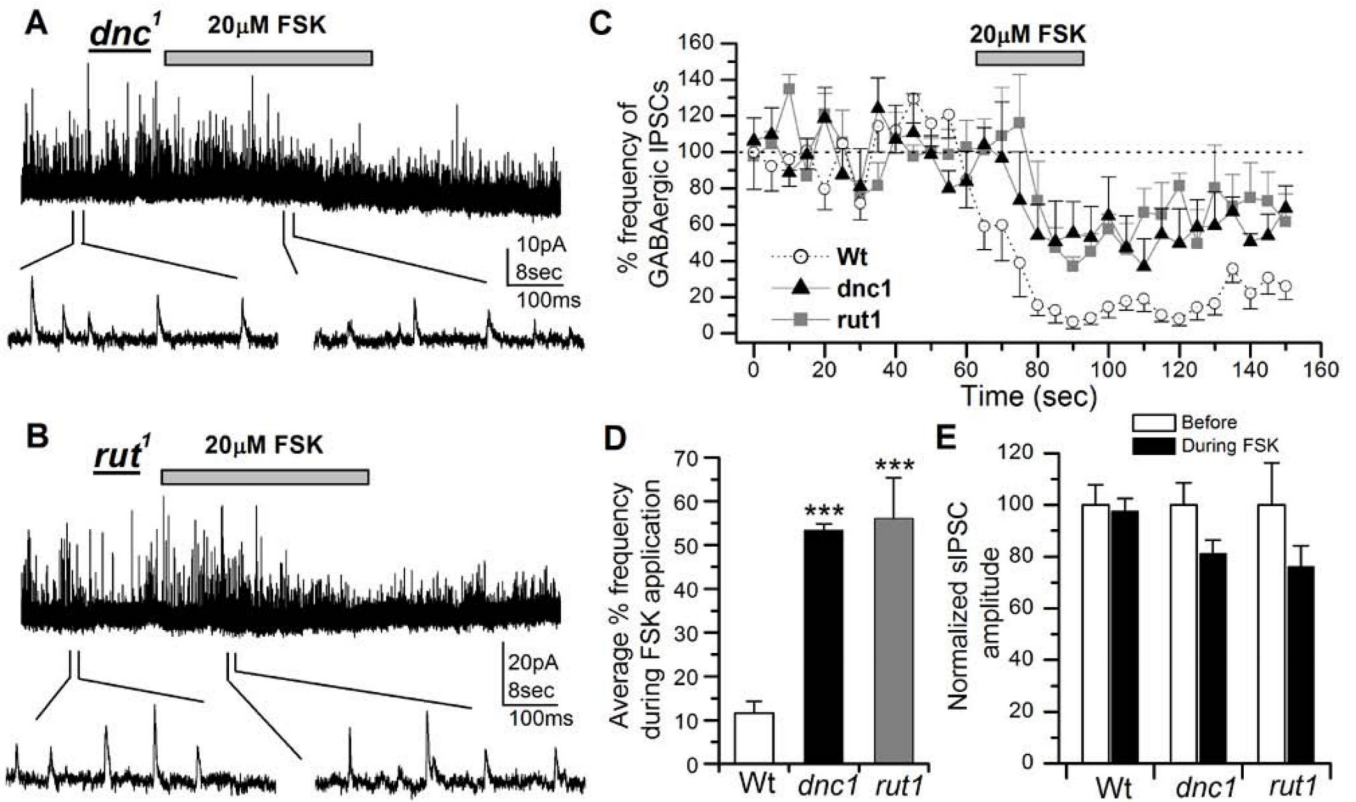


Figure 7. Modulatory actions of forskolin (FSK) on inhibitory GABAergic postsynaptic currents (IPSCs) is attenuated in learning and memory mutants

A, Effects of FSK on GABAergic IPSCs in a *dnc1* neuron. After obtaining a stable IPSC trace from a 3 day old *dnc1* neuron, 20 μ M FSK was focally applied for 30 seconds (indicated by bar). FSK suppression of GABAergic IPSC frequency was significantly attenuated compared to wild type (see Figure 1). GABAergic IPSCs are shown on an expanded time scale below the entire recording trace. $V_H = 0$ mV. **B**, Effects of FSK on GABAergic IPSCs in a *rut1* neuron (3 days old). **C**, The graph shows a comparison of the reduction of GABAergic IPSCs by 20 μ M FSK in wild type ($n=9$, circles), *dnc1* ($n=5$, triangles) and *rut1* ($n=7$, squares) neurons. **D**, The graph shows reduction in percent frequency of GABAergic IPSCs during the focal application of 20 μ M FSK in *dnc1* and *rut1* neurons compared to wild type. Bars indicate SEM. Students t-test, *** $P < 0.001$. **E**, The graph shows average sIPSC amplitude before and during FSK application from GABAergic IPSCs described in **C**. Although the amplitude in *dnc1* and *rut1* was slightly reduced the difference was not significant. Paired T-test was used to compare before and after treatment amplitudes in each genotype.

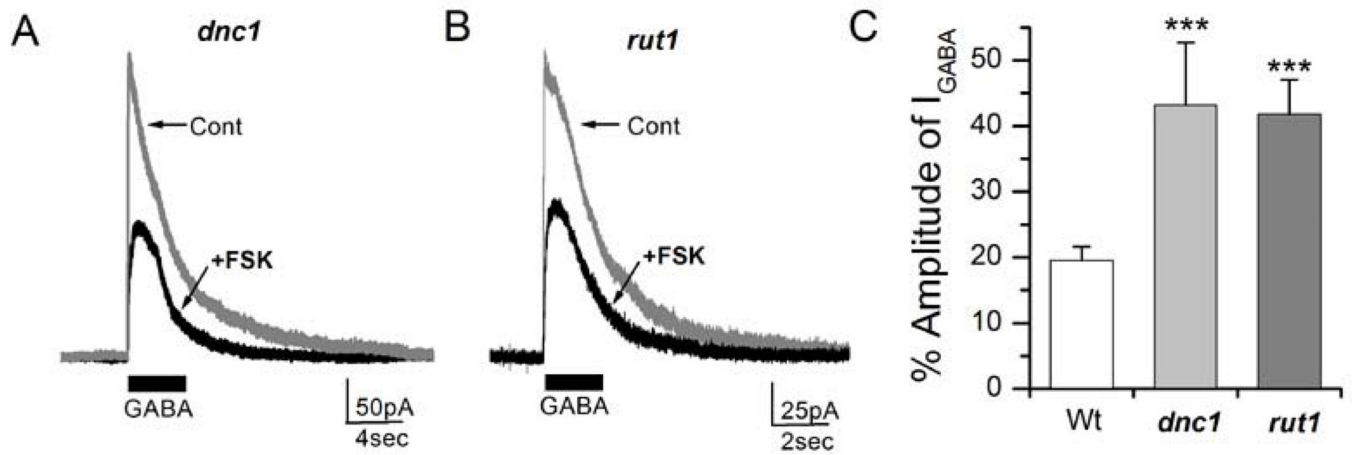


Figure 8. Forskolin effects on GABA-evoked currents (I_{GABA}) are attenuated in learning and memory mutants

A, GABA-evoked currents (I_{GABA}) were recorded from a *dnc1* neuron before and after perfusion of FSK (20 μ M). 100 μ M GABA was focally applied for 2 sec as indicated. **B**, Perfusion of FSK (20 μ M) suppressed I_{GABA} in a *rut1* neuron. **C**, Percent amplitude of I_{GABA} after perfusion of FSK. Data for wild type was from Figure 5C for comparison. Bars show SEM. Students t-test, *** P < 0.001. Number of replicates: Wt (n=5), *dnc1* (n=8), *rut1* (n=8).

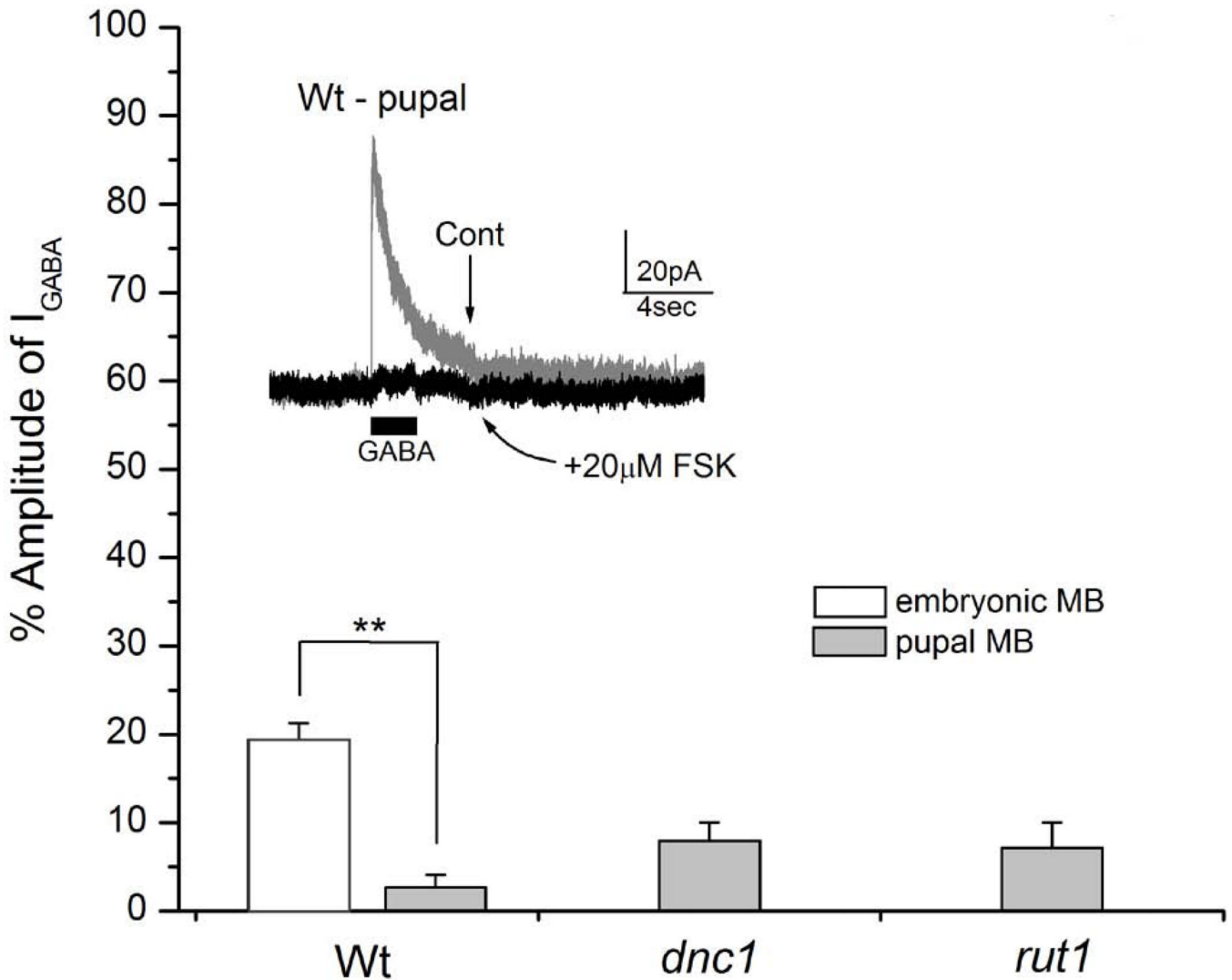


Figure 9. 20 μ M forskolin (FSK) markedly suppresses GABA-evoked currents (I_{GABA}) recorded in wild type, *dnc1* and *rut1* pupal mushroom body (MB) neurons

Cultured pupal MB neurons were prepared from wild type, *dnc1* and *rut1*. MB neurons were identified by GFP expression under the control of a MB-specific driver *c309-Gal4*. GABA-evoked currents (I_{GABA}) were suppressed in neurons derived from all three strains. Suppression for wild type, *dnc1* and *rut1* was 97.3, 92.0 and 92.9%, respectively. Although this suppression was slightly higher in wild type, there was no statistical difference between the three strains. In contrast, GABA-evoked currents (I_{GABA}) from wild type embryonic MB neurons showed reduced sensitivity to FSK as it suppressed only 80.7%. *Inset* I_{GABA} recorded from a wild type pupal MB neurons before and after 20 μ M FSK perfusion. Students t-test, ** $P < 0.01$. Number of replicates: Wt embryonic (n=7) and pupal (n=4), *dnc1* (n=4), *rut1* (n=6).

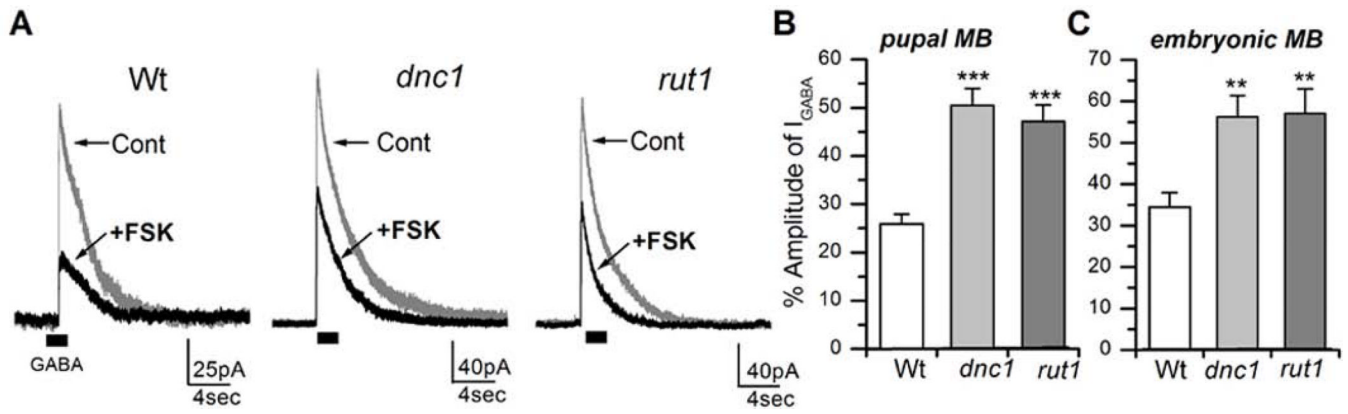


Figure 10. FSK (2 μ M) effects on GABA-evoked currents (I_{GABA}) are attenuated in pupal and embryonic mushroom body (MB) neurons prepared from learning and memory mutants
A, GABA-evoked currents (I_{GABA}) were recorded from wild type, *dnc1* and *rut1* pupal MB neurons before and after perfusion of 2 μ M FSK. 100 μ M GABA was focally applied for 2 seconds as indicated. **B**, Percent amplitude of I_{GABA} in pupal MB neurons after perfusion of FSK. **C**, Percent amplitude of I_{GABA} in embryonic MB neurons after perfusion of FSK. Bar = SEM. Students t-test, ** $P < 0.01$ and *** $P < 0.001$. Number of replicates: Wt (n=6), *dnc1* (n=6), *rut1* (n=6).

Table 1

Functional properties of GABAergic sIPSCs in wild type, *Rdl1* null, *dnc1* and *rut1* neuronal cultures.

strain	Incidence rate	Frequency (Mean±SEM)	Amplitude (Mean±SEM)
wild type	29.4% (10/34)	3.0±0.2 Hz (n=9)	10.3±1.4 pA (n=9)
<i>Rdl1</i> null	5.7% (3/52)	0.5±0.3 Hz (n=3)	9.4±1.0pA (n=3)
<i>dnc1</i>	20.3% (11/54)	1.7±0.2 Hz (n=11)	10.2±1.9 pA (n=9)
<i>rut1</i>	27.9% (12/43)	2.2±0.1Hz (n=11)	9.4±1.0 pA (n=9)