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Serotonin Inhibition of Synaptic Transmission: $G\alpha_o$ Decreases the Abundance of UNC-13 at Release Sites

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Summary

We show that serotonin inhibits synaptic transmission at *C. elegans* neuromuscular junctions, and we describe a signaling pathway that mediates this effect. Release of acetylcholine from motor neurons was assayed by measuring the sensitivity of intact animals to the acetylcholinesterase inhibitor aldicarb. By this assay, exogenous serotonin inhibited acetylcholine release, whereas serotonin antagonists stimulated release. The effects of serotonin on synaptic transmission were mediated by GOA-1 (a $G\alpha_o$ subunit) and DGK-1 (a diacylglycerol [DAG] kinase), both of which act in the ventral cord motor neurons. Mutants lacking *goa-1* $G\alpha_o$ accumulated abnormally high levels of the DAG-binding protein UNC-13 at motor neuron nerve terminals, suggesting that serotonin inhibits synaptic transmission by decreasing the abundance of UNC-13 at release sites.

Introduction

Monoamines are thought to play a pivotal role in modulating the activity of neural circuits, often controlling global behavioral states of an animal. For example, serotonin has been implicated in several aspects of mood and behavior, including depression (Maes and Meltzer, 1994), eating disorders (Tecott et al., 1995), alcohol consumption (Crabbe et al., 1996), and aggression (Berman et al., 1997; Saudou et al., 1994). This physiological function has been conserved across phylogeny, as serotonin controls the behavioral states of animals ranging from lobsters (Edwards and Kravitz, 1997) to the nematode *C. elegans* (Sawin, 1996; Waggoner et al., 1998) to humans (Soubrie, 1988; Maes and Meltzer, 1994; Berman et al., 1997).

C. elegans has been used as a genetic model to study serotonin signaling in vivo. *C. elegans* has only five classes of serotonergic neurons (Desai et al., 1988), one of which (NSM) is a neurosecretory cell that produces serotonin as a neurohormone (Albertson and Thomson, 1976). Several behaviors are modulated by serotonin, including locomotion (Horvitz et al., 1982; Ségalat et al., 1995), egg laying (Trent et al., 1983; Waggoner et al., 1998), and feeding (Avery and Horvitz, 1990). Six genes are required for serotonin stimulation of the egg laying muscles (Trent et al., 1983). Mutations in *unc-2*, which encodes a voltage-dependent calcium channel subunit, prevent adaptation of animals to serotonin-induced inhibition of locomotion (Schafer and Kenyon, 1995). Mutations that reduce the activity of *goa-1* $G\alpha_o$ confer partial resistance to serotonin-induced inhibition of locomotion (Mendel et al., 1995; Ségalat et al., 1995). Taken together, these studies define a set of genes that play some role in serotonin signal transduction. However, it remains unclear what cells are regulated by serotonin and how serotonin regulates the activity of these cells.

Serotonin often acts as a neuromodulator regulating transmission by other neurotransmitters. In some cases, serotonin has been shown to enhance synaptic transmission (Byrne and Kandel, 1996; Wang and Zucker, 1998), whereas, in other cases, serotonin inhibits synaptic transmission (Singer et al., 1996; McDearmid et al., 1997). We show here that serotonin inhibits synaptic transmission at *C. elegans* neuromuscular junctions and that this effect is mediated (at least in part) by reducing the abundance of presynaptic DAG-binding protein UNC-13 at release sites.

Results

cat-1 and *dgk-1* Mutants Have Defects in Serotonin Signaling

Exogenous serotonin reduces the rate of locomotion (Horvitz et al., 1982; Ségalat et al., 1995), whereas it stimulates the rate of egg laying (Trent et al., 1983; Weinshenker et al., 1995). The effects of serotonin on these two behaviors are likely mediated by distinct pathways, as particular mutations differentially alter these two aspects of serotonin function. For example, *goa-1* $G\alpha_o$ is required for serotonin-mediated inhibition of locomotion but not for serotonin-mediated stimulation of egg laying (Mendel et al., 1995; Ségalat et al., 1995). To identify new components of the serotonin signaling pathway, we isolated mutations that block serotonin inhibition of locomotion. We describe here two genes identified in this screen, *cat-1* and *dgk-1*.

The *cat-1* gene encodes a vesicular monoamine transporter (VMAT), which pumps serotonin and dopamine into synaptic vesicles (Duerr et al., 1999). We isolated *cat-1(nu90)* as a mutation that causes hyperactive locomotion (Figure 1A) and reduced egg laying rates (S. N. and J. M. K., unpublished data; Duerr et al., 1999), both consistent with an underlying defect in serotonin signaling. The *cat-1(e1111)* allele (Sulston et al., 1975; Desai et al., 1988) corresponds to a nonsense mutation (Duerr et al., 1999) and causes similar behavioral defects to those observed in *cat-1(nu90)* mutants (Duerr et al., 1999).

The *dgk-1* gene was defined by five recessive alleles. Homozygous *dgk-1* mutants had hyperactive locomotion (Figure 1A) and laid eggs constitutively (data not

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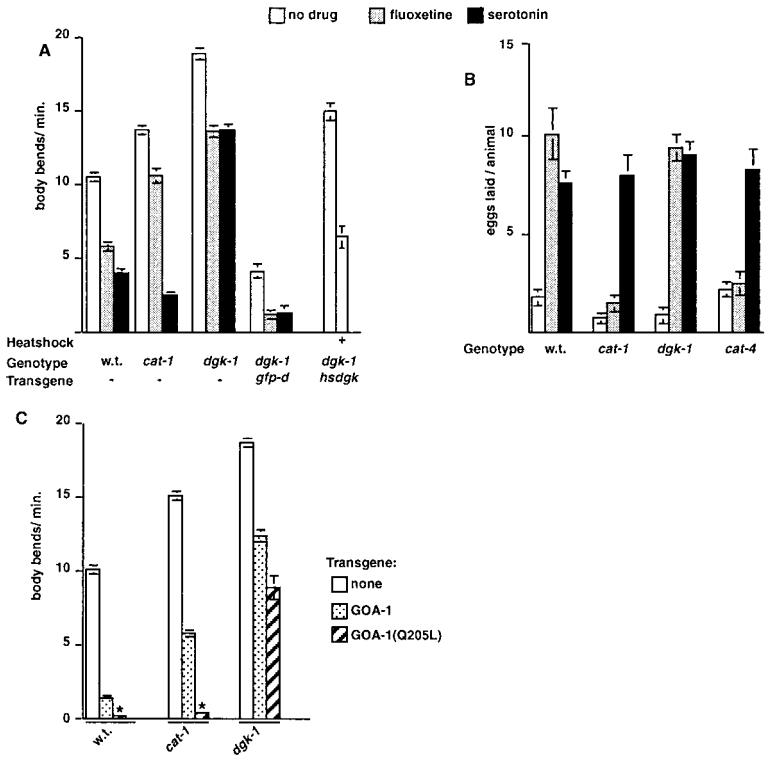

Figure 1. Serotonin and GOA-1 Signaling in *cat-1* and *dgk-1* Mutants

Behavioral assays and drug treatments and transgenic animals were as described in the Experimental Procedures. Drug treatments are indicated by the legends.

(A) Locomotion of *cat-1(e1111)* was partially resistant to fluoxetine but not to serotonin, whereas locomotion of *dgk-1(nu199)* was partially resistant to both. Animals (*nuIs45*) expressing *gfp::dgk-1* (*gfp-d*) or a heat shock–promoted *dgk1a* cDNA (*hsdgk*) had slower locomotion rates than wild type.

(B) Egg laying of *cat-1(e1111)* mutants was partially resistant to fluoxetine but was sensitive to serotonin, whereas that of *dgk-1(nu199)* mutants was fully sensitive to both.

(C) Effects of GOA-1 activity on locomotion. GOA-1 signaling was increased by expressing transgenes containing wild-type GOA-1 or GOA-1(Q205L). In *cat-1(e1111)* mutants, GOA-1 inhibited locomotion while the effect of GOA-1 activity on locomotion was significantly reduced in *dgk-1(nu199)* mutants. Errors indicate the standard error of the mean. Asterisk indicates animals expressing GOA-1(Q205L) whose standard errors were less than 0.12.

shown). In addition to these phenotypes, homozygous *dgk-1* mutants also tend to form large clumps of animals at the edges of the bacterial lawn (data not shown), which has been termed the bordering phenotype (de Bono and Bargmann, 1998). The bordering phenotype occurs in mutants lacking a putative neuropeptide receptor (NPR-1) (de Bono and Bargmann, 1998) but is not seen in other serotonin-defective mutants (e.g., *cat-1* and *cat-4*), suggesting that the *dgk-1* gene acts both in serotonin and in peptidergic signaling pathways.

dgk-1 Mutations Cause Defects in Serotonin Target Cells

The behavioral deficits observed in *dgk-1* mutants could reflect impaired function of either the serotonergic neurons or the serotonin target cells. In principle, one can distinguish between these possibilities by testing the response of mutant animals to exogenous serotonin or to fluoxetine, which exaggerates the effects of endogenous serotonin by inhibiting plasma membrane serotonin reuptake pumps. A normal response to fluoxetine requires function of both the serotonergic neurons and the serotonin-responding cells, whereas a normal response to serotonin only requires the latter (Trent et al., 1983; Weinshenker et al., 1995); therefore, responsiveness to these drugs allowed us to distinguish mutants with defects in these two cell types.

To confirm the validity of this approach, we tested whether responsiveness to these drugs correctly predicts the site of *cat-1* function. The *cat-1* VMAT is expressed in serotonergic and dopaminergic neurons, where it mediates transport of these monoamines into synaptic vesicles (Duerr et al., 1999). The locomotion and egg laying behaviors of *cat-1* mutants were partially resistant to fluoxetine but remained sensitive to exogenous serotonin (Figures 1A and 1B). Therefore, *cat-1* mutants fit the pharmacological profile predicted for a defect in the serotonergic neurons. Similarly, *cat-4* mutants, which have abnormally low levels of endogenous serotonin (Desai et al., 1988), also fit the profile for a defect in the serotonergic neurons (Figure 1B).

By contrast, the hyperactive locomotion of *dgk-1* mutants was partially resistant to both serotonin and fluoxetine (Figure 1A). These results suggested that *dgk-1* acts in the serotonin target tissues. Interestingly, not all serotonin targets required *dgk-1*, since the egg laying of *dgk-1* mutants was still stimulated by serotonin (Figure 1B). Thus, *dgk-1* is required for serotonin inhibition of locomotion but not for serotonin stimulation of egg laying.

dgk-1 Encodes a Diacylglycerol Kinase

We mapped the *dgk-1* gene to a small region on the left arm of the X chromosome between *aex-3* and *unc-1*. A cosmid clone (C06G11) from this region corrected the locomotion and egg laying defects of *dgk-1* mutants in transgenic animals (data not shown). All five *dgk-1* alleles corresponded to mutations in a gene (C09E10.2, accession U49946), which encodes a protein that is 38% identical to a human diacylglycerol (DAG) kinase, DGKθ (Houssa et al., 1997) (Figures 2A and 2B). Four *dgk-1* alleles caused very similar behavioral defects and potentially correspond to severe loss-of-function mutations. The alleles *nu62* and *n892* are nonsense mutations, while *nu199* and *n2949* disrupt donor and acceptor splicing consensus signals in intron 7, all of which should reduce DGK-1 activity. A fifth allele (*nu76*)

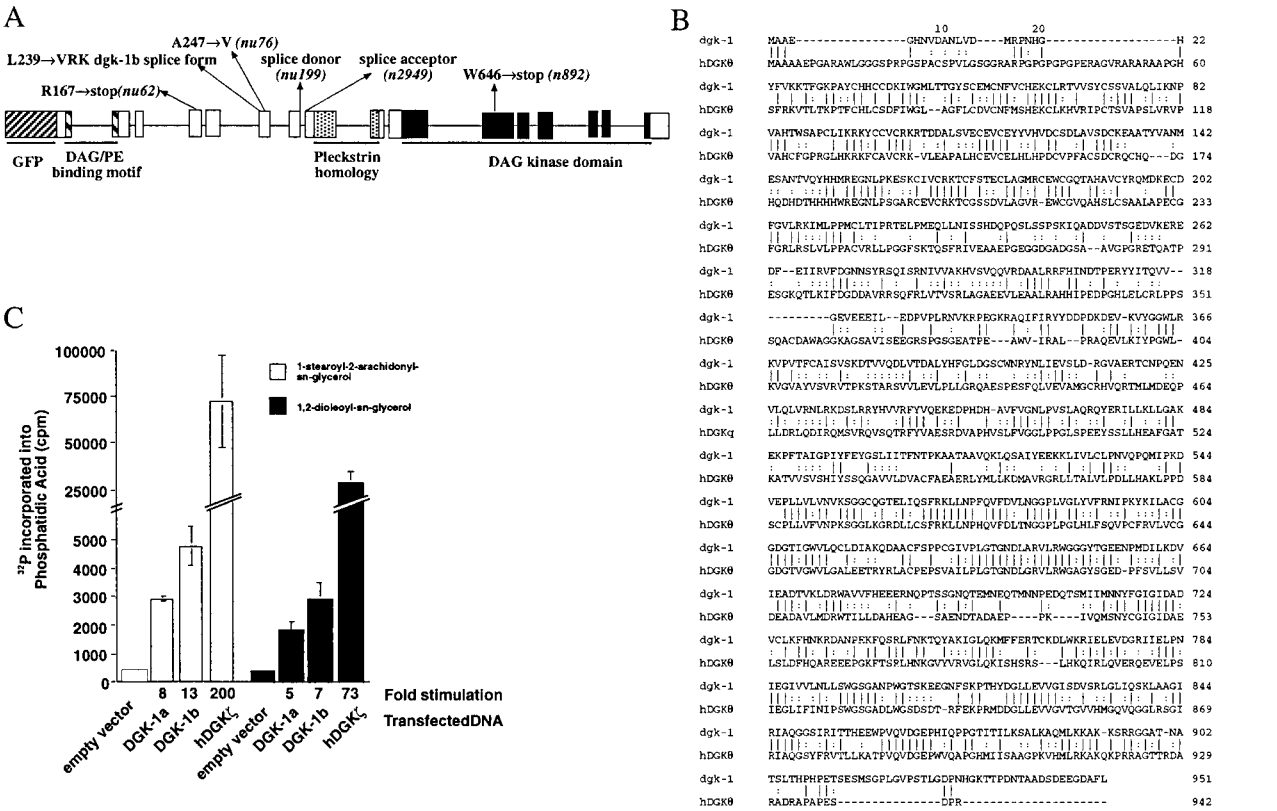

Figure 2. Cloning of *dgk-1*

(A) Genome structure of the *dgk-1* gene. Putative domains based on homology to DGKθ, *dgk-1b* splice form, and sequence changes in *dgk-1* alleles are indicated. The site of the GFP fusion is shown.

(B) Translated *dgk-1a* cDNA (KP#137) aligned with human DGKθ protein. Identities (38%) are indicated by vertical lines, similarities by dots.

(C) DGK-1 expressed in HEK 293 cells has DAG kinase activity using two different diglyceride substrates. Phosphatidic acid was isolated by thin layer chromatography and incorporation of ^{32}P into the phosphatidic acid band is a quantitative measure of the activity of diacylglycerol kinase activity. The activity of human DGKζ isoform is shown for comparison.

corresponds to a missense mutation (A247V) in a non-catalytic domain and causes less severe hyperactivity. Expression of a full-length *gfp::dgk-1* translational fusion construct (Figure 2A) in *dgk-1* mutants reduced the locomotion rate below that of wild-type animals and restored responsiveness to exogenous serotonin and fluoxetine (Figure 1A). These results demonstrated that the gene identified by these mutations encodes a putative DAG kinase. Furthermore, since wild-type transgenes rescued *dgk-1* mutant phenotypes, these results also suggest that *dgk-1* alleles reduce gene activity.

We isolated full-length DGK-1 cDNA clones and determined their sequence (Figure 2B). The predicted DGK-1 protein contains 950 amino acids, which share several salient features with human DGKθ. Like DGKθ, DGK-1 has three amino terminal cysteine-rich domains (which are thought to bind DAG), a potential pleckstrin homology domain, and a catalytic domain. Although it is highly expressed in the brain, the function of DGKθ in neural signaling has not been determined. Two alternatively spliced forms (DGK-1a and DGK-1b) were detected, which differ by three amino acids in a region of unknown function (Figure 2A). Transient expression of the DGK-1a cDNA in adult *dgk-1* mutants, utilizing a heat shock expression vector, restored normal locomotion rates, demonstrating that DGK-1a is a functional form of *dgk-1* DAG kinase and can act in adults (Figure 1A).

DAG kinases are encoded by a large gene family that has been conserved across phylogeny. The genome database predicts at least five distinct *C. elegans* DAG kinase isoforms, and twelve mammalian isoforms have been described (Topham and Prescott, 1999). All DAG kinases share a common enzymatic activity, phosphorylation of DAG to produce phosphatidic acid. To confirm that *dgk-1* encodes a catalytically active DAG kinase, we expressed full-length DGK-1 cDNAs in HEK293 cells (Figure 2C). Cells transfected with the DGK-1 expression construct produced significantly more DAG kinase activity than nontransfected cells, and this kinase was active with two different diglyceride substrates.

cat-1 VMAT Acts Upstream of *goa-1* $G\alpha_o$

Prior results suggested that *goa-1* $G\alpha_o$ mediates many of the effects of serotonin (Mendel et al., 1995; Ségalat et al., 1995). The similarity of the behavioral defects observed in *cat-1* and *dgk-1* mutants to those observed in *goa-1* mutants suggested that these genes are part of a common signaling pathway. To further examine the role of GOA-1 in serotonin signaling, we tested whether

Table 1. Analysis of Behaviors

Effect of CAT-1 on GOA-1 Signaling

	Body Bends/Minute ± SEM (Number of Animals)
Wild type	10.6 ± 0.2 (115)
cat-1(e1111)	14.1 ± 0.3 (70)
egl-10(md176)	2.8 ± 0.2 (20)
egl-10(md176); *cat-1(e1111)*	4.1 ± 0.4 (10)

DGK-1 Is Required for GOA-1 Regulation of Locomotion

	Body Bends/Minute ± SEM (Number of Animals)	
Genotype	No Transgene	GFP::DGK-1
Wild type	10.6 ± 0.2 (115)	ND
dgk-1(nu199)	18.7 ± 0.3 (80)	4.1 ± 0.4 (15)
goa-1(n1134)	18.1 ± 0.6 (10)	10.7 ± 1.0 (10)
egl-10(md176)	2.8 ± 0.2 (20)	ND
egl-10(md176); *dgk-1(nu199)*	15.3 ± 0.7 (10)	ND

Locomotion rates were compared in various strains, as detailed in the Experimental Procedures. The mutation *egl-10(md176)* eliminates a negative regulator of GOA-1, the RGS protein EGL-10 (Koelle and Horvitz, 1996). Values reported are means ± standard errors.

exaggerated GOA-1 activity would compensate for reduced release of endogenous serotonin (in *cat-1* mutants) or reduced serotonin signaling (in *dgk-1* mutants). Exaggerated GOA-1 activity was produced by three independent methods—eliminating an endogenous RGS protein (EGL-10) that negatively regulates GOA-1 (Koelle and Horvitz, 1996), overexpression of wild-type GOA-1 or of a GTPase-defective mutant GOA-1(Q205L). In all three cases, enhanced GOA-1 activity significantly reduced the locomotion rate of *cat-1* mutants, suggesting that *goa-1* $G\alpha_o$ acts downstream of *cat-1* VMAT (Figure 1C and Table 1), as would be predicted if *goa-1* $G\alpha_o$ mediates the effects of monoamine neurotransmitters on locomotion.

dgk-1 DAG Kinase Acts Downstream of or in Parallel with *goa-1* $G\alpha_o$

In contrast to the *cat-1* VMAT results, *dgk-1* loss-of-function mutations partially blocked the inhibition of locomotion caused by exaggerated *goa-1* $G\alpha_o$ signaling. Conversely, overexpression of GFP::DGK-1 reduced locomotion rates even in homozygous *goa-1* $G\alpha_o$ mutants (Figure 1C and Table 1). One simple model for DGK-1 function in locomotion would be that *goa-1* $G\alpha_o$ regulates *dgk-1* DAG kinase activity. Coexpression of GOA-1(Q205L) did not alter the catalytic activity of DGK-1 in transfected cells (data not shown); however, these results do not exclude the possibility that *goa-1* $G\alpha_o$ regulates *dgk-1* DAG kinase by a less direct mechanism. These results suggest that the *dgk-1* DAG kinase acts downstream of or in parallel with *goa-1* $G\alpha_o$ in serotonin target tissues.

The Serotonin Pathway Inhibits Acetylcholine Release by Motor Neurons

To test if serotonin modulates neurotransmission in *C. elegans*, we assayed synaptic release of acetylcholine by measuring sensitivity of animals to aldicarb (Figure 3A). Aldicarb enhances the effects of endogenously released acetylcholine by inhibiting acetylcholinesterase. The ventral cord motor neurons, which drive locomotion, utilize acetylcholine as their neurotransmitter (Rand and Nonet, 1997). Consequently, aldicarb treatment causes hypercontraction of the body wall muscles and paralysis in wild-type animals but not in mutants (e.g., synaptotagmin mutants) that are unable to release acetylcholine by exocytosis (Nonet et al., 1993; Nguyen et al., 1995; Miller et al., 1996). Wild-type animals treated with serotonin became resistant to aldicarb (Figure 3A; Table 2, line 2). By contrast, *cat-4* mutants, which have abnormally low levels of endogenous serotonin and dopamine (Sulston et al., 1975; Desai et al., 1988), were hypersensitive to aldicarb (Figure 3A). To confirm that the absence of serotonin is sufficient to cause aldicarb sensitivity, we also examined the effect of serotonin antagonists on aldicarb responsiveness. Wild-type animals treated with either of two serotonin antagonists, methiothepin (Figure 3A) or ketanserin (Table 2, line 4), also became hypersensitive to aldicarb, whereas treatment with the dopamine antagonist haloperidol (Table 2, line 5) had no effect. The effect of methiothepin on aldicarb sensitivity was mediated by a change in synaptic transmission at neuromuscular junctions, since *unc-29* mutants, which lack a body muscle acetylcholine receptor (AChR), were resistant to paralysis induced by treatment with methiothepin and aldicarb (Figure 3A). These results suggest that endogenous serotonin inhibits synaptic transmission at neuromuscular junctions.

The effect of serotonin on synaptic transmission could reflect inhibition of acetylcholine release by motor neurons or inhibition of the response of body muscles to acetylcholine. To distinguish between these possibilities, we tested the effect of serotonin treatment on the response to the acetylcholine agonist levamisole (Figure 3B). We found that the paralysis induced by levamisole in *cat-4* mutants and in wild-type animals treated with serotonin or serotonin antagonists was indistinguishable from that seen in untreated wild-type controls (Figure 3B). By contrast, *unc-29* AChR mutants were resistant to both aldicarb and levamisole. These results

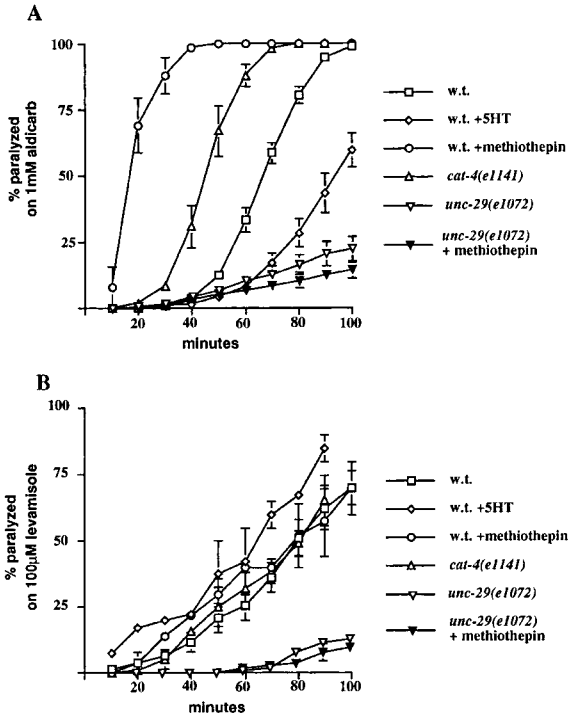

Figure 3. Serotonin Regulation of Neurotransmission

Synaptic release of endogenous acetylcholine (Ach) was measured by determining the onset of paralysis induced by the acetylcholinsterase inhibitor aldicarb.

(A) The *cat-4(e1141)* mutation reduces endogenous levels of serotonin and dopamine (Sulston et al., 1975; Desai et al., 1988) and caused hypersensitivity to aldicarb. Similarly, the serotonin antagonist methiothepin caused hypersensitivity to aldicarb. By contrast, treatment with exogenous serotonin (5-HT) reduced aldicarb sensitivity. Homozygous *unc-29(e1072am)* AChR mutants were resistant to paralysis induced by treatment with methiothepin and aldicarb.

(B) The sensitivity of body muscles to acetylcholine was measured by determining the onset of paralysis in response to the acetylcholine agonist levamisole. By this assay, untreated wild-type controls, serotonin-treated, methiothepin-treated, and untreated *cat-4* mutants were equally sensitive to levamisole, whereas homozygous *unc-29(e1072am)* AChR mutants were resistant to levamisole.

suggest that serotonin regulates synaptic transmission by inhibiting release of acetylcholine at neuromuscular junctions.

The Effects of Serotonin on Acetylcholine Release Require *goa-1* $G\alpha_o$ and *dgk-1* DAG Kinase

Since *goa-1* $G\alpha_o$ and *dgk-1* DAG kinase are required for serotonin inhibition of locomotion (Figure 1A), we wondered whether these genes are also required for serotonin inhibition of acetylcholine release. Serotonin treatment did not cause aldicarb resistance in mutants lacking either *goa-1* $G\alpha_o$ or *dgk-1* DAG kinase (Table 2, lines 7 and 14). In fact, homozygous *goa-1* $G\alpha_o$ (line 6) or *dgk-1* DAG kinase (line 13) mutants were hypersensitive to aldicarb compared to wild-type controls (line 1), consistent with an increased release of acetylcholine. Transient expression of either GOA-1(Q205L) or DGK-1a (utilizing a heat shock expression vector) made animals resistant to aldicarb (lines 9 and 16). In all cases, responses of mutant and treated animals to the acetylcholine agonist levamisole were not significantly different from controls (data not shown), suggesting that responsiveness of the body wall muscles to acetylcholine was not altered. Taken together, these results suggest that the serotonin/GOA-1/DGK-1 pathway regulates release of acetylcholine by the ventral cord motor neurons.

goa-1 $G\alpha_o$ and *dgk-1* DAG Kinase Act in Ventral Cord Motor Neurons

goa-1 $G\alpha_o$ is expressed in most neurons, including the ventral cord motor neurons that drive locomotion (Mendel et al., 1995; Ségalat et al., 1995). Both a transcriptional GFP reporter and a full-length *gfp::dgk-1* translational fusion were also expressed in most neurons, including the ventral cord motor neurons (Figures 4A and 4B). In addition to the neurons, the GFP reporters were also expressed in the excretory canals (Figure 4B). The GFP::DGK-1 fusion protein was localized to an organellar structure in the cell bodies of neurons and appeared to be diffusely distributed throughout the cell bodies and axons (Figure 4C). Thus, *goa-1* $G\alpha_o$ and *dgk-1* DAG kinase were both ubiquitously expressed in the worm nervous system.

Given their expression patterns, we wondered if *goa-1* $G\alpha_o$ and *dgk-1* DAG kinase act in the ventral cord motor neurons. We tested this hypothesis by specifically expressing transgenes that alter the activity of these proteins in motor neurons, utilizing a motor neuron specific expression vector. Expression of a constitutively active form of *goa-1* $G\alpha_o$ (Q205L) in motor neurons inhibited locomotion rate and made animals aldicarb resistant (Table 2, line 11). The *goa-1* $G\alpha_o$ subunit is predicted to by a substrate for ADP-ribosylation by pertussis toxin (Lochrie et al., 1991). Expression of the catalytic subunit of pertussis toxin in motor neurons made animals hypersensitive to aldicarb (Table 2, line 12) and produced hyperactive locomotion rates (data not shown). Similarly, expression of the GFP::DGK-1 fusion protein in motor neurons restored normal aldicarb responsiveness to homozygous *dgk-1* mutants (Table 2, line 17). These results suggest that *goa-1* $G\alpha_o$ and *dgk-1* DAG kinase act in motor neurons, directly or indirectly regulating release of acetylcholine at neuromuscular junctions. Given their broad expression patterns, these genes probably act in many other neural circuits as well.

goa-1 $G\alpha_o$ Regulates the Abundance of UNC-13 at Nerve Terminals

The *dgk-1* DAG kinase phosphorylates DAG, converting it into phosphatidic acid (Figure 2C). Therefore, eliminating the *dgk-1* DAG kinase is predicted to cause an increased abundance of DAG, which could explain the aldicarb hypersensitivity caused by *dgk-1* mutations. Consistent with this idea, we showed that treating wild-type animals with phorbol esters induced hypersensitivity to aldicarb (Table 2, line 3), suggesting an increased release of acetylcholine. Since *dgk-1* mutations also prevented *goa-1* $G\alpha_o$ induced paralysis, we wondered whether phorbol ester treatment would block *goa-1* $G\alpha_o$ inhibition of acetylcholine release. Consistent with this idea, we found that phorbol ester treatment produced

Table 2. Aldicarb Sensitivity of Serotonin Signaling Mutants

Serotonin Modulates ACh Release			
		Percent of Animals Paralyzed on 1 mM Aldicarb ± SEM	
Genotype [number of trials]	Drug	40 min	80 min
1. wt [28]		4 ± 1	81 ± 3
2. wt [11]	5-HT	2 ± 1	29 ± 5
3. wt [10]	PMA	99 ± 1	100 ± 0
4. wt [3]	ketanserin	32 ± 5	100 ± 0
5. wt [3]	haloperidol	7 ± 1	97 ± 1
GOA-1 Modulates ACh Release at the Motorneurons			
		Percent of Animals Paralyzed on 1 mM Aldicarb ± SEM	
Genotype [number of trials]	Drug	40 min	80 min
6. *goa-1(n1134)* [3]		100 ± 0	100 ± 0
7. *goa-1(n1134)* [3]	5-HT	100 ± 0	100 ± 0
8. *hs goa-1(Q205L)* − hs [3]		12 ± 5	100 ± 0
9. *hs goa-1(Q205L)* + hs [5]		0 ± 0	12 ± 3
10. *hs goa-1(Q205L)* + hs [3]	PMA	95 ± 3	100 ± 0
11. *p.acr-2::goa-1(Q205L)* [3]		8 ± 3	39 ± 2
12. *p.acr-2::ptx* [4]		39 ± 4	100 ± 0
DGK-1 Modulates ACh Release at the Motorneurons			
		Percent of Animals Paralyzed on 1 mM Aldicarb ± SEM	
Genotype [number of trials]	Drug	40 min	80 min
13. *dgk-1(nu199)* [6]		77 ± 9	100 ± 0
14. *dgk-1(nu199)* [3]	5-HT	72 ± 5	100 ± 0
15. *hs::dgk-1a* − hs [3]		11 ± 6	77 ± 3
16. *hs::dgk-1a* + hs [3]		1 ± 1	40 ± 4
17. *dgk-1(nu199)*; *p.acr-2::gfp-dgk-1* [4]		19 ± 3	88 ± 4

The time course of aldicarb-induced paralysis was compared for the indicated strains and drug treatments, as detailed in the Experimental Procedures. Two informative time points are shown for each condition. An early time point (40 min) is shown to illustrate hypersensitivity to aldicarb. A late time point (80 min) is shown to illustrate resistance to aldicarb. Abbreviations are as follows: 5-HT, serotonin; PMA, phorbol esters; *acr-2*, motor neuron specific expression vector; and hs, heat shock. Values reported are means ± standard errors.

hypersensitivity to aldicarb even when the constitutive form of *goa-1* $G\alpha_o$ (Q205L) was expressed (Table 2, line 10). One possible model to explain these results is that *goa-1* $G\alpha_o$ inhibits acetylcholine release by reducing the levels of DAG at nerve terminals. However, these results do not exclude alternative models where DAG and *goa-1* $G\alpha_o$ antagonistically regulate acetycholine release even though *goa-1* $G\alpha_o$ does not regulate the abundance of DAG.

If *goa-1* $G\alpha_o$ regulates the abundance of DAG in motor neurons, then we might expect that *goa-1* $G\alpha_o$ would regulate the subcellular distribution of DAG-binding proteins in motor neurons. Phorbol esters (which directly bind to C1 domains) have been shown to promote membrane association of several DAG-binding proteins (Betz et al., 1998; Oancea et al., 1998). UNC-13 is a presynaptic DAG-binding protein that stimulates neurotransmitter release (Maruyama and Brenner, 1991; Ahmed et al., 1992; Kazanietz et al., 1995). These results suggested that a local change in DAG levels at neuromuscular junctions could be visualized by observing a change in the abundance of UNC-13 at nerve terminals.

To test this idea, we constructed an expression vector for the short form of UNC-13 (UNC-13S, accession 3881735), which contains 1293 amino acids including the C1 domain, both C2 domains, and the domain predicted to bind syntaxin (Betz et al., 1997), fused to GFP at the carboxy terminus of UNC-13S. Expression of UNC-13S::GFP in *unc-13(e51)* mutants restored normal locomotion rates and normal responsiveness to aldicarb, suggesting that UNC-13S activity is sufficient to reconstitute synaptic transmission (data not shown). We compared the distribution of UNC-13S::GFP in wild-type animals and those with various defects in the serotonin and GOA-1 signaling pathway (Figure 5). In wild-type animals, UNC-13S::GFP was found in a diffuse staining pattern in both the cell bodies and in the axons of the ventral nerve cord, with a few punctate structures in the axons (Figure 5A; Table 3). The distribution of UNC-13S::GFP was not significantly altered in mutants lacking *dgk-1* DAG kinase (Figure 5E; Table 3), nor in animals treated with phorbol esters (data not shown). We observed a significant increase in the number of UNC-13S::GFP punctate structures in the ventral nerve cords of homozygous *goa-1* mutants compared to wild-type controls (Figure 5C; Table 3). Mutants that lack endogenous serotonin (e.g., *cat-4*) and wild-type animals treated with the serotonin antagonist methiothepin had a slight increase in the number of UNC-13S::GFP puncta (Figure 5B and Table 3). To test the importance of DAG

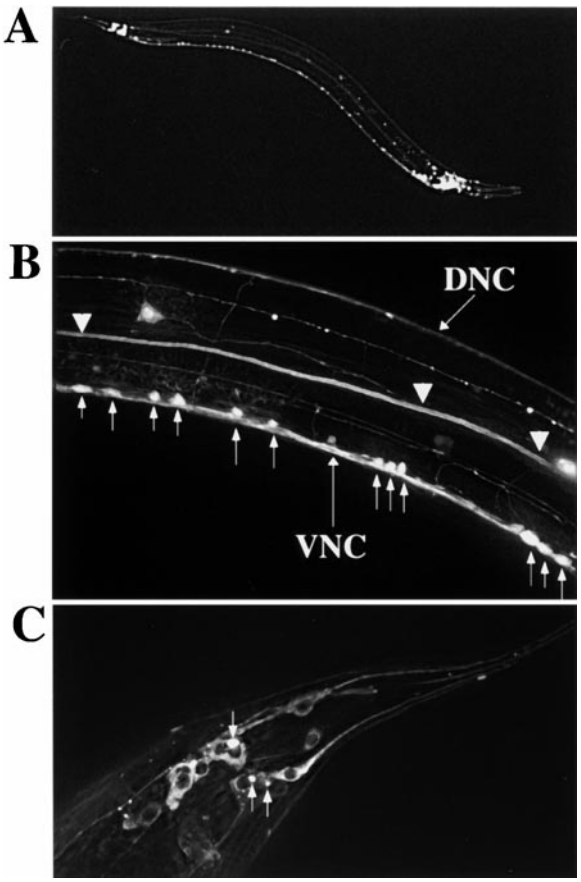

Figure 4. Expression of DGK-1

(A) A transcriptional GFP reporter construct (*nuIs15*) is expressed in many or all neurons, including cells in the in the head and tail ganglia, (B) the ventral cord motor neurons (arrows), axons in the ventral and dorsal nerve cords (VNC and DNC), and in the excretory canals (arrowheads). The full-length *gfp::dgk-1* translational fusion (*nuIs45*) has a similar expression pattern (data not shown). (C) Subcellular localization of the DGK-1 protein was examined with the full-length *gfp::dgk-1* translational fusion in *nuIs45* animals. This fusion construct rescues the *dgk-1* mutant phenotype. In general, GFP::DGK-1 is uniformly distributed throughout the cell body and axons of expresssing cells. In cell bodies, GFP::DGK-1 is localized in a large perinuclear structure (arrows).

binding for UNC-13S localization, we expressed a mutant form of UNC-13S, H173K, in which we altered a conserved histidine residue in the C1 domain that is required for binding to phorbol esters (Betz et al., 1998). Unlike the wild-type protein, UNC-13S(H173K)::GFP remained diffusely distributed in *goa-1* $G\alpha_o$ mutants (Figure 5D and Table 3). Thus, *goa-1* $G\alpha_o$ regulates the distribution of UNC-13S::GFP in the ventral nerve cord, and this effect depends upon the ability of UNC-13S to bind to DAG.

To determine if the punctate structures containing UNC-13S::GFP corresponded to acetylcholine release sites at neuromuscular junctions, we conducted double labeling experiments. Neuromuscular junctions were labeled by expressing a cyan fluorescent protein tagged synaptobrevin construct (SNB-1::CFP) in motor neurons (Rongo et al., 1998). UNC-13S was visualized by expressing a yellow fluorescent protein tagged UNC-13S under the control of the *unc-13S* promoter. In animals coexpressing these two constructs, we see a clear colocalization of the UNC-13S::YFP punctate structures with clusters of SNB-1::CFP (Figure 5F). In homozygous *goa-1* $G\alpha_o$ mutants, 81% of the SNB-1::CFP puncta at motor neuron nerve terminals colocalized with a corresponding UNC-13S::YFP punctum (n = 162 SNB-1::CFP puncta and nine animals). These results confirm that UNC-13S accumulates at acetylcholine release sites of motor neurons in mutants lacking *goa-1* $G\alpha_o$. Our data are most consistent with the model that serotonin and GOA-1 specifically regulate DAG levels at nerve terminals and, as a consequence control, the abundance of UNC-13 at acetylcholine release sites.

Discussion

We showed that serotonin modulates *C. elegans* locomotion by inhibiting release of acetylcholine at neuromuscular junctions. The effects of serotonin on acetylcholine release require *goa-1* $G\alpha_o$ and *dgk-1* DAG kinase, which act in the ventral cord motor neurons. Finally, we showed that *goa-1* $G\alpha_o$ regulates the abundance of the presynaptic protein UNC-13S at motor neuron nerve terminals. Based on these results, we propose a model for presynaptic inhibition by serotonin (Figure 6), which suggests that serotonin directly regulates some aspect of synaptic vesicle exocytosis or recycling, at least in this case.

The Role of *goa-1* $G\alpha_o$ in Aminergic Signaling

Prior work (Mendel et al., 1995; Ségalat et al., 1995) showed that *goa-1* mutants behave like animals that lack serotonin and are partially resistant to the behavioral effects of exogenous serotonin. While these results suggest that GOA-1 is required in some manner for serotonin signaling, they do not allow one to distinguish whether GOA-1 is directly involved in serotonin signal transduction or is a component of an interacting pathway. Our current results provide further support for the idea that GOA-1 plays a direct role in serotonin signaling, perhaps by coupling directly to serotonin receptors. First, *goa-1* $G\alpha_o$ acts downstream or parallel to serotonin release (as evidenced by epistasis experiments with *cat-1* VMAT mutations). Second, GOA-1 is required for serotonin-mediated inhibition of synaptic transmission. Third, both serotonin and GOA-1 regulate the localization of UNC-13 at motor neuron nerve terminals, suggesting that both act directly at the ventral cord neuromuscular junctions.

On the other hand, it is likely that GOA-1 also plays an important role in other neurotransmitter pathways. The effects of GOA-1 on egg laying cannot be explained by a defect in serotonin signaling. Serotonin is an excitatory neurotransmitter acting at the egg laying neuromuscular junctions, yet *goa-1* $G\alpha_o$ inhibits egg laying (Mendel et al., 1995; Ségalat et al., 1995). In addition, the behavioral defects observed in *goa-1* mutants are much more severe than those seen in *cat-1* mutants. These results suggest that GOA-1 also mediates the effects of other unidentified neuromodulators. Consistent with this idea, we found a substantially larger number of UNC-13S::GFP containing puncta in the ventral

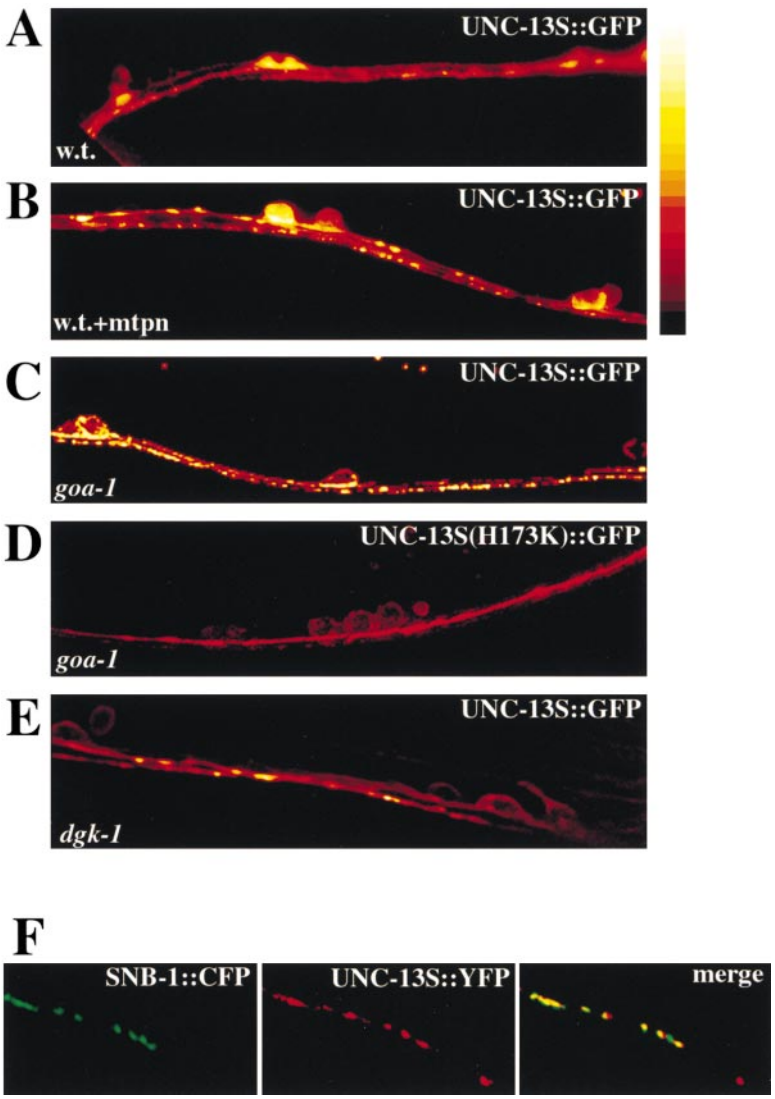

Figure 5. Serotonin Regulates the Distribution of UNC-13S::GFP in the Ventral Nerve Cord

(A–C) UNC-13S::GFP (expressed from the internal *unc-13S* promoter) was primarily diffusely distributed in the ventral nerve cord axons of untreated wild-type animals (A) but became more punctate in the ventral cords of animals treated with methiothepin (B), in *cat-4(e1141)* mutants (data not shown), and in homozygous *goa-1(n1134)* mutants (C).

(D) Unlike the wild-type protein, UNC-13S (H173K)::GFP, which is predicted not to bind to DAG (Betz et al., 1998), remains diffusely distributed in homozygous *goa-1(n1134)* mutants.

(E) UNC-13S::GFP remained primarily diffuse in homozygous *dgk-1* mutants.

(F) UNC-13S is localized to acetylcholine release sites in homozygous *goa-1(n1134)* $G\alpha_o$ mutants. Motor neuron nerve terminals and UNC-13S were simultaneously visualized in *goa-1(n1134)* mutants animals with CFP tagged synaptobrevin (left), and YFP tagged UNC-13S (middle). In the merged image (right), it is clear that most SNB-1::CFP puncta are colocalized with a UNC-13S::YFP punctum. The SNB-1::CFP construct was specifically expressed in motor neurons (with the *acr-2* promoter). The UNC-13S::YFP construct was expressed throughout the nervous system with the *unc-13S* promoter.

In (A)–(C) and (E), the integrated transgene *nuIs46* was used; in (D) and (F), transgenes were carried as extrachromosomal arrays. In (A)–(E), digital images were converted from grayscale into a 32 color look-up table (NIH Image) to visualize pixel intensities.

nerve cords of *goa-1* mutants than in *cat-4* mutants, which have reduced levels of serotonin and dopamine.

The Mechanism of *goa-1* $G\alpha_o$ Action

Our genetic results are most consistent with the model that *goa-1* $G\alpha_o$ decreases the abundance of DAG at nerve terminals. This conclusion is based on two results. First, manipulations that increase DAG levels (treatment with phorbol esters, *dgk-1* mutations) restored acetylcholine release to animals expressing a constitutive form of *goa-1* $G\alpha_o$. Second, wild-type UNC-13S::GFP accumulated at nerve terminals in *goa-1* homozygotes, but UNC-13S(H173K)::GFP did not.

Our results are equally consistent with either of two models for *goa-1* $G\alpha_o$ function. First, *goa-1* $G\alpha_o$ could stimulate the activity of the *dgk-1* DAG kinase, thereby

Table 3. Analysis of UNC-13::GFP Puncta

Genotype [Number of animals]	Transgene	GFP Puncta/10 μm ± SEM
1. wild type [11]	UNC-13S::GFP	1.0 ± 0.2
2. wild type + methiothepin [10]	UNC-13S::GFP	1.8 ± 0.2
3. *cat-4(e1141)* [5]	UNC-13S::GFP	1.5 ± 0.1
4. *goa-1(n1134)* [11]	UNC-13S::GFP	2.5 ± 0.2
5. *goa-1(n1134)* [7]	UNC-13S(H173K)::GFP	0.7 ± 0.1
6. *dgk-1(nu199)* [10]	UNC-13S::GFP	1.4 ± 0.3

Localization of UNC-13S::GFP is controlled by serotonin and *goa-1* $G\alpha_o$. Numbers of UNC-13S::GFP puncta in the ventral nerve cords were compared for the indicated strains and drug treatments, as detailed in the Experimental Procedures. For comparison, the number of puncta containing a mutant form of UNC-13 that is predicted not to bind DAG [UNC-13S(H173K)::GFP] is also shown. Digital images were thresholded according to pixel intensity, and puncta were counted manually. Values reported are means ± standard errors.

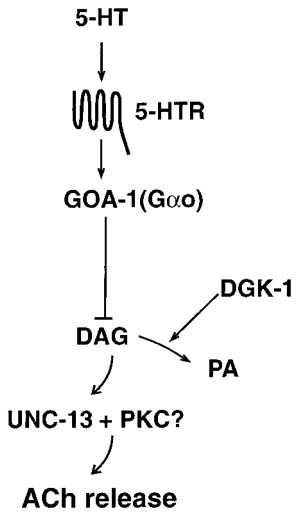

Figure 6. A Model for Serotonin Inhibition of Synaptic Transmission

We propose that presynaptic DAG is a critical second messenger, determining the amount of transmitter released by exocytosis. Exogenous phorbol esters cause hypersensitivity to aldicarb, implying that DAG at motor neuron nerve terminals facilitates acetylcholine release. The inhibitory neuromodulator serotonin activates GOA-1, thereby decreasing DAG levels at nerve terminals. Presynaptic DAG recruits UNC-13 to the neurotransmitter release sites, promoting association with the nerve terminal membrane. Accumulation of UNC-13 at release sites could provide a mechanism for DAG-mediated stimulation of synaptic transmission. Other published experiments suggest that presynaptic DAG also stimulates release via protein kinase C (Stevens and Sullivan, 1998). DGK-1 depletes DAG levels at nerve terminals by converting DAG into phosphatidic acid (PA).

decreasing DAG levels at nerve terminals. We did not detect GOA-1 regulation of DGK-1 catalytic activity in transfected cells. Nonetheless, it remains possible that GOA-1 regulates DGK-1 through a less direct mechanism. For example, GOA-1 might regulate DGK-1 activity through an intervening small GTP-binding protein (e.g., Rac or Rho) (Tolias et al., 1998; Houssa et al., 1999). Alternatively, *goa-1* $G\alpha_o$ could regulate DAG levels by another mechanism with *dgk-1* DAG kinase acting in parallel to regulate DAG levels. For example, *goa-1* $G\alpha_o$ might inhibit *egl-30* $G\alpha_q$, a GTP-binding protein that stimulates phospholipase C activity (Brundage et al., 1996). Both of these possibilities are consistent with our genetic analysis. Since these pathways are both biosynthetic (producing DAG) and regulatory, mutations in the biosynthetic pathway (*egl-30* $G\alpha_q$) are predicted to be epistatic to those in the *goa-1* $G\alpha_o$ pathway in either of these scenarios, which we have observed (M. Lackner and J. M. K., unpublished data). Therefore, these models can only be distinguished by biochemical experiments.

Why doesn't the distribution of UNC-13S change in *dgk-1* DAG kinase mutants or in animals treated with phorbol esters? In both of these cases, acetylcholine release is enhanced, yet UNC-13S::GFP remained diffusely distributed. The diffuse pattern of UNC-13S::GFP in these cases could reflect the fact that DAG levels are elevated uniformly throughout the ventral cord axons, rather than locally at neuromuscular junctions. For example, since DGK-1::GFP appears to be uniformly distributed throughout the ventral cord, then it is likely that DAG is elevated throughout the ventral cord in *dgk-1* mutants. Although DAG is diffusely distributed in these cases, its increased levels at release sites would nonetheless stimulate acetylcholine release. By contrast, DAG could be selectively elevated at neuromuscular junctions in *goa-1* mutants, leading to the punctate distribution of UNC-13S::GFP. Alternatively, localization of UNC-13S could require the coincidence of two distinct signals. For example, since it contains both C1 and two C2 domains, it is possible that UNC-13S localization is dependent on both DAG and calcium. In this scenario, *dgk-1* DAG kinase mutants would only elevate DAG levels and, hence, would not alter UNC-13S distribution. By contrast, if *goa-1* $G\alpha_o$ regulates both DAG and calcium levels, then changes in *goa-1* $G\alpha_o$ activity would alter the distribution of UNC-13S.

Implications for the Mechanism of Presynaptic Inhibition

Serotonin, *goa-1* $G\alpha_o$, and *dgk-1* DAG kinase all appear to inhibit synaptic transmission at neuromuscular junctions. As both *goa-1* $G\alpha_o$ and *dgk-1* DAG kinase act in motor neurons in this process, we propose that serotonin acts directly on motor neuron nerve terminals, inhibiting synaptic transmission and thereby reducing locomotion rates. Consistent with this idea, a GFP reporter construct for a candidate *C. elegans* serotonin receptor gene is expressed in a subset of the ventral cord motor neurons (T. Niacaris and L. Avery, personal communication), suggesting that serotonin does in fact act directly on these cells. Serotonin is likely acting as a neurohormone in this case, since none of the serotonergic neurons are predicted to provide direct synaptic input to the motor neurons (White et al., 1986). Although this is the simplest explanation of our results, it remains possible that serotonin acts more centrally (e.g., in sensory or interneurons), even though *goa-1* $G\alpha_o$ and *dgk-1* DAG kinase act in the motor neurons.

How does serotonin inhibit neurotransmission? Our results suggest that DAG is an important second messenger in this process (Figure 6). One potential target of DAG in synaptic transmission is the DAG-binding protein UNC-13, which is required for exocytosis of synaptic vesicles (Maruyama and Brenner, 1991; Ahmed et al., 1992; Kazanietz et al., 1995). Recently published results showed that overexpression of munc13–1, a mammalian ortholog of UNC-13, confers enhanced DAG-stimulated transmission at frog neuromuscular junctions (Betz et al., 1998). Our results also support the model that facilitation of synaptic transmission by DAG is mediated (in part) by UNC-13S. We propose that in mutants lacking either GOA-1 or serotonin, DAG levels at the acetylcholine release sites increase thereby recruiting UNC-13S to neuromuscular junctions (Figure 6). Once localized, UNC-13S could promote release of acetylcholine, perhaps through its interactions with the target SNARE syntaxin (Betz et al., 1997). Thus, both of these results suggest that DAG directly regulates some aspect of synaptic vesicle exocytosis or recycling. On the other hand, our results do not exclude the possibility

that other DAG-binding proteins (e.g., PKC) also contribute to the behavioral effects of serotonin.

Several published reports suggested that presynaptic DAG stimulates release of transmitter (Malenka et al., 1986; Shapira et al., 1987; Segal, 1989; Stevens and Sullivan, 1998). Similarly, we showed that exogenous phorbol esters stimulate acetylcholine release in *C. elegans*. Here, we show that an inhibitory neuromodulator also relies on DAG as a second messenger; however, in this case, by reducing presynaptic DAG. Thus, we speculate that parallel pathways for production and consumption of DAG at synaptic release sites could provide a simple, elegant mechanism for positive and negative modulation of synaptic transmission (Figure 6).

Interestingly, all of the components in the serotonin pathway are conserved in mammals—including CAT-1 (VMAT2), GOA-1 ($G\alpha_o$), DGK-1 (DGKθ), and UNC-13 (munc13–1). Therefore, it is possible that this DAG-sensing mechanism represents a conserved signaling cassette for modulation of synaptic transmission. In particular, given that the DAG kinases constitute a family of at least 12 genes in mammals (Topham and Prescott, 1999), we speculate that the different DAG kinase isoforms are utilized to couple different inhibitory neuromodulators to synaptic transmission. If this were the case, DAG kinases might represent ideal targets for drugs to manipulate the effects of different neuromodulators.

Experimental Procedures

Isolation of *cat-1* and *dgk-1* Mutations

The *cat-1* allele *nu90* and three *dgk-1* alleles (*nu199*, *nu76*, and *nu62*) were isolated as hyperactive mutants following mutagenesis with ethylmethane sulfonate. *cat-1(e1111)* was isolated previously in a screen for mutations that reduce the abundance of endogenous dopamine (Sulston et al., 1975). The *nu90* allele is a missense mutation in the *cat-1* VMAT (A406V). Two *dgk-1* alleles were provided by M. Finney (*n892*) and E. Sawin (*n2949*). All of these alleles have a purely recessive pattern of heredity. Allelism was determined by complementation tests and by meiotic mapping.

Positional Cloning of *dgk-1*

We mapped the *dgk-1* gene to a small region on the left arm of the X chromosome between *aex-3* and *unc-1*. A cosmid clone (C06G11) from this region corrected the locomotion and egg laying defects of *dgk-1* mutants in transgenic animals (data not shown). Sequence changes in mutant alleles were determined by amplifying exons and exon/intron boundaries from mutant strains and direct sequencing of the amplified products by cycle sequencing.

Transgenes and Germline Transformation

Plasmids were constructed by standard techniques, and sequences were verified where appropriate; full details are available on request. Transgenic strains were isolated by microinjecting various plasmids (typically at either 50 or 100 ng/μl) using *ttx-3::gfp* (a gift of O. Hobert) as a marker. Plasmids and transgenic strains were constructed as follows.

DGK-1 Constructs

KP#174, a rescuing full-length *gfp::dgk-1* fusion gene, contains GFP fused in-frame at the amino terminus. *nuIs45* contains an integrated version of the KP#174 transgene. KP#329, a *dgk-1::gfp* fusion construct, contains GFP fused in-frame at a unique XhoI site in exon 8 of *dgk-1*. *nuIs15* contains an integrated version of the KP#329 transgene. KP#137 and KP#143 are full-length (2.85 kb) DGK-1a and DGK-1b cDNA clones in the cytomegalovirus expression vector GW1 (Choi et al., 1991). KP#207 is a heat shock–promoted DGK-1a cDNA expression vector derived from PD49.78 (a gift of A. Fire), and *nuIs41* contains an integrated version of the KP#207 transgene.

GOA-1 Transgenes

nuIs8 (a gift of A. Berger) contains an integrated wild-type GOA-1 transgene. *syIs17* (a gift of J. Mendel) contains an integrated heat shock–promoted GTPase-defective GOA-1(Q205L) transgene (Mendel et al., 1995).

UNC-13 Transgenes

KP#268 encodes a full-length *unc-13S::gfp* fusion gene, which restored normal locomotion to animals carrying two different *unc-13* alleles (*e51* and *e450*). It contains a genomic fragment encompassing residues 1074–18913 of the ZC524 cosmid sequence (accession Z73912); GFP was fused in frame at the carboxy terminus. *nuIs46* contains an integrated version of the KP#268 transgene. KP#291 is identical to KP#268, except GFP is replaced by YFP (Rongo et al., 1998). KP#273 is also identical to KP#268, except it contains a point mutation resulting in the amino acid change H173K.

acr-2 *Transgenes*

Expression of trangenes in ventral cord motor neurons was accomplished with the *acr-2* promoter (a gift of Y. Jin), which is expressed in VA, DA, VB, DB, DA, IL1, RMD, and PVQ neurons (Y. Jin, personal communication). KP#305 contains the mature S1 subunit of PTX (a gift of T. Wilkie) in an *acr-2* expression vector, KP#338 contains a *gfp-dgk-1a* minigene containing the DGK-1a cDNA and the first intron of DGK-1 fused to GFP at the N terminus in the *acr-2* vector, KP#284 contains a *goa-1* $G\alpha_o$(Q205L) cDNA in the *acr-2* vector, and KP#282 contains a SNB-1::CFP fusion (a gift of C. Rongo) in the *acr-2* vector. The pattern of cells expressing GFP in animals carrying the *acr-2::gfp-dgk-1* construct was the same as that described for the *acr-2* promoter.

Analysis of Behaviors and Drug Sensitivities

Drugs were purchased from Research Biochemicals or Sigma. Locomotion and egg laying assays were performed as described (Trent et al., 1983; Ségalat et al., 1995), except we measured eggs laid in liquid medium (M9 salts) over 2 hr. Drug treatments were as follows (in mg/ml): for locomotion, 3.8 serotonin creatine sulfate, 0.125 fluoxetine; for egg laying, 3.8 serotonin creatine sulfate, 0.25 fluoxetine. Behaviors were assayed 2 hr after exposure to drugs. For locomotion rates, each animal was assayed three times for 1 min each. At least ten animals were tested in all cases. For animals expressing GOA-1(Q205L) (*syIs17*), behaviors were analyzed 1 hr after a 30 min heat shock at 33°C.

Sensitivity to aldicarb and levamisole was determined by analyzing the onset of paralysis following treatment with either 1 mM aldicarb (Chem Services) or 100 μM levamisole (Sigma). Locomotion was assessed by prodding animals with a platinum wire every 10 min following exposure to drug. Where indicated, animals were pretreated (for 2 hr) prior to aldicarb treatment together with a second drug, as follows: with 3.8 mg/ml serotonin creatine sulfate; with 2 μg/ml Phorbol 12-myristate 13-acetate (PMA); with 1 mg/ml ketanserin or 50 μg/ml methiothepin or 1 mg/ml haloperidol; with transient expression of GOA-1(Q205L) or DGK-1a from heat shock expression vectors for 30 min at 33°C. For each experiment, 25 animals were tested blind with respect to the genotype or drug treatment. Each experiment was repeated at least three times.

Diacylglycerol Kinase Assays

HEK 293 cells were transfected with 1 μg of either GW1, KP#137, KP#143, or FLAG DGKζ (a gift of S. Prescott) using lipofectamine (GIBCO). DAG kinase activity in 7.5 μg of cell extract was assayed 48 hr after transfection, essentially as described (Tolias et al., 1998). Phosphorylated lipids were quantified by scintillation counting.

Fluorescence Microscopy

GFP containing strains were mounted on agarose pads and viewed on an Aviovert microscopes, using a Planapo 63× (NA = 1.4), Planneo 40× (NA = 1.3) (Figure 4B), or Planneo 10× (NA = 0.3) (Figure 4A) objective. Images were captured with a Hamamatsu ORCA digital camera. Digital images were processed to remove out of focus light and to give maximum intensity projections of a z series, using Metamorph 4.0 image processing software (Universal Imaging Corporation). UNC-13 puncta were thresholded using Metamorph and

counted by eye. UNC-13S::GFP and UNC-13S(H173K)::GFP in *goa-1* mutants were quantified together blind to the identity of the transgene.

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Note Added in Proof

While our work was in review, Sternberg and colleagues published a paper describing a genetic locus, *sag-1*, which is required for GOA-1-mediated regulation of locomotion: Hadju-Cronin, Y.M., Chen, W.J., Patikoglou, G., Koelle, M.R., and Sternberg, P.W. (1999). Antagonism between $G_o\alpha$ and $G_q\alpha$ in *Caenorhabditis elegans*: the RGS protein EAT-16 is necessary for $G_o\alpha$ signaling and regulates $G_q\alpha$ activity. Genes Dev. *13*, 1780–1793. In collaboration with the Sternberg lab, we have shown that *sag-1* and *dgk-1* are the same gene.