Not all RGC axonal arbors responded to either BDNF or anti-BDNF. A subset of arbors in tadpoles treated with BDNF or anti-BDNF showed behaviours and rates of branch elaboration similar to those in control-treated tadpoles (Figs 3/and 4). Thus presenting the quantitative data as averages (see Fig. *3ci)* underrepresents the magnitude of either BDNF or anti-BDNF effects on arborization in the most affected cases. The lack of noticeable effects on a subgroup of RGC axons is consistent with our previous findings that not all RGCs express the ***trkB*** gene'?. This predicts that a subpopulation of RGCs should be neither responsive to. nor dependent on. BDNF. Indeed. a subpopulation of RGCs survives and differentiates in culture. even in the presence of neutralizing antibodies to BDNF (not shown). Consistent with this prediction of a mixed response, in some cases in which two arbors were followed simultaneously in the same animal. one arbor responded to the BDNF or anti-BDNF treatment but the other did not (in three of four animals). Thus those cases in which the neurotrophin or antibodies had no effect appear to result from a lack of neurotrophin responsiveness by individual RGCs, rather than to failed access of the agents. Consequently, our *in vivo* data indicate that BDNF has a direct effect on axon arborization in most RGCs. probably affecting those expressing TrkB. the specific receptor for BDNF.

The present results show a rapid and significant effect of altering tectal BDNF levels on the dynamics of optic axon terminals *ill vivo*. BDNF stimulates the elaboration and extension of axonal branches and spikes; branch elimination continues at the control values. The significant decrease in branch addition in the presence of function-blocking antibodies to BDNF suggests that endogenous tectal BDNF influences the djnamic branching of axonal arbors. Thus these results provide evidence for the involvement of neurotrophins in developmental events other than survival. and suggest a role for BDNF in the interaction of axon terminals with their targets. The rapid BDNF effect on axon arborization and remodelling is consistent with a direct action on optic axons and with possible effects on growth-cone motility, synapse formation. and or synapse stabilization. Previous studies have shown that axons mustdramatically rearrange during retinotectal map formationx~y~-at~a time of active transmission of visual information between the retina and optic tectum? Given that neurotrophins have been implicated in modulation of synaptic effi~acy'~and that roles have been proposed for coordinated synaptic activity in the formation and refinement of the neuronal maps'^^ ". an attractive hypothesis is that reciprocal interactions between neuronal activity and neurotrophic factor^^^'^" provide a direct means to couple neuronal morphology and function during development of the central nervous system. ●

Received 30 June: accepted 13 October 1995

1. Korshing S. *1* Neurosci. **13,** 2739 (I993\
2. Korshing. S. & Thoenen, H. Devl Blol. **126,** 40 (19881,
3. Davies, A. M J Neurobiol. **25,** 1334 (1994).
4. Schnell, L., Schneider. R , Kolbeck. R , Barde, Y. A & Schwab M E Nature **367,** 170 (1994).
5. Diamond. J , Holmes, M. & Coughlin. M. J. Neurosci. **12,** 1454 (1992)
6. Cabelli, R J.. Hohn. A & Shatz, C J, Science **267,** 1662 (19951
7. Harris, W A,. Holt. C. E, & Bonhoeffer. F Develornent **101,** 123 (19871
8. O'Rourke, N. A. & Fraser, S. E, Neuron **5,** 159 (1991)
9. O'Rourke. N. A,, Cline, H. T. & Fraser, 8. E. Neuron **12,** 921 (1994).
10 Squinto, S P. et a1 Cell **65,** 885 (1991)
11 Soppet, 0 *et al.* Cell **65,** 895 (1991).
12. Cohen-Cory, S. & Fraser, S. E Neuron **12,** 747 (1994)
13. Johnson. J. E., Barde. Y. A,. Schwab M. & Thoenen, H J Neurosci. **6,** 3031 (19861
14 Rodriguez-Tébar, A.. Jeffrey. P L., Thoenen, H. & Barde, Y A *Devl Biol* **136,** 296 (19891.
15 Nawa. H.. Pelleymounter, M A & Carnahan, J J Neurosci. **14,** 3751 (1994).
16 Ghosh. A., Carnahan, J. & Greenberg, M E. Science **263,** 1618 (1994).
17. Kaplan, 0.. Hernpstead. B., Martin-Zanca, D., Chao, M. & Parada, L. Science **252,** 554 (1991).
18. Ernfors, P , Merlio, J-P. & Peerson, H. Eur *J Neursci.* **4,** 1140 (19921
19 Lamballe, F.. Klein, R. & Barbacid, M. Cell **66,** 967 (1991)
20 Berkemeier, L. R. et al Neuron **7,** 857 (1991).
21. McAllister, A. K., Lo, D. C. & Katz, L. C. Neuron **15,** 791 (1995).
22. Nakarnura. H. & O'Leary, D D M *J.* Neurosci **9,** 3776 119891
23. Simon. D. K., Roskies, A. L. & O'Leary, D D M. *Devl Biol.* **162,** 384 (1994)
24. Lohof, A. M., Ip, N. Y. & Poo, M -m. Nature **363,** 350 (1993)
25. Cline, H. T , Debski. E A & Constantine-Paton M *Proc natn Acad* So. U S A **84,** 4342 (1987)
26 Cline. H T & Constantine Paton. M Neuron **3,** 413 '1989;
27 Goodman, C S. & Shatz, C J *Cell* **72,** 77 (1993).
28 Lindhom. D.. Castren, E , Berzaghi M., Blöchl A. & Thoenen, H *J. Neurobiol.* **25,** 1362 (19941
29. Dugich-Djordjevic M. M. *et al.* Neuron **8,** 1127 (1992)
30 Cohen Cory S., Fraser Q C Dreyfus, C F & Black I B Exo! *'WJW* **119,** 165 81993

ACKNOWLEDGEMENTS We thank J Carnahan for the BDNF ant bodies Genentech for recombi nant neurotrophins and A Coilazo 8 Dobbins J Shih K Woo B Trevarrow and D De Sha for helpful comments to the manuscript This work was supported by the Helen Hay Wh tney Foundation and NIMH

Defective recycling of synaptic vesicles in synaptotagmin mutants of *Caenorhabditis elegans*

Erik M. Jorgensen*', Erika Hartwieg, Kim Schuske*, Michael L. Nonet‡, Yishi Jin† & H. Robert Horvitz†

Department of Biology, University of Utah, Salt Lake City. Utah 84112, USA
† Howard Hughes Medical Institute, Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, USA
‡ Department of Anatomy and Neurobiology, Washington University School of Medicine, St Louis, Missouri 63110, USA

S\M~PTOT~GnMIntegral membrane protein of the synaptic vesicle'[2], binds calcium and interacts with proteins of the plasma membrane[4]. These observations suggest several possible functions for synaptotagmin in synaptic vesicle dynamics: it could facilitate exocytosis by promoting calcium-dependent fusion[3], inhibit exocytosis by preventing fusion[7], or facilitate endocytosis of synaptic vesicles from the plasma membrane by acting as a receptor for the endocytotic proteins of the clathrin AP2 complex[8]. Here we show that synaptic vesicles are depleted at synaptic terminals in synaptotagmin mutants of the nematode *Caenorhabditis elegans*. This depletion is not caused by a defect in transport or by increased synaptic vesicle release, but rather by a defect in retrieval of synaptic vesicles from the plasma membrane. Thus we propose that, as well as being involved in exocytosis, synaptotagmin functions in vesicular recycling.

In the nematode *Caenorhabditis elegans,* synaptotagmin is encoded by the gene *snt-1* (ref. 9). To characterize the role of synaptotagmin. we examined the synaptic ultrastructure of both wild-type worms and mutants deficient in *snt-1* function, using electron microscopy to determine the number and localization of synaptic vesicles at neuromuscular junctions. We analysed junctions involving three cell types in the ventral nerve cord. the VA. VB and VD motor neurons. Neuromuscular junctions were depleted of synaptic vesicles in *snt-1* mutants compared to wild-type worms (Fig. 1), which had an average of 40 vesicles per synaptic profile. whereas *snt-1*(*n2665*) mutants had an average of seven vesicles and *snt-1*(*md290*) mutants had an average of nine vesicles per synaptic profile. Moreover, there was no redistribution of vesicles to the intersynaptic regions: wild-type and *snt-1*(*md290*) worms both had an average of two vesicles per section between neuromuscular junctions (Fig. 1 legend).

These data suggest that synaptic vesicles may be accumulating in the plasma membrane in *snt-1* mutants. However. alternative explanations are possible. One possible explanation is that the depletion of vesicles at the synapse might be a consequence of the general sickness of the *snt-1* mutants. which have a very slow rate of pharyngeal pumping'" and. as a result of chronic starvation. are small and have a transparent gut. To determine

whether starved worms are generally depleted in synaptic vesicles, we sectioned a mutant defective in acetylcholine synthesis, *cha-1*(*p1152*)[11]. Like *snt-1* mutants, *cha-1* mutants pump slowly and are starved in appearance. Starvation of both the ***snt-1*** and *cha-1* mutants is probably caused by the absence of acetylcholine-stimulated pharyngeal pumping[10]. The *cha-1* mutant was not greatly depleted in synaptic vesicles, as *cha-1* animals had an average of 32 vesicles per synaptic profile (Fig. 1). **A** second possible explanation is that the vesicle depletion in ***snt-1*** mutants might be caused by defects in vesicle transport along the axon. It is believed that immature vesicles are generated from the Golgi and transported to the synapse, and mature synaptic vesicles are formed by an endosomal compartment[12]. One possibility is that synaptotagmin is required for the transport of immature vesicles from the Golgi to the synapse. However, *snt-1* mutants did not accumulate an excess of vesicles in the cell bodies of neurons (Fig. 2*a*). ***A*** third explanation is that *snt-1* mutants might be depleted of vesicles because synaptotagmin is involved in vesicular biogenesis: the Golgi may simply fail to generate synaptic vesicle components. However, synaptic vesicles are present in a ***snt-1*** strain that also carries a mutation in *unc-104*, a gene that encodes a *C. elegans* kinesin heavy-chain-like molecule'. Because of the defect in kinesin, vesicles are not transported along the axon in *unc-104* mutants[14]. Vesicles accumulated in the cell bodies of ***snt-1 unc-104*** double mutants, as visualized using electron microscopy (Fig. 2b). Therefore, vesicles appeared to be generated normally at the cell body in ***snt-1*** mutants.

To demonstrate more directly that synaptic vesicle components were transported to the synapse in *snt-1* single mutants, we used an expression construct in which the green fluorescent

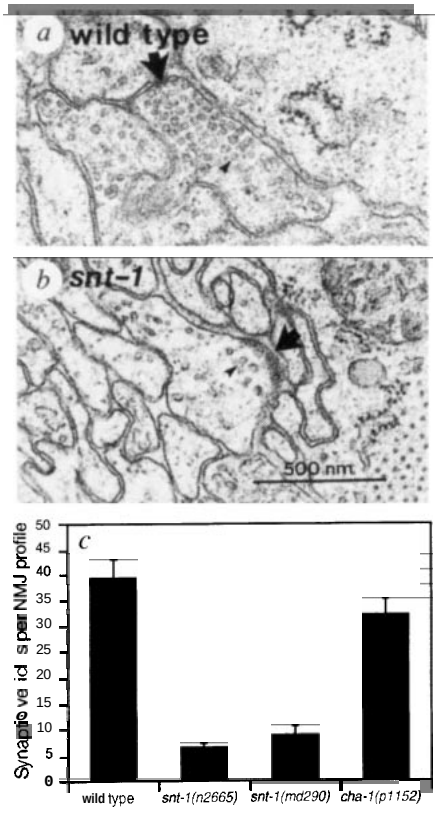

FIG. 1 Synaptic vesicles are depleted at neuromuscular junctions in snt-1 mutants. a, Electron micrograph of a wild-type neuromuscular junction (NMJ). Large arrow, dark thickening of an active zone; arrowhead, one synaptic vesicle. b, Electron micrograph of a *snt-1*(*md290*) neuromuscular junction. c, Synaptic vesicles in wild-type (15 NMJs from five worms), *snt-1*(*n2665*) (15 NMJs from two worms), *snt-1*(*md290*) (17 NMJs from four worms), and cha-l(pll52) worms (17 NMJs from four worms). Synaptic vesicles were counted in a single section that passed through the midpoint of the presynaptic specialization. Error bars represent s.e.m. The *snt-1*(*md290*) allele should generate a synaptotagmin protein deleted for all but the first 7 1 amino acids[9] and thus eliminates part of the transmembrane domain and the two C2 domains. The *snt-1*(*n2665*) mutation causes a frameshift after Lys 267 and introduces a termination codon 1 9 codons later, which should generate a truncated protein containing only the first C2 domain (J. A Crowell and J. B. Rand, personal communication).
METHODS. Worms were cut in 0.8% glutaraldehyde and 0.7% osmium tetroxide in 0.1 M cacodylate (pH 7.4) on ice. After 2 h, they were moved to 2% osmium tetroxide in 0.1 M cacodylate and left at 4 °C overnight. Processing and sectioning were as described previously[24]. We obtained serial sections from the region of the animal near the anterior reflex of the gonad. We examined the ventral nerve cords for regions containing an active zone or an accumulation of synaptic vesicles and photographed such regions, and counted vesicles at synapses. To quantitate vesicles in intersynaptic regions, we analysed a wild-type motor neuron spanning 50 sections and two active zones, and examined two *snt-1* motor neurons spanning 65 sections containing four active zones each. Sections containing an active zone and two sections on either side of an active zone were eliminated from the data set and vesicles counted in the intervening sections. In wild-type worms there were 2.0 ± 0.3 synaptic vesicles (25–35 nm), 0.9 ± 0.2 large spherical vesicles (−45 nm), and 0.5 ± 0.1 oblong cisternae per section. In *snt-1*(*md290*) mutants there were 1.7 ± 0.2 synaptic vesicles, 1.2 ± 0.4 large vesicles, and 0.5 ± 0.1 cisternae per section.

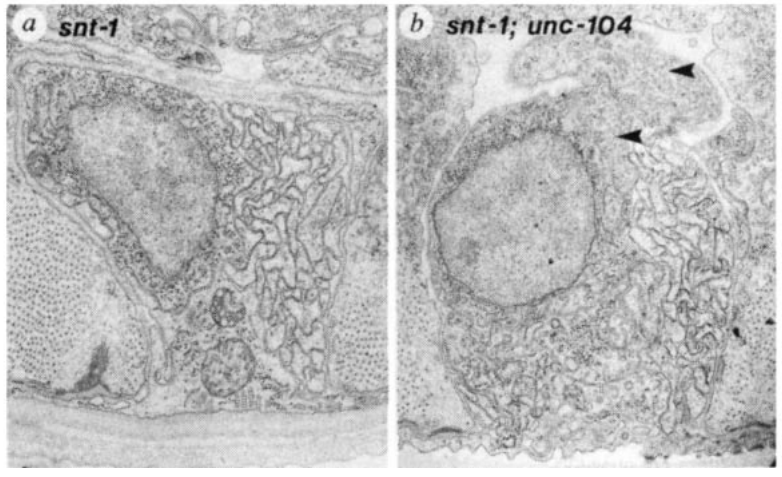

FIG. 2 Biogenesis and transport of vesicles are normal in snt-1 mutants. a, *snt-1*(*md290*) motor neuron cell body in the ventral nerve cord. There was no accumulation of synaptic vesicles in the cell body. Similar results were observed for 13 cell bodies. *b*, *snt-1*(*md290*) *unc-104*(*e1265*) neuronal cell body in the ventral nerve cord. Note accumulation of vesicles in the axonal hillock (arrowheads) of two motor neurons. Similar results were observed for seven cell bodies. To ensure that the vesicles accumulating in the cell bodies contained synaptic vesicle proteins, we demonstrated that two proteins associated with synaptic vesicles are also located in the cell bodies in the snt-1 *unc-104* mutant. First, RAB-3 colocalized with neuromuscular junctions in wild-type animals as visualized with antiRAB-3 antisera. Second, an expression construct in which GFP was fused to the C. *elegans* homologue of the synaptic vesicle protein VAMP (see below) colocalized with synaptic vesicles. Both of these synaptic vesicle markers were found in cell bodies in snt-1 *unc-104* mutants (data not shown).

protein (GFP)[15] was fused to the C. *elegans* homologue of the synaptic vesicle protein VAMP, also called synaptobrevin[16,17], and expressed this construct in the DD and VD motor neurons. The DD cell bodies are in the ventral nerve cord; these neurons extend a commissure dorsally and form neuromuscular junctions along a process in the dorsal nerve cord. In wild-type worms the fluorescently tagged VAMP molecule was found in clusters along a process in the dorsal nerve cord (Fig. 3*a*). The distribution of the fluorescent clusters was consistent in frequency and position with localization at neuromuscular junctions when compared with a reconstructed section of the nerve cord from electron micrographs (data not shown). Although fluorescence was more diffuse in *snt-1* mutants than in wild-type worms, similar levels of fluorescence appeared to be present in the dorsal cords of *snt-1* mutants and wild-type animals (Fig. 3). Additionally, we demonstrated that RAB-3 is transported normally in a *snt-1* mutant. RAB-3 is a C. *elegans* protein related to the vertebrate protein Rab3a, and is expressed by all ventral-cord motor neurons (M.L.N., unpublished observations). As visualized with antiRAB-3 antisera, RAB-3 was localized along the dorsal cord

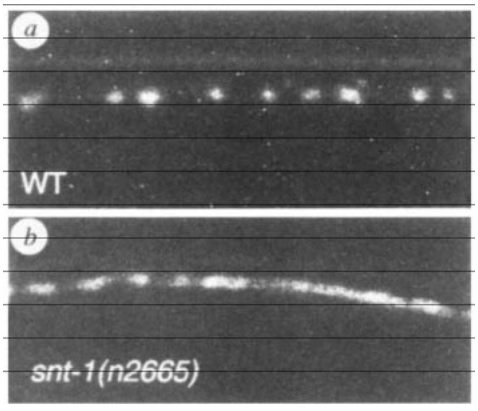

FIG. 3 In *snt-1* mutants, vesicular components are transported to the dorsal cord but localization is diffuse. a, *b*, Distribution of fluorescently tagged VAMP/synaptobrevin in the dorsal cord of wild-type (a)and *snt-1*(*n2665*) (b) worms. Both wild-type and mutant worms showed normal transport of the tagged VAMP molecule to the axon terminals. In the *snt-1* mutants the fluorescently tagged VAMP protein appeared diffusely localized along processes instead of at discrete positions along the dorsal cord. Photographs of mutant and wild-type dorsal cords at the level of the anterior reflex of the gonad were mixed and scored in a double-blind test as diffuse or punctuate. *snt-1* was significantly more diffuse: 1 of 10 wild-type and 9 of 10 mutant nerve cords were scored as diffuse (Fisher'sexact test, $P = 0.0011$). Similar results were obtained with *snt-1*(*md290*). These results suggested that VAMP was sequestered in the membrane of the axon and had diffused laterally from the neuromuscular junctions, but we have no direct evidence that VAMP is localized in the plasma membrane.

METHODS. A C. *elegans* VAMP-like gene was cloned using the polymerase chain reaction (PCR).The open reading frame of GFP[15] was inserted at the penultimate codon of a genomic clone of the VAMP gene to yield a fusion to the lumenal domain of VAMP (M.L.N., unpublished results). This fusion protein was placed under the control of the *unc*-25 promoter, which causes expression in a subset of the neurons which use the neurotransmitterGABA (y-aminobutyricacid) (RMEs, DDs and VDs; Y.J., unpublished observations).A fin-15 mutation was used to generate a non-neuronal mutant phenotype to assay successful transformation of co-injected DNA fragments. A fin-15 rescuing fragment[25] and the VAMP–GFP expression construct were co-injected[26] into the gonad of *lin*-15(*n765ts*) mutants. An extrachromosomal array rescuing the Un-15 phenotype and expressing GFP was stabilized by γ-ray integration into the X chromosome (Y.J., unpublished observations). This fluorescent construct appeared to label synaptic vesicles, as it forms clusters in the ventral and dorsal cords at frequencies approximately equivalent to those of neuromuscular junctions, and because in unc-104 mutants fluorescence was coincidentally shifted with synaptic vesicles to the cell body (data not shown).

in *snt-1*(*md290*) animals, as it is in wild-type animals (data not shown). Thus proteins associated with synaptic vesicles were transported normally from the cell body to neuromuscular junctions.

The depletion of synaptic vesicles could be caused by an increased rate of exocytosis, which should increase the efflux of neurotransmitter. To assay acetylcholine release we tested for sensitivity to aldicarb, an inhibitor of acetylcholinesterase[18]. Blocking acetylcholinesterase causes toxic levels of acetylcholine to accumulate in the junctional cleft in wild-type worms and causes paralysis; an increase in neurotransmitter release in the mutant should cause hypersensitivity to this drug. We found that *snt-1* adults can tolerate fivefold higher concentrations of aldicarb than can wild-type worms before being paralysed (Fig. 4). These results suggest that acetylcholine release is decreased at neuromuscular junctions in *snt-1* mutants. In support of this conclusion, electrophysiological recordings from pharyngeal muscles of intact C. *elegans* indicated a decrease in neurotransmission from a class of glutamatergic motor neurons in *snt-1* mutants[19] (J. A. Dent, M. W. Davis and L. Avery, personal communication). In addition, acetylcholine accumulates at abnormally high levels in *snt-1* mutants[9]. These observations are inconsistent with there being an increased rate of exocytosis in *snt-1* mutants and suggest that these mutants are deficient in neurotransmitter release. We propose that a null mutation in synaptotagmin leads to a decrease in transmitter release by blocking the regeneration of synaptic vesicles and depleting the synaptic termini of vesicles.

Synaptic vesicle components are recovered from the plasma membrane by endocytosis[20,21]. The simplest interpretation of our data is that synaptotagmin is required for endocytosis of synaptic vesicles. This possibility is supported by the finding that rat neuronal synaptotagmin I binds clathrin AP-2 (ref. 8), a complex of proteins required for the formation of coated pits. Moreover, other synaptotagmin genes expressed in non-neuronal tissues also bind clathrin AP-2 (ref. 22). These data indicate that synaptotagmin might function as a plasma membrane receptor for the endocytotic machinery in many tissues. After endocytosis, vesicular components are sorted and reformed into vesicles from an endosomal compartment[12]. It is also possible that synaptotagmin functions in the regeneration of synaptic vesicles from

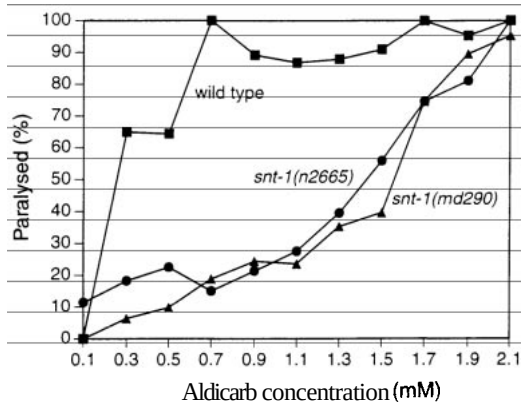

FIG. 4 Synaptotagmin mutants are resistant to an inhibitor of acetylcholinesterase.

METHODS. We placed 20 *snt*-1(*n*2665), *snt*-1(*md*290) or wild-type worms on agar plates seeded with bacteria and containing aldicarb, an inhibitor of acetylcholinesterase. Chronic application of aldicarb does not severely affect the generation time of snt-1 mutants[9]. To assay directly the effect of aldicarb at neuromuscular junctions, we assayed acute paralysis in adult animals. Worms were assayed for movement and pharyngeal pumping 8 h after exposure to the drug. Paralysis is defined as lacking both movement of the body and pumping of the pharynx.

the endosome rather than in membrane retrieval at the plasma membrane.

Our conclusion that synaptotagmin has a role in vesicular recycling does not preclude synaptotagmin from also having a role in exocytosis; we simply find that the primary defect seen in the *C. elegans* mutant is at the step of vesicular recycling. We propose that synaptotagmin functions to regulate both exocytosis and the recycling of synaptic vesicles[2,3]. □

Received 3 May; accepted 30 August 1995

1. Matthew, W. D., Tsavaler, L. & Reichardt, L. F. *J. Cell Biol.* 91, 257–269 (1981).
2. Perin, M. S., Fried, V. A., Mignery, G. A., Jahn, R. & Sudhof, T. C. Nature 345, 260–263 (1990).
3. Brose, N., Petrenko, A. G., Sudhof, T. C. & Jahn, R. Science 256, 1021–1025 (1992).
4. Bennett. M. K., Calakos, N. & Scheller, R. H. Science 257, 255–259 (1992).
5. Hata, Y., Davletov, B., Petrenko, A. G., Jahn, R. & Sudhof, T. C. Neuron 10,307–315 (1993).
6. Leveque. C. et *al*. Proc. natn. Acad. Sci. U.S.A. 89, 3625–3629 (1992).
7. Sollner, T., Bennett. M. K., Whiteheart. S. W., Scheller. R. H. & Rothman, J. E. *Cell* **75,** 409–418 (1993).
8. Zhang, J. Z., Davletov. B. A., Sudhof, T. C & Anderson, R. G. *Cell* 78, 751–760 (1994)
9. Nonet, M. L., Grundahl, K., Meyer, B. J. & Rand, J. B. *Cell* 73, 1291–1306 (1993).
10. Avery, L. *Genetics* 133,897–917 (1993).
11. Rand, J. B. & Russell. R. L. Genetics 106, 227–248 (1984).
12. Mundigl, O. & De Camilli, P. Curr. Opin. *Cell Biol.* **6,** 561–567 (1994).
13. Otsuka, A. J. et *al*. Neuron 6, 113–122 (1991).
14. Hall, D. H. & Hedgecock, E. M. *Cell* 65, 837–847 (1991).
15. Chalfie, M., Tu, Y., Euskirchen, G., Ward. W. W. & Prasher, D. C. Science 263, 802–805 (1994).
16. Trimble, W. S., Cowan, D. M. & Scheller, R. H. Proc. natn. Acad. Sci. U.S.A. 85, 4538–4542 (1988).
17. Sudhof, T. C., Baumert, M., Perin, M. S. & Jahn, R. Neuron **2,** 1475–1481(1989).
18. Rand, J. B. & Russell. R. L. Psychopharmac. *Bull.* 21, 623–630 (1985).
19. Raizen, D. M. & Avery. L. Neuron 12, 483–495 (1994).
20. Miller, T. M. & Heuser, J. E J. *Cell Biol.* 98, 685–698 (1984).
21. Mavcox, P. R., Link, E., Reetz, A., Morris. S. A & Jahn. R. J. *Cell Biol.* 118, 1379–1388 (1992).
22. Li. C. et *al*. Nature **375,** 594–599 (1995).
23. Sudhof, T. C. Nature 375, 645–653 (1995).
24. McIntire. S. L., Garriga. G., White. J., Jacobson. D. & Horvitz. H. R. Neuron 8. 307–322 (1992).
25. Clark. S. G., Lu, X W. & Horvitz, H. R. Genetics 137, 987–997 (1994).
26. Mello, C. C., Kramer, J. M., Stinchcomb, D. & Ambros, V. EMBOJ. 10, 3959–3970 (1991).

ACKNOWLEDGEMENTS. We thank J. Rand for the *snt-1*(*md290*) strain and for sharing unpublished results. E.J. was supported by the Damon Runyon-Walter Winchell Cancer Research Fund and the Howard Hughes Medical Institute. Y.J. was supported by the Jane Coffin Childs Foundation. H.R.H. is an investigator of the Howard Hughes Medical Institute. This work was supported by a U.S. Public Health Service research grant.

Role of *SUPERMAN* in maintaining *Arabidopsis* floral whorl boundaries

Hajime Sakai, Leonard J. Medrano & Elliot M. Meyerowitz

Division of Biology, 156-29, California Institute of Technology, Pasadena, California 91125, USA

THE Arabidopsis gene SUPERMAN (SUP) is necessary for the proper spatial development of reproductive floral tissues[1–3]. Recessive mutations cause extra stamens to form interior to the normal third whorl stamens, at the expense of fourth whorl carpel development[3]. The mutant phenotype is associated with the ectopic expression of the B function genes, AP3 and PI, in the altered floral region, closer to the centre of the flower than in the wild type[3], and *ap3* sup and *pi* sup double mutants exhibit a phenotype similar to *ap3* and pi single mutants. These findings led to SUP being interpreted as an upstream negative regulator of the B function organ-identity genes, acting in the fourth whorl[2,3], to establish a boundary between stamen and carpel whorls. Here we show, using molecular cloning and analysis, that it is expressed in the third whorl and acts to maintain this boundary in developing flowers. The putative SUPERMAN protein contains one zinc-finger and a region resembling a basic leucine zipper motif, suggesting a function in transcriptional regulation.

The phenotypes of wild-type and *SUP* mutant flowers are shown in Fig. 1a and b. The *SUP* gene was mapped to the middle of chromosome 3 between the restriction-fragment length polymorphism (RFLP) markers λAt105 and KG-23, in close vicinity to λAt255, with a distance of 1–2 cM[3]. We thus used λAt255 (ref. 4) as the starting point for a chromosome walk towards the *SUP* locus. We isolated 600 kb of contiguous genomic DNA by screening yeast artificial chromosome (YAC)

FIG. 1 a, Wild-type flower; *b*, superman-I mutant; and *c*, *superman-1* with transgenic SUP gene. a, Wild-type flower consists of four whorls, occupied by four sepals, four petals, six stamens and two fused carpels from the outermost first whorl to the innermost fourth whorl. *b*, The flower of superman forms extra stamens interior to the third whorl stamens and a reduced carpelloid organ in the centre of the flower. c, superman-I plants carrying the transgenic wild-type SUP gene produce flowers indistinguishable from wild type.

METHODS. For the complementation test the genomic clone pHS-QY6.7 in a pCGN1547 vector (Calgene), which contains the SUP coding region with 5.3 kb of upstream and 0.7 kb of downstream sequences, was introduced into the Agrobacterium tumefaciens strain ASE (Monsanto). This strain was used to infect wild-type No-0 plants by the vacuum infiltration method[2]. Two independently isolated kanamycin-resistant transformants were then crossed to *sup-1 gl1-1* strains. The *gl1-1* recessive mutation, which is approximately 12 cM away from *sup*[2,3], was used to screen potential *sup-1* homozygous plants in the F_2 progeny. All 28 Km^R *gl1* plants from these crosses produced flowers indistinguishable from wild type. The homozygous state of *sup-1* in these *gl1* plants was confirmed by amplification of the sup locus by the polymerase chain reaction (PCR) using one primer from the 5' region of SUP and another from 1.2 kb downstream of SUP, which was not present in pHS-QY6.7, and followed by genotyping through cleaving DNA with *Nco*I, which has a cleavage site in the wild-type sequence but not in the sup-1 mutant allele used.