The *Drosophila* homolog of Down's syndrome critical region 1 gene regulates learning: Implications for mental retardation

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Mental retardation is the most common phenotypic abnormality seen in Down's syndrome (DS) patients, yet the underlying mechanism remains mysterious. DS critical region 1 (DSCR1), located on chromosome 21, is overexpressed in the brain of DS fetus and encodes an inhibitor of calcineurin, but its physiological significance is unknown. To study its functional importance and role in mental retardation in DS, we generated *Drosophila* mutants of *nebula*, an ortholog of human DSCR1. Here, we report that both *nebula* loss-of-function and overexpression mutants exhibit severe learning defects that are attributed by biochemical perturbations rather than maldevelopment of the brain. These results, combined with our data showing that the same biochemical signaling pathway is altered in human DS fetal brain tissue overexpressing DSCR1, suggest that alteration of DSCR1 expression could contribute to mental retardation in DS.

Down's syndrome (DS), a disorder that affects ≈1 in 700 live births across all ethnic groups, is the leading genetic cause of mental retardation (1). Other clinical abnormalities associated with DS include congenital heart disease, hypotonia, facial dismorphology, immune system defects, gastrointestinal anomalies, and the early development of the pathological and neurochemical changes of Alzheimer's disease (1, 2). Extensive studies on DS patients with partial trisomy 21 have allowed researchers to narrow the search for genes associated with the phenotypic features of DS to a segment of chromosome 21, the DS region (2). Among the genes located in the DS region is the DS critical region 1 (*DSCR1*) gene. *DSCR1* is normally expressed in the central nervous system and the heart, but overexpressed in fetal DS brain (3, 4). DSCR1 belongs to a highly conserved calcineurin inhibitor family called calcipressin, which contains RCN1P in yeast (5), CBP1 in fungus (6), Nebula in *Drosophila* (GenBank accession no. AAD33987), and Dscr1 in mouse (7). Biochemical studies have shown that DSCR1 and other proteins in the calcipressin family can bind to and inhibit calcineurin, a serine/threonine protein phosphatase important for learning and memory (4–6, 8–12). The physiological significance of DSCR1 and its role in DS, however, are not well understood. Here, we generated *Drosophila* mutants of *nebula*, a homolog of human DSCR1, to address: (*i*) what is the role of *nebula* in learning and memory; and (*ii*) is mental retardation in DS related to DSCR1 overexpression?

Materials and Methods

Generation of Mutant Flies. nla^1 was generated by using random hop with *Drosophila* lines carrying $P\{lacZ,w^+\}$. The site of *P* element insertion was determined by plasmid rescue (13), and the presence of a single *P* element insertion was confirmed by genomic Southern blots. To excise the *P* element, we crossed nla^1 with ry K_i $P\{ry^+\ \Delta 2\text{–}3\}$. The male jump-starters were then crossed to w;TM3/TM6 flies. Progeny with white eyes were made homozygous, and lines were established. Imprecise and precise *P* element excision lines were determined by PCR and confirmed by sequence analyses. All fly lines were outcrossed to Canton-S (CS) (Bloomington Stock Center, Bloomington, IN) background for several rounds. nla^{t1} and nla^{t2} transgenic flies were generated by a germ-line transformation method (14). The *nebula* transgene construct was generated by subcloning the coding region of *nebula* into the pINDY6 vector (L. Seroude and S. Benzer, personal communication). To drive overexpression of *nebula*, transgenic flies were crossed to either Act5C-GAL4/Tb (15), Elav-GAL4 3E (16), c739-GAL4 (17), Elav-GeneSwitch (18), or hsp70-GAL4 (19) fly lines.

Pavlovian Olfactory Learning and Memory Assays. Flies 2–7 days old were used in the Pavlovian olfactory conditioning assay (20). Before conditioning, the training apparatus was humidified for 2 h with a vacuum-connected air bubbler to allow saturation of humidity. Before training, flies were ensured to distribute equally between 3-octanol (OCT) and 4-methylcyclohexanol (MCH) used for training. Groups of ≈50–100 flies were trained with one odor (MCH) for 60 s paired with electric shock (at 60 V), and then exposed subsequently to the second odor (OCT) for 60 s without electric shock. Immediately after training, learning was measured by giving flies 120 s to choose between the two odors used during training in T maze arms. To examine long-term memory, flies were subjected to spaced training (10 training sessions with 15-min rest intervals between sessions) (21). Flies were then tested both immediately and 24 h after spaced training. All training was performed under dim red light, and performance tests were carried out in the absence of light. Performance index was calculated as the fraction of flies that avoided the conditioned stimulus (odor with electric shock) minus the fraction of flies that avoided the unconditioned stimulus (second odor) multiplied by 100. For odor acuity tests, flies were transported to the choice point of the T maze and allowed 120 s to choose between odor and air. For shock reactivity, flies were allowed 120 s to choose between the tube with electric shock pulses and the tube without shock. Individual performance index was calculated as reported (22).

RU486 Induction. RU486 (10 mg/ml, Mifepristone, Sigma) was dissolved in ethanol. RU486 was diluted and mixed with fly food to get a final concentration of 100 μg/ml for adult feeding and 20 μg/ml for larval feeding. The final ethanol concentration in both cases was 4%. To activate GeneSwitch transiently in adults, 1- to 5-day-old adult flies were transferred to fly food containing 100 μg/ml RU486, and Pavlovian olfactory learning tests were performed 24 h after feeding. To activate GeneSwitch during development, larvae were raised on fly food containing 20 μg/ml RU486; eclosed adults were promptly removed to normal food media. Adult flies that had been transferred to normal fly food media for 3–6 days were

Abbreviations: DS, Down's syndrome; DSCR1, DS critical region 1; PKA, cAMP-dependent protein kinase; CREB, cAMP-responsive element binding protein; pCREB, phosphorylated CREB; CS, Canton-S.

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used in Pavlovian olfactory learning assays. For mock feeding, flies were raised as described above, except fly food contained 4% ethanol, but no RU486.

Materials. Human normal and trisomy 21 fetal brain tissues were obtained from the Brain and Tissue Bank at the University of Maryland, Baltimore.

Supporting Methods. For details on methods used, see *Supporting Text*, which is published as supporting information on the PNAS web site.

Results and Discussion

Generation of *nebula* Loss-of-Function Mutants. We created a *Drosophila* mutant with a *P* element insertion between the 5′ UTR and the translation start site of the *nebula* gene (nla^1; Fig. 1*A*). Furthermore, by mobilizing the *P* element inserted in nla^1, we isolated precise and imprecise *P* element excision lines, nla^{PJ} and nla^2, respectively (Fig. 1*A*). The nebula protein shares 43% identity and 64% similarity in amino acid sequence with human DSCR1 (Fig. 5, which is published as supporting information on the PNAS web site). Quantitative real-time RT-PCR analyses revealed reduced expression of *nebula* in the mutants (Fig. 1*B*),

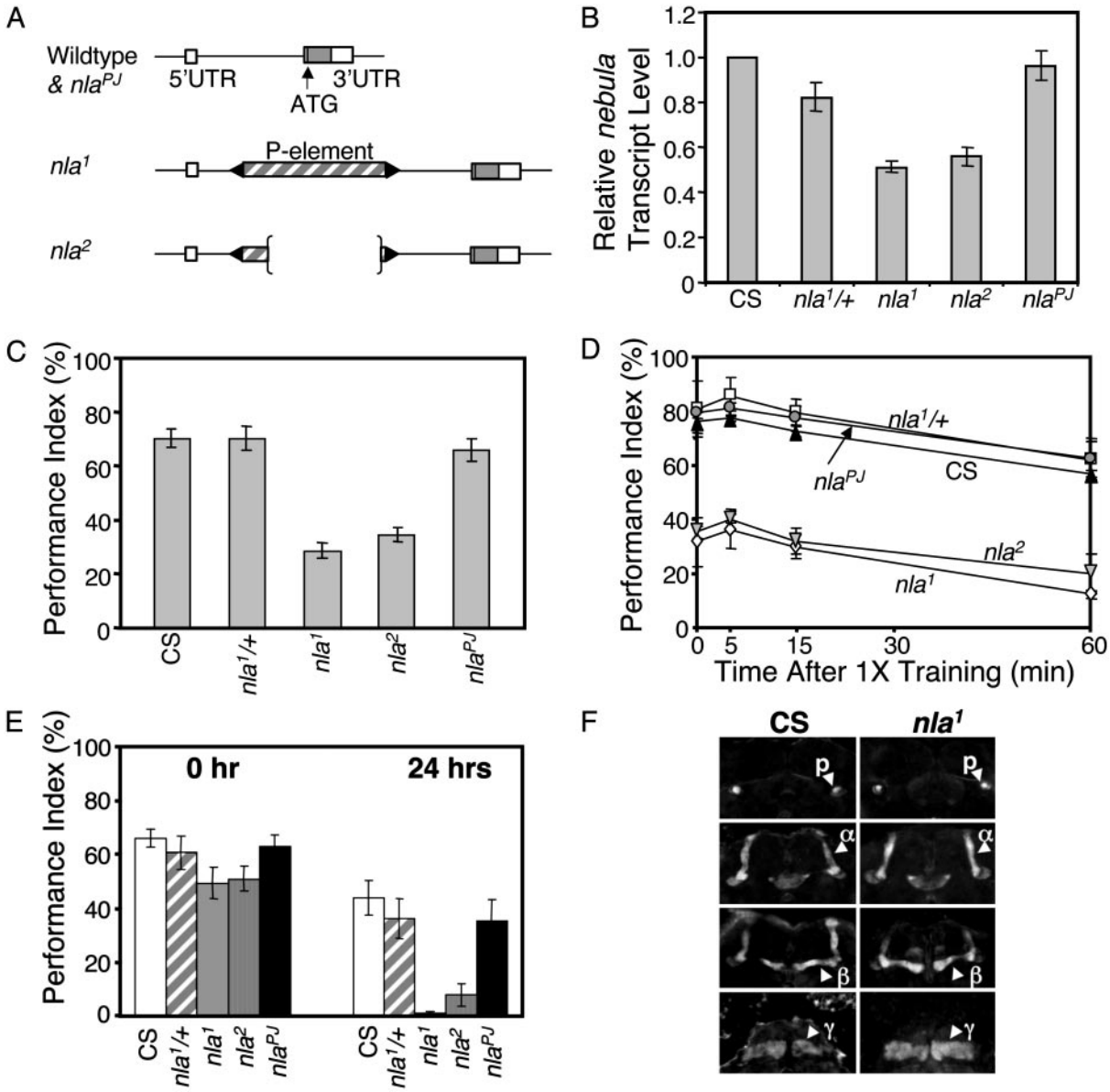

Fig. 1. *nebula* mutants show defective learning and long-term memory, but normal short-term memory decay. (*A*) Schematics of different *nebula* alleles. Empty and filled boxes indicate untranslated and coding sequences, respectively. nla^{PJ} and nla^2 show precise and imprecise *P* element excision, respectively, as determined by sequence analysis. (*B*) Quantitative real-time RT-PCR results showing relative fold change in *nebula* transcript level for each fly line. $n = 4$ independent experiments done in triplicate. Values represent mean ± SEM. (*C*) Performance of control (CS), nla^1 heterozygotes ($nla^1/+$), nla^1 homozygotes (nla^1), nla^2 homozygotes (nla^2), and nla^{PJ} homozygotes (nla^{PJ}) in Pavlovian olfactory conditioning test after training. nla^1 and nla^2 showed severe learning defects, whereas nla^{PJ} displayed normal learning. (*D*) Memory decay curves of flies after one training session. The same decay rate seen for all fly lines indicates that nebula is not required for short-term memory. (*E*) Long-term memory assayed immediately (0 h) or 24 h after spaced training. nla^1 and nla^2 showed a virtual absence of long-term memory, whereas CS, $nla^1/+$, and nla^{PJ} showed normal memory retention. For *C*–*E*, $n \geq 5$ for each fly strain and each time point. All values are expressed as mean ± SEM. (*F*) The nla^1 mutant has normal mushroom bodies as revealed by Fasciclin II antibody staining. There is no difference in the structures of the α, β, γ lobes and peduncles (p) of nla^1 flies as compared to the control (CS) flies.

suggesting that both *nla*^{1} and *nla*^{2} are hypomorphic alleles. The *nebula* mutants are viable and fertile without visible abnormality. *In situ* hybridization using an antisense probe to the *nebula* gene showed that *nebula* is expressed in the cortex of the normal fly brain (Fig. 6, which is published as supporting information on the PNAS web site).

Effects of *nebula* Loss-of-Function Mutations on Learning and Memory. To investigate the role of nebula in learning and memory, we used a Pavlovian olfactory associative learning and memory test (20). Significant defect in learning was observed after a single trial of training for homozygous *nla*^{1} and *nla*^{2} mutants, as compared to *nla*^{1} heterozygotes, precise excision line (*nla*^{PJ}), and CS flies (Fig. 1*C*). The learning defects are not attributed by abnormal sensorimotor responses, because both odor and shock avoidances were indistinguishable among the fly lines tested (Fig. 7, which is published as supporting information on the PNAS web site). Nevertheless, we cannot completely rule out the possibility that some of the performance defects seen in the *nebula* mutants could be caused by subtle alteration in sensitivity to stimuli.

Short-term memory is thought to last 60 min after training in *Drosophila* (23). We thus investigated short-term memory in different *nebula* alleles by examining the performance at different time points after training. The same rate of normal memory decay seen for the different fly lines (Fig. 1*D*) suggests that short-term memory is likely intact and that the low performance value obtained right after training may be a reflection of defective learning, albeit we do not rule out the possibility that nebula is required for immediate short-term memory (during the 3 min in between training and the first possible test time point). To further examine the role of nebula in the memory pathway, we tested long-term memory in the *nebula* mutants. Long-term memory in *Drosophila* is associated with phosphorylated cAMP-responsive element binding protein (pCREB) and protein synthesis and is evident 1 day after 10 trials of spaced training with 15-min rest intervals between trainings (21, 23). Strikingly, we found that homozygous *nebula* mutants showed a virtual absence of long-term memory, whereas control flies displayed ≈40% memory retention 24 h after training (Fig. 1*E*). The observed defect in long-term memory is not caused by the initial deficiency in acquisition, because the performance of *nla*^{1} and *nla*^{2} homozygotes immediately after spaced training did not differ significantly from CS flies ($P > 0.05$ for CS vs. *nla*^{1} and CS vs. *nla*^{2} as determined by Student's *t* tests). Together, our results indicate that increasing the number of training sessions can improve the learning performance of *nebula* mutants, and more importantly, nebula is required for effective learning and long-term memory.

***nebula* Mutants Have Normal Mushroom Bodies but Altered Calcineurin-Mediated Signaling.** Despite the overall normal brain structure (data not shown), it is possible that the learning and memory deficits seen for the *nebula* mutants are caused by structural defects specifically in the mushroom bodies, the center for learning and memory in *Drosophila* (24). To examine the integrity of the mushroom bodies, we visualized the α, β, and γ lobes with Fasciclin II antibody (25). Of all 60 *nla*^{1} homozygous mutant flies examined, no visible structural defect in the mushroom bodies was detected, and the α, β, and γ lobes were similar to the control flies (Fig. 1*F*). This finding implies that the *nebula* mutation does not cause maldevelopment of the mushroom bodies and that defects in learning and memory are not caused by gross structural defect.

To understand whether the mutation in *nebula* significantly alters the biochemical activity predicted for the gene, we examined calcineurin activity level in the *nebula* mutant flies. Calcineurin activity detected in the homogenate of *nla*^{1} homozygotes was ≈40% higher than CS control and *nla*^{1} heterozygotes, indicating that nebula functions as an endogeneous inhibitor of calcineurin as other members in the calcipressin family (4–6) (Fig. 2*A*). We further examined the signaling pathways downstream from calcineurin and found that homozygous *nla*^{1} mutants showed a substantial decrease in cAMP-dependent protein kinase (PKA) activity, the amount of pCREB, and the level of *d-jun* transcripts (Fig. 2). Given that PKA and pCREB are important for normal

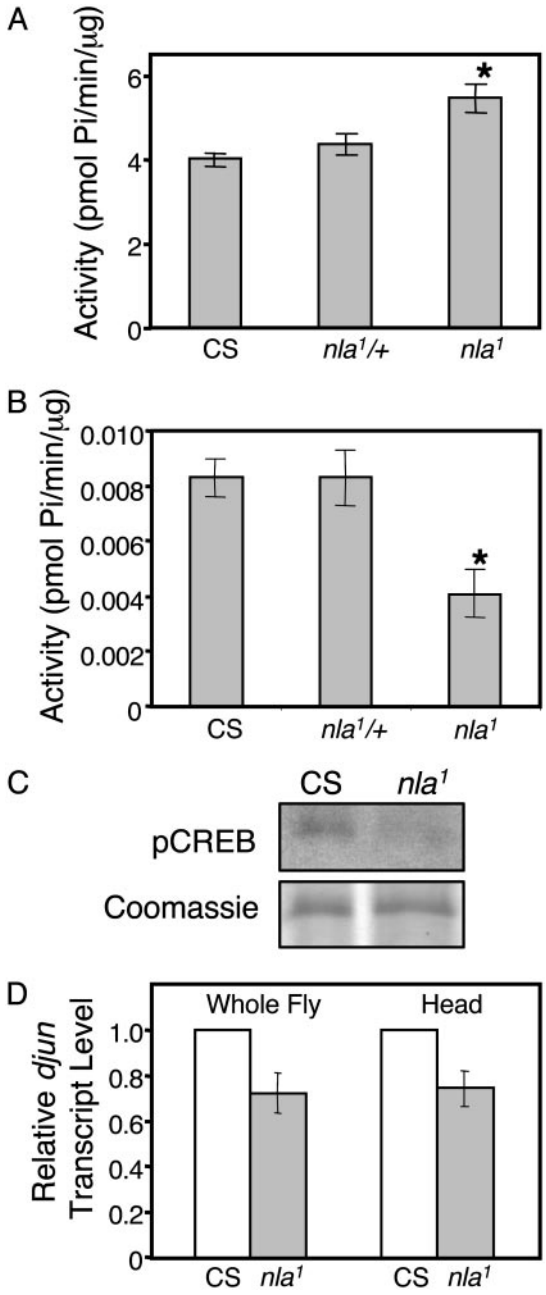

Fig. 2. *nebula* mutation causes perturbations in biochemical signaling. (*A*) Calcineurin activity determined from extracts of CS, *nla*^{1}/+, and *nla*^{1}. Calcineurin activity in *nla*^{1} increased by 40%. (*B*) PKA activity in *nla*^{1} is ≈50% of the CS flies. (*A* and *B*) $n = 3$ experiments done in triplicate (*, $P < 0.05$). (*C*) Western blot analysis with anti-pCREB antibody shows that the level of pCREB in *nla*^{1} is significantly reduced. Equal intensity bands seen by Coomassie blue staining of the SDS protein gel confirm equal loading. (*D*) Quantitative real-time RT-PCR results. *d-jun* transcript level in *nla*^{1} is ≈30% lower than in the CS flies whether total RNA from whole flies or adult heads was used. $n = 4$ independent experiments in triplicate. Values represent mean ± SEM.

learning and long-term memory (26–28), the observed biochemical perturbations in the *nebula* mutant may contribute to the observed deficiency in learning and memory.

Transgenic Flies Overexpressing *nebula* Exhibit Severe Defects in Learning. Our findings that nebula, the *Drosophila* homolog of human DSCR1, can regulate learning and long-term memory led us to hypothesize that overexpression of DSCR1 in DS may be responsible for mental retardation. To create a model for studying the role of DSCR1 in mental retardation in DS, we generated transgenic flies overexpressing *nebula* by using the UAS/GAL4 system (29). Flies containing the *nebula* transgene on the third chromosome (nla^{t1}) were crossed with both the Act5C-GAL4 (Act5C) and Elav-GAL4 (Elav) driver lines to yield *nebula* overexpression throughout the fly or specifically in the neurons, respectively (15, 16). Fig. 3*A* shows that the level of *nebula* transcripts was elevated ≈1.5-fold in transgenic flies containing the indicated driver. Remarkably, Pavlovian olfactory learning tests revealed that both Act5C/nla^{t1} and Elav/nla^{t1} had virtually no learning (Fig. 3*B*), whereas the flies showed no visible defect and responded normally to odor and electric shock (Fig. 7). To further restrict spatial expression of the *nebula*

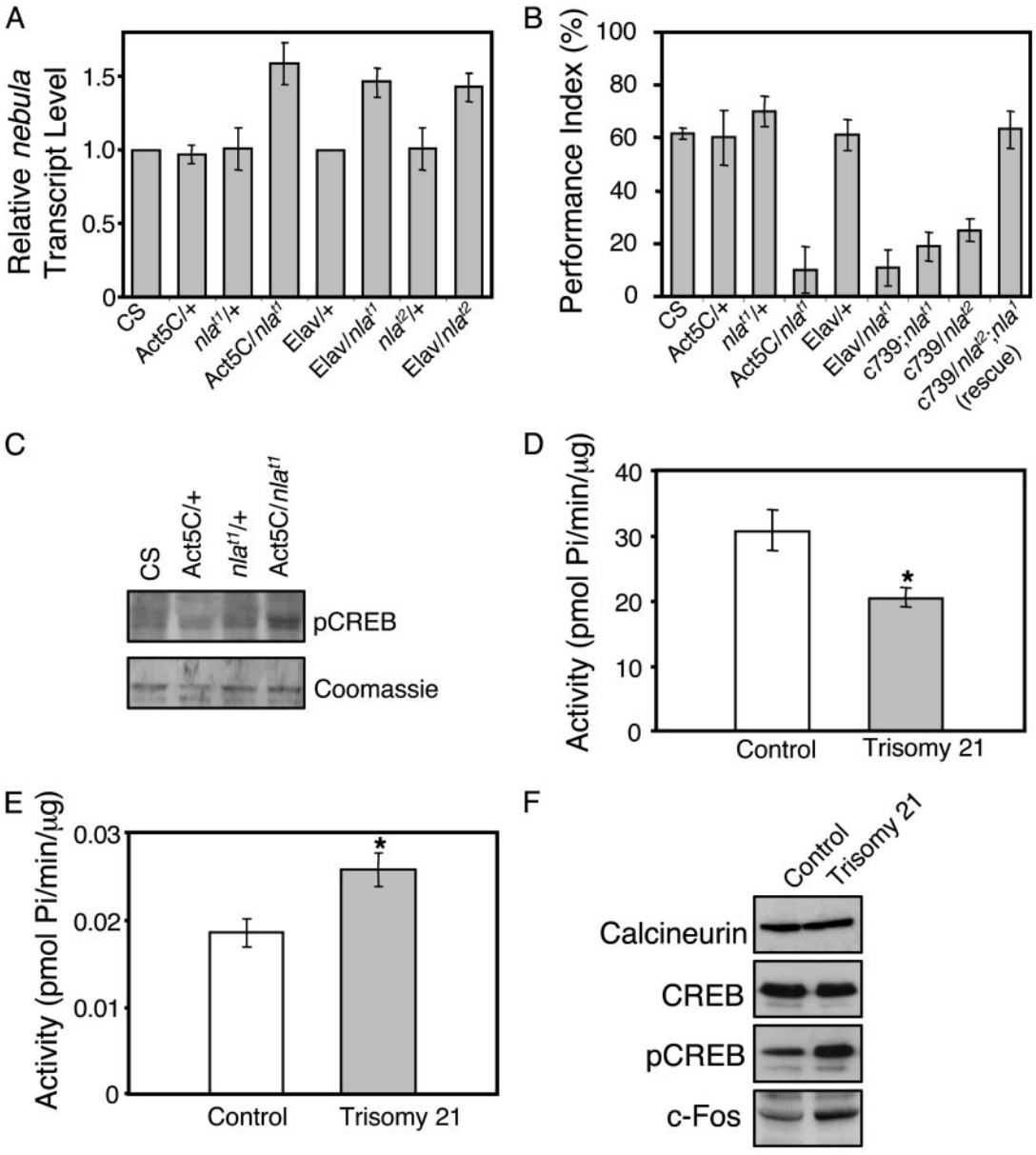

Fig. 3. Transgenic flies overexpressing *nebula* show defective learning, and human trisomy 21 fetal brain tissues overexpressing DSCR1 exhibit altered calcineurin-mediated signaling. (*A*) Quantitative real-time RT-PCR results. $n = 3$ independent experiments done in triplicate. Note that nla^{t1} and nla^{t2} transgenic flies show the same level of *nebula* overexpression in the presence of Elav-GAL4 driver. (*B*) Performance index values for Pavlovian olfactory learning. Flies overexpressing *nebula* by using the Act5C driver (Act5c/nla^{t1}), Elav driver (Elav/nla^{t1}), and mushroom bodies-specific driver (c739/nla^{t1} and c739/nla^{t2}) show severely impaired learning. Overexpression of *nebula* in the mushroom bodies of the homozygous *nebula* loss-of-function mutant (c739/nla^{t2};nla^{1}) shows complete rescue of the learning defect. (*C*) Western blot analysis shows that the level of pCREB in Act5C/nla^{t1} is higher than that of the control lines (CS, Act5C/+, nla^{t1}/+). Coomassie staining confirms the amount loaded. (*D*) Calcineurin activity in control and trisomy 21 fetal brain tissues. (*E*) PKA activity in control and trisomy 21 fetal brain tissues. (*F*) Western blots showing calcineurin, CREB, pCREB, and c-Fos protein levels in control and trisomy 21 fetal brain tissues. All values represent mean ± SEM. For *A*, $n = 4$ experiments done in triplicate. $n = 4$ for each fly strain in *B*. For *D* and *E*, $n = 3$ experiments done in triplicate. *, $P < 0.05$ as determined by Student's *t* test.

transgene, we used the c739-GAL4 driver, which drives UAS-transgene expression preferentially in the α and β lobes of the mushroom bodies (17, 30). Fig. 3*B* illustrates that overexpression of *nla*^{t1} or *nla*^{t2} (UAS-*nebula* transgene on the second chromosome) with the c739-GAL4 driver (c739;*nla*^{t1} and c739/*nla*^{t2}) also caused severe learning defects, implying that specific overexpression of *nebula* in the mushroom bodies is sufficient to disrupt learning. Furthermore, overexpression of *nebula* in the mushroom bodies on the *nebula* mutant background completely restored learning to normal, confirming that *nebula* is the gene responsible for the learning defects seen in the *nebula* mutants (Fig. 3*B*). Next, we examined the level of pCREB in *nebula* transgenic flies and found that Act5C/*nla*^{t1} showed an increase in pCREB level as compared to the control lines (Fig. 3*C*), indicating the existence of altered biochemical signaling. The result that defective learning in the transgenic flies is accompanied by an increase in pCREB is consistent with recent findings by Yuan *et al.* (31) that transient overexpression of CREB and the subsequent elevation in the level of pCREB before odor preference training interfered with learning in rats. This finding suggests that the global increase in the level of pCREB may saturate the signaling pathway and thereby make the signaling pathway less receptive to further regulation by kinases after training.

Overexpression of DSCR1 in Human Trisomy 21 Fetal Brain Tissue also Leads to Disturbed Calcineurin-Mediated Signaling. To directly correlate the findings obtained by using our *Drosophila* model with DSCR1 overexpression in DS, we examined the level of DSCR1 transcripts and DSCR1-mediated signaling in human trisomy 21 fetal brain tissues. Using quantitative real-time RT-PCR, we detected a 1.55 $\pm$ 0.14 (n = 3 experiments done in triplicate; values represent mean $\pm$ SEM)-fold increase in the level of *DSCR1* transcripts in human DS fetal brain. Compared to the control, trisomy 21 fetal brain tissue also demonstrated altered calcineurin-mediated signaling. Trisomy 21 fetal brain showed a 40% reduction in calcineurin activity (Fig. 3*D*), but 40% increase in PKA activity level (Fig. 3*E*). In addition, overexpression of DSCR1 led to a substantial increase in the amount of pCREB and c-Fos, whereas the overall level of CREB remained constant (Fig. 3*F*), consistent with altered biochemical signaling seen in the *nebula* transgenic flies.

GeneSwitch Expression System to Determine the Cause of Deficient Learning. To better understand whether the learning deficits in *nebula*-overexpressing flies are caused by excessive expression of *nebula* at the time of the learning test or by developmental defects, we used the inducible UAS/GeneSwitch expression system (18, 32). First, we overexpressed *nebula* transiently and specifically in the neurons of adult flies by feeding Elav-GeneSwitch/*nla*^{t1} flies (Elav-GS/*nla*^{t1}) RU486. In the presence of RU486, Elav-GS, a conditional GAL4-progesterone-receptor fusion protein controlled by the neuron-specific promoter Elav, could bind to the UAS and activate transcription of *nebula*. Second, we overexpressed *nebula* throughout development by feeding Elav-GS/*nla*^{t1} larvae with RU486 and examined the learning performance of eclosed adults 3–6 days after removal to normal food media. Fig. 4*A* shows adult Elav-GS/*nla*^{t1} flies that had been fed for 1 day with media containing RU486 showed a robust increase in *nebula* transcript level; Elav-GS/*nla*^{t1} larvae fed with RU486 showed elevated *nebula* transcript only during development (larvae), but not in adulthood (Fig. 4*B*). Pavlovian olfactory learning tests demonstrated that transient elevation of *nebula* transcripts in adult flies by RU486 feeding is sufficient to cause defective learning, similar to flies that constantly overexpress *nebula* (Figs. 3*B* and 4*C*). This result was further confirmed by transient overexpression of *nebula* by using the heat-shock driver. Note that the learning impairment

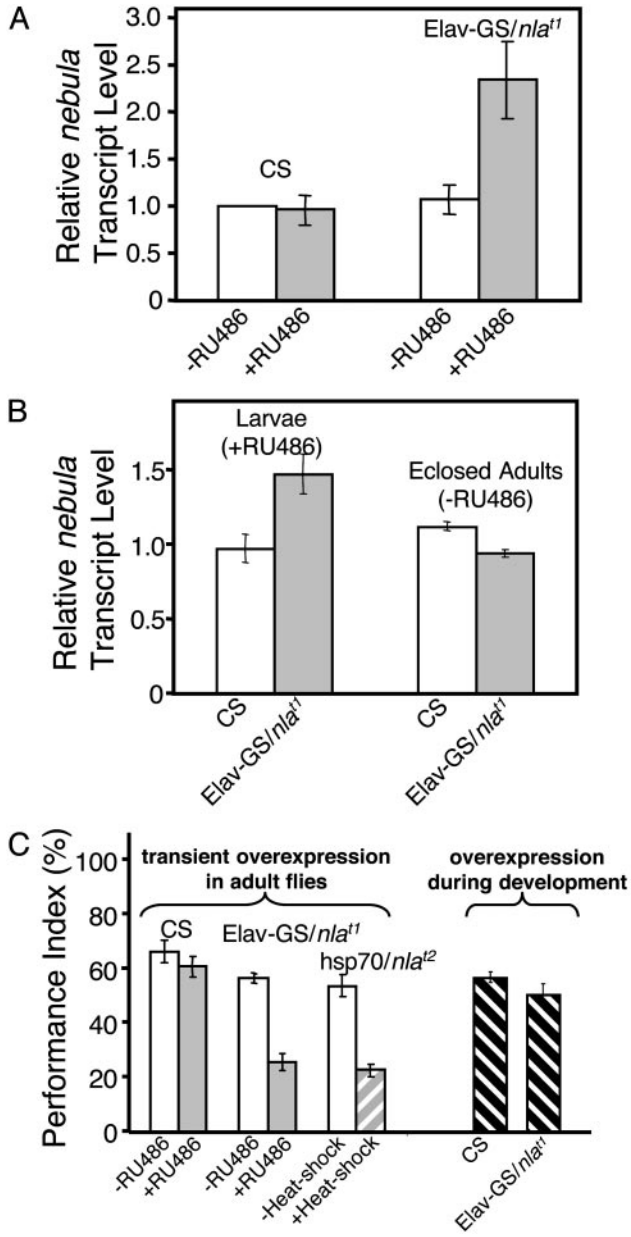

Fig. 4. Deficient learning in *nebula*-overexpressing flies is not caused by maldevelopment of the brain. (*A*) Quantitative real-time RT-PCR detects an increase in the level of *nebula* transcripts in adult heads 24 h after feeding Elav-GS/*nla*^{t1} flies with 100 μg/ml RU486 (+RU486), but not if flies were mock fed (−RU486). (*B*) Feeding Elav-GS/*nla*^{t1} larvae with 20 μg/ml RU486 during development induced *nebula* overexpression. Adults that had been transferred to normal food media for 3–6 days show normal *nebula* transcript levels. (*C*) Performance index values for Pavlovian olfactory learning tests. Transient overexpression of *nebula* in adult flies (Elav-GS/*nla*^{t1} and hsp70/*nla*^{t2}) impaired learning. However, learning performance of adult Elav-GS/*nla*^{t1} flies that had been fed with RU486 during development as described in *B* was not altered as compared to CS flies that had been raised the same way. For *A* and *B*, n = 4 experiments done in triplicate; for *C*, n = 4 for each strain. All values represent mean $\pm$ SEM.

is not caused by abnormal sensorimotor responses because both RU486-fed flies and heat-shocked flies responded normally to odor and shock avoidances (Fig. 7). In contrast, Elav-GS/*nla*^{t1} flies that overexpress *nebula* during development, but not at the time of olfactory learning tests, showed normal learning in comparison to control flies that had been raised the same way

(Fig. 4*C*). Together with data that nebula is a regulator of calcineurin-mediated signaling, our data strongly support that biochemical defects, rather than maldevelopment of the brain, are the cause of learning impairment in the transgenic flies.

Concluding Remarks. By using *Drosophila* as a simple model organism, we demonstrated that nebula mediates learning and long-term memory. Our observations that both *nebula* loss-of-function and overexpression mutants displayed learning defects suggest that precise regulation of nebula-mediated calcineurin signaling is necessary to maintain optimum learning. Furthermore, these results, along with data that the same biochemical pathway is disrupted in human trisomy 21 fetal brain tissue, strongly implicate the involvement of DSCR1 in mental retardation in DS.

Here, we focused our study on the function of DSCR1 in learning and memory to address its role in mental retardation associated with DS. However, DSCR1 is also expressed in other tissues such as the heart and skeletal muscle (3) and may thus regulate diverse calcineurin-dependent processes (33–35). Furthermore, calcineurin has been shown to regulate the transcription of DSCR1, suggesting the existence of a complex negative feedback regulation circuit (36). It will be interesting to investigate whether such regulatory mechanism also controls *nebula* expression in flies. Combined with its amenability to genetic manipulations and behavioral assays, *Drosophila* can be used to identify some of the genotype–phenotype relations in DS. In addition, our finding that defective learning in *nebula* overexpressing flies is likely caused by functional defects rather than developmental defects raises the exciting possibility of pharmacological intervention to ameliorate at least some of the cognitive deficits in DS patients. *Drosophila* may be used as a tool to rapidly screen for drugs that treat learning and memory deficits by restoring the balance of kinases and phosphatases. Genetic screens that identify suppressors of nebula in the learning and memory pathway will also provide insights into the underlying mechanism of mental retardation in DS.

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1. Reeves, R. H., Baxter, L. L. & Richtsmeier, J. T. (2001) *Trends Genet.* **17,** 83–88.
2. Epstein, C. J. (1995) in *The Metabolic and Molecular Bases of Inherited Disease*, eds. Scriver, C. R., Beaudet, A. L., Sly, W. S. & Vaile, D. (McGraw–Hill, New York), pp. 749–794.
3. Fuentes, J. J., Pritchard, M. A., Plana, A. M., Bosch, A., Ferrer, I. & Estivill, X. (1995) *Hum. Mol. Genet.* **4,** 1935–1944.
4. Fuentes, J. J., Genescà, L., Kingsbury, T. J., Cunningham, K. W., Pérez-Riba, M., Estivill, X. & de la Luna, S. (2000) *Hum. Mol. Genet.* **9,** 1681–1690.
5. Kingsbury, T. J. & Cunningham, K. W. (2000) *Genes Dev.* **14,** 1595–1604.
6. Görlach, J., Fox, D. S., Cutler, N. S., Cox, G. M., Perfect, J. R. & Heitman, J. (2000) *EMBO J.* **19,** 3618–3629.
7. Casas, C., Martínez, S., Pritchard, M. A., Fuentes, J. J., Nadal, M., Guimerà, J., Arbonés, M., Flórez, J., Soriano, E., Estivill, X., *et al.* (2000) *Mech. Dev.* **101,** 289–292.
8. Klee, C. B., Ren, H. & Wang, X. (1998) *J. Biol. Chem.* **273,** 13367–13370.
9. Mansuy, I. M., Mayford, M., Jacob, B., Kandel, E. R. & Bach, M. E. (1998) *Cell* **92,** 39–49.
10. Rothermel, B., Vega, R. B., Yang, J., Wu, H., Bassel-Duby, R. & Williams, R. S. (2000) *J. Biol. Chem.* **275,** 8719–8725.
11. Malleret, G., Haditsch, U., Genoux, D., Jones, M. W., Bliss, T. V. P., Vanhoose, A. M., Weitlaug, C., Kandel, E. R., Winder, D. G. & Mansuy, I. M. (2001) *Cell* **104,** 675–686.
12. Zeng, H., Chattarji, S., Barbarosie, M., Rondi-Reig, L., Philpot, B. D., Miyakawa, T., Bear, M. F. & Tonegawa, S. (2001) *Cell* **107,** 617–629.
13. Hamilton, B. A., Palazzolo, M. T., Chang, J. H., VijayRaghavan, K., Mayeda, C. A., Whitney, M. A. & Meyerowitz, E. M. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 2731–2735.
14. Montell, C., Jones, K., Hafen, E. & Rubin, G. (1985) *Science* **230,** 1040–1043.
15. Ito, K., Awano, W., Suzuki, K., Hirom, Y. & Yamamoto, D. (1997) *Development (Cambridge, U.K.)* **124,** 761–771.
16. Simpson, J. H., Kidd, T., Bland, K. S. & Goodman, C. S. (2000) *Neuron* **28,** 753–766.
17. Armstrong, J. D., de Belle, J. S., Wang, Z. & Kaiser, K. (1998) *Learn. Mem.* **5,** 102–114.
18. Osterwalder, T., Yoon, K. S., White, B. H. & Keshishian, H. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 12596–12601.
19. Brand, A. H., Manoukian, A. S. & Perrimon, N. (1994) *Methods Cell Biol.* **44,** 535–654.
20. Tully, T. & Quinn, W. G. (1985) *J. Comp. Physiol. A* **157,** 263–277.
21. Tully, T., Préat, T., Boynton, S. C. & Del Vecchio, M. (1994) *Cell* **79,** 35–47.
22. Dura, J. M., Préat, T. & Tully, T. (1993) *J. Neurogenet.* **9,** 1–14.
23. Dubnau, J. & Tully, T. (1998) *Annu. Rev. Neurosci.* **21,** 407–444.
24. de Belle, J. S. & Heisenberg, M. (1994) *Science* **263,** 692–695.
25. Crittenden, J. R., Skoulakis, E. M., Han, K. A., Kalderon, D. & Davis, R. L. (1998) *Learn. Mem.* **5,** 38–51.
26. Yin, J. C., Wallach, J. S., Del Vecchio, M., Wilder, E. L., Zhou, H., Quinn, W. G. & Tully, T. (1994) *Cell* **79,** 49–58.
27. Drain, P., Folkers, E. & Quinn, W. G. (1991) *Neuron* **6,** 71–82.
28. Goodwin, S. F., Del Vecchio, M., Velinzon, K., Hogel, C., Rossell, S. R. H., Rully, T. & Kaiser, K. (1997) *J. Neurosci.* **17,** 8817–8827.
29. Brand, A. H. & Perrimon, N. (1993) *Development (Cambridge, U.K.)* **118,** 401–415.
30. McGuire, S. E., Le, P. T. & Davis, R. L. (2001) *Science* **293,** 1271–1272.
31. Yuan, Q., Harley, C. W., Darby-King, A., Neve, R. L. & McLean, J. H. (2003) *J. Neurosci.* **23,** 4670–4675.
32. Roman, G., Endo, K., Zong, L. & Davis, R. L. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 12602–12607.
33. Crabtree, G. R. & Olson, E. N. (2002) *Cell* **109,** S67–79.
34. Ermak, G., Harris, C. D. & Davies, K. J. (2002) *FASEB J.* **16,** 814–824.
35. Vega, R. B., Rothermel, B. A., Weinheimer, C. J. Kovacs, A., Naseem, R. H., Bassel-Duby, R., Williams, R. S. & Olson, E. N. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 669–674.
36. Yang, J., Rothermel, B., Vega, R. B., Frey, N., McKinsey, T. A., Olson, E. N., Bassel-Duby, R. & Williams, R. S. (2000) *Circ. Res.* **87,** E61–E68.