Neuralized is expressed in the α/β lobes of adult *Drosophila* mushroom bodies and facilitates olfactory long-term memory formation

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Memory formation involves multiple molecular mechanisms, the nature and components of which are essential to understand these processes. *Drosophila* is a powerful model to identify genes important for the formation and storage of consolidated memories because the molecular mechanisms and dependence of these processes on particular brain regions appear to be generally conserved. We present evidence that the highly conserved ubiquitin ligase Neuralized (Neur) is expressed in the adult *Drosophila* mushroom body (MB) α/β lobe peripheral neurons and is a limiting factor for the formation of long-term memory (LTM). We show that loss of one copy of *neur* gene results in significant LTM impairment, whereas overexpression of Neur in the peripheral neurons of the α/β lobes of the adult MBs results in a dosage-dependent enhancement of LTM. In contrast, learning, early memories, or anesthesia-resistant memory are not affected. We also demonstrate that the role of Neuralized in LTM formation is restricted within the neurons of the periphery of the α/β lobes, and we suggest that this structural subdivision of the MBs participates in the formation of LTM.

Neurons respond and integrate external stimuli through changes in intracellular signaling, gene expression, and synaptic remodeling, which are the molecular hallmarks of the experience-dependent modifications of animal behavior referred to as learning and memory. *Drosophila* has played a cardinal role in defining the molecular mechanisms that subserve these processes (1, 2). Short-term memory and middle-term memory (MTM) are labile and last from minutes to 3–4 h, respectively. The long-lasting forms of memory (consolidated memory) last for many hours to several days and in *Drosophila* are distinguished into anesthesia-resistant memory (ARM) and long-term memory (LTM) (1, 3).

The major site for olfactory learning and memory in *Drosophila* are the mushroom bodies (MBs) (1). However, the exact role of particular neuronal subsets of the MBs in this process remains obscure (1). The MB neurons extend dendrites into a neuropil (calyces) ventral to the cell bodies where inputs arrive, conveying sensory information. The fasciculated axons of MB neurons form the pedunculus, which in the anterior part of the brain bifurcates to the medial (β, β', γ) and dorsal (α, α') axonal projections/lobes (4, 5).

Significant insight into the molecular mechanisms of olfactory learning and memory resulted from studies of *Drosophila* mutants and identification of genes critical for these processes (1). These analyses, however, are far from complete, especially with regard to the identification of genes that function specifically in the formation and storage of LTM, and have provided little information on the role of subpopulations of MB neurons in the process. This is because most of the genes studied so far are uniformly expressed in the MBs, or their expression profile is unknown (1). Thus, the identification of genes with restricted spatiotemporal expression in the MBs and the investigation of their role in memory will significantly advance the functional dissection of this brain structure and elucidate the contribution of each subregion to LTM formation.

We found that the gene encoding the E3 ubiquitin ligase Neuralized (Neur) is expressed in the neurons that extend their axons in the periphery of the adult MB α/β lobes. Neur is critical for the regulation of Notch (N) signaling during cell-fate decisions in *Drosophila* neurogenesis. Neur ubiquitinates the N ligand, Delta (Dl), and induces its endocytosis, necessary for the generation of the free intracellular domain of N (Nic), which translocates to the nucleus and modulates transcription (6). Neur protein is characterized by the NEUZ1 and NEUZ2 repeats, essential for its interaction with N ligands, and a C-terminal C3HC4 RING domain, important for Neur ubiquitin ligase function as well as Neur self-ubiquitination and proteosomal degradation (6–10).

Although the functional properties of Neur have been defined in *Drosophila*, evidence for a potential role in neuroplasticity came from studies of its mouse orthologue, *Neurl1*. *Neurl1* is expressed in the adult mouse forebrain, and its transcription is modulated by neuronal activity (11). In this report we provide evidence that *neur* is limiting for the formation of LTM and not its stability and retrieval. We also show that the action of Neur in LTM is restricted in the neurons within the α/β lobe periphery, and we suggest that this subpopulation of MB neurons participates in the formation of olfactory LTM.

Results

***neur* Is Expressed in the Adult MBs.** Preferential expression in the adult MBs of the GAL4 driver $neur^{GAL4\text{-}A101}$ (henceforth $neur^{GAL4}$) was identified in a screen for genes expressed in those neurons. β-Galactosidase reporter accumulated with exquisite specificity within the periphery of the α/β lobes, whereas it was notably absent from α'/β' neurons and barely detectable in the γ lobe [Fig. 1 *A–C* and supporting information (SI) Fig. S1]. We attempted detection of Neur itself, but neither published (7) nor newly derived antibodies (C. Delidakis, personal communication) were competent for immunohistochemistry. This likely reflects the low abundance of Neur in the MBs because it is detectable by these antibodies when overexpressed (7). The expression patterns of the $neur^{GAL4}$ driver and the endogenous *neur* during embryogenesis are identical (12). Furthermore, the transposon insertion at the 5′ of the gene disrupts *neur* function (12, 13). Thus, expression of $neur^{GAL4}$ in the MBs mimics, at least in part, the endogenous *neur* pattern without excluding expression in additional neurons. Nevertheless, the striking expression pattern of *neur* within adult MBs suggests a role in olfactory learning and memory.

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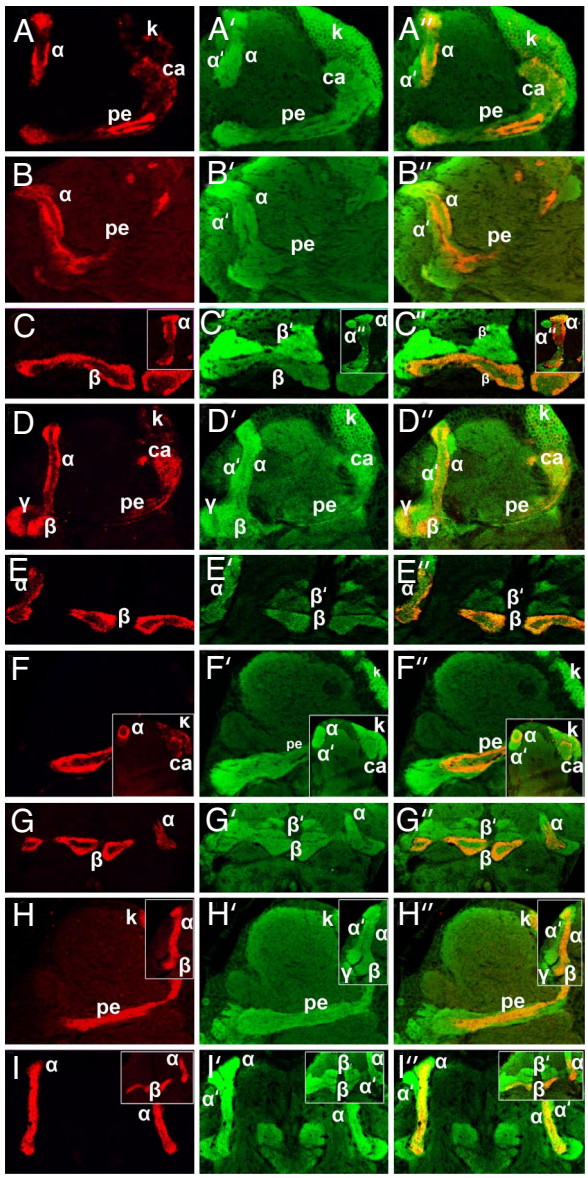

Fig. 1. Confocal images of immunohistochemical staining on sagittal (*A*, *B*, *D*, *F*, and *H*) and frontal (*C*, *E*, *G*, and *I*) paraffin brain sections from flies expressing a cytoplasmic UAS-*lacZ* reporter under the control of $neur^{GAL4}$ (*A–C*), c772 (*D* and *E*), MB247 (*F* and *G*), and c739 (*H* and *I*) GAL4 drivers. Red color represents the β-galactosidase distribution. MBs are detected with an anti-Leonardo antibody (green). The *Insets* in *C*, *F*, *H*, and *I* are images from a different section. ca, calyces; k, Kenyon cells; pe, pedunculus.

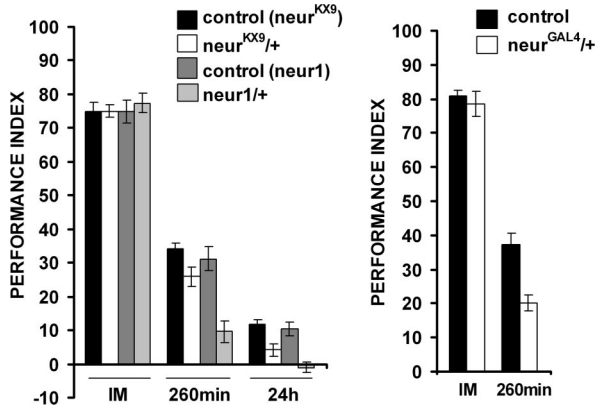

Fig. 2. Olfactory memory deficits in *neur* mutant flies. Two-hundred-sixty-minute and 24-h olfactory memories in *neur* mutant heterozygotes after 12 CS/US pairings ($n \geq 8$). Dunnett's tests revealed significant differences between the *neur* mutants and their controls for 260-min memory ($neur^{KX9}/+$, $P < 0.01$; $neur^{1}/+$, $P < 0.0001$; $neur^{GAL4}/+$, $P < 0.002$) and 24-h memory ($neur^{KX9}/+$, $P < 0.01$; $neur^{1}/+$, $P < 0.0001$).

Reduction of Neuralized Results in Memory Deficits. To investigate the putative role of Neur in behavioral plasticity, we tested *neur* heterozygous mutants in olfactory classical conditioning. We were not able to use *neur* mutant homozygotes because all known mutations of the gene are lethal (12, 14). Thus, $neur^{1}$/TM3, $neur^{KX9}$/TM3, or $neur^{GAL4}$/TM3 flies were mated *en masse* to the w^{1118} strain and heterozygous mutant progeny, and their TM3/+ siblings (controls) were tested. Learning and 90-min memory (MTM) of *neur* mutants appeared identical to that of controls (Fig. S2). However, these animals displayed significantly reduced 260-min memory that was pronounced in heterozygotes for the loss-of-function allele $neur^{1}$ and the presumed null $neur^{GAL4}$ (Fig. 2). The memory deficit was detectable 24 h later despite the low performance of controls after single-cycle training. In fact, memory in the mutants was not statistically different from zero (Fig. 2). The mutants did not exhibit deficiencies in the perception of the conditioned stimuli (CS) and unconditioned stimuli (US) (Table S1) or detectable aberrations in the architecture of the brain and the MBs (Fig. S3). These results suggest that the decrease in Neur levels reduces signaling necessary for memory formation or stability, indicating that the protein may be a limiting factor for these processes.

Overexpression of Neuralized in the MBs Enhances Consolidated Memory. To test whether Neur is limiting for memory, we investigated the effects of increasing its amount in the MBs using UAS-*neur* transgenes (6). We attempted to emulate the *neur* expression pattern with various MB-restricted GAL4 drivers because $neur^{GAL4}$-directed expression of *neur* transgenes precipitated embryonic lethality. This was not surprising given the role of *neur* in embryonic neurogenesis (12, 14) and the variable, dose-sensitive phenotypes upon *neur* overexpression in larvae (15). We determined the precise expression pattern of MB-specific GAL4 lines in relation to that of $neur^{GAL4}$ using a UAS-*lacz* reporter and immunostainings of adult brain sections. We found that the c772 and MB247 drivers directed expression in peripheral neurons of α/β lobes (Fig. 1 *D–G*), with much lower expression in the γ lobes. The pattern of these two GAL4 drivers in the α/β lobes was remarkably similar to that of $neur^{GAL4}$ (Fig. 1 *A–C*). In contrast, c739 was expressed in internal neurons of the α/β lobes (Fig. 1 *H* and *I*), complementary to that of c772, MB247, and $neur^{GAL4}$.

To determine whether *neur* overexpression affects learning, we assessed the immediate memory of animals expressing UAS-*neur* under c772 after training with increasing numbers of CS/US pairings (Fig. 3*A*). All strains performed equally, and performance improved to a plateau concomitantly with the number of CS/US pairings. Elevation of Neur in the MBs did not affect responses to the CS and US stimuli (Table S1) or the gross morphology of the MBs (Fig. S3). Because *neur*-overexpressing animals showed learning similar to that of controls, we used a submaximal training protocol of six CS/US pairings to increase the resolution of memory tests. Ninety-minute memory was similar in flies overexpressing *neur* and controls (Fig. 3*B*). In contrast, memory assessed 260 min later was significantly higher in the UAS-*neur*-expressing flies (Fig. 3 *B* and *C*). In addition, elevation of Neur in the MBs resulted in higher 24-h memory assessed after conditioning with a single cycle of 12 CS/US pairings (Fig. 3*D*). Identical results were obtained when we expressed an independent *neur* transgene (Fig. S4*C*) and UAS-*neur* with MB247 (Fig. 3 *C* and *D*). Because the c772 and MB247 show minor expression in the γ lobes and recent studies of

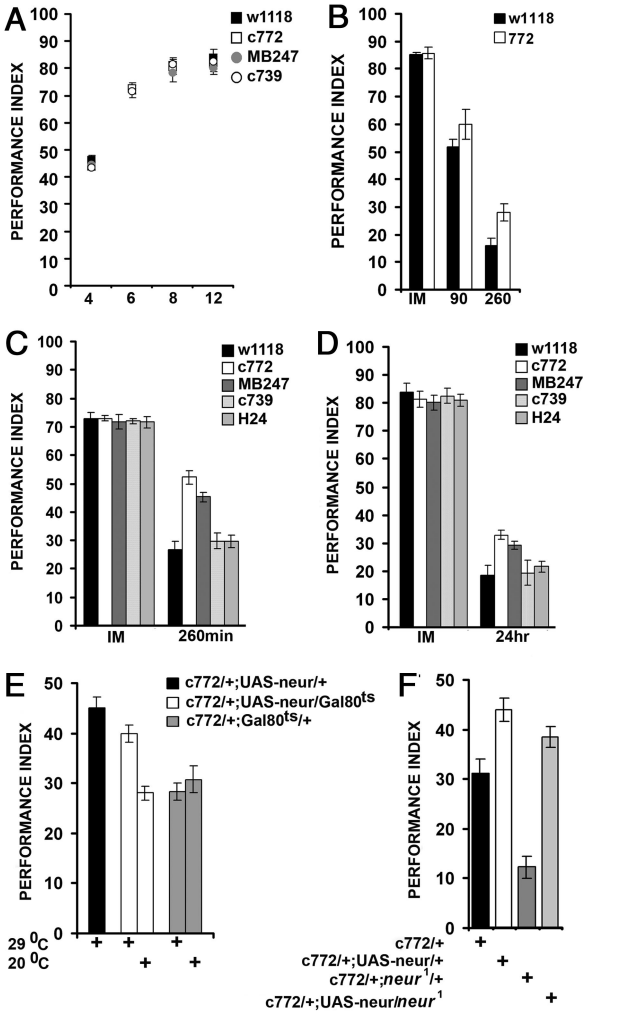

Fig. 3. Directed overexpression of *neur* in the MBs results in LTM enhancement and rescues the memory deficits observed in *neur* mutant flies. (*A*) Immediate memory (IM) elicited by different numbers of CS/US pairings for c772/+;UAS-*neur*/+ (c772), MB247/+;UAS-*neur*/+ (MB247), and c739/+;UAS-*neur*/+ (c739) flies and UAS-*neur*/+ (w1118) controls, derived by crossing the UAS-*neur* strain to w^{1118}. ANOVA indicated significant effects of the number of pairings ($P < 0.0001$) but not of genotype. (*B*) Olfactory memory after six CS/US pairings. Dunnett's tests showed significant differences between UAS-*neur*/+ and c772/+;UAS-*neur*/+ for 260-min memory ($P < 0.001$). (*C* and *D*) Two-hundred-sixty-minute memory (*C*) and 24-h memory (*D*) for c772/+;UAS-*neur*/+ (c772), MB247/+;UAS-*neur*/+ (MB247), c739/+;UAS-*neur*/+ (c739), H24/+;UAS-*neur*/+ (H24), and UAS-*neur*/+ flies after six CS/US (*C*) or 12 CS/US (*D*) pairings ($n \geq 9$). Dunnett's tests revealed highly significant differences only between controls and c772/+;UAS-*neur*/+ and MB247/+;UAS-*neur*/+ flies ($P < 0.001$). (*E*) Two-hundred-sixty-minute memory of c772/+;UAS-*neur*/+, c772/+;UAS-*neur/Gal80^{ts}*, and c772/+; *Gal80^{ts}*/+ flies after training with six US/CS pairings ($n \geq 9$). The performance of c772/+;UAS-*neur/Gal80^{ts}* flies kept at 29°C (Gal80 inactive, transgene active) and the c772/+;UAS-*neur*/+ strain was significantly different from that of c772/+;UAS-*neur/Gal80^{ts}* animals kept at 20°C (Gal80 active, transgene inactive) and c772/+;*Gal80^{ts}*/+ controls ($P < 0.0001$). No differences were observed between c772/+;UAS-*neur*/+ and c772/+;UAS-*neur/Gal80^{ts}* flies kept at 29°C. (*F*) Two-hundred-sixty-minute memory after six CS/US pairings for c772/+; *neur^{1}*/+ flies and flies expressing UAS-*neur* under c772 in wild-type (c772/+;UAS-*neur*/+) or *neur^{1}*/+ (c772/+;UAS-*neur/neur^{1}*) genetic background. The performance of c772/+;UAS-*neur/neur^{1}* animals was significantly different from both c772/+; *neur^{1}*/+ ($P < 0.0001$) and c772/+ ($P < 0.001$) flies. There was no difference between c772/+;UAS-neur/*neur^{1}* and c772/+;UAS-*neur*/+ flies.

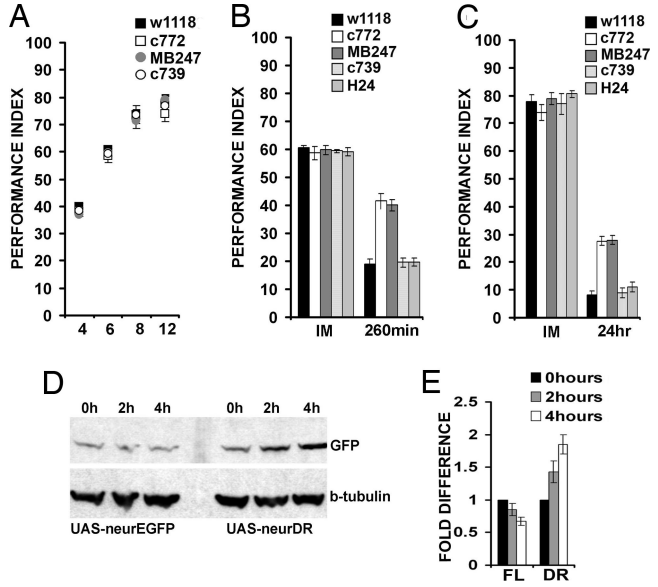

Fig. 4. Deletion of the RING domain enhances the effect of *neur* overexpression. (*A*) Immediate (IM) memory elicited by different numbers of CS/US pairings for c772/+;UAS-*neurDR*/+ (c772), MB247/+;UAS-*neurDR*/+ (MB247), and c739/+;UAS-*neurDR* (c739) flies and UAS-*neurDR*/+ (w1118) controls ($n \geq 9$). ANOVA showed significant effects of the number of pairings ($P < 0.0001$) but not of genotype. (*B* and *C*) Two-hundred-sixty-minute memory (*B*) and 24-h memory (*C*) of c772/+;UAS-*neurDR*/+, MB247/+;UAS-*neurDR*/+, c739/+;UAS-*neurDR*, H24/+;UAS-*neurDR*, and UAS-*neurDR*/+ flies ($n \geq 8$). Dunnett's tests showed highly significant differences of both 260-min and 24-h memory ($P < 0.0001$) of c772/+;UAS-*neurDR*/+ and MB247/+;UAS-*neurDR*/+ animals and controls. (*D*) Western blot of head lysates from flies expressing Neur or NeurDR fused to EGFP, under c772, at 0, 2, and 4 h at 18°C after 24 h of maximal induction at 29°C. β-Tubulin was used as loading control. Image quantification analysis showed that the levels of the full-length Neur (FL) are reduced over time whereas NeurDR (DR) shows increased accumulation (*E*).

the Orb2 gene implicated these lobes in courtship LTM (16), we targeted the UAS-*neur* transgene to these neurons with the H24 driver, but we did not observe any effect (Fig. 3 *C* and *D*). Because H24, like all existing γ lobe-specific drivers, expresses weakly, memory may have not been elevated because of insufficient increase of Neur levels. However, expression of UAS-*Orb2* under this driver sufficed to partially rescue the courtship memory deficits of *Orb2* mutants (16). This and the nearly undetectable expression of *neur^{GAL4}* in the γ lobes suggest that the effect of Neur in memory is due to its elevation in α/β lobes.

To confirm that the effect of Neur overexpression in memory was specific to the α/β lobe peripheral neurons rather than a nonspecific effect of the transgene, we directed its expression with c739. This driver exhibits similar expression levels with the c772 and MB247 (Fig. S5) but expresses in internal neurons of the α/β lobes (Fig. 1 and Fig. S1). Neither 260-min nor 24-h memories of the UAS-*neur*-expressing flies were different from those of controls (Fig. 3 *C* and *D*). To rule out any histologically undetectable developmental effects of *neur* overexpression, we expressed the UAS-*neur* with c772 and controlled its expression with the temperature-sensitive version of the GAL80 protein (GAL80^{ts}) that blocks GAL4 activity (17). The expression of *neur* transgene was blocked during development but was permitted in adults before training. The data in Fig. 3*E* demonstrate that elevation of Neur in the adult MBs suffices to enhance 24-h memory.

If Neur levels are limiting for consolidated memory and its effect is specific as our data suggest, we would expect that the memory deficits of the *neur* mutant heterozygotes would be reversed upon expression of UAS-*neur* with c772. Indeed, memory in *neur* mutants was not only fully rescued, but it also appeared consistently higher than that of controls (Fig. 3*F*), indicating that Neur has a limiting role in memory within adult MBs.

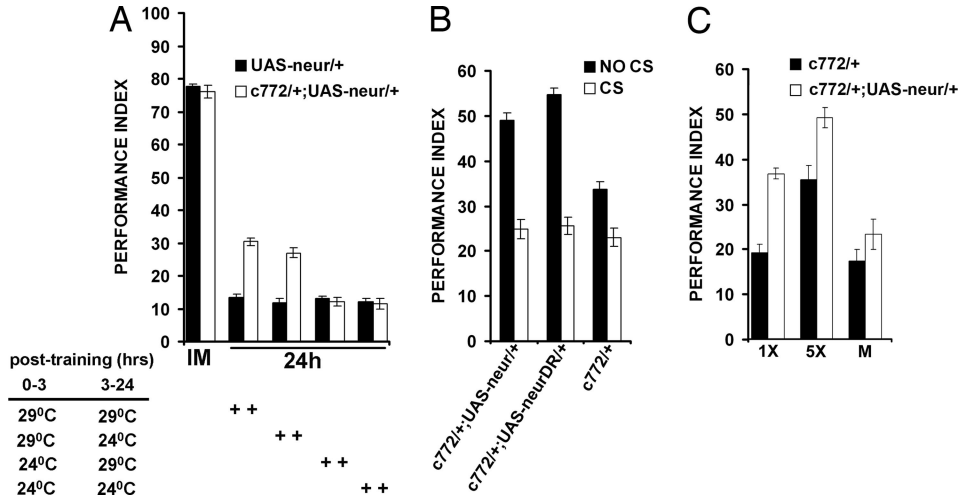

Fig. 5. *neuralized* is essential for the formation of LTM. (*A*) Immediate (IM) and 24-h memories after 12 CS/US pairings ($n \geq 10$). After conditioning, the animals were kept under the indicated conditions until testing. Student's *t* tests indicated highly significant differences of 24-h memory ($P < 0.0001$) between UAS-*neur*/+ (controls) and c772/+;UAS-*neur*/+ flies only when they were kept at 29°C either continuously or for the first 3 h after training. (*B*) Two-hundred-sixty-minute memory performance of c772/+;UAS-*neur*/+, c772/+;UAS-*neurDR*/+, and c772/+ (control) flies before and after a 10-min cold shock given 2 h after training. Two-way ANOVA showed significant effects of genotype and treatment ($n \geq 10$, $P < 0.0001$). Dunnett's tests revealed that compared with c772/+ the 260-min memory of c772/+;UAS-*neur*/+ and c772/+;UAS-*neurDR*/+ flies was highly different ($P < 0.0001$), but there was no difference in the performance after the cold shock. The difference between the 260-min memory of the controls before and after cold shock was significant ($P < 0.005$). (*C*) Twenty-four-hour memory of c772/+;UAS-*neur*/+ and c772/+ (control) animals after one (1X) and five (5X) spaced or massed (M) cycles of 12 CS/US conditioning. Statistically significant differences of the performance of c772/+;UAS-*neur*/+ and controls were found only for 1X and 5X trainings. The performance of the c772/+;UAS-*neur*/+ flies was higher than that of controls ($n \geq 10$; 1X, $P < 0.001$; 5X, $P < 0.01$).

Deletion of the RING Domain of Neuralized Results in Increased Protein Stability and Enhancement of Consolidated Memory. Because the RING domain possesses ubiquitin ligase activity, we expressed in the MBs a truncated form of Neur (NeurDR), with the RING replaced by the EGFP (6). Animals expressing UAS-*neurDR* under c772 exhibited normal learning (Fig. 4*A*). Surprisingly, 260-min and 24-h memories were significantly higher in UAS-*neurDR*-expressing flies than those of controls (Fig. 4 *B* and *C* and Fig. S4*A*). The observed memory increase was not due to position effects because the same results were obtained with a different UAS-*neurDR* line (Fig. S4*C*). Enhanced memory was also observed when the transgenes were expressed under MB247 but not c739 or H24. These results demonstrate that the effect of NeurDR is specific for neurons in the α/β lobe periphery. The UAS-*neurDR*-expressing flies responded normally to the stimuli, and the morphology of their MBs was indistinguishable from those of controls (Table S1 and Fig. S3). The observed memory enhancement resulted from deletion of the RING rather than the addition of the EGFP, because similar 24-h memory results were obtained (Fig. S4*C*) from flies expressing the full length protein without (UAS-*neur*) or with the EGFP fusion (UAS-*neurEGFP*). It is noteworthy that the expression of *neurDR* yielded 35% higher 24-h memory than that of animals expressing the full-length protein, when all such experiments were averaged (Fig. S4*B*). Therefore, deletion of the RING domain did not result in dominant negative effects, but rather augmented the memory-enhancing effects of Neur elevation in the MBs.

One explanation for the effect of NeurDR is that loss of the RING domain, which is involved in Neur degradation (8), results in its increased stability and accumulation to a higher level. To address this, flies carrying the UAS-*neurEGFP* and UAS-*neurDR* transgenes under c772 were raised to adulthood at 20–22°C, placed at 29°C for 24 h to induce maximal expression, and then kept at 18°C, where little new transcription of the transgenes would be expected (18), until killed. Thus, the level of the proteins in head lysates at different time points after maximal induction would reflect their relative stability. The results in Fig. 4 *D* and *E* are consistent with the above hypothesis and indicate that the levels of RINGless Neur remain high 4 h after maximal induction in contrast to those of the full-length protein, which decline over time. Our data confirm that, as in other contexts, the RING domain regulates Neur levels in the adult MBs.

Neuralized Is Required for LTM Formation. The memory enhancement observed upon Neur elevation in the MBs could result from increased memory formation, stability, or recall. To differentiate between these possibilities we capitalized on the apparent relative instability of the full-length protein and the requirement for elevated expression of the transgene at 29°C after training for consistent 24-h memory enhancement. 772/+;UAS-*neur*/+ flies and their controls kept at 29°C for 48 h were given a single cycle of 12 CS/US training and then either returned to 29°C or kept at 24°C until testing 24 h later. Another group of such animals was kept at 29°C for the first 3 h after training and then at 24°C until testing; a fourth group underwent the converse treatment, kept at 29°C for 21 h before testing, but not the initial three.

The results in Fig. 5*A* clearly demonstrate that, compared with controls, the *neur*-overexpressing flies showed enhanced 24-h memory when kept at 29°C either for the entire period or specifically for the first 3 h after training. Because processes that lead to consolidated memory are thought to be operant during these initial posttraining hours (1, 3, 19), the results suggest that Neur is limiting for processes required during memory formation in neurons of the α and β lobe periphery.

There are two forms of consolidated memory in the fly: ARM and LTM (2, 19). They can be differentiated operationally because ARM is resistant to cold shock anesthesia (19, 20). Additionally, a single training trial consisting of 12 CS/US pairings induces mostly ARM (19, 20). To investigate whether ARM is the memory affected by Neur overexpression, we trained the animals with a single 12 CS/US cycle, gave them a 10-min cold shock 2 h after training (19), and tested them 2 h later (260-min memory). The cold shock erased a fraction of 260-min memory of control animals (Fig. 5*B*), accordingly with the suggestion that one 12 CS/US training cycle yields consolidated memory composed mostly of ARM. In

contrast, the cold shock collapsed the enhanced 260-min memory of the *neur*-overexpressing flies (Fig. 5*B*), indicating that it is not ARM, but possibly LTM, that is elevated in these animals, thus attributing to Neur a role in facilitation of LTM formation. Consistent with the interpretation that elevation of Neur in the MBs facilitates *bona fide* LTM formation after a single training trial, the enhanced 24-h memory in Neur-overexpressing flies was eliminated by the protein synthesis inhibitor cycloheximide, and it was indistinguishable from the 24-h memory performance of control flies after training with a single cycle of 12 CS/US pairings (Fig. S6; compare with Fig. 3*D*). To further establish whether Neur overexpression enhances the formation of LTM, we used the spaced training paradigm, established to specifically yield this form of consolidated memory (19, 20). Twenty-four-hour memory of *neur*-overexpressing animals was significantly higher than that of controls after spaced training, similar to the increased memory after a single round of 12 CS/US pairings (Fig. 5*C*). In contrast, controls and *neur*-overexpressing flies exhibited equal performance after massed training that was similar to that of controls after one cycle of 12 CS/US pairings. Our data clearly demonstrate that Neur overexpression in the MBs enhances LTM but not ARM and predict that Neur participates in signaling involved in long-lasting changes of neuroplasticity (1, 21).

Discussion

Our results demonstrate that Neur, critical for neurogenesis in the fly, plays an essential and limiting role for LTM formation in differentiated neurons. This is supported by complementary experiments that demonstrate dramatic reduction of memory in *neur* mutant heterozygotes and significant enhancement of LTM upon elevation of Neur levels only within MB neurons that normally express the gene. Consistent with this, the memory deficit in *neur* mutant heterozygotes is reversed upon UAS-*neur* transgene expression specifically in the adult MB α/β lobe peripheral neurons. We also show that the enhancement of LTM is proportional to Neur dosage and does not have a developmental etiology. These observations strongly suggest that Neur acts to enhance LTM by modulating mechanisms within neurons of the α/β lobe periphery.

Recent evidence indicates functional specialization of the MB axonal projections, with an essential role for the α/β lobes in LTM (22–24). In agreement, our data strongly suggest that Neur is required for LTM formation within c772 and MB247 neurons in the periphery of the α/β lobes. In support of this, c772 and MB247 neurons were recently shown to be important for appetitive LTM (25). However, the c739 neurons in the center of the α lobes were shown to harbor a LTM cellular trace, evident as enhanced Ca^{2+} influx, 9 or 24 h after spaced training (23). In contrast, elevation of Neur within these neurons did not enhance LTM. Because the cellular memory trace within c739 neurons appeared 9 h after training, it may characterize a late phase of LTM consolidation or maintenance. This is distinct from the requirement for elevated Neur within the initial 3 h after training, likely representing involvement in LTM formation. Then, at least two different LTM generating systems may exist, one Neur-dependent in neurons at the periphery of the α/β lobes and another Neur-independent in the central c739 neurons of the α lobe. Alternatively, considering the spatiotemporal differences in the requirement for Neur function and cellular trace appearance, LTM formation may occur within c772 neurons and be later transferred to the central c739 neurons for storage and recall. Consistent with the later notion, neurotransmission from the α/β lobes and specifically from c739 neurons was reported to be essential for memory retrieval (26–28), and particular neuronal populations of the MBs seem to be used sequentially in distinct phases of aversive and appetitive memory processing (26). A recent report implicated the γ lobes in long-term courtship conditioning memory (16), and it is likely that, in contrast to olfactory LTM, these lobes are specifically required for this type of consolidated memory. It is noteworthy, however, that full rescue of the long-term courtship memory deficit in the *orb2* mutants in that study was achieved with 201Y and c772 drivers, which, in addition to γ lobes, also express in the α and α/β lobes, respectively (16). Therefore, in agreement with previous reports (24), at least the α lobe in addition to the γ appears to be required for efficient long-term courtship memory. Thus, our data support the proposed structural and functional division of the MBs, provide functional validation of the suggested model, and refine the role of neuronal subpopulations of the α/β lobes in LTM.

Interestingly, Neur overexpression yielded significant LTM with a single 12-pairing cycle in a dosage-dependent manner, whereas multiple spaced cycles are necessary for equivalent LTM in control animals (19, 20, 24). Therefore, Neur appears to facilitate LTM formation and to be limiting for this process. The limiting role of Neur in memory is in agreement with the low abundance of the protein in the MBs and indicates that its levels are tightly regulated. In fact, the RING domain of Neur has been shown to be critical for Neur proteosomal degradation (7, 8, 10). Consistently, deletion of the RING domain resulted in increased Neur levels, suggesting that the ubiquitin-proteasome system likely regulates the abundance of the endogenous Neur protein in the MBs.

Neur has an established role as an E3 ubiquitin ligase involved in the regulation of N signaling in developmental contexts, and this could explain its action in LTM. This is consistent with increasing evidence for the critical role of ubiquitination in the regulation of synaptic strength and long-term changes in plasticity (29) and in agreement with the potential role of N in LTM. In particular, conditional overexpression of N enhances LTM with a single 12-pairing cycle (30), whereas reduction of N within MBs results in impaired LTM (31). Surprisingly, expression of NeurDR yielded pronounced elevation of memory. The role of the NeurDR remains unclear because its function in N signaling in development is controversial. Studies report a dominant negative effect of NeurDR (7, 15), whereas others suggest a gain-of-function phenotype (6, 32). An explanation for this puzzling question comes from evidence for redundant function of Neur and the E3 ubiquitin ligase Mind bomb (Mib), which also regulates the endocytosis of N ligands and N activation. Although the endogenous requirements for Neur and Mib are quite distinct and the two ligases are mostly expressed in different cell types, they also coexist and act synergistically or redundantly for N activation in developmental contexts (32, 33). For example, the establishment of the wing DV boundary requires Mib but not Neur. In that context, absence of Mib is rescued by ectopic expression of Neur. Importantly, ectopic expression of NeurDR in the wing pouch results in overactivation of N signaling that could be due to potentiation by Mib (6), as the effect of NeurDR in $mib^{-/-}$ clones, in the same developmental context, is abolished (32). It is possible that NeurDR besides ubiquitination has additional, yet unknown, stimulatory activity, but this activity can be manifested only when Mib is present and the ubiquitination of N ligands is feasible (32). Potentiation of NeurDR by Mib in the adult MBs and its increased stability indicated by our data could explain the pronounced increase of memory, similar to what has been observed for N signaling activation in the wing pouch (6, 32). Elucidation of the yet unknown expression patterns of Mib, N, and its ligands in the adult *Drosophila* brain is intriguing and will help us validate the above model.

Neur function in LTM through an N-independent mechanism is an alternative possibility. One aspect of such Neur function is supported by detection of Neur in the nucleus in contexts where N and its ligands are absent (34). Nuclear localization of Neur does not depend on the RING but the Neuz1 domain, also essential for its interaction with N ligands, and it is abolished upon ectopic expression of Dl (34). Moreover, earlier studies have shown that the mouse and rat Neurl1 shuttle between cytoplasm and nucleus in Neuro2a cells, their nuclear localiza-

tion increases in response to neuronal differentiation, and they are potent inhibitors of transcription (11). Interestingly, the activity of Neurl1 in transcription was found to be dependent on the Neuz domains but not the RING. Consistent with our observations and given that LTM depends on transcription regulation, a novel function of Neur in the nucleus that does not involve its ubiquitin ligase activity and operates in LTM formation is challenging, and it is worth further investigation.

Although the exact role of Neur in LTM remains to be resolved, our findings emphasize the importance of Neur for this process. It seems that alternative mechanisms of Neur function may be operative in different contexts depending on the regulated compartmentalization of the protein and the function of its domains. The questions of what mechanisms operate in memory formation will be resolved by future work.

Materials and Methods

***Drosophila* Culture and Strains.** The lines c772, c739, and MB247 have been described before (35, 36). All transgenic strains expressing *neur* and its modified forms (6), as well as the $neur^{1}$/TM3, $neur^{KX9}$/TM3, and $neur^{GAL4}$/TM3 lines, were kind gifts of C. Delidakis (Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology, Crete). $Gal80^{ts}$ has been described previously (17).

Histology and Immunohistochemistry. Immunohistochemistry and histology were performed on 7-μm paraffin head sections as previously described (4, 37). The antibodies used and conditions can be found in *SI Materials and Methods*.

Western Blot Analysis. Dissected heads were homogenized in RIPA buffer with protease inhibitors, and proteins were separated by SDS/PAGE and blotted. The antibodies used, the conditions, and the information for detection of the Gal4 line expression levels can be found in *SI Materials and Methods*.

Behavioral Analyses. Learning and memory were assessed using the negatively reinforced olfactory assay as described previously (35). Immediate memory refers to performance assessed 3 min after training (37). Control experiments established that six CS/US pairings offered the best resolution for all assessments of immediate to 260-min memories, but 12 CS/US pairings were required to reliably measure 24-h memory. The detailed information for the conditions of the behavioral training and testing can be found in *SI Materials and Methods*.

To acquire the maximal expression of the transgenes, flies raised at 20–22°C were placed at 29°C for 48 h before behavioral experiments (35). The animals were placed back at 29°C in the dark after training until 30 min before testing. For the cold shock experiments, the vials were immersed in ice water in the dark for 10 min and recovered at 29°C until testing.

Animals bearing the $Gal80^{ts}$ were raised at 20°C, and UAS-*neur* transgenes were induced maximally by placing the flies at 29°C for 48 h. The animals were kept at the training temperature (25°C) for 30 min before training. After training, the flies were returned to 29°C for 3 h and then placed at 25°C until testing.

Statistical Analysis. Untransformed data were analyzed parametrically with the JMP5.1 statistical software package (SAS Institute) as described before (35).

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