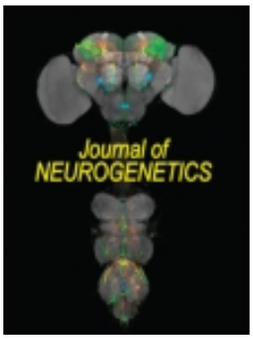

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Suppressor of Hairless Is Required for Long-Term Memory Formation in *Drosophila*

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Abstract: Suppressor of Hairless [Su(H)] is a DNA-binding protein of the Notch-signaling pathway, which is important for developmental processes and has been implicated in behavior plasticity. It acts as a transcriptional activator in the Notch pathway, but also as a repressor in the absence of Notch signaling. Our previous work has shown that Notch signaling contributes to long-term memory formation in the *Drosophila* adult brain. In the present report, we show that Su(H) null heterozygous mutants perform normally for learning, early memory, and anesthesia-resistant memory, whereas long-term memory is impaired. Interestingly, we find overexpressing wild- type Su(H) also causes long-term memory defect in *Drosophila*. Significantly, induction of a heat-shock inducible $Su(H)^{+}$ transgene before training can fully rescue the memory defect of Su(H) mutants, thereby demonstrating an acute role for Su(H) in behavioral plasticity. We show that Su(H) is widely expressed in the adult brain. Transgenic expression of wild-type Su(H) in the Mushroom Bodies is sufficient to rescue the memory defect of Su(H) mutants. Our data clearly demonstrate that transcriptional activity of Su(H) in Notch signaling in the mushroom bodies is critical for the formation of long-term memory.

Keywords: Suppressor of Hairless, long-term memory, Notch signaling, mushroom bodies

INTRODUCTION

The Notch (N)-signaling pathway is an evolutionarily conserved mechanism and it controls cell fate in both vertebrate and invertebrate development (Artavanis-Tsakonas et al., 1999). In N signaling, two kinds of ligands, Delta and Serrate, bind to the N extracellular domain of the neighbor cells, which causes the N intracellular domain (N^{ICD}) to translocate to the nucleus after proteolytic release (Brou et al., 2000; Struhl & Greenwald, 2001). Then, in the nucleus, N^{ICD} binds to a DNA-binding transcription factor, Suppressor of Hairless [Su(H)], to activate the transcription of various target genes (Delidakis & Artavanis-Tsakonas, 1992; Bailey & Posakony, 1995; Lecourtois & Schweisguth, 1995). In the absence of N signaling, Su(H) represses N target genes, and this ''default repression'' is essential for proper cell specification (Kao et al., 1998; Morel et al., 2001; Barolo et al., 2002). Previous studies have revealed other functions of N signaling besides its vital role in development. In *Drosophila*, molecular disruption of N specifically produces long-term memory deficits, while having no effect on other memory phases (Ge et al., 2004; Presente et al., 2004). Meanwhile, knock-out of Notch1, one of the N homologs in mice, results in deficits in spatial learning and memory without affecting other activities (Costa et al., 2003, 2005). These results suggest a role for the N-signaling pathway in learning and memory.

Previous studies indicated that RBP-J, the Su(H) homolog in mice, affects learning in vertebrates. Null heterozygous mice ($RBP\text{-}J^{+/-}$) have a spatial learning defect, which is similar to N mutants, suggesting the Su(H)-dependent N pathway affects spatial learning in mice (Costa et al., 2003). A major limitation of this work was that it could not distinguish an acute impairment of memory formation from a more chronic abnormality in neurodevelopment. Therefore, using *Drosophila melanogaster* to explore the function of Su(H)-dependent N signaling in learning and memory is important and necessary.

Learning and memory in *Drosophila* has been studied with a Pavlovian procedure, in which flies learn to associate a conditioned stimulus (CS; usually odors)

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with an unconditioned stimulus (US; usually an electrical footshock) (Tully & Quinn, 1985). Behavioral, pharmacological, and genetic analysis has dissected olfactory memory into four distinct phases: short-term memory (STM), middle-term memory (MTM), anesthesia-resistant memory (ARM), and long-term memory (LTM) (Tully et al., 1990, 1994). STM and MTM appear in the early stage after training and decay away within 5 hours, while ARM appears slowly, reaching asymptotic levels within 2 hours after training. STM, MTM, and ARM are insensitive to cycloheximide (CXM), a protein-synthesis inhibitor, and are present after single and massed training (Folkers et al., 1993; Tully et al., 1994). However, LTM is uniquely induced by spaced training, specifically depends on protein synthesis, and can last 7 days (Tully et al., 1994). Meanwhile, other studies on *Drosophila* indicated ARM and LTM cannot coexist, and they are formed by a different mechanism (Isabel et al., 2004).

In this report, we show that Su(H) null heterozygous mutants have normal learning, early memory, and ARM, but LTM is specifically defective. Overexpression of wild-type Su(H) also has similar effects. We show that Su(H) is widely expressed in the adult brain, and transgenic expression of wild-type Su(H) in the Mushroom Bodies (MBs) is sufficient to rescue memory defect in Su(H) mutants. Our data clearly demonstrate that activity of Su(H) in the MBs is critical for the formation of long-term memory.

MATERIALS AND METHODS

Fly Stocks

Flies were raised at room temperature (about 25°C) on standard cornmeal medium. The Su(H) mutants, $Su(H)^{SF8}$, $Su(H)^{IB115}$, and $Su(H)^{HG36}$, (Ashburner, 1982; Schweisguth & Posakony, 1992) were generously provided by M. Ashburner (University of Cambridge, Cambridge, UK) and were outcrossed with *CyO* balancer with w^{1118} (*isoCJ1*) background for five generations. Transgenic flies, heat-shock Su(H) (*hs-Su(H)*$^+$), and *UAS-Su(H)*$^+$ (Schweisguth & Posakony, 1994) were gifts from J. Posakony (University of California, San Diego, California, USA) and were outcrossed for five generations with our standard wild-type strain, w^{1118} (*isoCJ1*). Transgenic flies, $Gal80^{ts}$, *MB247-Gal4*, and *C232-Gal4*, were from Bloomington Stock Center (Indiana University, Bloomington, Indiana, USA) and were outcrossed for five generations with w^{1118} (*isoCJ1*). Then, we combined them to generate $Gal80^{ts}$*; MB247-Gal4* and $Gal80^{ts}$*; C232-Gal4*. For all behavior analyses, w^{1118} (*isoCJ1*) served as the control, if not specifically mentioned.

Heat-Shock Treatment

For heat-shock treatment to *hs-Su(H)*$^+$ and $Su(H)^{SF8}$/*hs-Su(H)*$^+$, flies were raised at 25°C. During a heat-shock session, flies were subjected to heat shock by placing them in empty vials in a 37°C incubator for 30 minutes, then they were transferred back to a bottle with a paper towel at 25°C for a 3-hour recovery period. For heat-shock treatment to $Gal80^{ts}$*; MB247-Gal4* and $Gal80^{ts}$*; C232-Gal4* lines, flies were raised at 18°C until they had grown to the adult stage. The 4-day-old flies were divided into two groups. In the heat-shock group, flies were subjected to heat shock by placing them in a bottle with a paper towel in a 30°C incubator for 3 days. In the non-heat-shock group, flies were placed in bottles with a paper towel at 18°C for 3 days.

Pavlovian Olfactory Learning and Memory

The training and testing procedures were the same as described previously (Tully & Quinn, 1985; Yin et al., 1994). During one training session, a group of $\sim$100 flies was exposed sequentially to two odors (octanol and methylcyclohexanol) for 60 seconds with a 45-second rest interval after each odor presentation. During exposure to the first odor, flies were simultaneously subjected to footshock (12 separate 1.5-second pulses with 3.5-second intervals; 60 V). To measure ''immediate memory'' (also referred to as ''learning''), flies were transferred immediately after training to the choice point of a T-maze and given a choice between the two odors for 2 minutes, after which they are trapped in their respective arms, anesthetized, and counted. A performance index (PI) was calculated from the distribution, whereas a PI of 100 represents 100% of flies avoiding the shock-paired odor by choosing the other T-maze arm. A 50:50 distribution means the PI is zero. Typically, after one training session, memory retention in normal flies dropped to near zero within 24 hours. To produce long-lasting memory, flies were subjected to repetitive training sessions either massed (10 training sessions with no rest interval) or spaced (10 training sessions with a 15-minute rest between each). After training, flies were transferred to food vials and stored at 18°C for 24 hours before testing. All Pavlovian training and testing experiments were preformed at 25°C and 70% humidity. For each experiment, the experimental and control groups were trained and tested in a balanced way.

Quantitative Real-Time PCR

For the quantitative real-time polymerase chain reaction (PCR) analysis of Su(H), total RNA was prepared from control flies or mutants, using TRIzol reagent and RNA

extraction protocol from Invitrogen (Carlsbad, CA), cDNA was synthesized by using the protocols of the ImProm^{TM}-II Reverse Transcription System (Promega, Madison, WI, USA). The resulting cDNA was used as templates in quantitative real-time PCR of Su(H) and ribosomal protein 49 (*rp49*) as the control. The Su(H) primers used were 5′-ccgcccgccattgcctacga-3′ and 5′-cgccagccacttccaaacagatag-3′, and the *rp49* specific primers were 5′-atgaccatccgcccagcatac-3′ and 5′-gagaacgcaggcgaccgttgg-3′. Quantitative real-time PCR was performed within the Stratagene Mx3000P system (La Jolla, CA, USA). For testing Su(H) RNA level after the heat-shock induction, flies were raised at 25°C. During a heat-shock session, flies were subjected to heat shock by placing them in empty vials in a 37°C incubator for 30 minutes. After 3-hour recovery in a bottle with food at 25°C, we collected mRNA to perform quantitative real-time PCR analysis.

Immunohistochemistry

Immunohistochemistry on adult flies' heads was performed on paraffin sections. The flies were fixed in Carnoy's fixative (60% ethanol, 30% chloroform, and 10% acetic acid) for 4 hours at room temperature, then washed twice in ethanol for 30 minutes each, washed in absolute ethanol (dehydrated) for 1 hour, treated with methylbenzoate overnight, and embedded in paraffin. We obtained 7-μm sections, deparaffinized them in a xylene bath for 75 minutes, rehydrated them through 100 ∼25% ethanol series and distilled water, blocked the tissue with goat serum in PBT (PBS, pH 7.2, and 0.2% Triton X-100) for 40 minutes at room temperature, and then incubated it with a 1:500 dilution of anti-Su(H) polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (Gho et al., 1996) overnight at 4°C. Sections were washed in PBT three times and incubated in horseradish peroxidase (HRP)-conjugated antirat secondary antibody for 1 hour at room temperature. Finally, sections were washed in phosphate-buffered saline (PBS) three times and stained by a DAB kit (Zhongshanjinqiao Corporation, Beijing, China) at room temperature (Balling et al., 2007). All the flies were prepared on the same slide.

RESULTS

Su(H) Mutation or Overexpression Causes LTM Defect

We chose one loss-of-function allele, $Su(H)^{SF8}$, to study the function of Su(H) (Ashburner, 1982; Furukawa et al., 1992). Since the homozygote of $Su(H)^{SF8}$ is lethal during the first day of pupal development, we used heterozygous adult flies to test 24-hour memory after spaced training. Null heterozygous mutants of $Su(H)^{SF8}$ showed significantly lower 24-hour memory after spaced training than control flies (Figure 1A). To rule out the possibility that this result was caused by differences of mutant and control flies' backgrounds, we also tested two other independently isolated mutants of Su(H) to confirm whether Su(H) mutation causes LTM deficit: $Su(H)^{IB115}$ and $Su(H)^{HG36}$ (Furukawa et al., 1992). Because of the lethality of homozygotes in pupal development, heterozygotes of both alleles were used in the behavior experiment. We found that heterozygotes of $Su(H)^{IB115}$ displayed a

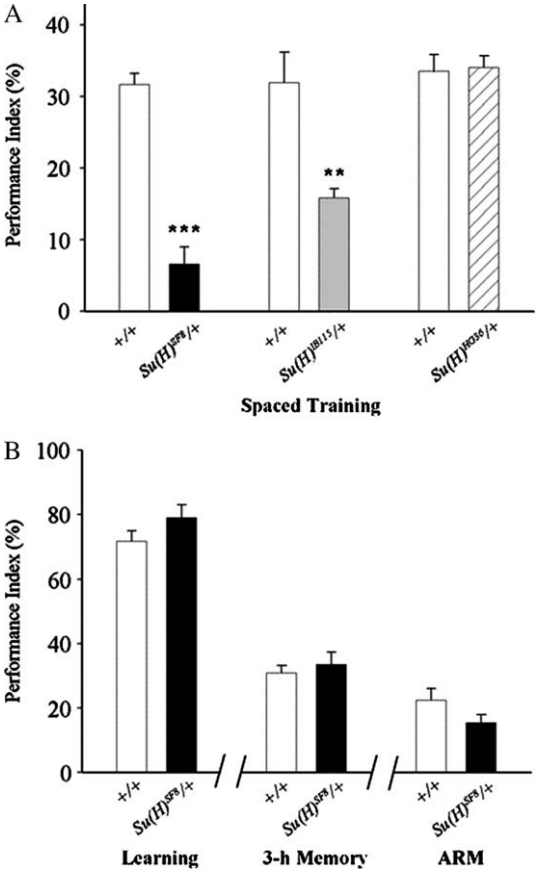

Figure 1. LTM is specifically impaired in Su(H) mutants. (**A**) Twenty-four–hour memory after spaced training was significantly impaired in $Su(H)^{SF8}$ mutants ($n=8$ PIs per group; $P<0.001$) and in $Su(H)^{IB115}$ mutants ($n=8$ PIs per group; $P<0.01$), but that was normal in $Su(H)^{HG36}$ mutants ($n=8$ per group; $P>0.05$). All PIs of these and the following behavioral experiments are means and standard errors of the mean. Statistical significances are determined from a *t*-test. Asterisks indicate critical values of: $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. (**B**) Memory retention, measured immediately or 3 hours after one training session, was normal in $Su(H)^{SF8}$ mutants ($n=6$ PIs per group; $P>0.05$). Twenty-four–hour memory after massed training of $Su(H)^{SF8}$ mutant was similar to control, too ($n=8$ PIs per group; $P>0.05$).

decrease in 24-hour memory after spaced training, while heterozygotes of $Su(H)^{HG36}$, a milder hypomorph allele, showed normal 24-hour memory, compared to the control (Figure 1A). To determine which memory phase is disrupted in Su(H) mutants, we first examined 24-hour memory after massed training, which is believed to generate ARM, and found there was no statistical difference between $Su(H)^{SF8}$ and control. Further, we examined memory both immediately and 3 hours after one training session, which assessed STM and MTM, respectively. For both tests, the performance of $Su(H)^{SF8}$ was statistically indistinguishable from control flies (Figure 1B). Thus, it is unlikely that the 24-hour memory defect of $Su(H)^{SF8}$ is due to olfactory acuity and shock reactivity. These results lead us to conclude that mutation of Su(H) could impair LTM formation in *Drosophila*.

To distinguish whether the LTM defect resulted from effects of developmental abnormality, we then used transgenic flies, $Su(H)^{SF8}$/*hs-Su(H)*$^{+}$, to see if the LTM defect in Su(H) mutant could be rescued by acute induction of wild-type Su(H). Flies were given a 30-minute heat shock, followed by a 3-hour rest. They were then subjected to the spaced training session. Transgenic flies with heat shock had no statistically distinguishable performance from control flies after the spaced training session, while those without heat shock still showed lower 24-hour memory. This suggests that inducing wild-type Su(H) in null heterozygous flies can fully rescue LTM defects (Figure 2). The rescue is not a result of the heat-shock treatment, because it could not cause 24-hour memory change in control flies. Thus, these results further confirm that Su(H) mutation is responsible for the LTM deficit observed in null heterozygous mutants and also suggest that Su(H) acts an acute role in LTM formation.

To further explore the function of Su(H) in learning and memory, we then evaluated effects of the overexpression of wild-type Su(H) in transgenic flies (*hs-Su(H)*$^{+}$) (Schweisguth & Posakony, 1994). Flies were given a 30-minute heat shock. And, after a 3-hour rest, they were subjected to the spaced training session. Quantitative real-time PCR and immunohistochemistry results confirmed the overexpression of Su(H) after heat shock (Figure 3A and unpublished data). We found 24-hour memory was significantly lower in *hs-Su(H)*$^{+}$ transgenic flies with heat-shock treatment, and the transgenic flies without heat shock had similar performance to the control. Our result shows that the overexpression of wild-type Su(H) impairs 24-hour memory after spaced training (Figure 3B). Thus, maintaining an optimal level of Su(H) is critical for LTM formation.

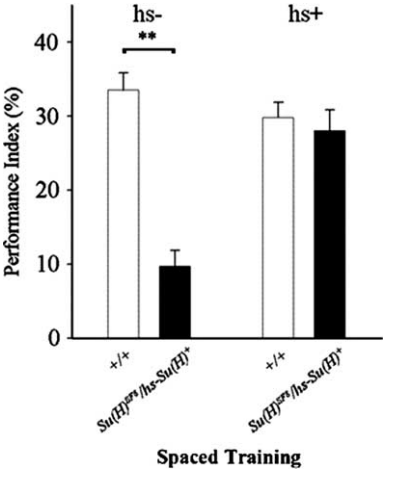

Figure 2. Acute expression of *Su(H)*$^{+}$ transgene in the mutant background can rescue the LTM defect. The defect in 24-hour memory after spaced training was rescued in $Su(H)^{SF8}$/*hs-Su(H)*$^{+}$ transgenic mutants when animals were trained 3 hours after a 30-minute 37°C heat-shock treatment ($n = 8$ PIs per group; $P < 0.01$).

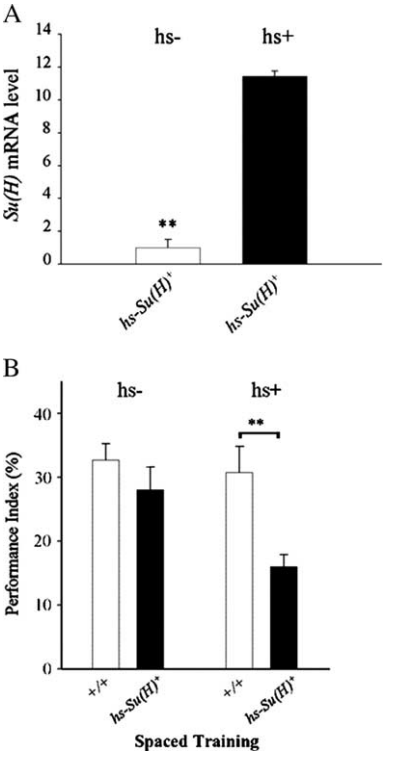

Figure 3. Acute overexpression of *Su(H)*$^{+}$ transgene in the adult stage can cause LTM defect. (**A**) Quantitative analysis of Su(H) mRNA in *hs-Su(H)*$^{+}$ transgenic flies before and after heat-shock treatment of independent samples. Quantitative real-time PCR revealed strong overexpression of Su(H) after a heat-shock treatment ($n = 6$ per group; $P < 0.01$). (**B**) Twenty-four–hour memory after spaced training was defective in *hs-Su(H)*$^{+}$ transgenic flies when animals were trained 3 hours after a 30-minute 37°C heat-shock treatment ($n = 8$ per group; $P < 0.01$).

Expression of Su(H) in the Mushroom Bodies Is Sufficient to Rescue the Memory Defect of Su(H) Mutants

Immunohistochemical analysis of Su(H) protein expression in wild-type adult brains was done on paraffin sections, using a polyclonal antibody (Gho et al., 1996). Antigen was detected throughout the brain, including the central complex region (Figure 4A), and the Mushroom Bodies (MBs) lobes and cell bodies (Figure 4B). These observations suggest immunostaining largely reflects the expression pattern of Su(H) protein. Since we used a commercial polyclonal antibody against Su(H), we could not rule out the possibility that there was cross-reactivity to other proteins in the immunohistochemical analysis. To localize the neuronal site within the *Drosophila* brain where Su(H) is required to regulate the particular memory phase after spaced training, we performed a spatiotemporal rescue by using the $Gal80^{ts}$ system in combination with MB247-Gal4 and C232-Gal4 drivers (McGuire et al., 2003). MB247 drives expression in ~700 α/β and γ-lobe MB neurons (Schwaerzel et al., 2002). C232 drives expression in the central complex region. In this experiment, homozygous *Gal80^{ts}; MB247-Gal4* and *Gal80^{ts}; C232-Gal4* were crossed to *Su(H)^{SF8}/CyO; UAS-Su(H)$^{+}$*. Non-*CyO* offspring were analyzed. After inducing for 3 days at 30°C, 24-hour memory of *Gal80^{ts}/Su(H)^{SF8}; 247-Gal4/UAS-Su(H)$^{+}$* was normal. In contrast, *Gal80^{ts}/Su(H)^{SF8}; MB247-Gal4/UAS-Su(H)$^{+}$* flies at 18°C still showed memory deficits (Figure 5). The rescue was not caused by temperature conditions, since *Gal80^{ts}/+; MB247-Gal4/+* flies had normal LTM at 18 and 30°C. Meanwhile, *Gal80^{ts}/Su(H)^{SF8}; C232-Gal4/UAS-Su(H)$^{+}$* flies had 24-hour memory deficit at 18 and 30°C (Figure 5). This observation led us to conclude that the function of Su(H) in the MBs is crucial for LTM formation.

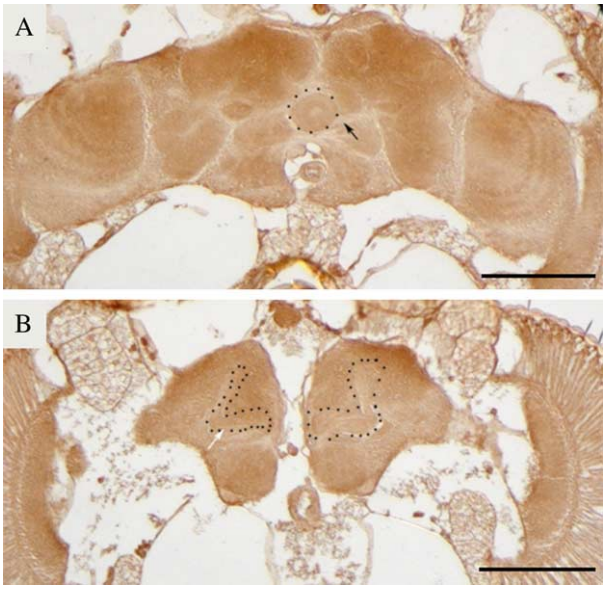

Figure 4. Su(H) protein is widely expressed in the nervous system of wild-type adult flies. Immunohistochemical detection of Su(H) protein in paraffin sections of adult brain in wild-type flies, revealing protein expressing widely in the adult brain, especially in central complex regions (**A**) and MBs (**B**). Black arrow with circle: central complex; white arrow with circle: MBs. Scale bars, 100 μm.

DISCUSSION

Previous studies have revealed that N mediates the formation of LTM in the adult brain, suggesting a role for N signaling in memory formation (Ge et al., 2004; Presente et al., 2004). In the present studies, we focus on

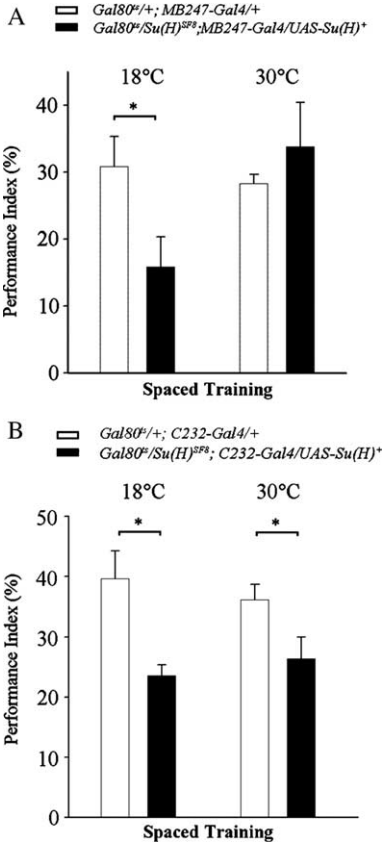

Figure 5. The LTM defect of mutants can be rescued by the expression of wild-type Su(H) in the MBs. (**A**) The defect in 24-hour memory after spaced training was rescued in *Gal80^{ts}/Su(H)^{SF8}; MB247-Gal4/UAS-Su(H)$^{+}$* transgenic mutants when animals were trained after a 3-day 30°C heat-shock treatment ($n = 8$ PIs per group; $P < 0.05$). (**B**) The defect in 24-hour memory after spaced training could not be rescued in *Gal80^{ts}/Su(H)^{SF8}; C232-Gal4/UAS-Su(H)$^{+}$* transgenic mutants when animals were trained after a 3-day 30°C heat-shock treatment ($n = 8$ PIs per group; $P < 0.05$).

an important component in the N-signaling pathway, Su(H). Behavior experiments showed that LTM specifically was impaired in Su(H) heterozygous mutants and *hs-Su(H)*$^{+}$ transgenic flies, while learning ability and other memory phases were intact. And, the LTM defect could be fully rescued by the acute induction of wild-type Su(H) widely, as well as specifically, in the MBs. The behavior experiments were conducted strictly in a balanced manner (see Materials and Methods). Thus, all these results provide strong evidence that Su(H) plays an acute role in LTM formation in *Drosophila*.

Our data showed that two loss-of-function alleles, *Su(H)*^{SF8} and *Su(H)*^{IB115}, can cause LTM defect, while a milder hypomorph allele, *Su(H)*^{HG36}, has no detectable effect in heterozygotes. The different behavior phenotypes might be caused by different mutation severity. The *IB115* allele has a mutation that replaces Lys-94 (AAG) with a stop codon (TAG). This lesion results in the production of a truncated Su(H) peptide that is only one sixth of the full length. The molecular nature of the *SF8* allele is unknown, but it also causes severe and loss-of-function mutation of Su(H) protein. However, in the *HG36* allele, the mutation leads to an amino-acid substitution of Lys-261 (AAG) for Glu-261 (GAG) (Furukawa et al., 1992). Further, structure studies have revealed that position 261 is outside the N-binding domain, providing another possible explanation of why we cannot observe any effect in *Su(H)*^{HG36} heterozygotes (Kovall & Hendrickson, 2004; Wilson & Kovall, 2006).

We found that the overexpression of wild-type Su(H) also caused LTM defect. This result is consistent with the canonical N/Su(H) interaction. On one side, when Su(H) is mutated, N^{ICD} cannot normally bind to Su(H) protein to form the activation complex to activate the transcription of downstream genes. On the other side, overexpression of Su(H) reinforces the ''default repression'' of N target genes. Thus, either reduction or overexpression of Su(H) could repress the transcriptional activity of downstream genes, then impair LTM formation, which is dependent on protein synthesis (Yin et al., 1994).

In this report, we showed that Su(H) was involved in LTM formation. The involvement acts through an acute mechanism, rather than developmental abnormity. Two different pieces of evidence support this conclusion. First, the acute induction of a *Su(H)*$^{+}$ transgene could fully rescue the LTM defect in Su(H) mutants or could cause LTM deficits if it is overexpressed. Second, we used a $Gal80^{ts}$, Gal4/UAS system (McGuire et al., 2003) to express wild-type Su(H) protein in the MBs in adult flies, and this could fully rescue the LTM deficit. The above evidence provides a strong demonstration of an acute role for Su(H) during LTM formation. Further, our data reveal Su(H) functions in the MBs, which is consistent with a previous report that the functional location of N might be in the MBs (Presente et al., 2004). All the evidence leads to the conclusion that the transcriptional activity of Su(H) mediated by N is involved in LTM formation. Further, previous studies have revealed several proteins were required for LTM formation in the MBs, such as CREB (Yin et al., 1994; Yu et al., 2006), neurotrypsin tequila (Didelot et al., 2006), and cathepsin (Comas et al., 2004). At the neuronal level, studies of α-lobe absent (*ala*) mutants and imaging of Ca^{2+} activity suggest *Drosophila* α/β MB neurons can form long-term cellular memory traces after spaced training (Pascual & Preat, 2001; Yu et al., 2006). Our findings about Su(H) emphasize the involvement of the MBs in LTM formation.

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