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Nitric oxide metabolism controlled by *formaldehyde dehydrogenase* (*fdh*, homolog of mammalian *GSNOR*) plays a crucial role in visual pattern memory in *Drosophila*

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ABSTRACT

Nitric oxide (NO) plays an important role in learning and memory which is essential for animals to adapt to the external environment. However, little is known about the role of NO metabolism in this process. S-nitrosoglutathione reductase (GSNOR) is a key protein in the control of NO metabolism and protein S-nitrosation. To study the relationship between NO metabolism and learning and memory, the expression of gene *fdh* which is homolog to mammalian *GSNOR* was modulated by the Gal4/UAS system in *Drosophila*. The over-expression of the *fdh* in the central nervous system significantly increased GSNOR activity and induced visual pattern memory defects of *Drosophila*. The role of *fdh* in learning and memory was independent of development and was neuron-specific: over-expression of the *fdh* in the fan-shaped body induced memory defect, while over-expression in the mushroom body did not. The visual pattern memory defect could be rescued by co-expression with exogenous cGMP-dependent protein kinase (PKG). Moreover, *fdh* over-expression resulted in denitrosation of multiple proteins functionally enriched in vesicle-mediated transport, which is important for learning and memory. These results showed that regulation of NO metabolism plays an important role in learning and memory, and the mechanism may involve both NO-cGMP-PKG signaling pathway and S-nitrosation modification.

Introduction

Nitric oxide (NO) is a small molecule that plays a vital role in a multitude of biological processes [1,2]. As a neurotransmitter, NO participates versatilely in learning and memory in the nervous system [3]. For example, NO acts as retrograde messenger to induce long-term potentiation (LTP), a prominent form of synaptic plasticity, which is associated with memory formation in the hippocampus [4,5]. The majority of NO bioactivity studies have focused on upstream regulation and NO synthesis controlled by nitric oxide synthases (NOSs) [6–9]. However, little is known regarding downstream regulation and the effect of NO metabolism on learning and memory.

In 1998, Jensen et al. reported that alcohol dehydrogenase class III (ADH III) blocks NO function by reducing S-nitrosoglutathione (GSNO) to NH_3 [10], and for this reason, ADH III is also named S-nitrosoglutathione reductase (GSNOR). GSNOR is highly conserved from bacteria to humans and is extensively expressed in organisms [11]. GSNO is the main reservoir for non-protein S-nitrosothiols (SNOs) [12] and temporally and spatially extends functions of the fragile NO. GSNO also induces S-nitrosation of protein cysteine thiols, a post-translational modification (PTM) [13,14], to regulate protein functions such as enzyme activity, protein stability, and protein localization. GSNOR turnover significantly influences the level of whole-cell S-nitrosation [11,15–18]. Therefore, high specificity of GSNOR toward GSNO and regulation of S-nitrosation [11,16] have established a direct relationship between GSNOR and NO metabolism. In addition to the general functions of NO in an organism [1], GSNOR is involved in the cardiovascular system [16], immune system [16,19], and respiratory system [17,20] by regulating NO metabolism. However, the role of GSNOR and NO metabolism in the nervous system remains poorly understood. Results have been shown that nitric oxide synthase (NOS) inhibition results in learning and memory defects [21]. Therefore, the present study was designed to determine the

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effect of GSNOR on learning and memory. GSNOR is the sole alcohol dehydrogenase isozyme in vertebrate brains, while the failure to detect any ethanol dehydrogenase activity makes its function in brains an interesting question [22].

The present study chose the *fdh* gene, the homolog of mammalian GSNOR, to generate UAS-*fdh* and *fdh* double-stranded RNA interference (ds-RNAi) transgenic *Drosophila*. These transgenic models were used to study the effect of GSNOR activity modulation on visual learning and memory and to explore the possible mechanisms of learning and memory mediated by NO metabolism.

Experimental procedures

Fly strains

All fruit flies were maintained at 25 °C and were fed standard corn meal/molasses medium [23] in a 12-h light/12-h dark cycle at 60% humidity. Three- to six-day-old flies were used in behavioral experiments. The following fly strains were used:

wild-type Canton-S (CS): wild type controls using in behavior test.

w^{1118}: white eyed background flies for constructing the transgenic flies and for controls using in screening the transgenic flies.
tubulin-Gal80^{ts}: temperature sensitive Gal80 driven by the *tubulin* promoter for temporally controlling gene expression.
actin-Gal4: a Gal4 line for controlling ubiquitously gene expression.
elav-Gal4: a Gal4 line for controlling pan-neurons gene expression.
c205-Gal4: a Gal4 line for controlling fan-shaped body F5 neurons gene expression.
mb247-Gal4: a Gal4 line for controlling mushroom body neurons gene expression.
UAS-*forP1* [24]: a UAS line for over-expression of *forP1* (coding for protein PKG).

All fly lines were back-crossed with w^{1118} for at least six generations to assure the same genomic background.

Plasmid construction and molecular biology

The cloning strategy of double-headed constructs for RNAi was performed according to previously described methods, with minor modifications [25]. To construct the *UAS-fdh*-RNAi lines, the *Bam*HI site-flanked upstream primer (5′-CAA*ggatcc*GCCGCTGGAGAAAG TCTGCCT-3′) and *Kpn*I site-flanked downstream primer (5′-TGC*ggtacc*CGGATACTCTCGCCCTTGTGC-3′) were used to clone the *fdh* cDNA fragment from a wildtype *CS* fly cDNA library. The PCR products were digested with *Bam*HI and *Kpn*I and the resulting cDNA fragments were subcloned into *Bam*HI/*Kpn*I sites of a pHIBS vector. The plasmids were sequenced by Invitrogen (Shanghai). The resulting plasmids were digested with *Sal*I and *Kpn*I and initially subcloned into *Xho*I/*Kpn*I sites of *pUAST*, following digestion of the previous pHIBS plasmid with *Bam*HI and *Eco*RI, and were subsequently subcloned into the *Eco*RI and *Bgl*II sites of the above-mentioned *pUAST* plasmid.

For constructing the UAS-*fdh* plasmid, the *Eco*RI site-flanked upstream primer (5′-AAACAA*gaattc*ATGTCTGCTACCGAGGGCAA-3′) and *Xho*I site-flanked downstream primer (5′-GGGCGG*ctcgag*TTA GTACTTAATAATGGATCGG-3′) were used to clone the coding region of the *fdh* gene. The PCR product was subcloned into *Eco*RI/*Xho*I sites of *pUAST*.

UAS-*fdh*-RNAi and UAS-*fdh* plasmid DNA were then purified and subjected to germline transformation into w^{1118} embryos according to previously described protocols [26].

Two transformants of the UAS-*fdh* plasmid (*UAS-fdh*1 and *UAS-fdh*31) and one transformant of the UAS-*fdh*-dsRNAi plasmid (*UAS-fdh*ri34) were used in this study.

Quantitative PCR

Quantitative PCR (qPCR) was used according to standard methods [24]. In brief, 0.5 μl cDNA, prepared from bodies of 3- to 5-day-old flies, as described previously [24], was used as templates and tested on a Chromo 4 system (MJ Research/Bio-Rad). For each fly strain, cDNA was obtained from three independent RNA preparations. The relative difference in *fdh* mRNA expression levels was quantified by comparing expression levels with standard curves, which were constructed using the corresponding recombinant plasmids. Expression levels were normalized to expression levels of *actin*. A two-tailed *t*-test was used for statistical analysis of relative mRNA levels. The qPCR primers were as follows:

qPCR-*actin* upper, 5′-CGTGACTAACTGGGACGACA-3′;
qPCR-*actin* lower, 5′-GGAGGCGTAGAGAGAAAGCA-3′;
qPCR-*fdh* upper, 5′-AGAGCGGCAAGACGAATCT-3′;
qPCR-*fdh* lower, 5′-AGCAGGCAGACTTTCTCCAG-3′.

GSNOR enzyme activity assay

Approximately, 100 3-day-old flies were collected and homogenized in 50 mM sodium phosphate buffer (pH 7.5)/1.1% isopropyl/1 mM PMSF on ice. The solution was then centrifuged at 13,000 rpm for 50 min at 4 °C. The supernatant was filtered through a 0.22-μm filter to remove fat prior to GSNOR activity measurements. GSNO reductase activity was measured as described previously [15]. In brief, 0.85–1.0 mg/mL lysate was incubated with 200 μmol/L GSNO in reaction buffer (50 mM sodium phosphate buffer (pH 7.5)/1.1% isopropyl/1 mM PMSF) with 0 or 100 μmol/L NADH at 25 °C for various times. The GSNO reductase activity was measured by GSNO-dependent NADH consumption using the change in absorbance at 340 nm per minute per mg protein. Protein concentrations were measured using a BCA™ protein assay kit (Pierce Biotechnology, Rockford, IL, USA) according to manufacturer's instructions.

Visual pattern memory assays

Visual learning experiments were performed in a flight simulator described by Wolf and Heisenberg [27]. Briefly, the tethered fly, which was suspended by a torque meter, flew stationarily in the center of the arena, which was illuminated from behind and contained two upright and two inverted alternating T-shaped patterns on the wall. During training, the computer switched a heat beam on when the fly was headed toward a quadrant with, for example, an upright T, and switched the heat beam off when the fly oriented toward one of the other two quadrants. Therefore, half of all possible orientations in the arena are paired with heat, the other half with ambient temperature. During testing, the heat was switched off. Angular position was recorded every 50 ms and orientation preferences were calculated for nine consecutive 2-min periods (performance index, PI1–9). Pattern A was paired to ambient temperature during training, and pattern B to heat. The two patterns alternated between A and B and between flies. If t_A represented time spent heading toward quadrants with pattern A, and t_B represented time spent heading toward pattern B quadrants, the performance index was calculated as $PI = (t_A - t_B)/(t_A + t_B)$. For

details of learning paradigms, procedures, and the definition of performance index (PI), see Ref. [27].

Identification of S-nitrosated proteins in Drosophila and data analysis

S-nitrosated proteins were purified using the biotin-switch method and were analyzed by mass spectrometry as previously described [28]. Briefly, approximately, 100 3- to 4-day-old whole fly bodies or dissected heads were homogenized in lysis buffer (20 mM Tris–Cl, pH 7.7/1 mM EDTA/0.1 mM neocuproine/protease inhibitor cocktail tablets/8 M urea/20 mM MMTS) on ice. All steps were performed in the dark unless otherwise noted. Protein concentration was determined using a BCA protein assay kit and adjusted to 1 mg/mL following removal of MMTS from the protein solution by TCA/acetone precipitation. Supernatant was collected following centrifugation at 13,200*g* at 4 °C for 30 min. Subsequently, urea-based biotin-switch method was performed [28]. Following trypsin digestion, peptide samples were purified using streptavidin agarose, alkylated with iodoacetamide, and diluted in 0.1% formic acid (FA) for liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis. Peptide samples were analyzed using a Thermo LTQ linear trap instrument equipped with a Thermo micro-electrospray source, a Thermo Surveyor pump, and an autosampler (Thermo Finnigan, San Jose, CA, USA). MS2 spectra, with a total ion currency (TIC) greater than 104, were used to search for matches against a maize protein sequence database from the National Center for Biotechnology Information (NCBI) (version: 2009. 1, plus 180 sequences of the most common contaminants, *e.g.*, keratins and proteases, provided by the Bioworks package) using a TurboSEQUEST algorithm. For detailed search parameters, see Ref. [28]. Gene ontology (GO) enrichment and analysis were performed via DAVID [29], and the preliminary analysis was restricted to a 179-term GO essential slim [30].

Statistical analysis

The two-independent sample *t*-test was used to compare experimental groups to controls. Error bars in the figures represent SEM (standard error mean), and asterisks indicate levels of significance ($^{***}p \leqslant 0.001$; $^{**}p \leqslant 0.01$; $^{*}p \leqslant 0.05$).

Results

GSNOR activity in transgenic flies

The structure of the *fdh* gene in *Drosophila melanogaster* is shown in Fig. 1A. The genome length of *fdh* is approximately 1.3 kb, with three exons and two introns, which form a single transcript. The relatively small size and simple structure allows for convenient modulation of gene expression. For over-expression of *fdh*, the p[UAS-*fdh*] plasmid was constructed. Microinjection of recombinant P element plasmids [31] (Fig. 1B) yielded seven independent transformants carrying UAS-*fdh*, from which three different transgenic lines (UAS-*fdh1*, UAS-*fdh22*, and UAS-*fdh31*) with a single insertion on the first, second, and third chromosomes were selected for further testing. The p[UAS-*fdh*RNAi] plasmid was constructed for reducing GSNOR activity of *fdh*. Six independent transformants carrying UAS-*fdh*RNAi transgenes were generated on the second or third chromosomes, from which two different transgenic lines (UAS-*fdhri22* and UAS-*fdhri34*), with a single insertion on the second and third chromosomes, respectively, were used for further experiments. All five transgenic lines were back-crossed for a minimum of six generations to a reference w^{1118} background to ensure genetic homogeneity. No visible developmental defects were observed in these transgenic flies (data not shown).

To assess effectiveness of the constructed transgenic fly strains, the constitutively expressed *actin*-Gal4 was selected to cross with the transgenic flies. Entire fly bodies were used to measure *fdh* mRNA expression by qPCR. Results showed that *fdh* mRNA expression was significantly greater in three over-expression lines (*act*>UAS-*fdh*1, *act*>UAS-*fdh*22, *act*>UAS-*fdh*31) compared with controls (*act*/+ or UAS-*fdh*22/+). One of the *fdh* RNAi lines (*act*>UAS-*fdhri*34) revealed significantly reduced *fdh* mRNA expression compared with the control line (UAS-*fdhri*34/+). However, there was no difference between *fdh* RNAi lines (*act*>UAS-*fdhri*22) and the control (UAS-*fdhri*22/+) (Fig. 1C).

GSNOR activity of *fdh* in the transgenic flies was assessed by GSNO-dependent NADH consumption. GSNOR activity in whole body homogenates increased by 3-fold in *act*>UAS-*fdh*1 flies compared with control flies (UAS-*fdh*1/+), and by 5-fold in *act*>UAS-*fdh*31 flies compared with control flies (UAS-*fdh*31/+) (Fig. 1D). However, GSNOR activity was reduced by approximately 50% in *act*>UAS-*fdhri*34 flies compared with control flies (UAS-*fdhri34*/+), and there was no significant difference between *act*>UAS-*fdhri*22 flies and control flies (UAS-*fdhri*22/+) (Fig. 1D). Subsequently, *elav*-Gal4 was utilized to regulate *fdh* expression in the nervous system and GSNOR activity in the brains of *Drosophila* was tested. Similar to the previous results, GSNOR activity in the two over-expression lines (*elav*>UAS-*fdh*1 and *elav*>UAS-*fdh*31) increased by 3-fold and 13-fold, respectively. However, GSNOR activity in the *elav*>UAS-*fdhri*34 line was reduced by approximately 50% compared with the control line (UAS-*fdhri34*/+) (Fig. 1E). According to these results, two *fdh* over-expression lines (*elav*>UAS-*fdh*1 and *elav*>UAS-*fdh*31) and one *fdh* RNAi line (UAS-*fdhri34*/+) were used to modulate GSNOR activity in the *Drosophila* central nervous system and employed for further behavior experiments.

Over-expression of fdh blocks visual pattern memory in Drosophila

First, *elav*-Gal4 was crossed with two UAS-*fdh* flies (UAS-*fdh*1 and UAS-*fdh*31) to over-express *fdh* in pan neurons. The progeny of these flies were then tested for visual pattern memory in a flight simulator. Two over-expression lines (*elav*>UAS-*fdh*1 and *elav*>UAS-*fdh*31) presented with visual pattern memory defect compared with their controls UAS-*fdh*1/+ and UAS-*fdh*31/+, respectively (Fig. 2A). Following analysis of behavioral data, the effects of thermo-tolerance and pattern discrimination were excluded in *fdh* over-expressing flies that presented with visual pattern memory defect (see Supplementary Fig. 1). These results suggested that increased GSNOR activity, as a result of *fdh* over-expression in the nervous system, could damage visual pattern memory in *Drosophila*.

Subsequently, *elav*-Gal4 and UAS-*fdhri*34 flies were crossed to reduce GSNOR activity in pan neurons. The progeny of these flies were tested for visual pattern memory. However, the partial reduction in GSNOR activity did not result in significantly different memory performance in *elav*>UAS-*fdhri*34 flies compared with control flies (UAS-*fdhri*34/+) (Fig. 2A).

Spatial–temporal regulation of fdh expression in visual pattern memory

Previous results showed that the fan-shaped body (FB), rather than the mushroom body (MB), is necessary for visual pattern memory in *Drosophila* [32,33]. To further elucidate the effect of NO metabolism on learning and memory in *Drosophila*, the Gal4 line c205 (FB F5-labeled neurons) and the Gal4 line mb247 (MB-labeled and serving as negative control) were utilized to drive local expression of the *fdh* gene. As expected, *fdh* over-expression in the c205-driven FB resulted in visual pattern memory defect, whereas *fdh* over-expression in the mb247-driven MB revealed

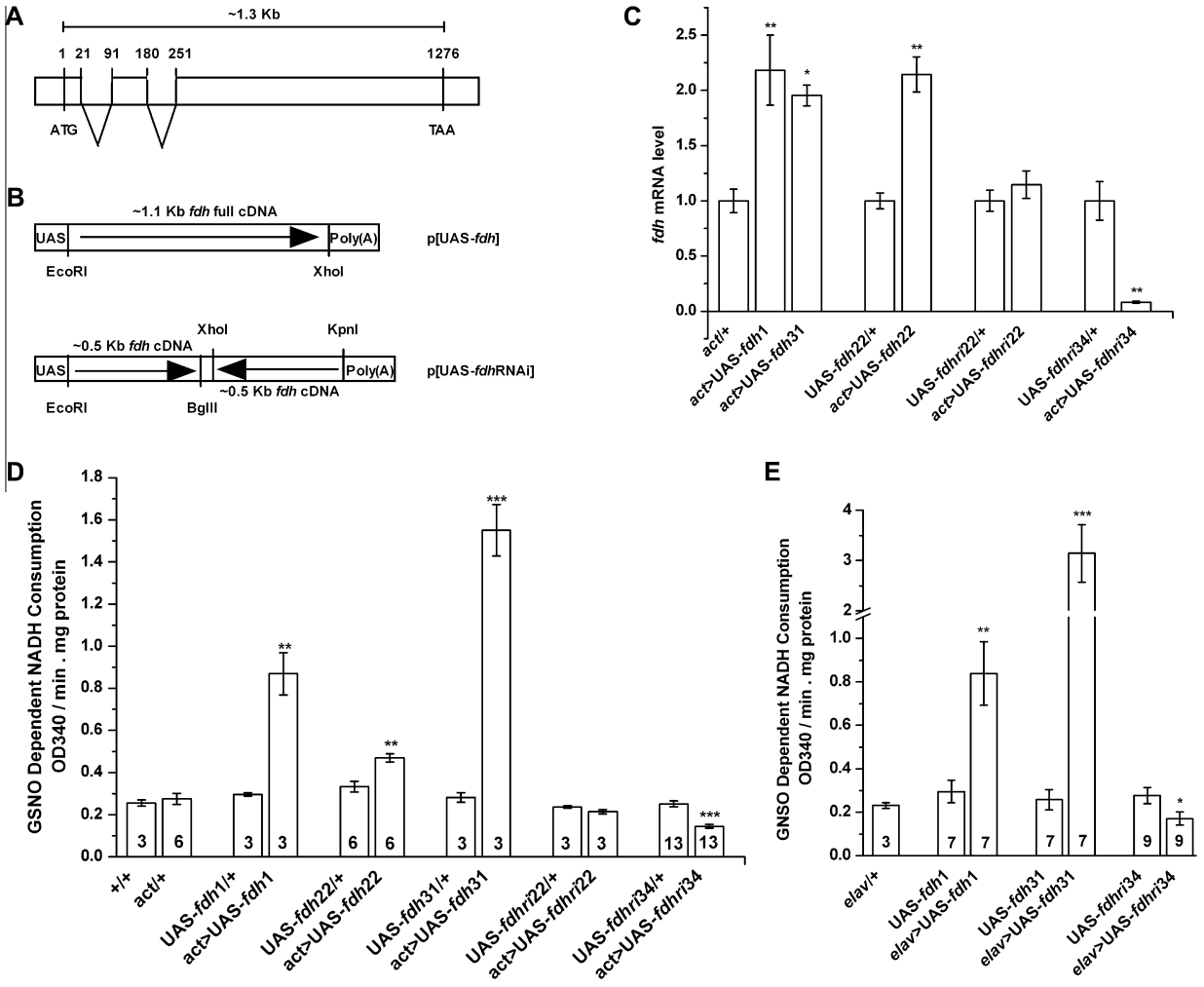

Fig. 1. Effectiveness of *fdh* over-expression and dsRNAi transgenic flies. (A) The genome length of *fdh* in *Drosophila* is approximately 1.3 kb and includes three exons and two introns, which are spliced by CU/AG intron/exon boundaries. (B) Schematic diagrams of the over-expressing *UAS-fdh* and dsRNAi *UAS-fdh-RNAi* constructs. (C) qPCR results reveal *fdh* mRNA levels when *fdh* is over-expressed by actin-GAL4 or downregulated by dsRNAi. *fdh* mRNA levels of three over-expression lines are significantly greater than control lines (*act*>UAS-*fdh*1 *vs*. *act*/+, $t = 6.73$, $p < 0.01$; *act*>UAS-*fdh*22 *vs*. UAS-*fdh*22/+, $t = 6.58$, $p < 0.01$; *act*>UAS-*fdh*31 *vs*. *act*/+, $t = 3.53$, $p < 0.05$). The *fdh* RNAi line (*act*>UAS-*fdh*ri34) has significantly reduced mRNA levels compared with the control line (UAS-*fdh*ri34/+, $t = -5.29$, $p < 0.01$). However, there is no difference between the *fdh* RNAi line (*act*>UAS-*fdh*ri22) and the control line (UAS-*fdh*ri22/+, $t = 0.93$, $p > 0.05$). $n = 3$ for each bar. (D) GSNOR activity of *fdh* in the entire flies. Three *fdh* over-expressing lines express significantly greater GSNO reductase activity compared with control lines (*act*>UAS-*fdh*1 *vs*. UAS-*fdh*1/+, $t = 5.75$, $p < 0.01$; *act*>UAS-*fdh*22 *vs*. UAS-*fdh*22/+, $t = 4.24$, $p < 0.01$; *act*>UAS-*fdh*31 *vs*. *UAS-fdh*31/+, $t = 10.20$, $p < 0.001$). In addition, GSNO reductase activity is significantly less in the dsRNAi line *act*>UAS-*fdhri*34 compared with the control line UAS-*fdhri*34/+ ($t = -6.55$, $p < 0.001$). However, there is no difference between line *act*>UAS-*fdhri*22 and the control line UAS-*fdhri*22/+ ($t = -2.10$, $p > 0.05$). (E) GSNOR activity of *fdh* in the fly brains. GSNOR activity in the brain is significantly increased in the two lines (*elav*>UAS-*fdh*1 and *elav*>UAS-*fdh*31) compared with the control lines (UAS-*fdh*1/+ and UAS-*fdh*31/+) ($t = 3.52$, $p < 0.01$; $t = -5.05$, $p < 0.001$, respectively). GSNOR activity in the brains of line *elav*>*UAS-fdhri*34 is significantly reduced compared with the control line (*UAS-fdhri34*/+, $t = -2.21$, $p < 0.05$). All graphs depict mean ± SEM; (*n*) number of independent experiments; *act*: *actin*-Gal4; *elav*: *elav*-Gal4.

normal visual pattern memory (Fig. 2B). These results indicated that increased GSNOR activity in the FB, but not in MB, resulted in visual pattern memory defect in *Drosophila*. These results were consistent with previous observations in *rut* and for *Drosophila* mutants [24,32].

To exclude possible developmental effects due to constitutive Gal4 activity, a temperature-sensitive protein, *tubulin*-Gal80^{ts}, was used to suppress Gal4 activity at a permissive temperature (19 °C) and to recover Gal4 activity at a restrictive temperature (30 °C) for temporary *fdh* expression in the *Drosophila* brain [34]. The temperature shift from 19 °C to 30 °C did not alter fly visual pattern memory (Fig. 2C and Ref. [24]). Subsequently, the flies were housed at 19 °C and then transferred to a 30 °C environment in adulthood for 12 h prior to memory testing in the flight simulator. Flies from the same genotype served as the control group and were maintained at 19 °C until visual pattern memory testing. As shown in Fig. 2C, both *elav*>*tubulin*-Gal80^{ts};UAS-*fdh*31 and c205>*tubulin*-Gal80^{ts};UAS-*fdh*31 flies, which were maintained at 30 °C in adulthood, showed memory defect which is significantly different compared with the same fly strains constitutively maintained at 19 °C. Moreover, the *tubulin*-Gal80^{ts}/+;UAS-*fdh*31/+ flies, which were maintained at 30 °C in adulthood, displayed normal visual pattern memory. These results excluded the possibility that *tubulin*-Gal80^{ts} or 30 °C treatment induced memory defect. Collectively, these data suggested that temporally restricted expression of *fdh* during adulthood is sufficient to inhibit visual pattern memory in *Drosophila*.

Regulation of protein S-nitrosation by fdh in transgenic Drosophila

GSNOR has been reported to be a key protein for the control of NO metabolism and protein S-nitrosation [35]. Therefore, the present study attempted to determine whether GSNOR-regulated protein S-nitrosation is involved in learning and memory. The

biotin-switch method was utilized to purify S-nitrosated proteins, and mass spectrometry (LC/MS/MS) was used to identify the targets. First, whole body homogenate protein SNOs were detected in over-expressing flies (*act*>UAS-*fdh*31), dsRNAi flies (*act*>UAS-*fdhri*34), and control flies (+/*act*). In *fdh* over-expressing flies, 61 S-nitrosated proteins were determined, whereas 139 and 122 were found in heterozygous *act*/+ control and *act*>UAS-*fdhri*34 dsRNAi flies, respectively (Fig. 3A). These results demonstrated that *fdh* over-expression reduced the level of protein S-nitrosation in *Drosophila*.

Moreover, the variation of protein S-nitrosation under adjusted expression levels of *fdh* in the nervous system of *Drosophila* was determined. Results of S-nitrosated proteins in the heads of *fdh* over-expressing flies (*elav*>UAS-*fdh*31), dsRNAi flies (*elav*>UAS-*fdhri*34), and control flies (+/*elav*) were shown in Fig. 3B and C. There were less S-nitrosated proteins (1st: 133; 2nd: 100) in *fdh* over-expressing flies than in control flies (1st: 176; 2nd: 136) or *fdh* RNAi flies (1st: 194; 2nd: 132), implying that the visual pattern memory defect in the *fdh* over-expression flies (*elav*>UAS-*fdh*31) may be related to the protein S-nitrosation, i.e., some S-nitrosated proteins in the control (+/*elav*) or *fdh* RNAi (*elav*>UAS-*fdhri*34) flies with normal visual pattern memory might be necessary for visual pattern memory. From the two independent experiments, we found that there was a total of 68 proteins de-nitrosated in *elav*-Gal4-driven *fdh* over-expressing flies with the exception of duplications (see Table S1). Further analysis of these denitrosated proteins with gene ontology (GO) clustering [29] in respect to molecular functions, subcellular localization, and biological processes [30,36,37] showed that these proteins enriched in the vesicle-mediated transport process, located in mitochondrion and cytoplasm and mainly related to antioxidant activity and GTPase activity (Fig. 3D).

fdh function in learning and memory via the NO-cGMP-PKG pathway

The most prominent natural target of NO is soluble guanylyl cyclase (sGC), which produces cyclic GMP (cGMP) [38]. cGMP, in turn, activates cGMP-dependent protein kinase (PKG). A recent study demonstrated activation of the NO-cGMP pathway through the use of a GSNOR inhibitor [39]. Therefore, GSNOR may also function in learning and memory by initially regulating this pathway. UAS-*forP1* was then used to over-express PKG in an attempt to rescue the visual pattern memory defect induced by *fdh*

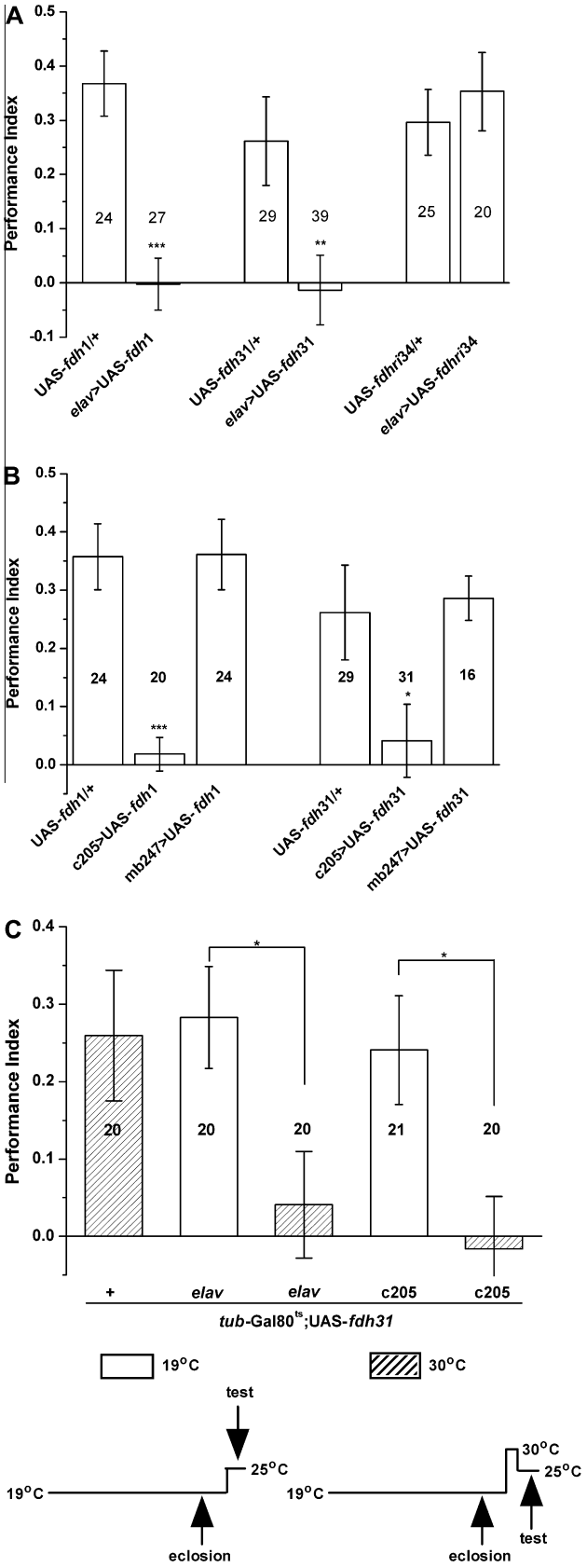

Fig. 2. Over-expression of *fdh* in the nervous system blocks visual pattern memory in *Drosophila*. (A) Over-expression of *fdh* in pan neurons induces memory defect in line *elav*>UAS-*fdh*1 and line *elav*>UAS-*fdh*31 compared with the control lines UAS-*fdh*1/+ ($t = -5.28$, $p < 0.001$) and UAS-*fdh*31/+ ($t = -2.67$, $p < 0.01$), respectively. In contrast, *fdh* dsRNAi flies (*elav*>UAS-*fdh*ri34) exhibit normal memory compared with the control line (UAS-*fdh*ri34/+, $t = 0.25$, $p > 0.05$). (B) Spatial regulation of *fdh* functions in visual pattern memory of *Drosophila*. In both UAS-*fdh*1 and UAS-*fdh*31, *fdh* over-expression in FB neurons, driven by c205-Gal4, inhibits memory (c205>*UAS-fdh*1 *vs.* UAS-*fdh*1/+, $t = -5.03$, $p < 0.001$; c205>UAS-*fdh*31 *vs.* UAS-*fdh*31/+, $t = -2.16$, $p < 0.05$), while *fdh* over-expression in MB neurons, driven by mb247-Gal4, does not affect visual pattern memory (mb247>UAS-*fdh*1 *vs.* UAS-*fdh*1/+, $t = 0.045$, $p > 0.05$; mb247>UAS-*fdh*31 *vs.* UAS-*fdh*31/+, $t = 0.22$, $p > 0.05$). (C) Temporal regulation of *fdh* expression in visual pattern memory of *Drosophila*. The temperature sensitive protein, Gal80^{ts}, which suppresses Gal4 activity at a permissive temperature (19 °C) and recovers Gal4 activity at a restrictive temperature (30 °C), was used to temporarily express *fdh* 12 h prior to memory testing in *Drosophila*. Temporarily expressed *fdh* in pan neurons (*elav*>*tubulin*-Gal80^{ts};UAS-*fdh*31, 30 °C, PI = 0.041 ± 0.069) following heat-shock treatment reveals significantly reduced memory levels compared with the same genotype flies maintained at 19 °C (*elav*>*tubulin*-Gal80^{ts};UAS-*fdh*31, 19 °C, PI = 0.28 ± 0.066, $t = -2.54$, $p < 0.05$). Temporarily expressed *fdh* in the FB (c205>*tubulin*-Gal80^{ts};UAS-*fdh*31, 30 °C, PI = −0.016 ± 0.068) following heat-shock treatment reveals memory defect, compared with the same genotype flies maintained at 19 °C (c205>*tubulin*-Gal80^{ts};UAS-*fdh*31, 19 °C, PI = 0.24 ± 0.070, $t = -2.50$, $p < 0.05$). However, flies (*tubulin*-Gal80^{ts}/+;UAS-*fdh*31/+, 30 °C) lacking the Gal4 driver, which were maintained at 30 °C prior to memory testing, reveal normal visual pattern memory that is significantly greater than zero (PI = 0.26 ± 0.084, $t = 3.07$, $p < 0.01$). All graphs depict mean ± SEM; (n) number of flies tested in experiments.

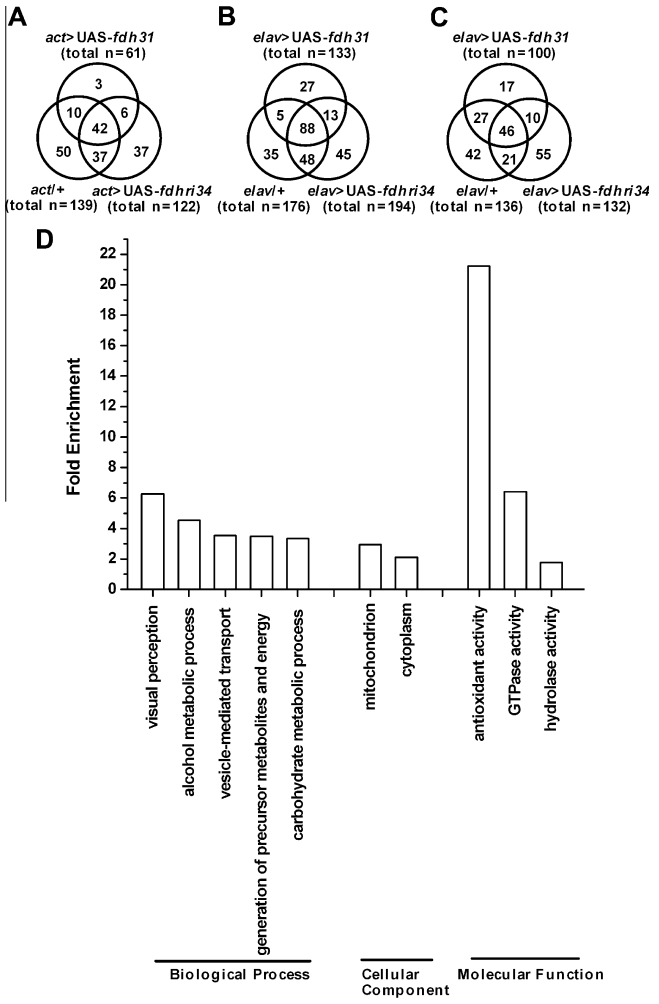

Fig. 3. The effects of *fdh* modulation on protein S-nitrosation/denitrosation and functional characterization of the modified proteins. (A) *act*-Gal4-driven S-nitrosated proteins from whole body homogenates. (B) *elav*-Gal4-driven S-nitrosated proteins from head homogenates. (C) Repeat experiment of (B). (D) Gene ontology analysis of denitrosated proteins in *fdh* over-expressing flies. The bars indicate fold enrichment of genes belonging to a particular GO term ($p < 0.05$) in the population of regulated genes. Genes were classified using a set of 179 GO categories for biological processes, cellular components, and molecular function (GO essential slim) [30]. *n*: the number of S-nitrosated proteins in three *Drosophila* lines.

over-expression. As expected, *elav*-Gal4 driven pan-neuronal GSNOR and PKG co-expression flies restored normal memory performance as good as the genetic control (+/UAS-*fdh*31 and UAS-*PKG*/UAS-*fdh31*) (Fig. 4), indicating that the memory defect was rescued by co-expression of exogenous PKG. These results demonstrated that *fdh* might affect visual pattern memory by the NO-cGMP-PKG pathway.

Discussion

The present study investigated the relationship between NO metabolism and visual pattern memory in *D. melanogaster*. Results demonstrated that over-expression of *fdh* in adulthood resulted in memory defect in a visual learning paradigm and *fdh* participated in visual pattern memory in specific neurons. PKG over expression could rescue the memory defect caused by over-expression of *fdh*. Furthermore, flies with downregulated bioactivity of NOS displayed the same visual pattern memory defects as *fdh* over-expression flies (Fig. S1). Meanwhile, there were multiple proteins denitrosated in the memory-deficient flies. No specific phenotype was detected in the *fdh* down-regulated flies, which could be due to the compensatory effect of other enzymes that exhibit GSNO reductase activity [40,41]. Based on results from the present study and previously reported data [42], we proposed a model for the role of NO in visual pattern memory in *Drosophila*: the homeostasis of NO is doubly-controlled by NOS/GSNOR; GSNOR controls NO metabolism via GSNO regulation and NOS controls NO synthesis by using arginine as a substrate. Modulation of either GSNOR or NOS can regulate NO bioactivity. Over-expression of *fdh* resulted in NO imbalance, thereby affecting the NO-cGMP-PKG pathway and protein S-nitrosation and, ultimately, learning and memory function (Fig. 5). Our results demonstrated that NO metabolism regulated by *fdh* is crucial in visual pattern memory.

In this study, both the NO-cGMP-PKG pathway and protein S-nitrosation may be involved in *fdh*-regulated learning and memory. PKG deficiency has been shown to result in learning and memory defects [24], and GSNOR inhibitors increase the soluble guanylyl cyclase (sGC) activity and enhance signal transduction of the NO-cGMP pathway [39]. Therefore, over-expression of the *fdh* gene in the present model may block sGC activity. In turn, it could lead to decreased PKG activity *via* the NO-cGMP-PKG pathway, resulting in visual pattern memory defect. Moreover, because NO bioactivity was exerted through cGMP-dependent and cGMP-independent pathways, protein S-nitrosation may also play a role in learning and memory. It has been reported that S-nitrosation of the N-ethylmaleimide-sensitive factor (NSF) induces cerebellar LTP by modulating surface expression of AMPA

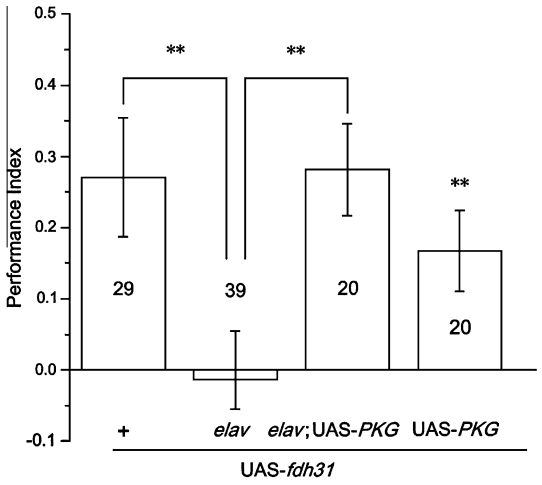

Fig. 4. *PKG* over-expression rescues visual pattern memory defect caused by *fdh* over-expression in flies. Over-expression of *fdh* gene driven by *elav*-Gal4 causes *Drosophila* visual pattern memory defect compared with control flies of UAS-*fdh31* alone or UAS-*fdh31* and UAS-*PKG* combined together. The behavioral defect was rescued by co-expressing of PKG. All graphs depict mean ± SEM; (*n*) number of flies tested in experiments.

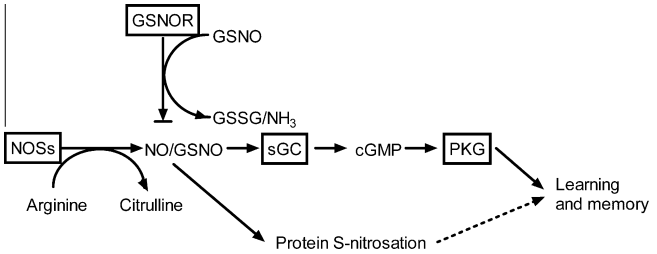

Fig. 5. NOS–GSNOR double-gated control of NO homeostasis in learning and memory. GSNOR which controls NO metabolism together with NOS which controls the generation of NO constitute a double-gated control of NO homeostasis. GSNOR is involved in learning and memory through cGMP-PKG pathway and possibly through protein S-nitrosation modification simultaneously.

receptors [43] and S-nitrosation of cyclic nucleotide-gated (CNG) channels, which could affect learning and memory by affecting olfactory and visual transduction [44]. In the present study, multiple vesicle-mediated transport proteins were denitrosated, such as Arr1, Syt1, and Rop. Syt1 is functionally related to neurotransmitter secretion and synaptic vesicle endocytosis, which are important in learning and memory mechanisms. These results suggested that protein S-nitrosation/denitrosation modification could contribute to memory defect in *fdh* over-expressing flies.

Results from the GO analysis revealed that some denitrosated proteins in *fdh* over-expressing flies were related to the process of visual perception, which could interfere with visual learning and memory. Therefore, Fourier analysis was used to evaluate the ability of flies to discriminate between patterns [24]. As shown in Supplementary Fig. S2A, visual discrimination was not significantly different between *fdh* over-expressing flies, *fdh* dsRNAi flies, and the genetic control flies. A defect in thermo-tolerance can induce low memory performance in *fdh* over-expressing flies. Therefore, the present study measured dwelling time of the transgenic flies during the first and last training [45]. As shown in Fig. S2B, there was no significant change in thermo-tolerance. These results excluded the effects of thermo-tolerance and pattern discrimination on visual pattern memory in *fdh* over-expressing flies.

In addition, for the function of GSNOR in brain, since GSNOR is the sole alcohol dehydrogenase isozyme in brain, the failure to detect ethanol dehydrogenase activity poses a problem if it is assumed that this enzyme has evolved and developed as a protective mechanism for ethanol detoxification in that organ [46]. Our findings that GSNOR participates in the regulation of learning and memory may provide an answer to this dilemma: control of NO metabolism and GSNO/SNO homeostasis as demonstrated in the present study suggested a novel theory for understanding the presence of GSNOR in the brain.

In conclusion, the present study demonstrated that *fdh*-controlled NO metabolism plays a key role in visual pattern memory of *Drosophila*. This mechanism may be involved in protein S-nitrosation modification and the NO-cGMP-PKG signaling pathway. GSNOR and NOS composed a double-control of NO bioactivity during synthesis and metabolism. GSNOR could be a potential novel target for curing dysfunctions related to NO bioactivity. Further studies are needed to determine how GSNOR regulates S-nitrosated proteins and thereby influences learning and memory.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.niox.2010.09.007.

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